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Original Research Article

Assessment of antimicrobial activity of *Meliponula ferrugica* in pathogenic wound samples

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ABSTRACT

Background: Honey is a naturally occurring sweet material that bees make from nectar from flowers, secretions from plant parts, or excretions from plants that suck insects from plant parts. Honey's antibacterial and antifungal qualities are widely known, and it has been used to heal burns, surgical wounds, and decubitus ulcers. Honey instantly sterilizes wounds affected by *Staphylococcus aureus*. The honey's ability to fight bacteria can be ascertained by the nectar's origin. Many antibiotic resistances among the bacteria that cause infections in humans have directly evolved as a result of the use of antibiotics in clinical practice.
Methods: This study found that Bergey's manual of determinative bacteriology can be used to identify *Pseudomonas aeruginosa* and *Staphylococcus aureus*. According to the study's findings, honey has a capacity to neutralise *Pseudomonas aeruginosa* and *Staphylococcus aureus* that have been isolated from infected wounds. Honey's antibacterial characteristics account for a significant portion of its antibacterial activity.
Results: *Staphylococcus aureus* and *Pseudomonas aeruginosa* were both susceptible to the antibacterial activity of the honey sample. *Pseudomonas aeruginosa* exhibited lower activity compared to *Staphylococcus aureus*.
Conclusions: The results of this investigation show that Bergey's handbook of determinative bacteriology can be used to identify *Staphylococcus aureus* and *Pseudomonas aeruginosa*. According to the study's findings, honey has the ability to neutralise *Pseudomonas aeruginosa* and *Staphylococcus aureus* that have been isolated from infected wounds.

Keywords: Antibacterial, Antifungal, Bactericidal, Bacteriostatic, Honey, Human infection, Wounds

INTRODUCTION

Honey is a naturally occurring sweet material that bees make from nectar from flowers, secretions from plant parts, or excretions from plants that suck insects from plant parts. Bees gather this material, modify it, combine it with other substances, and store it in the honeycomb until it is ripe and mature.[1] It also utilises floral nectar to create honey, a viscous, sweet liquid. Water, glucose, fructose, proteins, vitamins, and minerals are all contained.[2] However, the effect of honey on more pathogenic organisms, toxicological investigations, and further purifications, need to be carried out.

Apis ceranaindica and *Apis mellifera*, gathered by contemporary extraction techniques in apiaries, are known as apiary honey. Their surfaces are translucent and free of any external substances. In contrast, forest honey is acquired through the difficult procedure of squeezing the comb of the *Apis dorsata* rock bee or the *Apis ceranaindica* wild nest in forests.[3]

Honey's antibacterial and antifungal qualities are widely known, and it has been used to heal burns, surgical wounds, and decubitus ulcers.[4] A variety of bacteria, both Gram-positive and Gram-negative, are susceptible to the antibacterial activity of honey. Honey's inhibition is primarily responsible for its antibacterial properties.[5] Antimicrobial substances play an essential part in minimizing the worldwide epidemic of infectious illnesses. However, the effectiveness of the antibiotics diminishes when organisms that are resistant grow and proliferate. The public's health is seriously threatened by this kind of bacterial resistance to antibiotics.[6]

The majority of ancient cultures have used honey for nutritional and therapeutic purposes. The theory behind apitherapy, an area of alternative medicine, is that honey has medicinal qualities and can be applied externally, as an ointment, or as a dietary supplement.[7]

Wound infection is a very unpleasant condition because of the associated mortality and morbidity, extended hospital admissions, intense discomfort, and an immediate increase in healthcare expenses. A wound's infection can impede its healing process and result in wound breakdown, herniation, and total dehiscence.[8] The most common pathogens identified as the cause of delayed wound healing are aerobic ones, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and beta hemolytic streptococci.[9]

