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SYNERGISTIC EFFECTS OF *OCIMUM SANCTUM* EXTRACT AND ANTIBIOTICS ON METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATED FROM CLINICAL SPECIMENS

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ABSTRACT

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a worldwide increasing problem and difficult to cure because MRSA are resistant against almost all the clinically available antibiotics, Synergistic effects of *Ocimum sanctum* extract and antibiotics on MRSA isolated clinically from swabs from pus, wound, nasal cavity, burn wound at Swaroop Rani Nehru Hospital, and Nazareth Hospital, Allahabad, were explored.

MRSA were screened for resistance against selected antibiotics such as Penicillin, Gentamicin, Cephalexin, Ciprofloxacin and Tetracycline by using disc diffusion method..Synergetic effect of acetone extract of *Ocimum sanctum* and antibiotics were evaluated Zone of inhibition were increased significantly with all antibiotics.

KEY WORDS: Methicillin Resistant *Staphylococcus aureus*, *Ocimum sanctum,* Penicillin, Gentamicin, Cephalexin, Ciprofloxacin and Tetracycline

INTRODUCTION

Staphylococcus aureus is the most common cause of nosocomial infection and is of increasing concern because of their tendency to multiple antibiotic resistance which often complicates treatment. Many isolates of *Staphylococcus aureus* have been found to be resistant to new semi synthetic β-lactams (methicillin, oxacillin and flucloxacillin) known as Methicillin Resistant *Staphylococcus aureus* (MRSA) (Bhat, 1990).

Staphylococcal infections give rise to a wide spectrum of symptoms and diseases in human. *Staphylococcus aureus* skin infections were classified as primary or secondary, primary infections are those occurring on apparently normal skin, and mainly comprised impetigo, ecthyma, folluculitis, furuncles, sycosis barbae, cellulitis, abscesses, paronychia and whitlows. Secondary infections are those arising in damaged skin (traumatized skin, or a pre existing skin disease) (Giudice, 2006).

Few studies have found that the efficacy of antimicrobial agent can be improved by combining them with crude plant extracts against different pathogens including *S. aureus, P. aeruginosa*, *E. coli,* extended spectrum lactamases-producing multidrug resistant *E. coli* and vancomycin-resistant *Enterococcus faecalis* (Adwan, 2008). *Ocimum sanctum* is commonly used in Ayurvedic medicines to treat different diseases such as respiratory tract infection, diarrhoea, headache, skin diseases and pneumonia. It is antiallergic antihelminthic, antipyretic, antibacterial and carminative in nature(Phadke and Kulkarni., 1989).

Drug synergism between known antibiotic and bioactive plant extracts is a novel concept and could be benificial (synergistic or addition interaction) or deleterious (antagonistic or toxic outcome) (Nascimento, 2000). Antimicrobial drugs effective for treatment of patients infected with MRSA are limited. Thus, it is important and

valuable to find compounds that potentiate antimicrobial activity of antibiotics.

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MATERIALS AND METHODS

Study samples

Clinical samples (n=100) consisting of pus, wounds, nasal swabs, burn and ear swabs etc were collected from Swaroop Rani Nehru Hospital Allahabad, Nazareth Hospital Allahabad. Sterile cotton wool swabs moistened with Stuart's Transport Medium were used to collect the specimens and applied to freshly prepared slant of nutrient agar and mannitol salt agar. The cultures were incubated at 37±1ºC for 24 hours (Kolawole, 1997).

Identification of the isolates

The identification of the isolates was done on the basis of morphological characterstics and biochemical tests as given in the Bergey's Manual of Systematic Bacteriology **(Holt *et al*., 1984).**

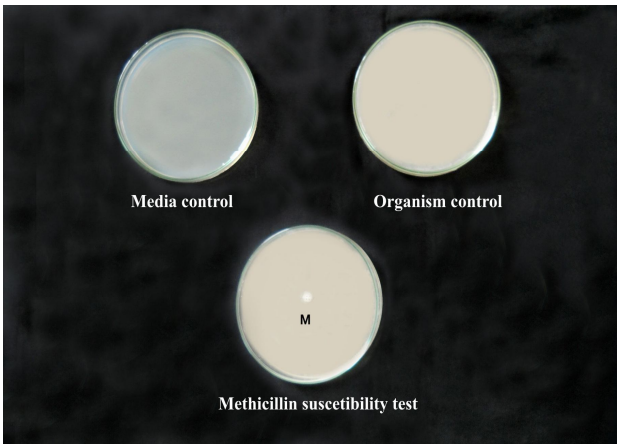
Cultural, morphological and biochemical characteristics of clinical isolates of *Staphylococccus aureus* .

