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Evaluation of Antimicrobial and Antibiofilm Activity of Seriphidium chitralense Extracts Against Staphylococcus aureus

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Abstract: In this study, the crude extract of the plant of Mulkhow valley district Chitral, Seriphidium chitralense was investigated for its antibacterial and antibiofilm activity against S. aureus. Crude extract was prepared using two solvent system methanol, and ethyl acetate. The result of the antibacterial activity of ethyl acetate extract was good having greater zone of inhibition against S. aureus, but the MIC and MBC value of ethyl acetate was greater than the methanolic extract. Methanolic extract shows better MIC value. The extracts were further evaluated for their ability in inhibiting biofilm formation by S. aureus, both extracts showed a dose dependent antibiofilm activity against S. aureus. The effect of plant extracts on the growth curves of S. aureus showed a slight difference in treated cells as compared to positive control. Antihemolytic activity of the extracts was performed against human erythrocytes, the hemolytic percentage of the extracts observed was low as 15% and 15.7% for methanolic and ethyl acetate respectively at the concentration of 1000 µg/mL. S. chitralense can be used for further study for isolation of active compounds for their antimicrobial activity against wide range of pathogens and could be useful in pharmaceutical industries for drug discoveries. Evaluation of Antimicrobial and Antibiofilm Activity of Seriphidium chitralense Extracts Against Staphylococcus aureus.
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1. Introduction

Most of the bacteria in the biofilm are 10,000 times more resistant to agents used as an antimicrobial, host immunity, and external stimuli, and thus helping microbes to persist in chronic infections. Bacteria in biofilm gain resistance by enzymes that degrade antibiotics, and some other genetic variation in these bacteria which make it dormant inside the body (Stoodley et al., 2002; Kirmusaoglu et al., 2017). Due to resistance to antibiotics, environmental stresses make microorganism that exist in biofilm a major threat to global healthcare as 60-80% infections in human caused by biofilm associated pathogens (Hoibya et al., 2010; Hu et al., 1999). Community of bacteria embedded in the biofilm are heterogeneous population of cells that have different mode of interaction with each antimicrobial agent, therefore different antimicrobial agents have different effects against different cells in the community that are present in various layers of biofilm and these cells persist and has great survival rate in the biofilm. Staphylococci can be transmitted from human skin to the indwelling devices, through these contaminated devices Staphylococci transmitted to the human body during implantation (Kukhtyn et al., 2017).

S. aureus is a member firmicutes and it is a gram-positive coccus, usually found on the skin and the upper respiratory tract as the normal microbiota of the body. It is facultative anaerobe that can be survived in the absence of oxygen and catalase positive organism. Although S. aureus usually found as the commensal of normal microflora of the human, but it can also be switched to opportunistic mode and become a pathogen and could lead to different type of skin infections like abscesses, respiratory tract infections like sinusitis and food poisoning. Pathogenic strains of S. aureus produce different kind of virulence factors like potent protein toxins, and the proteins expressed on the surface of cell which inactivates antibodies by binding with it.

Various Staphylococcus species causes biofilm related diseases like osteomyelitis, endocarditis, urinary tract infections (UTIs), soft tissues and skin infections, colonization of nasal and complication of cystic fibrosis as well as infections associated with the implantation of medical devices (Paharik et al., 2016). In many cases the formation of

biofilm, makes S. aureus more resistant and causes chronic infections. The colonization on medical devices of S. aureus is one of highest problem which could lead to spread of infections. Different medical materials such as prosthetic medical devices, cerebrospinal fluid shunts, prosthetic joints, catheters, and cardiac pacemakers are usually colonized by S. aureus. Regarding this, after the transformation of these medical devices inside the host, these devices become coated with the host proteins which favor the colonization of S. aureus and formation of biofilm (Lister et al., 2014).

