2. MATERIALS AND METHODS
2.1 Experimental insect

*M. domestica*, the common housefly, a member of the family Muscidae and order Diptera, was the experimental organism of choice in the present study. This insect is known to have relatively short reproductive cycle lasting about 10 days from egg to adult stage and can be mass-cultured at a minimal cost. Despite its smallness, the fleshy larvae yield, on careful puncturing and withdrawing, 5\(\mu\)l of haemolymph. Active and wandering larvae can be immobilized by cold anaesthesia without any damage to the tissues. The maintenance of the mass cultures is also considerably easy. The above reasons were responsible for the selection of housefly as the experimental organism.

2.1.1 Collection and rearing of houseflies

Adult houseflies were initially collected from market places using hand nets and brought to the laboratory in polythene bags. Flies were maintained in cubical plastic containers (30 x 30 x 30 cm) at 28 ± 2°C and 50 ± 5% relative humidity. A dish with small amount of bran was kept inside the container for egg deposition and was replaced each day. Adult flies were fed *ad libitum* with sugar solution prepared using diluted milk, through soaked cotton.

2.1.2 Culture procedure

Housefly culture was maintained following the procedure of Grady (1928).

The culture medium consisted of

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice bran</td>
<td>1575.00 g</td>
</tr>
<tr>
<td>Chicken mash</td>
<td>1425.00 g</td>
</tr>
<tr>
<td>Yeast granules</td>
<td>7.50 g</td>
</tr>
<tr>
<td>Glass distilled water (GDW)</td>
<td>2600.00 ml</td>
</tr>
</tbody>
</table>
The above ingredients were mixed thoroughly. Cylindrical jars of size 15 x 20 cm were filled with the medium approximately to three-fourth of the volume. Eggs laid afresh were collected gently with no.1 painting brush and were placed on the top of the medium. About 600 eggs were placed in each jar. The containers were covered with cheese-cloth held in position by a rubber band. The eggs hatched after about 24h and the life-cycle, with two larval moults, was completed in 8-10 days. Pupae collected by sieving the medium were kept in cylindrical containers (15 x 20 cm) layered with a moist sand at the bottom. Each container had about 500 pupae. For all experimental studies third instar larvae were used.

2.2 Bacterial strains

Bacterial species used in present studies and the sources from which they were obtained are as follows:

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> K12 strain</td>
<td>National Chemical Laboratory, Pune, India.</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>Department of Biotechnology, Madurai</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Kamaraj University, Madurai, India.</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus polymyxa</em></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Thomas Diagnostic and Research Centre</td>
</tr>
<tr>
<td><em>E. coli</em> from human source</td>
<td>Tiruchirappalli, India.</td>
</tr>
</tbody>
</table>

2.2.1 Culture of bacteria

Glassware used in culturing of bacteria were well cleaned using detergents, washed in water and thoroughly rinsed in GDW. All glass and
metal apparatus were sterilized in a hot air oven at 180°C for a minimum period of 3h. Luria broth (LB) was the medium used for growing the bacterial cultures.

**Luria broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.00 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.00 g</td>
</tr>
<tr>
<td>GDW</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

The medium pH was adjusted to 7.2. The solid medium, in addition to the above ingredients, contained 1 g of agar per 100 ml of medium. The solid and liquid media were sterilised in an autoclave at 15 psi for 20 min.

The sterilized liquid medium was inoculated with the specific bacterium with a platinum loop and kept in a shaker-incubator at 37°C for overnight. 1 ml of overnight culture contained approximately $10^9$ bacteria.

Of the different bacterial species listed earlier, *E. coli* K12 strain, *P. aeruginosa* and *M. luteus* were used exclusively for immunizing the housefly larvae. The other bacteria along with these three species were used for testing the antibacterial activity of the immunized haemolymph.

### 2.3 Preparation of larvae for immunization

Third instar larvae obtained from housefly culture were washed twice with water followed by 80% iodinated alcohol. The larvae were blotted dry on filter paper and were immobilized by cold anaesthesia. When the larvae were returned to ambient temperature, they exhibited normal activity and mobility.
2.3.1 Immunization

Third instar larvae were inoculated with 2 µl of diluted log phase bacterial culture in 0.8% saline containing about 5000 viable cells. Bacterial concentrations were determined by optical density (OD) measurements at 740 nm using a spectrophotometer (Spectronic 21-Bausch and Lomb, USA) followed by plate counting. Inoculations were done with a 10 µl Hamilton microsyringe. Extreme care was taken to avoid blocking of the needle of the syringe by trachea or by coagulated haemolymph by flushing the needle repeatedly with saline water. Blocked needles were regularly cleaned in saturated solution of sodium dodecyl sulphate (SDS) or in acetone. Nevertheless, blocking of the needles with coagulated haemolymph was a major problem encountered while immunizing the larvae.

Immunization effects could also be achieved by pricking the larvae with sterile hypodermic needle dipped in a specific bacterial culture. This procedure modified from the one adopted by Okada and Natori (1983) was practised for collecting immunized haemolymph at a relatively shorter time and obviated the need for elaborate culturing of bacteria. The immunized larvae, either by injection or by mere injury, returned to the same culture medium from which they were collected earlier.

2.4 Collection of haemolymph

24h after immunization, the larvae were collected and sterilized by washing twice in fresh water and once in 80% iodinated alcohol. An incision was made in the larval skin with corneal scissors and the oozing haemolymph was collected using a fine-tipped capillary tube. The haemolymph was transferred to ice-cold Eppendorff tube containing a few crystals of phenylthiourea (PTU) that prevented the melanization of haemolymph. Care was taken to flush the tips of the capillary tubes with diluted PTU to prevent blackening of the tip. The haemolymph thus collected was centrifuged at 5000 g at 4°C for 5 min. The
cell-free, clear supernatant was regarded as antibacterial serum. Haemolymph collected from non-immunized larvae served as control serum.

