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Apis cerena indica honey's potentiality to combat bacterial infections in diabetic wounds with an emphasis on vancomycin-resistant coagulase-negative Staphylococcus aureus

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ABSTRACT: Diabetic wounds often have a distinct bacterial population compared to non-diabetic wounds due to the altered physiological conditions that favor bacterial growth. Factors such as lesion size, wound severity, type of diabetes, and other medical conditions can influence the bacterial community in these wounds. Bacteria in diabetic wounds frequently form biofilms, which contribute to their resistance to drugs. This study highlights the effectiveness of traditional medicinal honey in combating these challenges, demonstrating its potential as a treatment for biofilm-forming, drug-resistant bacteria in diabetic wounds. Honey collected from A. cerena indica bees in Dharmapuri district, Tamil Nadu, India, was analyzed for its bactericidal effects against vancomycin-resistant coagulase-negative Staphylococcus aureus and multidrug-resistant bacteria from wound samples. The study focused on assessing the ability of these bacterial isolates to produce biofilms. On Trypticase soy agar, all 24 bacterial isolates exhibited diverse colony morphologies. The study assessed the ability of bacterial isolates from wounds to form biofilms using the Congo Red Agar (CRA) and tube methods (TM). Out of 24 isolates, 21 formed biofilms, with 13 producing strong biofilms. Antibiotic resistance profiles revealed: 10 isolates resistant to vancomycin, 11 resistant to Gentamicin, Oxytetracycline, and Kanamycin 12 resistant to Erythromycin and Amoxicillin10 resistant to Tetracycline and Chloramphenicol and 8 showing resistance to all tested antibiotics. Vancomycin-resistant coagulase-negative S. aureus was confirmed using various tests. When forest honey was diluted 1:1 with distilled water and tested, it effectively inhibited all bacterial species, including S. aureus, which showed a significant inhibition zone of 29 mm in diameter. Other bacteria, such as Proteus, Proteus mirabilis, Aeromonas caviae, Klebsiella, and Vibrio, also showed inhibition. The study highlights honey's potential as an alternative treatment for infections caused by antibiotic-resistant bacteria, including VRSA and other biofilm-forming pathogens. Despite the availability of modern antibiotics, honey offers a promising, natural option with significant bactericidal activity.

KEYWORDS: Apis cerena indica, Biofilm, Vancomycin, Diabetic wound, Bactericidal activity

I. INTRODUCTION

Under a natural environmental condition, microbes are survived mostly by attaching and growing upon a solid substratum like living and nonliving thing. Sessile microbes are able to produce complex of extracellular polysaccharide and progressed as biofilm communities. Biofilm development is extremely composite progressions in which microbes are metamorphose from free living stage to the sessile mode of growth [1]. These sessile bacteria form a community which is comprised of a diverse bacterial group and special cell types that provide additional benefits to the community. In some of the literature suggested that biofilm development is relies on the expression of particular genes that determine the growth of biofilm [2, 3]. Formation of biofilm processes have subsequent stages such as surface attachment, growth of micro-colony, three dimensional structure formation, after that a complete biofilm community formation, maturation and dispersion [4]

A chronic diabetic wound showed a low-grade inflammatory phase of healing, because all proinflammatory factors are prevented. This unreceptive environment allows bacteria to multiply and colonize in the wound by constructing confined colonies known as biofilm. More than

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90% of chronic wounds hold with bacteria living inside and construct biofilm. Elimination of biofilm is one of the complicated processes to do, since it is confidently adhere to adjacent tissue and it is not allow penetrating antibiotics, subsequently developed a resistance against many antibiotics. As a consequence of these processes leads to development of multidrug resistant strains (MDR) [5].

The condensed nature of biofilm structures, the supposed reduced rates of cellular growth and respiration of biofilm bacteria and the protection conferred by biofilm matrix polymers, natural and artificial chemical agents are not capable to effectively attack and wipe out infectious biofilm bacterial populations [6]. The nature and condition of the biofilm put forth for development of many multidrug bacterial pathogens. At present MDR strain development is serious problem in hospital environment. Presently need an alternate drug to destroy resistant bacteria and also a strong strategy is requiring to addressing this problem. Therefore this study focused on bactericidal effect of honey against MDR bacteria [7].Honey is the oldest wound-healing agent identified to mankind when some modern chemicals have failed in this regard. In many investigational research represented more proposal suggested that supporting its usage in wound healing because of its bioactivities including antibacterial, antiviral, anti-inflammatory, and **antioxidant activities [**8].

II. METHODOLOGY

2.1 Collection of wound sample

The diabetic wound swab was collected from the patient with the help of rayon swab and the pre analysis procedure of the sample was performed. Then the swab was placed in the culture medium for bacterial pathogen isolation. All samples were collected from SRM Medical College, Kattankulathur, Chennai and Tamil Nadu.

