EFFICACY OF THE SPIDER WEB METABOLITES ACTIVITY AGAINST MULTI DRUG RESISTANCE (MDR) BACTERIA

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Abstract. Antibiotic resistance, which is emerging among pathogenic bacteria, is a leading cause of treatment failure worldwide with particular reference to developing countries. Thus, there is a need to explore and discover novel antimicrobial agents. The objective of this study was to investigate antibacterial activity of spider web metabolites and characterize certain antibacterial compounds. Web solvent extract (methanol, ethanol and acetone) was biologically screened for antibacterial activity against seven multiple drug resistant bacteria including three Gram negative bacteria (*E. coli, A. baumannii,* and *S. typhi*) and four gram positive bacteria (*S. aureus, Bacillus, E. faecalis and S. pneumonae*) using well diffusion test. Solvent extracts with the concentrations of (3.3 mg/ml 4.95 mg/ml 6.6 mg/ml 8.25 mg/ml 9.9 mg/ml and 11.55 mg/ml) were used to check the efficiency against MDR bacteria. Solvent extract of web metabolites exhibited significant activity against all seven MDR bacteria, by producing clear growth inhibition zones ranging in diameter from 04 to 22 mm. Considerable activities was exhibited against *E. faecalis* (22 mm), *E. coli* (14 mm) *and S. typhi* (15 mm). Acetone solvent extracts showed high antibacterial activity against most MDR bacteria. Solvent extracts with concentrations less than 5 mg/ml did not exhibit sufficient activity. Higher activities were related to concentrations higher than 6 mg/ml. Findings of the current study indicated that spider web metabolites may show promising antimicrobial activity against MDR bacteria. Additionally, the results suggest that spider web may be a broad spectrum antimicrobial agent, wherein its individual constituents contribute to its antimicrobial activity.
Keywords: *antibiotics resistance, antibacterial metabolites, solvent extracts, MDR, Pakistan*

Introduction

A lack of proper antimicrobial agents capable of controlling the incidence of infectious diseases is a major issue facing the health sector worldwide. An increase in

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microbial diseases resistant to antibiotics has reached worrisome proportions in the biosciences field **(**Cassini et al., 2018). Discovery of the first antibiotic, followed by technological advances in antimicrobial drug research, led to the protection of millions from microbial diseases. In the history of antibiotics, the first ever contribution was made by Sir Alexander Fleming who, in 1928, discovered that Penicillin derived from molds was capable of inhibiting bacteria. Following the discovery of this highly appreciated antibiotic, several other antibiotics were also discovered and used to treat infectious diseases (Davies et al., 2010)**.** However, towards the end of the 1900s, emergence of antimicrobial resistant species together with the capacity for resistant gene transfer between species became a cause for major concern in the life sciences community (Amare et al., 2011). The Emergence of MDR bacteria capable of resisting antibiotics directly and indirectly is the direct result of all these factors. Furthermore, extensive use of antibiotics during the past few decades, led to the application of selective pressure on vulnerable bacteria, favouring the survival of resistant strains, few of which are resilient to more than one antibiotic (Arason et al., 2002).

