Aquaculture Practices – *Vallisneria spiralis* L.

5.1 TAXONOMY

Vallisneria spiralis L., (the name Vallisneria is in honour of Antonio Vallisneri) belonging to the family Hydrocharitaceae is a submerged exotic macrophyte with its origin in the African and European continents. The aquatic rooted macrophyte spreads invasively in wetlands, ponds, marshes with its vegetative reproduction. It is also abundant in Ponds and wetlands of West Bengal specially following monsoon or rather from the mid of monsoon with rapid increase towards the end of the season. With clustered phyllotaxy and multiple parallel foliar venations, the plant establishes itself as an extensive colonizer, suppressing all other rooted macrophytes with its horizontal runners (Fig.7). Economically, it is the most common aquarium plant providing dissolved oxygen and shelter to fish fingerlings.

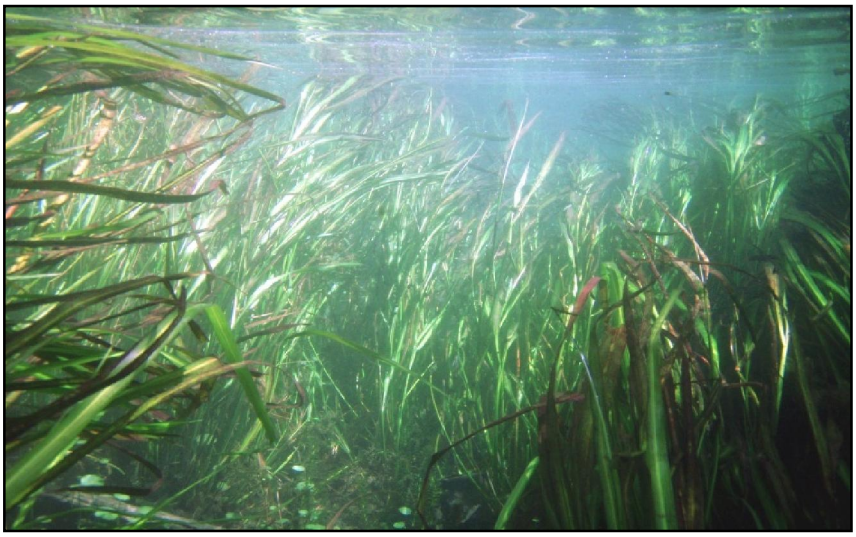

Fig. 7 ***Vallisneria spiralis* in natural habitat**

5.2 MATERIALS AND METHODS

The plant samples (7 kg fresh weight) were obtained from intensely growing area of the wetlands of Nadia district and also from East Kolkata wetland and Captain Bherry. Following uprooting, the leaf samples were preliminarily washed under running water to remove the dirt from the roots and the snails that stick to the leaves are carefully placed on other leaves without causing any diversity loss. The samples are carried to the laboratory in ice boxes with ice bags where they are subjected to three fold wash, chopping and shade dry. The powdered leaves are stored in 4°C for further use.

5.2.1 Extraction and isolation of bioactive compounds

- Method 1 (Immersion Extraction)
- Method 2 (Soxhlet Extraction)

5.2.1 (A) Method 1 (Immersion Extraction)

Following the preparative steps, the powdered leaf samples were homogenised with the solvent system C_2H_5OH: ddH_2O with in ascending ratios from 0% - 80%. Each of the experimental set up was sonicated at equal intervals for 48hrs.

↓

The ratio of the solvent to the dry weight of the sample was maintained at 10:1 in each 500ml flat bottom flask with B24 standard joint. The experiment was performed in duplicate to obtain sufficient quantity of the extract.

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The flasks were kept away from direct sunlight at room temperature. The flasks containing higher ratios of ddH_2O were checked for the typical foul odour emitted due to microbial growth.

↓

The samples were vacuum filtered through sintered disc filter G4 grade with 5-10 micron and the solvent is removed using a rotary vacuum evaporator attached to a cold water circulator.

↓

The extract obtained ruin the rotary flask is subjected to biochemical assay

↓

The fraction with highest amount of phenol is screened for antimicrobial potential.

↓

The bioactive fraction is subjected to Spectrometric and spectroscopic analysis

5.2.1 (B) Method 2 (Soxhlet Extraction)

The dried leaves were powdered and soaked in methanol for 48hrs in room temperature

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Eluted fractions	Solvent System	Ratio
Fraction 1 (F1)	**H:EtOAc**	**7:3**
Fraction 2 (F2)	**H: EtOAc**	**5:5**
Fraction 3 (F3)	**H: EtOAc**	**3:7**
Fraction 4 (F4)	**DCM: EtOAc**	**9:1**
Fraction 5 (F5)	**DCM: EtOAc**	**1:1**

Fraction 6 (F6)	**EtOAc**	**1**
Fraction 7 (F7)	**Chl: MeOH**	**1%**
Fraction 8 (F8)	**Chl: MeOH**	**10%**

↓

The extract was filtered by vacuum filtration using vacuum pump and sintered disc funnel fitted in 250ml conical.

↓

Subjected to Column Chromatography Solvent System as shown below

↓

Bioassay

↓

Isolation of pure compound

↓

F7 methanol fraction isolated and put for a separate column; TLC Solvent System: Toulene : DCM: 9:1; R_f: 0.8 (Fig. 8)

↓

The fraction eluted from the column had a pleasant aroma. It was chosen for further work and named as VsF7

Fig. 8 **TLC of VsF7 of Vallisneria leaf extract.**

5.2.2 BIOCHEMICAL ANALYSIS

5.2.2 (A) Total Phenol Content:

The total phenolic content of four hydro-ethanolic fractions obtained from method 1 and VsF7 fraction from method 2 of the *V.spiralis* leaf extract was estimated using Folin- Ciocalteu method (Antony, 2003).The extracts were prepared at six different concentrations from 0.05mg/ml - 0.3 mg/ml and 0.1 ml of each was added with Folin-Ciocalteu reagent followed by 2ml of Sodium carbonate (Na_2CO_3). The solution was incubated for 30mins at room temperature and the change in colour was observed. The quantification was done by spectro-photometric reading at 765nm and compared to standard phenol Gallic acid. It is used as the positive control and the phenol content in the samples are measured in Gallic acid Equivalent (GAE) mg/gm of dry plant sample.

5.2.2 (B) Total Flavonoid Content:

The total flavonoid estimation of the plant samples were done by calorimetric method following Jia *et al*. (Jia *et al*., 1999). Briefly, 0.1ml of the test samples at concentration range 0.05 mg/ml – 0.3 mg/ml was added to 0.12ml of 5% $NaNO_2$.Aluminium chloride (10%) was added to each sample at 0.12ml volume after 5mins incubation at room temperature to observe the colour change. Finally, 0.8ml of NaOH (1mM) mixed with ddH_2O was supplemented to the test samples following spectral reading at 510 nm. Similar to Gallic acid, a standard flavonoid called Quercetin was used and the total flavonoids content was calibrated against Quercetin equivalent (mg/g) using the calibration curve.

5.2.2 (C) Total Tannin Content:

The assessment of the tannin content of the leaf extracts were also done by Folin-Ciocalteu method where 0.1ml of the test sample was homogeneously mixed with 0.5ml Folin- Ciocalteu solution and 7.5ml of ddH_2O. 1ml of diluted (35%) Sodium carbonate (Na_2CO_3).The mixture was measured at 725nm pursuing 30mins incubation at room temperature supplemented with periodic shaking (Tambe and Bhambar, 2014; Europeenne Commission, 2000). Gallic acid at all the above mentioned concentration similar to the test samples was prepared and the tannin content was estimated by the Gallic acid calibration curve.

5.2.2 (D) Antioxidant Assay by DPPH:

The antioxidant estimation by DPPH (2, 2-diphenyl-1-picrylhydrazyl) method was invented by Blois (Blios, 1958). DPPH ($C_{18}H_{12}N_5O_6$) is a commercially available free radical which works on hydrogen atom transfer principle. The test samples at concentrations 0.05mg/ml - 0.3 mg/ml are assayed for their free radical scavenging potential. The freshly prepared DPPH solution at 0.1mM with 25mg/L is prepared in methanol at concentration ranges 0.05mg/ml - 0.3 mg/ml and the qualitative estimation is drawn by absorbance reading at 517nm following 30mins incubation. Butylated Hydroxy Toluene (BHT) is a commercial antioxidant and used as a positive control to compare the efficiency of the test samples (Blois, 1958; Schofield, 1989). The concentration of the test sample which causes 50% the free radical or DPPH to scavenge is called the IC50 and measures the competence of an antioxidant. The test sample scavenging potential and the IC50 are stated by the following equations:

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

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

5.2.3 IN-VITRO BIOLOGICAL ACTIVITY

5.2.3 (A) Anti-microbial Screening

(i) Anti-fungal Activity

The fungal culture was grown in sabouraud dextrose agar and the single pure colony was sub-cultured to check for contamination. The antifungal activity assayed with Agar Well Diffusion method as described by Mayser with negligible modification (Boekhout *et al*., 2010; Murray *et al*., 1995). The fungal culture was adjusted at 0.5 McFarland with 10^6 cells/ml of the sabouraud dextrose broth using sterilized cotton swabs used for the assay. Prior to the experiment, the glassware and culture media were sterilized at 121°C for 15mins and dried in hot air oven where required. The test sample at dilution range 1000 µg/ml, 500µg/ml and 1 µg/ml was prepared in 5% DMSO (Akinyemi *et al*., 2005) which is a non-toxic and aprotic solvent which dissolves compounds of diverse polarity. The experimental set up was carried out entirely under laminar air flow bio-safety cabinet class II. The agar plates were spread uniformly with 75µl of the microbial culture and kept for 15mins.The wells were bored with a cork borer with 6mm diameter and 50µl of the test sample was poured in the pre sealed wells using agar. DMSO was used as a positive standard to nullify the inhibition or stimulation effects caused by the dissolving solvents. The plates were kept for 3hrs in the laminar air flow and thereby incubated at 28°C for 36hrs to check the zone of inhibition. The MIC and MFC was worked out to find the potency of the antifungal agent (Olurinola, 1996; Chea *et al*., 2007; Khare, 2007).

(ii) Anti-bacterial Activity

Agar well diffusion method with Muller Hilton Agar (MHA) (Dahiya & Purkayastha, 2012) was used against gram negative bacteria *Aeromonas popoffi* and *Aeromonas hydrophila.* The bacterial identification was confirmed by 16S rRNA sequencing using

primer [16S univ 5'-GAG TTT GAT CCT GGC TCA G-3' 27f, 5'-TAC GGT TAC CTT GTT ACG AC-3' 1492r]. The annular radii of the inhibition zone (mm) were measured for two broad dilution of the sample at 1000 ppm and 100 ppm along with control. DMSO is used as the test sample dissolving solvent and as the control. In brief, 8mm wells were bored with a cork borer and filled with 20µl of the plant sample on a pre-inoculated agar plate. Prior to inoculation, the bacterial culture were adjusted to 0.5 McFarland {$1.5x10^8$ (CFU)/ml}, with final dilution to $5x10^5$ CFU/ml. The cultured plates were allowed to diffuse the plant samples for 2hrs in the bio safety chamber and finally incubated for 18hrs at 28°C. The two significant factors of assessment, the MIC and the MBC were calculated.

(iii) Minimum inhibitory concentration & Minimum microcidal concentration

The MIC concentration range was decided based on the bacterial inhibition in the well diffusion assay showing static or cidal activity. The lowest possible concentration of the the test sample displaying the inhibition zone was taken as a marker and at least two dilutions down fold to it were considered to trace the minimum inhibiting concentration. The test samples were prepared in the sabouraud dextrose broth and the experiment was repeated thrice to reach the exact data representation. The broth dilution method with 20µl of the inoculum at 5×10^5 CFU/ml was transferred to each dilution tubes containing 4.8ml of the test sample broth. After 18hrs of incubation at 28°C, the tubes were checked for visible growth and the optical density at 660nm was checked to find the MIC for fungal growth and 600nm for bacterial growth. The tubes which showed no visible growth were plated with 10µl of the inoculums to find the MFC/MBC. The plates were incubated for 24hrs to 36hrs and the test sample with smallest possible concentration of the plant sample displaying zone of inhibition be conferred as MFC/MBC (Coulidiati *et al*., 2011).

5.2.3 (B) Cytotoxicity Assay

(i) Brine Shrimp lethality Test

This assay assessed the acute toxicity of the bioactive fractions of leaf sample of *V.spiralis* by a static bench top bioassay. The methodology determines the LC50 values in µg/ml of active compounds from different fractions of leaves by screening the mortality rate of brine shrimp (*Artemia salina* L.) nauplii in brine media. The shrimp used in the experiment had shown a PHR of 90% in controlled laboratory conditions of pH (7.5), Light intensity (1200 lumen) and Salinity (25-32 ppt). Brine shrimp eggs were brought 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 (Fig. 9). 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 Five different concentrations 10000, 1000, 100, 10, 1 µg/ml respectively of compounds were assayed on the nauplii at 6hr time interval of exposure time from 1h, 6h, 12h, 18h and 24h using brine media which was also used as the control solution for true interpretation (Solis *et al*., 1993). The solvent dissolving the test plant sample was used as the negative control to nullify mortality caused due to the solvent used. Binomial Probit Regression analysis was performed to validate the model and triplicates of each concentration were tested at 95% confidence level by Finney's Probit analysis method or alternatively by IBM SPSS statistical software 20 (Chakraborty *et al*., 2018).

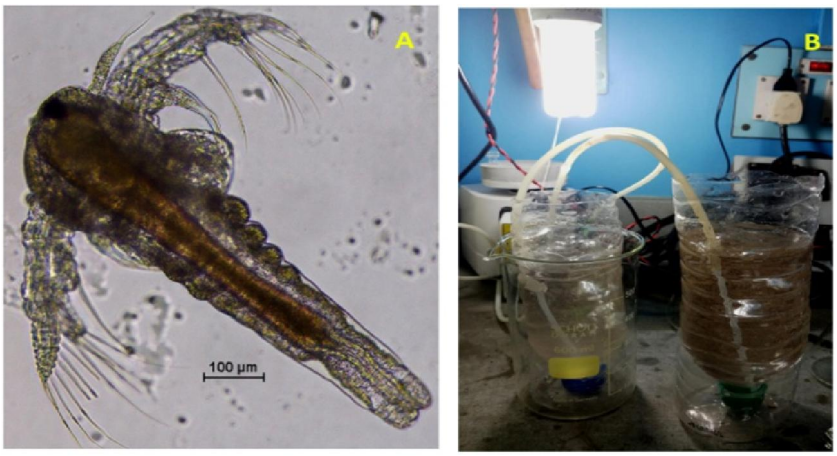

Fig. 9 **A: Microscopic view of nauplii stage of *Artemia salina*; and B: Hatching of dormant cysts of *A. salina.***

(ii) Anti-algal activity

The antialgal experiment was performed with the most harmful blue-green alga, *Microcystis aeruginosa*. The algal culture was purified as per the protocol provided by the Centre for Conservation and Utilization of Blue-green algae, Indian Agricultural Research Institute (IARI, New Delhi). BG11 (pH 7.5-7.8) was used as the culture media specific for blue-green alga. *M. aeruginosa* cultured with sterilized BG11 medium in 1000 ml of distilled water. An exponentially growing algal slant culture was used as source inoculums for subculture and the final volume was adjusted at 10^6 cells/liter of the test solution. The anti-algal potential of the biologically significant fractions of the plant sample were assessed at four different concentration ranges of 50µg/ml to 500 µg/ml and control without the plant extracts. Each test sample was mixed to Erlenmeyer flasks with 3 ml of the algal inoculums, 142 ml of the BG11 culture medium and 2ml of extract shown (Fig. 10). The experiment was maintained in triplicate for each treatment set and the optimum growth conditions of 90 µmol of photons m^{-2} s^{-1} (light-dark = 14hrs:10 hrs) 25°C temperature was maintained for 96 hrs for growth confirmations. The anti-algal activity was visually observed for pigment colouration and the

quantitative analysis was done by chlorophyll quantification of chl-a, chl-b and chl-c and total chlorophyll.

