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"To Study the Antimicrobial Activity of Honey against Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* with special reference to Mec A gene, from Various Clinical Samples at a Tertiary Care Centre, Uttar Pradesh, India".

Syed Shadman Ahmad[1], Nilam Nigam[2], Deepak Kumar[3], Ritika Tiwari[4], Nashra Afaq[5], Vikas Mishra[6], Rana Firdaus[7], Suraiya Khanam Ansari[8], Mohammad Suhaib[9]*

[1]Associate Professor, Department of Pharmacology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India
[2]Professor and Head, Department of Pharmacology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India
[3]Assistant Professor and Head, Department of Microbiology, Uma Nath Singh Autonomous State Medical College, Jaunpur, India
[4]Tutor, Department of Microbiology, Uma Nath Singh Autonomous State Medical College, Jaunpur, India
[5]Research Associate, Department of Microbiology and Central Research Laboratory, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India
[6]Professor, Department of Microbiology, G.S.V.M. Medical College, Kanpur, Uttar Pradesh, India

[7]Assistant Professor, Department of Pharmacology, Sharda School of Pharmacy, Greater Noida, India
[8]Associate Professor, Department of Microbiology, G.S.V.M. Medical College, Kanpur, Uttar Pradesh, India
[9*]Associate Professor, Department of Microbiology, Sharda School of Medical Science and Research, Noida, India
***Corresponding author:** Dr. Mohammad Suhaib
*Email ID: 2011sohaib@gmail.com

Abstract

Introduction: Multiple antibiotic resistances in the bacteria that cause infections in humans have been directly caused by the ongoing use of antibiotics in clinical practise. In undeveloped and impoverished nations, the overuse and inappropriate use of antibiotics may increase the risk of resistant bacteria emerging and proliferating throughout the population. Consequently, the antibiotics' effectiveness is declining. Therefore, there is now more interest in the therapeutic use of natural materials due to the need for innovative alternative antibacterial techniques.

Aim and Objective: To study the antimicrobial activity of honey against clinical isolates of Methicillin-Resistant *Staphylococcus aureus* with special reference to Mec A gene , from various clinical samples at a tertiary care centre, Uttar Pradesh, India.

Material and Methods: This was a prospective study carried out in the Department of Pharmacology and the Microbiology Department for a period of 1 year i.e, August 2022 to August 2023 at a Tertiary care centre, Uttar pradesh. The antibacterial activity of Dabur pasteurised honey was evaluated against the bacterial strains of *Methicillin Resistant Staphylococcus aureus* and *Methicillin Sensitive Staphylococcus aureus*. Their antibacterial sensitivity pattern was tested using Kirby-Bauer disc diffusion susceptibility testing technique according to the CLSI guidelines 2022 along with other commonly used antimicrobials. .

Results: A total of 200 clinical isolates were isolated, out of which 72 isolates were of *S.aureus* in which a total of 24 MRSA isolates were were identified by CX, OX, and E-test. The DNA was extracted using the DNA extraction Qiagen Kit and the Mec A gene was detected by the PCR. In the present study there were 24 isolates of MRSA out of which 15(62.5%) isolates were sensitive to honey and 48 isolates of (MSSA) Methicillin sensitive *S. aureus* out of which 42(87.5%) isolates were sensitive to honey. It was observed that all the Methicillin resistance isolates were resistant to Cefoxitin and Oxacillin whereas sensitive to linezolid, Teicoplanin and Vancomycin.

In the present study the results of antibacterial activity of honey towards the two microorganisms tested were performed. MRSA as well as MSSA were sensitive to undiluted honey samples tested with an average zone of inhibition of 30.03± 0.1 and 41.06±0.2 respectively.
Both MRSA and MSSA isolates were sensitive to honey. But MRSA were resistant to all antimicrobials tested except linezolid where as MSSA were sensitive to all except penicillin.

Conclusion: Honey is undoubtedly a viable candidate for further research and testing as an antibacterial in the future. With more research on its mode of action at the molecular level, honey could find extensive application as an antibacterial agent in the future.

