
Materials
and
Methods

CHAPTER II

MATERIALS AND METHODS

2.1 COLLECTION OF SPECIMENS (*SALVINIA MOLESTA*)

The whole plant of fresh *Salvinia molesta* was collected from the lakes of Kaliyakkavilai, Kanyakumari district (Figs. 2 and 3). It was authenticated by Prof. P. Jayaraman, Institute of Herbal Botany, and Plant Anatomy Research Centre. Tambaram, Chennai. *S.molesta* D.S.Mitchell belongs to a monogeneric family (*Salviniaceae*) of free-floating aquatic ferns coated with velvety hairs on the leaf surfaces (Fig. 4). Upto 12 species of the genus (*Salvinia*) have been reported worldwide (McFarland *et al*., 2004). The species was initially identified as a form of *S. auriculata,* but reclassified and nomenclatured as *S.molesta* in 1972 based on details of the male sporocarps or fruiting bodies by D.S. Mitchell. Giant *Salvinia* is a commonly occurring invasive weed in warm climates (Fig. 5). The plant originated in South eastern Brazil and was exported as a part of the pet industry to be used in aquaria and garden ponds. There are around 12 species of *Salvinia* found in nature and their distribution varies according to favourable weather conditions.

Scientific classification of *Salvinia molesta*

Kingdom	:	Plantae
Order	:	Salviniales
Family	:	*Salviniaceae*
Genus	:	*Salvinia*
Species	:	*molesta*
Binomial name	:	*Salvinia molesta* D.S.Mitchell (1972.

2.2 PREPARATION OF *SALVINIA MOLESTA* SAMPLE

The collected leaves were cleaned and cut into small pieces before being dried under shade at room temperature (Fig. 6). The dried material were ground to fine powder using a mechanical blender and passed through 24 mesh sieve (Fig. 7). The powdered sample was further used to make different solvent extractions.

2.2.1 PREPARATION OF FIVE DIFFERENT PLANT EXTRACTS

Plant extracts were prepared by standard methods (Sofowora, 1993). One gram of dried leaf powder of *Salvinia molesta* was extracted with 20 ml ethanol (75%), acetone, chloroform, aqueous and petroleum ether (Merck, extra pure) for 1 min using an ultra turax mixer (13,000 rpm) and soaked overnight at room temperature. The sample was then filtered through Whatman No.1 filter paper in a Buchner funnel. The filtered solution was evaporated

under vacuum in a rotator at 40°C and then dissolved in respective solvents. The dissolving rate of the crude extracts was approximately 100 %. The solution was stored at 18 °C until further use.

2.3 PRELIMINARY PHYTOCHEMICAL SCREENING

The phytochemical screening of all the five leaf extracts was assayed by standard methods (Harborne and Trease, 1978). The screening was carried out on the leaf extracts using five different solvents *viz.*, aqueous, ethanol, acetone, chloroform and petroleum ether to identify the significant bioactive components such as saponins, phenols, terpenoids, tannins, alkaloids, glycosides, cardiac glycosides, flavonoids, coumarins and steroids.

TEST FOR TANNINS

To 1 ml of the plant extract, 1 ml of ferric chloride (5% $FeCl_3$) was added. Dark blue or greenish black colour was formed indicating the presence of tannins.

TEST FOR SAPONINS

2ml of plant extract and 2ml of distilled water was mixed and shaken in graduated cylinder for 15 mins. Presence of saponins was confirmed by the formation of 1cm layer of foam on the surface.

TEST FOR QUINONES

To 1ml of plant extract, 1ml of concentrated sulphuric acid (H_2SO_4) was added. Presence of quinones was confirmed by the formation of red colour.

TEST FOR FLAVONOIDS

To 2ml of plant extract, 1ml of 2N sodium hydroxide (NaOH) was added. Formation of yellow colour confirmed the presence of flavonoids.

TEST FOR ALKALOIDS

To 2ml of plant extract, 2ml of concentrated hydrochloric acid (HCl) was added. To the mixture few drops of Mayer's reagent was added**.** Formation of green colour or white precipitate indicated the presence of alkaloids.

TEST FOR GLYCOSIDES

To 2ml of the plant extract, 3ml of chloroform and 10% ammonium solution was added. Formation of pink colour indicated the presence of glycosides.

TEST FOR CARDIAC GLYCOSIDES

To 0.5 ml of the plant extract, 2 ml of glacial acetic acid and few drops of 5 % ferric chloride were added. 1 ml of concentrated sulphuric acid was

added to the mixture. Formation of brown ring at interface confirmed the presence of cardiac glycosides.

TEST FOR TERPENOIDS

To 0.5 ml of the plant extract, 2 ml of chloroform and concentrated sulphuric acid was added. Formation of red brown colour at the interface confirmed the presence of terpenoids.

TEST FOR PHENOLS

To 1ml of the plant extract, 2ml of distilled water was added followed by few drops of 10% ferric chloride ($FeCl_3$). Dark blue or deep green colour confirmed the presence of phenols.

TEST FOR STEROIDS

To 0.5 ml of the plant extract, 2 ml of chloroform and 1 ml of sulphuric acid ($H_2\ SO_4$) was added. Formation of reddish brown ring at interface indicated the presence of steroids.

TEST FOR COUMARINS

For coumarins identification, to 1 ml of plant extract, 1 ml of 10 % sodium hydroxide (NaOH) was added. Formation of yellow colour indicated the presence of coumarins.

TEST FOR ANTHOCYANIN AND BETA CYANIN

To 2ml of the plant extract, 1 ml of 2N sodium hydroxide (NaOH) was added and heated for 5 min at 100 °C. Formation of bluish green colour confirmed the presence of anthocyanin and formation of yellow colour confirmed the presence of betacyanin.

2.4 QUANTIFICATION OF SECONDARY METABOLITES

2.4.1 ESTIMATION OF TOTAL PHENOL CONTENT

Total phenolic content in the leaf extracts of *S.molesta* was determined by Folin– Ciocalteau colorimetric method (Slinkard and Singleton, 1977). 0.5 ml of aliquot of sample was added to 0.5 ml of Folin–Ciocalteau reagent (0.5 N) and the contents of the flask were mixed thoroughly. To the mixture, 2.5 ml of sodium carbonate (2%) was added, and the contents was allowed to stand for 30 minutes after mixing. The absorbance was measured at 760 nm using UV-Visible spectrophotometer. The total phenolic contents were expressed as mg Gallic acid equivalents (GAE)/g extract.

