The influence of different amounts of *Escherichia coli* inducement on the antibacterial activity of *Periplaneta Americana* hemolymph against common nosocomial pathogens

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Abstract— *Insects, such as P. americana, have the capability to produce antimicrobial proteins (AMPs) that play as an effector substance in the innate immunity of vertebrates and invertebrates. However, there were no studies yet on the influence of different amounts of E. coli inducement on the variability of its antibacterial activity that would be relevant for the selection of representative hemolymph in this growing discovery of research. Five groups of P. americana were induced with 50 μL, 75 μL, 100 μL, 125 μL, 150 μL of E. coli and crude hemolymph extracts were collected 6-12 hours post inducement. The variability of hemolymphs' antibacterial activity was tested against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, and Pseudomonas aeruginosa ATCC 27853. The protein content and molecular weight of the AMPs were determined through Bradford Assay and native SDS-PAGE. Comparative results of the antibacterial activity showed significant p-value of 0.049 in hemolymph from 150 μL induced P. americana against S. aureus ATCC 25923 with its molecular weight of 75kDa while 75 μL induced hemolymph provided the significant p-value of 0.024 against E. coli ATCC 25922 correlating its protein content of 2212.77 μg/mL. However, the hemolymph is not effective against P. aeruginosa since the values obtained were close to negative control. The resulting effective antibacterial activity of the hemolymph has an advantage when induced 6-12hours at the highest tolerable amount of E. coli as the inducible biologic material, presence of increased amounts of active AMPs while considering the strain it acts upon.*

Keywords: *Escherichia coli inducement, antimicrobial proteins, hemolymph, Periplaneta americana*

INTRODUCTION

Health care-associated infections (HCAIs) also referred to as nosocomial or hospital infection [1] are infections of patients in which the etiologic agent is acquired in a hospital or long-term health care center of facility [2] during their treatment for surgical or medical conditions with most frequent adverse occurrences during care delivery [3]. HCAIs pose a major risk factor for serious health [4] and also account for the increase rate of morbidity of admitted patients [5]. Prevalence of HCAIs is universal and pervades every health-care facility and system worldwide [1]. Common nosocomial pathogens of concern are *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli*. *P. aeruginosa* is a gram-negative bacillus that causes infections usually in people in the hospital or with immune compromised conditions [6]. Ten to twenty percent of the reported cases of *P. aeuruginosa* infection results in septicemia in intensive care units, cystic fibrosis, burn and wound infections [7]. It poses a serious therapeutic challenge due to very limited treatment options [8]. *S. aureus* is also one of the common causes of infections in both the community and hospital with approximately 30% of the human population is infected [9]. *S. aureus* is the most virulent species of staphylococci encountered [2]. This may lead to bacteremia and infective endocarditis as well as osteo articular, skin and soft tissue, pleura pulmonary, and device-related infections [10]. On the other hand, *E. coli* is an opportunistic microorganism which consists of diverse group categorized into pathotypes [6]. It is also one of the considered frequent bacteria causing HCAIs such as urinary tract infection in mixed patient populations (WHO, 2017). Moreover, production of variety β-lactamases is a way by which it becomes multi

resistant [11]. Improving the local surveillance data using standardized antimicrobial susceptibility testing methods and validation of diagnostic algorithms against microbial findings are essential as part of preventive measures and solutions to worldwide HCAIs [12].

Figure 1. *Periplaneta Americana* cockroach. Retrieved from https://bugguide.net/node/view/1264505

In the advent of these evolving microorganisms, there is a need for a better antibacterial agent in the form of antimicrobial peptides (AMPs) from synthetic and natural sources by using alternative antimicrobial strategies [13]. Medicinal plants, marine and terrestrial organisms are among the known sources of natural compounds with valuable antimicrobial peptides [14]. Of these various sources, researchers are now in line of studying insect's metabolites and proteins. AMPs and proteins are essential components of innate immunity against pathogens [15]. AMPs are short peptidic molecules with cationic and hydrophobic sequences within a linear or cyclic structure [16] and are inducible [17]. AMPs could be in the form of defensins, cecropins, crustins, attacin-like (glycine-rich) proteins, proline rich peptides and anti-lipopolysaccharide factors [18]. It is evident that invertebrate innate immune mechanisms are effective [19] against bacteria, viruses and fungi [20]. They resist these infections during symbiotic interactions [21] by the action of cellular and humoral processes [22] and through activation of a complex genetic cascade to express these peptides [23]. AMPs can be mediated by the insects' hemocytes, the fat body, the midgut, the salivary glands and other tissues [24] which are then secreted in their hemolymph and supplied to their whole body [13].

