Research Article

Evidence of Antibacterial Potential of Spider Silk (Araneae: Araneidae) as Borrowed Property

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Abstract | The notion that spider silk possesses antibacterial potential dates back to the time of ancient Greece when it was frequently used as wound dressings. In recent years, several studies have investigated the alleged antibacterial potential of spider silk but the results have largely been inconclusive. The studies either failed to consider the inhibitory effects of the processing chemicals or did not investigate whether the inhibitory potential is an intrinsic property of silk or is due to the aggregate gland secretion coating its surface, hence a borrowed property. Our study used direction application and disk diffusion method to investigate the antibacterial potential of *Neoscona mukherji* and *N. theisi* egg and web silk. For disk diffusion method, silk was first degummed to form degummed silk solution' (DgS), then the degummed silk was dissolved to form the dissolved silk solution (DSS). The DgS and DSS solutions were tested against selected common pathogenic bacteria (*Escherichia coli*, *Salmonella typhimurium* SJW1102, and *Staphylococcus aureus*). According to the study, the DgS and DSS solutions of egg-sac silk exhibited no inhibitory potential by either of the two methods. However, application of web silk using the DSS treated discs showed no growth inhibition compared to the DgS treated discs, where increased inhibition zones were observed against all three bacteria. This study suggested that spider silk does possess antibacterial potential, however, this is not an intrinsic property and is type specific as well as species specific. Use of spider silk for coating of medical devices such as stents and catheters can decrease transmission of pathogenic bacteria forming biofilms as well as aid in wound healing.

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1. Introduction

Over the course of thousands of years, spiders have evolved to use their silk for various purposes e.g. building web, capturing prey, making egg cases and lining burrows (Weissbach *et al.*, 2021). The ability of spiders to spin intricate webs has always fascinated humans. Not only have we endeavoured to understand the spinning mechanism, but also the possible applications of spider silk due to its unique properties (Tahir *et al.*, 2019). In recent times, spider silk has catapulted to the centre stage of biopolymer research. The main cause of this piqued interest is the extraordinary properties possessed by this tiny piece of thread. Its remarkable strength, toughness, flexibility, elasticity, extensibility, biodegradability, biocompatibility and antimicrobial potential are the various aspects that have captivated the scientific community (Tokareva *et al.*, 2014; Jastrzebska *et al.*, 2015; Marelli *et al.*, 2016). However, current study simply focused on the antibacterial potential of spider silk against common pathogenic bacteria.

The use of spider silk for wound dressing dates back to the time of ancient Greece (Lewis, 2006). The spider silk was wrapped around wounds to help speed up the healing process. Over the years, various researchers have reported spider silk to have an inhibiting effect on bacterial growth (Wright and Goodacre, 2012; Keiser, 2016; Al-Kalifawi *et al.*, 2017). They found that spider silk was an effective inhibitor of both Gram-positive and Gram-negative bacteria. However, these studies have failed to provide conclusive evidence. The solvents used in the studies to extract spider webs included acetone, ethanol, methanol and tween80 (Roozbahani *et al.*, 2014; Al-Kalifawi *et al.*, 2017). Therefore, the studies failed to rule out the possibility that the inhibitory effect may actually be due to the solvents used, instead of the silk.

Another major ambiguity left unanswered is that whether the antibacterial potential is an intrinsic property of silk, i.e. due to the structure and composition of silk, or is a consequence of the aggregate silk secretions coating the strand. Previous studies either left the question unexplored or asserted that the antibacterial potential was due to the presence of various compounds e.g. peptide phosphonates, glycoproteins, sulphur containing compounds, 12-methyltetradecanic acid and 14-methylhexadecanoic acid present in the silk (Kiseleva *et al.*, 2020).

