academicJournals

Vol. 10(27), pp. 1036-1043, 21July, 2016
DOI: 10.5897/AJMR2016.8102
Article Number: 98D977359471
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro antimicrobial activity of maggot excretions/secretions of *Sarcophaga* (*Liopygia*) *argyrostoma* (Robineau-Desvoidy)

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Received 10 May, 2016; Accepted 26 May, 2016

The excessive usage of conventional antibiotics leads to the emergence of multidrug-resistant bacterial strains which threaten public health and stimulates searching for new sources of bio-therapeutic drugs. The aim of this study was to investigate the antimicrobial activity of maggot excretions/secretions from larvae of *Sarcophaga argyrostoma*, a common species of the family Sarcophagidae in Egypt. The excretions/secretions (ES) produced by third instar larvae were sterile filtered and tested against selected pathogenic strains of Gram positive (Gram+ve) bacteria, *Staphylococcus aureus* and *Bacillus subtilis;* Gram negative (Gram-ve) bacteria, *Escherichia coli* and *Pseudomonous aeruginosa*; and the filamentous fungus *Aspergillus flavus*. The ES product produced by third instar maggots proved to be more effective against Gram-ve bacteria. Larval ES, at 0.125 mg/ml concentration, were significantly potent towards *P. aeruginosa, E. coli* and *S. aureus* in a descending sequence. The minimum inhibitory concentrations of *S. argyrostoma* ES were 0.125 mg/ml for *P. aeruginosa* and *E. coli*, using the turbidimetric assay method. Twice and four times this concentration were required to inhibit growth of *S. aureus* (0.25 mg/ml) and *B. subtilis* (0.5 mg/ml), respectively. The antibacterial properties of *S. argyrostoma* ES were not affected by heating or freeze-thaw cycles when tested against *E. coli*.

Key words: *Sarcophaga argyrostoma*, antimicrobial activity, larval excretions, larval secretions, minimum inhibitory concentration.

INTRODUCTION

As necrophagous flies are living in the filthy environment filled with microorganisms, they must possess robust immune cellular and humoral components to counter infection (Wang, 2010; Hall et al., 2016). These components, dried bodies and secretions, have been used in folk medicine to treat many diseases including

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different types of infections and cancer (Ratcliffe et al., 2011). It's unclear whether the maggots produce and secrete antimicrobial molecules as a defensive mechanism or produce them to enhance the survival of their symbiotic bacteria internally and/or on vertebrate carrion. However, these facts together encourage entomologists to study these promising antimicrobial factors from larval products as health reports estimated that, 1-2% of third-world populations would experience chronic skin wounds (Brem et al., 2000).

During maggot debridement therapy (MDT), success is partly due to bactericidal properties of the fly's gut and/or exo-secretions which include the salivary gland secretions and fecal waste *(*Mumcuoglu et al., 2001; Kerridge et al., 2005*)*. Maggot's ES contains several proteases and antimicrobial substances which aid in debridement, disinfecting and accelerating wound healing (Nigam et al., 2006). Maggot excretions/secretions (ES) anti-microbial components have been shown to disinfect wounds by destroying bacteria that often do not respond to commercially available antibiotics, such as methicillin-resistant *S. aureus* (MRSA) (Bexfield et al., 2004). Furthermore, maggot ES inhibited the pro-inflammatory response of human neutrophils and monocytes in the wound healing process (van der Plas et al., 2007, 2009a, b). These secretions contained factors that could break down and inhibit *S. aureus* and *P. aeruginosa* biofilms, which colonized wounds and protected harmful bacteria from both the host immune system and therapeutic antibiotics. Lately, Pöppel et al. (2016) proved that *Lucilia sericata* maggot excretion products could accelerate the wound healing by excreting a pro-coagulant chymotrypsin-like serine protease. This protease was able to reduce the clotting time and showed a potential mechanism of wound debridement by digesting the extracellular matrix proteins.

