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UNCONTAMINATED ACTION OF THERAPEUTIC PLANT EXTRACT IN CONTRADICTION OF MULTIPLE DRUG RESISTANT BACTERIA-STAPHYLOCOCCUS AUREUS

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ABSTRACT

Some bacteria become resistant to many different antibioticsare called as multidrug- resistant. Multidrug-resistant bacteria isdifficult to treat andspread the antibiotic resistance gene. Methicillin, as the first beta-lactamase resistant penicillin, was used to treat Staphylococcusaureus infection in 1961. The first methicillin-resistant S. aureus (MRSA) was identified in the United Kingdom in the same year. It appeared in the United States in 1981 among intravenous drug users. Methicillin-resistant Staphylococcus aureus (MRSA) is an important agent of hospital-acquired infection. Nasal swabs samples were collected from Anand civil hospital patients and all swabs samples were cultured on Mannitol salt agar. Age, sex, name, ward/infection, date of admission to hospital were recorded. Gram's staining, Catalase test, DNase test, coagulase tests were done.Out of 20 nasal samples, 12Staphylococcus aureus were recovered. HIMEDIAHiCromeMeReSa Agar Base (M1674) + MeReSa Selective Supplement (FD229) were used in order to detect MRSA, only 03 Staphylococcus aureus were recovered out of total 12 isolates. Antibiotic susceptibility was tested by using the disk diffusion technique on Mueller-Hinton agar. The antibiotics tested were **Methicillin (5µg), Oxacillin (5µg), Cephalexin (30 µg), Gentamycin (50µg), Tetracycline (10 µg), Kanamycin (5 µg), Amikacin (10 µg), Cefotaxime (10 µg), Amoxicillin (10 µg),** Chloramphenicol (10**µg**), Ciprofloxacin (10**µg**),Cloxacillin (5**µg**) **Vancomycin (30 µg) and Erythromycin (15 µg).**Almost all the MRSA strains (91.3%) screened from nasal samples were resistant to Amikacin, 86.95% to kanamycin and Cloxacillin, 78.26% to ciprofloxacin, 52.17% to chloramphenicol, and 34.78% to both tetracycline and gentamycin. Also, MRSA samples were screened for the vancomycin resistance.The present study reveals the emergence of MRSAand also indicates the magnitude of antibiotic resistance in and around the study area.The major cause of this may be unawareness and indiscriminate use of broad-spectrum antibiotics.Our study reveals the presence of MRSA in Anand hospitals this might also be prevalent in other parts of India as antibiotic misuse is equally common there.

Many plants have been used against multiple drug resistant bacteria due to presence of bioactive compound.

The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body. Medicinal plants are relied upon by 80% of the world's population and in India there is a rich tradition of using herbal medicine for the treatment of various bacterial diseases, inflammations, injuries and other diseases. Many of the plant materials used in traditional medicine are generally proved more effective and relatively cheaper than modern medicine and have lessside effects that are often associated with synthetic antimicrobials.

Murraya koenigii, Eucalyptus globulus, Dodonaea viscose & Mentha spicata water extracts used against Methicillin-resistant Staphylococcus aureus (MRSA).Out of which Murraya koenigii (18mm) shows maximum zone of inhibition.

Keywords: MRSA, prevalenceand antibiotics.

INTRODUCTION

Large amounts of antibiotics used for human therapy, as well as for farm animals and even for fish in aquaculture, resulted in the selection of pathogenic bacteria resistant to multiple drugs. Multidrug resistance in bacteria may be generated by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids. Second, multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs. This review discusses our current knowledge on the molecular mechanisms involved in both types of resistance. MRSA is an important cause of hospital-acquired infections leading to high morbidity and mortality. MRSA infection places an addition burden on the patient care budget due to prolonged hospital stays. Further, it adds an inestimable human suffering, which could be avoided by taking the proper infection control precautions[1]. According to a European Antimicrobial Resistance Surveillance System (EARSS) report,MRSA was responsible for 0.5% to 44% of cases of staphylococcal bacteraemia in Europe, with the highest incidence of 44% in Greece and lowest of 0.5% in iceland[2]. According to National Nosocomial Infection Surveillance System (NNIS) report, 50% of hospital acquired infections in ICUs in the USA are due to MRSA[3]. Apart from high transmissibility in the hospital, MRSA also carries certain virulence factors that are associated with toxic shock syndrome, necrotizing pneumonia and skin infections[4]. MRSA is of greater concern in females due to its higher prevalence rate in comparison to males[5-6]. Nasal colonization with MRSA is a significant risk factor for hospital acquired infections. Nasal colonization of MRSA among 1% to 3% of the out patients population in the USA has been reported, [7-9] whereas nasal colonization of MRSA was absent among the out patient population in Turkey[10]. A high prevalence of nasal colonization (18.1%) among healthy community individuals has been reported in India[11]. The prevalence of MRSA nasal colonization in the community needs to be determined before instituting measures to prevent the transmission of MRSA.Vancomycin, a

