CHAPTER 3

MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Maintenance of mosquito colonies:

Wild caught mosquitoes viz. *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* (Lin) were collected by sucking tubes and mechanical aspirators (fig. 2) and reared cyclically in separate cages (18" x 18" x 18") following the method of Dash et al. (1988) at 26-28°C temperature and 86% relative humidity (RH). Glucose solution (10% in tap water) and plain water soaked in separate absorbent cotton pads were given *ad libitum* in each cage. For completion of gonotrophic cycles, *An. stephensi* and *Ae. aegypti* were provided with rabbit blood meals, while *Cx. quinquefasciatus* with fowl blood meals. Ovitraps were kept in each cage for the collection of eggs. (fig. 3 to 9).

Eggs collected from respective cages were put into separate enamel trays (12" x 12" x 3") containing 2 litres of tap water for further development (fig. 10 to 13). After eclosion of the larvae, the water was regularly changed every 24 hours and about 10 mg of Dog biscuit and Yeast powder (2:1) mixture was put into each tray for feeding of larvae. The pupae, after development were separated by pasteure pipettes into 250 ml beakers containing plain water and kept in respective cages for adult emergence.
Fig: 2. Photograph showing (a) Sucking tube  
               (b) Mechanical aspirator.

Fig: 3. Rearing of Mosquitoes.
Fig: 4 and 5. Photographs showing rearing of *Aedes aegypti* (Linn) (4) and *Anopheles stephensi* Liston (5): (a) ovitrap (b) feeding dish
Fig: 6. Photograph showing rearing of *Culex quinquefasciatus* Say.

Fig: 7. Photograph showing *Aedes aegypti* (Linn) taking a blood meal.
Fig. 8 and 9. Photographs showing *Anopheles stephensi* Liston and *Culex quinquefasciatus* Say taking a blood meal.
Fig: 10. Photograph showing eggs of *Aedes aegypti* (Lin) collected in whatman filter paper no 3.

Fig: 11. Photograph showing eggs of *Anopheles stephensi* Liston.
Fig. 12. Photograph showing Egg rafts of *Culex quinquefasciatus* Say.

Fig. 13. Photograph showing rearing of mosquito larvae.
Fig: 14. Microphotograph of *Aphis craccivora* Kotch nymphs (16x).

Fig: 15. Microphotograph of *Aphis gossypii* Glover nymphs (16x).
Fig: 16. Photograph of *Aphis craccivora* Kotch nymphs infesting bean flowers.
Fig: 17. Photograph of aphid extracts: TAEag (Extracts from *Aphis gossypii* Glover) and TAEac (Extracts from *Aphis craccivora* Kotch).

Fig: 18. Photograph of juvenoids (Methoprene, Neporex, OMS 3007, OMS 3019 and DPE-28) used in the study.
3.2 Preparation of Aphid Extracts:

Insects: The aphids, *Aphis craccivora* Kotch (fig. 14) and *A. gossypii* Glover (fig. 15) were sampled from the infested bean (fig. 16) and bitter guouard plants respectively and were subjected to solvent extraction for the isolation of JHs following the methods of Bergot et al. (1981) with slight modification (about 1.5 kg. of *A. craccivora* and 1.57 kg. of *A. gossypii*).

Reagents: 1) Acetonitrile (E MERK), 2) Pentane (LOBA) 3) Diethyl ether (E MERK), 4) Methanol 99.9% (GLAXO) 5) Benzene (BDH), 6) Ethylacetate (BDH) (all the solvents were distilled before use) 7) Neutral alumina, 8) Sodium chloride (GLAXO), 9) Celite.

Standards: JHI, JHII and JHIII were obtained from Sigma Chemical Co. St. Louis. USA.

