

EXPLORING THE ANTIMICROBIAL POTENTIALS OF SPIDER SILK FROM *PHOLCUS PHALANGIOIDES* FUESSLIN, 1775 AND *HIPPASA* SP. SIMON, 1885

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ABSTRACT

As the efficacy of traditional antimicrobial therapies diminishes, the need for novel, safe, and effective antimicrobial agents has become increasingly urgent. An unconventional yet promising source of such agents is spider silk. Traditionally recognized for its remarkable mechanical attributes, including high tensile strength and elasticity, spider silk has recently garnered attention for its potential biomedical applications, particularly its intrinsic antimicrobial activity. This study investigated the antimicrobial potential of silk from two spider species: *Pholcus phalangioides* and *Hippasa* sp. Silk samples were collected, extracted using methanol and formic acid, and subsequently filtered. The resulting extracts were assessed for antimicrobial activity using both disc diffusion and well diffusion assays against a panel of pathogenic microorganisms, including *Candida albicans*, *Circinotrichum* sp., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The methanolic extract of *P. phalangioides* silk demonstrated significant antimicrobial activity ($P < 0.05$), with the most considerable effect observed against *Circinotrichum* sp. In contrast, the formic acid extract of *Hippasa* sp. displayed greater antimicrobial efficacy compared to its methanolic counterpart, with more substantial activity against bacterial strains than fungal pathogens. Notably, inhibition zones observed in the disc diffusion assays were generally larger than those measured in the well diffusion assays. These findings suggest that spider silk from *P. phalangioides* and *Hippasa* sp. harbors bioactive compounds, potentially antimicrobial peptides that exhibit significant inhibitory effects against pathogenic microbes. The results underscore the potential of spider silk as a novel reservoir for developing next-generation antimicrobial agents.

Keywords: Spider, Venoms, *Hippasa* sp., *Pholcus Phalangioides*, Antimicrobial assay

INTRODUCTION

Antimicrobial resistance (AMR) is a rapidly escalating global health threat, driven by the accelerated evolution of microbial resistance mechanisms. This phenomenon renders previously effective antimicrobial agents ineffective against a broad spectrum of pathogens, including bacteria, fungi, viruses, and parasites (Nikaido, 2009; Larsson and Flach, 2022). Beyond complicating the treatment of infections, AMR significantly heightens the risks associated with standard medical interventions such as surgical procedures, by increasing vulnerability to severe complications and mortality (Magiorakos *et al.*, 2012).

Notably, Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella* spp., and members of the Enterobacteriaceae family, as well as Gram-positive pathogens like *Staphylococcus aureus*, have demonstrated increasing resistance, limiting the efficacy of current antibiotic therapies (WHO, 2017). According to data from the Global Research on Antimicrobial Resistance (GRAM), Nigeria ranks 20th globally in age-standardized mortality attributed to AMR among 204 countries. In 2019 alone, over 185,000 deaths in Nigeria were associated with infections caused by *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and Group B *Streptococcus* (GRAM, 2019).

Despite the pressing global demand for novel antibiotics, investment in antibiotic drug discovery has remained limited, thereby constraining the therapeutic options available to manage multidrug-resistant (MDR) infections (Morehead and Scarbrough, 2018). This shortfall has prompted researchers to investigate natural sources, including plants, microorganisms,

and animals for novel bioactive compounds with antimicrobial activity (Goy *et al.*, 2016; Ciriminna *et al.*, 2020; Kumar, 2020; Stan *et al.*, 2021; Lee *et al.*, 2022; Ouertani *et al.*, 2024). Among these, spider silk has emerged as a promising biomaterial due to its documented wound healing properties and potential biomedical applications (Newman and Newman, 1995; Phartale *et al.*, 2019; Deshmukh and Pansare, 2019; Tsiareshyna *et al.*, 2024). A distinguishing characteristic that enhances the suitability of spider silk for biomedical applications is its inherent capacity to inhibit microbial growth and resist bacterial degradation (Lammel *et al.*, 2010).