2.4.2 ESTIMATION OF TOTAL FLAVONOID CONTENT

Total flavonoid content in leaf extracts of *Salvinia molesta* was determined by aluminium chloride colorimetric method (Mervat *et al.,* 2009). 0.5 ml of leaf extracts of *S.molesta* at a concentration of 1mg/ ml were taken and the volume was made up to 3ml with methanol. To the mixture, 0.1ml

$AlCl_3$ (10%), 0.1ml of potassium acetate and 2.8 ml distilled water were added sequentially. The mixture was shaken vigorously. The absorbance range was recorded at 415 nm after 30 minutes of incubation. A standard calibration plot was generated at 415nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated by the calibration plot and expressed as mg quercetin equivalent /gm of sample.

2.4.3 ESTIMATION OF TOTAL TANNIN CONTENT

Levels of tannins in leaf extracts of *S.molesta* were estimated by adapting the method of Broadhurst and Jones (2008). To 1 ml of leaf extract, 0.5ml of Folin-Ciocalteau's reagent was added, followed by the addition of 1 ml of saturated sodium carbonate (Na_2CO_3) solution and 8 ml of distilled water. The reaction mixture was allowed to stand for 30 min at room temperature. On centrifugation, the supernatant was collected and absorbance of the supernatant solution was recorded at 725 nm using UV-visible spectrophotometer. A standard graph was plotted using different concentrations of standard tannic acid and relative absorbance. The tannin content was expressed as µg tannic acid equivalent (TAE) per gram of the sample.

2.4.4 ESTIMATION OF TOTAL SAPONIN CONTENT

Determination of total saponin was performed by the method followed by Obadoni and Ochuko (2001) with slight modifications. To 1 gm of

powdered leaf, 100 ml of 20% aqueous ethanol was added and stored in a flask and stirred for half an hour and was heated for 4 h at 45 °C with mixing. The mixture was filtered by using whatman filter paper no. 1 and the residue was again extracted with another 100 ml of 25% aqueous ethanol. The combined mixtures were concentrated by using rotary evaporator in 40 °C to 40 ml approximately. The concentrate was then transferred into separator funnel and extracted twice with 20 ml diethyl ether. The aqueous layer was retained and the ether layer was discarded .The aqueous layer was re-extracted with 30 ml n-butanol. The n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated. After evaporation, the samples were dried in the oven at 40°C to a constant weight and the total saponin content was calculated using standard formula.

2.4.5 ESTIMATION OF TOTAL ALKALOIDS

The quantification of alkaloids was performed by standard methods (Harborne, 1973). 100 ml of 10% acetic acid in ethanol was added to 1 gram of leaf extracts of *S.molesta* and then the extracts were covered and allowed to stand for 4 hrs. The extracts were then filtered and concentrated using a water bath to one fourth of its original volume. The droplets of concentrated ammonium hydroxide were added to the extract until the whole solution was allowed to settle, and then the precipitates were washed with dilute ammonium hydroxide and then filtered using whatman filter paper. The alkaloid content was quantified using the following formula:

$$\textbf{\% of alkaloid} = \frac{\text{Final weight of the sample}}{\text{Initial weight of the extract}} \times 100$$

2.5 DETERMINATION OF ANTIOXIDANT ACTIVITY

2.5.1 QUALITATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY OF *S.MOLESTA*

The antioxidant activity of leaf extracts of *S.molesta* was determined by standard method (George *et al*., 1996). 50µl of leaf extracts of *Salvinia molesta* was taken in microtiter plate. To that, 100µl of 0.1% methanolic DPPH was added and incubated for 30 minutes in dark condition. Change in colour was observed; changing from purple to yellow and to pale pink were considered as strong and weak positive respectively. The antioxidant positive samples were subjected for further quantitative analysis.

2.5.2 QUANTITATIVE ANALYSIS OF FREE RADICAL SCAVENGING ACTIVITY OF *S.MOLESTA*

The antioxidant activity was determined using DPPH (Sigma-Aldrich) as a free radical. To 100µl of leaf extracts, 2.7ml of methanol and then 200µl of 0.1 % methanolic DPPH was added. The suspension was incubated for 30 minutes in dark condition. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured as control

(Lee *et al.*, 2003).The antioxidant activity of the sample was then compared with known synthetic standard of 0.16% Butylated Hydroxy Toluene (BHT).

Free radical scavenging activity was calculated by the following formula:

$$\textbf{\% DPPH radical-scavenging} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6 ASSAY OF ANTIBACTERIAL ACTIVITY OF *S.MOLESTA*

A general antibacterial assay of ethanolic leaf extract of *S.molesta* was performed against a group of selected gram positive and gram negative pathogens *viz., Pseudomonas aeruginosa, Aeromonas hydrophila, Escherichia coli, Bacillus cereus, Bacillus subtilis, and Staphylococcus aureus* in varying concentrations using standard methods (Mounyr Balouiri *et al*., 2016) to assay the efficacy of antibacterial activity against both gram positive and gram negative pathogens and the results were recorded.

2.6.1 GROWTH AND MAINTENANCE OF TEST MICRO ORGANISM FOR ANTIBACTERIAL STUDIES

To perform the antibacterial activity, bacterial cultures of *Aeromonas hydrophila, Pseudomonas aeruginosa , Escherichia coli, Bacillus cereus, Bacillus subtilis, and Staphylococcus aureus* were maintained in nutrient agar (NA) slants at 4°C. For further study, cultures were grown in nutrient broth (NB) for 24hrs as overnight cultures.

2.6.2 PREPARATION OF INOCULUMS

The pure cultures of bacteria were grown on nutrient agar slants and incubated at 37°C for 24hrs. Nutrient broth and the slants were stored at 4°C and maintained in active state by regular sub-culturing for further use.

2.6.3 DISC DIFFUSION METHOD

The antibacterial assay of ethanolic leaf extract of *S.molesta* was performed by standard disc diffusion method (Bauer *et al.*, 1966). The nutrient agar media was poured into sterilized petri dishes and left to solidify at room temperature. The overnight bacterial cultures were spread plated on petridishes using sterile L rod. Different concentrations (10, 20 and 30 mg/ml) of the concentrated ethanolic *S.molesta* leaf extracts was tested for its antimicrobial strains against selected pathogens. The filter paper discs were placed equidistantly on inoculated media and diffusion of the extracts was allowed to occur for 30 minutes at room temperature. Plates were incubated at 37°C for 24 hours. The average zone of inhibition was recorded. Sterile distilled water and ethanol was maintained as control. The diameters of the inhibition zones were measured in mm.

2.7 EXTRACTION OF BEST ANTIOXIDANT FRACTION BY COLUMN CHROMATOGRAPHY

The concentrated ethanolic leaf extract of *S.molesta* was separated by standard methods and analysed by column chromatography process to identify the best fraction possessing maximum antioxidant activity among the collected fractions.