Honey sterilises *Staphylococcus aureus*-infected wounds fast. There are various kinds of honey, depending on where the nectar used to make it came from. The source of the nectar influences honey's antimicrobial capacity.[10] Many antibiotic resistances among the bacteria that cause infections in humans have directly evolved as a result of the widespread use of antibiotics in clinical practice.[11] Scientists have identified naturally occurring sources of non-antibiotic drugs with antibacterial potential, such as medicinal plants, to combat antibiotic resistance.[12] Honey has been demonstrated to have antibacterial effect against a range of potentially fatal pathogens, in addition to medicinal plants.[13] Tests were conducted on the efficacy of 13 different honey samples, including three commercially available antibacterial honeys, against *Pseudomonas aeruginosa* and *Escherichia coli*.[14] Studies have indicated that honey has the ability to inhibit gram-positive and gram-negative pathogens through its antifungal, bacteriostatic, and bactericidal characteristics.[15] Clinical isolates of *Salmonella enterica serovar Typhi*, *P. aeruginosa*, and minimum bactericidal concentrations (MBC) and minimum inhibitory concentrations (MIC) of honey for *Escherichia coli* range from 0.625 to 5.000 mg/ml.[16] Honey was shown to have a minimum inhibitory concentration (MIC) of 11% for isolates of Pseudomonas. Moreover, honey is among the oldest home cures for a wide range of illnesses, including gastrointestinal infections and respiratory problems. It works well as a dressing to quickly lessen discomfort and odour from burns, wounds (including surgical wounds), and skin ulcers.

METHODS

Collection of samples

Bee keepers in the Thiruvananthapuram district's Parassala panchayath provided the honey sample (*Meliponula ferruginca*) used in the study. After being aseptically collected in sterile screw-capped tubes, the honey samples were brought to the lab for additional processing.

Preparation of extract

Separate samples of honey were taken and placed in airtight bottles. 50 ml of each of the following solvents- acetone, methanol, ethanol, and hexane- were then added, and the bottles were stored in the dark. After two days, the substances were well combined and filtered using Whatman No. 1 filter paper. For additional research, the filtrate was gathered and kept in sterile glass beakers.

Confirmatory test of sample

To a glass of warm water, add a teaspoon of honey and slowly mix. This was useful in figuring out whether it dissolved in the water. The majority of raw honey sinks as a solid lump or stays stuck as a lump on the spoon after adhering together. A candle wick soaked in honey was placed over a fire to check for any more water that would have stopped the honey from burning.

Collection of test sample

The pathogenic wound samples were collected from SP Multispecality Hospital, Parasala, Trivandrum and the samples were analysed for a period of June 2022 to April 2023. Following that, the samples were sub-cultured in nutritious broth and incubated for 24 hours at 37°C.

Separation and detection

A nutrient agar plate was inoculated with samples obtained from swabs containing pus from patients. After that, the plate was incubated at 37°C for 24 hours. Subculturing allowed for the pure isolation of each colony for upcoming research and identification. Each pure culture was found to have distinct morphological characteristics, including colony morphology, colony elevation, and colony margin. The pus and swab samples were inoculated on cetrimide agar and mannitol salt agar (MSA), respectively, in order to isolate *Staphylococcus aureus* and *Pseudomonas aeruginosa* with selectivity.

Microscopic examination

A loopful of culture was applied on a sanitised glass slide, heat-fixed, and then inspected using an oil immersion

objective microscope, along with a motility test, using Gram's staining procedure.

Confirmatory test using biochemistry

Berger's manual of determinative bacteriology was followed in the identification of the bacteria, which was done using biochemical tests and macroscopic and microscopic examination.

Test for Indole

Tryptophan broth was added to the test culture, and following that, it was incubated at 37°C for 24 hours. All culture tubes were given a few drops of Kovac's reagent after incubation, but none of them produced a cherry red layer, which would have indicated an unfavourable result.

Test for methyl red

The test cultures were inoculated into MR-VP broth and then cultivated for 24 hours at 37°C. A couple of drops of methyl red indicator were applied to the test tube following the incubation period. Results that are positive are indicated by the reddening of methyl red at a pH of 4. At pH 6, the indicator becomes yellow and yields a negative result, still indicating the presence of acid but with a reduced concentration of hydrogen ions. This test gives the negative results with a yellow colour.

