Antibacterial Effects of Tulsi (*Ocimum Sanctum*) on Isolated Resistant Bacteria *Staphylococcus Aureus* from Infected Cow

Md. Shimul Hosen[1], Misrat Masuma Parvez[1], Rakibul Islam[1], Md. Bazlar Rashid[1], Md. Mahmudul Hasan[1] and Md. Gausur Rahman[2*]

[1]Department of Physiology and Pharmacology, Hajee Mohammad Danesh Science and Technology University, Dinajpur 5200, Bangladesh

[2*]Department of Pathology and Parasitology, Hajee Mohammad Danesh Science and Technology University, Dinajpur 5200, Bangladesh

***Corresponding Author**

DOI: https://doi.org/10.51584/IJRIAS.2024.908021

Received: 11 July 2024; Revised: 24 July 2024; Accepted: 29 July 2024; Published: 04 September 2024

ABSTRACT

The current research work was carried out to screen and evaluate antimicrobial activity of leaf extracts of *Ocimum sanctum* (common name: Tulsi) on Multidrug resistant *Staphylococcus aureus* organism. Tulsi materials which were used during this study were collected from local garden whereas the bacterial strains were isolated from infected cow. Fresh leaves were processed and extracted by absolute ethanol and methanol before testing. Besides, antibiogram study performed and Minimum Inhibitory Concentration (MIC) was determined by diffusion method. Disk diffusion methods were used to determine zone diameter of inhibition (ZDI). 'Four S. aureus presented resistance to these antibiotics: oxytetracycline, penicillin, streptomycin, amoxicillin, and erythromycin. These isolates were susceptible to tulsi extracted pellet'. The results showed that the effectiveness of the extracts was dependent of the concentration used thus the increase of extract concentration increased the inhibition zone against multidrug-resistant S. aureus (MDRSA) as antimicrobial agents against MDRSA and can be used to explore novel antibacterial compounds against MDRSA. However, Maximum ZDI (17mm) value was observed for methanol extracts 0.8g/ml against MDRSA and minimum ZDI (7mm) value was observed for methanol extracts 0.4g/ml against MDRSA and no zone was found 0.2g/ml of methanol etract of tulsi. From the current research, it can be concluded that the selected *O. sanctum* have great potential as antimicrobial agents against MDRSA *Staphylococcus aureus* and can be used to explore novel antibacterial compounds against MDRSA.

Keywords: Tulsi extract, Staphylococcus aureus, Antimicrobial susceptibility, Multi-drug resistant S. aureus.

INTRODUCTION

The Mammary Microenvironment in Mastitis in Humans, Dairy Ruminants, Rabbits and Rodents: A One Health Focus. J Mammary Gland Biol Neoplasia (Hughes K, Watson CJ, 2018). Staphylococcus aureus Exotoxins and Their Detection in the Dairy Industry and Mastitis. Toxins (Basel) (G Abril A *et al.*,2020). 'Wide varieties of bacteria, both Gram-positive and Gram negative, aerobes and anaerobes, have been isolated from bovine uterus post-partum. The bacterial metabolites and uterine inflammatory exudates produced from endometritic uterus alter the normal uterine environment required for fertilization, conception and birth of a live progeny. Misuse of antibiotic is now common mater. For this reason, antimicrobial resistance occur. Antibiotic resistance is the ability of microorganism to withstand the effects of an antibiotic. The emerging of antibiotic-resistant pathogens has become a major global threat but no new antibiotic is available. In this scenario, scrutinizing for some alternatives yet effective antibacterial therapeutics like herbs, nutritional immunomodulators, bacteriophages, avian egged antibodies and others have become need of the day. Herbs