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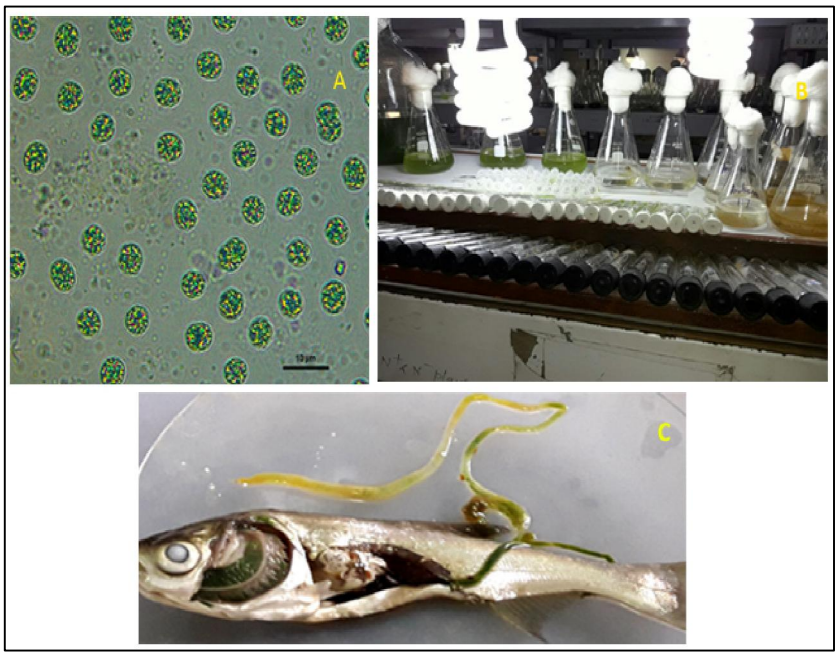


Fig. 10 **A: Microscopic view of *Microcystis aeruginosa*; B: Culture of *M. aeruginosa* under laboratory conditions and C: View of Microcystis bloom infested fish gut.**

The algal cultures both treated and control is vacuum filtered by a 47mm glass fibre filter (Whatman GF/C filter or Gelman AE filter) made of Gelman polycarbonate with pressure < 8 PSI and stored in 10ml acetone in a stopper joint glass tube. 0.15 ml of the buffer solution of saturated magnesium carbonate solution is added to the filter just before initiating the filtration to prevent phaeophytinization. The amount of the buffer is to be maintained precisely to prevent hindrance in filtration. The test sample is tethered with homogenizer and centrifuged at 2000rpm for 20mins. Thereafter the extracts are determined spectrometrically at 750 nm to correct turbidity disturbances and the pigment absorbance at 664 nm, 647 nm and at 630nm. The two treatment groups one with extract as prophylaxis (early application) (Vp) and the one with application after

48hrs as therapeutics (Vt) are considered. 90% acetone solution was treated as blank solution.

The standard equations for the calculation of Chlorophyll a, b and c are as follows:

$$\textit{Chl-a} = 11.85*(Abs\ 664) - 1.54*(Abs647) - 0.08*(Abs\ 630)$$

$$\textit{Chl-b} = 21.03*(Abs\ 647) - 5.43*(Abs664) - 2.66*(Abs\ 630)$$

$$\textit{Chl-c} = 24.52*(Abs\ 630) - 7.60*(Abs647) - 1.67*(Abs\ 664)$$

Where Chl-a, Chl-b and Chl-c are concentrations of Chlorophyll a, Chlorophyll b and Chlorophyll c respectively; the optical density at three different wavelengths of 664nm, 647nm and 630nm are measured at 1cm path length of the light.

$$\text{Chl-(a/b/c)} = \frac{\text{Chl –a/b/c * extract volume (l)}}{\text{Volume of the sample (m3)}}$$

(iii) Duckweed Assay

The duckweed assay was performed by root cuttings of *Lemna minor*. The plant samples were collected from East Kolkata Wetland, Kolkata and cultured in laboratory using Steinberg medium. The roots of the laboratory grown macrophyte was 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.

5.2.4 IN-VIVO FISH BIOASSAY

5.2.4 (A) Biological Classification

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

5.2.4 (B) 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).

5.2.4 (C) Experimental Design

(i) Fish Bioassay set up

The juvenile Pangasius fish used for experiment were maintained of almost uniform length of 4 -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 (Fig. 11).

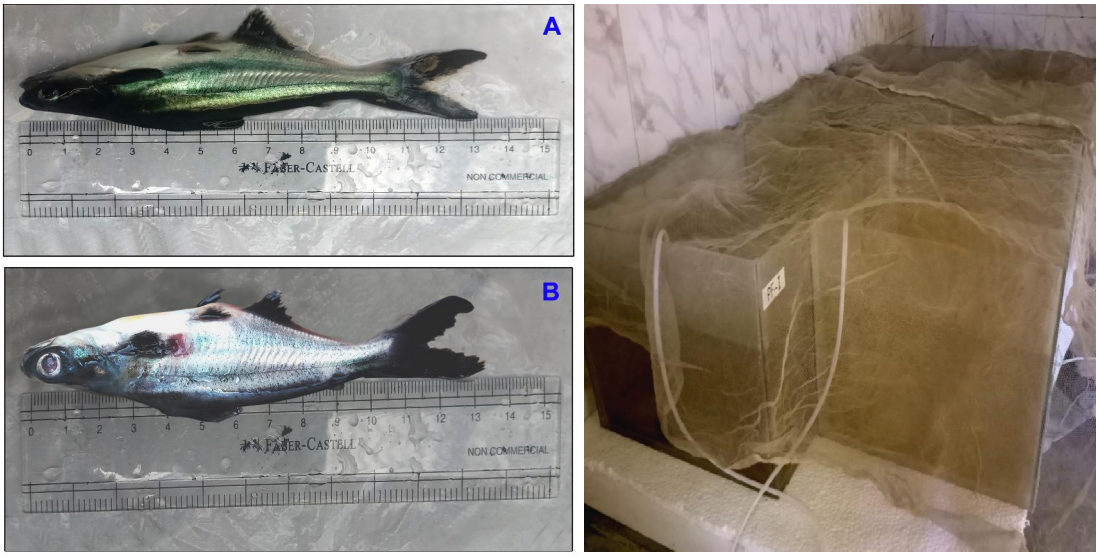


Fig. 11: **Left: - *P. hypophthalmus* uninfected (A) and infected (B) at the beginning of the experiment; Right: - Experimental set up for KP (+), PF-I, PI-F & KP (-)**

(ii) Challenging the fish samples with Bacterial injection

P. hypophthalmus was 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 adding 0.05ml of 1% of Barium Chloride solution 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.

5.2.4 (D) 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.

5.2.4 (E) Histopathology

(i) Fixation of the tissue samples

The live fishes were sacrificed to obtain the liver and kidney. The removed organs were slice to 2-3mm size tissue sample and immediately placed in the fixative 10% Neutral buffer formaldehyde for 24hrs at room temperature. For long span storage the tissue samples were placed in 70% alcohol and refrigerated in glass vials. The tissue sample to fixative volume was made to 1:20. For instantaneously processing, the tissue samples were transferred from formaldehyde to ascending hydro-alcohol ratios.

(ii) Dehydration of the tissue samples

The tissue samples were dehydrated by alcohol to remove the water content before paraffin bedding. The repetitions for each ratio are made in fresh glass vials. The ascending hydro-alcohol ratios are as follows:

Hydro-Alcohol Ratio	Time Span	Repetitions
Alcohol (50%)	1hr	4
Alcohol (70%)	30mins	5
Alcohol (90%)	30mins	5
Alcohol (100%)	15mins	5

(iii) Clearing of alcohol

The alcohol from the tissue samples are removed by Xylene. The tissues are dipped in Xylene for 15mins with three repetitions.

(iv) Paraffin impregnation

The tissue samples are enclosed in plastic tissue cassettes and dipped in paraffin chamber in hot water bath with molten paraffin: xylene :: 1:1 for 1hr followed by only paraffin for 1hr with another change in paraffin for 1hr.

(v) Embedding in paraffin block

The tissue samples are transferred in molten paraffin laid in stainless steel base molds by a preheated forceps. The tissue has to be placed while the paraffin in the mold is still in a molten state. The tissue sample may be pressed to the base of the mold for proper orientation. The molds are refrigerated overnight following which the paraffin block is fetched out of the mold for tissue sectioning.

(vi) Microtome sectioning and slide preparation

The paraffin block is placed in the block holder of the microtome and the knife is positioned with clearance angle of 3-5degree and slope of 90 degree. The section thickness is adjusted to 5 micron. The paraffin ribbons with embedded tissue sections are obtained in suitable size and placed in pre heated water placed on a water bath (not exceeding 50°C) to prevent shrinkage. The floating paraffin ribbons are lifted to the slides smeared with adhesives Mayer's albumin and placed on tissue papers in room temperature for drying overnight.

(vii) Staining of the slides

The staining is done in coupling jars as tabulated below:

Order (Down grade)	Cleansing Solution	Time span
1	Xylene	5mins
2	Alcohol (100%)	5mins
3	Alcohol (90%)	5mins
4	Alcohol (70%)	10mins
5	Alcohol (50%)	5mins
6	ddH2O	1min
Staining		
7	Haematoxylin	2mins
8	ddH2O	30secs
9	ddH2O	1 min (wash)
Order (Up- grade)	**Cleansing Solution**	**Time span**
10	Alcohol (50%)	10mins
11	Alcohol (70%)	15mins
12	Alcohol (90%)	10mins
Staining		
13	Eosin	30secs
14	Alcohol (100%)	1 min (wash)
15	Alcohol (100%)	10mins
16	Xylene	10mins

(viii) Mounting of the tissue sections

The slides are permanently mounted with a cover slip using DPX moutant solution. 2-3 drops are poured on the sections and the cover slips are slided with 45 degree angle to prevent bubble formation. Following drying the slides are observed under microscope and stored in slide box with proper tags (Fig. 12).

Fig. 12 **Paraffin molds for tissue sectioning by microtome and staining accessories in coupling jars.**

5.2.5 STRUCTURAL IDENTIFICATION

The structural characterization of the bioactive compound or the constituents of the bioactive fraction was done by spectrometric and spectroscopic analysis. The analysis includes: Mass Spectrometry (MS), Gas-Chromatography Mass Spectrometry (GC-MS), Fourier-Transform Infra-Red Spectroscopy (FT-IR) and Nuclear Magnetic Resonance Spectroscopy (NMR).

5.3 STATISTICAL ANALYSIS

The statistical analysis of the biological activity is evaluated to elucidate the utility of the plants considered as good aquaculture practise and brief the conclusive comparisons. Curve fitting models are evaluated to precisely predict the interpolation of data series in a mathematical equation.

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 a range of 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-aquare, Z-statistics and F- value finds the goodness fit model and difference of means of the variables under consideration.

5.4 RESULTS

5.4.1 BIOCHEMICAL ANALYSIS

The biochemical content analysis done for the four different hydro-ethanolic fractions of the plant sample are tabulated below (Peret-Almeida *et al*., 2005). The fourth fraction was found to have highest consistent content of phenols, flavonoids, tannins and antioxidants through the concentration ranges from 0.05mg/ml to 0.30mg/ml (Table 1).

Fractions (% Ethanol)	Phyto-Chemical Assay	Concentration range (mg/ml)					
		0.05	0.10	0.15	0.20	0.25	0.30
Ethanol 0%	TPC	0.16	3.24	7.59	6.01	11.88	15.43
	TFC	0.43	0.68	1.92	1.28	2.60	3.77
	T	0.11	0.19	0.36	0.85	1.03	1.21
	A_{DPPH}	2.53	6.47	12.58	10.01	16.01	20.22
Ethanol 20%	TPC	0.12	2.18	5.37	2.61	6.77	8.40
	TFC	0.18	0.32	0.69	1.34	2.18	4.82
	T	0.10	0.39	0.33	0.46	0.89	1.06
	A_{DPPH}	2.68	5.37	7.98	9.56	21.32	26.49
Ethanol 40%	TPC	0.18	3.73	4.51	4.98	9.85	15.61
	TFC	0.10	0.32	0.38	0.08	1.06	3.49
	T	0.28	0.25	0.32	0.37	1.06	1.59
	A_{DPPH}	1.65	5.29	9.00	9.82	22.91	25.66
Ethanol 80%	TPC	0.38	5.29	8.64	6.78	13.56	19.23
	TFC	0.68	0.76	2.64	3.53	3.58	6.91
	T	0.56	0.58	0.64	0.49	1.27	1.88
	A_{DPPH}	5.20	9.56	15.46	11.28	21.01	28.97

Table 1: **Biochemical contents of the hydro-ethanolic fractions of the leaf extract of *V. spiralis* obtained from Immersion Extraction (Method 1)**

A comparative analysis of the Ethanol 80% (VsE80) from Method 1 and F7 (VsF7) methanol fraction obtained from Method 2 of leaf sample of *V.spiralis* shows that the phenol content (TPC), flavonoids content (TFC) along with antioxidants were higher in F7. However, the tannins (TTC) were higher in the hydro-ethanolic fraction. The graph depicts the radical scavenging activity of the F7 fraction is comparable with that the commercially available antioxidant, BHT.

5.4.1 (A) Total Phenol Content

The figure (Fig.13) depicts a concentration dependent increase in total phenol content with the lowest recorded phenol content at 0.05mg/ml with 0.38 GAE mg/g for Ethanol 80% and 2.81 GAE mg/g for VsF7 fraction and the highest of 19.23 GAE mg/g and 21.08 GAE mg/g at 0.3mg/ml for VsE80 and VsF7 respectively. A sudden rise and fall in the phenol content at 0.15mg/ml and 0.20mg/ml respectively of the plant sample concentration is observed as a graphical trend characteristic to most of the biochemical parameters under consideration. The curve fit model followed a cubic polynomial equation $y = 0.405x^3 - 3.945x^2 + 14.01x - 10.04$ and $y = 4012.x^3 - 1944.x^2 + 322.3x - 8.996$ for Ethanol 80% and F7 respectively with coefficient of determination $R^2 = 0.963$ and 0.998.

