Detection of Antibacterial Properties of *Musca domestica, Drosophila melanogaster*, and Sarcophaga nodosa Using Resazurin as A Growth Indicator in Bacterial Cells

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ABSTRACT

Background: Due to the side effects of chemical and synthetic antibiotics and the increase in bacterial resistance, extensive research has been conducted to obtain natural compounds without side effects from natural sources such as insects, especially Diptera order, because these insects require to live and survive on rotting food and be in direct contact with pathogenic and dangerous microorganisms because of their special diet.

Materials & Methods: In this study, hemolymphs were extracted from *Musca domestica, Drosophila melanogaster*, and *Sarcophaga nodosa*, and then the anti-bactericidal activity of these extracts against important pathogenic bacteria was investigated separately by the minimum inhibitory concentration (MIC) method using resazurin indicator.

Findings: *S. nodosa* and *M. domestica* larvae were not able to prevent the growth of any of the bacteria. *D. melanogaster* larvae extract completely inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa* bacteria at all densities, while *Staphylococcus aureus* was completely resistant to all concentrations. The minimum inhibitory concentration of *D. melanogaster* larvae extract against two bacteria of *Listeria monocytogenes* and *Salmonella typhimurium* was determined as 125 and 500 μL/mL, respectively.

D. melanogaster pupae extract was unable to inhibit the growth of *E. coli* and *S. typhimurium* but prevented the growth of *P. aeruginosa* at all concentrations. Also, the minimum inhibitory concentration of this extract against both *S. aureus* and *L. monocytogenes* was determined as 1000 μL/mL.

Conclusion: These outcomes show that *D. melanogaster* holds a high potential of antibacterial effects, and the purification and evaluation of this extract active substances are recommended for future utilization as antibacterial agents and food preservatives to fight pathogenic and toxigenicmicroorganisms.

Keywords: *Musca domestica, Drosophila melanogaster, Sarcophaga nodosa,* Antibacterial, Resazurin.

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Introduction

Foods might be contaminated by various pathogenic microorganisms, in which case they are defined as the main source of many human diseases. The survival and growth of microorganisms in food products cause spoilage and reduction of their quality [1]. Nowadays, the emergence of diverse drug resistance in pathogenic microorganisms has become an important challenge in both human and veterinary health. Thus, there is an urgent need to find new antimicrobial compounds and reduce drug resistance in microorganisms [2-3]. Various biological and physiological compounds have been shown to have a very high potential for use as new pharmaceutical compounds in promoting health and treating human and animal diseases, and due to their antimicrobial, anti-cancer, antioxidant, and free radical scavenging properties, they could be considered as one of the major sources of natural medicinal compounds [1-4]. The antimicrobial properties of hemolymph extracted from insects have been proven, and several major antimicrobial peptides have been identified in hemolymph. A vital side of the insect systemic responses to a microbial infection seems to be the generation of peptides which have direct antimicrobial activity against invading microorganisms.

In recent years, various advances have been made to recognize antimicrobial peptides and signaling pathways responsible for regulating their synthesis, very limited information has been found about the control of cellular responses of defense system. This is due to the presence of large to small sections in numerous insects, which make it difficult to collect hemocytes and identify effective molecules produced by them. It is often hard to manipulate hemocyte-dependent defense reactions under "*In vivo*" conditions or to isolate specific groups of hemocytes for use in "*In vitro*" experiments. This study was done based on cellular immune reactions in insects with an emphasis on Diptera in such a way that required the least amount of hemolymph of larvae and small pupae to perform the tests. Insect hemocytes are derived from mesoderm stem cells which are subdivided into specific lineages characterized by morphology, function, and molecular markers. In the family Lepidopteran, granular cells and plasma cells are involved in almost all cellular defense responses, while in the Drosophilidae family, primary plasma cells and lamellocytes are involved [5]. Both humoral and cellular receptors have a significant role in identifying external factors. As soon as a target is identified as an external factor, cellular defense reactions (cell-mediated defense) are regulated by signaling factors and then by molecules effective in controlling pathway of cell adhesion and cytotoxicity. Some factors demonstrate that both humoral and cellular defense factors are well coordinated. Such interactive processes between the immune and nervous systems could regulate insect's inflammatory responses to infection. Multicellular animals defend themselves against organisms with both innate and acquired immune systems. The innate immune system depends on coded genetic factors to identify and kill foreign invaders, while acquired immunity generates receptors by rearranging somatic genes, which identify certain antigens letting the organisms to make an immunological memory [3].