S.no.	Characteristics	Observations
1.	**Cultural**	
a.	On Nutrient agar	colonies are large (2-4mm diameter), Circular, convex, smooth, shiny, opaque and golden yellow.
b.	On Mannitol salt agar	smooth, elevated and yellow pigmented colonies showing mannitol fermentation
c.	On Blood agar	β- haemolysis
2.	**Morphological**	gram positive, spherical cocci, arranged in grape-like clusters
3.	**Biochemical**	
(i)	*Sugar fermentation Test*	
a.	Glucose	A
b.	Sucrose	A
c.	Lactose	A
d.	Maltose	A
e.	Mannitol	A
(ii)	Catalase activity	+
(iii)	Coagulase activity	+
(iv)	Nitrate reduction	+
(v)	Acetoin production	+
(vi)	H_2S production on TSI agar	-
(vii)	Starch hydrolysis test	-
(viii)	Oxidase test	-
(ix)	Gelatin liqiefaction test	+
(x)	Methyl red reaction	+
(xi) a.	H/L oxidation (aerobic)	+
(xi) b.	H/L fermentation (anacrobic)	+

A= acid production, - = negative, + = positive.

Detection of Methicillin resistance

Staphylococcus aureus strains were tested for methicillin resistance using the disc diffusion method (Bauer *et al*., 1966). Nutrient agar plates were swabbed with overnight broth culture of *Staphylococcus aureus* and Methicillin (5µg) disc obtained from Hi-media, Mumbai were impregnated. These plates were incubated at 37±1ºC for 48h. Zones of inhibition were determined recorded in millimeters and interpreted in accordance with the CLSI standards (CLSI, 2009). *Staphylococcus aureus* isolates are considered to be resistant if inhibition zones are <14 mm. The resistant strains were further used for other evaluations. The isolates obtained were periodically sub cultured in nutrient agar plates and stored under refrigerated conditions (4°C).



Plant Materials

The plant materials used in the study consisted of *Ocimum sanctum* (Leaves) which is growing in Allahabad.

Preparation of *Ocimum sanctum* (Leaves) Extract

Ocimum sanctum (leaves) were dried in an open air protected from direct exposure to sun light, and 50 gm dried plant material was separately powdered, extracted by adding 200ml acetone and then was kept over night in a rotatory shaker. Then extract was filtered through whatman no.2 filter paper and there after it was kept in DMSO (Di methyl sulphoxide) solution and it was air dried.

Preparation of *Ocimum sanctum* (Leaves) extract discs

Circular filter paper discs (6 mm diameter) were prepared with the aid of an office paper perforator. The discs were placed in a Petri dish and sterilized in an autoclave. Dilutions of plant extract were made in test-tubes using sterile water. The paper discs were then aseptically transferred into the tubes containing the plant extract solution and allowed to absorb the solution for about 15 seconds. The discs were then aseptically transferred to empty sterile test tubes and allowed to air dry while the mouth of the test tubes is still plugged with cotton wool. Each of the test tubes containing the dried discs was labeled with the strength of the solution of plant extract in which the paper discs were dipped. (Esimone *et al*., 2006).

Antibacterial activity tests of *Ocimum sanctum* extract

Petri plates containing approximately 25-30 ml of Nutrient agar medium were inoculated using a cotton swab with a 4-6 hour old culture of bacterial isolates (Bauer *et al*., 1996). Discs containing *Ocimum sanctum* plant extracts soaked in solution of 100 µg/ml (equivalent to 1000 µg/disc) were aseptically placed on the seeded nutrient agar plates. Replicate of each plate were prepared. After a 30 minutes pre-diffusion time interval, the plates were incubated at 37°C for 24 hrs. There after the diameters of zones of inhibition surrounding the discs were accurately measured and their relative susceptibility pattern was deduced (Esimone *et al*., 2006).

Antibiotic sensitivity pattern of isolates

Five antimicrobial agents were evaluated for the sensitivity pattern of isolates. These are as follows-

Penicillin (10 U)
Gentamicin (30 µg)
Cephalexin (30 µg)
Ciprofloxacin (30 µg)
Tetracycline (30 µg)

All these antimicrobial agents were produced by Hi-media laboratories; India was used as per CLSI recommendation. All the above antibiotics were aseptically placed on seeded nutrient agar plates. These procedures were performed in 2 replicate plates for each antibiotic. Thereafter all the plates were incubated at 37°C for 24 hrs. Thereafter, the diameters of zones of inhibition surrounding the discs were accurately measured and their relative susceptibility pattern was deduced.