2.2 Processing of wound sample

Totally four clinical swabs were collected and subjected to detect the biofilm formation. All swabs had been initially inoculated in nutrient broth and to be kept for a 24-hour bacterial growth incubation period at 37° C. Then the broth culture was transferred to nutrient agar and Trypticase soya agar plates for isolation as well as detection of biofilm forming ability of the organisms.

2.3 Identification of organism

Each isolate have been identified through conventional methods. All pathogens were thoroughly tested using the subsequent testing procedures. Gram staining, motility, and capsule tests, as well as other preliminary identification procedures, were used. The genus level of the test isolates was determined after all other biochemical tests had been completed.

2.4 Isolation of biofilm bacteria

2.4.1Qualitative method: Tube method (TM)

The tubes were cultured at 37° C for 24 hours after a loopful of the test culture was added to 10 ml of Trypticase soy broth and one percent glucose. After the incubation period, tubes were air-dried and rinsed in phosphate buffered saline (pH 7.3). Then 0.1% crystal violet was used to dye the tubes. Upon removing the excess stain with distilled water, the tubes have been dried upside-down. A visibly thick coating that covered the tube's wall and bottom was observed as a sign that a biofilm had successfully developed. Test isolate findings were examined for the development of biofilms (9).

2.4.2 Congo Red Agar method (CRA)

The CRA medium has been prepared and incorporates the following components such as 50 g/L of sucrose, 10 g/L of agar, 37 g/L of brain heart infusion broth, and 8 g/L of Congo red dye. The stain was made into a separate aqueous solution and autoclaved . Then, along with sucrose , the indicator had to be added to the Brain Heart Fusion Agar. Bacterial pathogens used in tests were inoculation on CRA medium and cultured aerobically at 37° C for 24 hours. The development of black colonies denotes the production of biofilms. (10).

2.5 Bacterial resistant profile

Based on the Clinical and Laboratory Standards Institute (CLSI) recommendation, the Kirby-Bauer disk diffusion approach has been employed to determine the antibiotic-resistance profile. The prepared Müeller-Hinlton solid medium was heated in an autoclave at 121°C for 20 minutes. In an aseptic condition, 15 ml of the sterilized media were poured onto the dishes (65 15 mm).

0.1ml of turbidity adjusted bacterial culture was inoculated on the surface of the plate and spread with L rod. The bacterial culture inoculated plates to dry for five minutes. The

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following twelve antibiotic discs have been used to study the sensitivity pattern of test organism, including Tetracycline (T 30 µg), Clindamycin (CD 2 µg), ceftriaxone (CRO 30 µg), Erythromycin (E 15 µg), penicillin (PG 10 µg), Rifampicin (RP 5µg), Ciprofloxacin (CIP 5µg), kanamycin (K 30 µg), Amikacin (AK 30 µg), Gentamicin (GM 10 µg) and Vancomycin. All the antibiotic discs were placed in the culture inoculated plates and incubate plate at 37˚C for 18- 24 hrs. Plates were examined for the zone of inhibition in nm and results were recorded for the further assay [11, 12 & 13].

2.6 Staphylococcus special test

2.6.1 Coagulase test (Patrick Boerlin)

The test strains are being cultured in brain-heart infusion broth for an overnight period at a temperature of 35 °C. The overnight brain-heart infusion was mixed with 0.2 ml of the tube coagulase test solution, and a total of 0.5 ml of coagulase and EDTA was added to the tube. The samples were gently mixed and then incubated in an incubator at 35 °C for 2 and 4 hours. The tube is left at room temperature for overnight incubation if the test is negative after four hours at 37 °C (14).

2.6.2 DNAse test

The DNAse medium of agar was prepared, and then sterilized at 121°C for 20 minutes. In an aseptic setting, 15 ml of the prepared medium was transferred onto the petridish. On the bottom of each plate, lines were drawn to create portions. In order to construct a line 3–4 cm long between the outer edge and the center of the DNAse test agar plate, a heavy inoculum that was used for the bacteria was inoculated into the agar medium. All streaked plates underwent an 18–24 hour incubation period at 37 °C. After incubation, immerse the plates in 1N hydrochloric acid and let them sit for a short while to enable the solution that was used to be absorbed into a plate**.** After draining the excess hydrochloric acid, observe the plates against a dark backdrop for five minutes. Methyl green was emitted and the medium turned colorless around the test organism if DNA was hydrolyzed. The assay was run in triplicate and included the necessary controls [15].