The emergence of MDR bacterial species increased gradually over the years. Currently numerous studies have described various pathogenic bacterial species including *Salmonella typhi, Acinetobacter baumannii, Staphylococcus aureus, Enterococcus faecalis, Streptococcus pneumonae* and *Escherichia coli* as highly antimicrobial resistant. These bacteria, which cause many different infectious diseases, are resistant to multiple antibiotics and cannot be treated with traditional antibiotics as reported in many studies (Singhal, 2014; Doi, 2015; Da Silva, 2016; Megan, 2016; Nilsson, 2012; Wattanathum, 2003; Noble, 1992). These strains may be untreatable in the near future unless substitute or novel antimicrobials are developed. Novel antibiotics may be derived from natural sources such as plants, animals or their secretions in the form of molecules and compounds. Spider web metabolites comprise different molecules which appear to be antimicrobial according to studies (Vollrath et al., 2000). Spider silk is also attracting attention due to its antimicrobial properties. There have been unsubstantiated reports regarding the antimicrobial potential of spider silk (Wright et al., 2012). This report indicated that microbes were unable to grow on spider silk due to its acidic properties, suggesting that spider silk either prevented. Biofilm formation by microorganisms or were bacteriostatic or bactericidal in nature (Saleem et al., 2010). Additionally, bacteriostatic activity of spider silk has also been attributed to the presence of potassium nitrate, which inhibits the growth of microbes such as *Bacillus subtilis* and *E. coli* (Chakraborty et al., 2009). Antibacterial and antifungal activity of spider webs is mentioned in literature. However, antimicrobial potential of spider webs against multi drug resistant bacteria (MDR) has not yet been examined in Pakistan. Multi-drug resistant (MDR) organisms are a global threat. Antibiotic resistance accounts for hundreds of thousands of deaths annually (Johan et al., 2018). Spider web been traditionally used as an antiseptic in rural areas of Pakistan, while Atypus spider webs were used by people in the Carpathian Mountains as a topical antiseptic (Wright et al., 2012). This was considered to be helpful as a disinfectant.

The aim of this study was to investigate antibacterial activity of spider web metabolites and characterize certain antibacterial compounds. Different concentrations of web extract were used against previously identified MDR bacteria. This study will be helpful to kill drug resistant bacteria and discovery of the novel antibiotics.

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Materials and methods

Sampling

The current study and sampling of web were done in the district Kohat, Khyberpakhtunkhwa, Pakistan. Previously identified and characterized clinical bacterial isolates of *Salmonella typhi, Staphylococcus aureus, Enterococcus faecalis, Acinetobacter baumannii, bacillus, Streptococcus pneumonae,* and *Escherichia coli* were recruited for this study. These previously identified isolates were basically from chronic wounds origin. The samples were forwarded for re-culturing and re-identification. Biochemical test used for identification are given in details in *Table 1*.

Table 1. *Biochemical identification of test isolates*

Pathogens	Cell morphology		Biochemical tests				
	Shape	Gram	Cat	Oxi	Ind	DNase	Cit
Bacillus	Rod	+ ve	+ ve	variable	-ve	-ve	+ ve
A. baumannii	Rod	-ve	+ ve	-ve	-ve	-ve	+ ve
S. Aureus	Cocci	+ ve	+ ve	-ve	-ve	+ ve	+ ve
S. Typhi	Rod	-ve	+ ve	-ve	-ve	-ve	-ve
E. coli	Rod	-ve	+ ve	-ve	+ ve	-ve	-ve
S. pneumonae	Cocci	+ ve	-ve	-ve	-ve	-ve	-ve
E. faecalis	Cocci	+ ve	+ ve	-ve	-ve	-ve	+ ve

S. aureus = staphylococcus aureus, S. Typhi = salmonella typhi, S. Pneumonae = streptococcus Pneumonae, E. faecalis = Enterococcus faecalis. Cat = catalase, Cit = citrate, Ind = indole, Oxi = oxidase

Antibiotic resistance patterns

Antibiotic resistance patterns of the isolates were determined via the Kirby-Bauer disk diffusion method (Bauer et al., 1966) according to the recommendations of the Clinical and Laboratory Standard Institute (CLSI) (Shallu et al., 2015). The isolates were treated with a total of 11 antibiotics as follows; Clarithromycin, Doxycycline, Cefoperazone sulbactam, Cefoxitin, Kanamycin, Nitrofurantoin, Penicillin, Levofloxacin, Ciprofloxacin, Oxacillin and Clarithromycin. Briefly, Mullen-Hinton agar was prepared. A 0.5 McFarland turbidity standard equivalent to inoculation of a bacterial suspension was prepared and inoculated. Following 48 h of incubation, the diameters of inhibition zones around the disks were measured using a graduated ruler and results were interpreted according to CLSI guidelines.