Test for Voges-Proskauer

This test shows if some organisms can convert the organic acids that come from the metabolism of glucose into neutral or non-acidic end products, like acetyl methyl carbine. Following injection with MR-VP broth, the test cultures were incubated at 37°C for a duration of 24 hours. After the tubes were incubated, a few drops of Barrit's reagent were added, and they were let to stand for fifteen minutes. Positive results are indicated by a pink hue, and negative results are indicated by no colour change. This test yields negative findings and exhibits no colour change.

Test of citrate utilization

Certain microbes can use citrate as a carbon source for energy when fermentable glucose or lactose is present. This capacity is reliant on the existence of a citrate permease, which makes citrate easier to move throughout the cell. Simmon citrate agar was streaked with the test organism, and it was incubated at 37°C for the whole day. Following incubation, a blue hue that denotes citrate positivity is present along with development on the slant's surface. The medium will stay green in negative cultures and they will not grow.

This test indicates that there is growth present on the slant's surface, along with a shade of blue. Declare the outcome to be positive.

Test for oxidase

The oxidase test examines the presence of oxidised discs. The discs were placed on a glass slide that had been cleaned, and a culture loop was placed over them. The outcomes were recorded after a short period of time.

Test for catalase

Catalase-producing organisms break down hydrogen peroxide quickly into oxygen and water. We added 3% hydrogen peroxide to test cultures. There is no information on free oxygen gas bubbles in the positive results. The lack of bubble development is an unfavourable outcome. It produces a bubble in this test and shows that the results are positive.

Test for urease

A hydrolytic enzyme called urease breaks down the nitrogen and carbon bonds in amide compounds like urea to produce the alkaline byproduct ammonia. The organisms cultivated in a urea broth medium including pH indicator phenol red allow for the detection of urease. The presence of ammonia produces an alkaline environment that allows the urea substrate to break into its components, resulting in the deep pink coloration of phenol red. This is a response to urease that is positive. The absence of a rich pink hue is indicative of a negative response.

Hydrolysis of starch

Glucose molecules joined by glycosidic linkages form the high molecular weight branched polymer known as starch. It is composed of two glucans, amylase and amylopectin. Some organisms produce extracellular amylases it is the centre of the chain. Because of the rapid breakdown of the macromolecules, starch reacts with iodine to give blue colour. The culture was streaked once on the starch agar plates, and then they were incubated at 37°C for 24 hours. After the incubation period, the plates were dipped in iodine solution and let to stand for 30 seconds. A distinct zone surrounding the growth indicates successful starch hydrolysis. This test indicates the positive and displays a separate area surrounding the growth.

Hydrolysis of gelatine

Collagen is hydrolyzed by a protein called gelatine, which is a key element of tendons and connective tissue in both humans and other animals. Certain organisms have the ability to produce gelatinase, an extracellular proteolytic enzyme that hydrolyzes proteins into amino acids and facilitates liquefaction. Gelatine agar plates were streaked with the test organism and incubated for 24 hours at 37°C. Positive findings are indicated by the hydrolysis of gelatine produced by a clear zone surrounding the growth after the incubation time. This test yields favourable findings and displays a distinct zone surrounding the growth.

The antibacterial properties of honey against wound pathogens

The agar well diffusion technique was used to investigate honey's antibacterial properties. Muller Hinton agar plate was inoculated with the standard inoculums, and the media was evenly distributed using a sterile glass. Using a sterile cork borer, five wells were created, and each well was filled with a different honey extract (ethanol, methanol, acetone, and hexane). The zone of inhibition was seen when the plate was incubated for 24 hours at 37°C. The antibacterial activity was expressed as a mean diameter of the inhibitory zone (mm) formed by the honey.

Phytochemical analysis of honey

Using conventional laboratory methods, the sample was screened for the reducing sugar, alkaloids, flavonoids, glycosides, phenol, saponins, and tannins.

Test for alkaloids

Two millilitres each of the extract and strong hydrochloric acid. The Mayer's reagent was then added in a few drops. White precipitate or green coloration suggest the presence of alkaloids.

Test for terpenoids

To 0.5 ml of the extract, a solution of 2 ml chloroform and conc. sulfuric acid was added. When a reddish-brown hue appears at the place of contact, terpenoids are present.

