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ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF LARVAL EXTRACT OF *MUSCA DOMESTICA* (HOUSE FLY)

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ABSTRACT

The principle objective of this study was to investigate the presence of antimicrobial activities in the larvae of insect *Musca domestica* (house fly). For this purpose, the body wall of the insect larvae were injured by a sterile hypodermic needle and then the larval extract were prepared in acidic (pH 5.0), neutral (pH 7.0) and alkaline (pH 8.0) buffers or water. These extracts were used for the screening of antimicrobial activities against 11 bacteria and 10 fungi by an agar disc diffusion assay and by poisoned food technique respectively. All the three extracts showed inhibition against 2 bacterial species viz., *Salmonela typhi*, *Bacillus subtilis* and 5 fungi viz., *Macrophomia phaseolina* , *Fusarium equiseti* , *Colletrotrichum corchori*, *Alternaria alternate* and *Rhizopus species*. The acidic extract also showed inhibition against bacteria *Escherichia coli*. Of the three extracts, acidic extract showed highest inhibition against all the microbes. Cytotoxic activity (LC_{50}) of the acidic, neutral and alkaline extract was found to be 141.2 µg/ml, 152.2 µg/ml and 149.5 µg/ml respectively.

Keywords: Antimicrobial, Cytotoxic, Extract, inhibition, *Musca domestica.*

INTRODUCTION

Microorganisms have developed resistant to many antibiotics and this has created immense clinical problems in the treatment of infectious diseases [1]. The increase of microbial resistance to antibiotics has led to a continuing search for newer and more effective drugs [2].

Insects are among the oldest and the most successful groups of animals occupying this planet. An essential feature of the success of insects has been their ability to invade and to exploit a diverse range of ecological niches. Insects have evolved and, in many situations, thrived in environments replete with potentially parasitic and pathogenic competitors. Disease in insect populations may reduce vigor, productivity, and ultimately survival of individuals and thus may contribute to the regulation of population levels. The ubiquitous presence of both infectious organisms and metazoan parasites in ecosystems occupied by insects has exerted a strong selection pressure for insects resistant to infection. Thus modern species of insects may represent the most extensive summary of successful defensive strategies against infection in the animal kingdom. Indeed, several early studies of antimicrobial defense mechanisms in animals were performed using insect models [3].

The presence of antimicrobial activities and the purification of many peptides responsible for these activities in numerous insects have been reported so far [3]. Antibacterial peptides have been isolated from the haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae [4] and from the haemolymph of immunized pupae of *Hyalophora cecropia* (cecropia moth) [5]. The housefly (*Musca domestica*) belonging to the Diptera grows and survives in dirty places. So

it is reasonable to think that the insect could have strong antimicrobial activities. The aim of the present study is to test the antimicrobial and cytotoxic activities in the housefly larvae as a part of our attempt to isolate and purify new antimicrobial agents.

MATERIALS AND METHODS

Collection of larvae and preparation of extract:

Larvae of *Musca domestica* was collected from the rotten cow dung from Chittagong University campus. Larvae were washed in tap water first and then in distilled water in a sieve. Then the larvae were anesthetized by (after) keeping them on ice for 5 min. The larvae were then injured with a sterile stainless steel hypodermic needle. One prick per animal was used to induce the antimicrobial activity. 2 gm of larvae were homogenized separately in 5 ml of distilled water (A), 0.1 M phosphate buffer, pH 8.0 (B) and 0.1 M sodium acetate buffer, pH 5.0 (C) for 10 min in a glass homogenizer keeping it at 4^0 C. The homogenates were centrifuged at 10 000 rpm for 10 min at 4^0 C in a sorvall refrigerated centrifuge. Supernatant was collected and stored at -20^0 C until use.

Antibacterial activity test of the extract:

The antibacterial activity was determined by disc diffusion method [6] against eleven bacterial strains, which included four gram positive and seven gram negative organisms and kindly provided by the Department of Microbiology, University of Chittagong. The strains were *Escherichia coli* ATCC25922, *Shigella dysenteriae* AE14612, *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* BTTC17, *Bacillus cereus* BTTC19, *Bacillus megaterium* BTTC18, *Shigella sonnei* CRL, *Salmonella typhi* AE 14296, *Salmonella paratyphi* AE14631, *Vibrio cholera* AE14748 and *Pseudomonus aeruginosa*. In brief, filter paper discs of 5 mm in diameter were individually impregnated with 20 µl/disc of the larval extracts (each group) and then placed onto the solidified agar plates previously inoculated with tested microorganisms. The plates were incubated at 37^0 C for 24 h. The antimicrobial activities were measured from zone of inhibition expressed in mm. Tetracycline was used as standard at the dose of 35 µg/disc as positive control. The experiments were carried out in triplicate.