It was suggested that cockroaches are good sources of these AMPs since under polluted and unsanitary settings, they encounter different types of bacteria, including superbugs and that they were immunologically challenged [25]. Among the species of cockroaches being studied is the *Periplaneta Americana* (American cockroach). It is commonly considered a home and hospital pest. It has the ability to produce AMPs against Gram-positive bacteria (*Micrococcus luteus*) when induced [26]. It is also proven that induced hemolymph has activity against *S. aureus* and *E. coli* whereas no reported activity against *P. aeruginosa* [27]. A time-induction study showed that antibacterial peptides were induced as early as ½ an hour to between 6 to 12 hours with a peak at 9 hours which started to decline around 24 hours [28]. Though the antibacterial activity in hemolymph can be induced by injecting different matter into *P. americana*, studies have shown that induced by *E. coli* strains (*E. coli* K88) gives better activity [29].

With these discoveries, it can be a way to uplift the problem concerning nosocomial infections and minimizing indiscriminate use of chemical agents turning limiting pests and insects into means of medical treatment. However, there are still no studies on the effect of different amounts of *E. coli* inducement correlating the trends and variations of antibacterial activity. Thus, a need to support other inducement studies focusing other factors such that of amount of *E. coli* inducement is the primary purpose of this research. This study aims to determine how much *E. coli* inducement would be tolerable for *P. americana*; to observe and compare the effect of different amounts of *E. coli* inducement for its corresponding antibacterial activity against select bacterial strains; to quantify and separate the protein content of hemolymph from induced *P. americana* to know its molecular weight and correlate such findings believed to be the AMPs. Therefore, this research would be relevant for selection of representative hemolymph exerting the best of its antibacterial activity against different strains of nosocomial pathogens.

MATERIALS AND METHOD

Insects

Adult American cockroaches, *P. americana*, were commercially procured from the Institute of Weed Science, Entomology and Plant Pathology, University of the Philippines-Los Baños, Laguna day before the experimentation. The cockroaches were maintained in a clean container at room temperature (25±2°C) with a 12h light/dark ratio and supplied with distilled water and biscuits [26].

Toxicity Assay

Three groups of *P. americana* were prepared, each with ten members and induced with different amounts of *Escherichia coli* ATCC 25922 suspension (10^8cells/mL) at the coelom part of the body. The first group was induced with 200μL of the *E. coli* suspension while the second and third groups were injected with 150μL and 100μL of the *E. coli* suspension, respectively. The number of mortality from each group was observed after 24 hours and was indicated by the absence of physical movement. This assay was performed to determine how much *E. coli* inducement would be tolerable for the *P. americana* and so as basis for selection of how much E. coli inducement for the succeeding assay [29], [30].

Inducement of *E. coli* suspension

Two hundred cockroaches were used and were divided into five groups each with forty members. *E. coli* ATCC 25922 suspension (10^8cells/mL) was injected into the cockroaches' coelom at different amount (Group 1: 50 μL; Group 2: 75 μL; Group 3: 100 μL; Group 4: 125μL; Group 5: 150 μL) as determined by the toxicity assay [26], [28].

Collection of *P. Americana* hemolymph

Collection of *P. americana* hemolymph induced with *E. coli* was done between 6 to 12 hours after inducement. *P. Americana* cockroaches were first anaesthetized with ice and sterilized with 70% ethyl alcohol in the ventral surface of the sternum. The hind pairs of legs were pricked with sterile lancets and the hemolymph was collected using plain blue-tipped capillary tubes. The upper body part of the cockroaches was then subjected to centrifugation at 1800xg for 10 minutes to extrude the remaining hemolymph. The extracted hemolymph was immediately transferred to Eppendorf tubes with few phenyl thiourea (PTU) crystals and was then again subjected to centrifugation at 1800x g for 10 minutes. PTU was used to prevent melanization of the extracted hemolymph. The resulting extracts were then kept at -20°C until used [26] [28].

Bacterial Strains

Commercially obtained cultured stocks of gram-positive and gram-negative bacteria namely *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC25922and *Pseudomonas aeruginosa* ATCC 27853were used to assess the antibacterial activity of the hemolymph of *P. Americana* depending on the level of inducement.

