Original Article

Methicillin-Resistant Staphy.Aureus

Study on Alternative Therapeutic Agents on Methicillin-Resistant Staphylococcus Aureus from Clinical and Environmental Isolates

1. YasmeenTaj 2. Aftab Ahmad Soomro 3. Syed Wajid Ali 4. Shahana Urooj Kazmi

1. Prof. of Pathology, Dow Medical College, DUHS, Karachi 2. Prof. of Pathology, SMBBMC, Lyari, Karachi
3. Final Year MBBS, Student, DMC, DUHS, Karachi 4. Prof. of Microbiology, University of Karachi

ABSTRACT

Introduction: The in vitro antimicrobial activity of Sea buckthorn (SBT) (Hippophaerhamnoides), Green tea (Camellia sinensis) and Dandasa (Juglansregia) on selected methicillin resistant Staphylococcus aureus (MRSA) isolates from clinical samples was tested. The in vitro antimicrobial activity of six antiseptics/disinfectants against MRSA isolated from environmental samples was also evaluated.

Study Design: Experimental Observational.

Place and Duration of study: This study was conducted in Immunology and Infectious Diseases Research Laboratory (IIDRL) Lab, University of Karachi from January 2011 to July 2011.

Materials and Methods: Minimum Inhibitory Concentration (MICs) of plant extracts was determined by micro-broth dilution method and, susceptibility of MRSA isolates from environmental samples against antiseptics/ disinfectants was estimated by the agar disk diffusion and agar well diffusion methods.

Results: None of the plant extracts inhibited the isolates originating from blood samples. SBT offered comparatively more inhibitory zones and among the antiseptics/disinfectants, savlon was the most effective.

Conclusions: In view of the rising antibiotic resistance, exploring possible natural plant extracts for their antimicrobial action seems like an attractive substitute. The results showed some degree of susceptibility and can be suggested for use in vivoafter standard clinical trials.

Key Words: Antiseptics/Disinfectants, Methicillin Resistant, Plant Extracts.

INTRODUCTION

Pharmaceutical companies have a task of manufacturing multitudes of new antimicrobials with broad spectrum activity but the bacteria in turn become rapidly resistant. Therapeutic actions of plants and herbs have been investigated since ancient times due to their availability as locally grown, inexpensive, and applicable in a large spectrum of medical conditions. Medicinal plants are the only source of treatment for some communities[1], and especially have a role in primary health care. The practice of employing herbal medicine is widespread in Pakistan, India, Sri Lanka and the Far East. In United States, the use of herbal products has also increased from 33.8% to 42.1% between the years 1990 and 1997[2]. World Health Organization (WHO) estimates that 80% of world population uses herbal medicine for some form of primary healthcare[3]. Around the world, 35,000 to 70,000 plant species are used as traditional medicine, out of these, 20,000 species are commonly used in the developing countries. Approximately 6,000 species of plants grow in Pakistan and about 700 of them are known to have therapeutic properties, but less than 5% have been evaluated.[1] SBT has been used in traditional Chinese therapeutics since the Tang Dynasty, dating back more than 1,000 years. The leaves, flowers, fruit and roots of SBT are used in Pakistan for the last 2,000 years, for their anti-spasmodic and anti-helminthic properties[2]. The root extract of Glycyrrhiza is widely used as a cough medicine in Pakistan. Hyssop extract derived from Hyssopusofficinalis is used in the treatment of stomach ailments.[1] In our study, anti-microbial action was tested for Greentea (Camellia sinensis)[4] a widely consumed beverage; Dandasa (Juglansregia)[5], the stem of walnut tree, used as a common tooth cleaning agent; and, SBT (Hippophaerhamnoides)[6], the leaves and berries of this plant are widely eaten in the northern areas of Pakistan.

Contact spread puts a massive burden for infection control in the hospital and the environment. In, some studies staphylococcus organisms were retrieved for a period of up to two months after contaminating hospital environment.[7,8] It was therefore, instructive to assess commonly employed antiseptics and disinfectants in healthcare facilities for their antimicrobial potentials.

MATERIALS AND METHODS

The three plants used in this study were dried bark of the walnut tree: Dandasa(Juglansregia), Green tea (Camelliasinensis), berries of SBT (Hippopphaer-hamnoides).

Four strains of MRSA isolated from urine, pus, high vaginal swab and blood clinical samples were selected.