Keywords: Honey, Antimicrobial activity, Kirby-bauer disc diffusion, Zone of Inhibition, MRSA, MSSA

INTRODUCTION

One of the microorganisms that is well-known to cause infections in human skin, soft tissues, deep-seated tissues, pneumonia, and surgical sites is *Staphylococcus aureus* (*S. aureus*). The two main strain variants of the Gram-positive cocci bacteria *Staphylococcus aureus* are *Methicillin-susceptible Staphylococcus aureus* (MSSA) and *Methicillin-resistant Staphylococcus aureus* (MRSA) [1].
The term methicillin-resistance is a classic term that implies resistance to all beta-lactam antibiotics, except for recently introduced anti-MRSA cephalosporin's, such as ceftobiprole. MRSA, which was first reported in the 1960s [2] has become endemic in hospitals and health-care settings worldwide. The frequency of methicillin-resistant *S. aureus* (MRSA) isolates is increasing [3, 4] and this issue can lead to severe therapeutic dilemmas and exacerbate the control of infections in hospitals settings [5]. The mecA gene, which encodes for a modified penicillin-binding protein, PBP2a, with decreased beta-lactams affinity [6] is responsible for methicillin-resistance among bacteria, including MRSA.
Additionally, MRSA has been identified as the source of community- and hospital-acquired (CA-MRSA) infections [7]. Numerous severe infections, including nosocomial, necrotizing fasciitis, potentially deadly illnesses, pneumonia, osteomyelitis, endocarditis, severe sepsis, and toxic shock syndrome, have been linked to MRSA in recent years [8,9]. MRSA have created a big challenges about to cure an infected person due to resistance to multiple classes of antibiotics including penicillin, and methicillin. Furthermore, MRSA has been found co-resistance with vancomycin, linezolid (oxazolidinone), and tigecycline [10]. Only new classes of antibiotic such as Rifampicin etc have been used to cure MRSA and biofilm infections. However, vancomycin-resistant *S. aureus* and rifampicin-resistant *S. aureus* strains have been recorded in China [11] may be due to mutations in rpoB gene.
Drug of choice to treat these multidrug resistant MRSA are glycopeptide antibiotics such as vancomycin [12]. The increase in the resistance to MRSA with the decreases susceptibility of the glycopeptides antibiotics is a worrisome problem observed worldwide. With the irrational and excessive use of antibiotics in underdeveloped and developing countries the developed resistance may spread in the community making the strains as super bugs causing difficulties in eradication [13]. As a result, the effectiveness of the antibiotics is diminished [14].
Therefore, the need for novel alternative antimicrobial strategies has renewed interest in natural products like turmeric, honey, ginger etc., exhibiting antibacterial properties. This situation has led to a re-evaluation of the therapeutic use of ancient remedies including honey [15-17]. Honey is well known as a magic drug for almost all kinds of diseases, not to mention the fact that many people do depend more on folk medicine and natural remedies which are cheap that have been known for their therapeutic effects over the past decades [15]. Honey has well established function as an effective antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria [18-20]. The application of honey can promote the healing of infected wounds that do not respond to the conventional therapy, i.e., antibiotics and antiseptics [21] including wounds infected

with methicillin-resistant *S. aureus* [22,23]. Laboratory studies have revealed that honey is effective against MRSA, β-haemolytic streptococci and *Vancomycin Resistant Enterococci* (VRE) [24,25]. The beneficial role of honey is attributed to its antibacterial property with regards to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H2O2) and non-peroxide components, i.e., the presence of phytochemical components like Methylglyoxal (MGO). The antimicrobial agents in honey are predominantly hydrogen peroxide, of which the concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen [26].
Therefore, the present study was undertaken to study the antimicrobial activity of honey against clinical isolates of methicillin-resistant *staphylococcus aureus* with special reference to MecA gene, from various clinical samples at a tertiary care centre with an attempt to find out the efficacy of locally available honey against MRSA which emerged as a superbug and MSSA and their antimicrobial activity to commonly used antimicrobials.

MATERIAL AND METHODS

This was a prospective study carried out in the Department of Pharmacology and the Microbiology Department for a period of 1 year i.e, August 2022 to August 2023 at a Tertiary care centre, Uttar pradesh. The antibacterial activity of Dabur pasteurised honey was evaluated against the bacterial strains of *Methicillin Resistant Staphylococcus aureus* and *Methicillin Sensitive Staphylococcus aureus*. Their antibacterial sensitivity pattern was tested using Kirby-Bauer disc diffusion susceptibility testing technique according to the CLSI guidelines 2022 [27] along with other commonly used antimicrobials. .
The different Phenotypic methods including cefoxitin , oxacillin disc diffusion test, E test were carried out. The samples were processed immediately to the laboratory and tested for their biochemical test for the identification according to the CLSI guidelines 2022 [27]. In case of delay the samples were kept at 4^0C. The patients demographic profile along with the written consents were obtained from all the participants involved in this study. Ethical Clearance was duly obtained from the Ethical committee before the start of the study.

