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The Regulatory Events Associated with The Induction of Antimicrobial Activity Following Injection of *Escherichia coli* into *Galleria mellonella* Larvae

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ABSTRACT

The present investigation used *Galleria mellonella* larvae as an infection model to describe the regulatory events associated with the induction of antimicrobial activity following injection of a sub-lethal dose of *Escherichia coli* into the larval hemocoel. The ability of larval hemolymph to generate this activity as natural antibiotics was examined at different time periods postinjection against both the target bacteria (Gram-negative, *E. coli*) and Gram-positive, *Staphylococcus aureus*, as well as against some selected fungi such as *Candida albicans* and *Aspergillus fumigatus*. The results indicated that the highest response was found against G -ve bacteria (*E. coli*) than any other pathogens tested, and the highest activity was obtained at 24 h post-treatment. This may indicate that the antimicrobial is specific to the inoculated bacteria. The presence of such activity in larval plasma was verified using microbiological and biochemical tests. *E. coli*-growth tests on solid agar were carried out, and the results showed drastic effects represented by bacterial mortality that exceeds 74.5% in bacteria-treated with immune plasma, while weak effects were evident in normal (untreated) and control (water-treated) plasma. These tests have confirmed this activity is bactericidal. Also, hemolysis tests were conducted using human erythrocytes as target cells, and the results indicated that all plasma samples possess a weak hemolysis activity, which confirms its safety on human and animals health. Additional biochemical tests of the mode of action were performed to determine the ability of the induced antimicrobial agent of the immune plasma to alter the cell membrane integrity of the *E. coli* bacterial cell by analysis of proteins. The results confirmed that the immune plasma is capable of inducing leakage of these intracellular molecules in the tested bacterial cells. Scanning electron microscopy (SEM) was also used to visualize the morphological effects resulting from treating the target bacterium with this antimicrobial agent and the results indicated a weak growth and an irregular pitted surface of the treated bacterial cells as compared to dense growth and regular smooth surface exhibited in the controls. These results may provide us with essential data for the development and production of new means of protecting humans and animals from infectious diseases.

INTRODUCTION

Despite the developments in controlling infectious diseases around the world, they are still the second biggest cause of morbidity and mortality due, in part, to the increase in drug resistance among large numbers of microbial pathogens. This means that new strategies are needed to prevent and treat infectious diseases. As a result, several ancient methods have been re-evaluated and the substances/procedures employed historically to cure diseases are now attracting renewed scientific attention. The use of natural products such as honey and essential oils, extracted from aromatic plants, and their single constituents as alternative antimicrobial and pharmaceutical agents has attracted considerable interest recently and are now being re-assessed as antimicrobial agents (Allocati *et al.,* 2013; Elshafie *et al.*, 2017; Albaridi, 2019; Rady *et al.,* 2020).

Invertebrates, which include insects, present an extreme diversity and a potential source of a large variety of antimicrobial substances. The main source of these substances is hemolymph which bears various immunity-related products that function in protecting the insect against systemic infection (Meshrif *et al.,* 2010). However, most of the antimicrobial molecules are not present in the hemolymph of normal animals but are induced by an injury or an infection of microbes (Rady *et al.,* 2020). Active immunization of individuals gives protection that varies considerably, and is never as complete as desired (Barakat, 2000) but has a wide spectrum against various microbes. Peptide molecules constitute most of these antimicrobial compounds (Barakat *et al.,* 2002). Additionally, insect antimicrobial compounds are not specialized for specific pathogens (i.e., don`t need a long start-up phase). Because of this broad effect, they are only capable, to a certain degree, of stopping infection (Retschnig *et al.*, 2014).

Therefore, the search for new natural antibiotics as an alternative to the commonly used antibiotics must be parallel to research on understanding physiological immunity against pathogens to avoid infection or alleviate disease symptoms. In addition, the mode of action of these antibiotics and the hypothesis that the induced antimicrobial compounds have a wide spectrum against various microbes needs to be investigated.

The insect immune and mammalian innate immune systems show vast similarities in the cellular and humoral responses (Champion *et al.,* 2009). Previous studies have shown a strong and positive correlation between the virulence of different pathogens in mouse infection systems and *G. mellonella* (Alghoribi *et al.,* 2014). Hence, employing *G. mellonella* as a model system for the determination of microbial pathogenesis, toxicity testing and antimicrobial therapies is extremely suitable and relevant (Leuko and Raivio, 2012; Rady *et al.,* 2020; Chen and Keddie, 2021; Ménard *et al.*, 2021).

The bacterium, *Escherichia coli,* the major cause of both acute and persistent diarrhea in all human ages worldwide, was chosen as the pathogen for challenge in this study because its pathogenesis is not yet fully understood. Understanding the complex relationship between the host and the bacterium is a crucial step for revealing the pathogenicity of a certain strain.

Although the increasing interest in *G. mellonella-E. coli* model system is evidenced by the number of studies: Leuko and Raivio (2012), Rady *et al.* (2020), Chen and Keddie (2021), nevertheless, the mechanisms of *E. coli* virulence in *G. mellonella* remain unknown and nothing is known about the sublethal effects of *E. coli* to *G. mellonella*, which could be used as metrics for measuring virulence in addition to mortality. In addition, almost nothing is known about the events occurring inside the insect postinjection, which are important in understanding both *E. coli* pathogenesis

and *Galleria* immunity for the establishment of the *G. mellonella-E. coli* model system.

The present work generally aimed to obtain a clearer understanding of the regulatory events involved in the synthesis and release of antimicrobial activity, adapted and employed by *G. mellonella* larvae during the course of infection by *E. coli*. In addition, the mode of action and spectrum activity against different microbes was also investigated. In order to elicit an initial response, priming of larvae involves the exposure to dead or a sub-lethal dose of bacteria. A better understanding of these mechanisms may provide us with bassline data about the advanced development and production of new means of protection of man and animals against infectious diseases, and finding out new agents for survival and recovery from combat infections.

