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ANTIMICROBIAL ACTIVITY AND MICRO-FLORA QUALITY EVALUATION OF COMMONLY USED TOOTHPASTES

ORANUSI US*AND AKHIGBE EDI

Biological Sciences Department, Covenant University, Ota, Ogun State, Nigeria

***Corresponding Author: E Mail: orasol2002@yahoo.com.au; Ph.: +2348152215183**

ABSTRACT

To determine the microbiological quality and antimicrobial activity and effectiveness of commonly used toothpaste, thirty products consisting of eight brands of toothpaste were evaluated using standard methods and *Staphylococcus aureus* and *Candida albicans* as test organisms. All the toothpastes were sterile, and had some levels of antimicrobial activity at neat and 10^{-1} dilutions. Colgate and Signal had the highest zones of inhibition 20mm and 12mm against *Staphylococcus aureus*. Colgate and Macleans herbal neat concentration had the highest inhibition of 11mm and 10mm on *Candida albicans*. Colgate and Macleans had a minimum inhibitory concentration greater than 10^{-3} for *Staphylococcus aureus*. The other toothpastes showed minimum inhibitory concentration of 10^{-1} and 10^{-2}. Close Up herbal and Colgate had minimum inhibitory concentrations of greater than 10^{-3} for *Candida albicans.* At 10^{-2} dilution, total bacteria count of colonies increased as the time of exposure increased for most of the toothpastes. There was however, a general decline in the number of Candida colonies as the time of exposure increased. The toothpastes reduced and inhibited the test organisms mainly as neat and at 5 and 10 minutes. It is advocated that brushing the teeth for 5 to 10 minutes will allow for enough contact time for toothpaste to act on oral microbes and importantly pathogens for maximum result of good oral hygiene. Further studies on the relationship of brushing mannerism and toothpaste use culture are necessary. Regular survey of personal care products at the consumer level is advised to help keep the consumers informed of quality of products and

checkmate producers of fake product and thus help stamp out unwholesome product from our markets.

Key words: Antimicrobial Activity, Microbiological Quality, Minimum Inhibitory Concentration, Oral Microbes, Oral Hygiene, Pathogens

INTRODUCTION

The oral cavity (mouth) is the primary orifice of entry for dietary and respiratory elements. The mouth is continually bombarded by the various rudiments of nature, and when coupled with a perpetual warm and moist climate, serves as a hot bed for the residence and development of numerous microbial populations **[1]**. Waking up in the morning will not be complete without the duo of the toothbrush and toothpaste. The toothpaste has a resounding popularity with an example in every home. Ranging from animated adverts on the screen introducing new products with the claims of restoring blood flow to the teeth, breaking down food particles and even killing gems to billboards showing perfect white teeth , such preposterous adverts brings to mind toothpastes in their diverse shades and colors. It is a paste or gel used to clean the teeth. According to the American Dental Association-ADA (2013) **[2]**, toothpastes are pastes, gels or powders that help remove plaque, a film of bacteria that forms on teeth and gums. Toothpaste, also called dentifrice, is essential to daily oral hygiene routine. However, when cleaning teeth with a toothbrush and toothpaste, the essential cleaning is done by the mechanical brushing, and not by the active toothpaste chemicals. The toothpaste mainly improves the mechanical brushing and cleaning power of a toothbrush **[2]**. The purpose of oral hygiene using toothpaste is to reduce oral bacterial flora and deliver fluoride to the teeth. This is because fluoride has been proven to protect teeth against attack from bacteria, it helps remove plaque, prevent tooth decay by strengthening tooth enamel **[2]** and can be found naturally in many everyday things including food and drinking water. Toothpaste that efficiently reduces oral bacterial flora should contribute to dental health. Triclosan is a constituent used to avert gum disease (gingivitis), reduce build up of hardened plaque, called tartar and mitigate oral malodor and bad breath because of its antibacterial properties **[2,3,4]**.

The invention of collapsible tube for tooth paste and the introduction of fluoride to toothpaste in the 1960 have revolutionized the toothpaste industry and since then, the global market has been a recipient of various

toothpaste brands and this has never been slowed down. New brands roll in year in year out, some flourish while others fail **[5, 6]**.

Modern toothpaste was invented to aid in the removal of foreign particle and food substance in addition to cleaning of tooth. Many of the innovations in today's toothpaste were made after the fluoride break through which involved the addition of ingredients with special abilities to toothpaste and toothpaste packaging **[7, 8, 9]**. For effectiveness against various dental conditions, active ingredients have been added. Fluoride, antimicrobial agents e.g. triclosan and zinc, surfactants, anti-tartar agents, de-sensitising agents, abrasives, baking soda, teeth whitening agents, pyrophosphates, enzymes and flavors are some of the ingredients now added **[1, 10]**.