Synergistic activity determination of antibiotic and *Ocimum sanctum* extract

Synergistic activity was determined by stacked disc technique. One disc containing antibiotic and another with plant extract concentration showing maximum antibacterial activity were stacked one above the other and impregnated on *Staphylococcus aureus* plates. The plates were incubated at 37°C for 24-48 hours. The antibacterial activity were assessed by measuring the inhibition zone (diameter in mm) around each disc. The average of three replicates for each extract, antibiotic and combination were calculated

Statistical analysis

The data recorded during the course of investigation was subjected to Chi-square analysis and the conclusions were drawn accordingly. The data recorded during the course of investigation was statistically analyzed by using χ^2 test (chi square test) and the interpretation was done accordingly. The calculated value χ^2 test was compared with the tabulated value at 5% probability level of significance for appropriate degree of freedom

The formula used for χ^2 test is :

$$\chi^2 = \frac{(O_{ij} - E_{ij})^2}{Eij}$$

RESULTS

The different clinical samples such as swabs of burn wound, ear, nasal, pus and wound studied for the presence of *Staphylococcus aureus* showed significant incidence. The higher incidence of *Staphylococcus aureus* was observed in Burn wound (26%) followed by Nasal cavity (23.33%), wounds (15%), pus (12%) and least in ear canal (10%). When these isolates were tested for susceptibility towards methicillin higher incidence of Methicillin resistance was recorded in the isolates obtained from Burn wound (50%) followed by nasal cavity (42.86%), pus and wound swabs (33.33%). However, none of the *Staphylococcus aureus*

isolates obtained from ear canal were found to be positive for MRSA (Table 1, Fig 1).

Table 1: Incidence of methicillin resistant *Staphylocuccus aureus* (MRSA) in different clinical samples.

S. No.	Clinical samples	No. of samples	No. of positive samples	MRSA (%)
1.	Burn wound	15	4 (26%)	2 (50%)
2.	Ear swab	10	1 (10%)	0 (0.00%)
3.	Nasal swab	30	7 (23.33%)	3 (42.86%)
4.	Pus	25	3 (12%)	1 (33.33%)
5.	Wound	20	3(15%)	1 (33.33%)
Total		**100**	**18(18%)**	**7 (38.89%)**

χ^2 (Cal) = 1.735 ; χ^2 (Cal) < χ^2(5%) (tab 9.4) (NS) χ^2 (Cal) = 0.529 ; χ^2 (Cal) < χ^2(5%) (tab 9.4) (NS)

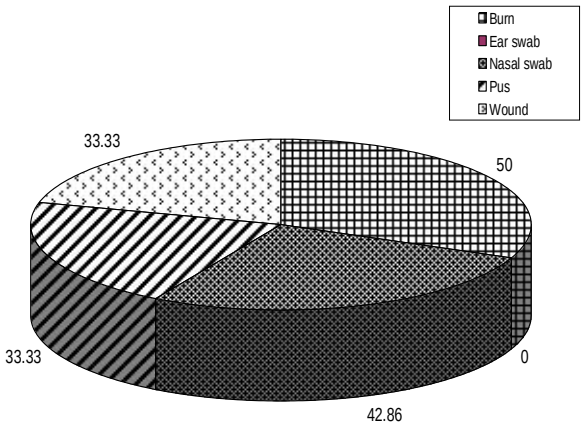


Fig. 1 : Incidence of methicillin resistant *S. aureus* in different clinical samples.

The methicillin resistant *Staphylococcus aureus* (MRSA) isolates from different clinical samples when subjected to antibiotic sensitivity testing showed higher degree of resistance towards the antibiotics tested (Table 2). All the isolates were found to be resistant towards Penicillin (100%). Greater degree of resistance was also observed against Cephalexin (85.7%), Ciprofloxacin and Tetracycline (71.42%) and 42.85% isolates showed resistance towards Gentamicin (Fig 2).

