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***In Vitro* Study to Investigate the Antimicrobial Efficacy of Different Toothpastes and Mouth Rinses.**

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ABSTRACT

The aim of the present *in vitro* study was to investigate the antimicrobial efficacy of different dentifrices (toothpastes and mouth rinses) and to study the variations in their effectiveness against the test microorganisms. *Escherichia coli* and *Candida albicans* were selected as test microorganisms against which the different toothpastes and mouth rinses were tested. The procedure included the evaluation of dentifrices, antimicrobial assay by modified agar well diffusion method and finally statistical analysis. This investigation showed that toothpaste having natural formulation gave maximum zones of inhibition against *Candida albicans* at all dilutions and *E. coli* at 1:1 dilution. Results from our study have shown that toothpaste formulation containing natural antimicrobial agents were more effective in controlling the oral microflora compared to toothpastes containing synthetic antimicrobial agents.

Keywords: Toothpastes and mouth rinses, *Escherichia coli*, *Candida albicans*, zone of inhibition, natural antimicrobial agents.

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INTRODUCTION

Dental caries and related oral diseases like gingivitis and periodontitis are most common oral diseases throughout the world including both developed and developing countries, affecting people from all ages of life. The frequency of these oral diseases is continuously increasing with change in eating habit of among peoples of different age group and increased consumption of sugar [1]. The occurrence of dental caries is approximately 60 - 65% among the Indian population [2, 3]. The epidemiological studies evidently reflect a noticeable increase in the prevalence of dental caries in many developed and developing countries [4]. Dental problems are of three types, formation of dental plaques, dental caries and periodontal diseases [5]. Dental caries, also known as tooth decay or a cavity, is an infection, generally bacterial in origin, localized and transmissible, that results in the destruction of hard dental tissue. It results from accumulation of plaque on the surface of the teeth and biochemical activities of complex micro-communities. *Streptococcus mutans* is one of the main opportunistic pathogens of dental caries [6]. The pathogens of dental caries, play a central role in fermenting carbohydrates resulting in acid production, and leading to the demineralization of the tooth enamel [7]. In addition, other microflora like *Escherichia coli* and *Candida* are also associated with active caries lesions. *C. albicans* is the most common yeast isolated from the oral cavity. It is by far the fungal species most commonly isolated from infected root canals, showing resistance to inter-canal medication [8]. Poor oral hygiene is one of the reasons for growth of these microbes and their harmful activities.

Periodontal diseases are bacterial infections that affect the supporting structure of the teeth (gingival, cementum, periodontal membrane and alveolar bone). The endotoxins, hydrolytic enzymes and toxic bacterial metabolites are involved in this disease. Gingivitis, an inflammatory condition of gum, is the most common form of periodontal disease. Serious forms of periodontal disease that affect the periodontal membrane and alveolar bone may results in tooth loss. *Streptococci*, *spirochetes* and *bacteroides* are found to be the possible pathogens responsible for the disease.

Toothpaste is a gel dentifrice used with a toothbrush as an accessory to clean and maintain the aesthetics and health of teeth. Toothpaste is used to promote oral hygiene [9]. Triclosan, an antibacterial agent, is a common toothpaste ingredient in the United Kingdom. Triclosan or zinc chloride prevents gingivitis and, according to the American Dental Association, helps reduce tartar and bad breath [10]. Herbal toothpastes contain baking soda, aloe, eucalyptus oil, myrrh, plant extract, and essential oils. Various Oral microflora includes most commonly *Escherichia coli* and *Candida albicans*.

Action of fungus

Like *Candida albicans* causes Oral thrush in which curd-like white patches formed inside the mouth, on the tongue and palate and around the lips. It may also cause cracked, red, moist areas of skin at the corners of the mouth. *Candida* species causes infections in immuno-compromised patients, individuals on drug therapy, and the chronically ill

Action of bacteria

Like *Streptococcus mutans* and *E. coli*, they accumulate on both the tooth surface and gingival epithelium. The endotoxins, hydrolytic enzymes and toxic bacterial metabolites are released by them causing demineralization and destruction of tooth. At the sites with a low rate of salivary flow, grooves on the occlusal surfaces of molar and premolar teeth provide microscopic retention sites for plaque bacteria.

