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Screening of antibacterial activity and biochemical assay from haemolymph of cockroach *Blatta orientalis* (Linnaeus, 1758)

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Abstract
The present study deals with the antibacterial activity of *B. orientalis* haemolymph against five bacterial strains namely, *Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhi.* The results of the present study showed the maximum zone of inhibition against *Escherichia coli*, moderate effects were observed in *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Proteus mirabilis,* whereas the less effect was observed in *Salmonella typhi.* In SDS-PAGE analysis the prominent bands were observed at 18 kDa, 30 kDa and 66 kDa. The results of FTIR analysis revealed the peak ranges from3299 cm^{-1} to 546 cm^{-1}. The FTIR spectral studies clearly indicate the presence of various functional groups such as Secondary sulphonamide, Secondary amide, Cyclopeptane, Vinyl halides, Aromatics compound, Triazoles, Thiophenes, Aldehyde group, Methylene compound, Sulphinic acid group, Bromo compounds which may be attributed to the antibacterial activity properties. The results of present study showed that the haemolymph of *B. orientalis* may have the potential antimicrobial activity.

Keywords: *B. orientalis*–haemolymph-antibacterial activity -SDS-PAGE -FTIR spectrum

1. Introduction
Infectious diseases is a significant threat to human health, contributing to more than 14 million annual deaths, more than half of them occurring in children, even with advances in antimicrobial chemotherapy. For example, tuberculosis caused by *Mycobacterium tuberculosis* alone contributes to approximately 1.6 million deaths per year [1].Also some other bacteria's causing acute respiratory infections, food bore illnesses, coetaneous and central nervous system infections which are all a major threat to human health. In the recent years, the burden of infectious diseases has exacerbated with the emergence of antimicrobial resistance and lessening efficacy of the available antimicrobial compounds [2, 3].

Since early times, insects and the substances extracted from them have been used as therapeutic resources in the medical systems of many cultures. And they are commonly considered to be disgusting and filthy animals. Many insect species have been used in infusions, in plasters, in salves and as ointments, both in curative and preventive medicines, as well as in magico-religious rituals [4]. The therapeutic use of insects and insect-derived products is known as entomotherapy.

During the seventeenth century, people in Europe believed that many kinds of insects had some healing power [5].The oil obtained from the larvae of the May beetle, *Melolontha vulgaris* (L.) 1758, can be used topically on scratches and other wounds and as a cure for rheumatism, the adult beetles soaked in wine are helpful in treating anemia, the pulverized cockroaches were used for treatment of epilepsy; and earwigs can be used for cure earaches [6]. Marie [7] gave an outstanding contribution to the history of entomotherapy by describing the use of 33 kinds of medicinal insects. A recent research project studied the anti-asthmatic and anti-anaphylactic activities of *B. orientalis* mother tincture and concluded that it revealed "nonselective anti-asthmatic activity [44]

Cockroaches are nocturnal omnivore insect that live in damp places throughout the world and belong to the Order: Blattodea with most species associated with human habitats. The most commonly known as household pests includes *Periplaneta americana* L., *Blattella germanica* L., *Blattella asahinai* M., and *B. orientalis* [8].

B. orientalis Cockroach is found to be present both outdoors and indoors. It can be found outside during warm weather in trash cans, sewers, or even under old leaves or stones. In Asia it has been found in caves where bats live. During autumn, however, Oriental Cockroaches may move in large groups into unheated buildings, since they prefer a cooler environment. The preferred temperature ranges from about twenty to twenty-nine degrees Celsius [9-11]. It is mostly feeds on garbage or any sort of decaying organic matter, although its favorite foods consist of anything that is either sugary or starchy. Therefore, it is likely to defend itself against invading pathogens by means of antimicrobial compounds. Hence the present study was carried out to find the antibacterial activity of selected bacterial species and determines the possible bioactive protein isolation and chemical composition of *B. orientalis* haemolymph.

2. Materials and Methods

2.1 Collection of cockroach

In the present study *B. orientalis* were directly caught from households, kitchen store rooms, and corners of buildings, household man-holes, rubbish dumps by using sterile surgical gloves. The collected insects were caged into sterile jars with small holes on the top of the lid to provide air.

2.2 Study period: The study was carried out at Annamalai University and A.V.C College, Department of zoology during November 2016 to February 2017.