Essential oils, such as eucalyptol, menthol and thymol, frequently used for flavoring in oral products, can also contribute to the antiseptic properties of these products **[11]**. When these substances are added to oral products, they kill microorganisms by disrupting their cell walls and inhibiting their enzymatic activity. They prevent bacterial aggregation, slow multiplication and release endotoxins **[12]**. Thus toothpastes are designed to solve specific/certain dental problem e.g. anticaries/cavity protection, plaque and gingivitis prevention, tooth whitening, tooth sensitivity relief, tartar control, fresh breath and to prevent fluorosis in children.

Recently there has been an increase in the number of herbal toothpaste in the market with the claims of being made from natural sources. Toothpaste is classified as drugs not cosmetics because drugs should contain an ingredient to achieve the effect the consumer desires. As a sterile pharmaceutical product, great care must be taken both during production and use.

It is known that a balance exists in each person's oral microbial population. If that balance is lost, opportunistic microorganisms can proliferate, enabling the initiation of disease processes **[5]**. Different bacterial populations have been found in association with healthy and diseased areas in human oral cavity. The oral cavity is a highly contaminated area; it has approximately 10^8 bacteria/ml of saliva. *Streptococcus* sp, *Neisseria* sp, *Veillonella* sp, *Actinomyces* sp and *Lactobacillus* sp are more often related to carcinogenesis **[13, 14]**. In the human communities, dental problems are the most common health issues. Dental infections are mainly of three types, viz.: formation of dental plaques, dental caries and periodontal diseases **[7, 9]**.

The effects of toothpastes on the residual microbial contamination of toothbrushes has been explored extensively by various scientist, however, there is the need for consumer level evaluation to verify the claims of manufacturers as regards their antimicrobial activity and effectiveness. This work therefore aim to determine the microbiological quality and antimicrobial activity of different brands of toothpastes in Nigeria, to ascertain their sterility and ability to effectively reduce oral bacteria, destroy pathogens and improve dental health.

MATERIALS AND METHODS

Sources of Samples

Thirty (30) products consisting of 8 brands of toothpaste were bought from markets in three Nigerian cities; Lagos, Benin City in Edo State and Ota in Ogun State. The samples were collected between the months of February and March. All samples collected are within the expiry date from manufacture.

Sample Preparation

A calculated amount (1ml) of the toothpaste was collected via the use of a sterile syringe. The toothpaste was then diluted using sterile water following the method as described by Ogunledun *et al*, (2008) **[15]** to obtain dilutions of 10^{-1} and 10^{-2}.

Assessment of Microbiological Quality of Toothpaste

In the assessment of the microbiological quality of toothpaste, the method described by Ogunledun *et al.*, 2010, **[15]** and Okpalugo *et al.*, 2009, **[4]** was used with slight modifications. Aliquot 1ml of the paste was added to 9ml of peptone water and serial dilutions were made to 10^{-2}. The test tube was agitated manually for about 1 minute, 1mL of each dilution (10^{-1} and 10^{-2}) was applied onto Nutrient Agar, MacConkey agar and Sabouraud Dextrose Agar (all from Biomark, India) via the spread plate method. The raw pastes were also streaked on the different media plates. The Nutrient Agar, MacConkey agar and Sabouraud Dextrose Agar plates were incubated upside down at 37°C and ambient room temperature of 28±2°C respectively for 24 hours.

Assessment of Antimicrobial Effect

Sources of Test Isolates

Pathogenic strains of *Candida albicans* and *Staphylococcus aureus* were obtained from the Department of Biological Sciences Covenant University. *E. coli* (ATCC 25322) and standard *S. aureus* also obtained from Department of Biological Sciences Covenant University were used as controls. Test isolates were maintained on nutrient agar slants at refrigeration temperature of 4°C. Prior to experiment, the organisms to be used were

inoculated in nutrient broth in McCartney bottles and incubated at 37°C for 24 hours.

Standardization of Inocula

Overnight broth culture of test organisms were placed in test tubes of uniform and equal diameter to one containing McFarland standards. Dilutions with sterile water was made of the overnight broth cultures till the solution appeared the same in turbidity when compared to the McFarland standard 0.5 equivalent to $1.5x10^8$ CFU/ml.