Action of dentifrices on fungal and bacterial activity: They contain remineralization factors such as calcium and fluoridated components maintaining mineral balance. They also contain antibacterial and antifungal agents which kills them by disrupting their cell wall. Proper method of tooth brushing helps to control growth of pathogenic oral microflora. Toothpaste is a gel dentifrice and mouthrinse is a solution dentifrice used as an accessory to clean and maintain the aesthetics and health of teeth.

Though similar studies have been reported in literature, the studies are comparable as the constituent herbs of the dentifrices used in the present study are different from those of the other published data. Though it has been reported that the herbal dentifrices are inferior in inhibiting cariogenic microbes, the ingredient herbs of these study are different [11].

MATERIALS AND METHODS

For the present study, two strains of micro-organisms- *Escherichia coli* [MTCC- 68] and *Candida albicans* [MTCC- 227] were taken. The media used were Nutrient agar media and Yeast peptone dextrose agar media respectively for each microorganism. We prepared dilutions of several brands of toothpastes with different compositions and also mouth rinses (**TABLE 1**). Other important materials required were double distilled water, pyrogen free water, test tubes, petri plates, micro pipettes, gel puncher.

TABLE 1: Ingredients of Common Toothpastes and Mouth Rinses marked as A to I

A	Anacyclus pyrethrum (akkalkara), Embeliaribes, Azadirachta indica (neem), Curcuma longa (haldi) Acacia arabica (babool), Salvadora persica (misvak) Xantho xylumalatum (tomar), Quercus infectoria Syzygium aromaticum (lavang) Piper sylvaticum (pepal) Barleria prionitis (bajradanti) Mimusp selengi
B	Carrageenan, Calcium carbonate, Silica, Titanium dioxide, Sodium silicate, Sodium bicarbonate, Sodium monoflourophosphate, Sodium saccharin, Flavour, Sorbitol, Triclosan, Sodium lauryl sulfate or SLS
C	Sodium fluoride, Hydrated silica, Zinc sulphate, Water, Sodium saccharin, Sorbitol Triclosan, PEG-32, Sodium lauryl sulfate or SLS Cocamidopropylbetain, cellulose gum and flavor
D	Hydrated silica, Calcium carbonate, Water, Potassium nitrate, Sorbitol, flavour, Cellulose gum, Sodium silicate, Benzyl alcohol, Sodium lauryl sulfate or SLS, Sodium saccharin, Perlite, Sodium monofluorophosphate CI 74160

	CI 74260 CI 77891
E	sodium Fluoride 0.21%. Inactive Ingredients: Sorbitol, Water, Hydrated Silica, Glycerin, Propylene Glycol, Sodium Lauryl Sulfate, Xylitol, PEG-8, Flavor, Titanium Dioxide, Xanthan Gum, Sodium Saccharin, Methylparaben, Propylparaben, FD&C Blue No. 1. , CI 77891, CI 42090, Sodium carboxymethylcellulose
F	Sorbitol, Aqua, Hydrated silica, Glycerin, Silica, Sodium lauryl sulphate, Flavour, Xanthan gum, Titanium dioxide, Sodium saccharin, Menthol, Sodium benzoate
G	Marichapippali, Sunthi, Karpoor, Tomarbeej, Lvang oil, Sugandhitravaya, Pudina, Satva, Kasmi, Haritaki, Shudha
H	Purified water, Sorbitol, Alcohol, Poloxamer 407, Benzoic acid, Sodium saccharin, Mouthwash flavour, Eucalyptol, Methyl salicylate, Thymol, Sodium benzoate, Menthol, CL 47005, CL 42053
I	Povidone Iodine

METHOD

Our method was divided into three phases:

Evaluation of dentifrices

Antimicrobial Assay by modified agar well diffusion method

Statistical Analysis

Evaluation of dentifrices

Solutions of selected toothpastes and mouthrinses made by mixing 3gm of toothpaste/mouthrinses in 3 ml of pyrogen free distilled water to give 1:1 dilution. Then further dilutions made were 1:2, 1:4, 1:8, 1:16.

Antimicrobial Assay

NAM and YPDA plates were prepared to assess the antimicrobial activity.

NAM and YPDA plates were seeded with 0.5 ml of 24 h broth cultures of *Escherichia coli* and *Candida albicans*.

Plates were allowed to dry for 1 h.

Sterile gel puncher is used to cut one central and five wells at equidistance in each of the plates. 0.02 ml of dentifrices dilutions was introduced into each of the five wells.

