

ANTIBACTERIAL ACTIVITY OF IMMUNE HEMOLYMPH FROM A NATURAL DIET REARING EUPTEROTE MOLLIFERA LARVAE

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ABSTRACT - A Natural diet and rearing system was standardized for Eupterote Mollifera under natural environmental conditions. Larvae of the moth Eupterote Mollifera were reared on a natural diet of moringa oleifera leaves. The last instar larvae were reared till the larvae were formed into moth. And third and fourth instar larvae were immune-challenged by an injection of live Escherichia Coli D31. Hemolymph were collected from the larvae and prepared hemocyte free hemolymph by centrifugation process. The extra cellular protein/peptide was concentrated by AMICON (MWCO-3kDa) ultra-centrifugal filters. The protein concentration was estimated by Bradford dye binding assay. The Antibacterial activity of purified hemolymph was tested against Gram-positive, Gram-negative bacteria of B.subtilis, E-coli, Saureus, K.pneumoniae by well diffusion assay and colony counting assay using Escherichia Coli, final average CFU values of two different samples (>3kDa and <3kDa) were calculated by two different dilution ratio.

Keywords: Natural diet, rearing, Eupterote Mollifera, immune-challenge, Hemolymph, CFU, Antibacterial

INTRODUCTION

Insects play many important roles in nature. They aid bacteria, fungi, and other organisms in the decomposition of organic matter and in soil formation. The decay of carrion, for example, brought about mainly by bacteria, is accelerated by the maggots of flesh flies and blowflies. The activities of these larvae, which distribute and consume bacteria, are followed by those of moths and beetles, which break down hair and feathers (Hultmark., 1983). Insects are particularly resistant to microbial infections, although they do not have an acquired immune system that is capable of specifically recognizing and selectively eliminating foreign microorganisms and molecules (i.e., foreign antigens) (Mak, 2001). The protein, which is synthesized in various tissues during metabolism, is transported to the haemolymph (Fingerman et al., 1994). Antimicrobial peptides have been isolated from insects (Otvos., 2000). Antimicrobial peptides are synthesized in response to microbial infection or septic body injury mainly in insect fat body (functional equivalent of mammalian liver) and in certain blood cells, and then rapidly released into hemolymph where they act synergistically against microorganisms (Hetru., et al, 1998; Tzou., et al, 2002; Irving.,et al,2004). Antimicrobial peptides are a major component of the innate immune defense system in marine invertebrates. They are defined as molecules less than 10 kDa in mass which show antimicrobial properties (Boman., 1995, Matsuzaki, 1999), and provide an immediate and rapid response to invading microorganisms. These peptides possess broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Zasloff, 1987; Nakamura et al., 1988; Selsted et al., 1992). The present investigation was taken up to study of haemolymph from Eupterote molifera moth (Eupterotidae: Lepidoptera), a common pest of Moringa oleifera tree.

INSECT SELECTION

Eupterote molifera moth Adults are large-sized moths with light yellowish-brown wings having faint lines. Moths appear with onset of monsoon and lay eggs in clusters on leaves and tender stems. Egg period lasts for 6 days. Full-grown caterpillars are brownish in colour and densely hairy. Hairs are irritating to touch. Larval and pupal periods last for 12 to 14 and 8 to 10 weeks respectively. Pupation takes place in soil. Only one generation per year (Hetru C, 1998; Hoffmann,1999).

MATERIAL AND METHODS

SAMPLE COLLECTION

Eupterote mollifera larvae were used in this project as a test insect. Initially the larvae were collected from the Morniga oleifera fields in and around Tiruchirappalli District, Tamil Nadu, India. The collected E. mollifera larvae were transferred to the laboratory and reared in M. oleifera leaves for experimental purpose.

FEEDING ON NATURAL DIET

For experimental control, all larval stages of Eupterote Mollifera moth were reared on Moringa oleifera leaves in to the plastic container with in 20ml vials containing water. Observations were taken 8gr Moringa leaves/ day infested

with 10-15 larvae each. Fresh leaves changed every day and the larvae from the old leaves were transferred on the new leaves for feeding. Clean the containers every day, to avoid the larval death. The rearing was carried out in the natural environment.

INSECT- IMMUNE CHALLENGE

Larvae of the moth *E. Mollifera* were reared on a natural diet of *moringa oleifera* leaves at 30°C. Final instar larvae weighing 350-450mg were selected for this study. The larvae were immune-challenged by an injection of live *Escherichia Coli* D31. After the treatment, larvae were kept at 30°C in the sterile containers, and Hemolymph was collected after 48 hours.

