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Antimicrobial Study of Chitosan-Based Crosslinked Hydrogel Against *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*

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Abstract

Gingivitis is a pathogenic disorder caused by the growth of bacterial flora inside the gum cavity, also known as the gingival cavity. The formation of hard plaque is initiated by the deposition of a biofilm of microbes on the surface of teeth at the age of gums. These plaques, when developed, turn into tarter and are difficult to remove. These hard structures formed around the teeth caused severe damage to soft tissue and affected teeth, eventually causing permanent tooth loss. Gingivitis in an advanced stage, called periodontitis, causes permanent damage to the gum and teeth. Periodontal disease is caused by the bacteria *Staphylococcus aureus, Porphyromonas gingivalis, Pseudomonas aeruginosa, and Streptococcus mutans.* The aim of the investigation is to study how well chitosan-based crosslinked hydrogels containing Chlorhexidine, Metronidazole, and Lignocaine hydrochloride inhibit the growth of selected bacteria isolated from oral microbial flora such as *Staphylococcus aureus, Porphyromonas Gingivalis, Pseudomonas Aeruginosa, and Streptococcus Mutans* in-vitro. Chitosan-based crosslinked hydrogel 2 % combined with all the active ingredients prepared and tested for antibacterial activity in SCAN-Laboratory Indrepuri, Bhopal. Bacterial samples were taken from freshly extracted teeth provided by District Hospital Raise in Madhya Pradesh, India, and then preserved in a saline solution. Isolate and culture each bacterium colony separately before performing an identification test for each of them. The hydrogel was then diluted at 25%, 50%, 75%, and 100% concentrations up to 10 power -9 to perform the antibacterial activity. All isolated bacteria colonies were separately cultured and introduced to a paper disc etched with antimicrobial gel. The media were then incubated in the incubator, and the (ZoI) Zone of inhibition was checked for the gel's effectiveness. The mean diameter of the zone of inhibition against all isolated bacterial colonies was noticed to be in the 10-33 mm range, indicating the presence of strong anti-bacterial activity. A considerable qualitative difference ($p = 0.000$) was found between the inhibitory effects of each concentration of hydrogel on the bacteria *Staphylococcus aureus, Porphyromonas gingivalis, Pseudomonas aeruginosa, and Streptococcus mutans.* Because of the largest diameter of the inhibition zone, the most effective antibacterial activity was found in 2% chitosan-based hydrogels.

Keywords: Gingivitis, Antimicrobial, Metronidazole, Crosslinked Hydrogel, Periodontitis.

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Introduction

According to a World Health Organization survey, nearly 10-15% of the global population suffers from dental problems, specifically periodontitis. Periodontitis is a pathogenic infection that damages the teeth's supporting tissues and thus can lead to permanent tooth loss. (1). The cause of periodontal disease is an increase in the population of pathogenic disease-causing agents in the oral cavity debris of microorganisms deposit over the surface of teeth and form a thick layer known as excessive biofilm deposition will indeed be plaque (2). The formation of biofilm, which can be exacerbated by poor dental hygiene and leads to plaque formation, is one of the major causes of the disease. Plaque buildup around teeth irritates surrounding tissues and, if left untreated, destroys tooth attachments (3). The general host response influenced by subgingival bacterial growth and the severity of infection left unnoticed exacerbates the symptoms of infection. Furthermore, if the pathogen grows rapidly, the host will simultaneously increase the pathogenic response and destroy the periodontium. (4). The disease is more severe and degenerative, affecting soft tissue around the teeth such as gingiva, ligaments, cementum, and supporting bones; in advanced stages, it may cause permanent loss. Pathogenic colonies proliferate quickly and deposit their dead remains on the upper surface of teeth, forming a permanent layer called biofilm and many additional layers collectively known as calculus. The disease concept is based on some considerations that may cause the disease to persist and proliferate, such as a susceptible host, an excess of pathogenic microbes due to poor hygiene, and the absence of beneficial bacteria. The elaborated growth of Gram-negative and mobile bacteria increases the prognosis. [5,6] Pathogens of various types are associated with the disease and are the main factor in its pathogenesis. Porphyromonas Gingivalis, Bacteroides forsythias, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, and Eubacterium sp. are among the bacteria responsible for chronic periodontitis. However, microaerophile bacteria such as Actinomyces mycomitans, Campylobacter rectus, and Eikenella corrodens cause chronic periodontitis (7 8). Functional groups on Chitosan tend to bind negatively charged groups on the cell wall of bacteria, affecting the physiology and permeability of the cell wall. They also interact with DNA, limiting replication and resulting in cell death (9). Metronidazole has broad antimicrobial activity against protozoan infections as well as anaerobic bacteria. Metronidazole was first used to treat trichomoniasis before its broad antimicrobial activity was discovered in 1950. Metronidazole is now a widely used periodontal disease treatment (10). Metronidazole is a highly effective drug for reducing the deposition of dead cells in periodontal pockets, removing calculus, and scaling. It can effectively limit anaerobic bacteria growth (11) Because of a three-dimensional network of polymer chains formed by physical or chemical interaction between functional groups, the hydrogel of the crosslinked polymer has a tendency to adsorb and hold a large amount of water more than natural polymer. Hydrogel has numerous benefits and improved properties. Tissue engineering, biosensors, regenerative agents, and improving adhesion properties are just a few of the applications. Chlorhexidine is antiseptic and disinfectant in nature and is also used as a cleaning agent for the oral cavity in low concentrations. Chlorhexidine destroys bacteria's cell walls by binding negatively charged phosphate groups, disrupts cellular integrity, and interacts with cytoplasm, resulting in cell death. Because biofilm reforms on a regular basis, surgical methods for removing calculus are less effective; however, nonsurgical methods such as antimicrobial therapy are quite effective because it provides active ingredients at the site and effectively inhibit microbe growth. Local drug delivery is advantageous because it delivers to the infected site, but it is also gaining popularity because it avoids many of the drawbacks of systemic applications (12,13) It has the possibility of causing hypersensitivity reactions, gastrointestinal intoler-