2.7.1 SEPARATION OF FRACTIONS BY COLUMN CHROMATOGRAPHY

Silica gel (100 - 200 mesh - Fisher Scientific – India) was washed thoroughly using methanol solvent for 3 times. The cleaned 10gm of silica gel was dissolved with 20 ml of double distilled water; the slurry of semisolid / liquid silica gel was carefully poured in to column without any air bubbles. Concentrated ethanolic leaf extract of *S.molesta* (10mg /ml) was carefully transferred on to the upper surface of silica gel. Mobile phase was maintained in the ratio of 2:1 with methanol and chloroform. The concentrated extract is slowly passed through the column and the eluent is collected and stored in equal intervals to perform antioxidant activity of the individual fractions. Nine fractions were collected freshly in a continuous manner and assessed for antioxidant activity (Shimizu *et al*., 1997).

2.7.2 ANTIOXIDANT ACTIVITY ASSAY OF ISOLATED BEST FRACTION OF ETHANOLIC LEAF EXTRACT OF *S.MOLESTA*

Antioxidant assay of ethanolic leaf extract of *S.molesta* chromatography extract was analysed for their free radical scavenging activity using DPPH (1,1, Diphenyl – 2picryl – hydrazyl) as free radicals (Chandhini *et al*., 2014). All the fractions were subjected to antioxidant assay and the levels were recorded.

2.7.3 GAS CHROMATOGRAPHY MASS SPECTROMETRY ANALYSIS OF ETHANOLIC LEAF EXTRACT OF *S.MOLESTA*

To identify the significant compounds in the best antioxidant activity extract, the extract was subjected to GC-MS analysis (Kalpana Devi Rajesh *et al.*, 2016). The data was obtained on Elite-1(100% Dimethyl poly siloxane) column (30 0.25mm 1μmdf). Helium (99.999%) was used as the carrier gas with a flow rate of 1ml/min in the split mode (10:1). An aliquot of 2μl of ethanol solution of the sample was injected into the column with the injector temperature of 250ºC. GC oven temperature was set at 110ºC and holding for 2min and it was raised to 200ºC at the rate of 10ºC/min, without holding. Holding was allowed at 280ºC for 9 min with program rate of 5ºC/min. The injector and detector temperatures were set at 250ºC and 280ºC respectively. Ion source temperature was maintained at 200ºC. The mass spectrum of compounds in samples was obtained by electron ionization at 70 eV and the

detector was operated in scan mode from 45-450amu (atomic mass units). A scan interval of 0.5seconds and fragments from 45 to 450 Da was maintained. The total running time was 36minutes.

2.8 ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUND FROM ETHANOLIC LEAF EXTRACT OF *SALVINIA MOLESTA* BY ANALYTICAL METHODS

The compounds separated by GC-MS are further characterised for the presence of active compound and essential groups or bonds which are responsible for the potent activity of *S.molesta* extract. The identification process includes the separation of the compound by TLC method which will separate the compounds in to individual fractions and then the fractions will be scrapped off to identify the matching compounds based on retardation factor and then followed by NMR technique to elucidate the structure of active compound based on the peaks obtained. FTIR technique was performed to find out the functional groups.

2.8.1 THIN LAYER CHROMATOGRAPHY -TLC

TLC was performed by method of Jiri and Jaroslav (1978). 500µl of ethanol extract of *S.molesta* was dissolved in chloroform and the solution is spotted on thin layer chromatography plates. Then the TLC plates were run by specific solvent system (Hexane: Acetone in ratio 8:2) and were viewed

individually in iodine chamber and with ethanolic-50% H_2SO_4 spraying reagent. Identification and calculation of retardation factor (Rf) value was calculated by the following formula,

$$\text{Rf value} = \frac{Distance\ travelled\ by\ sample}{Distance\ travelled\ by\ solvent}$$

2.8.2 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY – NMR

A nuclear magnetic resonance spectrum gives the largest amount of information about the structure of a compound. In NMR Spectroscopic method, a substance is placed in a strong magnetic field that affects the spin of the atomic nuclei. A radio wave passes through the substance, and reorients these nuclei. When the wave is turned off, the nuclei release a pulse of energy that provides data on the molecular structure of the substance and that can be transformed into an image by computer techniques (Anand *et al*., 2011). The ^{1}H and ^{13}C NMR spectral analysis of the purified *S.molesta* isolated fraction was performed with Bruker DRX-500 (500 MHZ) and data was recorded. This technique would infer the possible structure of the active compound present in the crude fraction.

2.8.3 FOURIER TRANSFORM INFRARED SPECTROSCOPY – FT-IR

2 mg of dried fraction of *S.molesta* ethanolic extract was mixed with KBr salt, using mortar and pestle and compressed into a thin pellet. Infrared

spectra were recorded as KBr pellets on a thermoscientific nicot iS5 iD1 transmission, between 4000 − 400 cm −1 (Kareru *et al.*, 2008). The FTIR spectra will show the presence of the functional group present in the extract solution thereby supporting the data obtained by NMR studies.

2.9 COLLECTION OF BACTERIAL CULTURES FOR BACTERIAL CHALLENGE STUDY IN *OZIOTELPHUSA SENEX SENEX*

The microbial culture of *Aeromonas hydrophila and Pseudomonas aeruginosa* was derived from Poonga biotech and research laboratory, Vadapalani, Chennai, Tamilnadu and confirmed by biochemical characterisation using standard methods.

2.9.1 SELECTION OF BACTERIAL PATHOGENS FOR ANIMAL STUDY

A.hydrophila and *P.aeruginosa* was sub-cultured and characterised to study their morphological, biochemical and physiological parameters by biochemical methods.

2.9.2 BIOCHEMICAL CHARACTERISATION OF BACTERIAL COLONIES

Morphological characteristics features was observed for each bacterial colony after 24 hrs of growth includes colony appearance, size, shape,

elevation, edge, optical characteristics, consistency, colony surface and pigmentation. Biochemical characterizations were done according to the method of Fawole and Oso (2004).

GRAM STAINING TECHNIQUES

A thin smear of each of the pure 24 hr old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smear were again flooded with Lugol's iodine for 30 sec and rinsed with water, decolourized with 70% alcohol for 15 sec and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple.

MOTILITY TEST

A sterile needle was used to pick a loop of a 24 hr old culture and was stabbed onto nutrient agar in glass vials. The vials were incubated at 37°C for 24-48 h. Non-motile bacteria were grown confined to the stab line with definite margins without spreading to surroundings area while motile bacteria gave diffused growth extending from the surface (Olutiola *et al*., 2000).

INDOLE TEST

Tryptone broth (5 ml) was placed into different test tubes after which a loopful of the bacterial isolates was inoculated into the test tubes. The test tubes were then incubated at 37°C for 48 h. After incubation, 0.5 ml of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 min to permit the reagent to rise. A red or red-violet colour at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result (Cheesbrough, 2006).

METHYL RED TEST

Five millimetres of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 ml distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 hrs. At the end of incubation, few drops of methyl red solution were added to each test tube and the colour change was observed. A red colour indicates a positive reaction.