Web weaving species of spiders build webs of various shapes and sizes to maximize their feeding capacity. The ability of spider web to capture prey efficiency depends on the location, mesh type, capture thread and area, location alongwith orientation (Mishra and Rastogi, 2020). There are various types of silk web spiders however orb web is the most common type. The orb web forming spiders are able to construct seven different types of webs keeping their elasticity while maintaining tough structure even during contraction using free molecular chains (Saravanan, 2006). Various families of spiders such as *Nephilia* and *Araneus* construct orb spiders while other families of spiders build sheet and tangle webs. Spiders who build orb webs spend less energy to search prey and more on synthesis of silk and web construction. The two species of spiders used in this study build orb web. The design of orb web varies depending upon the type of species, within the individuals of the species, with in the web. Even the web of young spiders differs from the adult ones being more circular (Becker *et al.*, 2003). Orb type webs are usually constructed in an orientation to avoid web damage caused by air drag used to capture prey. Also, hungry spiders bring smaller webs. Web asymmetry also differs depending upon species, age, weight, and size of the web, however web upper half is necessary to maintain a stable functional web. Several spokes laid outward from a common point in an orb web depending upon the spider species (Higgins *et al.*, 2001).

Neoscona mukherji is an orb weaver nocturnal spider commonly found in Pakistan and India. These are mostly found in gardens amongst bushes and shrubs underneath leaf surface during daytime. These have different colors and banding pattern but each with same type of cephalothorax. Cephalothorax is yellow brown, elongated and has hairs. Cephalic region is 'V' shaped with dark brown patch. Abdomen is triangular, grey, and hairy with posterior tapering end and pubescence. Mid longitudinal part of abdomen has bands. Epigynal scape has indistinct lateral lobes with profound constriction. Among eight eyes, anterior median eyes are larger and encircled with black rings compared to posterior median eyes. Lateral median eyes are located on projecting tubercles. Chelicerae are yellow and solid. Sigilla are present in five pairs. Legs are long, yellow covered with spines and hairs. Distal end of leg segments except for coxa has transverse bands (Figure 1A).

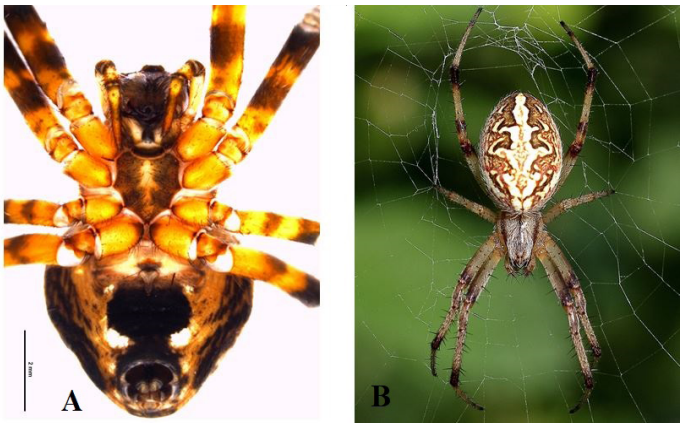

Figure 1: Spider species belonging to the family Araneidae (a). *Neoscona mukherji* © (Tikaderbk and Malhotra, 1980), (b). *Neoscona theisi* ©Walckenaer, 1842.

Neoscona theisi are non toxic and found in wheat crop, gardens, in Pakistan, India and Bangladesh. *N. theisi* are yellow brown and have hairs with dark bands. Cephalothorax is comparatively longer than wide. Anterior median eyes are bigger compared to

posterior median eyes while lateral is close. Sternum is dark brown in color and has hairs. Padipalps are brown yellow with medium size. Maxillae are longer and brown. Chelicerae are hairy, strong and have spines. Abdomen is sub-oval with hairs. From dorsal side, abdomen has white-chalk bar pattern and ventral side is brown. Middle side of epigynum has visible rim (Walckenaer, 1842) (Figure 1B).

