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RESEARCH PAPER

Antimicrobial Effect of *Syzygium cumini* Extract Against Methicillin Non-Susceptible *Staphylococcus aureus*

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of infections in Community-Associated (CA) as well as Hospital-Associated (HA) settings. Identification of new antibacterial agents from natural sources takes the forefront in research.

Objectives: The aim of the present study was to identify the resistance pattern of *S. aureus* in the clinical samples causing disease in Dhaka city, molecular typing of the methicillin non-susceptible *S. aureus* isolates and identifying new herbal components with anti-microbial effect against *S. aureus*.

Methods: We screened total of 78 clinical specimen of various nature (pus, urine, tracheal aspirate, conjunctiva and wound swab) with confirm *S. aureus* infection between March 2018 to October 2018. The specimen were cultured on mannitol salt agar to isolate *S. aurues*, which were later tested for antibiotic resistance according to disc diffusion method. The MRSA isolates were confirmed with PCR and typed for SCC*mec* element to know the distribution of hospital-associated and community-associated strains. Finally, the MRSA isolates were cultured in the presence of leaf extract and fruit extract of *Syzygium cumini* for observing the antibacterial potential.

Result: A total of 12 isolates of *S. aureus* were found to be non-susceptible to methicillin, 34%, 25%, 17% out of these were from pus, blood and urine respectively and 8% isolates were from wound swab, conjunctiva and tracheal aspirates each. Out of methicillin non-susceptible isolates, 25% and 16% were HA-MRSA and CA-MRSA respectively, as seen from PCR analysis of the SCC*mec* gene cassette of the *S. aureus* genome. The rest of the 59% of the isolates were untypable. Overall, higher concentration of leaf and fruit extract reduced the optical density of MRSA culture and reduced bacterial growth in drop plate significantly.

Conclusion: Dhaka population has *S. aureus* with varying sensitivity against methicillin, which needs further characterization by molecular epidemiology methods.

Keywords: Methicillin, Methicillin-resistant *Staph. aureus*, Syzygium extract, Non-susceptible to methicillin

Introduction

Methicillin is a â-lactam antibiotic, chemically related to semi-synthetic penicillin.[1] It was called Staphcillin due to effect against pathogenic staphylococci resistant to penicillin.[2] The resistance against penicillin in *Staphylococcus aureus* (*S. aureus*) came from the enzyme penicillinase.[3] Methicillin-resistant *S. aureus* (MRSA) are a class of genetically similar strains of *S. aureus* that are resistant to methicillin, and are a leading cause of skin and soft-tissue infections in hospital patients as well as in healthy persons.[4] MRSA strains differ from methicillin-sensitive *S. aureus* (MSSA) strains with insertion of a mobile genetic element, SCCmec into on the chromosome gene *orfX*.[5] MRSA produces mutant Penicillin binding protein 2a (PBP2a') (encoded by *mecA* gene) upon exposure to a β-lactam antibiotics.[6] MRSA also produces â-lactamase enzymes (encoded by extracellular enzyme *blaZ*) decreases â-lactam antibiotic activity.[7] *Staphylococcus* is the major pathogen in family Staphylococcaceae that accounts for the majority of the abscesses, large boils and wound infections.[8] *S. aureus* cause many other human infections of skin, soft tissue, respiratory tissue and bone joints, causing endovascular infections like bacteremia, endocarditis, sepsis, and toxic shock syndrome, thus termed one

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of the major human pathogens.[9] In the previous decade, the MRSA strains have expanded worldwide and currently became a concern for nosocomial diseases.[10] The annual death frequency from MRSA is raising quickly surpassing human immunodeficiency virus/ acquired immune deficiency syndrome.[11] Bangladesh is a densely populated developing country and many people suffer from unawareness, illiteracy, indiscriminate use of antibiotics and malnutrition.[12] Inadequate number of toilets and lack of anal hygiene, life style, poor economic status, lack of safe disposal of excreta also play a role in the spread of bacterial infection. MRSA was found to be the second most abundant critical drug-resistant pathogen in Bangladesh.[13] The high rate of nasal carriage of MRSA in apparently healthy adults and association with bovids and poultry creates a high bioburden of MRSA in our population.[14] A specific lineage of MRSA is in circulation in Bangladesh (ST772), that originated in India in 2004.[15]