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ance, and bacterial resistance. The goal of the research is to establish a more precise delivery system for delivering drugs to local tissues and maintaining their concentration in order to provide more effective results while overcoming the limitations of existing systemic approaches (14). To accomplish this, a crosslinked hydrogel containing a drug combination was created. The polymeric base was chosen to extend the time spent in the oral cavity. Oral cavity has many limitations due to the high flow of saliva. The approach is to develop a gel with natural polymer crosslinking to overcome the limitation. To some extent, hydrogel protects against factors such as pH and Enzyme and prevents drug exposure with biological content. By releasing the drug at a predetermined rate, you can extend the drug's residence time at the site of application and allow it to absorb for a longer period of time (15). The goal of this research is to create a more precise delivery system for controlled release to local tissues while maintaining concentration, resulting in more effective results while overcoming the limitations of current systemic approaches. A crosslinked hydrogel containing a drug combination was created to accomplish this. The polymeric base was selected to increase the amount of time spent in the oral cavity. Because of the high flow of saliva, the oral cavity has many limitations. To overcome the limitation, the approach is to create a gel with natural polymer cross-linking. Chitosan is a natural mucoadhesive polysaccharide derived from various sources. Chitosan is a natural mucoadhesive polysaccharide derived from various sources. Chitosan, Polyacrylamide, Polyvinyl alcohol, Polyethylene glycol, and Alginate are some examples of natural and synthetic polymers used to make hydrogel (16). Hydrogels are insoluble in water and have a high affinity to absorb water within the matrix (17). Polymer macromolecules interact and form a crosslinking network between polymeric chains. The reaction between polymeric chains is initiated by crosslinking agents, forming a network of water-repelling molecules. A chemical reaction in which natural or synthetic polymeric monomers react to form copolymers or homopolymers (18). As a result, the complex structure retains a significant amount of water or biological fluid; high water content sustainably increases bioadhesive by increasing the contact angle between the mucus layer and molecules. It improves hydrogels' similarity to soft tissue and makes them more biocompatible, bioadhesive, and nonirritant, allowing its diverse applications to deliver drugs more precisely at the site of application (19).

Materials and Methods

Isolation and identification of organisms from extracted human tooth

Collection of sample

A fresh tooth sample was donated by District Hospital Raise in Madhya Pradesh, India, and stored in saline solution. The sample was then immersed in sterile saline and allowed to incubate for 24 hours.

Preparation of serial dilutions

1 ml of the gel was diluted in 9 ml of nutrient broth to make a standard sample. Subsequently, serial dilutions were done to create crosslinked hydrogel at concentrations of 25%, 50%, 75%, and 100% up to 10 power 9 to perform the antibacterial activity.

Spread Plate technique

With a sterile pipet or metallic loop, bacteria suspended in a solution are poured over prepared media. A sufficient number of bacteria spread across the surface of the culture media allows them to grow (20). The technique is stable and reliable, and it is aligned with the SOP to avoid contamination.

1. Preparation of samples to achieve a suitable concentration of the bacterial solution; the sample was serially diluted.
2. Agar plates are prepared by pouring sterile agar solution into Petri - dishes and continuing to allow it to solidify.

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3. By pipetting out 0.1 ml diluted solution from a series of dilutions, a sterile glass spreader or pipet is used to spread the sample on the surface of prepared agar plates.
4. Incubate prepared plates by placing the Petri dish in a moderate-temperature incubator for a duration that allows the bacterial colony to grow.
5. Incubate the plate at 37 °C for 24 hours.
6. Count the bacterial colonies that have grown on the surface of the agar plates after the incubation period.
7. The CFU value of the sample will be given in CFU/ml of the prepared sample if the CFU value is calculated using the Dilution factor and the colony count.

Streak Plate technique

It is a technique for separating and purifying bacterial colonies from a mixed culture. Select the separate wells in an agar plate, then collect the sample after successive dilutions of a mixture of bacterial colonies. The inoculum dilutes in successive sections of a plate until only one bacterial cell remains, with no other cells in the few MM area surrounding the cell. It enables each cell to culture independently, and the pure colony can grow in a specific area. Pure colonies can now be isolated by selecting isolated colony walls and re-striking them in different culture media to select individual colonies for further examination.