Collection, processing and metabolites of spider web

Spider webs were collected from garages and ceilings of unused buildings in the district of Kohat, Pakistan. Webs were washed with distilled water in the laboratory and cleansed of other redundant ingredients and dust. Clean and wrapped web was air-dried at room temperature and preserved in ampoules. Organic solvents, such as methanol, ethanol and acetone, were screened for possible extraction of web metabolites which were to be tested against MDR species or specific pathogens considered sufficiently important or suitable for exploration (Tshipamba et al., 2018).

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Pure web metabolites were extracted using standard methodology. Pure web portions weighing 6 g each were introduced into falcon tubes, each containing 60 mL of acetone, methanol, ethanol and distilled water, and set on an orbital shaker at different angles for 2 weeks. After shaking liberated excessive amounts of metabolites, solvents were filtered into a beaker. The filtrate of solvent extract was placed in a large beaker and allowed to evaporate for one week. Following evaporation, the solidified extract was dissolved in 6 mL DMSO and kept it in sterile falcon tubes.

Screening of webs

Biological screening of antimicrobial properties of the web metabolites was performed via the well diffusion method. Muller Hinton Agar (MHA) media was used to assess susceptibility patterns of isolated bacteria by applying solvent extracts using well diffusion (Jaja et al., 2018).

Results

All isolates of *S. typhi, S. aureus, E. faecalis, A. baumannii, bacillus, S. pneumonae* and *E. coli* were re-identified via culture characterization, gram staining and biochemical testing.

Antimicrobial sensitivity pattern of bacterial isolates

Antimicrobial patterns of isolated bacterial pathogens were determined using 11 different antibiotic groups. *E. coli, S. typhi* and *A. baumannii* showed resistance to 7 antibiotics widely used against these pathogens, whereas *S. aureus* was resistant to 8 antibiotics. *S. pneumonae* was resistant to 6 antibiotics. However, *Bacillus* and *E. faecalis* were resistant to 6 antibiotics (*Table 2*). After confirming MDR patterns of these pathogens, aqueous acetone, ethanol, and methanol extracts of spider web were evaluated in *vitro* against *Acinetobacter, bacillus, S. aureus, S. typhi, E. faecalis, S. pneumonae* and *E. coli.*

Table 2. *Assessment of test isolates*

Antibiotics	*E. coli*	*E. faecalis*	*S. aureus*	*Bacillus*	*Acinetobacter*	*S. typhi*	*S. pneumonae*
Chloramphenicol (30 µg)	NA	R	R	I	NA	R	R
Clarithromycin (2 µg)	S	I	I	R	R	I	I
Oxacillin (5 µg)	S	R	R	R	R	R	R
Doxycycline (30 µg)	R	R	R	R	I	R	R
SCF (105 µg)	I	S	S	S	R	S	S
Cefoxitin (30 µg)	R	I	I	R	R	R	R
Kanamycin (30 µg)	R	I	R	R	R	R	R
Nitroforantoin (300 µg)	I	R	R	I	I	I	I
Penicillin (6 µg)	R	R	R	R	R	R	R
Levofloxacin (5 µg)	R	S	S	S	I	S	S
Ciprofloxacin (5 µg)	S	S	R	S	R	S	I

R = Resistant, I = Intermediate, S = Sensitive, SCF = Cefoperazonesulbactum. NA = not applicable

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Antimicrobial activity of solvent extract against MDR bacteria

A total of 6 trials were conducted to test the anti-MDR effects of web, using different solvent concentrations against each MDR bacterium. Following 24 h, a significant portion of 9.9 mg/ml and 11.55 mg/ml solvent extract concentrations showed prolific activity while those concentrations that were less than 9.9 mg/ml did not display sufficient activity. Activity was measured by zone of inhibition around the wells. The highest inhibition zone (22 mm) was recorded for *E. faecalis* while the lowest zone (04 mm) was formed against *E. coli* (*Fig. 1*).