Antibacterial Susceptibility Assay

Agar disk diffusion assay was used to determine the variability of antibacterial activity of the hemolymph depending on the level of inducement. Preparation of bacterial suspensions were done by selecting four well-isolated colonies of the same morphological type from MacConkey for both *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 and BAP colonies for *S. aureus* ATCC25923. The select colonies for specific bacterial strains were transferred to 4mL sterile saline solution. The prepared suspension was incubated at 35°C to 37°C until 0.5 MacFarland turbidity standard were achieved. The prepared suspensions of bacterial strains were inoculated in the MHA II by streaking the swab across the entire surface. The surface of the medium should be allowed to dry for 3-5 minutes but no longer than 15 minutes, with the lid tipped. Sterile 6mm Whatman filter paper No. 1 is impregnated with 20μl (at a concentration of 1.21mg/ml) of the representative hemolymph designated from each group [31]. Standard antibiotics, such as vancomycin for *S. aureus*, piperacillin-tazobactam for *P. aeruginosa* and aztreonam for *E. coli*were used as positive control while distilled water was used as negative control. Further, they were aseptically placed on the agar plates swabbed with the test microorganisms. Within 15 minutes after discs placement, the plates are incubated at 35°C to 37°C overnight. The formation of inhibition zone (the lack of growth) represented the antibacterial hemolymph effect in the mediumand the variability of activity was compared [27], [32].

Bradford Assay

Quantification of protein content of the hemolymph was done through Bradford assay. Twenty microliters of 2000 μg/ml bovine serum albumin standard (BSA) and sample were transferred into separate disposable cuvettes. The 1x dye reagent were added to each cuvette and mixed. The solutions were incubated for at least 5minutes and no longer than 1 hour at room temperature. The spectrophotometer was set at 595 nm. The absorbance of the standards and samples were measured [33].

SDS-PAGE

The assay, as described by Laemmli [34], was run in non-reducing conditions. Twenty microliters of

sample was mixed with 20µl 2x Laemmli sample buffer. The solution was centrifuged at 10000g for 5 minutes and heated at 90-100 degree Celsius for 10 minutes. Twenty microliters of each solution was loaded in Bio-Rad any kD™ Mini-Protean® TGX™ precast protein gel (Lifeline Diagnostics Supplies Inc. Quezon City, Philippines). Precision Plus Protein™ Dual XtraPrestained protein standards were also loaded into the gel. Electrophoresis was performed at a constant voltage of 200v, 45 minutes with 1x Laemmli SDS-PAGE running buffer. After separation, the gel was fixed and stained for 1 hour with Coomassie Brilliant Blue G-250 and analyzed with densitometer [26], [34].

RESULTS

Toxicity Assay

Table 1 shows three groups of *P. americana*, each with 10 members each and induced with different amounts of *E. coli* suspension. Six out of ten from the first group that was induced with 200 µL (2 x10^7*E. coli*) did not survive or there were no physical movements observed. All members from the second and third groups that were induced with 150 µL (1.5x10^7*E. coli*) and 100 µL (1x10^7 *E. coli*), respectively, did not die or there were still observable physical movements. Since there were observable deaths on the highest amount of *E. coli* inducement from the third group, this means that 200 µL (2 x10^7*E. coli)* inducement could make the *P. americana* die.

Table 1. Mortality result after inducement

GROUP	No. *P. americana* alive before inducement	No. of deaths 24 hours after inducement
*P. americana*induced with 200µL (2x10^7 *E. coli*)	10	6
*P. americana*induced with150µL(1.5x10^7 *E. coli*)	10	0
*P. americana*induced with 100µL (1.0 x10^7 *E. coli*)	10	0

For the succeeding assays in this research paper, therefore only 0-150 µL (1.5x10^7*E. coli*) of *E. coli* could be tolerable and can be basis for the amount of bacterial density that is not toxic for the *P. americana.* The deaths could be due to the number of *E. coli* induced and the mechanism by which the *P. americana* respond with the bacteria. Upon entry of the *E. coli* to *P. americana*, the bacteria could persist and colonize the insect specially the gut portion and the circulatory canal, having larger quantities of bacteria blocking the circulation [36]. In addition, the bacteria could make a way to modify the production of AMPs when the bacterial density is increased and so the production of enzymes such as metalloproteases which were found to inhibit AMPs and degrade host tissues making the host inactive [35], [30].

Based from the probit analysis seen in Figure 2, the level of *E. coli* inducement from which *P. americana* would survive in 196.792 µL at 50% after 24 hours. This means that less than 50% of the population of *P. americana* would survive when induced with less than 196.792 µL and would give the total survival rate of 100% at 150 µL of inducement after 24 hours. Therefore, results suggested 0 µL-150 µl level range of *E. coli* inducement only for the *P. americana* would withstand the toxicity. Studies of Basseri et al. [26] and Latifi et al. [27] used 100µL and 20 µL of *E. coli* inducements that gave 100% survival rate. Thus, the assay was run at 100 µL, 150 µL and 200 µL of *E. coli* inducements to assess the level from which the *P. americana* would not survive.