5.4.1 (B) Total Flavonoids Content

The graph for the total flavonoids content displays a zig-zag pattern with consistent increase with increasing concentration of the plant sample (Fig.13). The VsF7 fraction was however observed to be stable with a steady increase. The lowest concentration at 0.05mg/ml recorded the least flavonoids content with 0.68 QE mg/g for VsE80 and 0.55 QE mg/g for VsF7 and the highest concentration of the plant extract at 0.3mg/ml showed the maximum QE mg/g with 6.91 and 7.28 for the former and the latter fraction

of the leaf sample. The graphs followed a polynomial regression model with the equation $y = 0.072x^3 - 0.603x^2 + 2.345x - 1.376$ and $y = 217.7x^3 - 131.4x^2 + 49.51x - 1.75$ and $R^2 = 0.935$ and 0.985.

The VsE80 fraction had higher tannin content with 1.88 TAE mg/g observed at 0.3mg/ml and with 0.91 TAE mg/g for VsF7 (Fig. 13). The characteristic rise and fall at 0.15mg/ml with 2.64 QEmg/g and 0.20mg/ml with 3.53 QEmg/g respectively for the former fraction was not observed. The best curve fit model was polynomial in nature with $R^2 = 0.959$ and 0.986 with polynomial equation $y = 0.021x^3 - 0.126x^2 + 0.210x + 0.47$ and $y = 55.55x^3 - 25.52x^2 + 6.156x - 0.13$ for VsE80 and VsF7 respectively.

5.4.1 (D) Antioxidant activity by DPPH method

The DPPH free radical inhibition assay followed a graphical pattern alike that of total phenol content. It showed a linear correlation with concentration of the plant samples except for following the characteristic rise and fall of the graph at 0.15 mg/ml and 0.2mg/ml respectively. The highest scavenging activity for both the fraction was observed at 1 mg/ml with 36.77% and 77.11% inhibition for VsE80 and VsF7 respectively as compared to the commercial antioxidant with 89.28%. 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. The % scavenging activity followed an exponential curve fit model for both with $y = 0.049e^{0.074x}$ with IC50 at 0.981mg/ml for the former (Fig.13) and $y = 0.035e^{0.04x}$ and IC50 at 0.258 mg/ml for the VsF7 fraction. The latter proved to be a better free radical scavenger.

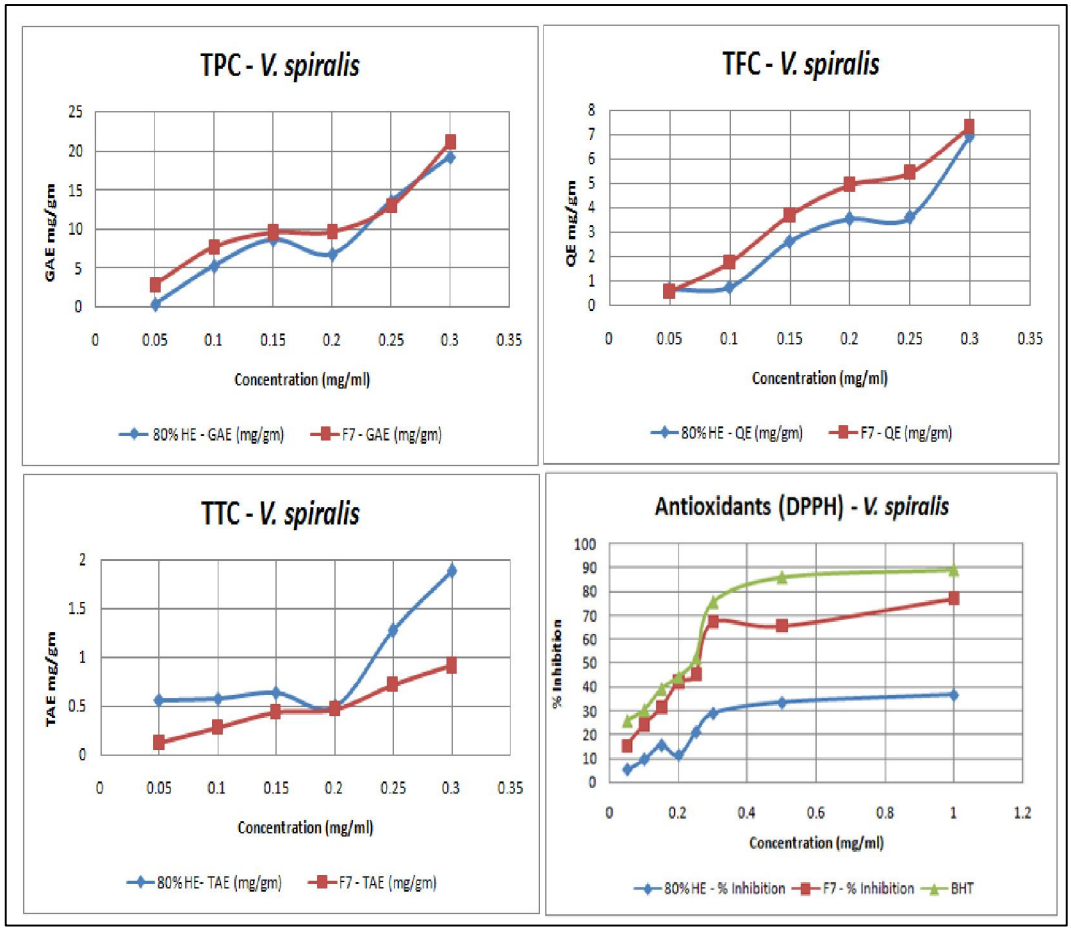


Fig. 13: **Graphical representation of the biochemical parameters of the 80%HE fraction (VsE80) and VsF7 of *V. spiralis*.**

5.4.2 IN-VITRO BIOLOGICAL ACTIVITY

5.4.2 (A) Anti-microbial Screening

The VsE80 fraction was found to possess antifungal activity whereas the VsF7 fraction of methanol base had antibacterial activity against fish disease causing pathogens.

(i) Antifungal Activity of VsE80

The antifungal assay was conducted against *Malassezia globosa,* a dermatitis fungus, at three broad dilutions 1000µg/ml, 500µg/ml and 100µg/ml. The inhibition zone was observed by well diffusion assay which revealed the highest annular radii of 12mm for

1000 µg/ml followed by 500µg/ml with 7mm and 100µg/ml with 3.2mm. The annular radius was defined by the distance between the edges of the zone of inhibition and the well with the plant sample (Fig. 14)

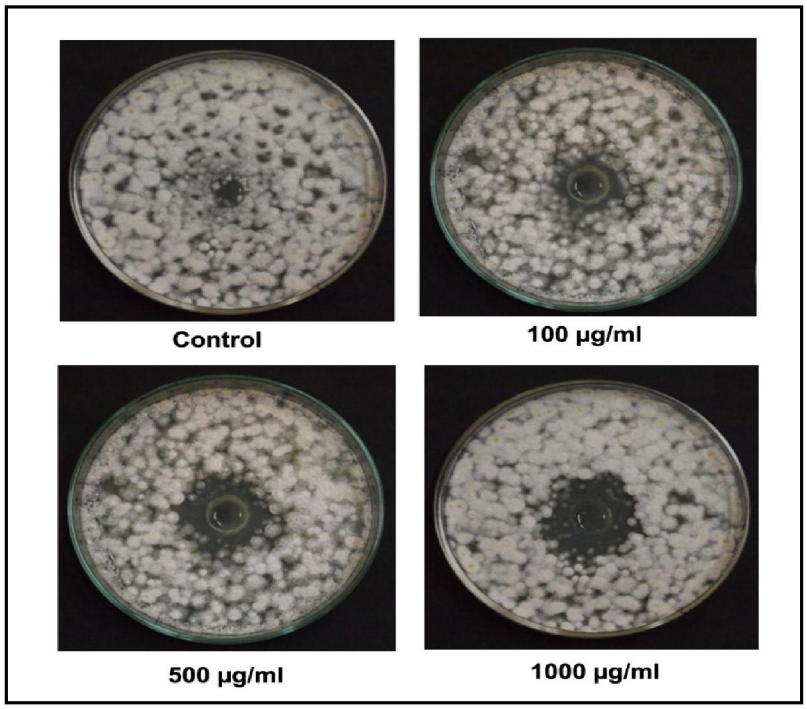


Fig. 14: **Pictorial representation of the zone of inhibition of the Ethanol 80% of *V. spiralis* against *M. globosa*.**

(ii) Antibacterial Activity of VsF7

Bacterial species	Sample Concentration (µg/ml)	Zone of Inhibition ZOI(mm)
A. popoffi	Control (C)	5
	100 (B)	7
	1000 (A)	12
A. hydrophila	Control (C)	0
	100 (B)	8
	1000 (A)	14

Table 2: **Zone of Inhibition (mm) by *Aeromonas popoffi* & *Aeromonas hydrophila* by VsF7**

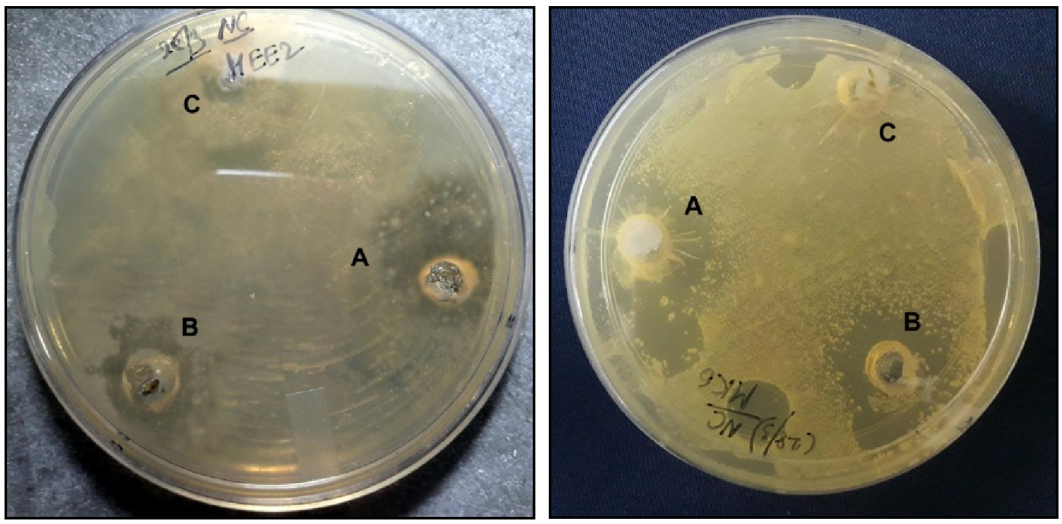


Fig. 15: **Well diffusion assay of VsF7 fraction against *Aeromonas popoffi* (MEE2) and *Aeromonas hydrophila* (MK6)**

(iii) Minimum inhibitory concentration & Minimum microcidal concentration

For the VsE80 fraction the MIC and MFC had to be repetitively done due to false reading caused by the mycelia of the fungal culture. The absorbance at 660nm reveals the MIC 156.2 µg/ml and 5000µg/ml was revealed as the MFC (Fig. 16).

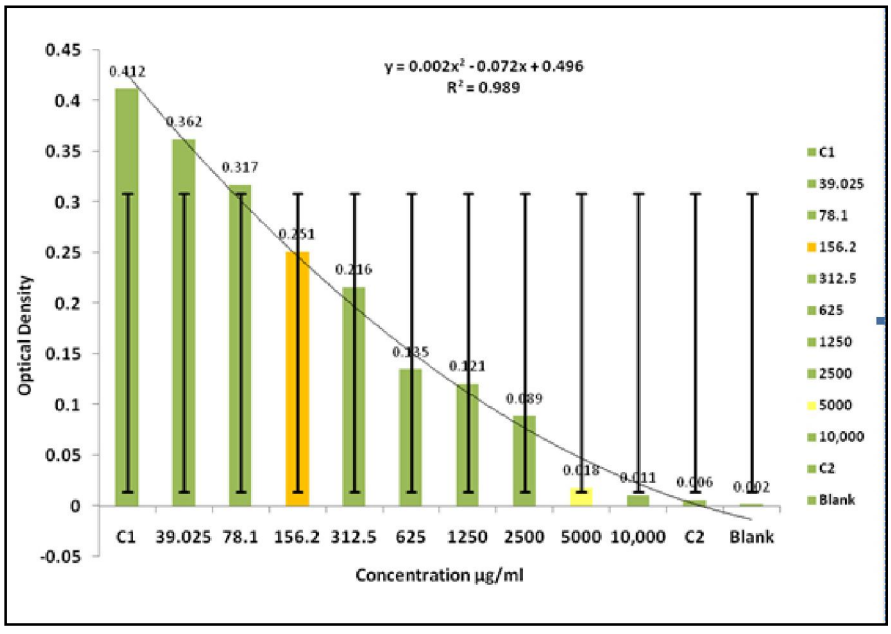


Fig. 16: **Pictograph representation for MIC & MFC of the VsE80 fraction of *V.spiralis* on *Mallesizia globosa*.**

The minimum inhibitory concentration (MIC) of VsF7 fraction was 125µg/ml against *A. popoffi* with minimum bactericidal concentration (MBC) at 5000µg/ml and MIC 15.625µg/ml and MBC 10000µg/ml against *A. hydrophila*.

The non-monotonic pattern (Fig. 17) (Kohn and Melnick, 2002) of the antimicrobial graph of the leaf extract against microbial growth is represented with cubic curve estimation pattern. It shows the efficiency of plant based drugs as lower doses and the threshold dose.

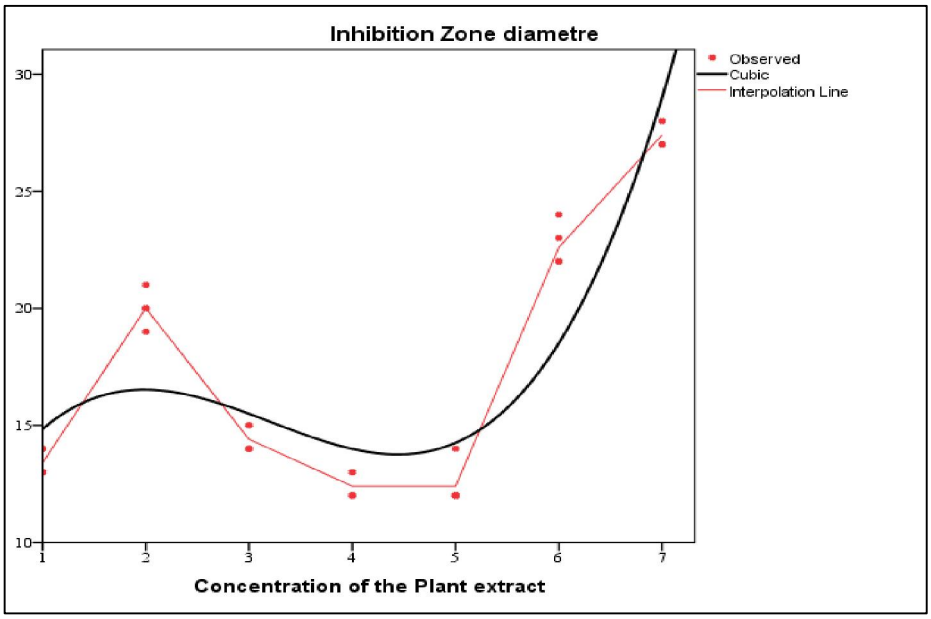


Fig. 17: **Non-Monotonic curve - Inhibition Zone diameter *vs* plant sample concentration**

5.4.2 (B) Cytotoxicity Activity

(i) Brine Shrimp Lethality Assay

The toxicity assay of the Ethanol 80% fraction of Method 1 and the F7 methanol fraction of Method 2 was assessed by brine shrimp lethality assay. The median lethal concentration, LC50 for Ethanol 80% at 1hr exposure time is 398µg/ml and that of methanol fraction is 0.407µg/ml. There was acute lethality for methanol fraction

beyond 1hr exposure while the hydro-ethanolic fraction survived the nauplii for another 6 hrs with LC50 33.424 µg/ml. Brine solution served as the control where no lethality was observed. The probit analysis (Fig.18) of three values (LC25, LC50 & LC75) is plotted against log of concentration.