HEMOLYMPH COLLECTION

Hemolymph samples were collected by puncturing larval head with sterile needle. Out-flowing hemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing an equal volume of an ice cold anti-coagulant (pH 4.5).

PREPARATION OF HEMOCYTE FREE HEMOLYMPH

The hemocyte free hemolymph was obtained by centrifugation for 5 minutes to pellet hemocytes and subsequently the supernatant was spun down for 15 minutes at 4°C to pellet cell debris. Finally 5ml of hemolymph samples were collected. The obtained hemocyte free hemolymph was stored at -20°C until use.

ULTRA-CENTRIFUGATION

Ultra-centrifugation of hemolymph was done using amicon ultra-centrifugal filter (MWCO-3kDa). 0.5ml sample centrifuged at 7000g for 45min at 4°C. Centrifugation resulted in 0.1ml (>3kDa) of retentate and 0.4ml (<3kDa) of filtrate.

ESTIMATION OF PROTEIN BY BRADFORD METHOD

The protein was determined according to the method of Bradford (1976). To the 1 ml of sample, 5ml of CBB G-250 was added, mixed thoroughly and incubated for 5 min at room temperature. The absorbance of the reaction mixture was then read at 595 nm.

COLONY COUNTING ASSAY

One microliter of freeze-dried fraction re-dissolved in apyrogenic water was added to 10µl of suspension containing 10⁵ CFU of *E.coli* D31, prepared in LB medium. The mixtures were incubated for 1hr at 37°C (*E.coli*), serial dilutions were prepared and plated on solid agar plates. After incubation at appropriate temperature for 24hr, bacterial colonies were counted. The antibacterial activity was expressed as percent of bacterial growth inhibition in comparison to control (bacterial suspension incubated without addition of fractions) (Malgorzata et al., 2007).

WELL DIFFUSION ASSAY

Antibacterial activity was estimated by agar disk diffusion method with minor modifications (Favel et al., 1994 and Alaa Eddeen et al., 2009). Nutrient agar was prepared and poured onto sterile Petri dishes and allowed to solidify. 24 h growing bacterial cultures (*E. coli*, *K. pneumoniae*, *S.aureus* and *B. subtilis*) were swabbed on it. Then, 5 wells were made by using a sterile cork borer. The samples of varying concentration were loaded in the wells. Amoxicillin served as standard. The plates were then incubated at 37°C for 24h. After incubation the inhibition diameter was measured.

RESULTS

REARING OF LARVAE

The fifth instar larvae were separated in to other containers for rearing. In this stage there is no supplement of foods to the larvae and kept those containers without any disturbance. Finally from 1-2 weeks the larvae were converted in to moth. Pupation takes place in soil. Only one generation per year. The rearing was carried out in the natural environment.



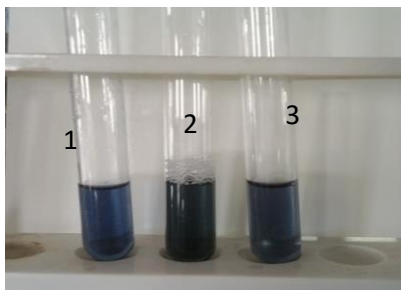
Figure 1: Eupterote Mollifera Larvae



Figure 2: Eupterote Mollifera Moth

ESTIMATION OF PROTEIN by Bradford Method

The concentration of the retentate protein (>3kDa) sample is 33.12 mg/ml, and the concentration of the filtrate protein (<3kDa) sample is 23.34 mg/ml of protein is determined.



- 1 – Control (CBB)
- 2 – Sample (>3kDa)
- 3 – Sample (<3kDa)

Figure 3: Estimation of Protein by Bradford method

Table 1: Estimation of Protein by Bradford method

Sl. No	Sample	Protein Concentration (mg/ml)
1	>3kDa	33.12
2	<3kDa	23.34

COLONY COUNTING ASSAY

The counting of E.coli was taken into the cell counting technique. The cell count was carried out by using 24hrs of E.coli culture.

Table 2: Colony Counting Assay

S.No	Sample	No of CFU	
		10 ⁻⁵	10 ⁻³
1	>3KDa	204	256
2	<3KDa	304	392
3	Control	298	451

WELL DIFFUSION ASSAY:

The hemolymph of E.mollifera moth antibacterial activities were tested by Gram Positive and Gram Negative Bacteria. The selective bacteria's are B.subtilis, E-coli, S.aureus, K.pneumoniae. Amoxicillin were served as standard. After incubation, the inhibition diameters were observed in E-coli and B.subtilis. The diameters of zone of inhibition were given in the table 3.

