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Research Article

ANTIMICROBIAL EFFICACY OF ESSENTIAL OILS OF SELECTED PLANTS AND VACCINE DESIGN AGAINST mecA PROTEIN OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Objective- Emergence of Multi Drug Resistance indicates a dire to understand the bacterial involvement in infections and find out new alternative approaches in its therapeutics and prevention. The present study was undertaken to study the antimicrobial resistance patterns of *S. aureus* isolated from various samples collected from Hospitals of Gwalior. During the present study an effort was made to find out the information about mecA protein of *Staphylococcus* and their conserved regions were analyzed in order to assess their antigenic potential.

Methods- In the present study, a total of 872 samples were collected and processed for MRSA screening. Conventional methods were used for the isolation and identification of bacteria. Thereafter, antibacterial property of 20 various drugs as well as aromatic compounds of 18 herbal plants was performed against multiple resistant *Staphylococcus aureus* (MRSA) isolates according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS). *In silico* prediction of vaccine candidates in *mecA* through bioinformatics approach was also performed.

Results-The study revealed that drug resistance pattern of MRSA isolates is increasing. But the major concern is the development of resistance against Vancomycin which is thought be the most effective drug against *Staphylococcus*. In comparison to antibiotics, essential oils showed very good activity against the test bacteria with few exceptions.

Conclusion- The essential oils of Clove and Cinnamon were found to be more active against the test organism. We predicted multi-epitope peptide which was having very good potential to induce B cell response and a very good candidate for binding to MHC II molecule and thus can act as a suitable vaccine target against *S. aureus*.

Keywords: Multidrug Resistance, essential oils, *in silico*, multi-epitope peptide.

INTRODUCTION

The genus "*Staphylococcus*" is common inhabitant of the skin and mucous membrane and it account for a considerable proportion of human infections. Humans are a natural reservoir for *Staphylococcus aureus*. Approximately 20–30% of the general population is "staph carriers" [1]. *Staphylococcus aureus* has been reported as a major cause of community and hospital acquired infection. It is still one of the four most common causes of nosocomial infections, often causing post-surgical wound infections. The emergence of antibiotic resistance in microorganisms and their spread is threatening the medical community. This is particularly true in case of *Staphylococcus aureus*.

The resistance development in *Staphylococcus aureus* dates back to 1940s. Multiple drug resistance of *Staphylococcus aureus* is due to the presence of *mecA* gene coding for penicillin binding protein (PBP2a) with a low affinity for β- lactam antibiotics. This gene is carried on Staphylococcal Cassette Chromosome (SCC) *mec,* a unique mobile genetic element that harbors the methicillin resistant gene (*mecA*) and other antibiotic resistant determinants. Thus, it mediates drug resistance in Staphylococci [2].

The increasing gain of resistance to available antimicrobials and side effects associated with the drugs have attracted the attention of scientific community towards the search and development of new cost effective drugs of natural and synthetic origin [3]. In the present scenario, interest of biologists to find antimicrobial properties in aromatic plants especially essential oils is continually increasing. Some studies demonstrated that plant derived essential oils may be an effective alternative to overcome microbial resistance to conventional drugs [4, 5]. Essential oils are complex mixtures of volatile secondary metabolites that mainly consist of mono and sesquiterpenes including carbohydrates, alcohols, aldehydes, ketones and ethers. These are responsible for both fragrant and biological effects of aromatic medicinal plants [6, 7, 8]. So, we also tried to analyse whether application of essential oils can be used to design a therapeutic measure to address this problem. In comparison to antibiotics, essential oils showed very good activity against the test bacteria with few exceptions. Varieties of aromatic medicinal plants are tested for treating infectious diseases [9]. These plants have been mentioned in different phytotherapy manuals due to their availability, fewer side effects and reduced toxicity. An important characteristic of essential oils and their constituents is their hydrophobicity, which enables them to permeate in the lipids of bacterial cell membranes & mitochondria, thus disturbing the structures and rendering them more permeable [10, 11].