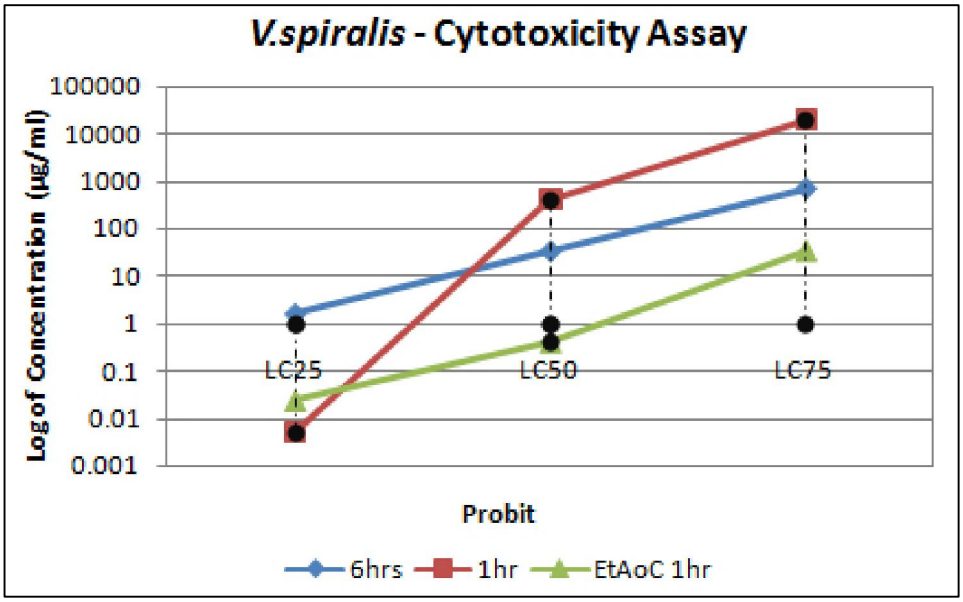


Fig. 18: **Graphical representation of LC25, LC50 and LC75 against log of concentration for VsE80 and VsF7 of *V. spiralis* at 1hr, 6hrs, 12hrs, 18hrs and 24hrs exposure time.**

(ii) Anti-algal activity

The Ethanol 80% fraction (VsE80) was found to inhibit the dense growth of *Microcytis aeruginosa* (Fig.19, 20) but the VsF7 fraction did not show any such activity The anti-algal activity by chlorophyll assessment depicts an increased chlorophyll count in samples containing extract at 0hrs (Vp) than the samples with extracts added after 48hrs (Vt). The control sample with 0µg/ml of extract has the highest chlorophyll content of 0.727 mg/m^3. (Fig.21)

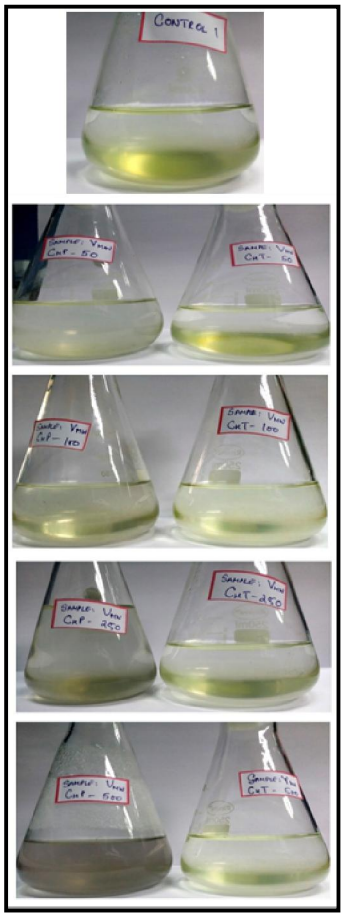


Fig. 19: **Anti-algal activity of VsE80 of *V. spiralis***

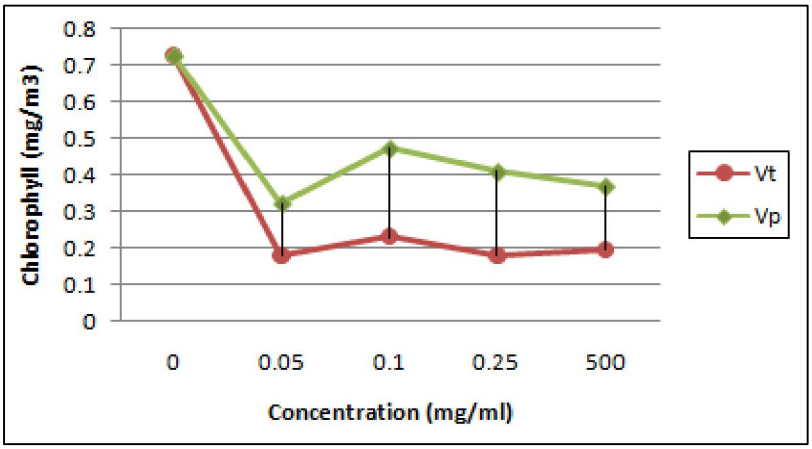


Fig. 20: : Graphical representation of anti-algal assay of Vt & Vp of VsE80 of *V. spiralis*

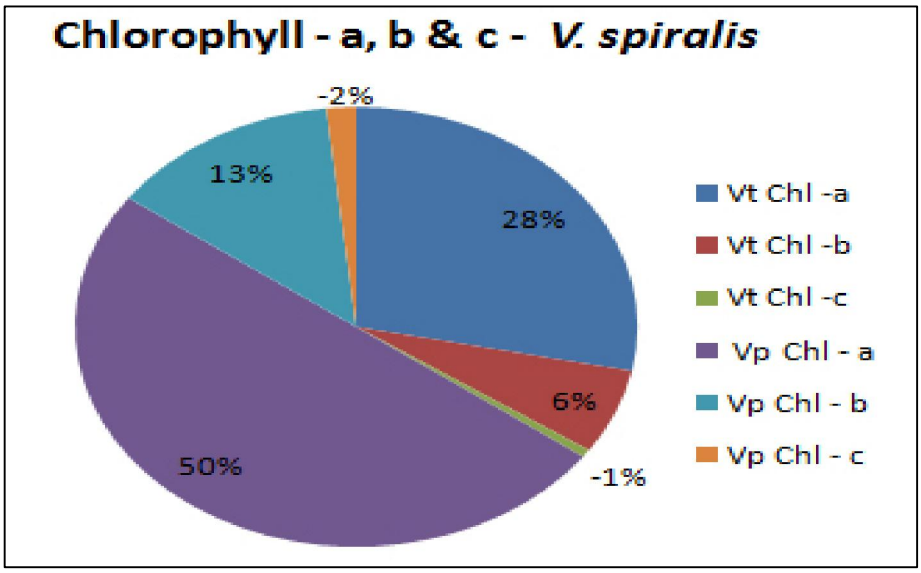


Fig. 21: **A pie chart representation of the distribution of Chlorophyll a, b & c for Vt and Vp against *Microcystis aeruginosa*.**

(iii) Duckweed Assay

The toxicity assay against duckweeds was found to be nullified in VsE80 fraction as no visible change was seen. For VsF7 fraction, the assay was carried out in four different concentrations viz; 1000ppm, 500ppm, 100ppm and 10ppm with control. The growth of the fronds decreased with increase in concentration (Fig. 22, 23).

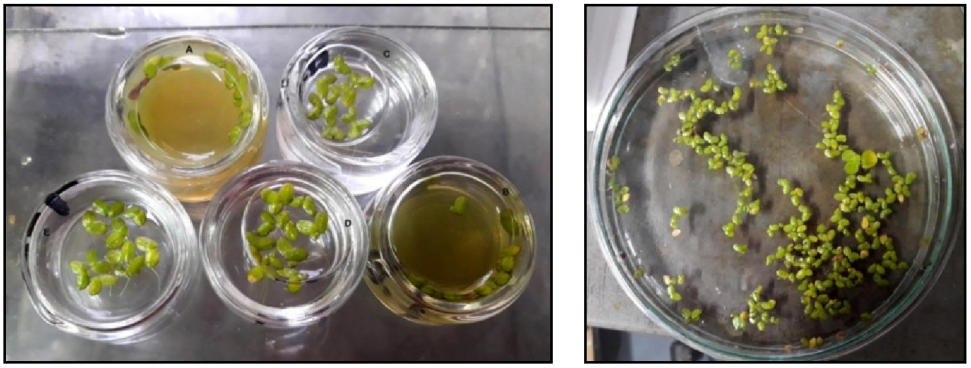


Fig. 22: **Left: *L. minor* bioassay, A- 1000ppm, B- 500ppm, C- Control, D-100ppm and E- 10ppm; Right: Laboratory growth of *Lemna minor*.**

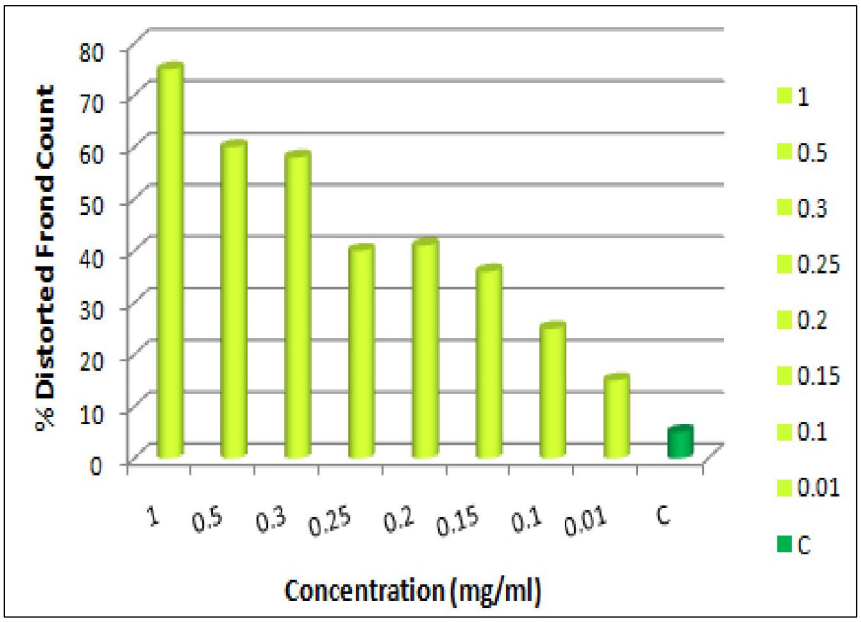


Fig. 23: **The picto-graphical representation of the % distorted frond count against the concentration of the VsF7 fraction of the leaf extract of *V. spiralis*.**

5.4.3 IN-VIVO FISH BIOASSAY

Mortality Chart:

Treatment Sets	Total No	Mortality	Recovery Percentage
KP (+)	10	0	100
PF-I	10	7	30
PI-F	10	4 (1sinking)	55
KP (-)	10	10	0

Haematological parameters:

Parameters	Fresh Fish	PF-I	PI-F	Infected fish
White Blood cells ($x10^3$ µl)	5.41±0.2	7.41±0.6	6.26±0.1	7.53±0.5
Red Blood Cells ($x10^6$ µl)	2.15±0.5	1.79±0.06	2.18±0.07	1.72
Haemoglobin (g/dL)	7.58±0.5	6.94±0.05	7.29±0.09	5.21±0.4

Histopathology:

Liver

The Normal Liver section (Fig. 24A) is seen with central lobule and portal vein. The intra hepatic pancreatic tissue is prominent. There is some collagen deposition seen. The PI-F is shown (Fig. 24B) which has shown better haematological factors and less mortality as compared to PF-I. However, pyknotic nucleus is seen in many hepatocytes which conforms the traces of infection. The PF-I (Fig. 24C) is prominent with aggregations of melano-macrophage centres with dilated sinusoids. The clinical symptoms of infected tissue sample (Fig. 24D) include excess of enlarged melano-macrophage centres, granulated cytoplasm, necrosis and dilated sinusoids.

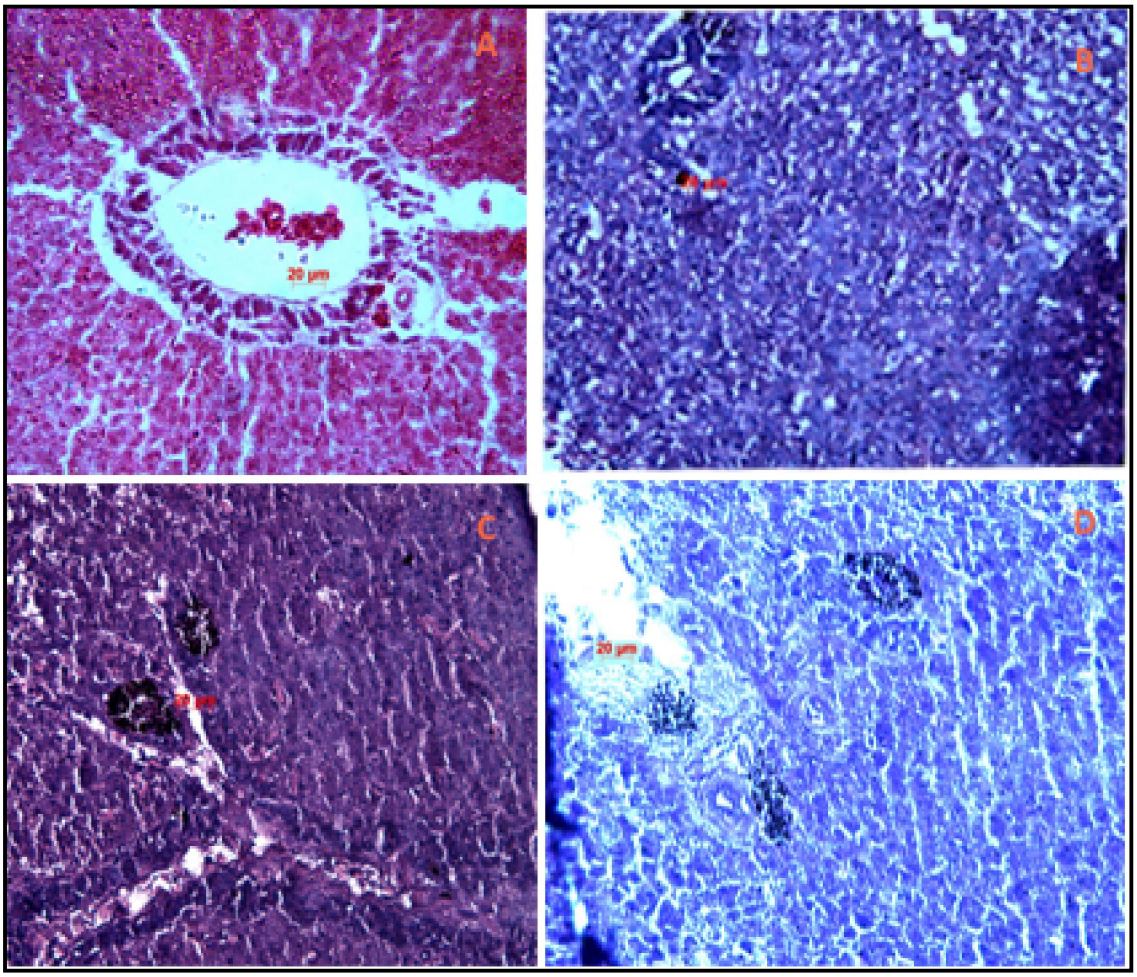


Fig. 24 : **Sections of *P.hypophthalmus* liver (40X) from four experimental sets. A: Normal Liver (KP+); B: PI-F liver; C: PF-I liver & D: Infected Liver (KP-)**

Kidney

The Normal Kidney section (Fig. 25A) is seen with sharp Bowman's capsule and Glomerulus with brush lined renal tubules. The PI-F (Fig. 25B) shows almost similar conditions as normal uninfected fish samples with negligible alterations in increase in diameter of the tubules and a few disintegrated proximal tubules. The PF-I (Fig. 25C) shows the strong traces of infection with cellular disintegration, Collapse of renal tubules and Bowman's capsule with hardly any intact ones. The infected tissue sample (Fig. 25D) showed broad melano-macrophage aggregation, shrinkage of Glomerulus and extensive degeneration of cells.