glycopeptide antibiotic continues to be an important antimicrobial agent to treat MRSA but resistance finally emerge. In 1996, a *S. aureus* strain with intermediate resistance to vancomycin (VISA) (vancomycin MIC= 8µg /ml) was first isolated from a patient in Japan[12]. Shortly afterward, VISA strains were isolated in USA, Europe and other Asian countries [13-14]. Characterization of these VISA strains indicates that the mechanisms of resistance are complex and involve changes in cell wall content and composition [14-15]. In June 2002, the World's first reported clinical infection due to *S. aureus* with high resistance to vancomycin (VRSA) (vancomycin MIC>128 µg /ml) was diagnosed in a patient in the USA[16].

We determined the prevalence of MRSA of nasal colonization among patients at the time of admission to the hospital and examined possible risk factors involved in the nasal carriage of *Staphylococcus aureus*.

MATERIAL AND METHODS

(A) Collection and Enrichment of Samples

Patients admitted in the hospitals were examined for MRSA infection. Nasal samples were taken from each patient. A total of 20 patients were monitored for the proposed study of nasal carrier state of Methicillin Resistant *Staphylococcus aureus.*Autoclaved cotton swabs (dipped in normal saline 0.9%) were used for nasal swabbing of the anterior nares of the patients. The swabs were rubbed very well by rotating 5 times over the inner wall of the ala and nasal septum and immediately processed for culture and isolation.The nasal swabs collected were cultured on Mannitol Salt agar (selective medium for *Staphylococcus aureus*) within one hour after collection by spreading as per the conventional technique. The culture plates were incubated at 37^0C for 24-48 hours in the incubator.

(B) Identification of *Staphylococcus aureus*:

The suspected *Staphylococcus aureus* yellow-colouredcolonies showing Mannitol fermentation were selected and subjected to **Gram staining** and sub-cultured into nutrient agar slopes. The isolates showing gram-positive cocci in clusters were subjected to**Catalase test**, **DNase, Coagulase test** by slide and test tube technique using undiluted and 1: 5 diluted human plasma respectively.

(C) Identification of MRSA by "Crome agar" plate method:

For the identification of the MRSA among the isolates of *Staphylococcus aureus*, the Hi- Media (India) made **HIMEDIAHiCromeMeReSa Agar Base** (M1674) was used. The media was prepared by mixing 41.65 gms of the media into 500 ml of the distilled water. The medium is cooled to around 50-55^{0}C and **MeReSa Selective Supplement** (FD229)After checking the plates for sterility by keeping at 37^0C overnight the *Staphylococcus aureus* strains were streaked onto the Hi Crome Me Re Sa agar and incubated at 35^0C for 24 hours. The MRSA only grew on this Hi Crome Me Re Sa agar, while the MSSA was inhibited on the same agar plate. All cultures showing bright blue colored growth were taken as MRSA positive strains, while all others are recorded as MSSA strains.

(D) Antibiotic susceptibility testing:

All isolates of *Staphylococcus aureus* were subjected to in vitro anti-bacterial testing method on Muller-Hinton agar containing 2-3% NaCl, using 2-hour-old nutrient broth culture and HIMEDIA make antibiotic discs as per the method described by Kirby and Bauer (1966). The zone of inhibition around the discs were measured and interpreted as sensitive, moderately sensitive and resistant using the interpretation chart supplied by the antibiotic disc manufacturers (HIMEDIA, Mumbai).

PREPARATION OF PLANT EXTRACTS COLLECTION OF PLANTS

*Murraya koenigii, Eucalyptus globulus, Dodonaea viscose &Mentha spicata*were collected from local area of Deharadun and identified by botanical survey of India.

PREPARATION OF EXTRACT

For this purpose, dried powdered of leaves were used for aqueous extractionaqueous by sonicator. Then water extract heated on water bath at 70-80˚C in china dish to get semisolid crude extract.