Test for saponins

In a graduated cylinder, after adding two millilitres of extract and two millilitres of distilled water, the mixture was shaken for fifteen minutes lengthwise. As a result, a layer of foam measuring one centimetre developed, signifying the presence of saponins.

Confirmatory test for flavonoids

2 ml of extract and 1 ml of 2N sodium hydroxide were added. Flavonoids are indicated by the presence of yellow colour.

Confirmatory test for tannins

1 ml of extract was mixed with 2 ml of 5% ferric chloride. When tannins are present, dark blue or greenish black coloration is produced.

Confirmatory test for cardiac glycoside

Two millilitres of glacial acetic acid and a few drops of ferric chloride were added to the extract to dilute it. This was covered by 1 millilitre of sulfuric acid solution. When a brown ring forms at the point of contact, heart glycosides are discovered.

Confirmatory test for phenols

One millilitre of the extract was combined with two millilitres of distilled water and a few drops of 10% ferric chloride. The development of a green or blue tint suggests the presence of phenols.

Confirmatory test for steroids

When the formation of a bluish brown ring indicates the presence of phytosteroids, the presence of a brown ring indicates the presence of steroids. One millilitre of the extract is added together with an equivalent amount of sulfuric acid and a few drops of powerful chloroform.

Test for reducing sugars

The filtrate was heated using Fehling's A and B solutions, neutralised with alkali, then acidified with diluted hydrochloric acid. The formation of crimson precipitate indicated the presence of reducing carbohydrates.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the honey was determined by the broth dilution method. A solution containing 50 v/v of the extract was created by adding 2 ml of the honey (100 v/v) to 2 ml of nutritional broth in a test tube. This allowed for the preparation of two-fold serial dilutions of the honey. The concentrations that were produced were 50, 25, 12.5, 6.25, and 3.125 v/v as a outcome of carrying out the procedure successively up to test tube number five. As a negative control, test tube number six was devoid of extracts. Within the test tubes, after carefully adding 0.5 ml of 0.5 McFarland equivalent standards of test organisms, the tubes were incubated at 37°C for a whole day. Following the incubation period, the turbidity of the test tubes was checked to look for growth.

Minimum bacterial concentration (MBC) determination

The concentration at which no bacterial growth is observed is known as the minimal bacterial concentration. broth dilution obtained from the MIC tubes was used to determine this, and Rubin et al stated subculturing to antimicrobial-free agar was followed.[8] Using a sterile wire loop, the contents of the rest tubes that resulted from the MIC were smeared on an agar plate devoid of bacteria and incubated at 37°C for a full day to cultivate bacteria. The MBC was identified as the extract concentration with the lowest bacterial growth seen.

Techniques for thin layer chromatography

A slurry of silica gel was prepared with distilled water in a 1:2 (w/v) ratio and applied to a glass slide to create a 500µm-thick coating of silica gel. Activation of the coated plates was done at 80°C for three hours. The solvent system employed in this investigation included ethanol, acetic acid, and water (4:1:5). The concentrated filtrate

(0.05 ml) was added to the TLC plates using a capillary tube, slightly above 2 cm from the bottom. The developing jar containing the solvent combination was filled with the plates. After removing the plates and letting them sit at room temperature for half an hour, the outcomes were checked by misting ninhydrin solution on them. The value of Rf was computed.

$$Rf \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Design of study or work plan

Study design or work plan is presented in the following flowchart.

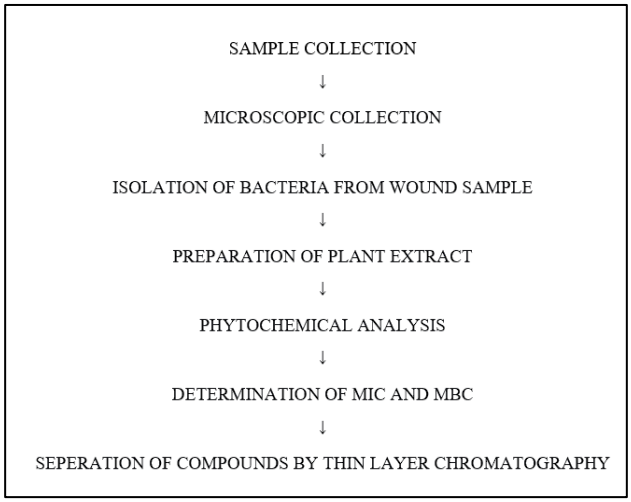

Figure 1: Design of study.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was evaluated by one-way ANOVA followed by Tukey HSD test. Values with $p<0.05$ were considered statistically significant.