Screening of the MRSA:

Phenotypic screening:

On the basis of colony morphology, mannitol fermentation, Gram staining, catalase test, coagulase test and DNase activity MRSA isolates were identified. The phenotypic MRSA was performed using the cefoxitin, oxacillin disk diffusion test, E-test as per the protocol of the Clinical and Laboratory Standard Institute guidelines (CLSI) .
The Disc diffusion test with Cefoxitin 30 µg disc (obtained by Himedia, Mumbai) was used to differentiate MRSA and MSSA isolates and the interpretation was done by MSSA if zone size was 22 mm. The strain was considered as MRSA if zone size was <22 mm. (ref. CLSI M100-S23) [27] and genotypically (DNA sequencing was done for MecA gene at Eurofins Genomics India Pvt., Ltd. Bangalore, Karnataka, India).

Genotypic screening

The molecular characterization for the detection of MecA gene of the clinical isolates was performed. The DNA was extracted using the DNA extraction Qiagen Kit (Germany. The MecA gene was identified as gold standard test for the identification of MRSA by using polymerase chain reaction (PCR) [28]. Cefoxitin was considered as an inducer of MecA gene expression.
The primers for MecA gene was synthesized by Chromous Biotech. Pvt. Ltd. (Bangaluru).

Figure No.1: The DNA Extraction kit

Figure No.2: The Reagents used for the DNA Extraction

Gene	Primer sequence	Length (bp)	Reference
MecA gene	5'- GTTGTAGTTGTCGGGTTTGG-3' 5'- CTTCCACATACCATCTTCTTTAAC -3'	335	[29]

Table No. 1: The Primers used for the MecA gene fragment

Figure No. 3: The MecA gene primers synthesized by Chromous Biotech

Polymerase Chain Reaction (PCR)
The obtained DNA fragment were amplified in PCR (BIO-RAD T100 Thermal Cycler, Singapore) (volume 20 µl) by mixing 10µl master mix (Takara), 5µl nuclease free water, 1 µl forward and reveres primer each and 3µl DNA as a template for PCR conditions with initial denaturation at 94oC for 5 min, then for 34 cycle at 94oC for 30 sec for cycle denaturation, 50oC for 45 sec for annealing for MecA gene.

Step	**Program**		
	MecA gene		
	Time	**Temperature**	**Cycles**
Initial denaturation	15 min	95 ºC	
Denaturation	30 s	94 ºC	
Annealing	1 min30 s	59 ºC	
Extension	1 min 30 s	72º C	30
Final extension	10 min	72º C	

Table No. 2 : The PCR cycling conditions to amplify Mec A gene fragments.

The Agarose gel preparation and visualized by Gel Doc™ EZ Gel Documentation System
The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1% agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 1 kb DNA Ladder (Thermo Fisher Scientific ™, Waltham, MA, USA) was used as the marker to evaluate the PCR product of the sample [30,31].

Antimicrobial Testing of Honey
Their antibacterial sensitivity pattern was tested using Kirby-Bauer disc diffusion susceptibility testing technique of CLSI [27]. MRSA ATCC strain No. 43300, MSSA ATCC 25923 were included as a positive control strains and MRSA ATCC 33591, MSSA ATCC 29213 were used as negative control strains. A six hour incubated bacterial culture suspension matching with 0.5 Mc-Farland scale standard was prepared equivalent to 1.5 x 108 CFU/ ml organisms in 5 ml peptone water and spread onto the sterile Mueller-Hinton agar (Himedia, Mumbai) plates to prepare a lawn culture. Dried in the incubator for half an hour and three such plates were prepared for each bacterial strain. Honey disks were prepared by using Watman No.1 filter paper and discs were punched with a office paper hole punching machine of 6 mm diameter and such 100 discs were taken in small glass bottle and sterilized at 160OC for two hours in a hot air oven. To these 100 sterile discs, 1 ml of pasteurized 100% V/V undiluted honey obtained for this study was added and kept for overnight for equal absorption of honey by all discs [32].