Insects do not possess acquired immune system while they possess an organized innate immune system. Early defenses compromise physical barriers of the skin or gastrointestinal tract, hematopoietic chelating reactions, and different cytotoxic

molecules at the site of injury. External organisms that cross such barriers and enter inside the hem cell should fight against more cytotoxic molecules, including a group of diverse hemocytes. The immune system of insects is divided into two parts; cellular and humoral defense reactions. Lots of humoral factors influence the function of hemocytes, which are a vital source of many humoral molecules. Furthermore, there is a notable overlap between humoral and cellular defense responses in processes like the identification of external intruders. Hemocytes detect external factors directly through the interactions between the receptors on the surface of hemocytes and molecules of the invading organism or indirectly by identifying humoral receptor-binding, which marks the invader's surface. Then intracranial and extracellular signaling events need to coordinate receptive responses such as phagocytosis and encapsulation. The most important origin of antimicrobial peptides is fat bodies; however, hemocytes generate these molecules in some insects. Several types of hemocytes are produce by insects [6]. Lately, in certain insects, antibodies and genetic markers have been recognized, which more reliably identify different hemocyte sorts from one another. The most common hemocytes reported by researchers are prohemocytes, granular cells (granulocytes), plasmatocytes, spherule cells (spherocytosis), and oenocytoids. The mentioned hemocytes have been characterized in species of the families Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola [7].

Objectives: The aim of this study was to evaluate the antibacterial properties of hemolymph of insects by emphasizing on Diptera order because most studies of insect hemocytes have been performed on this order.

Materials and Methods
Resazurin preparation: To prepare resazurin reagent, 270 mg of resazurin reagent powder (Sigma, USA) was dissolved in 40 mL of sterile distilled water. Sterile stocks were stored and refrigerated.

The test was done twice, and the results were presented based on the mean value. MICs of *D. melanogaster* pupae and larvae were determined and compared with each other, and their difference was presented in the results.

Bacterial strains: To evaluate the antibiotic resistance of bacteria to the extracts used in this study, at first, five standard bacterial strains including *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 644, *Pseudomonas aeruginosa* ATCC3 27853, *Salmonella typhimurium* ATCC 13311, and *Escherichia coli* ATCC 43894 were prepared from the pathology department of the Faculty of Veterinary Medicine at Shahid Bahonar University of Kerman, and the antimicrobial ability of the extracts against these bacteria was evaluated. In this study, only one sample of each bacterium was used. Thus, the use of statistical analysis was not possible.

Resazurin based minimum inhibitory concentration (MIC): To perform the test, a sterile 96-well microtiter plate was provided for each extract. First, 100 µL of essential oil at a concentration of 1000 µL/mL (volume/volume) in 10% solution of dimethyl sulfoxide was poured into the first row. To the rest of the wells, 50 µL of sterile Mueller-Hinton broth liquid medium was added. Then in each column, 8 houses, from the first house to the last house, were serialized and doubled. Next, 2 µL of resazurin was added to each cell. Finally, 10 µL of bacterial suspension with a turbidity equivalent to 0.5 McFarland turbidity was added to the cells, which was equal to 8×10^8 CFU/mL. Therefore, the final number of bacteria in each well was approximately

equal to 1.3×10^8 CFU/mL. Each column was assigned to a bacterium. No extract was added to a column, and it was considered as a bacterial control group. The plates were incubated at 37 °C for 24 hours. Any color change from red was considered as bacterial growth, and the last well without color change was considered as MIC (minimal inhibitory concentration) or the least growth-inhibitory concentration [8-9].

In this study, the susceptibility of the studied bacteria to kanamycin and ampicillin antibiotics was evaluated by resazurin at densities of 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL. All the tests were performed twice, and the results were presented based on their mean.