Table 2. Antibiotic resistant profile of methicillin resistant *Staphylococcus aureus* (MRSA) isolates

S. No.	Antibiotics	Disc concentration	Susceptibility	
			Resistant	Sensitive
1	Gentamicin	30 µg	4 (57.14%)	**3(42.85 %)**
2	Penicillin	10 U	7(100%)	**0.00**
3	Tetracycline	30 µg	5(71.42%)	**2 (28.57%)**
4	Ciproflox acin	30 µg	5 (71.42%)	**2(28.57 %)**
5	**Cephalexin**	**30 µg**	**6 (85.7%)**	**1(14.28 %)**

χ^2 (Cal) = 4.213 ; χ^2 (Cal) < χ^2(5%) (tab 9.4) (NS)

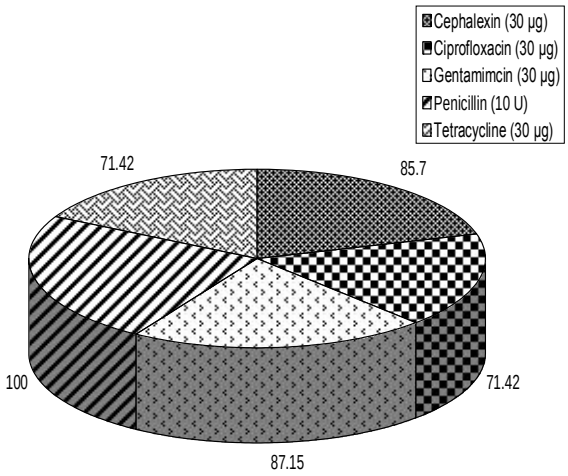


Fig. 2: Antibiotic resistant pattern of methicillin resistant *Staphylococcus aureus* (MRSA) isolates

Synergistic activity of *Ocimum sanctum* leaf extract and antibiotics against methicillin resistant *Staphylococcus aureus* (MRSA) isolates

Concentration dependent inhibition of the Methicillin Resistant *Staphylococcus aureus* isolates was observed with *Ocimum sanctum* extract. At 62.5 µg/ml concentration, the MRSA isolates were inhibited however from 125 µg/ml towards 1000 µg/ml increasing trend in the inhibitory activity was observed (Fig. 5). In Table. 3 Synergistic activity determination of *Ocimum sanctum* leaf extract and antibiotics on methicillin resistant *Staphylococcus aureus* strain isolated from clinical specimen

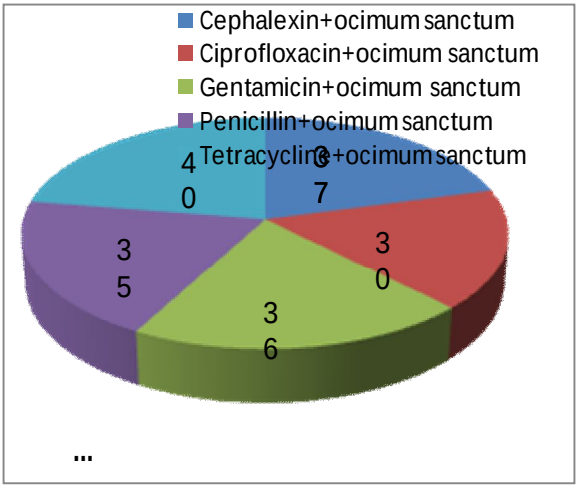


Fig-3

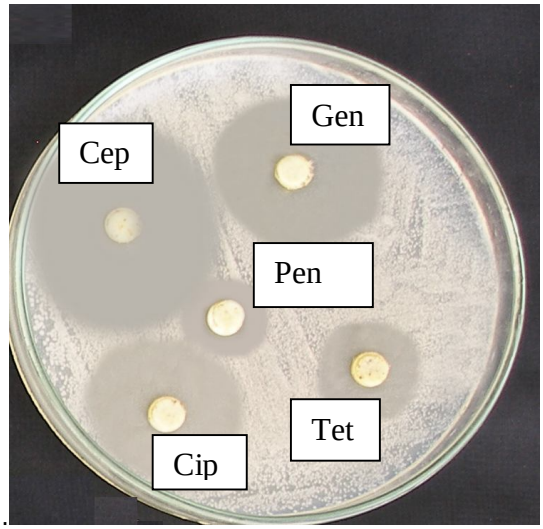


Fig.4 antibacterial activity of antibiotics.