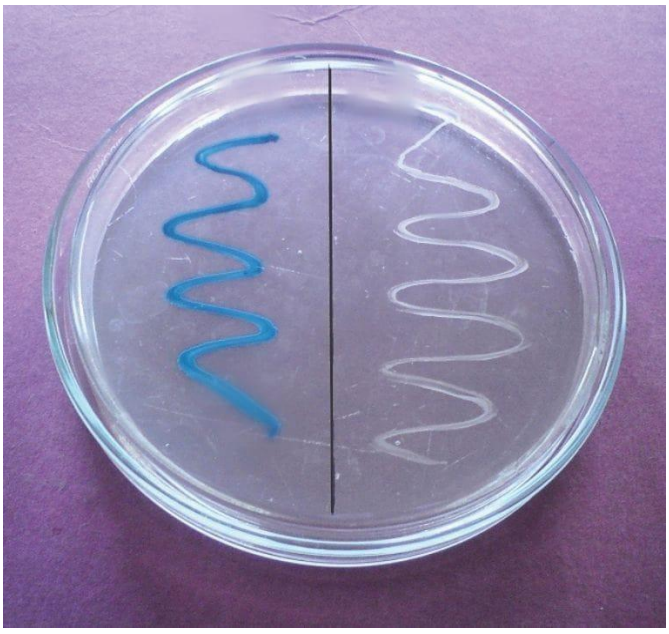
DETERMINATION OF THE ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANTS

From the crude extract, the 500mg/ml dilution of plant paste was prepared for antibacterial assay. The modified agar well diffusion method was employed to determine the antibacterial activity of plant extracts, 200µl of the extract (500mg/ml) were poured in to the well. All the plates were incubated at 37˚C for 24 hrs and zone of inhibition were observed in the form of mm (mili meter).

OBSERVATIONSAND RESULTS

Out of 20 nasal samples, 12*Staphylococcus aureus*were recovered and were further subjected to biochemical testing. The detection of MRSA among the isolates of the *Staphylococcus aureus* was carried out using the Hi-Crome MeReSa agar medium.

Fig 1: MRSA positive (on left) and negative (on right) on Hi-Crome MeReSa agar medium.



A total of 12*Staphylococcus aureus* strains were tested on this medium. The strains were inoculated on this

special medium and incubated at 35^0 C for full 24 hours. The cultures showing bright blue color were taken as MRSA positive and the color less growth was recorded as MSSA strains (shown in fig 1). A total of 03*Staphylococcus aureus* were found to be MRSA.The prevalence of MRSA from nasal samples of Anand hospital was 25%. The details of the results are given in Table- 1.

Table 1: The prevalence of MRSA from nasal samples of Anand hospitals

No. of samples	Staphylococcus aureus	MRSA
20	12	03

ANTIBIOGRAM

We have tested 14 different types of antibiotics for the susceptibility pattern of Methicillin resistant staphylococcus aureus isolates on Mueller-Hinton agar (MHA) plates[17].The drug resistance patterns of MRSA isolated from clinical specimens and carrier screening samples were found to be highly variable. Almost all the MRSA strains screened from nasal samples were 100%resistant to Amikacin, 86.95% to kanamycin and Cloxacillin, 78.26% to ciprofloxacin, 56.52% to erythromycin, 52.17% to chloramphenicol, and 34.78% to both tetracycline and gentamycin. In, general all MRSA provided were multidrug resistant (as shown in Table 3).

Table 3: Antibiogram of MRSA

Antimicrobial Agent	Disc Potency (µg)	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ciprofloxacin	10	78.26%	21.73%	0.01%
Amoxicillin	10	34.78%	4.34%	60.86%
Cephalexin	30	60.86%	4.34%	34.78%
Cloxacillin	5	73.91%	17.39%	8.69%
Methicillin	5	100%	-	-
Cefotaxime	10	100%	-	-
Oxacillin	5	86.95%	-	13.05
Gentamicin	50	34.78%	39.13%	26.09%
Kanamycin	5	86.95%	13.04%	0.01%
Tetracycline	10	34.78%	52.17%	13.04%
Chloramphenicol	10	52.17%	43.47%	4.36%
Amikacin	10	91.30%	4.34%	4.36%
Erythromycin	15	56.52%	43.47%	-

