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Preliminary *In Vitro* Assessment of Antimicrobial and Haemostatic Activity of Spider Web Silk Extract

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Abstract: Spider web is made up of a material which is known as silk. Silk contains protein which shows various pharmacological action against various pathogens. The objective of this research work was to investigate antimicrobial as well as haemostatic action of spider web metabolized and to identify certain haemostatic and antimicrobial compounds. Web solvent extract (acetone, methanol and ethyl acetate) was screened for *in vitro* antimicrobial activity. Most of the extracts demonstrated potent antimicrobial activity at 400 µg/ml and 800 µg/ml by cup plate method. Acetone extract of spider silk showed profound antimicrobial activity against gram positive organism *Bacillus subtilis*, ethyl acetate extract against gram negative test organism *Escherichia coli*, methanol extract against *Pseudomonas aeruginosa* and in ethyl acetate extract against gram positive organism *Staphylococcus aureus* and antifungal activity against test organism *Candida albicans*. The evaluation of *in vitro* hemostatic activity of the web solvent extract demonstrated the partial effect on promoting hemostasis, which may be partly contributed to the platelet aggregation and subsequent activation of the primary hemostasis. So further experiments should be designed to isolate the active ingredients and investigate possible hemostatic activities and mechanisms of web extract.

Keywords: Spider silk, Extraction, Antimicrobial activity, Haemostatic activity, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli*

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Introduction

Morphologically all spiders have particular common anatomical features. They have two body segments, called the abdomen and the cephalothorax, which are connected by a narrow trail called a pedicel. The parts which are of most importance of spider morphology are the silk glands and the spinnerets, where the silk is manufactured. Spider silk is used for a variety of different purposes such as web spinning, cocoon construction and for depositing sperm. Spider silk is initially in a liquid form inside the spider, and becomes solidified once exposed to the air. The weight of spider silk's in liquid form is ten times less than its solid form, and whilst it is initially soluble in its liquid form, once solidified it becomes insoluble (Garb and Hayashi, 2010). Silks are largely composed of non-essential amino acids but the precise composition varies (Vollrath and Knight, 1995). The major use of spider webs by human is wound healing due to the antiseptic properties of the spider silk (Newman and Newman, 1995). Biotechnological application of spider silk described is for using recombinant spider web silk components as drug delivery medium (Lammel *et al.*, 2011). Nowadays spider silk is also magnetizing attention due to its antimicrobial properties and there are unsubstantiated reports regarding the antimicrobial potential of spider silk (Wright and Goodacre, 2012) as its acidic properties either prevent/inhibit the growth of the microbes. Bacteriostatic activity of spider silk has also been attributed to the presence of potassium nitrate, which inhibit the growth of microbes such as *Bacillus subtilis* and *Escherichia coli* (Chakraborty and Das, 2009). According to reports on antibiotic resistance, more than a hundred thousand deaths occur each year (Bengtsson-Palme *et al.*, 2018). Traditional uses for spider web as an antiseptic include atypus spider webs as topical antiseptics in the Carpathian Mountains and other rural areas of various countries (Wright and Goodacre, 2012). The goal of this study was to investigate the antibacterial and hemostatic activity of spider web

metabolites with different concentrations of web extract used against various gram positive, gram negative, and some fungal strains as well as to investigate the haemostatic activity against standard drugs.

Materials and Methods

The web of silk from *Tegenaria domestica*, a common house spider, was collected from Dhubri, Assam, India during January, 2022. After collection of webs it was separated from dust and other particles. Associated macro and micro particles were removed manually, washing with distilled water and dried in shadow. Sunlight drying has to be avoided as the thermostatic substance present in spider net will get degraded as a result reduction of the pharmacological action. Before making extraction of spider net, made sure that the nets were clean, dry and free from all other microorganism. To make free from microorganism, sterilization of dry spider net should be done. Moist heat sterilization with help of autoclave is widely used for purpose of sterilization.

Extraction Procedure:

Ethyl acetate and acetone extract were obtained by dipping dry web silk for about 48 h. About 20 g of dry web silk was added to 100 ml solvent in the brown glass sealed jar. It was filtered and pure extract was stored in sealed bottle. Methanol extract was obtained through Soxhlet extractor (Mirghani *et al.*, 2012).