2.5 Immunological procedures

To ascertain whether any immunological identity existed between the immune serum obtained from the housefly larvae and the bacterial proteins, conventional immunodiffusion and immunoelectrophoretic procedures were carried out in agar plates.

2.5.1 Immunodiffusion

Qualitative immunodiffusion was done following the method of Ouchterlony (1949).

2.5.1.1 Reagents

a) 0.5% Agarose solution (w/v)

Agarose - B (Pharmacia) - 0.5 g
GDW to - 100.0 ml
Prepared by heating in a boiling water bath.

b) 0.025M Barbital buffer (pH 8.6)

Barbitone - 4.6 g
Sodium barbitone - 5.2 g
GDW to - 1000.0 ml

c) 1% Agarose solution (w/v)

Agarose - B - 1.0 g
Barbital buffer to - 100.0 ml
Prepared by heating in a boiling water bath.
2.5.1.2 Preparation of immunodiffusion plate

Microslides (75 x 25 cm) were coated with a thin film of agarose by dipping a sandwich of them in a hot solution (50°C) of 0.5% agarose. 1 mm thick spacers were sandwiched between a precoated plate and a silicon-grease coated plate which were held together by metal clamps. 5 ml of hot solution (50°C) of 1% agarose was poured in the space between these plates and thus an immunodiffusion plate of uniform thickness was obtained.

Soon after agar gel solidified, circular wells (1 centre and 5 to 6 peripheral) were punched out with a 2 mm gel punch. Small pieces of agarose sticking to the well were removed by a Pasteur pipette.

2.5.1.3 The antigen and antiserum used

The antigen solution was prepared by shaking a thick suspension of bacteria (E. coli, M. luteus and B. thuringiensis in 1% SDS solution containing 50 mM ethylenediaminetetraacetic acid (EDTA). The immune haemolymph obtained from housefly larvae served as the antiserum.

2.5.1.4 Procedure

In one preparation, serial dilutions of the immune serum was used as the antiserum source to determine the threshold concentration of antiserum required to elicit a precipitation reaction. The centre well was filled with antigen.

In another preparation again the central well served as antigen well. The five peripheral wells were filled with control haemolymph and variously treated immune serum. They were (i) control haemolymph obtained from non-immunized insects (ii) immune serum (iii) immune serum heated in a boiling water bath at 100°C for 5 min (iv) immune serum incubated with equal
volume of 0.001% trypsin at 37°C for 1h and (v) immune serum adsorbed with *M. luteus* at a concentration of 2x10^6 cells for 1h.

The agar plates were left in a humid chamber at 37°C and observed over a period of 48h.

### 2.5.2 Immunoelectrophoresis

Immunoelectrophoresis was carried out following the method of Feinstein (1976).

#### 2.5.2.1 Reagents

a) 0.025 M Barbital buffer (pH 8.2)

- Sodium barbitone: 3.96 g
- 0.1N HCl: 57.50 ml
- Sodium azide: 1.00 g
- GDW to 1000.00 ml

b) 1% Agarose solution (w/v)

- Agarose - B: 1.00 g
- Barbital buffer to 100.00 ml

Prepared by heating in a boiling water bath.

#### 2.5.2.2 Preparation of plates, antigens and antiserum

Precoated microslides of 75 x 25 mm were used for preparation of plates and the procedure was similar to the one described in section 2.5.1.2.

Two antigen wells (2 mm diameter) were cut in the middle of the plate and an antiserum trough was cut in the centre of agar gel. The antigens were
prepared as described in section 2.5.1.3. The immune serum of housefly larvae served as antiserum source.

2.5.2.3 Procedure

The plates were placed in the electrophoresis chamber and connected to buffer chambers through paper wicks. The wells were filled with bacterial antigen and electrophoresis was carried out for 100 min at 7 to 8 v/cm width of the slides. At the end of electrophoresis, plates were taken out and the central agar strip was removed from the antiserum trough. The trough was filled with the immune serum and the plates were incubated in a humid chamber for 48h.

2.5.3 Staining procedure

Both the immunodiffusion and immunoelectrophoretic plates were washed extensively in 0.9% sodium chloride solution (w/v) for 6h to remove the excess untreated proteins. Subsequently the plates were washed in GDW and air dried. The precipitin arcs were stained with 0.025% Coomassie brilliant blue (w/v) for 15 min and destained in a solution of methanol, GDW and acetic acid (5:5:1 v/v). The plates were finally washed in 7% acetic acid (v/v), dried and photographed.

2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS - PAGE)

SDS - PAGE was resorted to determine the polypeptide composition of the immune serum. The procedure adopted was according to the method of Laemmli (1970).
2.6.1 Preparation of sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.625 M Tris (hydroxymethyl) aminomethane (Tris)</td>
<td>- HCl buffer (pH 6.8)</td>
<td>20 ml</td>
</tr>
<tr>
<td>20% SDS (w/v)</td>
<td></td>
<td>20 ml</td>
</tr>
<tr>
<td>50% β - mercaptoethanol (v/v)</td>
<td></td>
<td>20 ml</td>
</tr>
<tr>
<td>100% Glycerol</td>
<td></td>
<td>20 ml</td>
</tr>
<tr>
<td>0.02% Bromophenol blue (w/v)</td>
<td></td>
<td>20 ml</td>
</tr>
</tbody>
</table>

Proteins in the normal and immune haemolymph were estimated by following Lowry et al. (1951). From each sample, 2 μl was collected and diluted 10 times with Tris-HCl buffer pH 6.8. From this diluted sample, a volume equivalent to 25 μg protein was collected, mixed with 10 μl of sample buffer and was heated for about 2 min in a boiling water bath. The protein standard mixture was dissolved in 100 μl of Tris-HCl buffer and heated similar manner and out of it 25 μl was loaded on a separate well.