MATERIALS AND METHODS

1. The Experimental Insect:

The greater wax moth, *G. mellonella* (L.) was maintained and reared for several generations in constant darkness at 30± 2°C in a 2-liter screen jar with the diet mixture containing pollen and bee wax. The small hatched larvae started to develop by feeding on an artificial diet, consisting of maize flour, 200g; wheat flour, wheat bran and milk powder, 100g each; glycerol, 125mL; honey, 225g and yeast, 25g as reported by Kulkarni *et al.* (2012). Fully grown larvae (~ 0.33g), when they became motionless and just started spinning the cocoon in which they would pupate, were used in this study.

2. The Test Pathogens:

2.1. Bacterial Pathogens:

Two bacterial strains; Gram -ve *E. coli*, ATCC 8739 isolated from human feces, and Gram +ve *S. aureus*, ATCC 29213 (methicillin-sensitive *S. aureus* or MSSA), obtained as actively growing cultures from the Microbiological Resources Centre (Cairo MIRCEN). Bacterial suspensions were prepared as described in the National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 method (NCCLS, 2002a). A concentration of 10^6 CFU/mL sterile distilled water was used directly for inoculation of Mueller–Hinton agar plate (MHA, HiMedia™ Laboratories Pvt. Ltd., India) and 0.5 McFarland Turbidity Standard from (BBL^{TM}, Becton, Dickinson and Company, USA).

2.2. Fungal Pathogens:

Two pathogenic fungi, *C. albicans* and *A. fumigatus*, were obtained from Ain Shams Specialized Hospital, and identified at the Microbiology Department, Faculty of Science, Ain Shams University. These strains were routinely grown and cultured on the same medium for bacterial growth. Yeast and fungi inoculum suspensions were prepared as described in the NCCLS M27-A2 method (NCCLS, 2002b).

3. Immunization of *G. mellonella* Larvae:

A stock suspension of a sub-lethal concentration (10^6 CFU/mL) of *E. coli* that produces 20% mortality was prepared as described by our previous study (Rady *et al.*, 2020). Groups of the experimental larvae, each containing 10 individuals, were injected with 10µl of this bacterial suspension using a 10µl Hamilton micro-syringe fitted with a 26-gauge needle through the ventral surface in the intersegmental region between the 3rd and the 4th prologs according to Miranpuri and Khachatourians (1993). Control insects were injected with equivalent volumes of sterile distilled water only. Another negative control group is kept without injection.

4. Collection and Processing of *G. mellonella* Larval Hemolymph:

Hemolymph samples from normal, control and bacteria-injected insects were collected at 6, 12, 24 and 48 h postinjection. Larvae were bled by puncturing the cuticle on the first hind leg with a fine sterile dissecting needle. According to Hoffmann (1980), about 3-5 insects were transferred to a micro-centrifuge tube (1mL) with a finely

perforated bottom, fitted in another (2mL) micro-centrifuge tube previously cooled on an ice bath. The hemocytes were removed by centrifugation (Human Centrifuge, TGL-16XYJ-2, 16000 rpm, Korea) in cold at 3000 rpm for 10 min at 4ºC. The supernatant (referred to as plasma) was removed from the hemocyte pellet, and immediately transferred into sterile and chilled Eppendorf tubes and stored at -18 ºC until use. Under such conditions, the pure plasma did not show any coagulation for several days at room temperature but coagulation could be triggered if hemocytes were added.

5. Estimation of the Antimicrobial Activity of *G. mellonella* Larval Plasma:

The inhibition-zone assay was conducted to determine the presence of antimicrobial (antibacterial and antifungal) compounds in the hemolymph plasma of *E. coli*-injected larvae compared to those of control groups. The well diffusion method presented here has been carefully standardized by the National Committee for Clinical Laboratory Standards (Jorgensen *et al.*, 1999).

The antibacterial activity of larval plasma against *E. coli* and *S. aureus* was tested in this assay. The antibacterial drugs used in this study as positive control were Ciprofar for G +ve bacteria and Curisafe for G -ve bacteria (obtained from Farco B International, Egypt). These drugs were dissolved in distilled water and used instantly. In addition, the antifungal activity of larval plasma was also tested against *C. albicans* and *A. fumigatus.* The antifungal positive drug control was Fluconazole obtained from Pfizer, Egypt. It was dissolved in distilled dimethyl sulphoxide (DMSO) and stored at -20°C until used.

For screening antimicrobial activities, the MHA powder was dissolved in distilled water and sterilized in an autoclave. After cooling to 37ºC, the mixture was pipetted and streaked into a sterile Petri dish (~ 1 mm thick). According to Faye and Wyatt (1980), the agar plate was surface inoculated by spreading the microbial inoculum over the entire agar surface using a sterile swap. A hole with a 6-8 mm diameter was then punched aseptically with a sterile Cork borer and 80µL volume of the tested plasma was introduced into the well. The plates were then incubated at 37°C for 24 and 48 h for bacteria and at 20°C for fungi. When the plate was held obliquely to transmit light, a distinct clear zone was seen surrounding the well that contained immunized hemolymph. The actual zone width was measured (including the diameter of the well) in millimeters as the following:

Zone width = zone diameter - well diameter

The measurements were replicated five times for each time interval post-injection and the average was calculated.

6. Antibacterial Activity Assays of *G. mellonella* Larval Plasma against *E. coli*:

To evaluate the presence of antibacterial activity in *G. mellonella* larval plasma, bacteria (*E. coli*) growth tests on solid agar were carried out using broth microdilution assay according to the Clinical Laboratory Standards Institute (CLSI) in 96-wells microtiter plate. According to Mastore *et al.* (2015) bacterial cells were diluted with Mueller Hinton broth (NHB) media to 10^6 CFU/ml and aliquots (20µL) of insect plasma (with different amounts of antibacterial compounds), were added to 180µL of bacterial suspension and then incubated for 3 h at 37°C under shaking. After incubation, 100µL of each sample (normal, control and immune plasma) was placed in a test well of the microtiter plate. Samples were serially diluted with phosphate buffer (61.4 mM K_2HPO_4, 38.4 mM H_2PO_4) and finally, they were plated on solid agar and bacteria colonies were counted after incubation at 37°C for 24 h using plate count technique. The antibacterial activity was intended as the percentage of survival bacteria compared with the controls (bacterial suspension incubated without plasma). This experiment was replicated five times.

