MATERIAL AND METHODS

3.1 MATERIALS

Chemicals

Most of the chemicals used in the present study were of analytical grade and were purchased from Sigma Chemical Co., Merck, Qualigen, Ranbaxy fine chemicals, SISCO Research Laboratories and LobaChemie, India. Technical grade Chloropyrifos was received on complimentary basis from Meghmani Organics Limited.

Animals

Adult male rats of Wistar strain, weighing 120-180 g were purchased from the central animal house of Panjab University, Chandigarh. All the animals were housed in clean polypropylene cages and were fed standard diet ad libitum (Ashirwad Industries). They had free access of water on a 12 h light/dark cycle. The experimental protocols were approved by the Institutional Animal Ethic Committee (IAEC) and efforts were made to minimize animal suffering.

3.2 METHODS

3.2.1 Treatment of Animals

The animals were randomly segregated into following groups with each experimental group/subgroup having six animals. Corn oil was used as a vehicle for oral administration of Chloropyrifos. For plant materials (gum in case of Commiphora mukul, powdered stem in case of Tinospora cordifolia and powdered fruit in case of Myristica fragans), water was used as a vehicle.

- Control (Vehicle): Animals were administered corn oil.
- Chloropyrifos treated: Animals were further divided into 3 subgroups. subgroup I animals were administered with 10 mg chloropyrifos/kg b. wt., subgroup II animals were administered with 20 mg/kg b. wt. and subgroup III animals 30 mg chloropyrifos /kg b. wt., with reference to the LD₅₀ value of Chloropyrifos for 28 days.
Material and Methods

- *Tinospora cordifolia* + Chloropyrifos treated: Subgroup I animals were administered with 2.0 g/kg b. wt, subgroup II animals were administered with 2.5 g/kg b.wt, subgroup III animals were administered with 3.0 g/kg b.wt. of *Tinospora cordifolia* along with 30 mg chloropyrifos /kg b. wt. and subgroup IV (3.0 g/kg b.wt.) of *Tinospora cordifolia*.

- *Comniphora mukul* + Chloropyrifostreated: Subgroup I animals were administered with 150 mg/kg b. wt, subgroup II animals were administered with 200 mg/kg b.wt and subgroup III animals were administered with 250 mg/kg b.wt. of *Comniphora mukul* along with 30 mg chloropyrifos /kg b. wt. and subgroup IV (250 mg/kg b.wt.) of *Comniphora mukul*.

- *Myristica fragnas* + Chloropyrifos treated: Subgroup I animals were administered with 200 mg/kg b. wt, subgroup II animals were administered with 300 mg/kg b.wt and subgroup III animals were administered with 400 mg/kg b.wt. of *Myristica fragnas* along with 30 mg chloropyrifos /kg b. wt. and subgroup IV (400 mg/kg b.wt.) of *Myristica fragnas*.

3.2.2 Tissue Homogenate

All the treatments were continued for a total duration of 28 days. After 28 days, animals were fasted overnight and sacrificed by decapitation. Their livers were removed and rinsed in ice cold 0.9% (w/v) normal saline, blotted dry, weighed and processed for various biochemical studies. A 10% (w/v) tissue homogenate was prepared in 50mM TrisHCl (pH 7.4) using Potter-Elvehjem glass homogenizer. Post nuclear supernatant was prepared by centrifuging the homogenate at 1000 x g for 10 minutes at 4°C and then supernatant was again centrifuged at 12,000 x g for 20 minutes at 4°C to obtain post mitochondrial supernatant (PMS).

3.3 Antioxidant Defense system

Superoxide dismutase (SOD)

The activity of super oxide dismutase was assayed in the post mitochondrial supernatant according to the method of Kono (1978).
**Material and Methods**

**Principle:** Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride (NH$_2$OH·HCl). The reduction of nitro blue tetrazolium (NBT) dye to blue formazan mediated by superoxide anions was measured at 560 nm under aerobic condition. Addition of super oxide dismutase inhibits the reduction of NBT mediated by hydroxylamine hydrochloride. The extent of inhibition is taken as a measure of enzyme activity.

