

Leaves of *Vallisneria* Finds Source to Anti Dermatitis: Enriching Wetland Ecosystem

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Abstract

The renaissances of phyto-medicines lead to a resolute search of potential medicinal plants worldwide. In this perspective, predominantly, terrestrial allelochemistry has been an imperative tool to evaluate the drug potentiality of a plant. However, in aquatic ecosphere, the pharmaceutical evaluation of the aquatic macrophytes is an emerging aspect. Our study seeks to explore the curative traits of the exotic macrophyte, *Vallisneria spiralis* L. (Hydrocharitaceae), a perennial stoloniferous species and a key ecological community in freshwater wetland ecosystem. It is commonly used as aquarium plant and inhabits both in lentic and lotic environment of tropical and sub-tropical regions worldwide. Being rapid colonizers of aquatic ecosystem, literature study states the leaves of the macrophyte can excrete special function groups that can absorb, filter and precipitate chemical compound of water and through the auxiliary function of the microorganism. Considering these significances, we have extracted the leaf leachates of *Vallisneria* in 80% ethanol which had been purified by solid-liquid extraction process and further crystallized and subjected to biochemical analysis viz; phenols, flavonoids and tannins followed by antioxidant scavenging and microbial screening. Further the isolated compound was subjected to Mass and Fourier Infra-red Spectrometry. Comprehensively, all the experimental assays infer that *Vallisneria* leaves contain bioactive compound with mol wt. m/z 359 structured with microbial growth inhibiting functional groups which were found fungicidal against *Malassezia globosa* - the dandruff causing dermatitis fungus.

Keywords: *Vallisneria spiralis*, Wetlands; *Malassezia globosa*; Fungicidal

Abbreviations:

MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicidal Concentration; GAE: Gallic Acid Equivalents; QE: Quercetin Equivalent; TAE: Tannic Acid Equivalent; DPPH: 2, 2-diphenyl-1-Picrylhydrazyl; DMSO: Dimethyl Sulphoxide; FT-IR: Fourier Infra-red

Introduction

An integrative ecosystem demands biological interactions which accords for survival values amongst life. The competition between different photoautotroph [1-3] for resources in water body changes the succession of species which is otherwise an outcome of interactive secondary metabolites in progressive plant populations. Virtually, various theoretical and experimental citations have been stated decades back on the role of these metabolites not only for synthetic innovations as drug adjuvant [4,5] but also on natural ecospheres including aquatic macrophyte and their indulged impact on algal bloom and phytoplankton [6,7]. Field evidences and literature studies confer that all primary producing organisms (cyanobacteria, micro-and macroalgae as well as angiosperms) are capable of producing and releasing these active compounds [8]. However, utilizing there *in situ* exudations in outward pharmaceutical implications is a fascinating aspect and a fetch for ayurvedic sciences [9] Some Indigenous

technical knowledge (ITK) and a few chemical perceptions through experimentation lead to various knowledge outsourcing to insight into the medicinal traits of many plants.

Vallisneria spiralis L. is a common submerged rooted macrophyte found in many wetlands, shallow ponds, lakes, marshes and streams of West Bengal. It is an extensively wide stretched colonizer [10-12] and the dispersal of the species can take place both through human and natural means via wind or water. Literature cites its role as appetizer, refrigerant, demulcent and women complaint (leucorrhoea) and used for stomach ache [13,14]. The plant is also found to bio remediate the tannery effluents [15]. The fact that the species is also capable of changing the pore water chemistry towards a more oxidized state [16] reveals its microbial static traits. This study aims to use the leaf leachates of *Vallisneria* against the most troubleshoot dermatitis fungi *Malassezia globosa*. The experiment was initiated with four different fractions with 0%, 40%, 80% and 100% ethanol in water but only the 80% ethanol fraction was found to show activity and in accordance to that this paper demonstrates only the data relevant to this fraction.

Methods and Materials

Collection and preparation of plant sample

The plant samples were collected from wetland water bodies of Nadia district of West Bengal, India, where the growth was intense. The plants after uprooting were washed in tap water and carried to the laboratory in sterile polypropylene and rewashed thoroughly with

double distilled water, air dried and weighed in sterile bags and processed within 24 hrs of collection [17].