The emergence of pathogenic microorganisms with drug resistance is a cause of global concern because they threaten to bring back the death tolls of pre-antibiotic era. The long and resource-intensive process of making new antibiotics calls for search in alternate directions. Systematic study is necessary to identify natural compounds that can inhibit Methicillin-resistant *S. aureus* (MRSA) or Methicillin-intermediate *S. aureus* (MISA) isolates *in vivo*. The rationale behind the study was to employ bioinformatics to identify natural molecules with binding capacity to the resistance factors in MRSA *in vitro,* so that we have alternate to expensive synthetic antibiotics in the time of rise of the super-bugs. The aim of the present study was to identify the resistance pattern of *S. aureus* in the clinical samples causing disease in Dhaka city, molecular typing of the methicillin non-susceptible *S. aureus* isolates and identifying new herbal components with anti-microbial effect against *S. aureus*. We hypothesized about antimicrobial activity of herbal compound *Syzygium cumini* (Family: Myrtaceae) against MRSA/MISA. After initial characterization of targeted bacteria with molecular methods, the organism was subjected to confirmation of Hospital-associated MRSA (HA-MRSA) and Community-associated MRSA (CA-MRSA) by conventional molecular technique (PCR). We determined minimum inhibitory concentration of the natural molecule by broth dilution method, allowing antibacterial activity of *Syzygium cumini* fruit and leaf extracts against clinical MRSA and MISA isolates. Such studies are important to keep track of genotypes of emerging pathogens so that effective containment strategies could be implemented to save lives both in and out of healthcare settings.

Materials and Methods

Bioinformatics Analysis: Mutant â-lactamase (*bla*) and mutant penicillin binding protein (PBP-2a') are responsible for resistance against methicillin in *S. aureus*. [16] We retrieved the 3D structure of these 2 proteins from Protein Databank (https://www.rcsb.org/structure/3ZFZ, https://www.rcsb.org/structure/1mwu), subjected each of them to ligand-binding search using ZINC 15 database (https://zinc.docking.org/), which turned up a number natural molecule that bind to different domains of the target proteins (figure 1).

Collection of Samples: The samples were collected between the periods of March to October, 2018, from Dr. Sirajul Islam Medical College, Asgar Ali Hospital, Rusmono General Hospital, Popular Diagnostic Center located in Dhaka city, Bangladesh. Initial processing of sample and transportation was maintained as per WHO guideline (Official Website, 2010).[17]

Phenotypic Screening, antibiotic sensitivity test and selection of MRSA: Molecular (PCR) confirmed isolates (*nuc* gene positive *S. aureus*) were subjected to Kirby-Bauer disk diffusion assay for determination of antibiotic sensitivity according to Clinical and Laboratory Standards Institute (CLSI) standards M02-A12 and M07-A10.[18,19] Isolates demonstrated resistance or intermediate-resistance to Methicillin antibiotic was defined as methicillin resistant *S. aureus* (MRSA) or methicillin intermediate-resistance *S. aureus* (MISA) respectively (table I).

SCCmec typing of MRSA isolates: Around 3× 10^8 CFU/ml of bacterial suspension was used for DNA extraction following the protocol from Qiagen, Germany. Polymerase Chain Reaction (PCR) was done according to the established protocol as described in Ghaznavi-Rad *et al*, 2010 with a slight modification.[19] A volume of 25µlPCR-mix was prepared using 10 pmol of corresponding forward and reverse primers, 200ng of template DNA, nuclease free water and TaqMan® universal PCR Mastermix (Thermo-Fisher Scientific, USA). Initial denaturation of 95ºC for 15 minutes, then 30 cycles with denaturation of 94ºC for 30 s, annealing at 57ºC for 1.5 minutes,

elongation at 72ºC for 1.5 minutes and a final elongation step of 72ºC for 10 minutes in a Mx3005P platform (Strategene, USA). The PCR products were resolved in a 0.7% agarose gel (Merck, Germany) (Figure 2) with 100bp Invitrogen DNA ladder (Thermo Fisher Scientific, USA). List of primer and amplified target DNA sized used in this study are provided in (table III).

Preparation of *S. cumini* Leaf and Fruit Extract: Disinfected leaf and fruit samples were stored at -4°C, homogenized without water and filtered through 0.22µm Whatmann filter using gravitational flow, concentrated with a vacuum membrane distillation system at 120 psi (AVMD Inc. Denmark).

Minimum Inhibitory Concentration (MIC) and drop plate assay: The isolate suspensions were adjusted to 0.5 McFarland, further diluted to 10^7 CFU/ml. The final reaction mix contained 5%, 2.5% and 1.25% extracts of *S. cumini* leaf or fruit. The test tubes were incubated for further 24 h, and observed visually for any change in color indicating bacterial growth. The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) was recorded as MIC value. The efficacy of the extracts was assayed with drop-plate technique and subsequent colony count. From every test tube used in MIC test, 50 µl broth was inoculated into MSA agar for drop plate assay. After 18-24 hours of incubation, micro-colonies appearing on test drops were enumerated with magnifying glass. The numbers of colonies appearing on the controls and the test reactions were analyzed statistically (figure 3).