2.7 Vancomycin test

2.7.1 Detection of VRSA isolates (Merck, Germany).

To identify vancomycin-resistant organisms, all S.aureus isolates underwent screening on vancomycin agar media and it was prepared by addition of 6 mg/L vancomycin to Brain heart infusion (BHI) agar and then all inoculated plates were incubated at 37℃ for 24hours. Spot inoculation was used to evaluate bacterial ability to develop on BHI. Growth was taken into account as an excellent result. Vibrio sp. were employed as the control since it's sensitive to vancomycin. To demonstrate their resistance, the isolates that had developed on vancomycin agar screening medium and performed further analysis.

2.7.2 MIC

The MIC (minimum inhibitory concentration) of vancomycin-resistant S. aureus isolates was determined using the E-test method in accordance with the CLSI guidelines. In each experiment run, the strains of S. aureus and Vibrio were employed as vancomycin susceptible and resistant quality control strains. Vancomycin MIC breakpoints were specified as follows per the CLSI guidelines: Three levels of resistance exist: Susceptible (less than 2 g/ml), Intermediate (between 4 and 8 g/ml), and Resistant (more than 16 g/ml) [16, 17].

2.8 Honey

2.8.1 Honey sample collection

Samples of honey were gathered in the Dharmapuri district. An adapted 20 ml syringe with a sterile tube was used to extract the honey from the pots because Apsis cerena indica species store their honey in compartments within the hives known as pots. In order to prevent photo-degradation, the honey was stored promptly in sterile, dark glass containers and kept at 5°C. In this experiment, commercial honey purchased from a nearby market was utilized to test the antibacterial activity of the substance.

2.8.2 Antibacterial potential of the honey

The isolates of bacteria were cultured overnight in Mueller-Hilton broth at 36.5°C for carrying out antibacterial tests. The quantity of bacteria present was standardized and 15 ml of autoclaved media was transferred onto the plates with an aseptic method. After inoculating the agarized medium with the bacterial strains, the 3 mm wells were created.

Bidistilled water was used to dilute the honey samples in ratios of 1:1, 1:2, 1:3, 1:4, and 1:8 (v/v). 30µl of the honey samples were dropped in the wells. Diluted samples, non-diluted samples, and bidistilled samples have all been added to the wells, respectively. In this test, bidistilled water

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serves as the negative control and the inoculated plates were incubated at 36.5°C for 24 hours before the zone of inhibition was visually evaluated. The diameter of the clear zones of microbial growth around the wells in the agar was measured in order to quantify the inhibition of microbial growth. Three times the experiment was conducted [8].

2.8.3Micro dilution method:

The lowest inhibitory concentration of the honeys has been determined using the broth dilution technique (tube method). Ten sterile test tubes with the numbers 1 to 8 were arranged in a rack. Honey control tubes (HC) and growth control tubes (GC) were used as a control. Before being sterilized, each tube pored with newly prepared nutrient broth. After that, test tubes 1 and HC were added one millilitre of a 100% pure honey solution using a sterile micropipette and tips [18].The second tube was filled with 1 ml of undiluted honey, and two separate sterile micropipettes, tips, and vortexes were used to homogenize the mixture before doing two fold serial dilutions. After complete mixing, 1 ml was transferred from tubes 2 and 3 using a sterile micropipette. Up until the seventh tube attained a dilution of 1:128, the same steps were followed. At that point, one ml was discarded from 8thtube. The HC tube was employed as a honey control with no bacterial inoculums, while the GC tube served as a growth control with no honey added. After an incubation period, growth was assessed using a spectrophotometer, and the results were recorded.

III. RESULTS

3.1 Preliminary identification of biofilm bacteria

Four diabetic wound samples collected from SRM medical college and hospital. Pre analyses of the samples were performed under the laboratory conditions. The pre-processed samples were inoculated to Trypticase soya agar. Based on the colony morphology the biofilm formed bacteria were identified from TSA. Totally 24 isolates have been isolated from all the four samples. The results were tabulated (Table No.1).

3.2 Qualitative method

3.2.1Congo red method

Black colour colony formation and crystalline consistency indicated the biofilm production. According to this identification 21 biofilm producers and 3 non biofilm (plank tonic bacteria) producers were identified. Based on the visible intensity, very strong, Strong and moderate Biofilm producer have been identified. The results were noted in Table no 2

S.No	Isolates number	Medium	Colony Morphology
1	1	TSA1	Dry colonies
2	2	TSA1	White colour shade colonies
3	3	TSA 1	Pink colour colony
4	4	TSA 1	Convex colony
5	5	TSA3	Brown with yellowish colour colony
6	6	TSA 3	White colour isolated colony
7	7	TSA 3	Irregular large colony
8	8	TSA 3	Orange colour colony
9	9	TSA4	Brown with yellowish isolated colony
10	10	TSA 4	Small dry colonies
11	11	TSA 4	Pinkish colour colonies
12	12	TSA 4	White colour colony
13	13	TSA2	Yellowish orange colour colony
14	14	TSA 2	Irregular whitish pink colour colony
15	15	TSA1	Black colur colony

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16	16	TSA 1	Yellowish orange
17	17	TSA 1	Small isolated
18	18	TSA2	Small round yellow isolated colonies
19	19	TSA2	Small irregular colonies
20	20	TSA 2	Circular whitish colour colonies
21	21	TSA3	Irregular shiny colonies
22	22	TSA 3	Translucent colonies
23	23	TSA4	Irregular large shiny colonies
24	24	TSA 4	Rhizoid colonies

Table 1: The various colony morphology on Trypticase soya agar.

