Aquaculture Practices – *Ipomoea aquatica* Forssk.

6.1 TAXONOMY

Ipomoea aquatic Forssk. is commonly named as water spinach, is a tropical plant with broad sharp end leaves and stems with nodes for propagation (Fig.32) belonging to the family convolvulaceae. Its tender leaves are used as green vegetable and serve as a nutritious food with high content of calcium and vitamin C. It has diverse habitat from aquatic to swamp areas and cultivated space [Wikipedia]

Fig. 32: ***Ipomoea aquatica*** **in natural habitat**

6.2 MATERIALS AND METHODS

6.2.1 Extraction and isolation of bioactive compounds

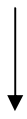
The plant samples were uprooted and washed to remove the debris attached to roots. After preliminary washing the sample was transferred to laboratory and lyophilised. The dried leaves were powdered and stored in -20˚C until use. The subsequent steps following washing and preliminary process of excision of the roots and stems, around 5kg leaves were processed by shade dry and powered using electrical mixer to obtain a dry weight of approximately 1.7kg. The plant material was initially defatted using n-Hexane in the ratio of 1:10 w/v then subjected to soxhlet extraction with chloroform,

ethyl acetate and methanol with 1:5 w/v each. The methanol fraction was dispensed to vacuum filtration to sieve any colloidal particles and subjected to column chromatography.

6.2.1 (A) Chromatography

The column chromatography was done partially with silica gel for less polar fractions and mostly with neutral alumina as stationery phase with a thin bed of 20% florisil as the stationery phase. The fractions obtained are tabulated below. The thin layer chromatography (TLC) solvent system on Silica Gel G with 13% $CaSO_4.0.5\ H_2O$ as binder was run through CH_3Cl: MeOH: H_2O :: 8:1.9:0.1 (Fig. 33).The plant fraction was allowed to stand for a week time and the hexagonal crystals formed were cleared with Petroleum Ether: Chloroform :: 1:1 flush. Borosilicate glass Columns (50 cm) are tightly packed with Silica gel 60-120 mess size with 20% Florisil up to 35 cm. The material coated silica gel in dried form is poured into it. The selection of suitable solvent system depends much on the type of material packed. Usually the solvent which dissolves the column fraction more easily is taken in lesser fraction than the sparsely soluble one. It not only helps in retention of the different fraction but also aids in clarity of segregation of spots in following thin layer chromatography. The following are the steps for extraction and isolation.

Column chromatography with 20% Florisil eluted fractions



Fractions	Solvent/Solvent System	Ratio	Quantity (ml)
SILICA GEL			
Fraction 1 (F1)	H:EtOAc	7:3	300
Fraction 2 (F2)	H: EtOAc	6:4	400
Fraction 3 (F3)	Pet Ether : CH_3Cl	4:6	450
ALUMINUM OXIDE (neutral)			
Fraction 2a (F2a)	EtOAc : MeOH	10%	250
Fraction 2b (F2b)	EtOAc : MeOH	15%	250
Fraction $2a_1$ ($F2a_1$)	CH_3Cl:MeOH	9.9:0.1	300
Fraction $2a_2$ ($F2a_2$)	CH_2Cl_2:MeOH	4.5:0.5	200
***Fraction $2a_{1a}$ ($F2a_{1a}$)**	**CH_3Cl:H_2O**	**4:1**	**300**

↓

F2a, $F2a_1$ and $F2a_{1a}$ are the subsequent fractions of F2 run on separate columns each. The F2 and F3 fractions upon elution was concentration on rotary vacuum evaporator were made moisture free by running through granular calcium chloride column.

↓

Biochemical Test → Spectral analysis

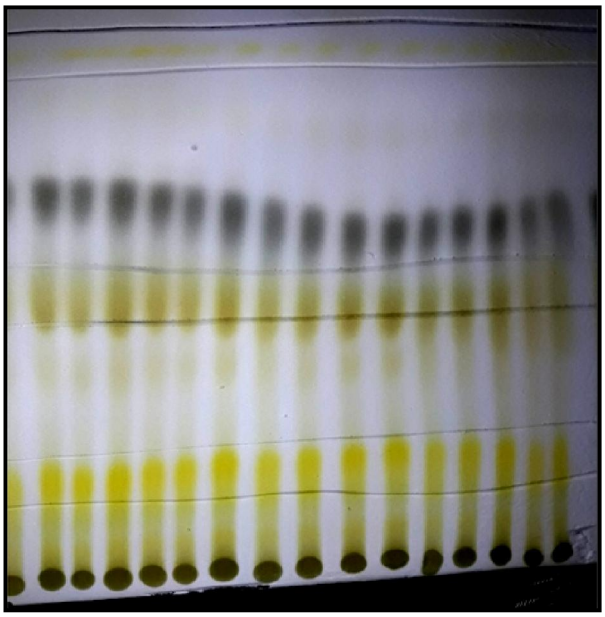


Fig. 33: **Preparative TLC of F2 fraction of Ipomoea leaves**

6.2.2 Bio-autography

The F2 and F3 fraction was subjected to direct bio-autography (DB) to screen the fraction having antimicrobial property in TLC plates (Choma & Grzelak, 2011; Cieśla, 2015; Suleiman *et al.*, 2010). After running the TLC in suitable solvent system, the TLC strip is left open in room temperature for 1-2hrs to allow complete evaporation of the solvents. Thereafter, the strip is layered with bacterial broth suspension and incubated for 24-30 hrs in humid condition. The humidity was maintained by using saturated NaCl solution. The strips are then sprayed with 2mg/ml of 2, 3, 5-triphenyl tetrazolium chloride solution which has a unique property to stain brick red in contact with live cells. The fraction having antimicrobial trait will have a white encircled cavity. The assay is a preliminary check to guide the isolation and purification of bioactive fraction from many other isolates.

6.2.3 BIOCHEMICAL ANALYSIS

6.2.3 (A) Total Phenol Content

The Folin-Ciocalteu method applied for the quantification of phenols present in the F2 fraction. The concentration range from 0.05mg/ml - 0.3 mg/ml was considered for the analysis. The results were expressed in terms of Gallic acid equivalent/gram of the powdered leaf sample, Gallic acid taken as standard. 2ml of diluted Folin-Ciocalteu reagent followed by Sodium carbonate was added to 0.1ml of the test samples was and absorbance taken at 765nm.

6.2.3 (B) Total Flavonoids Content

The flavonoids were quantified by calorimetric method as described by Jia *et al*., at 0.05mg/ml - 0.3 mg/ml with 0.1ml of the fraction. Quercetin was taken as the standard and the results expressed in Quercetin equivalent/gram of the leaf sample. The absorbance was taken at 510nm.

6.2.3 (C) Total Tannin content

The tannin content was also estimated by Folin-Ciocalteu method with 0.5ml of the reagent diluted with ddH_2O and 2ml of the F2 fraction mixed with sodium carbonate. The test samples was incubated for 30mins with periodic shaking and the sprectral reading was taken at 725 nm. The results were expressed in tannic acid equivalent/gm of the leaf sample using tannic acid as the standard.

6.2.3 (D) Antioxidant assay by DPPH method

The antioxidant estimation by DPPH (2, 2-diphenyl-1-picrylhydrazyl) was invented by Blois in 1958. The scavenging activity of DPPH by antioxidants is executed by (Hydrogen Atom Transfer) HAT process. 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 standard to maesure 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:

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

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

The antioxidant activity index (AAI) is given by equation:

Concentration of DPPH free radical (µg ml^{-1})/IC50 (µg ml^{-1}) (Figueroa *et al.*, 2014)

6.2.4 IN-VITRO BIOLOGICAL ACTIVITY

6.2.4 (A) Anti-microbial Screening

(i) Antibacterial activity

The bioactive fraction was evaluated for the antibacterial activity against facultative gram-negative bacteria *Citrobacter freundii and Edwardsiella tarda* by the agar well diffusion method (Dahiya & Purkayastha, 2012) using Muller Hilton Agar (MHA). The bacterial identification was confirmed by 16S rRNA sequencing. The annular radii of the inhibition zone (mm) were measured for two-fold dilutions of the plant sample starting from 1000 µg/ml - 62.5 µg/ml. Deionised water with 5% MeOH is used as the dissolving solvent and negative control. In brief, 8mm wells were bored 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. After 18hrs incubation at 28°C, the glass petri plates were settled for 2hrs diffusion in the bio safety chamber.

(ii) Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The two significant factors of assessment, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were calculated.

6.2.4 (B) Cytotoxicity assay - Brine Shrimp lethality Assay

Shrimp mortality tset of the *Ipomoea aquatica* leaf extract determines the LC50 values in µg/ml of active fraction 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. 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 exposure time of 1h, 6h, 12h, 18h and 24h using brine solution as the control. The results were modelled by Binomial Probit Regression analysis. To validate the model, triplicates of each concentration were tested at 95% confidence level by Finney's Probit analysis method or alternatively by IBM SPSS statistical software 20.

6.2.5 IN-VIVO FISH BIOACTIVITY

6.2.5 (A) Scientific Classification

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

6.2.5 (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).

6.2.5 (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.

(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 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%. Three different experimental groups were maintained viz; a control group with normal feed KP(+) and without being challenged, an infected or challenged group which was fed with a feed formulated (PI-F) with plant compound at 80mg/kg of feed finally a negative control KP(-) which was challenged and maintained with normal feed.

6.2.5 (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.

6.2.5 (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 in the following page:

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.

6.2.6 STRUCTURAL IDENTIFICATION

6.2.6 (A) Atomic Force Microscopy

The atomic force microscopy data was obtained from Indian statistical Institute (ISI), Kolkata. The image of the crystal was captured by an Atomic Force Microscope XE-70 (Park System, Korea) in an air conditioned room maintaining 20°C temperature. The

image was taken in Non-Contact mode with a pyramidal shaped long (aspect ratio 4:1) silicon tip. The spring constant of the cantilever was ±40 to 50 N/m and the resonance frequency was ±300 kHz. The collected image data was analysed by XEI and XEP software (Park System, Korea). The details about sample topography and fine differences of sample surface as well as amplitude profiles are vividly highlighted by the resolution of Non-Contact mode of the instrument. A suitable tiny area of 1.5 μm^2 was taken to analyse and emphasize the average roughness of the upper surface of dispersed sample (Guo *et al.*, 2002).

6.2.6 (B) Mass-Spectrometry

The mass spectrometry was outsourced from Kalyani University, Nadia, West Bengal. The Tandem Time of Flight Mass analyzer (TOF-MS) with Electro spray ionization (ES) was used for detecting the mass/charge ratio of ions with highest intensities.

6.2.6 (C) ^{1}H-NMR (Proton Nuclear magnetic Resonance, 300 MHz, D_20) Analysis

It is used for detecting the number of hydrogen atoms present in the sample. The ^{1}H-NMR was performed from Organic Chemistry Department, Calcutta University. A reference was taken for the study (Subashini & Kumar, 2017).

6.2.6 (D) FT-IR (Fourier Transform Infra-red Spectroscopy) Analysis

It detects the important organic functional groups present in sample. The characteristic function group present in the crystals was analyzed using JASCO-SP-Model Spectrometer from CSIR-IICB, Kolkata.

