Antibacterial activity of crude methanolic extract and various fractions of *Vitex agnus castus* and *Myrsine africana* against clinical isolates of Methicillin Resistant *Staphylococcus aureus*

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Abstract: *Staphylococcus aureus* is a nosocomial pathogen that resides in the soft tissues causing many diseases. The current study was conducted to determine the prevalence of Methicillin Resistant *S. aureus* (MRSA) in ear discharge and pus of patients and antibacterial activity of crude methanolic extract (Cr. MeOH Ext.) and various fractions of *M. Africana* and *V. agnus castus* against clinical isolates of MRSA. A total of 40 samples were collected from ear, nose and throat (ENT) outpatient department and wards of Khyber Teaching Hospital (KTH), Peshawar. Out of 40 samples, 36 (90%) samples showed growth on Mannitol Salt Agar (MSA) media out of which 9(25%) were MRSA and the remaining 27(75%) were methicillin susceptible *S. aureus* (MSSA). A good antibacterial activity was observed for the Cr. MeOH Ext. (76.1%) and ethyl acetate (EtOAc) fraction of *V. agnus castus* against S_{11} (71.4%). The *n*-hexane fraction also showed good antibacterial effect (70%) against S_{26}. The chloroform ($CHCl_3$), butanol (BuOH) and aqueous fractions of *M. africana* showed good antibacterial activity against S_{11} (71.4%), S_{32} (70%) and S_{26} (75%), respectively. The above results revealed that the selected plants can be further utilized for isolation of the active ingredients as the crude extracts were found good for inhibition of MRSA.

Keywords: Antibacterial activity, Methicillin Resistant *Staphylococcus aureus*, *Myrsine africana, Vitex agnus castus*.

INTRODUCTION

Human body serves as a natural habitat for various microorganisms; fungi, bacteria, yeast and some types of viruses; collectively known as the microflora or normal flora of the body. Cerumen or ear wax, present in the outer ear, stops the entry of different microorganisms into the inner ear. *Pseudomonas aeruginosa* and *S. aureus* are both pathogenic and their growth is prevented by the antimicrobial substances produced by cerumen. Otherwise outer ear being moist and warm would be an ideal place for the microbial growth (Sonali B, 2011).

A study was conducted for the detection of bacteria in ear cavity. No bacteria was isolated from the ear cavity of 50% individuals because it is coated by a protective layer of wax. The viral, bacterial or fungal infections most commonly result in the otitis media. Ear infections in older, adolescents and young adults are commonly caused by *Haemophilus influenzae,* respiratory syncytial virus and also the viruses associated with the common cold. These result in the otitis media by damaging the normal defenses of the epithelial cells in the upper respiratory tract (Sophie *et al.,* 2004). *H. influenza* also helps in setting in which biofilms contribute to bacterial persistence and disease (Costerton *et al.,* 1999). *S. aureus* is another infectious bacterial agent that is responsible for spreading various diseases in the human. It is one of the main sources of skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections and sepsis. Among the variety of *S. aureus* species, one of the most destructive specie is the MRSA (Michael ZD and Robert SD, 2010). The researchers have also documented novel MRSA variants, which were considered accountable for the augmentation of the disease load. These were later pronounced as community associated MRSA variants (CA-MRSA variants). Another type of *S. aureus*; Namely MSSA is reported to cause illnesses; less severe than those induced by their MRSA counterparts and they are easy to treat (Michael ZD and Robert SD, 2010). For about 25% of human population, *S. aureus* is a commensal of the anterior nares and its carriage is responsible for the infection and transmission of *S. aureus* in hospitals (Kluytmans *et al.,* 1997). Similarly surgical wound infections, pneumonia and sepsis are severe *S. aureus* infections that lead to illnesses and even death cases (Chin J 2000). Colonization with *S. aureus* can result at any instant of time; even just after birth (Moran *et al.,* 2006). Sources of transmission of CA-MRSA infections are direct contact with infected patients (Seguin *et al.,* 1999), colonized subjects (Ellis *et al.,* 2004 and Cook *et al.,* 2007) or a contaminated environment (Begier *et al.,* 2004 and Baggett *et al.,* 2004).

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Table 1: Biochemical tests (Catalase, Coagulase and DNase) for *S. aureus* confirmation.