Antimicrobial Susceptibility Testing
The antimicrobial susceptibility testing was performed by modified Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (HiMedia laboratories private limited, India) .Antibiotic discs used were ciprofloxacin (5µg), clindamycin (2µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), tetracycline (30µg), cotrimoxazole (25µg), rifampin (5µg), mupirocin (200µg), and penicillin G (10 units) cefoxitin, oxacillin as per the Clinical and Laboratory Standards Institute (CLSI) guidelines , and the prepared honey discs were placed aseptically on the Mueller Hinton agar. Plates were left for one hour at 25OC to allow a period of preincubation diffusion in order to minimize the effect of variation in time between the placements of different discs. The plates were then incubated aerobically at 37OC over night to allow bacterial growth. After incubated plates were observed and the zone of inhibition was measured to evaluate the antimicrobial activity for each of the tested antibiotics and honey samples using a special scale obtained from Himedia Laboratories, Mumbai, India. The sensitivity testing plates were done in triplicates for each strain of MRSA and MSSA isolates and the zone of inhibition were measured to the nearest millimeters [33].

All the isolates were tested by making a lawn culture of 0.5 Mc Farland suspensions of isolates on Muller Hinton Agar (MHA) plate. Plates were analysed after incubation at 37°C for 18 h. The zone diameter of ≤19 mm was considered as antibiotic-resistant for MRSA as per the CLSI guidelines [27].
To calculate the mean and standard deviation of each strain statistically using Statistical Package for the Social Sciences (SPSS) software. We took the criteria for sensitivity was as the zone of inhibition for honey is bigger than the zones any of the antimicrobials used for testing the MRSA and MSSA isolates.

RESULTS

A total of 200 clinical isolates were isolated. The different Phenotypic Methods including Cefoxitin ,Oxacillin Disc Diffusion test E test and the genotypic method including MecA gene detection for Methicillin Resistant *Staphylococcus aureus* isolates for the MecA gene was performed. Out of which 72 isolates were of *S.aureus* in which a total of 24 MRSA isolates were
were identified by CX, OX, and E-test. The DNA was extracted using the DNA extraction Qiagen Kit and the Mec A gene was detected by the PCR.

Microscopic observation	Gram's test	Catalase test	Coagulase test		Urease test	Cefoxitin(cx) and Oxacillin(ox)	DNAase Test
Cocci form (For all 220 cases)	+	+	Slide +	Tube +	+	+	+

Table No.3: Phenotypic Identification of *S.aureus* with the use of different test

Type of Clinical Isolates	Number of Ioslates	Percentage
S.aureus	72	36%
Others clinical isolates	200	64%

Table No. 4: The type and the total number of clinical isolates

From the Table no.4 it was observed that the *S.aureus* isolates were 36%, and the other clinical isolates were 64%.

Molecular analysis:
The authentic confirmation of MRSA was decided by the molecular analysis. The presence of MecA gene was detected in all the 24 isolates of MRSA The gene sequences of MecA gene was obtained and it was confirmed by homology of sequences.

Detection of Mec A gene: In this study, 24 MRSA isolates were subjected for the molecular analysis. We extracted a good quality fragment of the DNA of all the isolates.The Gel photographs of the DNA samples are mentioned below observed by Gel documentation system.