**Reagents:**
- Solution A: 50mM Sodium carbonate in 0.1mM EDTA (pH 10.8)
- Solution B: 96 µM nitro blue tetrazolium in solution A
- Solution C: 0.6% (v/v) Triton X-100 in Solution A
- Solution D: 20mM hydroxylamine hydrochloride (pH 5.0)

**Procedure:**

In the reference sample (non-enzymatic) 2.0 ml of solution A, 0.7 ml of solution B, 0.15 ml of solution C were added to the cuvette successively and the reaction was initiated by the addition of 0.15 ml of solution D. The development of blue colored complex was followed at 560 nm for 3 minutes at 30 seconds interval.

In the test sample (enzymatic) 1.99 ml of solution A, 0.7 ml of solution B, 0.15 ml of solution C were added to the cuvette successively and the reaction was initiated by the addition of 0.15 ml of solution D and 10µl of appropriately diluted PMS.

**Calculations:**

The activity of enzyme was expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

**Catalase (CAT)**

Catalase activity was assayed in the post mitochondrial supernatant by the method of Luck (1971).
Material and Methods

Principle:

Catalase is enzyme that catalyze the breakdown of hydrogen peroxide to H$_2$O and O$_2$. The rate of decomposition of H$_2$O$_2$ by catalase is measured spectrophotometrically at 240nm.

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2.
\]

Reagents:

- 0.067 M Sodium phosphate buffer (pH 7.0)
- 12.5 mM H$_2$O$_2$ in 0.067 M Sodium phosphate buffer (pH 7.0)

Procedure:

3.0 ml of H$_2$O$_2$ - phosphate buffer was pipetted directly into the cuvette. Appropriate amount of post mitochondrial supernatant was added to it and the contents were mixed thoroughly. The decrease in absorbance was followed at 240 nm for 3 minutes at 30 seconds interval.

Calculations:

The activity of enzyme was expressed as µmoles of H$_2$O$_2$ decomposed/min/mg protein, using molar extinction coefficient of H$_2$O$_2$ (71 M$^{-1}$ cm$^{-1}$).

Glutathione reductase (GR)

The Glutathione reductase (GR) enzyme activity was measured in the postmitochondrial supernatant by the method of Horn (1971).

Principle:

GSSG is reduced to GSH by NADPH through the glutathione reductase catalyzed reaction:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+.
\]

The activity of the enzyme was measured by following the oxidation of NADPH spectrophotometrically at 340nm.
Material and Methods

Reagents:

- 0.067 M Potassium phosphate buffer (pH 6.6)
- 15mM EDTA
- 6mM NADPH (tetra sodium salt) in 1% (w/v) NaHCO$_3$
- 7.5 mM oxidized glutathione

Procedure:

The assay mixture consisted of 2.7 ml Potassium phosphate buffer, 0.1 ml EDTA, 0.05 ml GSSG, 0.1 ml NADPH. The reaction was initiated by the addition of 0.05 ml of postmitochondrial supernatant. Reduction of oxidized glutathione to reduced glutathione by NADPH was followed at 340 nm for 3 minutes at 30 seconds interval.

Calculations:

The activity of enzyme was expressed as nmoles of NADPH oxidized/ min/mg protein, using molar extinction coefficient of NADPH ($6.22 \times 10^6$ M$^{-1}$ cm$^{-1}$).

3.4 Biochemical Estimations

Lactate dehydrogenase (LDH)

Activity of LDH was determined spectrophotometrically by the method of Schatz and Segal (1969) by following the changes in absorbance at 340nm for 3 minutes.

Principle:

The enzyme LDH catalyses the reduction of pyruvate to lactate with simultaneous oxidation of NADH to NAD$^+$ causing a decrease in absorbance at 340nm.