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

being a valuable source of medication due to their important microbial principles, easy availability, cost effectiveness and wider therapeutic potentials may offer a potent alternative to antibiotic and hormonal therapy (Jadav and Butani, 2005). In modern complementary and alternative medical practice, plants are the primary source of therapeutics and each part of the plant, including the seeds, root, stem, leaves, and fruit, potentially contains bioactive components (Jiang *et al.,* 2014, 2015; Sun *et al.,* 2014). Medicinal plant extracts have phytochemicals with antifungal and antibacterial properties (Kumar *et al.,* 2017). Among the medicinal plants, aromatic herbs are a rich source of biologically active compounds useful both in agriculture and medicine (Mathela, 1991; Cutler and Cutler, 1999). Of these, *Ocimum tenuiflorum*, also known as Tulsi, or Holy Basil from the family Lamiaceae has been described as the "Queen of plants" and the "mother medicine of nature" due to its perceived medicinal qualities (Singh *et al.,* 2010). It has been one of the most valued and holistic herbs used over years in traditional medicine in India and almost every part of the plant has been found to possess therapeutic properties (Singh *et al.,* 2010). Traditionally, Tulsi is used in different forms; aqueous extracts from the leaves (fresh or dried as powder) are used in herbal teas or mixed with other herbs or honey to enhance the medicinal value. Traditional uses of Tulsi aqueous extracts include the treatment of different types of poisoning, stomach-ache, common colds, headaches, malaria, inflammation, and heart disease (Pattanayak *et al.,* 2010).

The Objectives of this study were:

1. To isolate and identify antibiotic resistant Staphylococcus aureus from mastitic cow

2. To assess the antibacterial activity of Tulsi leaf extracts against antibiotic resistant S. aureus'.

MATERIALS AND METHODS

Study area

This study was carried out at AG Agro lab in Gazipur during July 2021 to December 2021 under the department of Physiology and Pharmacology, HSTU, Dinajpur.

Collection and preparation of tulsi leaf extracts

The fresh tulsi leaves were collected directly from the plants, at HSTU campus area.

About 250 ml of methanol was added in a separate conical flask with 50 g of each leaves powder to make a stock solution of 0.2 g/ml concentration using a cold maceration extraction process. Afterward, working solution of each extract at concentrations such as 0.2 g/ml, 0.4 g/ml, 0.6 g/ml, and 0.8 g/ml was prepared on the basis of the formula

Collection of sample and identification of bacteria

A total of 42 milk samples were collected from mastitis positive cow. The positive samples were subjected to bacteriological examination for isolation, identification and characterization of bacteria. Bacterial identification was carried out by conventional cultural and biochemical methods according to standard microbiological techniques in Microbiology laboratory, HSTU.

Detection of bacterial susceptibility of S. aureus to tulsi leaf extracts

'Antibacterial activity of tulsi leaf extracts against of S aureus isolates was determined by disc diffusion method in nutrient agar medium. Antibacterial activities of tulsi leaf extracts were tested following agar well diffusion method in Muller Hinton agar. Whatman No. 1 filter paper discs of 6 mm diameter were made with the help of a punching machine and these discs were sterilized by hot airoven (Andrews, 2001) and impregnated discs were placed on the media, The wells were filled individually with 0.2g/ml, 0.4g/ml, 0.6g/ml and 0.8g/ml concentration using a micropipette. The plates were then kept for 1 hour at room temperature to allow diffusion of the extract into the medium and then incubated aerobically at 37°C for overnight. The solvent was checked for its antibacterial activity.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

Determination of minimum inhibitory concentration (MIC) on the test organisms

After incubation, the diameters of the zone of inhibitions were measured by using transparent ruler and the mean values of three readings were recorded. Diameters of zone of inhibition were measured in millimeter. Minimum Inhibitory Concentration (MIC) was determined by similar well diffusion method in Muller Hinton agar.

RESULTS

The present research was carried out to the isolation, identification and characterization of multidrug resistance *Staphylococcus aureus* The study also presents antibacterial effects of tulsi leaf extracts on obtained bacteria. The results are presented bellow in detail:

Table 3: General culture media and colony characters

Name of the media	**Colony character**
Nutrient broth	Uniform turbidity
Nutrient agar	Circular, small, smooth, convex, and yellowish colonies.
MacConkey agar	Pink color colony
Manitol salt agar base	Golden yellow manitol fermenting colony.
Staphylococcus agar media 110	Pale opaque in color colonies and sometimes Golden yellowish colonies
Blood agar media	Smooth, shiny, round and convex colony with hemolysis. Complete hemolytic zone (β hemolysis) in sheep and ox blood agar. Sometimes partial hemolysis appeared