2.6.2 Stock solutions

a) Monomer

- Acrylamide: 30.0 g
- Methylene Bis acrylamide: 0.8 g

GDW to 100.0 ml

b) Resolving gel buffer (Tris - HCl pH 8.3)

- Tris: 36.30 g
- 1N HCl: 48.00 ml

GDW to 100.00 ml
c) Stacking gel buffer (Tris - HCl pH 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6.00 g</td>
</tr>
<tr>
<td>1N HCl</td>
<td>48.00 ml</td>
</tr>
<tr>
<td>GDW to</td>
<td>100.00 ml</td>
</tr>
</tbody>
</table>

d) 10% SDS

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>1.00 g</td>
</tr>
<tr>
<td>GDW to</td>
<td>10.00 ml</td>
</tr>
</tbody>
</table>

e) Catalyst

N, N, N, N' - tetramethylethylenediamine (TEMED)

f) Initiator

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>0.15 g</td>
</tr>
<tr>
<td>GDW to</td>
<td>10.00 ml</td>
</tr>
</tbody>
</table>

This solution was prepared just before use.

g) Reservoir buffer (Tris - glycine pH 8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.00 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.40 g</td>
</tr>
<tr>
<td>SDS</td>
<td>1.00 g</td>
</tr>
<tr>
<td>GDW to</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

2.6.3 Preparation of gels

The resolving and stacking gels were of the following composition:
(i) 10% Resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>10.00 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Initiator</td>
<td>1.50 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>GDW to</td>
<td>30.00 ml</td>
</tr>
<tr>
<td>Catalyst</td>
<td>0.015 ml</td>
</tr>
</tbody>
</table>

The solution was degassed for 2 min before the addition of catalyst.

(ii) 5% Stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>1.67 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>Initiator</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>GDW to</td>
<td>10.00 ml</td>
</tr>
<tr>
<td>Catalyst</td>
<td>0.015 ml</td>
</tr>
</tbody>
</table>

2.6.4 Procedure

Two rectangular glass plates (17 x 17 cm), one notched and the other unnotched were perfectly cleaned and dried. The plates were sandwiched with 1.5 mm thick perspex spacers using silicon grease and sealed with special adhesive tapes (Pharmacia Fine Chemicals) along the two sides. The plate assembly was clamped to electrophoresis chamber with strong metal clips. The bottom of the assembly was sealed by filling the lower tank with 15 ml of resolving gel mixture. The resolving gel mixture was then poured on to the space between the plates to a height of 15 cm leaving sufficient space for stacking gel. About 2 ml of water was gently added on to the surface and the gel
was allowed to polymerize. After polymerization the water above the gel was drained and blotted dry with filter paper. The stacking gel was poured and 13 teeth comb plate was used to make wells. After polymerization the wells were rinsed and filled with reservoir buffer and connected to electrophoretic chamber. The upper and lower reservoirs were each filled with 250 ml of reservoir buffer.

After checking leakages samples were carefully loaded on to the wells using a lambda pipette, fitted with disposable tips. The unused wells were filled with an equal volume of sample buffer.

The apparatus was connected to the power pack and a constant current of 15 mA was supplied. As the samples entered the resolving gel, the current was increased to 30 mA. After 6 to 7h the electrophoresis was stopped, when the tracking dye approached the bottom of the gel. The slab gel assembly was removed and the gel was separated from the glass plates gently.

**2.6.5 Staining**

The gels were stained using silver nitrate solution following the procedure adopted by Merril et al. (1981). The gels were fixed in 12% acetic acid (v/v) containing 100 μl of formaldehyde. After about 2h, the gels were washed in GDW and transferred to 50% methanol (v/v). The gels were then washed thrice with GDW and then were transferred to a solution of 0.1% silver nitrate (w/v) in GDW containing 0.08 ml of formaldehyde. Subsequently the gels were washed with GDW and transferred to developer solution.

The developer solution was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate</td>
<td>3.00 g</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>25.00 ml</td>
</tr>
<tr>
<td>GDW</td>
<td>100.00 ml</td>
</tr>
</tbody>
</table>
2.7 SDS-PAGE procedure for purified fractions

SDS-PAGE was also carried out at each stage of purification procedure with the following modifications recommended by Okada and Natori (1983) using 8 x 8 cm gels containing 3% stacking gel and 15% resolving gel. The protein samples were denatured by heating them in 1% SDS (w/v) containing 2% β-mercaptoethanol (v/v) for 20 min at 75°C. The molecular weight standards used were cytochrome c and aprotinin. The gels were stained according to the method of Fairbanks et al. (1971). Overnight staining was done in 0.05% Coomassie brilliant blue (w/v) prepared in 25% isopropanol (v/v) and 10% acetic acid (v/v). This is followed by 6-9h destaining in 10% acetic acid (v/v). The gels were further stained for overnight in 0.005% Coomassie brilliant blue (w/v) in 10% acetic acid. Finally the gels were destained for several hours in 10% acetic acid till the background became clear.

2.8 Assessment of antibacterial activity

Antibacterial activity of bulk serum of housefly larvae was assessed by four different procedures viz., (1) inhibition zone assay (IZA), (2) lytic assay, (3) killing assay and (4) assay using acidic polyacrylamide gel electrophoresis (acidic PAGE).