Bar graph showing different Antibiotic resistant pattern of MRSA

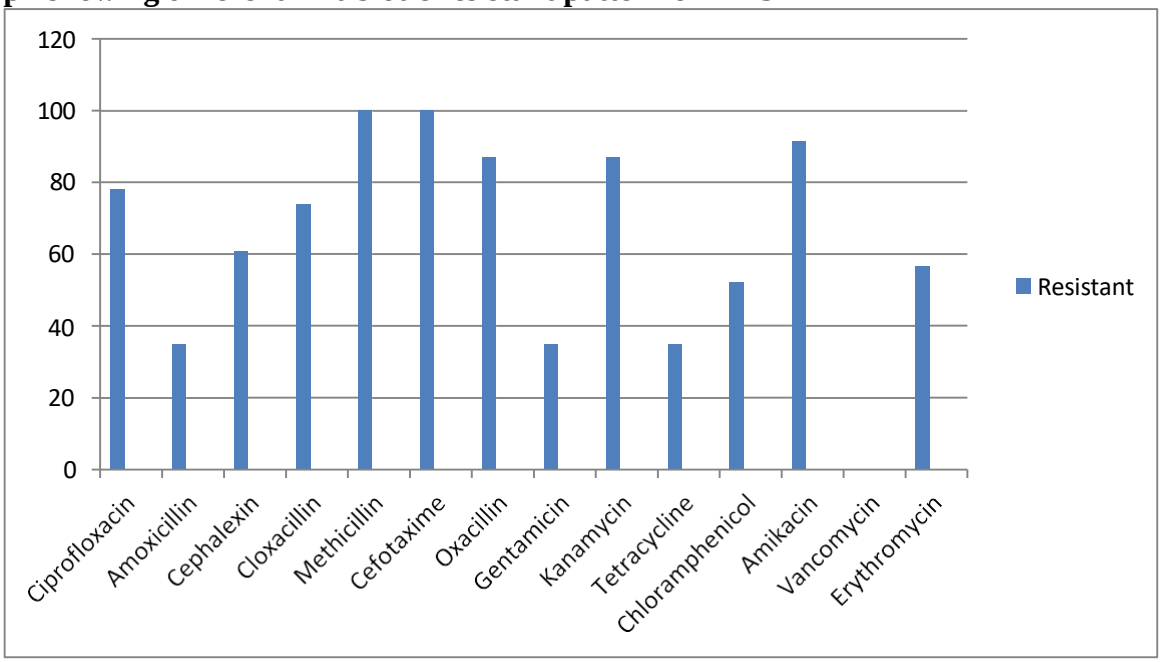
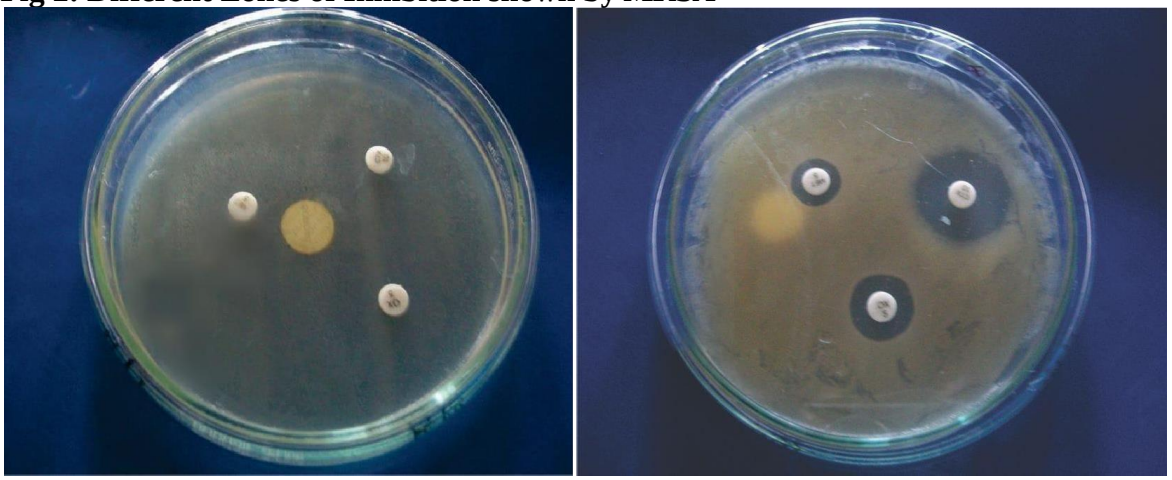
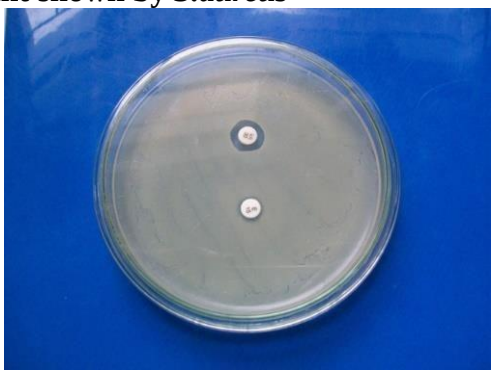


Fig 2: Different Zones of Inhibition shown by MRSA



If the zone of MRSA against Vancomycin is less than or equal to 14mm then it will be considered as VRSA, if the zone diameter is 15-16mm then it will be considered as VISA (Vancomycin Intermediate *S.aureus).* Out of 03 MRSA samples, 1 (13.3%)strain were resistant to vancomycin and these strains were also resistant to all other antibiotics as shown in table 4.

