Chowdhury et al., International Current Pharmaceutical Journal, October 2013, 2(11): 185-188
http://www.icpjonline.com/documents/Vol2Issue11/01.pdf

International Current Pharmaceutical Journal

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

Prevalence of multidrug resistance in human pathogenic *Staphylococcus aureus* and their sensitivity to *Allamanda cathartica* L. leaf extract

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ABSTRACT
Staphylococcus aureus is one of the major pathogen responsible for skin infection, urinary tract infection (UTI) and endocarditis in human. The study was performed to determine the prevalence of multidrug resistant *S. aureus* in human clinical sample and to evaluate their sensitivity to *Allamanda cathartica L.* leaf extract. A total of 12 isolates were identified belongs to *S. aureus* by performing several physiological and biochemical tests. The isolates exhibited highest resistant (75%) to streptomycin and lowest (33.33%) against co-trimoxazole followed by disc diffusion assay of eight antibiotics tested. The other four antibiotics such as azithromycin, chloramphenicol, gentamycin and erythromycin exhibited 50 to 66.67% resistant to present isolates. Here we found that 75% of *S. aureus* isolates were multidrug-resistant (MDR). The crude leaf extract of *A. cathartica L.* found to possess antibacterial properties at the rate of 83.33% against *S. aureus* isolates with 12-22 mm zone of inhibition. Results of TLC states that Benzene : Ethyl acetate (1:1) solvent system was more effective for initial separation of compound from crude leaf extract resulted three distinct bands with different R_f values ranging from 0.53 to 0.89. The result of this study refers that *A. cathartica L.* leaf extract would be useful to develop effective drugs that would reduce the higher prevalence of multidrug resistance *S. aureus* causing clinical infection in human.

***Key Words*:** *Staphylococcus aureus,* biochemical identification, antibiogram, disk diffusion assay, leaf extract, TLC.

INTRODUCTION

Staphylococcus aureus is a gram positive, round shaped, small, non-motile bacteria belongs to the family *Staphylococcaceae*. It is the leading bacteria in the normal flora of humans especially, in the skin and nasal vestibule and causes a variety of clinical infections including septicemia, pneumonia, osteomyelitis, Endocarditis, UTI, wound sepsis, septic arthritis, and post-surgical toxic shock syndrome with substantial rates of morbidity and mortality (Boyce, 1997; Engemann, 2003). Recently, this organism is being acquired resistance to different antibiotics and development of resistance to antimicrobial agents by Staphylococci is still frequently associated with hospital and community acquired infections (Locksley *et al.*, 1982). Strains of *S. aureus* resistant to β-lactam antibiotics are known as methicillin-resistant *S. aureus* (MRSA). It is also called multidrug-resistant (MDR) *S. aureus*. The emergence of Multi- drug resistant bacteria is a major problem for treatment of diseases using antibiotics. In recent years it has been reported that the clinical administration of antibiotics, against the pathogenic bacteria be gradually prohibited due to emergence of MDR bacterial strains including *S. aureus*. (Kumar *et al.*, 2010; Akindele *et al.*, 2010; Hoerlle and Brandelli, 2010, Efuntoye *et al.*, 2011) As a result, searching of alternative and effective medicine against MDR pathogen has become an important concern, all over the world. On the other hand, herbal medicine has been used for the medication of different bacterial disease and and many plant extracts and plant products have been reported to be valuable antimicrobial agent against human pathogens (Maity *et al.*, 2009; Arya et el., 2010; Fuad *et al.*, 2012) *A. cathartica* belongs to family Apocynaceae is notable for its medicinal properties. It has been found that the plant extract contain "allamandin", a toxic iridoid lactone and milky sap having antibacterial and possibly anticancer properties (Liogier, 1995). Although the antibacterial effects of *A. cathartica* to different human pathogen have been studied by the previous researchers but the information especially against MDR *S. aureus* is still not sufficient. As a result, the present study has been carried out to investigate prevalence of MDR *S. aureus* in human clinical sample and to evaluate the antibacterial effects of *A. cathartica* leaf extract against the MDR *S. aureus*.