Preparation of larval and pupal extracts:
Preparation of larval and pupal extracts was carried out by the protocol published by Teh et al. [10] with some modifications and minor changes. Almost 200 non-sterile insect

larvae and pupae were collected (2 to 3-day-old larvae and 1 to 2-day-old pupae)

These larvae and pupae were isolated from the breeding medium and transferred to sterile tubes. Non-sterile larvae and pupae were disinfected using 40 mL of ethyl alcohol (%70) and subsequently washed three times by distilled water. Afterward they were dried on sterile paper towels and then transferred to the disinfected neat tubes. The larvae and pupae of each insect were isolated in a labeled manner and homogenized by absolute methanol (200 larvae/100 mL methanol). This homogenate was transferred to 2 mL centrifuge tubes and centrifuged by a refrigerated centrifuge at 4,000 × g for 30 minutes. The resulting yellow supernatant was then collected and transferred to sterile disinfected glass vials. At last, in order to separate the alcohol phase from the extract, the supernatants were concentrated by a

Table 1) Minimum inhibitory concentration (uL/mL volume/volume) of the extracts used in this study

Extracts Names	S. aureus ATCC 25923	L. monocytogenes ATCC 644	P. aeruginosa ATCC3 27853	S. typhimurium ATCC 13311	E. coli ATCC 43894
D. melanogaster larvae	<1000 (NA[a])	125 (forth well)	7.8 (growth inhibition[b])	500 (second well)	7.8 (growth inhibition[b])
D. melanogaster pupae	1000 (first well)	1000 (first well)	7.8 (growth inhibition[b])	<1000 (NA)	<1000 (NA)
S. nodosa larvae	<1000 (NA)	<1000 (NA)	<1000 (NA)	<1000 (NA)	<1000 (NA)
M. domestica larvae	<1000 (NA)	<1000 (NA)	<1000 (NA)	<1000 (NA)	<1000 (NA)

a. Able to grow in all dilutions of the extract.
b. Unable to grow in any of the dilutions of the organism extract.
D. melanogaster larvae extract had antibacterial properties on *P. aeruginosa* and *E. coli.*
D. melanogaster pupae extract had antibacterial properties on *P. aeruginosa.*

Table 2) Minimum inhibitory concentration (µg/mL) of the antimicrobial controls.

| Antibiotics	*S. aureus* ATCC 25923	*L. monocytogenes* ATCC 644	*P. aeruginosa* ATCC3 27853	*S. typhimurium* ATCC 13311	*E. coli* ATCC 43894
Kanamycin	2	16	1	8	1
Ampicillin	0.5	4	64	1	2

centrifugal vacuum concentrator. In the last step, the extracts were weighed and stored at -80 °C. Prior to antibacterial assay, 200 mg of larval extract was re-suspended in 1 mL of sterile distilled water and filter-sterilized with Minisart® cellulose acetate membrane syringe filter with a pore size of 0.2 µm.

Findings

According to the results of this study, *S. nodosa* and *M. domestica* larvae extracts were not able to prevent the growth of any of the bacteria. *D. melanogaster* larvae extract completely inhibited the growth of *E. coli* and *P. aeruginosa* bacteria at all densities, while *S. aureus* was completely resistant to all concentrations. The minimum inhibitory concentration of *D. melanogaster* larvae extract against two bacteria of *L. monocytogenes* and *S. typhimurium* was determined as 125 and 500 µL/mL, respectively. *D. melanogaster* pupae extract was unable to inhibit the growth of *E. coli* and *S. typhimurium* but in all concentrations prevented the growth of *P. aeruginosa*. Also, the minimum concentration of this extract against both *S. aureus* and *L. monocytogenes* was 1000 µL/mL.