Statistics and data Analysis: Zone of inhibition data from the Kirby-Bauer disk diffusion test compiled using spreadsheet (MS Excel, Microsoft Corporation, USA) and used as input file in BacLink software to format the data for further analysis using WHONET-2019 software (WHO Collaborating Centre for Surveillance of Antimicrobial Resistance, USA).[20]

In the drop plate assay, One-way ANOVA was done between number of colonies from both controls and those from test samples in SPSS (v21). The study was approved by the Department of Microbiology, Jagannath University, Dhaka and the research work was done in Bangladesh Livestock Research Institute, Savar.

Results

In community and hospital environment, one of the most common causes of serious infection is *Staphylococcus aureus.* This study had been designed to identify natural compound from *Syzygium cumini* that can inhibit MRSA and MISA isolates *in vitro*.

In bioinformatic analysis; the simplified docking analysis of 3-dimentional structures of â-lactamase and PBP-2a' molecules from MRSA strains retrieved from protein data bank (www.rcsb.org) were used to identify ligands from natural molecule database ZiNC15 (www.zinc.docking.org). The mutant Penicillin Binding Protein 2a' (PBP-2a') responsible for penicillin-resistance binds to molecules belonging to azolidine, harzol and phenanthrene groups (figure 1). The â-lactamase binds to molecules belonging to pyrrolidine, phenolic compounds, phenanthrene group and L-arabinose (data not shown).

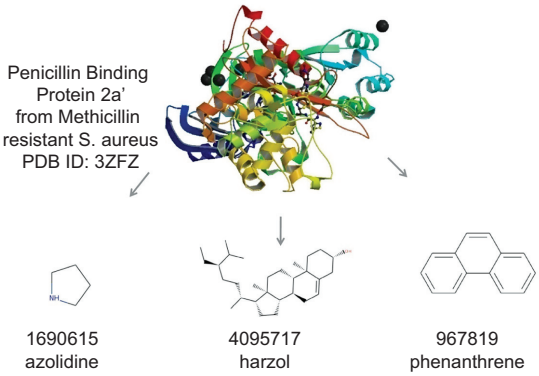

Figure 1: Three-dimensional structure of penicillin-binding protein 2a' (PP-2a') from methicillin-resistant S. aureus (MRSA) and it's binding partners among natural molecule. There is potential ligand-epitope interaction predicted between mutant PBP-2a' and small molecule inhibitor interaction.

All the natural molecules shown to bind to PBP-2a' and â-lactamase are found in Indian Blackberry plant (*S. cumini*), as reported by HPLC analysis by other groups. Therefore, we proceeded to study the effect of the fruit extract and leaf extract on Methicillin non-susceptible clinical isolates of *S. aureus* from Bangladesh.

In phenotypic antimicrobial sensitivity test, among the 12 isolates, all tested resistant against amoxicillin, most were resistant linezolid 91.7% (n=11; 95%CI: 60-99%) and cefoxitin 75% (n=09; 95% CI: 43.4-93) (table I). Sensitivity to vancomycin was higher (40%).

Overall 83.3% (n=10/12) isolates (including 5/5; 100% MISA and 5/7; 71.4% MRSA isolates) were termed as MDR isolate according to the widely used standardized international terminology and WHONET 2019 analysis.[21] Among the all isolates, 75% and 41.6% isolates were classified as possible-XDR (extreme drug resistant) and possible-PDR (pan-drug resistant) isolate (Table II) according to the alert level definition for *S. aureus* provided by the expert panel of European Centre for Disease Prevention and Control

(ECDC) and Centers for Disease Control and Prevention (CDC).

MRSA and MISA isolates were examined with different primers Type I, Type II, Type III, Type IVa, Type IVb, Type IVc, Type IVd, Type IVh and Type V. Analysis of SCC*mec* gene cassette using multiplex-PCR technique revealed 25% HA-MRSA (hospital associated) and 25% CA-MRSA (community associated) was confirmed based on the result of Type I-613bp, Type III-243bp and Type IVa-776bp, Type IVh-663bp respectively. Whereas 50% isolates was unidentified (figure 2).

The assessment of minimum inhibitory concentration relies on the ability of leaf and fruit extracts concentration to inhibit drug resistant clinical strains. Under the treatment conditions used here, when the organism was treated with by at least 1.25% v/v fruit or leaf extract compared to positive control, inhibited growth proliferation. A concentration of 2.5% maintained growth at control level compared to 1.25%, while 5.0% inhibited growth proliferation following 24 h exposure (figure 3).