Blow flies and flesh flies were previously searched for their value for MDT and whether they are good or poor candidates (Sherman et al., 2000). The antimicrobial actions against a variety of Gram+ve and Gram-ve bacteria were studied from maggot's ES of *L. sericata* (Daeschlein et al., 2007; Huberman et al., 2007; Jaklic et al., 2008), *Lucilia cuprina* (Arora et al., 2010; El Shazely et al., 2013), *Calliphora vicina* (Barnes et al., 2010) and three *Chrysomya* species (Ratcliffe et al., 2015). Currently, there is a tendency to use modern biosurgery (that is, maggot therapy without maggots) instead of traditional biosurgery (Vilcinskas, 2011), where maggot's derivatives or active molecules could be therapeutically used in either their native or recombinant/synthetic form to face the antibiotic-resistant bacteria in hospitals and communities. Some antimicrobial factors from muscoid flies were developed as new antimicrobial and anti-tumor drugs using peptide combining patterns (Ratcliffe et al., 2011; Chernysh and Kozuharova, 2013). Pöppel et al. (2015) used insect biotechnology to characterize the transcriptomes of antimicrobial peptides (AMPs), which are synthesized in *L. sericata* larval tissues. Previous authors identified 47 genes which encode putative AMPs and they produced 23 synthetic AMPs that showed activity against broad spectrum Gram-ve and Gram+ve bacteria. The objective of this study was to evaluate the antibacterial properties of maggot's ES of *Sarcophaga argyrostoma* on five pathogenic microbial strains as a first step in a process to find novel antibiotic-like compounds that may be used to overcome the bacterial resistance problems, and to provide insight into the maggot's antimicrobial action.

MATERIALS AND METHODS

Rearing laboratory colony

S. argyrostoma was captured from Abu Rawash, Giza provenance, Egypt. The colony was maintained for one year under a 16L:8D h cycle at 28 ± 2°C and 50% RH in the Entomology Department, Faculty of Science, Cairo University. Adults had continual access to water and granulated sugar in 45x45x45 metal cages. Females were allowed to oviposit on fresh beef meat and the larvae were reared on the same food source. Early third instar larvae were used in the experiments.

Maggot ES extraction

A modified method from Kerridge et al. (2005) was used for extraction. Briefly, approximately 200 larvae (~ 35 g) were used in each assay. Larvae were washed with ethyl alcohol for 5 min, replaced with 0.5% formaldehyde for another 5 min and finally rinsed two times with sterile phosphate saline (PBS) buffer (pH 7.2) (Dulbecco's). Larvae were incubated with 2 ml of PBS for 60 min at 27°C and 50% RH in darkness. The resultant liquid was then extracted using a pipette and centrifuged at 8,000 g for 10 min at 4°C. The supernatant was filter sterilized through a 0.22 µm membrane (Xi'an Zenlab) for antibacterial screening, collected in disinfected Eppendorf vials, and stored at -20°C. The protein concentration of the extract was determined by BCA* protein kit (Thermo Scientific) and bovine serum albumin was used as the standard.

Microbial cultures

Strains of *S. aureus* (ATCC 12600)*, B. subtilis* (ATCC 6051)*, E. coli* (ATCC 11775)*, P. aeruginosa* (ATCC 10145) and *A. flavus* (IMI 111023) were isolated from Abo-Elrish hospital and were used to assess the biological activity of the extract. The optimal testing methods approved by the National Committee for Clinical Laboratory Standards (NCCLS) were used to evaluate the susceptibility of bacteria and filamentous fungus (NCCLS, 1993, 1997, 2002). Bacterial strains were incubated in Luria-Bertani medium (LB) agar broth at 37°C for 24 h while the fungus *A. flavus* was reactivated by incubation in potato dextrose agar at 27°C for 12–15 h.

Disc diffusion assay

The modified Kirby-Bauer method (Bauer et al., 1966) was used to evaluate the susceptibility of both bacteria and filamentous fungus to the ES extract. 100 µl of each microbial culture solution, about

Table 1. Antimicrobial activity of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by disc diffusion method.