Qualitative analysis of web silk extracts:

The spider web preliminarily underwent the 20 h hydrolysis with hydrochloric acid at 110°C. The amino acid division was performed (Wright, 2011) on silica gel plates, using a phase consisting of the following solvents: acetone: butanol: acetic acid: water in a ratio of 7: 7: 2: 4. The chromatograms took 4 hours to develop, and 0.5% ninhydrin solution was used to stain the plates (Fig. 1). Following preliminary examination, TLC qualitatively contains the following amino acids:

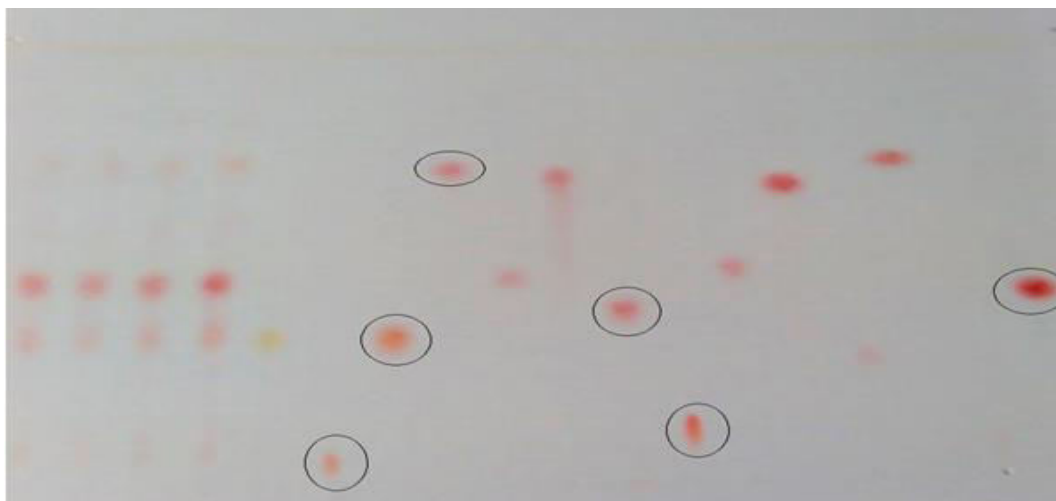


Fig. 1: Developed TLC plate in daylight caused by 0.5% ninhydrin solution.

glutamic acid ($R_f=0.398$), serine ($R_f=0.375$), threonine ($R_f=0.458$), arginine ($R_f=0.093$), phenylalanine ($R_f=0.712$) and aspartic acid ($R_f=0.244$).

In-vitro antimicrobial activity (Alam et al., 2014):

The agar test was used to determine the synthetic compounds' antibacterial properties. All bacteria were grown on Mueller-Hinton agar (Hi-media) plates (37 °C, 24 h). All the extracts were subjected to antimicrobial screening by cup-plate method for zone of inhibition. The antibacterial activity was tested against various gram-positive and gram negative bacteria compared with standard drug Ampicillin and Ketoconazole using solvent control. The microorganisms selected for antimicrobial activity were-- *Bacillus subtilis* (ATCC11774), *Staphylococcus aureus* (NTCC6571), *Escherichia coli* (TG14) and *Pseudomonas aeruginosa* (ATCC9027) and two fungal stains viz. *Candida albicans* and *Aspergillus nigricans*.

Minimum inhibitory concentration (MIC) (Alam, 2015):

The lowest concentration (highest dilution) of extract preventing the appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the extract is known to be bacteriostatic. Bacterial strains were