Evaluation of Antimicrobial Activity

The antimicrobial activity of the toothpastes was evaluated via three (3) methods:

i. **Susceptibility Test by Agar Well Diffusion Method**

This was done using the method of Lee *et al.*, 2004, **[5]** with modifications. Mueller-Hinton agar (Oxoid, England) prepared in accordance with the manufacturers instruction was seeded with standardized culture of the test organisms. A sterile cork borer was used to cut uniform wells of 5mm diameter on the surface of the agar, using a sterile syringe, a drop of the neat paste was spotted onto the seeded plates and the different dilutions 10^{-1} and 10^{-2} was filled into the wells. Sterile distilled water and 10µg Gentamicin (Oxoid) was used as negative and positive controls. The plates were allowed to stand for diffusion of the pastes and were incubated at 37°C for 24h. The antibacterial susceptibility was indicated by the zone diameter of inhibition and was measured using a transparent ruler.

ii. **Linear Regression Method (Time Kill Test)**

The method of Bou-Chacra *et al.*, 2005, **[16]**, Ogunledun *et al*., 2008, **[15]** was adopted with slight modifications. To 5mL of the neat paste, was added 5mL of the standardized 1.5 $x10^8$ organism. The tube was swirled to mix and agitated constantly at 37°C in shaker incubator (Guangzhon healthy ling- HZQ-X300) for two minutes. Aliquot 0.1ml was plated out on Nutrient agar and Mueller-Hinton agar respectively. The same procedure was repeated at five and ten minutes. Plates were incubated at 37°C for 24h, colonies observed on plates at the expiration of incubation time were counted using colony counter (HCY 560-Vision Scientific, Japan). To the different dilutions of

10^{-1} and 10^{-2}, 1ml of the standardized test organisms was added and agitated for 2 minutes, 5minutes and 10 minutes as in neat. Approximate 0.1ml sample was collected and treated as in neat for colony enumeration.

iii. Determination of Minimum Inhibitory Concentration

The method as described by Candido *et al*., 1996, **[17]**, was adopted with little modification. To 10ml of the neat and dilutions of toothpastes in test tubes was added 1ml of standardized test organisms. The tubes were incubated for 24h at 37°C and then examined for growth evidenced by turbidity of medium. The MIC was recorded as the lowest dilution of the toothpaste that inhibited the growth of the test organisms evidenced by lack of turbidity. Tubes showing no growth were plated out on Nutrient agar, the highest dilution that yielded no growth of bacteria colonies after 24h incubation was recorded as minimum bactericidal concentration (MBC)

RESULTS

None of the toothpaste had growth of microbial colonies after 24 to 48h incubation at 37°C. **Table 1** shows the susceptibility of test organisms to different dilutions of toothpastes. At neat, Colgate had the highest zone of 20mm followed by Signal with a zone of 12mm. The lowest zones were recorded by Maxam and Close Up red hot with 7mm each. At 10^{-1}, Colgate and MyMy had the highest zones of 16mm and 8mm. Maxam and Dabur had the lowest zones of 3mm each. At 10^{-2}, Colgate and Macleans had the highest zones of 5mm each while Maxam had the lowest of 2mm. On *Candida albicans,* Colgate and Macleans herbal neat had the highest inhibition of 11mm and 10mm respectively, while the least zone of 5mm was given by Maxam. At 10^{-1}, Colgate had the highest of 8mm, Close Up red hot and Dabur had 5mm. Macleans and Macleans herbal had no effect. However, at 10^{-2}, only Close Up red hot and Signal had inhibition zones of 3mm each while Dabur had 1mm. The control organisms are more susceptible than the clinical test organisms; however, the zone of inhibition reduces with dilution as in the test organisms.

The MIC test **Table 2** shows that Colgate and Macleans showed no growth at 10^{-3} for *Staphylococcus aureus*, hence having a minimum inhibitory concentration greater than 10^{-3}. The other toothpastes showed minimum inhibitory concentration of 10^{-1} and 10^{-2}. On *Candida albicans,* Close Up herbal and Colgate had minimum inhibitory

concentrations of greater than 10^{-3} while Dabur showed growth when diluted.

Tables 3 and 4 show the time kill test, within the time of exposure, Colgate showed no growth of the organism. Macleans herbal at10^{-2} had a total bacteria count of 1.1x 10^2 colonies in two minutes, this increased as the time of exposure increased. This was so for Florish gel, Maxam, Close Up herbal and Dabur. For Close Up red hot, there was a reduction from 2.5x 10^2 colonies to no growth at 10 minutes. MyMy had a decrease at 2 minutes (4 colonies) which remained constant at 5 minutes and 10 minutes. Colgate showed no growth of *Candida albicans* throughout the time of exposure. Close Up red hot had no growth at 10^{-1} and a decline at 10^{-2} from 1.6x10^2 colonies to no growth. There was a general decline in the number of Candida colonies as the time of exposure increased. At 10 minutes of exposure, and at neat and 10^{-1}, all toothpaste showed no growth for *Candida albicans*, i.e. all the toothpastes were more active on *Candida albicans* at neat and 10^{-1}.