Nutrient broth inoculated with the sample revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and was indicated by the presence of uniform turbidity. (Plate 1). Nutrient agar plates streaked separately with the sample revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically on nutrient agar the organism were produced circular, small, smooth, convex, and yellowish colonies (Plate 2). On MacConkey agar produced pink color colony. Manitol salt base agar plates were streaked separately with the sample revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically the organism *Staphylococcus aureus* produced golden yellow manitol fermentation colony with yellowish media (Plate 3). On the other hand, On *Staphylococcus* agar media 110 produced Pale opaque in color colonies and sometimes Golden yellowish colonies (Plate 4). In blood agar media the organism *Staphylococcus aureus* produced smooth, shiny, round and convex colony with β-hemolysis (Plate 5).

Table 4: Isolation of *Staphylococcus* sp. by morphological and different biochemical tests

Test performed	**Observation**	**Response**				**Indication**
		Positive isolates	**% of positive isolates**	**Negative isolates**	**% of negative isolates**	
Microscopic examination by grams staining	Showing gram positive, cocci shape, grape like cluster	38	90.47	4	9.53	*Staphylococcus sp*
TSI agar slant	Acid production becomes	38	90.47	4	9.53	*Staphylococ*

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reaction	uniformly yellow color					*cus sp*
Motility test by MIU medium	Absence of turbidity	38	90.47	4	9.53	*Staphylococcus sp*
Indole test	No pink color ring at the adjacent	38	90.47	4	9.53	*Staphylococcus sp*
MR test	Red color indicate MR test positive	38	90.47	4	9.53	*Staphylococcus sp*
VP test	no color change indicates VP test negative	38	90.47	4	9.53	*Staphylococcus sp*
Manitol salt fermentation	The medium color becomes yellow	38	90.47	4	9.53	*Staphylococcus aureus*
Catalase test	Presence of bubbles	38	0	4	9.53	*Staphylococcus aureus*

(Legends: S=Slant, B=Butt, SC = Simmon's Citrate Test, IT = Indole test, TSI = Triple sugar iron test, MR = Methyl-Red test, VP = Voges-Proskauer test, C= Catalase Test**,** + = Positive reaction, — = Negative reaction).

Results of antibiotic sensitivity pattern of isolated bacteria

Out of 38 positive isolates, four isolates of *Staphylococcus aureus* were selected randomly for antibiotic sensitivity and resistance pattern against commonly used antibiotics. The result of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistance (-), less sensitive (+), moderate sensitive (++), and highly sensitive (+++). The results of antibiotic sensitivity are given in Table 5 and Plate 6-8.

Table 5: Results of antibiotic sensitivity test of the isolated bacteria

Antibiotics (µg)	**Resistant (mm)**	**Intermediate (mm)**	**Sensitive (mm)**	**Inhibition zone of identified isolates**
Ciprofloxacin (CIP)	≤15	16-20	≥21	32 mm (+++)
Penicillin (P)	≤20	-	≥24	0 mm (-)
Amoxicillin (AMX)	≤13	14-17	≥18	0 mm (-)
Doxycycline (DO)	≤28	-	≥29	20 mm (-)
Gentamicin (GEN)	≤12	13-14	≥15	13mm (++)
Erythromycin (E)	≤13	14-22	≥23	0 mm (-)
Cefalexin (CN)	≤15	13-15	≥19	19 mm (+++)
oxytetracyclin (o)	≤14	15-18	≥19	25 mm (+++)
Streptomycin (S)	≤11	12-14	≥15	13 mm (++)

Here: µg= micro gram, +++ = Highly sensitive, ++ = Intermediate sensitive, - = Resistance

The antibiogram of various isolates of *Staphylococcus aureus* were found to be highly sensitive (+++) to Cipro floxacillin (5), oxytetracycline (30), Cefalexin (30), and Intermediate sensitive (++) to Streptomycin (10),

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Gentamycin (10). Resistance to Erythromycin (15), Penicillin (10) and Amoxicillin (30) From the table tested organism were MDR.

