INTRODUCTION

1.1 BACKGROUND STUDY

Biological interactions are indispensible for an integrative and functional ecosystem. The science of herbal medicines or Ayurvedic, with its origin in India, serves as a depiction of plant-animal intercommunication. Even in the advanced scientific era, more than 80% of human population depends on traditional medicinal resources either largely for their primary health care and to an inconsequential extends for infectious diseases, as read by the World Health Organization (WHO) database (Sowmya *et al*., 2015). In context to drug adjutants, there are plentiful theoretical as well experimental inputs on the role of secondary metabolites or allelochemicals of plants being used independently as drug or as an additive or bioactive component with the synthetic counterpart (Mendoza, 1980; Ayethan *et al*., 1996; Chakraborty *et al*., 2015). The competition for resources in aquatic ecosystem and establishment of the autotrophic climax community by succession is also a reflection of the efficient release of secondary metabolites in the dominant plant populations (Rahman *et al*., 2007; Dewanji, 1993). There are literatures stating the impact of aquatic plants on growth and regeneration of algae, phytoplankton and zooplankton (Thakare *et al*., 2011; Lundholm *et al*., 2005).It has been found that primary producers including plants of highest order are capable of synthesizing as well exudation of the bioactive compounds either by leaf leaching, root exudation or by the stem/bark aroma. (Wium-Andersen *et al*., 1982). The in-vivo production of these secondary metabolites and accretion of the same in the exogenous environment is a fascinating aspect of nature to pharmaceutical with agriculture and allied sciences (Elisabeth, 2003; Anjaria, 2002). Scientific perception added with natural chemistry helps indigenous technical knowledge (ITK) to host an outsourcing for medicinal many plants.

1.2 ALLELOCHEMICALS – GREEN SOLUTION

Allelochemicals consigns the not so essential secondary metabolites of plants which are released into their surroundings (Rice, 1984; Duke et al., 2000; Inderjit et al., 1995). Various chemical groups of flavonoids, alkaloids, tannins and phenols form the potent plant based pesticide, herbicide or insecticides against weeds, plant pathogens and insects. These low molecular mass chemicals exert a bipolar function of stimulation and inhibition on other neighbouring plants or microorganisms in the environment or the nearest ambience of source organs such as rhizosphere from roots. (Inderjit, 1996).They also plays a role in plant defence mechanisms. The continuous application of synthetic chemicals for better and fast yield have led to hazardous consequences such as pathogen resistance against chemicals, bioaccumulation of toxic residues, and contamination of the fish population yield from a stock water body and finally served as food exerts harmful effects on the entire biota. Holistically a small amount of chemical applied in any ecosystem results in bio-magnification and the final yield in the food chain of the producers are almost 10 times magnified. Bio-intensive aquaculture practises is the best way to safeguard the environment, encouraging fish health management. The environment-friendly approach can serve as a source of natural herbicides and fungicides (Inderjit and Mukerji, 2006; Khanh et al., 2005) which are degradable and least toxic. The green solution has fetched much attention and helped to conserve natural fauna of a habitat.

1.3 POPULATION ECOLOGY COMPLIMENTATION – WETLAND ECOLOGY

There is a growing notion that population and ecosystem based approaches complement each other and in view of this concept, allelopathy can be used as an efficient tool to