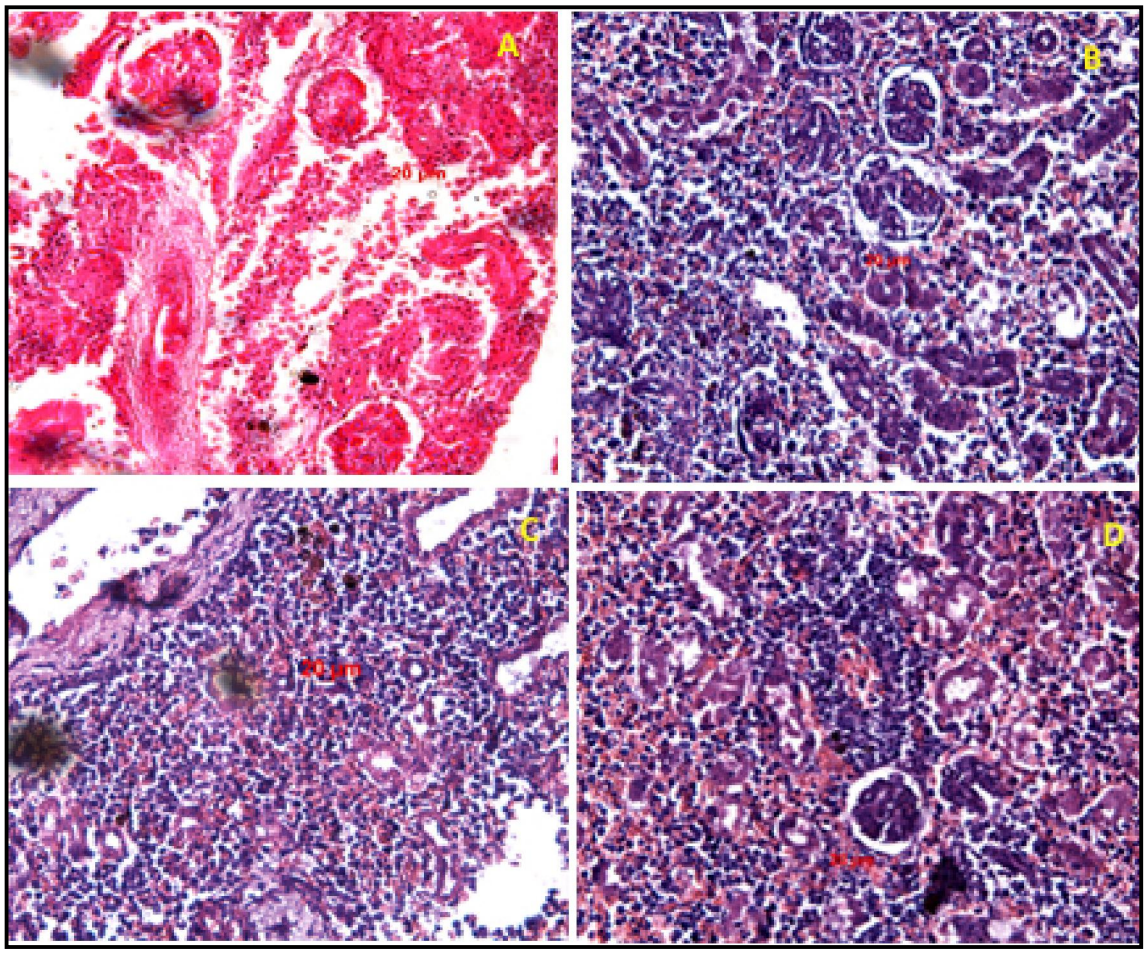


Fig. 25: **Section of *P.hypophthalmus* kidney (40X) from four experimental sets. A: Normal Kidney; B: PI-F Kidney; C: PF-I Kidney & D: Infected Kidney**

5.4.4 STRUCTURAL IDENTIFICATION

5.4.4 (A) Mass Spectrometry

The mass spectroscopy was used to find molecular mass of the chemical compound pertaining to bioactivity of the Ethanol 80% or VsE80 fraction. The column fraction eluted with 1% MeOH in EtAoC from a neutral aluminium oxide column was considered bioactive which accumulated crystals at the bottom when kept undisturbed for a week. Upon adding low polarity solvents as pet ether the crystals could be easily isolated and recovered. The crystal formed a major part of the fraction. The sticky

substances on the crystals are removed with sequential wash of methanol followed by toluene (Vulfson and Zaikin, 1976). The Time of Flight Mass Spectrometry (TOF-MS) with electro spray ionization was obtained from Department of Chemistry, Kalyani University, Nadia, West Bengal (Fig. 26). The spectrum revealed the mass/charge (m/z) = 359 [M^+] which corresponds to base peak spectrum 360.12 at 100%.

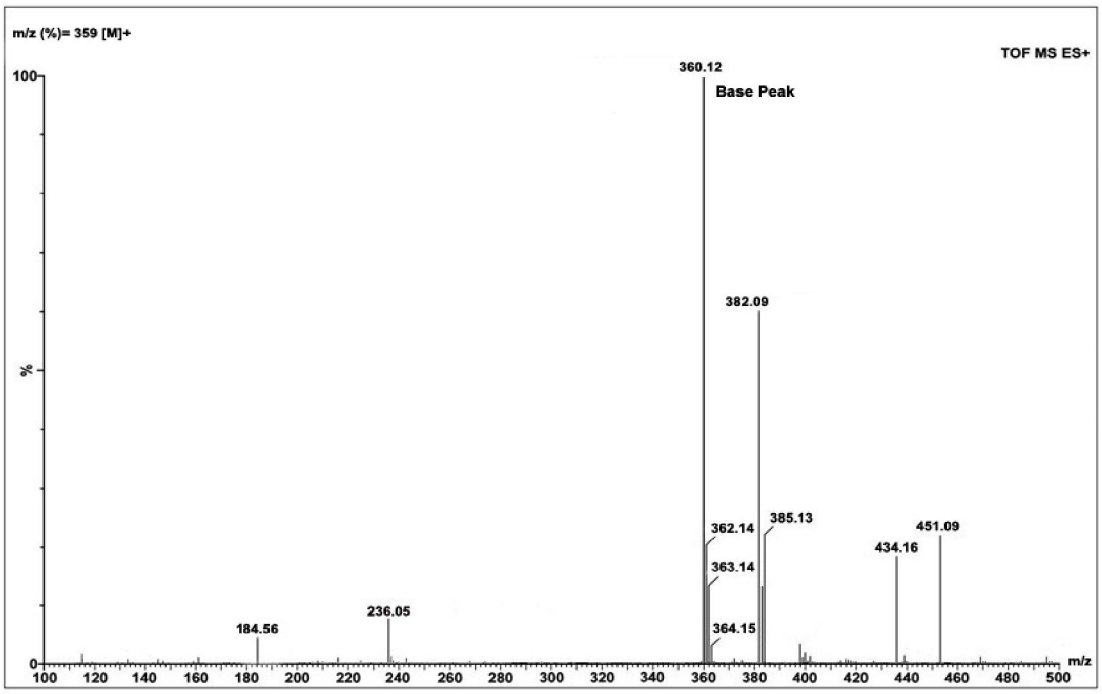


Fig. 26: **TOF-MS of the crystal obtained from VsE80 fraction of the leaf sample of *V. spiralis*.**

5.4.4 (B) Infra-Red Spectroscopy

The Fourier Transform Infra Red (FT-IR) spectrum of the crystal of VsE80 fraction was obtained from Central Instrumentation Facility, Indian Institute of Chemical Biology, Kolkata, West Bengal. The spectrum helped to reveal the chemical functional groups which could show parity with the bioactivity of the fraction. The finger print region on the right side of the spectrum from 1500cm^{-1} to 500cm^{-1} is unique to each compound. Ten major bands have been studied. The free hydroxyl group corresponds to 3642.10cm^{-1} (Fig. 27). The sp^3 C-H stretching is represented by 2965.43 cm^{-1} and

2928.54 cm^{-1}. The corresponding bending is represented by 1432cm^{-1}(Table 3). The presence of the carbonyl group (RC=O) is represented by the band at 1665.22 cm^{-1}. The presumption of the presence of aromatic group is comprehended by the bands 1554.80 cm^{-1} and 779.64 cm^{-1} within the fingerprint region. Another significant band within the fingerprint region is the 1360.0cm^{-1} which corresponds to the sulphoxides (S=O). It is studied to be an active composition in compounds reported to have antimicrobial and anti parasitic properties (Kim *et al*., 2006; Lynett *et al*., 2011) which includes the study of A*llium cepa* (Akujobi *et al*., 2004; Anon, 2005).

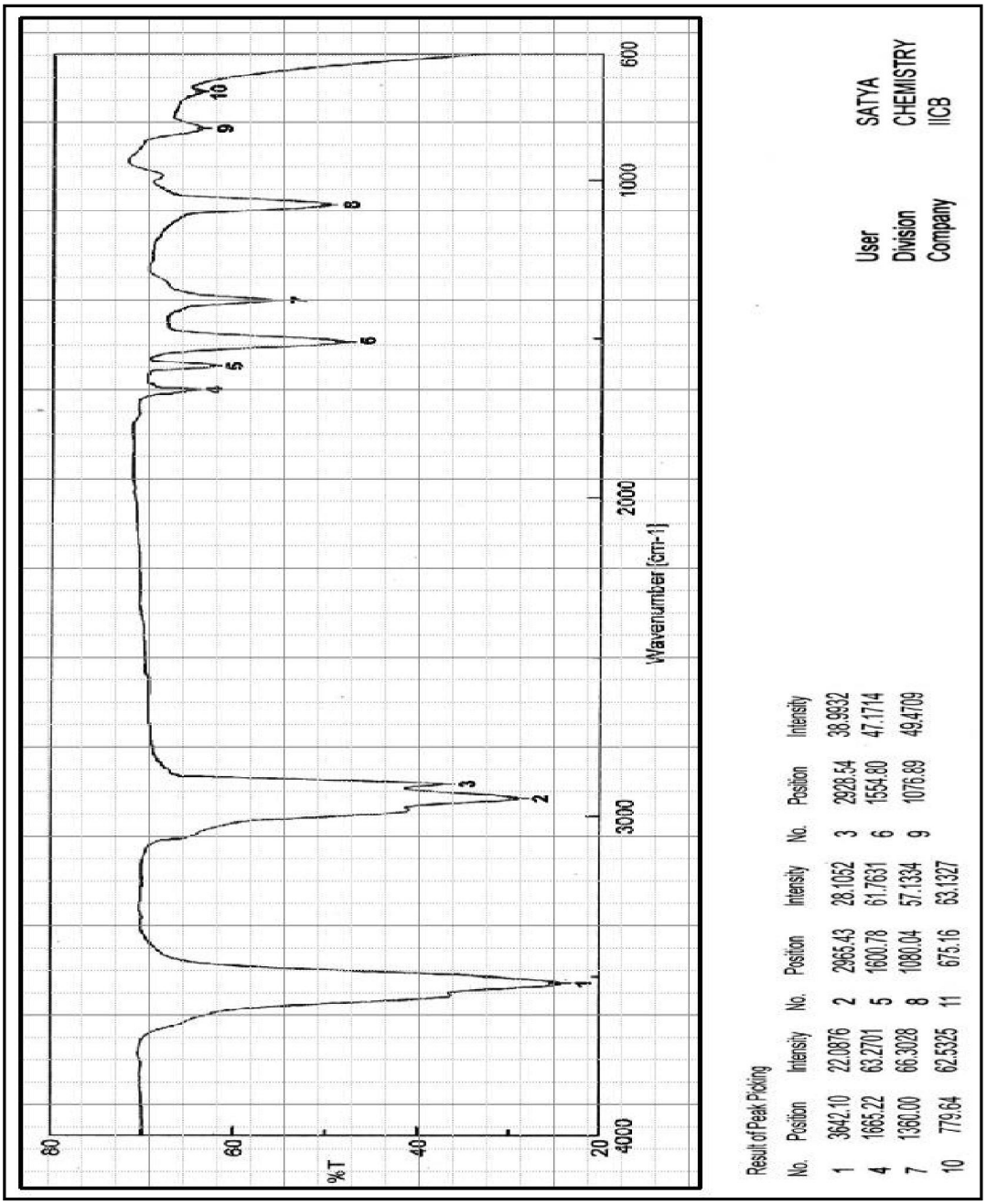


Fig. 27: **FT-IR spectrum of VsE80 fraction of the leaf sample of *V. spiralis* (Chakraborty *et al.*, 2015).**

Peak value (cm^{-1})	Stretching of function groups	Functional groups
3642.10	O-H stretching	Alcohols/Phenols
2965.43	C-H stretching	Alkanes
2928.54	C-H stretching	Alkanes
1665.22	C=O stretching	C-O NH
1600.78	C=C stretching	Alkenes
1554.80	C=C stretching of aromatic ring	Aromatic Compounds
1360.00	S=O, sym	Sulphoxide
1080.04	C-O	Ethers
1076.89	C-O	Ethers
779.64	C-H stretching	Aromatics

Table 3: **FT-IR analysis of the VsE80 fraction *V. spiralis* leaves (Chakraborty *et al.*, 2015)**

[**Fig. 27 & Table 3 are published data from this thesis;** ***Chakraborty N, Mandal B, Das AK (2015) Leaves of Vallisneria Finds Source to Anti Dermatitis: Enriching Wetland Ecosystem. J Ecosys Ecograph 5:169***.]