Plate 1: Nutrient broth (Right last one is control)

Plate 2: Nutrient agar with bacterial colony (Right). Left one is control

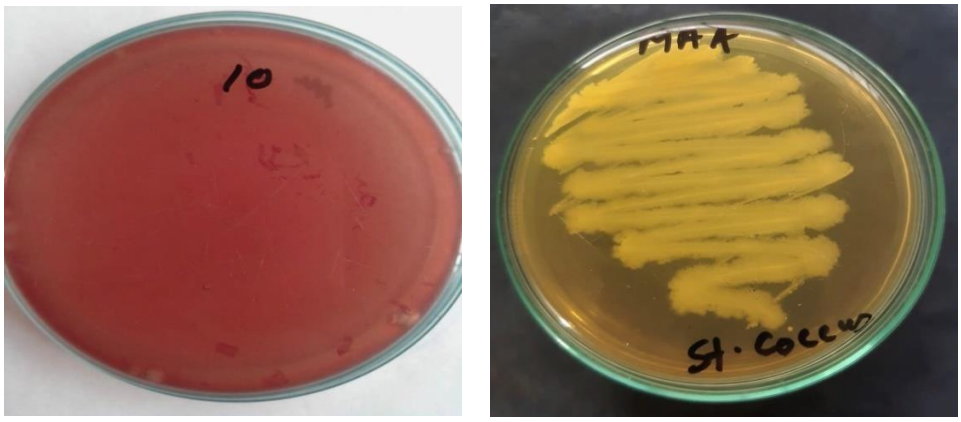

Plate 3: Manitol salt agar base with bacterial colony (Right). Left one is control

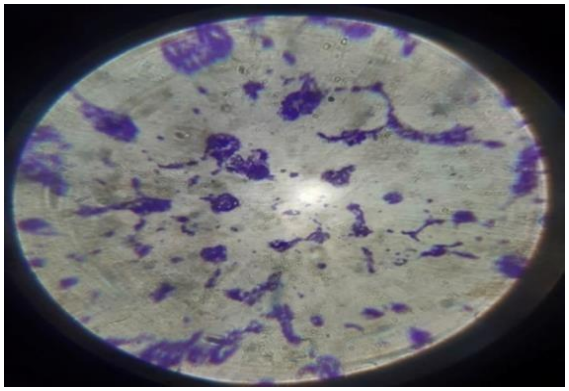

Plate 4: Microscopic view of gram positive *Staphylococcus aureus* showing cocci shaped, grape like cluster.

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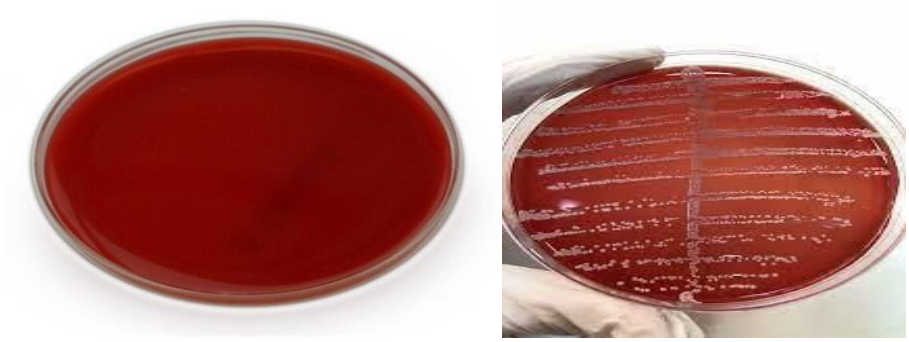

Plate 5: Bacteria produced smooth, shinny, round colony with hemolysis on blood agar media (Right). Left one is control