More than 70-80% of the population around the globe depends on medicinal plants for curing their diseases and the allelopathic medicine is expressed with high expense in cost, such as Germany (40-45%), Australia (48%), USA (42%) and France with 49%. For Pakistan, 5521 species of flowering plants have been documented. Furthermore, most of them are reported from mountainous area of Pakistan. Of these, only 1500 (27.16%) taxa of flowering plants are found in Chitral. Many plants are reported as ancient plants for medical purposes in Chitral. Chitral is located in the extreme northern part of Khyber Pukhtoon Khwa province, Pakistan. Administratively Chitral has been divided into two sub division i.e sub division Chitral and sub division Mastuj. In subdivision Mastuj, the Mulkhow valley situated with the driest climatic condition and covered approximately 1250 km², at the distance of 84 km from lower Chitral (Headquater). Mulkhow Valley, District Chitral has a rich flora of medicinal plants. About 60 taxa are documented as being used by ethic group for medicinal purposes. It is reported that about 60% of the population of Mulkhow Valley depends upon the use of medicinal plants for the treatment of various diseases.

The genus Seriphidium belongs to Asteraceae (Daisy family) which consist of about 400 different species. Plants of the genus Seriphidium are mostly herbs and shrubs, and they are known for their volatile oils. They are found in temperate climates usually in dry or semi-dry habitats. Many species of this genus are used for medicinal purposes like in folk medicine as antihelminthics. Due to the presence of sesquiterpene, lactones and terpenoids in plants of genus Seriphidium, these plants have bitter tastes, which help them in adaptation in environment and discourage herbivores. Leaves of many species are used in medicines and flavoring agents. Some species were used to repel fleas and moths, and in brewing. Some of the plants of this genus are used as a food.
Seriphidium chitralense is the medicinal plant of Mulkhow valley, chitral and is used traditionally by the ethics group from thousands of years likeworm repellent, abdominal pain, stomach pain also used for sweeping the floor.Seriphidium chitralense, Family: Asteraceae. Genus: Seriphidium
Specie: Seriphidium chitralense
Vernacular name: Daroon
Altitude: 2005 m (Kifayatullah et al., 2017)

Because of the increasing spread of antibiotic resistant strains around the globe, the need of novel and effective antimicrobial agents is of great importance. In this study antibacterial and antibiofilm activity of S. chitralense extracts against S. aureus were evaluated.

2. Material and Methods

S. chitralense plant was collected from Mulkhow valley District Chitral, Pakistan. Plant was air dried at room temperature. The whole plant (including leaves, flower and stem) was chopped into small pieces and grinded using mechanical grinder into a fine powder. The powdered plant material was weighed and stored at room temperature.

Two solvents system were used (methanol and ethyl acetate) for the extraction of plant material (Betoni et al., 2006).Colonies of bacterium were picked from the culture plate with the wire loop and sub cultured in the nutrient broth prior to use in subsequent experiments (Hwang et al., 2016).S. aureus inoculum was prepared and compared to 0.5 McFarland turbidity standards (1 × 108 CFU/mL) and spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension to make a lawn (Dahiya et al., 2012).The MIC values were determined for each plant extract by a broth microdilution method using 96 well round bottom microtiter plates (Das et al., 2016). Biofilm inhibition assay was performed based on method described previously (O'Toole et al., 2000).

For staining of biofilm, 110 μL of 0.1% crystal violet solution was added in each well of 96 well plate. Plates were incubated at room temperature for 15 minutes. Then crystal violet was discarded, plates were washed and dried. For quantification of biofilm 110 μL of 30% acetic acid was added in each well and incubated at room temperature for 15 minutes, for dissolving of biofilm. Then acetic acid from each well was transferred to another 96 well flat bottom plate. Absorbance of the plates was observed at optical density of 570 nm using microplate reader.For determination of growth curves of bacteria treated with plant extract (Kim et al., 2011).Hemolytic activity of S. chitralense plant extracts was performed by spectrophotometer method as described by (Yang et al., 2005).