2.8.1 Preparation of haemolymph for antibacterial assay

The antibacterial serum prepared as described under section 2.4 could not be directly used for assessing the activity as it contained other bacterial pathogens. To remove the pathogens, the serum was filtered using a microfilter under vacuum. This procedure ensured that nearly 95% of the serum was recovered. Subsequently the sample was sterilized by placing it under ultra-violet light for about 4h in ice cold conditions.

The bacterial pathogens were removed by heat treatment as well. The antibacterial serum was mixed with equal volume of acetate buffer pH 3.6
[40 mM ammonium acetate and 1M acetic acid (2:1 v/v)] and was left in a boiling water bath. The contents were shaken constantly. This procedure resulted in the precipitation of all other proteins except the basic (cationic) proteins. The precipitate was removed by centrifugation at 12000 g for 20 min in a refrigerated centrifuge and its supernatant was used for assessing antibacterial activity.

2.8.2 Inhibition zone assay

In this procedure the antibacterial activity of the serum of housefly larvae was assessed by following the method of Hoffmann et al. (1981). Thin agar plates were prepared by pouring 8 ml of 1% melted agar to sterile petriplates and the plates were inoculated with $2 \times 10^8$ cells of log phase *M. luteus*. On these agar plates 2 mm diameter wells were punched using a sterile gel punch. The wells were filled with 2 µl immune serum and the petridishes were incubated at 37°C for about 15-20h. The bacterial multiplication was accompanied by a slow diffusion of the serum into the agar. This procedure was a modification of the paper disc method of Kirby and Bauer (Benson, 1994) used to determine the susceptibility of bacterial isolates to antibiotics. The area surrounding the well where no bacterial growth occurred, was regarded as zone of inhibition of bacterial growth. The diameter of the zone was measured and recorded.

2.8.3 Lytic assay

Log phase culture of *M. luteus* grown in nutrient broth prepared in 0.2M phosphate buffer pH 6.4 was adjusted with sterile broth to yield an OD of 0.06 at 740 nm in a spectrophotometer. At this OD, the cell count was approximately $2.5 \times 10^8$ cells/ml. To 2 ml of such a bacterial suspension in a sterile tube, 200 µl of the serum immunized against *M. luteus* was added. The test tube was plugged with sterilized cotton and incubated overnight at 37°C. The OD of the suspension was measured at every 4h interval upto the first 12h,
then at 20h and 24h. Other preparations in which the immunized haemolymph was either substituted by haemolymph from the normal larvae or by LB medium, served as controls. The OD readings of experimental and control preparations obtained at various time intervals were plotted against time.

2.8.4 Killing assay

The procedure for killing assay was based on the viable count of bacteria when they were incubated with serum obtained from immunized housefly larvae. The assay was adopted from the one developed by Hultmark et al. (1980).

10 µl of immunized haemolymph was added to 100 µl of LB medium prepared in 0.01M phosphate buffer solution pH 6.4, containing 4x10^5 bacteria. The mixture was incubated for a period of 4h. At every 1h interval, 10 µl of the mixture was drawn, diluted suitably and then transferred to soft agar before being poured into agar containing petriplates. The plates were incubated overnight at 37°C and the number of colony forming units (CFU) were counted. In control cultures, either the immune serum or the nutrient medium was omitted.

The number of colonies formed after overnight incubation multiplied by dilution factor constituted the number of viable CFU in control as well as immune serum treated cultures. The number of CFU counted at each hour, over a period of 4h, were plotted against time.

2.8.5 Assay using acidic PAGE

The bulk immunized serum was subjected to acidic PAGE and at the end of electrophoresis the gel was placed on an agar plate inoculated with a bacterial suspension. The zones on the gel where there was a distinct inhibition
of bacterial growth were indicative of the protein fractions of immunized serum that possessed antibacterial activity.

Acidic PAGE was carried out to resolve the fractions with antibacterial activity. Electrophoresis was carried out under non-dissociating condition in 15% polyacrylamide gel (pH 4) in a discontinuous buffer system. The procedure followed was that of Gabriel (1971) with minor modifications as suggested by Hultmark et al. (1980). The stacking gel was omitted and the acrylamide-bis acrylamide ratio was 60:0.8.

### 2.8.5.1 Stock solutions

a) **Monomer**

Acrylamide  
60.0 g

Bis acrylamide  
0.8 g

GDW to  
100.0 ml

b) **Resolving gel buffer (pH 4.3)**

1N Potassium hydroxide  
48.0 ml

Acetic acid  
17.2 ml

TEMED  
4.0 ml

GDW to  
100.0 ml

c) **Initiator**

Ammonium persulphate  
0.28 g

GDW to  
100.00 ml
d. Reservoir buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>31.20 g</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>8.00 ml</td>
</tr>
<tr>
<td>GDW to</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

2.8.5.2 Preparations of gels

The gel was prepared by mixing the stock solutions in the following ratio:

One part of (a); two parts of (b); four parts of (c); and one part of GDW.

2.8.5.3 Casting of gel

A sandwich of one notched and other unnotched clean dry gel plates (8 x 8 cm) was made with 1 mm thick spacers, using vacuum grease and adhesive tapes. The plate assembly was fitted to electrophoresis chamber with the notch of the glass plate aligned with the edge of the upper reservoir. The bottom of the assembly was sealed with a small volume of resolving gel mixture. The gel mixture was poured between the glass plates, a teflon comb to create wells of appropriate size was placed into it and the gel was allowed to polymerize. At the end of polymerization the comb was removed and sample wells of 0.2 ml volume were formed. The reservoir chambers were filled with equal amount of alanine-acetic acid buffer.

