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The Antibacterial Activity of Induced and Non-induced Usherhopper, *Poekilocerus bufonius* Hemolymph

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Authors' contributions

This work was carried out in collaboration among authors. Author SS designed the study and performed the statistical analysis. Authors OA and AA managed the analyses of the study and wrote the first draft of the manuscript. All authors wrote the protocol and managed the literature searches. Author SS proofread the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Insects secret antimicrobial peptides into the hemolymph to protect themselves against bacterial infection. This study aimed to test the antibacterial activity of the usherhopper, *Poekilocerus bufonius* hemolymph against positive (two species) and negative (six species) gram bacteria.
Study Design: Non-induced and induced hemolymph (for six and twelve hours) by *Escherichia coli* were used for this study.
Place of Study and Duration: Department of Biotechnology, Faculty of Science, Taif University, Taif, Saudi Arabia, between March and July 2016.
Methodology: Thirty usherhopper adults were collected from Taif Governorate, Saudi Arabia. 10 insects were used for each treatment and twenty microliter of *E. coli* was injected into the coelom to stimulate the usherhopper immunity. Hemolymph antibacterial assay was carried out using ten microliter hemolymph on the pieces of filter paper and then the media culture was incubated at

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37℃ for 24 hours. The formation of inhibition zone represented the antibacterial hemolymph effect in the medium.

Results: It was found that bacteria-induced hemolymph and non-induced hemolymph of usherhopper had antibacterial effect against eight species of bacteria. The inhibition zones diameter of tested bacteria species were significantly differed at all treatments of the non-induced, induced for 6 h and induced hemolymph for 12 h. In general, the higher antibacterial activity was recorded against *E. coli* whether the hemolymph was induced or non-induced. With the exception of three tested bacteria species i.e., *Proteus mirabills*, *Bordetella petrii* and *Entrobacter hormaechei*, the inhibition zone diameter not significantly affected by the non-induced, induced for 6h and induced for 12 h.

Conclusion: Usherhopper immune system against pathogens can be replacing the natural defensive mechanisms against invading microorganisms and can prevent indiscriminate use of chemical agents. We suggest that the extraction and definition of peptides from usherhopper hemolymph could be useful in defensive strategy against the pathogenic bacteria for human, plants and animals.

Keywords: Antibacterial activity; Insects; usherhopper; hemolymph.

1. INTRODUCTION

Many of organisms are sources of numerous cationic peptide with antimicrobial behaviors [1]. The quest for the next generation of antibiotics is focused recently on natural peptides produced by animals or insects which are known for their ability to resist infection. Insects protect themselves against bacterial infection by secreting a battery of antimicrobial peptides into the hemolymph. Insect hemolymph constitutes 16-40% of the body weight of certain insects. The volume and component of hemolymph are different among species of insects and their developmental stages. The hemolymph circulation would help to transport the antimicrobial peptide to its target site [2,3]. Insects survive against pathogenic infection depending on innate immunity because they lack the adaptive immunity of vertebrates that provides a host defense mechanism which is more potent and specific. Insects have various types of barriers which can be physical, chemical or biological weapons constituting first line of defense against invasion by pathogenic organisms. Upon a successful entry into the insects, the second line of innate immunity at the cellular and molecular levels is activated in the hemocoel. When this is successful, pathogens are eliminated from the infected insects, thus preventing their propagation and spread. Instead, in the hemocoel, pathogens have advanced variation of mechanism against host defense and inhibit the activated natural immune system [4]. However, the innate immune responses are found to be very efficient mechanisms for safeguarding the insects against any kind of infection, given the lifespan of the insects and their vast territorial successes in all ecospheres [5,6]. The insect immune system includes cell-mediated and humoral-based immune reactions which need synergic actions of hemocytes, fat body and freely circulating hemolymph proteins and peptides to contain or deactivate any invading pathogen. For such a coordinated response, insect cytokines come to play the fundamental role in mediation of the immune reactions [7,8,9]. The cytokines along with hormones are assumed to regulate mobilization of resources during the course of infection, especially at times energy trade-offs between immune and non-immune (e.g. growth) responses are essential for host survival [10,11].

The polyphagous Usherhopper, *Poekilocerus bufonius* (Orthoptera: Pyrgomorphidae), was recorded in different regions in Saudi Arabia with high presence in western regions [12,13] and have variable enzymatic patterns within and between the different habitats [14].

Therefore, the present study aimed to evaluate the antibacterial activity of the induced and non-induced usherhopper hemolymph for the first time against various gram positive and gram negative bacteria.