3.2.2 Tube method

The tube method allowed for the observation of the thick film inside the tube wall and at its bottom. According to this method 18 biofilm producers and 6 non biofilm (plank tonic bacteria) producers were identified. 18 isolates were showed the thick film formation in wall as well as in the bottom of the tubes. The results were located in table no 3.

Isolates number	Congo red method
1	++++
2	+++
3	-
4	+
5	++
6	+++
7	+++
8	+++
9	++++
10	+
11	+
12	++++
13	+
14	+++
15	++++
16	+++
17	+++
18	++++
19	+++
20	+++
21	-
22	_
23	+++
24	_

Table 2: The indication of very strong (++++), strong (+++), moderate (++), week (+) and non-biofilm (-) producers on Congo red medium.

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3.2.3A Comparative result of qualitative method

According to the results of the Congo red and tube techniques (table 4), 13 substantial biofilm-forming isolates were discovered, while 11 bacteria showed only weak or non-biofilm activity.

3.3 Primary analysis

The isolated bacterial species were identified through traditional biochemical methods and the results were confirmed and compared with Berge's manual .The results were noted in Table no 5& 6.

3.4 **Antibacterial susceptibility test**

Thirteen Strong biofilm isolates were identified and performed the antibacterial susceptibility test to determine resistant pattern of the bacterial species. Through antibacterial test all isolates were found to be highly resistant to various antibiotics. Among the 13 isolates, 10 were resistant to vancomycin, 11 were resistant to Gentamicin, Oxytetracycline and Kanamycin. 12 were resistant to Erythromycin and Amoxicillin. Chloramphenicol and tetracycline showed resistant towards 10 bacteria. Among the thirteen biofilm bacteria the eight isolates were showed resistant against all antibiotics. According to this result eight multidrug resistant isolates were identified. The isolate number 2 staphylococcus was selected for further analysis to confirm the vancomycin resistant property. The results were showed in Table no 7**.**

Isolates number	Tube method
1	+++
2	++++
3	++
4	++
5	+
6	-
7	-
8	++++
9	++++
10	-
11	-
12	++++
13	+
14	+
15	+++
16	+
17	-
18	++++
19	+
20	++++
21	+
22	+
23	++
24	-

Table 3: The indication of very strong (++++), strong (+++), moderate (++), week (+) and non-biofilm (-) producers on tube method.

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Isolates number	Congo red method	Tube method
1	++++	+++
2	+++	++++
3	-	++
4	+	++
5	++	+
6	+++	-
7	+++	-
8	+++	++++
9	++++	++++
10	+	-
11	+	-
12	++++	++++
13	+	+
14	+++	+
15	++++	+++
16	+++	+
17	+++	-
18	++++	++++
19	+++	+
20	+++	++++
21	-	+
22	_	+
23	+++	++
24	_	-

Table 4: The comparative analysis of qualitative method.

3.5 Staphylococcus aureus special test

3.5.1 Coagulase test:

There was no clump formation observed over the 4 hours incubation further it was check with overnight that showed the coagulase negative.

3.5. 2 DNAse test

The DNAse test confirms that the strains are S. aureus based on the positive result.

3.6 Detection of the vancomycin resistant staphylococcus

Vancomycin resistant S. aureus was confirmed based on the growth observed at different concentrations of vancomycin on BHA medium. Among the different concentrations, growth was noticed at 16 g/ml and 20 g/ml, and no growth was seen at low concentrations of vancomycin (≤ 2 and 4–8 g/ml). Detection test and MIC test were tabulated in table no 8 and Table no8 & 9.

Isolates	Gram staining	Motility	Capsule	Catalase	Oxidase
1	-Ve rods	Motile	Capsulated	+	-
2	+ve	Non	Non	+	-

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	cocci	motile	capsulated		
3	-ve rod	motile	Non motile	+	+
4	-ve rod	Motile	+	+	-
5	-ve rod	+	-	+	-
6	-ve rod	+	-	_	+
7	-ve rod	+	-	_	+
8	-ve rod	-	--	+	-
9	-ve rod	Non motile	Non capsulated	-	-
10	-ve rod	Motile	+	+	-
11	-ve rod	+	-	+	-
12	-ve rod	Motile	+	+	-
13	-ve rod	+	_	+	-
14	-ve rod	+	-	+	-
15	-ve rod	motile	Non motile	+	+
16					
17	-ve rod	Motile	+	+	-
18	-ve rod	Motile	+	+	-
19	-ve rod	+	-	_	+
20	-ve rod	+	-	_	+
21	-ve rod	Motile	+	+	-
22	-ve rod	+	_	+	-
23	-ve rod	+	-	_	+
24	-ve rod	Motile	+	+	-

Table 5: The preliminary characteristics of all isolates.

