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MULTIDRUG RESISTANT PATHOGENIC STAPHYLOCOCCUS AUREUS IN PUS FROM POST-OPERATIVE WOUNDS OF HOSPITALIZED PATIENTS AND ANTIMICROBIAL ACTIVITY OF NATURAL HERBS

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ABSTRACT

Objectives: *Due to increased indiscriminate use of different antibiotics the Staphylococcus aureus becomes multidrug resistant leaving few therapeutic options for the treatment against it. Present study is conducted to detect Staphylococcus aureus as predominating organism including their haemolytic activity, antibiotic susceptibility as well as to study the antimicrobial effect of natural herbs (leaf extract of Andrographis paniculata, Ocimum tenuiflorum and honey). Materials and methods: Pus samples from postoperative wounds of the hospitalized patients are collected and several biochemical tests are used to detect Staphylococcus aureus. Antibiotic susceptibility and antimicrobial effect of natural herbs (leaf extract of Andrographis paniculata, Ocimum tenuiflorum and honey) are studied. Result: Pathogenic as well as multidrug resistant Staphylococcus aureus is present in the post-operative wounds of the hospitalized patients as a predominating organism. Andrographis paniculata and the Ocimum tenuiflorum have antimicrobial role against Vancomycin Resistant Staphylococcus aureus which are also resistant to Methicillin, while honey is less important in the organisms. Conclusion: Andrographis paniculata and the Ocimum tenuiflorum can be used against the Vancomycin Resistant Staphylococcus aureus.*

KEY WORDS

Staphylococcus aureus, haemolytic activity, Antibiotic susceptibility, natural herbs

INTRODUCTION:

Staphylococcus aureus has been reported to be a major human pathogen causing skin and tissue infections, pneumonia, septicemia and device associated infections [1, 2]. On the other hand, the organisms are reported to have ability to colonize healthy individuals asymptomatically [3]. Suggestion is there that indiscriminate use of antibiotics has become the major factor for the emergence and dissemination of multidrug resistant strains of several groups of microorganisms [4]. The emergence of methicillin (oxacillin) resistant *Staphylococcus aureus* (MRSA) creates a serious health problem in developing as well as developed countries [1, 2]. Several reports suggest that the frequency of methicillin resistant *Staphylococcus aureus* (MRSA) infections increases continuously in hospital and methicillin resistant *Staphylococcus aureus* (MRSA) is now endemic in India [5, 6]. Beside this it is reported that methicillin resistance is mediated by PBP-2a, a penicillin binding protein encoded by the *mecA* gene that permits the organism to grow and divide in the presence of methicillin and other beta-lactam antibiotics [7]. Additionally, emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) has also been reported to be increased globally leads to increased morbidity and mortality [8, 9].

Due to rapid global spread of resistant clinical isolates, it is necessary to find new antimicrobial agents of paramount importance. In this situation researchers are busy to give attention toward the herbal product looking for new to develop better drugs against MDR microbe strains [10] because antimicrobial agents will have a short life expectancy [11].

On the other hand, natural products have been reported to be used traditionally in medicine all over the world [12] and it is suggested by WHO that medicinal plants would be the best source for obtaining a variety of drugs [13]. Several reports have also given suggestion about the antimicrobial activity of different herbal extracts [14, 15]. Moreover, it is mentioned that various medicinal plants can be used to treat infectious diseases due to their availability, fewer side effects and reduced toxicity [16]. In recent years considerable effort has been given to discover plant-derived antibacterial active against methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which have developed resistance to most antibiotics. In this context the present study is carried out to detect *Staphylococcus aureus* as predominating organism in the post-operative wounds of hospitalized patients including their haemolytic activity, antibiotic susceptibility as well as to study the antimicrobial effect of natural herbs (leaf extract of *Andrographis paniculata* (Kalmegh), *Ocimum tenuiflorum* (Krishna Tulsi) and honey) on drug resistant *Staphylococcus aureus*.

MATERIALS AND METHODS:

Collection of samples:

Pus samples are collected from postoperative wounds of the hospitalized patients of both sexes of age group of 40-60 years from Berhampore Medical College Hospital, Murshidabad, India during the period of April 2017 to August 2017. This study is carried out as per the human ethical guidelines and pus samples are collected with informed consent from the subjects and institutional authority.

Bacterial strains and culture method:

Pus samples are collected aseptically, spread on the LB agar media and incubated overnight at 37°C. The bacterial colonies obtained from the pus samples are cultured on sheep blood agar and incubated overnight at 37°C.