No effective vaccine is generally available that stimulates active immunity against staphylococcal infections in humans. However, vaccine therapies represent a new and innovative approach in broadening the available clinical tools against the global health problem of community and healthcare-associated *S. aureus* bacterial infections. Moreover it is cardinal to focus over mecA protein to design a multi-epitope antigenic target specific against multidrug resistant *Staphylococcus* that may provide protection from variety of infections caused by this deadly bacterium.

The objective of this research was to evaluate the potential of drugs and essential oils on standard microorganism strains as well as multi-drug resistant bacteria, which were isolated from hospitals. Moreover, we investigated the possible vaccine candidate for the test organism using bioinformatics tools.

MATERIALS & METHODS

Micro-organisms used in this study were isolated from patients suffering from various infections. The present study comprised 872

cases among which 500 samples were taken from patients, 266 cases were from healthy and 106 samples were taken from environment. All these cases were selected out of the peoples attending the local hospitals of Gwalior and the interior environment of the hospitals' wards. The candidates for this study were selected randomly.

The control group was selected from the persons who were not having any infection or any other visual symptom of diseases. The subjects for this study were between 5 to 75 years of age. Patients were examined by the doctors. Data were recorded, analysed and subjected to statistical analysis.

Culture media and antibiotics

Brain Heart Infusion agar and Mueller-Hinton agar were used for growing the organisms. Antibiotic discs used in the study are given in table no. 1. Test bacteria were characterized by their biochemical characteristics as well as by standard microbiological techniques. The identified bacterial pathogens were tested against 20 different antibiotics using disc diffusion method of Bauer *et al,* 1966 [12]. MRSA was determined by the reference broth micro-dilution methods using established National Committee for Clinical Laboratory Standards break points.

Eighteen essential oils obtained from Department of pharmaceutical Science, Jiwaji University, Gwalior, M.P were used in this study. These oils were selected on the basis of their use in traditional and conventional medicine.

Screening of essential oils for their antimicrobial potential

Each bacterial culture, to be tested was streaked onto Brain Heart Infusion (BHI) non-inhibitory agar medium to obtain isolated colonies. Each colony was put into BHI broth medium. After 6 hrs of growth, microorganisms at a concentration of 10^6cells/mL were layered on the surface of Mueller-Hinton agar plates. Subsequently, filter paper discs (6 mm dia.) saturated with essential oils (40 µL) was placed on the surface of each inoculated plate. In order to evaluate the efficiency of the methodology, 40µL of each essential oil was instilled simultaneously in the wells of another plate. The plates were incubated at 37 °C. After 24 h. it was possible to observe the inhibition zones. The diameter of the zones of complete inhibition was measured in millimetres with the help of antibiotic zone scale (HiMediaPvt. Ltd., Mumbai, India). Bacterial growth with halos equal to or greater than 10 mm was considered susceptible to essential oils. Controls were maintained along with each test.

Vaccine designing using bioinformatics tools

After the analysis of therapeutic potential of various natural products against MRSA, an effort was made to analyze preventive measure against it. The development of vaccine against several diseases is a very ancient practice. Recently this practice is also going to be evaluated for the development of preventive measure against MRSA infections. Molecular pathogenesis of MRSA infections involve several phases, each of which may offer targets for immunological intervention.

So we performed *in silico* prediction of vaccine candidates of *mecA* through bioinformatics approach. In this context, we have done multiple sequence alignment of *mecA* sequences using database.

RESULTS & DISCUSSION

The threat to the human population is that reservoirs of drug-resistant bacteria is abound. The rotational use of vancomycin in the treatment of life-threatening infections due to MRSA is of last resort to preserve the efficacy of this antibiotic. The spectra of high-level vancomycin resistance transposed into MRSA are one of the doomsday scenarios in infectious diseases. There has also been the call to consider wider use of combinations of antibiotics in primary therapy, not because of their synergistic approaches where two components work together to neutralize a single target, but combinations of distinct antibiotic classes that work on different targets concurrently.