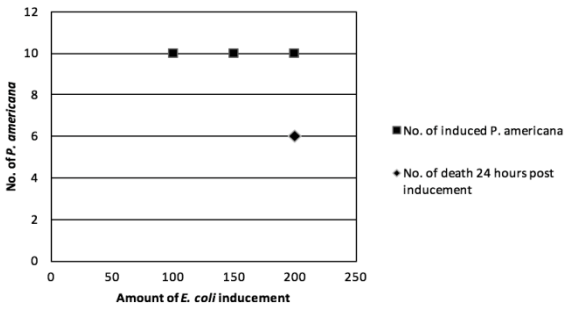

Figure 2. Statistical graph of *P. Americana* toxicity assay

P. americana hemolymph extraction

In the collection of *P. Americana* hemolymph, approximately 700 µL, 600 µL, 800 µL, 700 µL and 900 µL of hemolymph were collected from each group with 40 cockroaches induced with 50 µL, 75 µL, 100 µL, 125 µL, and 150 µL of *E. coli*, respectively. The collected hemolymph is slightly pale yellow to orange in color, clear and non-viscous. The color is not red because *P. americana*does not use haemoglobin to carry oxygen rather they use spiracles [37]. The contributory component of the color of hemolymph is due to its fat body and melanin content; thus, phenylthiourea was used to prevent further melanization and blackening [26].

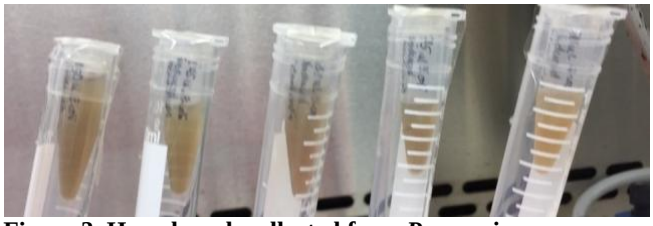

Figure 3. Hemolymph collected from *P. americana*

Table 2. Antibacterial susceptibility assay result for *S. aureus* ATCC 25923

Hemolymph	Zone of inhibition	Positive control	Negative control
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. Americana*	6.53mm	17.11mm	6 mm
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. Americana*	7.11mm	17.60mm	6 mm
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	7.63mm	17.47mm	6 mm
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	8.01mm	17.70mm	6 mm
Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	9.67mm	17.40mm	6 mm

Table 2 shows the result of antibacterial susceptibility assay for *S. aureus* ATCC 25923 strain. The zones of inhibition were measured after 24hours of incubation. Vancomycin was used as positive control and distilled water as negative control. Based from the result, the hemolymph collected from 150 µl (1.5x10^7*E. coli*) induced *P. americana* exhibited the largest zone of inhibition with 9.67mm (Figure 4.D) against *S. aureus* ATCC 25923 while the hemolymph collected from 50 µL (5x10^6 *E. coli*) induced *P. americana* has the lowest antibacterial activity with 6.53mm zone of inhibition (Figure 4.A). There is also a trend of increasing inhibitory activity as the amount of*E. coli* inducement increases. The zones of inhibition of vancomycin (Figure 4. A, B, C, and D) are almost the same at 17mm. Hemolymph from *Blattaorientalis* and *P. americana* have an activity against *S. aureus* ATCC 25923 due to the AMPs present; thus, the activity observed could be due to the AMPs present in sample hemolymph. Cockroaches were reported to have plenty source of this active AMPs [38].

Table 3 shows the statistical multiple comparison of inhibition on *S. aureus* ATCC 25923 across each hemolymph collected from different amount of *E. coli* induced *P. americana.* The results were significant at p-value of less than 0.05. It is statistically shown that when hemolymph collected from 50 µL of *E. coli* induced *P. americana* is compared to the hemolymph collected from 75 µL, 100 µL and 125 µL of *E. coli* induced *P. americana* produced insignificant p-values of greater than 0.05. This implies that the antibacterial activity of hemolymph collected from 50 µL induced *P. americana* is the same as the antibacterial activity of the hemolymph collected from 75 µL, 100 µL, and 125 µL of *E. coli* induced *P. americana*. Same antibacterial activity was also observed when hemolymph from 75µL, 100µL, and 125 µL of *E. coli* induced *P. americana* were compared across each other. However, there is significant difference in the antibacterial activity of the hemolymph collected from 150 µL of *E. coli* induced *P. americana as* compared to the hemolymph collected from 50 µL *E. coli* induced *P. Americana* at the p-value of 0.049. This implies that the hemolymph collected from 150 µL*E. coli* induced *P. Americana* gave the best antibacterial activity against *S. aureus* ATCC 25923 with significant variation as observed from statistics and the largest zone of inhibition shown from Table 2 and Figure 4.D correlating that an induced *P. americana* has antibacterial activity against *S. aureus* compared to non-induced *P. americana* [26].