50 samples for culture from hospital environment were obtained from Civil Hospital Karachi, from door knobs, privacy curtains and toilets. Pre-moistened sterile swabs were applied to a 25 cm^2 area followed by direct plating on blood agar plates (Oxoid). Out of these, 11 (22.0%) isolates were characterized as MRSA.The isolates were tested as MRSA by Cefoxitin disk diffusion test and molecular characterization for mecA gene was done by Polymerase Chain Reaction (PCR) method of Geha et al[9]. ATCC 43300 was used as positive control for mecA gene.

Preparation of Aqueous Plant Extracts: A 5% solution of each of the dried plant material was prepared by heating in sterile distilled water at temperature of 95°C in water bath for two minutes followed by cooling for 2 minutes. The extracts obtained were centrifuged at 10,000 rpm for half an hour. Supernatants were filtered through sterile membrane filter 0.22 µm filter unit (Millex-GS, Millipore), stored at -20°C.

Determination of MICs of Plant Material by Microbroth Dilution Method: Concentrations tested ranged from "neat" (undiluted) in well #1 (concentration: 5000 µg/ml) to 1:512 in well #10 (concentration: 9.77 µg/ml). In sterile flat-bottomed 96 well plates (Becton-Dickinson, Oxford, England), two fold serial dilutions of each extract were prepared in Mueller Hinton Broth (Oxoid). The inoculum size was $5x10^5$ CFU/ml, final volume of broth achieved in each well was 100 µl. Well inoculated with the control strains (ATCC 43300) (#12) were taken as positive control. Negative control wells (#11) consisted of plant extracts only. Highest dilution of the plant extracts showing no turbidity were recorded as MIC.

Effect of Antiseptics and Disinfectants on selected S. aureus Isolates: Dettol: Antiseptic. (Chloroxylenol solution: Para-chloro-metaxylenol 1.44% w/v, terpineol 1.8% w/v) (Reckitt Benckiser, Lysol: Antiseptic. (Cresol 500ml, linseed oil 180 gm, potassium hydroxide 42 gm, solution) (LCPW/Howards), Pyodine: Antiseptic. (Povidone-iodine 10%, aqueous solution) (Brooks Pharmaceutical Lab,), Hibiscrub: Disinfectant (Chlorhexidinegluconate 4%) (ICI), SteriliumVirugard: Antiseptic/disinfectant. (Ethanol) (BODE), and Savlon: Disinfectant. (0.3% w/v Chlorhexidinegluconate, 3% w/v Cetrimide, aqueous solution).

a) **Agar-Disk Diffusion Method:** Whatman's 12.6'' filter paper disks (6 mm diameter) were punched out and placed in Petri dishes at a distance of 2 to 4 mm, and sterilized in hot air oven at 160°C for 1 hour. 30 µl of the antiseptic/disinfectant were transferred onto one of these disks, and dried in incubator at 37°C for 1 hour. The disks filled with antiseptic/disinfectant were labeled and stored at 4°C. MRSA isolated from hospital environment (n=11) were prepared for the inoculum turbidity to match 0.5 McFarland standard. A lawn of the isolate was made on a Mueller Hinton media, disks of each antiseptic/ disinfectant were placed at a distance of 15 mm. The plates were incubated at 37°C for 24 hours.

b) Agar-Well Diffusion Method: A lawn of the bacterialsuspensions was made on (MHA) plates six wells of 3 mm diameter spaced at equal distances were punched out. 30 µl from each antiseptic/disinfectant were transferred to the corresponding well. The plates were incubated at 37°C for 24 hours. The inhibitory zone around each well was measured.

RESULTS

The isolates positive for mecA gene 310 bp are shown in Fig 1.The MICs by microbroth dilution method using aqueous extracts Dandasa (Juglansregia), SBT (Hippophaerhamnoides) and Green tea (Camellia sinensis) in serial dilutions ranging from a concentration of 5000 µg/ml (undiluted) to 1:512 (concentration: 9.77 µg/ml) using the flat bottomed 96-well microtube tray were determined and shown in (Table 1a, 1b, 1c).