S. No	Catalase	Coagulase	DNase	*S. aureus*	S. No	Catalase	Coagulase	DNase	*S. aureus*
1	-	-	-	-	21	+	-	+	-
2	-	-	-	-	22	+	-	+	-
3	+	+	+	+	23	+	+	+	+
4	-	-	+	-	24	-	-	-	-
5	+	+	+	+	25	+	+	+	+
6	-	-	-	-	26	+	+	+	+
7	+	+	+	+	27	+	+	+	+
8	+	+	+	+	28	+	+	+	+
9	-	-	-	-	29	-	-	-	-
10	+	+	+	+	30	+	+	+	+
11	+	+	+	+	31	+	+	+	+
12	+	+	+	+	32	+	+	+	+
13	+	+	+	+	33	+	+	+	+
14	+	+	+	+	34	+	+	+	+
15	+	+	-	-	35	+	+	+	+
16	+	+	+	+	36	+	+	+	+
17	+	-	+	-	37	+	-	-	-
18	+	+	+	+	38	+	+	+	+
19	-	-	-	-	39	+	+	+	+
20	+	-	+	-	40	-	-	-	-

+; Positive, -; negative

In both Western and Eastern cultures, developed and undeveloped countries, the evidence of plant's uses for medicinal purposes dates as far back as 60 000 years ago. The emperor Shen Nungpharmacopoeia (2730-3000 BC), defines the medicinal use of plants; Opium, Aconite and Opium. The Pharmacopoeia of Ebers Papyrus (Egyptian) (1500 BC), describes the medicinal use of plant extracts; poppy of Opium and oil of Castor beans. A number of plants are commonly used nowadays; rue (*Ruta graveolens*), sage (*Horminum pyrenaicum*), poppy (*Papaver somniferum),* rosemary (*Hyssopus officinalis*), mugwort (*Artemisia vulgaris*), peppermint (*Mentha piperita*) and verbena (*Verbena officinalis*) are well documented in the "*Materiamedica*" of the great physician Hippocrates (460-370 BC) (Solecki and Shanidar 1975, Ackerknecht EH 1973 and Strange RL 1977).

According to World Health Organization (WHO), 4 billion people throughout the world use herbal remedies for primary healthcare (Farnsworth *et al.,* 1985). This gratitude of the importance of medicinal plants use has resulted in a WHO decision to produce 28 standard pharmacopoeia documenting information on selected plants (WHO 1999).

Plants contain various biologically active constituents and most of these constituents possess antimicrobial properties (Cowan MM 1999). The plant based medicines is a part of traditional healthcare in many parts of the world for thousands of years and there is snowballing interest in plants as sources of constituents to fight infectious microbial diseases (Chariandy *et al.,* 1999). There is a constant need for new effective therapeutic agents due to alarming frequency of antibiotic resistance in bacteria of medical importance (Monroe and Polk, 2000 and Bhavnani and Ballow 2000). *V. agnus castus* belongs to family *Verbenaceae* and is distributed in subtropical, warm and temperate regions (Soule JA 2012). The plant along with its ripened seeds and flowers have been used in alternative medicine for the cure of several diseases. Also the berries, flowers and leaves have been used as syrup, decoction, tincture and elixir (Hartung T 2000). The fruit of *V. agnus castus* is considered as tonic for both male and female reproductive systems (Chevallier A 2000). *M. africana* belongs to family *Myrsinaceae* and is widely distributed in subtropical and tropical regions (Nasir and Ali 1979). The plant has been traditionally used as carminative, appetizer, flavoring agent and as fragrant in tea and spices. Fruits of the plant are anthelminthic and edible (Desta 1995). Also the fruit possess potent anti-diarrheal activity and is used to relieve toothache, rheumatism, haemmorhage and pulmonary tuberculosis (Zhong 1985).

MATERIALS AND METHODS

Collection of the plant material

Aerial parts of *V. agnus castus* (*Verbenaceae*), *M. africana* (*Myrsinaceae*), were collected from Hazara division, Khyber Pakhtunkhwa, Pakistan. The plants were identified by Professor Dr. Habib Ahmad, Plant

Taxonomist, Hazara University, Khyber Pakhtunkhwa, Pakistan.

Extraction and fractionation

The plant material dried in shade was crushed into small pieces and ground to fine powder in an electric grinder. Powdered plant materials were soaked in methanol for 15 days at room temperature with occasional shaking. After soaking, filtration of methanol soluble material was done. The filtrates were combined and concentrated in rotary evaporator under vacuum below 40°C. As a result, Cr. MeOH extracts of *V. agnus castus* and *M. africana* were obtained, respectively.