primarily inoculated into Mueller-Hinton agar for overnight growth. To adjust the inoculum size for the test strain to 108 CFU ml⁻¹ (Colony Forming Unit per millilitre) per well, the turbidity of a sample was compared to that of the McFarland standard before a number of colonies were directly suspended in saline solution (Turbidimetric method). Similar inoculation techniques and inoculum standardisation steps were used while inoculating fungus onto Sabouraud Dextrose broth. The extracts were dissolved in DMSO and the solutions were diluted with a culture medium in order to get the necessary concentration of the extract and standard medicines for testing upon standard microbiological strains. Each extract and the standard medications were diluted to create a stock solution with a 2000 mg/ml concentration. The necessary concentrations for primary and secondary screening were attained through additional incremental dilutions using the test medium. In primary screening, 0.1 ml of culture (bacterial and fungal) was added to the solution of 1000 µg/ml, 500 µg/ml and 250 µg/ml concentrations of the extracts. The active extracts found in this primary screening were further diluted to obtain 200 µg/ml, 100 µg/ml, 62.5 µg/ml, 50, 25 µg/ml, 12.5 µg/ml and 6.250 µg/ml concentrations for secondary screening to test in a

second set of dilution against all microorganisms. Eight MIC tubes were taken and labeled it as number 1-8. Briefly, the control tube (8th tube) containing no antibiotic is immediately sub-cultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of the tested organism. The tubes are then kept for incubation at 37 °C for 24 h for bacteria and 48 h for fungi. Growth or a lack of growth in the tubes containing the antimicrobial agent was determined by comparison with the growth control, indicated by turbidity. The lowest concentration that completely inhibited visible growth of the organism was recorded as the minimal inhibitory concentration (MIC, µg/ml), i.e., the amount of growth from the control tube before incubation (which represents the original inoculum) is compared. To ensure that the solvent had no impact on strain growth, a set of tubes containing only seeded broth and the solvent controls were kept under similar circumstances. The size of the inoculums has a significant impact on the outcome. The test combination must include 10⁸ CFU ml⁻¹ organisms. The interpretation of the results was based on amphotericin B break points for the fungi and also on amoxicillin for bacterial pathogens. Minimal inhibitory concentration (MIC) values.

Haemostatic Activity (Shital et al., 2013):

Clotting time of blood in presence of various synthesized compound was determined *in vitro* using Lee White's method as follows:

In a sterile and dry corning glass tube, human venous blood was drawn. Clotting times were calculated and compared in both the presence and absence of different substances. 100 mg of dry extract was dissolved in 0.5 ml of distilled water. In experimental sets, this extract preparation was utilised. A clean, grease- and detergent-free corning glass tube with a 1 cm diameter was used to collect 1 ml of freshly drawn human venous blood, which was then added to a 0.5 ml mixture of various extract preparations. The reference drug for hemostatic was Heparin

at a concentration of 1 IU/ml and control determination was performed using 0.5 ml of distilled water instead of solution of extract. The clotting time of the different test extracts and control drug was recorded.

Results and Discussion

We learn a lot of facts about the strength of spider silk that sometimes seem contradictory, such as that not all spiders can produce all the types of silk that can be produced, and that scientists typically refer to draglines when evaluating the strengths of silk. The data we do have applies to the strongest spider silk we are aware of contradictory, although, spider silk is way stronger than any other silk or natural material silks (Hu *et al.*, 2006; Huemmerich *et al.*, 2008), like many other biomaterials, have a hierarchical structure, The amino acid sequence of a protein's basic structure is amino acid analysis. From the qualitative analysis of the extracts it has been found that the spider silk is made up of amino acid sequence. After preliminary analysis TLC method, the following amino acids has qualitatively evaluated: glutamic acid ($R_f=0.398$), serine ($R_f=0.375$), threonine ($R_f=0.458$), arginine ($R_f=0.093$), phenylalanine ($R_f=0.712$) and aspartic acid ($R_f=0.244$) (Fig. 1). As we know that Potassium nitrate is one of the major web constituent that can be prevented from denaturing in the acidic condition, so above proteins components make spider silk a good agent for preservation.

The spider silk which is obtained from kitchen or fuel smoke area showed a potential inhibition towards both gram positive and gram negative bacteria. It has been found that the gram positive bacteria get more affected by silk extract as compared to the gram negative bacteria. The inhibition zone was measured in mm scale (Table 1). Table 1 shows that the inhibition zone in case of gram positive bacteria is greater than the gram negative bacteria. The silk extract produce inhibition zone like 15 mm and 24 mm at conc. of 400 µg/ml and 800 µg/ml in gram positive bacteria (*B. subtilis*) of acetone extract and in

Table 1: The antibacterial and antifungal properties of various spider web silk extracts

