

The Antibacterial Effect of American Cockroach Hemolymph on the Nosocomial Pathogenic Bacteria

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Background: Insects are able to recognize many pathogenic microorganisms and defend against them due to their long evolutionary history. Due to the development of resistance to synthetic antibiotics, researchers are trying to apply insect immune-derived products.

Objectives: The current study aimed to investigate the antibacterial effect of the American cockroach (*Periplaneta americana*) hemolymph on susceptible and resistant strains of nosocomial bacteria.

Materials and Methods: To stimulate adult cockroaches' immune system, *Escherichia coli* cells were injected. The antimicrobial effect of the extracted induced and non-induced hemolymph was assayed on many susceptible and resistant pathogenic bacteria.

Results: The comparison of antimicrobial effects of the induced and non-induced hemolymph strains showed that about 43% of bacteria were sensitive to induced hemolymph ($P < 0.001$), whereas non-induced hemolymph showed no inhibitory effect on the bacteria. Also, evaluation of induced hemolymph effect on the types of strains showed that induced hemolymph affected about 75% of the susceptible bacterial strains ($P < 0.001$); whereas, it did not affect the resistant strains. Among the tested bacteria, ceftazidime-sensitive *E. coli* and methicillin-sensitive *Staphylococcus aureus* showed sensitivity to the induced hemolymph ($P < 0.001$).

Conclusions: The study results showed that stimulation of the American cockroach's immunity system lead to production of antibacterial proteins and peptides which had inhibitory effect on the bacteria, depending on the bacterial strains and their sensitivity. Likely this feature of insects can be used as therapeutic strategies to produce natural antimicrobial compounds against the pathogenic bacteria.

Keywords: Hemolymph; *Periplaneta americana*; Anti-Bacterial Agents

1. Background

Today, a great number of scholars and scientists consider the use of insects' metabolites and proteins. This group of organisms, accounted for nearly three quarters of all animal species, is able to acquire the various physiological and morphological adaptations with their local ecological conditions. Therefore, insects are increasingly used to meet human needs by limiting pests from biological control to human disease treatment (1). By discovering the natural compounds such as lipids and proteins that exhibit antimicrobial activity in the invertebrates, researchers are constantly looking for new and effective antibacterial agents from natural sources like insects (2). Many insect species are able to induce antimicrobial proteins in response to external stimuli in their hemolymph, according to this feature; among other animals, insects have led researchers to conduct many studies (3). So far, more than 150 antibacterial peptides are identified and purified from different species of insects (4, 5). Antimicrobial peptides were identified and isolated from the hemolymph of the *Cecropia pupae* (A large North American silkworm moth), after challenging with live bacteria (6). Already, more than five ma-

ior groups of antimicrobial peptides have been identified, including cecropin, insect defensins, attacin-like (glycinerich) proteins, proline-rich peptides, and lysozymes. Furthermore, extensive studies have been conducted on the antimicrobial activity of cecropin and insect defensins (7). Although, the discovery of penicillin of green mold (*Penicillium* sp.), the first antibiotic discovered by Alexander Fleming, saved the lives of millions of people in the world, widespread use of antibiotics increased resistance to antibiotics. Therefore, repeated use of antibiotics has caused some human and animal bacteria to become resistant to them by utilizing multiple mechanisms. While it was expected to eradicate pathogenic infections using antibiotics, many bacteria gradually became resistant to them through evolutionary process (8). Increased resistance to antibiotics may depend on the factors such as genetic characteristics of bacteria, intensity of induced resistance in bacteria, and the degree of tolerance of host cells to bacterial resistance mechanisms (9). Repeated use of antibiotics has led to transmission of the antibiotic-resistant microorganisms; thus their use is somehow restricted

(10). As recent studies showed the repeated use of antibiotics in medicine, veterinary medicine and agriculture are associated with increased bacterial resistance against antibiotics. Resistance to antibiotics is a growing problem, particularly in the medical procedures; therefore, it has limited the treatment of nosocomial infections caused by Gram-positive and Gram-negative pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (11). For example, infections caused by methicillin-resistant *S. aureus* (MRSA) is a major cause of infections in hospitals and communities in most parts of the world (12). Therefore, using natural products, there have been many efforts to produce medicines and antibacterial compounds to overcome resistance problems. Insects are carriers of many types of pathogens, among which cockroaches are identified as carriers of various species of bacteria, viruses, protozoa and fungi due to travelling between hospital waste and dirty areas; therefore, they potentially transmit a variety of infectious diseases (13). In the studies on cockroaches, about 40 species of pathogenic bacteria were isolated (14). Cockroaches identify pathogenic bacteria by proteins such as lectins as the most important components of innate immunity response and there by inhibit pathogenic activities of bacteria in hemolymph (15). In general, such molecules in invertebrate hemolymph play important roles in the immune responses such as healing wounds, phagocytosis, hemolymph coagulation, encapsulation, and elimination of bacteria and parasites (16, 17). It was found that introducing *Streptomyces griseus* into the hemolymph of American cockroaches increased the lectin activities and led to *S. griseus* agglutination (18).