6.3 STATISTICAL ANALYSIS

The procedure validation of an aquatic macrophyte in a wet lab experiment is authenticated by statistical analysis of the biological activity asserted to add to good aquaculture practise. 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 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. The analysis of variance is predicted by F-value and Z-statistics.

6.4 RESULTS

6.4.1 BIO-AUTOGRAPHY

It serves as an important tool to qualify a compound as bioactive in stationery phase as silica. The thin layer chromatogram shown below is of F2 fraction which was run in the solvent system EtOAc: MeOH 10% which displayed five spots including the base. The 4^{th} spot ($F2a_{Ia}$) with R_f value 0.76 as encircled in red has a distinct zone of inhibition (Fig. 34).Hence considering the bioassay guided fractionation, $F2a_{Ia}$ was isolated for further work.

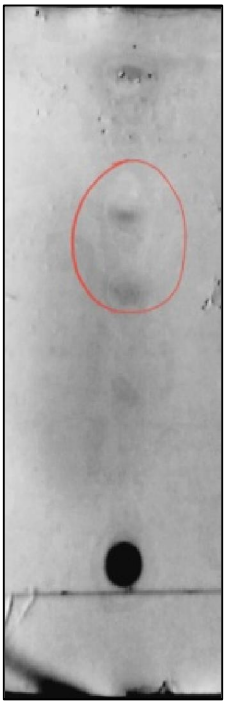


Fig. 34: **Bio-autography of F2a$_{II}$ fraction (R_f – 0.76). Zone of Inhibition (ZOI) shown within encircled area.**

6.4.2 BIOCHEMICAL TEST

The biochemical analysis consisted of phenols, flavonoids, tannins and antioxidants to evaluate the free radical inhibition potential of the test samples.

6.4.2 (A) Total Phenol Content (TPC)

Gallic acid was taken as the standard for phenol estimation and the results expressed Gallic Acid Equivalent, GAE mg/g of the leaf sample. The highest quantity of phenol was found in 0.3mg/ml with 18.45 GAE mg/g and lowest was found in the lowest concentration of the leaf sample at 0.05mg/ml with 0.83GAE mg/g of the sample. A sudden decrease in 0.2mg/ml was observed. The coefficient of determination $R^2 = 0.919$ validated the polynomial curve fit model $y= 0.769x^2+ 2.959x$ (Fig. 35).

6.4.2 (B) Total Flavonoids Content (TFC)

The graph for flavonoids in the entire plant samples followed a steady trend in proportion with the concentration of plant sample compared with Quercetin and expressed as Quercetin equivalent/g of the sample. The maximum flavonoid concentration was recorded at 0.3mg/ml with 5.21 QE/g of the sample. The alkaloids and glycosides present in the plant samples also contributes to flavonoid count. The best fit regression line followed a polynomial equation $y = -0.451x^2+1.024x$ with $R^2 = 0.984$ (Fig. 35)

6.4.2 (C) Total Tannin Content (TTC)

The tannins recorded highest quantity at 0.3mg/ml with 2.54 Tannic acid equivalent (TAE) mg/g of the sample. A general comparison of the tannic acid content reveals the $F2a_{1a}$ leaf extract of *I. aquatica* with very less tannins. The polynomial equation $y = -0.131x^2+0.299x$ shows the regression coefficient $R^2 = 0.986$ (Fig.35)

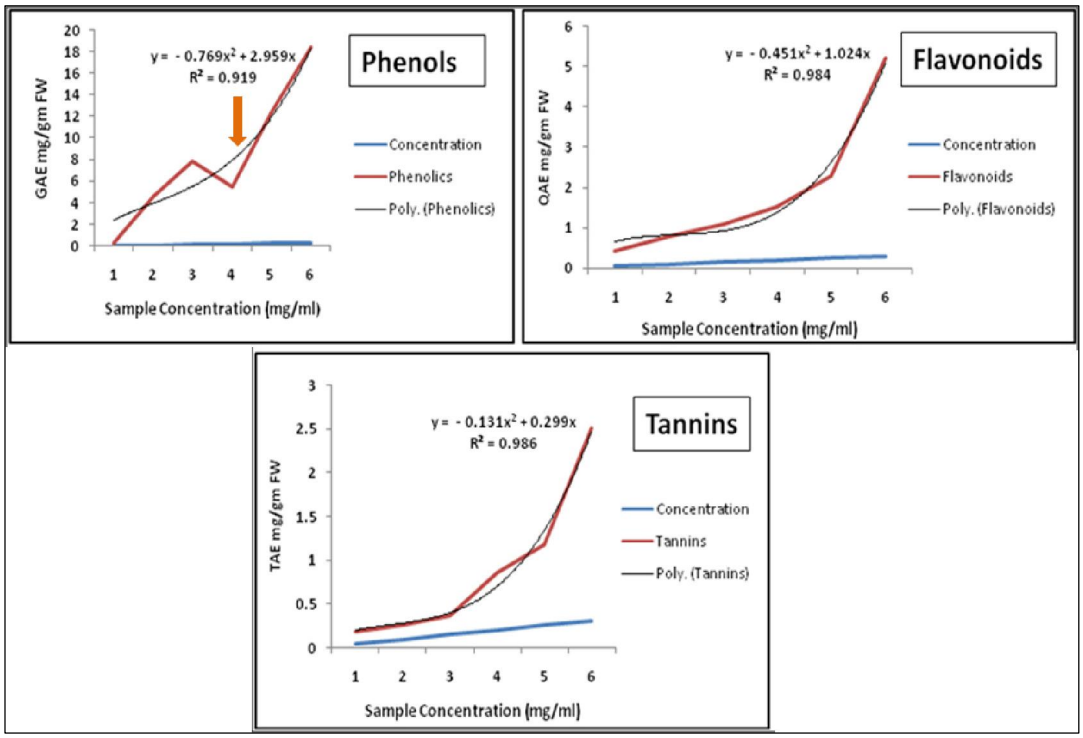


Fig. 35: **Picto-graphical display of phenols, flavonoids & tannins of $F2a_{1a}$ fraction of *Ipomoea aquatica*.**

6.4.2 (D) DPPH Radical Scavenging Assay

The comparative scavenging by DPPH (Rehman *et al.*, 2013) for $F2a_{1a}$ and F3 fractions is compared with standard antioxidant BHT. The highest scavenging % of 73.9 was found at 1 mg/ml of $F2a_{1a}$ as compared to 91.5% of the standard antioxidant. The lowest scavenging at 0.05mg/ml was recorded to 40.6%. For F3 fraction, 14.59% and 19.82% scavenging activity was recorded at the lowest and the highest concentration respectively (Fig. 36). The IC50 for $F2a_{1a}$ was 0.144 mg/ml and 5.65mg/ml for F3.Hence, it can be concluded that $F2a_{1a}$ shows prospects of a strong radical scavenger as compared to the F3 fraction. Additively, it can also be stated that the $F2a_{1a}$ fraction possesses proton donating ability preventing tissue damage. The power curve estimation model is validated by $R^2 = 0.933$ following the equation, $y = 2E\text{-}09x^{4.624}$.

The F3 fraction however followed a linear equation *y = 0.160x-2.350.* The AAI for $F2a_{1a}$ was found to be 2.41 and 1.38 for F3.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.

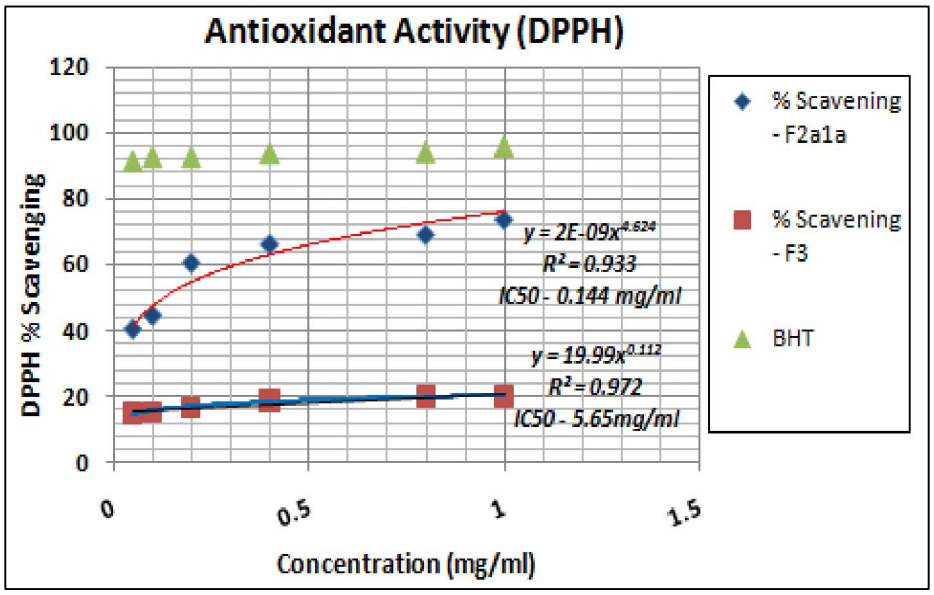


Fig. 36: **% scavenging activity of $F2a_{1a}$ and F3; Cubic (polynomial) equation model.**

6.4.3 IN-VITRO BIOLOGICAL ACTIVITY

6.4.3 (A) Anti-microbial screening

(i) Antibacterial activity

The antibacterial activity was tested against three fish pathogens, *Edwardsiella tarda* (CGH9); *Citrobacter freundii* (M7) and *Bacillus safensis* (MOH1). Two broad dilutions of the compounds (1000 µg/ml and 100 µg/ml) are tested along with the control against the 7 above mentioned bacterial species. For MIC and MBC determination a broad range of 10 dilutions ranging from 10,000 µg/ml-15.625 µg/ml was considered (Fig.

37). The compounds $F2a_{1a}$ and F3 showed inhibitory activity against three bacteria as tabulated below: (Table 5).

(ii) Minimum Inhibitory Concentration (MIC) & Minimum Bactericidal Concentration (MBC)

$F2a_{1a}$ fraction showed MIC at 62.5µg/ml and 31.25µg/ml for *E. tarda* and *C .fruendii* respectively and MBC at 10,000µg/ml and 2500µg/ml. The absorbance at 600nm reveals the MIC at 62.5 µg/ml and MBC at 5000µg/ml for F3 fraction against B. *Safensis*.

