Antibacterial potential of venom extracted from wolf spider, *Lycosa terrestris* (Araneae: Lycosiade)

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**ABSTRACT**

The wolf spider *Lycosa terrestris* (Araneae: Lycosiidae) is a well known arthropod containing toxic compounds. It has significant predatory potential in addition to its uses in medicinal and insecticidal formulation. Current investigations were aimed to extract and partially characterize the venom of *L. terrestris* and the susceptibility tests to evaluate antibacterial potential of venom supernatant and venom pellets against four pathogenic bacterial strains i.e., Gram negative *Acinetobacter sp.* and *Pasteurella sp.* and Gram Positive *Staphylococcus sp.* and *Streptococcus sp.* Results of this study revealed that the venom of *L. terrestris* contained six relatively high molecular weight peptides ranging from 125 kDa to 35 kDa. Moreover, results of the susceptibility test confirmed the bacteriostatic action of venom supernatant against aerobic Gram negative *Acinetobacter sp.* in dose dependent manner. A reduced trend of bacteriostatic inhibition was also observed for venom pellets against *Acinetobacter sp.*

**Key words**: Bacteria, Characterization, *Lycosa terrestris*, Spiders, Toxicity, Venom.

**INTRODUCTION**

The massive use of antibiotics in the mid-20th century resulted in dramatic decline in the death rate across the globe (Bi *et al.*, 2003). However, prolonged and intensive use of broad-spectrum antibiotics has eventually led to the rise of multi-resistant bacterial types (Wright, 2007). Today antibiotic resistance has emerged as one of the most tenacious and alarming health concern (Hoffman *et al.*, 2015). Recently, almost all the clinically important bacterial pathogens in Pakistan and throughout the world are increasingly becoming resistant to antibiotics (Riaz *et al.*, 2011). This decline in efficacy of antibiotics in treating ordinary infections indicates that we are standing at the verge of post antibiotic era (Alanis, 2005). The solution to this dilemma and to cope with the upcoming challenges related to increased resistance among microbes, the adoption of novel therapeutic approaches and drugs is urgently required. Thus, the natural products are outpacing and surpassing the synthetic materials (Chen *et al.*, 2005). They provide a major source of new chemical diversities (Cragg and Newman, 2013). They have novel sites of action and such unique mechanisms against microbes that it is unlikely for them to acquire resistance towards these natural killers (Ding and Ho, 2004). The natural compounds when used as antibiotics are not only effective but also have fewer side-effects on human health and environment. They are also much safer than the commercially used synthetic antimicrobial drugs (Cragg and Newman, 2013).

Among the natural resources for retrieving a potent and more effective antimicrobial drug, spiders have excelled all others. Spiders are considered as one of the most successful and abundant terrestrial predators with approximately 100,000 extant species (Windley *et al.*, 2012). They are capable of producing venom that holds a diverse array of biologically active compounds. Spider venom comprises of heterogeneous compounds targeting a diverse range of sites including membranes, receptors, channels, and enzymes in a vast variety of invertebrate and vertebrate species (Saenz *et al.*, 2010). These compounds provide spiders a competitive edge and can be utilized by humans for immunity enhancing and encourage resistance mechanisms in human body.

Previously, a limited investigation is done for the identification and application of peptides which have been isolated from spider venom. However, despite being less studied, spiders venom has the most diverse range of impressive biological activities including the potential application in therapeutics (Vassilevski *et al.*, 2009). Spider venom is enriched with small polypeptides known as antimicrobial peptides (AMPs) (Vassilevski *et al.*, 2009).
The AMPs have attracted much attention in recent years as novel antimicrobial agents (Rafael et al., 2015). Mainly, they cause structural and functional disruptions in cell membranes even at micromolar concentrations by directly binding to plasma membrane of the target cell (Jenssen et al., 2006). The AMPs cause permeation and lysis of the cell followed by the interaction of microbe’s receptor with the peptide (Zasloff, 2002). Therefore, in regards to the antibacterial potential of spider venom, this study was conducted and its purpose was to partially characterize the venom extracted from a lycosid spider, Lycosa terrestris and also to evaluate the antibacterial potential of venom against some common pathogenic bacteria.