The method is based on the idea that a single bacteria can multiply multiple times and form a colony of uniform cells. The precise technique for obtaining pure bacteria culture has a wide range of applications in fields such as diagnosis, identification, characterization, and genetics.

1. Sterilize the inoculation loop with a spirit lamp or Bunsen burner and allow it to cool.
2. Divide the agar media on a plate into four equal sections and streak a single colony in each.
3. Strew the inoculating loop across a quarter of the plate quickly, softly, and back and forth.
4. Spread it in a pentagon shape on the opposite side of the agar plate quadrant.

Identification of microorganisms

Gram staining

Microbiologists use this tool to identify and classify bacterial species. It is widely used in medical microbiology to diagnose bacterial infections and determine the best antibiotic treatment. It is the first test commonly used to diagnose any bacteria using methylene blue or crystal violet as a primary stain, followed by safranin. (21). Gram-positive bacteria retain crystal violet and appear purple after decolorization. In contrast, Gram-negative bacteria retain safranin instead of crystal violate due to a thick cell wall and appear pink under a microscope.

Preparation of a slide smear

- Apply a drop of culture suspension to the glass slide using the inoculation loop.
- Examine the slide under a microscope. If a bacterial colony appears, move a small amount of the suspension to the next slide for further examination.
- If culture is visible on the loop, it indicates that a large amount of culture was taken; it will disappear over 15mm diameter; if more than one culture is taken for the examination, a thin coating using the loop must be crate; some slides can have up to four tiny stains.
- Keep the slide over a spirit lamp or in the oven to dry quickly and form a smear. Keep the slide moving to avoid overheating. Heating will bind the cells to the slide and prevent them from draining while washing.
- For staining, crystal violate stain is applied over a fixed smear of a culture. After a while, pour water over the slide and gently wash it to remove any extra stain. This will remove

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any traces of stains and bacteria that may have remained on the slide.

- To fix the color, an iodine solution is applied over the smear. After that, the slide is rinsed under running water to remove the iodine solution. To absorb excess water from a slide, tissue paper is used.
- Solvent treatment is the process of removing the color from a smear by using ethanol and acetone solution as a decolorizing agent. After some time, the slide is rinsed again with distilled water. To avoid over-decolorizing, we must stop adding decolorizing agents once the smear has stopped changing color. After soaking in water, the smear is treated with a fuchsine solution for 50-60 seconds before being washed with clean water, excess water removed with a tissue paper slide, and air dried.

Characteristic Identification

S. Aureas can be identified and isolated using the MSA (Mannitol Salt Agar) test. MSA contains a high concentration of sodium chloride (7.5% NaCl), which inhibits bacterial growth. Only Halophile bacteria can withstand such high salinity levels. Mannitol and sugar alcohol are both added to culture media as ingredients. Bacteria ferment mannitol to produce acid, lowering the pH and turning phenol red. The color change from red to yellow indicates the presence of bacteria. In a high salt medium, other halophile bacteria may also survive to distinguish S aureus from them. Mannitol was added to the culture because staphylococcus and other halophile bacteria are incapable of fermenting it, resulting in a pH drop. Characteristic Yellow The color of the medium indicates the presence of S. aureus. Another method for confirming the presence of S. Aureas is the Coagulase test, in which bacteria produce the enzyme coagulase, which causes blood collected from humans to clot quickly. If S. Aureas is present in the sample, it will coagulate the blood; otherwise, it will remain liquified. Snyder test for *S. Mutans* The microbes collected from the teeth's surface are transferred to a special agar medium called Snyder agar media. The medium contains a high concentration of glucose and is acidic in pH. *S. Mutans*, unlike other bacteria, can grow in acidic environments, which is why they are referred to as acidophilus. If bacteria are present in a sample, they will multiply and form successive colonies, which may be translucent or slightly yellow in color. *P. Aeruginous* Pyocyanin production test: bacteria are known to produce blue-green pigment, which is thought to be a characteristic feature. A variety of factors influence bacterial pyocyanin production, including environmental and oxygen availability in addition to other nutrients. Pigments act as electron donors in the absence of oxygen, allowing bacteria to use oxygen more efficiently. Pigment also plays a role in virulence damage tissue and inflammation during biofilm formation. Blood microbe culture Pigments are commonly seen on agar plates as small, black, dark brown spots that resemble fried eggs. This is because of pyocyanin. Under a microscope, it appears to be a comma-shaped rod. Because the bacteria are immobile, nonfunctional flagella tufts appear. Under a microscope, *P. Gingivalis* appears as a curved or comma-shaped rod with a polar tuft of flagella. However, the bacterium is not motile in its natural environment, and the flagella are not functional.

Biochemical test

IMVIC Test

The IMViC test is a series of four biochemical tests used to differentiate between different groups of bacteria, specifically Enterobacteriaceae. A series of four tests, each with its own characteristics used to identify different types of bacteria based on their metabolic characteristics. Different Enterobacteriaceae family species have different metabolic properties; they can identify easily on the basis of these tests. The test is named after the first letters of each of the four tests, which are the Indole test, Methyl Red test, Voges Proskauer test, and Citrate Utilization Test. The test is used to

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distinguish between bacterial colonies that are closely related and may have similar metabolic properties. The Indole test is positive if bacteria break down the amino acid tryptophan to produce indole, the Methyl red test is positive if bacteria ferment glucose to produce acidic byproducts, the Voges-Proskauer test is positive if cells can produce acetone during glucose fermentation, and the citrate test is positive if cells use citrate as a carbon source (22-23).