In the present study, *S. aureus* was indicated by the yellow halo produced around the colonies on Mannitol Salt Agar. This is caused by the ability of *S. aureus* (and not *S. epidermidis* or most other osmotolerant bacteria) to ferment mannitol to acids which is detected by a change of pH indicator from red to yellow [13]. Mannitol salt agar is often a selective medium for *Staphylococcus* due to the high concentration of sodium chloride the agar contains.

The observed colonies were circular and pale to dark yellow in colour. The diameter of the colonies was about 1 to 1.5 millimeters. Gram staining of various colonies grown on different plates showed that the bacteria were Gram positive cocci, and arranged in irregular, grape like clusters.

Even though pharmaceutical companies have produced a number of new antibacterial drugs over the years, resistance to these drugs by *Staphylococcus aureus* has increased manifold and has now become a global concern. In general, *Staphylococcus aureus* have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. Therefore the identification of new effective antimicrobial agents is of paramount importance. Medicinal plants have long been investigated as the potential sources among new agents,

Results on the antibiotic sensitivity of MRSA isolates are presented in Table No.1. Overall study indicated that the antibiotics Cefuroxime, Ciprofloxacin, Piperacillin, Linezolid, Teicoplanin, Co-Trimoxazole, Ofloxacin, Tetracycline, Amikacin, CefaperazoneSulbactam and Vancomycin should be used judiciously against most of the staphylococcal infections in the region. Ampicillin, Cefazolin, Chloramphenicol, Erythromycin, Gentamycin, Oxacillin and Penicillin, however, should not be used any more by general medical practitioner while giving treatment to the patients because organisms are resistant to these antibiotics.

Table 1: It shows Antibiotic resistance pattern of test bacterial organism isolated from various samples included in the study

S. No.	Antibiotics (Concentration in µg)	Resistance (%)	Sensitive (%)
1.	Amikacin (30)	24 (25%)	70 (75%)
2.	Ampicillin (10)	94 (100%)	0 (0%)
3.	CefaperazoneSulbactam (75/30)	14 (15%)	80 (85%)
4.	Cefazolin (30)	94 (100%)	0 (0%)
5.	Cefuroxime (30)	75 (80%)	19 (20%)
6.	Cephalothin (30)	38 (40%)	56 (60%)
7.	Chloramphenicol (30)	94 (100%)	0 (0%)
8.	Ciprofloxacin (05)	78 (83%)	16 (17%)
9.	Clindamycin (02)	38 (40%)	56 (60%)
10.	Co-Trimoxazole (25)	56 (60%)	38 (40%)
11.	Erythromycin (15)	94 (100%)	0 (0%)
12.	Gentamycin (10)	94 (100%)	0 (0%)
13.	Linezolid (30)	62 (66%)	32 (34%)
14.	Ofloxacin (01)	56 (60%)	38 (40%)
15.	Oxacillin (01)	94 (100%)	0 (0%)
16.	Pazubid 25 (25)	0 (0%)	94 (100%)
17.	Penicillin (10 units)	94 (100%)	0 (0%)
18.	Piperacillin (100)	78 (83%)	16 (17%)
19.	Teicoplanin (30)	62 (66%)	32 (34%)
20.	Tetracycline (30)	56 (60%)	38 (40%)
21.	Vancomycin (30)	09 (10%)	85 (90%)

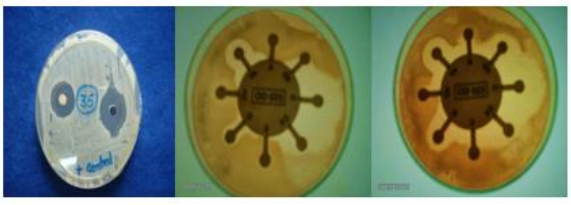

Fig. 1 It shows resistance pattern of test bacteria against the various drugs used in the study. Zone diameter measured with the antibiotic susceptibility zone diameter measurement scale

(HiMediaPvt. Ltd., Mumbai). The clear zone (absence of growth) indicates sensitivity of the test isolate against the drug. Zone diameter less than 10mm is considered to be resistant and more than 10mm is sensitive.

