ORIGINAL ARTICLE

Antibacterial Effect of *Camellia, Juglans, and Hippophae* spp. on Methicillin-Resistant Strains of *S. epidermidis* and *S. hemolyticus* in Urine Samples

Farhan E. Abdullah,¹ Maryam Zulfiqar,² Midhat Lakhani² and Abu Talib³

ABSTRACT

Object: To investigate the antibacterial activity of *Camellia sinensis*, *Juglans regia* and *Hippophae rhamnoides* extracts on methicillin-resistant strains of *Staphylococcus epidermidis* and *Staphylococcus hemolyticus*.

Background: Coagulase negative staphylococci (CoNS) which were once considered avirulent and usually contaminants when isolated from urine samples are becoming increasingly recognized as agents of clinically significant nosocomial infections. These pathogens are also steadily becoming resistant to conventional antibiotics available as well as combination drugs, highlighting the need for alternative or synergistic natural antimicrobial products that are effective, inexpensive and non-toxic.

Methods: 300 urine samples from hospitalized patients with indwelling catheters were tested for CoNS. Methicillin resistant strain of *S. hemolyticus* and methicillin resistant strain of *S. epidermidis* were found amongst others using standard microbiological procedures. Identification was done by API-staph and 16S rRNA gene sequencing using ATCC cultures as control. The antibacterial activity of aqueous extracts of green tea leaves (*Camellia* spp.), dandasa: dried bark of the walnut tree (*Juglans* spp.), and sea-buckthorn berries (*Hippophae* spp.) were investigated on the CoNS isolates, in duplicate, using Microtube-dilution method for Minimum Inhibitory Concentration (MIC) estimation.

Results: Amongst the CoNS isolated, 19% were *S. epidermidis* and 15% were *S. hemolyticus*. Methicillin resistance observed was above 60%. Of which, 51% of the *S. epidermidis* strains and 89% of the *S. hemolyticus* strains were found to be sensitive to the tested extracts. The average MICs of extracts for the *S. epidermidis* were found to be 1250 ug/ml for green tea, 312.5 ug/ml for Dandasa and 156.25 ug/ml for sea-buckthorn. *S. hemolyticus* strain, however, showed the average MICs of 625ug/ml for green tea, 2500ug/ml for dandasa and 39.06 ug/ml for sea-buckthorn.

Conclusion: Our results indicate that extracts of green tea leaves, dried bark of walnut tree, and sea-buckthorn berries may contain compounds with important therapeutic activities, encouraging further study.

INTRODUCTION

Human skin is inhabited by a number of microorganisms, but the most frequent constituents of human skin are the CoNS.¹ Once considered avirulent and usually contaminants when isolated from a clinical specimen, these organisms have become increasingly recognized as agents of clinically significant nosocomial bloodstream, urinary tract infections and other infections. As many as 18 species have been isolated from human skin, but *S. epidermidis* accounts for more than half the resident staphylococci² with extensive distribution over the body surface. In terms of clinical isolates too, *S. epidermidis* accounts for 75% of all isolates;³ this may be in part due to its excessive colonization of the human skin, although it may possess virulence determinants that other CoNS lack.⁴

In a study, *S. epidermidis*, *S. hemolyticus*, *S. warneri* and *S. hominis* were indicated as the most prevalent CoNS in a hospital setting.⁵ Among all clinical isolates of CoNS species, *S. hemolyticus* is second only to *S. epidermidis* in frequency.⁶ Coagulase-negative infections generally occur in immunocompromised patients, patients with implanted foreign bodies like catheters, or neonates. The main cause of pathogenesis is formation of biofilm, which promotes persistence in host leading to infections such as bacteremia, endocarditis, otitis media, vascular graft infection, prosthetic joint infections, CSF shunt infections, ocular infections & urinary tract infections.