Extraction and Purification of JH:

An amount of 100-150 g of aphids were placed each time in a mortar containing 150 ml of acetonitrile and an amount of celite =1/3 of the weight of aphid samples. The mortar was, then, placed in an ice bath and the contents were homogenized with a pestle. The homogenate was filtered through a Buchner funnel (Ace Glass 9439-08). The cake was returned to the mortar and the above process was repeated
thrice. The filtrates were evaporated in vacuo (cautiously to avoid bumping or foaming) to 30 ml, then decanted into a separatory funnel containing 80 ml pentane and 300 ml 4% sodium chloride. The mixture in the separatory funnel was then shaken for 5 minutes and left to stand still for 30 minutes. Upon removal of the separated aqueous layer, an oily pentane soluble residue was obtained. The pentane solution was carefully transferred with rinses to a glass column (2 x 16 cm) which was packed with 4 g alumina, previously washed with ether. A 5.0 ml portion of diethyl ether was then percolated through the column and the eluent containing the JHs was bulked and dried in vacuo. The weight of the eluent was determined gravimetrically.

Thin layer Chromatography:

Thin layer chromatography (TLC) were performed on (4 x 9 cm) 250 µm thick precoated plates of silica gel (5-25 µm mean particle size, 60oA mean pore diameter) obtained from Sigma chemical Company, St. Louis, USA. Before use the plates were prewashed by development in methanol and activated at 60°C for 12 hours. After the application of the test samples along with the JH standard in separate vertical rows, the plates were developed in the solvent of Benzene: Ethylacetate (15:1). After the development, the plates were air dried and stained with iodine vapour (iodine kept in a chamber). (Novak. 1975)
3.3 Bio-assay:

Bioassay of the two aphid extracts (TAEac = Total aphid extracts of *Aphis craccivora* and TAEag = Total aphid extracts of *A. gossypii*) (fig.17). Five juvenoids (Methoprene, Neporex, OMS 3007, OMS 3019 and DPE-28) (fig.18) were carried out in the Laboratory (28°C and RH 86%) against three species of mosquitoes: *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* following the procedure of WHO (1981). A single compound was tested at a time against a single species. One percent (W/V) stock solution of each compound was prepared by dissolving in 100 % acetone and stored at -20°C. Final concentrations of 0.0001 ppm to 10 ppm were prepared for each compound during the study by adding appropriate volume of stock solution in 250 ml of distilled water placed in a 500 ml beaker. The water was then stirred vigorously with a glass rod for 30-60 seconds. There were two replicates of each concentration and two control replicates.

About 20-25 early fourth instar larvae were released in to each test concentration including control, for assay, within 15-30 minutes of the preparation of test concentrations. After an exposure of six hours, each lot of larvae were removed to separate 500 ml beakers containing 250 ml of plain water. Pupae formed during this period, if any, were removed / discarded and not considered in the
calculations of the results. A pinch of yeast tablet was added into each beaker as feeding for the larvae and held in dimlight until mortality counts were made.

The duration of the observation period was that required for a complete adult emergence in the control beakers (WHO 1981). Mortalities and non-emerged adults were taken into consideration to assess the effects. The successfully emerged adults were counted from the remaining empty pupal cases. The mortality was corrected using " Abbott's formula " (Abbot. 1925). whenever necessary.

Tests with a control effect of 20 % or more were repeated. The EC₅₀ and EC₉₀ doses were calculated by probit regression equation (Finney, 1953) and the relative activity between test compounds were computed according to UNEP (1982).

3.4 Treatment of eggs with Aphid Extracts and Juvenoids :

Test concentrations of EC₅₀ and EC₉₀ doses were prepared for TAEac. TAEag. Methoprene. Neporex. OMS 3007. OMS 3019 and DPE-28 by adding appropriate volumes of stock solutions (1 μg/10 μl) and vigorously stirring in 1 litre of tap water kept in 12" x 12" x 3" white enamel tray. Five replicates were taken for each concentration of each compounds.
Two different age groups of eggs (*An. stephensi*: 0 - < 12/ > 12 hours, *Cx. quinquefasciatus*: 0 - < 12/ > 12 hours and *Ae. aegypti*: 0 - < 24/>24 hours) were selected for the study. In each set of experiments, eggs of desired age/number/species were taken and released into the treatment tray. Separate controls (water mixed with acetone) were maintained for comparison. After eclosion of the larvae, about 10 mg of the yeast tablet was given in each tray for feeding of the larvae.

