Comparative antimicrobial study of different toothpaste against pathogenic bacteria

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Abstract : Toothpaste is a paste or gel dentifrice used with a toothbrush as an accessory to clean and maintain the aesthetics and health of teeth. The study investigated antimicrobial activities from 12 different toothpaste against Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Streptococcus pyogenes*) and Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) bacteria. All bacteria were isolated from dirty water and identified with Gram staining and various biochemical test. Based on the results, all toothpaste having antimicrobial activity against maximum bacteria but pepsodent, sensodyne and patanjali of more effective toothpaste.

Keywords : Toothpaste, Bacteria, Gram positive, Gram negative, Biochemical test.

INTRODUCTION

Toothpaste is used to promote oral sanitation: it serves as an abrasive that aids in removing the dental plaque and food from the teeth, assists in suppressing halitosis, and delivers active ingredients to help prevent tooth decay and gum disease (gingivitis)[1]. Salt and sodium bicarbonate are among materials that can be substituted for commercial toothpaste. Toothpaste is not intended to be swallowed due to the fluoride content, but is generally not very unsafe if accidentally swallowed in small amounts; however, one should seek health attention after swallowing abnormally large amounts[2].

Abrasives are the ingredients that actually eliminate food waste and stains from teeth. Abrasives constitute at least 50% of typical toothpaste. These insoluble particles help remove plaque from the teeth. The removal of plaque and calculus helps reduce cavities and periodontal infection. Representative abrasives include particles of different silica and zeolites, calcium hydrogen phosphates, aluminum hydroxide, calcium carbonat. Abrasives were originally very rough and included things such as crushed egg shells, which were used by the ancient Egyptians, or crushed oyster shells, which were favored by the Romans. Today's abrasives are a bit gentler and typically include calcium carbonate, dehydrated silica gels and hydrated aluminium oxides. It's a mineral that helps strengthen the enamel on your teeth, making them less susceptible to cavities and less likely to wear down from acidic foods and drinks. The extra fluoride in toothpaste has beneficial effects on the development of dental enamel and bones. Sodium fluoride is the most common source of fluoride. Fluoride and abrasives – these ingredients might help you clean and protect your teeth. What they won't do is taste delicious on their own. That's why you'll typically find a number of flavoring ingredients in toothpaste. Toothpaste flavors typically come from sweetening agents, such as saccharin or sorbitol. Toothpaste comes in a variety of colors, and flavors intended to encourage use of the product. Three most common flavorants are peppermint, spearmint, and wintergreen. A number of of those flavoring agents like sorbitol actually play two roles. Sorbitol is an example of a humectant, an ingredient that protects loss of water in the toothpaste. A humectant traps water in the toothpaste so that when you squeeze the tube, you get a nice, smooth matter. Along with sorbitol, other examples of humectants include glycol and glycerol. It is significant to have detergents in toothpaste because they help to provide foaming to occur when you brush your teeth[3].

Toothpastes contain potential antimicrobial agents which could have a beneficial effect in the prevention of plaque and gingivitis. If these preparations were to be effective clinically, some effect on salivary bacteria would also be expected. This cross-over study measured salivary bacterial counts and the presence or absence of residual antibacterial activity in saliva following tooth brushing with different commercially available toothpastes, and moreover, compared their effect with that produced by a chlorhexidine gel. Generally, all toothpaste products produced a reduction in aerobic, anaerobic and streptococcal counts with a hexetidine containing toothpaste producing the largest and longest lasting reduction. In contrast, enzyme containing toothpaste and amine fluoride toothpaste had little effect on bacterial counts. The chlorhexidine gel produced the largest reduction in salivary counts. Residual antibacterial activity in saliva was only evident immediately following brushing with the hexetidine toothpaste, but for the chlorhexidine gel, was present up to 90 min following brushing. The findings of this study have illustrated the limited antibacterial activity of presently available toothpastes on the salivary flora compared to chlorhexidine, and as such, would tend to question the relative benefit of toothpaste in preventing periodontal disease through an antimicrobial effect.

DIFFERENT TOOTHPASTES USED IN THIS STUDY

Himalaya, Pepsodent, Dabur red, Close up max fresh, Colgate active salt, Colgate max fresh blue gel, Colgate cavity protection, Close up visible white, Sensodyne, Close-up deep action, Patanjali and Oral-B.