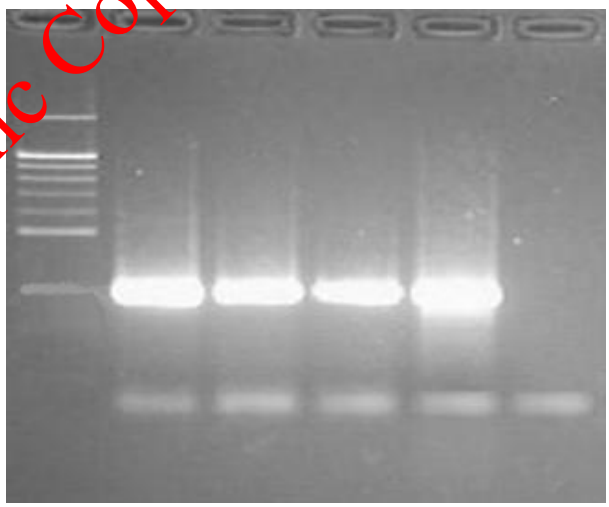

Figure No.1: Agarose Gel showing 310 bp products of *mecA* gene by PCR

Lane 1 --- DNA ladder
Lane 2 --- mecA gene positive control
Lane 3-5 --- mecA gene positive isolates
Lane 6 --- mecA negative control

The highest dilutions of extracts showing no visual turbidity in the microtubes were recorded as the MIC.None of the plant extracts, in concentration of 5000 µg/ml inhibited MRSAstrainsoriginating from blood samples (Table 1a, 1b, 1c). SBT offered comparatively more inhibitory zones as compared to Green tea and Dandasa on isolates from high vaginal swab (MIC ≤ 156.25µg/ml) and significant effects in a dilution of 1:256 (MIC ≤ 39.06µg/ml) on urine and pus

strains. Green tea extract had a (MIC ≤ 1250 mg/ml) on strain high vaginal swab and (MIC ≤ 625µg/ml) on both strains urine and pus. The Dandasa extract was the less effective (MIC ≤ 2500µg/ml) on strains urine and pus, but more on strain high vaginal swab (MIC ≤ 312.5 µg/ml).

The inhibitory action of antiseptics/disinfectants by well-diffusion in a Petri dish is illustrated in (Fig. 2). Savlon was the most effective antiseptic/disinfectant. Lysol, Hibiscrub, Dettol and Iodine displayed "intermediate" zones of inhibition. Sterilium was the least effective.

Table 1a: MIC Estimation Sea buckthorn against MRSA

Sr.	Dilution	MIC (µg/ml)	A*	B*	C*	D*
1	Neat	5000	-	-	-	+
2	1:2	2500	-	-	-	+
3	1:4	1250	-	-	-	+
4	1:8	625	-	-	-	+
5	1:16	312.5	-	-	-	+
6	1:32	156.25	-	-	-	+
7	1:64	78.13	-	-	+	+
8	1:128	39.06	-	-	+	+
9	1:256	19.53	+	+	+	+
10	1:512	9.77	+	+	+	+
11	Negative control	-	-	-	-	-
12	Positive control	+	+	+	+	+

*Isolates used;
A- Code No.m-7723 (Urine)
B- Code No. m-7709 (Pus)
C- Code No. m-7936 (High Vaginal Swab)
D- Code No. m-8376 (Blood)

Table 1b: MIC Estimation of Green Tea against MRSA isolates.

Sr.	Dilution	MIC µg/ml	A*	B*	C*	D*
1	Neat	5000	-	-	-	+
2	1:2	2500	-	-	-	+
3	1:4	1250	-	-	-	+
4	1:8	625	-	-	+	+
5	1:16	312.5	+	+	+	+
6	1:32	156.25	+	+	+	+
7	1:64	78.13	+	+	+	+
8	1:128	39.06	+	+	+	+
9	1:256	19.53	+	+	+	+
10	1:512	9.77	+	+	+	+
11	Negative control		-	-	-	-
12	Positive control		+	+	+	+

*Isolates used;
A- Code No.m-7723 (Urine)
B- Code No. m-7709 (Pus)
C- Code No. m-7936 (High Vaginal Swab)
D- Code No. m-8376 (Blood)

Table 1c. MIC Estimationof Dandasa against MRSA isolates.

Sr.	Dilution	MIC µg/ml	A*	B*	C*	D*
1	Neat	5000	-	-	-	+
2	1:2	2500	-	-	-	+
3	1:4	1250	+	-	-	+
4	1:8	625	+	-	-	+
5	1:16	312.5	+	+	-	+
6	1:32	156.25	+	+	+	+
7	1:64	78.13	+	+	+	+
8	1:128	39.06	+	+	+	+
9	1:256	19.53	+	+	+	+
10	1:512	9.77	+	+	+	+
11	Negative control		-	-	-	-
12	Postive control		+	+	+	+

*Isolates used;
A- Code No.m-7723 (Urine)
B- Code No. m-7709 (Pus)
C- Code No. m-7936 (High Vaginal Swab)
D- Code No. m-8376 (Blood)