The antimicrobial results of the extracts were comparable with each other; extracts of *S. nodosa* and *M. domestica* larvae had no antimicrobial effects on the studied bacteria. *D. melanogaster* larvae had more antimicrobial effects in comparison to its pupae especially against *L. monocytogenes, S. typhimurium*, and *E. coli*. The antimicrobial effects of *D. melanogaster* larvae and pupae on *P. aeruginosa* were similar.
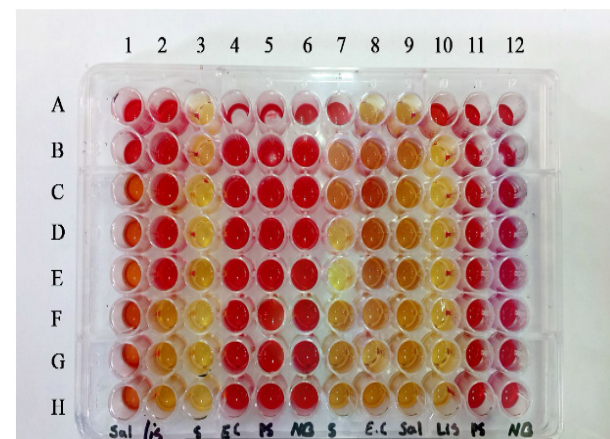
Fig. 1) A 96-cell plate after 24 hours of incubation. Red color means preventing the growth of the organism, and colors other than red mean the growth of the organism. Row A: dilution of 1000 ul/mL, Row B: dilution of 500 uL/mL, Row C: dilution of 250 uL/mL, Row D: dilution of 125 uL/mL, Row E: dilution of 62.5 uL/mL, Row F: dilution of 31.25 uL/mL, Row G: dilution of 15.62 uL/mL, and Row H: dilution of 7.81 uL/mL. Column 1: *S. typhimurium* and *D. melanogaster* larvae extract, Column 2: *L. monocytogenes* and *D. melanogaster* larvae extract, Column 3: *S. aureus* and *D. melanogaster* larvae extract, Column 4: *E. coli* and *D. melanogaster* larvae extract, Column 5: *P. aeruginosa* and *D. melanogaster* larvae extract, Column 6: control of *D. melanogaster* larvae extract without bacteria, Column 7: *S. aureus* and *D. melanogaster* pupae extract, Column 8: *E. coli* and *D. melanogaster* pupae extract, Column 9: *S. typhimurium* and *D. melanogaster* pupae extract, Column 10: *L. monocytogenes* and *D. melanogaster* pupae extract, Column 11: *P. aeruginosa* and *D. melanogaster* pupae extract, and Column 12: control of *D. melanogaster* pupae extract without bacteria.
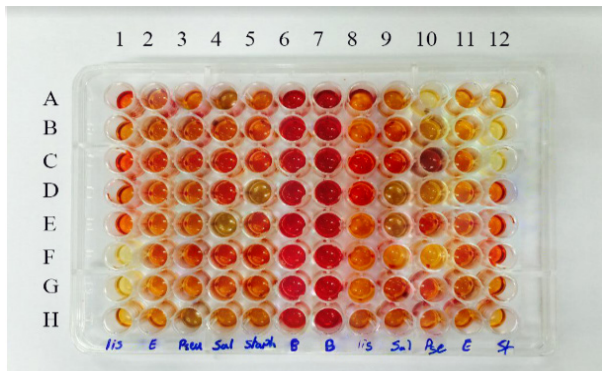
Fig. 2) A 96-cell plate after 24 hours of incubation. Red color means preventing the growth of the organism, and colors other than red mean the growth of the organism. Row A: dilution of 1000 ul/mL, Row B: dilution of 500 uL/mL, Row C: dilution of 250 uL/mL, Row D: dilution of 125 uL/mL, Row E: dilution of 62.5 uL/mL, Row F: dilution of 31.25 uL/mL, Row G: dilution of 15.62 uL/mL, and Row H: dilution of 7.81 uL/mL. Column 1: *L. monocytogenes* and *S. nodosa* larvae extract, Column 2: *E. coli* and *S. nodosa* larvae extract, Column 3: *P. aeruginosa* and *S. nodosa* larvae extract, Column 4: *S. typhimurium* and *S. nodosa* larvae extract, Column 5: *S. aureus* and *S. nodosa* larvae extract, Column 6: control of *S. nodosa* larvae extract without bacteria, Column 7: control of *M. domestica* larvae extract without bacteria, Column 8: *L. monocytogenes* and *M. domestica* larvae extract, Column 9: *S. typhimurium* and *M. domestica* larvae extract, Column 10: *P. aeruginosa* and *M. domestica* larvae extract, Column 11: *E. coli* and *M. domestica* larvae extract, and Column 12: *S. aureus* and *M. domestica* larvae extract.

Discussion

Hemolymph of insects has been scientifically proven to contain many antimicrobial substances like melanin, immune proteins, and AMPs to protect them against invading pathogens as they have no adaptive immune system. Many AMPs, like cecropin [10], attacin, defensin, and gloverin, have been characterized in diverse orders of insects, such as Lepidoptera [11]. This study was focused on the hemolymph of *S. nodosa* larva, *D. melanogaster* pupa and larva, and *M. domestica* larva. Natural exposure of flies or larvae to Gram-negative and Gram-positive bacteria triggers the inducible expression of AMPs in some surface epithelia in a tissue-specific way. Totally, the induction of AMPs expression depends on the immune

