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Activity of *Argiope catenulata* (Grass Cross Spider) web againstselected microorganisms

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Abstract

This study investigated the antimicrobial properties of *Argiope catenulata*, an endemic spider species due to the increasing incidence of antimicrobial resistance against clinically significant and commonly isolated organisms. Spider web has the promising potential to be the source of antimicrobials, however studies regarding endemic spider species in the Philippines were limited. In this study, the spider web was dissolved in 2.5% NaOH solution and was tested using the Kirby Bauer Method, Macro Broth Dilution and Agar Diffusion Method to determine the inhibitory properties of the spider web. A zone of inhibition was performed as a screening test to determine the minimum inhibitory and microbicidal concentration of the web solution. The starting stock solution of 500ug/mL was filtered and was used to prepare web solutions as it was serially diluted on the succeeding tubes. One-fold decrease in the starting solution was used on the whole process. A concentration of 31.25 ug/mL was found to be inhibitory and 62.5ug/mL was microbicidal against *Staphylococcus aureus, Escherichia coli* and *Candida albicans* whereas, a concentration of 15.63ug/mL was inhibitory and 32.25ug/mL was microbicidal against *Aspergillus niger* and *Neisseria gonorrheae*. On the other hand, a concentration of 62.5ug/mL was inhibitory, and 500ug/mL was microbicidal against *Bacillus subtilis*. Therefore, this study proved that *Argiope catenulata's* web could be a new source of antimicrobial agent.

Keywords: *Antimicrobial, Argiope catenulata, , Spiders, Spider web,*

INTRODUCTION

The natural environment is a great source of curative and preventive products for many diseases that arise in our health today. Because of the increased likelihood of new cases and the increased risk of antibiotic resistance (Roozbahani, Asmar, Ghaemi and Issazadeh, 2014), the investigation of various chemical structures and new antimicrobial compound mechanisms is highly encouraged (Zuridah, Fairuz, Zakri and Rahim, 2008). The explosive growth of resistant strains globally jeopardizes the efficacy of antibiotics, which have transformed medicine and the efficacy to save thousands of lives. Unfortunately, resistance to these antibiotics ultimately has increased overtime (Ventola, 2015). Many therapeutic compounds derived from natural products at different stages of clinical development illustrated the existing soundness and importance of the use of natural products as potential future sources of new drug candidates worldwide (Veeresham, 2012). Existing antibiotics have been proven to fight and cure diseases all over the world, but the cheap and reliable antimicrobial compounds are already losing their power because of misuse. Since spider cobweb do not cause an immune response in humans, it is then regarded as potentialcandidate for antimicrobial research (Al-kalifawi and Kadem, 2017).

Argiope catenulata (Figure 1) is an orb weaver which is colorful in appearance and makes circular webs in rice canopy and are prey catchers. It lays 600-800 eggs in its whole lifespan of 2-3 months. When the day is hot, this spider hides under leaves beside the web and when the day is cloudy, the female waits on the center of the web while the male waits nearby (Shepard, Barrion and Litsinger, n.d.). There are several published studies already regarding on the antimicrobial properties of a spider web (Al- kalifawi and Kadem, 2017; Amaley, Gawali and Akarte, n.d.; Ayroza, Ferreira, Sayegh, Tashima and da Silva Junior, 2012; Rameshwar Maheshwari and Zumberlal Chopda, 2018; Roozbahani et al., 2014; Saravanan, 2006; Schulz, 1997; Tahir et al., 2017; Vierra, Hsia, Gnesa, Tang and Jeffery, 2011; Wright and Goodacre, 2012). On closer examination only, a small number of spider species are dangerous to man. Serious bites are caused often by small inconspicuous spiders such as the black widow (Latrodectus sp.). Other dangerous genera are *Atrax, Loxosceles, Phoneutria*, and *Harpactirella.* The spider venom often creates intense pain, but if treated properly, seldom leads to death (Schulz, 1997).

Silk proteins are produced and excreted in nature by

specialized cells lining a gland's epithelium; this is the major ampullate gland in the case of dragline silks. Proteins are stored in the lumen of the glands at remarkably high concentrations without aggregation as the so-called dope and assembled in a stable and tough way through the spinning duct (Spiess, Lammel and Scheibel, n.d.). The variety of physical properties noted is likely to reflect variances in the relative proportions and combinations of specific amino acidssuch as alanine, glycine and proline (Wright and Goodacre, 2012).

