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"To Study the Isolation, Identification of Bacteria from Wound Infection and Antimicrobial Activity of Honey Against It"

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Abstract:
Honey possess diverse range of applications in medicine because of its antioxidant, antimicrobial and anti-inflammatory activities. Honey gains more significance due to the antibacterial effect shown by it against various pathogenic species of bacteria which are of major concern to human health. To check the antimicrobial activity shown by honey, standard method of well diffusion technique was followed. Along with WDT, Minimum Inhibitory Concentration (MIC) was performed. Various pathogenic bacteria (Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus spp. and Salmonella spp.) isolated from clinical samples like wound, blood and pus, were subjected to different concentrations (dilutions 10% -100%) of honey. This helps us to select an appropriate concentration of honey, which will show maximum antibacterial activity. S.aureus and Pseudomonas show maximum zone of inhibition at all dilutions of honey followed by E. coli, Proteus spp. and Salmonella spp. The antibacterial effect increase along with increase in concentration of dilution.
Keywords: *Honey, Well diffusion, Antimicrobial activity, MIC, Wound infection.*

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I. Introduction:

Honey is sweet, thick form of liquid made by bees (Apis mellifera) from the nectar of the flowers. Honey is complex, sweet ailment with well-established antimicrobial and anti-oxidant properties. Inadequate wound management compounded with secondary infection is still remaining a major public health problem in developing countries. Therefore, wound management has remained as research focus so far. Because of increased drug resistance, the interest in using alternative therapies and natural remedies in wound management has rapidly increased (H. Maghsoudi et al., 2011).The antibacterial activity of honey was first recognized in 1892; however, it has a limited use in modern medicine due to lack of scientific support. Honey is the nectar collected from flowers by bees. It contains 15% to 20% water and 80% to 85% sugar. The remainder of the honey is made up of proteins, enzymes, and nonessential amino acids. Several properties of honey like enzymes are responsible for its bactericidal effect and wound healing. (D. Mohapatra., 2011)

Honey is composed of approximately 82.4% total carbohydrates which include Mainly 38.5% fructose and 31.5% glucose. Bacterial resistance is less likely to develop as a result of treatment of bacteria with honey this is because of the composition of honey which contain a number of different component Gram negative organisms such as Pseudomonas aeruginosa have been a major problem in hospital acquired infection and caused most severe wound and burn infections. (Alaa A. M. Al-Nahari et al, 2015)

Biotic resistant bacteria possess a very serious threat to public health. There is a large variation in the antimicrobial activity of honey collected from natural environments, which is a concern from the view of clinical applications. The first observations of the antimicrobial activity of honey were made in 1892, and since then honey has been observed to have a broad spectrum of activity, and inhibiting both Gram positive and Gram negative organisms including is 32, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus, Salmonella enterica serovar typhi* (Victoria C. Nolan, *et al,*. 2019) . Wound infection plays an important role in the development of chronicity, delaying wound healing. Lucinda J Bessa *et al,*. 2015, Researched on identification of bacterial pathogens present in infected wounds and characterize their resistance profile to the most common antibiotics used in therapy most common bacterial species detected were *Staphylococcus aureus* (37%),, followed by *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%). Based on Piotr Szweda et al,. (2015) experience, they would rather recommend a serial dilution method for investigation of antimicrobial potential of honey this method allows quantitative determination of both bacteriostatic and bactericidal activity of tested honey dilutions. The bacteriostatic activity is characterized with MIC i.e., Minimal Inhibitory Concentration

(lowest concentration of honey that inhibits the growth of tested strains of microorganisms) parameter, while bactericidal activity is characterized with MBC (Minimal Bactericidal Concentration, the lowest concentration of an antibacterial agent required to kill a particular bacterium).

II. Materials and Methods:

Sample collection:

The sample were taken from the patients visiting private clinics, pathological labs and Government Medical College, Akola., after taking informed consent form all patients. Total 52 samples were collected. Patients with diverse type of wounds were included in the study. These samples were collected before the patient undergo treatment with antibiotics. The samples were collected

- By swabbing the surface of an infected wound by sterile swab.
- By collecting pus sample
- By collecting blood from the site of injury

Collection of honey:

Honey sample collected from local market in Satav sq. Akola. The processed honey was used for the study. The honey was sterilized in autoclave at 120°C for 10 mins and then it was streaked on nutrient agar plate to check any contamination left. The pH of honey was checked under the working condition and then stored at 4°C until use.