METHODOLOGY

ISOLATION AND IDENTIFICATION OF BACTERIA[4,5]

Preparation of NAM (Nutrient Agar) Media:
0.5% Peptone: It is an enzymatic digest of animal protein. Peptone is the principal source of organic nitrogen for the growing bacteria.
0.3% beef extract/yeast extract: It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.
1.5% agar: It is the solidifying agent.
0.5% NaCl: The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.
Distilled water: Water is essential for the growth of and reproduction of micro-organisms and also provides the medium through which various nutrients can be transported.

- pH is adjusted to 6.8±0.2 at 25 °C.
- Heat this mixture while stirring to fully dissolve all components.
- Autoclave the dissolved mixture at 121°C for 15 minutes.
- Once the nutrient agar has been autoclaved, allow it to cool but not solidify.
- Pour nutrient agar into Petri plate and leave plates on the sterile surface until the agar has solidified.
- Take dirty water sample was collected from the nearby area.
- 10µl of dirty water was spreader over the surface of media.
- Petri plate was then incubated for 24 hrs at 30°C.

Identification of Bacteria

Gram Staining

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer whereas the peptidoglycan layer in gram negative cells is much thinner and surrounded by outer lipid-containing layers.

- One clean glass slide was taken.
- A smear was prepared by placing a drop of water on the slide and then transferring microorganism to the drop of water with a sterile cooled loop. It was mixed and spread by means a circular motion of the inoculating loop.
- Smear was air dried and heat fixed.
- Smear was gently flooded with crystal violet for 1min.
- Gently washed with tap water.
- Smear was gently flooded with Gram's iodine and left for 1 min.
- Gently washed with tap water.
- Decolorized with 95% ethyl alcohol reagent. It was added drop by drop until no further violet colour comes out.
- Gently wash with tap water.
- Counterstained with safranin for 45 seconds.
- Gently washed with tap water.
- It was dried with bibulous paper and examined under oil immersion.

Motility Test

The motility test is not a biochemical test since we are not looking at metabolic properties of the bacteria. Rather, this test can be used to check for the ability of bacteria to migrate away from a line of inoculation thanks to physical features like flagella.
To perform this test, the bacterial sample is inoculated into mannitol motility agar media (Composition -Peptone 20g,Mannitol 2g, Potassium Nitrate 1g, Phenol Red 0.04 g and Agar 5 g per litre maintained at a pH 7.3 ± 0.2) using a needle. Simply stab the media in as straight a line as possible and withdraw the needle very carefully to avoid destroying the straight line. After incubating the sample for 24-48 hours observations can be made. Check to see if the bacteria have migrated away from the original line of inoculation. If migration away from the line of inoculation is evident then you can conclude that the test organism is motile (positive test). Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

BIOCHEMICAL TESTING OF ISOLATED BACTERIA

Catalase Test

Used to test for the presence of enzyme catalase. Hydrogen peroxide (H_2O_2) is formed as an end product of the aerobic breakdown of sugars. When H_2O_2 accumulates, it becomes toxic to the organism. Catalase decomposes H_2O_2 and enables the organism to survive. Only obligate anaerobes lack this enzyme.

Procedure: Streak nutrient agar slant with the organism

- Incubate at optimum temperature for 24-48 hours.
- Place a few drops of 3% H_2O_2 on the slant culture

Interpretation:

- Positive-Bubbling (O_2 gas is liberated from the H_2O_2)
- Negative- No bubbling.

Starch Hydrolysis

Used to determine the ability of an organism to hydrolyze (break down) starch. The enzyme amylase breaks starch down into components more easily metabolized by the organism.

Procedure: Make a single streak of the organism on a starch agar plate

- Incubate at optimal temperature for 24-48 hrs.
- Drop a small amount of IKI (Gram's Iodine) onto the plate and rotate the plate gently. (Iodine is an indicator of starch; in the presence of starch the iodine will turn blue/black)

Interpretation:

- Positive-A zone of clearing appears adjacent to the streak line.
- Negative- No clearing; only a blue/black area surrounding the streak line.

Citrate Utilization (Simmons Citrate Agar)

Used to determine if an organism is capable of using citrate as the sole source of carbon with production of the enzyme citrase.

The media contains sodium citrate as the carbon source, and ammonium salts as the nitrogen source, with bromothymol blue as the pH indicator. An organism that uses citrate breaks down the ammonium salts to ammonia, which creates an alkaline pH.