address ecosystem level aspects. The branch of science which deals with the detrimental or beneficial impact of plant or microorganisms on the growth and survival of other (especially neighbour) plant or microorganisms through release of low mass compounds is called allelopathy and the released chemicals are the allele-chemicals (Duraipandiyan et al., 2006). The application of allelopathy in agriculture and aquaculture dates back to 3rd century B.C. (Theophrastus) but major progress in this field occurred in the twentieth century. The allelopathic interaction in floodplain wetlands holds immense significance as wetlands are highly productive and most biologically diverse (Ramsar convention, 2011) land which is flooded either permanently or only during monsoons. 'Floodplain wetlands' represent the wetlands situated along the floodplains of rivers; mostly lentic in nature and excludes the lotic component of the river such as the main river channel, the levee region and the flats. The ecosystem better known as 'Liquid treasures' or 'Kidneys of the Landscape', provides the world with nearly 2/3rd of its fish harvest. Wetlands hold immense significance as compared to other water bodies in their characteristic vegetation and fauna that is specifically adapted to the unique soil condition. They arise with flood water producing flood plain conquered by anaerobic processes with rooted plants adapted to such growing conditions. It is the vital factor (Paul, 2010) for producing wetlands with other associated factors being fertility, natural disturbance, competition, herbivore etc. Wetlands consist mainly of hydric soil supporting anaerobic ambience with aquatic plants capable of tolerating the salinity. Floodplain wetlands vary widely in size, shape and the extent of riverine connection, offering tremendous scope for expanding fisheries if managed scientifically (Ramsar convention, 2011). They provide strong arguments for integration of traditional knowledge of local communities of West Bengal into conservation and management practices and provide livelihood support to a

large economically underprivileged population for sustenance. However, majority of these water-bodies are currently reeling under environment perturbation, swampification, shift in species spectrum and anthropogenic threats, adversely affecting their productivity. There is ample number of wetlands in West Bengal due to the lower stretch of river Ganga. More than 150 floodplain wetlands are distributed in the north, comprising in Nadia, Cooch-Behar, Murshidabad, Maida, Midnapore and 24 Parganas with an approximate area of 42,500 ha which accounts of more than 20% of the total area covered under fresh water ecosystem under the state. The use of intensive synthetic chemicals as disinfectant or antimicrobial agent in floodplain wetlands of west Bengal and algal blooms especially of East Kolkata wetlands are the key factors that have degraded the resources economically (Sugunan and Mukhopadhyaya, 1995).

Literature studies support that aquatic plants do exert an interference that tailors the aquatic vegetation and fish community structural dynamics in floodplain wetland ecosystem but very scanty work in India has been done in this regard. Hence this study can be a great step towards harnessing this dynamic ecosystem as natural resources for fisheries and aquaculture and with consistency.

Additively, the botanical antimicrobial therapeutics exemplify a wide range of plant secondary metabolites (Walters and Namchuk, 2003; Yang et al., 2001; Yu et al., 2003) asserting diverse effects from extreme deadly to immense beneficial. The plant products may be phyto-anticipins or phyto-alexins (Grayer and Kokubun, 2001). Though finding healing powers in plants is an ancient idea but in most cases lack defined protocol for formulations and quality control. There are numerous reviews on ethno pharmaceuticals on human, but in our study we have utilized the antimicrobial trait of plants against fish pathogen (Rajandra, 1990).

1.4 AQUATIC MACROPHYTES FOR THIS STUDY

For the purpose of this study, primly aquatic macrophytes and related plant is chosen which includes leaves of *Vallisneria spiralis* (Hydrocharitaceae), *Ipomoea aquatic* (Convolvulaceae), and the inflorescence of *Cyperus rotandus* (Cyperaceae). And out of context, the abscessed leaves of *Musa sp*. (Musaceae) from terrestrial ecosystem are also included. *Spirodela polyrhiza* (Araceae), the floating macrophyte is considered as the model organism for toxicity assay. Differential working principals are studied depending on the application source which can vary from pelagic zone to benthic substrate associated habitat. It is supported to some extend by convincing visual evidences that these plants do exert an interference that tailors the aquatic vegetation and fish community structural dynamics in the ecosystem. The chosen plants are subjected to extraction processes usually after shade dry with various elutropic solvents through the polarity scale. Once extracted the target fraction with maximum lead compounds are analysed and assayed for bioactivity. The bioactive fraction is further attempted to purify as a single pure compound along with molecular structure elucidation via Mass Spectrometer, Nuclear Magnetic Resonance Spectroscopy and Infra Red spectroscopy.

1.4.1 *Vallisneria spiralis* L.