Isolation:

The collected samples (blood, pus and wound swab) were inoculated on the nutrient media. Nutrient media plates were made by adding 28gm of nutrient agar in 1000 ml of distilled water. The media was sterilized in autoclave along with petri plates at 121°C for 15 min. After autoclaving, the media was cooled and poured in the plates. The plates were kept to solidify, later the samples were inoculated. The inoculated plates were kept for incubation for 37°C for 24 hrs. The growth was observed. The colonies appeared on the plate were categorized on the basis of the colony characters and microscopy. These selected colonies were then inoculated on selective and differential media (Mannitol Salt Agar, Eosin Methylene Blue agar, CLED agar, Cetrimide Agar and Bismuth Sulphite Agar). These plates were incubated at 37°C for 24 hrs.

Identification:

The colonies appeared on the selective media were subjected to biochemical tests including IMViC test, Sugar fermentation (Glucose, Fructose, Lactose and Mannitol) and Enzyme tests (Amylase, Oxidase, Catalase, De-sulphurase and Urease). The outcome of these tests were checked out of *Bergey's Manual of Systemic Bacteriology.*

Dilutions of honey:

The honey which was collected and sterilized is then mixed with distilled water to prepare series of dilutions to form different concentrations such as 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%.

Antimicrobial activity of Honey:

The cultivated organisms on the selective media were subjected to inoculation in liquid media i.e., Nutrient broth. The nutrient broth was made by adding 13 gm of nutrient broth in 1000 ml distilled water. Autoclaved the media at 121°C for 15 min. The lawn was prepared through the liquid media using the sterile cotton swab. The well diffusion method was followed to check the antimicrobial activity of honey. The wells were made using sterilized borer. The different concentration of honey was poured in the well and then the plates were allowed to incubate at 37°C for 24 hrs.

Minimum Inhibitory Concentration (MIC):

Honey dilutions were prepared in the series such as 20%, 40%, 60%, 80%, 100%. Minimum inhibitory concentration was determined among all these concentration. The isolates were separately grown in nutrient broth supplement with different concentration of honey. The tubes were incubated at 37°C for 24 hrs. The minimum inhibitory concentration was determined by measuring the optical density in the spectrophometer at 620 nm.

Antibiotic Susceptibility profile:

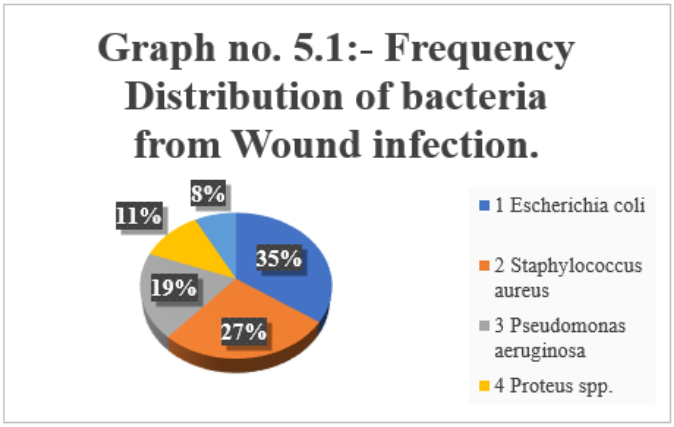
The isolates were subjected to the disc diffusion method to check the antibiotic sensitivity test against the commonly available antibiotics. The nutrient broth was prepared by adding 13 gm of nutrient broth in 1000 ml distil water. Autoclaved the broth at 121°C for 15 min and inoculate the broth tubes with the loop full culture of the isolates in sterile condition. Incubate the tubes over-night. Nutrient agar plates were prepared by adding 28gm of nutrient agar in 1000 ml of distilled water. It was autoclaved at 121°C for 15 min. After the media gets

cool, it is poured in the plates. The solidified plates were inoculated with the overnight incubated nutrient broth. Lawn of the isolates were prepared on the plates. The antibiotic discs were placed on the plates keeping equal distance between them. The plates were kept for incubation at 37°C for 24 hrs. Zone of inhibition was observed, measured and recorded.