2.3 Collection of haemolymph

The collected American cockroaches, *B. orientalis*, were maintained in an in sectary at 25±2 °C temperature with a 12 hours light/dark ratio, and fed on dried bred, dates and water. During experimental period, Cockroaches were subjected to a single bleed collection. Haemolymph (approx. 3mL) was collected by cutting the leg of live animal with fine sterile scissors. To avoid haemocytes degranulation and coagulation, the haemolymph was collected with sodium citrate buffer pH 4.6 (2.1 v/v) and equal volume of physiological saline (0.85% Nacl, w/v). To remove the haemocytes from haemolymph, the haemolymph was centrifuged at 2000 g for 15min at 4◦C. Supernatant was collected and stored at 4◦C until use.

2.3.1 Microbial Strains Used: Antibacterial activity of *B. orientalis* haemolymph was determined against Five pathogenic bacterial strains namely, *Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhi.*

2.3.2 Antibacterial Assay: The spectrum of antimicrobial activity was studied using the above-mentioned bacteria. Erythromycin was used as a positive control for pathogenic bacteria. *In vitro* antibacterial assay was carried out by the disc diffusion technique by Bauer [12]. Whatman No. 1 filter paper discs with 4mm diameter were impregnated with known 10 μL of test sample (haemolymph), and 10 μg/mL positive control contained a standard antibiotic disc. Negative controls (sterile disc only) are also used. The impregnated discs along with control were kept on nutrient agar plates, seeded with test bacterial cultures. At room temperature (37◦C), the bacterial plates were incubated for 24 hrs. They were expressed in terms of diameter of zone of inhibition, measured in mm using cm scale. In each strain, 6 replicates were maintained, and mean was tabulated along with their standard deviation.

2.4 SDS-Page

The molecular weight of active fractions in the sample was analyzed by SDS-PAGE in 12%separating gels, as method described by Laemmli [13].

2.5 Fourier transform-infrared spectroscopy (FT-IR)

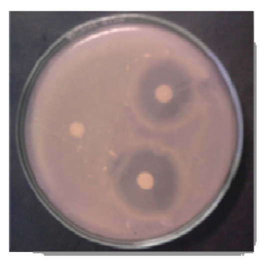
FT-IR was used to identify the chemical identities in a wide range of compounds. Infrared spectroscopy was a useful analytical technique for detection of functional groups in organic compounds. 10 mg sample was mixed with 100mg of dried Potassium bromide (KBr) and it was compressed to prepare as a salt disc (10 mm dia) for spectrometer reading. The infrared absorption bands, molecular components and structures were identified as method described by Akkas [14] using the Nicolet Avatar-360.

3. Results

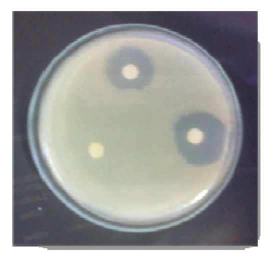
3.1 Antibacterial activity of haemolymph from *B. orientalis*

In this study *B. orientalis* crude haemolymph were collected and used to screen the antibacterial activities. Five bacterial strains have been selected for the present study and they are *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, and Salmonella typhi.* Antibacterial activity (Zone of inhibition) of the haemolymph is tabulated in the Table 1 Plate 1 and the same is presented in Fig 1.

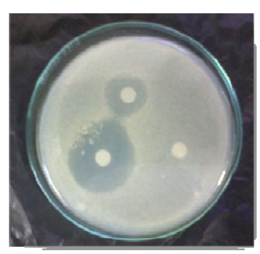
Crude haemolymph from *B. orientalis* showed maximum zone of inhibition against *Escherichia coli* (16.15±0.10), moderate effects were observed in *Pseudomonas aeruginosa* (7.97±0.43), *Staphylococcus aureus* (7.20±0.09) and *Proteus mirabilis* (4.38±0.28).Whereas less effect was observed in controlling the growth of *Salmonella typhi* (3.20±0.23).



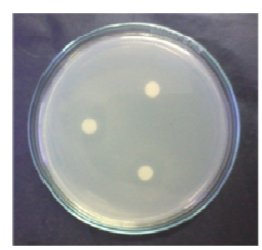
1-a. *Escherichia coli*



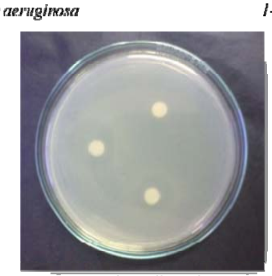
1-b. *Staphylococcus aureus*



1-c. *Pseudomonas aeruginosa*



1-d. *Proteus mirabilis*



1-e. *Salmonella typhi*

Plate. 1(1-a, 1-b,1-c, 1-d, and 1-e) Antibacterial activity of crude haemolymph from *B. orientalis.*