VOGES-PROSKAEUR TEST

Five millimetre of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 ml distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 h. After incubation, 6% α-naphhtol and 6% Sodium

hydroxide were added to about 1 ml of the broth culture. A strong red colouration formed within 30 min indicates positive reaction.

CITRATE TEST

This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. About ten millilitre (10 ml) of citrate medium was dispensed into each tubes and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. A change from green to blue indicates utilization of the citrate.

NITRATE REDUCTION TEST

Many microorganisms have the capacity to reduce nitrates to nitrites as they possess the enzyme nitrate reductase. The nitrate is reduced to nitrite and further into ammonia or to molecular nitrogen and this process is called denitrification. Nitrate reduction is indicated by the addition of alphanaphthylamine and sulphanilic acid in acidic environment with the formation of a red diazonium dye, P-sulfobenzene-azo-alpha naphthylamine. The organism was inoculated into nitrate broth and incubated overnight at room temperature. After 24 hrs, the nitrate reagents A and B were added in equal amounts one after other. Formation of red colour was noted.

OXIDASE TEST

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour

CATALASE TEST

A small quantity of 24 hrs old culture was transferred into a drop of 3% hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Evolved gas as white froth indicated the presence of catalase enzyme.

BACTERIAL DENSITY ESTIMATION

The barium chloride and sulphuric acid were added in different concentrations in a sterile test tube and the final volume was made up to 3ml and the contents were read at 630nm using calorimeter. The overnight grown bacterial culture was taken in a sterile cuvette and the readings were read at 630nm and all the readings were tabulated. The concentration of the bacterial cultures was determined by comparing with the Mc Farland standard (Adriana Zapata and Sandra Ramirez, 2015). Mc Farland turbidity standards were used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of a test suspension with the turbidity of a Mc Farland standard.

2.9.3 PREPARATION OF *A.HYDROPHILA AND P.AERUGINOSA* INOCULUMS

Both the bacterial inoculum was prepared by the procedure adopted by Lightner and Lewis (1975) with slight modification. The live bacteria was harvested from 24 hrs culture using sterile bacterial loop and mixed with double distilled water. This was then diluted to two-fold serial dilutions of the bacterial suspension, which was made into different dilutions *viz.*, 10^9, 10^8, 10^7, 10^6, and 10^5.

2.10 EXPERIMENTAL ANIMAL STUDY

The experimental animal for study, *Oziotelpusa senex senex*, fresh water crabs was collected, acclimatized and made in to different groups separately for male and female crabs and their physiological and pathological parameters were assayed and recorded.

2.10.1 COLLECTION SITE OF THE ANIMAL

Male and female freshwater crabs, *Oziotelphusa senex senex* were collected from paddy fields in Tirukazhukundram village near Chengalpatu, Kanchipuram district, Tamil Nadu (Figs. 8, 9 and 10) and were brought to the laboratory and maintained in plastic tubs. Crabs were fed with beef mutton and the water was changed daily and was acclimatized for 15 days at existing room temperature (Fig.11).

2.10.2 PHYSICAL CHANGES IN EXPERIMENTAL ANIMAL

WEIGHT

The weight of male and female crabs was weighed individually.

MORPHOLOGY

The appearance and morphological variations were studied for both male and_female crabs. The morphological changes during acclimatization, infection and treatment periods were monitored to understand the physical changes happening to the animal groups.

MOVEMENT AND RESPONSE

After acclimatization the movement in aquatic environment and responses to feed and other climatic conditions were studied.

2.10.3 DETERMINATION OF ACUTE TOXICITY BY TEST PATHOGENS

2.10.3.1 ACUTE TOXICITY TEST WITH *A.HYDROPHILA* INOCULUMS

Acute toxicity bioassay to determine the LD_{50} of the inoculums was carried out by the method described by Akhila *et al.,* (2007). About 4 groups of crabs, each consisting of 10 crabs was selected for acute toxicity studies. The crab in each group was inoculated with *A.hydrophila* bacterial suspension at

varying concentrations *viz*., 10^9, 10^8, 10^7, 10^6, and 10^5. The bacterial suspension (about 0.1 ml) was injected into the infrabranchial sinus present at the base of the walking leg. This was taken as the test groups for the determination of the lethal dosage of *A.hydrophila* in *Oziotelphusa sense senex.* The cumulative percentage of mortality at the intervals of 24hrs, 48hrs, 72hrs and 96 hrs was noted. From the data on mortality, the LD_{50} for 96 hours was calculated. The death of the crabs was determined by observing the crabs for the following changes: blackening of the carapace, avoiding the feed (loss of appetite), lethargic movement, formation of black spots on the exoskeleton. Before death the crabs become moribund and will fail to respond for physical disturbance.

2.10.3.2 ACUTE TOXICITY TEST WITH *P.AERUGINOSA* INOCULUMS

Acute toxicity bioassay to determine the LD_{50} of the inoculums was carried out by the method described by Reed and Muench (1938). About 4 groups of crabs, each consisting of 10 crabs was selected for acute toxicity studies. The crab in each group was inoculated with bacterial suspension (*Pseudomonas aeruginosa*) at varying concentrations *viz*., 10^8, 10^7, 10^6, and 10^5. The bacterial suspension (about 0.1 ml) was injected into the infrabranchial sinus present at the base of the walking leg. This was taken as the test groups for the determination of the lethal dosage of *Pseudomonas aeruginosa* in *Oziotelpphusa sense senex.* The cumulative percentage of

mortality at the intervals of 24hrs, 48hrs, 72hrs and 96 hrs was noted. From the data on mortality, the LD_{50} for 96 hours was calculated. The death of the crabs was determined by observing similar changes as mentioned in *A.hydrophila* groups.

2.10.4 TOXICITY TEST BY ETHANOLIC EXTRACT OF *S.MOLESTA* ON *O.SENEX SENEX* (LC $_{50}$)

The best antioxidant fraction of *S.molesta* leaf extract was mixed with saline solution and three different concentrations were prepared *viz*.., 0.1ml, 0.15ml and 0.2 ml with 0.1 mg, 0.15mg and 0.2mg of *S.molesta* concentration respectively. Three groups of each consisting of ten male and ten female animals were administrated with all the three concentrations separately and observed for mortality rates. The weight of the animal was considered in each criteria to assess the mortality and survival rates.