III. Results and Discussion:

Sample collection:-

The various type of clinical samples from wound infection were collected i.e., blood, pus and wound. Among these samples, 22 were blood samples, 17 were pus samples and 13 wound samples. In total 52 samples were collected. Out of 52 collected samples 33 samples were found to be positive for bacteria from wound infection. From these positive samples 26 different kinds of isolates were found. The frequency distribution of total 26 isolates, out of which total 5 bacteria isolated and encoded as Isolate 1, Isolate 2, Isolate 3, Isolate 4 and Isolate 5.



Isolation:-

All of the above-mentioned samples were collected from different private hospitals, pathological labs and Government Medical College of Akola. Some of the bacteria were isolated by using Nutrient Agar media. It is the general medium which is suitable for cultivation of wide variety of microorganisms.Out of all inoculated plates, 19 plates showed no growth of any microorganism. While the rest of the plates i.e., 33 plates showed positive growth of organisms. Among those 33 positive growth of inoculated sample, five different colonies were selected and their morphological and biochemical tests were done to identify the organism. These colonies were selected on the basis of the colony characters. The selected colonies were labelled as Isolate 1, Isolate 2, Isolate3, Isolate 4, and Isolate 5. Each isolate was separately inoculated on the nutrient agar plate for pure growth of the isolate.

Identification of bacteria:-

Colony morphology and Gram characters

Table 5.2:- Colony morphology and Gram characters

sr. no.	Colony characters	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
1	Size	2 - 4 mm	1-2 mm	Large	1-2 mm	2-3 mm
2	Shape	Circular	circular	Circular	Circular	Circular
3	Margin	Undulated	Entire	Entire	Irregular	Entire
4	Elevation	slightly raised	Convex	Umbonated	Convex / effuse	Convex
5	Colour	White	Golden yellow	Green	Greyish white or pale	Jet black or brown
6	Opacity	Translucent	Opaque	Opaque	Translucent	Opaque
7	Texture	Smooth	Smooth and sticky	Smooth	Glistening	Smooth
8	Motility	Motile	Non motile	Motile	Motile	Motile
9	Gram character	Gram Negative (short rod)	Gram Positive	Gram Negative (rod)	Gram Negative	Gram Negative
10	Organism identified as	*E. coli*	*S. aureus*	*Pseudomonas aeruginosa*	*Proteus spp.*	*Salmonella spp.*

All the selected isolates were inoculated on selective media like EMB, MSA, C.L.E.D., BSA and Cetrimide agar.

Table 5.3:- IMViC test

IMViC Test						
Sr. no	**IMViC**	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
1	Indole	positive	negative	negative	positive	negative
2	Methyl Red	positive	negative	negative	positive	positive
3	Voges Proskauer	negative	positive	negative	negative	negative
4	Citrate	negative	negative	positive	negative	positive

Table 5.4:- Sugar fermentation test

SUGAR FERMENTATION						
Sr. no.	**Sugar test**	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
1	Glucose					
	Acid	Positive	Positive	negative	Positive	Positive
	Gas	Positive	Negative	negative	Positive	Positive
2	Fructose					
	Acid	Positive	Positive	negative	Positive	Positive
	Gas	Positive	Negative	negative	Positive	Positive
3	lactose					
	Acid	Positive	Positive	negative	negative	negative
	Gas	Positive	Negative	negative	negative	negative
4	Mannitol					
	Acid	Positive	Positive	Positive	Positive	Positive
	Gas	Positive	Negative	Positive	Positive	Positive

Table 5.5:- Enzyme test

ENZYME TEST						
Sr. No	**Enzymes**	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
1	Amylase	Negative	Positive	Positive	Positive	Negative
2	Oxidase	Negative	Negative	Positive	Negative	Negative
3	Catalase	Positive	Positive	Positive	Positive	Positive
4	Desulphurase	Positive	Positive	Negative	Positive	Positive
5	Urease	Negative	Positive	Negative	Positive	Negative

Antimicrobial activity of Honey:

The table below shows the zone of inhibition shown by isolates against various dilutions of honey:

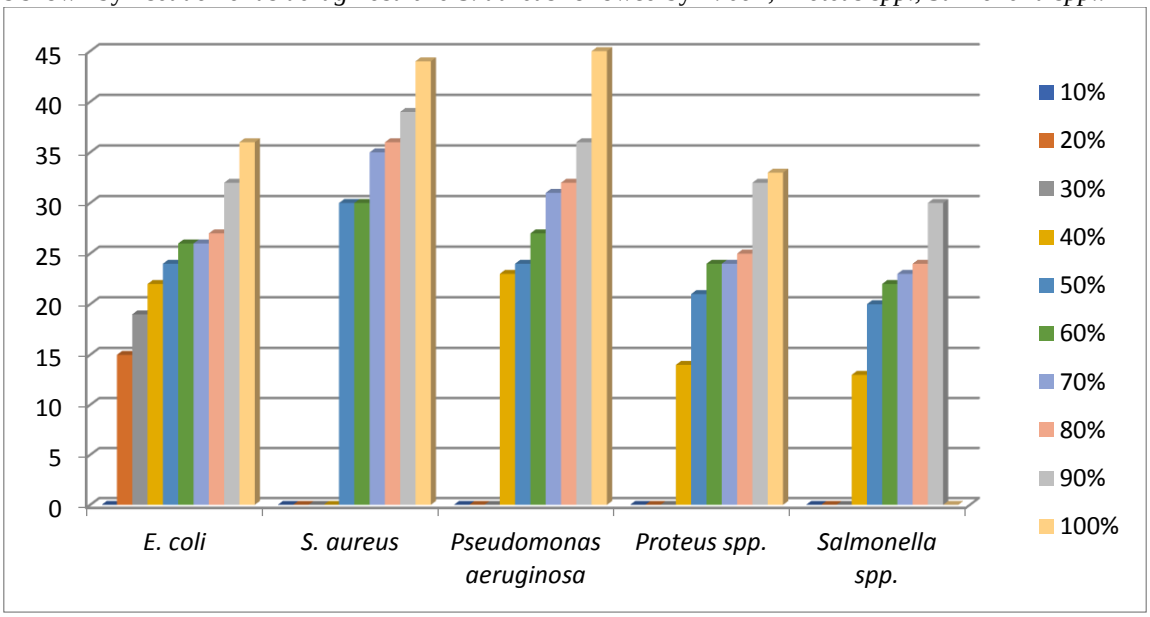
Table 5.6:- Antimicrobial activity of honey

Concentration of honey	***E. coli***	***S. aureus***	***Pseudomonas aeruginosa***	***Proteus spp.***	***Salmonella spp.***
10%	-	-	-	-	-
20%	**15mm**	-	-	-	-
30%	19mm	-	-	-	-
40%	22mm	-	23mm	**14mm**	**13mm**
50%	24mm	30mm	24mm	21mm	20mm
60%	26mm	30mm	27mm	24mm	22mm

70%	26mm	35mm	31mm	24mm	23mm
80%	27mm	36mm	32mm	25mm	24mm
90%	32mm	**39mm**	**36mm**	32mm	30mm
100%	**36mm**	**44mm**	**45mm**	33mm	30mm

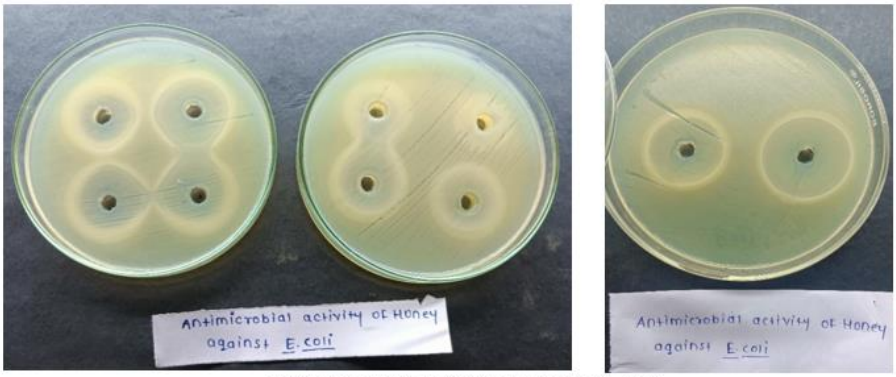
The isolated and identified microorganisms were then checked for their antimicrobial activity pattern against honey. Honey exhibited the antimicrobial activity against *Escherichia coli* by showing zone of inhibition at 20% (15mm), 30% (19mm), 40% (22mm), 50% (24mm), 60% (26mm), 80% (27mm), 90% (32mm), 100% (36mm). Similarly for *Staphylococcus aureus*, upto 40% concentration of honey doesn't showed any zone of inhibition, while at 50%(30mm), 60% (30mm), 70% (35mm), 80% (36mm), 90% (39mm), 100% (44mm). Likewise, *Pseudomonas aeruginosa* inhibited by Honey having 40% concentration of Honey by showing zone of inhibition (23mm), at 50% (24mm), 60% (27mm), 70% (31mm), 80% (32mm), 90% (36mm), 100% (45mm). Honey showed zone of inhibition against *Proteus spp.* at 10%, 20%, 30% is zero, but at 40% (14mm), 50% (21mm), 60% (24mm), 70% (24mm), 80% (25mm), 90% (32mm), 100% (33mm). *Salmonella* regulated by Honey dilutions from 40% of concentration (13mm), 50% (20mm), 60% (22mm), 70% (23mm), 80% (24mm), 90% (30mm), 100% (30mm).