A comprehensive review of studies indexed in the Google Scholar database reveals the absence of studies regarding *Argiope catenulata* and its spider web.

The supporting references directly stated that there are evidences of antimicrobial activities but with other kind of spiders used. The choice to investigate on the *Argiope catenulata* is because it is ubiquitous in nature and to add to its usefulness. There is a need to develop alternative antimicrobials because of increasing incidence of patterns of resistance to commercially available antibiotics.

Figure 1. *Argiope catenulata*

Thus, this study aims to determine the effects of the *Argiope catenulata*'s web against selected organisms. The determination of the minimum microbicidal effects of the *Argiope catenulata's* web will then allow us to prove that there are antibacterial and antifungal compounds present on the spider silk. Macro broth dilution assay was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the web solution. It utilized liquid growth medium with decreasing concentrations

(typically a onefold dilution series) of the antimicrobial solution and then inoculated with a definite volume of microbial suspension (Wiegand, Hilpert and Hancock, 2008) According to Wiegand et. al (2008), the minimum inhibitory concentration is the least concentration of the antimicrobial solution which inhibits the occurrence of visible growth in a maintained condition. Furthermore, minimum bactericidal concentration is defined as the lowest concentration that exhibits exhaustion of microbial growth (Chikezie, 2017). The purpose of the study is to investigate the potential of *Argiope catenulata*'s web as an alternative to other antimicrobial regimens. This study will then pave way to more diverse and eco-friendly way of discovering useful and helpful products naturally, as antimicrobial resistance is being alarmingly reported with increasing frequency around the world.

MATERIALS AND METHODS

Collection of *Argiope catenulate*

The collection of *Argiope catenulata* was done through hand picking method from San Juan, Batangas. We were assisted by the municipal agriculturist throughout the collection to ensure safety and observance of proper protocol. These spiders were authenticated by University of the Philippines Los Baños Museum of National History.

Preparation of *Argiope catenulata* websolution

Preparation of *Argiope catenulata* web solution was based on the protocol of Tahir, Qamar, Sattar, Shaheen, and Samiullah (2017) with slight modifications. Inour method, a clean box was used to rear the spiders in the laboratory foe the recoveryof *Argiope catenulata* webs, instead of usingplastic containers. The spiders were given fruit flies ad libitum. The collection process took only about 1 to 2 hours for 50 mg of web. For the preparation of the stock solution, spider webs were collected by allowing a sterile glass rod inside thecontainer for the spiders to weave on it. We measured the mass of the glass rod before and after it was weaved to get the mass of the collected spider web. A 50 mg of *Argiope catenulata* was dissolved in a 100 mL of 2.5% NaOH solution. This stock solution was used to prepare web solutions with different concentrations. To ensure that there will be no contamination in the solution, the web solution was passed through a filter paper with a pore size of 0.4

micrometer (Roozbahani et al., 2014).

Bacterial and fungal strains

Commercially obtained pure culture of gram-positive and gram-negative cocci and bacilli namely *Bacillus subtilis* (*ATCC 6633*), *Escherichia coli* (ATCC 25922), *Neisseria gonorrheae* (ATCC 49226), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 14053), and *Aspergillus niger* were used to determine theantimicrobial activity of *Argiope catenulata*spider web. These strains were collected from University of the Philippines-Manila, Department of Medical Microbiology and Batangas Medical Center, PathologyDepartment.

Media Preparation

Preparation of Mueller Hinton Agar (MHA). Thirty-eight grams of the agar was dissolved in one liter of distilled water which was then subjected to heat to dissolve. Upon autoclaving at 121 degree Celsius for 15 minutes, the mixture was cooled at room temperature and was transferred to 100-millimeter plastic petri plates

Preparation of Mueller Hinton Broth (MHB). An amount of thirty grams of themedium was suspended in one liter of distilled water, mixed well and then dissolved by heating. The solution was dispensed into twelve screw capped tubes and then autoclaved at 121 degrees Celsius for 15 minutes.

Preparation of Sabouraud'sDextrose Agar (SDA). Sixty-five grams ofthe medium was suspended in one liter of distilled water, mixed and then heated until dissolved. It was then autoclaved at 121 degrees Celsius for 15 minutes.

Preparation of Saboraud's Dextrose Broth (SDB). Thirty-five grams of the medium is dissolved in one liter of distilled water, mixed thoroughly until dissolved. The medium was then autoclaved at 121 degrees Celsius for 15 minutes and thendispensed onto plates.