Figure No. 4: The DNA isolated from *S. aureus* isolates

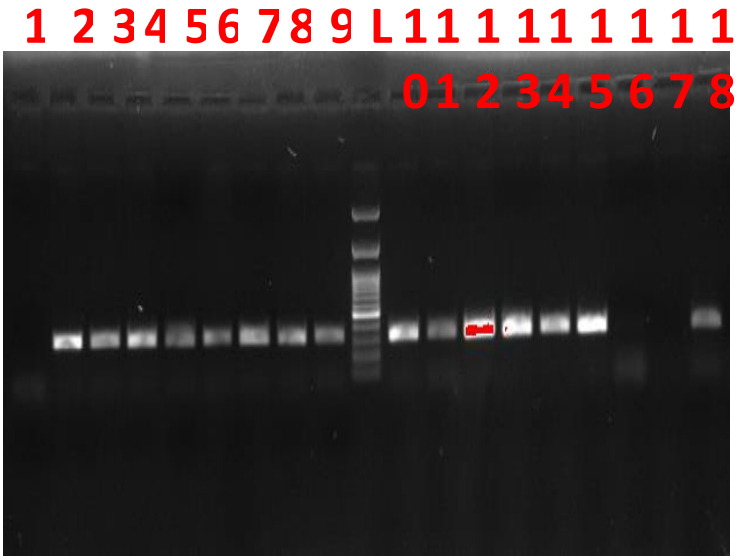


Figure No. 5: The Photograph of the amplified Mec A gene in *S. aureus*, the amplified DNA band size was obtained 336 bp, L corresponding to 100bp ladder used, where Lane 15 is the positive control , Lane 16 - Lane 17a Negative control, and Lane 1-9, L10-L14 and Lane 18 is the sample positive for the MecA gene detection.

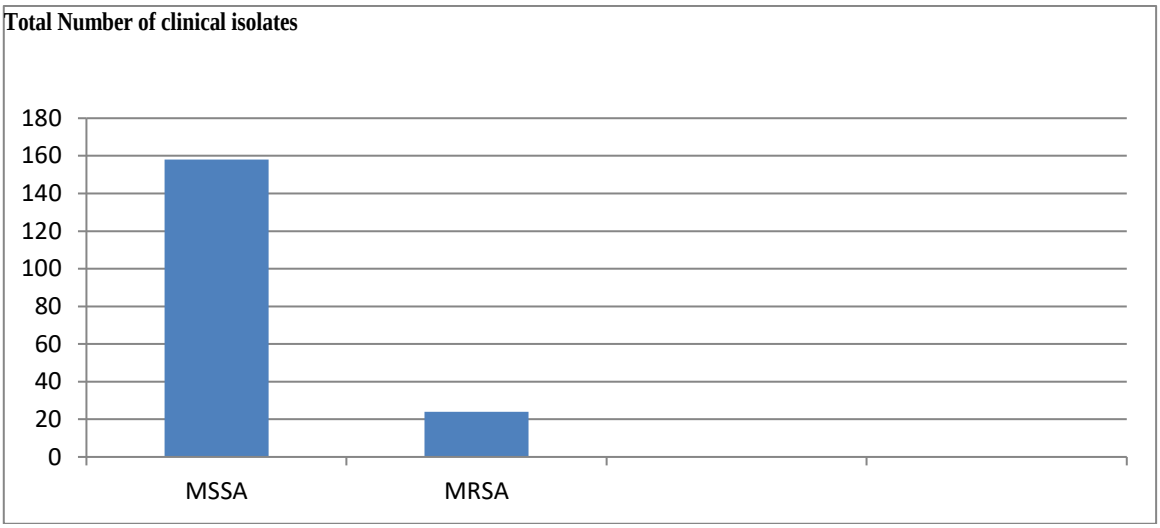
GTTGTAGTTGTCGGGTTTGGTATATATTTTTATGCTTCAAAAGATAAAGAAATTAATAA
TACTATTGATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAAAGATAGCAGTTAT
ATTTCTAAAAGCGATAATGGTGAAGTAGAAATGACTGAACGTCCGATAAAAATATATA
ATAGTTTAGGCGTTAAAGATATAAACATTCAGGATCGTAAAATAAAAAAAGTATCTAA
AAATAAAAAACGAGTAGATGCTCAATATAAAATTAAAACAAACTACGGTAACATTGAT
CGCAACGTTCAATTTAATTTTGTTAAAGAAGATGGTATGTGGAAG

Figure No. 6: Obtained gene sequences of MecA gene in *S. aureus*

In the molecular study, we have obtained 100% prevalence of MecA gene recorded.