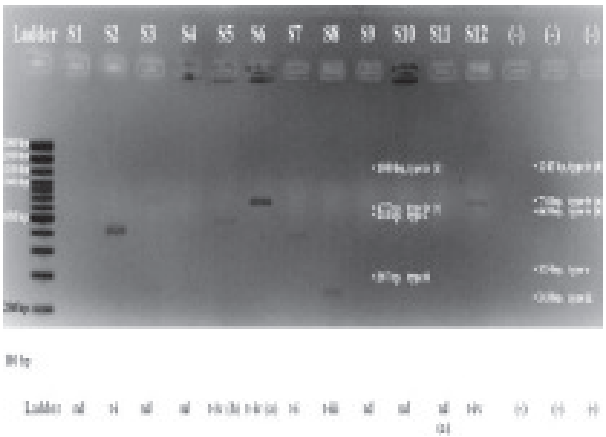

Figure 2: Gel electrophoresis of multiplex-PCR product of each isolates' DNA extracts amplified using sccmec gene typing primers. 0.7% Agarose gel was quantify using 100bp DNA Ladder (Invitrogen) on the first well followed by isolates 1to 12 and then negative well includes only dye as negative control; nd: not detected.

Table I: Antibiotic resistance of MISA and MRSA isolates against regularly used antibiotics to treat Staphylococcal infection.

Antibiotics	Code	Break Points	MISA (n=5)		MRSA (n=7)		Overall (n=12)	
			% R	95% C.I.	% R	95% C.I.	% R	95% C.I.
Cefoxitin	FOX	S >= 22	80	30-99	71.4	30-95	75	43-93
Linezolid	LNZ	S >= 21	100	46-100	85.7	42-99	91.7	60-99
Trimethoprim/ Sulfamethoxazole	SXT	11 – 15	80	30-99	57.1	20-88	66.7	35-89
Vancomycin	VAN	15 – 16	40	7.0-83	42.9	12-80	41.7	16-71
Azithromycin	AZM	14 – 17	60	17-93	57.1	20-88	58.3	29-83
Amoxicillin	AMX	S >= 29	100	46-100	100	56-100	100	70-100

Table-II: Antibiotic resistance profile of MRSA and MISA isolates

Isolate	Source	Resistance profile	Number of classes non-susceptible	Resistance level according to WHONET & CDC alert		
				MDR Profile	XDR Profile	PDR Profile
S12	Blood	MET* FOX§ AMXθ	2	MDRΩ		
S04	Pus	MET LNZΨ SXT† AZM© AMX	4			
S09	Wound Swab	MET LNZ SXT AZM AMX	4			
S10	Pus	MET FOX LNZ SXT AMX	4	MDR	Possible XDRω	
S11	Urine	MET FOX LNZ SXT AMX	4	MDR	Possible XDR	
S01	Blood	MET LNZ SXT VAN¡ AZM AMX	5	MDR	Possible XDR	
S05	Urine	MET FOX LNZ SXT AZM AMX	5	MDR	Possible XDR	
S02	Pus	MET FOX LNZ SXT VAN AZM AMX	6	MDR	Possible XDR	Possible PDR¶
S03	Pus	MET FOX LNZ SXT VAN AZM AMX	6	MDR	Possible XDR	Possible PDR
S06	Blood	MET FOX LNZ SXT VAN AZM AMX	6	MDR	Possible XDR	Possible PDR
S07	Tracheal	MET FOX LNZ SXT VAN AZM AMX	6	MDR	Possible XDR	Possible PDR
S08	Conjunctiva	MET FOX LNZ SXT VAN AZM AMX	6	MDR	Possible XDR	Possible PDR

MET: Methicillin, FOX: Cefoxitin, LNZ: Linezolid, SXT: Trimethoprim/ Sulfamethoxazole, VAN: Vancomycin, AZM: Azithromycin, AMX: Amoxicillin, MDR: multidrug-resistant, XDR: extensively drug-resistant, PDR: pandrug-resistant.