2. Objectives

Since cockroaches are mechanical vectors and their immune system activation prevents the transfer of biological pathogens, the current study aimed to evaluate the American cockroach hemolymph antibacterial properties against a number of nosocomial infectious pathogenic bacteria.

3. Materials and Methods

3.1. Sampling

Totally 100 cockroaches were collected from public places and residential areas. The insects were kept in insectariums of Hamadan Medical Entomology Department, and the other matters related to the study including insect-killing and hemolymph extraction were done in the microbiological and entomological laboratories.

3.2. Cockroaches Rearing Condition

To adapt the insects, they were kept in the aquarium (30 insects per aquarium) and had free access to food, biscuits and water, and were under the terms of $40 \pm 5\%$ moisture, 12 hours light, 12 hours dark and, $24 \pm 2^\circ\text{C}$ temperature for a week.

3.3. Hemolymph Collection (Non-Induced)

Cockroaches were anesthetized with CO_2 and their body surface 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 phenyl thiourea (PTU). Hemolymph was centrifuged at $10000 \times g$ for 10 minutes and the supernatant was collected for the antibacterial testing and stored in 4°C (19).

3.4. Induction of Hemolymph

Twenty microliter of *Escherichia coli* (10^6 cells/mL) was injected into the cockroaches' coelom to stimulate the cockroaches' immunity. The time required to produce antimicrobial proteins was between 6 to 12 hours after injection of bacteria into the cockroaches' bodies.

3.5. Antibacterial Assay

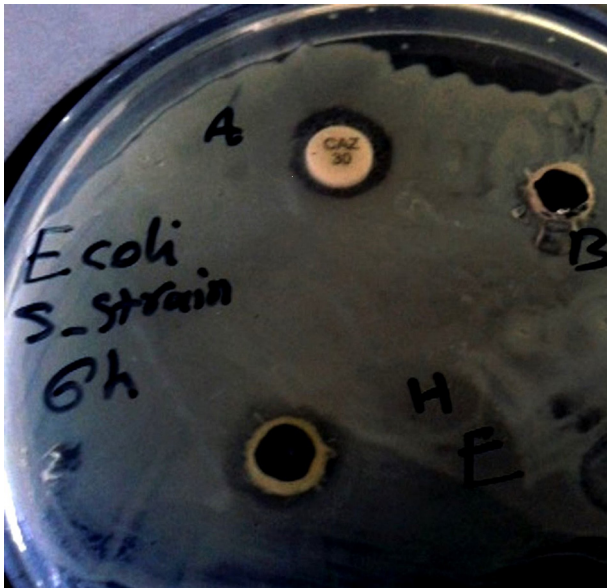
Hemolymph antibacterial assay was carried out using agar diffusion method. The 20 mL melted Luria agar media (LA. pH:7) was placed in the petri dishes (100 mm diameter), then the medium surface was impregnated with the 24-hour-grown strains (1.5×10^6 cells per mL). Twenty microliter hemolymph was poured in the wells of the plate and then the media culture was incubated at 37°C for 24 hours. The formation of inhibition zone (the lack of growth) represented the antibacterial hemolymph effect in the medium. Antibacterial assay was conducted on susceptible and resistant bacteria to ceftazidime, imipenem, and methicillin including susceptible *Escherichia coli* ATCC 25922 (standard strain) and PTCCIBRC-M 10708 (native strain), *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC 27853 and, resistant strains of *Escherichia coli*, *Pseudomonas aeruginosa* and, *Staphylococcus aureus* isolated from patients and stored in bank of bacteria. Also, non-induced hemolymph effect was evaluated on susceptible and resistant strains of the *Escherichia coli*, *Pseudomonas aeruginosa*, and, *Staphylococcus aureus* (19).