RESULTS

Isolation characterization and identification of test organisms

The bacterial strains isolated were *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Table 1 displays the outcome of the test isolates' identification. The morphological characters were observed on nutrient agar plates by spread plate technique and are identified by quadrant streaking on respective selective media. Gramme staining, indole, methyl red, Voges-Proskauer, citrate, catalyse, oxidase, urease, starch hydrolysis, and gelatine hydrolysis were among the tests that were carried out. After the identification and isolation of bacteria, agar well diffusion methods were performed by extracting honey.

Table 1: Isolation description and identification of test organisms.

Tests	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
Gram staining	+	-
Test for indole	-	-
Test for methyl red	+	-
Test for Voges-Proskauer	+	-
Test for citrate	+	+
Test for catalyse	+	+
Test for oxidase	-	+
Test for urease	+	-
Hydrolysis starch	+	-
Gelatine hydrolysis	+	+

Meliponula ferruginca's antibacterial activity

The outcome demonstrated that honey's ethanolic extract exhibited a greater inhibitory impact than its acetonic extract. The highest zone of inhibition was recorded in *Meliponula ferruginca* ethanolic extract with a zone of 12 mm on *Pseudomonas aeruginosa* and whereas the ethanolic extract of *Meliponula ferruginca* showed a zone of inhibition of 11 mm on *Staphylococcus aureus*. In the acetone extracts, the highest zone of inhibition was recorded in honey with a zone of 9 mm on *Staphylococcus aureus* and a zone of 6 mm on *Pseudomonas aeruginosa* (Table 2).

Table 2: *Meliponula Ferruginca's* antibacterial efficacy against microorganisms which trigger wounds.

Organisms	Zone of inhibition (diameter in mm)	
	Ethanol	**Acetone**
Staphylococcus aureus	11	9
Pseudomonas aeroginosa	12	6

Phytochemical screening

Terpenoids, saponins, flavonoids, tannins, and reducing sugar were detected in *Meliponula ferruginca* phytochemical examination (Table 3).

Table 3: Phytochemical analysis of *Meliponula ferruginca*.

Compounds	Result
Alkaloid	-
Terponoids	+
Saponins	+
Flavonoids	+
Tannins	+
Steroids	-
Reducing sugar	+

Thin layer chromatography (TLC)

Thin-layer chromatography analysis was performed on the extract, and the findings were tabulated (Table 4). Figure 7 illustrates the substances that have been separated.

Table 4: TLC analysis of *Meliponula ferruginca*.

Compound position	*Meliponula ferruginca* extract (ethanol)
1	0.15
2	0.35
3	0.44
4	0.33
5	0.54
6	0.34
7, 8	0.52

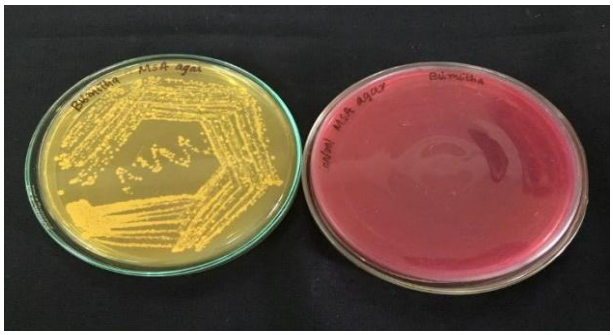

Figure 2: *Staphylococcus aureus* isolated from wound sample.