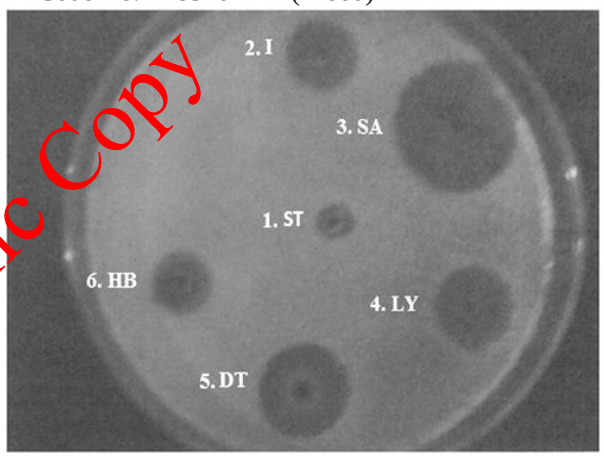

Figure No.2: Zones of Inhibition by Disk Diffusion method of antiseptics/disinfectants against MRSA.
[1. Sterillium (ST), 2. Iodine (I), 3. Savlon (SA), 4. Lysol (LY), 5. Dettol (DT), 6. Hibiscrub (HB)].

DISCUSSION

Pakistan has an abundant plant flora, including those with medicinal properties that are being used for centuries for therapeutic purposes.[10] Our study evaluated the efficacy of crude extracts of three locally grown plants on MRSA isolates retrieved from clinical samples. These included Green tea(Camellia sinensis), Dandasa (Juglansregia), and SBT (Hippophaer-hamnoides). A chemical analysis of active fractions from SBT leaf extracts has led to the finding of a phytochemical drug Hiporamin that possesses a wide spectrum of anti-microbial and anti-viral activity. Hiporamin is a purified form of polyphenol fraction, containing hydrolysable tannins. Green Tea (Camellia Sinensis) has been reported to consist of a variety of components, including polyphenols like catechins and

flavonols.Some of the important actions include its ability to activate leucocytes, and act as an antioxidant, antimutagenic[11], it also reduces plasma cholesterol levels[12]. Walnut (Juglansregia) has anti-inflammatory, antidiarrheic, antihelmintic, antiseptic and astringent properties.[13,14] In our study, none of these extracts had any inhibitory action on the isolate recovered from blood. It may be that when a pathogen enters the blood it is a lethal strain combining multidrug resistance and other virulent properties. SBT had relatively more antibacterial because it is a good source of antioxidants and contains lipophilic and polyunsaturated fatty acids.[15] In a medicinal research, the consumption of SBT berries in 229 healthy individuals markedly raised the fasting plasma concentration of flavonols which has been reported to possess antifungal, antiviral and antibacterial activities[16]. Antioxidant potential of fractions is due to dienes, along with hydroxyl groups, which penetrate through the bacterial wall, disrupting and inhibiting the growth of bacteria[15]. The Total Phenolic Content (TPC) of crude SBT stem extracts has been calculated by a study to be 84 ± 29 mg gallic acid equivalent/g dry extract.[17,18] Studies have shown that SBT inhibits the growth of Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus faecalis It decreasesTumor Necrosis Factor (TNF-α),and increased Interferon (IFN-γ) secretion from macrophages[19]. In a study MRSAwas isolated from the quarters of 73% infected patients, and 69% of colonized patients. It was concluded that every-day articles used by infected or colonized patients may well be converted into sources of dissemination.[20] In light of the above mentioned facts, the evaluation of six selected antiseptics/ disinfectants on 11 (22%) of MRSA strains retrieved from hospitalenvironment was valuable. Savlon was most and Sterillium was the leasteffective. Savlonhas both bacteriostatic and bactericidal activities, its main mechanism of action being membrane disruption. Its antimicrobial action is associated with the attractions between chlorhexidine (cation) and negatively charged bacterial cells membranes. Cetrimide is a cationic quarternary ammonium compound, which acts a surfactant. Sterilium was the least effective a fact that should be noted by hospitals[21]. The action of biocides varies by concentration, time and temperature[22].

CONCLUSION

In view of the fact, that there is a growing demand for finding new effective drugs due to the rising resistance to existing antibiotics, derivatives from some plants look like an attractive substitute. These plant derivatives do not have any side effects, are easily available and can be used for treatment as eye/ear drops, or topical treatments. They have an important role in the prevention of biofilm formation on medical devices and catheters.