Procedure: Stab and streak Simmons citrate agar slant with the organism.

- Incubate at the optimum temperature for 24-48 hours.

Interpretation:

- Positive-Alkaline pH causes media to change from green to Prussian blue
- Negative- No color change

Urease Test

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide.
Many organisms especially those that infect the urinary tract, have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Procedure for urease test

1. The broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.
2. Leave the cap on loosely and incubate the test tube at 35°C in ambient air for 24-48 hrs; unless specified for longer incubation.

Interpretation:

- Positive-Pink colour
- Negative- Orange colour

Methyl Red Test

Media and Reagents used in Methyl Red (MR) Test

Ingredients per liter of deionized water:

buffered peptone	=	7.0 gm
glucose	=	5.0 gm
dipotassium phosphate	=	5.0 gm

Biochemicals test	1	2	3	4	5	6	7

Methyl red solution, 0.02%

a. Dissolve 0.1 g of methyl red in 300 ml of ethyl alcohol, 95%.
b. Add sufficient distilled water to make 500 ml.
c. Store at 4-8°C in a brown bottle. Solution is stable for 1 year.

Procedure of Methyl Red (MR) Test

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24 hrs pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37°C. for 24 hrs.
4. Following 24 hrs of incubation, aliquot 1ml of the broth to a clean test tube.
5. Reincubate the remaining broth for an additional 24 hrs.
6. Add 2 to 3 drops of methyl red indicator to aliquot.
7. Observe for red color immediately.

Antibacterial activity test[5,7]

Antibiotics, also called antibacterials, are a type of antimicrobial drug used in the treatment and prevention of bacterial infection. They may either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal activity. Antibiotic sensitivity or antibiotic susceptibility is the susceptibility of bacteria to antibiotics. Because susceptibility can vary even within a species (with some strains being more resistant than others), antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo. Testing for antibiotic sensitivity is often done by the Kirby-Bauer method. Small wafers containing antibiotics are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth.

The disc diffusion method (Bauer et al., 1966), using NAM, was employed to screen for antimicrobial activities. The plates were prepared by pouring 25 ml of media into sterile 90 mm Petri dish. Within 15 min after adjusting the turbidity of the inoculums prepared according to the method described above, a sterile cotton swab was dipped into the suspension and was spread uniformly on agar plates. Then sterile Whatman number one filter papers (6 mm diameter) were placed on the spread-surface of the Petri dishes and 3µl of dried extract was spotted on each of the filter papers. After this, the plates were incubated at 37°C for 24 hr . Subsequently, the inhibition zones formed around the discs were measured in millimeter.

RESULTS AND DISCUSSION

Spreading of dirty water on NAM plate

Figure 1: Colony growth of bacteria on NAM plate

IDENTIFICATION OF ISOLATED BACTRIA

Table 1. Biotechemical tests result of isolated bacteria

Gram staining	+ve	-ve	+ve	-ve	+ve	+ve	-ve
Shape	Rod	Rod	Cocci	Rod	Cocci	Cocci	Rod
Catalase	+	+	+	+	+	-	+
Motality	+	+	-	+	-	-	-
Urease	-	-	+	-	-	-	+
MR	+	+	-	-	-		-
Citrate	+	-	+	+	-	-	+
Starch hydrolysis	+	-	-	+	-	+	+
Possible identify bacteria	*B. subtilis*	*E. coli*	*S. aureus*	*P. aeruginosa*	*S. pneumoniae*	*S. pyogenes*	*K. pneumoniae*