Solvent Extract	Dose ($\mu\text{g/ml}$)	Mean Zone of inhibition (mm)					
		<i>B.s</i>	<i>S. a</i>	<i>E.c</i>	<i>P.a</i>	<i>A. n</i>	<i>C.a</i>
Acetone	400	15 $\pm$ 0.23*	-----	-----	-----	10 $\pm$ 0.07	5 $\pm$ 0.26
	800	24 $\pm$ 0.90**	8 $\pm$ 0.09	9 $\pm$ 0.23	8 $\pm$ 0.64**	16 $\pm$ 0.87*	11 $\pm$ 0.55*
Ethyl A	400	-----	14 $\pm$ 0.08	13 $\pm$ 0.34**	-----	9 $\pm$ 0.45**	16 $\pm$ 0.56
	800	6 $\pm$ 0.50	20 $\pm$ 0.29	23 $\pm$ 0.45*	7 $\pm$ 0.12	12 $\pm$ 0.34	20 $\pm$ 0.81*
Meth.	400	10 $\pm$ 0.23**	-----	7 $\pm$ 0.56	19 $\pm$ 0.27**	11 $\pm$ 0.77**	7 $\pm$ 0.74**
	800	13 $\pm$ 0.65**	5 $\pm$ 0.02**	10 $\pm$ 0.67**	22 $\pm$ 0.33*	14 $\pm$ 0.56**	9 $\pm$ 0.54
Amp	1000	28 $\pm$ 0.80*	25 $\pm$ 0.19**	27 $\pm$ 0.07*	24 $\pm$ 0.87*	-----	-----
Nystatin	8	-----	-----	-----	-----	22 $\pm$ 0.25*	23 $\pm$ 0.41*
DMSO	-----	-----	-----	-----	-----	-----	-----

Values are mean ($n = 3$). *B.s*: *Bacillus subtilis*; *S.a*: *Staphylococcus aureus*; *E.c*: *Escherichia coli*; *P.a*: *Pseudomonas aeruginosa*; *A.n*: *Aspergillus niger*; *C.a*: *Candida albicans*; Am: Ampicillin; Meth: Methanol; Ethyl A: Ethyl acetate; ‘-’ indicates no sensitivity or mean inhibition zone diameter lower than 4 mm. * $p < 0.05$, ** $p < 0.01$

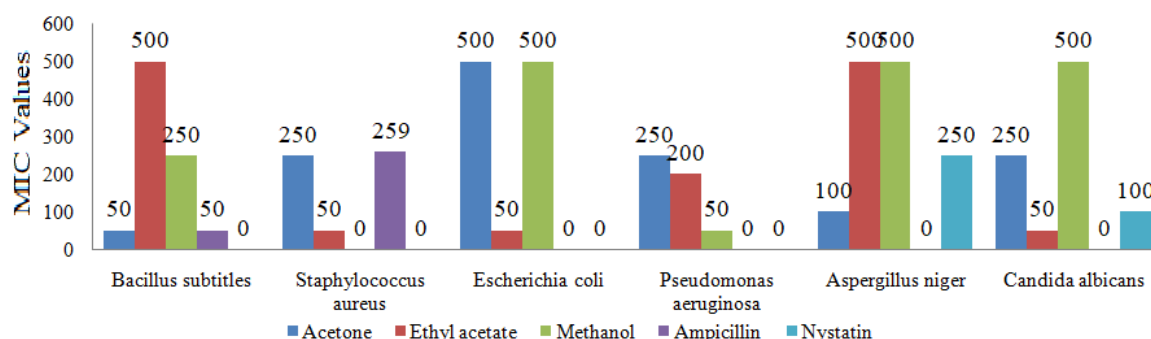


Fig. 2: Minimum inhibitory concentration (MIC, $\mu\text{g/ml}$). Data are expressed as mean $\pm$ standard deviation ($n = 3$).