Extraction of bioactive compounds

Following the preparative steps, the plant leaves (7 kg fresh weight/set; excised of roots) were completely immersed in glass jars with perforated lids in increasing ratios from 0%, 20%, 40% and 80% ethanol using milipore water. The amount of solvent added was in the ratio of 10:1 with respect to the fresh weight of the plants in each jar. The jars were kept in room temperature with sufficient sunlight initially for a span of not more than three days to prevent auto toxicity resulting into decolouration and foul odour. Around 3 sets were required to obtain desirable amount. The crude extracts of the four fractions were collected and concentrated to dryness by rota vap and subjected to the following biochemical analysis.

Biochemical assay

Phenols

The total phenolic content was determined following Folin-Ciocalteu method [16] using 0.1 ml of the extract with a concentration range of 0.05-0.3 mg/ml of the leaf leachate. The extracts were mixed with Folin-Ciocalteu reagent and Sodium carbonate (Na_2CO_3) following incubation for 30 mins at room temperature. The change of colour was measured in spectrophotometer with absorbance reading at 765 nm. Gallic acid in the same concentration as the sample was used as positive control. The total phenolic content was expressed as GAE in milligram per gram of dry material using the calibration curve, where X was the absorbance and Y was GAE (mg/g).

Flavonoids

Flavonoid estimation was carried out following the method of Jia et al. [18]. The preferred concentration range for the leaf leachates were 0.2-1.2 mg/ml with 0.1 ml of the extract. Later the extract was added with 1.2 ml distilled water, 0.12 ml of 5% Sodium nitrite (NaNO_2) with uniform intermixing. Following incubation for 5 mins at 25°C temperatures, 0.12 ml of 10% AlCl_3 solution was added and mixed thoroughly. Then the tubes were further incubated at room temperature for 5 minutes and added with 0.8 ml of 1 mM Sodium hydroxide (NaOH) solution and 1.16 ml of distilled water. The absorbance was measured at 510 nm. Methodically, quercetin in the same concentration as the sample was used as positive control. Total flavonoids content as calculated as Quercetin (mg/g) using the calibration curve, where X was the absorbance and Y was QE (mg/g).

Tannins

European Commission [19] reference method was used to determine the total tannins content using tannic acid as standard curve. Briefly, 200 μL of extracts of 0.05 – 0.3 mg/ml was mixed with 200 μL of ferric ammonium citrate (0.35%) prepared freshly and 200 μL of ammoniac (0.8%). The absorbance of the mixture was measured at 525 nm. The results were expressed as TAE mg of per gram of extracts or fractions.

Antioxidant assay

DPPH radical scavenging activity: The free radical scavenging activity of extracts and fractions for the radical DPPH was measured as described [20,21]. Freshly prepared DPPH solution (25 mg/L) in

methanol was prepared and 3.9 ml of this solution was mixed with 0.1 ml of extract in methanol containing different concentration range (0.05-0.3 mg/ml conc.) of the extract. 30 minutes later, the absorbance was measured at 517 nm using Spectrophotometer. Butylated Hydroxy Toluene (BHT) in the same concentration as the sample was used as positive control. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\{Ac - At\}}{Ac} \times 100$$

Where Ac is the absorbance of the blank reaction and At is the absorbance in presence of the sample of the extracts.

IC50 which defines the concentration of the plant extract that's needed to scavenge 50% of the radical present was calculated by the following equation:

$$\text{IC50} = \frac{\{\text{Percentage Inhibition}\}}{\{\text{Concentration of the sample}\}} \times 50$$

Microbiological screening

Antimicrobial activities of different extracts were evaluated by the agar well diffusion method Mayser et al. [22] modified by Murray [23] and Minimum inhibitory concentration [24-26].

Sample and Media preparation

The crude plant extracts were concentrated to dryness and resuspended in 20% DMSO [27]. A dilution range of 1000 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ was prepared with DMSO as a control. For agar well diffusion method, the fungal sample was collected from human and cultured in sabouraud agar. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined by serial broth dilution assay using sabouraud dextrose broth. All the media prepared was sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method

The well diffusion method was used to determine the antifungal activity. The plates were swabbed (sterile cotton swabs) with the overnight fungal culture from sabouraud dextrose broth. Single well was punched in a petri plate using sterile cork borer. 100 μl of each concentrations of plant extracts were added into the wells and allowed to diffuse at room temperature for 3 hrs along with the control plates. The plates were incubated at 28°C for 36-48 hrs.