Chocolate Agar Plate. Commerciallyprepared chocolate agar plates enhancedwith glycerol were obtained from STD/AIDS Cooperative Central Laboratory (SACCL) in San Lazaro Hospital, Sta. Cruz, Manila.

Disc Diffusion Assay

Disc diffusion assay was performed to screen for the inhibitory properties of the spider web in accordance to the method of Al-kalifawi and Kadem, (2017). The bacterialand fungal isolates were streaked on Mueller Hinton agar and Sabouraud's Dextrose agar plates respectively. Chocolate agar plates were used for *Neisseria gonorrheae* due to its fastidious nature. Each plate contained blank discs which were infused with distilled water, NaOH, 500ug/mL web solution and the designated antibiotic control for eachorganism. The concentrations used for the spider web solution and antibiotics were patterned with the panel recommended by Clinical Laboratory and Sciences Institute (CLSI) Guidelines. After incubation of theplates for 18-24 hours at 35 degrees Celsius, zones of inhibitions were measured using micro caliper.

Determination of Minimum Inhibitory Concentration (MIC) of the prepared web solution

Sterilized 13 screw capped test tubes were used and numbered accordingly. One milliliter of Mueller Hinton broth was introduced into tubes 2 to 11. On tube 12, 2 mL of MHB was poured. One mL of the diluted bacterial and/or fungal suspension was introduced on tubes 1 to 11 and tube 13.To the 13th tube, additional 1mL of antibiotic was poured. The tightly screwed tubes were gently shaken. All the tubes were incubated for 18-24 hours at 37degrees Celsius. The growth was checked by looking at the turbidity of the tubes which appeared as whitish pellet at the bottom of the tube. The tube having the lowest concentration of the web solution that have no growth or turbidity observed was regarded as the MIC of the web solutionagainst either bacterial or fungal isolates.

An MIC will be valid if tube 11 or thenegative control tube showed visible growth.However, tubes 12 and 13, the media and antibiotic or positive control respectively must show no growth as well.

Determination of Minimal Bactericidal or Fungicidal Concentration of the Prepared Web Solution

The minimum inhibitory concentration and the first tube which showed visible growth on the Mueller Hinton Broth were subcultured by streaking or spreading 0.01 mL on Mueller Hinton Agar plates. The plates were numbered according to where the subcultures originated. The plates were inverted and incubated at 37 degrees Celsius for 18 - 24 hours. Growth was observed after the required incubation period and the concentration of the web

solution producing one or no colony at all on Mueller Hinton agar plates was reported either as the Minimal Bactericidal Concentration (MBC) of the prepared solution as to the bacterial isolates or as the Minimal Fungicidal Concentration (MFC) as to the fungal isolates.

