Pharmacophore 2010, Vol. 1 (2), 123-131. ISSN 2229 – 5402

Pharmacophore

(An International Research Journal)

Available online at http://www.pharmacophorejournal.com/

Original Research Paper

IN VITRO ANTIBACTERIAL ACTIVITIES OF *KIRGANELIA RETICULATA* BAILL. AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Methanol, chloroform and hexane extracts from leaves of *Kirganelia reticulata*, used in Indian ayurvedic medicine for the treatment of several ailments of microbial and non-microbial origin were evaluated for potential antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Antibacterial activity and biofilm production of crude extracts against MRSA (ATCC 25923) isolated from clinical specimen was studied. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the methanol, chloroform and hexane extracts were in the range of 12.5 to 50.0 mg/ml and 25.0 to 100.0 mg/ml, respectively. Amongst the evaluated extracts, the methanolic extract showed the strongest antibacterial effect as well as biofilm inhibition. Micro plate screening used for detection of biofilm formation by *Staphylococci* is a quantitative model to study its adherence level and has been a sensitive method.

Keywords: Antibacterial, biofilm, leaf extracts, methicillin-resistant *Staphylococcus aureus*, MIC, MBC.

INTRODUCTION

The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms.[1] The substances that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another big concern is the development of resistance to the antibiotics in current clinical use. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in AIDS and cancer patients.[2,3] Moreover, excessive budget is currently spent on import of antibiotics manufactured abroad. Therefore, antibacterial activity of local medicinal plants should be studied to provide alternative and locally available antibacterial regimens.[4]

Methicillin-resistant *Staphylococcus aureus* (MRSA) is now common in many areas of the world. The frequencies of infections and outbreaks due to MRSA have continued to increase. It is often multidrug resistant and therapeutic options are limited.[5, 6, 7, 8] MRSA is a type of *Staphylococcus* that is resistant to the antibiotics that are often used to treat *staphylococcal* infections. *Osteomyelitis* is one such infection which is particularly difficult to treat. The options for treatment of infections caused by these microorganisms are limited: the sensitivity of clinical strains to quinolones, clindamycin, co-trimoxazole and rifampicin is variable, and the sensitivity is often limited to glycopeptides, which must be administered by the parenteral route. Novel drugs for the treatment of methicillin-resistant *staphylococcal* infections, such as quinupristin–dalfopristin and linezolid, have recently been introduced in clinical practice.[9,10] However; none has been fully investigated in clinical studies on the treatment of *osteomyelitis*.

S. aureus cause disease through the production of virulence factors. They are the part of our normal flora, but they can cause fatal diseases as a result of the expression of multiple virulence factors. These factors include adhesins, exotoxins, enterotoxins, hemolysins, and leukocidin, as well as proteases that enable the bacteria to spread within the host.[11,12,13] Strains defective in their ability to form a biofilm or produce toxins show diminished virulence[14], suggesting that a novel approach for therapy development would be to interfere with the production of virulence factors. TRAP is a membrane-associated 167-amino acid residue protein that is highly conserved among *Staphylococci*. When TRAP is not expressed or not phosphorylated, the bacteria do not adhere, do not form a biofilm, do not express toxins, and do not cause disease. TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches peak levels in the mid-exponential phase of growth[15,16,14], followed by activation of *agr* and induction of SQS2 components. RAP is a 277-amino acid residue protein that activates the *agr* system by inducing the phosphorylation of TRAP. RAP is an ortholog of the 50S ribosomal protein L2 that is secreted by *S. aureus.*

Kirganelia reticulata Baill. (Synonym- *Phyllanthus reticulatus* Poir.) is a large, often scandent, shrub of the family *Euphorbiaceae*. The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands[17]. The leaves and bark are used as

astringent and diuretic. Juice of leaves is used for the treatment of diarrhea in children.[18] The bark showed significant antiviral[19] and antiplasmodial activity.[20] The antibacterial potential of the aerial parts of this plant has been evaluated.[21] The bark is used to treat rheumatism, dysentery and venereal diseases. [22] The plant is used for a variety of ailments, including *smallpox, syphilis, asthma, diarrhoea,* bleeding from gums.[23,24] It is also claimed to have antidiabetic activity in tribal areas, which has been validated by Kumar *et al*.[25] The antibacterial potential of the leaf extracts of this plant has been evaluated recently.[26]