MATERIALS AND METHODS

Collection of bacterial isolates

A total of 25 presumptive clinical isolates of *S. aureus* were collected from patient suffering Skin infection, Urinary Tract infection (UTI) and Endocarditis, during February 2012, from Popular Diagnostic Center, Sylhet, Bangladesh and aseptically transferred to Laboratory of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology for present studies. The isolates were collected with the proper concern of the authority of the diagnostic center as well as permission form each individual patient. Source of the bacterial isolates with information of the patients were recorded (table 1). The isolates were cultured on Nutrient Agar (NA) medium and incubated at 37°C for 24 hours. Individual colonies were separated from the plates on the basis of color, shape and size and sub-cultured on the relevant media to obtain pure culture. Then the individual isolates were subjected to biochemical identification.

Identification of bacteria from human clinical sample

For the identification of bacterial isolates, several morphological, physiological and biochemical tests were conducted. Morphological appearance of colonies of all isolates was observed after 24h incubation (shape, size and color) and subjected to gram staining. The tests

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Table 1: Presumptive *S. aureus* isolates with their source and patient background.

Isolates Name	Source	Patient Background		
		Disease of the patient	Sex	Age (year)
S1	Puss	Skin infection	Female	28
S2	Blood	Endocarditis	Male	43
S3	Puss	Skin infection	Male	37
S4	Puss	Skin infection	Male	43
S5	Puss	Skin infection	Female	37
S6	Blood	Endocarditis	Male	43
S7	Urine	UTI	Female	45
S8	Puss	Skin infection	Male	29
S9	Urine	UTI	Female	51
S10	Blood	Endocarditis	Male	39
S11	Puss	Skin infection	Male	56
S12	Urine	UTI	Female	38

included motility, oxidase activity, catalase test, acid production from glucose, oxidation-fermentation (OF) Voges-Proskauer Test (VP) and Hydrogen Sulfide Production etc. All of the bacterial isolates were identified up to genus level following the Bergey's Manual of Determinative Bacteriology (Bergey and John, 1994).

Antibiogram profiling of bacterial isolates

Antibiogram assay of present isolates were determined by disc diffusion assay using commercial antibiotic discs followed by Rahman and Hossain (2010). The individual isolates were cultured into nutrient broth and incubated at 37°C for 24 hours. Fifty micro liter of individual broth culture was dropped on the NA plate with micropipette. The broth on the plate was spread aseptically by a sterile 'L' shaped glass rod. Eight commercial antibiotics were used in the present study, *viz.* chloramphenicol (25µg/disc), azithromycin (15µg/disc), erythromycin (15 µg/disc), streptomycin (10µg/disc), co-trimoxazole (25µg/disc), ciprofloxacin (5µg/disc), cephradine (30µg/disc), centamicin (10µg/disc). The plates were again incubated at 37°C for 24 h. After incubation, each plate was examined for the determination of diameter of zone of inhibition by the antibiotics in millimeter and compared with British Society for Antimicrobial Chemotherapy (BSAC) guideline for antimicrobial susceptibility testing (Andrews, 2012) to determine *S. aureus* isolates as resistant, intermediate or sensitive.

Antibacterial activity

The filter paper disk diffusion assay was performed to determine the antibacterial activity of leaf extract of *A. cathartica L.* leaf extract followed by Sharmeen *et al.* 2012. The 24 h incubated broth culture of individual bacterial isolates were spread aseptically by 'L' shaped glass rod and subjected to prepared filter paper disc of *A. cathartica* leaf extract. The plates then incubated at 37°C for overnight and antibacterial activity was determined by measuring the diameter of zone of inhibition with a millimeter scale.

Table 3: Antibiogram profile of S. aureus isolates to different antibiotic disc used.