Further, 750g Cr. MeOH extract of *M. africana* was suspended in 400ml distilled water and subsequently partitioned with *n*-hexane (3 x 400ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 x 400ml) and BuOH (3 x 400ml) to yield *n*-hexane (50g), $CHCl_3$ (45g), EtOAc (255g), BuOH (190g) and aqueous (210g) fractions. Similarly, *V. agnus castus* was fractionated by suspending 850g Cr. MeOH extract in 400ml distilled water and subsequently partitioned with *n*-hexane (3 x 400ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 x 400ml) and BuOH (3 x 400ml) to yield; *n*-hexane (100g), $CHCl_3$ (80g), EtOAc (140g), BuOH (80g) and aqueous (155g) fractions respectively.

Sample collection from hospital

40 samples were collected from ear, nose and throat (ENT) outpatient department and wards of KTH, Peshawar by utilizing sterile swabs.

Isolation of S. aureus

Mannitol Salt Agar (MSA) media is both selective and differential media that helps in the distinction among different bacteria depending upon their ability to ferment mannitol. The presence of 7.5% salt in this media helps *staphylococcal* strains to grow (Difco 1984). Media was autoclaved at 121ºC for 15 minutes. After the solidification of the media, the samples collected through culture swabs were streaked directly on the selective media in laminar flow hood (LFH). The plates were then incubated at 37ºC for 24 hrs. Colonies and change in color of the media were then observed.

Identification of MRSA isolates

Biochemical tests

Catalase test

Catalase test was performed by taking a clean slide and adding a small amount of inoculum; collected from 18-24 hrs culture, with the help of a sterile wire loop. 3% H_2O_2 was added through a dropper. Appearance of bubbles showed positive test (Rollins DM 2000).

Coagulase test

In coagulase test, a clean slide was taken and a small amount of plasma was added to it. A sterile wire loop was used to pick a bacterial colony. Colony was added on the slide and mixed with plasma. Clumping was observed within 10 seconds for positive result (Bayliss and Hall 1965).

DNase test

DNase test was performed in DNase agar media. After sterilization of media, on solidification, sterile loop was used to make compartments. These compartments were then labeled. With the help of sterile loop, a colony was picked and sub-cultured into respective labeled compartment. Incubation was done for 24 hrs at 37ºC. About 15 ml of 1N HCl solution was drained after some time. Clear zones around the bacterial colonies indicated positive result (Bayliss and Hall 1965).

Antibiotic sensitivity test

Determination of MRSA and MSSA isolates was done by antibiotic sensitivity test using cefoxitin discs on the cultured isolates. Nutrient agar media was prepared, sterilized and poured into petri plates. On solidification, sterile swab was taken to pick a colony and uniform bacterial lawn was prepared. Cefoxitin disc was taken with the help of sterile forceps and placed on petri plate. The plates were then incubated for 24 hrs at 37ºC. Zone of inhibition was measured after 24 hrs. Zone of inhibition of diameter <19 mm indicated MRSA and >19 mm showed the presence of MSSA strains (Wayne PA 2005).

Antibacterial activity

The Cr. MeOH extract and various fractions of *M. africana* and *V. agnus castus* were screened for their possible antibacterial activity against clinical MRSA isolates as per our reported procedure (Bashir *et al.*, 2010). Nutrient agar media was prepared, sterilized, poured in petri plates and incubated at 37ºC for 24 hrs to check sterility. Next day, bacterial lawn was made on the labeled plates with their respective isolates. Wells were dug with the help of a sterile borer (6 mm). Stock solution(s) of the test samples were prepared in DMSO (≤1%) at concentration of 3mg/ml. 100µl of the solution was then transferred from the stock solution to the respective wells through micropipette. For the better diffusion, the plates were left for 2-3 hrs inside the Laminar Flow Hood. Finally, the plates were incubated for 24 hrs at 37ºC. Zone of inhibition around each well was observed and measured after incubation period in comparison with the positive control.