Spider silk may exhibit antimicrobial properties primarily for two functional reasons: the protection of the spider and the preservation of the silk itself. Many spiders inhabit silk-lined environments and deposit their nutrient-rich eggs within silk casings, rendering both the silk and the eggs highly susceptible to microbial colonization and degradation (Babczynska *et al.*, 2019; Makover *et al.*, 2019). Furthermore, spider silk is composed mainly of proteinaceous fibers rich in hydrophobic amino acids, which could serve as substrates for microbial activity (Vollrath, 2000; Romer and Scheibel, 2008), thus necessitating intrinsic antimicrobial defenses. Despite the potential of spider silk as a novel antimicrobial agent, research efforts in Nigeria remain predominantly focused on plant-derived materials (Ugboku *et al.*, 2020; Ojah *et al.*, 2021; Zailani *et al.*, 2024), with comparatively limited attention given to animal-derived products such as spider silk. In light of this, the present study aims to investigate the antimicrobial properties of silk from *Pholcus phalangioides* and *Hippasa* sp.

MATERIALS AND METHODS

Spider Silk Collection and Extraction

Spiders were collected from their natural habitats within the premises of Ahmadu Bello University, Zaria, Nigeria, and taxonomically identified based on morphological characteristics following the keys provided by Dippenaar-Schoeman and Jocqué (1997). To ensure the authenticity of silk production, the spiders were maintained under controlled laboratory conditions in individual cages. Silk samples were obtained by gently drawing a sterile pipette through freshly spun webs, which were then transferred into a sterile, cleaned Eppendorf tube for extraction. Silk from *Hippasa* sp. and *Pholcus phalangioides* was weighed (0.2 g) and placed into borosilicate test tubes, followed by the addition of 5 mL each of formic acid and methanol in separate preparations. The mixtures were allowed to stand for 24 hours. Subsequently, the samples were filtered, and the resulting extracts were used for antimicrobial assays (Phartale et al., 2019).

Antibacterial Assay Formic and Methanolic Silk Extract

The antimicrobial activity of both formic acid and methanolic spider silk extracts was evaluated against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) using disc diffusion and well diffusion methods. For the disc diffusion assay, Mueller-Hinton agar was prepared, and 100 μ L of standardized microbial suspension was evenly spread across the surface of each agar plate using a sterile glass spreader. Sterile filter paper discs were impregnated with varying volumes of silk extract (37.5, 150, and 300 μ g/ μ L), labeled accordingly, and placed onto the inoculated agar surfaces. Plates were incubated at 37 ± 1 °C for 24 hours, after which antimicrobial activity was assessed by measuring the diameter of the inhibition zones in millimeters (Phartale et al., 2019).

For the well diffusion assay, agar plates were similarly prepared and inoculated, and uniform wells were bored into the agar using a sterile cork borer. Each well was labeled and filled with (37.5, 150, and 300 μ g/ μ L) if the respective silk extract. Plates were incubated at 37 ± 1 °C for 24 hours, and the resulting zones of inhibition were measured to determine antimicrobial efficacy (Deshmukh and Pansare, 2019). Formic acid and methanol served as negative controls, while 10 μ g gentamicin was employed as the positive control. All experimental assays were conducted in triplicate.

Antifungal Assay of Formic and Methanolic Silk Extract

The antifungal activity of formic acid and methanolic spider silk extracts was assessed against *Candida albicans* and *Circinotrichum* sp. using both disc diffusion and well diffusion methods. Sabouraud Dextrose Agar (SDA) plates were prepared, and upon solidification, 100 μ L of

standardized fungal spore suspension was inoculated onto the center of each plate and evenly spread using a sterile glass spreader. For the disc diffusion assay, sterile filter paper discs impregnated with different volumes of silk extract (37.5, 150, and 300 μ g/ μ L) were aseptically placed on the inoculated agar surfaces. In the well diffusion assay, wells were created in the agar using a sterile cork borer, labeled accordingly, and filled with the same volumes of silk extract. All plates were incubated at 28 ± 1 °C for 24 to 72 hours. Antifungal activity was determined by measuring the diameter of the inhibition zones (in millimeters). Each experiment was performed in triplicate, and formic acid and methanol were negative controls to validate the specificity of the silk extracts' antifungal effect, while 10 μ g econazole was used as the positive control.