Indole test

Principle: Some bacteria have the ability to convert the amino acid tryptophane to indole due to the presence of an enzyme; the presence of indole can be confirmed using Ehrlich's or Kovac's reagents. It will generate a red precipitate, which will appear as a ring in the alcoholic layer.

Steps: Transfer the bacteria suspension to a water and peptone solution containing the amino acid tryptophan and culture the mixture overnight in an incubation chamber at 37 degrees Celsius. Add a few drops of Kavac's reagent to the mixture the next day. After being inoculated in peptone water, which contains the amino acid tryptophan, the bacteria are cultured overnight at 37°C. After incubation, Kovac's reagent is added. Kovac's reagent is composed of para-dimethyl amino benzaldehyde, isoamyl alcohol, and concentrated hydrochloric acid. The reagent detects the presence of indole in anaerobic conditions by forming a red or pink ring at the surface.

Methyl red (MR) test

Principle: The methyl red test assesses an organism's ability to metabolize glucose into an acidic byproduct. Because some bacteria have a high capacity for producing acid byproducts, the pH of the system may be affected, reducing the system's ability to maintain a buffer. Methyl red is a pH indicator that turns red when a strong acid is present in a system.

Procedure: The test must be carried out on a bacterial culture that has been grown for 48 hours at 37 degrees Celsius in a culture containing glucose. Following the completion of the incubation period, some media is transferred to a separate tube containing methyl red indicator. If the bacteria produce enough acidic byproduct, the mixture will immediately turn red. The positive test result aids in the differentiation of the bacterial colonies in the sample. When a few drops of Methyl red indicator are added, the red color indicates the presence of bacteria, while the yellow color indicates the absence of bacteria.

Voges proskauer (VP) test

Principle: The Voges-Proskauer (VP) test relies on bacteria's ability to convert glucose into (butylene glycol) acetoin for identification. Some Enterobacteriaceae members, such as *E. coli* and Salmonella, are pathogenic to humans. The test employs two indicators. Alpha-naphthol and Potassium Hydroxide (KOH) When added to the culture in the presence of excess air, both reagents will react with the acetoin (Butylene Glycol), and the diacetyl will react with the guanidine components of peptone due to the presence of naphthol. Alpha-naphthol is a catalyst and color intensifier that produces a red color. The presence of color indicates that the cell is producing acetoin. The yellow color indicates the absence of bacteria.

Procedure: The colony of unknown bacteria transferred to the culture media after 48 hours of incubation in culture media containing glucose phosphate broth at 370C, 0.6 ccs of Alpha naphthol added to the diluted sample taken from the plate, and gently shake the test tube, then transfer 0.2 ml of 40% KOH solution drop by drop while continuous stirring. Keep the test tube aside for about 15 minutes. The appearance of red color will confirm the presence of bacteria in the negative tube. Negative results are indicated by no or slightly low color.

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Citrate utilization test

Principle: Citrate utilization testing identifies microorganisms based on their proclivity to consume citrate as a carbon source and produce energy. Some Enterobacteriaceae pathogens are commonly harmful to humans. The experiment continues by inoculating microorganisms on summon citrate agar. Other nutrients and indicators are also introduced into the culture. In an acidic medium, indicators such as bromothymol blue change color from green to blue. If a cell consumes citrate, it will produce a large amount of citrate lyase, which breaks citrate down into pyruvate and other alkaline products, causing the ph to rise and the indicator to turn blue from green. If it does not turn blue, it means that the microorganism is not present. Microbes that can use the citrate Klebsiella Pneumonia and Enterobacterium Aerogenes include *Escherichia Coli* and Salmonella Typhi.

Antimicrobial activity of a prepared hydrogel on isolated microorganisms

The ability of a substance to kill pathogenic bacteria is referred to as antimicrobial activity. Microbe-killing agents can be synthetic or natural, disrupting the cell wall or interfering with the metabolic pathway. The nature of the microbe or the agent used heavily influences the effectiveness of an antimicrobial agent. Different concentrations of 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml were used to test the antimicrobial effect of the prepared hydrogel agar well method.

Preparation of media

The gel's antimicrobial activity was tested using agar well diffusion at three different concentrations 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml. The culture of bacteria and fungus requires the use of specific growth media formulations for cloning, plasmid DNA synthesis, and protein production. A Nutrient agar medium was made by combining nutrient broth, agar, and distilled water, and it was used to cultivate the bacterial culture. Nutrient broth contains peptone, sodium chloride, yeast extract, vitamins, and carbohydrates, all of which are required for bacterial growth. Agar is used as a solidifying agent (24).