This study also showed that antimicrobial resistance of *Staphylococcus aureus* was high and alarming. The major concern is the development of resistance against Vancomycin which is thought be the most effective drug against *Staphylococcus*. Resistant bacteria could be the mutant form of common bacteria due to non-judicial use of broad spectrum antibiotics. Antibiotics are frequently prescribed in hospital and general practice. However they are often administered before the pathogen's culture and sensitivity results are known. Oxacillin resistance is primarily mediated by the production of penicillin binding protein 2a (PBP2a), which is encoded by the *mecA* gene. Because its production is under complex control by regulatory genes and in some cases also by *mecA*-negative resistance mechanisms, phenotypic or even genotypic evaluation is not completely straightforward. Furthermore, genetic testing requires significant technical and financial resources which might be lacking in many clinical laboratories. It is possible that plasmid DNA represents the transposon-associated *van*A gene cluster found in vancomycin-resistant enterococci, which could be horizontally transferred to other species, consequently conferring resistance to the antibiotic in that species [14]. It is also likely that strain's resistance to vancomycin was a result of a thickened cell wall, which allows the bacteria to inhibit vancomycin molecules from reaching its major target on the cytoplasmic membrane of the cell [15]. Plasmid borne antibiotic resistance was beyond the scope of the present study.

As the distribution of causative organisms and bacterial resistance rates vary according to time and place, the recent local data will be conducive to the clinicians for the best choicest treatment. As a result, not only the patients will be treated with the correct antibiotics but the misuse and overuse of antibiotics, which lead to rapid development and spread of resistance, will be minimized.

Since the development of antibiotic resistance in the pathogenic strains of *Staphylococcus aureus* is an ever increasing problem, suitable and possible alternate chemotherapeutic phytochemical compounds of plant origin such as alkaloids, terpenoids, polyphenols, flavonoids and steroids may be tried for effective control of drug resistant bacteria like MRSA [16].

The antibacterial activity of essential oils of 18 plant species was assayed *in vitro* by disc diffusion and agar well diffusion method against the test bacteria. The maximum antibacterial activity was shown by *Cinnamomumzeylanicum*, followed by *Syzigiumaromaticum*. The essential oils of olive, mustard, castor and coconut could not inhibit any of the bacterial isolates studied. It is quite possible that some of the essential oils that are ineffective in this study do not possess antibacterial properties or the essential oils might have contained antibacterial constituents in insufficient concentration so as to be effective. The activity of cinnamon oil was found to be more than vancomycin drug. So this can be a better agent for inhibition of MRSA after suitable toxicological analysis. All other oils showed moderate activity.

Table 2: It shows Antimicrobial resistance pattern of test bacterial organism against the selected plant essential oils included in the study

S. No.	Plant essential oils	Resistant	Sensitive
1	Rasna oil	17 (18%)	77 (82%)
2	Ajwain oil	17 (18%)	77 (82%)
3	Tulsi oil	17 (18%)	77 (82%)
4	Peppermint oil	0 (0%)	94 (100%)
5	Garlic oil	77 (82%)	17 (18%)
6	Til oil	77 (82%)	17 (18%)
7	Cinnamon oil	0 (0%)	94 (100%)
8	Turpentine oil	77 (82%)	17 (18%)
9	Eucalyptus oil	42 (45%)	52 (55%)
10	Clove oil	19 (20%)	75 (80%)
11	Mentha oil	42 (45%)	52 (55%)
12	Olive oil	17 (18%)	77 (82%)
13	Mustard oil	17 (18%)	77 (82%)
14	Castor oil	77 (82%)	17 (18%)
15	Coconut oil	77 (82%)	17 (18%)
16	Camphor oil	94 (100%)	0 (0%)
17	Amla oil	58 (55%)	36 (45%)
18	Neem oil	19 (20%)	75 (80%)
	Control (Vancomycin 40µl)	9 (9.57%)	85 (90.43%)

Fig. 2 It shows antibacterial effectiveness of essential oil against the test bacterial isolates. Zone diameter measured with the antibiotic susceptibility zone diameter measurement scale (HiMediaPvt. Ltd., Mumbai). The clear zone (absence of growth) indicates sensitivity of the test isolate against the essential oil. Zone diameter less than 10mm is considered to be resistant and more than 10mm is sensitive.