Minimal Inhibitory Concentration (MIC) and Minimal Fungal Concentration (MFC)

Broth dilution method was preferred to perform the MIC assay. The dilutions were chosen depending on the results of the well diffusion assay. The inoculum was loop transferred to 0.85% saline solution and the turbidity adjusted to 0.5 McFarland to ensure 5×10^5 CFU/ml after addition to the dilution tubes. 20 μl of the inoculums was transferred from saline solution to Sabouraud dextrose broth. 5 μl of the inoculums was transferred to all the experimental tubes within fifteen minutes after transferring in the broth. The tubes were incubated in 28°C for 18 hrs and the lowest concentrations without visible growth were the MIC. The higher concentrations above to MIC were plated in sabouraud agar and incubated in 28°C for 48 hrs; the lowest concentration without any visible growth was conferred as the MFC indicating 99.5% killing of the original inoculums [28].

Purification and Instrumental Analysis

The compound was isolated by chromatographic technique and deposited for Mass and FT-IR spectrometry to confer the molecular weight and functional groups.

Results

Biochemical analysis

The results of the biochemical assay for the four different fractions are tabulated in Table 1. Since fraction 4 was found to contain considerable amount of antioxidants, phenols and flavonoids [29], further work was carried out only with fraction 4 as well the data description for fraction 4 is stated below.

Fractio ns	Bio- Chemical Assay	Concentration (mg/ml)					
		0.05/0. 2	0.10/0. 4	0.15/0. 6	0.20/0.8	0.25/1.0	0.30/1. 2
0% ethanol	A	0.16	3.24	7.59	6.01	11.88	15.43
	B	0.43	0.68	1.92	1.28	2.6	3.77
	C	0.11	0.19	0.36	0.85	1.03	1.21
	D	2.53	6.47	12.58	10.01	16.01	20.22
20% ethanol	A	0.12	2.18	5.37	2.61	6.77	8.4
	B	0.18	0.32	0.69	1.34	2.18	4.82
	C	0.1	0.39	0.33	0.46	0.89	1.06
	D	2.68	5.37	7.98	9.56	21.32	26.49
40% ethanol	A	0.18	3.73	4.51	4.98	9.85	15.61
	B	0.1	0.32	0.38	0.08	1.06	3.49
	C	0.28	0.25	0.32	0.37	1.06	1.59
	D	1.65	5.29	9	9.82	22.91	25.66
80% ethanol	A	0.38	5.29	8.64	6.78	13.56	19.23
	B	0.68	0.76	2.64	3.53	3.58	6.91
	C	0.56	0.58	0.64	0.49	1.27	1.88
	D	5.2	9.56	15.46	11.28	21.01	28.97

Biochemical analysis of the four different fractions extracted from the leaves of *Vallisneria spiralis* L. A- Phenols; B-Flavonoids; C-Tannins and D-Antioxidant activity. The concentrations 0.5 mg/ml-3.0 mg/ml are for phenols, Tannins and Antioxidant activity and 0.2 mg/ml-1.2 mg/ml is specific for Flavonoid assay.

Table 1: Biochemical Analysis.

Phenols

The Total Phenol Content was expressed as Gallic acid equivalents (GAE) in mg/g fresh weight. The experimental data exhibits (Figure 1A) a proportionate increase along the concentration gradient although a sudden fall at 0.2 mg/ml with 6.78 GAE mg/g and highest noted at 0.3 mg/ml with 19.23 GAE mg/g of (0.05 – 0.3 mg/ml) extracts concentration. The result is validated with the coefficient of

determination ($R^2=0.963$) which reads the data as best fit along the regression line.

Flavonoids

The Total Flavonoid Content was expressed as Quercetin equivalents (QE) in mg/g fresh weight. The flavonoid content showed a consistent data (Figure 1B) evenly throughout the concentration range. It increased thoroughly with raise in concentration and 1.2 mg/ml extract had the highest flavonoid content with 6.91 mg/gm QE. Presence of flavonoid may result as alkaloids and glycosides in the plant extract. The R^2 value 0.935 was found to be significant and defines the goodness of fit of the data along the regression line.

Tannins

The Tannin Content was expressed as Tannic acid equivalents (TAE) in mg/g fresh weight. The progress of the data for tannin and flavonoid was found to be quite similar by way of steady increase with concentration of the leaf extract. The highest tannin content was recorded at 0.3 mg/ml of the leaf extract with 1.88 mg/gm TAE (Figure 1C). The strength of the response variables and the model was found to be significant with $R^2 = 0.959$.