RESULTS

Identification

A total of 40 samples were screened for isolation of *S. aureus*, 36 samples showed growth on the MSA media. Yellow growth, characteristic of *S. aureus* was observed in 16 samples. However, yellow growth on pink

Table 2: *S. aureus* zone of inhibition against Cefoxitin disc for detection of MRSA and MSSA

Isolate No.	Zone of Inhibition (mm)	MRSA / MSSA	Isolate No.	Zone of Inhibition (mm)	MRSA / MSSA
3	21	MSSA	26	22	MSSA
5	35	MSSA	27	34	MSSA
7	18	MRSA	28	39	MSSA
8	26	MSSA	30	10	MRSA
10	29	MSSA	31	11	MRSA
11	11	MRSA	32	26	MSSA
12	28	MSSA	33	29	MSSA
13	37	MSSA	34	17	MRSA
14	10	MRSA	35	22	MSSA
16	30	MSSA	36	15	MRSA
18	28	MSSA	38	10	MRSA
23	26	MSSA	39	35	MSSA
25	17	MRSA	40	24	MSSA

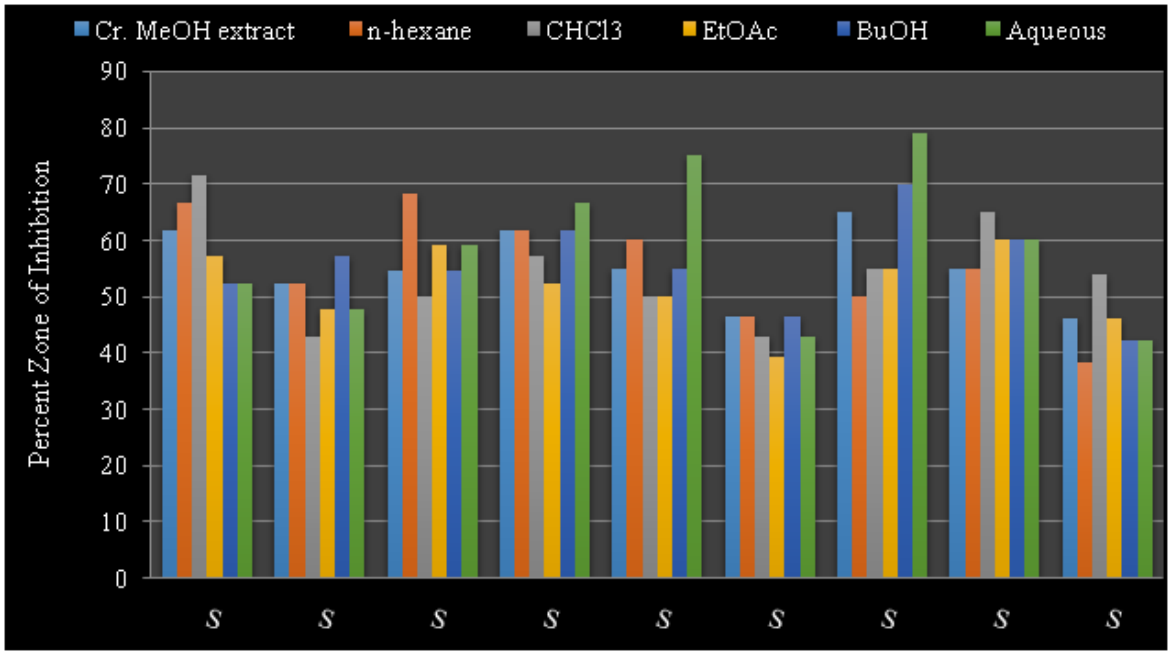

Fig. 1: Antibacterial activity of Cr. MeOH and fractions of *M. africana* against MRSA isolates

background was observed in 19 samples. In two samples, only color of the media was changed from red to pink whereas no growth was observed in three samples.

Biochemical tests

The biochemical tests were then performed for further identification of the 36 isolates. Results are summarized in table 1.

Catalase test

31 (86.1%) samples showed positive catalase test result as observed by the appearance of bubbles due to the production of oxygen when 3% H_2O_2 reacted with *Staphylococcus* strains.

Coagulase test

26 (72.2%) samples showed positive results for coagulase as observed by the clump(s) formation on the addition of plasma to *Staphylococcal* colonies. 10 (27.7%) samples showed coagulase negative results as no clumps were observed.

DNase test

28 (77.7%) samples were DNase positive. The positive result was observed because of the appearance of transparent media on pouring of 1N HCl solution. The remaining 8 (22.2%) samples were DNase negative.

Determination of MRSA and MSSA Isolates

The MRSA and MSSA isolates were distinguished by the antibiotic sensitivity test using cefoxitin disc. Out of 26 *S. aureus* isolates, 9 (34.61%) isolates were observed to have a zone of inhibition ≤19 mm. These 9 isolates were MRSA. Results are shown in table 2.