Table 1: Susceptibility of Test Organisms to Different Dilutions of Toothpastes

Toothpaste	Zone diameter of inhibition (mm)					
	Staphylococcus aureus			*Candida albicans*		
	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}
Close Up herbal	10	4	3	8	3	5
Close Up red hot	7	5	4	7	5	3
Colgate	20	16	5	11	8	6
Dabur	11	3	3	8	5	1
Florish gel	8	4	3	9	4	-
Macleans	9	6	5	8	-	-
Macleans herbal	11	4	3	10	-	-
Maxam	7	3	2	5	3	-
MyMy	9	8	4	7	4	-
Signal	12	5	3	6	3	3

NOTE: - = No Inhibitory Effect

Table 2 Minimum Inhibitory Dilution for Test Organisms

Toothpaste	Dilutions							
	Staphylococcus aureus				*Candida albicans*			
	Neat	10^{-1}	10^{-2}	10^{-3}	*Neat*	10^{-1}	10^{-2}	10^{-3}
Close Up herbal	N	N	G	G	N	N	N	N
Close Up red hot	N	N	N	G	N	N	N	G
Colgate	N	N	N	N	N	N	N	N
Dabur	N	N	G	G	N	G	G	G
Florish gel	N	N	G	G	N	N	N	G
Macleans	N	N	N	N	N	N	G	G
Macleans herbal	N	N	G	G	N	N	G	G
Maxam	N	N	G	G	N	N	G	G
MyMy	N	N	N	G	N	N	N	G
Signal	N	N	N	G	N	N	N	G

NOTE: N = No Growth; G= Growth

Table 3: Time Kill Test for *Staphylococcus aureus*

Time (min)	Toothpaste dilutions								
	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}
	Close Up herbal			Close Up red hot			Colgate		
2	5	4	3	N	N	$2.5x\ 10^2$	N	N	N
5	N	N	$6.4x10^1$	N	4	$1.5x\ 10^1$	N	N	N
10	N	N	$9.7x\ 10^1$	N	N	N	N	N	N
	Dabur			Florish gel			Macleans		
2	N	8	1	N	8	7	N	N	N
5	N	3	9	3	N	$2.8x10^1$	N	N	N
10	N	N	$1.0x\ 10^1$	N	N	$3.1x\ 10^2$	N	N	N
	Macleans herbal			Maxam			MyMy		
2	N	3	$1.1x\ 10^2$	4	2	$1.0x\ 10^1$	N	6	4
5	N	N	$2.3x\ 10^2$	2	N	$5.1x10^1$	N	4	$1.0x\ 10^1$
10	N	N	$3.5x\ 10^3$	N	N	$1.0x\ 10^2$	N	N	$1.0x\ 10^1$
	Signal								
2	N			6			$1.3x\ 10^2$		
5	N			N			$2.5x\ 10^2$		
10	N			N			$3.3x\ 10^3$		

NOTE: N = No Growth

Table 4: Time Kill Test for *Candida albicans*

Time (min)	Toothpaste dilutions								
	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}
	Close Up herbal			Close Up red hot			Colgate		
2	$2.2x10^2$	9	$1.3x\ 10^1$	$1.4x\ 10^1$	$1,5x\ 10^2$	$1.6x10^2$	N	N	N
5	8	N	8	N	$1.5x\ 10^1$	$5.5x\ 10^1$	N	N	N
10	N	N	N	N	N	N	N	N	N
	Dabur			Florish gel			Macleans		
2	$4.5x\ 10^1$	$1.5x\ 10^1$	$5.4x10^2$	$4.0x\ 10^2$	$9.3x\ 10^1$	$2.4x10^2$	26	6	9
5	5	N	$1.2x10^2$	N	8	6	N	$1.0x\ 10^1$	$1.1x\ 10^1$
10	9	N	$3.0x\ 10^1$	N	N	$1.0x10^1$	N	8	$1.0x\ 10^1$
	Macleans herbal			Maxam			MyMy		
2	$1.0x10^1$	18	$1.9x10^1$	N	$1.2x10^1$	$2.2x10^2$	8	$3.9x10^1$	$3.5x\ 10^2$
5	$2.3x10^1$	N	9	N	$3.2x10^1$	5	N	$2.9x10^2$	$2.7x\ 10^1$
10	N	N	9	N	N	$1.0x10^1$	N	N	$1.4x10^1$
	Signal								
2	N			$1.7x\ 10^2$					$1.0x10^1$
5	N			$3.5x\ 10^1$					8
10	N			N					2

NOTE: N= No Growth

DISCUSSION

The microbiological analysis of all the samples yielded no growth, hence were sterile in compliance with the sterility standard required of such sanitary personal care products **[2, 18]**. These findings also corroborate the reports of **[4]**.