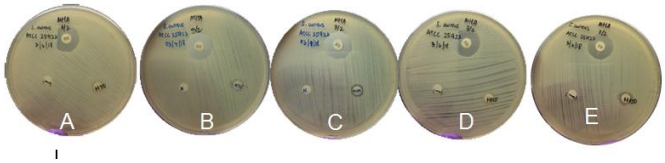

Figure 4. Antibacterial Assay for *S. aureus* ATCC 25923 via Agar Disk Diffusion A) 50 µL Induced Hemolymph, B) 75 µL Induced Hemolymph, C) 100 µL Induced Hemolymph, D) 125 µL Induced Hemolymph, E) 150µL Induced Hemolymph

Table 3. Multiple Comparison of Inhibition on *Staphylococcus aureus* ATCC 25923 Across Each Concentration

Concentration	Concentration	p-value
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.936
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.455
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.417
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.049*
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.936
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.816
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced*P. americana*	0.774
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.1.00
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.455
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.816
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	1.00
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.302
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.417
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.774
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	1.00
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.331
Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.049*
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced*P. americana*	0.100
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.302
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.331

**Significant at P<0.05*

Table 4 shows the result of antibacterial susceptibility testing for *E. coli* ATCC 25922 strain. The zones of inhibition were taken after 24 hours of incubation. Aztreonam was used as positive control and distilled water as negative control. Based from the result, the hemolymph collected 75 µL(7.5x10^6 *E. coli*)induced *P. americana* exhibited the largest zone of inhibition with 9.07 mm while the hemolymph collected from 50 µL(5 x10^6 *E. coli*) induced *P. americana* exhibited the lowest antibacterial activity with 6.92 mm zone of inhibition.

Table 4. Antibacterial susceptibility assay result for *E. coli* ATCC 25922

Hemolymph	Zone of inhibition	Positive control	Negative control
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	6.92mm	37.99mm	6 mm
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	9.07mm	38.25mm	6 mm
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	8.50mm	37.67mm	6 mm
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	8.47mm	36.46mm	6 mm
Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	8.69mm	37.57mm	6 mm

The hemolymph collected from 100 µL(7.5x10^6 *E. coli*), 125 µL (1 x10^7 *E. coli)*, and 150 µL(1.25x10^7 *E. coli*) induced *P. americana* each exhibited a zone of inhibition of 8.50mm, 8.47mm, and 8.69mm, respectively. The zones of inhibition of aztreonam range from 36.46mm-38.25mm. The resulting zones of inhibition are different from each other. However, there is no observable trend of increasing or decreasing antibacterial activity as the amount of *E. coli* inducement increases. The antibacterial activity could be contributed by other factors like protein concentration or the molecular weight of the AMPs present in the sample hemolymph and not only depends on the amount of *E. coli* inducement [38]. It was reported that the larger portions of the AMPs produced upon inducement of *P. americana* were for antibacterials and the smaller portions could be for antifungals and antivirals [39].Therefore, an increased production of AMPs gave favorable antibacterial activity exhibited by the hemolymph collected from 75 µL (7.5x10^6 *E. coli*)induced *P. americana.*

Table 5 shows the statistical multiple comparison of inhibition on *E. coli* ATCC 25922 across each hemolymph collected from different amount of *E. coli* induced *P. americana.* The results were significant at p-value of less than 0.05. It is statistically shown that there is significant value of difference in inhibitory activity of the hemolymph collected from 150 µL (1.5x10^7*E. coli*) induced *P. Americana* compared to hemolymph collected from 50µL (5x10^6 *E. coli*) induced *P. Americana* at the p-value of 0.05.

Table 5. Multiple Comparison of Inhibition on *Escherichia coli* ATCC 25922 Across Each Concentration

Concentration	Concentration	p-value
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.024*
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.076
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.081
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.05*
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.024*
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.709
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.675
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.900
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.076
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.709
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	1.00
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.991
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.081
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.675
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	1.00
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.984
Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. american*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.05*
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.900
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.991
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.984

. There is also a significant value of difference in inhibitory activity of the hemolymph from 75µL(7.5x10^6*E. coli*) induced *P. americana* compared to the hemolymph from 50µL(5 x10^6 *E. coli*)induced *P. americana* at the p-value of 0.024. The hemolymph from 100 µL (1 x10^7*E. coli*), and 125 µL (1.25 x10^7*E. coli*) induced *P. americana*did not show significant difference in the inhibitory activity when compared across each other. The p-value 0.024 of inducement has a greater significant difference compared to the p-value of 0.05. This suggests that hemolymph from 75 µL (7.5x10^6*E. coli*) induced *P. americana* has the best inhibitory activity against *E. coli* ATCC 25922. Studies proved that hemolymph from induced *P. americana* has an inhibitory activity against *E. coli* ATCC 25922 [26].The resulting activity is due to the AMPs but the main factor of the said process did not only depend on the amount of inducement but on the nature of the AMPs against the select bacterial strain it acts upon since AMPs although from the same species, may differ by its binding capacity with the microorganism and the activity it may elicit [38].