Table-3

S. No	Antibiotics	Zone of inhibition (mm)		
		Antibiotic alone	*Ocimum sanctum* leaf extract alone	*Ocimum sanctum* leaf extract + Antibiotics
1	Cephalexin (30 µg)	25	5 (62.5µg/ml)	37
2	Ciprofloxacin (30 µg)	17	10 (125µg/ml)	30
3	Gentamicin (30 µg)	26	15 (250µg/ml)	36
4	Penicillin (10 IU)	14	20 (500µg/ml)	35
5	Tetracycline (30 µg)	20	25 (1000µg/ml)	40

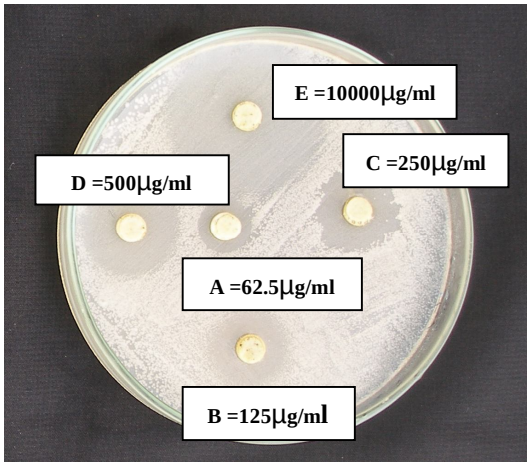


Fig.5 antibacterial activity of *Ocimum sanctum* extract.

Comparison to activity of antibiotics alone, the *Ocimum sanctum* extract showed lower inhibition with the exception observed against Tetracyline. Considerable l increase in the inhibitory activity of of *Ocimum sanctum* extract and antibiotics against the MRSA isolates was recorded (Fig 6). Synergistic activity determination of *Ocimum sanctum* leaf extract and antibiotics on methicillin resistant *Staphylococcus aureus* strain plate.

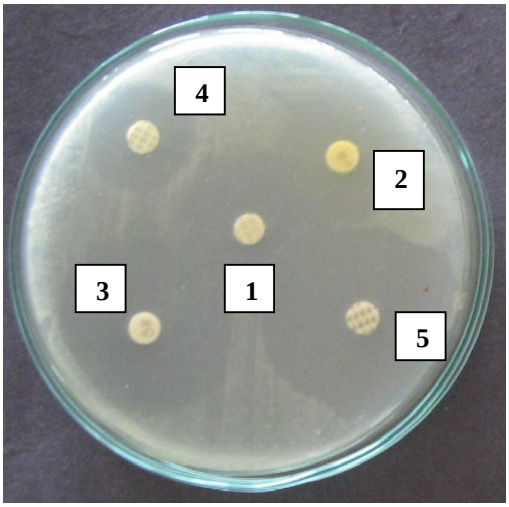


Fig. 6 Synergistic effects of *Ocimum sanctum* extract and antibiotics.

DISCUSSION

The different clinical samples from burn wound, ear nasal and wound were studied for the presence of S*taphylococcus aureus* showed noticeable incidence. The higher incidence of *S. aureus* was observed in burn wound (26%) followed by Nasal cavity (23.33%) wounds (15%) pus (12%) and least in ear canal (10%). The data when analyzed statistically was found to be non-significant (P>0.05) Similar findings were also reported by either workers where burn samples were found to show higher prevalence of *S. aureus* infection as compared to other samples (Cafferky et al, 1983; Aravind et al, 2000, Kaushik *et al*, 2001, Samy, 2003; Macedo and Santos, 2005, Orrett and Land. 2006) In the other studies (Sundarrajan and Kale, 1984; Tahnkiwale et al, 2002; shittu *et al*, 2006 and Mulla *et al*; 2007), recovery rate of *S. aureus* was reported to be more from pus and wound Samples in the absence of burn samples and nasal swabs.

As suggested by Macedo and Sontos (2005), Susceptibility of burn wound to such colonization by bacteria results from several factors including the presence of coagulated proteins, the absence of blood borne immune factors and the avascularity of the burn wound. According to them burns provide a suitable site for bacterial multiplication and infection, mainly because of the larger area involved and longer duration of patient stay in hospital. In present study. We isolated 7 MRSA and 11 MSSA. among 18 *Staphylococcus aureus* strains from 100 clinical specimens obtained from various patients. The prevalence of MRSA was 38.89% the data when analyzed statistically was found to be Non-significant (P>0.05) .