MATERIALS AND METHODS
Spider collection and venom extraction: The wolf spiders, (Lycosa terrestris) were collected from the citrus orchards and agricultural fields in Sargodha (32.08°N, 72.67°E), Punjab, Pakistan. Collected spiders were placed in separate ventilated containers (7 × 8.5 × 250 cm) to avoid cannibalism. To retrieve maximum amount of venom, the specimens were starved for two days (Nagaraju et al., 2007).

The spiders were temporarily immobilized by placing them at 0-4°C for 6 minutes in the freezer (Khan et al., 2013) and dissected under a stereomicroscope (Labomed-7GA9). The dissected chelicerae along with the venom gland (Guerrero et al., 2010) were immediately transferred in eppendorfs (1.5 ml) containing 0.5 ml of tris HCI buffer of pH 8.2 and 0.05 molarity (Frontali et al., 1976). After manual maceration of venom glands, they were centrifuged at 15,000 rpm for 20 minutes at 4°C by using refrigerated centrifuge (MPW-352R). Both the lighter and denser supernatants were collected in separate eppendorfs by using a micropipette. The venom fractions consisting of supernatant and pellets were then kept at -20°C in Ultra Freezer (Arkteco ULTF 220) until use (Frontali et al., 1976).

Culturing and maintenance of bacterial strains: Two gram negative (Acinetobacter sp. and Pasteurella sp.) and two gram positive (Staphylococcus sp. and Streptococcus sp.) bacterial species were used. These strains were cultured on nutrient agar and maintained in liquid nutrient broth at 37°C. For the preparation of liquid broth, 3.7 grams of Brain Heart Infusion Broth powder was dissolved in 100 ml of distilled water and autoclaved for 20 minutes. The required quantity of autoclaved nutrient (5 ml) broth was added with bacterial colony of each strain and isolated from the culture medium. These tubes containing bacterial colony along with nutrient broth were incubated at 37°C for 24 hours and stored at 4°C in liquid broth for further studies.

Characterization of venom by Sodium Dodecyl Sulphate Gel (SDS) electrophoresis: For analysis of various protein fractions present in spider venom, SDS polyacrylamide gel electrophoresis was performed (Sambrook and Russle, 2001) and different venom peptides were separated according to their molecular weights. Glass plates were assembled in a sealed gel apparatus (MSmidi10) by placing them 1mm apart. Two gels of different percentages (5% staking gels and 12% resolving gel) were prepared. Separating the 12% resolving gel (10 ml) was prepared by mixing 30% polyacrylamide (acrylamide 29: bisacrylamide 1) (3,300 µl), distilled water (4 ml), tris-Cl buffer (pH 8.8, 1.5M) (2,500 µl), 10% SDS (100 µl), 10% ammonium per sulphate (300 µl) and Tetramethylethylenediamine (TEMED) (100 µl). This solution was poured in the provided space between two glass plates for staking gel (0.5 inch below the edges of comb) and allowed to polymerize for 30 minutes. Stacking gel (3 ml, 5%) was prepared by mixing 2.1 ml distilled water, 500 µl acrylamide (30%), 380 µl tris buffer (pH 6.8, 1M), 30 µl SDS (10%), 90 µl ammonium per sulphate (10%) and 21 µl TEMED. Supernatant containing venom sample was thawed at room temperature. An equal ratio 1:1 of supernatant (20 µl) and 2X SDS loading buffer (100 mM tris-Cl, pH 6.8) (20 µl) were mixed in an eppendorf and heated at 100°C for 30 minutes in dry heating chamber to denature the proteins. The gel was kept in staining solution (1% Coomass brilliant blue R 250, 10% ethanol, 40% acetic acid) for an hour and then in destaining solution for 6-8 hours. Destaining solution was a mixture of 40% methanol and 30% acetic acid. Stained gels were photographed and approximate molecular weights of different fractions were determined by comparing with reference protein standards.