Antimicrobial activity of different toothpaste

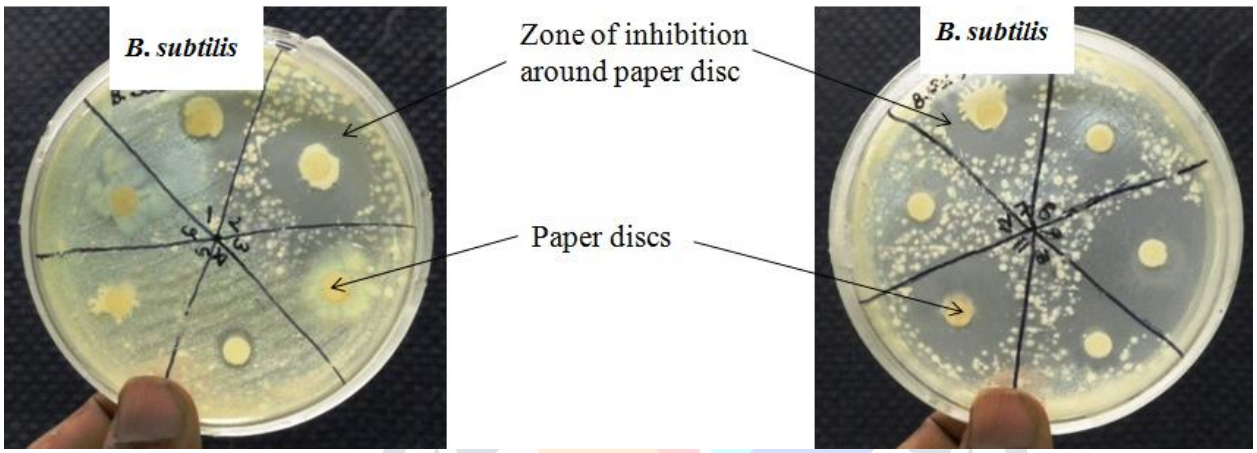

Figure 2: Antimicribial activity of 12 different toothpaste against *B. subtilis*.

