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AN ASSESSMENT OF THE ANTIMICROBIAL ACTIVITY OF *SARCOPHAGA ARGYROSTOMA* HEMOLYMPH AGAINST PATHOGENIC MICROORGANISMS

By

WALAA A. MOSELHY[1*] and MARWA M. ABDEL-AZIZ[2]

Department of Zoology[1], Faculty of Science, Al-Azhar University (Girls)
Regional Center of Mycology and Biotechnology[2], Al-Azhar University, Cairo, Egypt (*correspondence: walaa_moselhi@yahoo.com)

Abstract

The pathogenic microorganisms are exceptionally adept to acquiring resistance to antibiotic and antiseptic drugs, so new strategies are there for needed to deal with this threat. Antimicrobial peptides from a wide spectrum of insects possess potent microbicidal properties against disease-related microorganisms. This study investigated the antimicrobial activity of maggot hemolymph from *Sarcophaga argyrostama,* a common species of flesh flies in Egypt. The crude hemolymph of the third instar larvae was tested against selected pathogenic strains of Gram positive bacteria, *Streptococcus pyogenes, Staphylococcus aureus, S. epidermidis, Bacillus subtilis and Micrococcus lutea;* Gram negative bacteria, *Pseudomonas aeruginosa, Proteous vulgaris, Salmonella typhimurium, Klebsiella pneumo-niae* and *Escherichia coli* and *Fungi, Aspergillus niger, Syncephalastrum racemosum, Geotrichum candidum, Candida albicans, Microsporum canis* and *Trichophyton mentagro-phytes.* The results showed that hemolymph was more effective against Gram positive bacteria especially *Bacillus subtilis* and effective against the filamentous fungus *Geotrichum candidum*. The most affected bacterial and fungal species were examined by TEM to study the antimicrobial peptides action in the hemolymph. Also, crude hemolymph was subjected to SDS-PAGE to estimate the proteins number and molecular weight.

Key words: *Sarcophaga Argyrostoma,* Hemolymph, Antimicrobial Activity, Microorganisms

Introduction

Insects lack the specific immune system that found in vertebrates, and depend on their effective and complex innate immune strategies (Vilmos and Kurucz, 1998). The quick and intensive production of potent antimicrobial peptides (AMPs) is the main defense weapon generated by insects against the invading pathogens (Elhag *et al*, 2017).

Since sarcophagid flies are living in filthy environment filled with microorganisms, they must develop cellular and humeral immune components to counter infection (Wang, 2010; Hall *et al*, 2016). Their defensive components, secretions as well as their dried bodies have been used in folk medicine for treatment of several diseases (Ratcliffe *et al*, 2011). Recently, there is a tendency to use maggot's derivatives or active molecules therapeutically in either their native or synthetic from to overcome the antibacterial-resistant bacteria in hospitals and communities (El-Bassiony and Stoffolano, 2016).

Members of orders Diptera and Lepidoptera synthesize a battery of defense proteins in response to infectious challenges (Boman, 1991). Okada and Natori (1985); Matsuyama and Natori (1988); Kanai and Natori (1990) and Ishikawa *et al.* (1992) proved that cells of *Sarcophaga* induce at least five groups of antimicrobial proteins: sarcotoxin I, II & III, sapecin and diptericin. Each group contains three to five structurally related proteins. All of these proteins were isolated and characterized from larval hemolymph.

This study aimed to evaluate the antimicrobial activity of maggot's hemolymph of *Sarcophaga argyrostoma* against different pathogenic microbial strains as a step to provide insight into the maggat's antimicrobial action.

Materials and Methods

Insects rearing: The laboratory colony was established from *Sarcophaga argyrostoma* maggots that were collected from Giza Governorate, Egypt, using a piece of meat in an open wooden box. The colony was maintained under 14L: 10D at 28±2°C and 60± 5% RH in Zoology Department, Faculty of Science, Al-Azhar University. Adult flies were feed on sucrose and water (1:2) and larvae were reared on bovine meat. The third instar larvae were used in the experiment.

Preparation of the hemolymph: Larval body wall was pricked with a needle, hemolymph was collected by cutting off its anterior tip with sterile fine scissors and placed in an ice-cold eppendorf containing phenylmethyl surlfonylfluoride (Natori, 2010). The crude hemolymph was freeze-dried and stored at -20°C till assayed.