2.11 EXPERIMENTAL PROTOCOL

2.11.1 EXPERIMENTAL GROUPS OF *O.SENEX SENEX* FOR *BACTERIAL* CHALLENGE BY *A.HYDROPHILA*

The crabs were divided into six groups of thirty crabs each. Group A (male) and Group B (female) are kept as saline treated control. Group C (male) and group D (female) are infected with 0.1 ml of 10^9 CFU/ml standard concentration of *A.hydrophila*. Both the groups are allowed to withstand the

infection for 96 hrs. After 96 hrs, hemolymph was collected from ten crabs of each group for haematological and immunological assays for control group assays. Remaining twenty bacterial infected crabs were treated with 100 µl of ethanolic leaf extract of *S.molesta*. The treated groups are maintained as group E, E_1, E_2 and E_3 (male), F, F_1, F_2 and F_3 (female) in respective time interval of 24 hrs, 48 hrs, 72 hrs and 96 hrs. After 96 hrs, the hematological and immunological assays were performed for infected and treated groups.

2.11.2 EXPERIMENTAL GROUPS OF *O.SENEX SENEX* FOR BACTERIAL CHALLENGE BY *P.AERUGINOSA*

The crabs were divided into six groups of thirty crabs each. Group A and B are kept as saline treated control. Group C and D are infected with 0.1 ml of 10^7 CFU/ml standard concentration of *P.aeruginosa*. Both the groups are allowed to withstand infection for 96 hrs. After 96 hrs hemolymph was collected from ten crabs of each group for haematological and Immunological assays. Remaining twenty bacterial infected crabs were treated with 100 µl of ethanolic leaf extract of *S.molesta* .The treated groups are maintained as group E, E_1, E_2 and E_3 (male) and F, F_1, F_2 and F_3 (female) in respective time interval of 24 hrs, 48 hrs, 72 hrs and 96 hrs. After 96 hrs, the hematological and immunological assays were performed for infected and treated groups (Table 1).

2.12 COLLECTION OF HEMOLYMPH

Hemolymph of *O. senex senex* was collected aseptically from the base of one of the second walking legs using a sterile syringe with ice-cold citrate EDTA buffer (0.45 M NaCl; 0.1M glucose; 30mM trisodium citrate; 20mM citric acid; 100mM EDTA, pH 4.6) as anticoagulant. The collected hemolymph was stored for further analysis in aseptic conditions.

2.13 ASSAY OF BIOCHEMICAL PARAMETERS IN COLLECTED HEMOLYMPH

2.13.1 ESTIMATION OF CARBOHYDRATES

Principle

The carbohydrate content of the tissue and hemolymph extracts was estimated by the method of Roe (1955). Sulphuric acid hydrolyses the di and oligosaccharides in to monosaccharides and converts the monosaccharides in to furfural or furfural derivatives, which react with anthrone, and development of a complex blue color is proportional to the concentration of carbohydrates.

Reagents required

- Preparation of 10% TCA: 10gms of TCA added in 100ml of distilled water.

- Anthrone Reagent: 50ml of anthrone powder was dissolved in 100ml of 66% H_2SO_4 and 1gm of Thiourea was added to stabilize the color.

- Standard: 1mg of glucose was dissolved in 10ml of saturated benzoic acid to obtained standard solution.

Procedure

Series of test tubes containing 0.1ml, 0.2ml, 0.3ml to 1.0ml of standard solutions for plotting the standard curve. Volume in all the test tubes was made up to 1 ml with saturated benzoic acid. Then 5 ml of anthrone reagent was added in the test tubes and were kept in boiling water bath for 15 minutes. Then it was cooled to room temperature in darkness to protect from the light. The blue color developed was read at 620 nm against a reagent blank in spectronic - 21 (Bausch and Lomb, U.S.A.). The standard graph was drawn by plotting the concentration of standard solution on the X-axis and the optical density on the Y-axis.

For estimating the carbohydrate in the sample, about 0.1ml of sample was taken in a clean test tube and was made up to 1ml with 10% TCA, and then the same procedure as described for the standard was followed. By referring to the standard curve obtained, the amount of carbohydrates present in the hemolymph and tissues were calculated. The carbohydrate concentration was expressed as µg /mg tissue and µg /ml of hemolymph.

Calculation

$$\text{Carbohydrate} = \frac{\text{OD of unknown}}{\text{OD of known}} \text{ x mg of Standard}$$

2.13.2 ESTIMATION OF PROTEINS

Principle

Total proteins were analyzed by Biuret method (Gornall, 1949). Proteins, in an alkaline medium which binds with cupric ions present in the Biuret reagent to form a blue-violet colour complex. Intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

Reagent required

- Sodium hydroxide 600mM
- Copper sulphate 12mM
- Sodium potassium tartrate 32mM
- Potassium iodide 30mM
- Protein standard concentration 8gm/dl

Procedure

In three test tubes, test specimen, standard and blank was taken. They were labelled as ‘T’, ‘S’ and ‘B’ respectively. 1 ml of Biuret reagent was pipette out into all the labelled test tubes. 20µl of sample/standard was added into “T” and “S”. 20µl of distilled water was added into “B”. The test tubes were incubated for minutes at RT. The absorbance (optical density) was

measured for “T” and “S” against “B” at the wavelength of 520nm. The colour of the complex formed would be stable for more than one hour at room temperature. Therefore, absorbance should be measured within one hour.

Calculation

$$\text{Protein} = \frac{\text{Abs.Test}}{\text{Abs.Standard}} \text{ x } 8$$

2.13.3 ESTIMATION OF CHOLESTEROL

The Cholesterol content of the hemolymph was estimated by the method of CHOD-PAP method (Herbert *et al*., 1984).

Principle

Cholesterol esterase hydrolyses and esterifies cholesterols to free cholesterol. The free cholesterol is oxidized to form hydrogen peroxide to form a red colored quinoneimine dye complex. Intensity of the color formed is directly related to the amount of cholesterol present in the test sample.

Reagents required

Cholesterol Reagent

- Good Buffer pH 6.7 50mMol/L
- Cholesterol Esterase ≥ 100mMol/L
- Cholesterol Oxidase Peroxidase ≥ 50mMol/L

- 4- Aminoantipyrine 0.4mMol/L
- Sodium Azide 0.10%
- Zinc Sulphate 0.3mMol/L
- Cholesterol Standard concentration 200mg/dl

Procedure

The test specimen, standard and blank was taken in three test tubes. It was labelled as 'T', 'S' and 'B' respectively. 1 ml of cholesterol was pipette out into all the test tubes.20µl of standard was added into "T" and "S". 20µlof distilled water added into the test tube "B". The test tubes were incubated for 15 minutes at room temperature. The absorbance was measured for "T" and "S" against "B" at the wavelength of 520nm. The colour of the complex formed would be stable for more than two hours at room temperature. Therefore, absorbance should be measured within two hours.

Calculation

$$\text{Cholesterol} = \frac{\text{OD Test} - \text{OD Blank}}{\text{OD Standard} - \text{OD Blank}} \text{ x } 100$$

2.13.4 ESTIMATION OF MICROPROTEINS

The test specimen, standard and blank was taken in three test tubes. They were marked as 'T', 'S' and 'B' respectively. 1 ml of microprotein working reagent was pipetted out into each of the test tubes.20µl standard was added into "T" and "S" followed by 10µl of distilled water into "B". The test

tubes were incubated for 5 minutes at room temperature. The absorbance (optical density) was measured for "T" and "S" against "B" at the wavelength of 578nm. The colour of the complex formed will be stable for more than 20 minutes at room temperature. Therefore, absorbance should be measured within 20 minutes (Devakumar *et al.*, 2015).