2.8.5.4 Procedure

The gel was subjected to a prerun, prior to loading the samples to remove the excess ammonium persulphate that may be present on the gel after polymerization. During prerun, resolving gel buffer was used in buffer compartments of electrophoresis chamber. After 90 min of preelectrophoresis at a constant current of 100 mA, the apparatus was disconnected and the buffer
was removed from both the chambers. The wells were emptied and rinsed with reservoir buffer. The chambers were then filled with fresh reservoir buffer. Prior to loading, the control, heat treated control, immunized serum and heat treated immunized haemolymph were acidified with 1/10 volume of 1M acetic acid. Samples containing 150 μg of proteins were mixed with equal volume of 40% sucrose (w/v) containing 0.001% of methyl green (w/v) that served as tracking dye.

The wells were carefully loaded with the different samples using a lambda pipette, fitted with disposable tips. Separate tips were used for each sample. The apparatus was connected to power pack in such a way that the cathode of the power supply was connected to the anode of reservoir and vice-versa. Constant current of 200 mA was supplied till the methyl green ran off the gel.

At the end of the run, the gel plate assembly was dismantled. The gel was separated and transferred to a nutrient broth prepared with 0.2M phosphate buffer (pH 7.4) and buffered for about an hour. Meanwhile, a 12 cm diameter petridish layered with 10 ml of soft agar containing $2 \times 10^5$ viable *M. luteus* was prepared and kept. The gel was then carefully placed over the solidified agar and another layer of soft agar *san* bacteria was poured over the gel and incubated overnight at 37°C. The entire experiment was carried out in a sterile bacteria free environment. After 24h the gel was examined for zones of antibacterial activity.

### 2.9 Induction kinetics

To determine the time of commencement of the antibacterial activity of immune serum, haemolymph was collected from immunized larvae at every 1h interval upto 4h and at 8, 12, 18, 21, 25 and 48h after immunization.

The haemolymph thus collected was processed in a similar way as mentioned in section 2.8.1 and subsequently the immune serum was assessed
for antibacterial activity using IZA as well as for an assay involving acidic PAGE. As mentioned earlier this experiment was useful in determining the time of the induction of antibacterial activity.

### 2.10 Purification procedures

After establishing the antibacterial property of the bulk immune serum of housefly larvae, attempts were made to isolate and purify the basic proteins responsible for antibacterial activity using appropriate purification procedure (Fig. 1). This procedure consisted of

1. the separation of the basic proteins by cation exchange chromatography using carboxy-methyl cellulose (CM-cellulose),

2. removal of heat-labile basic proteins that do not possess the antibacterial activity,

3. the subsequent gel permeation chromatography of heat treated serum using Sephadex G-50,

4. finer separation of antibacterial proteins by cation exchange chromatography using CM-cellulose and

5. the final purification in hydroxyapatite column.

#### 2.10.1 Cation exchange chromatography - Step I

Cation exchange chromatography was carried out in 3 x 4 cm columns using CM-cellulose as the medium. About 30 ml of haemolymph was collected from 9000 larvae. Haemocytes were removed by centrifuging the haemolymph at 5000 g for 5 min. The cell-free haemolymph was stored at -20°C.
PURIFICATION REGIME

30 ml cell free haemolymph
↓
Diluted to 4 fold with 10 mM phosphate buffer pH 6.0
↓
Step I

CM-cellulose
3 cm x 4 cm

Washed with 25 mM NaCl in buffer
↓
Eluted with 250 mM NaCl in buffer and assayed
↓
Pooled
↓
Step II

Heat treatment at 100°C for 5 min
↓
Centrifuged and freeze dried
↓
Step III

Sephadex G-50
(1.5 cm x 60 cm)

Eluted with 130 mM NaCl in buffer
↓
Peak I assayed
↓
Peak II assayed for antibacterial activity
↓
Peak III assayed
↓
diluted with 4 volumes of buffer
↓
Step IV

CM-cellulose
2 cm x 4 cm

Eluted stepwise
↓
at 130 mM NaCl
MIIA - assayed
↓
at 260 mM NaCl
MIIB - assayed
↓
diluted 10 fold with buffer
↓
Step V

Hydraxyapatite
2 cm x 2 cm

Washed with 50 mM phosphate buffer
↓
Eluted with 100 mM buffer
↓
purified MIIB assayed and characterized
↓

Fig. 1: Flow chart showing the various steps involved in the purification procedure.
Separation in ion-exchange chromatography was achieved by reversible adsorption in a two stage procedure. In the first stage, the sample was adsorbed on the ion-exchange medium and substances which were not bound to the exchanger were washed out using a buffer. In the second step the adsorbed substances were eluted from the ion-exchanger by varying the ionic strength of the eluent.

CM-cellulose is an ideal medium for separating cations. It has a peak affinity value of about 3.5 in 0.5M NaCl. In water or in dilute salt solution its peak value is slightly above 4. At pH value below 5.0, its affinity for binding polycations progressively diminishes. In the present work an elution buffer with a pH value of 6.0 was used for equilibrating the medium as well as for elution.

2.10.1.1 Preparation of CM-cellulose

4.5 g of dry CM-cellulose was added to 70 ml of 0.5N NaOH in a beaker and the powder was allowed to sink into the liquid slowly. The medium was left in NaOH for nearly 30 min so that there could be a maximum accessibility of the ionized groups. The medium was then filtered and resuspended in 70 ml of water acidified with an equal volume of 0.5N NaCl. After 30 min it was again filtered and resuspended in 0.5N NaOH. This causes cellulosic material to hydrated condition and ionized sites would have adequate accessibility to the ionized sites of the proteins. The equilibration of CM-cellulose was done with 10 mM Na2HPO4 / NaH2PO4 buffer at pH 6.0. The fines were removed by suspending the swollen adsorbent in the same buffer.