Antimicrobial activity assay: The antimicrobial activity was determined using agar well diffusion method (Smania *et al*, 1999). the sample was tested *in vitro* for their antibacterial activity using nutrient agar medium against *Streptococcus pyogenes* (RCMB 000101), *Staphylococcus aureus* (RCMB 000102), *Staphylococcus epidermidis* (RCMB 000103), *Bacillis subtilis* (RCMB 000105) and *Micrococcus lutea* (RCMB 000104) (Gram positive bacteria); while the used Gram negative were bacteria *Pseudomonas aeruginosa* (RCMB 000106), *Proteous vulgaris* (RCMB 000107), *Salmonella typhimurium* (RCMB 000108), *Klebsiella pneumoniae* (RCMB 000109) and *Escherichia coli* (RCMB 000110). Antifungal activity was carried out against *Aspergillus niger* (RCMB 002007), *Syncephalastrum racemosum* (RCMB 016001), *Geotrichum candidum* (RCMB 052006), *Candida albicans* (RCMB 005002), *Microsporum canis* (RCMB 026001), *Trichophyton menta grophytes* (RCMB 025001) using Sabouraud dextrose agar medium. Ampicillin, Gentamycine and Amphotricine B were used as standard drugs against Gram positive, Gram negative and fungi, respectively. Dimethyl sulfoxide (DMSO) was used as solvent and the hemolymph sample was tested at a concentration 1mg/ml against both bacterial and fungal strains.

The sterilized media was poured onto the sterilized Petri dishes (20ml, each) and allowed to solidify. Wells of 6mm diameter were made in the solidified media by sterile borer. A sterile swab was used to evenly distribute microbial suspension over the surface of solidified media and solutions of the tested sample were added to each well with the help of micropipette. Plates were incubated at 37°C for 24hrs for bacteria and at 25°C for 48-72hrs for fungi. This experiment was carried out in triplicate and zones of inhibition were measured in mm scale.

Determining minimum inhibitory concentration (MIC): MIC was determined by the broth micro dilution method using 96-well micro-plates (Saini *et al,* 2005; Bhuiyan *et al*, 2011). The inoculum of the microbial strains was prepared from 24hr broth cultures and suspensions were adjusted to 0.5McFarland standard turbidity. Hemolymph sample (1.0mg) was dissolved in DMSO (1 ml) to obtain 1mg/ml stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µl) was added to the well from row B to H. The stock solutions of samples (100µl) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100µl) in row B was transferred to each well in order to obtain a two-fold serial dilution of the stock samples (concentration of 500, 250, 125, 62.5, 31.3, 15.6 & 7.81,3.9,1.95, 0.98 and 0.49µg/ml). The inocula (100µl) were added to each well and a final volume 200µl was obtained in each well. Plates were incubated at 37°C for 24hrs in case of antibacterial activity and 48hrs at 25°C for antifungal activity. Microbial growth was indicated by presence of turbidity and a pellet at the well's bottom.

Transmission electron microscopy: *Bacillus subtilis* and *Geotrichum candidum* (The most susceptible tested pathogens) were ex-

amined by the transmission electron microscope.

Approximately 10^7 *bacteria* cells treated with hemolymph at 0.25x MIC (detected from previous analysis), shaken well, and left undisturbed for 20hr. Suspension was centrifuged down to a pellet and washed twice with phosphate buffer saline. For Transmission Electron Microscopy (TEM), postfixation was carried out in 1% osmium tetroxide in cacodylate buffer at room temperature for 1hr. Cells were dehydrated in acetone and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate (Suwalak and Voravutikunchai, 2009) and examined with the GEOL GEM-1010 TEM at Regional Center for Mycology and Biotechnology (RCMB)

Electrophoretic analysis: Proteins in crude hemolymph of *S. argyrosoma* were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed (Laemmli, 1970). A molecular weight 10: 250 kDa kit (Fermentas) was used as standard. The separated proteins' molecular weight was determined via gel scanning using gel protein analyzer Ver. 3 (Media cybernetice, USA) software.