$F2a_{1a}$		
Bacterial species	**Plant Sample Concentration(µg/ml)**	**Annular radii of Zone of Inhibition (mm)**
E. tarda	Control (C)	0
	100 (B)	4.5
	1000 (A)	7.5
C. fruendii	Control (C)	3.5
	100 (B)	5
	1000 (A)	8.5
F3		
B. safensis	Control (C)	5
	100 (B)	6
	1000 (A)	8

Table 5: Bacterial zone of inhibition of $F2a_{1a}$

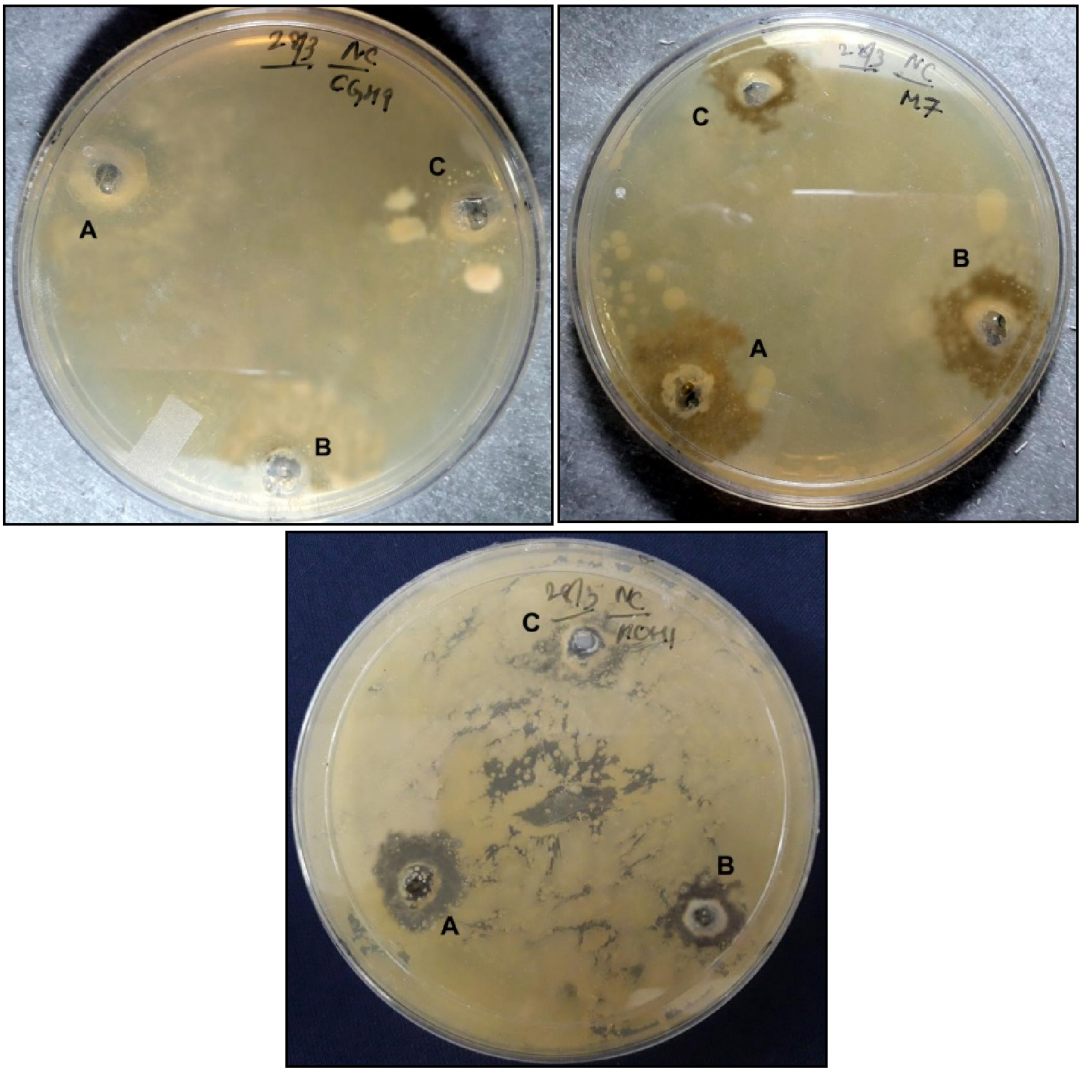


Fig. 37: **Zone of Inhibition (mm) clockwise: *Edwardsiella tarda* (CGH9); *Citrobacter freundii* (M7) and *Bacillus safensis* (MOH1)**

6.4.3 (B) Cytotoxicity Screening - Brine Shrimp Lethality Assay

The brine shrimp toxicity test of Hexane, ethyl acetate, Chloroform of which F3 is the main constituent, methanol and water which forms the $F2a_{1a}$ fraction. As the 6h, 12h, 18h and 24h-LC50 test of Hexane, Chloroform and Ethyl acetate was < 1000 µg/ml. The LC25 was highly toxic for Hexane, Chloroform and Ethyl acetate fraction (chi square 13.2-0.57; $P < 0.05$). The lowest LC50 0.027 µg/ml was recorded for Ethyl Acetate at 24h exposure rendering it the most toxic. Notably, for each fraction there was

a significant decrease in nauplii count from 100 µg/ml to 10 µg/ml on concentration scale and 12h to 28 h on time scale. For non polar fractions the lethality was more time dependent but for polar fractions it was concentration dependent (Fig.38). The scales LC25 and LC75 give us a perspective towards the toxicity pattern with concentration and the ideal dosage in context to polarity of the compounds.

1. **F3 (Chloroform fraction) -** Exposure time of 1h, 6h, 12h, 18h and 24h was recorded in the non polar fraction. The 1hr exposure recoded a *LC50 of 460.7µg/ml* and lowest at 24hr with 0.003µg/ml (Fig. 52, 54).
2. **F2a$_{1a}$ (Water fraction) -** Exposure time of 1h, 6h, 12h, 18h and 24h was recorded the aqueous fraction. The 1hr exposure recoded a *LC50 of 20000µg/ml* and lowest at 24hr with *98.45 µg/ml* (Fig. 52, 57).

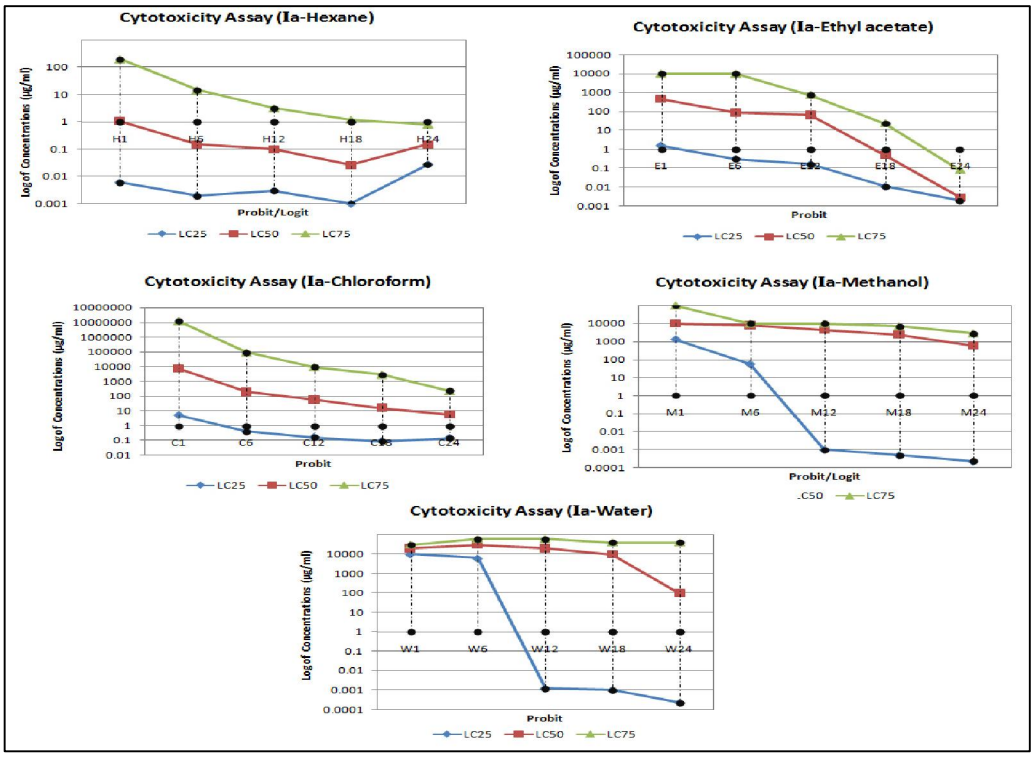
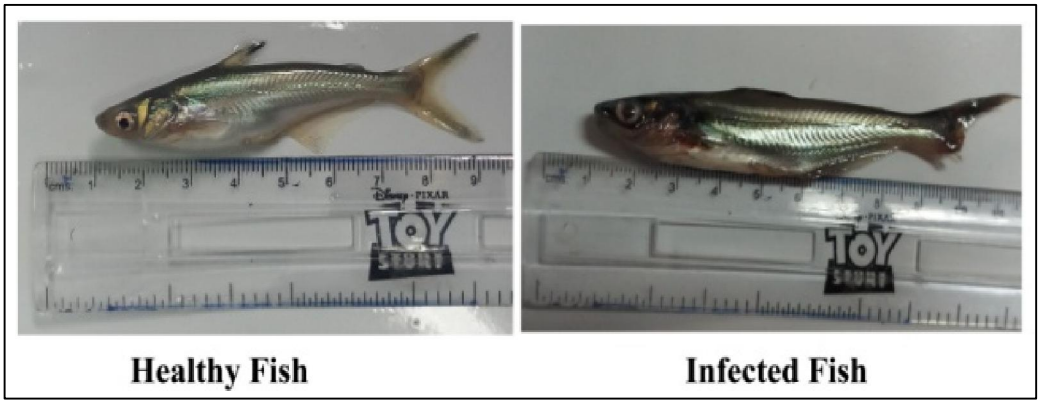


Fig. 38: **Picto-graphical representation of LC25, LC50 and LC75 for *I. aquatica* at 1hr, 6hrs, 12hrs, 18hrs and 24hrs exposure time.**

6.4.4 IN-VIVO FISH BIOASSAY



Technically better results were observed in the formulated feed (PI-F) and the clinical symptoms for the disease appeared after almost 17days as compared to 54hrs 100% mortality for the third group with normal feed (KP-). To understand the differences better, the liver and kidney histopathology of the normal, infected fish fed with formulated feed and infected fish fed with normal feed was compared in Haematoxylin eosin stain after 54 hrs.

Mortality Chart:

Treatment Sets	Total No	Mortality	Recovery Percentage
KP (+)	10	1	90
PI-F	10	5 (1 sinking)	45
KP (-)	10	10	0

Haematological parameters:

Parameters	Fresh Fish	PI-F	Infected fish
White Blood cells ($x10^3$ µl)	5.76 ±0.3	5.98±0.03	7.43±0.5
Red Blood Cells ($x10^6$ µl)	2.41±0.07	3.21±0.05	2.52±0.2
Haemoglobin (g/dL)	7.52±0.5	6.71±0.05	6.18±0.04

Liver

The liver histopathology (Fig. 39) reveals the normal liver section with intact portal triad, distinct bile duct and proportionate hepatocyte and pancerocyte (A); the infected fish fed with plant material had almost the same histopathology like the normal with only exception of cytoplasmic vacuoles (B); the infected fish with normal feed showed disintegrated portal triad, disintegrated hepatocyte and dissociation of the hepatocyte and pancerocyte (C).