Dried plant powder was extracted in methanol and ethyl acetate. The extracts were filtered and concentrated with the help of rotary evaporator. Dried extract was dissolved in DMSO and stored at

room temperature for further use in the experiment. Blood (3 mL) was collected from a healthy volunteer in EDTA tube. The blood was subjected to centrifugation in laboratory centrifuge machine for 3 minutes at 1500 rpm. After centrifugation blood plasma supernatant was discarded and the pellet was washed thrice with sterile phosphate buffer solution (pH 7.2±0.2) through laboratory centrifugation for 5 min at 1500 rpm. The cells were resuspended in normal saline to 2%.For hemolytic activity 0.5 mL of the erythrocyte suspension was added in 0.5 mL of plant extracts (250, 500 and 1000 µg/mL concentrations in DMSO). The mixture of cell suspension and plant extract was centrifuged for 10 minutes at 1500 rpm. The hemoglobin that is free in the suspension supernatant was collected and measured in UV-Vis spectrophotometer at 540 nm. Saline solution of phosphate buffer was taken as a negative control while 0.1% triton X100 was taken as a positive control for complete hemolysis. Experiment was performed in triplicates for each plant extract concentration. Percentage hemolysis by the plant extracts was calculated by the formula:

% Hemolysis = (Absorbance of the test sample/absorbance of the positive control) × 100.

3. Results

Plant extract yield was obtained in mg as shown in the Table 1.

Dried powder (g)	Solvent	Solvent volume	Soaking time	Yield obtained
6.6	Methanol	200 mL	2 days	550 mg
8.3	Ethyl acetate	250 mL	3 days	525 mg

Percentage of the extract yield

Percentage of the yield obtained was calculated according to the formula.

`Yield (%) = weight of the dry extract after solvents have been removed/ weight of the dry plant before extraction × 100, the yield in this case is crude not fractionated.

Methanolic extract:

Yield (%) = [(550/ (6CSZ.6×1000)] ×100 = 8.33%

Ethyl acetate extract:

Yield (%) = [(525/ (8.3×1000)] ×100 = 6.32%

Agar well diffusion assay of methanolic and ethyl acetate extracts

The antimicrobial activity of *S. chitralense* was evaluated according to their zone of inhibition against *S. aureus,* and the results inhibition zones were compared with the antibiotic standard used (ciprofloxacin). The agar well diffusion assay is defined as a qualitative method and is mostly used for the selection of extracts with potential antibacterial or antimicrobial efficiency, mostly when the inhibition zones are equivalent or greater than 10 mm. The results revealed that both extracts were potent antimicrobials against microorganism studied. In the present investigation, the ethyl acetate and methanolic extracts were found to have the antibacterial activity against *S. aureus*, as the zones of inhibition were recorded 18 mm and 19 mm respectively when 100 mg/ml of the extract were used. The diameter of inhibition zones was reduced when extract concentration was used 50 mg/mL. Results are illustrated in Table 2.

Table 2: Agar well diffusion assay of Ethyl acetate and Methanolic extract.

Extract	Concentration	Zone of inhibition mean ± SD	Positive control	Negative Control
Ethyl acetate	100 mg/mL	19.33667±0.090738	24±0.264575	0
	50 mg/mL	15.33333±0.208167		
Methanol	100 mg/mL	18.6667 ± 0.4	24±0.264575	0
	50 mg/mL	18± 0.2		

Antibacterial activity of methanolic extract, zone of inhibition (mean ± standard deviation) of 100 mg/mL and 50 mg/mL, positive control (Ciprofloxacin disk), negative control (DMSO) and Antibacterial activity of ethyl acetate extract, zone of inhibition (mean ± standard deviation) of 100 mg/mL and 50 mg/mL, positive control (Ciprofloxacin disk), negative control (DMSO).

MIC and MBC of methanol and ethyl acetate extract

MIC was defined as the highest dilution range or lowest extract concentration that inhibit the growth of bacteria. The results obtained in MIC are comparable with that of the results of agar well diffusion assay because least MIC obtained from the experiment shows best antimicrobial activity. The

MBC were determined by sub-culturing of the dilution range of the extract used in MIC plate on to agar medium and incubated further for 24 h. The concentration of the plant extract that completely killed the bacteria has been taken as MBC. All the experiments were repeated three times. Results revealed that the methanolic extract has lower value of MIC and MBC then the ethyl acetate extract which means that methanolic extract could kill or inhibit the bacterial growth at low concentration than the ethyl acetate extract.