Identification and pathogenicity:

Bacterial isolates obtained from pus samples identified as *Staphylococcus aureus* through Gram stain, colony morphology, catalase, coagulase, heat-stable nuclease production on thermonuclease test agar, mannitol salt agar fermentation [17, 18, 19]. Haemolytic activity is tested on sheep blood agar (15 ml of 5% sheep blood in Trypticase soy agar is overlaid on 10 ml of blood agar base) according to Rodger et al. (1999) [20]. *Staphylococcus aureus* ATCC 25923 was used as the positive
control.

Antibiotic susceptibility:

Antibiotic susceptibility test is carried out against the *Staphylococcus aureus* isolates by disc diffusion method. *Staphylococcus aureus* isolates are spread on tryptic soy agar and antibiotic discs are used for Erythromycin (15 µg), Clindamycin (2 µg), and Vancomycin (30 µg) (HiMedia Laboratories Pvt. Ltd., India) according to standard recommendation [21]. Isolates are categorized as susceptible and resistant according to interpretative criteria given by the Clinical and Laboratory Standards Institute (CLSI) (2005) [22].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is detected according to the method described by the CLSI, using Tryptic soy agar plate supplemented with NaCl (0.68 mol/L) and Oxacillin (6 mg/ml, w/v) (HiMedia Laboratories Pvt. Ltd., India) [23].

Preparation of extract from herbs

Selection of plant material:

Leaves of locally growing plants viz. *Ocimum tenuiflorum* and *Andrographis paniculata* are selected for the evaluation of their antimicrobial properties against the antibiotic resistant *Staphylococcus aureus* isolates (VRSA which are also resistant to Erythromycin, Clindamycin and Methicillin).

Preparation of leaf extract:

100 g of air dried leaves of each plant are powdered and soaked in 100 ml of methanol for 36 h. Each mixture is stirred after every 18 h using a sterile glass rod. At the end of extraction each extract is passed through Whatman Filter Paper No. 1. The methanolic filtrates obtained are concentrated at 30 °C and then kept at 4 °C. Thus, methanolic leaf extracts of the selected plants are prepared [24, 25].

Honey: Natural raw honey is taken and warmed at 28°C and dilutions are made with sterile distilled water as follows – 100%, 75% and 50% [26, 27].

Antimicrobial assay:

The antimicrobial activities of the plant extracts and honey are determined using Agar Well Diffusion Method [28] with further modifications in our laboratory. 0.1 ml of diluted inoculum (105 CFU/ml) of the pathogenic *Staphylococcus aureus* isolates which are resistant to Erythromycin, Clindamycin, Methicillin and Vancomycin is spread on the Nutrient agar plates and also MSA

plates. Wells of 2 mm diameter are punched into the agar plates and also MSA plates. 40 µl of the plant extracts are added to the wells made in the plate. The plates are incubated aerobically at 37°C for 24-48 h. Antimicrobial activity is evaluated by measuring the zone of inhibition (mm) against the VRSA (which are also resistant to Erythromycin, Clindamycin and Methicillin) strains. The test is performed in triplicates with controls.

Determination of minimum inhibitory concentration (MIC):

The plant extracts having antimicrobial activity, are further tested to estimate MIC for each Vancomycin-resistant *Staphylococcus aureus* (VRSA) (which are also resistant to Erythromycin, Clindamycin and Methicillin). The extracts are diluted for the final concentrations of 75, 37.5, 18.8, 9.4, 4.7, 2.4, 1.2, 0.6 mg/ml. 100 µl of (10^5 CFU/ml) of each of the *Staphylococcus aureus* strains described previously is transferred into tubes with equal volume of nutrient broth and plant extracts. Then the tubes are incubated aerobically at 37 °C for 24-48 h. Media control, organism control and extract control are used for each strain. After inoculation plates are incubated aerobically at 37 °C for 24-48 h. The lowest concentration (highest dilution) of the extract that produces no growth (no turbidity) after comparing with the control tubes in 48h is regarded as MIC.

RESULT AND DISCUSSION:

Staphylococcus aureus is a common pathogen responsible for nosocomial and community infection [1, 2]. Now occurrence of multidrug drug resistant pathogenic bacteria falls the developing as well as developed countries in alarming condition due to indiscriminate use of antibiotics [29, 30]. Treatment for this organism associated with infections using the antibiotic includes various other factors such as high cost and severe side effects. On the other hand, it is suggested that herbal medicine has a long history of healing effects and more over provides alternative way to treat this etiological agent [31]. In the present study, out of the 120 bacterial isolates collected from the postoperative wounds from the hospitalized patient, 85 (70.83%) bacterial isolates are found to be positive for *Staphylococcus aureus* through Gram staining, Catalase test, coagulase test, heat-stable nuclease production on thermonuclease test agar, Mannitol salt fermentation and it indicates that predominating organism is *Staphylococcus aureus*. Several reports also suggest that the incidence of post-operative wound infection with *Staphylococcus aureus* is significantly higher in patients carrying these bacteria on the skin with wound infections as they stayed, on an average, 15 days in hospital sharing the bed always occupied by infected patients [32, 33]. Beside this, 68 (80%) of the *Staphylococcus aureus* isolates are found to produce clearing zone surrounding their growth on blood agar media (Table 1) demonstrating that they can produce hemolysin. It is also reported that *Staphylococcus aureus* produces hemolysins for contribution to its pathogenicity [34].