Vallisneria spiralis L. is an extensively invasive submerged macrophyte which is deep rooted to the soil bed beneath water bodies. They can be seen wide spread in the stagnant ponds, rivers, wetlands in West Bengal. By routine observation it shows antagonism against surface growers and can be said to be a colonizer with seed dispersal by natural means (Wichitnithad et al., 2009; Bowmer et al., 1995).*V. spiralis* has also gained significance in water chemistry by increasing the dissolved oxygen

which serves as a boon to aquatic organisms (Singleton and Rossi, 1965; Mithun and Shashidhara, 2011 Everitt et al., 2007). Besides being reported with antimicrobial traits, *V.spiralis* is also reported to be used to cure leucorrhoea and stomach ache resulting in appetite and acting as a natural refrigerant (Chopra et al., 1968; Vajpayee et al., 2001).

1.4.2 *Ipomoea aquatica* Forssk.

Ipomoea aquatica Forssk. (Water Spinach) belongs to the family Convolvulaceae and is a widely consumed vegetable, commonly known as "kalmi" in West Bengal. It usually grows in wild areas in wetlands. The water spinach has wide disease preventive applications such as urinary infection, constipation or even broad aspects of gynaecological disorder (Prasad et al., 2008) due its laxative and psychedelic properties (Uva et al., 1997). It is also endowed to be anti-cancerous, oxytocic with hepatic medicinal contents (Tart and Charles, 1990; Khatiwora et al., 2012) along with antibiotic traits against fungal and bacterial infections against human infections and rheumatism (Ruckmani et al., 2010). One such example is the ethanol extract of *I. Cairica* L. obtained from the database of medicinal plants used in Brazilian folk medicines (Ferreira et al., 2005; Rong-Jyh et al., 2008; Paska et al., 2002).

1.4.3 *Cyperus rotandus* L.

Cyperus rotandus or most commonly known as nut grass is exotic sedge to India with its origin in Africa and southern Asia. It is a perennial plant with fleshy rhizomes. The phyllotaxy is alternate with leaf apex acuminate-cuspidate. The inflorescence is a terminal, open umbel by several leaf bracts. The fruits is a three angled achene often referred to as seeds. It is one of the important herbs in traditional Chinese medicine and also mentioned in ancient medicine Charaka Samhita for fever, digestive system

disorder and bruises. It is highly invasive and commonly competes in paddy fields. At the same time curse turned boon in oil contaminated soil where raising Cyperus sedge makes the soil fit for any cultivation. Quite a appreciable number of reports have been witnessed for allelopathy of Cyperus leaves. This study however is done on its inflorescence (seeds) which is one of the vital factors imparting invasions (Fransworth et al., 1985; Al-Snafi, 2016).

1.4.4 Associated terrestrial plant – *Musa paradisiaca* L.

A very frequent sight surrounding the local water bodies such as ponds, lakes & wetlands are numerous banana plants (*Musa paradisiaca*) .Banana is one of the most commonly found alkaloid resource in nature. With this state of fact an attempt has been initiated to study the antimicrobial activity of banana leaves against fish pathogens followed by cytotoxicity effect (Direkbusarakom, 2000). Most of the banana leaves in such locations after abscission falls off and keeps floating on water bodies and many a times they are intentionally place on water bodies which is evident to balance the pH of the water providing a cool environment to the aquatic lives. In view of this interaction, this experiment aims to further conquer the beneficial effects that the leaves impart to the aquatic ecosystem.

1.5 ISOLATION OF BIOACTIVE FRACTION

Extraction is the fundamental process for utilizing plant resources with proper isolation and characterization of bioactive fraction followed by compounds. The basics of extraction include collection and processing of the plant samples to improve the kinetics of analytical recovery of desired compound and increase the amalgamation of solute and solvent. The polarity of the compounds and their thermal stability determines the

process of extraction such as microwave assisted recovery, sonification, heating, soxhlet, lyophilisation etc. Dried plant material may also be subjected to mechanical maceration and hydraulic percolation as an alternate applied to obtain plant extracts with suitable organic solvent or even as an aqueous extract.