Name of Antibiotic	Conc. (µg/disc)	Antibiogram of *S. aureus* isolates (%)		
		Resistant	Intermediate	Sensitive
Chloramphenicol	25	50	16.67	33.33
Azithromycin	15	50	-	50
Erythromycin	15	66.67	25	8.33
Streptomycin	10	75	25	-
Co-Trimoxazole	25	33.33	16.67	50
Ciprofloxacin	5	41.67	8.33	50
Cephradine	30	41.67	-	58.33
Gentamicin	10	58.33	25	16.67

Table 2: Physiological and biochemical characteristics of *S. aureus*.

Tests	Characteristics
Gram stain	+
Motility	-
Grow Aerobically	+
Grow Anaerobically	+
O-F test	Fermentative
Utilization of Sugars:-	
Glucose	+
Lactose	+
Sucrose	+
Mannitol	+
Oxidase test	-
Catalase test	+
MR test	+
VP test	+
Indole test	-
H_2S production	+
Citrate test	+
Urease test	+

+ = positive, - = negative

Thin layer chromatography

The leaf extract of *A. cathartica L.* were subjected to thin layer chromatography (TLC) with three different eluting solvents for the effective separation of antimicrobial compounds (Masuduzzaman *et al.*, 2008). TLC plates were removed from hot air oven and sample extract spotted on the activated silica gel allowing capillary action of solvent up the plate until approximately 1 cm from the end and allowed for air drying. R_f value of the compound was measured as the ratio of mobility for bioactive compound to the total length of run. The TLC plates were stained with iodine vapor (Kruiswijk, 2005) and observed under bright light and the separated spots were marked.

RESULTS

Identification of *Staphylococcus aureus* isolates

Among 25 isolates a total of 12 isolates were identified belong to *S. aureus* on the basis of their morphological, physiological and biochemical characteristics. All of the isolates were Gram negative, round shaped, non-motile, catalase positive, oxidase negative, able to utilize sugar (glucose, lactose, sucrose manitol), Methyl-Red (MR) positive Urease positive, Citrate positive and H_2S positive (table 2).

Table 4: Sensitivity of S. aureus isolates to Allamanda cathartica L. leaf extracts.

Isolates Name	Zone of inhibition (mm)
S1	22
S2	18
S3	21
S4	-
S5	19
S6	19
S7	-
S8	20
S9	17
S10	21
S11	-
S12	18

Antibiogram profiling of bacterial isolates

In the present study, the *S. aureus* isolates found to be variable in their resistance pattern against eight antibiotic discs tested. The streptomycin (10µg/disc) was found to be highest resistant (75%) but co-trimoxazole (5µg/disc) exhibited lowest (33.33%) to present isolates. The antibiogram profiles of *S. aureus* isolates to different antibiotics are given in table 3. Other four antibiotics such as erythromycin (15µg/disc), gentamycin (10µg/disc), chloramphenicol (25µg/disc) and azithromycin (15µg/disc) were notably resistant (50-66.67%) against *S. aureus* isolates. On the other hand, Cephradine (30µg/disc) showed as maximum 58% sensitive to present isolates and three antibiotics such as azithromycin (15µg/disc), ciprofloxacin (5µg/disc) and co-trimoxazole (25µg/disc) were 50% sensitive. Here we found that 75% of *S. aureus* isolates were resistant to at least two antibiotics and determined as multidrug resistant (MDR) organism.

Sensitivity study

In this study 83.33% *S. aureus* were found to be sensitive to *A. cathartica* leaf extracts. The leaf extract exhibited inhibition zone, ranging from 12-22 mm followed filter paper disc diffusion assay. Except the two isolates of *S. aureus* (S4 and S11), all of the isolates were found to be sensitive to the leaf extract (table 4).