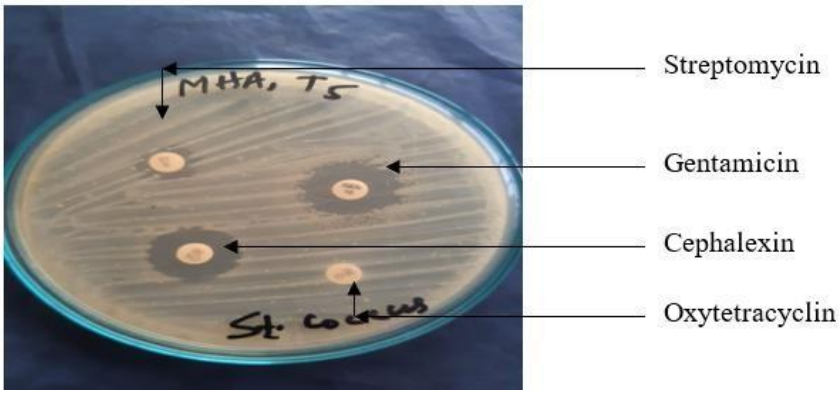

Plate 6: Antibiotic sensitivity test for *Staphylococcus aureus*

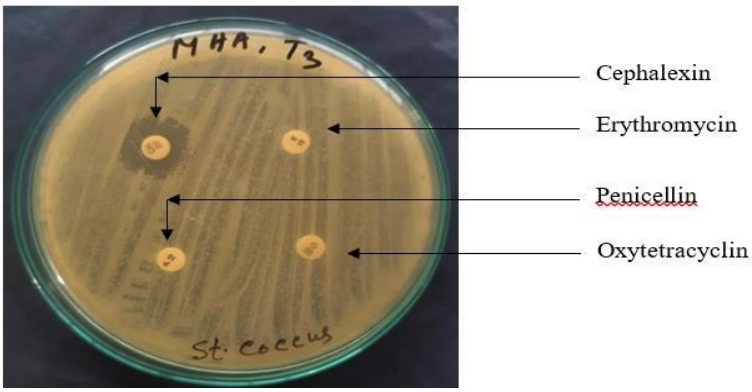

Plate 7: Antibiotic sensitivity test for *Staphylococcus aureus*

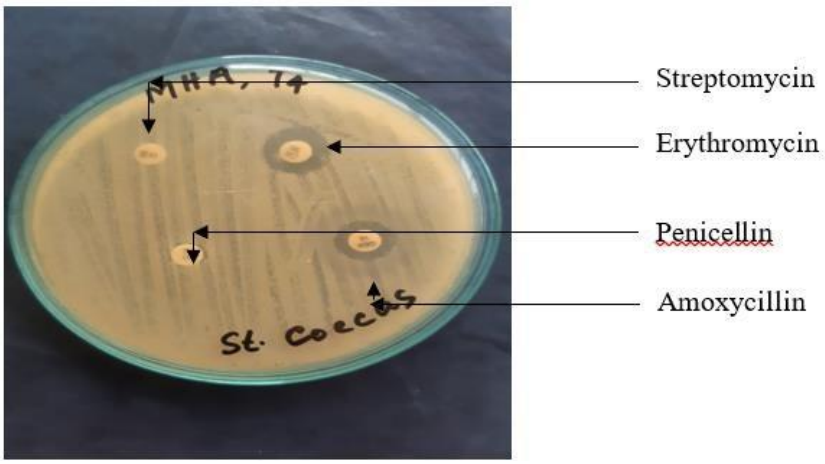

Plate 8: Antibiotic sensitivity test for *Staphylococcus aureus*

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Results of antibacterial effects of tulsi leaf extracts at different concentrations against *Staphylococcus aureus*

The result of sensitivity of tulsi leaf extracts against *Staphylococcus aureus* were measured Zone of Diameter of Inhibition (mm). The results of antibiotic sensitivity are given in Table 6 and Plate 13-15).