5.4.4 (C) Microscopic View of the Crystal

Status: White crystalline compound.

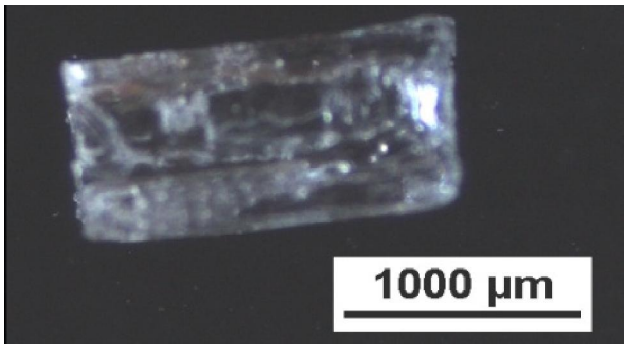


Fig. 28: **Microscopic view of an isolated white cubic crystal from VsE80 fraction of *V. spiralis* leaf extract.**

5.4.4 (D) Gas- Chromatography Mass Spectrometry of the VsF7 fraction

The Gas Chromatography Mass spectrometry for VsF7 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 were from NIST standard reference database 1A. The sample VsF7 was submitted as VsM.

GC – Parameters:

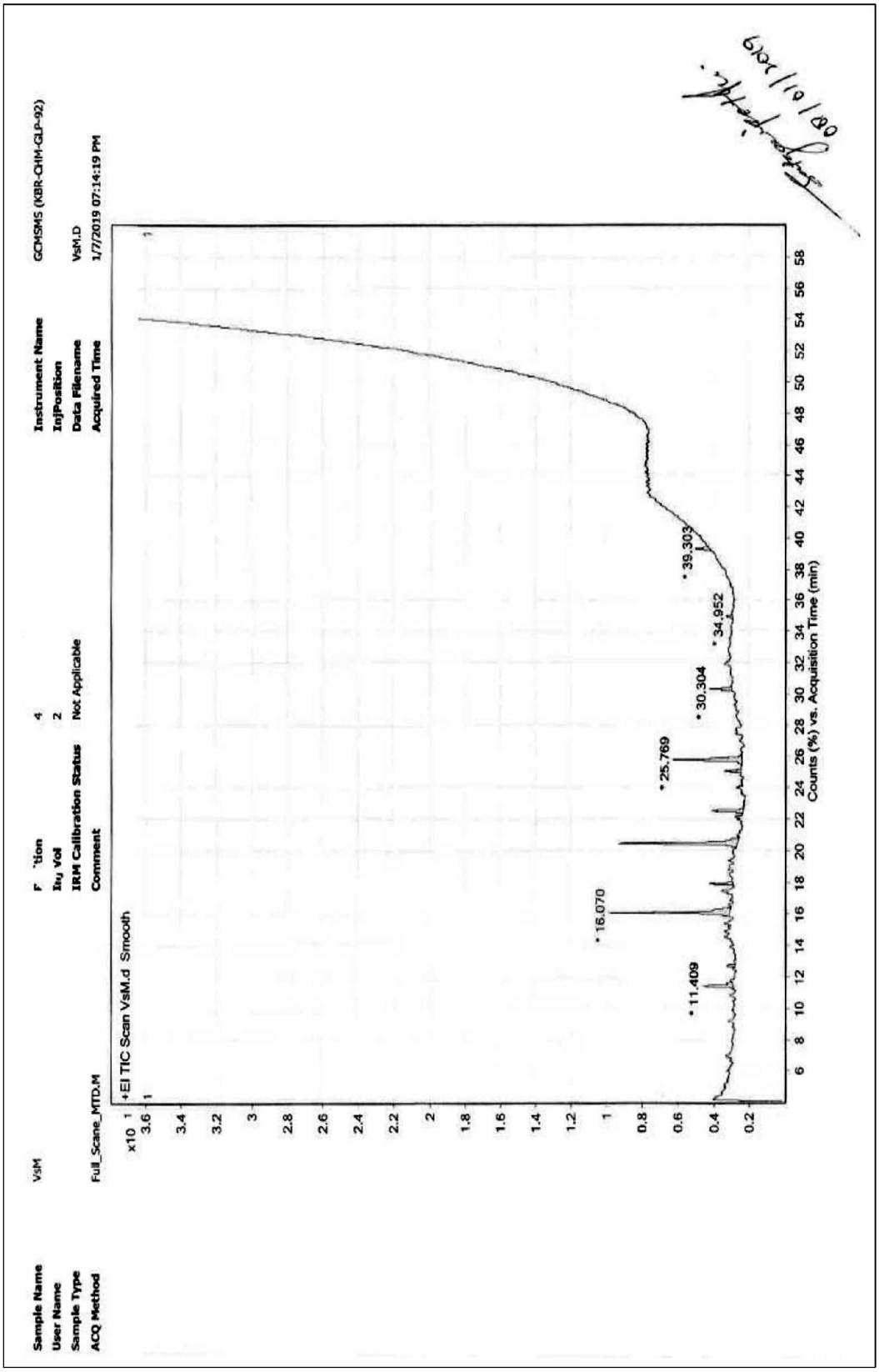
Column	HP-5 Capillary Column
Carrier Gas Flow	He, 1.2ml/min
Injection Volume	2µl
Injection Mode	Spilt Mode; 2:1
Source Temperature	230°C
Electron Energy	70eV
Detector	Mass Spectrometry

Table 4: GC-MS Column parameters

The following peaks are identified as important constituent of the bioactive fraction VsF7 isolated from the leaves of the *Vallisneria spiralis*.

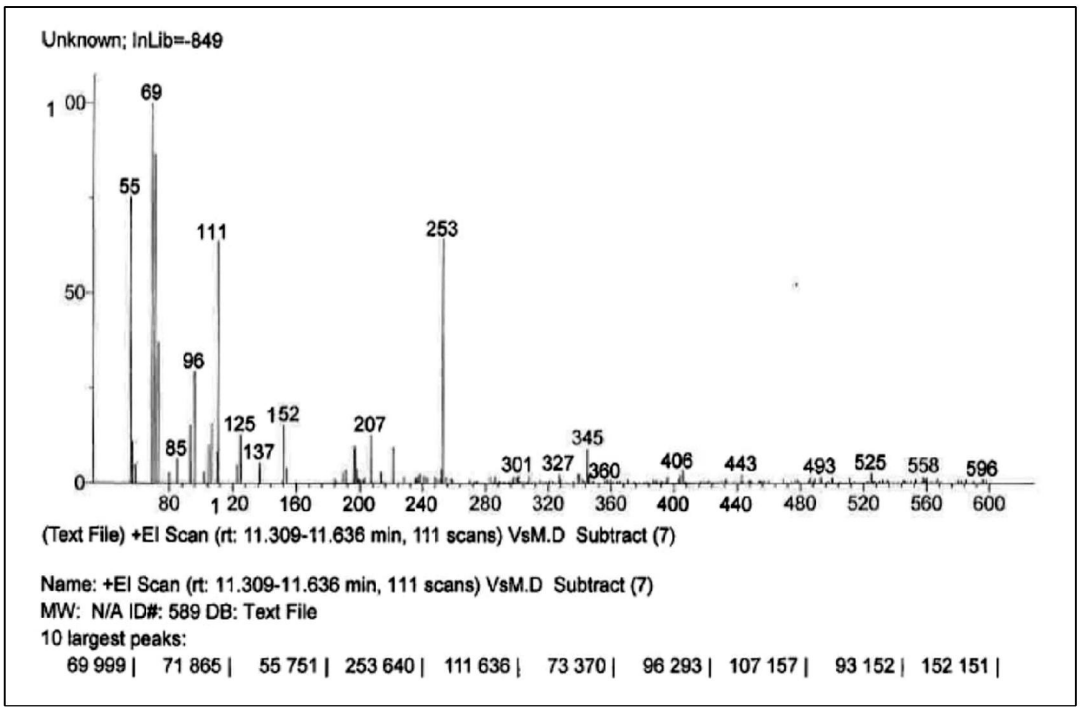
Peaks: +Total Ion Chromatogram Scan – VsF7							
Peaks	**RT (mins)**	**MF**	**RMF**	**Area%**	**MW**	**Chemical formula**	**Chemical Name**
1	11.409	443	501	25.22	364	$C_{26}H_{52}$	1-Hexacosene
2	16.07	701	792	99.04	182	$C_{13}H_{26}$	1-Tridecene
4	20.432	689	715	100	266	$C_{19}H_{38}$	1-Nonadecene
5	22.493	385	576	23.26	314	$C_{17}H_{30}O_5$	Botrydiol
7	25.035	799	842	10.89	362	$C_{22}H_{34}O_4$	Phthalic Acid Diheptyl Ester

The Chromatogram and the Mass spectra of the important above mentioned peaks are given below:



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

	Peak	RT	Area	Area %	Height	Symmetry
	1	11.409	7468337.1	25.22	774138.7	2.38
	2	16.07	29332456.	99.04	3051373.	2.3
	3	17.904	5147797.3	17.38	609308.6	1.24
	4	20.432	29615464.	100	3001163.	1.38
	5	22.493	6887763.1	23.26	777067.3	0.85
	6	25.035	3226302.1	10.89	409390.1	1.16
	7	25.769	18367464.	62.02	1730470.	1.57
	8	30.304	5077192.8	17.14	583219.5	1.33
	9	34.952	1687182.0	5.7	159776.4	1.06
	10	39.303	3018684.2	10.19	365870.9	2.18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96
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525
558
596
80
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160
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240
280
320
360
400
440
480
520
560
600
(Text File) +EI Scan (rt: 11.309-11.636 min, 111 scans) VsM.D Subtract (7)
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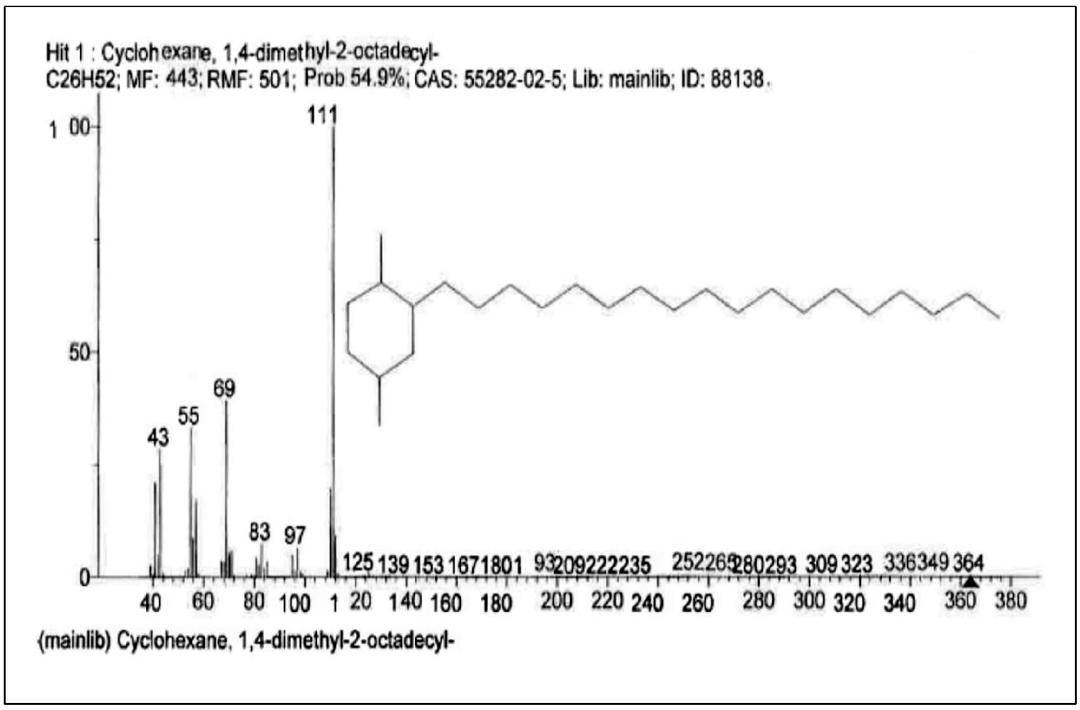
Hit 1 : Cyclohexane, 1,4-dimethyl-2-octadecyl-
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100
50
0
111
69
55
43
83
97
125
139
153
167
180
193
209
222
235
252
265
280
293
309
323
336
349
364
40
60
80
100
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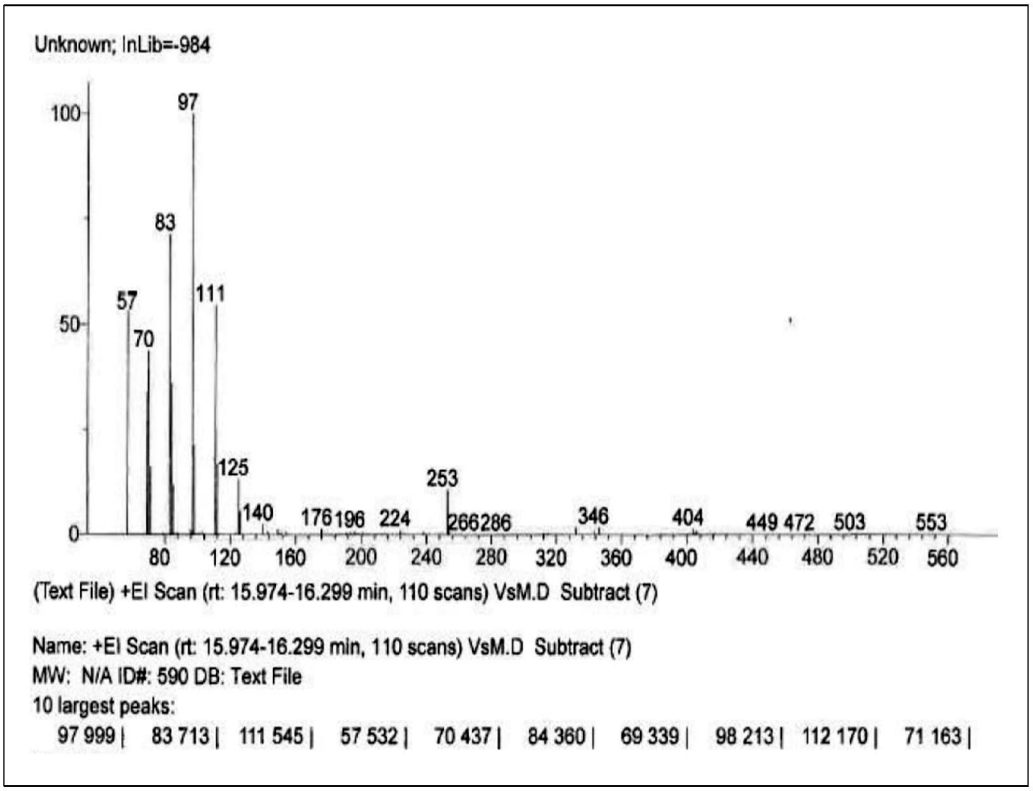
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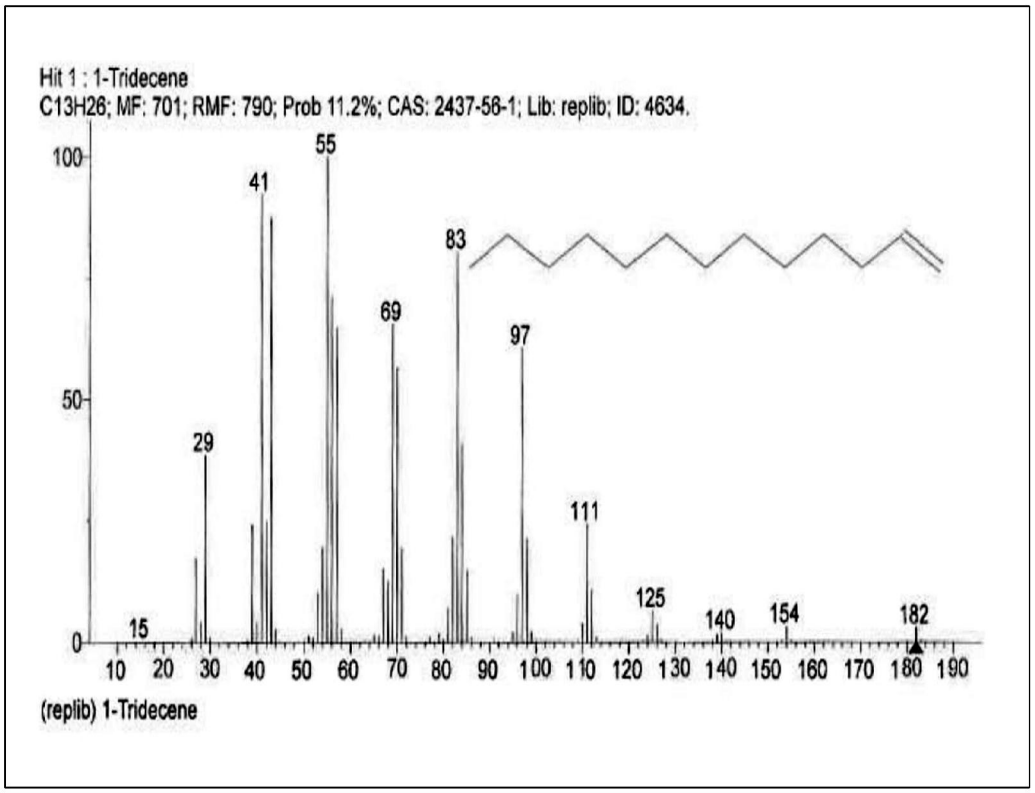
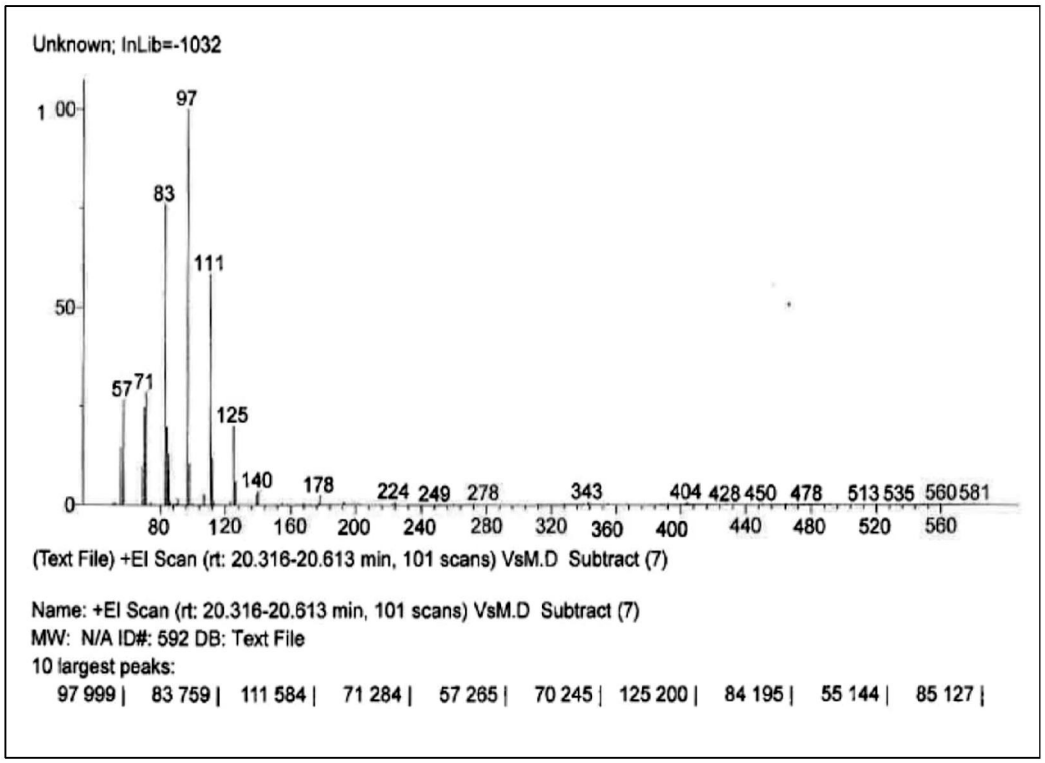
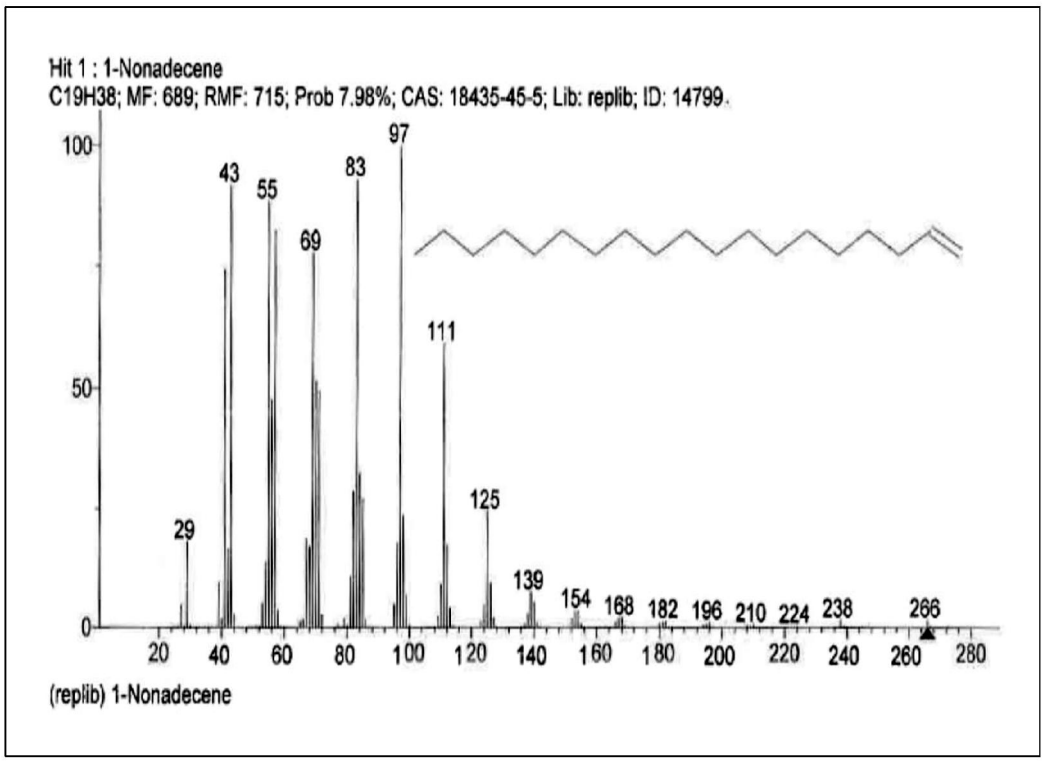
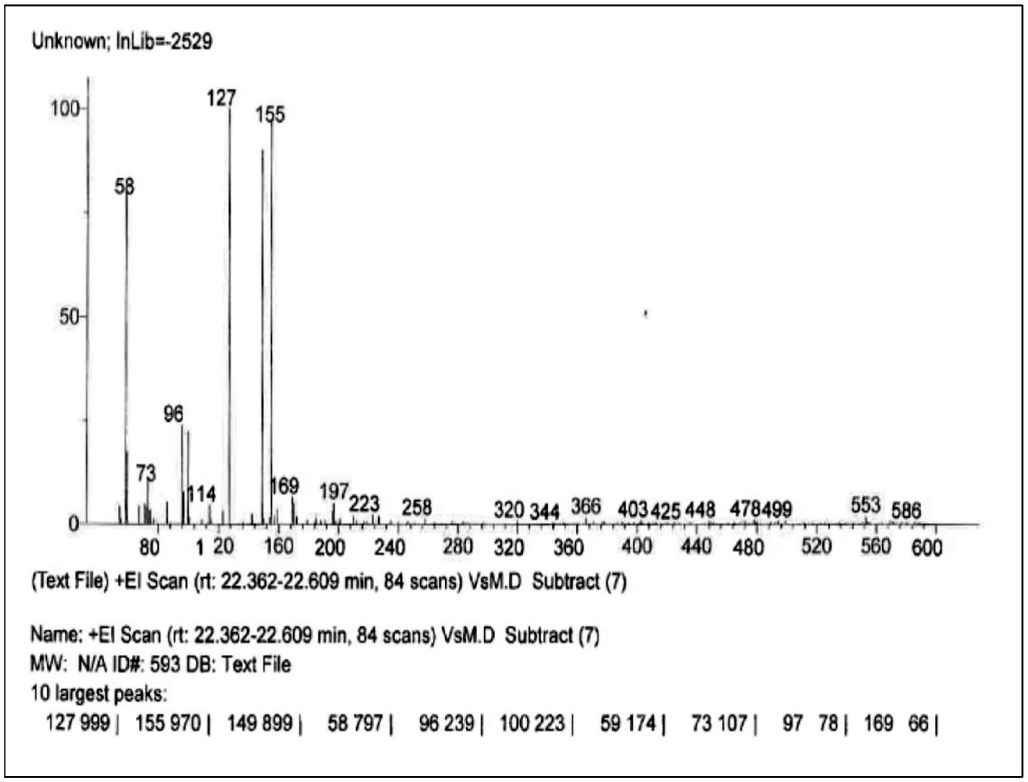
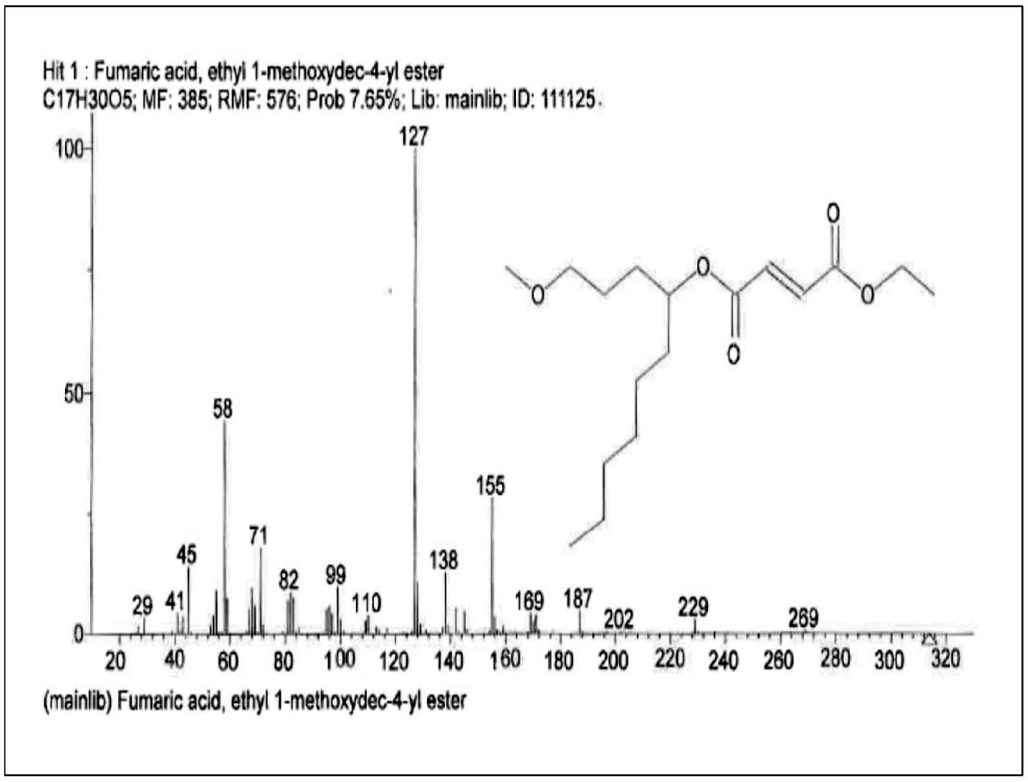
50
0
97
83
57
70
111
125
140
253
176 196 224
266 286
346
404
449 472
503
553
80 120 160 200 240 280 320 360 400 440 480 520 560
(Text File) +EI Scan (rt: 15.974-16.299 min, 110 scans) VsM.D Subtract (7)
Name: +EI Scan (rt: 15.974-16.299 min, 110 scans) VsM.D Subtract (7)
MW: N/A ID#: 590 DB: Text File
10 largest peaks:
97 999 | 83 713 | 111 545 | 57 532 | 70 437 | 84 360 | 69 339 | 98 213 | 112 170 | 71 163 |

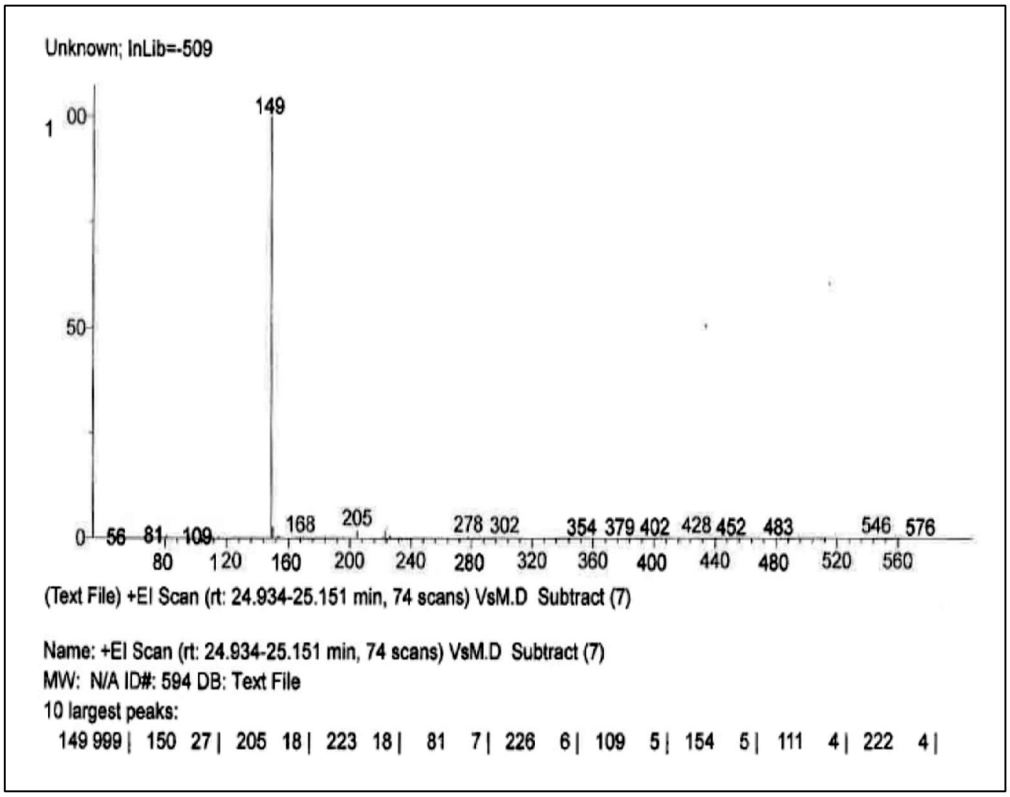

Hit 1 : 1-Tridecene
C13H26; MF: 701; RMF: 790; Prob 11.2%; CAS: 2437-56-1; Lib: replib; ID: 4634.
100
50
0
55
41
83
69
97
29
111
125
140
154
182
15
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
(replib) 1-Tridecene