Fig 4: Vancomycin resistant shown by *S.aureus*



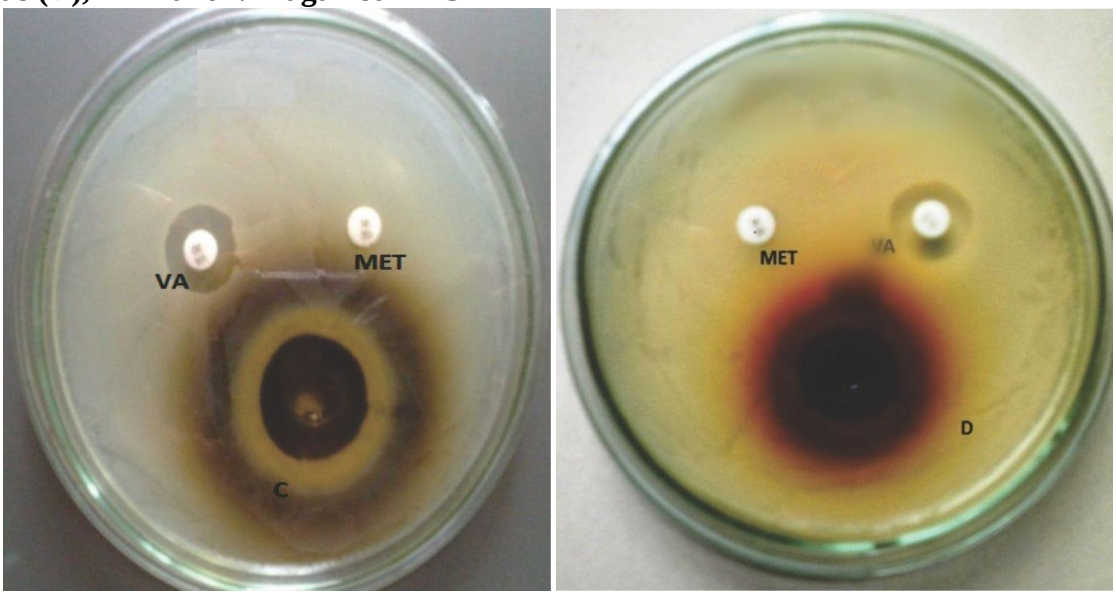
ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANT EXTRACT AGAINST MRSA

Aqueous extract of four plants were used against MRSA strain. The maximum zone of inhibition was observed by*Murraya koenigii, (18 mm), Eucalyptus globulus (15mm),Dodonaea viscose (14mm) and Mentha spicata (12mm) as shown in table 1and slide 3,4,5 and 6*

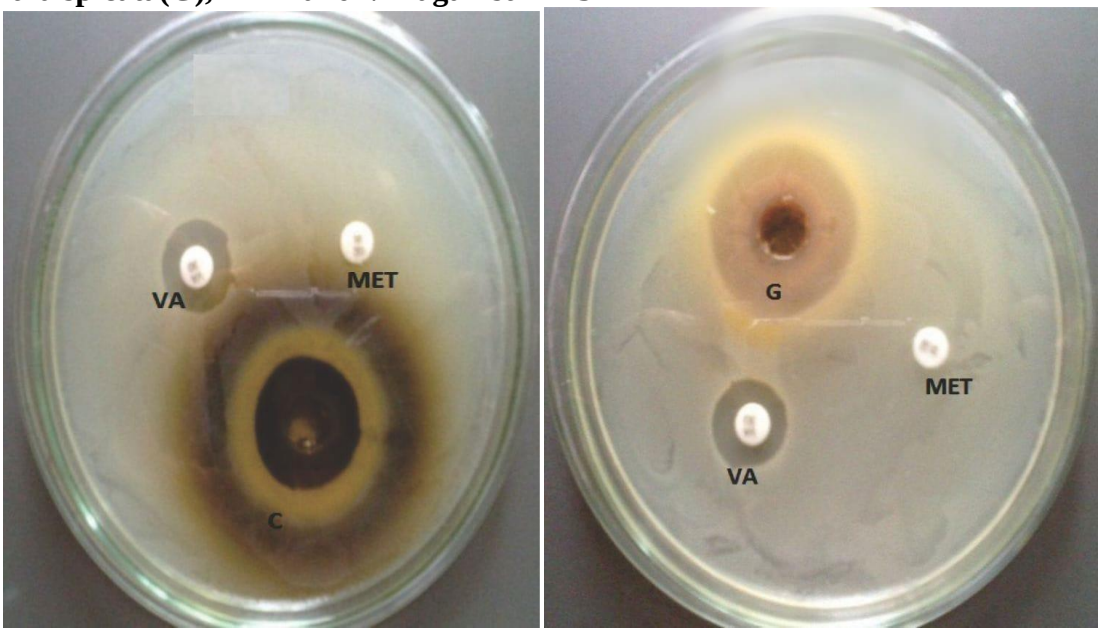
Table: 1 Zone of inhibition of aqueous extracts of medicinal plants and along with Methicillin and Vancomycin against VRSA:

S.NO	Name of plants	Aqueous extract (mg/ml)	Methicillin (mm)	Vancomycin (mm)
A	*Murraya koenigii*	18mm	0mm	6mm
B	*Eucalyptus globulus*	15 mm	0mm	6mm
c	*Dodonaea viscose*	14mm	0mm	6mm
D	*Mentha spicata*	12 mm	0mm	6mm

Slide 1: Murraya koenigii(C),MET and VA against MRSA Slide 2: Eucalyptus (D),MET and VA against MRSA



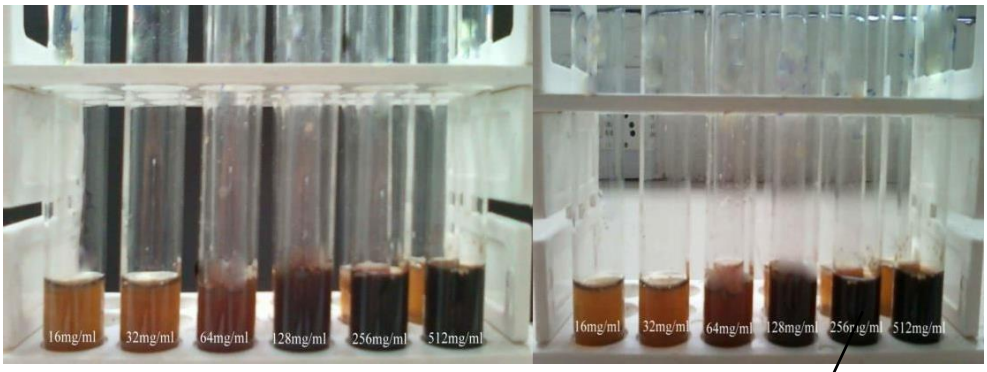
Slide 3: Dodonaea viscose (C), MET and VA against MRSA Slide 4: Mentha spicata(G), MET and VA against MRSA



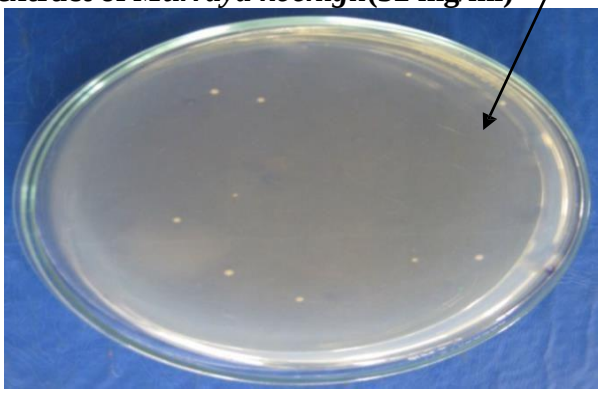
DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC is defined as the lowest concentration of extracts that completely inhibits the growth of the microorganism in 24 hrs (Thongson C, 2004). MIC of four medicinal plants of aqueous extractwere determined.*Murraya koenigii* (32mg/ml),*Eucalyptus globulus*(32mg/ml), *Dodonaea viscose* (64mg/ml) and*Mentha spicata*.(64mg/ml) as shown below.

Slide 5.26: Before incubation of 24hrs **Slide 5.26: After incubation of 24hrs**



Slide 5.27: MIC of aqueous extract of *Murraya koenigii*(32 mg/ml)



POLYHERBAL FORMULATION

Aqueous extract of *Murraya koenigii* (32mg/ml), *Eucalyptus globulus* (32mg/ml), *Dodonaea viscose* (64mg/ml) and *Mentha spicata*.(64mg/ml) is highly effective against recovered isolates. So polyherbal formulation is formulated by using aqueous extract of *Murraya koenigii* (32mg/ml), *Eucalyptus globulus* (32mg/ml),*Dodonaea viscose* (64mg/ml) and *Mentha spicata*.(64mg/ml) against multidrug resistant strain (MRSA).