The isolated and identified microorganisms were then checked for their antimicrobial activity pattern against honey. The inoculated plates of isolates were subjected to various dilutions of honey. Well diffusion method was used for this process. After the incubation of the plates at 37°C for 24 hrs., zone of inhibition appeared. These zone of inhibition were observed, measured and recorded. 10% concentration of honey showed no zone of inhibition against any isolate. While 20% and 30% concentration of honey showed zone of inhibition against *E. coli.* Rest of the concentrations were prominently effective against all isolates. The maximum zone of inhibition was shown by *Pseudomonas aeruginosa* and *S. aureus* followed by *E. coli, Proteus spp., Salmonella spp.*.



Graph 5.2:- Antimicrobial activity of honey

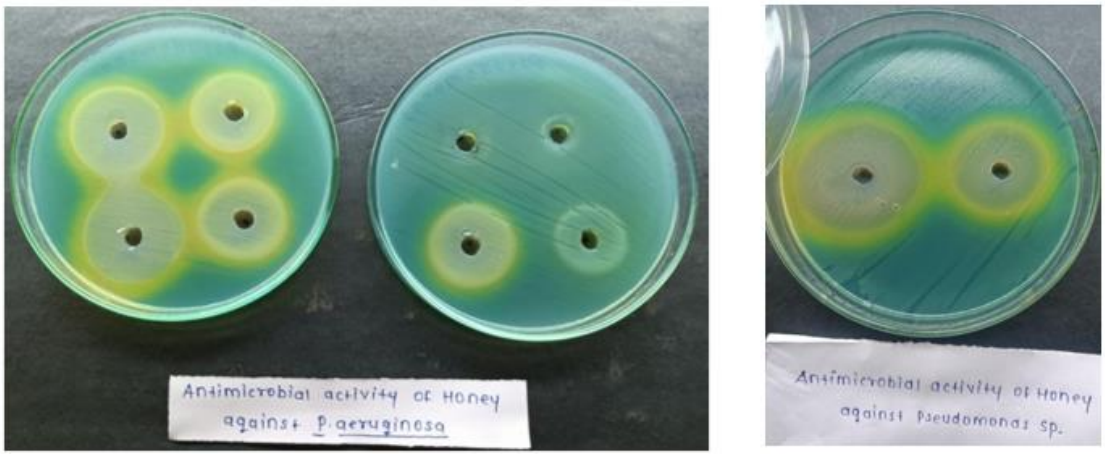
ANTIMICROBIAL ACTIVITY OF HONEY



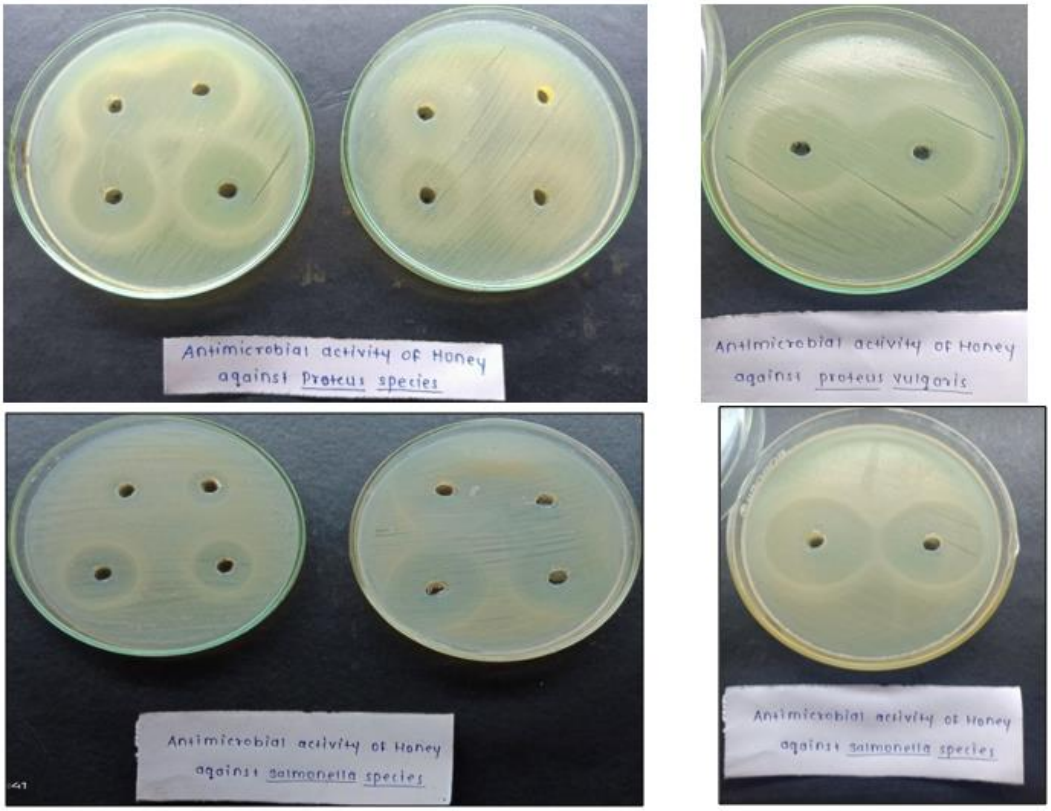
Antimicrobial activity against *E. coli*

Antimicrobial activity against *S. aureus*

Antimicrobial activity against *Pseudomonas aeruginosa*



Antimicrobial activity against *Proteus spp.*



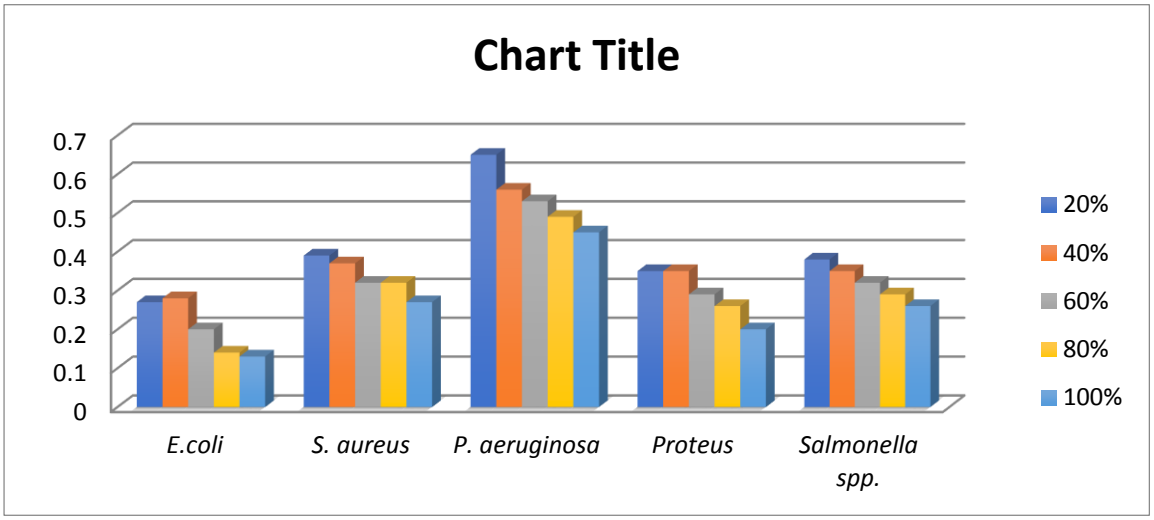
Antimicrobial activity against *Salmonella spp.*

Minimal Inhibitory Concentration:

Minimum Inhibitory Concentration (MIC) was done to evaluate in-vitro levels of susceptibility or resistance of isolated microorganisms against honey.