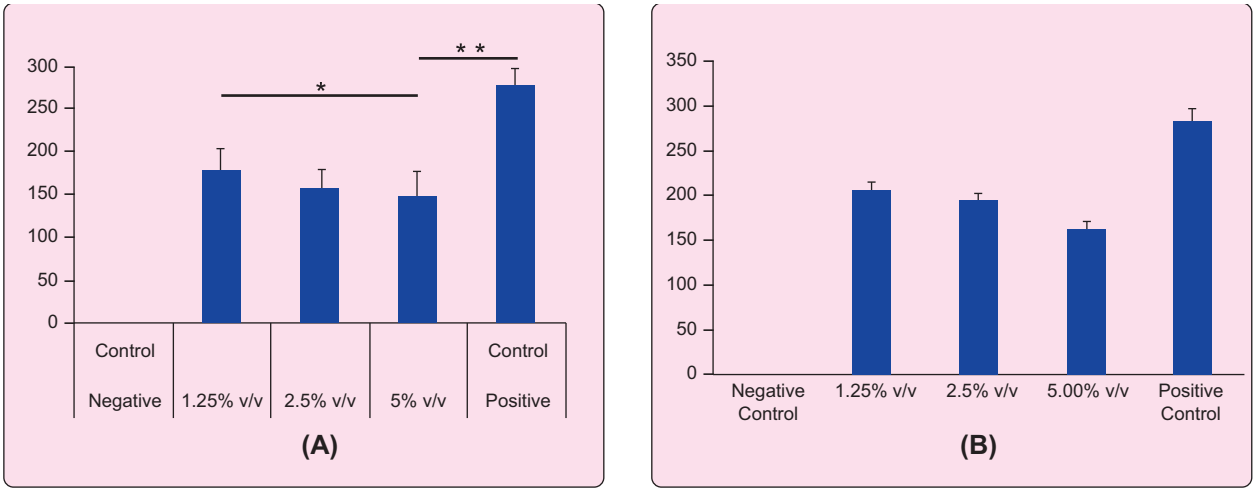

Figure 3: Ability of fruit extracts [A] and leaf extracts [B] to inhibit MRSA. Negative control = 1.25% test substance in culture media. Positive control = organism without the test substance in media. Results represent means and SD of different concentration. Significantly different values determined by one-way ANOVA (*p< 0.05, **p<0.01).

Table-III: *List of All Primers Used in this Study*

Purpose	Primer	Primer (5'-3')	ProductSize (bp)	T_m Value(°C)
Identification	nu-F3	TCGCTTGCTATGATTGTGG	359	52
	nu-nucR	GCCAATGTTCTACCATAGC		
Methicillin	mecA-1F	GTAGAAATGACTGAACGTCCGATAA	310	50
resistant gene	mecA-2F	CCAATTCCACATTGTTTCGGTCTAA		
SCCmec	TypeI_f	GCTTTAAAGAGTGTCGTTACAGG	613	57
Typing	TypeI_r	GTTCTCTCATAGTATGACGTCC		
(mPlex PCR)	TypeII_f	GATTACTTCAGAACCAGGTCAT	287	
	TypeII_r	TAAACTGTGTCACACGATCCAT		
	TypeIII_f	CATTTGTGAAACACAGTACG	243	
	TypeIII_r	GTTATTGAGACTCCTAAAGC		
	TypeIVa_f	GCCTTATTCGAAGAAACCG	776	
	TypeIVa_r	CTACTCTTCTGAAAAGCGTCG		
	TypeIVb_f	AGTACATTTTATCTTTGCGTA	1000	
	TypeIVb_r	AGTCATCTTCAATATGGAGAAAGTA		
	TypeIVc_f	TCTATTCAATCGTTCTCGTATT	677	
	TypeIVc_r	TCGTTGTCATTTAATTCTGAACT		
	TypeIVd_f	AATTCACCCGTACCTGAGAA	1242	
	TypeIVd_r	AGAATGTGGTTATAAGATAGCTA		
	TypeIVh_f	TTCCTCGTTTTTTCTGAACG	663	
	TypeIVh_r	CAAACACTGATATTGTGTCG		
	TypeV_f	GAACATTGTTACTTAAATGAGCG	325	
	TypeV_r	TGAAAGTTGTACCCTTGACACC		

Discussion

S. aureus occupies the list of multi-disease pathogens because of myriads of virulence factors.[22] Methicillin-resistant *S. aureus* was reported in 1990 by Matthews.[23] The pathogens become more dangerous with acquisition of multi-drug resistance genes.[16] Methicillin non-susceptible *S. aureus* was declared a highly critical pathogen in 2016 by the World Health Organization due to the high cases of fatality associated with bloodstream infections, pneumonia and post-surgical infections, dialysis recipients and long-term inmates of the intensive care units.[24] As of 2005, 20% of all clinical isolates of *S. aureus* were resistant to methicillin.[25] In USA, 90% of all hospital-associated infections by methicillin non-susceptible *S. aureus* (MnsSA) occurred to patients in post-operative units with a 50% rate of mortality.[26]