Our study ensured to negate the effects of any chemicals used in processing of silk by using dialysis tubing. Furthermore, the study also examined the possibility that the antibacterial potential might be due to the sticky secretions of aggregate silk gland which coat the silk strand. The sticky secretion consists of glycoproteins, low molecular weight ionic compounds and neurotransmitters such as choline, GAB amide, etc. which are collectively referred to as salts (Collin *et al.*, 2016). Thereby providing definitive answer to whether the antibacterial potential is an intrinsic or borrowed property.

2. Materials and Methods

2.1 Spider silk collection

The collection sites for silk were the rice and wheat fields of Muridke, Punjab, Pakistan. The silk was collected from spider species belonging to the family Araneidae, *Neoscona mukherji* (Tikaderbk and Malhotra, 1980) and *Neoscona theisi* (Walckenaer, 1842). The silk was collected using sterile glass rods, at dawn and dusk when the spiders built fresh webs. The egg-sac silk was also gathered through field collection of 30 egg-sacs of Neoscona after removal of the embryo. The glass rods and the egg-sacs were stored in separate air tight jars and exposed to UV light in a safety cabinet for fifteen minutes to kill all forms of microbial contaminants.

2.2 Degumming of silk

Before dissolution, degumming of spider silk was performed according to the process outlined by Kim *et al.* (2003). A slight modification to the process was done; the temperature for degumming was reduced to 70°C to prevent denaturation of any surface proteins which may be responsible for the antimicrobial property. 100 mg of silk was dissolved in 100 mL of solution (1.0 mg/mL). The solution left after degumming was referred to as the degummed silk solution (DgS). The DgS was then dialyzed for three days with distilled water using dialysis tubing (Thermo Fisher Scientific; molecular weight cut off 3.5 kDa) to remove the chemical residues. The distilled water was changed three times per day, while the removal of sodium bicarbonate was confirmed by the neutral pH of the solution. The DgS solution contained the aggregate gland secretions striped from the silk strands.

2.3 Dissolution of silk

A combination of two solutions, calcium chloride solution and urea solution were used as a solvent to dissolve the silk. The calcium chloride solution was made as described by Ajisawa (1998). The 8M urea solution was made by dissolving 48 g in 100 mL distilled water. 100 mg of degummed silk was dissolved in 100 mL of solvent (1.0 mg/mL) at 70 °C with constant stirring for two hours. The dissolved silk solution (DSS) was dialyzed for three days with distilled water (changing water thrice per day) using dialysis tubing to remove chemical residues. The removal of dissolution solvent was confirmed by the neutral pH of the solution. The DSS solution was used to test whether the inhibitory activity is an intrinsic property of silk.

2.4 Bacterial strains

Three bacterial strains including *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (KY698020) and *S. typhimurium* SJW1102 (Liaqat *et al.*, 2021) were tested to monitor the antibacterial potential of spider silk.

2.5 Direct application of egg-sac silk

Selected pathogenic bacteria were streaked on an agar plate and left for five minutes to dry. Afterwards, egg sac silk was directly applied onto the centre of the agar plate. The plate was left incubated overnight at room temperature and observed for inhibition zones around the silk next day.

2.6 Disk diffusion method

Antibacterial susceptibility test was performed by the disc-diffusion method as outlined by Liaqat *et al.* (2017). The turbidity of bacterial culture was adjusted to 0.5 McFarland standards to ensure that the same dose of bacteria was inoculated each time. The culture was then spread on the agar plates and left for fifteen minutes of drying period. Two sterile blank paper discs (6 mm diameter) each impregnated with 30 µL of DSS and DgS at final concentration 1 mg/1mL were placed on plates under sterile conditions. Tetracycline

(30 µg/µL) and streptomycin (10 µg/µL) were run in parallel as controls. Plates were incubated at 37°C for 24-hours and the zones of inhibition were measured. The experiment was carried out with egg-sac silk and web silk in triplicate.