Beside this, present study shows that 55 (64.7%) of *Staphylococcus aureus* isolates are resistant to Erythromycin and 59 (69.41%) are resistant to Clindamycin (Table 1). Moreover 41 (48.24%) *Staphylococcus aureus* are resistant to both Erythromycin and Clindamycin (Table 1) those are usually used for the treatment of skin infection [35, 36]. Moreover, 21 (51.22%) of these *Staphylococcus aureus* isolates (resistant to both Erythromycin and Clindamycin) are also found to be resistant to Methicillin (Table 1) on agar plate containing Oxacillin (Table 1) this may be due to long staying in hospital. Suggestion is there that prolonged hospital stays, indiscriminate use of antibiotics, lack of awareness, and receipt of antibiotics before coming to the hospital are some of the possible predisposing factors of MRSA emergence [37]. Additionally, present study has shown that 13 (61.9%) of the Methicillin-resistant *Staphylococcus aureus* isolates are also resistant to Vancomycin (Table 1). According to several reports low level and intermediate Vancomycin-resistant *Staphylococcus aureus* (VRSA) have been found to be emerged in north India [38, 39].

Table 1: Haemolytic activity and antibiotic susceptibility of *Staphylococcus aureus*

Haemolytic activity (%)	Antibiotic susceptibility (%)				
	Erythro^R strain	Clind^R strain	Both Erythro^R Clin^R strain	Meth^R among Erythro^R Clin^R strains	Vanc^R strain among Meth^R Erythro^R Clin^R strains
80.0% (68)	64.7% (55)	69.41% (59)	48.24%(41)	51.22% (21)	61.9% (13)

N.B: R means resistant; Erythro=Erythromycin; Clin=Clindamycin; Meth=Methicillin; Vanc=Vancomycin

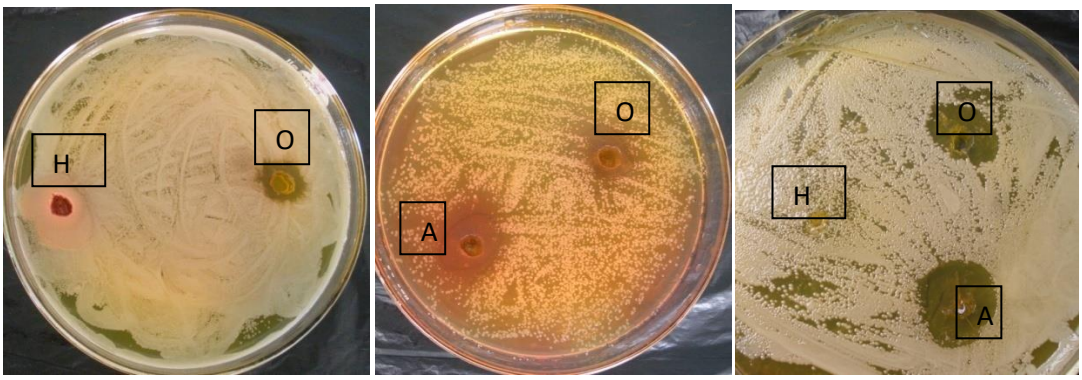
Table 2: Antimicrobial activity of the natural herbs against "Erythro^R Clin^R Meth^R Vanc^R"*Staphylococcus aureus* isolates.

Strains	Leaf (Methanolic) extract of *Andrographis paniculata* (Zone of inhibition in mm)	Leaf (Methanolic) extract of *Ocimum tenuiflorum* (Zone of inhibition in mm)	Honey (Zone of inhibition in mm)
R1	17.5mm	14.5mm	No zone
R2	16mm	13.0mm	No zone
R3	14.5.mm	11.0mm	4mm
R4	15.5mm	14.5mm	No zone
R5	17.5mm	12.5mm	No zone
R6	14.5mm	11.5mm	No zone
R7	16mm	14.0mm	No zone
R8	14.0mm	10mm	No zone
R9	14.0mm	10.5mm	No zone
R10	13.5mm	10mm	No zone
R11	13.0mm	12.5mm	No zone
R12	15.0mm	12mm	No zone
R13	14.5mm	13.5mm	No zone