Antifungal activity test of the extract:

The *in vitro* antifungal activities were tested by poisoned food technique [7]. Ten fungi, collected from the Department of Microbiology, University of Chittagong, were used for this study. They were *Macrophomina phaseolina*, *Alternaria alternate*, *Curvularia lunata*, *Colletotrichum* corchori, *Penicillium sp*, *Aspergillus niger Aspergillus fumigates*, *Aspergillus flavus*, *Rhizopus sp* and *Fusarium equiseti*. The potato dextrose agar (PDA) was used as a culture medium. The larval extract of every group was mixed separately with sterilized PDA to obtain a final concentration of 15 µg of protein/ml. From this, 20 ml medium was poured into separate petridish and allowed to solidify. For each fungus, inoculum was done by placing a 5 mm mycelium block obtained from a five days old culture of the test fungi on PDA at the centre of each plate in an inverted position. The inoculated plates were incubated at 28 ± 2^0 C for 5-7days. A control set was maintained in each experiment using only PDA as growth medium. The antifungal activities were measured from the diameter of fungal colony expressed in mm. All the antifungal results were compared with the standard antifungal antibiotic Nystatin (100 µg/ml PDA). The

percentage inhibition of radial mycelia growth of the test fungus was calculated as follows:

% Inhibition = (C-T/C) × 100

Where, C = diameter of the fungal colony in the control petridish

T = diameter of the fungal colony in the treated petridish.

Determination of cytotoxic activity or lethal concentration 50 (LC_{50}):

In vitro lethality bioassay of the larval extract of *M. domestica* was performed to detect cytotoxicity following the method described by

[8]. Brine shrimp (*Artemia salina* Leach) eggs were hatched in seawater (3.8 gm sea salt per liter distilled water) under the light of a lamp for 48 h yielding large number of larvae (nauplii). Then 3 ml of seawater was given to 10 vials. Afterwards, with the help of micropipette specific volumes (3, 6, 9, 12, 15, 18, 21, 24, 27, 30 µl) of each extract (A- 29.2 µg/µl, B-34.2 µg/µl and C- 20.0 µg/µl of protein) were transferred from the stock solutions to the vials to get final sample concentrations summarized in table 3. With the help of the Pasteur pipette 10 living nauplii were kept to each of the vials. Alive nauplii were counted after 24 h and the median lethal concentrations (LC_{50}) were calculated by trend linefit linear regression analysis of the obtained data. The experiments for each extract were carried out in triplicate.

Acute toxicity studies upon swiss albino mice:

The acute toxicity evaluation was performed on mice with 30–35 g and 6–8 weeks in age of either sex. Ten mice were randomly divided into two equal groups: control group in which water was administered and experimental group in which extract was administered. Larval extracts were administered by intraperitoneal route at a high dose of 2 gm protein/kg body weight. The animals were observed during the following 10 days for detection of any behavioral change, poisonous symptoms, food consumption, growth rate and mortality.

Determination of protein concentration in the larval extract:

The protein concentration in the extract was measured by Lowry's method [9]. In brief, different aliquots of standard BSA solution (250 µg/ml) were pipetted out in different tubes along with the aliquots of extract in separate tubes. Equal amount of alkaline copper sulphate was added in each tube and then allowed to stand for 15 min. Equal amount of Folin-ciocalteau reagent was added in each tube and the tubes were left for 30 min. Blue color was formed which was measured at 650 nm against a proper blank where no protein solution was used. The protein concentration of the extract was calculated from the standard curve.

RESULTS AND DISCUSSION

The antibacterial and the antifungal activities of injured larval extract, prepared in different pH, of *Musca domenistica* were determined against eleven bacterial species and ten fungi. In antibacterial activity assay, the results were compared with those produced by the standard antibiotic tetracycline (35 µg/disc).The sensitivity of the bacteria towards the extract and standard have been shown in table 1. All the extracts showed antibacterial activities against both gram positive and gram negative bacteria but in a very species specific way. In case of extract C, highest activities were found against gram positive bacteria, *Bacillus subtilis* (19 mm) and gram negative bacteria, *Salmonella typhi* (20 mm) whereas relative weak activities were found against gram positive bacteria, *Echerichia coli* (10 mm). Similarly the extract A exhibited the zone of inhibition of 16 mm against *Bacillus subtilis* and 17 mm against *Salmonella typhi*, whereas extract B produced that of 5 mm and 15 mm respectively. In comparison, the standard drug tetracycline produced zone of inhibition against *Bacillus subtilis* (20.6 mm), *Salmonella typhi* (23.3 mm) and *Echerichia coli* (16.4 mm). No activities were found against the other tested bacteria. Similar antibacterial activities of the flesh-fly larval extract against strain specific bacteria have been reported previously [4]. The chemical compositions of housefly larvae (dry weight/dry starting material weight, w/w) are protein (54.47%), carbohydrate (12.04%), fat (11.60%), crude fiber (5.70%) and ashes (11.43%), indicating a high content of protein [10]. We suppose that the active molecules in housefly larvae may be some peptides. Chernysh *et al*. (2002) [11] isolated two anti-viral and anti-tumor peptides from the blow fly *Calliphora vicina* (Diptera). Lepidoptera have been reported to produce several antibacterial peptides in response to septic injury [12]. The