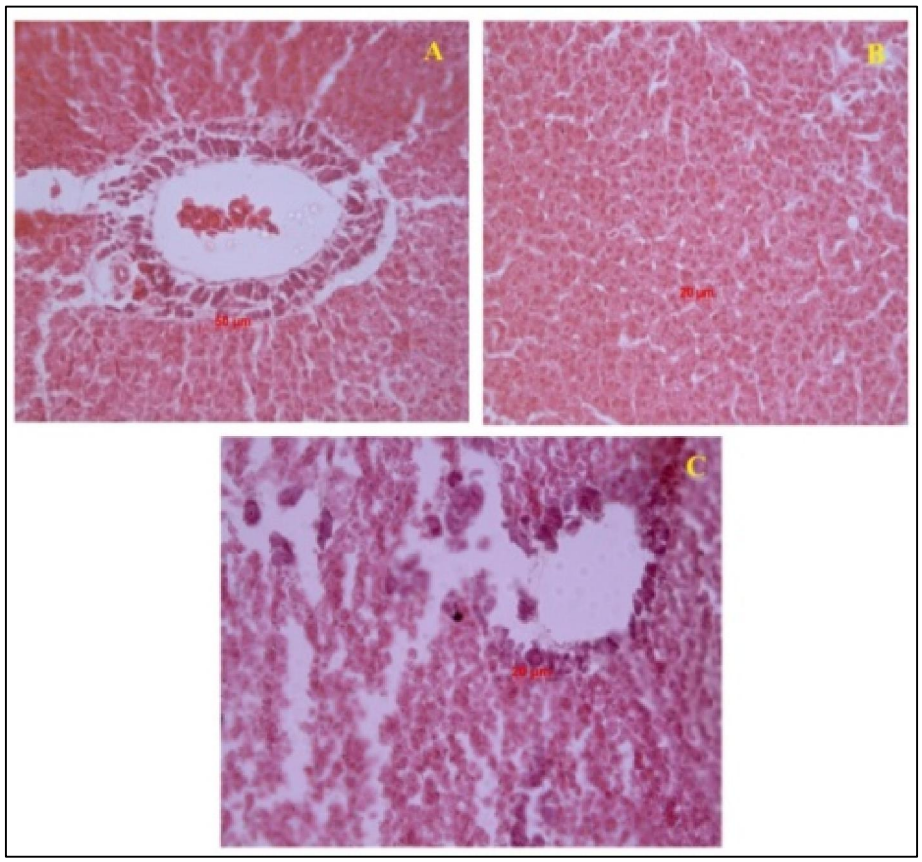


Fig. 39: Histopathology of Liver tissues of *P. hypophthalmus.*

Kidney

The kidney histopathology (Fig. 40) reveals the normal kidney section with intact Bowman's capsule with Glomerulus with intact proximal tubules (A); the infected fish

fed with plant material also was seen with prominent Bowman's capsule but some tubules were seen dilated (B); the infected fish with normal feed showed disintegrated Bowman's capsule and the sloughing of the epithelial lining of the proximal and distal tubules.

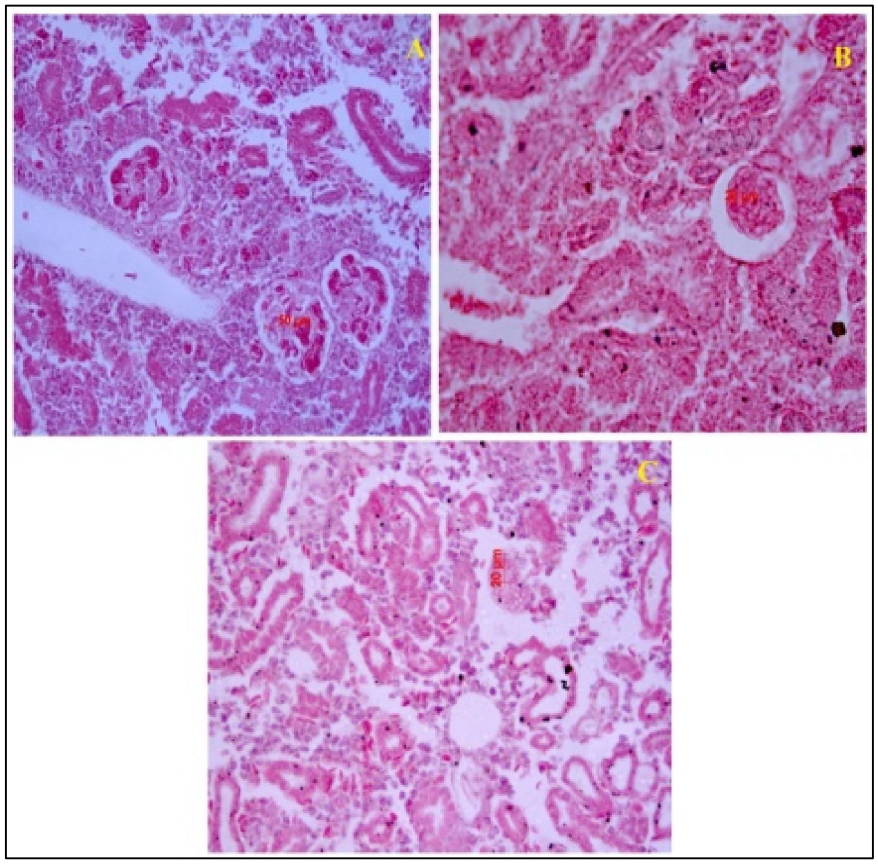


Fig. 40: Histopathology of kidney tissues of *P. hypophthalmus.*

6.4.5 STRUCTURAL IDENTIFICATION

The structural elucidation the two bioactive fractions of the leaf sample of *I. aquatica* are stated below.

6.4.5 (A) F3 fraction

(i) Mass Spectrometry

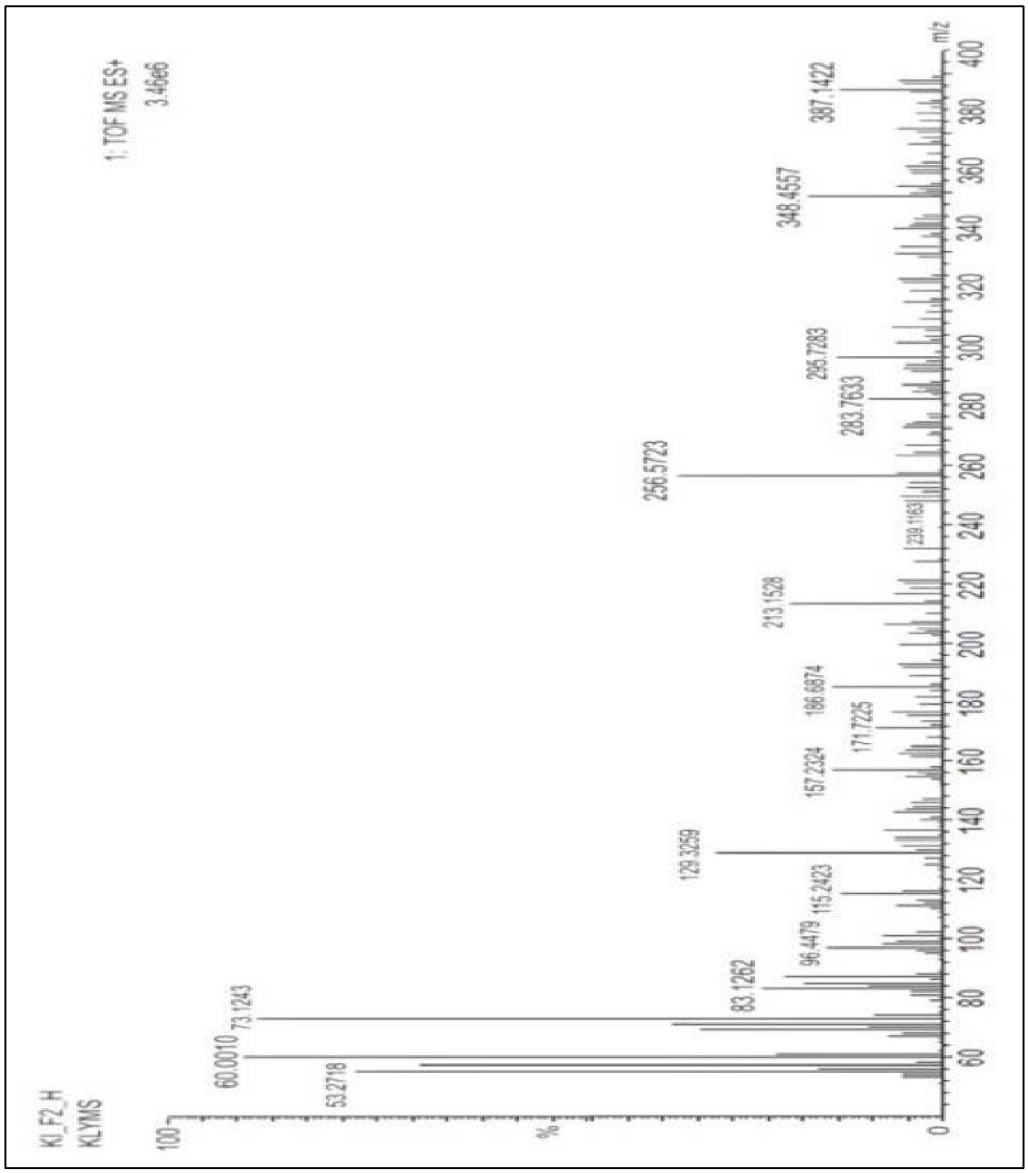


Fig. 41: **Mass spectrum of F3 fraction of *Ipomoea aquatica.***

(ii) Fourier Transform Infra-red Spectroscopy (FT-IR)