The medicinal plant, which have been used as folk medicine for several diseases, were selected for this research to study antibacterial activity and biofilm inhibition of their crude methanolic, chloroform and hexane extracts against methicillin-resistant *Staphylococcus aureus*. In India, multi drug resistance has clearly emerged as a serious problem with MRSA. Hence, the basis of the study is in order to overcome this and to prove the folkloric claims of the plant.

MATERIALS AND METHODS

Collection of material and preparation of extracts

Fresh leaf materials of plant were collected in winter season locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in December 2009. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429). Freshly collected leaves of *K. reticulata* were shade-dried and then powdered using a mechanical grinder. The shade dried leaves were pulverised and subjected for successive extraction using hexane, chloroform and methanol (LR grade, Merck, India) separately using soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). A portion of the residue was used for the further activities.

Microorganisms and Antibacterial activity

The MRSA strains used in this study were clinical isolates from patients presenting with symptoms of *S. aureus* associated diseases. The isolates were identified as *S. aureus* according to colonial and microscopic morphology, positive catalase and coagulase production. All the isolates were tested for methicillin resistance. The disk diffusion method outlined by the National Committee for Clinical Laboratory Standards (NCCLS) was used with a 1 µg oxacillin disk (Oxoid). Zone sizes were read after incubation at 35^0C for 24h. Isolates with zone sizes $\pm$ 10 mm were considered methicillin resistant. The antibacterial activity was determined by the well diffusion method according to NCCLS.[27] Three to five identical colonies from each agar plate were lifted with a sterile wire loop and transferred into a tube containing 5ml of tryptic soy broth (TSB). The turbidity of each bacterial suspension was adjusted to reach an optical comparison to that of a 0.5 McFarland standard, resulting in a suspension containing approximately 1 to 2 x 108 CFU/ml. Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60^0 each time to ensure even distribution of the inoculum. As a final step, the rim of the agar was also swabbed. After allowing the inoculum to dry at room temperature, 6 mm diameter wells were bored in the agar. Each extract was checked for antibacterial activity by introducing 50 µl of a 100 mg/ml concentration into triplicate wells. The extracts were dissolved in 10% aqueous

dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml. Pure DMSO was taken as the negative control and 0.05% Ciprofloxacin as the positive control. The plates were allowed to stand at room temperature for 1h for extract to diffuse into the agar and then they were incubated at 37^0c for 18 h. Subsequently, the plates were examined for bacterial growth inhibition and the inhibition zone diameter (IZD) measured to the nearest millimeter.

MIC and MBC determination

To measure the MIC values, micro-broth dilution method was used.[28] The reconstituted extracts was serially diluted 2-fold in Mueller-Hinton broth medium to obtain various concentrations of the stock, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781 mg/ml and were assayed against the test organism. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth.[29,30] Equal volume of the various concentration of each extract and Mueller-Hinton broth were mixed in micro-tubes to make up 0.5 ml of solution. 0.5 ml of McFarland standard of the organism suspension was added to each tube.[30] The tubes were incubated aerobically at 37^oC for 24 h. Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller-Hinton agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the minimum bactericidal concentration.[31]

Biofilm production

Staphylococci are also a common cause of infections related to bacterial biofilm formation on implanted devices. Isolates from fresh agar plates were inoculated in respective media and incubated for 18 h at 37^oc in stationary condition and diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) wells were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 18 h at 37°c. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic bacteria. Biofilm formed by adherent sessile organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent *Staphylococcal* cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (LISA Plus, Micro plate reader, Aspen Diagnostics Pvt. Ltd, Delhi) at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbence, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD value obtained from media control well was deducted from all the test OD values.[32]

Statistical analysis

The results of the experiment are expressed as mean ± SE of three replicates in each test. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple pairwise comparison tests to assess

the statistical significance. P≤0.05 was considered as statistically significant, using software ezANOVA ver. 0.98.