3. Results and Discussion

3.1 Direct application of spider egg-sac silk to investigate its antibacterial potential

The inhibitory potential of spider egg-sac silk was directly tested against *E. coli*, *S. aureus* and *S. typhimurium* 1102. The three strains were streaked onto a petri plate and egg-sac was directly applied. No inhibition of bacterial growth was observed for either of the three strains.

3.2 Investigation of antibacterial potential of spider egg-sac silk

The inhibitory potential of spider egg-sac silk was also investigated using the disk diffusion method. Figure 2 shows no inhibition zones observed around either DSS or DgS. Table 1 shows and compares the results of inhibition zones of positive controls and silk treated discs. Likewise, no inhibition zones against *S. aureus* and *S. typhimurium* 1102 were observed around either DSS or DgS (Figure 2A-C and Table 1). The reported size of inhibition zones was 6 mm in diameter which was not considered as inhibition zone following standard criteria by Liaqat *et al.* (2017).

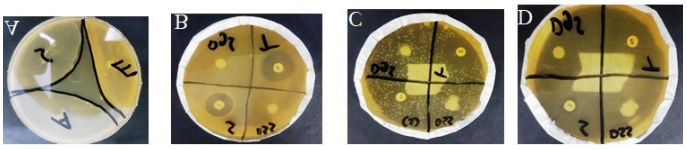

Figure 2: (A) Direct application of spider egg sac silk to investigate its inhibitory potential against *E. coli*, *S. aureus* and *S. typhimurium*; (B), Antibacterial potential of egg silk against *E. coli*; (C) *S. aureus* and (D) *S. typhimurium* using disc diffusion method.

3.3 Investigation of antibacterial potential of spider web silk

The disk diffusion method was also employed to test the inhibitory potential of spider web silk against selected bacteria. Inhibition zones were observed around the positive controls and the DgS treated disk for all three strains. The zone was largest against *S. typhimurium* 1102 (27 mm), smallest against *E. coli* (20 mm), and intermediate against *S. aureus* (24 mm) (Figure 3A-C). The size of reported inhibition zones around DgS discs is close to that of positive controls (Table 2). These results therefore highlight the effectiveness of the antimicrobial compounds against the pathogenic bacteria. However, no inhibition zone was formed around the DSS, against either of three strains.

Table 1: Antibacterial activity of spider egg silk against *E. coli*, *S. aureus* and *S. typhimurium*.

Diffusion disk	Size of inhibition zone (mm)		
	E. coli	*S. aureus*	*S. typhimurium*
Streptomycin	17.0	12.0	11.0
Tetracycline	20.0	20.0	6.0
Dissolved silk solution (DSS)	6.0	6.0	6.0
Degummed silk solution (DgS)	6.0	6.0	6.0

*The reported size of inhibition zones includes the 6mm diameter of diffusion discs as well (Bauer *et al.*, 1966). Therefore the 6 mm inhibition zone means no reported inhibition.

Table 2: Antibacterial activity of spider web silk against *E. coli*, *S. aureus* and *S. typhimurium*.

Diffusion disk	Size of inhibition zone (mm)		
	E. coli	*S. aureus*	*S. typhimurium*
Streptomycin	30.0	35.0	35.0
Tetracycline	30.0	45.0	40.0
Dissolved silk solution (DSS)	6.0	6.0	6.0
Degummed silk solution (DgS)	20.0	24.0	27.0

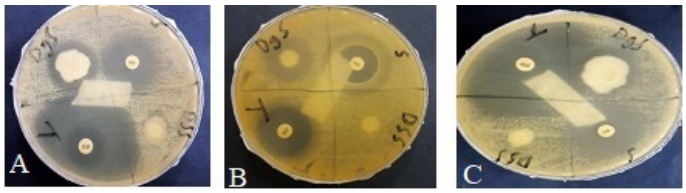

Figure 3: Antibacterial potential of spider web silk against. (A) *E. coli*; (B) *S. aureus* and (C) *S. typhimurium* using disc diffusion method.