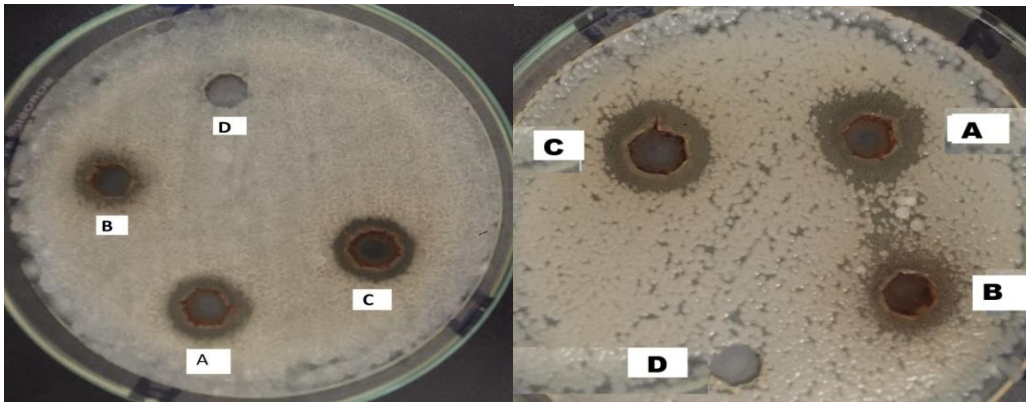

Figure 1. *Antimicrobial activity of the spider web was evaluated by means of well diffusion method by using different organic solvents acetone, ethanol and methanol as compared with control respectively (A, B, C and D). The web extract residues with different concentration (3.3 mg/ml 4.95 mg/ml 6.6 mg/ml 8.25 mg/ml 9.9 mg/ml and 11.55 mg/ml) showed promising activity. The presence of inhibition zones was measured, recorded and considered as indication for antibacterial activity. After 24 h a significant fragment of trials 9.9 mg/ml and 11.55 mg/ml solvent extract concentration showed proficient activity while the rest less than 9.9 mg/ml did not show enough activity. Activity was measured by zone of inhibition around the wells. The highest zone of inhibition (22 mm) was recorded for Enterococcus faecalis while lowest zone (04 mm) was formed against E. coli*

Anti-MDR bacterial potential of spider web metabolite extracts of the organic solvents acetone, ethanol, and methanol was analyzed. Solidified solvent extracts were dissolved in DMSO. An extract of distilled water was incorporated as the negative control**.** Results were described based on two parameters; organic solvents, and solvent extract concentrations.

Screening based on organic solvents

Spider silk was screened using different organic solvents to extract the web metabolites.

(i) Acetone

Acetone extract showed activity against all MDR bacteria, but higher activity was recorded against *E. coli, E. faecalis, and S. aureus*. The highest zone of inhibition (15 mm) was recorded for *E. coli,* whereas *E. faecalis, S. aureus, and S. typhi* each

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exhibited 12 mm inhibitory zones on average. Inhibitory zones of 10, 09, and 07 mm were recorded for *Bacillus, S. pneumonae* and *A. baumannii,* respectively (*Table 3*).

Table 3. *Antimicrobial activity of web with the acetone extract*

Pathogens	Zone of inhibition (mm) of acetone extract					
	3.3 mg/ml	**4.95 mg/ml**	**6.6 mg/ml**	**8.25 mg/ml**	**9.9 mg/ml**	**11.55 mg/ml**
Bacillus	-	-	-	-	-	10
S. aureus	-	-	-	7	9	12
S. typhi	-	-	-	8	-	12
Acinetobacter	-	-	-	-	7	9
E. faecalis	-	-	-	13	13	13
E. coli	-	-	-	14	14	15
S. pneumonae	-	-	-	-	05	07