case of gram negative bacteria (*E. coli*) it produce 16 mm and 20 mm at conc. 400 $\mu\text{g/ml}$ and 800 $\mu\text{g/ml}$, respectively of ethyl acetate extract after 24 h of incubation at 37 °C. Acetone extract of spider silk showed lowest MIC value as compared to the other extracts in case of *B. subtilis*, while in case of *E.coli* it gives more profound MIC value against ethyl acetate extract (Fig. 2). Only methanol extract give significant MIC value against *P. aeruginosa*, while *Aspergillus niger* has no significant activity against all the tested fungal strains. It indicated that spider silk against fungal strains does not give similar activity as compared to the bacterial strains. Moreover, antimicrobial peptides create an acidic environment above pH 4 which is not suitable for fungal growth. It was estimated that fungus types are resistant to silk or bacteria present are masking the antifungal

properties. The result of zone of inhibition and MIC by serial dilution method suggested that some strains sample show positive result but after 24 h no antifungal activity was detected. Nystatin (8 $\mu\text{g/ml}$) was used as standard in antifungal activity. Results show that action of spider silk oil is specific against fungus, against some fungal strain result was positive and against some strains was negative. Thus, it could be used against specific fungal strains.

All the extracts were subjected for haemostatic activity determined *in vitro* using Lee White's method. In a sterile, dry, and corning glass tube, human venous blood was drawn. Clotting time was evaluated and compared in the presence and absence of different substances. Distilled water has been taken as control instead of synthesized compound solution. It was discovered that acetone

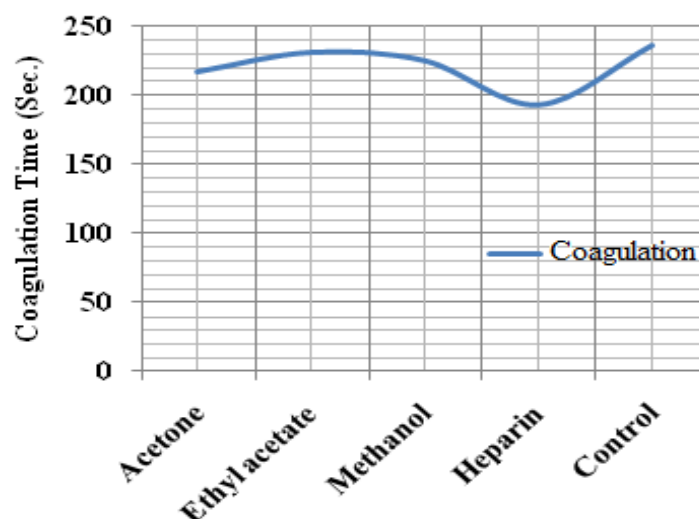


Fig. 3: Study of haemostatic activity and amount of time required for clot formation was measured. Data are expressed as means $\pm$ standard deviation ($n = 3$). *Coagulation time was under 236s. * $p < 0.05$; ** $p < 0.01$.

extract was better among all the synthesized compounds when compared to distilled water taken as control solution whereas remaining compounds found to be moderate to less active (Fig. 3). It was found that the ability of acetone extract of spider silk in platelet micro aggregation was nearer to that of the standards. GP IIb/IIIa, a glycoprotein on the platelets, plays an important role in the platelet aggregation during hemostasis. Upon activation of platelets, a series of signaling molecules (prostanoids such as thromboxane A₂, PGE₂, etc.) are released in order to induce conformational changes to the GP IIb/IIIa receptors and increase their binding affinity to fibrinogen. This procedure promotes platelet interaction and their subsequent aggregation.

Conclusion

Some *in vitro* experiments examining the potential of spider silk as an antimicrobial agent have been less than robust. Very low-powered activity in the presence of a gram-positive bacteria strain appeared to be sensitive to degradation over time, indicating a possible bacteriostatic action rather than a bactericidal action. Activity against a chosen strain of gram-negative bacteria was weak and decreased over time. The high amount of variation in the data meant that the inhibitory

effect of the silk was not shown on every single sample. When examined after 24 h of growth there was a trend in the samples with silk present showing less growth of bacteria. Its fundamental structure is its amino acid sequence, which primarily consists of blocks of glycine and alanine that are extremely repetitive which provide the antioxidant property of spider silk. 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*. The current study demonstrated the partial effect of web silk of spider on promoting hemostasis, which may be partly contributed to the platelet aggregation and subsequent activation of the primary hemostasis. However, the involvement of the other mechanisms cannot be neglected and needs to be studied.

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