For >3kDa Retentate protein/peptide sample- Antibacterial activity

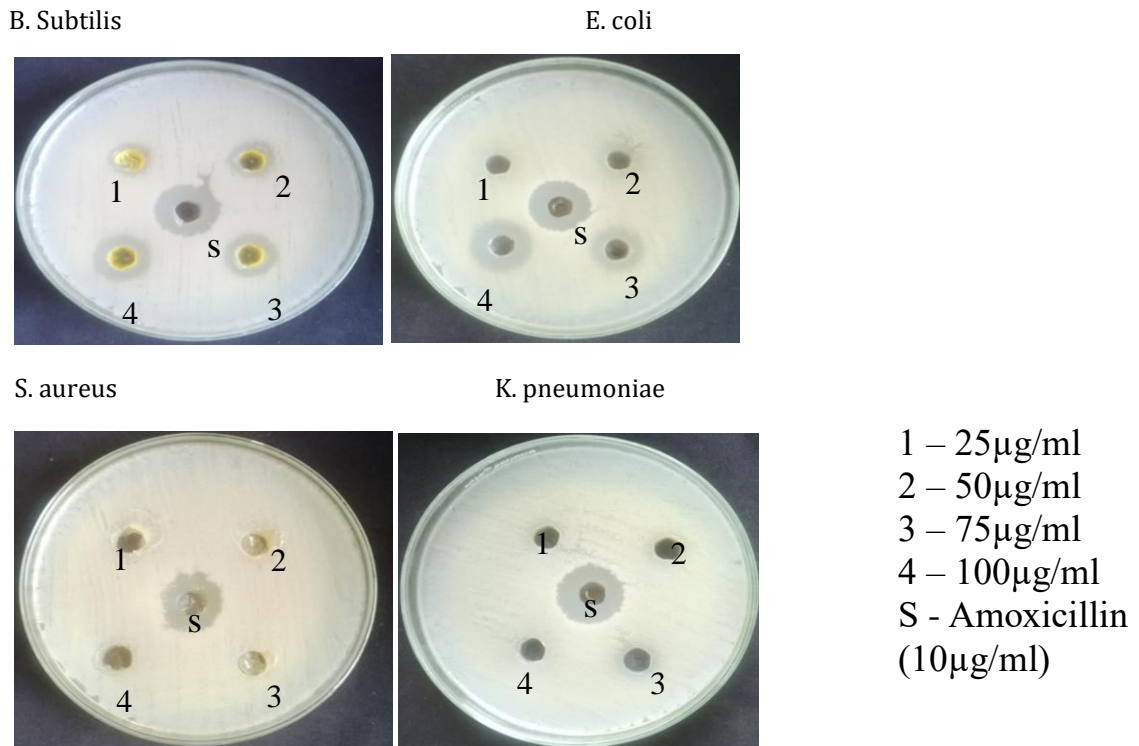


Figure 3: Antibacterial activity of >3kDa protein/peptide sample

For <3kDa Filtrate protein/peptide sample- Antibacterial activity

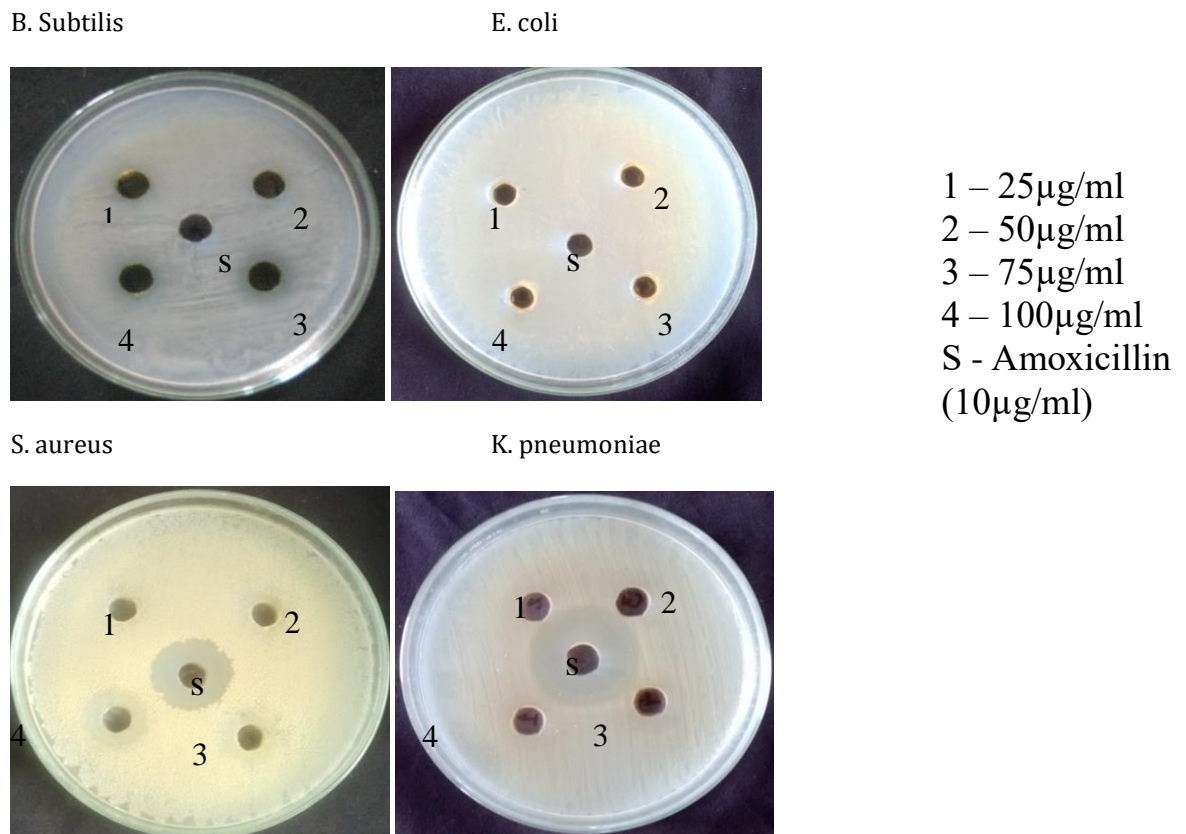


Figure 4: Antibacterial Activity of <3kDa protein/peptide sample

Table 3: Antibacterial activity- Well Diffusion Assay

Sl.No	Organisms	Zone of inhibition (mm)								
		Retentate (mg/ml)				Filtrate (mg/ml)				Std
		25	50	75	100	25	50	75	100	
1	B.subtilis	9	11	12	14	-	-	9	10	18
2	E-coli	-	9	11	13	-	-	-	-	18
3	S.aureus	-	-	-	-	-	-	-	-	19
4	K.pneumoniae	-	-	-	-	-	-	-	-	18

DISCUSSION

After inject the E-coli, the larvae are live healthy. The hemolymph was collected after 48hrs. The hemolymph sample of the moth *Eupterote molliefera* were divided into two components by the ultra-centrifugation process. One is retentate (>3kDa) and another one is filtrate (<3kDa). The concentration of the retentate protein (>3kDa) sample is 33.12 mg/ml, and the concentration of the filtrate protein (<3kDa) sample is 23.34 mg/ml of protein is determined.