All samples showed some level of antimicrobial activity and efficacy. This could be attributed to the presence of antimicrobial substances in toothpaste. More activity was shown against *Staphylococcus aureus* than to *Candida albicans*. Colgate had a better activity on the test organisms as compared to

other toothpaste. This is not in tandem to reports of **[19]** stating that "Close Up® is the best toothpaste out of the 7 pastes tested, followed by Aquafresh®, Pepsodent®, DailyNeed®, Colgate®, Macleans® and Minta® in that order." The findings of **[4]** and **[20]** depict Colgate as having resounding antimicrobial activity compared to other toothpastes. The results obtained also showed a concurrence with the work of **[5]** where Colgate was used as the standard.

As the concentration was reduced i.e. increased dilution, the zone of inhibition decreased in all the toothpastes. The diluted paste to 10^{-3} does not really have any effect. This was true for all except for Colgate and Close Up herbal which showed that they would be effective at 10^{-3} and their minimum inhibitory concentration will be greater than 10^{-3} for *Staphylococcus aureus* and *Candida albicans.* The toothpastes had better activity against *Staphylococcus aureus* than on *Candida albicans* this could be attributed to the stronger antibacterial other than antifungal activities of the active ingredients. Yigit *et al*., 2008, **[21]** showed that herbal toothpaste exhibited good antifungal activity against all *Candida* species. This was corroborated by the results obtained (Dabur and Close Up herbal), Macleans herbal however, prove to the contrary.

The time kill test showed the time taken for the organism to be reduced or killed completely. It can be deduced that if the toothpaste has contact with the organism at the time T, it will have a static or cidal effect. Result obtained in this report shows that at neat (undiluted toothpaste) the microbial load of test isolate *Staphylococcus aureus* decreases with time from 2 to 10 minutes. However, at 10^{-2} dilutions, there was increase in microbial load with time in most of the toothpaste. This could be explained by the fact that dilution erodes the antimicrobial factor making the toothpaste available for metabolism by the organism. Similarly the ingredients sodium saccharin and other sweeteners in the toothpastes could support microbial proliferation **[4]**. The concentration of Candida albican was reduced through time and in all dilutions. Only Colgate and Macleans inhibited/killed the test organisms at all dilution and exposure time. This indicates that apart from Colgate and to some extent Macleans, no brand of the toothpastes sampled could completely remove or destroy the test organisms. Okalugo *et al*., 2009, **[4]** reported that no brand of toothpaste evaluated in their work removed teeth bacteria by up to 50%, Adenike *et al.*, 2012, **[20]**, reported a susceptibility rate of 18.6% to 100% for *candida spp*.

Tiwari *et al.*, 2008, **[9]**, showed that the toothpastes containing triclosan as a major chemical ingredient pose significant antibacterial activity while Okpalugo *et al.*, 2009, **[4]**, showed that toothpaste brands that contained only fluoride were the least effective in reducing mouth bacteria. Although this work was done without considering the chemical composition of the toothpaste used, Colgate toothpaste which has triclosan as one of its active ingredients showed better activity than other toothpaste. This was in correspondence with the work of Tiwari *et al*., 2008, **[9]**.

The toothpastes reduced and inhibited the test organisms mainly as neat and at 5 and 10 minutes. It is advocated that brushing the teeth for 5 to 10 minutes will allow for enough contact time for toothpaste to act on oral microbes and more importantly pathogens for maximum result of good oral hygiene. Brushing for this long could equally mean that saliva will dilute the toothpaste to as much as 10^{-2} and the effect of increase in microbial load established in this work may not be ruled out. Further studies should be carried out on the relationship of brushing mannerism and toothpaste use culture. Regular survey of personal care products at the consumer level is advised this will help keep the consumers informed of quality of products and checkmate producers of fake product to prevent a repeat of the ugly experiences of 1990, 2007 and 2009 when many innocent children were killed by Diethylene glycol (DEG) in a formulae, the discovery of DEG in some imported toothpastes and the introduction into the market of toothpaste of poor microbial quality that led to the 2nd of March, 2009 recall of some batches of *Macleans* toothpaste. According to a consumer website, "Although the microbial load of the tooth paste pose little risk to most people, those with a weakened immune system may be at risk of infection" **[22, 23]**.

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