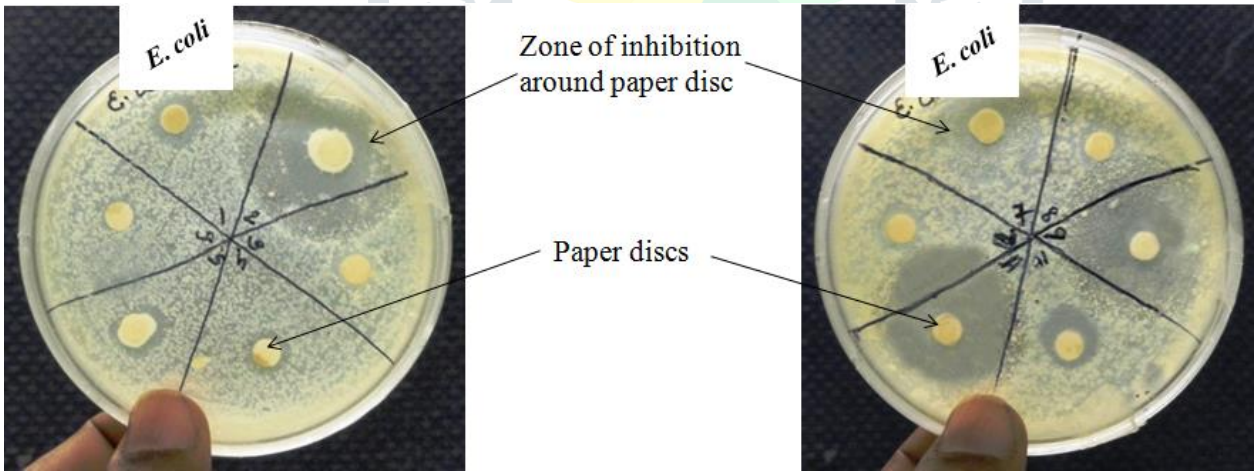

Figure 3: Antimicrobial activity of 12 different toothpaste against *E. coli.*

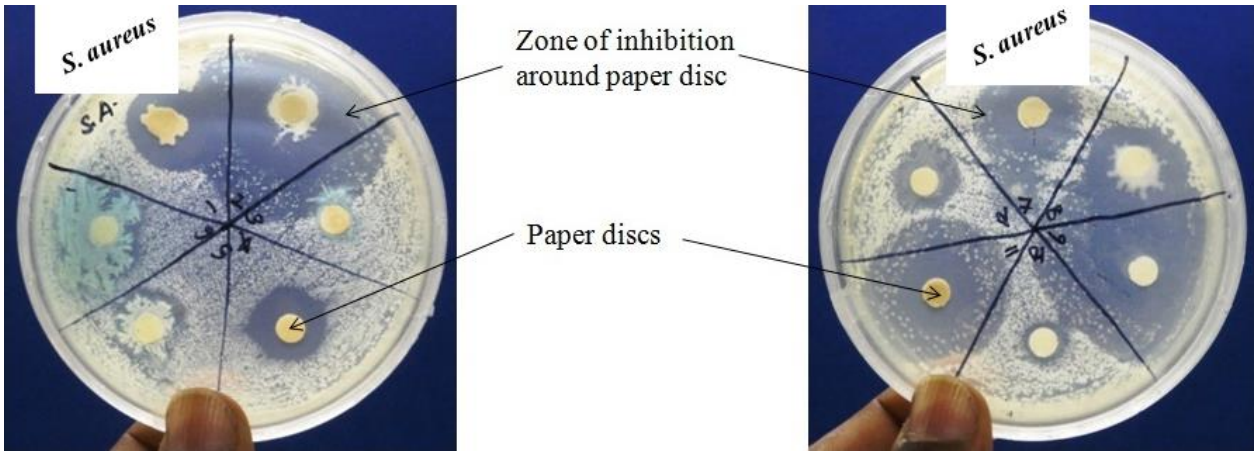

Figure 4: Antimicrobial activity of 12 different toothpaste against *S. aureus.*

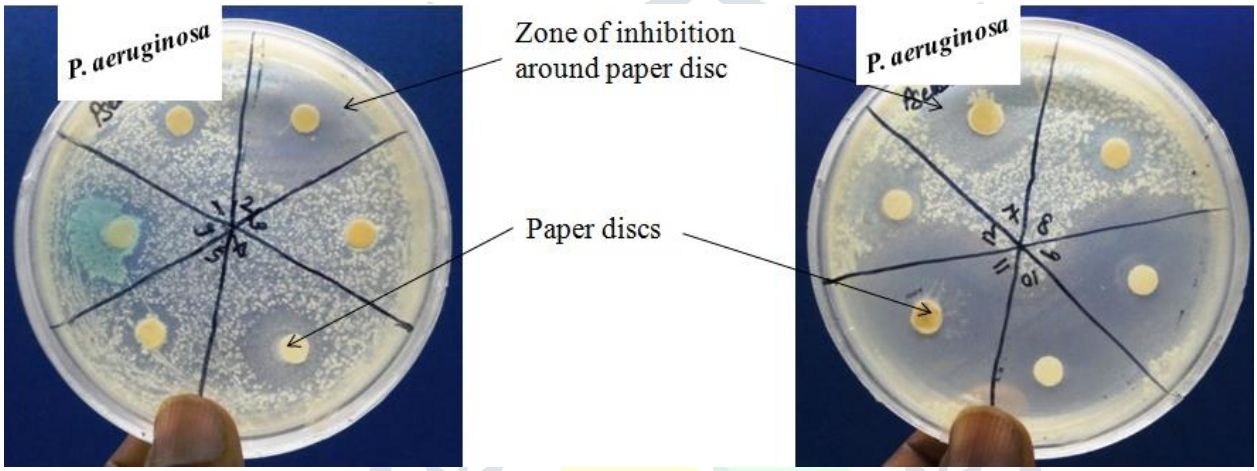

Figure 5: Antimicrobial activity of 12 different toothpaste against *P. aeruginosa.*