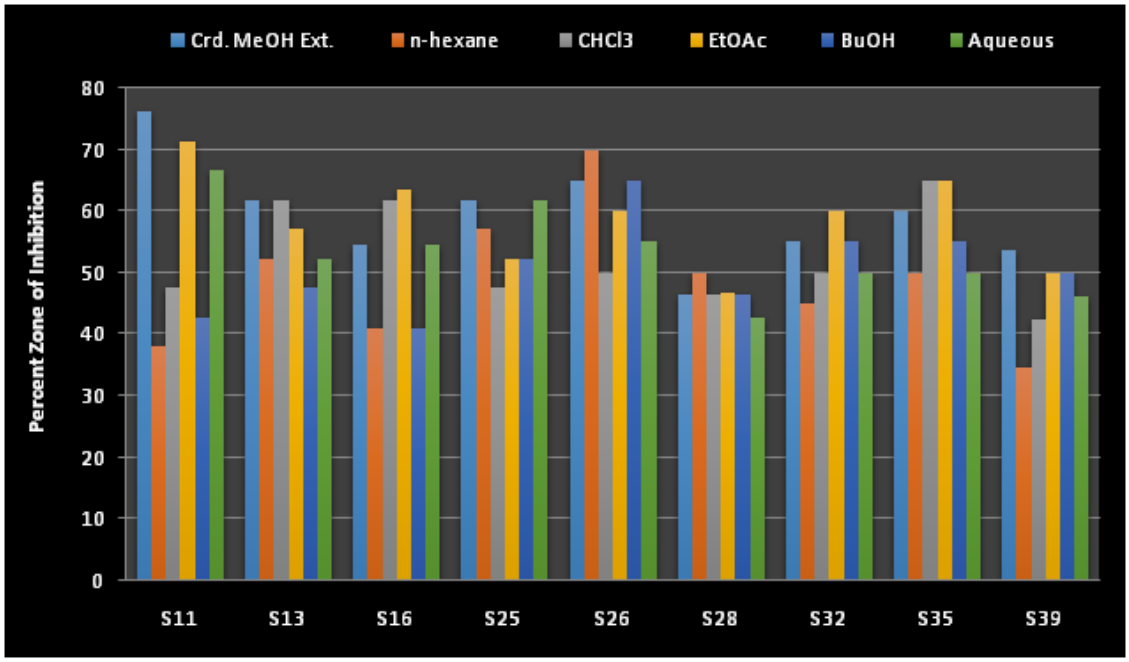

Fig. 2: Antibacterial activity of Cr. MeOH and fractions of *V. agnus castus* against MRSA isolates

Screening of plant extracts against MRSA

Antibacterial activity of Myrsine africana

The Cr. MeOH extracts and various fractions of *M. africana* were screened against clinical MRSA isolates. Good antibacterial activity was shown by the aqueous fraction of *M. africana* against clinical MRSA isolate S_{32} (79.0%), S_{26} (75.0%) and S_{25} (66.6%) while rest of the isolates were moderately inhibited by this fraction. The $CHCl_3$ fraction also showed good antibacterial activity against S_{11} (71.4%) and S_{35} (65.0%) rest of the isolates were moderately inhibited. The BuOH fraction of *M. africana* also showed good antibacterial activity against S_{32} (70%), S_{25} (61.9%) and S_{35} (60%) rest of the isolates were moderately inhibited. The *n*-hexane fraction of the plant also showed good antibacterial activity against S_{16} (68.1%), S_{11} (66.6%), S_{25} (61.9%) and S_{26} (60.0%). Rest of the clinical MRSA isolates were moderately inhibited except S_{39} which showed more resistance to the *n*-hexane fractions. The Cr. MeOH extract of *M. africana* showed good antibacterial activity against some of the MRSA isolates; S_{32} (65.0%), S_{11} (61.9%) and S_{25} (61.9%). EtOAc fraction *M. africana* was found to show good antibacterial activity against S_{35} (60.0%) while low inhibition was observed for S_{28} (39.2%) and the remaining isolates were moderately inhibited. Results are presented in fig. 1.

