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Antimicrobial potential of Indian cooperative spider, *Stegodyphus sarasinorum*, and Golden orb spider, *Nephila pilipes* hemolymph against pathogenic bacteria

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ABSTRACT

The hemolymph of spiders contains several antimicrobial peptides (AMPs), which are one of the potential sources of antibiotics against drug resistant microbes. In this context, we have studied antimicrobial potential in the hemolymph of the spiders *Stegodyphus sarasinorum* and *Nephila pilipes* against ten pathogenic bacteria. The hemolymph of *S. sarasinorum* and *N. pilipes* yielded maximum Zone of Inhibition against *Streptococcus pneumoniae* (15 mm) and *Staphylococcus aureus* (10.5 mm), respectively. The total hemolymph protein content of *N. pilipes* and *S. sarasinorum* was 91.8 µg/mL and 16.10 µg/mL, respectively. The dominant band on the protein profile of *S. sarasinorum* and *N. pilipes* was in the range of 60–100 kDa and 50–120 kDa, respectively. The study showed that the spider's hemolymph is a promising source of AMPs.

1. INTRODUCTION

Antibiotics saved millions of lives from the pathogenic infections. However, after the advent of multidrug resistance microorganisms, the conventional antibiotics become ineffective against pathogenic microbes [1]. Multidrug resistance, a global crisis, has necessitated the search of an effective antimicrobial agents to control the resistant bacteria. Drug-resistant microorganisms have emerged mainly due to the indiscriminate usage of broad-spectrum antibiotics against microbes [2,3]. This is a growing problem, particularly in hospitals where pathogenic bacteria get antibiotic resistance [4], which include methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Mycobacterium tuberculosis* [5].

Nature provides us many therapeutic products that are able to inhibit the growth of pathogenic microbes [6]. One of the natural products, the antibacterial peptides, has attracted much attention in recent days as a new therapeutic agent against the drug resistant microbial pathogens. These antimicrobial peptides (AMPs) have been reported to found in broad spectrum of organisms which include microbes, plants, and whole of the animal kingdom including invertebrates and vertebrates [7]. It is interesting that the diversity, complexity, and variety of the AMPs seem to be much wider than expected [8].

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Among the animal kingdom, Phylum Arthropoda is the largest phylum with diverse group of organisms and account for more than 80% of all known living animal species [9]. The first AMP from arthropods was isolated from the moth *Hyalophora cecropia*, known as Cecropins [10]. After that, many AMP from other arthropods came to limelight in pharmaceutical industry and several AMPs have been reported in several scorpions and spiders [11]. Gomesin is the first AMP identified from the hemolymph of spiders [12]. Interestingly, spider AMPs showed sequence similarities to Protegrins, an AMP from porcine leukocytes [8].

Due to their potent antibacterial activity against antibiotic-resistant bacteria, AMPs have emerged as a novel class of antibiotics and a promising therapeutic option for many infections caused by multidrug-resistant bacteria [13]. The persistence of different invertebrate populations in a contaminated environmental niche supports the development of disease-fighting AMPs [14-16]. For example, invertebrates such as cockroaches and centipedes thrive in polluted settings and frequently exposed to dangerous bacteria and disease-causing substances, showing the presence of natural AMPs in their bodies to nullify the pathogens [17-19]. There are limited publications on the spider hemolymph's antibacterial properties [8,20,21]. Spiders are regarded as one of the most abundant and technologically advanced suppliers of these peptide compounds due to their diversity [17,22].

Several studies have been attempted to test the spider's hemolymph's potential as antimicrobial agent, which includes the hemolymph of the spiders such as *Lycosa singoriensis* [23], *Agelena*

labyrinthica [8], *Loxosceles intermedia* [24], and *Acanthoscurria rondoniae* [25]. However, the full potential of AMPs in spiders is yet to be explored. In this context, we studied the antimicrobial potential of hemolymph of Indian cooperative spider *Stegodyphus sarasinorum* and Golden orb web spider *Nephila pilipes* against microbial pathogens. Further, we quantify the proteins present in the spider's hemolymph and made an effort to separate the proteins from the spider hemolymph.

2. MATERIALS AND METHODS

2.1. Study Species

The Indian cooperative spider *S. sarasinorum* belongs to the family Eresidae. It is widely distributed in India, Nepal, Sri Lanka, and Myanmar. The female of *S. sarasinorum* is nearly 1 cm and the male is nearly 0.5 cm (excluding the legs). This spider exhibits communal predation and feeding [26], where individuals live in large cooperatively built nests [27].