The transmission, management, prevalence, morbidity and mortality of community-associated methicillin-resistant *S. aureus* (CA-MRSA), hospital-associated methicillin-resistant *S. aureus* (HA-MRSA) and, a third category reported very recently, livestock-associated MRSA (LA-MRSA) are very different.[27] Proper identification of the category of clinical MRSA is important for effective infection control. HA-MRSA is spread by infected patients, contaminated fomites and clinical personnel whereas CA-MRSA is transmitted by poor hygiene and drug-overuse.[28] The occurrence of 5-10% MRSA in the community and 1% MRSA in a healthcare facility defines an endemic situation requiring implementation of specialized disinfection protocol.[29] All these emphasis on the clinical categorization of MRSA prompted us to analyze the 12 clinical isolates of methicillin non-susceptible *S. aureus* with PCR typing (Figure 2). The short time period of sample collection and occurrence of too few MRSA is insufficient for any conclusive information for public health informatics. However, the non-susceptibility of methicillin-intermediate isolates indicate an increase in evolution of resistance against methicillin. Distribution of MnsSA isolates across all age/sex groups and all anatomical sites indicate redundancy of the infection. Our findings are consistent with the other reports from Bangladesh by Parvez et al. and Ahmed et al.[30,31] Yet, the PCR typing of SCC*mec* gene cluster for clinical categorization does not match ours, because the other groups could determine all their clinical isolates with the common primers for HA-MRSA and CA-MRSA typing (table III) and no report emerges on untypable SCC*mec* cluster from Bangladesh.[32,33] We rationalize that livestock-associated MRSA might have spread widely in the Dhaka population in the recent years (2017-18), showing up in our study, which probably was negligible before.

The high labour, effort and cost in developing new generations of antibiotics pressed scientists to look for affordable and sustainable options such as identification of antimicrobial molecules from natural sources. *S. cumini* is an interesting target for potential anti-microbial activity because it is traditionally known to heal infections.[34] There are scores of published articles from Asia showing inhibitory effect of *S. cumini* on *S. aurues*. Ethanolic extract of *S cumini* leaves was shown to disrupt quorum sensing and biofilm development of *S. aurues*.[35] Holoacetic acid extract of *S. cumini* leaves has an MIC value of 70µg/ml on *S. aureus*.[36] Methanolic extact of *S. cumini* leaves had an MBC of 1.56-50 mg/ml on *S. aureus*.[37] Ethanolic fraction of *S. cumini* leaf and seed extract inhibited drug-sensitive and drug-resistant isolates of *S. aurues*.[38] Methanolic extract of *S. cumini* leaves and fruit was shown to be more effective against *S. aurues* than aqueous extracts.[27] Our study adds some more experimental data on the effect of *S. cumini* on MnsSA: aqueous extracts of both fruits and leaf extract from *S. cumini* have inhibitory effect on multi-drug resistant clinical isolates of *S. aurues* at an MIC of 1.25-5% v/v (figure 3). A preliminary bioinformatic analysis shows that the major active ingredients in *S. cumini* leaf and fruits extracts have potential molecular targets on *S. aureus* (figure 1). Though rudimentary, these experimental data show great future prospect of *S. cumini* as an antibacterial molecule.

Conclusion

Findings of this study could serve as very strong background data for advanced studies, such as conformational dynamics of the bioactive ingredients of *S. cumini* on ligand-binding sites on *S. aurues*. Such studies have been done on other natural molecules and their chemical modifications have been done to construct a substrate-delivered antibacterial agent that has increased bioavailability against *S. aureus*. The half-maximal inhibitory concentration (IC_{50}) of *S. cumini* leaf, seed and fruit extract needs to be determined. Pathologic profile of the clinical isolates should be done using LukS/F-PV virulence determinants. Finally, a thorough PCR typing of the

SCCmec gene cassette should be done using HA-, CA- and LA- specific primers of *S. aureus* to identify all available clinical isolates properly, in case an infection control protocol needs to be established. The variants of *mec*A gene also need to be typed to understand the evolving resistance of methicillin-intermediate clinical strains of *S. aurues.*