Table 5.10: MIC of four medicinal plants

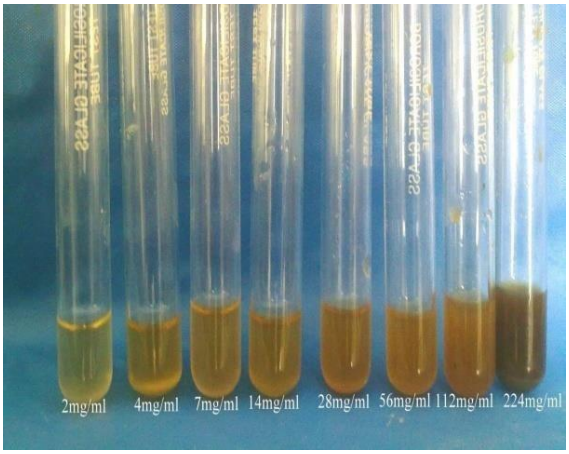
MIC of four medicinal plants		
Plants	**Extract**	**MIC**
Murraya koenigii	Aqueous	32 mg/ml
Eucalyptus globulus	Aqueous	32 mg/ml
Dodonaea viscose	Aqueous	64 mg/ml
Mentha spicata.	Aqueous	64 mg/ml

BY COMBINING THE ABOVE MIC OF FOUR MEDICINAL PLANTS WE FORMULATED THE DRUG AGAINST MRSA

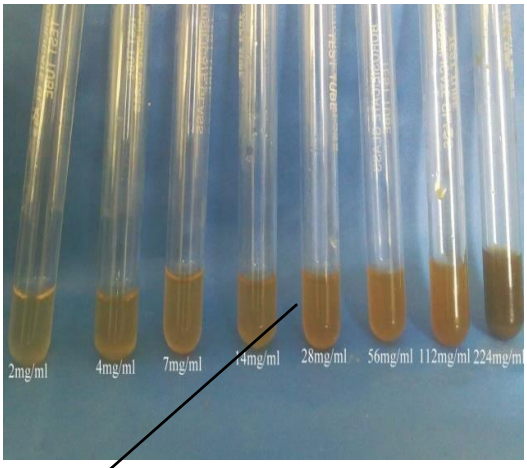
Table 5.9: By combining the MIC of four medicinal plants

32 mg + 32 mg + 64 mg + 64 mg =192mg

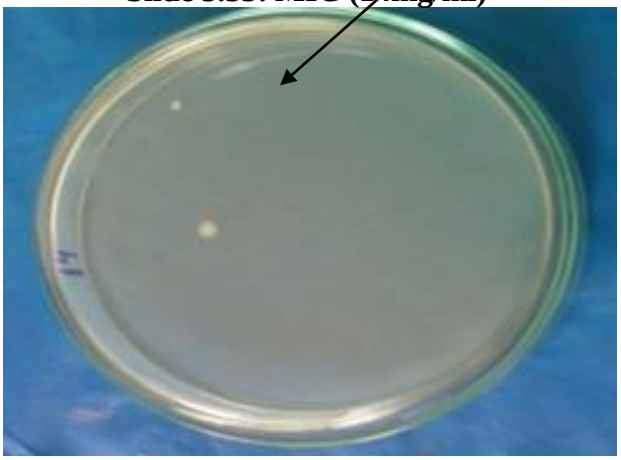
Slide 5.31: Before 24hrs of incubation



Slide 5.32: After 24hrs of incubation

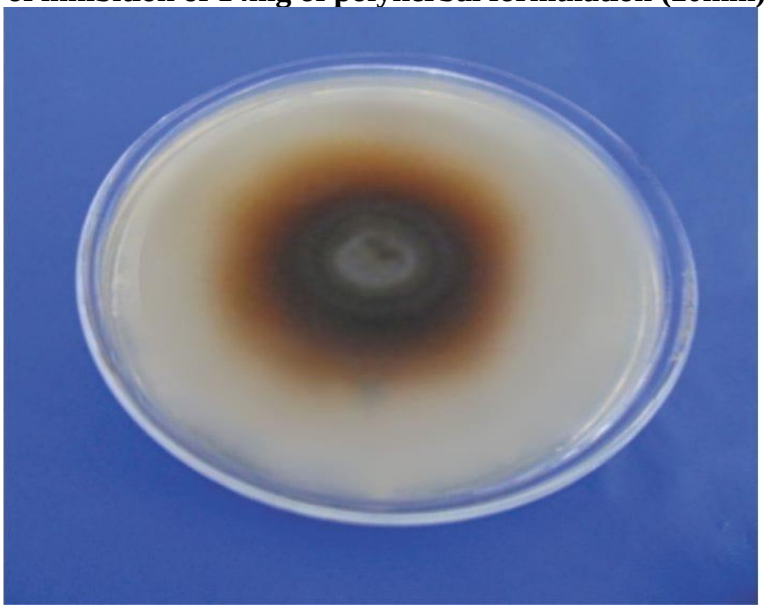


Slide 5.33: MIC (14mg/ml)