Microorganism	Inhibition area (mm)		
	Tetracycline	Amphotericin B	ES
Gram-positive bacteria			
Staphylococcus aureus	16.00 ± 0.1	-	11.87* ± 1.4
Bacillus subtilis	14.03 ± 0.2	-	8.50* ± 1.1
Gram-negative bacteria			
Escherichia coli	24.13 ± 0.1	-	17.77* ± 0.8
Pseudomonous aeruginosa	21.10 ± 0.1	-	19.53 ± 1.06
Filamentous fungus			
Aspergillus flavus	-	28.83 ± 1.41	13.07* ± 2.1

Data expressed as mean ± S.E. Three replicates were carried out for each experiment. Tetracycline and amphotericin B were used as positive controls for bacteria and fungus, respectively; and sterile ddH_2O was used as a negative control. *Refers to significance ($p \leq 0.0001$) between control and ES on each organism.

$1x10^5$ cells/ml, was spread onto Mueller-Hinton agar (BDH Laboratory Supplies, England) plates. 10 µl of the tested extract (2.0 or 10.0 mg/ml concentration) was added to 6.0 mm blank paper disc (Schleicher & Schuell BioScience GmbH) and discs were allowed to dry for 3 h at room temperature. Discs were then placed on agar and plates were incubated at 37°C for 24 h for bacteria and 25°C for 72 h for filamentous fungus. The radial zones of inhibition (mm) were measured. Tetracycline (10 µg/ml) and amphotericin B (40 µg/ml) were used as positive controls for bacteria and fungus, respectively; and sterile ddH_2O was used as a negative control. All the assays were done in triplicate.

Determining minimum inhibitory concentration (MIC) by turbidimetry

The MIC was determined according to the micro plate method (Bhuiyan et al., 2011) with modification. Briefly, ES extract was diluted in 1:2 serial dilutions using dimethyl sulfoxide (LY303366) as solvent. The initial concentration of the ES extract was 1.0 mg/ml. 100 µl of each dilution was dispensed into the wells of a 96-well, flat-bottom microtitre plate. 100 µl of each of the four bacterial culture solutions containing $1x10^5$ cell/ml were dispensed into the ES wells. Tetracycline (10 µg/ml) served as control and was diluted following the above mentioned procedure using dimethyl sulfoxide while dimethyl sulfoxide served as negative control. The plates were incubated at 37°C for 24 h. The optical density (OD) of each well was read at 600 nm wavelength at zero (OD_1) and 24 h (OD_2). Each concentration was tested in triplicate and repeated three times (n=9). The bacterial growth ratio was calculated as OD_2/OD_1 using the 0.125 mg/ml concentration. The MIC was calculated as the lowest concentration of larval ES extract that inhibited bacterial growth after 24 h incubation (Wiegand et al., 2008).

Thermal stability of ES extract

A tube containing 1 ml of ES extract (0.125 mg/ml concentration) was heated in a water bath at 100°C for 5 min while another tube of ES extract was cycled from freezing to room temperature 10 times, allowing for freezing and thawing of the sample. Then, the tubes were centrifuged at 8000 *g* for 5 min and the collected supernatants were assayed against *E. coli* for antibacterial activity using the turbidimetric assay. Five replicates were used for each experiment and tetracycline was used as control.

Statistical analysis

Data were expressed as arithmetic means ± standard error (S.E.) using SPSS 16.0 statistical software. The significance of differences between the two values was assessed using a two-tailed unpaired Student's *t*-test with significance set at $p \leq 0.05$. Tukey's post hoc test was used to analyze multiple comparisons and $p \leq 0.05$ was considered as significant.

RESULTS

Disc diffusion assay failed to show any antimicrobial activity of *S. argyrostoma* ES against the five selected microbes (*S. aureus, B. subtilis, E. coli, P. aeruginosa* and *A. flavus*) at 2.0 mg/ml concentration. No zones of growth inhibition were noticed around wells containing the larval extract. However, at 10.0 mg/ml concentration, the larval extract exhibited high potency against *P. aeruginosa* almost similar to the control (Table 1). The other four organisms showed moderate potency which were significantly ($p \leq 0.0001$, n=3) lower than the antibiotics. The highest zone of growth inhibition (mm) was recorded in *P. aeruginosa* assay and the lowest one in *B. subtilis* (Table 1).