Sterilization

The killing of harmful microorganisms from the surface of any object is referred to as sterilization. It plays an important role in preventing the growth of microorganisms that we do not want. There are several methods for killing microbes, including physical methods such as heat, filtration, and radiation. Another method is chemical, which employs disinfectant chemicals. Some common examples include alcohol, bleach, ethylene oxide, hydrogen peroxide, glutaraldehyde, and formaldehyde solutions. Following that, the prepared media undergoes sterilization. The media were autoclaved for 15-20 minutes at 121°C. (25)

Inoculation of microbial culture

Aseptically placed media in a Petri dish up to 90mm high. Allow the media to settle for a while. The media was then spread evenly in clean cotton plates before being placed in the incubation chamber.

Sample preparation

In a test tube, a measured amount of prepared hydrogel was placed, followed by the preparation of a standard sample and dilution up to a certain level. The standard sample was used to make samples with concentrations of 25, 50, 75, and 100 mg/ml. Fill each well halfway with the diluted sample.

In-Vitro antimicrobial activity

Chitosan was used as the base for the cross-linked hydrogel, which was then tested for antimicrobial properties. Four different strains of bacteria were used to test the hydrogel's activity *S. Aureus, P. Gingivalis, P. Aeruginosa, and S. Mutans* were among them. At Raisen District Hospital, all microorganisms were collected from the patient's freshly extracted tooth, and

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isolation and antimicrobial studies were performed (5) at the SCAN laboratory in Indrapuri, Bhopal, Madhya Pradesh, India. The agar well diffusion method is commonly used to investigate the antimicrobial properties of gels. Wells were prepared on agar media plates and filled with a suitable amount of diluted gel solution. Each bacterial strain was placed on a separate agar plate. Following that, the antimicrobial gel was applied to the cultured colonies to see if it could inhibit the growth of bacteria. Following that, the zone of inhibition was measured in millimeters (26).

Incubation

After the plate has been colonized with bacterial colonies, a gel of varying concentrations is applied, and the plate is kept in an incubation chamber at 37°C under aerobic conditions for about 24 hours. After incubation, the plates were examined for the zone of inhibition. When bacterial colonies come into contact with an antimicrobial substance that kills microbes, the inhibition zone forms, and it grows with increasing concentration which is expressed in millimeters (27).

Results and Discussion

Figure 1: Tooth sample for isolation of bacteria

Figure 2: Serial Dilutions of the Tooth Sample

Table 1. Determination of Colony Count

Sr. No.	Dilutions	Colony Count	Dilution Factor	Average
1.	10-5	233	105	3.841X 10-8 Cfu/ml
2.	10-6	189	106	
3.	10-7	94	107	

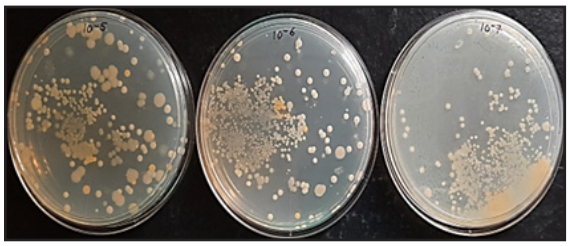
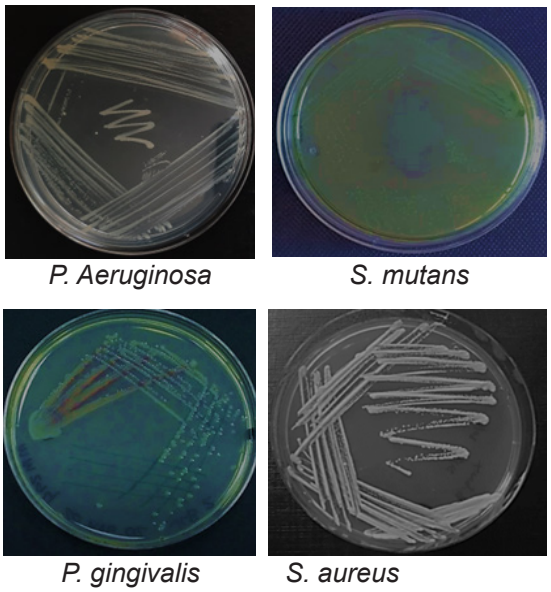