7. Determination of Hemolysis Activity of *G. mellonella* Larval Plasma:

The lysis of human RBCs was checked by recording hemoglobin release into the extracellular environment after the incubation of blood cells with immune plasma. 10mL of fresh human blood was drawn into K3-EDTA vacuum tubes (BD Vacutainer, Becton Dickinson Inc., USA). Blood testing solution was prepared by washing 3mL of blood with 7mL of pyrogen-free PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The suspension was centrifuged at 400 rpm for 10 min at room temperature and washed several times until the supernatant turned clear. RBC pellets were diluted to 20mL with PBS, and 180µL of diluted blood was added to 20µL of larval plasma. All the samples were incubated for 30 min at 37°C under gentle shaking. Positive or negative controls were performed by adding to blood samples 20µL of 0.2% Triton X-100 or PBS, respectively. Samples were centrifuged at 400 rpm for 5 min and 100µL of supernatants were diluted to 1 mL with PBS. Finally, cell lysis was assessed by measuring the OD (ƛ 404 nm) with a JASCO V-530 UV/VIS spectrophotometer. The percentage of hemolysis was calculated as follows:

$$[(A_{immune\ plasma} - A_{PBS})/(A_{Triton\ X100} - A_{PBS})] \times 100$$

8. Effect of *G. mellonella* Immune Plasma on *E. coli* Bacterial Cell Membrane Integrity:

A bacterial cell membrane integrity assay was carried out to investigate the antimicrobial mode of action of *G. mellonella* larval immune plasma components through its ability to alter the membrane integrity of *E. coli* bacterial cells, causing leakage of intracellular protein (Cordeiro *et al.,* 2014; Ibrahim *et al.,* 2018). The bacteria were treated with 20µL of each larval plasma, blank solution (culture medium control) and bacterial cells were also included in the test. 1mL from each tube was transferred to sterile microcentrifuge tubes and centrifuged at 13,400 rpm for 15 min. A volume (7µL) of the supernatant was removed from each tube to the cuvette and diluted 1:10 (0.7mL) with sterile distilled water and the content was subjected to a spectrophotometric reading at a wavelength of 280 nm for analysis of the presence of proteins **(**Kubo *et al.,* 2004).

9. Effect of *G. mellonella* Immune Plasma on Bacterial Cell Wall Integrity:

The changes in cell morphology induced by tested immune plasma with its antimicrobial agent were detected using a scanning electron microscope (SEM). The tested bacterial cells (*E. coli*) were incubated in nutrient broth at 37ºC for 10 h. The bacterial cells were then treated with immune plasma and incubated at 37ºC for 6 h (control culture was left untreated). Bacterial cells were harvested by centrifugation at 1500 rpm for 10 min. The precipitated cells were washed 3 times and re-suspended in 0.1 M PBS (pH 7.4). The bacterial suspensions (20uL) were spread onto a microscopic slide and air-dried. The samples were coated with gold particles under a vacuum followed by microscopic examination using SEM (Hitachi S-3400N, Tokyo, Japan). The samples were fixated by glutaraldehyde 2.5% and dehydrated by serial dilution of ethanol with agitation using an automatic tissue processor (Leica EM TP, Leica Microsystems, Austria). Then, the samples were dried using CO_2 critical point drier (Model: Audosamdri-815, Tousimis; Rockville, Maryland, USA). The samples were coated by a gold sputter coater (SPI-Module, USA), and observed by SEM by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

10. Statistical Analysis of The Data:

All data were expressed as mean values $\pm$ standard error (SE). One-way analysis of variance (ANOVA) was carried out to determine significant differences ($P< 0.05$ were considered statistically significant) between means by using SPSS statistical software package (SPSS, version 16.0, SPSS Inc., Chicago, IL, USA).

RESULTS

1. Screening Antimicrobial Activity Using the Agar Diffusion Method:

Results presented in Table (1) indicated that the normal (untreated) plasma attained a weak antibacterial activity to the all-tested pathogens without receiving the antigenic challenge. The control (water-treated) plasma had antibacterial activity nearly identical to normal plasma at all post-injection intervals. There was a slight increase ($P \leq 0.05$) of antibacterial activity of control plasma above normal one against the tested bacteria, while there were no significant changes ($P \geq 0.01$) between the normal and control plasma against fungal pathogens. Significant induction ($P \leq 0.025$) of antimicrobial activities against the all-tested pathogens was observed with immune plasma as compared with controls. Great activity was observed against Gram – ve *E. coli* (the challenged bacteria) than all the other tested pathogens. Time-dependent differences were shown in the produced antimicrobial activity. In general, the antimicrobial activity exhibited a largely time-dependent increase up to 24 h post-treatment (Fig. 1), as this immune response was most effective, after that, it showed an insignificant decrease.