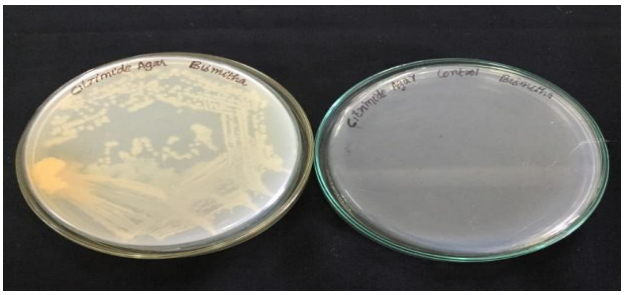

Figure 3: *Pseudomonas aeruginosa* isolated from wound sample.

Figure 4: Extraction of *Meliponula ferruginca*.

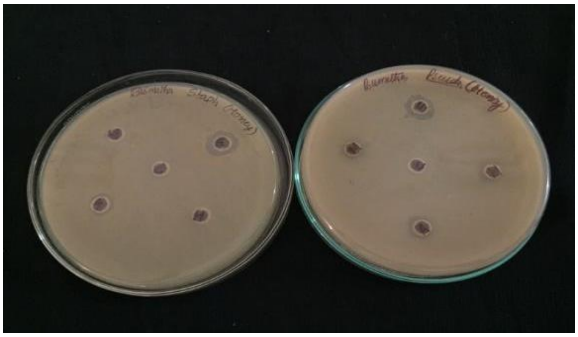

Figure 5: Antibacterial activity of *Meliponula ferruginca* against wound pathogens.

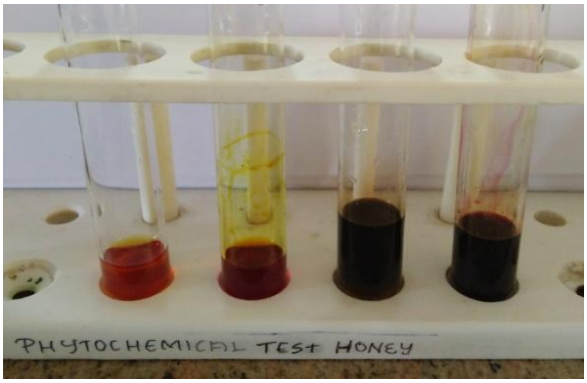

Figure 6: Phytochemical analysis of *Meliponula ferruginca*.

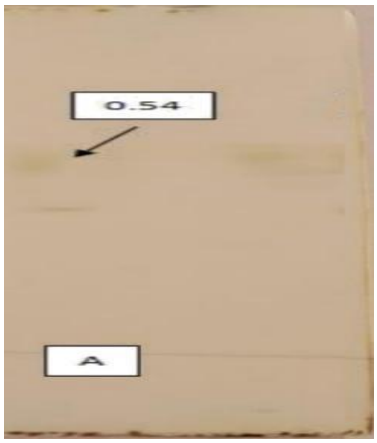

Figure 7: Thin layer chromatography.

DISCUSSION

Staphylococcus aureus and *Pseudomonas aeroginosa* were both susceptible to the antibacterial activity of the honey sample. *Pseudomonas aeroginosa* exhibited lower activity compared to *Staphylococcus aureus*. This is because *Pseudomonas aeroginosa* is starting to show signs of resistance. Terponoids, saponins, flavonoids, tannins, and reducing sugar were detected by phytochemical analysis. Similar results were noted for saponins, which were found in honey and were shown to be an antibacterial material on numerous organisms' cell walls (Harborne,

1992).[17] According to Thompson (1988), the potential of flavonoids to neutralise the acidity of wounds and inflammation aids in the healing of wounds and the treatment of skin diseases. Alkaloids-containing plants are used to cure coughs, colds, and malaria.[18] According to Macrae et al, flavonoids, saponins, and glycosides-particularly saponins that stay in the gastrointestinal tract-may stimulate the heart and be responsible for the therapy of heart disorders. Certain compounds directly interact with dietary cholesterol to form an insoluble complex that obstructs the cholesterol's absorption. In primates, dietary saponins lower plasma cholesterol levels, which may minimise the risk of coronary heart disease in humans.[19]

Willix et al observed that the suppression of the development of Pseudomonas, *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* corroborates the study's findings.[20] Previous studies have indicated that, rather than the species of bees, the type of honey sample and the plant source from which nectar was taken are related with honey's antibacterial effect. In a study conducted in Turkey, *Staphylococcus aureus* was shown to be inhibited by the majority of honey samples at concentrations more than 50%.[21]