deficiency (Imd) pathway [12-13]. Particularly, the expression of drosomycin (regulated by the Toll pathway during the systemic response) is regulated by Imd in the respiratory tract. On the other hand, it demonstrates the existence of distinct regulatory mechanisms for local and systemic induction of AMPs in Drosophila. In special tissues, the expression of AMPs is constitutive [14]. Seven families of antimicrobial peptides have been characterized in *D. melanogaster* with a separate inductive effect [15-16]. The mentioned peptides are divided into three families according to antimicrobial "*in vitro*" activities or according to reported activities and their association with peptides isolated from other insects. The first family which is affected by Gram-negative bacteria includes drosocin, cecropin (s), diptericin (s), and attacin (s), and the second family which works primarily on Gram-positive bacteria is defensive. The third group of peptides includes dromycin and metchnikowins, which work against fungi. There are two kinds of signaling pathways (Toll and immune deficiency), controlling the expression of these antibacterial peptides [17, 18]. The Toll pathway plays a role in defense against fungi and bacteria, while the Imd pathway is involved in defense against Gram-negative bacteria [19]. Many studies have demonstrated the antibacterial effect of *D. melanogaster* on various bacteria. In a study by Elred et al. (2000) [20], the hemolymph of *D. melanogaster* was shown to be able to inhibit the growth of *E.coli*. [17] Also, *P. aeruginosa* bacteria inhibition by *D. melanogaster* has been demonstrated in many studies such as YJ Heo et al. (2009) [21], FJ Bikker et al. (2006) [22], TA Markow et al. (2014) [23], and Y Apidianakis et al. (2006) [24] which is similar to the present study results, demonstrating the inhibitory effect of *D. melanogaster* larvae extract on the growth of *P. aeruginosa* and *E.coli*. *D. melnogaster* pupae extract also inhibited the growth of *P. aeruginosa*. Zhihua Pei et al. (2014) [25] also showed that hemolymph of *M.*

domestica had antimicrobial peptides. In their study, *M. domestica* larvae produced efficient immune responses to protect them against the damaging effects of microbial and eukaryotic invaders. After being infected by microbes, insects produce a wide range of antimicrobial proteins and peptides. This process has a significant role in protecting them against the invading microorganisms. Antimicrobial peptides derived from *M. domestica* larvae demonstrate bactericidal, antifungal, antiviral [26], antitumor [27], immunomodulatory [28], antioxidant [29], anti-inflammatory [30], anti-atherosclerosis [31] and hepatoprotective [32] activities. There are also studies that have been done on Sarcophagidae as the family of Diptera, sapecin and sarcotoxin antibacterial peptides have been identified in *S. peregrina* species [33-34]. The poor activity of *D. melanogaster* pupae and larvae extracts against *S. typhimurium*, *L. monocytogenes*, and *S. aureus* in our *in vitro* assays could be explained by the different effects of AMPs extracted from *D. melanogaster* and also the amount of AMPs, which depends on the growth stage of this insect. Dissimilar inhibitory effects of *M. domestica* and *S. nodosa* hemolymph extracts could also be associated with the differences in the dietary regimes used in *M. domestica* breeding as well as the differences between the species studied.

Conclusion

In conclusion, the hemolymphs of *D. melanogaster* pupae and larvae have effective antimicrobial peptides. Such peptides pose actions against clinically isolated MDR pathogenic bacteria. Thus, they could be used as potent alternative therapies. The findings of this study showed that the hemolymphs of *D. melanogaster* pupae and larvae have a good antibacterial activity against pathogenic microorganisms. Due to the important role of pathogenic microorganisms in causing infection diseases as well as the significant drug resistance

of bacteria used in this study, especially *P. aeruginosa*, the use of natural compounds of *D. melanogaster* along with other protective methods could be useful in improving and promoting the health of food products and subsequently the health of people in the community. The use of resazurin-based microtiter-plate method for evaluating the antibacterial activity of natural compounds such as essential oils and plant extracts is more accurate and sensitive; also, in this method, there is no need to use laboratory equipment such as spectrophotometer or elliptical device to determine the extent of inhibition of microorganism growth.

Ethical Permissions: There are no ethical permissions.
Conflicts of Interests: There are no conflicts of interest.
Author's contribution: Conceptualization: KA, MM, YSA; Data curation and formal analysis: KA, MM, AP, MJ, YSA; Investigation: KA, MM; Methodology and project administration: KA, MM; Supervision: KA, MM, AP, MJ, YSA; Validation: KA, MM; Writing of original draft: KA, MM; Writing, reviewing, and editing: KA, MM.
Consent to participate: Not applicable.

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