Characterization of the antimicrobialproperty of the web of *Argiope catenulate*

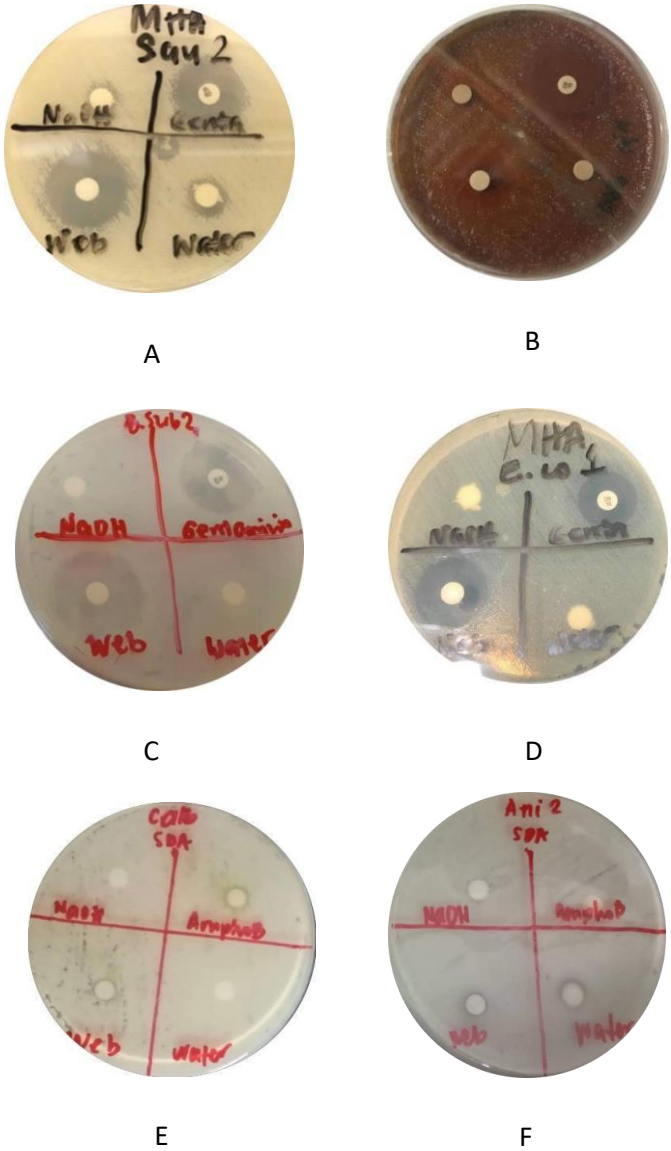
This study used the protocol by Wright and Goodacre (2012) to further characterize the active agents on the spider silk that inhibit microbial growth. The silk was subjected to a variety of treatments; ultraviolet exposure (254 nm) for 20 minutes, 1-2 hours soaking of spider silk in distilled water, and incubation with proteinase K for 1-2 hours before use. Pre- treated spider silk was dissolved using 2.5% NaOH and was tested using the macro broth dilution assay again. Proteinase K was used to check if the agent present on the silk behaved as if it was a protein i.e. under the conditions applied to be destroyed or denatured. The exposure to ultraviolet light was expected to damage DNA and thus affect the microorganisms already present in the silk sample, but not necessarily to denature protein. Water soaking was used to determine whether an active agent is present and is soluble on the silk surface.

RESULTS AND DISCUSSION

Screening for Antimicrobial activity using *Argiope catenulata* web Table 1 shows the results of the disc diffusion assay. Evident zones of inhibition were observed in the antibiotic disc control and the web solution. No inhibition was observed on the disk which was 2.5% NaOH and the disk infused with distilled water.

However, in our study, sensitivity was at 15 mm (Figure 2 B) which is considered resistant by CLSI. Figure 2 (C) shows inhibition of *o* at 22 mm which also complies with CLSI standard of sensitivity at 20 mm. On the other hand, CLSI standard for Escherichia coli renders sensitivity at 19 – 26 mm. As shown in Figure 2 (D), the spider web solution was effective for E. coli at 21 mm. According to Roozbahani, et al. (2014), spider web solution prevents bacterial growth of gram-positive bacteria more than gram-negative bacteria.

Consequently, this assay shows Staphylococcus aureus and *Bacillus subtilis* were more significantly inhibited as compared to *Escherichia coli and Neisseria gonorrheae.* Moreover, sensitivity to Candida albicans and Aspergillus niger in Figure 2 (E) & (F) showed a diameter of 7 mm which is considered resistant by CLSI since the



A B C D E F

Figure 2. Zones of inhibition against selected microorganisms in millimeter A) *Staphylococcus aureus; B) Neisseria gonorrheae; C) Bacillus subtilis; D) Escherichia coli; E) Candida albicans; F) Aspergillus niger*

Table 1
Antibacterial Susceptibility Assay

Microorganisms	Argiope catenulate web solution 500ug/mL	Antibiotic	Distilled water	NaOH
Staphylococcus aureus ATCC 6538	19 mm	15 mm	6 mm	6 mm
Neisseria gonorrheae ATCC 49226	15 mm	47 mm	6 mm	6 mm
Bacillus subtilis ATCC 6633	22mm	20 mm	6 mm	6 mm
Escherichia coli ATCC 25922	21 mm	19 mm	6 mm	6 mm
Candida albicans ATCC 14053	7 mm	9 mm	6 mm	6 mm
Aspergillus niger	7 mm	8 mm	6 mm	6 mm

standard diameter of discs used measures 6 mm only. Once the zones of inhibition were interpreted for each microorganism, further tests were performed through Macro Broth dilution and Agar Diffusion method for the determination of the minimum inhibitory and minimum microbicidal concentrations (MIC/MBC). However, since *Neisseria gonorrheae, Aspergillus niger* and Candida albicans showed resistance on the spider web solution, determination of MIC and MBC/MFC were not necessary anymore.