4. Results

4.1. Determining the Time of Hemolymph Stimulation

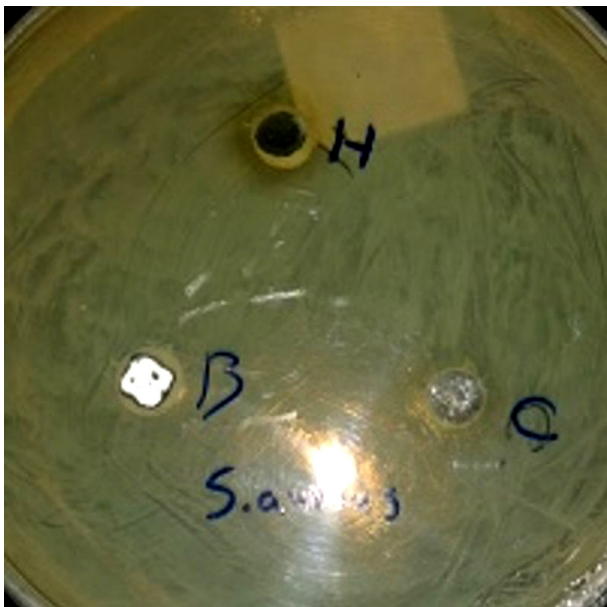
To determine the exact activation time of the cockroach immune system, stimulated with 20 μL of *E. coli* (at a concentration of 1.5×10^6 cells/mL), hemolymph was collected at different times after injection (2, 6, 12 and 24 hours), respectively. Based on the observations, the maximum effect of induced hemolymph on *E. coli* and *S. aureus* medium was observed at six to nine hours after injection (Figure 1). The antimicrobial effect of the extracted hemolymph was observed in the agar diffusion method on susceptible and resistant bacteria to ceftazidime, imipenem, and methicillin (Figures 2, 3 and 4).

Figure 1. The Emergence of Antimicrobial Activity Time of Hemolymph on *Staphylococcus aureus* Medium Six to Nine Hours After Injection



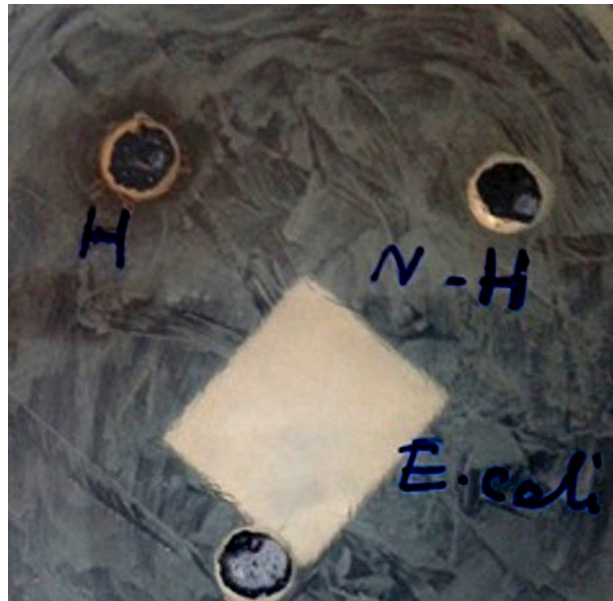
Hemolymph showed anti-bacterial properties. H, hemolymph; B, buffer; C, control.

Figure 2. Induced Hemolymph Antimicrobial Effect on Ceftazidime-Sensitive *Escherichia coli* Atcc



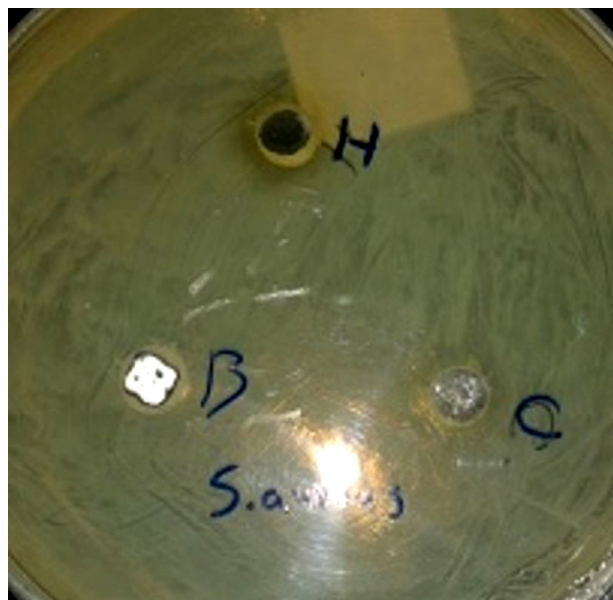
H, induced hemolymph; C, buffer; N-H, non-induced hemolymph (control).