3.7 Antibacterial efficacy of honey against bacteria

Honey has the potential to be utilized as an antibacterial agent to prevent and control infection with multidrug resistant bacteria, particularly pattern of tested

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Isolates	Indole	MR	VP	Citrate	Urease	Nitrate	TSI	Organism
1	-	-	-	-	-	+	A/A	*Escherichia blattae*
2	-	+	+	+	+	+	A/A	*Staphylococcus*
3	-	-	-	+	-	+	A/A	*Pseudomonas*
4	+	+	+	+	+	+	A/A	*K.oxytoca*
5	-	+	-	+	+	+	A/A	*Proteus*
6	+	-	+	+	-	+	A/A	*V.alginolyticus*
7	+	-	-	+	-	+	A/A	*Vibrio*
8	-	+	+	+	-	+	A/A	*Ewingellaamericana*
9	-	-	+	+	-	-	A/A	*Shigella*
10	-	+	-	+	+	+	A/A	*Klebsiella*
11	-	+	-	+	+	+	A/A	*Proteus mirabilis*
12	-	+	-	+	+	+	A/G	*Klebsiella*
13	-	+	+	+	+	+	A/A	*Enterobacter*
14	+	-	-	+	+	+	A/A	*Proteus vulgaris*
15	-	+	-	+	-	-	A/A	*Pseudomonas*
16	+	+	-	+	-	-	A/A	*Aeromonascaviae*
17	+	+	+	+	-	-	A/A	*Klebsiella*
18	+	+	+	+	+	+	A/A	*K .planticola*
19	+	+	+	+	-	+	A/A	*Vibrio*
20	-	+	-	+	-	+	A/A	*Vibrio*
21	+	+	+	+	+	+	A/G	*K .ozaenae*
22	-	-	+	+	+	-	A/A	*Enterobacter*
23	+	-	+	+	-	+	A/A	*Vibrio*
24	+	+	+	+	+	-	A/A	*Klebsiella*

Table 6: The biochemical results of isolates.

Amoxycillin		Chloramphenicol		Kanamycin		Vancomycin		Ampicillin		Penicillin	
NZ	Resistant	NZ	Resistant	6	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	8	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	15	Resistant	14	Resistant	16	Sensitive	NZ	Resistant	NZ	Resistant
10	Resistant	17	Resistant	19	Sensitive	15	Resistant	NZ	Resistant	27	Resistant
NZ	Resistant	24	Resistant	13	Resistant	17	Sensitive	NZ	Resistant	NZ	Resistant
NZ	Resistant	NZ	Resistant	8	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	10	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	12	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	NZ	Resistant	18	Resistant	6	Resistant	10	Resistant	16	Resistant
NZ	Resistant	20	Intermediate	8	Resistant	10	Resistant	NZ	Resistant	NZ	Resistant
NZ	Resistant	21	Intermediate	10	Resistant	15	Resistant	12	Resistant	15	Resistant
12	Resistant	NZ	Resistant	18	Sensitive	6	Resistant	10	Resistant	16	Resistant
23	Sensitive	20	Intermediate	NZ	Resistant	12	Resistant	20	Resistant	8	Resistant
NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant	NZ	Resistant

Table 7: The antimicrobial resistance pattern of the biofilm isolates.

vancomycin-resistant coagulase-negative staphylococci, according to the results of this study. The susceptibility

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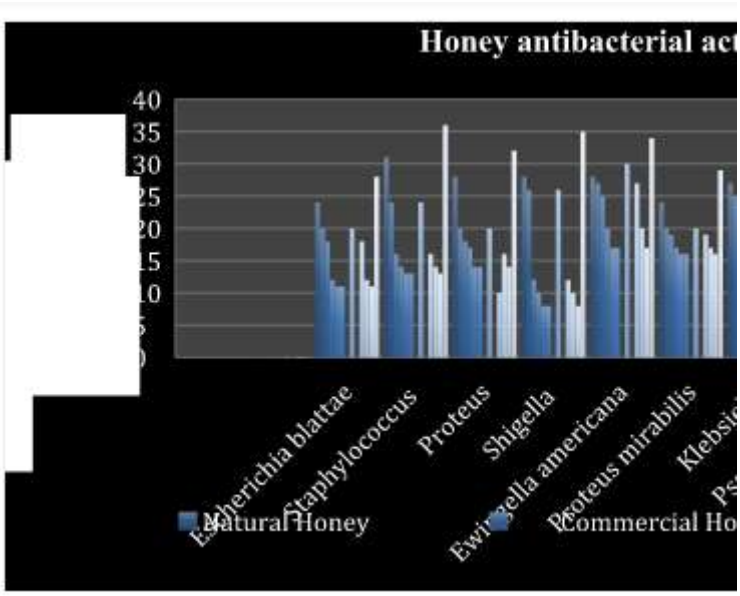

Fig 1: Graphical presentation of the result of the natural and commercial honey inhibitory activity.