Results

Hemolymph antimicrobial activity: The antimicrobial activity of the crude hemolymph of *S. argyrostma* maggots against ten bacterial, six fungal pathogens was given (Tab. 1). The sample demonstrated varied activity against the tested bacteria. *Bacilus subitilis* was the most sensitive species among Gram positive bacteria (23.2±0.58 mm) and *Escherichia coli* among the Gram negative bacteria (16.9±1.2mm). Also, the *Staphylococcus aureus* was the lowest Gram positive bacterial species that affected by crude hemolymph (14.2±0.63mm) and the Gram negative bacteria *Pseudomonas aeruginosa* was completely insensitive to the tested sample. Crude hemolymph gave weak antifungal activity that was detected only for half of tested pathogenic fungi if compared with positive control. *Geotrichum candidum* was the most susceptible fungus under the used screening conditions (18.3±1.2mm).

Table 1: Antimicrobial activities of *S. argyrostma* crude hemolymph against pathogenic bacteria and fungi.

Pathogenic microorganisms	Hemolymph	Positive control
	Means of inhibition zone diameters (mm) ± S. D.	
Gram Positive Bacteria		Ampicillin
Streptococcus pyogenes (RCMB 000101)	17.4 ± 1.5	21.3 ± 1.2
Staphylococcus aureus (RCMB 000102)	14.2 ±0.63	22.4 ± 1.5
Staphylococcus epidermidis (RCMB 000103)	18.3 ± 1.2	23.3± 0.58
Bacillus subtilis (RCMB 000105)	23.2 ±0.58	27.4 ± 0.72
Micrococcus lutea (RCMB 000104)	15.4± 1.2	19.8± 1.2
Gram Negative Bacteria		Gentamycine
Pseudomonas aeruginosa (RCMB 000106)	NA	20.4 ± 1.2
Proteous vulgaris (RCMB 000107)	9.8 ± 0.72	22.3 ± 1.4
Salmonella typhimurium (RCMB 000108)	13.6±0.63	21.6± 1.2
Klebsiella pneumoniae (RCMB 000109)	14.3 ±0.58	20.3 ± 0.58
Escherichia coli (RCMB 000110)	16.9 ± 1.2	24.6 ± 0.3
Fungi		Amphotricine B
Aspergillus niger (RCMB 002007)	12.6± 1.5	22.3 ± 0.58
Syncephalastrum racemosum (RCMB 016001)	NA	18.2 ±1.5
Geotrichum candidum (RCMB 052006)	18.3± 1.2	25.7 ±1.2
Candida albicans (RCMB 005002)	13.6± 0.58	21.2±0.58
Microsporum canis (RCMB 026001)	NA	22.1 ± 1.3
Trichophyton mentagrophytes (RCMB 025001)	NA	19.8 ± 1.2

The values of MICs of the crude hemolymph confirmed the data obtained when the antimicrobial activity were investigated, the lowest MICs were recorded against *Bacillus subtilis* (1.95µg/ml) and *Geotricum candidum* (7.81µg/ml) among the tested bacterial and fungal pathogens, respectively (Tab. 2).

Table 2: Minimum inhibitory concentration of *S. argyrostma* crude hemolymph against pathogenic bacteria and fungi.

Pathogenic microorganisms	Hemolymph	Positive control
	MIC (µg/ml).	
Gram Positive Bacteria		Ampicillin
Streptococcus pyogenes (RCMB 000101)	15.63	3.9
Staphylococcus aureus (RCMB 000102)	31.25	1.95
Staphylococcus epidermidis (RCMB 000103)	7.81	0.98
Bacillus subtilis (RCMB 000105)	1.95	0.49
Micrococcus lutea (RCMB 000104)	31.25	3.9
Gram Negative Bacteria		Gentamycine
Pseudomonas aeruginosa (RCMB 000106)	NA	3.9
Proteous vulgaris (RCMB 000107)	125	0.98
Salmonella typhimurium (RCMB 000108)	62.5	3.9
Klebsiella pneumoniae (RCMB 000109)	62.5	3.9
Escherichia coli (RCMB 000110)	15.63	0.98
Fungi		Amphotricine B
Aspergillus niger (RCMB 002007)	62.5	1.95
Syncephalastrum racemosum (RCMB 016001)	NA	7.81
Geotrichum candidum (RCMB 052006)	7.81	0.49
Candida albicans (RCMB 005002)	62.5	3.9
Microsporum canis (RCMB 026001)	NA	1.95
Trichophyton mentagrophytes (RCMB 025001)	NA	3.9