It has often been speculated that among the different types of spider silk, the egg-sac silk is most likely to possess antimicrobial potential because it serves the purpose of protecting the egg (Wright and Goodacre, 2012; Gu *et al.*, 2020). Furthermore, the peptide phosphonates present in the egg-sac silk were speculated to possess antibacterial property (Kiseleva *et al.*, 2020). Therefore, this study investigated its antibacterial potential by two means *i.e.* one by direct application an inoculated agar plate and secondly, using disc diffusion method

Our results clearly indicated that direct application of egg-sac silk provided no evidence of its antibacterial potential. Therefore, the disk diffusion method using DSS and DgS discs was used to counter-verify the acquired results and similar observations were made. The egg-sac silk (tubiliform in nature) secreted by female spider is meant to protect the egg and prevent any microbial entry to invade the egg-sac. The antibacterial ability of egg-sac silk is not common to all spiders and its often possible sometimes it has quite strong antibacterial activity depending upon the length of incubation period, however, this does not necessarily associates with all the species and at all times. The fact that egg silk of spiders has unique molecular architecture with different amino acid make up (Gu *et al.*, 2020), thus has different properties compared to previously studied spider silks. Additionally, highly robust nature of egg silk to resist attack of predators, parasites or other environmental fluctuations might explain that why it doesn't need to have antimicrobial activity in some species. There is currently very minor published literature available on this aspect, so more studies are required to further elaborate findings.

Spider silk being antibacterial as observed using DgS treated discs would confer potential benefits to spider since it invest large number of resources to capture prey and it would favour the spider if its prey is not decomposed or consumed by other predators in surroundings. We observed almost similar inhibition zones (20-27 mm) in both Gram positive (*S. aureus*) and Gram negative bacteria (*E. coli* and *S. typhimurium* 1102). This is in agreement to previous findings where authors reported that spider web possess has broad spectrum antimicrobial activity against different microorganisms, including both Gram positive and Gram negative bacteria, viruses, fungi and protozoa (Gomes *et al.*, 2011; Sarkar *et al.*, 2021). Wright and Goodacre (2012), compared antibacterial property of spider silk group against *B. subtilis* and *E. coli* in broth media and reported that the spider silk have bacteriostatic action on *B. subtilis* but no significant effect on *E. coli*. This antimicrobial activity was found to be associated with various substances such as peptides and proteins present in web silk (Huang *et al.*, 2020). The bactericidal activity of these molecules have been reported due to their direct binding with lipid bilayer of bacterial cell membrane and acquiring an amphiphilic three-dimensional conformation, thus leading to membrane porosity (Liaqat *et al.*, 2009; Nilebäck, 2013).

Conclusions and Recommendations

The results acquired from the present study provided concrete evidences that antibacterial potential of spider web is not an intrinsic property of spider silk, rather it is due to the sticky aggregate gland secretions coating the silk, hence might be evolving with change in environmental conditions. Additionally, observed antibacterial potential of web silk might not even be selected for silk that's protects eggs. Though this study demonstrated that inhibitory potential of silk is both type specific and species specific, however further studies using silks from wide range of species against diverse microbes will be required to understand the evolution of protection strategy traits as a result of exposure to microbial challenge. Use of spider silk based biomaterials might be helpful in reducing resistance by the bacteria in community mode called as biofilm. Use of spider silk for coating of medical devices such as stents and catheters can decrease transmission of pathogenic bacteria forming biofilms as well as aid in wound healing (Salehi *et al.*, 2020).

Novelty Statement

The current study provided solid evidence that spider silk (Araneae: Araneidae) does possess antibacterial potential, however, this is not an intrinsic property and is type specific as well as species specific.

Author's Contribution

HMT and IL conducted the study and wrote the manuscript. JN and HA did experimental work. SA helped in data collection while HMT, IL and SA analyzed the data and critically reviewed the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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