(ii) Ethanol

Ethanolic extract showed the highest inhibition zone (17 mm) against *bacillus.* The second highest zone of inhibition (15 mm) was recorded for *A. baumannii*. However, the inhibitory zones of *S. aureus, S. typhi* and *E. coli* were 12, 10 and 10 mm, while *S. pneumonae* showed resistance upto some extent, its zone of inhibition was 08 mm. *E. faecalis* appeared to be resistant to ethanol extract as shown (*Table 4*)

Table 4. *Antimicrobial activity of web with the ethanol extract*

Pathogens	Zone of inhibition (mm) of ethanolic extract					
	3.3 mg/ml	**4.95 mg/ml**	**6.6 mg/ml**	**8.25 mg/ml**	**9.9 mg/ml**	**11.55 mg/ml**
Bacillus	-	-	-	-	16	17
S. aureus	-	-	07	09	10	12
S. typhi	-	-	-	05	05	10
Acinetobacter	-	-	-	-	-	15
E. faecalis	-	-	-	-	-	-
E. coli	-	-	-	-	7	10
S. pneumonae	-	-	-	-	5	8

(iii) Methanol

The highest zone with methanol was recorded against *E. faecalis* (22 mm). Methanolic extracts showed average class inhibition zones against other MDR bacteria as follows; *S. aureus* (12 mm), *Bacillus* (13 mm), and *A. baumannii* and *S. typhi* 11 mm). The lowest inhibitory zones (06 mm) were recorded for *E. coli* and *S. pneumonae* (*Table 4*).

Screening based on solvent concentration

Web solvent extracts with the following solvent concentrations were tested; 3.3 mg/ml, 4.95 mg/ml, 6.6 mg/ml, 8.25 mg/ml, 9.9 mg/ml and 11.55 mg/ml. Sufficient activity was recorded only for concentrations ranging from 9.9 to 11.55 mg/ml.

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(i) Inhibition zones with 11.55 mg/ml

The highest inhibition zone (22 mm) was recorded for the concentration of 11.55 mg/ml for *E. faecalis* with the methanolic extract, whereas the second highest zone of inhibition was recorded for *Bacillus* (17 mm) with the ethanolic extract. Both *A. baumannii* and *E. coli* showed 15 mm inhibitory zones while the pathogens *S. aureus* and *S. typhi* showed zones of 12 mm. Additionally, *S. pneumonae* showed a10 mm zone (*Table 4*).

(ii) Inhibition zones with 9.9 mg/ml

Solvent extracts with concentrations of 9.9–11.55 mg/ml showed high efficiency. The highest inhibition zone (16 mm) was recorded for *Bacillus* with ethanolic extract. *E coli* appeared to be more susceptible to acetone extract of 9.9 mg/ml as it produced a zone of 14 mm. Another high inhibition zone (13 mm) was recorded for *E. faecalis,* while11 mm inhibitory zones were observed against *S. typhi and S. aureus* and 06 and 07 mm inhibitory zones were recorded for *A. baumannii* and *S. pneumonae,* respectively (*Tables 3, 4* and *5*).

Table 5. *Antimicrobial activity of web with the methanol extract*

Pathogens	Zone of inhibition (mm) of methanol extract					
	3.3 mg/ml	4.95 mg/ml	6.6 mg/ml	8.25 mg/ml	9.9 mg/ml	11.55 mg/ml
Bacillus					10	12
S. aureus					11	13
S. typhi	-	-	-	07	11	11
Acinetobacter	-	-	-	-	-	11
E. faecalis	-	-	-	-	08	22
E. coli	-	-	-	-	04	06
S. pneumonae	-	-	-	-	06	06