Table 3 MIC and MBC of methanol and ethyl acetate extract.

Extract	MIC (mg/mL)	MBC(mg/mL)
Methanol	14.63 ± 0.3005	30.21 ± 0.003
Ethyl acetate	28.3 ± 0.14	44.7 ± 0.1

Antibiofilm activity of plant extracts

Antibiofilm activity of plant extracts was evaluated in 96 well microtiter plate. Biofilm inhibition and formation was concentration dependent of plant extract. A significant decrease of biofilm was observed when concentration of the plant extract was increased. Biofilm formation was decreased when the extract concentration was increased. Both of the extracts were observed that showed potent antibiofilm activity. Biofilm formation and inhibition was compared with positive control (M63 media + bacterial culture) and negative control (blank M63 media). Results of the antibiofilm activity of methanolic and ethyl acetate extract shown fig 1 and 2.

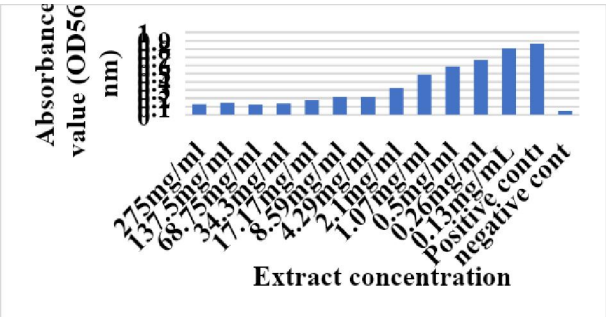

Figure 1. Effect of Ethyl acetate extract against biofilm formation of S. aureus, biofilm biomass was determined by crystal violet assay after 24 h of growth with M63 media, Postive control (M63 media + inoculum), negative control (M63 media).

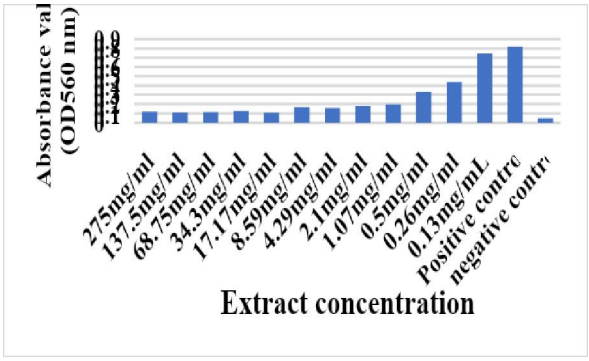

Figure 2 Effect methanolic extract against biofilm formation of S. aureus, biofilm biomass was determined by crystal violet assay after 24 h growth in M63 media, positive control (M63 media + inoculum), negative control (M63 media).

Growth curves

To examine the growth curves of bacteria exposed to plant extract nutrient broth with different concentration of plant extract (40, 50, 60 mg/mL) was used. The growth of the treated cells was compared with those of positive control, without the addition of plant extract. The growth curves of bacterial cells treated with plant extracts is the indication that the plant extract can inhibit the growth and division of bacteria. When the extract concentration was higher there is significant decrease in the bacterial cells was recorded with different time interval of incubation. Result revealed that both extracts were potent growth inhibitors of bacterial cells, however methanolic extract shows better activity compared to ethyl acetate extract the results are illustrated in fig 3 and 4.