Organism	Disc diffusion test	E-test	Molecular Test (Mec A gene)
MSSA	48 (%)	-	-
MRSA	24 (CX, OX) (%)	24	24
Total	200		

Table No. 5: Identification of staphylococcal strains with the use of different microbiological tests

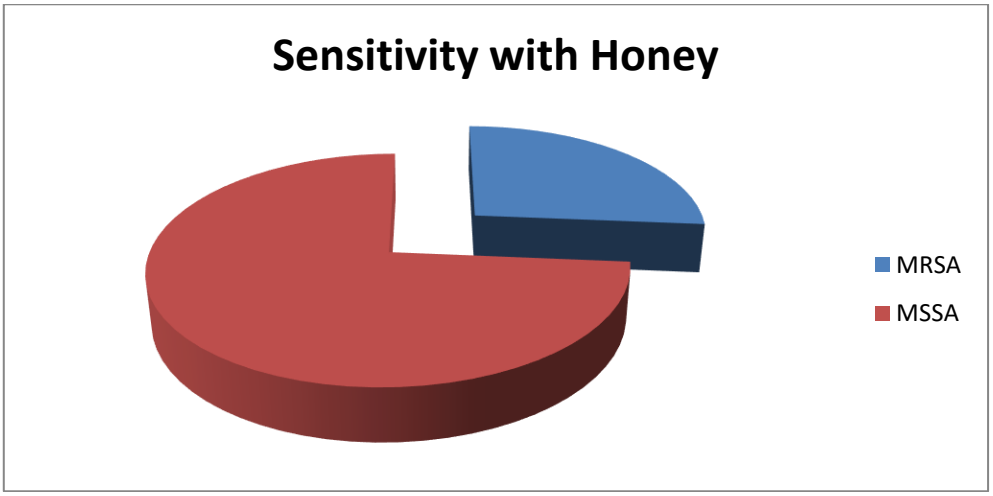


Graph No.1 : The graphical representation of the distribution of MSSA and MRSA isolates

In the present study there were 24 isolates of MRSA out of which 15(62.5%) isolates were sensitive to honey and 48 isolates of (MSSA) Methicillin sensitive *S. aureus* out of which 42(87.5%) isolates were sensitive to honey. Antibiotics susceptibility testing was also carried out by using other commonly used antibiotics according to the CLSI guidelines. It was observed that all the Methicillin resistance isolates were resistant to Cefoxitin and Oxacillin whereas sensitive to linezolid, Teicoplanin and Vancomycin.

Total no. of *S.aureus*	**MRSA**	**MSSA**
72	24	48
Isolates sensitive to honey	15 (62.5%)	42 (87.5%)

Table No. 6: Total Number of Isolates



Graph No.2 : The graphical representation of the sensitivity of honey to MSSA and MRSA isolates

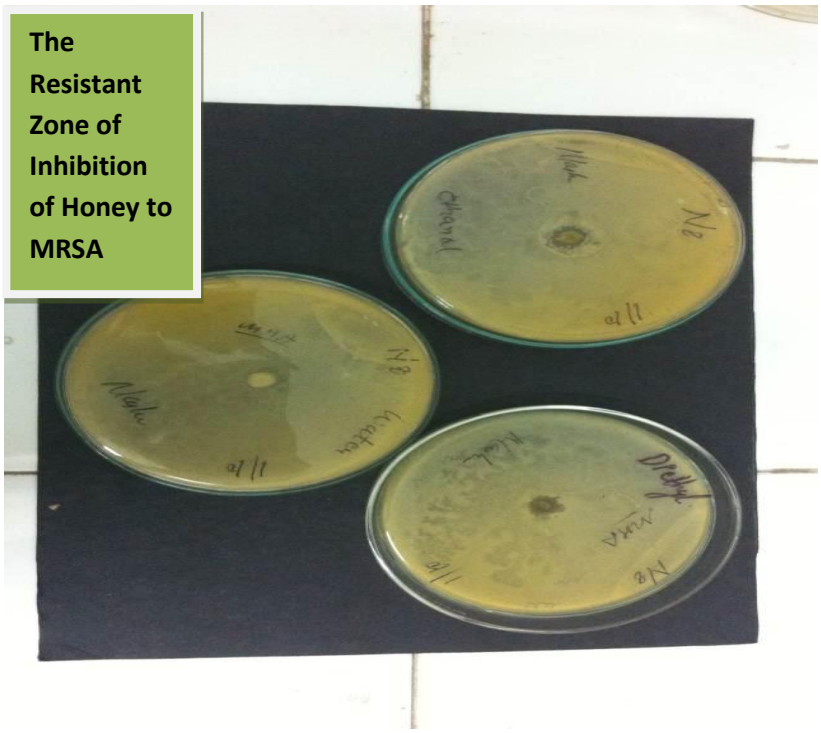


Figure No. 7: The Resistant Zone of Inhibition of Honey to MRSA

From the Figure 7 and Figure 8, it was clear that 24 isolates of MRSA out of which 15(62.5%) isolates were sensitive to honey and 48 isolates of (MSSA) Methicillin sensitive *S. aureus* out of which 42(87.5%) isolates were sensitive to honey.