Antioxidant assay

To determine the free radical scavenging as an index of antioxidant ability of the investigated plant extracts, DPPH assay was performed. The data followed that of the Total phenol content with a fall at 0.2 mg/ml of the extract with percent inhibition of 11.28. The highest percent inhibition of 28.97 was found for 0.3 mg/ml concentration. The co-linearity of the data trend (Figure 1D) for phenols and antioxidants reveals the fact that the active phenolics present in the plant extract are the antioxidants. The positive control BHT recorded the highest percent inhibition as 35.6 at 0.3 mg/ml concentration.

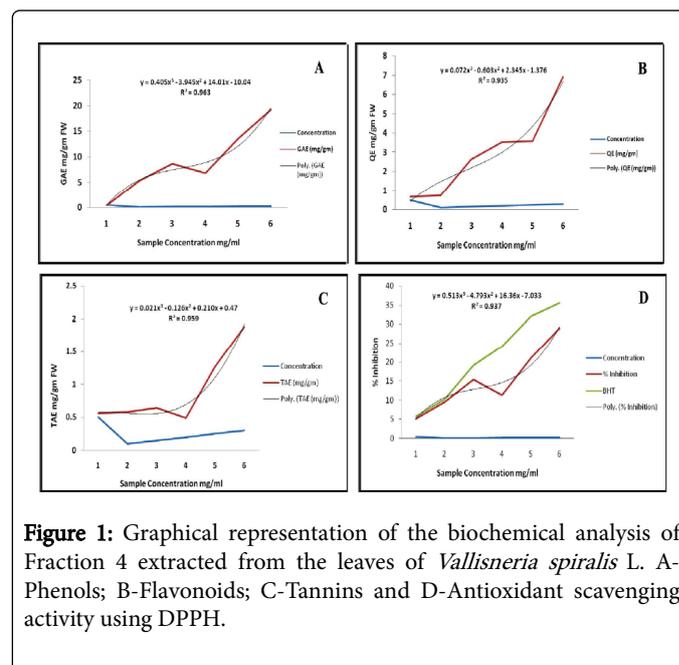


Figure 1: Graphical representation of the biochemical analysis of Fraction 4 extracted from the leaves of *Vallisneria spiralis* L. A- Phenols; B-Flavonoids; C-Tannins and D-Antioxidant scavenging activity using DPPH.

Microbial screening

The zone of inhibition was found to initiate at 100 µg/ml but the clear zone could be seen at 500 µg/ml and 1000 µg/ml (Figure 2). No anti-fungal activity was noticed in the other four concentrations. Hence forth, eleven tubes ranging from 10000 µg/ml to 39.0625 µg/ml of the extract and two controls one without inoculum and the other without extract was maintained for experimental assurance along with 1 ml of broth in each of the tube for MIC and MFC. The optical density at 600 nm was taken for all the samples. The data tabulated below shows MIC 156.25 µg/ml (Figure 3). All the six concentrations beyond that were streaked and kept in incubator at 37°C for 24 hrs/48 hrs and the MFC was found at 5000 µg/ml.

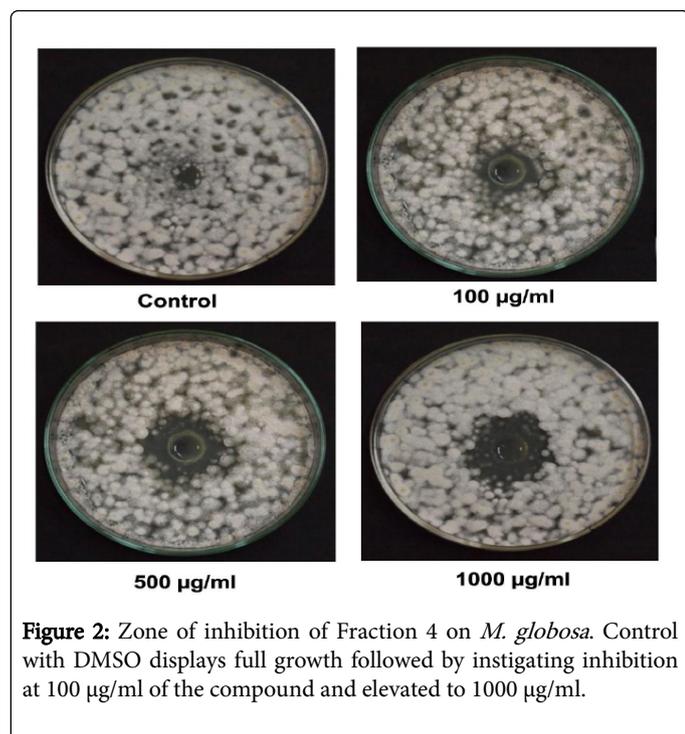


Figure 2: Zone of inhibition of Fraction 4 on *M. globosa*. Control with DMSO displays full growth followed by instigating inhibition at 100 µg/ml of the compound and elevated to 1000 µg/ml.