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References

1. Fishovitz J, Hermoso JA, Chang M, Mobashery S. Penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus. IUBMB Life. 2014; 66: 572-77. DOI:10.1002/iub.1289
2. Matsuhashi M, Song MD, Ishino F,Wachi M, Doi M, Inoue M, et al. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in Staphylococcus aureus. J Bacteriol. 1986; 167: 975-80. DOI: 10.1128/jb.167.3.975-980.1986
3. Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? Yale J Biol Med. 2017; 90: 135-45. PMID: 28356901
4. Kennedy AD, Otto M, Braughton KR, Whitney AR, Chen L, Mathema B, et al. Epidemic community-associated methicillin-resistant Staphylococcus aureus: recent clonal expansion and diversification. Proc. Natl. Acad. Sci 2008; 105: 1327-32. DOI: 10.1073/pnas.0710217105
5. Galia L, Ligozzi M, Bertoncelli A, Mazzariol A. Real-time PCR assay for detection of Staphylococcus aureus, Panton-Valentine Leucocidin and Methicillin Resistance directly from clinical samples. AIMS Microbiol 2019; 5:138-46. DOI:10.3934/microbiol.2019.2.138
6. Bæk KT, Gründling A, Mogensen RG, Thøgersen L, Petersen A, Paulander W, et al. â-Lactam resistance in methicillin-resistant Staphylococcus aureus USA 300 is increased by inactivation of the ClpXP protease. Antimicrob Agents Chemother. 2014; 58: 4593-603. DOI: 10.1128/AAC.02802-14
7. Stapleton PD, Taylor PW. Methicillin resistance in *Staphylococcus aureus:* mechanisms and modulation. *Sci Prog* 2002; 85: 57-72. DOI: 10.3184/003685002783238870
8. Lina G, Piémont Y, Godail-Gamot F,Bes M, Peter MO, Gauduchon V, et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 1999; 29: 1128–32. DOI: 10.1086/313461
9. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med 1999; 339: 520-32. DOI: 10.1056/NEJM199808203390806
10. Tiemersma EW, Bronzwaer SL, Lyytikäinen O,Degener JE, Schrijnemakers P, Bruinsma N, et al. Methicillin-resistant Staphylococcus aureus in Europe. *Emerg* Infect Dis. 2004; 10: 1627-34. DOI: 10.3201/eid1009.040069
11. Bancroft AE. Antimicrobial Resistance: It's Not Just for Hospitals. The Journal of the American Medical Association 2007; 298: 1803-04. DOI: 10.1001/jama.298.15.1803
12. Haseen F. Malnutrition among Bangladeshi Women in Ultra Poor Households: Prevalence and Determinants. LAP Lambert Academic Publishing. 2010; 52. DOI:55797099.1369456281.1593432785
13. Ahmed A, Rabbi MB, Sultana S. Antimicrobial resistance in Bangladesh: A Systematic Review. Int J Infect Dis. 2019; 80:54-61. DOI: 10.1016/j.ijid.2018.12.017
14. Taz KA, Jobayer M, Shamsuzzaman SM. Nasal Colonization of MRSA among Healthcare Providers in Tertiary Care Hospital Bangladesh. Mymensingh Med J. 2019; 28:627-33. PMID: 31391436
15. Steining EJ, Duchene S, Robinson DA, Monecke S, Yokoyama M, Laabei M, Slickers P. Evolution and Global Transmissionof a Multi-drug resistant Community-associated MRSA lineage from the Indian Subcontinent. mBio. 2019; 10: e01105-19. DOI: 10.1128/mBio.01105-19.
16. Katayama Y, Zhang Z, Chambers HF. PBP-2a mutations produce very high-level resistance to beta-lactams. Antimicrobial Agents and Chemotherapy. 2004; 48:453-459. DOI: 10.1128/aac.48.2.453-459.2004.
17. Drew WL, Barry A, O'Toole R, Sherris JC. Reliability of the Kirby-Bauer disc diffusion method for detecting methicillin-resistant strains of *Staphylococcus aureus.* Appl Environ Microbiol. 1972; 24: 240-47. PMID: 5071651
18. Gerbig DG, Engohang-Ndong J, Aubihl H. A new twist to the Kirby-Bauer antibiotic ssceptibiliy test activity-increasing abtibiotic sensivity of Pseudomonas fluorscens through thermal stress. J Microbiol. Biol. Educ. 2013. 14:269-270 PMC: 3867773
19. Ghaznavi-Rad E, Shamsudin MN, Sekawi Z, Neela V. A simplified multiplex PCR assay for fast and easy discrimination of globally distributed staphylococcal cassette chromosome mec types in meticillin-resistant *Staphylococcus aureus.* J med microbiol. 2010; 59: 1135-1139. DOI: 10.1099/jmm.0.021956-0

20. Stelling JM, Kulldorff M, O'Brien TF. WHONET and BacLink: software tools for laboratory-based surveillance of infectious diseases and antimicrobial resistance. Adv Dis Surveill 2007; 2:121. PMC: 4093803

21. Magiorakos AP, Srinivasan S, Carey RB, Carmeli Y, Falagas ME, Giske CG et al. Multidrug resistant, extensively drug resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012; 18: 268–81. DOI: 10.1111/j.1469-0691.2011.03570.x.

22. Ayliff GAJ. Recommendations for the Control of Methicillin-Resistant *Staphylococcus aureus* (MRSA). World Health Organization Reference Document for Emerging and other Communicable Diseases, Surveillance and Control. 2015. DOI: apps.who.int/iris/handle/10665/62984

23. Matthews P, Tomasz A. Insertional inactivation of the *mec* gene in a transposon mutant of a methicillin-resistant clinical isolate of *Staphylococcus aureus*. Antimicrob Agents Chemother. 1990; 34:1777–9. DOI: 10.1128/aac.34.9.1777.