Table 1: Antibacterial activity of crude hemolymph from *B. orientalis*

Sl. No		Zone of Inhibition (mm)		
	Pathogens	**Positive Control**	**Sample**	**Negative Control**
1	*Escherichia coli*	19.27±0.08	16.15±0.10	-
2	*Staphylococcus aureus*	18.82±0.08	7.20±0.09	-
3	*Pseudomonas aeruginosa*	23.61±0.31	7.97±0.43	-
4	*Proteus mirabilis*	8.23±0.18	4.38±0.28	-
5	*Salmonella typhi*	5.25±0.10	3.20±0.23	-

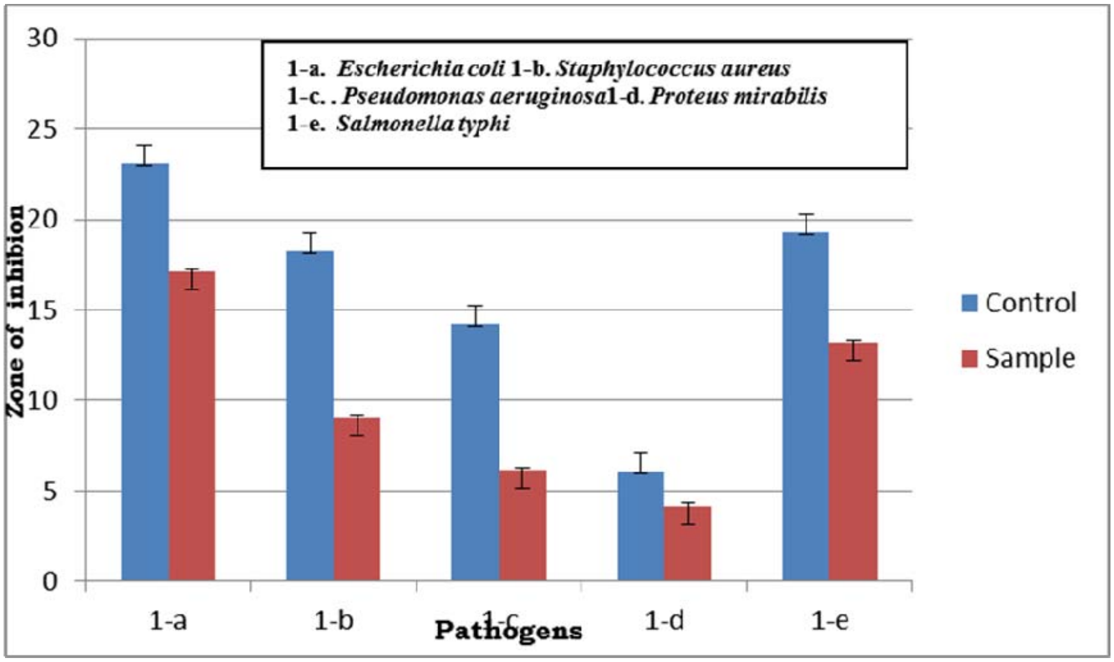


Fig 1: Antibacterial activity of crude haemolymph from *B. orientalis*

The antibacterial activity of positive control Erythromycin showed a maximum activity against *Pseudomonas aeruginosa* 23.61±0.31 and for others it showed the effect in the following rates *Escherichia coli* 19.27±0.08, *Staphylococcus aureus*18.82±0.08, *Proteus mirabilis*8.23±0.18and *Salmonella typhi*5.25±0.10.

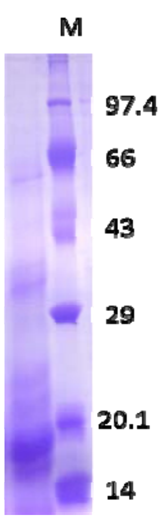


Fig 2: Separation of protein fractions by SDS-PAGE

The haemolymph of *B. orientalis* was subjected to SDS-PAGE analysis and it is results were shown in Fig.2.The prominent bands were observed at 18 kDa, 30 kDa, and 66 kDa. The prominent bands 18 kDa molecular weight indicates low molecular protein. Whereas 30 kDa indictes the bands with moderate molecular weight and 66 kDa high molecular weight.