2.14 ASSAY OF IMMUNOLOGICAL PARAMETERS

2.14.1 ESTIMATION OF TOTAL HEMOCYTE COUNT (THC)

Total hemocyte count was determined by using standard methods of haemocytometer. The hemolymph was drawn upto 0.5 mark in WBC pipette and immediately drawn up to 11 mark EDTA solution, and the pipette was rotated between the thumb and the forefinger. The cover glass was placed carefully on the ruled area of hemocytometer. The suspension was mixed thoroughly for a minute and expelled from the pipette immediately. The suspension was allowed to settle for 3 minutes .The cells were counted in the four corner blocks. (Each of these 4 square millimeter area is sub –divided in to 16 squares) by using the low power objective microscope. (Dacie and Lewis, 1968).

CALCULATIONS

$$\text{THC per mm}^3 = \frac{\text{Total cells count x blood dilution x chamber depth}}{\text{Area of chamber counted}}$$

2.14.2 ESTIMATION OF DIFFERENTIAL HEMOCYTES COUNT (DHC)

Differential counts of hemocytes were performed by the method followed by Kondo (2003). The smears were prepared carefully by spreading a drop of hemolymph and then thoroughly mixed with haemocyte suspension on glass slides. These films were then air dried, incubated for 5 min in methanol. Washed in distilled water and washed with Giemsa stain solution for 20 min and finally rinsed with distilled water. Presence of Large granule cells (LGC), small granule cells (SGC) and hyaline cells (HC) were determined.

2.14.3 ESTIMATION OF PROPHENOL OXIDASE ACTIVITY (ProPo)

Prophenol oxidase activity in haemolymph samples was determined using L-dihydroxyphenylalanine (L-DOPA) as a substrate.TBS (30 µl) was added to the experimental cuvette containing 30µl of haemolymph sample. Then 60 µl L-Dopa solution (1.6 mg/ml in TMS) was added followed by immediate mixing and 200 µl of TBS was added as a dilutent and enzyme activity was determined by measuring the absorbance of Dopachrome at 490 nm against a blank containing 260 µl of TBS and 60 µl of L.DOPA.The absorbance value at 1 and 3 minutes after the addition of 200 µl of TBS was recorded. Enzyme activity was expressed in units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per

min/mg/protein (Takahashi *et al*., 2000). All the obtained data were expressed as mean ± standard error of mean (S.E.M).

2.15 ESTIMATION OF MARKER ENZYMES

2.15.1 ESTIMATION OF ACID PHOSPHATASE (ACP)

Acid phosphatase (ACP) was estimated by the method as described by International federation of clinical chemistry and laboratory medicine, Kaplan *et al.,* (1984).

Principle

ACP at an acidic pH hydrolyses α- naphthylphosphate to form α-naphthol and inorganic phosphate. The α-naphthol formed is coupled with fast red TR salt to form a diazo dye complex. The rate of formation of this complex is measured as an increase in absorbance which is proportional to the ACP activity in the sample. Tartrate inhibits prostatic ACP and the testing in its sample is done to find the non-prostatic ACP. The difference between the activities of the total and non-prostatic ACP gives the activity of the prostatic ACP.

Reagents required

Acid phosphatase reagent

- α-naphthylphosphate 3mM
- Fast red TR 1mM

- Citric acid 20mM
- Sodium citrate 60mM, pH 5.3
- L-tartrate reagent (concentration refers to reconstituted reagent):
- Sodium L-tartrate reagent 2M
- Citric acid 70mM
- Sodium citrate 10mM, pH 5.3

Preparation of Sample for ACP

ACP is unstable in a collected sample hence the hemolymph was tested as soon as possible. In case of a delay in testing, the hemolymph should be acidified to a pH of 5.0 with 0.02ml Acetate Buffer (5M) to each ml of hemolymph and homogenized tissue supernatant.

Procedure

1.0ml ACP reagent was pipette out into the appropriate test tubes and pre-warmed at 37^0C for five minutes. The spectrophotometer was adjusted to zero with water at the wavelength 405nm. 0.1ml of sample was transferred to the reagent. The contents were mixed well and incubated at 37˚C for 5 minutes. The absorbance was measured after 10 minutes. The absorbance was recorded every minute for the next two minutes. The average absorbance per minute was calculated and then multiplied by the factor 853 which will yield results in IU/L.

Calculation

$$IU/L = \frac{\Delta abs/min \times 10\text{^}6 \times 1.1}{2.9 \times 10^3 \times 1.0 \times 0.1} \times \Delta abs/min \times 853$$

Where,

- Δ abs/min= Absorbance change per min
- 10^6= Conversion of moles to Millimoles
- 1.1= Total reaction value (ml)
- $12.9x10^3$=Molar absorptivity of α-naphthol fast red TR complex
- 1.0= light path in cm, 0.10= Sample value (ml)
- IU/L=International unit per litre

2.15.2 ESTIMATION OF ALKALINE PHOSPHATASE (ALP)

Alkaline phosphatase (ALP) was determined by the method described by Bablock (1988).

Principle

ALP at an alkaline pH hydrolyses p-nitrophenylphosphate to form p-nitrophenol and phosphate. The rate of formation of p-nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

Reagents required

- AMP pH 7.5 - 3.5 Mol/L
- Magnesium Chloride - 0.6mMol/L
- p-nitrophenyl phosphate - 0.070mMol/L
- Sodiumazide - 0.10%
- Zinc Sulphate - 0.3mMol/L

Procedure

1.0ml of ALP reagent was pipette out into the appropriate test tubes and pre-warmed at 37^0C for five minutes. The spectrophotometer was adjusted to zero with water at the wavelength 405nm. 25µl of sample was transferred to the reagent. The content were mixed well and incubated at 37^0C for one minute. The absorbance was measured after one minute. The absorbance was recorded every minute for the next two minutes. The average absorbance per minute was calculated and then multiplied by the factor 2187 which will yield results in IU/L.

Calculation

$$IU/L = \frac{\Delta abs/min x\ 1000\ x\ 1.025}{18.75\ x\ 1x\ 0.25} = \Delta abs/mIn\ x\ 2187$$

Whereas,

- Δabs/min= absorbance change per min
- 1000= conversion if IU/ml to IU/L
- 1.025= total reaction value (ml)
- 2187 = Conversion factor
- 18.75=millimolar absorptivity of p-nitrophenol
- 1= light path in cm
- 0.25= sample value (ml)

2.16 ESTIMATION OF ENZYMATIC ANTIOXIDANTS

2.16.1 ESTIMATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The enzyme was estimated by the method of Marklund and Marklund (1974).