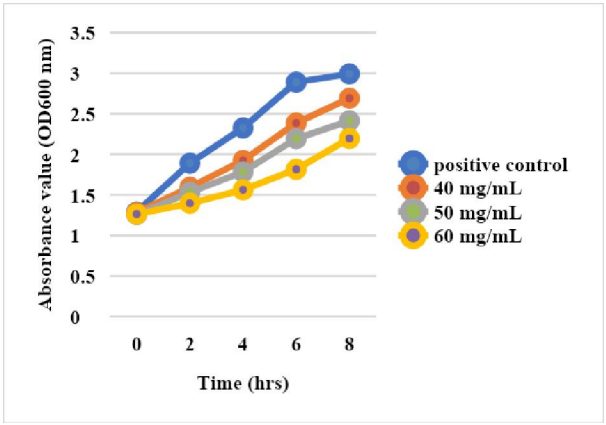

Figure 3 Growth curves of S. aureus treated with methanolic plant extract. The OD600 nm was measured at 0, 2, 4, 6, 8 h.

Hemolytic activity

In vitro hemolytic activity on human erythrocytes of various concentrations extracts was evaluated. Triton X-100 (0.1%) shows 100% hemolysis while phosphate buffer was used as a negative control which shows 0% hemolysis. Each concentration of plant extract was tested on erythrocytes in triplicates and results were analyzed as mean hemolysis percentage. Hemolysis percentage of the extract against red blood cells was concentration dependent, but all concentration of the extracts tested shows lower hemolytic activity. The hemolytic percentage 5.1%, 8.76%, and 15.17% were obtained for a dose of 250 µg/mL, 500 µg/mL, 1000 µg/mL respectively for ethyl acetate extract, while 6.6%, 10% and 15% were recorded from extract concentration of 250 µg/ml, 500 µg/ml, 1000 µg/ml respectively for methanolic extract. Hemolysis percentage of the plant extracts is shown in Fig 4.

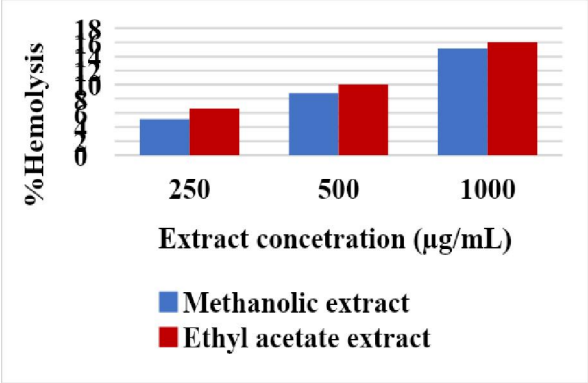

Figure 4: Haemolytic percentage of the methanolic and ethyl acetate plant extract at the concentration of 250, 500 and 1000 µg/mL.

4. Discussions

Microbial resistance to the antimicrobial therapeutic agents appears as a continuous process since the discovery of antibiotics. Scientists realized that natural products from medicinal plants have immense potential to combat infections in human body and can be used as alternate of antibiotics because these products have low cost and side effects compared to the synthetic compounds. As increasing of antibiotics resistance of microorganisms is of great concern because of its deleterious effects on healthcare system so that some natural agents should be used against those organisms (Hu et al., 1999). Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance. As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibit by bacterial pathogens (Kukhtyn et al., 2017). Microbial biofilm is a worldwide threat to the healthcare system due to high difficulty in treatments because of resistance to antimicrobial agents. In this study, the activities of the plant extracts were tested against the biofilms of the S. aureus. Hence searching of novel effective and economical therapeutic agents to overcome this problem is a priority. This would be the first study in evaluating the antibacterial and antibiofilm activity of the extract of the selected plant used against this organism. The potency of the plant extract to inhibit the biofilms would be used in the healthcare system for the treatment of chronic and life-threatening diseases. Bacteria are found in natural environments in two predominant forms, i.e., free living planktonic cells and in the form of biofilm. Planktonic cells are freely motile, metabolically active, susceptible to environment and other antimicrobial agents while the biofilm form of the bacteria is protected in membrane called extracellular polymeric substance EPS, thus resistant to antibiotic and disinfectants, and are metabolically less active (Limoli et al., 2015). Bacterial communities in the biofilm are hypothesized to have formed during the evolutionary development of earth as a mechanism of defense for many prokaryotic organisms, as the conditions were too harsh during primitive earth for the survival of prokaryotic organisms. Biofilms are found everywhere in natural environment. There are about every species of microbes have some mechanisms in which they undergo from free living life to attach to some surfaces and form a complex community where they interact with each other (Minaev et al., 2007). Most of the bacteria in the biofilm are 10,000 times more resistant to agents used as an antimicrobial, host immunity, and external stimuli, and thus helping microbes to persist in chronic infections. Microbial biofilms are largely responsible for the resistance of many infections to conventional antimicrobial therapies. Biofilms is the major mode of microorganisms that exhibit tolerance against multiple drugs, and almost all resistance mechanisms of pathogens are due to biofilm that prevent the antimicrobial agents from hitting its target sites. Studies in the past few decades indicated that targeting biofilms is the basic approach for drugs development used for infectious diseases. Due to resistance to antibiotics, environmental stresses make microorganism that exist in biofilm a major threat to global healthcare as 60-80% infections in human caused by biofilm associated pathogens (Høiby et al., 2010). S. aureus is a member firmicutes and it is a Gram positive round shaped bacterium, usually found on the skin and the upper respiratory tract as the normal microbiota of the body. S. aureus is the most common pathogen of nosocomial and community infections, that causes infection in bloodstream and