Figure 3: Image of Spread Plate Technique



P. Aeruginosa *S. mutans*

P. gingivalis *S. aureus*

Figure 4: Image of Pentagonal Streaking

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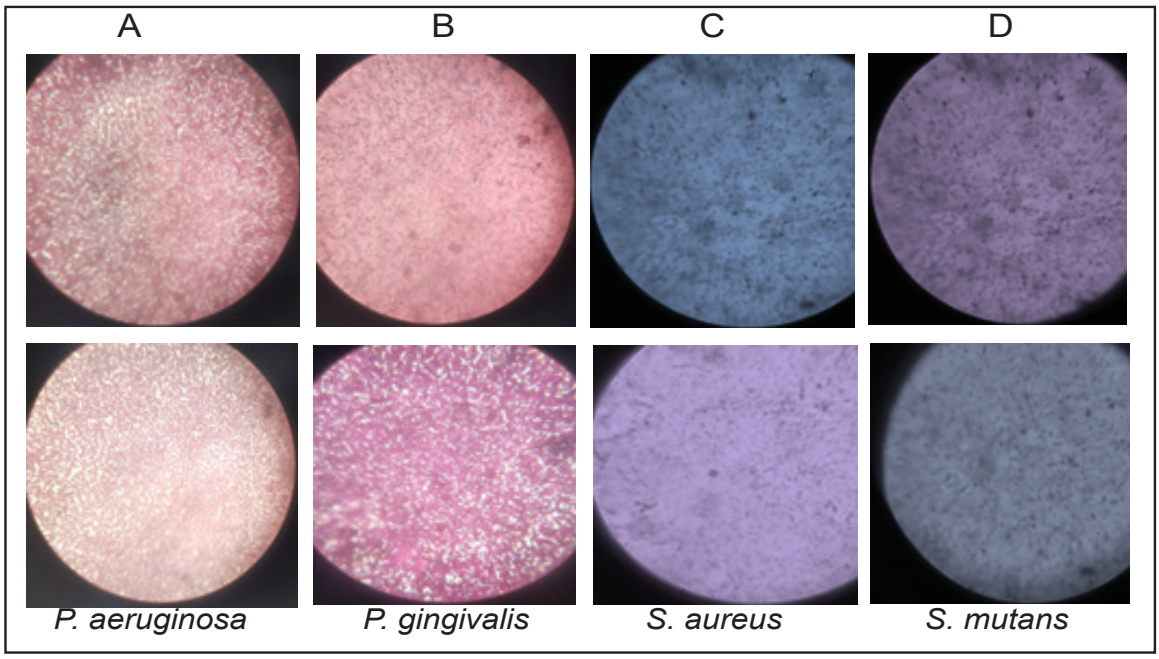

Fig. 5 Image of Gram Staining 100x

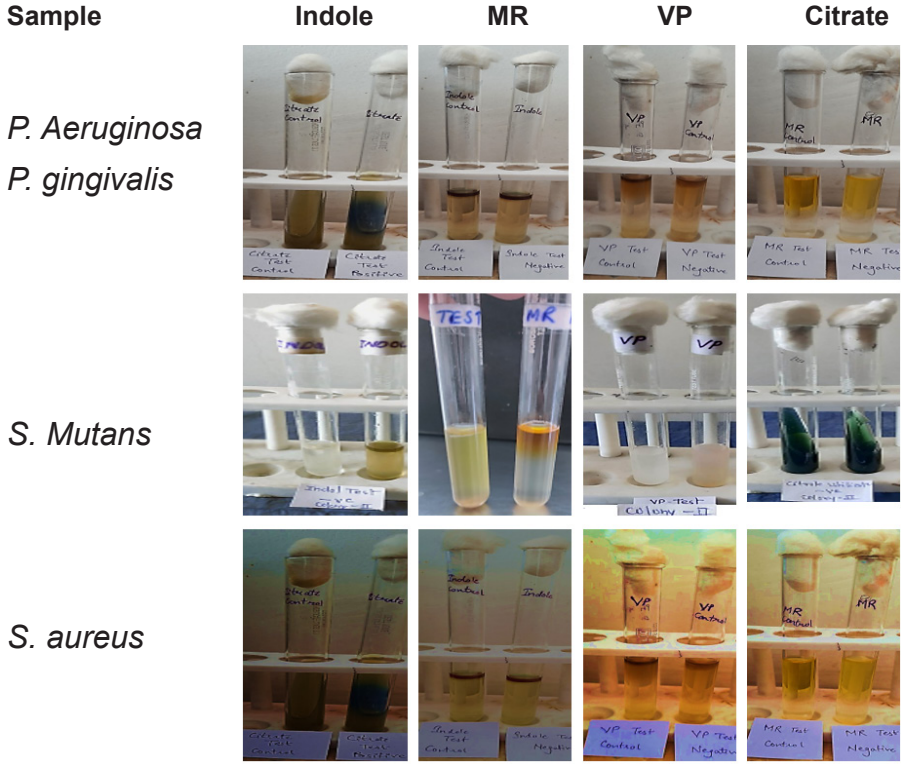

Fig. 6 Results of IMViC Tests for all the Isolated Colonies

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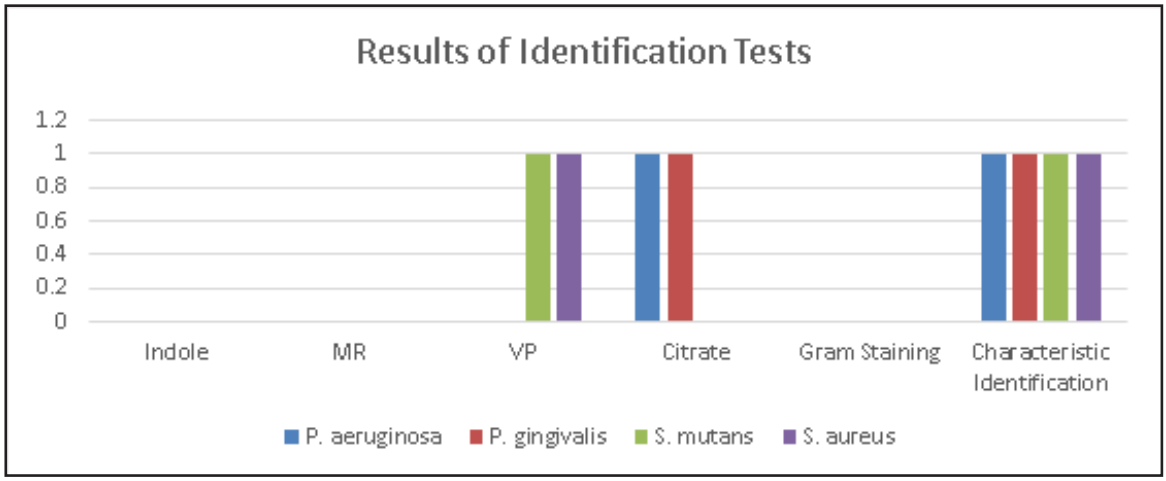