Data Analyses

Leven's homogeneity of variance and Shapiro-Wilk test were used to check the data's homogeneity and normal distribution. Analysis of variance (ANOVA) was conducted to determine statistically significant differences in the zones of inhibition among the tested microbial species at a significance level of $P < 0.05$. Boxplots were used to compare inhibition zones between the two assay methods (disc and well diffusion). Furthermore, group principal component analysis (PCA) was employed to assess the relationship between spider species based on the assay method and inhibition zone patterns. All statistical analyses were performed using Microsoft Excel 365 (2024) and R for Windows version 4.3.2.

RESULTS AND DISCUSSION

The methanolic extract of *Pholcus phalangioides* silk exhibited significantly greater antimicrobial activity ($P < 0.05$) compared to the formic acid extract across the tested microbial strains. Notably, no zones of inhibition were observed for the formic acid extract against fungal organism. In contrast, both methanolic and formic acid extracts demonstrated significant antimicrobial effects against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The highest antimicrobial activity for *P. phalangioides* silk was observed in the methanolic extract against *Circinotrichum* sp (Figure 1).

Conversely, the formic acid extract of *Hippasa* sp. silk exhibited significantly higher antimicrobial efficacy ($P < 0.05$) than its methanolic counterpart. The greatest zone of inhibition was recorded for the formic acid extract against *S. aureus*, while the lowest activity was observed with the methanolic extract against *C. albicans* and *Circinotrichum* sp (Figure 2). Generally, the fungal isolates were less susceptible to the silk extract as compared to the bacterial isolates.

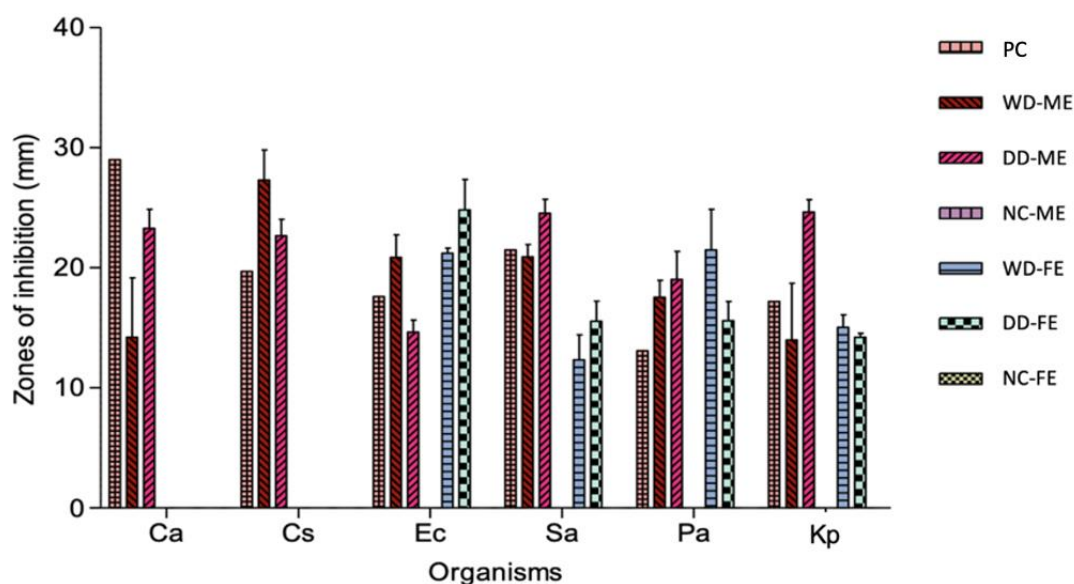


Figure 1: Antimicrobial activity of *Pholcus phalangioides* using disc and well diffusion assay PC: positive control; WD: well diffusion; DD: disc diffusion; NC: Negative control; ME: methanolic extract; FE: formic acid extract; Ca: *Candida albicans*; Cs: *Circinotrichum* sp.; Ec: *Escherichia coli*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Kp: *Klebsiella pneumoniae*