The biochemical parameters of any plant extract play a vital role in asserting its bioactivity and its interaction with the surrounding ecology. The role of the fraction on plant defence or fragrance or strong taste can be holistically be concluded by observing the values of the biochemical contents. In my work I have included four primary biochemical parameters

Phenols: these are carbon containing compound in most plants and play a prominent role in plant defence system. Plant poly-phenolics are the secondary metabolites of plants with aliphatic or aromatic phenolic rings (Maqsood, 2014; Parr and Bolwell, 2000). Phenol containing food items of plant origin comprises of fruits, vegetables, hot and cold beverages as tea (Lakenbrink et al., 2000), coffee (Clifford, 1999), beer and also chocolate (Hollman and Katan, 1999). The polarity index of the extracting solvent and the chemical interaction determines the nature of the phenolic compounds and their polymerization.

Flavonoids are considered as a subclass of phenols. It also constitute the plant secondary metabolites, widely distributed, fulfilling many functions. Flavonoids are the most important for plant pigments and eye catching colouration of flowers to attract pollinator animals. They are more of a chemical messenger. The prime subgroup of flavonoids which has been identified from plant sources includes flavone, isoflavonoid, flavanone and anthocyanidin (Harborne, 1998). Over past decades evidences have accumulated that plant polyphenols and especially, flavonoids are most

important classes of defence anti-oxidants. The plant secondary metabolites can be said to be the endogenous antioxidants which serves to protect the plant from environmental predators as reactive oxygen species as well biotic predators as herbivores.

Tannins are chemically varied poly-phenols which play an important role in nutritional and ecological aspects of plants. It is one of the oldest plant secondary component and also a strong antioxidant. But their amount is highly unstable within the same genus or type of processing of the plant materials.

Antioxidants or Reactive oxygen species (ROS) produced during several metabolic processes in live cells, plays a vital role in modifying various physiological functions and represent an essential part of life cycle for its existence. Several abiotic stresses due to excessive generation of these radicals disrupt the antioxidant system of the cell which may lead to so called oxidative stresses. A compound which serves as hydrogen atom transferring agents (HAT) can act as reducers in redox reaction (Medini, 2014) and are otherwise defined as antioxidants. The total antioxidant capability of a plant or its corresponding solvent fraction tells us its disease resistance capacity and its possible utilization as natural antimicrobial resource.

1.6 BIOLOGICAL ACTIVITY FOR THIS STUDY

It is an effective tool which measures the magnitude of the potency of our intended biological substance. Bioassay in vitro or in vivo has three vital dependable variables- the concentrations considered, time factor and ideal atmospheric conditions.

For my research I have chosen four bioassay detection methods based on likely biological responses and known amount of available biologically active molecules that could be extracted from the target plant sources.

The emergence of bacterial and fungal diseases and their resistance to most drugs has made the study of antimicrobial assay of natural compounds one of the most consistent bioassay methods. Inhibition zone test is done by well and disc diffusion assay for measuring antimicrobial activity of the extracted compounds to assay their ability to prevent the growth of the microorganism. A clear zone of inhibition surrounding the compound after standard incubation period indicates its antibiotic activity. Fish pathogens are considered for studies. Area of inhibition zone will be calculated as annular radius from the edge of the zone (Biswas et al., 2009). A commercially available antibiotic is treated as control. The bacterial were obtained from Dr. T Jawahar Abraham, West Bengal University of Animal and Fishery Sciences (WBUAFS), West Bengal.

The cytotoxicity activity is done to predict the toxicity of the extracted compound and its safety scale for implementation in target ecosystem. We have tested our experimental compounds by the Brine shrimp (*Artemia salina*) lethality test (BST) (Meyer et al., 1982) The BST procedure is simple, rapid and at most requires a very small amount of the compound. The mortality of hatched nauplii is plotted statistically to obtain the LC_{50} value with a correlation relative to the control taken.