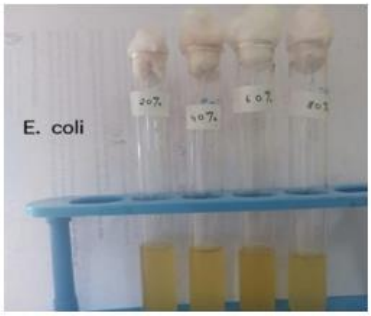
Table 5.8:- MIC of honey against selected isolates.

Minimal Inhibitory Concentration (MIC)						
Sr. no	**Honey Dilution**	***E. coli***	***S. aureus***	***Pseudomonas aeruginosa***	***Proteus spp.***	***Salmonella spp.***
1	20	0.35	0.39	0.65	0.35	0.38
2	40	0.28	0.37	0.56	0.35	0.35
3	60	0.20	0.33	0.50	0.29	0.32
4	80	0.14	0.32	0.39	0.26	0.29
5	100	0.13	0.26	0.27	0.2	0.26



The Minimum Inhibitory Concentration (MIC) of honey for all isolates examined. Dilutions were in the range of 20-100%. The MICs of honey against *Pseudomonas aeruginosa* were found nearly similar to those of *S. aureus.* In Minimal Inhibitory Concentration, the maximum microbial growth was observed in Honey (OD 620 : 0.65) at 20% concentration against *Pseudomonas aeruginosa*. The minimum microbial growth was observed in Honey (OD 620 : 0.13) at 100% concentration against *Escherichia coli.*

MINIMAL INHIBITORY CONCENTRATION OF HONEY (MIC) :

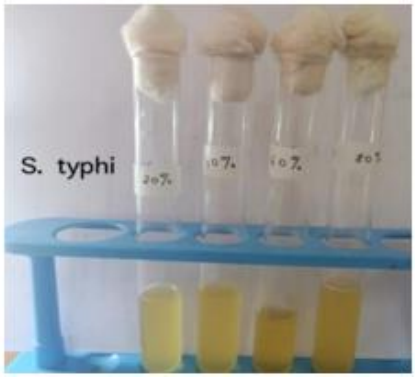


E. coli

S. aureus

Pseudomonas aeruginosa

Proteus spp.



Salmonella spp.

IV. Discussion:

The result demonstrated that the collected honey has potent antimicrobial activity against isolated bacteria from wound infections. There are many similar reports of antimicrobial activity of honey showed by Noori S. Al-Waili in 2004. Ahmed G. Hegazi *et al.*, (2017) performed an experiment where the Honey obtained from Izmir proved more effective as inhibitors against *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus.* Similar results also found in the present study.

By T. Selvamohan *et al.,* (2016), the inhibitory effect of honey sample was higher in *Escherichia coli* followed by *Staphylococcus aureus.* While in present study, the antimicrobial activity of honey exhibited the maximum zone of inhibition against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus.* Present study supports previous investigation that the low concentration of honey (10%) have no impact on antimicrobial activity. (Oinaala *et al.,* 2015)

Effect of conventionally produced honey against pathogenic bacteria has been scientifically demonstrated meanwhile, the present study staring at the antimicrobial activity of processed honey against wound infection causing bacteria. This study shows that commercially available processed honey possesses antimicrobial activity against various remarkable bacterial pathogens. This honey carry potential for consumer use with anti-infective preventative impact. It was similarly studied by Jackie K. Obey *et al., (2022)*

Finally it conclude that the variation in the activity of different dilutions of honey were assigned to some factors have an effect on antimicrobial activity like pH and its osmotic properties. These similar assumptions were found by Ahmed G. Hegazi *et al.,* (2022)

V. Conclusion:

Honey produced from some botanical sources exhibits high antimicrobial activity. Possibilities of application of this product for treatment on not only wound infections but also the other infections in clinical practice should be the subject of intensive investigations in the near future. Except of high activity, the most important advantages of this product are as follows:

- Lack of side effects for patients (important drawback of antibiotics);
- Low costs of therapies;
- The honey provides the body of the patient many health-promoting components, for example, antioxidants, microelements, trace elements and vitamins.
- Sustain a moist environment to the injury.
- Enhance tissue regeneration at the site of wound infection.

Summarizing we have no doubt that honey is an interesting and promising alternative to classical antibiotics and should be more seriously considered as therapeutic agents.

Acknowledgement:

I would like to express the gratitude and give a special thanks to my supervisor Dr. Monika S. Thakare, Assistant professor, Department of Microbiology, Shri. Shivaji College of Arts, Commerce and Science, for her valuable advice and making this work possible.

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