Table 3: MIC values of leaf extracts (methanolic) of the selected plants against "Erythro^R Clin^R Meth^R Vanc^R"*Staphylococcus aureus* isolates

Plant extracts	MIC values (mg/ml) of the plant extracts against"Erythro^R Clin^R Meth^R Vanc^R"*Staphylococcus aureus* isolates												
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13
Andrographis paniculata	4.7	4.7	9.4	9.4	4.7	9.4	4.7	18.8	18.8	18.8	18.8	9.4	9.4
Ocimum tenuiflorum	9.4	18.8	37.5	9.4	18.8	37.5	9.4	37.5	37.5	37.5	18.8	18.8	9.4

Fig. 1A, B & C. "Erythro^R Clin^R Meth^R Vanc^R"*Staphylococcus aureus* isolate on MSA plate and natural herbs i.e., extract of *Andrographis paniculata* (A), *Ocimum tenuiflorum* (O) and honey (H)



(A) (B) (C)

Then the effect of leaf extract of *Andrographis paniculata* and *Ocimum tenuiflorum,* and honey against the Vancomycin-resistant *Staphylococcus aureus* (VRSA) (which are also resistant to Erythromycin, Clindamycin and Methicillin) are studied. Present study shows that *Andrographis paniculata* (Kalmegh) possess good antimicrobial activity against all VRSA (Table 2 and Fig: 1B & C). Moreover, leaf extract of *Andrographis paniculata* has shown maximum zone of inhibition against R1 and R5 strains (Table 2). It is also previously reported that *Andrographis paniculata* possess extensively antimicrobial and antiparasitic activities against bacteria, viruses, and parasites [40, 41]. On the other hand it is found that all these drug resistant *Staphylococcus aureus* strains mentioned earlier (VRSA) are also sensitive towards the leaf extract of *Ocimum tenuiflorum* (Table 2 and Fig: 1A, B & C). Beside this maximum inhibitory zone is observed against R1 and R4 strains in case of leaf extract of *Ocimum tenuiflorum* (Table 2). These findings demonstrate that *Ocimum tenuiflorum* has antimicrobial activity against the antibiotic resistant strain mentioned earlier in the study. Several reports have also shown the antimicrobial activity of *Ocimum tenuiflorum* [24, 30]. Phytochemical analysis of the plant detects the antibacterial properties to be due to glycosides, phenols and tannins [30].

Moreover, it is found that honey shows antimicrobial activity against only one (6.65%) of them is found to be sensitive (Table 2). On the other hand, previous reports suggest [26, 27, 42] that honey has a potent role as antimicrobial active against *Staphylococcus aureus*. But in the present study antimicrobial activity of honey is found to be least (Table 2 and Fig: 1A & C). Beside this inhibitory zone for honey for the VRSA is less than the other plant extracts used in the study (Table 2). Hence these strains did not follow this which may be due to different geographic location [43].

Additionally, this present study revealed that inhibitory zone is larger in case of leaf extract of *Andrographis paniculata* than the *Ocimum tenuiflorum* (Table 2) against the VRSA (which are also resistant to Erythromycin, Clindamycin and Methicillin). Moreover, MIC values of them are also studied and present study has shown that the lowest concentration range i.e., 4.7-18.8mg/ml is in case of *Andrographis paniculata* and 9.4-37.5mg/ml is for *Ocimum tenuiflorum* respectively cause no visible growth which suggests that minimum inhibitory concentration (MIC) for *Andrographis paniculata* is 4.7-18.8mg/ml while 9.4-37.5mg/ml is for *Ocimum tenuiflorum* (Table 3) against these antibiotic resistant *Staphylococcus aureus* strains in the study. These findings indicate that herbal products containing *Andrographis paniculata* or the *Ocimum tenuiflorum* can be used for the treatment of post-operative wounds infected with VRSA and also MRSA and it can also be said that *Andrographis paniculata* is more effective as antimicrobial active than the *Ocimum tenuiflorum* for the VRSA in the present study. Numerous anti *Staphylococcus aureus* plant-derived antibacterials with good MIC values have been also identified in past decades by researchers. These evidences indicate the importance of screening natural products [24].

CONCLUSION:

It can be concluded that pathogenic as well as multidrug resistant *Staphylococcus aureus* is present in the post-

operative wounds of the hospitalized patients as a predominating organism. *Andrographis paniculata* and the *Ocimum tenuiflorum* have antimicrobial role against the Vancomycin Resistant *Staphylococcus aureus* (which are also MRSA) while honey is least important against the organisms. But *Andrographis paniculata* is more potent against pathogenic Vancomycin Resistant *Staphylococcus aureus* than the *Ocimum tenuiflorum*.

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