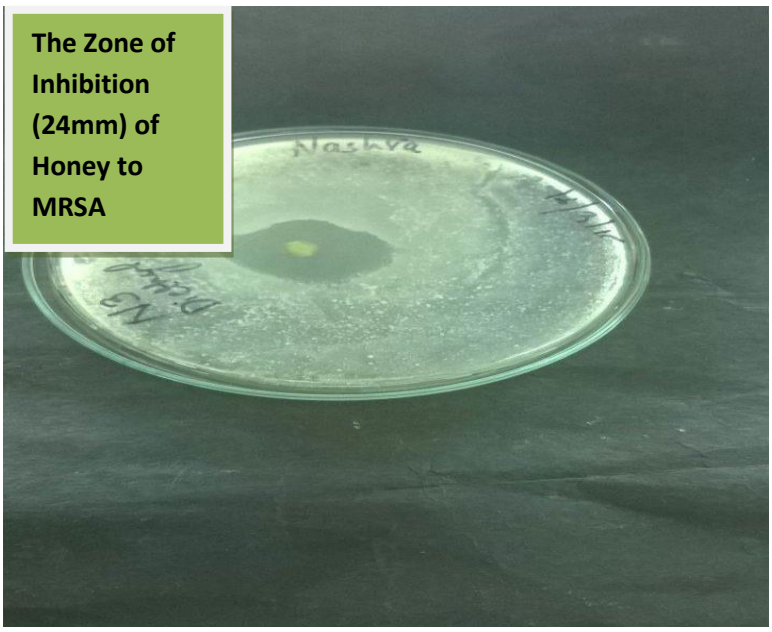


Figure No. 8: The Resistant Zone of Inhibition of Honey to MRSA

Type of Organism	Diameter of Zone of Inhibition (mm) (Mean ± S.D.)
MRSA	30.03± 0.1
MSSA	41.06±0.2

Table No. 7: The Diameter of Zone of Inhibition (mm)

In the present study the results of antibacterial activity of honey towards the two microorganisms tested were performed. MRSA as well as MSSA were sensitive to undiluted honey samples tested with an average zone of inhibition of 30.03± 0.1 and 41.06±0.2 respectively.

DISCUSSION

With antibacterial drugs being widely used in clinical settings, many microorganisms, especially methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*, have adapted to synthetic antibiotics and become highly resistant to these drugs over time [34]. Microorganisms with multi-drug resistance now cause thousands of deaths throughout the world each year [35,36] . Although some of these organisms can live harmlessly in humans and are carried in the nasal passage and on the skin, they can cause fatal infection in hospitals and nursing homes, where patients with open wounds, invasive devices and immunodeficiency are at higher risk of infection than healthy people [37.] Furthermore, resistance does make the infection more difficult to treat with standard antibiotics and thus more dangerous [38-40]. Therefore, the continuing spread of multi-drug resistant strains and the increased abuse of antibiotics highlight the need for alternative agents, thus medical plants and the natural source are the need of an hour.
It has been reported that honey showed both bacteriostatic and bactericidal effect against many Gram-positive as well as Gram-negative bacteria [41-45]. The use of natural products to enhance wound healing is a common practice in many parts of the world. Honey consists of a super saturated solution of sugars and has a low pH between 3.2 and 4.5. This pH together with honey's high osmolarity and the presence of H2O2 reduces the bacterial growth at the wound site. Honey in wound dressing has been reported to provide ideal environment for the rapid tissue repair and regeneration that are essential for growth of wound bed [46]. *Staphylococcus aureus* is the most frequently isolated wound pathogen and it is becoming increasingly resistant to antibiotics in common use. Honey has been reported to be effective in eradicating antibiotic resistant bacteria including MRSA [47] which is a super bug now.