Table 6: Tulsi methanolic leaf extracts at various concentrations (0.2 g/ml0.4g/ml, 0.6g/ml, 0.8g/ml) their particular ZOI for *S. aureus*

Concentration (Tulsi) (g/ml)	**ZOI (*S. aureus*) (mm)**
0.2	0 mm
0.4	7mm
0.6	13mm
0.8	17mm

S. aureus: Staphylococcus aureus, ZOI: Zone of inhibition

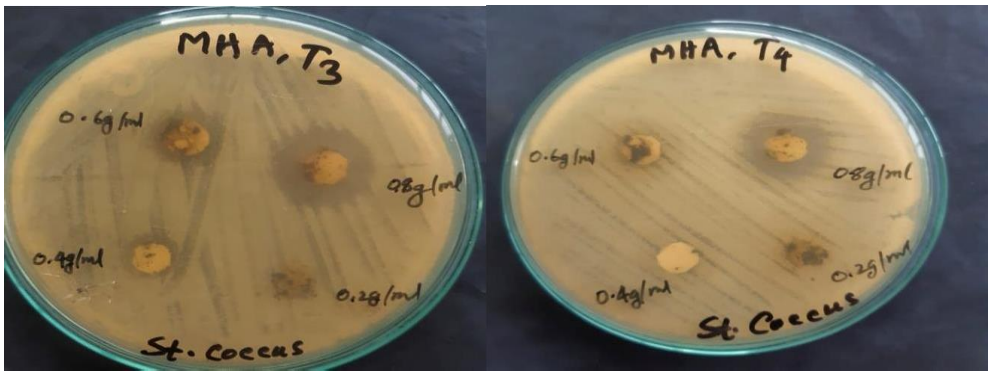

Plate 9: Antibiotic effects of Tulsi leaves on *Staphylococcus aureus*

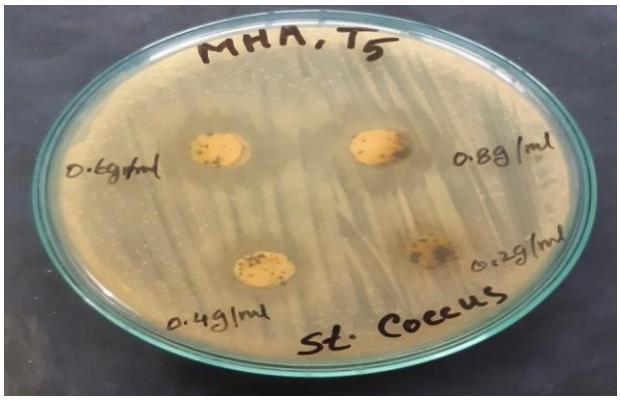

Plate 10: Antibiotic effects of Tulsi leaves on *Staphylococcus aureus*

In this work it was also observed that tulsi plant extracts were highly sensitive to *S. aureus* in comparison with local antibiotics. The antibacterial effect of different concentration of tulsi leaf extracts have significant effects on zone diameter of inhibition (ZDI) against *Staphylococcus aureus* (Table 6). Among the different treatment combination the maximum zone of inhibition 17mm against *Staphylococcus aureus* was observed under methanol extract of tulsi leaf at 0./8g/ml concentration. Whereas the minimum zone of inhibition 7mm against *Staphylococcus aureus* was measured in methanol extract in 0.4g/ml concentration. In addition the second highest zone of inhibition 13mm was found under methanol 0.6g/ml concentration. No zone of inhibition was recorded under at 0.2g/ml methanol extracts. In all cases, the activity of the extracts was compared with

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

antibiotic disc.

DISCUSSION

In the present study, it was observed that uniform turbidity in nutrient broth; Circular, small, smooth, convex and yellow color colonies in nutrient agar and golden yellow color colonies on manitol salt agar base with yellow color media.

It also produced yellow color colonies on *Staphylococcus* agar 118 and smooth, shiny, round, convex with β-hemolysis on blood agar media were might be *Staphylococcus aureus*. This observations was supported by a observation of Shareef *et al.,* (2009); Alkasir *et al.,* (2013) and Terzolo *et al.,* (1979).

In grams staining the morphology of the isolated bacteria exhibited gram positive, cocci shape and grape like cluster which was supported by several author Atalla *et al.,* (2008); Marchant and Packer *et al.,* (1967).