Reagents required

- Diethylene triaminepenta acetic acid Tris-HCl Buffer: 0.1M Tris-HCl buffer containing 2mM of diethylene triaminepenta acetic acid.
- Tris-HCl buffer: 50mM

- Pyrogallol stock solution: 25.2 milligrams of pyrogallol was dissolved in 1ml of 50mM Tris-HCl buffer in a black paper wrapped, Stoppered test tube.

- Pyrogallol working solution: At the time of assay 0.2ml was diluted to 20ml with 50mM Tris-HCl buffer

Procedure

The reaction mixture for auto oxidation consisted of 2ml of buffer containing diethylene triaminepenta acetic acid, 0.5ml of the diluted (2mM), pyrogallol solution and 1.5ml of double distilled water. The assay mixture contained 2ml of buffer, enzyme and double distilled water to give a final volume of 4ml. Diethylene tri amine penta acetic acid acts as a chelator and thus prevents the interference of Fe^{2+} as well as Cu^{2+} and Mn^{2+}. The enzyme activity was measured at 420 nm and was expressed as units/mg protein.

Calculation

$$SOD = \frac{\Delta \text{ O. D sample x O. D blank x 100}}{\Delta \text{ O. D sample x 50 x Vol. of sample mg protein}}$$

2.16.2 ESTIMATION OF CATALASE (CAT) ACTIVITY

The catalase activity was measured by the method of Claiborne (1985).

Reagents required

1. Sodium phosphate buffer (pH 7.0; 0.01M, w/v):

2. Hydrogen peroxide (H_2O_2) (0.2M, v/v): 1ml of 30% $H_2O_2$was made up to 45ml with distilled water.

3. Dichromate acetic acid reagent.

Procedure

The assay mixture contained 0.5ml H_2O_2, 1ml buffer and 0.4ml water, 0.1ml of 1:10 diluted tissue extract was added to initiate the reaction. 2ml Dichromate acetic acid reagent was added after 15, 30, 45 and 60 seconds to arrest the reaction to the control tube, the enzyme was added after the addition of the dichromate acetic acid reagent. The tubes were then heated for 10 minutes, allowed to cool and the green colour developed was read at 570nm.The catalase activity was calculated in terms of nmol H_2O_2 consumed/minute/mg protein, with the help of the following formula:

Calculation

$$CAT = \frac{\Delta \text{ O. D/ min x Vol. of assay}}{0.081 \text{ of Vol. of conjugate enzyme x protein (mg)}}$$

2.16.3 ESTIMATION OF GLUTATHIONE PEROXIDASE (GPX) ACTIVITY

The GPX activity was calculated by the method of (Mohandas *et al.*, 1984) with some slight modifications.

Reagents required

- Sodium phosphate buffer (pH 7.0, 0.4M, w/v):

  Solution A: 6.4 grams of Sodium dihydrogen phosphate was dissolved in 100ml of distilled water.

  Solution B: 7.11 grams of Disodium hydrogen phosphate was dissolved in 100ml of distilled water.

  39ml of solution A + 61ml of solution B were mixed and pH was adjusted to 7.0.

- Sodium azide (10mM, w/v): 65 milligram of Sodium azide was dissolved in 100ml of distilled water.

- Reduced glutathione (4mM, w/v): 12.3milligram of GSH was dissolved in 100ml of distilled water.

- Hydrogen peroxide 30% (2.5mM, v/v) (AR): 1ml of Hydrogen peroxide was dissolved in 44ml of water

- 10% TCA (w/v) (AR): 10 grams of TCA was dissolved in 100ml of distilled water.

- Disodium hydrogen phosphate solution (0.3M, w/v): 5.34 grams of Disodium hydrogen phosphate was dissolved in 100ml of distilled water.

- Dithio-bis-nitrobenzoic acid reagent: 40 milligrams of DTNB was dissolved in 100ml of 1% Tri sodium citrate solution.

- Standard Reduced glutathione: 20 milligrams of reduced glutathione was dissolved in 100ml of distilled water.

Procedure

0.5 millilitres buffer, 0.1ml Sodium azide, 0.2ml Reduced glutathione, 0.1ml H_2O_2 and 0.125ml sample were taken and the total volume was made up to 2.0ml with distilled water. The tubes were incubated at 37 ºC for 3 minutes and the reaction was terminated by the addition of 0.5ml 10% TCA. To determine the residual glutathione content, the supernatant was removed after centrifugation (1500 rpm for 8 minutes) and to this 4.0ml of disodium hydrogen phosphate (0.3 M) solution and 1ml of the DTNB reagent were added. The colour developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent. The standard was also treated similarly. The enzyme activity was calculated as µmol NADPH

oxidized/minute/mg protein with the molar extinction coefficient of 6.22 X 10^3 M^{-1} cm^{-1}.

Calculation

$$GPx = \frac{\Delta \text{ O.D x Vol. of assay x 1000}}{\text{6.22 x Vol. of enzyme x protein (mg)}}$$

2.16.4 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE (GST) ACTIVITY

With some slight medications Glutathione-S-Transferase was assayed by the method of Habig *et al*., (1974).

Reagents required

- Phosphate buffer: (pH 6.5; 0.5 M, w/v)

  a) 3.405 grams of Potassium dihydrogen phosphate was dissolved in 50ml of distilled water.

  b) 4.36 grams of Dipotassium hydrogen phosphate was dissolved in 50ml of distilled water.

  Equal volume of a and b were mixed and pH was adjusted to 6.5.

- 1 Chloro 2, 4 dinitrobenzene (CDNB; 30mM, w/v in ethanol): 6 milligrams of CDNB was dissolved in 1ml of ethanol

- Reduced glutathione (GSH; 30mM, w/v): 22.35 milligrams of GSH was dissolved in 3ml of distilled water.

Procedure

The reaction mixture containing 1.0ml buffer, 0.1ml of CDNB and 0.1ml of enzyme homogenate was made upto 2.5ml with water. The reaction mixture was pre incubated at 37°C for 5 minutes. 0.1ml of GSH was added and the change in OD was measured at 340nm for 3 minutes at 30 seconds interval. The enzyme activity was calculated as µmol. CDNB conjugate formed/minute/mg protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$ with the help of formula.

Calculation

$$GST = \frac{\Delta\ O.D/min \times Vol.\ of\ assay \times 100}{9.6 \times Vol.\ of\ conjugate\ enzyme \times protein\ (mg)}$$

2.16.5 ESTIMATION OF GLUTATHIONE REDUCTASE (GR) ACTIVITY

The GR activity was measured by the method of spectrophotometric described by Staal *et al*., (1969) with slight modifications.