To gain a more direct insight into the interaction of the larval hemolymph with the targeted pathogens, the most sensitive bacterial (*Bacillus subtilis*) and fungal (*Geotrichum candidum*) pathogens were examined by transmission electron microscope (Fig. 1). The control cells appeared intact and showed no morphological changes. Treated bacterial cells showed enlargement of cell size with rupture in cell membrane and cell contents burst out subsequently from the cell wall. But, there were morphological changes detected in the examined fungus include rounding of cells and appearance of vacuoles in some cells

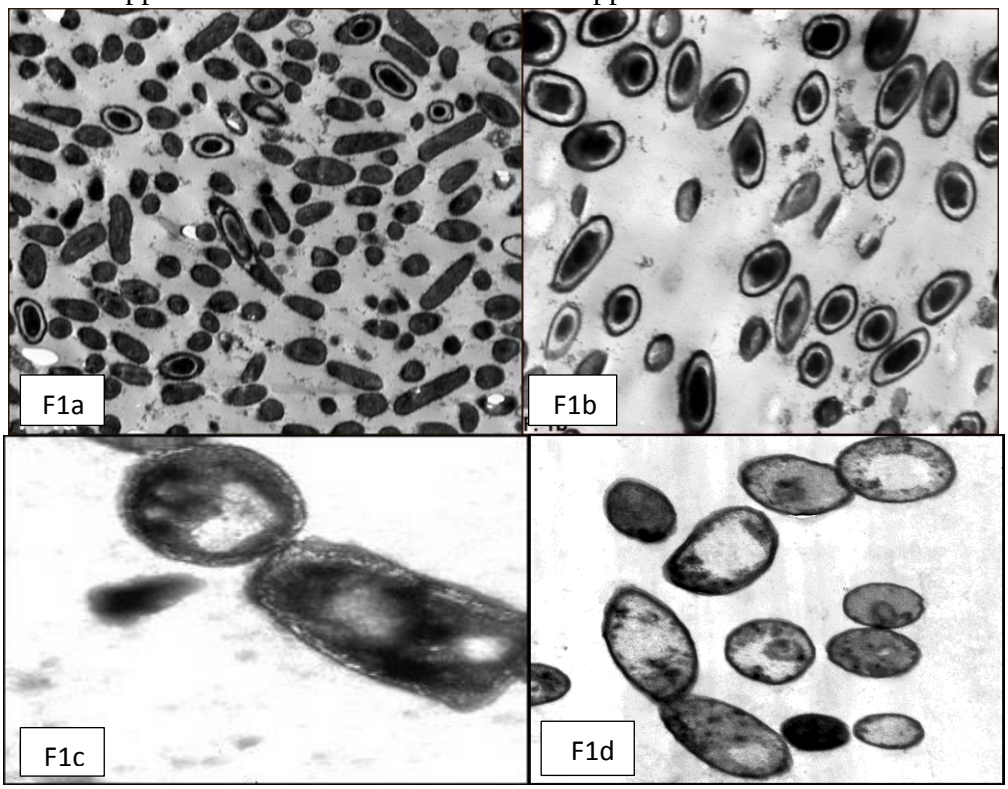

Fig. 1: (a) Photomicrographs of *Bacillus subtilis* and *Geotrichum candidum*, (a) normal *B.subtilus*. (b) *B.subtilus* treated with *S. argyrostma* crude hemolymph, (c) normal *G. candidum*, (d) *G. candidum* treated with *S. argyrostma* crude hemolymph Molecular weight of hemolymph proteins: Twenty proteins in crude larval hemolymph of *S. argyrostoma* using SDS-PAGE (Fig. 2).

Twenty proteins were detected in crude hemolymph of *S. argyrostoma* larvae using SDS-PAGE (Fig. 2). Protein bands molecular weight ranged from 205.127 to 11.234 kDa with varied intensity (Tab. 3). This weight was measured against standard used.