2.10.1.2 Packing the column

A siliconized column of 3 x 4 cm was used for packing. A bed support with the porosity of 70 μ was placed at the bottom of the column. The column was packed with the equilibrated CM-cellulose using a glass rod. The column outlet was closed and diluted adsorbent was slowly poured over the glass rod.
that was touching the wall of the column to avoid air bubbles. The column was attached to a 50 ml capacity reservoir at the top containing the buffer and the column outlet was opened. The flow rate was adjusted in such a way that the flow through the inlet was equivalent to flow through the outlet.

2.10.1.3 Sample application

The sample was prepared by diluting 30 ml of haemolymph with 4 volumes of phosphate buffer so that the conductivity of the haemolymph is at par with buffer used. The reservoir was removed and the sample was applied to the column with a pipette and care was taken not to disturb the bed surface. It was applied in increments of 1 ml, thus allowing the proteins to adsorb to the whole ion-exchanger medium. Approximately 20h were required for the loading and adsorption.

Subsequently the column was fitted with the reservoir filled with buffer, the flow rate adjusted appropriately (ml/min) and the adsorbed material was washed extensively with 25 mM NaCl in phosphate buffer pH 6.0.

2.10.1.4 Elution and absorbance in UV-spectrum

The adsorbed material was eluted with 250 mM NaCl in phosphate buffer pH 6.0. 2 ml fractions were collected manually in clean, sterilized test tubes and 80 such fractions were obtained. These fractions as well as the earlier washed solution were read in a Spectronic-1001 Plus-UV-spectrophotometer (Milton and Roy, USA) with deuterium and tungsten as UV-source. The absorbance profile was obtained at 280 nm. The fractions were assessed for their antibacterial activity.
2.10.1.5 Assay for antibacterial activity

IZA as described in section 2.8.2 was routinely carried out for assessing the antibacterial activity in the fractions obtained from cation exchange chromatography. *M. luteus* and *P. aeruginosa* were the bacterial organisms used in the assay. 5 µl of each fraction was used for the antibacterial assay.

2.10.2 Purification by heat treatment - Step II

The active fractions, that is the fractions which exhibited antibacterial activity were pooled and heated for about 10 min in a water bath at 100°C to denature all the heat-labile basic proteins. The precipitate formed due to denaturation was removed by centrifugation at 12000 g for 30 min. The supernatant was collected, freeze dried, assayed for antibacterial activity and was further purified by gel permeation chromatography. Acidic PAGE as well as SDS-PAGE were carried using the pooled and freeze dried fractions as described in sections 2.8.5 and 2.7 respectively.

2.10.3 Gel permeation chromatography - Step III

Gel permeation chromatography was carried out in a 60 x 1.5 cm column using Sephadex G-50 (Fine) with the particle diameter of 20-80 µ and fractionation range of 1,500-10,000 daltons.

2.10.3.1 Preparation of the column

12 g of Sephadex G-50 was suspended in phosphate buffer (pH 6.0) containing 130 mM NaCl. The gel was allowed to swell for a few hours. Care was taken not to allow the formation of lumps. In the bottom of the column a bed support of 70 µ porosity was placed. The bed volume of Sephadex G-50 was 9-11 ml/g. The gel was carefully poured into the column and allowed to settle gently. A 100 ml reservoir was fitted to the column and three to four column
volumes of phosphate buffer was allowed to flow through the column, thus ensuring a tight packing of the column.

2.10.3.2 Sample application

After closing the column outlet 2 ml of the concentrated sample obtained from step II treatment was loaded on the surface of the bed using a syringe fitted with a fine capillary tubing. The tip of the tubing was placed a few millimeter above the bed in the buffer solution and the sample was dispensed slowly without disturbing the bed surface. The column outlet was then opened allowing a free flow of the eluent. Phosphate buffer (pH 6.0) containing 130 mM of NaCl was the eluent used.

2.10.3.3 Elution and UV-absorbance of fractions

The flow rate of the column was adjusted to 40 ml/h and 2 ml fractions were collected. About 120 fractions equivalent to two column volumes were collected. The fractions were read in a UV-visual spectrophotometer at 280 nm. Fractions belonging to a specific peak were pooled together, freeze dried, dissolved in small volume of buffer and assessed for antibacterial activity.

2.10.3.4 Assay for antibacterial activity

Protein fractions obtained after the step III of purification were assayed for antibacterial activity of IZA technique using 5 µl of each fraction as described under section 2.8.2. *M. luteus* and *P. aeruginosa* were the test organisms used. Acidic PAGE as well as SDS-PAGE were carried out as described in sections 2.8.5 and 2.7 respectively.
2.10.4 Purification by CM-cellulose chromatography - Step IV

Gel permeation chromatography revealed three peaks that exhibited antibacterial activity. Of these three peaks, peak II included 14 fractions (from 40 to 53) and was the major peak. Further purification was confined to the fractions obtained from peak II. The fractions were pooled, freeze dried, dissolved in 120 ml of phosphate buffer and used as sample for ion-exchange chromatography.

The procedure for cation exchange chromatography was similar to the one outlined in section 2.10.1. The adsorbed substances were eluted stepwise, first in 130 mM NaCl followed by 260 mM NaCl. 3 ml fractions were collected in clean sterilized tubes and the absorbance was measured at 220 nm. Since the amount of protein was very small the elution of protein from the column was monitored at 220 nm, which is an indicator of the absorption due to peptide bonds (Okada and Natori, 1983).

2.10.4.1 Antibacterial assay

IZA was carried out to assess the antibacterial activity of the fractions collected from CM-cellulose chromatography, 10 μl from each fraction were used for assessing the antibacterial activity. Further purification was carried out with fractions obtained using higher ionic strength eluent.