S .No	BHI with vancomycin *S. aureus*	BHI with vancomycin in *Vibrio*.sp.(control)
1	Growth was observed	No growth

Table 8: The detection of vancomycin resistant S. aureus.

Concentration of antibiotics	Result	Control
≤ 2	No growth	No growth
4-8 µg/ml	No growth	No growth
≥16 µg/ml	Growth was observed	No growth
20 µg/ml	Growth was observed	No growth

Table 9: The vancomycin resistant strain against various concentrations.

organism results was illustrious in table no 10 and Fig.1. The 1:1 diluted honey showed the inhibitory activity towards all bacteria but the S.aureus showed a 29 nm in diameter zone of inhibition and it is a significant inhibitory activity followed by Proteus, Proteus mirabilis, Aeromonas caviae, Klebsiella, Vibrio sp, also showed inhibitory activity. The vancomycin resistant organisms Escherichia blattae, S.aureus, shigella, Ewingella americana, Klebsiella, Pseudomonas, Klebsiella planticola, Vibrio showed the inhibitory activity. In the above activity reveals that honey showed potential inhibitory activity on vancomycin resistance bacteria than the vancomycin sensitive bacteria. Commercial honey antibacterial activity assay showed less inhibitory when compared with natural honey**.** MIC experiment was performed in order to determine minimal concentration of honey. The assay revealed that tested honey has a significant MIC value against numerous pathogenic gram-positive and gram-negative bacteria as well as MDR isolates. Fig.2 contains an analysis of the inhibitory activity of both natural and commercial honey.

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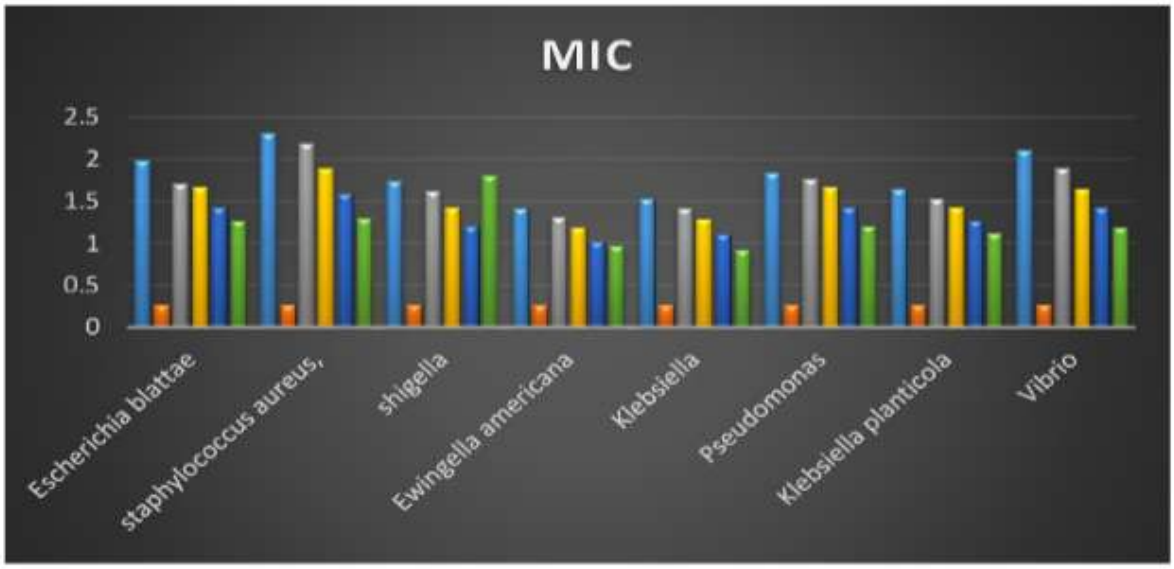

Fig 2: Graphical presentation of the result of the minimal inhibitory concentration of honey against different pathogens.