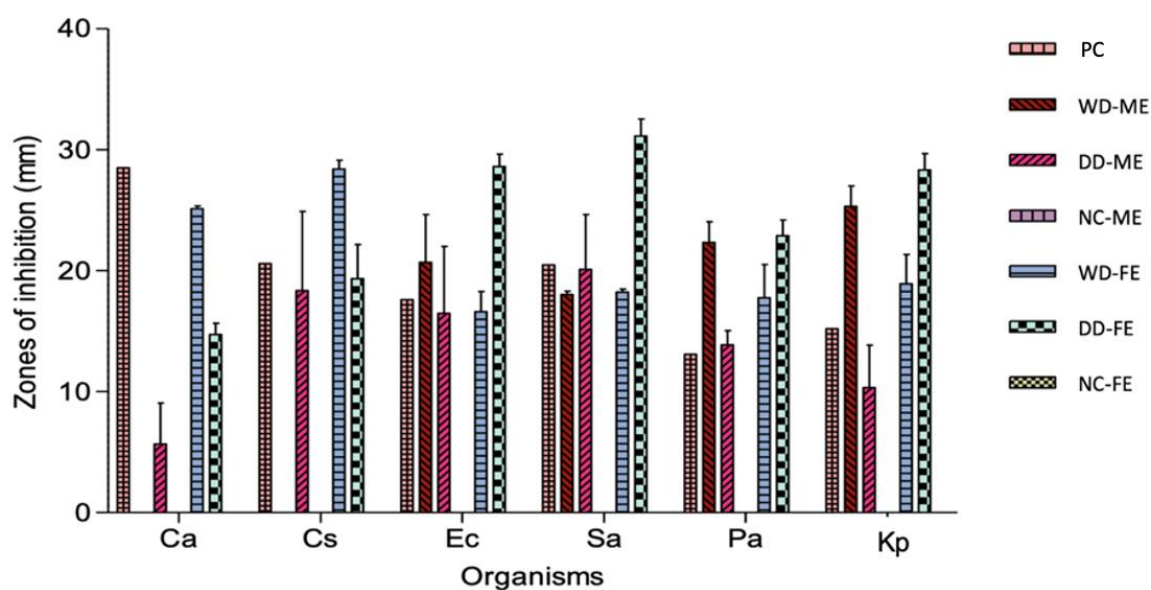


Figure 2: Antimicrobial activity of *Hippasa* sp. using disc and well diffusion assay PC: positive control; WD: well diffusion; DD: disc diffusion; NC: Negative control; ME: methanolic extract; FE: formic acid extract; Ca: *Candida albicans*; Cs: *Circinotrichum* sp.; Ec: *Escherichia coli*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Kp: *Klebsiella pneumoniae*

Figure 3 illustrates a comparative analysis of inhibition zones produced by two antimicrobial susceptibility testing methods: disc diffusion and well diffusion assays. Both methods display comparable median inhibition zones, as indicated by the central lines within each boxplot. However, the disc diffusion method shows a slightly higher median, suggesting

marginally greater antimicrobial effectiveness. The interquartile ranges (IQRs), represented by the heights of the boxes, are similar for both methods, reflecting comparable variability in the inhibition measurements. Additionally, the whiskers extend over nearly the same range for each method, indicating similar overall distributions.