The LC_{50}/median lethal concentration for the cytotoxicity test on brine shrimp (*Artemia salina*) lethality is statistically analysed by the logit and probit regression models. For generalized model, the dichotomous data normality distinguishes between probit and logit analysis. The normal distribution of data for probit on percent morality considered as the dependent variable, was done by Shapiro-Wilk significance test using stem and leaf model where $p>0.05$ specify normal distribution.

A world wide apprehended epidemic are the Harmful algal bloom or Phytoplankton blooms (ICES, 1984) which are the visible markers of water contamination with

discoloration, carpet growth, foul smell, foam formation and in extreme situations, fish mortality. The consumption of such water is toxic to human health. Hornell in 1980 (Hornell, 1917) recorded the algal blooms in Indian water bodies where high fish mortality in water bodies with high plankton content and Euglena and Noctiluca species were noted. The numerous reports of paralytic fish poisoning towards the southern part of India and Maharashtra has resulted in massive fish kills as well death and disease of consumers. Considering all these facts, the Ministry of Earth Sciences have launched a monitoring programme on Harmful Algal Blooms (HABs). Anthropgenic activities count the most for increase in organic load in water bodies and thereby increase in harmful algal bloom. Other reasons may include improper waste water management, unethical protocols of aqua-farming and eutrophications (Padmakumar et al., 2012). Ever since there are several synthetic remedies available along with physical endeavour which helps inhibiting algal growth. The discovery of application of plant secondary metabolites and the biological phenomenon of inhibiting neighbouring plants provided resources for presumed anti-algal properties possessed by aquatic macrophytes and utilization of the same as natural algaecides (Lee et al., 1996; Gross, 2003; Schrader et al., 2003; Vyvyan, 2002). In our study we have tried to use the aquatic macrophytes as sources of anti-algal compounds which would accelerate the probability of controlling growth pertaining to the fact that there is not much change of habitat in macro level.

The toxicity study has distinctive importance since the proposed use of any final product procured from natural resources depends on elimination of all toxicological constituents. The risk factor assessment parameter of any chemical compound be it naturally procured or synthetically designed, demands an ensured evaluation with proper sensitivity, rapid pace, uncomplicated economically viable and of ecological significance. The applicability of duckweeds as a biological indicator to measure the

phyto-toxicity of a biological compound specially intended to apply in aquatic habitat or its related components is a scientifically proven practise, generally with *Lemna minor*. Duckweed bioassay is considered as the most standardized plant bioassay for aquatic eco-toxicology as they are invasive in their carpet growth but tend to deteriorate swiftly with hostile habitat. They are used to assay the detrimental or benefice impact of phenoxy herbicides on plants (Blackman and Cuninghame, 1954; 1955). Lemna root elongation assay is of the extensively used to assign the toxicity scale in terrestrial ecosystem (Wang, 1991) because of their effortlessness invasion and swift elongation (Munzuroglu and Geckil, 2002).But to the extreme fragility of roots and for ease of handling the conventional roots regenerate ability is additive with the frond count and net wet weight.

In our experiment we have used *Spirodela polyrhiza* as the model plant precisely because the habitat which was considered by us for plant collection, belonging to the araceae family. The multivariate analysis of the percent growth and phytochemical constituents of aquatic flora showed massive growth of *S.polyrhiza* in spring and summer. It is free floating thalli with invasive growth in sprig and in summer. The use of this plant as a biological indicator dates back to 1978 (Mangi, 1978). Multiple roots emerge from each thallus which is oval and 0.6–5 mm broad; 1–8 mm long. The adaxial of the thalli are green in colour while the abaxial side ranges from red to deep purple.

The fronds multiply and the plant reproduces to a new individual. The roots are generally 1–2 cm. It is found worldwide in fresh water habitat growing perennial in dense colonies. The organism is characterized with substantial adaptative potential with rapid growth in aquatic habitat. Previous literatures report *S. polyrhiza* to be an excellent accumulator of heavy metals in water medium and having direct correlation with the concentration parameter contained in the in the contained water medium. The

simple anatomy and ease with which *S. polyrhiza* can be handled with excellent homogeneity makes them ideal test organisms.