The following parameters were noted after treatment:

(1) **Hatching**: The number and percentage of hatching of the larvae from the eggs treated with aphid extracts and juvenoids were recorded and were compared with the control ones to evaluate the ovicidal activity of the compounds tested.

(2) **Mortality in different stages and morphological abnormalities**: The pattern of mortality in different instars of larvae (I, II, III and IV), pupae, rate of adult emergence, rate of formation of morphologically abnormal larvae and adultoid formation were computed for different compounds against different mosquito species. At the fourth instar, each larva in the experiment group was examined for different morphological abnormalities and compared with natural ones.
(3) **Sex Ratio:** Number of successful males and females emerged after treatment with different test formulations were counted to calculate the sex ratio.

(4) **Developmental period:** Time taken for hatching of the larvae from the eggs, duration of different larval instars (I, II, III and IV), duration of the pupal stage and longevity of the adults (male/female) were noted in different groups of treatment and control ones to analyse the differences.

(5) **Fertility and Fecundity:** Adults emerged after the treatments (separate test for each compounds and species) were allowed to mate and lay eggs according to the procedure laid down in the para (3-1) of maintainance of mosquito colony. The number of female mosquitoes laid eggs were screened by examining the abdomens of the female mosquitoes to compute the fecundity rate. The eggs laid were counted and allowed for hatching to evaluate the fertility rate. The time taken from taking the blood to lay eggs (oviposition time) by the 0+ mosquitoes were noted in different treated groups and were compared with the control ones.

(6) **Dyar’s law/Morphometry:** From each instar, around 40 larvae were killed by putting into hot water (60° -70°C) and the length/width of head, thorax and abdomen were
measured with occular micrometer. Dyar’s factor was calculated following Kiatfunengfoo and Sucharit (1986). The mean measurements of the above characters were calculated and were transferred into logarithmic value to test whether it follows Dyar’s law.

The measurement of Winglength, length of proboscis, length and breadth of head, thorax and abdomen of adult male/female were taken separately in treated and control ones for comparison.

(7) Chaetotaxy: The larval chaetotaxy of An.stephensi, Cx. quinquirasciatus and Ae. aegypti were examined in different treated groups in comparison to the control ones.

(8) Each post-embryonic developmental stages were taken for the study of various biochemical parameters after treatment at the egg stage and the values were compared with the corresponding values of control groups.

(9) For histopathological examination of different organ systems after treatment, the 4th instar larvae and adult mosquitoes were taken.
3.5 Histological Techniques:

To study the histopathological changes of different organs of the mosquito larvae and adults, the 4th instar larvae and adults after treatment were processed as follows.

Processing of Larvae:

Fixation: The whole larvae were fixed in Bouin's fluid (water-190 ml, picric acid-2.5g, formalin - 60 ml and Glacial acetic acid-12.5 ml) for 12-15 hours at room temperature.

Dehydration: After fixation the tissues were dehydrated in graded ethyl alcohol
- .50% (Rinsed for 30 minutes)
- .70% (Kept for 24 hours)
- .90% (Kept for 12 hours)
- 1.00% (Three changes, first two were 30 minutes and last one was 60 minutes)

Clearing: Cleared with chloroform (two changes 30 minutes each)

Embedding: Embedded in molten paraffin wax (58°- 62°C)
Three changes: I=30 minutes, II=90 minutes, III=30 minutes).

Blocking: The tissues were blocked (of desired orientation) in paraffin wax (58° -62°C) with the help of Leukant's Mould:
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Processing of adult Mosquitoes:

**Fixation:** The adult mosquitoes were fixed in Duboscq-Brasil (80% alcohol: 150 ml, formalin: 60 ml, Glacial acetic acid: 15 ml, picric acid: 1 g) for 8-10 hours.

**Dehydration:** Dehydration of tissues were made in graded ethyl alcohol

- .70% (Washed thrice 15 minutes each)
- .70% (Kept for 12 hours)
- .90% (Kept for 12 hours)
- .100% (Three changes, first two were 30 minutes and last one was 60 minutes)

**Clearing:** Tissues were cleared in chloroform (two changes, 30 minutes each)

**Embedding:** Embedded in molten paraffin wax (58°-62°C) (three changes: 1st 30 minutes, 2nd 90 minutes and 3rd 30 minutes)

**Blocking:** The mosquitoes were blocked with desired orientation in the paraffin wax (58° - 62°C) with the help of Leukant's mould.