N. pilipes is commonly called as Giant Wood Spider. The female is 35–45 mm and the male is about 2–4 mm. It is distributed widely in East and Southeast Asia as well as Oceania [28]. These spiders construct gigantic orb webs, often reaching a length of 1.5–2 m in diameter. The males usually die soon after mating [29].

2.2. Hemolymph Collection

Permission was obtained from Periyar University Departmental Animal Ethical Committee before the collection of spiders hemolymph. These spiders were collected from the nearby scrub land and reared in cages under laboratory conditions. They were fed with insects such as grasshoppers, katydids, moths, and mosquitoes for 10 days before the hemolymph extraction. These spiders were narcotized with 70% ethanol, then, their fourth walking leg was separated from the coxa. The flowing whitish-brown hemolymph was collected with micropipettes as per Yigit and Benl, [8]. Nearly, 10 µL of hemolymph was obtained from each spider. The hemolymph is diluted in the range of 1/1 with insect saline solution (insect saline solution: 1.80 g NaCl, 1.88 g KCl, 0.16 g $CaCl_2$, 0.004 g $NaHCO_3$, and 100 mL distilled water).

2.3. Determination of Antimicrobial Potential

The antimicrobial activity of resultant hemolymph – saline solution mixture was tested against ten different pathogenic bacterial strains such as *S. aureus*, *Pseudomonas aeruginosa, Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Staphylococcus hominis*, *Streptococcus pneumoniae*, *Klebsiella pneumonia*, *Staphylococcus saprophyticus*, *Salmonella* spp., and *Shigella* spp. The strains were inoculated in Mueller–Hinton broth (MHB) (Merck) and incubated at 37°C for 24 h.

The antimicrobial potential was evaluated by the agar well diffusion method [30]. The bacterial culture medium was prepared by suspending 38 g of Muller–Hinton agar medium in 1 L of water. After sterilization, the media was poured into sterile glass Petri dishes followed by inoculation of 100 µL of the culture broth. The hemolymph-saline solution mixture was poured in one well of each agar plate containing microorganisms and incubated at 37°C for 24 h. The rest of the wells were loaded with gentamicin and saline solution for positive and negative control, respectively. The antagonistic activity of hemolymph was measured in mm called as zone of inhibition (ZI).

2.4. Minimum Inhibitory Concentration (MIC)

MIC was determined by broth dilution method [31]. About 0–120 µL/mL of hemolymph – saline solution was incubated in the pathogen inoculated broth for 24 h at 37°C. The conventional 96-well plates with MHB with different concentrations of hemolymph, against pathogens were incubated to find out the MIC. The MIC values were calculated to identify the lowest concentration of the extract that inhibits the growth of the pathogens [32,33]. After incubation, absorbance was measured at 595 nm using a microplate reader (Bio-Rad).

2.5. Electrophoretic Analysis of the Hemolymph Proteins

The protein profile of the hemolymph bands was analyzed by electrophoresis with continuous gradient 15% (w/v) polyacrylamide gels under reducing and non-reducing conditions following Laemmli [34]. They were visualized with the aid of Coomassie blue R-250 staining protocol [35] in Sodium dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) [34,36,37]. The electrophoresis was carried out at a constant voltage of 100 V for 3 h. In these analyses, a hemolymph pool from 20 spiders of *S. sarasinorum* and *N. pilipes* were used. The molecular mass markers were acquired from Sigma (Sigma Aldrich, USA).

2.6. Quantification of Total Hemolymph Proteins

A total of 60 µL clear supernatant mixture of hemolymph-saline solution (1:1 ratio) was inoculated by dye-binding method of

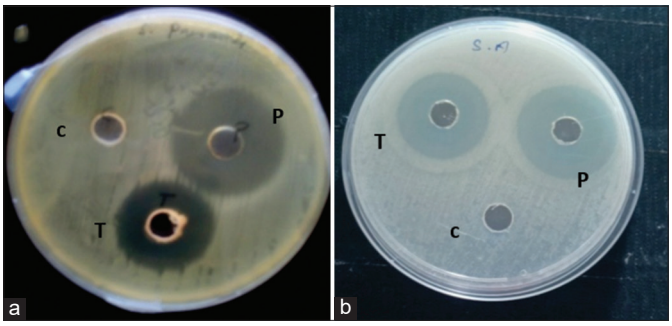

Figure 1: The maximum Zone of Inhibition formed due to the antibacterial activities of the colonial spider (a). *Stegodyphus sarasinorum* against *Streptococcus pneumoniae* and Golden orb web spider (b). *Nephila pilipes* hemolymph extract against *Staphylococcus aureus*.