Susceptibility test: Nutrient agar medium was prepared by dissolving 2 g nutrient agar, 0.3 g meat extract and 0.5 g peptone while maintaining the final volume up to 100 ml by adding distilled water. Sterilized apparatus having agar nutrients was placed in laminar air flow under flame zone and then placed at about 60-70°C. The agar nutrient medium was transferred in Petri dishes and was kept at 25°C to solidify the nutrients. The supernatant and pellets recovered from L. terrestris venom were investigated for their potential as an antibacterial agent. The protocols of Kirby-Bauer Disk Diffusion Susceptibility were used for analysis (Vineetha et al., 2015). The formation of inhibition zone was an indication of possession of marked antibacterial activity.

Statistical analyses: The mean and the standard error of mean (Mean± SE) were computed using Minitab 14. One-way ANOVA followed by Tukey’s test was applied to compare the zone of inhibitions of treatments using SPSS version 13 (SPSS, 2005).

RESULTS AND DISCUSSION
Characterization of venom and molecular weight determination of peptide fractions: The resolved venom protein bands were compared to protein bands of known molecular weight, separated on the same gel. Electrophoretic analysis of venom extracted from L. terrestris revealed presence of six different proteins bands. The venom of this
spider species contained bands of 125 kDa, 120 kDa, 80 kDa, 42 kDa, 38 kDa, and 35 kDa. The band of 80 kDa was densest and broad among all bands followed by 120 kDa, 38 kDa, 42 kDa and 35 kDa. The band of 125 kDa was less intense (Figure 1).

**Bactericidal action:** The venom fractions proved ineffective against all bacterial strains i.e. *Acinetobacter sp.*, *Staphylococcus sp.*, *Streptococcus sp* and *Pasteurella sp*. Thus, none of the venom fractions proved bactericidal in action. The clear zones of inhibition were formed against *Acinetobacter sp.* when treated with venom supernatant (Figure 2). The 50 µl dose of venom supernatant induced the largest inhibition zone (4.1 ± 0.45) followed by 20 µl venom supernatant (2.9 ± 0.23). The treatment of venom pellets remained least effective at minimum zone of inhibition. No other bacterial strain showed significant inhibition zones against venom fractions (P> 0.05).

**Partial characterization of spider venom using SDS PAGE:** The band of 80 kDa was denser and broader among all bands. However, the band of 125 kDa was less intense (P> 0.05). This might be because of lower concentration of this peptide. The venom peptides of more than 10 kDa molecular weight can act as a toxin (King et al., 2008). However, the peptide fractions present in the venom of tested species are expected to be neither cytolytic nor neurotoxic in action. The cytolytic peptides are typically rather small (~3 kDa) and the size of spider neurotoxins ranges from 3–7 kDa (Kuhn-Nentwig, 2003). It has also been reported that the venom of wolf spiders is enriched with potent antimicrobial peptides (Yan and Michael, 1998). The SDS-polyacrylamide gel electrophoresis on venom of *L. erythropoietina* showed a polypeptide band with apparent molecular weight of 8 kDa (Cruz et al., 1994) and a potent antimicrobial peptide, LycTx I (Santos et al., 2010). These peptides were named as lycocitin 1, 2 and 3 having molecular mass ranging from 2,000–3,000 Da (Xu et al., 1989) and antibacterial toxins, Lycotoxins I and II in another wolf spider *Hogna carolinensis* (Yan and Michael, 1998).