By using turbidimetry to assay the antibacterial activity of *S. argyrostoma* ES against four bacterial strains (*S. aureus, B. subtilis, E. coli* and *P. aeruginosa*), it was clear that the extract is significantly potent ($p \leq 0.001$, n=9) against *P. aeruginosa*, *E. coli* and *S. aureus* at 0.125 mg/ml concentration; the ES inhibited bacterial growth by 90, 76 and 61.09%, respectively, as compared to controls (Figure 1). *S. argyrostoma* ES failed to show a significant effect on *B. subtilis* ($p > 0.05$) at the same protein concentration, as its inhibition potency was less than 5% as compared to the control (Figure 1).

The MIC of *S. argyrostoma* larval ES was 0.125 mg/ml for *P. aeruginosa* and *E. coli* using the turbidimetric assay method. This concentration increased to inhibit the growth of *S. aureus* (0.25 mg/ml) and *B. subtilis* (0.5 mg/ml).

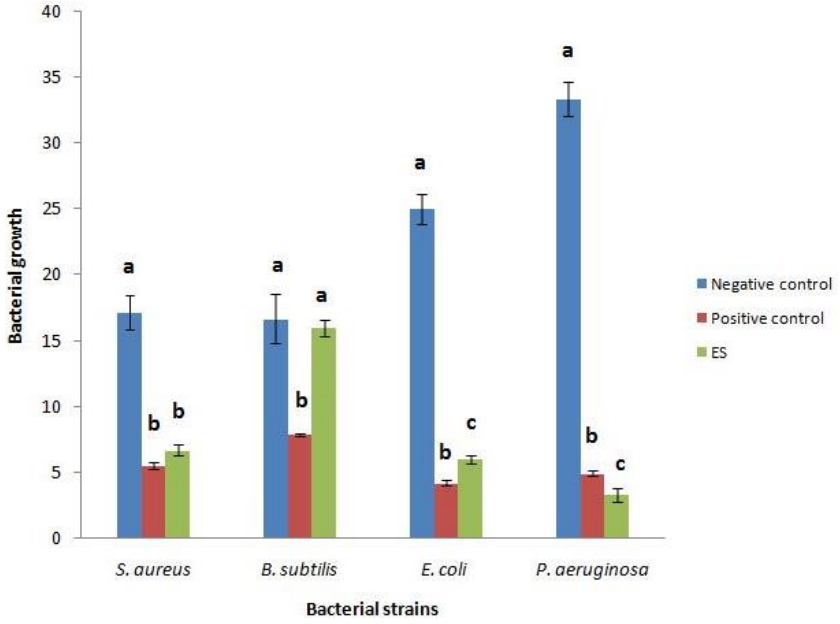

Figure 1. Bacterial growth ratio in the presence of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method at 0.125 mg/ml concentration. The bacterial growth ratio was expressed as OD_2/OD_1 for n=9. Data expressed as mean ± S.E. Means with different letters are significantly different from each other ($p \leq 0.05$) for the same bacterial strain. Tetracycline served as positive control and dimethyl sulfoxide served as negative control.