**Section cutting:** Tissue sections of 6-7 μ thickness were obtained using Reichert Jung, Auto cut 2040. The
sections were transferred to microscopic slides, with egg albumin and distilled water and spread, dried and left in a warm place overnight.

**Staining:** Both larval and adult tissue sections were stained with Haematoxylin and Eosin stain (H and E stain) as follows.

- The paraffin wax was dissolved by gentle warming (58°-62°C).
- Put into xylene, absolute alcohol, 90%, 70%, 50%, distilled water (3-4 minutes each).
- Stained in Ehrlich’s Haematoxylin, for 1-1/2 minutes.
- Rinsed in distilled water.
- Put into 1% hydrochloric acid in 70% alcohol for washing of the excess stain adhered to the tissues.
- Rinsed in distilled water for 5 sec.
- Kept in running water for 45 minutes for blueing.
- Transferred to 0.035% ammonium hydroxide for 3 minutes.
- Rinsed in distilled water for 5 secs.
- Counter stained with 5% aqueous Eosin for 20 minutes.
- Transferred through 70%, 95% and 100% ethanol (10 secs each) to xylene (two changes 10 minutes each).
- Mounted in DPX.

**Examination of slides:** Slides were examined with Nikon’s compound microscope having photomicrographic attachment (computerized). Photomicrographs were taken when necessary.
A minimum of 20 individuals from all the samples (i.e., control and treated larvae and adults of Ae. aegypti, An. stephensi and Cx. quinquefasciatus) were taken for histological studies.

3.6.1 Determination of total Glycogen and Glucose:

Quantitative estimation of glycogen and glucose were made following the procedure of Van Handel (1985)

Chemical/Reagents:

1. Anthrone reagent: One hundred and fifty ml of distilled water was poured into a 1000 ml Erlenmeyer flask. While cooling, 380 ml of concentrated sulphuric acid (H₂SO₄) was added carefully. In this diluted H₂SO₄ 750 mg anthrone was dissolved. This reagent can be stored at 4°C.

2. Standard solution: Glycogen (1 mg/ml) in distilled water and Glucose (1 mg/ml) in 25% ethanol (C₂H₅OH) was prepared.

3. Sodium sulphate: 2% solution in distilled water.

4. Methanol:

Procedure:

Calibration: In separate test tubes, 25, 50, 100, 150 and 200 μg (= μl) of glycogen solution (1 mg/ml) were placed. Appropriate volume of anthrone reagent was poured into individual tubes so as to make the final volume of the solution to 5 ml, mixed and heated in a water bath at 90°C -
92°C for 17 minutes. Then the solution was cooled and read directly in a LKB-spectrophotometer at 625 nm wavelength. The OD was plotted, glycogen vs OD, to form a calibration line. Following the similar procedure, the calibration line of the glucose (25, 50, 100, 150 and 200 μg (= μl) was prepared.

Separation and determination of Glycogen and Glucose:

Approximately 20-40 mg of larvae/pupae/adult mosquitoes were placed in a homogenizing tube. 0.4 ml of sodium sulphate solution was poured, and then homogenized. After the homogenization, 2 ml of methanol (CH₃OH) was added and then centrifuged in a centrifuge tube at 2.5 K for 5 minutes. The supernatant (containing glucose) was decanted to a test tube and the solvent was evaporated to 0.1-0.2 ml. The precipitate (containing glycogen) was washed with 80% methanol and then transferred to a second set of test tube. After this, the glycogen and glucose containing test tubes were filled with appropriate volume of anthrone reagent so as to make the final volume of the test samples to 5 ml. heated (at 90°C -92°C) for 17 min. cooled, mixed and the OD was taken at 625 nm. If the OD’s was above 2 these were diluted appropriately before taking the final reading. Glycogen and Glucose contents were read directly from the respective calibration line and then expressed in mg Glycogen/Glucose per gm of tissue.
3.6.2 Determination of total Lipid:

Total lipid in mosquitoes (larvae/pupae/adults) were determined by the method described by Van Handel (1985).