presence of multiple antibacterial activities in various Lepidoptera indicates that these proteins have different lethal effects or different antibacterial specificities and together construct a potent defense system to prevent bacterial infection [4].

The inhibition of fungal radial mycelial growth by the larval extract is presented in Table 2. Nystatin (100 µg/ml) was used as standard antibiotic. The results were compared with standard as 100% zone of inhibition. The extracts (15 µg of protein/ml) inhibited the growth of five fungi out of the tested ten. Maximum inhibition was found against *Rhizopus sp* (83.35%) and *Colletotrichum corchori* (80%) with extract C. Considerable inhibition was found against *Alternaria arternata* (75%), *Fusarium equiseti* (71.66%) and *Macrophomina phascolina* (60%). The extract A also showed inhibitions against *Rhizopus sp* (66.66%), *Fusarium equiseti* (62.50%), *Alternaria arternata* (14.28%) and *Colletotrichum corchori* (42.85%), whereas extract B showed little inhibition. No inhibition was found against the other tested fungi. Septic injury in *H. virescens* larvae results in the production of an antifungal peptide, heliomicin [12]. Lijima *et al*. (1993) [13] also reported the presence of an antifungal peptide (AFP) from the hemolymph of a *Sarcophaga peregrine* (flesh fly) larva.

Cytotoxic activities of larval extracts are presented in Table 3. The extracts exhibited toxicity towards brine shrimp. The extracts showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of protein concentration. A plot of log concentration of the sample versus percent of mortality showed an approximate linear correlation between them (plot not shown). The cytotoxic concentration of extract A that killed 50% of brine shrimps within 24 h of exposure was 152.2 µg/ml. None of the shrimps died on exposure to the 29.2 µg/ml concentration, while 7 out of 10 shrimps died at the 175.2 µg/ml concentration of this when exposed same time period. For extract B, the LC_{50} was calculated as 149.5 µg/ml. None of the shrimps died within the 24 h exposure to extract C up to 40 µg/ml concentrations. This extract produced an LC_{50} of 141.2 µg\ml. Cytotoxic test depicts the extracts may contain antitumor or pesticidal peptides. However, this cannot be confirmed without further higher and specific tests [14]. This is the first report of cytotoxicity of house fly larval extract on brine shrimp larvae.

House fly larval extract exhibited highest activity against bacteria and fungi around pH 5.0, but the activity continuously decreased when the pH was changed to alkaline values. On the contrary, the higher cytotoxic activity was showed at neutral pH, but the activity continuously decreased when the pH was changed either to acidic or to alkaline values (Fig 1), although very little difference in LC_{50} values was observed among extract A, B and C. This may be due to the fact that the charge forming, distribution and conformation of the molecules were different under different pH, which resulted in the different antimicrobial efficiency. A decrease in pH of the medium has been shown to cause protonation of the ionizable carboxylic acid side chain, leading to an increase in hydrophobicity and consequent aggregation [15]. It is generally accepted that increasing the hydrophobicity of the nonpolar face of the amphipathic α-helical peptides would also increase the antimicrobial activity [16].

In the studies of acute toxicity on mice, no external manifestation of toxic syndrome or death was observed within 10 days after injection of different extracts at a single dose of 2 gm/kg body weight through intraperitoneal route. This route was chosen as an effort to avoid interfering factors such as absorption, pH of the stomach and intestines, enzymatic activity of the digestive tract and first-pass metabolism [17]. At the moment nothing is known about the mechanism of the lethal effect of this extract on microbes. Because there is no acute toxicity against the extract on experimental mice, we only assume that the

insect could be used as a good source for developing antimicrobial agents and knowledge of these antimicrobial compounds will lead to better control of human pathogens.