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+
\]

Reagents:

- Tris-HCl (0.1M, pH8)
- Sodium pyruvate (1mM)
- NADH (0.15mM)

Procedure:

The reaction mixture in a total volume of 2.0 ml contained 0.1M Tris-HCl (pH 8), 1mM sodium pyruvate and 0.15mM NADH. The reaction was initiated by adding a suitable amount of the enzyme to produce a measureable decrease in absorbance at 340nm.
**Material and Methods**

**Calculation:**

The enzyme activity was expressed as \( \mu \)moles of NADH oxidized/min/mg protein. The extinction coefficient \( (6.3 \times 10^3 \ \mu \text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}) \) was used to calculate the enzyme activity.

**Acid phosphatase**

For the measurement of acid phosphatase, method of Linhart and Walter (1965) using p-nitrophenyl phosphate as the substrate was employed.

**Principle:**

p-nitrophenyl phosphate is used as substrate and the p-nitrophenol released by enzymic hydrolysis measured at 405nm.

**Reagents:**

- Acetate buffer (0.1 M, pH 4.8)
- Para nitro phenyl phosphate (PNPP)- Dissolved 1.65 mg of PNPP in 1 ml of distilled water.
- Para nitro phenol (Standard) – Dissolved 1.39 mg in 10 ml of distilled water.
- NaOH (0.4 N)

**Procedure:**

1 ml of an assay volume contained 0.9 ml of acetate buffer (0.1 M, pH 4.8), 0.05 ml of substrate PNPP (1.65 mg/ml) and 0.05 ml of sample. The reaction was terminated by the addition of 0.4 M sodium hydroxide after 30 min of incubation at 37°C. The yellow color developed due to the liberation of p-nitrophenol was read at 405 nm. Standards were run simultaneously to calculate the enzyme activity.

**Calculation:**

The phosphatase activity was expressed as \( \mu \) moles phenol produced /min /mg protein.
Material and Methods

Na\(^+\)/K\(^+\)-ATPase

The Na\(^+\)-K\(^+\)-ATPase enzyme activity was measured according to the method of Quigley and Gotterer (1969).

Principle:

Na\(^+\)-K\(^+\)-ATPase activity was measured in the presence of Mg\(^{2+}\), Na\(^+\) and K\(^+\) (total ATPase) and secondly in the presence of Mg\(^{2+}\), Na\(^+\), K\(^+\) and ouabain (oubain insensitive ATPase). The amount of inorganic phosphorus liberated by the action of ATPase on ATP was estimated using Fiske and Subbarow (1925) method. Inorganic phosphate reacts with molybdic acid to form phospho-molybdic acid. On treatment with 1,2,4-aminonaptholsulphonic acid, phosphor-molybdate is selectively reduced to give a deep blue color, which is a mixture of lower oxides of molybdenum. The color is compared with a standard phosphate solution treated in the same way.

Reagents:

- 75mM Tris-HCl buffer (pH 7.2)
- Buffer solution for total ATPase
  - a. 7.5mM Magnesium sulphate (MgSO\(_4\))
  - b. 20 mM Potassium chloride (KCl)
  - c. 120mM Sodium chloride (NaCl)

1.13g of a, 8.75g of b and 1.86g of c were dissolved in 75 mMTris-HCl buffer (pH 7.2) and volume was made to 500 ml with the same buffer.

- Buffer solution for Ouabain- sensitive ATPase. To the total ATPase buffer added 10mM ouabain.
- 10% Trichloro acetic acid.
- 7mM ATP (pH 7.2)
- Standard Phosphorus
- ANSA Reagent
- 2.5% Ammonium molybdate
Material and Methods

Procedure:

To obtain the inorganic phosphate liberated in case of total ATPase, 0.4 ml of the respective buffer was added to the tubes, followed by 0.1 ml of the test sample prepared, 0.4 ml DDW and 0.1 ml of ATP solution. To obtain the inorganic phosphate liberated in case of “Ouabain-sensitive ATPase” 0.4 ml of the respective buffer was added to the tubes, followed by 0.1 ml of the postmitochondrial supernatant, 0.1 ml ouabain, 0.3 ml double distilled water and 0.1 ml of ATP solution.