Principle

The enzyme activity was assayed by measuring the reduced glutathione (GSH) formed when the oxidized glutathione (GSSG) is reduced by Nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm.

Reagents required

- Sodium-phosphate buffer: (0.3M, pH 6.0)

- Ethylene diamine Tetra Acetic acid (EDTA) (25mM): 930 milligrams of EDTA, trisodium salt dissolved in 100ml distilled water.

- Glutathione (oxidized) (12.5mM): 765. 79 milligrams Oxidized glutathione was dissolved in 100ml distilled water, just before use.

- Nicotinamide adenine dinucleotide phosphate (reduced form) [NADPH] (3mM): 25 milligram of NADPH was dissolved in 25ml of distilled water before use.

Procedure

To 0.2ml tissue extract, 1.5ml buffer, 0.5ml EDTA, 0.2ml oxidized glutathione and 0.1ml NADPH were added. The decrease in OD at 340nm was then monitored for 3 minutes at 30 seconds interval in a spectrophotometer. The enzyme activity was assessed at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/minute/mg protein using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Calculation

$$GR = \frac{\Delta \text{ O. D x Vol. of assay x } 1000}{6.22 \text{ x Vol. of enzyme x protein (mg)}}$$

2.17 ESTIMATION OF NON - ENZYMATIC ANTIOXIDANTS

2.17.1 ESTIMATION OF REDUCED GLUTATHIONE (GSH)

Total reduced glutathione was determined by the method of Moron *et al*. (1979) with slight modifications.

Principle

Total reduced glutathione was estimated in the sample based on the reaction with 5, 5´- dithio-bis (2 nitro benzoic acid) (DTNB or Ellman's reagent) to give a yellow colored compound that absorbs at 412nm.

Reagents required

- Sodium phosphate buffer (0.2 M; pH, 8.0): Ninety four point seven milliliters of 0.2M disodium hydrogen phosphate was mixed with 5.3ml of 0.2M Sodium dihydrogen orthophosphate.

- 5, 5´ Dithio 2-nitrobenzoic acid (DTNB, 0.6mM): Two point three seven milligrams of DTNB was dissolved in 100ml of 0.2M Phosphate buffer.

- Trichloroacetic acid (TCA, 5%): Five grams of TCA was dissolved in 100ml of distilled water.

- Glutathione Standard: 10gms of reduced glutathione was dissolved in 100ml distilled water. The working standard contains 100µg/ml.

Procedure

0.1ml of the test sample was precipitated with 5% TCA. The precipitate was removed by centrifugation. To an aliquot of the supernatant was added 2.0ml of DTNB in 0.2M Phosphate buffer to a final volume of 3ml. The absorbance was read at 412nm against a blank containing TCA instead of sample. A series of standards treated in a similar manner were also run to determine the glutathione content. The GSH content was calculated as µmol DTNB conjugate formed/gram tissue using molar extinction coefficient of $13.6\times10^{3}\ M^{-1}\ cm^{-1}$.

Calculation

$$GSH = \frac{\Delta\ O.D/min\ x\ Vol.\ of\ assay\ x\ 100}{1.36\ of\ mole\ GSH\ conjugate/gm\ tissue}$$

2.18 FREE RADICAL SCAVENGING ACTIVITY ASSAY

2.18.1 LIPID PEROXIDATION ASSAY (LPO)

Tissue lipid peroxidation was measured by the method of Devasagayam and Tarachand (1987).

Principle

Malondialdehyde, an end product of lipid peroxidation reacts with Thiobarbituric acid (TBA) to form a pink chromogen ([TBA] 2-malondialdehyde adduct) and is measured by its absorbance at 532nm.

Reagents required

- Tris-HCl buffer (0.15M, pH 7.4): 1.815 grams of Tris was dissolved in distilled water and the p^H was adjusted to 7.4 with HCl then the volume was made up to 100ml with distilled water.

- Potassium dihydrogen phosphate (KH_2PO_4) (10mM, w/v):0.136grams of KH_2PO_4 was dissolved in 100ml of distilled water.

- Trichloro acetic acid (TCA) 10% (w/v): 10 grams of TCA dissolved in 100ml of distilled water.

- Thiobarbituric acid (TBA) 1% (w/v): 1gram of TBA was dissolved in 100ml of distilled water.

- Standard malondialdehyde: A stock solution of Malondialdehyde was prepared in distilled water, using 1,1,3,3 tetrahydroxypropane. This was stored at 4ºC and diluted just before use, to have a working standard containing 50 n moles /ml.

Procedure

The reaction mixture consisted of 1.0ml of 0.15M Tris- HCl buffer (pH 7.4), 0.3ml of 10mM KH_2PO_4 and 0.2ml of tissue extract in a total volume of 2ml. The tubes were incubated at 37ºC for 20 minutes with constant shaking. The reaction was stopped by the addition of 1ml of 10% TCA. The tubes were shaken well and 1.5ml TBA was added and were heated in a boiling water bath for 20 minutes. Standard tubes containing 10,20,30,40 and 50 nmoles/ml were also run simultaneously. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/gram tissue by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Calculation

$$\text{LPO} = \frac{\text{Vol. of assay} \times \text{O.D} \times 10^9}{1.56 \times 10^5 \times 10^3 \text{gm tissue}}$$

2.18.2 NITRIC OXIDE ASSAY

Principle

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions. Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl)

ethylenediamine, forming an azodye that can be spectrophotometrically quantitated based on its absorbance at 540 nm.

Reagents required

- Phosphate Buffer Saline (0.5 mM, pH 7.4)
- Griess Reagent
- *N*-(1-naphthyl) ethylenediamine dihydrochloride (Component A), 10 mL of a 0.1% (1mg/ml) solution
- Sulfanilic acid (Component B), 10 mL of 0.33% sulfanilic acid in 20% glacial acetic acid
- Equal volumes of *N*-(1-naphthyl) ethylenediamine dihydrochloride (Component A) and sulfanilic acid (Component B) were mixed to form the Griess Reagent

Procedure

The method of Ebrahimzadeh *et al* (2008) was adopted to determine the anti-radical activity of the test sample against nitric oxide radical. A volume of 2 ml of sodium nitroprusside prepared in 0.5mM phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of sample. The mixture was incubated at 25 °C for 150 min. An aliquot of 0.5 ml of the solution was added to 0.5 mL of Griess

reagent. The mixture was incubated at room temperature for 30 min. Ascorbic acid (AA)was used as standard

Calculation

$$NO\ radical\ scavenging\ activity\ = \frac{Abs\ control - Abs\ sample}{Abs\ control} x\ 100$$

2.19 STATISTICAL ANALYSIS

The SPSS software version 11.0 for Windows was used for the statistical analysis. Results are reported as mean ± SEM of three individuals per group per time point (n = 3). The data were processed by two-way analysis of variance (ANOVA).