In this study, morphologically and culturally identified isolates were then identified by using different biochemical tests (MR, VP, TSI, MIU, Manitol salt fermentation, Indole, Coagulase, Catalase). It was observed that MR test positive indicating red color; VP test negative; TSI positive indicating yellow color; MIU test negative; Manitol is fermented indicating yellow color; Indole test is negative and Catalase test is positive. These findings were also supported by Hogan *et al.,* 1986.

The recent antibiogram study revealed that the isolates were highly sensitive to Cipro floxacillin (5), oxytetracycline (30), Cefalexin (30), and Intermediate sensitive (++) to Streptomycin (10), Gentamycin (10). Resistance to Erythromycin (15), Penicillin (10) and Amoxicillin (30) From the table tested organism were MDR. The study is more or less similar to Miranda *et al.,* (2008); Nemati *et al.,* (2008); Zhou *et al.,* (2012). Moreover, which was supported by (Kumar *et al.,* 2011) and (Sikrodia *et al.,* 2020) and this is very much consistent with my work.

In this current research work we implement tulsi (*Ocimum sanctum*) leaf extract based on methanol. Besides we observed the resistance pattern against the bacteria notorious one *Staphylococcus aureus.* In our study tulsi (*Ocimum sanctum*) leaf chosen due to it has active ingredients like Oleanolic acid, Ursolic acid, Rosmarinic acid, Eugenol, Carvacnol, Linalool. That have the capability to fight against bacteria (Priya Panchal* and Nayyar Parvez2019); the leaf extract of both the A. indica and O. sanctum has shown antimicrobial activity against E. coli and S. aureus. The leaf extract of tulsi (*Ocimum sanctum*) has antimicrobial activity against both staphylococcus spp and E. coli which observed against tulsi (*Ocimum sanctum*) leaf extract (methanol) showed zone of inhibition 0mm, 0mm, 4mm, 12mm , 14mm and 20mm found against 0g/ml, 0.2g/ml, 0.3g/ml, 0.4g/ml,0.5g/ml,0.6g/ml and 0.7g/ml, concentration (Vipul kumar *et al.,* 2018).

On the other hand Tulsi oil at concentrations of 4.5 and 2.25% completely inhibited the growth of *S. aureus*, including MRSA and *E. coli*, while the same concentrations only partly inhibited the growth of *P. aeruginosa* (Hanaa A. Yamani *et al.,* 2016).

The activity of Tulsi extract against K. pneumonia & *Staphylococcus aureus* was found to be higher at a concentration of 100% followed by 75% & 50% respectively. The maximum zone of inhibition was found to be 21&11mm against leibsiella pneumonia and 18&15mm against Staphylococcus aureus (Mittal *et al.,* 2018).

The activity of Tulsi extract against *Staphylococcus aureus* was found to be higher at a concentration of 900µ/L, followed by 600µ/L&300µ/L, respectively zone of inhibition 17.98nm ,16.45 nm and 14.23nm (Gomathinayagam Subramanian *et al.,* 2014).This is similar to the results recorded in this study where the increase in the concentration of extracts corresponded to the increase of diameter of inhibition zone (Francine *et al.,* 2015). This results also supported by Pramod K. Raghav & Mitu Saini, 2018; Kumar *et al.,* 2019 and adhikari *et al.,* 2020.

Tulsi (*O. sanctum*) leaf extract showed good results than commercially available antibiotic disks. So, here we conclusively said that tulsi (*O. sanctum*) leaf extract is one of the good ingredients to combat against the super

bug *S. aureus.* Eventually tulsi (*O. sanctum*) leaf extracts methanol having better capacity than commercially available antibiotics. Here tulsi (*O. sanctum*) leaf extract methanol (0.8g/ml) is better than tulsi (*O. sanctum*) leaf extract methanol (0.4g/ml). The varying susceptibility of the bacterial isolates to commonly used antibiotics could be attributed to indiscriminate and irrational use of these drugs in the animals which usually results in resistance developed by the microbes.

CONCLUSION

It can be concluded that the tulsi leaves extract have great potential as an antibacterial agents against *Staphylococcus aureus.* The concentration of tulsi leaves extract 0.8g/ml showed better result compared to other concentrations.

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ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

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