Figure 3. Induced Hemolymph Antimicrobial Effect on Methicillin-Sensitive *Staphylococcus aureus* MSSA



H, induced hemolymph; B, buffer; C, control (non-induced hemolymph).

Figure 4. Induced Hemolymph Antimicrobial Effect on Ceftazidime-Sensitive *Escherichia coli* Ptcc



H, hemolymph; B, buffer; A, ceftazidime.

4.2. Comparison of Antimicrobial Effects of Induced and Non-Induced Hemolymph

Based on the type of hemolymph, results of the current study showed that the induced hemolymph had

antibacterial effect on 12 tests carried out on the bacteria (42.9 %, $P < 0.001$); while, the non-induced hemolymph did not show any antibacterial effects in these tests (Table 1). The results showed that induced hemolymph affected 100% of susceptible strains of *E. coli*, *S. aureus*, and the Iranian susceptible strain of *E. coli* (Ptcc); while, susceptible strains of *Pseudomonas aeruginosa* showed no sensitivity to the induced hemolymph ($P < 0.001$). Also, among the conducted tests, the induced hemolymph did not show any effect on the resistant strains (Table 2).

4.3. Comparative Sensitivity of Bacteria According to the Type of Tested Bacteria

The obtained results according to the type of bacteria and their sensitivity to hemolymph are as follows: *E. coli* (25%), *S. aureus* (25%), *E. coli* Ptcc (50%) ($P = 0.03$); whereas *Pseudomonas aeruginosa* did not show any sensitivity (Table 3).

4.4. Comparative Sensitivity of Bacteria According to the Tested Strains

Of the 32 tests conducted on susceptible strains, 37.5% of the total bacteria showed sensitivity to the hemolymph, while no effect was observed in 24 tests conducted on the resistant strains (Table 4).

4.5. The Comparison of Induced and Non-Induced Hemolymph Antibacterial Effects of the American Cockroach on the Tested Bacteria, Base on the Inhibition Zone Diameter

The current study evaluated the induced and non-induced hemolymph antibacterial effects on different strains of bacteria based on the inhibition zones. The results showed that the maximum and minimum diameter of inhibition zone was related to Gram-negative bacteria *S. aureus* and the Iranian susceptible *E. coli* strains, respectively (Table 5).

Table 1. The Absolute and Relative Frequency of the Antibacterial Effects of the Induced and Non-Induced Hemolymph on the Tested Bacteria ^{a,b}

Strain Type	Sensitivity		Total Count
	Yes	No	
Induce hemolymph	12 (42.9)	16 (57.1)	28 (100)
Non-Induce hemolymph	0 (0)	28 (100)	28 (100)
Total	12 (21.4)	44 (78.6)	56 (100)

^a Data are presented as No. (%).

^b The comparison between the induced and non-induced hemolymph anti-bacterial effect on bacterial sensitivity showed significant difference by chi-square test ($P < 0.001$, χ^2 : 15.2, df: 1).

Table 2. The Absolute and Relative Frequency of the Antibacterial Effect of Induced Hemolymph on Sensitivity of Susceptible Bacterial Strains ^{a,b}

Strain Type	Sensitivity		Total
	Yes	No	
<i>Escherichia coli</i> 25922Atcc	4 (100)	0 (0)	4 (100)
<i>Staphylococcus aureus</i> Atcc 25923	4 (100)	0 (0)	4 (100)
<i>Pseudomonas aeruginos</i> 27853Atcc	0 (0)	4 (100)	4 (100)
<i>Escherichia coli</i> (Ptcc) IBRC-M 10698	4 (100)	0 (0)	4 (100)
Total	12 (75)	4 (25)	16 (100)

^a Data are presented as No. (%).

^b There was significant difference between susceptible bacterial strains by chi-square test ($P < 0.05$, χ^2 : 16, df: 3).

Table 3. The Absolute and Relative Frequency of Sensitivity in Different Bacterial Species to the Antibacterial Property of Induced Hemolymph ^{a,b}

Strain Type	Sensitivity		Total
	Yes	No	
<i>Escherichia coli</i> 25922Atcc	4 (25)	12 (75)	16 (100)
<i>Staphylococcus aureus</i> Atcc 25923	4 (25)	12 (75)	16 (100)
<i>Pseudomonas aeruginosa</i> 27853Atcc	0 (0)	16 (100)	16 (100)
<i>Escherichia coli</i> (Ptcc) IBRC-M 10698	4 (50)	4 (50)	8 (100)
Total	12 (21.4)	4 (78.6)	56 (100)

^a Data are presented as No. (%).