Antibacterial activity of Vitex agnus castus

The Cr. MeOH extracts and various fractions of *V. agnus castus* were screened against clinical MRSA isolates results are presented in fig. 2. The Cr. MeOH extract of the plant possesses good antibacterial activity against; S_{11} (76.1%), S_{26} (65.0%), S_{13} (61.9%), S_{25} (61.9%) and S_{35} (60.0%), moderate antibacterial activity was observed against rest of the clinical MRSA isolates. The EtOAc fraction showed good antibacterial activity against 5 isolates; S_{11} (71.4%), S_{35} (65.0%), S_{16} (63.6%), S_{26} (60.0%) and S_{32} (60.0%) while rest of the isolates were moderately inhibited. The *n*-hexane fraction showed good antibacterial activity (70%) against S_{26}. Rest of the isolates were moderately inhibited except S_{11} (38.09%) and S_{39} (34.6%) which were more resistant to this fraction. Aqueous fraction of the plant also possess good antibacterial activity against S_{11} (66.6%) and S_{25} (61.9%). Moderate antibacterial activity was observed for the rest of the MRSA isolates. The $CHCl_3$ fraction of *V. agnus castus* showed good antibacterial activity against S_{35} (65%), S_{13} (61.9%) and S_{16} (61.9%) while rest of the isolates were moderately inhibited. The BuOH fraction of the plant showed good activity against S_{26} (65.0%) and remaining isolates were moderately inhibited. Results are shown in fig. 2.

DISCUSSION

Rosalie *et al.,* (1977) compared coagulase, DNase and heat stable nuclease test for identification of *S. aureus*. They observed 65 contradictory results when they compared coagulase and DNase tests. According to their research, nine (1%) isolates, which were coagulase negative were DNase positive and 56 (18%) DNase positive isolates were coagulase negative. On normal DNase agar containing 0.1% DNA, 91 DNase negative and 30 DNase positive results were observed. Out of these results, 91 DNase negative and three DNase positive isolates were coagulase negative. Similarly, it was also observed that the residual 27 DNase positive isolates were coagulase positive (Menzies and Rosalie 1977).

Rubeena *et al.,* (2002) carried out a study on the prevalence and antimicrobial susceptibility of MRSA. High frequency occurrence of MRSA was observed in the patients of intensive care units (ICU's) and special care wards. The findings showed highest yield of MRSA from endotracheal secretions (100%), bronchial washings (70%) and catheter tips (51.85%) followed by sputum (40.54%), ear swabs (40%), fluids (37.25%), pus / wound swabs (34.83%) and blood cultures (28.07%). Out of 55 ear samples, 22 were MRSA. Anand *et al.,* (2009) studied the comparison of cefoxitin disc diffusion test, oxacillin screen agar and PCR for *mec-A* gene for detection of MRSA. 50 clinical samples were collected. The verification of *S. aureus* isolates was done by performing variety of tests; growth observation on MSA, catalase, coagulase and antibiotic susceptibility tests against various antibiotics. Out of 50 samples, they found 32 MRSA isolates by cefoxitin disc diffusion test. According to their research, cefoxitin can be used as a true substitute for the vulnerability determination on regular basis at 37°C for 18-24 hrs. In our study, out of 36 samples in which growth was observed, 31 were catalase positive, 26 were coagulase positive and 10 were coagulase negative. In DNase test, 28 were DNase positive and 8 were DNase negative. No conflicting result was observed in 9 MRSA isolates. However, on the other hand out of 27 MSSA isolates, 9 isolates showed conflict in results; 6 MSSA isolates were coagulase negative but DNase positive and 3 MSSA isolates were coagulase positive and DNase negative, showing the percentage conflict of 66.66 and 33.33%, respectively. Coagulase is a proteinaceous enzyme produced by variety of microorganisms. It has the ability to convert fibrinogen to fibrin and is used to differentiate among different species *Staphylococcus* isolates. Though *S. aureus* is typically considered as coagulase positive yet researches have demonstrated that it is not necessary that every *S. aureus* isolate will strictly be coagulase positive (Ryan KJ and Ray CG 2004). As positive coagulase test shows the presence of coagulase enzyme. This coagulase has the tendency to react with prothrombin in the blood forming staphylothrombin complex. This complex causes coagulase enzyme to convert the liver fibrinogen into fibrin thereby resulting in the blood clotting. So the presence of coagulase in *S. aureus* helps it to evade the host defenses by forming blood clots. Thus, this blood clot protects the bacteria from phagocytosis and poorly affects host (Tortora Gerard *et al.,* 2013). DNase test helps to study the ability of microorganisms such as *S. aureus* to hydrolyze the DNA and utilize it as food and energy source. Rao *et al.*, observed the DNase negative *S. aureus* strains but couldn't provide the reason behind this DNase negative character (Rao *et al*., 2002).