RESULTS AND DISCUSSION

The results of antibacterial activity of all the crude leaf extracts of plant revealed that methanol extract showed good antibacterial activity against MRSA used. Whereas chloroform extract showed moderate effect on MRSA strain. On the other hand, the crude hexane extract was weakly effective against the cocci as judged by the zones of inhibition. The MIC and MBC values obtained for the extracts against the MRSA varied from one another. For instance, the MIC values of 25.0 and 50.0 mg/ml were obtained for chloroform and hexane extracts respectively, while the corresponding MBC values were 50.0 and 100.0 mg/ml. The MIC and MBC values of 12.5 and 25.0 mg/ml were recorded for methanol extract. Hence, this extract was bacteriostatic at lower concentrations and bactericidal at higher concentrations as revealed by MIC and MBC values. The inhibition levels of biofilm ranged from OD values 0.26, 0.18 and 0.17 for methanol, chloroform and hexane extracts respectively; whereas standard showed 0.30. Mathur *et al*.[32] explains the relationship between OD values and biofilm inhibition. The capacity is strong if OD value is >0.240, moderate if 0.120-0.240 and weak if <0.120. With reference to this, our methanol extract has got very strong inhibitory capacity which is followed by chloroform and hexane extracts.

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view of identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins (33). The results of the study indicated that the medicinal plant *Kirganelia reticulata* commonly used by traditional medical practitioners to cure venereal diseases and a variety of ailments, including *smallpox, syphilis* were active against hospital strains of MRSA. Previous studies by Shruthi *et al.*[26] indicated that the crude extracts of these plants were effective against *S. aureus*. The present study correlate to those findings but the only area of concern is that while those studies only dealt with the effect of crude extracts on *S. aureus*, while this study focused on the effect of crude extract on the MRSA, along with determination of both MIC and MBC values and biofilm inhibition of the extracts. It is worthy of note that traditional medical practitioners used this plant extracts solely without combining with other plant extracts for the treatment of microorganism associated skin and respiratory diseases.

The MIC value of active plant extract obtained in this study were lower than the MBC values suggesting that the plant extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. The methanolic extract exerted greater antibacterial activity than corresponding chloroform and hexane extract at the same concentrations. These observations may be attributed to two reasons; firstly, the nature of biological active components (saponins, tannins, alkaloids and anthraquinone) which could be enhanced in the presence of methanol. It has been documented that tannins, saponins and alkaloids are plants metabolites well known for antimicrobial activity.[34] Secondly, the stronger extraction capacity of methanol could have produced greater number of active constituents responsible for antibacterial activity.[31] Our investigation further revealed that methanol

extract showed good inhibition of biofilm formation by clinical isolates of MRSA. Biofilms are highly resistant to antibiotic treatment.[35,36,37,38,39] Infections may result in longer hospitalization time, or need for surgery, and they can even cause death. The spread of drug-resistant strains of *Staphylococci* and the ineffectiveness of treatments in cases of biofilm-related infections underscore the necessity to find new modes of prevention and effective alternatives to antibiotic treatment.

Table 1: Antibacterial, MIC, MBC and Biofilm inhibition values of *Kirganelia reticulata*

	Activities against MRSA			
	Antibacterial (mm)	MIC (mg/ml)	MBC (mg/ml)	Biofilm OD at 570 nm
Methanol Extract	17.60± 0.13**	12.5	25.0	0.26±0.01**
Chloroform Extract	14.60± 0.19**	25.0	50.0	0.18±0.01**
Hexane Extract	10.54±0.12**	50.0	100.0	0.17±0.01**
Ciprofloxacin	19.65±0.11**	6.25	12.5	0.30±0.01**

The values are the mean of triplicates ± S.E. * P<0.05, ** P<0.01 compared to standard.

CONCLUSION

Our results therefore offer a scientific basis for the traditional use of plant *Kirganelia reticulata* as a potential phytotherapeutic agent. The antimicrobial activities could be enhanced if the active components are purified and adequate dosage determined for proper administration, which is therefore employed in our further studies.

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