2.10.5 Purification by hydroxyapatite chromatography - Step V

The final purification was achieved by passing the antibacterial proteins through a column of hydroxyapatite. It is an organic adsorbent used to separate the basic proteins in native conditions. The principle behind this adsorbance chromatography is the firm retention of basic proteins to the anionic sites of the medium and the adsorbed proteins could be eluted with solutions of higher phosphate concentrations. Essentially the total ionic strength of the eluent is key to elution process.
2.10.5.1 Preparation of hydroxyapatite

Hydroxyapatite was prepared in the laboratory as per the procedure followed by Levin (1962).

200 ml of 0.5M CaCl₂ and 200 ml of 0.5M Na₂HPO₄ were allowed to flow at a rate of 12-15 ml per minute into one litre beaker, with constant stirring. The resulting Ca₃(PO₄)₂ precipitate was allowed to settle. The supernatant was removed using a 10 ml syringe fitted with a rubber tube without disturbing the precipitate layer. The precipitate was washed gently by shaking the beaker with 300 ml of GDW allowing the precipitate to complete settling. The same was repeated thrice and the final suspension was diluted with 300 ml of GDW. With this 10 ml of freshly prepared 40% NaOH (w/v) solution was added with constant stirring. The mixture was boiled over for 45 min and then gently boiled for 1h stirring through out.

After boiling it was allowed to settle for 5 min and the turbid supernatant sucked off. The precipitate was then washed four times with 300 ml of GDW allowing each time 5 min settling. After the decantation of supernatant 400 ml of 0.01 M sodium phosphate buffer pH 6.8 was added and the suspension was just allowed to boil with stirring. The boiled suspension was allowed to settle and the supernatant was sucked off. The boiling was repeated using 0.01M phosphate buffer. Further, the suspension was boiled twice each with 0.001M buffer for 15 min.

2.10.5.2 Packing, sample application and elution

A siliconized column of 2 x 2 cm was used for the chromatography. The bottom of column was fitted with the bed support and was packed with hydroxyapatite suspension equilibrated with phosphate buffer. 60 ml of the sample obtained from the step IV purification was diluted 10 fold with phosphate buffer and was applied to the column in increments as described in section 2.10.1. At the end of the adsorption procedure the column was
extensively washed with 50 mM phosphate buffer at a flow rate of 20 ml/h. Elution of the adsorbed material was done by increasing the phosphate buffer concentration to 100 mM and 6 ml fractions were collected.

### 2.10.5.3 Assay for antibacterial activity

The fractions were monitored at 280 nm in UV-visual spectrophotometer. The antibacterial activity was assessed by IZA using 5 μl of each fraction. Acidic PAGE as well as SDS-PAGE were carried out with purified sample as described in section 2.8.5 and 2.7 respectively. The purified fraction obtained from the last step of purification is designated as MIIB.

### 2.11 Estimation of protein

Protein content was determined at each step of purification of the antibacterial substance according to the procedure of Lowry et al. (1951).

### 2.11.1 Reagents

a) Solution A

2% Sodium bicarbonate (w/v) in 0.1N sodium hydroxide (w/v).

b) Solution B

0.5% Copper sulphate (w/v) in 1% aqueous potassium sodium tartrate solution (w/v)

c) Reagent mixture

| Solution A | - | 50.00 ml |
| Solution B | - | 1.00 ml |

d) Folin - Ciocalteu reagent

The reagent was diluted with an equal volume of GDW.
2.11.2 Procedure

10 μl of protein sample was diluted to 1 ml with 1N NaOH. 5 ml of reagent mixture was added. After 10 min, 0.5 ml of Folin-Ciocalteu reagent was added and mixed thoroughly. Simultaneously the reagent blank was prepared using 1N NaOH. The absorbance was measured in spectrophotometer at 740 nm and the concentration of protein was calculated from the standard curve obtained using bovine serum albumin (BSA).

2.12 Quantitative assay for antibacterial activity

Quantitative assay for antibacterial activity was routinely carried out at various steps of purification of antibacterial substance following the procedure of Okada and Natori (1983). *M. luteus* was grown in LB medium and the cells in the log phase were collected and suspended in insect saline. The concentration of bacteria in 1 ml of saline was adjusted to 2.5x10⁸ cells/ml that yielded a OD value of 0.06 at 740 nm in spectronic-21. The sample (500 μl), LB medium (390 μl) and *M. luteus* suspension (10 μl) were mixed in a test tube and incubated at 37°C for 2h with shaking. The mixture was rapidly chilled and the absorbance measured at 740 nm. For quantification of antibacterial activity the samples were serially diluted with 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl and 2.0% BSA (w/v). The antibacterial activity was assayed at each dilution. The amount of antibacterial substance that caused 50% inhibition of bacterial growth is equivalent to 1 unit of antibacterial activity relative to control. The number of units of antibacterial activity/mg of protein was then estimated and described as the specific activity of antibacterial substance.

2.13 Characterization of MIIB

The characterization of MIIB was done based on the thermostability, susceptibility to trypsin, molecular weight determination, assay to determine
minimal inhibitory concentration (MIC) and quantitative assay for antibacterial activity.

2.13.1 Thermostability

The thermal stability of the MIIB was tested by incubating the factor at

1. -20°C for several weeks in a deep freezer
2. 20°C for several weeks in a BOD incubator
3. 37°C for over 24h in an incubator
4. 60°C for 30 min in a hot water bath and
5. 100°C for 5 min in a boiling water bath.

At the end of the incubation period, the protein was assayed for antibacterial activity by IZA procedure.

2.13.2 Susceptibility to trypsin

The MIIB was subjected to proteolytic activity by incubating 10 mg of protein with trypsin (mg/ml) in phosphate buffer saline (PBS) at pH 7.0 for 1h at 37°C. At the end of the incubation, the activity was tested by IZA.