2. MATERIALS AND METHODS

2.1 Sampling

Thirty usherhopper adults were collected from different locations at Taif Governorate, Saudi Arabia. The insects were kept in plastic cages (2 Liters volume) and covered with tulle. They were fed on usher plant leaves (*Calotropis procera*).

Table 1. Bacteria species those used in antibacterial assay

Bacteria species	Family	+/- Gram	Sporulation
Bacillus subtilis	Bacillaceae	Positive	Sporulated
Escherichia coli	Enterobacteriaceae	Negative	Non-sporulated
Micrococcus luteus	Micrococcaceae	Positive	Non-sporulated
Pseudomonas aeruginosa	Pseudomonadaceae	Negative	Non-sporulated
Proteus mirabills	Enterobacteriaceae	Negative	Non-sporulated
Bordetella petrii	Alcaligenaceae	Negative	Non-sporulated
Entrobacter hormaechei	Enterobacteriaceae	Negative	Non-sporulated
Serratia nematodiphila	Enterobacteriaceae	Negative	Non-sporulated

2.2 Collection of Non-induced Hemolymph

The body surface of 10 insects were cleaned with 70% alcohol. Then, in order to collect hemolymph, hind pair legs were cut and hemolymph fluid was extracted with a capillary tube placed into micro tubes containing 50 mg of phenyl thiourea (PTU). Hemolymph was centrifuged at 10000 × g for 10 minutes and the supernatant was collected for the antibacterial testing and stored in 4℃ [15]. This centrifugation step gives cell-free hemolymph; the hemocytes (blood cells) and any undissolved PTU crystals will be pelleted. The hemolymph of these 10 insects was combined in one tube.

2.3 Hemolymph Induction

A volume of twenty microliter of *Escherichia coli* (10^6 cells/mL) was injected into the coelom of each of 20 usherhopper individuals to stimulate the usherhopper immunity.

2.4 Collection of Induced Hemolymph

The time required to produce antimicrobial proteins was between 6 to 12 hours after injection of bacteria into the usherhopper bodies. The same above mentioned method in collection of non-induced hemolymph was undertaken to collect the induced hemolymph from 10 individuals after 6 h. and from 10 individuals after 12 h. of *E. coli* injection. The hemolymph of both groups (each contains 10 insects) was combined in one tube.

2.5 Bacteria Species Tested

Antibacterial assay was conducted on eight bacteria species as presented in Table 1 above.

2.6 Antimicrobial Assay

Three treatments; non-induced, induced for 6 h and induced for 12 h, were replicated six times for each microorganism species. Hemolymph antibacterial assay was carried out using a piece of filter paper (5 mm in diameter) method. The 10 mL nutrient agar media was placed in the petri dishes (10 cm diameter), then the medium surface was impregnated with the 24-hour-grown strains (1.5×10^6 cells per mL). Ten microliter hemolymph was poured on the pieces of filter paper and then the media culture was incubated at 37℃ for 24 hours. The formation of inhibition zone (the lack of growth) represented the antibacterial hemolymph effect in the medium.

2.7 Statistics

Results were described as regular averages and standard deviations. One-way and Two-way ANOVA were conducted on all data. Following a significant differences, means were compared by Duncan test (P = 0.05). All the analyses were conducted using SPSS version 23 [16].

3. RESULTS AND DISCUSSION

Two-way of variance analysis revealed that bacteria species and induction effect significantly affected inhibition zone of eight bacteria species by usherhopper hemolymph. Moreover, there is significant interaction between bacteria species and induction effect (Table 2).

Table 2. Two-way ANOVA on bacteria species and induction effect affecting inhibition zone of eight bacteria species by usherhopper hemolymph

Source	df	Mean square	*F*	*P*
Bacteria species	7	70.377	83.891	<0.001
Induction effect	2	28.146	33.551	<0.001
Bacteria sp. x induction effect	14	5.154	6.144	<0.001
Error	120			

Table 3. Effect of induced and non-induced usherhopper hemolymph on eight tested bacteria species as inhibition zone in mm diameter (Mean±SD)