Table 1. In Vitro antimicrobial activity represented by inhibition zone diameter (mm) of *G. mellonella* larval plasma against selected bacterial and fungal microbial pathogens determined at different time intervals post-treatment with *E. coli.*

Hours post-injection	Inhibition zone diameter (mm) Mean ± SE											
	E. coli			*S. aureus*			*C. albicans*			*A. fumigatus*		
	Curisafe^{R}	Control	Test	Ciprofar^{R}	Control	Test	Flucona zole^{R}	Control	Test	Flucona zole^{R}	Control	Test
6	45.04 ±1.83^{d}	12.50 ±1.14^{i}	15.62 ±0.97^{h}	51.44 ±1.47^{c}	10.74 ±0.80^{fg}	11.24 ±0.77^{fg}	47.72 ±2.41^{c}	6.52 ±0.45^{i}	10.14 ±1.00^{h}	43.78 ±2.69^{c}	10.14 ±0.99gh	11.86 ±1.04^{fg}
12	59.08 ±2.39^{c}	16.26 ±1.52^{h}	18.02 ±1.71^{h}	60.12 ±1.90^{b}	11.86 ±1.09^{f}	12.24 ±0.51^{f}	55.40 ± 2.55^{b}	13.90 ±1.16^{fg}	16.36 ±1.44^{ef}	51.16 ±2.26^{b}	12.24 ±1.28^{fg}	12.12 ±1.22^{fg}
24	67.62 ±1.59^{a}	23.82 ±0.75f^{g}	29.16 ±1.92^{e}	65.44 ±1.94^{a}	16.06 ±1.14^{e}	18.76 ±0.98^{d}	62.76 ±2.08^{a}	18.40 ±0.98^{de}	20.58 ±1.54^{d}	63.52 ±1.97^{a}	16.66 ±1.48^{de}	18.66 ±1.29^{d}
48	62.68 ±1.48^{b}	21.68 ±1.99^{g}	25.30 ±2.64^{f}	63.70 ±1.73^{a}	14.82 ±0.59^{e}	16.64 ±0.67^{de}	59.92 ±2.08^{a}	17.96 ±1.23^{de}	18.02 ±0.66^{de}	60.64 ±2.32^{a}	14.88 ± 0.64^{ef}	15.76 ±0.83^{de}
Un-injected	10.72 ± 0.02^{i}			9.33± 0.63^{fgh}			9.11 ± 0.34^{ih}			9/00 ± 46^{ghi}		

n = 5 replicates per test, analyzed by using the SPSS11.5.0 software (SPSS Inc., 2012).
The differences between means were analyzed by one-way analysis of variance (ANOVA).
Means that don't share a letter within a column are significantly different.
Levels of significance of differences for each experiment were set at $P \leq 0.05$.

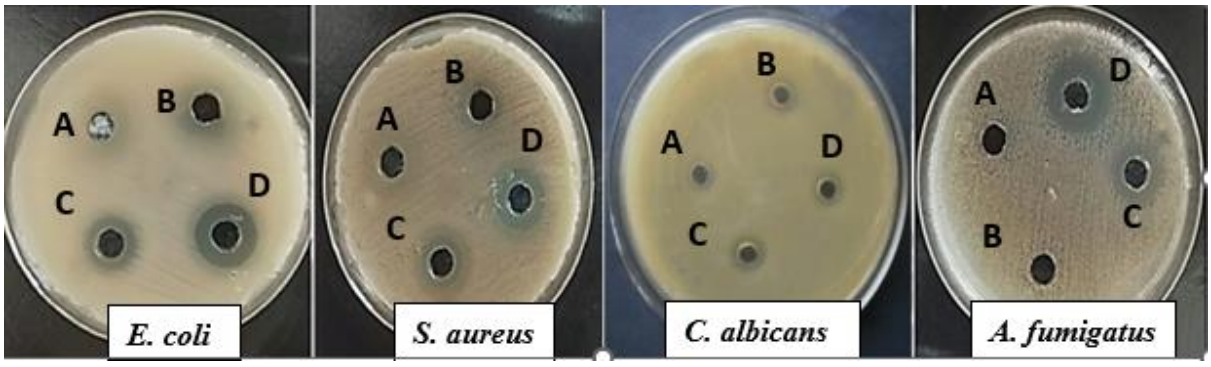
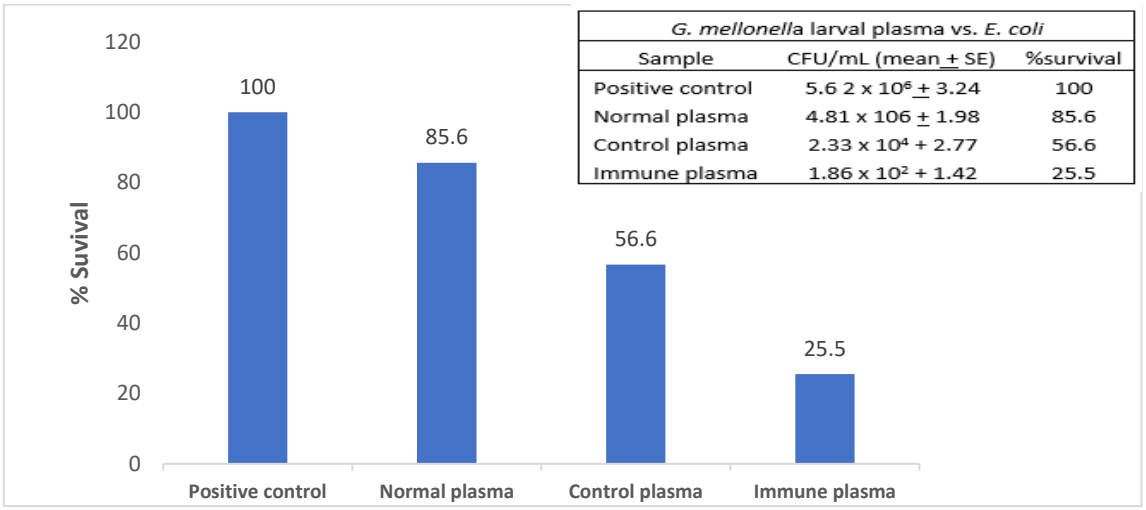


Fig. 1. Visible clear zone produced by immune plasma from *G. mellonella* larvae (collected at 24 h post-treatment with *E. coli*) against four selected microbial pathogens: *E. coli* ATCC 8739, *S. aureus*, ATCC 29213, *C. albicans*, and *A. fumigatus.* (A) normal (untreated) plasma, (B) water-treated (control) plasma, (C) bacteria-treated (immune) plasma, and (D) standard antibiotic drug.