Chemical/Reagents:

1. Vanillin-phosphoric acid reagent: 600 mg of Vanillin was dissolved in 100 ml of hot water. Then 400 ml of 85% phosphoric acid was added and stored in dark.
2. Sulphuric acid (95-98%)
3. Chloroform-Methanol (1:1)
4. Lipid standard: 100 mg/100 ml of a commercial vegetable oil (Soy bean oil) in chloroform.

Procedure:

Calibratrion line: In separate test tubes, 50, 100, 200 and 400 ul of the standard solution (1 mg/ml) were taken. Solvents were then evaporated keeping in a hot water bath (60-65°C). About 200 ul of sulphuric acid was added and then heated for 5-10 minutes in a boiling water bath. The test tubes were brought out after heating was over. After cooling, vanillin reagent was poured to make the final volume to 5 ml and allowed for 5-7 minutes to develop the reddish color. The tubes were then read directly in a LKB-spectrophotometer at 525 nm against the reagent blank. After this the calibration line was drawn taking μg lipid vs OD's.
Approximately 10-30 mg of mosquitoes (larvae / pupae / adults) were homogenized with 500 ul of chloroform: methanol solution, centrifuged at 2000 rpm for 4-5 minutes and the supernatant was used for lipid determination. Two hundred ul of sulphuric acid was added to 100 ul of supernatant and heated for 5-10 minutes in a boiling water bath. After cooling appropriate volume of Vanillin reagent was poured to make the final volume to 5 ml. The OD was read at 525 nm and amount of lipid was recorded from the calibration line. The quantity of the lipid estimated was expressed as milligram (mg) of lipid per gram (gm) of tissue.

3.6.3 Determination of total Protein:

The total protein of the mosquito larvae/pupae/adults were estimated following the method of Bradford(1976).

Chemicals and Reagents:

1. Approximately 100 mg of coomassie brilliant blue was dissolved in 50 ml 95 % ethanol and 100 ml of 85 % phosphoric acid. This was made upto 1000 ml with distilled water and stored at 4°C for use.

2. Standard solution: Bovine serum albumin (BSA) (1mg/ml) in sterile distilled water was prepared.
Procedure:

Calibration line: In separate test tubes 10, 20, 40, 50, 100, 150 \( \mu g \) of standard BSA solution was taken, and appropriate volume of Bradford reagent was mixed to make the final volume to 5 ml and vortexed for 1 minute. The OD's were read in the LKB spectrophotometer at 595 nm Wavelength. The calibration line was plotted taking \( \mu g \) of protein vs ODS.

Approximately 10-30 mg of larvae/pupae/adult mosquitoes were homogenized with 500 ul of sterile distilled water, centrifuged and supernatant was used for determination of total protein. To 100 ul of supernatant, 4.9 ml of Bradford reagent was mixed, vortexed for 1 minute. The OD was read after 2 minutes but before 60 minutes at 595 nm. The quantity of the protein was read off from the calibration line. The total protein was expressed in milligram (mg) of protein per gram (gm) of tissue.

3.6.4 Preparation of Mosquito Tissue Homogenates for Enzyme Assays.

Approximately, 60-70 mg of mosquito larvae/pupae/adults were taken in a homogenizing tube and were homogenized with 500 ul of 0.25 M Sucrose solution at 4°C. After homogenization for three to five minutes, the samples were collected in eppendorf tubes. To make the tissues completely soluble, these tubes were put into the liquid
nitrogen (-195° C) and taken out immediately for rapid freezing and thawing, to ensure that if any cells remained, would burst out. The crude homogenate was centrifuged at 2,500 rpm for 5 minutes. The supernatant (containing enzymes) was decanted to another set of eppendorf tube and preserved at -20°C. The residue was again homogenized with 500 ul 0.25 Sucrose solution and the above procedure was repeated thrice. The combined supernatants were then divided into three separate aliquots, for assay of three different enzyme systems (Alkaline phosphatase/Acid phosphatase, Glucose-6-phosphate dehydrogenase, and Acetyl choline esterase).