Table 2: Separation of protein fractions by SDS-PAGE

S. No	Molecular Weight of Proteins	Marker Proteins
1	18 kDa	Carbonic anhydrases
2	30kDa	Carbonic anhydrase
3	66kDa	Serum albumin

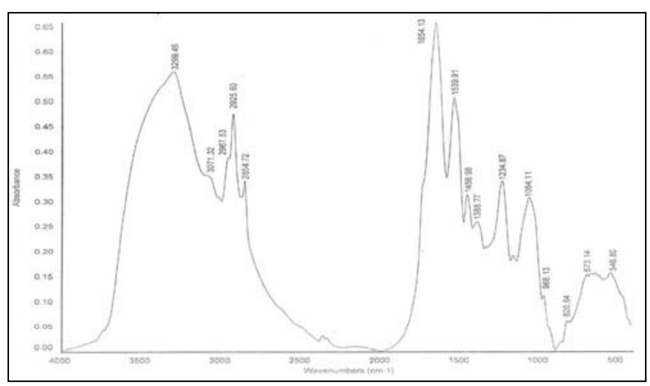


Fig 3: FT-IR spectra of crude Crude haemolymph from *B. orientalis*

The FT-IR spectra of crude hemolymph are shown in Fig. 3. The intense bands which are strong occurring at 3299 cm^{-1}, 3071 cm^{-1}, 2967 cm^{-1}, 2965 cm^{-1}, 2854 cm^{-1}, 1654 cm^{-1}, 1539 cm^{-1}, 1456 cm^{-1}, 1388 cm^{-1}, 1234 cm^{-1}, 1064 cm^{-1}, 968 cm^{-1}, 820 cm^{-1}, 673 cm^{-1}, and546 cm^{-1}. Their corresponding functional groups are given in the Table 3.

The peak at 3229 cm^{-1} which falls in the range of 3300-3260 is correspond to N-H stretching secondary sulphonamide. The spectrum show peak at 3071 cm^{-1} indicates the presence NH stretching - secondary amide. The bands between 2967 cm^{-1}2854 cm^{-1} and 2965 cm^{-1}represent Antisymmetic CH stretching - cyclopeptane compounds. The peak areas1654 cm^{-1} indicates the presence of mainly Vinyl halides.

Table 3: Analysis of chemical composition of crude haemolymph from *Blatta orientalis.*

S. No.	Frequency ranges	Intensity	Functional groups
1	3300-3260	s	N-H stretching secondary sulphonamide
2	3100-3077	m	NH stretching - secondary amide
3	2960-2950	vs	Antisymmetic CH stretching – cyclopeptane
4	2960-2980	vs	Antisymmetic CH stretching – cyclopeptane
5	2820-2810	s	CH_3 stretching
6	1660-1580	m	Vinyl halides
7	1600-1400	(m,s)	C-H stretching aromatics compound
8	1600-1400	(m,s)	C-H stretching aromatics compound
9	1400-1380	s	Triazoles
10	1250-1200	s	Thiophenes
11	1400-1000	v	Aldehyde group
12	950-810	m	Methylene compound
13	870-810	s	Sulphinic acid group
14	900-550	s	Bromo compounds

The band at 1539 $cm^{-1}$and 1456 cm^{-1}, showing the presence of C-H stretching aromatics compound. The peaks at 1388 cm^{-1}, indicating the thiazoles. The weak frequencies of 1234 cm^{-1}, are showing Thiophenes. The band at 1064 cm^{-1}, showing the presence of Aldehyde group. The spectrum show peak at 968 cm^{-1} indicates the presence Methylene compound. The peak at 820 cm^{-1} which is corresponds to Sulphinic acid group. The peak area at 673 cm^{-1}, and546 cm^{-1} are having Bromo compounds [15].

4. Discussion

Since insect blood or haemolymph, contains a number of effective antitoxins, one is inclined to believe that the reported remedies rely on more than sheer guesswork [16]. It was found that in the passage from the cuticle to the haemocoel (cavity of the insect's body) microorganisms confront several antimicrobial components such as proteins, lipids, hydrocarbons, diphenols, carbohydrates, chitin and melanin. These antimicrobials inhibit both the growth and the penetration of those agents into the haemocoel Insects, can supply important information for the fight against ills caused by microorganisms, such as malaria, dengue fever, trypanosomiasis and leishmaniasis [17].

In India, Several authors have described the local uses of insect-based remedies [18, 19, 20]. Sharma **[21]** has surveyed nine tribal communities in Rajasthan. The results provide data on ten species of invertebrates (including insects and crustaceans), with information on which parts, extracts, or secretions of the animals are used, the mode of administration, the ailments and diseases against which they are prescribed, and the tribe where they were reported to be used. Over 500 species of insects, mites, and spiders are used as medicines to cure both common and complicated ailments in the state of Chhattisgarh [45]. For example, locals immediately apply the fresh housefly (*Musca domestica*) as a first aid measure against scorpion bites. The oil from the red velvet mite (*Trombidium grandissimum* Koch) is useful for the treatment of more than ten important diseases including malaria, urogenital disorders, and paralysis. These mites are called ''Indian Viagra'' due to their ability to increase sexual desire [46].