2.13.3 Molecular weight determination

The approximate molecular weight of MIIB was determined by gel permeation chromatography following the method of Andrews (1965). Biologically active substances of known molecular weight were used as standards.
<table>
<thead>
<tr>
<th>S.No</th>
<th>Substances</th>
<th>Molecular weight (dalton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome c</td>
<td>12,400</td>
</tr>
<tr>
<td>2</td>
<td>Aprotinin</td>
<td>6,500</td>
</tr>
<tr>
<td>3</td>
<td>Insulin</td>
<td>5,734</td>
</tr>
<tr>
<td>4</td>
<td>Glucagon</td>
<td>3,500</td>
</tr>
</tbody>
</table>

0.5 mg/ml of each standard as well as the sample was dissolved individually in 130 mM phosphate buffer (pH 6.0) was layered to column with Sephadex G-50 (Fine). Gel filtration was performed as described in section 2.10.3.2. 2 ml fractions were collected and read in a UV-visual spectrophotometer at 280 nm. The ratio of the elution volume to the void volume ($V_e/V_o$) plotted against the log molecular weight of standards and apparent molecular weight of MIIB was calculated from the plot.

### 2.13.4 Assay to determine MIC

The MIC can be defined as the least concentration of the antibacterial substance at which there is no growth of the bacterium. The MIC was determined following the method of Okada and Natori (1983), by mixing the different concentrations of purified protein with 0.5 ml of LB medium inoculated with *M. luteus*. The protein concentration ranged between 0 and 3 µg of purified protein per millilitre. Incubation was generally done for 12h. LB medium with only different concentrations of purified protein served as control. At the end of 12h, the turbidity of the medium was measured in a spectrophotometer and the minimum concentration of the protein that inhibited the growth of the bacteria was determined.

### 2.13.5 Bactericidal effect of MIIB

The purified MIIB was tested for killing activity by viable count method using *M. luteus*. The bactericidal effect against various bacterial species and
yeast species was observed under phase contrast microscope and the antibacterial spectrum of MIIB determined using a variety of Gram positive and Gram negative bacteria.

2.13.5.1 Killing assay

Bacterial effect of MIIB was assayed as described in section 2.8.4 by incubating $4 \times 10^5$ log phase $M. \text{luteus}$ with 10 µg/ml of MIIB. The following controls were simultaneously maintained and assayed for CFU.

1. $M. \text{luteus}$ inoculated LB medium without MIIB at 37°C
2. $M. \text{luteus}$ inoculated LB medium with MIIB at 4°C

2.13.5.2 Phase contrast microscopic studies on antibacterial activity

The antibacterial proteins of housefly larvae obtained from the purification steps II and V were used extensively to assess the nature of their toxic effect. For this purpose the bacterial cultures treated with the antibacterial proteins were observed under a phase contrast microscope for their bactericidal effect.

Three bacterial species viz., $E. \text{coli}$, $M. \text{luteus}$ and $B. \text{thuringiensis}$ and eukaryotic yeast were the test organisms used for observations under phase contrast microscopy. Observations were limited to the details of morphological changes which the bacteria undergo on addition of toxins.

In such studies 10 µl of the log phase cultures were diluted with 100 µl of bacterial saline. 10 µl of the dilution was transferred to a clean slide and mixed with an equal volume of active fractions obtained from the steps II and V of the purification procedures. A clean cover glass was placed over the mixture, observed and photographed using Nikon photomicrographic unit.
2.13.6 Specificity of MIIB

In order to assess the efficacy of the purified antibacterial protein on the growth of a spectrum of bacteria, the following experiment was performed. *M. luteus*, *P. aeruginosa*, *E. coli* K12 strain, *E. cloacae*, *S. aureus*, *B. thuringiensis*, *B. polymyxa*, *B. megaterium* and *E. coli* from human source were the bacterial species used. As described in section 2.12 each species of bacteria was incubated with 0.5 ml LB medium containing varying concentrations of MIIB at 37°C for 12h. At the end of the incubation period, the minimum concentration that inhibited the growth was determined by measuring the turbidity using a spectrophotometer.

2.14 Statistical procedures

2.14.1 Analysis of variance

2.14.1.1 Two way analysis of variance

A two way analysis of variance was carried out to test whether viable count of *M. luteus* incubated with immunized haemolymph in LB medium, differ significantly from that of *M. luteus* suspended in phosphate buffer containing immunized haemolymph and LB medium without immunized haemolymph. For this purpose the number of CFU measured in terms of log concentration of cells/ml, were obtained at every hour of incubation till 4h. The values were tabulated. The columns representing the log concentration of cells/ml and the rows representing the time at which CFU were measured. The variance ratio was calculated at 1% or 5% level of significance and compared with critical F values.

Similarly, analysis of variance was carried out to test whether significant differences existed in the inhibition zone produced by haemolymph obtained from control, injured and *M. luteus* injected housfly larvae.
Two way analysis was also carried to test the bactericidal effect of MIIB on viable count of *M. luteus* in LB medium incubated with MIIB at 37°C and at 4°C and LB medium without MIIB at 37°C (Sokal and Rohlf, 1981).

### 2.14.1.2 Single classification analysis of variance

Single classification analysis of variance was carried out to find out whether significant differences existed in the lytic assay and killing assay against the haemolymph of normal and induced larvae.

For this purpose lytic assay was conducted to test the antibacterial activity of *M. luteus* induced haemolymph of housefly larvae. Similar tests were conducted with other bacterial species such as *P. aeruginosa, B. thuringiensis, E. coli* and *S. aureus*.

The values obtained in these experiments were tabulated and the variance ratio was calculated at 1% or 5% level of significance and compared with critical F values and inferences were drawn suitably (Sokal and Rohlf, 1981).