have capability of biofilm formation on various tissues of the host organism and medical devices, these biofilm are persistence and cause various mortality and morbidity associated diseases (Periasamy et al., 2012). Some major diseases caused by S. aureus are osteomyelitis, toxic shock syndrome, endocarditis, skin infections and food poisoning (Adam et al., 2002). Due to the shortage of current medicines and increase in the resistance of most of the antibiotics, which create a great impact on the global healthcare, therefore researchers are continuously searching for new effective and economical therapeutic agents against wide range of infectious agents. Natural products gained the popularity over the last few decades in pharmaceutical industries because researcher can easily process different compounds used in medicines from these products. (Stoodley et al., 2002). Plants are widely used in healthcare from millennia around the globe, because medicinal plants producing an amazing variety of secondary metabolites, like glycosides, steroids, terpenoids, quinones, saponins, flavonoids, coumarins. These secondary metabolites are the wide source of medicinal plants derived effective antimicrobial agents (Lowy et al., 2003). In contrast of synthetic compounds, natural products are highly effective in the treatment of infectious diseases (Fennell et al., 2004). Organisms in the biofilm showed resistance to the antibiotics is multifactorial and it is varied with the organisms. Therefore, treating biofilm is of great concern and difficult task. Interestingly, in some studies reported that plant extracts that show lower antimicrobial activity could be effective in destruction or prevention of microbial biofilms (Upadhyay et al., 2013). World population of about 70-80% depend on medicinal plants for curing their diseases and the allelopathic medicine is expressed with high expense in cost, such as France with 49%, Australia 48%, Germany 40-45%, and USA 42%. For Pakistan, 5521 species of flowering plants have been documented. Furthermore, most of them are reported from mountainous area of the Pakistan. Of these, only 1500 (27.16%) taxa of flowering plants are found in Chitral. Many plants are investigated as ancient plants for medicinal purposes in Chitral. Chitral is located in the extreme northern part of Khyber Pukhtoon Khwa province, Pakistan. Mulkhow Valley, District Chitral has a rich flora of medicinal plants. About 60 taxa are documented as being used by ethic group for medicinal purposes. It is reported that about 60% of the population of Mulkhow Valley depends upon the plants use as a medicine for the treatment of various diseases. Seriphidium chitralense is the medicinal plant of Mulkhow valley, chitral and is used traditionally by the ethics group from thousands of years, like worm repellent, abdominal pain, stomach pain also used for sweeping the floor. In the present study the antibacterial and antibiofilm activity of the plant of Mulkhow Valley district Chitral Pakistan S. chitralense against S. aureus was evaluated. The crude extract of plant was used. Plant was subjected for the solvent extraction. Two solvents system were used for extraction (methanol, ethyl acetate). Dried plant powder was at the concentration 1 gm/30 ml for both solvents. Extract yield of both solvents were approximately same respected to the solvent volume and plant material used. The yield obtained was 8.3% of methanolic extract and 6. 23% of ethyl acetate extract our results is comparable with that of the work of (Deng et al., 2004) the same yield was obtained with solvent extraction of the plant of genus Seriphidium. Plant extract was dissolved in the DMSO at the concentration of 275 mg/ml. DMSO (Dimethyl Sulfoxide) is an organosulfur compound with the formula (CH□)□SO. It is a colorless liquid and is a powerful solvent. It dissolves both polar and non-polar compounds. It is widely used in drug discovery in the pharmaceutical industry. Antibacterial activity of the crude extracts was determined by agar well diffusion assay. However the agar well diffusion assay is the technique used in qualitative analysis, mainly used in analyzing the agent with potency of killing of microorganisms, mostly when the inhibition zone diameter of the activity of the agent is ≥10 mm. it is in priority to observe that the inhibition zones size could be due to the polarity of the agents, the more the compound diffusible could give the greater zone size although it is not more active than the agent that is more active but non diffusible. Plant extracts dissolved in DMSO was used for its antibacterial activity against S. aureus. Antibiotic disc of ciprofloxacin was used as a positive control and DMSO was used as a negative control in the experiment. The agar well diffusion assay results shows that both of the crude extracts were potent antimicrobial against S. aureus. Extract concentrations 100 mg/mL and 50 mg/mL were used for both extracts. The agar well diffusion assay of ethyl acetate extracts shows 19.33 mm zone of inhibition, and methanolic extract shows 18.66 mm zone of inhibition against s. aureus when concentration of the extract was used 100 mg/mL. When the extract concentration was reduced to 50 mg/mL for both the extract, the zone of inhibition was 15 mm for ethyl acetate and 18 mm for methanolic extract was recorded. As the concentration of plant extract was changed a significance difference in zone of inhibition was observed. The inhibition zone results suggest that S. aureus was susceptible for both the extracts. The MIC, which is a major analyzer of an antimicrobial agent's potency, is defined as the concentration in (mg/mL) at which no visible growth of bacteria is observed under specific experimental