Table 3: Molecular weight of protein bands ranged from 205.127 to 11.234 kDa with varied intensity.

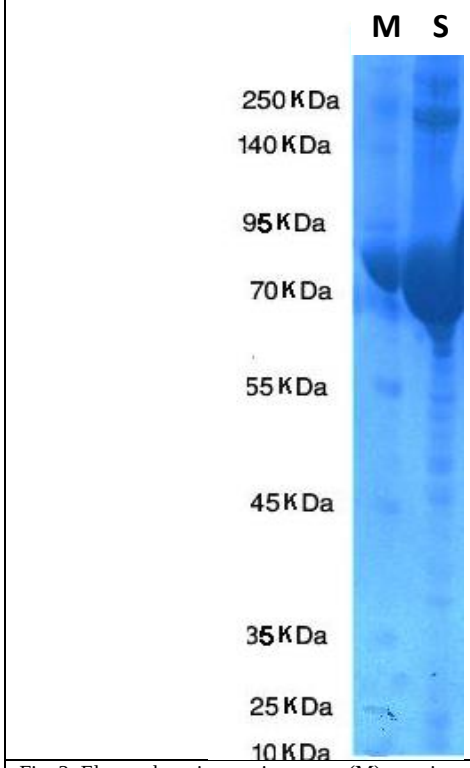

Fig. 2: Electrophoretic protein pattern (M) protein marker , (S) crude hemolymph of *S. argyrostoma*

M	Number	S
250		-
	1	205.127
	2	190.886
140		-
	3	137.462
		-
90		-
	4	79.604
70		-
	5	64.122
	6	60.279
	7	58.336
	8	56.123
55		-
	9	54.789
	10	52.322
	11	50.125
	12	48.203
	13	46.683
45		-
	14	44.763
	15	40.113
	16	38.234
35		-
	17	33.987
	18	28.356
	19	24.115
25		-
		-
		-
	20	11.234
10		-

Table 3: Molecular weight of protein bands, (M) protein marker , (S) crude hemolymph of *S. argyrostoma*

Discussion

The excessive usage of antibiotics leads to emergence of resistant microorganisms that obstructs human fight against infectious diseases. This dilemma makes the priority for this century is development of alternative drugs to fight pathogens (Vizioli and Salzet, 2002; El-Bassiony and Stoffolano, 2016). Progress in insect immunology gave new avenues to discover potential drugs meeting criteria of scientific medicine (Chernysh and Gordja, 2011).

In the present study, the crude hemolymoph of the flesh fly *S. argyrostoma* was screened for antimicrobial activity against 16 human pathogenic microorganisms such as *Staphylococcus aureus* (Gm+ve bacteria) which are fatal bacteria, that survive for long periods on the medical devices in the hospitals (Roder *et al.*, 1999), *Bacillus subtilis* (Gm +ve bacteria) which can contaminate food resulting in food poisoning as well as recording of some cases of fatal pneumonia and bacteraemia in some leukaemic patients due to the infection with this bacterial species (Logan, 1988), *Escherichia coli* (Gm-ve bacteria) which can cause serious food poisoning and responsible for a majority of cases of urinary tract infections (Vogt and Dippold, 2005) and *Geotrichum candidum* the causative agent of geotrichosis that affects the immunocompromised patients with neoplasms, diabetes mellitus, renal transplant and HIV (Mahendra *et al*, 2013). Natori (2010) found that several antimicrobial proteins were induced in hemolymph of *S. peregrine* when larval body wall was pricked. Since the present work was carried out under nonsterile conditions, body injury might be enough to induce bacteria into the larvae

which are equivalent to bacterial immunization. Antimicrobial proteins genes were expressed in response to bacterial infection to overcome the bacteria invasion to the body cavity (Kanai and Natori, 1990).