Any zone diameter having less than 7 mm shows that the organism is resistant to the honey sample but if the zone diameter is greater than 11 mm it suggests that the microorganism is sensitive to honey [48].
In the current study honey possess antimicrobial The findings of our study together with four other previous studies [49] show that honey promises to be an effective wound antiseptic with broad spectrum antimicrobial activity. Some topical antimicrobials adversely affect the human skin/tissue and repair process during the treatment of wounds where as there is no need for laboratory evaluation of honey as it does not adversely affect human skin/tissue [50]. The special character of honey is the potential to limit the growth of wound pathogens, but also there is evidence that honey has the potential to promote the healing [51,52] and no other antimicrobial agent possesses these characteristics. Honey is effective even it is diluted by burn wound exudates. In burns, honey's antimicrobial and anti inflammatory properties allow a moist healing environment to be maintained that protects the wounds from deterioration and fibrosis [53].

In the present study the results of antibacterial activity of honey towards the two microorganisms tested were performed. MRSA as well as MSSA were sensitive to undiluted honey samples tested with an average zone of inhibition of 30.03± 0.1 and 41.06±0.2 respectively.
There were other studies performed by the research investigators which states that 100% V/V undiluted honey inhibited the growth of MRSA with a zone of inhibition of 18 mm and 11 mm respectively [54,55] whereas Patel A et al., in their study observed that diluted honey of 20% V/V, 30% V/V and 40% V/V inhibited the growth of MRSA [56] whereas, 15% V/V also inhibited the growth of MSSA in addition to other concentrations used for MRSA and Almasaudi SB et al., observed the only 50% V/V concentrated honey inhibited the growth of both MRSA and MSSA [57],

whereas, Zakaria AS showed that 100% V/V undiluted honey inhibited MRSA with a zone of 14±2.83 mm and MSSA 15±2.83 mm for Yemen Sidr honey [58], MRSA with a zone of 15±0.71 mm and MSSA 17±1.35 mm for Southern Sidr honey and MRSA with a zone of 9±1.1 mm and MSSA 13±3.24 mm for multi-flower mountain honey.
There were many other studies performed which states that the antibacterial activity of honey is not known, but it is clear that the higher the concentration of honey the greater its usefulness as an antibacterial agent. However, it is expected thatthe clinical significance of the antibacterial activity in honey will be unequivocally proven only if a clinical trial is conducted to compare dressings of different sugars and selected honeys [54, 55] .
Well documented clinical trials and researches are going on honey and nanotechnology which may provide promising results on therapeutic use of honey in the future.

The honey can be applied directly to the surface of a wound. This provides a physical barrier between the wound and the environment, preventing contamination [59]. The secondary effects provided by application are the antimicrobial properties, including both bacteriostatic and bactericidal activity, further preventing wound contamination [60]. Additionally, an osmotic gradient is generated due to the high sugar content and low water activity, generating a flow of bacteria, necrotic tissue and debris out of the wound [61]. Finally, the phenolic content in honey aids in inflammation, helping to improve wound healing. Overall, this has been observed to improve both the healing of the wound, and the time taken to heal and reduce scarring [62]. This can reduce the use of antibiotics, while still aiding wound treatment.

Within a medical setting, honey can be used as an effective wound treatment, removing the need for antibiotics. Honey has the potential to vastly reduce the requirement of drugs of last resort for highly drug-resistant bacterial infections, since current resistance to antimicrobial mechanisms of honey is largely unseen [63]. This is likely due to the multiple mechanisms of antibacterial action from the plethora of antimicrobial compounds, resulting in a unique combination therapy, which has yet to be identified as a source of antimicrobial resistance. Therefore, if medical grade honeys were to be included in clinical treatment, it would reduce the demand for antibiotic usage

CONCLUSION

Honey should undoubtedly be considered as a viable future antibacterial to be researched and studied. The rediscovery of honey as a natural therapy for wound pathogens demonstrated its efficacy against antibiotic resistant types of bacteria such as MRSA. The current study attempted to focus further on whether honey can be utilised to treat Staphylococcal infections, specifically MRSA. Honey, a naturally blessed and environmentally friendly substance, may be used more extensively in the future with additional molecular research on its mechanism of action as an antibacterial agent.

Declarations:
Conflicts of interest: There is no any conflict of interest associated with this study
Consent to participate: We have consent to participate.
Consent for publication: We have consent for the publication of this paper.
Authors' contributions: All the authors equally contributed the work.

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