Cinnamon oil and clove oil are very ancient ingredients of Indian food. Their antimicrobial activity further supports the traditional natural medicinal system of India. Previous GC-MS study of cinnamon oil has shown Cinnamldehyde as the predominant active component [17, 18]. Cinnamldehyde is a natural anti-oxidant and the animal studies suggest that an extract of cinnamon bark taken orally may help to prevent stomach ulcer. Cinnamldehyde was completely inhibiting both sensitive and resistant strain of *Helicobacter pylori* [19]. Cinnamon oil was not harmful when consumed in food products and it inhibited the growth of molds, yeast and bacteria [20]. Cinnamon extract had a regulatory role in blood glucose level and lipids. This oil is also used in the treatment of cancer and other microbial diseases [21].

Clove oil has also been shown as potent antimicrobial agent against various pathogenic strains of bacteria and fungi including *Aspergillus, Penicillium*and yeast etc. The main constituents of the clove oil are phenyl propanoids such as carvacrol, thymol, eugenol and Cinnamldehyde [22]. The characteristic aroma of clove is due to the compound eugenol. It is the main component of clove essential oil, comprising 72-90% [23]. This can be helpful in designing antimicrobial agents for topical and oral applications.

The use of plants to heal diseases, including infectious one, has been extensively employed by people. Data from the literature as well as our own findings reveal that the plants have the great therapeutic potential, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search newer compounds. Once extracted, and before being used in new treatments, they should have their *in vivo* toxicity tested.

Furthermore, in a few cases, these plant aromatic oils were found active against antibiotic resistant bacteria in a very low concentration with minimum possible toxic effects, and can be used in the treatment of infectious diseases caused by the resistant microbes.

Vaccines have long been used to combat infectious diseases; however the last decade has witnessed a revolution in the approach to vaccine design and development. No longer is there a need to rely on the laborious classical methods such as attenuation or killing the pathogen.

Genome sequencing has become routine, and modern vaccine design is taking advantage of the accumulating genomic information. Reverse vaccinology is built on genome-based antigen discovery and has largely replaced classical vaccinology methods based on growing and dissecting the microorganism. The main advantage of the approach is the fast prediction of vaccine candidates. Most of the antigens will be surface exposed proteins, since these antigens are most likely accessible to antibodies. This approach can be applied to

non-cultivable microorganisms, something difficult or impossible to do with conventional approaches. When the first reverse vaccinology project was started, in the year 2000, antigen identification was mainly based on bioinformatics analysis of one genome. Since then, the technique has shown its full potential, with the first genome-derived vaccine now in clinical trials and several vaccines in preclinical studies. In the meantime the approach has been improved with the support of proteomics, functional genomics and comparative genomics. The complete process includes antigen prediction to high-throughput purification, screening and selection of the vaccine composition.

In the present study, it was found that mecA protein of *Staphylococcus* can be used as an effective candidate for the development of preventive measures against drastic diseases by blocking its resistance efficiency. In fact *in silico* approach of vaccine target prediction is definitely less labor intensive, rapid and economic in relation to search for a lead antigenic molecule against mecA protein [24].