Initial separation of *A. cathartica L.* leaf extract by Thin Layer Chromatography (TLC)

Compounds initially separated from *A. cathartica L.* leaf extracts by TLC were eluted by different solvents which were developed by iodine vapor. The experiment revealed that Benzene : Ethyl acetate (1:1) solvent systems exhibited most effective separation with three bands and their R_f value ranges from 0.53 to 0.89. On the other hand, solvent system Hexane : Benzene(1:1) gave two bands with R_f values 0.68 and 0.84. Hexane individually showed least effective separation with only one band of R_f value 0.68 (table 5).

Table 5: R_f values of different spots in TLC analysis using different solvent systems.

Solvent System	Number of bands	R_f values of different spots found
Benzene : Ethyl acetate (1:1)	3	0.53, 0.84, 0.89
Hexane	1	0.68
Hexane : Benzene (1:1)	2	0.68, 0.84

DISCUSSION

The infections, caused by *Staphylococcus aureus* are quite common all over the world and the emergence of antibiotic-resistant forms of pathogenic *S. aureus* is a worldwide problem in clinical medicine. Here, we collected a total of 25 isolates from clinical patients (table 1) and finally 12 isolates have been identified as *S. aureus* followed by several physiological and biochemical tests. Previously, Similar tests were used by the by the researchers (Silva *et al.*, 2000; Singh and Prakash, 2008; Narmeen and Jubrael, 2009) for the identification of *S. aureus.* In this study, we found that the streptomycin (10µg/disc) and erythromycin (15µg/disc) were highly resistant and it was assumed that these drugs would not be effective for medication against *S. aureus*. Although cephradine (30µg/disc) was maximum (58%) sensitive to present isolates but it was also less desirable for the disease treatment. In this study the 75% of isolates found as multidrug resistant (MDR) and refers that the rate of emergence of MDR *S. aureus* are very frequent in clinical patient. Recent studies also suggests that emergence of multidrug resistant *S. aureus* are increasing at alarming rate to different antibiotics like streptomycin, erythromycin, gentamicin, chloramphenicol and azithromycin (Hoerlle and Brandelli, 2009; Akindele *et al.*, 2010; Efuntoye *et al.*, 2011; Kumar *et al.*, 2011). Hence, these types of antibiotics would be quite ineffective in the future treatment of infection caused by *S. aureus.* So search for alternative and novel therapeutics is a must at this moment. On the other hand, most of the *S. aureus* isolates which were multidrug resistant (MDR) in antibiogram profiling but exhibited sensitive to *A. cathartica L.* leaf extracts (table 4). Medicinal plants have been used as traditional medication from the ancient times and their antibacterial properties has been reported against several human pathogens in recent research (Khan *et al.*, 2007; Maity *et al.*, 2009). Some recent reports are also available mentioning that the herbal extract can potentially inhibit multidrug resistant human pathogen (Fuad *et al.*, 2012; Sharmeen *et al.*, 2012). Several researchers also found that of *A. cathartica L.* leaf extract were able to kill human bacterial pathogen including *S. aureus* (Jeyachandran *et al.*, 2010; Islam *et al.*, 2010). Therefore, *A. cathartica L.* leaf extract may be useful to reduce the prevalence of multidrug resistant *S. aureus*. The crude leaf extract of *A. cathartica L.* was subjected to initial separation by TLC where Benzene : Ethyl acetate (1:1) solvent systems exhibited most effective separation with three bands. However, it was our primary attempt to separate active single compound from the crude leaf extract but to identify and characterize the bioactive compound more preciously, the crude leaf extract of *A. cathartica L.* can be further subjected to other chromatographic techniques (Mišan *et al.*, 2011; Sasidharan *et al.*, 2011; Patra *et al.*, 2012).

CONCLUSION

The present study revealed that, the prevalence of multidrug resistant *S. aureus* is emerging at an alarming rate. As a result, most of the antibiotics are quite ineffective for infection control. On the other hand, *A. cathartica L.* leaf extract was able to poses antibacterial effect including multidrug resistant (MDR) isolates. Therefore, potential antimicrobial drugs may be obtained from *Allaromanda cathartica L.* leaf extract against *S. aureus* associated infection in human.

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