The higher prevalence rate of MRSA was recorded by R.K. Sanjana *et al*. 2010 (39.6%) Nishi V *et al*; 2004 (35%) Mohanty S. *et al* 2008 (38.5%) Rajadurai pandi K *et al* 2006 (31.1%), Merlino *et al* 200 (34%) Pantazatou *et al*, 2003, (33.3%), where as a lesser prevalence rate was recorded by Chakravarthy *et al*, 1988 (69%) Pal N *et al*. 1990 (22.8%) Pulimood *et al*. 1993 (2.4%) Mehta A A *et. al*, 1996 (26.6%), Orrett FA *et al*, 1999 (9.8%), Naimi TS *et al*. 2004 (12%) and Adebayoo Shittu et al. 2006 (26.9%). A higher prevalence rate was obtained by some other workers in their study. Majumdar D *et al*. 2001(52.9%), Anupurba S. *et al* 2003 (54.8%), Dar Ja *et al*. 2006 (54.85%); and Bor M *et al*. 2006 (65%).Further reported that infection of MRSA is more compounded in the burn patients as they are severely immune compromised and receive numerous antibiotics. Morever care of these patients is often very labour intensive requiring many hours of hands on contact.

From the study conducted by steer et al (1995) the authors concluded that staphylococci can survive intracelluraly in Ploymorphonuclear leucocytes (PMNs). However in burn patients PMNs, bactericidal function is decreased allowing the organism to survive longer. The drug resistance patterns of MRSA isolated from clinical specimens were found to be highly variable. The methicillin resistant *Staphylococcus aureus* (MRSA) isolates from different clinical samples when subjected to antibiotic sensitivity testing showed higher degree of resistance towards the antibiotics tested . All the isolates were found to be resistant towards penicillin (100%) similar finding were also reported by other workers which include Gerald NKwelang *et al*. 2009 (100%) R.K. Sanjana *et al,* 2010 (100%), Iyad Naeem M., 2006 (100%), Ahamd S. 2010 (100%) Olowe O.A. *et al*.2007 (87.5%) Kumari N. 2008 (100%) Anupurba *et al* 2003 (100%) Dr. Bandaru Narasinga Rao. *et al*., 2010 (100%) Greater degree of resistance was also observed against cephalexin (85.7%) ciprofloxacin and Tetracycline (71.42%) and 42.85% isolates showed resistance towards gentamicin Similar obsrvations have been observed for Cephalexin Bandaru Narasinga Rao, et al. 2010 (85.80%), Muhammad Arafat Yameen *et al*., 2010 (90%) R.K. Sanjana, 2010 (81.81%).

Gentamicin resistance in MRSA is world wide. Mechanism of resistance is durg inactivation by cellular transferase enzyme.Ahmad *et al*. 2010 reported 100% resistance to Gentamicin. Olowe *et al.* 2007 (62.5%), Kumari et.al.2008 (68.37%), Dr. Bandaru Narasinga Rao *et al*., 2010 (85.80%) Garald NKwelang et. al., 2009 (83.5%).Muhammad Arfat Yameen, 2010 (34%) R.K. Sanjana *et al*. 2010 (38.09%) Iyad Naim, 2006 (92.80%), Dainio A*. et al.,* 2009) reported 32% resistance to Gentamicin. Kumari N, *et. al,* 2008 reported 67.35% resistance towards ceprofloxacin, Dr. Bandaru Narasinga Rao et al, 2010 reported 88.27% resistance to ciprofloxacin, Pulimood TB *et al*., (1988 reported 90% resistance and Majumdar D *et. al* (2001) reported 22.8% resistance and 12.8% by Rajadurai Pandi k *et. al* 2006. Dr. Bandaru Narasinga Rao *et. al*. 2010 reported 68.51% resistance to Tetracycline Ahmad S *et*. *al* (2010), reported 89.0% resistance to Tetracycline Kumari N. *et al*. 2008 reported 62.24%) resistance to tetracycline, however lower resistance was reported by KR Rijal *et al*. 2008 (15.6%) Muhammed Arfat Yameen et. al; 2010 (49%). All

the above studies showed that the MRSA isolates are often resistant to multiple antibiotics. Therefore treatment of infections due to this organism and its eradication is difficult and also use of beta lactam antibiotics in MRSA infections will increase antibiotic selection pressure. The variation in the antibiotic sensitivity pattern of isolated organisms may be due to several factors like difference in PH, nature and time of incubation and composition and nature of culture media, size of inoculum, source of isolated organism and perhaps in strain activity (Bhat et al. 1990).

As in the present study various workers have reported antibacterial properties of *Ocimum-sanctum*. (Ahmad et. al., 1988, Ahmad and Beig, 2001, Aqil et al. 2004).The phytochmical analysis of the plant revealed the antibacterial properties to be due to glycosides, Phenols and tannins (Ahmad and Beig, 2001).

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