Purification and Structure activity relationship by Mass and FT-IR spectroscopy

The compound was ideally isolated using solvent system of 1% methanol in ethyl acetate by column chromatography using silica gel 100-230 mesh size and obtained as crystals with chloroform: methanol washes and finally flushed with toluene. Mass spectrum [30] with Time of Flight analyzer (TOF) shows the (Figure 4) molecular weight of the isolated compound to be m/z (%) = 359 [M]⁺ with the base peak at 360 [M+H]⁺. Infra-red spectrum (Figure 5) analysis helped to sort the functional group of the active compound which is tabulated (Table 2). The absorption spectrum of the 80% ethanol fraction shows ten major bands; the band at 3642.10 cm⁻¹ corresponds to hydroxyl group usually free hydroxyls. The other dominating bands at 1665.22 cm⁻¹ and 1554.80 cm⁻¹ are those of carbonyls and aromatic compounds. The presence of aromatic compound is further confirmed at 779.64 cm⁻¹ with C-H stretching which if in case of flavonoids, corresponds to the first aromatic ring. The S=O bond usually includes compounds [31,32] which shows radical trapping antioxidant property and act as antimicrobial, antiparasitic and antitumor agents, a common example of this compound is contained in *Allium sps.* [33,34].

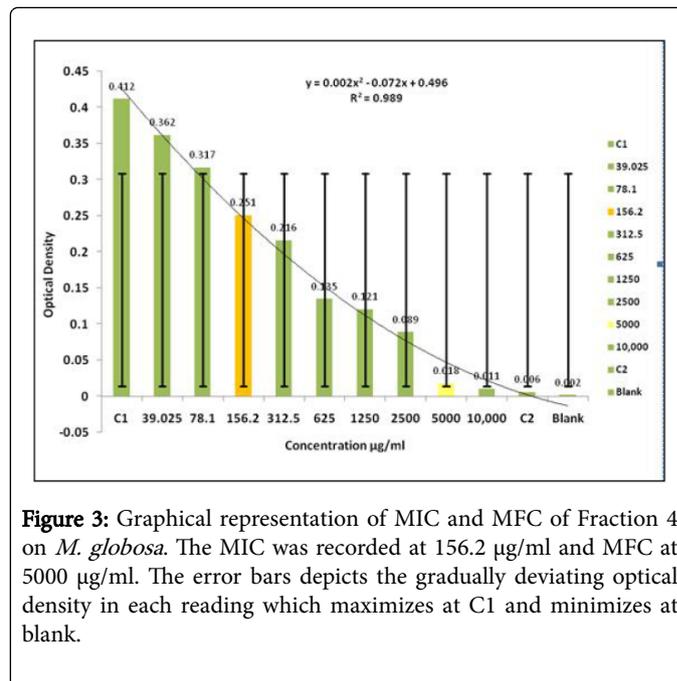


Figure 3: Graphical representation of MIC and MFC of Fraction 4 on *M. globosa*. The MIC was recorded at 156.2 µg/ml and MFC at 5000 µg/ml. The error bars depicts the gradually deviating optical density in each reading which maximizes at C1 and minimizes at blank.

SL No.	Peak value (cm ⁻¹)	Stretching	Inference
1	3642.1	O-H stretching	Alcohols/Phenols
2	2965.4	C-H stretching	Alkanes
3	2928.5	C-H stretching	Alkanes
4	1665.2	C=O stretching	C-O NH
5	1600.8	C=C stretching	Alkenes
6	1554.8	C=C stretching of aromatic ring	Aromatic Compounds
7	1360	S=O, sym	Sulphoxide
8	1080	C-O	Ethers
9	1076.9	C-O	Ethers
10	779.64	C-H stretching	Aromatics

Structure activity relationship of the different functional groups present in the bioactive compound extracted by 80% ethanol from leaves of *Vallisneria spiralis* L. by FT-IR Spectroscopy.

Table 2: FT-IR Peaks Interpretation.