Graph 1. Microorganism Identification Test

Table 2: Results of Identification Tests of *P. Aeruginosa and P. Gingivalis* Organism

Sample	Indole	MR	VP	Citrate	Gram Staining	Characteristic Identification
P. Aeruginosa	-ve	-ve	-ve	+ve	-ve	+ve
P. Gingivalis	-ve	-ve	-ve	+ve	-ve	+ve

Table 3: Results of Identification Tests of S. Mutans, S. Aureus Organism

Sample	Indole	MR	VP	Citrate	Gram staining	Characteristic Identification
S. Mutans	-ve	-ve	+ve	-ve	+ve	+ve
S. Aureus	-ve	-ve	+ve	-ve	+ve	+ve

Several laboratory methods such as the Spread plate Technique, Streak Plate Technique, Gram Staining, Characteristic Identification, and IMVIC Test were used during the study to isolate and identify the presence of *S. Aureus, P. Gingivalis, P. Aeruginosa, and S. Mutans* in a sample (Indole test, Methyl Red test, Voges Proskauer test, and Citrate Utilization Test). One common method for identifying *Staphylococcus aureus,* for example, is to perform a gram staining and then culture the bacteria on a selective media, such as Mannitol Salt Agar (MSA). *Staphylococcus Aureus* colonies will appear as yellow colonies on MSA due to their ability to ferment mannitol. Further tests, such as the coagulase test, can be used to confirm the identity of *S. Aureus. P. Gingivalis* is distinguished by its distinct colony morphology, which consists of black or brown pigmentation on blood *agar. P. Aeruginosa* is distinguished by its blue-green color on agar plates and fruity odor. *P. Gingivalis* looks like a curved or comma-shaped rod with a polar flagella tuft. However, in its natural environment, the bacterium is not motile, and the flagella are not functional. Snyder agar media is a *Streptococcus mutans*-specific agar medium. The medium has a high concentration of glucose and a low pH. *S. Mutans*, unlike other bacteria, can grow in acidic environments, hence the name acidophilus. If bacteria are present in a sample, they will multiply and form successive colonies on blood agar, which may be translucent or slightly yellow in color (28).

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Microbe culture in blood Pigments appears on agar plates as small, black, dark brown spots that resemble fried eggs. This is due to pyocyanin. Microbe culture in blood pigments appears on agar plates as small, black, dark brown spots that resemble fried eggs. This is due to pyocyanin. Once the colonies have been identified, an antimicrobial study can be performed to test the bacteria's susceptibility to various antimicrobial agents. This can be accomplished through a variety of methods such as disc diffusion, broth dilution, or agar dilution.

The MIC (Minimum Inhibitory Concentration) value recorded is the lowest concentration of the assayed antimicrobial agent that prevents observable growth of the microorganism tested, and it is often given in g/mL or mg/L. The gel produces positive results and successfully inhibits microbe growth. The zone of inhibition is measured using a ruler, calipers, or a template. Its dimensions are given in millimeters and are frequently rounded to the nearest millimeter. The diameter of the disc is also mentioned. These measurements are made with the naked eye without using any tools. The prepared gel was applied to each microorganism colony individually (29, 30).

Table 4: Zone of Inhibition Through Gel

Sr. No.	Isolated Organism	Zone of Inhibition		
		25 mg/ml (mm)	50mg/ml (mm)	100mg/ml (mm)
1.	*P. Aeruginosa*	14±0.47	21±0.5	25±0.47
2.	*S. Mutans*	10±0.94	12±0.47	17±0.47
3.	*P. Gingivalis*	11±0.94	19±0.5	29±0.47
4.	*S. Aureas*	13±0.94	24±0.5	33±0.47