The *S. epidermidis* capsule encourages the binding of other bacteria which promotes the development of a multilayer biofilm. This decreases the metabolic activity.
of bacteria within. The impaired diffusion of antibiotics along with decreased bacterial metabolism makes it a difficult job for antibiotics to eradicate infections caused by biofilm producing microbes. Investigators of a study published in 1994, suggested that hemolysins might be the factor responsible for the high virulence of this species. S. hemolyticus was found to be the causative factor of acute cystitis in males. It is one of the CoNS that dwell in the urethra or peri-urethra of both sexes.8

The rapid development of microbial resistance to conventional as well as newer antibiotics including combinations makes the problem graver.9 Infections caused by methicillin resistant organisms necessitate the use of a more powerful antibiotic like vancomycin. Resistance against vancomycin is also becoming a major problem, which is an alarming prospect. Thus the need to discover an alternative or synergistic compound is self-evident, derivatives from certain plants look like an attractive alternative, especially if they are non-toxic, inexpensive and can be used for treatment as in eye/ear drops, skin ointments, or the prevention of slime/biofilm formation on catheters and other surgical implants.

In vitro studies on plants used in traditional medicine10 have been explored in microbiology, especially on pathogenic bacterial growths & some of these studies were about the antimicrobial activity of Juglans regia, Camellia sinensis & Hippophae spp. Juglans regia is the bark of walnut tree known as dandasa & used for cleaning teeth in the Middle East & Asia. It has been proven to be an effective teeth cleanser and to prevent plaque formation & caries better than toothbrushes.11

After water, tea is the second most common beverage consumed worldwide, with a per capita consumption of 0.12 L/year. Green tea is an infusion of flavorful leaves of Camellia sinensis. The phytochemical screening of green tea reveals catechins, saponins, tannins, alkaloids & polyphenols. These have antibacterial, antioxidant & anticancer activities.

Hippophae rhamnoides (sea-buckthorn) is a deciduous plant with numerous greenish-yellow flowers & bright orange, globular, ellipsoid fruit, which belongs to family Elaeagnaceae. It is native to temperate regions of Asia and Europe. Sea buckthorn berries are abundant in nutrients and therapeutic compounds, such as flavonoids, carotenes, volatile oils, carbohydrates, vitamins, amino acids and mineral acids. The dietary intake of these berries may have a profound impact on human wellbeing.

Accordingly, a succinct trial evaluating the effect of crude extracts of three indigenous plants on selected methicillin resistant CoNS strains was attempted. These included green tea,12-13 dandasa14-15 and sea buckthorn.16-17 Studies all over the world investigating the antimicrobial actions of green tea, dandasa and sea buckthorn have shown promising results. There are no reports of severe toxicity in humans from eating, application or consumption of green tea, dandasa and sea buckthorn.

**MATERIALS AND METHODS**

The study was conducted at Dr. Essa’s Lab and Diagnostic Centre and at Immunology and Infectious Disease Research Laboratory, University of Karachi. It was a year long study ending in November 2011.

**Identification of isolates**

A total of 300 urine samples were taken from hospitalized patients with indwelling catheters. They were then tested for the presence of CoNS. The microbes were identified using API-Staph 20 & 16SrRNA gene sequencing using ATCC cultures as control. The pathogens were tested for antibiotic sensitivity by Kirby-Bauer method.

**Preparation of plant extracts**

A 5% solution of each dried plant material was prepared in sterile distilled water by heating at 95°C in a water bath for 2 minutes and cooling for 2 minutes. The procedure was repeated 3 times and the final resultant extracts were centrifuged at 10,000 rpm for 30 minutes. Supernatants were filtered through a sterile 0.22 um filter unit, stored at -20°C. They were thawed before use in the laboratory. Each of these aqueous extracts when thawed was used for not more than one week for conducting different bioassays.

Screening for susceptibility to the plant extracts

The three natural plant extracts were first screened for efficacy by using the Disc Diffusion method. 30 uL of each extract with a concentration of 5000 ug/ml was tested against the isolated pathogens. Zones of inhibition were observed.

**Minimum inhibitory concentrations of tested plant extracts**

This was done by using sterile flat-bottomed 96 well plates. Two fold serial dilutions of each extract were made in Muller-Hinton broth ranging from undiluted (5000 ug/ml) in well no. 1 to 1:512 (9.76 ug/ml) in well no. 6. The starting inoculums were 5 x 10² CFU/ml of each culture. The final volume achieved in each well was 100 ul and wells (no. 12) containing no extract but inoculated with test strains were considered as
positive control. Negative control was filled with serial dilutions of extract only. Plates were incubated at 37°C in the presence of oxygen for 18 hours. The highest dilution of plant extract showing no visible turbidity was recorded as the MIC.