2. Characterization of Antibacterial Activity of *G. mellonella* Immune Plasma Against *E. coli*:

Data of *E. coli* growth tests on solid agar after serial dilution of bacteria incubated with insect plasma were illustrated in Figure (2). In all assays, drastic antimicrobial effects are evident with immune plasma (bacteria mortality exceeds 74.5%) compared with the +ve control growth. Moreover, with normal and control plasma, low activity is present.



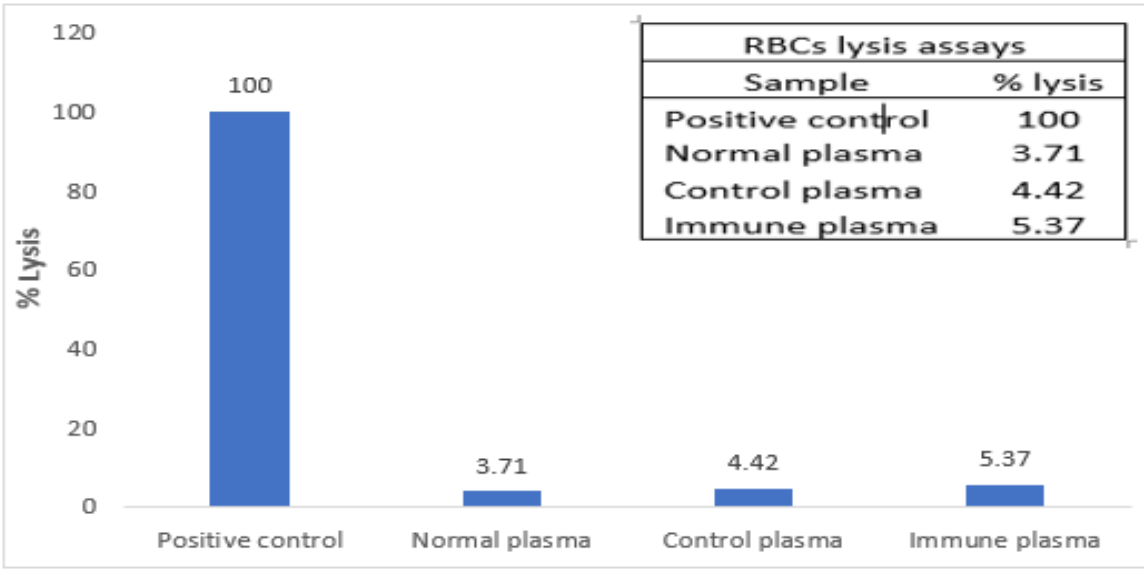
G. mellonella larval plasma vs. *E. coli*

Sample	CFU/mL (mean ± SE)	%survival
Positive control	$5.6\ 2 \times 10^{6} \pm 3.24$	100
Normal plasma	$4.81 \times 106 \pm 1.98$	85.6
Control plasma	$2.33 \times 10^{4} + 2.77$	56.6
Immune plasma	$1.86 \times 10^{2} + 1.42$	25.5

Fig. 2. Effects of *G. mellonella* larval plasma against Gram-negative bacteria *E. coli*. Bacterial survival was evaluated by CFU count (n = 5).

3. Hemolytic Activity of *G. mellonella* Larval Plasma:

Figure (3) shows the results of the hemolysis tests of *G. mellonella* larval plasma; (normal, control, and immune) against human erythrocytes as target cells. All plasma samples possess a weak hemolytic activity compared with Triton x-100 treatment (positive control).



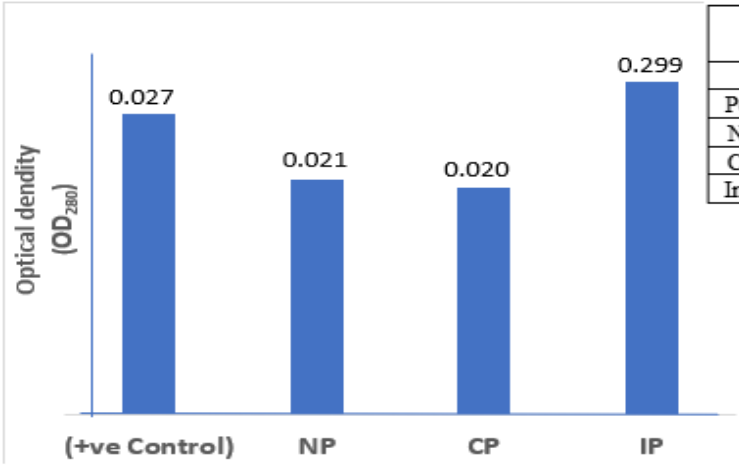
RBCs lysis assays

Sample	% lysis
Positive control	100
Normal plasma	3.71
Control plasma	4.42
Immune plasma	5.37

Fig. 3. Hemolytic activity (% lysis) of *G. mellonella* larval plasma against human RBCs. The graph shows the percentage of lysis referred to a 100% lysis positive control obtained by RBC incubation with 0.02% Triton X-100, (n = 5).

4. Cytoplasmic Membrane Permeability Assay:

Figure (4) shows the results of *E. coli* bacterial cell membrane integrity assays through the leakage of intracellular protein due to the action of *G. mellonella* larval immune plasma components. The results indicated that the immune plasma, with its induced antibacterial agents, was capable of inducing leakage of the intracellular molecules (protein) in the tested bacterial cells. Protein leakage caused an increase in absorbance reading (1.7), compared to that obtained from the bacterial growth control (0.069).