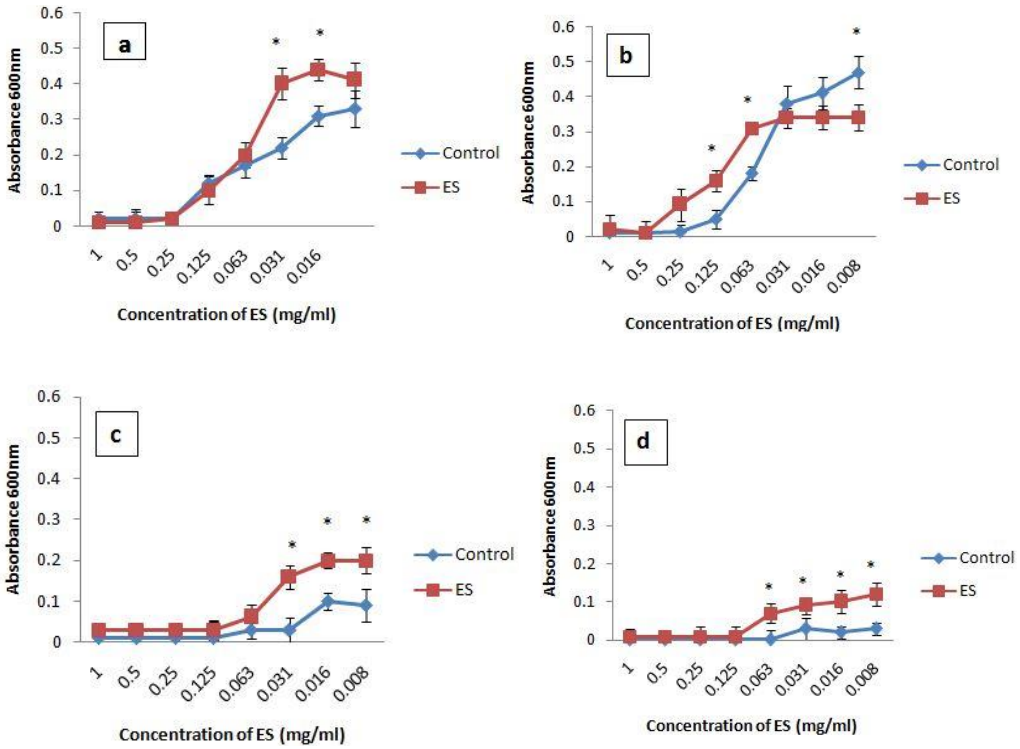

Figure 2. MICs of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method. Data expressed as mean ± S.E. and each point represented 3 experiments in triplicate wells (n=9). a= *S. aureus*, b= *B. subtilis*, c= *E. coli* and d= *P. aeruginosa*. *Refers to significance between control and ES $p \leq 0.05$. Tetracycline served as positive control.

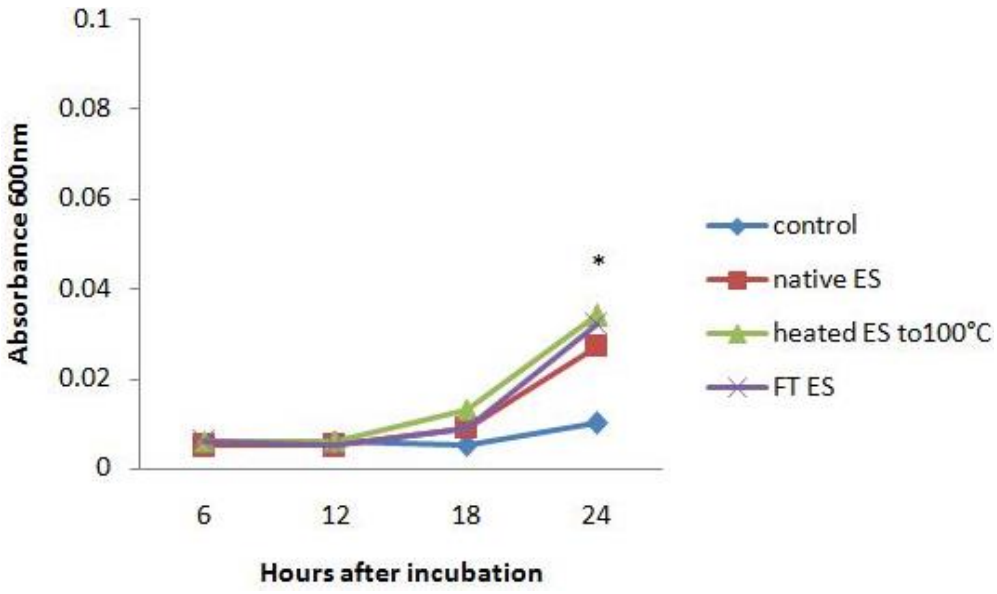

Figure 3. Heat stability of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method against *E. coli*. Each point represented 5 replicates. *Refers to significance ($p \leq 0.05$) between control and all tested ES after 24 h incubation period. Tetracycline served as positive control. FT ES = 10 cycles of freezing and thawing.