conditions. MIC of plant extract was performed in 96 well plates in order of twofold serial dilution of the plant extract. MIC of methanolic extract was recorded 14.63 mg/mL and for ethyl acetate 28.3 mg/mL. MIC results revealed that the methanolic extract shows better antimicrobial activity. MIC is generally stated as the basic measurement in the laboratory of the antimicrobial activity of the agent used against tested organisms. MIC value is most important in the diagnostic laboratory to determine the resistance of organisms to the agent and monitor the activity of agent used against organisms. MIC values helps clinician to choose antibiotic for patient in a certain dose (Wiegand et al., 2008). While the MBC was determined by sub cultivation of MIC plates on nutrient agar plate, after overnight incubation the visible growth of S. aureus on nutrient agar plate was observed. MBC was determined as the lowest extract concentration that inhibited the growth of bacteria. Agar plates was observed for bacterial growth corresponding to the wells in the MIC plates. Wells having no growth of bacteria on agar plate was taken as MBC of the plant extract. MBC of methanolic extract was 30.21 mg/mL and ethyl acetate was 44.7 mg/mL recorded. Methanolic extract shows better bactericidal activity. The plant extract shown remarkable antibacterial activity, so these extracts are further evaluated for the inhibition of biofilm growth of S. aureus. The 96 well-plates are used most frequently for quantifying biofilm biomass using different microorganisms and stains. Crystal violet is majorly used in observing biofilm biomass. Crystal violet was initially used in test tubes experiments but later it was optimized and used in 96 well microtiter plates (Stepanovic et al., 200). The results in this study shown the plant extract concentration dependent inhibition of biofilm formation. The data revealed that when higher concentration of the plant extract was used growth of biofilm was inhibited at higher degree, as observed by crystal violet staining. Extract concentration was used from 275-0.13 mg/mL maximum growth of biofilm was inhibited at 275 mg/mL for both extracts compared with the positive control after 24 h of treatment. S. aureus is known to be potent biofilm producer, which makes its clinical management difficult. It is investigated as causative agent of many infections in humans, ranging from superficial skin suppurations to life-threatening septicemias associated with visceral or bone infections. Successful treatment becomes more challenging by the increasing prevalence of methicillin-resistance and antibiotic in efficacy when such bacteria are involved in chronic infections. Many different natural products have been investigated that interact with bacterial biofilms, but many of these studies are still in the initial step of drug discovery. There are certain problems that need to be solved for the use of these agents for medical purpose against biofilm. Finally, expanding the classes of molecules to be explored may increase the likelihood of discovering novel anti-biofilm agents. The activity of plant extracts against bacterial biofilm formation used in this study, could be used in different medical purposes like for preventing microbial colonization on different tissues and surfaces which results in serious infections. These plants extracted are expected to become useful for study agents used against biofilm in future. As these experiments have been done invitro the next step would be further experiment in vivo to observe if the extract could be useful in management of different infectious diseases (Sandasi et al., 2010). The growth curves analysis of bacteria