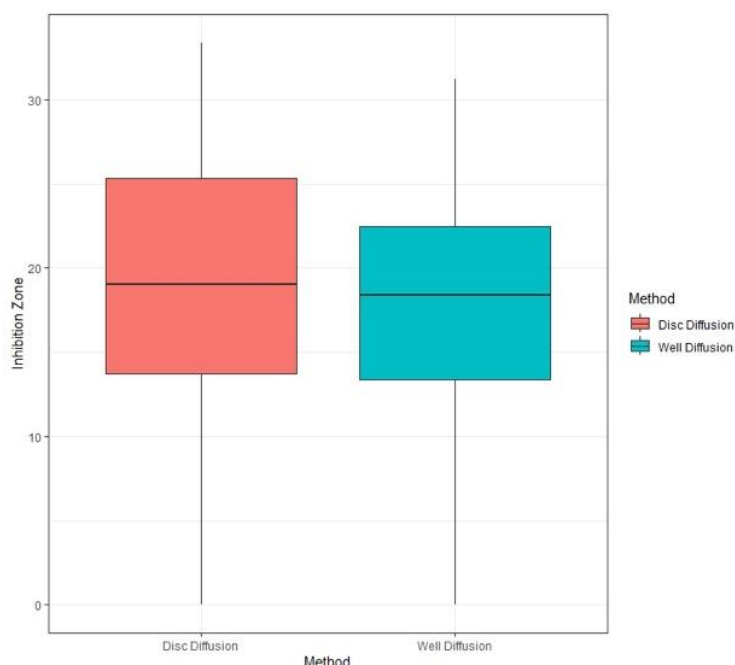


Figure 3: Comparison between the disc and well diffusion zones of inhibition

The Principal Component Analysis (PCA) plot (Figure 4) provides a multivariate assessment of the antimicrobial inhibition profiles of silk extracts from *Hippasa* sp. and *Pholcus phalangioides*, as influenced by different assay methods and extract types. The first principal component (PC1) accounts for 69.2% of the total variance, while the second principal component (PC2) explains an additional 14.7%, cumulatively representing 83.9% of the dataset's variation. This indicates that PC1 is the primary axis differentiating the antimicrobial responses of the two spider species. A clear separation is observed between *Hippasa* sp. and *P. phalangioides* along PC1, suggesting distinct differences in their antimicrobial efficacy. The clustering

patterns show *Hippasa* sp. forming a more compact group, while *P. phalangioides* exhibits a broader distribution, indicating greater variability in inhibition zones. Ellipses around each group reflect within-species variation, with a wider ellipse for *P. phalangioides* further supporting this observation. Vector orientation reveals differential contributions of the assay methods and extract types. Methanolic extracts (ME) are more strongly associated with *P. phalangioides*, whereas formic acid extracts (FE) align more closely with *Hippasa* sp. Additionally, the distinct positioning of the reference antimicrobials gentamicin and econazole suggests that their inhibition profiles differ markedly from those of the spider silk extracts.

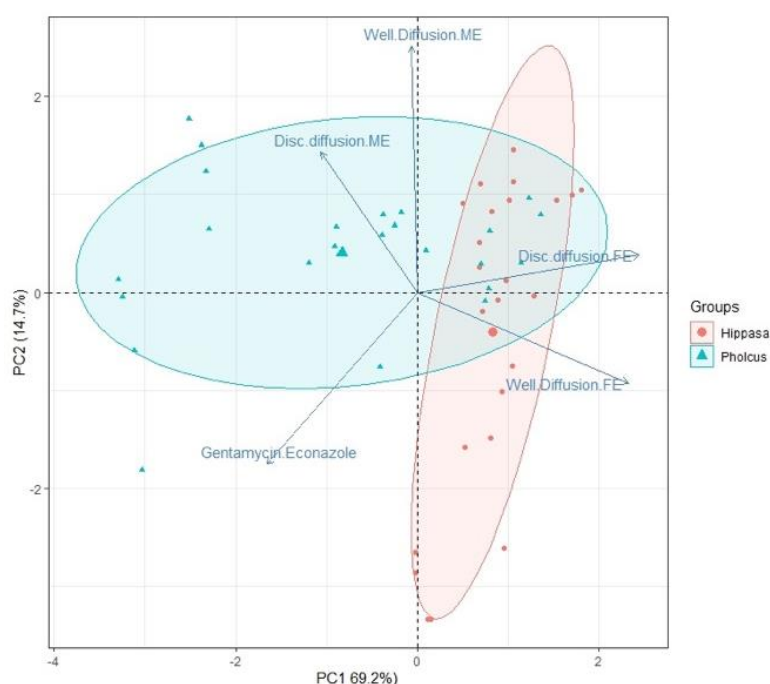


Figure 4: Relationship between spider species as influenced by methods and zones of inhibition