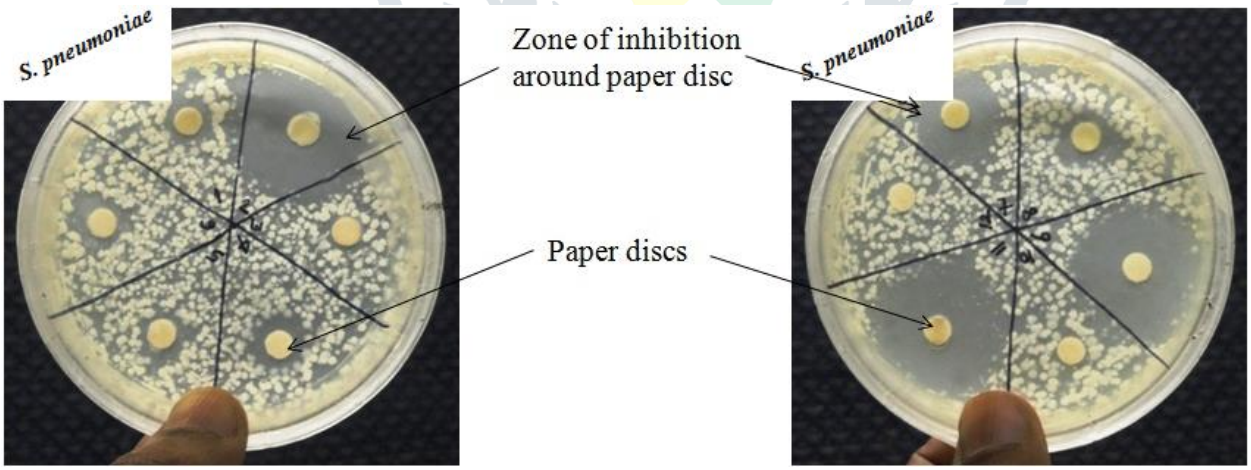

Figure 6: Antimicrobial activity of 12 different toothpaste against *S. pneumonia.*

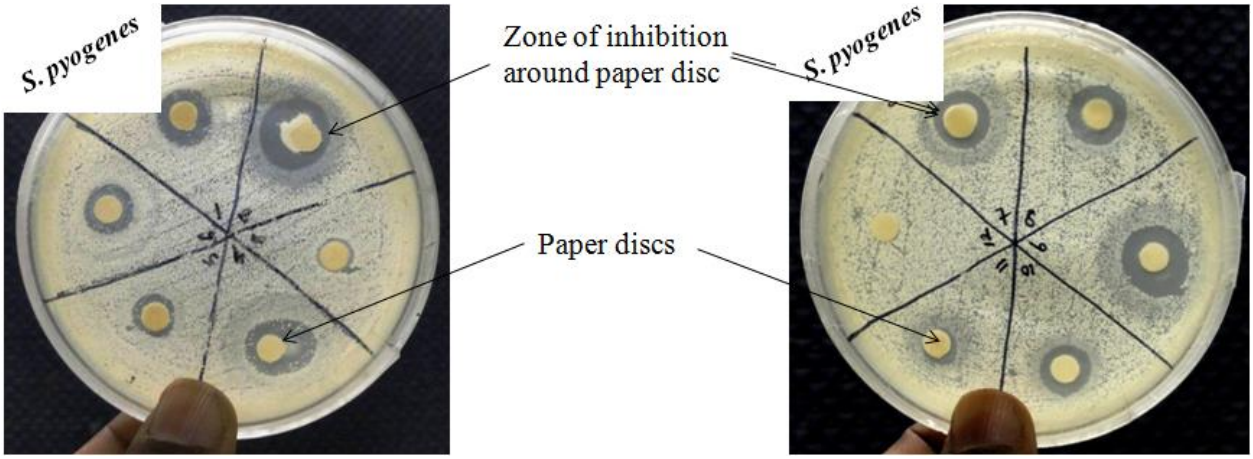

Figure 7: Antimicrobial activity of 12 different toothpaste against *S. pyogeness.*

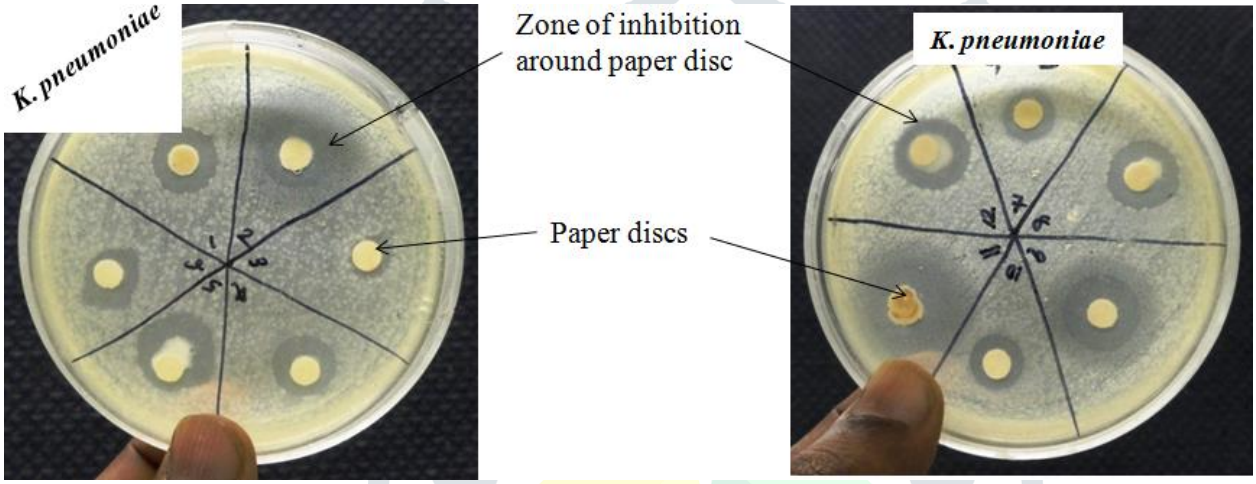

Figure 8: Antimicrobial activity of 12 different toothpaste against isolated bacteria.