The region found to be conserved in all mecA of Staphylococcus studied was

"mkkikivplilivvvvgfgiyfyaskdkeinntidaiedknfkqvykdssyisksdngevem terpikiynslgvkdiniqdrkikkvsknkkrvdaqykiktnygnidrnvqfnfvkedgmw kldwdhsviipgmqkdqsihienlksergkildrnnvelantgtayeigivpknvskkdyka iakelsisedyikqqmdqnwvqddtfvplktvkkmdeylsdfakkfhlttnetesrnyplgk atshllgyvgpinseelkqkeykgykddavigkkgleklydkklqhedgyrvtivddnsntia htliekkkkdgkdiqltidakvqksiynnmkndygsgtaihpqtgellalvstpsydvypfm ygmsneeynkltedkkepllnkfqittspgstqkiltamiglnnktlddktsykidgkgwqk dkswggynvtryevvngnidlkqaiessdniffarvalelgskkfekgmkklgvgedipsdy pfynaqisnknldneilladsgygqgeilinpvqilsiysalenngninaphllkdtknkvwk kniiskeninlltdgmqqvvnkthkediyrsyanligksgtaelkmkqgetgrqigwfisyd kdnpnmmmainvkdvqdkgmasynakisgkvydelyengnkkydide"

Multiple **s**equence alignment revealed various conserved regions of this protein. Analysis was done for finding out the potential of these conserved regions to work as B cell epitope and we found 41potential B cell epitopes among the conserved sequence. For any predicted epitope, it is cardinal that it should induce T and B cell response [25]. Such type of multi-epitope vaccine is a very recent experimental technique for predicting vaccine targets against HIV and Influenza virus [26]. We predicted one such type of multi-epitope peptide which was having very good potential to induce B cell response as well as a very good candidate for binding to MHC II molecule as found by Kohler (2000) also in his study [27].

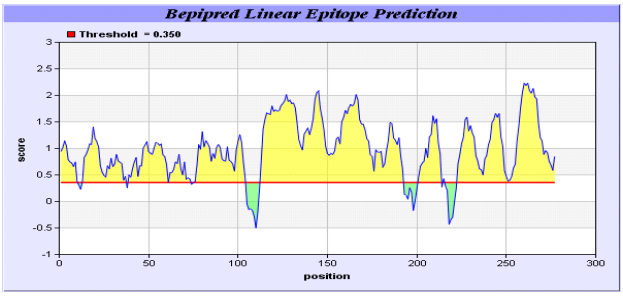

Fig. 3. It shows Predicted B-cell epitope by BepiPred Epitope prediction method. The peaks in yellow shaded area indicates the conserved sequences having value more than the threshold value and hence considered as the potential epitopes for vaccine designing.

Among 51 MHC II alleles analysed, the conserved region of protein *mecA* showed binding affinity with all the 51 alleles with one or more than one paratope found in each. As per the results of ProPred, 47 sequences were found to bind with MHC I alleles.During the last step of study, this protein sequence was also analysed for its binding affinity with T-cell receptors using the EpiJen server [28]. Structure prediction of this sequence by PSIPRED revealed that 22 helix are present in this sequence. This data can be very helpful for generating antigenic candidate by wet lab researchers.

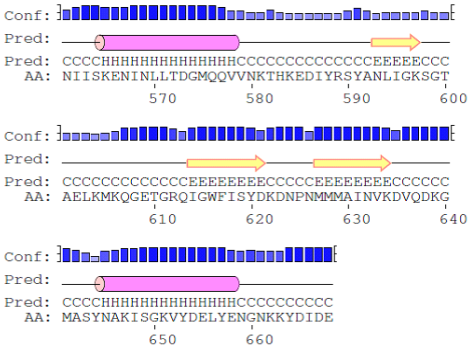

Fig.4. It shows a part of the structure of proposed vaccine target against MRSA predicted by PSIPRED. The pink cylindrical region indicates helix, black lines denotes coil and yellow arrow depicts strands in the predicted secondary structure of the vaccine target.

CONCLUSION

The results presented in this report were encouraging, although clinical controlled studies are required to define the real efficacy and possible toxic effects *in vivo*. It is also visualized that there is a need for extensive investigation concerning the molecular basis of understanding the mechanism which is fundamental in the development of pharmacological agents of medicinal plants used in the treatment of bacterial infections.

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