REFERENCES

1. Rojas JJ, Ochoa VJ, Ocampo SA, Munoz JF. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. BMC Complementary and Alternative Med 2006;6:2.
2. Eisenberg DM. Trends in Alternative Medicine Use in the United States, 1990-1997: Results of a Follow-up National Survey. J Am Med Assoc 1998;280(18):1569-75.
3. Shinwari ZKK, I.; Naz, S.; Hussain, A. Assessment of antibacterial activity of three plants used in Pakistan to cure respiratory diseases. Afri J Biotechnol 2009;8(24):7082-6.
4. Rasheed A, Haider M. Antibacterial activity ofCamellia sinensis extracts against dental caries. Arch Pharm Res 1998;21(3):348-52.
5. Craig WJ. Health-promoting properties of common herbs. Am J Clin Nutr 1999;70(3):491s-9s.
6. Zeb A. Important Therapeutic Uses of Sea Buckthorn (Hippophae): A Review. J Biolog Sci 2004;4(5):687-93.
7. Huang R, Mehta S, Weed D, Price CS. Methicillin-resistant Staphylococcus aureus survival on hospital fomites. Infect Control Hosp Epidemiol 2006;27(11):1267-9.
8. Neely AN, Maley MP. Survival of enterococci and staphylococci on hospital fabrics and plastic. J Clin Microbiol 2000;38(2):724-6.
9. Geha DJ, Uhl JR, Gustaferro CA, Persing DH. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J Clin Microbiol 1994;32(7):1768-72.
10. Shinwari ZK. Medicinal plants research in Pakistan. J Med Plant Res 2010;4:161-76.
11. Hayatsu H, Inada N, Kakutani T, Arimoto S, Negishi T, Mori K, et al. Suppression of genotoxicity of carcinogens by (-)-epigallocatechin gallate. Prev Med 1992;21(3):370-6.
12. Ikeda I, Imasato Y, Sasaki E, Nakayama M, Nagao H, Takeo T, et al. Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. Biochim Biophys Acta. 1992;1127(2):141-6.
13. Chrzanowski G, Leszczyński B, Czerniewicz P, Sytykiewicz H, Matok H, Krzyżanowski R. Phenolic acids of walnut (Juglans regia L.). Herba Polonica 2011;57(2).
14. Oliveira I, Sousa A, Ferreira IC, Bento A, Estevinho L, Pereira JA. Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglans regia L.) green husks. Food Chem Toxicol 2008;46(7):2326-31.
15. Chaman S, Syed NI, Danish Z, Khan FZ. Phytochemical analysis, antioxidant and

antibacterial effects of sea buckthorn berries. Pak J Pharm Sci 2011;24(3):345-51.
16. Larmo PS, Yang B, Hurme SA, Alin JA, Kallio HP, Salminen EK, et al. Effect of a low dose of sea buckthorn berries on circulating concentrations of cholesterol, triacylglycerols, and flavonols in healthy adults. Eur J Nutr 2009;48(5):277-82.
17. Michel T, Destandau E, Le Floch G, Lucchesi ME, Elfakir C. Antimicrobial, antioxidant and phytochemical investigations of sea buckthorn (Hippophaë rhamnoides L.) leaf, stem, root and seed. Food Chem 2012;131(3):754-60.
18. Upadhyay NK, Kumar MS, Gupta A. Antioxidant, cytoprotective and antibacterial effects of Sea buckthorn (Hippophae rhamnoides L.) leaves. Food Chem Toxicol 2010;48(12):3443-8.
19. Jain M, Ganju L, Katiyal A, Padwad Y, Mishra KP, Chanda S, et al. Effect of Hippophae rhamnoides leaf extract against Dengue virus infection in human blood-derived macrophages. Phytomedicine 2008;15(10):793-9.
20. Carling PC, Briggs J, Hylander D, Perkins J. An evaluation of patient area cleaning in 3 hospitals using a novel targeting methodolo gy. Am J Infect Control 2006;34(8):513-9.
21. Russell AD. Antibiotic and biocide resistance in bacteria: Introduction. J Appl Microbiol 2002;92: 1S-3S.
22. Idrees F, Jabeen K, Khan MS, Zafar A. Antimicrobial resistance profile of methicillin resistant staphylococcal aureus from skin and soft tissue isolates. J Pak Med Assoc 2009;59(5): 266-9.

Address for Corresponding Author:
Dr. Yasmeen Taj
Professor of Pathology
Dow Medical College, DUHS, Karachi
Karachi. 75530.
Cell No.: 03032094439
E-mail: y.taj@hotmail.com