24. World Health Organization Factsheet on Antimicrobial Resistance. (2017) who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance

25. Dickmann P, Keeping S, Döring N, Schmidt AE, Binder C, Ariño-Blasco S and Gil S. Communicating the Risk of MRSA: The Role of Clinical Practice, Regulation and Other Policies in Five European Countries. Front. Public Health. 2017; 5:44-49. DOI: 10.3389/fpubh.2017.00044/full

26. Sergelidis D, Angelidis AS. Methicillin-resistant *Staphylococcus aureus*: a controversial food-borne pathogen. Lett Appl Microbiol. 2017; 64:409-18. DOI: 10.1111/lam.12735

27. Mascaro V, Squillace L, Nobile CGA, Papadopoli R, Bosch T, Schouls LM, Casalinuovo F, Musarella R, Pavia M. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage and pattern of antibiotic resistance among sheep farmers from Southern Italy. Infect. Drug. Resist. 2019; 12: 2561-71. DOI: 10.2147/idr.s211629

28. Desai R, Pannaraj PS, Agopian J, Sugar CA, Liu GY, Miller LG. Survival and transmission of community-associated methicillin-resistant *Staphylococcus aureus* from fomites. Am J Infect Control. 2011; 39:219-25. DOI: 10.1016/j.ajic.2010.07.005.

29. Kinoshita T, Tokumasu H, Tanaka S, Kramer A, Kawakami K. Policy implementation for methicillin-resistant *Staphylococcus aureus* in seven European countries: a comparative analysis from 1999 to 2015. J Mark Access Health Policy. 2017; 5: 1351293. DOI: 10.1080/20016689.2017.1351293

30. Parvez MAK, Ferdous RN, Rahman MS, Islam S. Healthcare-associated (HA) and community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) in Bangladesh - Source, diagnosis and treatment. J Genet Eng Biotechnol. 2018; 16:473-78. DOI: 10.1016/j.jgeb.2018.05.004

31. Ahmad S, Alenzi FQ, Al-Juaid NF, Ahmed S. Prevalence and antibiotic susceptibility pattern of methicillin resistant *Staphylococcus aureus* at Armed Forces Hospital in Saudi Arabia. Bangladesh Med Res Counc Bull. 2009; 35:28-30. DOI: 10.3329/bmrcb.v35i1.1983

32. Zahan NA, Hossain MA, Musa AK, Shamsuzzaman AK, Mahamud MC, Mamun AA, Paul SK, Ahmed S, Sumona AA, Begum Z, Alam M, Yusuf MA, Uddin MS.PCR for mecA gene of methicillin resistant *Staphylococcus aureus*. Mymensingh Med J. 2009; 18:21-6. PMID:19182744

33. Gupta K, Singh SP, Manhar AK, Saikia D, Namsa ND, Konwar BK, Mandal M. Inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* Biofilm and Virulence by Active Fraction of *Syzygium cumini* (L.) Skeels Leaf Extract: In-Vitro and In Silico Studies. Indian J Microbiol. 2019; 59:13-21. DOI: 10.1007/s12088-018-0770-9

34. Jahan F, Lawrence R, Kumar V, Junaid M. Evaluation of antimicrobial activity of plant extracts on antibiotic susceptible and resistant *Staphylococcus aureus* strains. J. Chem. Pharm. Res. 2011; 3: 777-89. https://www.researchgate.net/publication/307509346

35. Imran M, Imran M, Khan S. Antibacterial activity of Syzygonium cumini leaves against multidrug-resistant pathogenic bacteria. J Appl. Pharma. Sci. 2017; 7:168-74 DOI: 10.7324/JAPS.2017.70327

36. Ramli S, Radu S, Shaari K, Rukayandi Y. Antibacterial Activity of Ethanolic Extract of Syzygium polyanthum L. (Salam) Leaves against Foodborne Pathogens and Application as Food Sanitizer. Biomed. Res. Int. 2017; e-9024246, p13. DOI: 10.1155/2017/9024246

37. Mohamed AA, Ali SI, El-Baz FK. Antioxidant and Antibacterial Activities of Crude Extracts and Essential Oils of *Syzygium cumini* Leaves. PLoS ONE. 2013; 8: e60269. DOI: 10.1371/journal.pone0660269

38. Sehwag anf Das M. Composition and functional of whole jamun based functional confection. J Food Sci Technol. 2016. 53:2569-79. PMC: 4951409/