Discussion

Plants have been serving us as versatile sources of our basic needs [35,36]. The art of utilising the plant associated aspects including various metabolites is intriguing and holds a great prospect in pharmaceuticals and drug crafty [37] mostly due to its nearly nullified negative impacts [38-41] However, the ecosystem that harbours the plant is a vital factor for its outsourcing to nature. Science have much studied the terrestrial ecosystem which orients soil as a resource variable and quiet easy to monitor and modify, but water being a diluting variable needs advanced techniques and resources, hence comparatively much less explored. In this paper, we have screened a

rapid wetland colonizer, *Vallisneria spiralis* L. for antifungal traits against dandruff dermatitis following its uses as refrigent and skin lesions. The fourth fraction (80% ethanol) was assayed for possible biochemical evaluation and interestingly displayed a co-linearity in peaks for phenol and antioxidants which confirms that the antioxidant compound is phenolic in nature [42]. Further it was found to be antifungal against *M. globosa* initiating at 100 µg/ml and showed highly remarkable zone of inhibition at 1000 µg/ml and the minimum fungicidal concentration was observed at 5000 µg/ml. The compound was crystalline in nature with molecular weight m/z (%) = 359 [M]⁺. The FT-IR Spectrum depicts phenols, carbonyls, aromatics, sulphoxides and alcohols as major functional groups [43,44] of the structure which are antifungal [45,46] or mostly antimicrobial [47] and shows prospective research in herbal drug analysis. The structure contains a polyphenolic group. Most antioxidants isolated from medicinal plants are polyphenols [48-50] which show biological activities include anti-bacterial, anti-inflammatory, anti-obesity, antiviral, anti-carcinogenic and immune stimulating effect. Additively, the S=O group of the compound is one of the major antioxidant imparting factor [51,52] and lysis of microbial cell wall integrity.

The concise attempt to utilize the aquatic macrophyte as an antidandruff source provides a platform for research with wetland colonizers in an innovative aspect. Further research with NMR spectroscopy is required for demonstration the chemical structure of the compound.

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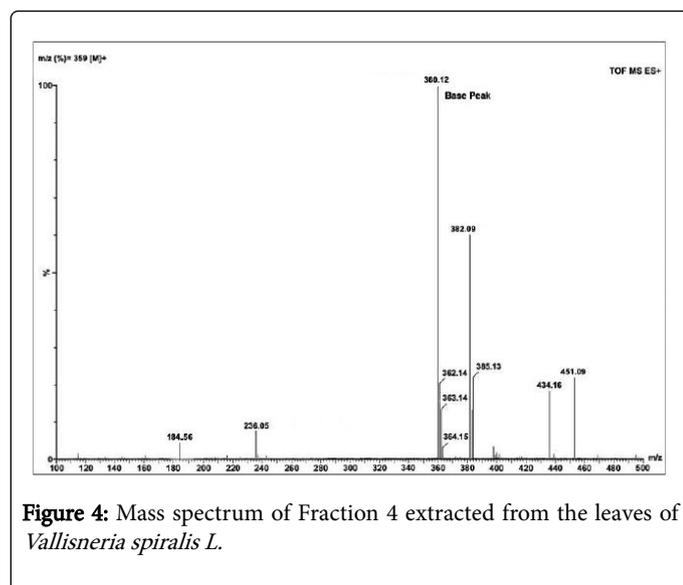


Figure 4: Mass spectrum of Fraction 4 extracted from the leaves of *Vallisneria spiralis* L.

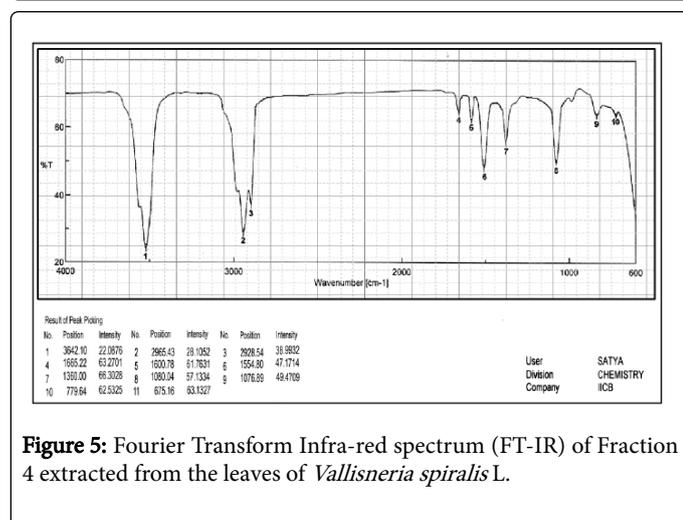


Figure 5: Fourier Transform Infra-red spectrum (FT-IR) of Fraction 4 extracted from the leaves of *Vallisneria spiralis* L.

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