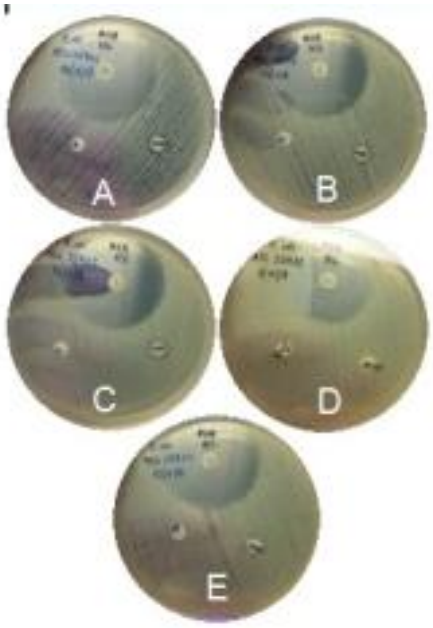

Figure 5. Antibacterial Assay for *E. coli* ATCC 25922 via Agar Disk Diffusion A) 50µL Induced Hemolymph, B) 75µL Induced Hemolymph, C) 100µL Induced Hemolymph, D) 125µL Induced Hemolymph, E) 150 µL Induced Hemolymph

Table 6. Antibacterial susceptibility assay result for *P. aeruginosa* ATCC 27853

Hemolymph	Zone of inhibition	Positive control	Negative control
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	6.13mm	34.82mm	6 mm
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	6.16mm	34.59mm	6 mm
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	6.21mm	35.49mm	6 mm
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	6.19mm	36.08mm	6 mm

Legend: significant at p-value <0.05

Table 6 shows the result of antibacterial susceptibility testing for *P. aeruginosa* ATCC 27853 strain. The zones of inhibition were taken after 24hours of incubation. Piperacillin-tazobactam was used as positive control and distilled water as negative control. Based from the result, the hemolymph collected from 150 µL(1.5 x10^7 *E. coli*) induced *P. americana* exhibited the largest zone of inhibition with 6.31mm while the hemolymph collected from 50 µL(5 x10^6 *E. coli*) induced *P. americana* exhibited the lowest antibacterial activity with 6.13mm zone of inhibition. The hemolymph collected from 75 µL(7.5x10^6 *E. coli*), 100 µL(1 x10^7 *E. coli)*, and 125µL(1.25x10^7 *E. coli*) induced *P. americana* each exhibited a zone of 6.16mm, 6.21mm, and 6.19mm, respectively. The zones of inhibition of piperacillin-tazobactam range from 34.59mm-36.08mm. The resulting zones of inhibition using the hemolymph collected were close to the zones of inhibition exhibited by the negative control with 6mm. Studies often shows no antibacterial activity against *P. aeruginosa* ATCC 27853 exhibiting 6mm zone of inhibition [27]. Although there were small variations, still the antibacterial activity of AMPs was not thatactive and observable against this bacterial strain.

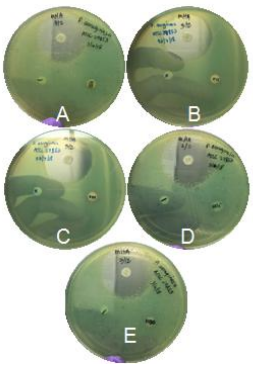

Figure 6. Antibacterial Assay for P. aeruginosa ATCC 27853 via Agar Disk Diffusion A) 50 µL Induced Hemolymph, B) 75 µL Induced Hemolymph, C) 100 µL Induced Hemolymph, D) 125 µL Induced Hemolymph, E) 150 µL Induced Hemolymph

Table 7. Multiple Comparison of Inhibition on *Pseudomonas aeruginosa* ATCC 27853 Across Each Concentration