**Effect of spider venom fractions on growth of bacterial strains:** The venom supernatant extracted from *L. terrestris* evidently constrains the growth of aerobic Gram negative *Acinetobacter sp.* via bacteriostatic means. The venom fractions did not show bactericidal activity against any of the tested strain. This is in accordance with Wang et al. (2016) regarding bacteriostatic effect of lycosin-II extracted from *L. singoriensis* against 18 multidrug-resistant bacterial strains. *Acinetobacter baumannii* was one of the most susceptible microbes to lycosin-II. *Klebsiella pneumoniae* and *Streptococcus pyogenes* that are either strictly anaerobe or facultative anaerobic organisms were less sensitive to this peptide. The bacteriostatic effect of lycosin-II might be correlated with its ability to bind to bacterial cell membrane. Lycosin-I is also found to potently inhibit the growth of multidrug-resistant *A. baumannii* (Wang et al., 2014). It is clear that the membrane permeabilization mechanism is the dominant mechanism through which AMPs restrict bacterial growth. The venom of *L. singoriensis* has anti-microbial activity (Xu et al., 1989) and contains low molecular weight AMP, lycocitin 1, 2 and 3 (Budnik et al., 2004). Both lycocitin 1 and 2 peptides show evident bactericidal effect against Gram-positive bacteria such as *S. aureus* and *Bacillus subtilis* along with Gram-negative bacteria, i.e., *Escherichia coli* and *P. aeruginosa* bacteria (Liu et al., 2009).

The 1/10th dilution of the venom of *Agelena labyrinthica* significantly inhibits the growth of *B. subtilis*, *E. coli*, *Shigella sp.*, *S. aureus*, and *P. aeruginosa* (Benli
and Yigit, 2008). The bacteriolytic activity was accompanied by shrinkage and the excessive loss of cytoplasm from bacterial cell. Furthermore, the venom of Lachesana tarabaevi spider possesses peptides known as latarcins (approximately 3 kDa). Latarcins are the cytolytic and antibacterial peptides that cause lysis in cells of various origins including cells of Gram-positive and Gram-negative bacteria along with fungal cell i.e., yeast (Kozlov et al., 2006). The lytic nature of peptides isolated from the venom of Cupiennius salei have marked antibacterial activity against Gram positive bacteria i.e., Staphylococcus epidermidis and B. subtilis and Gram negative bacteria i.e., E. coli, Pseudomonas putida and Paracoccus denitrificans (Kuhn-Nentwig et al., 1998). Rapid bacteriolytic effect of an acylpolyamine, VdTX-I isolated from the venom of tarantula, Vitalius dubius have been found, which possess significant broad spectrum antimicrobial activity (Rafael et al., 2015).

Besides this, the venom of the lynx spider, Oxyopes takobius contains modular toxins named as spiderines: OtTx1a, OtTx1b, OtTx2a and OtTx2b which show potent cytolytic effects on cells of various origins (Vassilevski et al., 2013). The antimicrobial or specifically bactericidal potential of spider venom is achieved only when it is enriched with low molecular weight peptides having molecular weight less than 3-4 kDa, as Kuhn-Nentwig (2003) reported typically rather small cytolytic peptides (~3 kDa). The partial characterization of venom of L. terrestris clearly demonstrates that the lowest molecular weight peptide present in the venom is approximately 35 kDa. This protein fraction is much larger to exhibit cytolytic or any kind of bactericidal effects and it cannot penetrate the cell membrane and induce lytic effects. However, it is capable of interaction with the cell membrane and causing membrane disruptions thereby restricting bacterial growth. Kuhn-Nentwig (2003) also defined that the size of spider neurotoxins is in ~3–7kDa range. While King et al. (2008) described that the venom peptides of more than 10kDa molecular weight acts as toxin. As a result, it can be concluded that the venom of L. terrestris can neither be cytolytic nor neurotoxic, but acts as bacteriostatic toxin.

DISCLOSURE
The authors declare that they have no conflict of interest.

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REFERENCES