RESULTS
In the 300 urine samples, the major pathogen component was S. saprophyticus at 30% (n=90) followed by S. epidermidis at 19% (n=57) and S. hemolyticus at 15% (n=45). Of the 19% (n=57) S. epidermidis isolated, 36% (n=19) were found to be susceptible to methicillin whereas 64% (n=38) were resistant. Accordingly, of the 15% (n=45) S. hemolyticus isolated, 33% (n=15) were found to be sensitive to methicillin whereas 67% (n=30) were resistant. However, all of these were susceptible to vancomycin.

When the isolates were tested with the plant extracts, 89% of the methicillin resistant S. hemolyticus and 51% of the methicillin resistant S. epidermidis were found to be susceptible. Sea Buckthorn was found to be the most effective with an average MIC of 39.06 ug/ml against S. hemolyticus & 156.25 ug/ml against S. epidermidis. Green tea had an intermediate antibacterial action against S. Hemolyticus at an average MIC of 625 ug/ml whereas against epidermidis it exhibited an average MIC of 1250 ug/ml. Dandasa was more effective against S. epidermidis at an average MIC of 312.5 ug/ml than against S. hemolyticus with an average MIC of 2500 ug/ml.

Figure 1 shows the MICs of the plant extracts effective against the two tested pathogens.

Thus the extract of sea-buckthorn was found to be the most efficacious of the three natural plant ingredients that were tested against both S. epidermidis & S. hemolyticus.

DISCUSSION
MRSA is a globally recognized and feared agent of nosocomial infections that are becoming increasingly difficult to combat because of emerging resistance to all current antibiotic classes. Much has been done to eradicate and prevent MRSA; however these measures have not been met with much success. The prevalence of MRSA in Pakistan has a frequency varying from 2-61%. But MRSA is not the only menace in a hospital setting; CoNS are also progressively emerging as a significant cause of invasive infections. Drug resistance is also a significant cause for worry with nosocomial CoNS infections. Furthermore, S. hemolyticus was demonstrated to be the most resistant among all CoNS species. The analysis of antibiotic susceptibility in relation to slime production demonstrated more frequent susceptibility with non-slime producing strains.

Hippophae has been demonstrated to have antioxidant activity. Mankind has always turned towards plants in their surrounding regions for finding the cure to their various illnesses, poisons & their antidotes, or anything that will prove helpful in combating sickness. The first antibiotic, penicillin was derived from a fungus. Thus it comes as no surprise that now when there are increasing legions of pathogens that are resistant to conventional medicines, with more being added to their ranks every day, scientists have again turned to natural substances some new and others used in everyday life. All over the world, the emphasis in research is now on finding substances that will overcome the resistance that the microorganisms have developed against the existing antibiotics. The goal is to find natural or synthetic substances acting synergistically with the most powerful antibiotics that we have or potent enough to act on their own. Several natural substances are being tested for antimicrobial activity against the most troublesome bugs that are plaguing us daily. Amongst them are common everyday things like green tea, honey, ginger, green chilies, lemon etc.

Indian studies on green tea have shown a significant antimicrobial effect against gram negative rods and enteropathogens. According to a study in Yangzhong, China, a 48% reduction in the risk of stomach cancer was observed in green tea drinkers. Nepalese studies have shown that Juglans regia which has been used for centuries as a teeth cleanser has significant bactericidal activity. In small villages in the Punjab region of Pakistan the powdered bark of walnut tree is packed in poultices and used to prevent burns from getting infected.
The results of our study were satisfactory and the plant extracts used in this study exhibited antibacterial activity against the two pathogens tested.

CONCLUSION

Thus it can be concluded that these three plant products could be a safer alternative to combat antibiotic resistance. This needs to be further researched for the development of an inexpensive cure for antibiotic resistant microbes.

REFERENCES