The in-vivo application of the isolated bioactive compounds was done on live striped catfishes *Pangasius hypophthalmus* Sauvage, 1878 belonging to the family Pangasiidae. It is a highly migratory fish and gained momentum in Indian aquaculture system. The fish is best suited in acidic to neutral pH from 6.5-7.5. [FAO] The juvenile fishes approximately 4-4.5inch in length and less than 15 gm body weight are selected for the experiment and the bioactive samples are fed to the segregated sample fish with control. The impact study includes preliminary haematology study and the histopathology of the organs; liver and kidney. The advantages of selecting this species over others are manifold. Most importantly, the acclimatization of this fish to changed ambience is fast and with less mortality and hence the chances of applying commercial antibiotics for their culture are minimum. This in turn assures the prevention of false response of the bioactive phyto-chemicals. Secondly, the skin lesions are other visible symptoms of diseases appears faster than scaled fishes and also the curative properties of applied drugs can be tracked. Thirdly, the economic viability of the fish in the Indian aquaculture system is worth mentioning.

1.7 STRUCTURAL CHARACTERIZATION

For the chemical characterization and structure depiction of the Lead Functional Components (LFCs) by peak analysis, the compounds are confirmed of <90% purity is subjected to instrumental analysis with its solubility tested.

Mass Spectrometry – It is used for detecting the molecular weight of the compound. The conclusion is drawn based on the appearance of the (M+H) and (M+Na) peaks in

the spectrum. The graph is plotted with mass/charge (m/z) ratio in the X-axis and the relative intensity of the peaks in Y-axis.

^{1}H-NMR (Proton Nuclear magnetic Resonance) Analysis – It is done in 300MHz or 600 MHz, mostly using D_2O or $CDCl_3$ solvents for detecting the number of hydrogen atoms present in a sample.

^{13}C-NMR (^{13}C Nuclear magnetic Resonance) Analysis – It is done in 150 MHz using $CDCl_3$ for detecting the carbon atoms present in a sample.

In NMR the graph is plotted with chemical shifts (ppm/ppb) in X-axis and intensity in Y-axis.

FT- IR (Fourier transform Infra-red spectroscopy) analysis is used for detecting the functional groups present in the sample. The finger print region 1500cm^{-1} to 500cm^{-1} are scanned to find function groups unique to the sample and could impart specific biological properties.

1.8 STATISTISTICAL ANALYSIS

The Statistical analysis was accomplished with IBM SPSS (Statistical package for social sciences) package version 20. The relationship study between different species was described by Spearman's rho or Pearson's correlation coefficients depending on parametric or non-parametric data obtained. Mean maximum and minimum value and standard deviation is done on the complete dataset. Student's t-test and post hoc test may be used to find the dependence of variables with respect to fixed factors which will interpret us the impact of the allelochemicals on fish growth and survival or on aquatic weeds. Model based curve estimation will further enumerate us the best fit curve for the experiment and the equation it follows. The normality check was done with

Kolmogorov-Smirnov's and Levene's tests were used to measure the homogeneity of variance.

1.9 OBJECTIVE OF THE STUDY

- Isolation of the bioactive allelopathic fraction (single pure allelopathic compounds may also be possible) from the crude plant extracts collected from the wetland macrophytes by possible sources viz: a) volatilization b) leaf leachates.

    a. *Vallisneria spiralis* L.

    b. *Ipomoea aquatica* Forsk.

    c. *Cyperus rotundus* L.

- Screening the antioxidant content and antimicrobial properties of the isolates against fish pathogens.
- Study of the bio-active allelo-compound on fish and its cytotoxicity against aquatic bio-indicators (Brine shrimp & Duckweed) and harmful algal growth.
- Identification, structural elucidation and chemical characterization of the bioactive allelochemicals.