can be examine to determine the extract concentration potency against the growth and death of bacteria, and evaluate the activity of certain antimicrobial agents against specific microorganisms over time (Khan et al., 2014). Time dependent bactericidal activity is shown by the agent when concentration of the antibacterial agent exceeds MIC level (Anantharaman et al., 2010). The growth curves results indicate that the antibacterial activity of 250 µg/mL of the extract could slightly affect bacterial growth but not enough to outpace the rate of division of the bacterial cells. Interestingly, in comparison of the bacterial growth curves, the growth curves of the plant extract treated bacteria indicated a faster growth inhibition of S. aureus. In the present study, there was only a slight reduction of growth observed in the strain compared to the control. However, natural product is passed through many steps for being used in the drug development. Toxicity of the active compounds is a major factor during development of drugs, and hemolytic activity of the agent is a key starting point to check the toxicity. It provides useful information about the interaction of compounds used and biological entities at cellular level. Hemolytic activity of the agent represents the general toxicity of the certain compounds against healthy cells (Da Silva et al., 2004). Hemolytic activity of the plant extracts in this study is expressed in percentage hemolysis. All the extract concentration of both solvent extracts used showed very low hemolytic effect toward human erythrocytes. Hemolytic percentage was observed higher with high extract concentration, a dose dependent response was observed. Maximum hemolytic activity 15.1 % was observed at the concentration of 1000 µg/mL for both extract while 5.1% and 6.6% hemolytic activity was recorded at the concentration of 250 µg/mL for ethyl acetate and methanolic extract respectively. The data in these results suggest that the extracts are non-toxic, these extracts would be safer and could be used in different drugs preparation used for different disease treatments.

It has been reported by many researchers the efficacy of plant extracts, and the effective compounds derived from these extracts used against pathogens. Some of the researchers suggested that plant derived components used as antimicrobial agent like (terpenoid, alkaloid and phenolic compounds) interact with basic cellular mechanisms of the microorganisms resulting in damaging their cell membranes or interact with different enzymatic pathways. Other researchers attributed the inhibitory effect of these plant extracts to hydrophobicity characters of these plants extracts which enable them to react with protein of microbial cell membrane and mitochondria disturbing their structures and changing their permeability. At the present time natural product is of great concern and the phytochemicals derived from plants may prove their activity and fighting against biofilms (Adnan et al., 2017). As related throughout this study, there is substantial evidence that plant extracts, plant derived secondary metabolites, phytochemicals or other natural chemicals have the potency to be developed as a potential antibiofilm therapeutics against biofilm associated infections.

5. Conclusion

The solvent extracts of the plant showed potential antibacterial and antibiofilm activities against the most common biofilm associated pathogen i.e., S. aureus. Percentage hemolysis was low with both the extracts. S. chitralense could be used for further study in drug discovery and in different pharmaceutical industries for drug development.

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