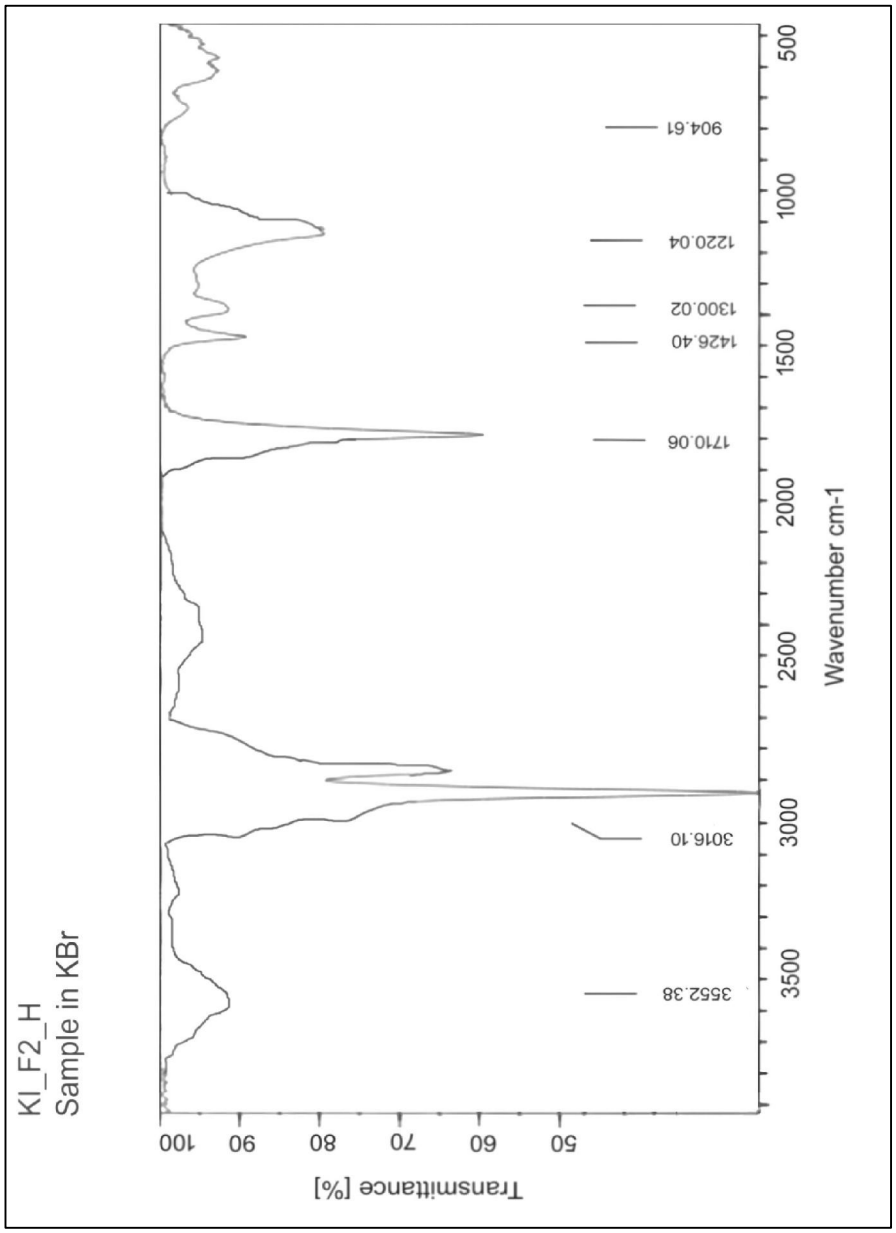


Fig. 42: **FT-IR of F3 fraction of *Ipomoea aquatica* leaves.**

(iii) ^{1}HNMR

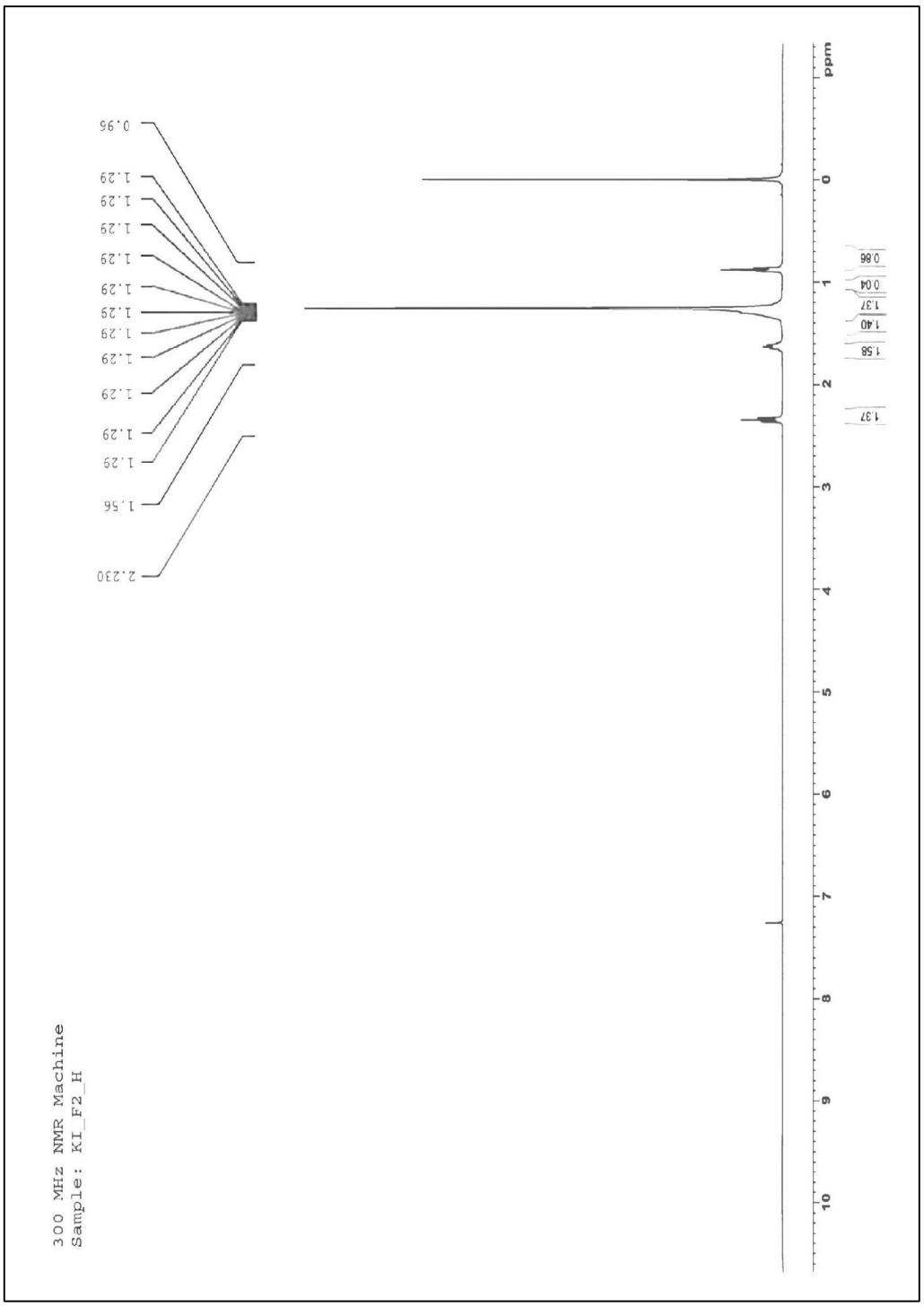


Fig. 43: **^{1}H-NMR of F3 fraction extracted from the leaves of *Ipomoea aquatica***

(iv) ^{13}CNMR

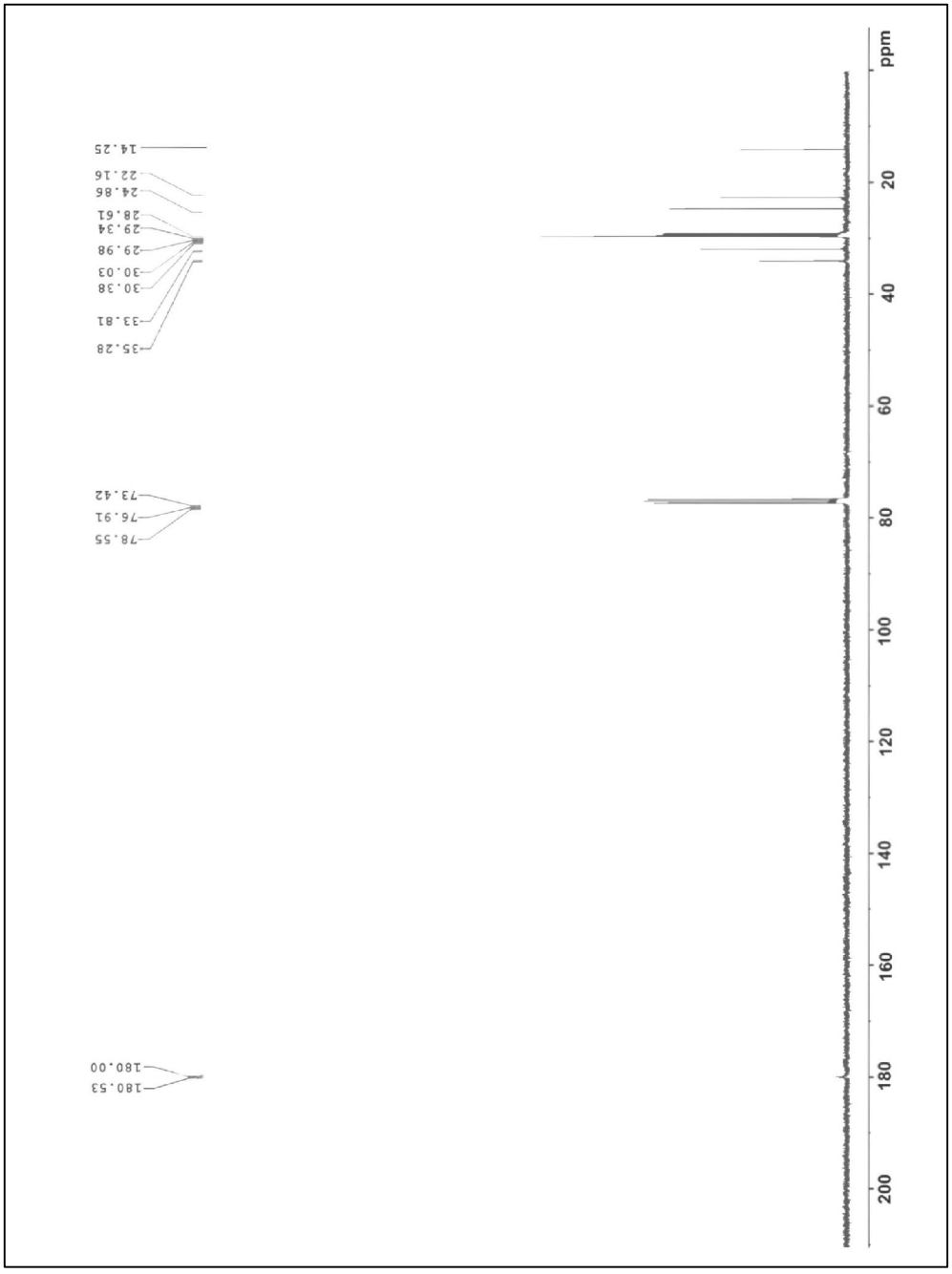


Fig. 44: **^{13}C-NMR of F3 fraction extracted from the leaves of *Ipomoea aquatica***

(v) Stereo Zoom Microscopic view (40X)

F3: N-hexa-Decanoic acid ($C_{16}H_{32}O_2$) (prismatic crystal; purity < 90%; Mol wt: m/z 256)

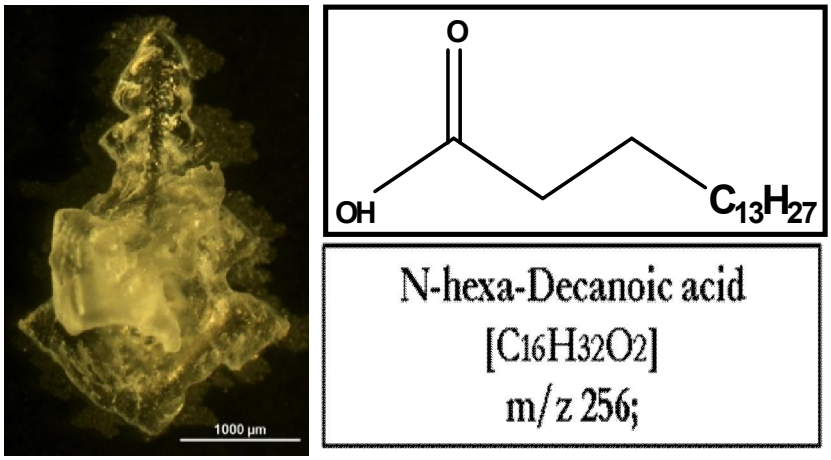


Fig. 45: **Right; Cubic crystal view under microscope, Left; structure of N-hexa - decanoic acid**

6.4.5 (B) F2a$_{1a}$ Fraction

(i) Atomic Force Microscopy

The sample was imaged (Fig: 46) by the help of an Atomic Force Microscope XE-70 (Park System, Korea). Surface phenomenon of the captured image resemblance the properties of crystalline structure. The average peaks of the crystals lies between 4 nm to 5 nm range with nearly homogeneity of dispersion. The sample was steadily exhibit in solid state although water soluble. Distribution of different types of peaks indicates presence of more than five molecules from more than two elements forming complex crystal. A crystal forming hydrous inorganic salt could be predicted. The size of the atoms shows prospects towards potent antibacterial property by bacterial cell membrane disruption.