Table 2: Antimicrobial activity test of different tooth paste.

S. No.	Tooth paste	Zone of inhibition (mm)						
		B. subtilis	*E. coli*	*S. aureus*	*P. aeruginosa*	*S. pneumoniae*	*S. pyogenes*	*K. pneumoniae*
1	Himalaya	10	9	11	7	9	9	12
2	Pepsodent	23.5	25	22	23	25	13.5	15
3	Dabur red	No	No	No	No	7.5	7.5	7.5
	Close up max fresh	7.5	8.5	15	14.5	12	7.5	11
5	Colgate active salt	No	10	No	7.5	11.5	10	15
6	Colgate max fresh blue gel	No	6.5	No	No	10.5	8.5	12
7	Colgate cavity protection	19.5	14	15	20	15	10	10
8	Close up visible white	No	7.5	12.5	11.5	12	12.5	13.5
9	Sensodyne	23.5	24.5	23	30	25	11	14.5
10	Close-up deep action	11.5	11	8	30	No	8	10.5
11	Patanjali	21.5	25	16	30	24	No	18.5
12	Oral-B	8	9	17.5	11	No	No	14

No = No any activity, Zone of inhibition included 6 mm diameter of paper disc.

It has already been reported earlier about the difference in sensitivity to antibiotics, external agents and detergents between Gram-positive and Gram-negative bacteria where Gram-negatives are reported to exhibit more resistance to these external agents. This

is due to the presence of lipopolysaccharides in their outer membranes which make these cells impermeable[8]. Whereas, the Grampositive bacteria possess an outer peptidoglycan layer, which is an inefficient permeability barrier[9] thus making the Gram-positives much more sensitive to external agents compared to Gram-negatives.

Conclusion

Toothpaste is a paste or geldentifrice used with a toothbrush as an accessory to clean and maintain the aesthetics and health of teeth. The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines, mostly plant drugs, for their primary health care needs. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. From the above experiment it is concluded that as 12 different toothpastes used for the comparison collected from local market are studied against the isolated bacteria from dirty water. All toothpaste showed antibacterial activity against *K.* pneumonia. Pepsodent, Sensodyne and Patanjali showed maximum antibacterial activity. Further studies will be needed to characterize the antibacterial activities of the components found in the toothpaste.

REFERENCE

1. American Dental Association Description of Toothpaste"Toothpaste", 2010.
2. National Library of Medicine. National Institutes of Health. Retrieved, 2014.
3. https://www.colgate.com/en-us/oral-health/basics/selecting-dental-products/what-is-in-toothpaste-five-ingredients-and-what-they-do-0814.
4. Abdulkadir M. and Waliyu S. 2012. Screening and Isolation of the Soil Bacteria for Ability to Produce Antibiotics. European Journal of Applied Sciences, 4(5):211-215.
5. Kumar R. S., Roymon M.G. and Ipe J.A. 2013. Isolation, biochemical and molecular characterization of strains of coliforms from the water sample collected from Shivnath river. Recent Research in Science and Technology, 5(2):57-63.
6. Bauer A.W., Kirby W.M.M. and Sherries T. 1966. Antibiotic susceptibility testing by a standard singlediscmethod.Americal Journal of Clinical Pathology, 45:493-96.
7. Gupta V. K, and Roy A. 2012. Comparative study of antimicrobial activities of some mangrove plants from Sundarban Estuarine Regions of India. Journal of Medicinal Plants Research, 6(42):5480-5488.
8. Nikaido H. and Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol Rev, 49:1-32.
9. Scherrer D. and Gerhardt P. 1971. Molecular sieving by the *Bacillus megatrium* cell wall and protoplast. J Bacteriol, 107:718-735.