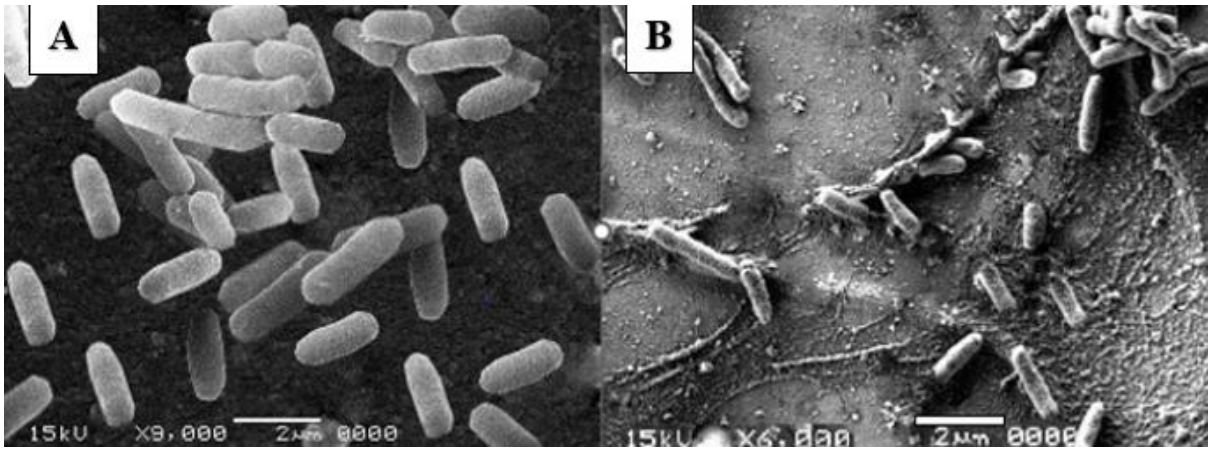


Cell membrane constituents' release	
Sample	OD_{260nm}
Positive control	0.0270 ± 0.003[a]
Normal plasma	0.0211 ± 0.002[a]
Control plasma	0.0203 ± 0.003[a]
Immune plasma	0.299 ± 0.019 [b]

Fig. 4. The effect of *G. mellonella* larval plasma on *E. coli* bacterial cell constituents' release. Values represent means of five independent replicates ± SE (different letters within a column indicate statistically significant differences at $P < 0.05$). NP: normal plasma, CP: control plasma, and IP: immune plasma.

5. Scanning of Bacterial Cells Using Scanning Electron Microscope:

The changes in cell morphology and the destructive effect induced by the tested immune plasma on *E. coli* were shown in Figure (5). The treated bacterial cells showed obvious morphological changes as compared to untreated cells. Growth of control *E. coli* cells without any treatment exhibited dense growth and regular smooth surface (Fig. 5A). Whereas treated bacterial cells with tested antimicrobial agent became deformed and broken and had weak growth and irregular pitted surface (Fig. 5B).



Fig, 5. Scanning Electron Microscope showing (A) control *E. coli* cells and (B) *E. coli* cells treated with immune plasma (antimicrobial compound).

DISCUSSION

One of the most commonly used models for studying microbial infections is the murine model. However, there are ethical, budgetary and logistical hurdles associated with the use of rodents as infection models. Firstly, maintaining a sufficient number of animals required to obtain statistically relevant data is expensive and often regarded as ethically objectionable. Secondly, mammals have lengthy reproduction times, which slow the progress of experimentation (Rejasse *et al.,* 2010).

Insects are undoubtedly becoming a popular model for studying microbial virulence and treatment options, as evidenced by both the expanding range of pathogens tested in the system and the growing number of reports utilizing the model. They can be easily and inexpensively obtained in large numbers and are simple to use as they don't require special lab equipment. There are no ethical constraints and their short life cycle makes them ideal for large-scale studies. Although insects lack an adaptive immune response, their innate immune response shows remarkable similarities with the immune response in vertebrates.

The innate immune systems of insects such as *G. mellonella* display a high degree of similarity to the mammalian immune systems, which makes them an attractive alternative to animal models for the investigation of microbial infection (Kavanagh and Reeves, 2004). In addition, it displays key advantages over other invertebrate models, such as its ability to be maintained at temperatures between 15°C and 37°C (Aperis *et al.,* 2007), which makes the larvae well suited to study pathogens at human body temperature, and the ease of handling and delivery of a precise infective dose. Furthermore, these attributes allow large numbers of larvae to be infected, therefore facilitating its use for the large-scale screening of virulence factors or antimicrobial activities of candidate drugs (Oliva *et al.,* 2018).

The present study tries to prove the ability of *G. mellonella* larvae to produce immune-related factors in their hemolymph, upon immunization (infection with a sub-lethal dose of bacterial pathogen, *E. coli*). In agreement with several studies e.g., Scully and Bidochka (2006), Lionakis (2011) Junqueira and Mylonakis (2019), and Rady *et al.* (2020), *Galleria* has proven to be successfully used as a model for ethical reasons that minimize the usage of mammals as models for studying and this substitution has several benefits. Within this frame, *G. mellonella* was used in this study to answer several questions; the most important are; (1) how can insects avoid destruction by disease agents? (2) What are the interactions that take place, within the insect body, between the microbial invaders and the immune system of the host, and (3) what is the role of hemolymph in the discrimination and elimination of foreignness?

Antimicrobial activity is a multifunctional component of the innate immune defense systems in prokaryotic and eukaryotic organisms (Bulet *et al.,* 1999). It has a range of antibacterial, antifungal, and antiviral activities. They have a promising capacity in therapeutic and prophylactic applications (Tonk *et al.,* 2016). Moreover, it also shows anticancer effects or has anticancer properties (Jin and Weinberg, 2018).

In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. Therefore, greater attention has been paid to antimicrobial activity screening and evaluating methods. Several bioassays such as disk diffusion and well diffusion are well known and commonly used (Balouiri *et al.,* 2016). Another and the most widely used experimental assay to determine the antibacterial activity of test compounds involves determining the

minimum inhibitory concentration (MIC) of a compound that is able to inhibit bacterial growth (Andrews, 2001). Formally, the MIC values are often used as quantitative indicators of the relative potency of new antibacterial agents. The agar and broth dilution methods are the most common protocols for determining MIC values (Wiegand *et al.,* 2008).