The final average CFU values of two different samples (>3kDa and <3kDa) were calculated by two different dilution ratio (10^{-5} and 10^{-3}). For >3kDa, no of colonies were found in 10^{-5} ratio is 204 and 10^{-3} is 256. For <3kDa, no of colonies were found in 10^{-5} ratio is 304 and 10^{-3} is 392.

The antibacterial activity of the >3kDa protein/peptide sample was showed the positive activity in E-coli and B.subtilis. In E-coli, there is an activity occurs in 50mg/ml, 75mg/ml & 100mg/ml dilution ratio, were 9mm, 11mm, 13 mm of zone of inhibition occurs. In B.subtilis, there is an activity occurs in 25mg/ml, 50mg/ml, 75mg/ml & 100mg/ml dilution ratio, were 9mm, 11mm, 12mm, 14 mm of zone of inhibition occurs.

The antibacterial activity of the <3kDa protein/peptide sample was showed the positive activity in B.subtilis. In B.subtilis, there is an activity occurs in 75mg/ml & 100mg/ml dilution ratio, were 9mm, 10mm of zone of inhibition occurs. The hemolymph of *E.mollifera* moth antibacterial activities were tested by Gram Positive and Gram Negative Bacteria. The selective bacteria's are B.subtilis, E-coli, S.aureus, K.pneumoniae. After incubation, the inhibition diameters were observed in E-coli and B.subtilis.

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