IV. DISCUSSION

Current antibiotic resistance conditions in wound bacterial pathogens present a significant issue. Bacterial species grow in the wound could be resisted all antibiotics. Since they can create a biofilm community, and inside this community, the bacteria start to naturally transfer genes among one another, there are two crucial factors that influence the drug resistance condition. Therefore the bacterial species acquire resistant property and also developed as multidrug resistant bacteria. Many commercial antibiotics are unsuccessful to prevent the growth of these bacteria. Hence we require a strong agent to destroy the antibiotic resistant bacteria. So this study focuses on the bactericidal effects of honey against multidrug-resistant bacteria. Previously much research work reported that the honey has efficient antimicrobial property but to know our knowledge first time the antimicrobial property against multidrug resistant of A.cerenia indica honey have been revealed through this research work.

Bacteria generally can fix it on the surfaces and build up biofilm. An extracellular polymeric substance (EPS) matrix is produced by bacteria that form biofilms, which can be separated from planktonic microorganisms. Biofilm forming ability of the bacteria would be assessed under laboratory conditions by qualitative tests. Among all bacterial isolates the efficient biofilm produces identified through Congo red and tube method. In Congo red method 21(87.5%) isolates were identified as biofilm producer and tube method only 18 (75%) isolates have identified.

In a recent study, Ruziicka et al. (2004) showed that out of 147 S. epidermidis isolates, TM identified biofilm formation in 79 (53.7%) isolates and CRA observed in 64 (43.5%) isolates. According to their study findings, TM is more effective method than CRA at detecting biofilms. In another research effort, Baqai et al. used TM to determine uropathogens that formed biofilms, and their findings showed that approximately 75% of the isolates exhibited the same. However, the current investigation found that the CRA method produced more biofilm isolates than the TM method. In this investigation, Knobloch et al. declined to recommend using the CRA approach to identify biofilms. As revealed by Vasudevan P. et al., 2003, TCP recognized 57.1% of the biofilm-producing bacteria among the 128 isolates of S. aureus, whereas CRA only detected 3.8% of them. Though the results of each work were different but the present study reveals that both methods are very useful to identify the efficient biofilm producer from wound sample. Thirteen efficient biofilm isolates have been identified based on the comparative accounts of these methods.

Strong biofilm producers showed a unique character i.e. antibiotic resistant property. Through this study thirteen drug resistant bacteria were isolated. Rodriguez-Banoetal., (2008) it has been previously noted that biofilm-forming isolates frequently develop antibiotic resistance, suggesting

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that these strains are less reliant on antimicrobial resistance than non-biofilm-forming pathogens for persistence.

Arciola et al., 2015 they developed a colorimetric scale for biofilm classification with six nuances. Koksal et al., 2009 found that CoNS (Coagulase-Negative Staphylococci) showed a high susceptibility to ciprofloxacin (52.72%) and resistance to gentamicin (14.5%). This indicates that CoNS are highly sensitive to ciprofloxacin but resistant to gentamicin. Palazzo et al.: They found that Vancomycin susceptibility was at 85.45%. However, it is now considered a last resort antibiotic due to the emergence of resistance in enterococci, S. aureus (Staphylococcus aureus), and coagulase-negative staphylococci. Qin et al.: They reported that Vancomycin had no effect on biofilm formation. This suggests that bacterial biofilm formation may serve as the first and possibly significant barrier in the development of multiple drug resistance. Overall, these findings highlight the importance of understanding biofilm formation and antibiotic susceptibility patterns in combating bacterial infections. It seems that there are challenges in treating certain bacterial infections due to the emergence of antibiotic resistance and the role of biofilm formation in providing a protective barrier.

Sushmita Roy et al., 2017 regarding wound infection and its bacterial pathogens. The study found that gram-positive bacteria showed high susceptibility to several antibiotics. Specifically, imipenem exhibited susceptibility in 90% of cases, followed by ceftriaxone at 85.5%, gentamycin at 81.8%, vancomycin at 80.8%, azithromycin at 76.5%, and other antibiotics at 75.0%.Regarding Gram-negative isolates, ceftazidime showed effectiveness against 79.0% of cases, followed by ceftriaxone at 71.8%, gentamicin at 70.7%, and other antibiotics at 70.0%.In the current investigation, the sensitivity pattern was revealed. Bacterial pathogens exhibited 76% resistance to vancomycin and 84.6% resistance to Gentamicin, Oxytetracycline, and Kanamycin. Amoxicillin and erythromycin resistance was present in 92%. Tetracycline and chloramphenicol both demonstrated 76.9% bacterial resistance. Eight (61.5) isolates of the thirteen biofilm bacteria were antibiotic-resistant across the board.