Concentration	Concentration	p-value
Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.935
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.380
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.603
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.032*
Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.935
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.729
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.935
	Hemolymph of 150 µL(1.5 x10^7*E. coli*) induced *P. americana*	0.064
Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.380
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.729
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.984
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.228
Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.603
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.935
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.984
	Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	0.135
Hemolymph of 150 µL(1.5 x10^7 *E. coli*) induced *P. americana*	Hemolymph of 50 µL(5 x10^6 *E. coli*) induced *P. americana*	0.032
	Hemolymph of 75 µL (7.5x10^6 *E. coli*) induced *P. americana*	0.064
	Hemolymph of 100 µL(1 x10^7 *E. coli)* induced *P. americana*	0.228
	Hemolymph of 125 µL(1.25x10^7 *E. coli*) induced *P. americana*	0.135

Legend: *significant at p-value <0.05

Table 7 shows the statistical multiple comparison of inhibition on *P. aeruginosa* ATCC 27853 across each hemolymph collected from different amount of *E. coli* induced *P. americana*. The results were significant at p-value of less than 0.05. It is statistically shown that there is significant value of difference in inhibitory activity of the hemolymph collected from 150µL (1.5x10^7*E. coli*) induced *P.*

americana compared to hemolymph collected from 50μL(5 x10^6 *E. coli*) induced *P. americana* at the p-value of 0.05. This implies that the hemolymph collected from 150μL (1.5x10^7*E. coli*) induced *P. americana* favor the most inhibitory activity against *P. aeruginosa* ATCC 27853 correlated to the largest zone of inhibition seen from Table 6. However, in comparison with the negative control, the observable inhibition of the hemolymph collected from *E. coli* induced *P. americana* is not effective against *P. aeruginosa* since the values obtained were close to each other.

Table 8. Bradford Assay result of Absorbance and Protein Concentration of hemolymph from different amount of *E. coli* induced *P. Americana*

Hemolymph	Absorbance of Hemolymph	Protein concentration of Hemolymph (μg/mL)	Absorbance of Standard	Protein concentration of Standard (μg/mL)
Hemolymph of 50 μL(5 x10^6 *E. coli*) induced *P. americana*	0.192	2121.55	0.181	2000
Hemolymph of 75 μL(7.5x10^6 *E. coli*) induced *P. americana*	0.208	2212.77	0.188	2000
Hemolymph of 100 μL(1 x10^7 *E. coli)*induced *P. americana*	0.186	2089.89	0.178	2000
Hemolymph of 125 μL(1.25x10^7 *E. coli*) induced *P. Americana*	0.187	2137.14	0.175	2000
Hemolymph of 150 μL(1.5 x10^7 *E. coli*) induced *P. americana*	0.211	2209.42	0.191	2000

Table 8 shows the protein concentration of the hemolymph collected from *P. americana* induced with different amounts of *E. coli*. There were variations in the concentration of protein content in each sample ranging from 2089.89-2212.77μg/mL. Studies shows that there could be 4.18 times or 520.85mg/mL increase in the protein content after 144 hours of inducement [29]. Thus, inducement of *P. americana* with varying amounts of *E. coli* has an effect on the protein content of its hemolymph. The protein content was computed based from the paralleled absorbance of the hemolymph and bovine serum albumin. The findings can be associated with the activity of the AMPs observed against select bacterial strains. Hemolymph collected from 75 μL of *E. coli* induced *P. americana* has the highest protein concentration of 2212.77 μg/mL and that could be attributed as a major factor to the effective antibacterial activity of this hemolymph against *E. coli* ATCC 25922. Although the protein content of the hemolymph collected from 150μL of *E. coli* induced *P. americana* which is 2209.42 μg/mL is lower than the hemolymph from 75 μL of *E. coli* induced *P. americana*, the difference is only minimal and could also be associated but not solely a major factor of its antibacterial activity against *S. aureus* ATCC 25923. However, in terms of the antibacterial activity of the hemolymph against *P. aeruginosa* ATCC 27853, it suggests that the quantity of the protein present in the hemolymph is of no value since the hemolymph exerts no significant antibacterial activity against it.

Table 9. Statistical Comparison of Protein Concentration of Hemolymph (μg/mL) Against 2000 μg/mL Bovine serum albumin

	Test Value = 2000		
	p-value	95% Confidence Interval of the Difference	
		Lower	Upper
protein	0.003**	86.22	222.08

*Legend: **Significant at p-vale <0.005*

Table 9 shows the statistical analysis result of protein concentration present in the hemolymph of *P. americana* induced with *E. coli* compared to the protein concentration of the 2000 μg/mL bovine serum albumin. With the Quick Start Bradford protein assay, dye color development is significantly greater with BSA than with most other proteins, including gamma-globulin. Therefore, the BSA standard was used as an appropriate standard for this assay. The statistical output revealed a significant p-value of 0.003.This means that there is observable difference between the protein concentrations of the hemolymph from *E. coli* induced *P. americana* and protein concentration of the standard. Such that differences encompasses the concentration of the standard. Some studies show that when an insect such as cockroach are induced, it

has the capability to increase its protein content up to 4.18 times or 520.85mg/ml after 144 hours of inducement [29].