Results obtained in the present study using the agar-well diffusion technique proved that the normal insects attained a weak antibacterial activity to virulent bacteria without they had received antigenic challenge. This result is supported by the findings of Heimpel and Angus (1959) and Yoon *et al.* (2018) who found antibacterial activity in some insects as a result of high pH and naturally occurring antimicrobial substances, that are contained in the food. Another explanation was suggested by Mirsch (1960) who mentioned that several substances such as fatty acids, lipids of sterol type, certain peptides and aliphatic amines are known to exert antibacterial action in vitro. One or more of these substances as integral parts of cells could be liberated to act on bacteria upon destruction of the cells or a change in the cell's natural environment. Accordingly, the amount of antibacterial activity in the blood of normal insect should be proportional to the amount of cell destruction or to the degree to which the environment was altered.

In our investigation, we obtained hemolymph for testing by centrifuging the insects after they had been incised through their bodies. Considerable cellular damage undoubtedly resulted from such treatment. These circumstances may explain the weak humoral antibacterial response in normal insects. These findings are in contrast to the findings of Walters and Ratcliffe (1983) and Meylaers *et al.* (2007) who stated that there was no evidence for any naturally occurring antibacterial factors in this species.

Another important point in the induction of immunity is the control injection, a phenomenon that is known to all investigators working on insect immunity. Earlier studies indicated that several insect species respond to injection of live bacteria by in vivo production of antibacterial activity and synthesis of immune proteins which are detectable in the hemolymph. However, this response is not specifically induced by bacteria, as injection with sterile insect saline solutions (Faye and Wyatt, 1980; Barakat *et al.*, 2002 and Meshrif *et al.*, 2010), distilled water (Momen *et al.*, 2019; Radwan, 2019), and latex bead (Wiesner, 1992) would cause a transient synthesis of an antibacterial activity. Also, as data illustrated, the *G. mellonella* larvae showed a weak antibacterial activity to water challenge (i.e. in control insects). This may be due to either a lower sensitivity or a higher induction-specificity of the larval immune system. However, it is also likely to be due to a lesser amount of physiological stress caused by injury of the cuticle and the injection of 10µl distilled water.

Bacterial susceptibility assays were performed to primarily compare the susceptibility of various bacterial and fungal pathogens against *G. mellonella* hemolymph plasma, a significant induction of antimicrobial activity was observed against *E. coli* as compared with the G +ve bacteria (*S. aureus*), fungal pathogens and negative controls. This may be attributed to the fact that *E. coli* is a virulent pathogen, that enhances the insect immune system to produce antimicrobial activity. This virulent effect comes from some cell wall components, such as lipopolysaccharides (LPS) which is one of the great virulence determinants of possessing a specific structure that might be a reliable indicator of virulence potential (Murray *et al.*, 2003). Time-dependent differences were shown in antibacterial activity, as this immune response was more effective in the late than the earlier examination periods. From these results, it should be suggested that the compounds that are responsible for the

appearance of this response need some time to be synthesized and released. These results are in agreement with the results of antibacterial susceptibility assays by Barakat (2003) worked on *G. mellonella* larvae. Similar results were also reported by Radwan *et al.* (2019) worked on *Spodoptera littoralis* larvae. Furthermore, in this respect, Meylaers *et al.* (2007) and Radwan *et al.* (2019) noted that the strength of the antibacterial activity in the hemolymph does not depend on the concentration of pathogens per hemolymph volume or body mass. If this was the case, the highest response would be in pupae and adults, which are characterized by a decrease in these parameters.

Most antibacterial or antifungal compounds exert their effects by interacting with and destabilizing both plasma cell membranes and bacterial cell walls, eventually leading to cell death. We confirmed the action of *G. mellonella* immune plasma on *E. coli* bacteria by evaluating the integrity of the bacterial cell plasma membrane, as well as the bacterial cell wall. As observed by bacterial cell membrane integrity assay through its ability to cause leakage of intracellular proteins, our results indicated that the tested immune plasma causes irreversible damage to the cell membrane, which leads to loss of cellular constituents and finally to cell death. The measurements of specific leakage markers after treatment with antibacterial agents, including nucleic acids and proteins, are an indicator of bacterial cell membrane integrity in comparison to unexposed cells (Bajpai *et al.,* 2013). These results corroborate previous reports (Borges *et al.,* 2013; Diao *et al.,* 2014; Sadiq *et al.,* 2017), indicating that irreversible damage might occur to bacterial membranes after treatment with medicinal plants, which could lead to loss of essential cellular components such as proteins and nucleic acids. Furthermore, the antimicrobial agents kill bacteria via a variety of mechanisms including membrane disruption, interference with bacterial metabolism, and targeting of cytoplasmic components (Shen *et al.,* 2018). The primary contact between the active compound and the target bacterium occurs via an electrostatic or hydrophobic interaction, which is strongly dependent on the lipid composition of the bacterial membrane (Yeaman and Yount, 2003).

Additionally, active compounds are capable of interacting with the surface of the cell membrane to alter the permeability of the membrane (Shen *et al.,* 2018). After their interaction with the cell membrane, the formed trans-membrane potential affects the osmotic pressure balance (Patocka *et al.,* 2018). In short, the interaction between these active components and the membrane is directly related to the antibacterial activity. At present, antimicrobial agents can also disrupt intracellular enzymes and DNA when they translocate into pathogens (Ongey *et al.,* 2018). Regarding the membrane activity, some issues need to be considered. For example, whether there is a specific membrane receptor, and whether there are other factors synergistically working in this context (Shen *et al.,* 2018).

Since the discovery of the first antibacterial peptide in insects in 1981 (Steiner *et al.,* 1981), large numbers of antimicrobial peptides have been characterized by representatives of the animal and plant realms and a large variety of techniques have been used to isolate, purify, and characterize such molecules (for reviews see Retschnig *et al.* (2014). Furthermore, antimicrobial lipids such as fatty acids and monoglycerides are promising antibacterial agents that destabilize bacterial cell membranes, causing a wide range of direct and indirect inhibitory effects (Yoon *et al.,* 2018). Kabara *et al.* (1972) conducted pioneering studies to evaluate the antibacterial activity of fatty acids and monoglycerides against a wide range of bacteria by using the broth dilution method.