Discussion

Accessibility to various antibiotics following the initial discovery of penicillin, initiated great enthusiasm in modern medicine (Sulaiman et al., 2014). The discovery of antimicrobials was one of the noteworthy discoveries in the history of microbial diseases. Following the discovery of antibiotics, humans became mostly free of harmful, deadly diseases. As a result of this medical miracle, people are able to live safe and healthy lives (Levy and Marshall, 2004). Currently, scientists are searching for natural sources of antibiotics that may help to develop antimicrobial agents against MDR bacteria. Novel antimicrobials have always been developed from natural sources. These sources may be microorganisms, plants and animals such as spiders and their metabolites. Spider silk is frequently cited as having antimicrobial properties in addition to several other scientifically verified biomedical properties (Chakraborty et al., 2009). Most studies on biomedical and mechanical properties of spider webs also mention their antimicrobial properties. Several studies describing antimicrobial properties of spider webs, may surface in literature reviews but these properties have not been tested against MDR bacteria. To our knowledge, this is the first study which addresses this crucial issue.

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In this study, silk of common house spiders was investigated for its qualitative antimicrobial potential. Aqueous, acetone, ethanol and methanol web extracts were tested against MDR bacteria including *S. typhi, S. aureus, E. faecalis, A. baumannii, bacillus, S. pneumonae* and *E. coli.* We determined that silk from the common house spider may act as an antimicrobial agent under various *in vitro* conditions. The studies related to antimicrobial potential of spider silk has been previously reported (Saleem, 2010; Laxminarayan, 2003). Web solvent extracts appear to be effective against all the MDR bacteria. Maximum inhibitory effects of all bacteria corresponded to concentrations ranging from 9.9 mg/ml and 12.55 mg/ml following after 24 h incubation period.

The most significant inhibition zone (17 mm) corresponding to increased activity was recorded against *bacillus* following 24 h of incubation. However, after 24 h, activity was not significant and decreased. Web extracts showed significant activity against *E. coli, Bacillus, S. aureus* and *S. pneumonae* as indicated by the diameter of their inhibition zones, which confirmed the findings of other studies, in which inhibition zones were; *E. coli* (8 mm) *B. subtilis* (14 mm), *S. aureus* (16 mm) and for *Streptococcus* species (12 mm) (Wright et al., 2012).

Among all MDR bacteria tested, *S. aureus* appears to be susceptible to minimum concentrations as well as maximum concentrations of solvent extracts. *E. faecalis* appears to be resistant to all solvent extracts to some extent.

Spider silk comprises certain constituents having antimicrobial potential such as potassium hydrogen phosphate, compound potassium nitrate and amino acids. These amino acids display broad spectrum activity, especially against a diverse group of microorganisms, including gram positive and gram negative bacteria and fungi (Gomes et al., 2011). Antimicrobial peptides bind to the lipid bilayer membrane of bacteria. Through this interaction bactericidal peptides attain an amphiphilic three-D configuration whereby its positive side interacts directly with negatively charged lipid head-groups resulting in the formation of pores in the bacterial membrane **(**Havard, 2006).

Thereby**,** potassium hydrogen phosphate lowers pH to approximately 4 by releasing protons into the aqueous solution, causing it to turn acidic. Such low pH prevents microorganisms from digesting web constituents (Case et al., 1999). Consequently, microorganisms are unable to degrade the web easily. Potassium nitrate is a web constituent that prevents protein from denaturing in the acidic condition**.** Therefore, the above stated components make spider silk a good agent for preservation.

Spider silk constituents show potential as novel preservative agents which may be useful in food, pharmaceutical and diary industries, among others. These are efficient and moderately in expensive with increased shelf life and reduced side effects. Silk producing genes of spiders may be manipulated to obtain higher yields of better quality silk, which may be utilized for industrial purposes.

Conclusion

Spider web metabolites used in this study displayed promising antimicrobial activity against MDR bacteria. Results indicate that spider web may function as a broad spectrum antimicrobial agent. Individual components responsible for its antimicrobial activity should be evaluated via cell culture assays for toxicity, as well as via NMR and HPLC for their molecular structure.

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