Plates were incubated at 37°C for 24 h for *E. coli* and 48 h for *C. albicans* .

Experiments were repeated thrice.

STATISTICAL ANALYSIS

Calculation of zone of inhibition

The average of vertically and horizontally measured diameter of obtained zone of inhibition were taken.

The size of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample or product - a larger zone of inhibition usually means that the antimicrobial is more potent.

Calculation of Standard deviation

It was calculated using MS-Excel.

RESULTS

The results of this investigation showed that toothpaste formulation A had maximum zones of inhibition against the test organism *Candida albicans* compared to all other toothpaste formulations. In *Escherichia coli* the zones of inhibition were less in comparison to *C. albicans* but were significantly different at higher dilutions (1:8, 1:16) for toothpaste formulation A. Mouthrinse formulation H showed maximum efficacy against the test organism, *Escherichia coli* compared to all other mouthrinse formulations. However, mouthrinse formulations I showed a very little anti-microbial activity. The mean values \pm standard deviations of zones of inhibition were calculated for all the test organisms. Each experiment was repeated thrice ($n = 3$). (Refer to TABLE 2 and TABLE 3)

TABLE 2: Antimicrobial activity of toothpastes and mouthrises against *E. coli*

Zone of inhibition in mm	1:1 Dilution Mean value \pm Std. Deviation	1:2 Dilution Mean value \pm Std. Deviation	1:4 Dilution Mean value \pm Std. Deviation	1:8 Dilution Mean value \pm Std. Deviation	1:16 Dilution Mean value \pm Std. Deviation
A	27.25 \pm 1.68	18.50 \pm 2.54	18.91 \pm 2.75	15.58 \pm 2.12	13.90 \pm 2.67
B	21.08 \pm 3.82	21.25 \pm 4.76	22.91 \pm 4.15	19.50 \pm 4.13	17.83 \pm 1.80
C	13.66 \pm 2.92	11.75 \pm 5.41	14.16 \pm 5.24	09.00 \pm 4.63	08.58 \pm 4.30
D	13.00 \pm 0.50	11.16 \pm 0.14	09.91 \pm 0.52	08.19 \pm 1.84	08.00 \pm 1.73
E	12.00 \pm 3.90	10.66 \pm 2.52	10.08 \pm 2.24	08.91 \pm 2.75	07.33 \pm 2.30
F	7.00 \pm 0.866	6.08 \pm 2.929	2.25 \pm 1.952	0.00 \pm 0.00	0.00 \pm 0.00
G	0.00 \pm 0.00				
H	8.33 \pm 1.607	3.50 \pm 3.041	2.33 \pm 2.020	0.50 \pm 0.866	0.00 \pm 0.00
I	3.00 \pm 0.23	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00

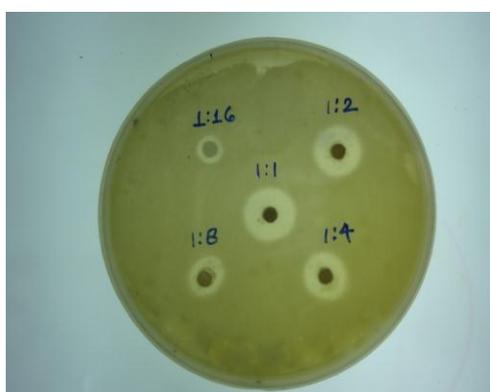
TABLE 3: Antimicrobial activity of toothpastes and mouthrises against *C. albicans*

Zone of inhibition in mm	1:1 Dilution Mean value \pm Std. Deviation	1:2 Dilution Mean value \pm Std. Deviation	1:4 Dilution Mean value \pm Std. Deviation	1:8 Dilution Mean value \pm Std. Deviation	1:16 Dilution Mean value \pm Std. Deviation
A	18.58 \pm 3.55	15.08 \pm 1.87	12.58 \pm 1.12	11.66 \pm 1.29	11.41 \pm 2.67
B	17.58 \pm 1.12	15.00 \pm 2.63	14.16 \pm 2.12	9.33 \pm 2.46	5.33 \pm 3.41
C	11.50 \pm 2.81	9.58 \pm 4.25	8.25 \pm 3.47	4.66 \pm 4.75	2.27 \pm 4.76
D	10.41 \pm 2.02	9.75 \pm 1.14	7.83 \pm 4.62	5.16 \pm 4.90	2.83 \pm 4.90
E	9.33 \pm 0.520	7.46 \pm 2.073	6.23 \pm 0.25	6.06 \pm 0.60	5.40 \pm 0.173
F	14.75 \pm 1.00	10.91 \pm 0.28	8.30 \pm 0.381	5.16 \pm 1.011	2.41 \pm 1.010
G	0.00 \pm 0.00				
H	4.16 \pm 0.577	0.50 \pm 0.866	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
I	0.00 \pm 0.00				