Our SEM data, which showed the ultrastructure of *E. coli* after treatment,

confirm the membranolytic activity of *G. mellonella* immune plasma. The results revealed that the immune plasma contains antibacterial compounds that led to deformation and broken of the bacterial cells, and also led to their weak growth and irregular pitted surface. These data were in agreement with other publications (Brogden, 2005; Brivio *et al.*, 2006; Mastore *et al.*, 2015) that had explained the occurrence of pores on the bacterial surface as a consequence of a distortion of the outer membrane resulting from the interaction between antibacterial compounds and the anionic surface of bacterial cells.

CONCLUSION

This study provides a rapid, inexpensive and reliable way to evaluate antimicrobials *in vivo*. The *G. mellonella*–*E. coli* infection model is ideal for studying the virulence and pathogenesis of *E. coli* alternative to the mammalian models that are constrained by the high costs of maintenance, low sample sizes, and ethical concerns compared to invertebrate models. Using this model, the efficacy and performance of anti-infective materials to prevent or combat infections were successfully verified. In this work, a dose (~ 10^3) of *E. coli* per larva was found suitable inoculum to activate the immune response in order to evaluate antimicrobial compounds generated in the hemolymph. The strength of the immune response, quantified at different time intervals after inoculation, indicated that this immune response was more effective in the later than the earlier examination periods, which suggests that the compounds which are responsible for the appearance of this response need some time to be synthesized and released. *E. coli*-induced *G. mellonella* hemolymph showed a broad antimicrobial profile against *E. coli*, *S. aureus*, as well as other fungal pathogens, which suggests its potential capability as a future drug candidate. More importantly, the potential activity against the challenging bacteria, *E. coli* rather than the weak activity observed against the other tested pathogens may indicate that the antimicrobial is specific to the inoculated bacteria. Additional microbiological and biochemical tests conducted to characterize and demonstrate the mode of action of antimicrobials have confirmed their bactericidal activity through membranolytic effects on bacteria, and at the same time, do not have hemolysis effects on human blood cells, which recommends their safe use on human and animal health.

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ARABIC SUMMARY

الأحداث التنظيمية المرتبطة بتحريض النشاط المضاد للميكروبات بعد حقن الإشريكية القولونية في يرقات غاليريا ميلونيلا

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استخدم البحث الحالي يرقات غاليريا ميلونيلا كنموذج للعدوى لوصف الأحداث التنظيمية المرتبطة بتحريض وإنتاج النشاط المضاد للميكروبات بعد حقن جرعة شبه مميتة من الإشريكية القولونية داخل التجويف الدموى لليرقات. ثم تم فحص قدرة بلازما دم اليرقات على توليد هذا النشاط، كمضادات حيوية طبيعية، في فترات زمنية مختلفة بعد الحقن ضد كل من البكتيريا المستهدفة (الإشريكية القولونية، سالبة الجرام) والمكورات العنقودية الذهبية (إيجابية الجرام)، وكذلك ضد بعض الفطريات المختارة مثل المبيضات البيضاء والرشاشيات الدخناء. أشارت النتائج إلى أن أعلى استجابة تم العثور عليها كانت ضد البكتيريا المستهدفة (سالبة الجرام) مقارنة بمسببات الأمراض الأخرى التي تم اختبارها، وتم الحصول على أعلى نشاط بعد 24 ساعة من المعالجة البكتيرية ضد جميع أنواع الميكروبات المختبرة، مما يدل على أن مضادات الميكروبات الناتجة متخصصة تجاه البكتيريا المستهدفة. كما تم التحقق من وجود هذا النشاط في بلازما دم اليرقات باستخدام الاختبارات الميكروبيولوجية والبيوكيميائية، حيث أجريت اختبارات نمو الإشريكية القولونية على الأجار الصلب، وأظهرت النتائج تأثيرات قوية تتمثل في معدل موت بكتيري يتجاوز 74.5% في البكتيريا المعاملة بالبلازما المناعية، بينما أظهرت تأثيرات ضعيفة في اختبارات المقارنة، وأكدت تلك الاختبارات أن هذا النشاط مبيد للجراثيم. وأيضا أجريت اختبارات لمعرفة قدرة المضادات الميكروبية الناتجة على إحداث التحلل الخلوى باستخدام كريات الدم الحمراء البشرية كخلايا مستهدفة، وأشارت النتائج إلى أن جميع عينات البلازما تمتلك نشاط دموى ضعيف، مما يؤكد على الأمان التام فى حال استخدامها على الإنسان والحيوان. ثم تم إجراء اختبارات إضافية لتحديد طريقة عمل وقدرة المضادات الميكروبية المستحثة والناتجة في البلازما المناعية على تغيير سلامة أغشية الخلايا البكتيرية (باستخدام الإشريكية القولونية كبكتيريا مستهدفة) عن طريق تحليل البروتينات، وأكدت النتائج قدرة البلازما المناعية على إحداث تسرب لهذه الجزيئات من داخل الخلايا البكتيرية المختبرة. كما تم استخدام المجهر الإلكتروني الماسح لتصور التأثيرات المورفولوجية الناتجة عن معالجة البكتيريا المستهدفة بهذه المضاد الميكروبية، وأشارت النتائج إلى نمو ضعيف وسطح منقر غير منتظم للخلايا البكتيرية المعالجة مقارنة بالنمو الكثيف والسطح الأملس المنتظم فى الخلايا البكتيرية غير المعالجة. وبهذا قد تزودنا هذه النتائج ببيانات أساسية لتطوير وإنتاج وسائل جديدة لحماية الإنسان والحيوان من الأمراض المعدية.