3.6.4.1. Alkaline Phosphatase (ALP) and Acid Phosphatase Enzyme (ACP) Assay

Alkaline phosphatase (ALP) and Acid phosphatase (ACP) activities were quantitatively estimated following the method of Bramley (1974) with slight modification.

Principle:

Alkaline phosphatase and Acid phosphatase catalyse the hydrolysis of P-nitrophenyl phosphate (P-NPP) in alkaline and acid medium respectively to form P-nitrophenol (P-NP) and inorganic phosphate (ip). The released P-NP is in the form of dissociated phenylate ion, which is yellow in colour. The intensity of the colour is proportional to the activity of
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Alkaline phosphatase/acid phosphatase, which is measured at 420 nm:

Alkaline phosphatase

(i) $\text{p-nitrophenyl phosphate} + \text{H}_2\text{O}$

(colour less)

alkaline medium

$\text{p-nitrophenol} + \text{iPr}$

(yellow colour)

Acid phosphatase

(ii) $\text{p-nitrophenyl phosphate} + \text{H}_2\text{O}$

(colour less)

Acid medium

$\text{p-nitrophenol} + \text{iPr}$

(yellow colour)

Reagents:

(i) **Alkaline phosphatase:**

0.1 M sodium carbonate-bicarbonate buffer PH 10.4.

0.5 $\text{mM}$ EDTA (adjusted to PH 10.4 with INNaOH)

3 $\text{mM}$ magnesium acetate

30 $\text{mM}$ $\text{p-nitrophenyl phosphate}$

mosquito (larvae/pupae/adults)

tissue homogenate containing 100-150 $\text{ug}$ of protein.

(ii) **Acid phosphatase:**

0.1 M sodium acetate-acetic acid buffer PH 6.2

1 $\text{mM}$ EDTA (disodium salt)

10 $\text{mM}$ magnesium acetate

30 $\text{mM}$ $\text{p-nitrophenyl phosphate}$

mosquito (larvae/pupae/adults)

tissue homogenate containing 100-500 $\text{ug}$ protein.
(iii) Standard solutions:

5 ug, 10 ug, 15 ug, 20 ug p-nitrophenol (p-NP)/100 ul of distilled water is prepared.

Procedure:

To draw a standard graph, the OD's of the known amount of p-NP are taken at 420 nm. Before taking the OD's the following reagent mixture is prepared.

Buffer (Alkaline PH for ALP/Acid PH for ACP) 700 ul
EDTA 0.5uM for ALP/1 uM for ACP) 100 ul
Magnesium acetate (3 uM for ALP/10 uM for ACP) 100 ul
p-NP (5 ug, 10 ug, 15 ug, 20 ug) 100 ul
0.5 (W/V) EDTA in 0.5M NaOH 3 ul

Then the OD's are taken at 420 nm and the standard graph is drawn taking, p-NP as OD.

For both the enzymes, first a reaction mixture is prepared to allow the enzyme catalysis. One ml of reaction mixture contains (respective reagents for alkaline phosphatase and acid phosphatase).

Buffer 600 ul
EDTA 100 ul
Magnesium 100 ul
Acetate
Homogenate 100 ul
p-NPP 100 ul
Then the reaction mixture is incubated at 30°C. Exactly after 30 minutes, the reaction (for both the enzymes) is stopped by adding 3 ml of 0.5% (W/v) EDTA in 0.5M NaOH. The OD is taken at 420 nm and the quantity of P-NP produced is read off from the standard graph.

The enzyme activity is then expressed in mole P-nitrophenol liberated/min/mg of protein.

Calculation:

Suppose X μg of P-NP is liberated
Then X μg of P-NP/0.1 ml homogenate (original)/30 min
X x 10 μg P-NP/1 ml/3 min
X x 10 xy μg P-NP/y ml of homogenate/30 min.
(where y = total homogenate of the tissue)
X x 10xy μg P-NP/p g of tissue
(where p= wet weight of the tissue)
X x 10xy
------------- μg P-NP/q g protein/30 min
p
(where q = g protein/ g tissue)
X x 10xy
------------- μg P-NP/mg protein/minute
pxq x 30
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All this divided by the molecular weight of P-NP (139.11) gives the mol activity.