Herbalism is a remedy based upon the utilization of plants and plant extracts. The Cr. MeOH Ext. of *V. agnus castus* possesses significant spasmolytic effect on rabbit's jejunum and at concentration of 3.0 mg/ml, it completely abolished the spontaneous tissue contractions (Sadiq *et al.,* 2012). The $CHCl_3$ fraction of this plant possess significant antibacterial activity against *Klebsiella pneumonia* (81% with MIC_{50}=2.19 mg/ml) (Sadiq *et al.,* 2012). The *n*-hexane fraction of the plant exhibit moderate (62.5%) phytotoxic effect against *Lemna minor* L at higher concentration (Sadiq *et al.,* 2012). The Cr. MeOH Ext. of *M. africana* showed a significant spasmolytic effect on rabbit's jejunum and completely abolished the tissue contraction at concentration of 5.0 mg/ml (Sadiq *et al.,* 2011). The $CHCl_3$ fraction of *M. africana* possesses moderate phytotoxic activity (31.25%) against *L. minor* at higher concentration (Bashir *et al.,* 2011). The Cr. MeOH Ext. and $CHCl_3$ fraction of *M. africana* showed good inhibitory effect against *K. pneumoniae* (MIC_{50}=2.45 and 2.1mg/ml), respectively (Bashir *et al.,* 2010). In the present study we also observed that the Cr. MeOH Ext. and various fractions of *M. africana* and *V. agnus castus* showed good and moderate antibacterial activity against the clinical isolates of MRSA.

REFERENCES

Ackerknecht EH (1973). Therapeutics from the Primitives to the Twentieth Century. Hafner Press: New York. pp.87-90.

Anand KB, Agrawal P, Kumar S and Kapila K (2009). Comparison of Cefoxitin disc diffusion test oxacillin screen agar and PCR for mec-A gene for detection of MRSA. *Ind. J. of Med. Microbiol.,* **27**: 27-29.

Baggett HC, Hennessy TW and Rudolph K (2004). Community-onset methicillin-resistant *S. aureus* associated with antibiotic use and the cytotoxin Panton-Valentine leukocidin during a furunculosis outbreak in rural Alaska. *The J. of Infec. Dis.,* **189**: 1565-1573.

Bashir A, Sadiq A, Shumaila B, Ibrar K, Achyut A and Choudhary MI (2010). Anti-inflammatory and enzyme inhibitory activities of a crude extract and a pterocarpan isolated from the aerial parts of *V. agnus castus*. *Biotechnol. J.,* **5**: 1207-1215.

Bashir A, Sadiq A, Shumaila B, Ibrar K, Niaz A and Chaudhary MI (2011). Phytotoxic, antibacterial and haemagglutination activities of the aerial parts of *Myrsine africana*. *Afr. J. of Biotechnol,* **10**: 97-102.

Bayliss BG and Hall ER (1965). Plasma Coagulation by organisms other than *Staphylococcus aureus*. *J. of Bacteriol.,* **89**(1): 101-105.

Begier EM, Frenette K and Barrett NL (2004). A high-morbidity outbreak of MRSA among players on a college football team, facilitated by cosmetic body shaving and turf burns. *Clin. Infec. Dis.,* **39**: 1446-1453.

Bhavnani SM and Ballow CH (2000). New agents for Gram-positive bacteria. *Curr. Opin. in Micro.,* **3**: 528-534.

Chariandy CM, Seaforth CE, Phelps RH, Pollard GV and Khambay BP (1999). Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. *J. Ethnopharmacol.,* **64**: 265-270.

Chevallier A (2000). *Encyclopedia of Herbal Medicine*. Dorling Kindersley. pp.294-296.

Chin J (2000). Control of Communicable Diseases Manual, 17th ed. Baltimore: United Book Press, Incorporation. pp. 534-540.

Cook HA, Furuya EY, Larson E, Vasquez G and Lowy FD (2007). Heterosexual transmission of community-associated methicillin-resistant *S. aureus*. *Clin. Infec. Dis.*, **44**: 410-413.

Costerton JW, Stewart PS and Greenberg EP (1999). Bacterial biofilms: A common cause of persistent infections. *Science,* **284**: 1318-1322.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin. Micro. Rev.,* **12**: 564-582.

Desta B (1995). Ethiopian traditional herbal drugs. Part I: Studies on the toxicity and therapeutic activity of local taenicidal medications. *J. Ethnopharm.*, **45**: 27-33.