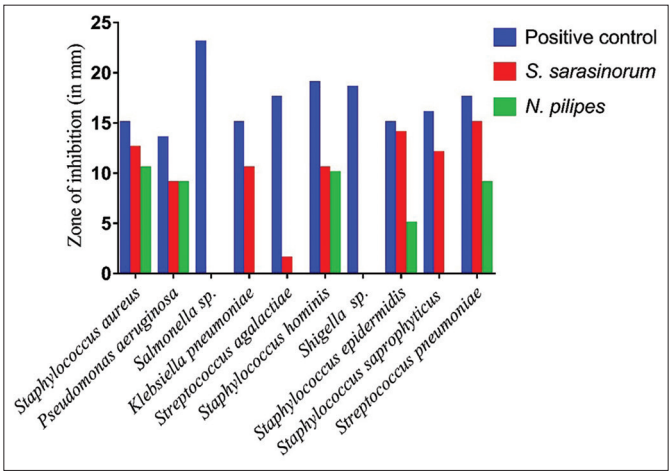

Figure 2: Effect of antimicrobial activities of spiders hemolymph against pathogenic bacteria.

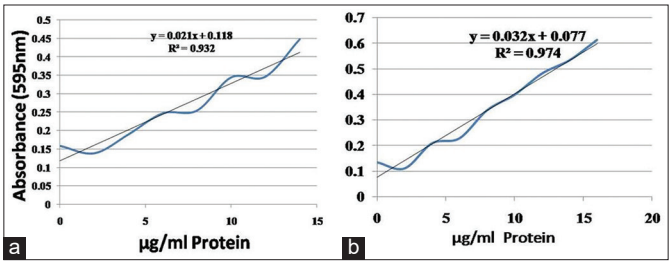

Figure 3: Quantification of total hemolymph proteins in bovine serum albumin (a). *Stegodyphus sarasinorum* and (b). *Nephila pilipes.*

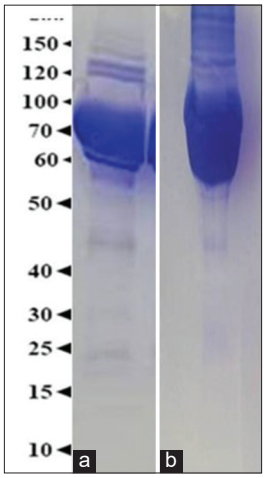

Figure 4: The analysis of proteins in the hemolymph of different spider species using 15% SDS-PAGE – (a) *Stegodyphus sarasinorum* and (b) *Nephila pilipes*.

Bradford (1976) with bovine serum albumin (BSA) as control. The total protein content of hemolymph was represented in mg/mL. The quantification was done using a spectrophotometer at 595 nm (UNICO Spectrophotometer, SP2100 UV, China) [38].

3. RESULTS

The hemolymph of *S. sarasinorum* demonstrated remarkable antibacterial activity against eight out of ten pathogenic test organisms. The maximum and minimum ZI obtained were against *S. pneumoniae* (15 mm) and *S. agalactiae* (1.5 mm), respectively, while the hemolymph of *N. pilipes* exhibited antimicrobial activity against five out of ten test organisms, with the maximum and minimum ZI in *S. aureus* (10.5 mm) and *S. epidermidis* (5 mm), respectively [Figure 1]. There is a significant difference observed between the ZI formed by S. *sarasinorum* and *N. pilipes* [Figure 2] ($F_{2, 27} = 19.41$, $P = 0.0001$).

The antibacterial activities of the hemolymph extract were identified against the test pathogens as *S. aureus*. Among the test bacteria, the MIC was 325 µg/mL for *S. aureus*. The protein content was determined using a standard curve prepared based on BSA with a Bradford Coomassie blue method the linear equation between protein content and absorption value was $y = 0.021\times + 0.118$, $R^2 = 0.932$ for *S. sarasinorum*, and $y = 0.032\times + 0.077$, $R^2 = 0.974$ for *N. pilipes* [Figure 3]. The protein quantified in *N. pilipes* and *S. sarasinorum* was 91.8 µg/mL and 16.10 µg/mL, respectively.