Bacteria species	Inhibition zone diameter in mm (Mean±SD)			df	*F*	*P*
	non-induced	induced for 6 h	induced for 12 h			
B. subtilis	8.5±0.84 Acd	9.67±0.52 Bb	9.33±1.21 ABc	2, 15	2.671	0.102
E. coli	14.17±1.47 Aa	14.33±0.82 Aa	14.67±0. 82 Aa	2, 15	0.333	0.722
M. luteus	9.50±0.55 Abc	10.50 ±0.55 Ab	10.0±1.27 Abc	2, 15	2.045	0.164
P. aeruginosa	8.17±0.98 Ade	7.67±0.82 Ac	7.50±1.05 Ad	2, 15	0.793	0.471
P. mirabills	7.17±0.75 Aef	8.50±1.05 Bc	10.50±0.55 Cbc	2, 15	25.763	<0.001
B. petrii	6.83±0.57 Af	9.67 ±1.03 Bb	11.0±1.41 Bb	2, 15	22.431	<0.001
E. hormaechei	7.50±0.55 Adef	10.17±0.75 Bb	10.33±1.03 Bbc	2, 15	23.534	<0.001
S. nematodiphila	9.67±0.82 Ab	9.67±0.82 Ab	10.00±0.63Abc	2, 15	0.385	0.687

- For each species, means within the same row bearing different capital letters Are significantly different according to Duncan test (P = 0.05).

- Means within each column bearing different small letters are significantly different according to Duncan test (P = 0.05)

Results showed that three tested bacteria (*Proteus mirabills*, *Bordetella petrii* and *Entrobacter hormaechei*) significantly affected by induced and non-induced usherhopper hemolymph (Table 3 above).

One-way of variance analysis revealed that inhibition zone diameter of bacteria species significantly affected by the non-induced ($F = 42.47$; df = 7, 40; $P < 0.001$), induced for 6 h ($F = 35.04$; df = 7, 40; $P < 0.001$) and induced for 12 h ($F = 22.68$; df = 7, 40; $P < 0.001$).

In the current study, by stimulating or non stimulating the usherhopper immune system, it was found that bacteria-induced hemolymph and non-induced hemolymph had antibacterial effect against eight species of bacteria specially *E. coli* that had the highly affect whether the hemolymph was induced or not.

On the other hand, lysozyme generally exhibits greater bactericidal lytic activity against Gram-positive bacteria [17]. However, some other insect lysozymes were reported to have moderate activities against Gram-negative bacteria [18]. Some insect lysozymes also exhibits antifungal activity by hydrolyzing the b-1,4-linkages of chitooligosaccharides in the fungal cell wall [19]. On the other hand, some c-type lysozymes have been evolutionarily adapted to digestive functions and are found in the midgut of larval cyclorrhaphans that live in highly contaminated decomposing matter [20,21]. So far, about 50 anti-bacterial molecules are isolated from the insects by stimulating their immune system [22]. It seems that the stimulations caused by the injury and damage lead to proteolytic responses in the arthropods and insects hemolymph and thus provoke their immune responses. Due to this point, several studies were conducted in this area.

In other study, by stimulating the immune system of bee (*Apis* spp.) hemolymph, apidaecin was produced which resulted in inhibitory effects on Gram-positive bacteria [23]. Another study revealed that the stimulation of sand fly, *Phlebotomus duboscqi,* by injected protozoan parasite or bacteria triggered the release of defensin family peptides with anti-bacterial activity in their hemolymph [24].

In our results, the higher antibacterial activity was recorded against *E. coli* whether the hemolymph was induced or non-induced. Previous study found that the maximum antimicrobial activity was observed 12 hours after injection of *E. coli* in the American cockroach (*Periplaneta americana*) hemolymph [25]. They also found that stimulation of the cockroaches' immune system by injecting *E. coli* was associated with the release of antibacterial compounds, and the induced hemolymph had inhibitory effect on the growth of two susceptible bacterial strains, including *E. coli* (gram negative) and *Staphylococcus aureus* (gram positive). However, non-induced hemolymph did not have antimicrobial activity against susceptible and resistant strains of bacteria [15]. Due to a long history of insect coexisting with a variety of microorganisms, may be a good choice to replace bacteria-resistant chemical compounds. Since these organisms are normally present in all animal habitats, they are exposed to many invasive and harmful microorganisms in the nature. The insect immune system is able to cope with invading microorganisms [25,26].

4. CONCLUSION

It was found that bacteria-induced hemolymph and non-induced hemolymph of usherhopper had antibacterial effect against eight species of bacteria including 2 positive gram bacteria and six negative gram bacteria. This result may be due to the hemolymph of usherhopper, *P. bufonius* naturally contains different toxic compounds such as cardiac glycocides [27] and cardenolides [28] which are coming from the host plant, *C. procera.* Moreover, usherhopper immune system against pathogens can be a defensive strategy against the pathogenic substances, and replacing the natural defensive mechanisms against invading microorganisms can prevent indiscriminate use of chemical agents. We suggest that the extraction and definition of peptides from usherhopper hemolymph could be useful in defensive strategy against the pathogenic bacteria for human, plants and animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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