The present agar well diffusion assay showed that *S. argyrostomai* hemolymph was active against Gm+ve & Gm–v bacteria expect *Pseudomonas aeruginosa,* but it affected Gm+ve bacteria more than negative ones especially *B. subtilus.* However, hemolymph was active against examined fungi. El-Bassiony and Stoffolano (2016) examined antimicrobial activity of excretions of this insect *in vitro* against some of microbal species. Their results contradicted with the present ones, as their sample was more potent against Gm–ve bacteria especially *E. coli*. Minimum inhibitory concentrations (MICs) are the gold standard to determine susceptibility of organisms to antimicrobials and are therefore used to judge performance of all other methods of susceptibility testing new antimicrobial (Andrews, 2001). The present study MICs showed that hemolymph recorded the lowest MIC value against *B. subtilus* and other values were lower against Gm+ve bacteria than Gm-ve ones. Also, hemolymph sample had lowest MIC value against *G. candidum*, the most susceptible species.

Several review articles summarized and categorized the insect antimicrobial petpides (AMPs) and tried to explain the mode of action such as Bulet *et al.* (1999); Ravi *et al.* (2011) and Yi *et al.* (2014). These authors established that the insect AMPs are belonging to several families; some of them were recognized in dipteran insects. Of these families insect defensins with sapacins were isolated from *S. pergrina* (Matsuyama and Natori, 1988a,b). Insect defensins are active against Gm+ve bacteria, including *Micrococcus luteus, Bacillus subtilis* and *Staphylococcus aureus* as well as Gm-ve bacteria *Escherichia coli* and some fungi which agree with the present results (Yamada and Natori, 1993; Lowen-berger *et al,* 1995; Ress *et al*, 1997; Vizioli *et al,* 2001; Lee *et al,* 2004; Ueda *et al,* 2005; Seufi *et al,* 2011). Cociancich *et al.* (1993) suggested that insect defensins kill bacteria by formation of channels in the cytoplasmic membrane. Maget-Dana and Ptak (1997) explained the bactericidal effect of insect defensins by interaction with phospholipids to induce microheterogenety in lipid membrane related to formation of channels. Another AMP family is cecropins includes sarcotoxins I was recorded in the reviews. Sarcotoxins I from *S. peregrina* have activity against Gm+ve & Gm–ve bacteria (Okada and Natori, 1985) via interaction with micelles and lipids of bacterial cells (Yagi-Utsumi *et al,* 2013) or by disruption of electrochemical of membrane potential, resulting in cessation of ATP synthesis and amino acid transport (Natori, 2010). Sarcotoxin II from *S. pergrina* belonged to attacins, AMP family (Ando *et al*, 1981). This family with relatively large molecular weight peptides (20-24 kDa) inhibited bacterial growth by targeting their outer membrane to increase permeability & inhibited synthesis of many bacterial outer membrane proteins (Engstrom *et al,* 1984; Carlsson *et al,* 1991). Natori (2010) reported another two antimicrobial proteins in *S. peregrina, Sarcophaga* lectin with a large molecule and antifungal protein (AFP). He found that lectin played a role in elimination of any foreign cell from larval body cavity, while, AFP is fungicidal protein normally in larval hemolymph without microbial challenge. Iijima *et al.* (1993) found that AFP had inhibitory effect against *Candida cans,* which agreed with the present data.

Bactericides of AMPs isolated from *Sarcophaga* explained morphological alterations observed in the *B. subtilis* photomicrograph. The changes in *G. candidum* were due to AMPs & AFP action (Lehrer *et al,* 1985; Terras *et al,* 1992; Van *et al,* 2010). They suggested that antifungal AMPs killed target cells by disrupting the integrity of membrane by increasing permeability of plasma membrane or by forming pores directly. Hemolymph protein pattern of *S. argyrostoma*

showed twenty proteins with varied molecular weight and intensity. Natori (2010) reported that *Sarcophaga* lectin and sacrotoxins II, antimicrobial proteins have molecular weight about 190 kDa & 24 kDa, respectively. Both molecular weights were recorded in the present work for bands number 2 & 19, respectively. All reviews declared that the other possibly induced antimicrobial proteins in *Sarcophaga* hemolymph have molecular weight less than 10 kDa.

Conclusion

Appearance of multi-resistant microbial strains obligates the scientists to discover alternatives to synthetic drugs.

The current study examined the hemolymph of one of insect species, *Sarcophaga argyrostoma* from the point of view of the antimicrobial potential. The results obtained proved that this insect efficiency as candidate for antimicrobial drugs extraction.

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