^b The sensitivity to the induced hemolymph among different bacterial species, based on chi-square test, was statistically significant ($P < 0.03$, χ^2 : 8.48, df: 3).

Table 4. The Absolute and Relative Frequency of the Bacterial Sensitivity to Induced Hemolymph ^{a,b}

Strain Type	Sensitivity		Total
	Yes	No	
Susceptible	12 (37.5)	20 (62.5)	32 (100)
Resistance	0 (0)	24 (100)	24 (100)
Total	12 (21.4)	44 (78.6)	56 (100)

^a Data are presented as No. (%).

^b According to the chi-square test, there was a significant difference between susceptible and resistant strains to the induced hemolymph ($P < 0.05$, χ^2 : 11, df: 1).

Table 5. The Absolute and Relative Frequency of the Tested Bacterial Sensitivity to Induced and non-Induced Hemolymph Antibacterial Properties Based on the Inhibition Zone Diameter

Bacteria/Hemolymph Type	Induced, mm	None-Induced
<i>Escherichia coli</i> 25922 Atcc	14	-
<i>Staphylococcus aureus</i> Atcc 25923	23	-
<i>Pseudomonas aeruginosa</i> 27853 Atcc	-	-
<i>Escherichia coli</i> (Ptcc) IBRC-M 10698	8	-
<i>Escherichia coli</i> resistance strain	-	-
<i>Staphylococcus</i> resistance strain	-	-
<i>pseudomonas aeruginosa</i> resistance strain	-	-

5. Discussion

Insects, due to a long history of 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. In the past century, researchers concluded that the insect immune system is able to cope with invading microorganisms (19-21). 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 a 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). It was reported that the presence and activity of antimicrobial cecropin proteins in *Galleria mellonella* hemolymph after an 18-hour infection by *Pseudomonas aeruginosa* (an insect pathogenic bacteria species) and *E. coli* (a non-pathogenic strain), the peak activity of the insect immune system was observed up to 48 hours after injection (25). It is known that infection of the American cockroach by the entomopathogenic fungus *Beauveria bassiana* led to phenylalanine oxidase (PO) activity in hemolymph, which is a part of the humoral immune defense mechanisms in insects (26). In another study on cockroaches, it was found that lectins, carbohydrate-binding proteins with high specificity, are not only able to identify foreign invaders but also activate PO system (18). By stimulating the American cockroach immune system, it was found that bacteria-induced hemolymph had antibacterial effect against 13 species of human pathogenic bacteria (19). On the other hand, it was found that the non-induced hemolymph had inhibitory effect on susceptible *E. coli*, while it had no effect on the others. In the current study, the maximum antimicrobial activity was observed 12 hours after injection. In the present study, adult male and female cockroaches (*Periplaneta Americana*) were used to examine the effect of hemolymph on different strains of bacteria. Hemolymph was stimulated by *E. coli* and was extracted in the specified time period. In this study, the effect of induced hemolymph was assessed on Gram-positive and Gram-negative bacteria, the highest antibacterial activity of hemolymph was observed six hours after bacteria inoculation. The differences between the emergence of antibacterial activity of hemolymph might be possibly due to nutritional conditions, moisture, ecomorphotype characteristics of species, and severity of bacteria inoculation. Similar to the previous studies, the current study 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 *S. aureus* (Gram-positive). However, unlike the previous study by Seraj, non-induced hemolymph did not have antimicrobial activity against susceptible and resistant strains of bacteria, probably due to resistance of the susceptible bacteria against lipopolysaccharide (LPS) of the cockroaches' hemolymph (19). Also, it was found that some compounds in the brains of cockroaches had antibacterial effects on the methicillin-resistant *S. aureus* (MRSA) and toxic strains of *E. coli* (27).

In conclusion, stimulation of the insects' 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.

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Authors' Contributions

Study concept and design: Milad Latifi and Amir Hossein Zahirnia. Analysis and interpretation of data: Milad Latifi. Manuscript drafting: Milad Latifi and Amir Hossein Zahirnia. Critical revision of the manuscript for important intellectual content: Milad Latifi, Amir Hossein Zahirnia, Mohammad Yousef Alikhani, Ali Reza Bandani, Aref Salehzadeh, Mansour Nazari. Statistical analysis: Milad Latifi and Amir Hossein Zahirnia.

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