Vancomycin has historically been recognized as a last-resort antibiotic for infections caused by multidrug-resistant staphylococci. Fortunately vancomycin resistance initially appeared in enterococci and since then has spread to S. aureus and coagulase-negative staphylococci. Present study isolated and identified vancomycin resistant S. aureus and it's a coagulase-negative bacterium from wound sample and based on concentration gradient of vancomycin antibiotic**.** Vancomycin susceptibility was reported to be 85% by Palazzo et al. in 2005, although in some experiments it was 100%. Ethiopian researchers Getaneh et al. (2013) evaluated the antibacterial activity of Ethiopian multiflora honey against Vancomycin resistant S. aureus (VRSA) in vitro. According to Qin et al. (2009), Vancomycin showed no impact upon the development of the bacterial biofilm, suggesting that this may be the first and most significant barrier to the development of drug resistance to numerous medicines. S. aureus is thus going to serve as a template bacterium to investigate the mechanisms underlying VRSA biofilm development.

A.cerenia indica honey was diluted at different concentration such as 1:1, 1:2, 1:3, 1:4 and 1:8 among all concentration 1:1 showed major inhibitory activity than other dilutions. Other diluted concentration also showed suitability pattern but zone measurement was lower than 1:1 concentration. Results for the antibacterial activity of honey from A.cerenia indica revealed that the honey gathered during the particular season inhibited bacteria that were multi-drug resistant. Numerous studies have verified that honey has antibacterial properties against both Gram negative and Gram positive microorganisms. [2,14].However, the majority of these studies used honey produced by Apis mellifera bees, and only a small number of research studies reported about A. cerena indica honey . Therefore the present study focused on the investigation of A.cerenia indica honey antibacterial activity on multidrug resistant bacteria. Throughout both the wet and dry seasons, M. compressipes manaosensis honey showed stronger antibacterial activity, inhibiting a total of five among six bacteria at various dilution rates. Antibacterial activity is influenced by the season. Antibiotic-resistant organisms like MRSA and MDR are effectively combated by the antibacterial properties of honey. Further studies are needed to determine local honey kill kinetics and in vivo applications, as unpredictable antibacterial activity may hinder their introduction. The osmotic effect brought about by honey's sugar content underlies its antibacterial effectiveness. The differences in susceptibility may be related to the pathogen's distinct rates of growth and reduced cell wall permeation, nutritional needs, humidity, the

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inoculum sizes, and various types of honeys, as well as the test method employed.

The findings of this study conclusively demonstrate that honey could have the ability to be used as an antibacterial agent in order to eradicate coagulase-negative staphylococci infection. As a result, honey would be an appropriate active for both therapeutic and preventative applications. The use of honey to the tissue that is damaged surrounding implanted devices has additional benefits. Serous exudates, which could serve as an ideal environment for bacteria, can be avoided by its anti-inflammatory effect. Additionally, its physical characteristics produce moist conditions suitable for healing and have a stimulatory effect on the formation of tissues for wound repair. Additionally, compared to other antiseptics, the substance has no adverse impact on tissues due to the slow enzymatic synthesis of the compound hydrogen peroxide, which results in a hydrogen peroxide solution with a 3% concentration of roughly a thousandth of that amount.

Using the broth micro dilution method, it was found that A. cerena indica honey inhibited the growth of bacteria at lower concentrations (25µl/ml) and against other strains, suggesting that the honey has antimicrobial properties and consists of elements or compounds that may prevent bacterial growth or eliminate it. However the present study suggested that since even low concentration of honey exhibited antibacterial activity against multidrug resistant and biofilm forming bacteria which indicating a broad-spectrum effect and property of honey.

V. CONCLUSION

It is widely acknowledged that honey has antibacterial properties and they are attributed to various factors, including its high sugar content, low water activity and the abundance of biologically active substances such enzymes, phenolic compounds, and hydrogen peroxide. These components can disrupt the bacterial cell membranes, inhibit enzymes essential for bacterial growth, and generate reactive oxygen species that are toxic to bacteria. It's crucial for one to comprehend that honey's antibacterial properties may fluctuate based on its botanical source and geographic region. Different types of honey may exhibit varying levels of antimicrobial potency. Therefore, the specific antimicrobial effects observed with A.cerenia indica honey may not be generalized to all types of honey. Since A.cerenia indica honey showed a board spectrum activity further this honey property and its mechanism evaluated in detail and much more investigation analysis is need to be addressed and then A.cerenia indica used as potential therapeutic agent specially resistant bacteria species present in diabetic wound.

SOME OF THE ADVANAGES FROM THE ABOVE RESULTS

a) A study in India found that traditional medicinal honey from A. cerena indica bees can effectively combat diabetes-related wounds, with 21 species producing biofilms out of the total number.

b)The study revealed 13 isolates producing biofilms, with ten showing vancomycin resistance, eleven showing Gentamicin, Oxytetracycline, Kanamycin, erythromycin, amoxicillin, tetracycline, Chloramphenicol resistance, and eight showing antibiotic resistance.

c) Honey shows potential bactericidal activity against various bacteria, including S. aureus, Proteus, Proteus mirabilis, Aeromonas caviae, Klebsiella, and Vibrio sp. with significant growth observed on vancomycin-resistant coagulase-negative S. aureus.

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