There were significant differences between potency of ES and the controls ($p \leq 0.05$, n=9) at some concentrations in all experiments (Figure 2). The ES has a significantly lower potency than the antibiotic control at more diluted concentrations against both Gram-ve bacteria.

The ES of *S. argyrostoma* proved to be heat stable (Figure 3). It was able to withstand both boiling at 100°C for 5 min and repeated freeze-thaw cycles without significant loss of potency ($p>0.05$, n=5). 24 h incubation led to significant loss of potency ($p \leq 0.05$) between control and both native and treated ES against *E. coli*.

DISCUSSION

The abusive usage of antibiotics leads to the emergence of multidrug resistant bacteria which form an obstacle in the battle of humans against infectious diseases. This dilemma promotes the development of new anti-infective drugs. Defensive peptides, the small molecular proteins which were extracted from different insect's species, constitute the key factors of biological antibiotics that work against several bacteria and fungi (Seufi et al., 2009). The importance of the maggot's ES is not only limited to their killing powers against several bacterial strains, but also to the factors which are contributing to the cleaning of infected wounds. Altincicek and Vilcinskas (2009) and Andersen et al. (2010) mentioned that *L. sericata* has 65 immune-inducible genes including lysozyme- and transferrin-likegenes and 3 proline-rich AMPs. Proteases induced by larval secretions may play a crucial role in wound healing process. Valachova et al. (2014) identified the full-length cDNAs of five novel putative salivary proteases of *L. sericata*, three of them from the serine protease families which could play a significant role in debridement of wounds. Also, recent studies investigated the potentials of sterile *L. sericata* ES to prevent the formation and disrupt bacterial biofilms of *S. aureus* and *P. aeruginosa* (van der Plas et al., 2008). It was found that ES prevented and disrupted *S. aureus* biofilms immediately and enhanced the formation of *P. aeruginosa* biofilms for 10 h after incubation, and then it began to breakdown *P. aeruginosa* biofilms (van der Plas et al., 2008). A combination of ES and conventional antibiotics could ensure complete breakdown of the biofilms (van der Plas et al., 2010).

The larval ES of *S. argyrostoma* possess one or more antimicrobial factors using disc well diffusion assay. ES showed a higher antibacterial activity against Gram-ve bacteria than Gram+ve bacteria. No previous reports have been found dealing with sarcophagid larval excretion's antibacterial activity, except for the work reviewed by Natori (2010) on *Sarcophaga peregrine* immunity molecules. Sapecin, a medium-sized cationic peptide belongs to the dipteran defensins, was isolated from the culture medium of the embryonic cell line of *S. peregrine* (Matsuyama and Natori, 1988). Defensins are 4-6 kDa cyclic peptides and are the most widespread insect's AMPs (Čeřovský and Bém, 2014). *S. peregrine* sapecin possesses an N-terminal flexible loop, a central α-helix and a C-terminal anti-parallel β-sheet (Hanzawa et al., 1990). *L. sericata* defensin (lucifensin) differs from

sapecin by five amino acid residues (Čeřovský and Bém, 2014). Sarcotoxin 1A, a cecropin antimicrobial peptide from *S. peregrine*, was found to be primarily active against Gram-negative bacteria but shows moderate activity towards Gram-positive bacteria (Natori, 2010), which agrees with our results. Pöppel et al. (2015) suggested that most insects produce a broad spectrum of AMPs during innate immune responses and that the complex interaction of these AMPs mediate the efficient antimicrobial defense.