3.6.4.2 Glucose-6 Phosphate Dehydrogenase (G6PD) Enzyme Assay:

G6PD enzyme assay has been done in mosquitoes (larvae/pupae/adults) according to WHO (1967).

Principle:

G6PD catalyses the oxidation of Glucose-6-phosphate to 6 phosphogluconic acid (6PG), in presence of coenzyme NADP. It is then reduced to NADPH, which has a maximum absorbance at 340 nm spectrum.

Reagents:

1.0 M Tris-Hcl buffer (PH8.0)
2 µM Nicotinamide adenine dinucleotide phosphate (NADP)
0.1M Magnesium chloride (MgCl2)
6 µM Glucose-6-phosphate (G6P)

Procedure:

A reaction mixture is prepared in the order given, just before the experiment in a 3ml test tube.

1. H2O 550 ul
2. NADP 100 ul
3. Tris-Hcl 100 ul
4. MgCl2 100 ul
5. Tissue homogenate 50 ul
The reaction is started by adding 100 ul of G6p to the reaction mixture and water to the blank. The OD of the final reaction mixture is taken at 340 nm at 25°C at one minute interval. After few minutes the rate of change of OD should become linear. The maximum linear rate is used in computing the enzyme activity. 

**Interpretation:**

International units are used in expressing enzyme activity. One unit of G6PD shall consists of the quantity of enzyme which reduces one umol of NADP per minute. One umol/ml of reduced NADP has an absorbance of 6.22 in a light path of one centimetre.

\[
\text{Activity (umol/min/mg of protein)} = \frac{\text{OD/min}}{6.22 \times x}
\]

(\text{where } x = \text{mg of protein per 50 ul of homogenate}).

**3.6.4.3. Assay of Acetyl Choline Esterase (AchE) activity:**

Acetyl choline esterase (AChE) enzyme assay was performed following the method described by NIN (1983).

Thio analogue of acetyl choline is used as a substrate and the -SH groups released are titrated with 5, 5-dithiobis-(2-nitro benzoic acid) - DTNB. The change in optical density (OD) can be followed spectrophotometrically at 412 nm.
Reagents:

1. Cocktail: The following solutions were mixed to give the cocktail reagent:
   - 1.0M sodium chloride (NaCl) 13.0 ml
   - 0.5M tris-HCl (pH 7.5) 10.0 ml
   - 1.0M Magnesium chloride (MgCl2) 2.0 ml
   - 0.2M EDTA (pH adjusted to 7.0 with NaOH) 10 ml.
2. 0.1M acetylcholine chloride (it is preserved in amber coloured bottle)
3. 1µM DTNB.
4. Reaction mixture: Cocktail - 10.5 ml
   (prepared freshly) DTNB - 3.0 ml
   Water - 6.5 ml

Procedure:

Two ml of reaction mixture was placed in a test tube and 0.03 ml of 0.1 M acetylcholine and 0.87 ml of distilled water (sterile) was added. The reaction of the enzyme activity is initiated by the addition of 100 ul of mosquito tissue homogenate.

A substrate blank without the enzyme is taken since acetylcholine always has 2-5% of free -SH groups (depending on the batch number and make) and is also unstable.
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Calculation:

Molar extinction co-efficient of -SH group of DTNB is \(14.3 \times 10^3\) at 412 nm.

For a 3.0 ml reaction volume it will be

\[
\frac{14.3}{3} = 4.43
\]

OD/min

Activity of AchE = \[
\frac{4.43 \times Y}{\text{umoles/min/mg of protein}}
\]

(Where \(Y\) = mg of protein in 100 ml of mosquito tissue homogenate).

3.7 Statistical analysis:

Data were analysed using

1) Test of significance for difference in proportion.
2) Students' 't' test.
3) Analysis of variance (ANOVA) or 'F' test.

Graphs were drawn using the Graphics softwares in IBM compatible Personal Computers with EPSON EX-1000 dot matrix printer having colour option.