Difco (1984). *Manual of Dehydrated Culture Media and Reagents for Microbiology,* 10thed. Difco Laboratories Inc. Detroit. MI., Pp.558-560

Ellis MW, Hospenthal DR, Dooley DP, Gray PJ, Murray CK (2004). Natural history of community-acquired methicillin-resistant *S. aureus* colonization and infection in soldiers. *Clin. Infec. Dis.,* **39**: 971-979.

Farnsworth NR, Akerele O, Bingel AS, Soejarta DD, Eno Z (1985). Medicinal plants in therapy. *Bull World Health Organization*, **63**: 965-981.

Hartung T (2000). Growing 101 Herbs That Heal. Storey Books. pp.187-188.

Kluytmans J, Van BA and Verbrugh H (1997). Nasal carriage of *S. aureus*: Epidemiology, underlying mechanisms and associated risks. *Clin. Microbiol. Rev.,* **10**: 505-520.

Menzies and Rosalie E (1977). Comparison of coagulase, deoxyribonuclease (DNase) and heat-stable nuclease tests for identification of *S. aureus. J. of Clini. Pathol,* **30**: 606-608.

Michael ZD and Robert SD (2010). Community-Associated MRSA: Epidemiology and Clinical Consequences of an Emerging Epidemic. *Clin. Microbiol. Rev.,* **23**: 617-687.

Monroe S and Polk R (2000). Antimicrobial use and bacterial resistance. *Curr. Opin. in Micro.,* **3**: 496-501.

Moran GJ, Krishnadasan A and Gorwitz RJ (2006). Methicillin-resistant *S. aureus* infections among patients in the emergency department. *The New England J. of Med.,* **355**: 666-674.

Nasir E and Ali SI (1979). Flora of West Pakistan; Myrsinaceae, complied by S.M.H. Jafri, Saida Qaiser. **89**: 5.

Rao JG, Qamruddin AO, Hassan IA, Burnie JP and Ganner M (2002). Cluster of clinical isolates of epidemic MRSA with a negative deoxyribonuclease (DNase) test-implications for laboratory diagnosis and infection control. *J. Hos. Infec.,* **51**: 238-239.

Rollins DM (2000). Bacterial Pathogen List. BSCI 424 Pathogenic Microbiology, University of Maryland. Retrieved on 2009-03-01.

Rubeena H, Chughtai AS, Aslam M (2002). Prevalence and antimicrobial susceptibility of MRSA. *Int. J. of Pathol,* **2**: 10-15.

Ryan KJ and Ray CG (2004). Sherris Medical Microbiology (4th ed.). New York: McGraw Hill. Pp.261-271.

Sadiq A, Shumaila B and Bashir A (2011). Anti-spasmodic action of crude methanolic extract and a new compound isolated from the aerial parts of *Myrsine africana*. *BMC Comp. and Alt. Med.*, **11**: 55.

Sadiq A, Shumaila B and Bashir A (2012). Anti-spasmodic action of crude methanolic extract of the aerial parts of *V. agnus castus*. *J. of Med. Plants Res.,* **6**: 461-464.

Seguin JC, Walker RD, Caron JP, Kloos WE, George CG, Hollis RJ, Jones RN and Pfaller MA (1999). MRSA outbreak in a veterinary teaching hospital: Potential human-to-animal transmission. *J. of Clin. Microbiol,* **37**: 1459-1463.

Solecki RS and Shanidar IV (1975). A neanderthal flower burial of Northern Iraq. *Science,* **190**: 880.

Sonali B (2011). Microbial flora of ENT (Ear, nose and throat). Biotech articles. com. 22: 01:27.

Sophie C, Traven L, Margaret C, Reinhold M and Richard M (2004). Management of chronic Suppurative otitis media. *The Med. J. Aus.,* **180**: 91-93.

Soule JA (2012). Butterfly Gardening in Southern Arizona. Tierra del Soule Press, Tucson, AZ. ISBN 0-9758554-3-3. P.59

Strange RL (1977). A History of Herbal Plants. Arco Publishing Company Incorporation, New York: pp.79-80.

Tortora GJ, Funke BR and Case CL (2013). Microbiology: An Introduction (11 ed.). Glenview, IL: Pearson Education Inc. p.434.

Wayne PA (2005). Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing; 15th Informational Supplement. *App. Std.,* **25**: M100-S15.

World Health Organization (1999). Monographs on selected medicinal plants, Geneva: WHO Library Cataloguing in Publication Data, **1**: 1.

Zhong HBC (1985). State administration of traditional Chinese medicine of the people's republic of China. *Shanghai Sci. Technol., Press: Shanghai.,* **3**: 245.