Table 2 shows the measurement of the antimicrobial concentrations of *Argiope catenulata* web solution on three bacteria which showed sensitivity on the screening test, disc diffusion. MIC is used by laboratories mainly to confirm resistance and as a research tool to discover new antimicrobials (Andrews, Working, & Testing, 2001).

Figure 3 depicts the different methods used for the determination of MIC and MBC for *Staphylococcus aureus.* Determination of MIC and MBC was done after knowing which of the microorganisms showed sensitivity on the spider web through the zones of inhibition. *Staphylococcus aureus* ATCC 6538 showed significant clearing up to 31.25□g/mL concentration of web solution, while bactericidal effect was noted on a one-fold increase, at 62.5□g/mL of concentration. No growth was observed on the plates after incubating it for 18-24 hours at 37 °C.

Table 2
MIC Minimum Inhibitory Concentration, MBC Minimum Bactericidal Concentration

Microorganisms	Argiope catenulate web solution		Gentamicin (ug/mL[2)]
	MIC (ug/mL)	MBC/MFC (ug/ML)	
Staphylococcus aureus ATCC 6538	31.25	62.5	500
Bacillus subtilis ATCC 6633	62.5	500	500
Escherichia coli ATCC 25922	31.25	62.5	500

Figure 4 is for *Bacillus subtilis* ATCC 6633, and 62.5μg/mL was noted as the minimum inhibitory concentration because this was the lowest concentration which displayed clearing on the tubes and showed visible growth on MHA plate after overnight incubation. The minimum bactericidal concentration for *Bacillus subtilis* was at 500□g/mL showing that it is effective on effect. This is because the bacteria grow this bacterium but is requiring the highest concentration to exert its bactericidal effect. This is because the bacteria grow in soil and remains in close contact with the spider silk leading to the development of its resistance to the web's antimicrobial properties. Another possible reason is that the antimicrobial agent is bacteriostatic in action thus slowing the bacteria's growth instead of killing them (Wright &Goodacre,2012). Because of this, inhibition against *Bacillus subtilis* is seen only after 24 hours and not more than 48 hours (Wright, 2011).

Characterization

Proteinase K and Distilled water

The results of the antimicrobial activity of the *Argiope catenulata* web soaked in both Proteinase K and distilled water against Staphylococcus aureus, *Neisseria gonorrheae*, *Bacillus subtilis*, *Escherichia coli* were obtained. In Figure 6, microwell serial dilution was performed and the absorbance was read at 640 nm. On the 13 tubes and concentrations, no inhibition was observed on all microorganisms tested as indicated by the increase in absorbance.

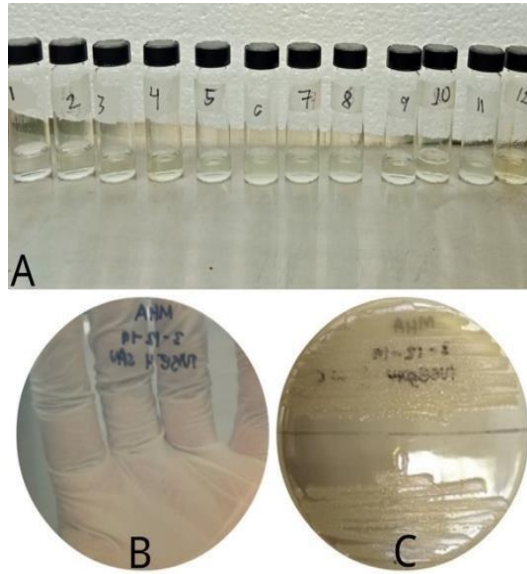


Figure 3. Determination of MIC and MBC on *S. aureus*. A) Macrobroth dilution B) MBC C) MIC

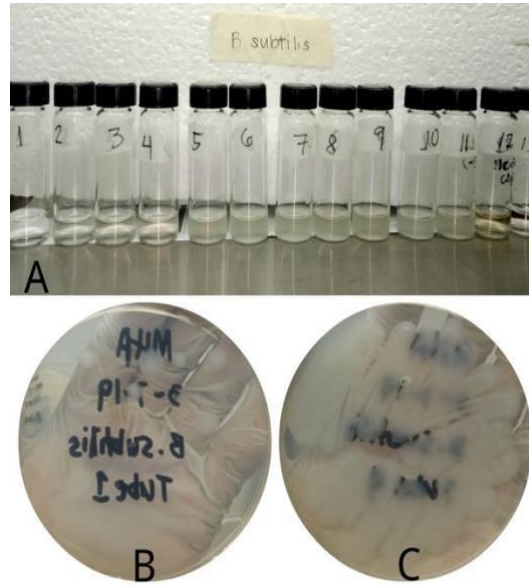


Figure 4. Determination of MIC and MBC on *B.subtilis*. A) Macrobroth dilution. B) MBC C) MIC