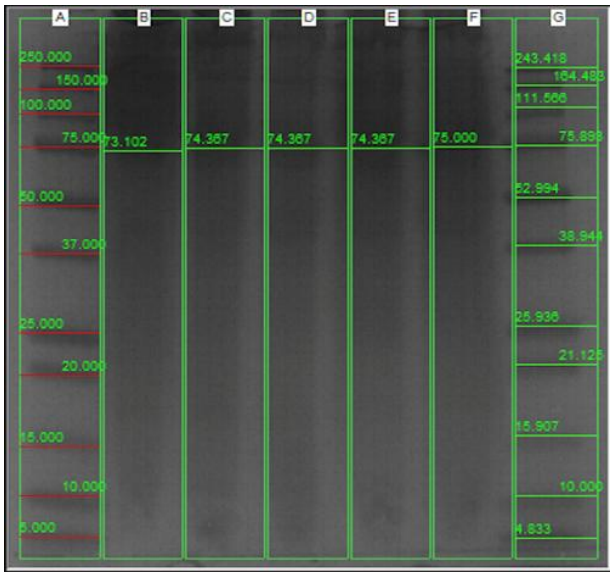

Figure 7. Native Polyacrylamide Agarose Gel Electrophoresis. A & G serve as the ladders, B) 50 µL *E. coli* Induced hemolymph, C) 75 µL *E. coli* Induced hemolymph, D) 100 µL *E. coli* Induced hemolymph, E) 125 µL *E. coli* Induced hemolymph, F) 150 µL *E. coli* Induced hemolymph

Figure 7 shows the result of SDS-PAGE in non-reducing conditions of the collected hemolymph from different amount of *E. coli* induced *P. americana.* The molecular weights of the proteins from the hemolymph were determined between 73-75kDa. Hemolymph sample from 150 µL (1.5 x10^7*E. coli*) induced *P. Americana* (Figure 7.F) exerted the heaviest molecular weight of 75kDa while the hemolymph sample from 50 µL (5 x10^6*E. coli*)induced *P. americana* (Figure 7.B) had the lightest molecular weight of 73kDa. The molecular weights for Fractions C, D, and E in Figure 7 corresponding to hemolymph sample from 75µL (7.5x10^6*E. coli*) induced induced *P. americana*100 µL (1 x107 *E. coli*) induced *P. americana*, and 125µL (1.25x10^7*E. coli*) induced *P. americana*, respectively, were the same with 74.367kDa. This reflects that the resulting molecular weight of the separated protein bands corresponds to the AMPs present in each sample. Since the hemolymph collected from the 150 µL (1.5 x10^7*E. coli*) induced *P. americana* (Figure 7.F)has the heaviest molecular weight of protein band, then it could be correlated to the inhibitory activity it may have against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. It was found out those AMPs present in the hemolymph of *P. americana* has a molecular weight of between 60-80kDa determined as 61kDa, 62kDa, 66kDa, and 72kDa [26], [28], [38]. These variations may due to the effect of the amount of *E. coli* inducement since previous studies only used 20-100 µL of *E. coli* inducement. The correlated studies and activity may not be the sole characteristic or factor that affects AMPs activity but also the amount of protein content and nature of the microorganisms they act upon [38].

CONCLUSION

The hemolymph collected from *P. americana* induced with different amounts of *E. coli* was found to have variable antibacterial activity against select common nosocomial bacterial strains. Influence of inducement proved variability observed in the activity of the hemolymph and the quantified protein content and its molecular weight suggest the presence of antimicrobial proteins. The comparative results suggest that 150 µL of *E.coli* induced hemolymph provides better activity against *Staphylococcus aureus* ATCC 25923, 75 µL of *E.coli* induced hemolymph was found to be the most effective antibacterial agent against *Escherichia coli* ATCC 25922. However, the hemolymph is not effective against *Pseudomonas aeruginosa* ATCC 27853. Results reflect that *P. americana* in the most unsanitary area is highly induced and has active AMPs. Selection of representative hemolymph has an advantage when induced at the highest tolerable concentration at 6-12hours with *E. coli* as the inducible biologic material.

RECOMMENDATION

Because the experiment revealed an influence on the antibacterial activity of hemolymph through different levels of inducement, the researchers highly recommend further analysis on the identity of specific antimicrobial proteins (AMPs) present in the hemolymph. Future researchers may also use different species of cockroaches to be tested against different strains of bacteria, or even fungi and viruses.

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