Discussion

This study employed both disc and well diffusion assays to evaluate the antimicrobial activity of methanolic and formic acid silk extracts derived from *Pholcus phalangioides* and *Hippasa* sp. against six microbial species. The results revealed significantly greater activity against bacterial strains compared to fungal isolates. This heightened susceptibility of bacteria particularly Gram-positive species is due to known biological and structural characteristics, including the presence of a peptidoglycan-rich cell wall/faster growth rate (Perfect, 2017), simpler membrane architecture (Cowen *et al.*, 2014), and diffusion assays that enable easy access to bacteria as compared to fungi due to fungal hyphal network (Rex *et al.*, 2001).

In contrast to the present findings, Phartale *et al.* (2019) reported significantly higher antifungal activity relative to antibacterial effects using dimethyl sulfoxide (DMSO) extracts of *Pardosa brevivulva* silk. The discrepancy may be attributed to differences in solvent polarity, which can influence the solubility and bioavailability of active compounds. Supporting the current results, Roozbahani observed greater inhibition of *Listeria monocytogenes* (Gram-positive) compared to *Escherichia coli* (Gram-negative) using silk extracts from *P. phalangioides*. The antimicrobial potential of spider silk is thought to be associated with its biochemical composition, as spiders are capable of storing excess food for months or even years by encapsulating them in silk (Tahir *et al.*, 2017).

The median inhibition zone observed in the disc diffusion method was marginally larger than that of the well diffusion method, suggesting its superior efficacy as an assay technique. This can be attributed to the enhanced diffusion from the disc, where antimicrobial agents are evenly dispersed radially from the paper disc across the agar surface, forming uniform concentration gradients (Balouiri *et al.*, 2016). Additionally, filter paper discs effectively retain the antimicrobial agent, allowing for a gradual release over time, which facilitates diffusion and ensures sustained contact with the test organism (Cheesbrough, 2006). This method is particularly advantageous for nonpolar or volatile extracts, as it minimizes issues related to evaporation and surface tension (Thakur & Juneja, 2021). Similar results have been reported by Kazemipoor *et al.* (2012), Baihaqi-Othman *et al.* (2019), and Kub *et al.* (2021). In contrast, Nurkhaliza *et al.* (2024) found the well diffusion method to be more effective, attributing this to the silver nanoparticles potentially adsorbing to the surface of the disc rather than diffusing into the agar.

The PCA plot facilitates the comparison of antimicrobial activities of silk extracts from two spider species, *Hippasa* sp. and *Pholcus phalangioides*, across different extraction solvents and assay methods. The solvent type (methanol or formic acid) significantly influences antimicrobial efficacy, with methanolic extracts showing greater activity in *P. phalangioides*, while formic acid extracts are more effective in *Hippasa* sp. This variation may stem from differences in silk type, as the two species produce distinct silk forms (e.g., dragline vs. capture silk), each characterized by unique protein compositions and concentrations (Branković *et al.*, 2024). Moreover, solvent selection has been shown to induce conformational changes in silk proteins, altering their structural properties and modulating interactions with microbial targets, thereby impacting antimicrobial potency (Kiseleva *et al.*, 2021). Similar observations have also been reported by Phartale *et al.* (2019).

CONCLUSION

This study provides the first report of the antimicrobial activity of *Hippasa* sp. silk and further evaluates the activity of *Pholcus phalangioides* silk using both disc diffusion and well diffusion assays. The silk extracts from both species demonstrated notable antimicrobial effects against the tested bacterial isolates, with inhibition zones observed to be slightly larger in the disc diffusion assay compared to the well diffusion method. These findings highlight the potential of spider silk, particularly from these species, as a promising source for the development of novel antimicrobial agents.

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