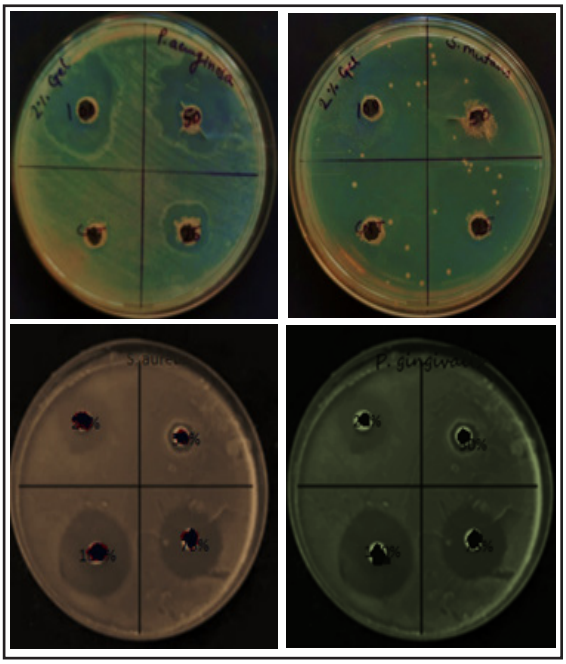

Figure 7: Antimicrobial Activity of 2% hydrogel

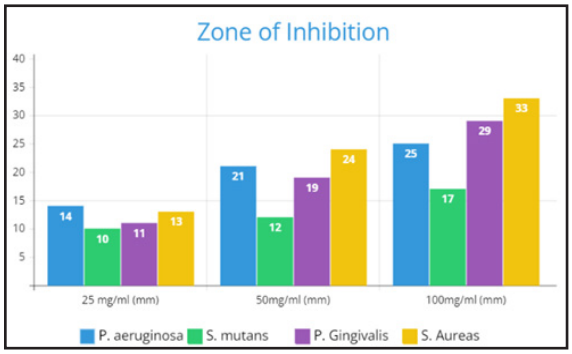

Figure 8: Zone of inhibition

Discussion

Based on their metabolic characteristics, the IMViC test may be used to distinguish between several types of bacteria belonging to the Enterobacteriaceae family. In order to distinguish between bacterial colonies that are closely related, each test offers a special property. To offer a more complete identification of the bacteria under examination, the findings of each test

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might be merged. If bacteria can convert tryptophan to indole, the Indole test is positive. A crimson or pink ring will appear on the surface when indole is present. Suppose the bacteria create acidic byproducts from the fermentation of glucose, causing a drop in pH and the appearance of a red color when methyl red indicator is added. In that case, the methyl red test is positive. If the fermentation of glucose by the bacteria produces acetoin, which reacts with alpha-naphthol and potassium hydroxide to give a red color, the Voges-Proskauer test is positive. If the bacteria can use citrate as a carbon source, raising the pH and changing the color of the sample from green to blue, therefore the Citrate Utilization Test is positive.

The results of the study indicated that the prepared hydrogel had significant antimicrobial activity against all four tested microorganisms. The zone of inhibition increased with increasing concentration of the hydrogel. The highest zone of inhibition was observed at a concentration of 100 mg/ml, indicating that higher concentrations of the hydrogel were more effective in inhibiting bacterial growth. The study also revealed that the hydrogel was more effective against gram-positive bacteria (*S. aureus and S. mutans*) than gram-negative bacteria (*P. gingivalis and P. aeruginosa*). This is likely due to differences in the cell wall structure of gram-negative and gram-positive bacteria, making it easier for the hydrogel to penetrate and disrupt the cell wall of gram-positive bacteria. Overall, the study suggests that the prepared hydrogel has potential as an effective antimicrobial agent against pathogenic microorganisms. Further studies are needed to evaluate the safety and efficacy of the hydrogel in clinical settings.

Conclusion

Gram staining revealed Gram-negative *P. Aeruginosa and P. Gingivalis* in the first and second isolates, and Gram-positive *S. Mutans and S. Aureus* cocci in the third and fourth isolates. Since the IMVIC test confirms the absence of the Enterobacteriaceae family, which is not found in the oral cavity but is found in the GI tract, all tests are negative. Finally, a characteristic identification test was performed to isolate and confirm the presence of all four strands for further investigation. The results of the tests show that *P. aeruginosa, S. mutans, and S. aureus* were obtained and isolated from the tooth sample. *S. aureus* was cultured in MSA (Mannitol salt agar) media, and the bacteria's presence was confirmed by a change in the color of the media from red to yellow due to the bacteria's ability to reduce acidity through metabolism. When *S. mutans* is cultured in Snyder agar media with a high concentration of glucose, its tendency to metabolize the glucose raises the pH and decreases acidity, turning the medium yellow. *P. aeruginosa* culture in agar media, where its ability to produce pyocyanin, a blue color pigment, turns the media blue. In agar media, *P. Gingivalis* produces pyocyanin, which causes brown or black spots that resemble fried eggs. Because of its comma rod shape and nonmotile flagella tuft, a microscopic examination of the bacteria will confirm its presence. *S. Mutans* is a Gram-positive bacterium with a thick cell wall and the gentian violet pigment. The cell wall's peptidoglycan (murein) and teichoic acids provide stiffness and structure by preventing protoplast osmotic lysis. The gel yields positive results, indicating that it can be used to inhibit microbes. Different concentrations of antimicrobial hydrogel were introduced into each isolated colony and the zone of inhibition was checked after incubation. The results of the preceding study show that the formulated hydrogel has a good antimicrobial effect. The area of the inhibition zone increased with increasing concentration, demonstrating the effectiveness of the gel at low and high concentrations. The prepared gel is capable of killing pathogenic bacteria. Further research into the approach's compatibility in oral mucosa for the treatment of gingivitis and periodontitis could be beneficial.

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