In fact, antimicrobial peptides were first discovered in insect larvae of the Karolinska Institute **[22]**.Chemical screening applied to 14 insect species has confirmed the presence of proteins, terpenoids (triterpenoids and steroids, carotenoids, iridoids, tropolones), sugars, polyols and mucilages, saponins, polyphenolic glycosides, quinones, anthraquinone glycosides, cyanogenic glycosides, and alkaloids [23]. Chitosan, a compound derived from chitin, has been used as an anticoagulant and to lower serum cholesterol level, as well as to repair tissues, and even in the fabrication of contact lenses **[24]**. Kunin and Lawton **[25]** have recorded that promising anticancer drugs have been isolated from the wings of Asian sulfur butterflies *Catopsilia Crocale* [4] and Oldfield [26] records that about 4% of the extracts evaluated in the 1970s from 800 species of terrestrial arthropods (insects included) showed some anticancer activity.

Insect AMPs have a broad spectrum of activity against bacteria, fungi, some parasites and viruses. Even AMPs from the same class but different insect species may have activity against different microorganisms. This may be because AMPs from different insect species may differ in the ability of binding to microorganisms. Insects such as cockroaches represent a plentiful and untapped potential source of new antimicrobial drugs prompting us to investigate the antibacterial activity of their various tissues in American cockroach [27]. Our studies have shown that *B. orientali s*have antibacterial properties and may serve as potential sources of antimicrobials in the future. They often inhabit environment niches that present a significant threat in terms of microbial infection and have evolved defenses to counter this threat.

Most AMPs are synthesized as inactive precursor proteins or pro-proteins, and active peptides (20–50 residues) are generated by limited proteolysis. But, active gloverins (~14 kDa) and attacins (~20 kDa) are large proteins. In *D. melanogaster*, seven classes of AMPs (cecropin, attacin, defensin, drosomycin, diptericin, drosocin, and metchnikowin) have been identified, and regulation of Drosophila antimicrobial peptides (AMP) genes by the Toll and immune deficiency (IMD) signaling pathways has been well studied. There have been many reports on AMPs from various insect species AMPs [28, 29].

Rethna Priya [30] reported the 27.4 to 77kDa were have the antibacterial protein molecules found in *Clibanarius clibanarius*. Gloverin is a basic, glycine-rich and heat-stable antibacterial protein of ~14-kDa, first purified from the haemolymph of *Hyalophora gloveri* pupae [31]. Based on the above report and present study report we can conclude that the haemolymph show the variation in the bands of protein. It may be assumed that the proteins identified in this study might play an important role in their self-defense against bacterial infection in *B. orientalis.*

The Fourier Transform Infrared (FTIR) spectroscopy has proven to be a valuable tool for characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of animal and plant extracts. Moreover, FTIR spectroscopy is an established time saving method to characterize and identify functional groups [32].The present study was carried out to analyze the biomolecule in the haemolymph of *B. orientalis* by using FTIR. The intensity and/or more accurately the area of the absorption bands in FTIR spectrum are directly related to the concentration of the molecules.

The results of FTIR analysis confirmed the presence of N-H stretching secondary sulphonamide, NH stretching ciscrel trans secondary amide, Antisymmetic CH stretching – cyclopeptane, Antisymmetic CH stretching – cyclopeptane), CH_3 stretching compound, Vinyl halides, C-H stretching aromatics compound, C-H stretching aromatics compound, Triazoles, Thiophenes, Aldehyde group, Methylene compound, Sulphinic acid group, Bromo compounds for the responsible of antibacterial activity of Crude haemolymph.

Antibacterial activity of triozoles was studied by Chanda [33] and they added that triozole compounds have pharmacological activites. 1, 2, 4-triazole compounds are known to exhibit a wide range of biological activities such as antibacterial and antifungal activity [34], anticancer [33] and antiviral **[35]**. A large number of 1, 2, 4-triazole, a heterocyclic derivative exhibits important therapeutic activities such as antifungal, [36] anti-tubercular, [37] antioxidant, [38] anti-inflammatory, [39] COX-2 inhibition, [40] anticancer, [41] and antimicrobial activity [42].

Furthermore, 1, 2, 4-triazole ring system has been incorporated into a wide variety of therapeutically interesting drug candidates like ribavirin (antiviral agent), rizatriptan (antimigraine agent) and fluconazole, itraconazole (an antifungal agent) [43]. Hence the present study it can be concluded that the haemolymph of *B. orientalis* is having broad spectrum of antibacterial activities by the presence of triazoles. Further study on this hemolymph of the bio active compound will pave the way for the formulation of new antibacterial drugs.

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