According to Subramanian et al, honey's inhibines are mostly responsible for its antibacterial properties. Along with numerous other unknown inhibines, these include hydrogen peroxide, flavonoids, and phenolic acids. The fermentation of honey, which yields alcohol, and a low pH of 3.6 have been suggested as explanations. Other concepts include the formation of an unfavourable environment with low water activity, which restricts bacterial growth, and shrinking breakdown of the bacterial cell wall caused by the osmotic action of the sugar content.[22] Molan found that *Staphylococcus aureus* was one of the bacterial species most responsive to honey's antibacterial activity. The results of this study's investigation into the antibacterial activity of honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa* are consistent with that finding.[23] According to Baltrusaitis et al, these could be caused by the osmotic effect, the pH effect, or the organisms' sensitivity to hydrogen peroxide, which is an inhibitory component in honey.[24]

According to Willix et al Pseudomonas sp., *E. coli*, and *Staphylococcus aureus* were all inhibited in growth by honey.[20] The findings of this investigation corroborated those of Bilal et al, who found that honey exhibited a reasonable level of antibacterial activity against both Gram-positive and -negative bacteria, with *Pseudomonas aeruginosa* and *Staphylococcus aureus* demonstrating especially noteworthy outcomes.[25] Depending on the diet and overall health of the bee, honey's glucosidase levels can change.[26] On the basis of Weston, hydrogen peroxide can degrade or prevent the harmful effects of chemicals like gallic and caffeic acids on microorganisms within the cell. Honey can also contain catalase, peroxidases, and antioxidants. However, the amount of hydrogen peroxide produced in a honey sample is not exclusively determined by the amounts of glucosidase.[27] According to Majtan, there have been publications indicating that methylglyoxal (MGO) can alter some proteinaceous molecules found in honey, which in turn can have an impact on glucosidase function.[28] According to research by Weston, the concentration of peroxide in honey is also influenced by the enzyme catalase, which is derived from plant pollen.[27] The effects of light, temperature, and oxygen on hydrogen peroxide content have been demonstrated by Dustman, and these effects may differ depending on how honey is processed and stored. Insufficient amounts can inactivate the primary enzyme responsible for producing hydrogen peroxide, which could potentially diminish the antibacterial properties of hydrogen peroxide.[29] In *Meliponula ferruginca*, eight compounds were found, according to the TLC analysis. It is possible that the molecule in fifth place, fructose or sucrose, has the highest RF value (0.54).

The limitation of this study is that clinical trials have shown that honey promotes autolytic debridement, stimulates growth of wound tissues and stimulates anti-inflammatory activities thus accelerates the wound healing processes. Honey stimulates leukocytes to release cytokines, which what initiates the tissue repair process.

CONCLUSION

The results of this investigation show that Bergey's handbook of determinative bacteriology can be used to identify *Staphylococcus aureus* and *Pseudomonas aeruginosa*. According to the study's findings, honey has the ability to neutralise *Pseudomonas aerugenosa* and *Staphylococcus aureus* that have been isolated from infected wounds. Some of honey's antibacterial qualities account for a considerable portion of its antibacterial effectiveness. Certain gluco-oxidase enzymes, which produce aseptic chemicals like hydrogen peroxide, are among them, as is low water activity, low pH, and high osmotic pressure. Terpenoids, saponins, flavonoids, tannins, and reducing sugars were all found in the current investigation.

A powerful antibacterial agent with a wide range of effects is honey. Honey has antibacterial properties due to a number of factors, including its sugar content, hydrogen peroxide, polyphenolic chemicals, 1,2-dicarbonyl compounds, and bee defensin-1. The degree of each of these varies based on the nectar source, honey bee, and storage. Based on the results of this investigation, honey has potential use in the treatment of wound infections. *Meliponula ferruginca* contained eight different compounds, according to the TLC study. The molecule at position five, which may be fructose or sucrose, has the greatest RF value, or 0.54.

However, more research on the effects of honey on pathogenic organisms, toxicological analyses, and purifying procedures are necessary.

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