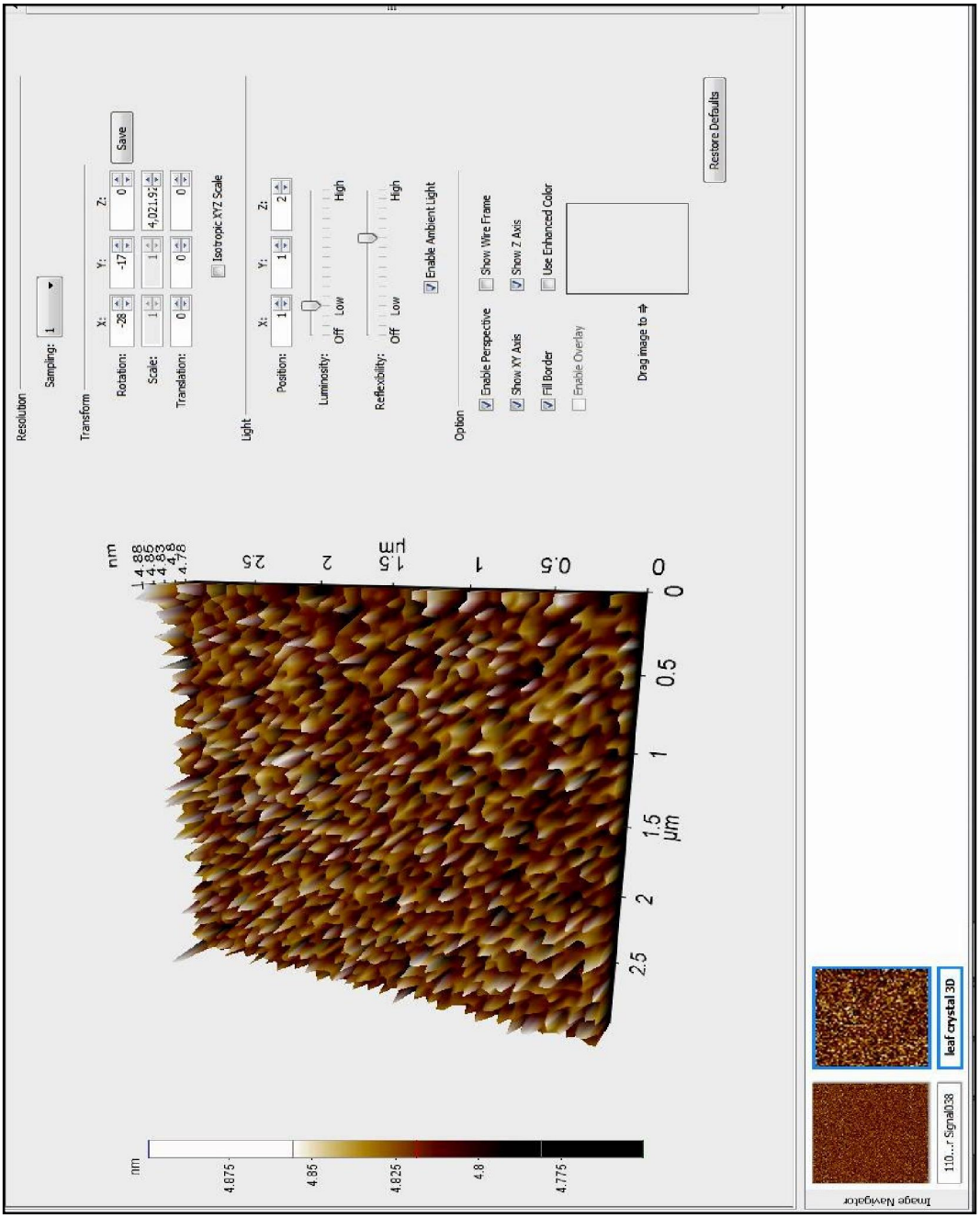
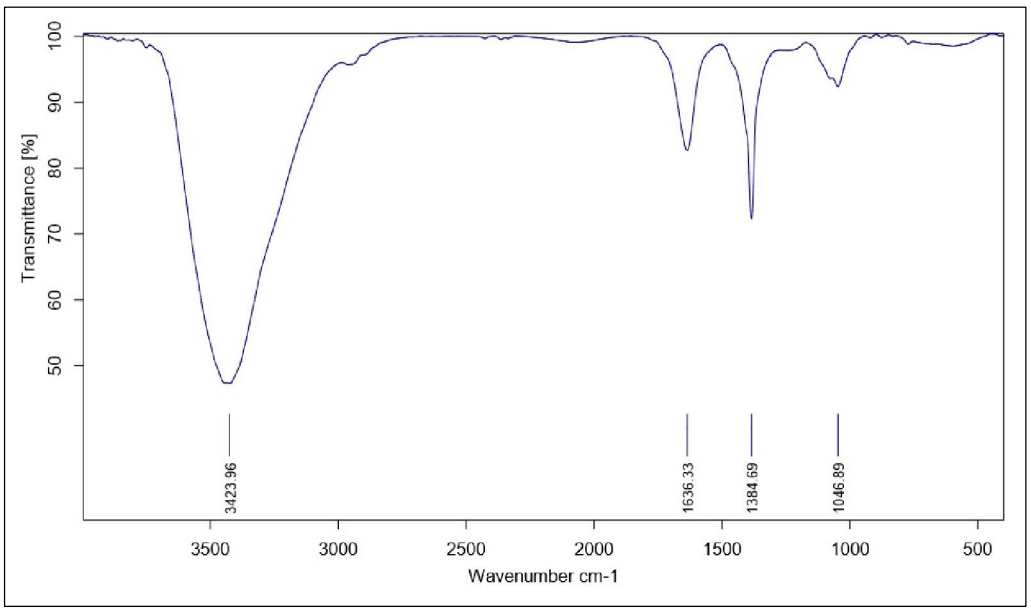


Fig. 46: **Atomic force microscopy of the crystals obtained from F2a1a fraction of the *I. aquatica* leaves.**

(ii) Fourier Transform Infra-red Spectroscopy (FT-IR)



Peak Values (cm^{-1})	Chemical groups
3423.96	OH
1636.33	Presence of water molecules
1384.69	C-H (bending/scissoring)
1046.89	C-O stretch

Fig. 47: FT-IR of F2a_{1a} fraction of the leaf sample of *Ipomoea aquatica.*

The broad and strong peak at 3423.96cm^{-1} corresponds to free hydroxyl group. The compound is of low molecular weight and probable to be inorganic salt lacking functional groups. Due its hydrophilic nature it absorbed water in room temperature which gave a peak at 1636cm^{-1}. The spectrum is see-through in the 1480cm^{-1}, 850 cm^{-1} region and 3500-3000 cm^{-1} region indicating absence of C=C and C-heteroatom. The IR spectrum therefore indicates the compound to be of aliphatic (Inderjit and Mukerji, 2006) (Fig. 47)

(iii) **Mass Spectrometry**

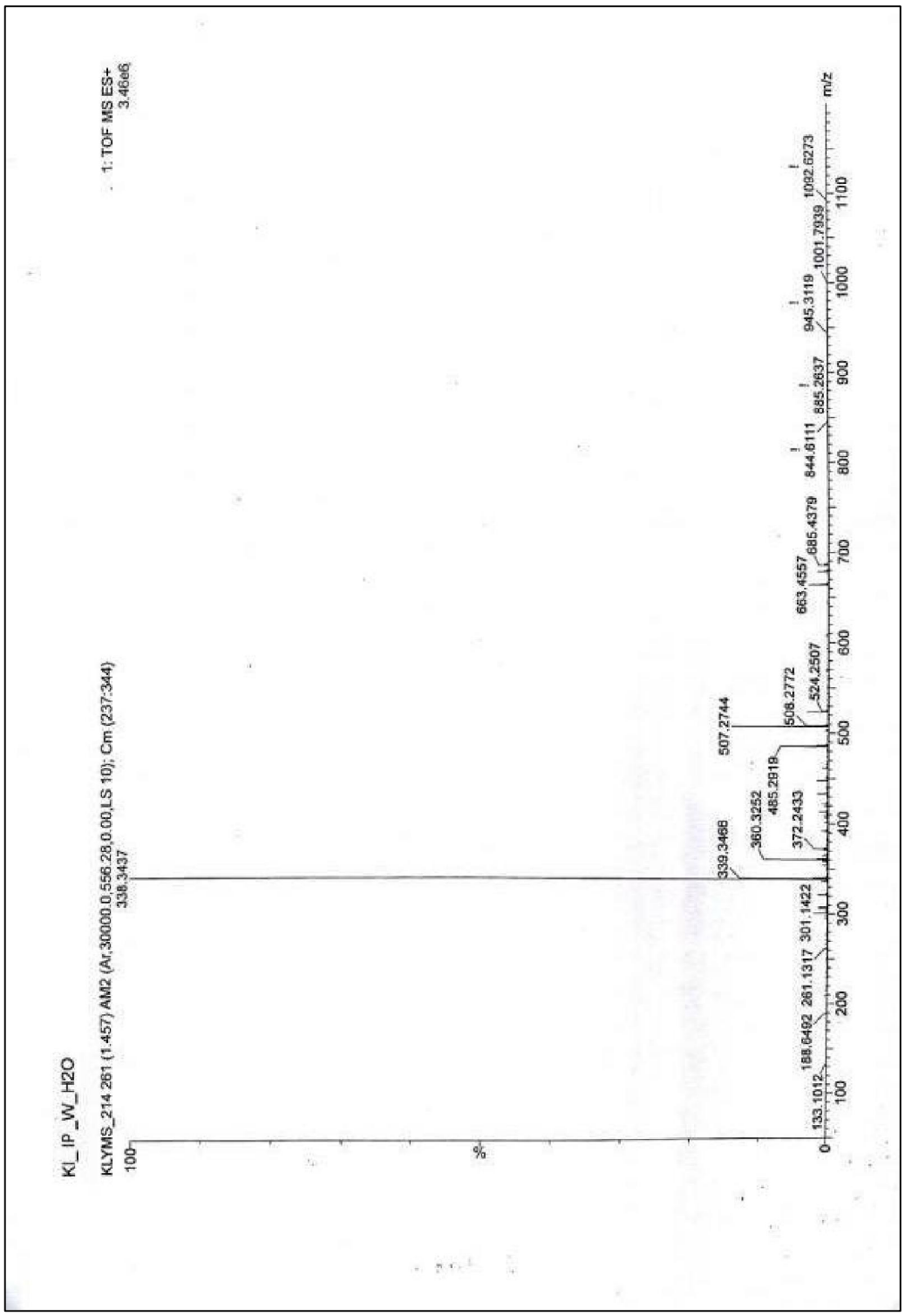


Fig. 48: **Mass spectrum of the F2a$_{1a}$ fraction of the leaf extract of *Ipomoea aquatica***