The protein profile of *S. sarasinorum* and *N. pilipes* hemolymph reveals that they are rich in several electrophoretic mobility-associated protein bands, ranging between 15 kDa and 200 kDa. The dominant bands in the protein profile of *S. sarasinorum* and *N. pilipes* were in the range of 60–100 kDa and 50–120 kDa respectively [Figure 4].

4. DISCUSSION

In this study, antimicrobial activities of *S. sarasinorum* and *N. pilipes's* hemolymph were tested against ten pathogenic microbes. Our analysis showed that *S. sarasinorum* showed antimicrobial activity against eight pathogenic bacteria, whereas *N. pilipes* showed antimicrobial activity against five pathogenic bacteria. This showed that *S. sarasinorum* AMPs have wide spectrum antimicrobial activity compared to the hemolymph AMPs of *N. pilipes*. Hemolymph of *S. sarasinorum* showed the maximum (ZI) against the pathogenic bacterium *S. pneumoniae*, while the hemolymph of *N. pilipes* exhibited maximum ZI against the pathogenic bacterium *S. aureus* (10.5 mm). Similarly, Yigit and Benli [8] reported that the hemolymph of the spider *A. labyrinthica* showed antimicrobial activity against five out of ten pathogenic bacteria. This showed that our two species of spiders hemolymph showed promising antimicrobial activities. Further, this also revealed that antimicrobial activity of spiders hemolymph vary between the species. The hemolymph of *Lasiodora* sp. exhibited antagonistic behavior against *Enterococcus faecalis* and *Bacillus subtilis* with MIC of 3400 µg/mL [39]. While, in our study, the growth of *S. aureus* was inhibited at MIC of 325 µg/mL of *S. sarasinorum* hemolymph. The MIC of Gomesin purified from *Acanthocurria gomesiana*'s hemolymph against a wide range of bacteria, fungi and eukaryotic cells represent their effective antagonistic nature [25]. Not only the antibacterial peptides, even an effective antifungal peptide, have been isolated from the hemolymph of *A. rondoniae* [25]. However, we analyze only the antibacterial properties of the spider hemolymph.

In the present study, the protein profile of *S. sarasinorum* and *N. pilipes* hemolymph reveals that they are rich in several electrophoretic mobility-associated protein bands, ranges between 15 kDa and 200 kDa. Our findings corroborate with the analysis of hemolymph of other spider species such as *Nephila inaurata* [40], *Cupiennus salei, Eurypelmacali fornicum,* and *Eurypelma helluo* [41]. Similarly, Jalal [42] analyzed hemolymph of ten species of spiders in citrus orchard and found 200 kDa and 60 kDa bands in all species of spiders.

This study demonstrated that the hemolymph of *S. sarasinorum* and *N. pilipes* was effective against most of the tested pathogenic bacteria. Consequently, to develop effective new drugs against antibiotic-resistant microorganisms, the hemolymph of spiders may be used as a potentially new source of natural antibacterial agents. The protein profile of the hemolymph has been studied. However, the active AMPs from the hemolymph need to be isolated and purified for further studies.

5. CONCLUSION

We reported the antimicrobial activities of hemolymph of two species of spiders *S. sarasinorum* and *N. pilipes*. In this study, *S. sarasinorum* showed antimicrobial activity against eight pathogenic bacteria and *N. pilipes* against five. This study clearly portrays the wide spectrum antimicrobial activity of *S. sarasinorum* hemolymph. Further, we estimated the molecular weight of the hemolymph proteins and isolated them with SDS PAGE. The protein profile of hemolymph revealed that their dominant band in the range of 60–100 kDa in *S. sarasinorum* and 50–120 kDa in *N. pilipes*. Further studies regarding separation of active AMPs from the hemolymph need to be done for the future

studies. Studies of AMPs produce novel perspectives on pathogenicity and provide new perspectives on the interrelations between pathogens and their hosts. This study will serve as the foundation for our future research on enhanced purification, protein type characterization, and the action mechanisms of their active ingredients into the molecular pathways will be highly beneficial in the development of novel medications that utilize spider hemolymph.

6. ACKNOWLEDGMENT

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7. AUTHORS' CONTRIBUTIONS

PTN contributed to the conception of the research idea. While the acquisition, analysis, or interpretation of data was carried out by DS and PSA. The laboratory facilities and chemicals were provided by DJHS. The initial draft was written by DS and PSA. The final draft was prepared by DS, PSA, and PTN.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

Data are available with the corresponding author of this publication upon reasonable request.

12. PUBLISHER'S NOTE

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