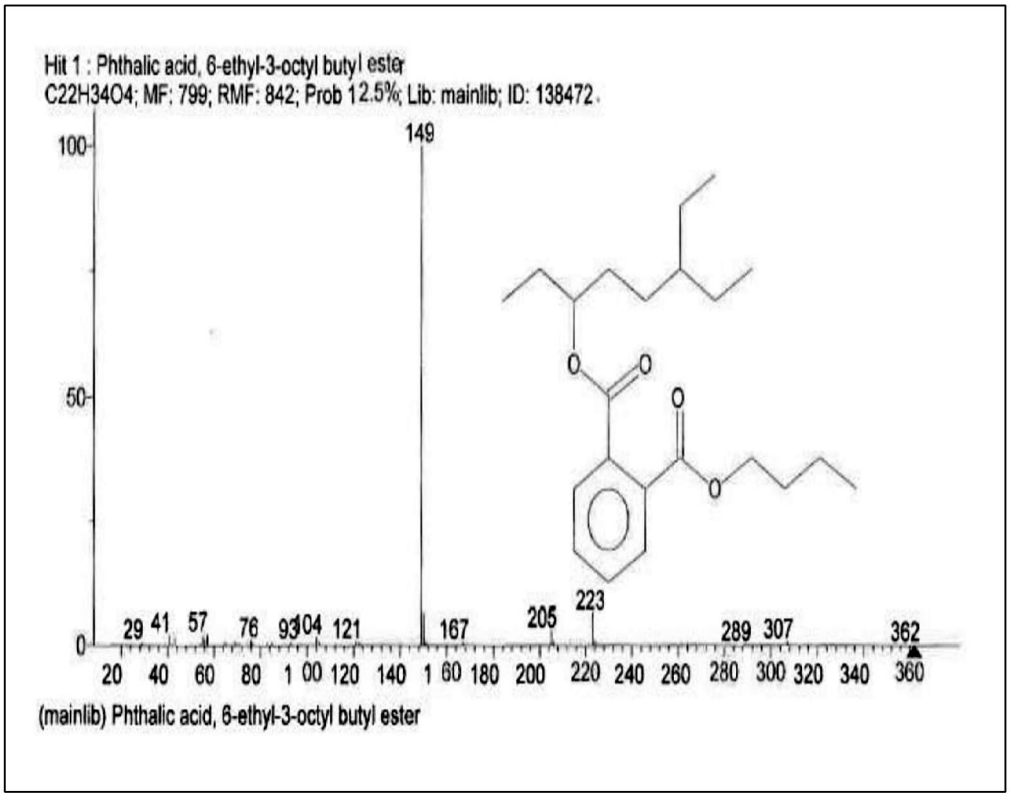

Unknown; InLib=-1032
1 00
50
0
97
83
111
57
71
125
140
178
224
249
278
343
404
428
450
478
513
535
560
581
80
120
160
200
240
280
320
360
400
440
480
520
560
(Text File) +EI Scan (rt: 20.316-20.613 min, 101 scans) VsM.D Subtract (7)
Name: +EI Scan (rt: 20.316-20.613 min, 101 scans) VsM.D Subtract (7)
MW: N/A ID#: 592 DB: Text File
10 largest peaks:
97 999 | 83 759 | 111 584 | 71 284 | 57 265 | 70 245 | 125 200 | 84 195 | 55 144 | 85 127 |


Hit 1 : 1-Nonadecene
C19H38; MF: 689; RMF: 715; Prob 7.98%; CAS: 18435-45-5; Lib: replib; ID: 14799.
100
50
0
97
43
55
83
69
111
125
29
139
154
168
182
196
210
224
238
266
20
40
60
80
100
120
140
160
180
200
220
240
260
280
(replib) 1-Nonadecene


Unknown; InLib=-2529
100
50
0
127
155
58
96
73
114
169
197
223
258
320 344
366
403 425 448
478499
553
586
80
1 20
160
200
240
280
320
360
400
440
480
520
560
600
(Text File) +EI Scan (rt: 22.362-22.609 min, 84 scans) VsM.D Subtract (7)
Name: +EI Scan (rt: 22.362-22.609 min, 84 scans) VsM.D Subtract (7)
MW: N/A ID#: 593 DB: Text File
10 largest peaks:
127 999 | 155 970 | 149 899 | 58 797 | 96 239 | 100 223 | 59 174 | 73 107 | 97 78 | 169 66 |


Hit 1 : Fumaric acid, ethyl 1-methoxydec-4-yl ester
C17H30O5; MF: 385; RMF: 576; Prob 7.65%; Lib: mainlib; ID: 111125
100
50
0
127
58
155
71
45
138
82
99
29
41
110
169
187
202
229
269
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
O
O
O
O
(mainlib) Fumaric acid, ethyl 1-methoxydec-4-yl ester


Unknown; InLib=-509
149
(Text File) +EI Scan (rt: 24.934-25.151 min, 74 scans) VsM.D Subtract (7)
Name: +EI Scan (rt: 24.934-25.151 min, 74 scans) VsM.D Subtract (7)
MW: N/A ID#: 594 DB: Text File
10 largest peaks:
149 999 | 150 27 | 205 18 | 223 18 | 81 7 | 226 6 | 109 5 | 154 5 | 111 4 | 222 4 |


Hit 1 : Phthalic acid, 6-ethyl-3-octyl butyl ester
C22H34O4; MF: 799; RMF: 842; Prob 12.5%; Lib: mainlib; ID: 138472
149
223
(mainlib) Phthalic acid, 6-ethyl-3-octyl butyl ester

5.5 STATISTICAL ANALYSIS

The Pearson's r correlation analysis depicts the curve fit model of the VsE80 and VsF7 fraction which follow exponential models with $y = 0.049e^{0.074x}$ and $y = 0.035e^{0.04x}$ for its free radical scavenging assay. As the statistical analysis involves at least one parameter with definite upper limit, exponential and power curves is more explanatory for interpolation of data in the equations. The shrunken R^2 value in *Vallisneria* leaves (88.6% ± 4.2). The Standard Error of the Estimate is the divergence of the residuals, all at 95% confidence level. In principal increase in R^2 with decreasing SEE signifies both the parameters with inverse proportionality. The SEE for Ethanol 80% was 4.270 with $R^2 = 0.886$ and that of VsF7 was 0.392 with $R^2 = 0.582$. The ANOVA tables shows the variable values have difference and is statistically significant group means at eight different concentrations with test samples and positive control as evident with (i.e., $p < 0.05$). The F value is found to be significant $p<0.05$ in both the fractions. Thus variance between the mean of two variables (% inhibition of sample and BHT) is significant concerning differential concentration and hence the null hypothesis is rejected (Fig. 29).

A.

- Model: Exponential
- Eq.: $y = 0.049e^{0.074x}$
- **F (1, 6) = 47.1, p<0.05**
- ***IC_{50} = 0.981µg/ml***

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.942	.886	.868	4.270

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	860.147	1	860.147	47.181	.000
Residual	109.384	6	18.231		
Total	969.530	7			

Correlations

		Con	Pinh	Control
Con	Pearson Correlation	1	.855**	.961**
	Sig. (2-tailed)		.007	.000
	N	8	8	8
Pinh	Pearson Correlation	.855**	1	.942**
	Sig. (2-tailed)	.007		.000
	N	8	8	8
Control	Pearson Correlation	.961**	.942**	1
	Sig. (2-tailed)	.000	.000	
	N	8	8	8

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

B.

- Model: Exponential
- Eq.: $y = \boldsymbol{0.035e^{0.04x}}$
- **F (1, 6) = 8.340, p<0.05**
- ***IC_{50}: 0.258 µg/ml***

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.763	.582	.512	.392

The independent variable is concentration.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	1.282	1	1.282	8.340	.028
Residual	.922	6	.154		
Total	2.204	7			

The independent variable is concentration.

Correlations

		% inhibition	concentration	BHT as positive control
percentage of inhibition	Pearson Correlation	1	.842**	.981**
	Sig. (2-tailed)		.009	.000
	N	8	8	8
concentration	Pearson Correlation	.842**	1	.850**
	Sig. (2-tailed)	.009		.007
	N	8	8	8
BHT as positive control	Pearson Correlation	.981**	.850**	1
	Sig. (2-tailed)	.000	.007	
	N	8	8	8

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

Fig. 29: **Graphical representation of the model summary and IC50 value of antioxidant activity of VsE80 (A) and VsF7 (B) fractions extracted from the leaves of *V.spiralis*.**

As in brine shrimp toxicity assay, the normal distribution of data on percent morality considered as the dependent variable, displayed the stem and leaf model Shapiro-Wilk significance test of 0.350, 0.306 and 0.655 ($p>0.05$), hence probit analysis for normal distribution was persuaded. In the typical cytotoxicity experiments, to assess the similarity of situations in data collection obtained one from control set and the other from the treatment set under experimentation assumed in different situation parameters and intending to observe the differences in results out of such differences in experimental condition. Kolmogorov-Smirnov Test is performed. The significance

assures the two situations are different and further that the %mortality of the treatments is solely the effects of the plant fractions introduced in the control situations. In both the fractions, Kolmogorov-Smirnov Test displayed $p>0.05$, thus validating ideal test situations. The probit analysis also test the goodness fit statistics by chi-square test to assess the adequate fit data, $p<0.05$.The best fit model was that of ethyl acetate fraction of Vallisneria leaf extract. Finally, the z-statistics is used for relationship evaluation of X-variable (Concentration) with Y-variable (Percent mortality), the significance of z-value $p\leq0.05$ in the three cases strongly supports the importance of concentration in the model and the probit/logit output. (Fig. 30, 31).

Tests of Normality

Time	1hr-Hydroethanol fraction	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.254	5	.200*	.889	5	.350

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

a. Lilliefors Significance Correction

Parameter Estimates

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.401	.044	9.083	.000	.315	.488
	Intercept	-1.043	.110	-9.441	.000	-1.154	-.933

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

Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	10.457	3	.015[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.

Tests of Normality

Time	Total	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	100	.228	5	.200*	.938	5	.655

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

a. Lilliefors Significance Correction

Parameter Estimates

Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.351	.050	6.992	.000	.253	.450
	Intercept	.137	.103	1.336	.182	.034	.240

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

Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	14.442	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.

Fig. 30: **Test of normality, Chi-square test and Z-statistics of *V. spiralis* at 1hr and 6hrs interval.**

Tests of Normality

Time	6hr-Hydroethanol fraction	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.256	5	.200*	.879	5	.306

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

a. Lilliefors Significance Correction

Parameter Estimates

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.508	.047	10.793	.000	.416	.600
	Intercept	-.774	.105	-7.340	.000	-.880	-.669

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

Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	11.972	3	.007[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. 31: **Test of normality, Chi-square test and Z-statistics of VsF7 fraction of *V. spiralis* at 6hrs interval.**

5.6 DISCUSSION

The use of plants in pharmaceuticals (Ray and Nagaiah, 2011; Simeón *et al*., 1990) dates back to Vedic ages harbouring a branch of science dealing with plant based drugs called Ayurveda. But it was only in the 19th century that the ayurvedic science has been extensively accepted worldwide and considered as an arm to modern drug therapy (Chika *et al*., 2007; Duke and Ayensu, 1985; Salgueiro *et al*., 2004), exploiting the possible antimicrobial, anti-tumour or anti-parasitic properties of various medicinal plants through Indigenous traditional knowledge (ITK) (Husain, 1992). In this aspect, the use of medicinal plants has been mostly and in principle for human treatment and its utilization in veterinary sciences has been scanty. Hence there is meagre literature support for treating fish diseases from plant based drugs. The two profound challenges for fish treatment with a non commercial drug resource are the habitat water which is a continuous variable state and second is feeding withdrawal of diseased fish. Therefore the formulation of the phyto-drugs is destined to be a disinfectant to be added in the water with infected fish or prophylactic for healthy fish. These limitations can however be nullified when the application is in the terrestrial ecosystem.

In this study, *Vallisneria spiralis* L., a population of fresh water habitat is used as resource which could be advantageous in terms of toxicity issues as it is already considered as an aquarium plant and also its availability along with habitat complementation with the target organism. 80% hydro-ethanolic fraction (VsE80) and methanol fraction (VsF7) has been isolated and purified from the leaf extract of the aquatic macrophyte. The former was found to possess anti dermatitis activity against *Mallesezia globosa* which is not only identified as a dandruff causing fungus but also a threat to deep water dwelling fish species. The latter was found to possess antibacterial activity against fresh water fish pathogens. The molecular characterization of the

compounds reveal a prospect for a potential disinfectant (Hili *et al*., 1997; Hosni *et al*., 2008) with sulphur containing (S=O) function groups which is the featured composition of commercially available plant based drugs (Bandara *et al*., 1992; Bautista *et al*., 1992; Baumgartner *et al*., 1992). The methanol fraction was found to be an active free radical scavenger with high total phenol content. The GCMS revealed the frcation to conatin long chain alkene hexacosene which is a constituent of plant essential oils. 1-Tridecene and 1- Nonadecene were found in abundance with 99% and 100% peak area. Literature studies reveal Tridecene to be a major constituent of coconut flesh (*Cocos nucifera*) and a flavouring agent (Human Metabolome Database (HMDB)). Additionally, botrydiol and ester of phthalic Acid has also been found. The polyphenol antioxidants are key to herbal drug research and development (Brader *et al*., 1997; Schultz *et al*., 1992; Sepúlveda *et al*., 2003; Mandal *et al*., 2010) with IC50 < 0.3 mg/ml but the fraction also exhibited an acute toxicity to brine shrimps with LC50 0.407 µg/ml at 1hr exposure period which decipher its use as bathing agent and not to be used as an oral treatment. Duckweed toxicity assay and the brine shrimp lethality test utilizes its role as biological indicators and serves as pre-application assessment criterion for any unfamiliar substance to be introduced in aquatic ecosystem. The inhibition of the microcystis by the plant sample was an additional gain to restore the fish habitat which would not only increase the dissolved oxygen in water but also decrease the harmful toxins (Matsui *et al*., 2007; Burits and Bucar, 2007) released by the blue green algae causing fish mortality. The study thus realizes the innovative facet of a wetland colonizer.