(iv) ^{1}HNMR

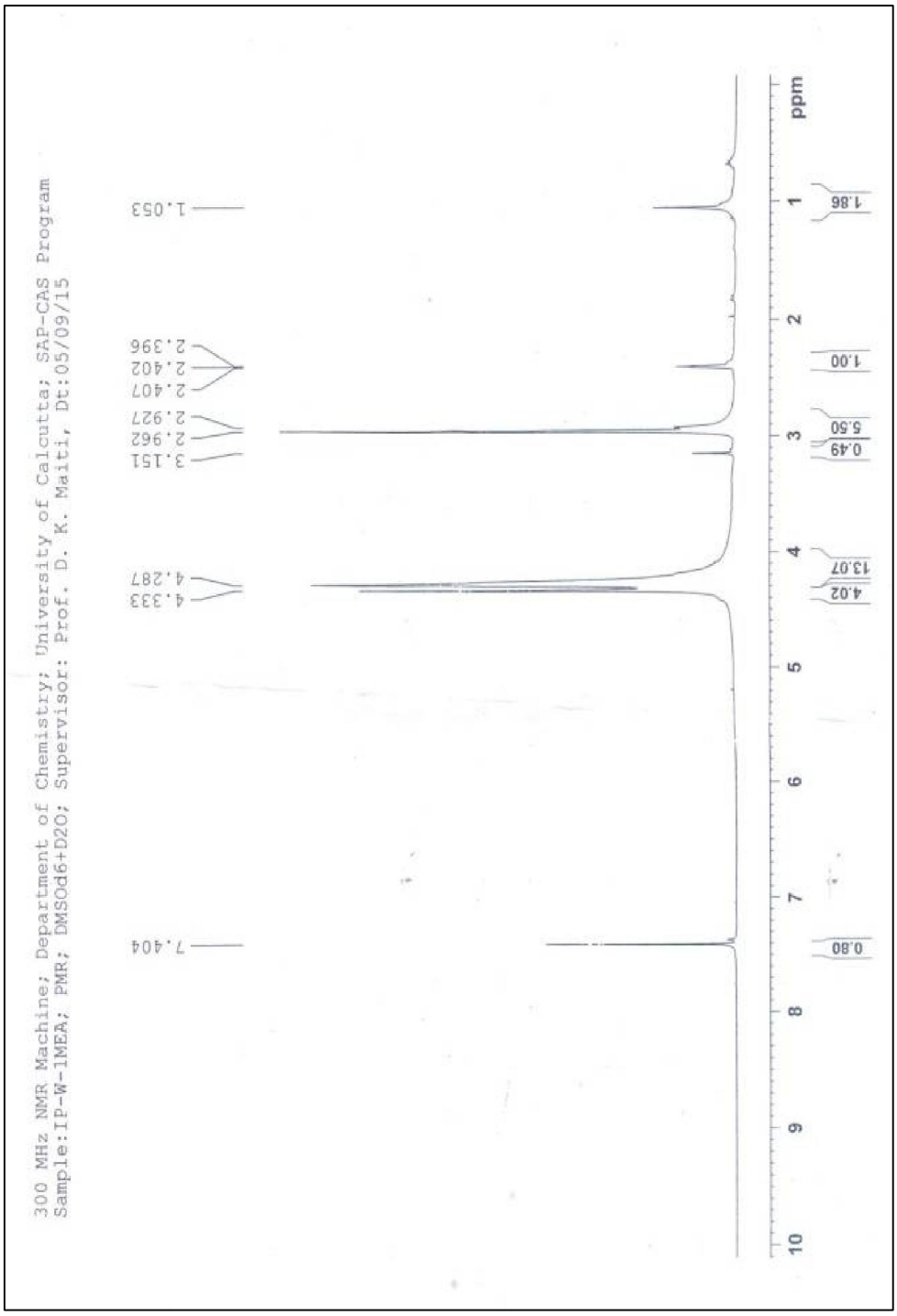


Fig. 49: **^{1}H-NMR of F2a$_{1a}$ fraction extracted from the leaves of *Ipomoea aquatica***

F8: Calcium oxalate [$CaC_2O_4 \bullet 2H_2O$]

The $F2a_{1a}$ crystal (white cubic crystal; purity < 99%) has a H shaped hexagonal edge

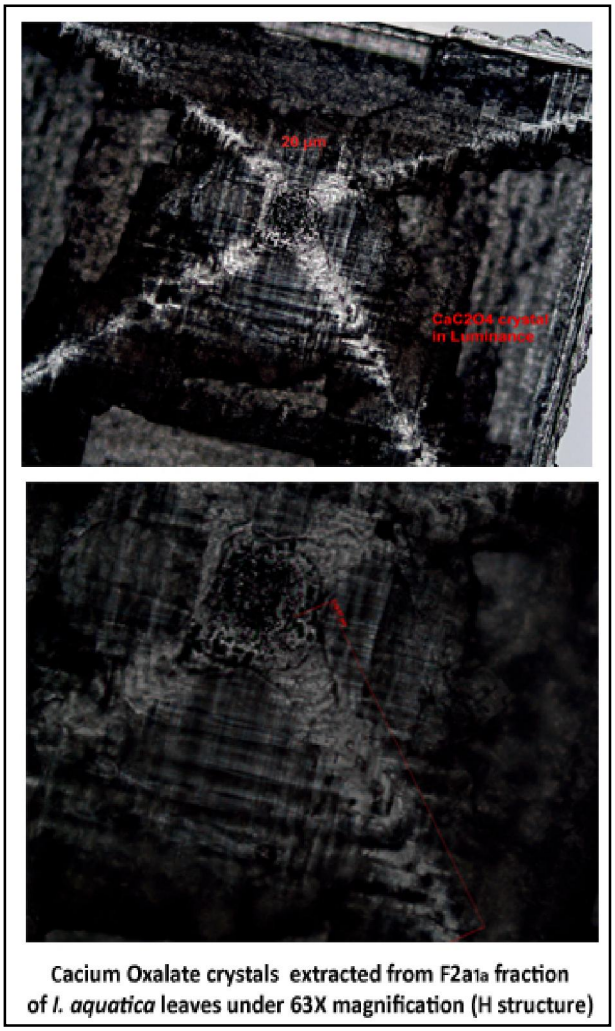


Fig. 50: Cubic crystal view under microscope 63X magnification

6.5 STATISTICAL ANALYSIS

The Pearson's r correlation analysis depicts the curve fit model for parametric data of the antioxidant test of *I. aquatica* leaves. There is a strong bivariate correlation between F3 and $F2a_{1a}$ fraction and also between the plant samples and the control, BHT.

Fraction: F3	**Fraction: F2a$_{1a}$**
• Model: Linear • Eq.: $y = 0.160x-2.350$ • F (1, 4) = 31.32, p<0.05 • IC_{50}: 5.65µg/ml	• Model: Power • Eq.: $y = 2E-09x^{4.624}$ • F (1, 6) = 56.28, p<0.05 • IC_{50}: 0.144µg/ml

F3

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.942	.887	.858	.867

The independent variable is Concentration.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	23.556	1	23.556	31.320	.005
Residual	3.008	4	.752		
Total	26.564	5			

The independent variable is Concentration.

F2a1a

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.966	.934	.917	.071

The independent variable is Concentration.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	.284	1	.284	56.282	.002
Residual	.020	4	.005		
Total	.304	5			

The independent variable is Concentration.

Correlations

		Concentration	PInS1	Control	PInS2
Concentration	Pearson Correlation	1	.942**	.945**	.876*
	Sig. (2-tailed)		.005	.004	.022
	N	6	6	6	6
F3	Pearson Correlation	.942**	1	.918**	.957**
	Sig. (2-tailed)	.005		.010	.003
	N	6	6	6	6
Control	Pearson Correlation	.945**	.918**	1	.895*
	Sig. (2-tailed)	.004	.010		.016
	N	6	6	6	6
F2a1a	Pearson Correlation	.876*	.957**	.895*	1
	Sig. (2-tailed)	.022	.003	.016	
	N	6	6	6	6

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

*. Correlation is significant at the 0.05 level (2-tailed).

Fig. 51: **Model summary and IC50 value of antioxidant activity of F3 & F2a$_{1a}$ fraction**

It shows the functioning efficiency comparable to a commercially available antioxidant. Largely followed is the power equation model as in $F2a_{1a}$ fraction of *Ipomoea aquatica* $y = 2E\text{-}09x^{4.624}$ with $R^2 = 0.934$ and linear fit model estimates the F3 fraction $y = 0.160x\text{-}2.350$ with $R^2 = 0.887$ (Fig. 51).

The shrimp nauplii mortality model depicts the Hexane fraction for 24hrs mortality test and Methanol fraction 1hr and 18hrs test of *Ipomoea aquatica* which follows non-normal distribution and hence logit analysis is followed. In case the control (sea water) has ≤10% mortality, the data correction, prior computation was done by Schneider-Orelli's formula (Schneider and Orelli, 1947)

$$\frac{\%\text{ Responded - \%Responded in Control}}{100 - \%\text{Responded in control}*100}$$

In the typical cytotoxicity experiments, to assess the similarity of situations in data collection from control set to test set in a diverse situation and further the results produced due to the differences in situations the Kolmogorov-Smirnov Test is performed (Fig. 52). The significance $p \leq 0.05$ 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 all the tests performed Kolmogorov-Smirnov Test displayed $p < 0.05$, thus validating ideal test situations. Worth mentioning, the non-normal data distribution masks this difference giving biased results. The probit analysis also test the goodness fit statistics by chi-square test to assess the adequate fit data, $p < 0.05$. The least difference in observed responses (number of cases observed in the data file that are in the cross-classification) and the expected responses (number of cases expected to see in the cell if the model is correct) shows extend of correctness of

the data as in residual. Large residuals can indicate cells that are not well fit by the model. The z-statistics is used for relationship evaluation of X-variable (Concentration) with Y-variable (Percent mortality), the significance of z-value $p \leq 0.05$ strongly supports the importance of concentration in the model and the probit/logit output. Pearson's chi-squared test measures three levels of comparison parameters: goodness of fit, heterogeneity and independence. When the p-value is too small, the variances & covariance are adjusted by a heterogeneity factor which is the chi-square to degrees of freedom. In all the cases the p value for goodness of fit test is $p \leq 0.15$; hence a heterogeneity factor is calculated.