All the tubes were incubated at 37°C for exactly 15 minutes and 1.0 ml TCA was added to each tube. All the tubes were centrifuged at 300 rpm. 0.5 ml supernatant and standard were taken and volume was made to 1.0 ml with DDW. 7 ml of DDW was added followed by 0.5 ml acidic ammonium molybdate and 0.2 ml of ANSA reagent and mixed well. After 10 minutes, absorbance was measured at 740 nm.

Calculation:

The Na\(^+\)-K\(^+\)-ATPase activity was computed by subtracting the ouabain insensitive ATPase from the total ATPase. The results were expressed as nmoles of iP liberated/min/mg protein.

Acetyl cholinesterase (AChE)

The activity of Acetyl choline esterase was determined in the homogenate according to the method of Ellman et al. (1961).

Principle:

The substrate used in the assay system is acetylthiocholine iodide, the ester of thiocholine and acetic acid. The substrate, acetylcholine is hydrolyzed into thiocholine and acetate by the enzyme. Thiocholine forms a mercaptan, which reacts with the oxidizing agent 5,5\(^\prime\)-dithio-bis 2- nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoate, which has maximum absorption at 412 nm. Thus the activity of AchE can be measured by following the increase in absorbance at 412 nm.
Material and Methods

\[(\text{CH}_3)_3\text{N}^+(\text{CH}_2)\text{-S-CH}_3 + \text{H}_2\text{O} \rightarrow (\text{CH}_3)_3\text{N}^+(\text{CH}_2)\text{-SH} + \text{CH}_3\text{COOH}\]

Thiocholine  Acetic acid

\[(\text{CH}_3)_3\text{N}^+(\text{CH}_2)\text{-SH} + \text{DTNB} \rightarrow 5\text{-thio-2-nitrobenzoate+Mixeddisulfide (colored)}\]

Reagents:
- 0.1 M Sodium phosphate buffer (pH 8.0)
- 10 mM DTNB (Ellman’s reagent)
- 14.9 mM Acetylthiocholine iodide

Procedure:

The reaction mixture consisted of 2.8 ml of 0.1M phosphate buffer (pH 8.0), 0.1ml of Ellman’s reagent and requisite amount of homogenate. The reaction was initiated by the addition of 0.1ml of Acetylthiocholine iodide and the rate of change in absorbance was followed at 412nm for 2 minutes.

Calculations:

AchE activity was calculated using molar extinction coefficient of 5-thio-2-nitrobenzoic acid \((13.6 \times 10^3 \text{M}^{-1}\text{cm}^{-1})\). The results were expressed as nmoles of Acetylthiocholine iodide hydrolyzed/min/mg protein.

3.5 Lipid peroxidation (LPO)

The lipid peroxidation was assayed according to the method of Buege and Aust (1978).

Principle:

Lipid components in the tissue, mainly the PUFAs are highly susceptible to peroxidation by various oxidizing free radicals which are formed from various sources such as enzymatic as well as non-enzymatic oxidation reactions promoted by Fe\(^{2+}\) species etc. Cycloperoxides are formed as a result of these peroxidation reactions, which give
Material and Methods

Malondialdehyde (MDA) by cleavage. MDA forms pink colored complex with thiobarbiturate, which can be measured at 532nm.