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TABLE 1. ANTIBACTERIAL ACTIVITIES OF LARVAL EXTRACT OF *MUSCA DOMESTICA*

Name of bacteria	Mean zone of inhibition (mm ± SD) by larval extract (20 µl/disc)			
	Extract in A*	Extract in B*	Extract in C*	Tetracycline [a] (35 µg/disc)
Gram positive				
Bacillus subtilis	16±0.31	5±0.30	19±0.22	20.6±0.12
Gram negative				
Salmonella typhi	17±0.14	15±0.23	20±0.41	23.3±0.31
Escherchia coli	-	-	10±0.53	16.4±0.42

'*'A= water, B= 0.1 M phosphate buffer, pH 8.0, C= 0.1M sodium acetate buffer, pH 5.0
'a' Standard antibacterial antibiotic

TABLE 2. ANTIFUNGAL ACTIVITIES BY LARVAL EXTRACT OF *MUSCA DOMESTICA*

Name of fungi	Inhibition of fungal radial mycelial growth (%) (15 µg/ml media) Nystatin (100 µg/ml) [a] as control (100%)		
	Extract in A*	Extract in B*	Extract in C*
Macrophomina phaseolina	-	-	60.00
Alternaria alternate	14.28	-	75.00
Colletotrichum corchori	42.85	33.33	80.00
Rhizopus sp	66.66	21.42	83.35
Fusarium equiseti	62.50	25.00	71.66

'*'A= water, B= 0.1 M phosphate buffer, pH 8.0, C= 0.1M sodium acetate buffer, pH 5.0
'a' Standard antifungal antibiotic

TABLE 3: CYTOTOXIC ACTIVITY (LC_{50}) OF LARVAL EXTRACT OF *MUSCA DOMESTICA* ON BRINE SHRIMP

Test sample	Conc.(µg/ml)	Log. Conc.	Average death	% mortality	LC_{50} (µg/ml)
Extract in A*	29.2	1.50	0	0	152.2
	58.4	1.80	1.3	13	
	87.6	2.00	2.0	20	
	116.8	2.10	2.0	20	
	146.0	2.20	5.0	50	
	175.2	2.24	7.0	70	
	204.4	2.31	7.3	73	
	233.6	2.40	9.0	90	
	262.8	2.42	10	100	
	292.0	2.50	10	100	
Extract in B*	34.2	1.53	0	0	149.5
	68.4	1.84	1.7	17	
	102.6	2.01	3.3	33	
	136.8	2.14	5.0	50	
	171.0	2.23	6.3	63	
	205.2	2.31	8.0	80	
	239.4	2.40	9.7	97	
	273.6	2.44	10.0	100	
	307.8	2.50	10.0	100	
	342.0	2.53	10.0	100	
Extract in C*	20.0	1.30	0	0	141.2
	40.0	1.60	0	0	
	60.0	1.77	1.3	13	
	80.0	1.90	2.0	20	
	100.0	2.00	3.3	33	
	120.0	2.08	3.3	33	
	140.0	2.15	4.0	40	
	160.0	2.20	6.0	60	
	180.0	2.25	7.0	70	
	200.0	2.30	7.7	77	
	220.0	2.34	10.0	100	

'*'A= water, B= 0.1 M phosphate buffer, pH 8.0, C= 0.1M sodium acetate buffer, pH 5.0

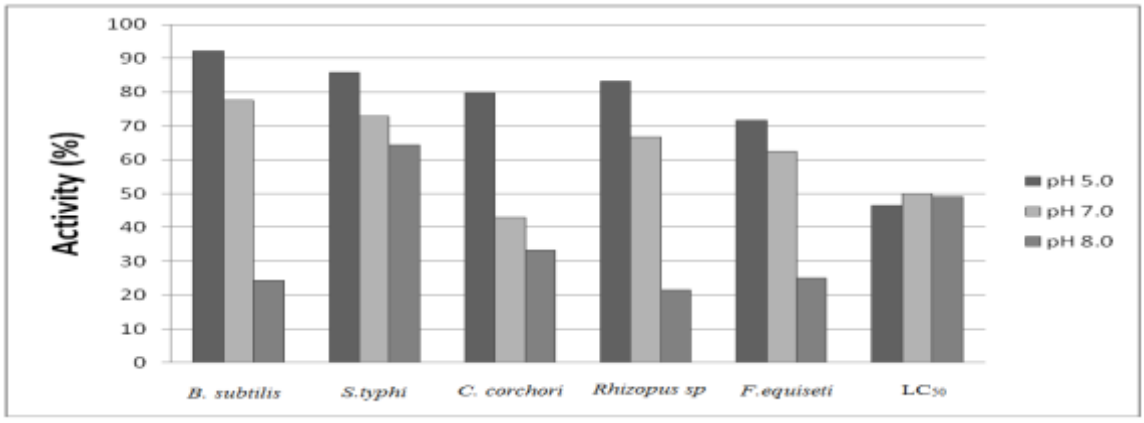

Fig 1: Effect of pH on antibacterial, antifungal and cytotoxic activity. Antibacterial and antifungal activities are shown comparing with standard antibiotic (100%), but cytotoxicity with highest LC_{50} as 50%.