This showed that the antimicrobial property was lost after being soaked in Proteinase K and distilled water, which action was to denature proteins and to remove soluble contaminants on the web, respectively (Augsten, Mühlig and Herrmann, 2010). Peptides or proteins that affect the pathogenic microorganisms comprise the spider web (Coutinho, Lobo, Bezerra and Lobo, 2008) or may be soluble to distilled water (Wright, 2011) ergo its antimicrobial activity did not manifest

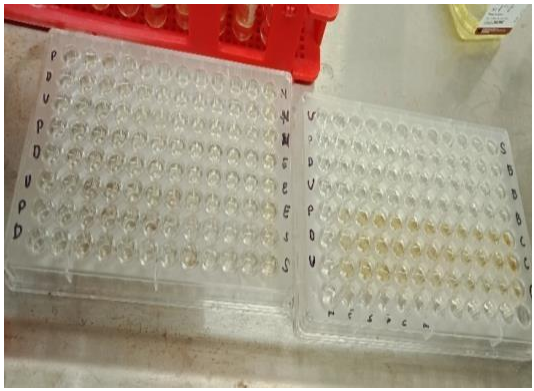


Figure 6. Characterization of active agents on the spider web measured at 640nm

Ultraviolet Light

The results showed decreased absorbance as the concentration of the web decreases therefore, UV exposure Spider web is subjected to UV light in the environment therefore, it is expected that exposure to UV rays will not remove its antimicrobial property. The reason is that theUV-treated spider web showed a more consistent and stronger reduction in the growth of the test microbe than untreated spider web. It is possible since the UV rays killed some of the bacteria present on the spider web. This reduced the effect on the absorbance rates of the other bacteria and could show a better representation of the effect on the tested microbes of spider web (Wright, 2011). It indicates that its antimicrobial property is not affected by subjecting the spider web to ultravioletlight. A decrease in absorbance on the first three tubes of *S. aureus* indicated that the spider web was able to inhibit the growth of the *Staphylococcus aureus* as well as *Neisseria gonorrheae.*

In this study, the results obtained for *Bacillus subtilis* were insignificant and highlysuggests that the *Argiope catenulata* spider

web solution was least effective on it compared to other bacterial isolates used.

On the other hand, *Escherichia coli* showed greatly significant inhibitions. In comparison to the macrobroth dilution, the results shouldbe the same or nearly the same because theprinciple of the ultraviolet light was merely todestroy the DNA of pre-existing organisms on the spider web which may or may not be present on the spider web itself.

CONCLUSION

The results of the present study show that the majority of microorganisms isolated such as *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* were effective on the spider web of *Argiope catenulata*. Whereas, *Neisseria gonorrheae, Aspergillus niger* and *Candida albicans* were resistant to the inhibitions induced by spider web. In contrary to the other research studies which was effective on *S.aureus* and*E.coli* or ineffective to *B.subtilis* or vice versa, this explains that different antimicrobial activity of spider web obtained from different spider species varies depending on their own unique properties. The characterization of spider web of *Argiope catenulata* denoted the antimicrobialproperty was really present on it since exposure of spider web under UV light correlated with the Macro Broth dilution results. However, the spider web was denatured in both proteinase K and distilled water thus resulted into no inhibition on the tubes at all. Antimicrobial property recoveredfrom *Argiope catenulata* have created thehope for the effective treatment of emerging spread of multiple antimicrobial-resistantpathogenic bacteria.

RECOMMENDATIONS

As per other researcherssuggestion, the properties and composition of spider web vary between and even within spider species (Tahir et al., 2017).Thus, use of other species of spider is necessary. Furthermore, toxicity studies also need to becarried out to determine the safety profile of *Argiope catenulata* spider web as an alternative antimicrobial agent. Lastly, identification of glycoproteins covering thesurface of the spider web should be investigated in determining both the stickiness and stretchiness of the silk**.**

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