The LC25, LC50 and LC75 values are represented graphically along with $\log_{10}$ of concentrations. In Ipomoea leaf extract, the most toxic exposure was of Hexane 24hrs (Fig. 53) (H24) followed by Ethyl acetate 24hrs (E24) (Fig. 55) and chloroform 24(C24) (Fig. 54).In these fractions LC25, LC50 and LC75 meet at very low concentration. Water fraction with 1hr exposure time (W1) followed by Methanol fraction (M1) (Fig. 56) was found least toxic with median concentration parameters at much higher concentrations. The 18hrs effect phenomenon is more prominent in non-polar fractions. The Chloroform fraction did not follow much of the trend and was found to be showing anonymous results at all three repetitions. The orders of median lethal concentration were highest for water fraction (Fig. 57) exposed for 6hrs and lowest for Ethyl acetate fraction exposed for 24hrs.

Hexane

Tests of Normality

Time	1hr-Hexane	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.183	7	.200*	.958	7	.801

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

a. Lilliefors Significance Correction

Tests of Normality

Time	6 hours- Hexane	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6.00	.168	5	.200*	.984	5	.953

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

a. Lilliefors Significance Correction

Tests of Normality

Time	12 hours- Hexane	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6.00	.241	5	.200*	.902	5	.421

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

a. Lilliefors Significance Correction

Tests of Normality

Time	18 hours- Hexane	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6.00	.232	5	.200*	.904	5	.432

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

a. Lilliefors Significance Correction

Tests of Normality

Time	24 hours- Hexane	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6.00	.349	5	.046	.771	5	.046

a. Lilliefors Significance Correction

Ethylacetate

Tests of Normality

Time	1hr hour- Ethylacetate	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1.00	.277	5	.200*	.846	5	.183

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

a. Lilliefors Significance Correction

Tests of Normality

Time	6hr hour- Ethylacetate	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.300	5	.159	.812	5	.100

a. Lilliefors Significance Correction

Tests of Normality

e	12hrs hour- Ethylacetate	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
rtality	12	.308	5	.137	.860	5	.229

illiefors Significance Correction

Tests of Normality

ne	18hrs hour- Ethylacetate	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ortality	18	.245	5	.200*	.944	5	.696

This is a lower bound of the true significance.

Lilliefors Significance Correction

Tests of Normality

ne	24hrs hour- Ethylacetate	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ortality	24	.261	5	.200*	.862	5	.236

This is a lower bound of the true significance.

Lilliefors Significance Correction

Chloroform

Tests of Normality

Time	1hrs - Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.209	5	.200*	.949	5	.733

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

a. Lilliefors Significance Correction

Tests of Normality

Time	6hrs - Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.262	5	.200*	.890	5	.357

*. 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	.267	5	.200*	.918	5	.519

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

a. Lilliefors Significance Correction

Tests of Normality

Time	18hrs - Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	18	.366	5	.027	.800	5	.081

a. Lilliefors Significance Correction

Tests of Normality

Time	24hrs - Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	24	.213	5	.200*	.958	5	.794

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

a. Lilliefors Significance Correction

Methanol

Tests of Normality

Time	1 hr hour- Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.403	5	.008	.692	5	.008

a. Lilliefors Significance Correction

Tests of Normality

Time	1 hr hour- Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.403	5	.008	.692	5	.008

a. Lilliefors Significance Correction

Tests of Normality

Time	12hrs - Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	12	.319	5	.106	.799	5	.080

a. Lilliefors Significance Correction

Tests of Normality

Time	18hrs - Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	18	.421	5	.004	.712	5	.013

a. Lilliefors Significance Correction

Tests of Normality

Time	24hrs - Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	24	.261	5	.200*	.862	5	.236

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

Lilliefors Significance Correction

Fig. 52: **Test of normality (Shapiro Wilk) and Kolmogorov-Smirnov Test of Hexane, Ethylacetate, Chloroform and Methanol fraction of *Ipomoea aquatica.***

Hexane

Parameter Estimates

Time: 1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.297	.046	6.491	.000	.207	.387
	Intercept	-.005	.100	-.049	.961	-.105	.095

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	31.784	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: 8hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBITa	Concentration	.339	.052	6.489	.000	.237	.442
	Intercept	.281	.105	2.685	.007	.176	.386

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

Chi-Square Tests

Parameter		Chi-Square	dfb	Sig.
PROBIT	Pearson Goodness-of-Fit Test	10.378	3	.016a

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
LOGIT[a]	Concentration	1.503	.297	5.060	.000	.920	2.085
	Intercept	1.252	.232	5.394	.000	1.020	1.484

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	4.959	3	.175[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	.448	.068	6.639	.000	.316	.581
	Intercept	.450	.112	4.007	.000	.337	.562

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	21.293	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. 53: **Probit and logit analysis of hexane fraction of *I.aquatica* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Chloroform

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.214	.042	5.086	.000	.131	.296
	Intercept	-.825	.107	-7.751	.000	-.932	-.719

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	2.133	3	.545[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	.253	.041	6.120	.000	.172	.334
	Intercept	-.580	.101	-5.727	.000	-.681	-.479

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	2.519	3	.472[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	.267	.042	6.406	.000	.185	.348
	Intercept	-.469	.100	-4.690	.000	-.569	-.369

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	7.092	3	.069[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	.299	.043	6.960	.000	.215	.383
	Intercept	-.362	.099	-3.643	.000	-.461	-.263

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	29.022	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	.423	.048	8.871	.000	.330	.516
	Intercept	-.326	.101	-3.220	.001	-.427	-.224

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	19.289	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. 54: **Probit and logit analysis of chloroform fraction of *I.aquatica* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Ethyl acetate

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.273	.042	6.579	.000	.192	.355
	Intercept	-.728	.103	-7.048	.000	-.831	-.624

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	8.375	3	.039[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	.290	.042	6.990	.000	.209	.372
	Intercept	-.528	.100	-5.268	.000	-.628	-.428

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	16.910	3	.001[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	.001	.000	5.749	.000	.001	.001
	Intercept	-.085	.075	-1.131	.258	-.161	-.010

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	.527	3	.913[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

Parameter Estimates

Time:18hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.402	.053	7.523	.000	.297	.506
	Intercept	.116	.104	1.122	.262	.013	.220

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	11.939	3	.008[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	.420	.102	4.127	.000	.220	.619
	Intercept	1.130	.143	7.895	.000	.986	1.273

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	4.051	3	.256[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

Fig. 55: **Probit and logit analysis of Ethyl acetate fraction of *I. aquatica* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Methanol

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LOGIT[a]	Concentration	.707	.102	6.952	.000	.508	.906
	Intercept	-3.184	.309	-10.302	.000	-3.493	-2.875

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[a]	Sig.
LOGIT	Pearson Goodness-of-Fit Test	14.726	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	.000	.000	7.093	.000	.000	.000
	Intercept	-.821	.073	-11.323	.000	-.894	-.749

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	3.949	3	.267[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	.000	.000	7.842	.000	.000	.000
	Intercept	-.534	.068	-7.899	.000	-.602	-.466

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	6.178	3	.103[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
LOGIT[a]	Concentration	.486	.070	6.975	.000	.350	.623
	Intercept	-1.093	.171	-6.376	.000	-1.265	-.922

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	35.549	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	.000	.000	4.682	.000	.000	.000
	Intercept	-.175	.067	-2.617	.009	-.241	-.108

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	1.550	3	.671[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.

Fig. 56: **Probit and logit analysis of Methanol fraction of *I.aquatica* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Water

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.000	.000	3.284	.001	.000	.000
	Intercept	-1.341	.090	-14.883	.000	-1.431	-1.251

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	4.049	3	.256[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	.000	.000	1.567	.117	.000	.000
	Intercept	-.821	.073	-11.255	.000	-.894	-.748

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	3.708	3	.295[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	.000	.000	1.171	.241	.000	.000
	Intercept	-.459	.067	-6.859	.000	-.526	-.392

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	.320	3	.956[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:18hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.000	.000	1.482	.138	.000	.000
	Intercept	-.263	.065	-4.036	.000	-.329	-.198

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	.528	3	.913[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

Parameter Estimates

Time:24hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.000	.000	1.180	.238	.000	.000
	Intercept	-.167	.065	-2.575	.010	-.232	-.102

a. PROBIT model: PROBIT(p) = Intercept + BX

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	.306	3	.959[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.

Fig. 57: **Probit and logit analysis of Water fraction of *I.aquatica* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

6.6 DISCUSSION

A plenteous research has been made on calcium oxalate crystallization from plant sources (Parekh & Chanda, 2007) and most of its implications have been stated in the host plant. It was in 1976 when Zindler Frank reported the role of oxalate in host photosynthesis pathway (Zindler-Frank, 1976). Following which, in 1980, Franceschi and Horner have stated calcium oxalate to be a metabolite formed in plants to eradicate the toxicity caused by excess cellular calcium accumulation (Çalişkan, 2000). There are also reports on calcium oxalate crystals serving as plant defence means against herbivores (Korth *et al.*, 2006; Konno *et al.*, 2014; Faheed, *et al.*, 2013). Also there are studies which assess the correlation of occurrence of calcium oxalate in poisonous plants (Konyar *et al*., 2014).

In this study, 1.6 % of calcium oxalate crystals were extracted from the aqueous fraction of the dried *I. aquatica* leaves and an endeavour has been made to explore the alternate applications of the inorganic salt crystals beyond the producer plant. The crystals were screened for their free radical scavenging capacity which was found to be strong with antioxidant activity index (AAI) of 1.38, however IC50 at 0.144 mg/ml is considered to be moderate (Emelda, 2015). The Antioxidant Activity Unit was however found to be strong. In contrary there are reports where excess of calcium oxalate crystals in mammals have induced lipid per oxidation and free radical generation (Abhirama and ShanmugaSundaram, 2018).This shows that calcium oxalate crystals in excess serves versatile task both in plants and animals, imparting protection against predators in the former and generating reactive oxygen species in the later.

Direct bio-autography on a silica (SiO_2) strip demonstrated a swift, economical and sensitive technique for objective based fractionation and isolation of the bioactive

compound. The crystals displayed a strong antibacterial activity against *C. fruendii* with MIC at 31.25 ppm. *C. freundii* is a fish pathogen which is facultative in nature and causes injuries and high rate moartality in fish. It leads to generative anemia, leukopenia, lymphocytosis, and leukoblastosis (Thi *et al.*, 2013; Junior; *et al.,* 2018).The atomic size of the crystals measuring 4-5nm as revealed by the atomic force microscopy assumes that the antimicrobial potency of the crystal is apparently due to bacterial cell membrane disruption. In addition to the known traditional values of *I. aquatica*, this study further contributes towards its virtue of beneficial weed. The isolation & structural characterization of this compound was generated by nuclear magnetic resonance and infra-red spectroscopy.