Slide 5.34: 14mg/ml

Slide 5.35: Zone of inhibition of 14mg of polyherbal formulation (20mm)



DISCUSSION

Methicillin resistant *Staphylococcus aureus* has become an enormous problem for health care providers because it is hard to treat and is sometimes called super bug.Multiple studies have been carried out on growing concern over multidrug resistance including India.MRSA is becoming a problem in paediatric population including hospital setting. The previous inclination of MRSA is in high intensity in the surgical and intensive care services, where antibiotic usage is the greatest. According to our study, there is high occurrence of MRSA in surgical wound infection, due to overcrowding, workload, and understaffing of wards. The MRSA could be prevented by identifying and screening MRSA carriers inside high-risk wards. A study from Eritrea revealed low MRSA (9%) prevalence[18] which is less than the prevalence observed in our study.The rate of prevalence ofMRSA isolates have increased over the years as reported by a study where they found 85.9% MRSA in 2003, decreasing to 57.8% in 2005 and again increasing to 90.8% in 2006[19].In our study the frequency of MRSA is 23% which is less than that reported from Karnataka (77.9%), Delhi (44%) and Uttar Pradesh (38.44%)[20-22].Frequency of MRSA in our study is comparable with another study done in Kashmir, where MRSA prevalence was 23.9%[23].

Regarding the carriage rate in relation to the age group, the prevalence of the MRSA carriage in our study was different than reported by other workers[24]. A much higher prevalence rate was seen in (52%) old persons, i.e. more than 55 years than (30%) those aged 35-55 years.

A total of 23 patients who developed MRSA infection/colonization were evaluated in our study. Out of which 9 were males (39.13%) and 14 were females (68.9%).A higher prevalence rate was seen in females than in the males which are similar to studies conducted by other workers[24].A majority of the MRSA isolates showed multiple drug resistance. A majority of the strains were resistant to Oxacillin, Ciprofloxacin, Kanamycin and Amikacin.Throughout the study, 23 nasal samples were collected from nearby Hospitals;

samples were transported to the laboratory within 30 minutes of collection and were screened for vancomycin resistance. The prevalence of VRSA shown in our study is 7.5%.A study from Kashmir revealed low VRSA (3.3%) prevalence[26] which is less than the prevalence observed in our study. In our study the frequency of VRSA is 7.5% which is less than that reported from West Bengal (26.67%) [27] and Kolkata (27.14%).

Water extract of our polyherbal formulation is effective against Methicillin resistant Staphylococcus aureus. 20 mm Zone of inhibition of our polyherbal formulation recorded against multiple drug resistant bacteria. Ethanolic extract of Moringa oleiferahave shown more zone of inhibition against MRSA (25mm) as compare to our study [28] . Melianthus comosus, Melianthus major, and Dodonaea viscose, shown good antibacterial activity against MRSA[29]by H. M. Heyman*etal* Pharmaceutical Biology, 2009; 47(1): 67–71.

CONCLUSION

Extracts of *Murraya koenigii*, *Eucalyptus globulus*, *Dodonaea viscose* and *Mentha spicata* were highly effective against the recovered isolates, purification and toxicological studies of theplant. It can be used as a potential sourcefor the development of a phytomedicine toact against multidrug resistant strain.MRSAare persistent and ever-growing problem for healthcare institutions. Minimizing the emergence of this organism and its spread remain to be the challenges that need to be addressed. The usual hygienic methods such as hand disinfection after each contact with patients, and the use of masks when is appropriate, must be performed by all workers in hospitals to protect the patients from nosocomial infections. Alcohol hand rub must be placed at every bedside in hospitals and promotional materials must be used to remind health workers and visitors to use the hand rub.

The antibacterial activities can be enhanced by making poly herbal formulation and adequate dosage determinedfor proper administration. As the globalscenario is now changing towards the use ofnontoxic plant products having traditionalmedicinal use, development of moderndrugs by using *Murraya koenigii*, *Eucalyptus globulus*, *Dodonaea viscose* and *Mentha spicata* should be emphasized for the control of infection caused by MDR bacteria.

In Morden time, beta lactam antibiotic becoming resistant day by day. To overcome burden of allopathic antibiotics poly herbal formulation can be a good alternate. Moreover, polyherbal formulation have no side effect like allopathic antibiotics. Our poly herbal formulation can be manufactured in the form of gel, cream, tablet and ointment to treat multiple drug resistant bacteria causing skin disease.

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