Reagents:
- Tris Buffer (150mM, pH 7.1)
- FeSO₄ (1mM)
- Ascorbic acid (1.5mM)
- TCA (10%)
- 5. TBA (0.375%)

Procedure:
To 0.1ml of the sample was added 0.1ml of Tris-HCl buffer, 0.1ml of FeSO₄ and 0.1ml of ascorbic acid in a reaction mixture. The volume was made to 1.0 ml. It was mixed well and incubated at 37°C for 15 minutes. After the completion of the incubation 1ml of TCA and 2 ml of TBA was added and then kept for 15 minutes on the boiling water bath. After that, centrifugation for 15 minutes at 3000 rpm was done. In the blank tube the sample was replaced by water. The absorbance was read at 532nm.

Calculation:
The molar extinction coefficient for MDA is 1.56 x 10⁵ M⁻¹ cm⁻¹. The results were expressed as nmoles of MDA formed /min /mg protein.

3.6 Scanning Electron microscopy
Scanning electron microscope (SEM) is a type of microscope that images the sample surface by scanning it with a high energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample’s surface topography, composition and other properties such as electrical conductivity. Morphological alterations in the erythrocytes were measured by scanning electron microscopy.

Reagents:
- 2.5% glutaraldehyde
Material and Methods

- 0.2M phosphate buffer (pH 7.2-7.4)
- Triple distilled water
- Conductive silver tape

Procedure:

Blood cells were fixed in 2.5% glutaraldehyde made in 0.2M phosphate buffer (pH 7.2). After 1-2 hr of fixation, the cells were separated by centrifugation at 1000-1500 rpm. The fixative was discarded and the pellet was resuspended in the phosphate buffer. This process was repeated two times, every time the supernatant was discarded. The pellets were suspended in triple distilled water, centrifuged and reconstituted two times in triple distilled water. Finally the pellet was suspended in minimum amount of triple distilled water and a drop of sample was smeared on the metallic SEM stubs, which were loaded with a conductive silver tape on its top. These stubs were then coated with gold to a thickness of 100 Å using sputter ion coater, with gold source for 4-5 min. These specimens were finally observed under electron microscope (JSM-6100, Jeol, Japan) at Regional Sophisticated Instrumentation Center (RSIC), Panjab University, Chandigarh, India.

Protein

The protein content was estimated according to the method of Lowry et al., 1951.

Principle:

This method is based on the color reactions of amino acids tryptophan and tyrosine with the Folin’s phenol reagent. By the reaction of these amino acids with molybdidic and phosphotungstic acid, a blue color is formed which is estimated at spectrophotometer. This color is the result of reduction of phosphomolybidic acid and phosphotungstic acid and Biuret reaction of proteins with Cu^{2+} ions in alkaline medium.

Reagents:

- Reagent A: 1% Copper Sulphate.
- Reagent B: 2% Sodium Potassium tartarate.
Material and Methods

- Reagent C: 2% Sodium Carbonate in 0.1N Sodium hydroxide.
- Lowry Reagent: It was prepared just before use by mixing reagents A, B and C in ratio of 1:1:98 prior to use.
- Folin-Ciocalteau reagent: It was prepared fresh by diluting the commercial 2N Folin’s reagent with DDW (1:1, v/v).
- Standard BSA stock solution (1.0 mg/ml).
- Standard working BSA solution: Stock was diluted to make the BSA solution in the range of 20-100µg/ml.

Procedure:

0.1ml of diluted sample, BSA standard, water (blank) was taken in different test tubes and the final volume was made to 1.0 ml with DDW. 3.0 ml of Lowry’s reagent was added to each of the tubes, vortexed and allowed to stand for 10 minutes at room temperature. Further, 0.5 ml of Folin-phenol reagent was added to each tube at room temperature, vortexed for 30 seconds and kept in the tubes at room temperature for 30 minutes. The absorbance was measured at 750 nm. The protein concentration was calculated by comparing the absorbance of test sample with that of standard concentrations (20-100µg/ml).

3.7 STATISTICAL ANALYSIS

All values were expressed as mean ± standard deviation of six observations. Data were analyzed using one way analysis of variance (ANOVA). Values with p≤0.05 were considered as statistically significant.