The current study showed that the larval ES has about half the lethal effect of amphotericin B on *A. flavus*. Further work, using different extraction and bioassay methods, should be done on other fungi and yeasts. A previous study succeeded in purifying an antifungal protein from the hemolymph of *S. peregrine* larvae, which worked successfully against *Candida albicans* and the protein's lethal potentials were greatly enhanced by adding sarcotoxin IA (Iijima at al., 1993). The differences between our findings and the Iijima team may be due to the differences in antimicrobial properties of ES and the insect's haemolymph. Also, Pöppel et al. (2014) separated an antifungal peptide, lucimycin, from the *L. sericata* cDNA library of genes. Lucimycin was effective against the phyla: Ascomycota, Basidiomycota and Zygomycota, but inactive against bacteria.

In the current work, the turbidimetric assay was more effective in demonstrating the potent antibacterial activity of *S. argyrostoma* larval extract by the significant inhibition of bacterial growth. Using the turbidimetric method, *L. sericata* ES showed significant activity against *S. aureus*, *Bacillus thuringiensis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa* (Bexfield et al., 2004). On the contrary, using the standard agar diffusion to assay *L. sericata* ES showed no activity against *P. aeruginosa* and *E. coli* (Kerridge et al., 2005). Previous authors suggested that contradictory results for the same species could be due to different extraction techniques, different bioassay methods or heavier bacterial inoculates.

The MIC assay evidently demonstrated that, the larval extract of *S. argyrostoma* was more potent towards *P. aeruginosa* and *E. coli* than *S. aureus*. This is in concordance with findings of Huberman et al. (2007) and Barnes et al. (2010) on *L. sericata* hemolymph extract and ES, respectively, where significant bactericidal activity against *P. aeruginosa* than against *S. aureus* were recorded. Also, Teh et al. (2013) found that *L. cuprina* larval methanol extracts at 0.78 and 1.56 mg/ml concentrations were able to inhibit more than 50% of *P. aeruginosa* and *E. coli*, respectively, while 3.13 mg/ml was necessary to inhibit 50% bacterial growth of *Klebsiella pneumonia*.

In the heat stability test, *S. argyrostoma* ES is resistant to heating when tested against *E. coli*. The current findings are consistent with Simmons (1935) and Bexfield et al. (2004) for *L. sericata* larval extracts; Simmons concluded that the active factors in maggot's ES may not be of a viable nature. On the contrary, Kerridge et al. (2005) recorded a complete loss of antibacterial activity of *L. sericata* boiled extracts against methicillin-resistant *S. aureus* (MRSA). Our current freeze–thaw stability test is in agreement with the findings of both Bexfield et al. (2004) and Kerridge et al. (2005) who mentioned that the antibacterial properties of *L. sericata* ES were not significantly affected by freeze–thaw cycles. Hundreds of insect's antimicrobial peptides were tested for their resistance to heating and freezing, many of them were unstable and susceptible to temperature and other factors which prohibited their development as new drugs (Kang et al., 2012; El-Bassiony et al., 2016).

Calliphorid flies received attention since they were used in MDT by ancient cultures. Continuous research efforts discovered and developed two low molecular weight antibacterial peptides in *C. vicina*, namely, the alloferons, which were found to be active as antiviral and anti-tumor factors (Chernysh and Kozuharova, 2013). Due to the biodiversity of the Sarcophagidae in Egypt and the scavenging mode of larval life, this family should produce numerous novel antimicrobial peptides or factors. At the moment, more research is needed on Sarcophagidae larval ES as suggested by this study.

In conclusion, the ES of *S. argyrostoma* has shown to be highly effective against both Gram-ve and Gram+ve bacteria. The extract's heat stability is encouraging for further investigations. Following additional isolation and characterization, this extract could potentially yield new antibacterial and/or antifungal drugs.

Conflict of interests

The authors declare that there is no conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Dr. Magdy S. El-Hawagry for identifying the fly and Professor Dr. Neveen S. Gadallah for her help during the work. Thanks to the two reviewers for their suggestions, additions and comments. Thanks to Dr. Vaclav Čeřovský for reading the manuscript. This research is based upon work supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, the Massachusetts Agricultural Experiment Station and the Stockbridge School of Agriculture at the University of Massachusetts, Amherst, under project number MAS00448 to J.G.S. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA or NIFA.

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