Figure 1: Antimicrobial activity of toothpastes and mouthrises against *C. albicans* (YPDA media)



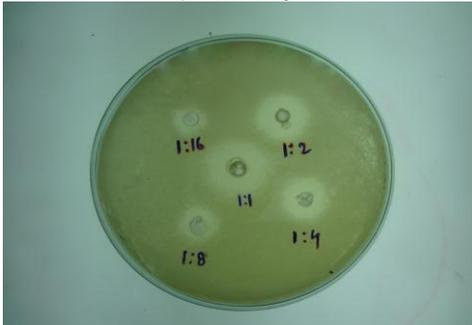
a) Control



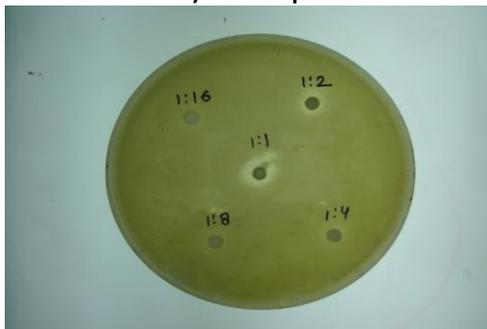
b) Toothpaste A



c) Toothpaste B



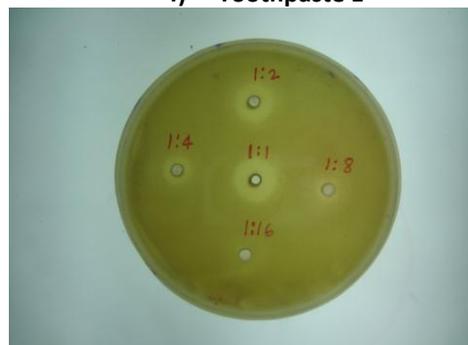
d) Toothpaste C



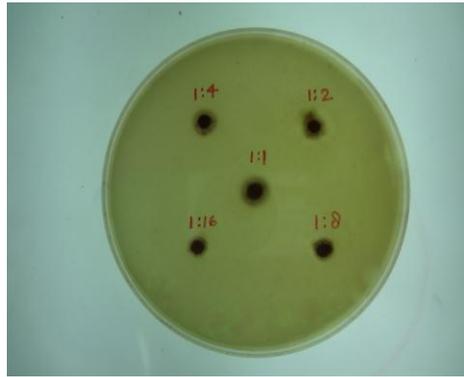
e) Toothpaste D



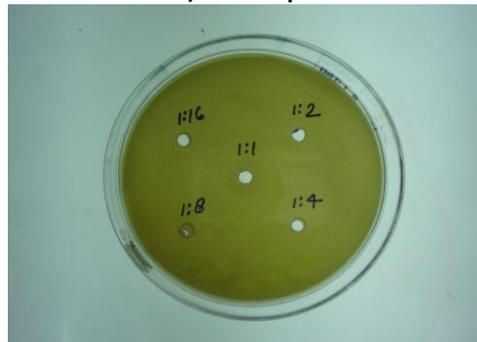
f) Toothpaste E



g) Toothpaste F



h) Toothpaste G



i) Mouthrinse H



j) Mouthrinse I

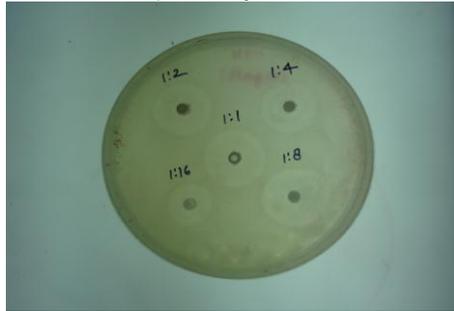
Figure 2: Antimicrobial activity of toothpastes and mouthrinses against *E. coli* (NAM media)



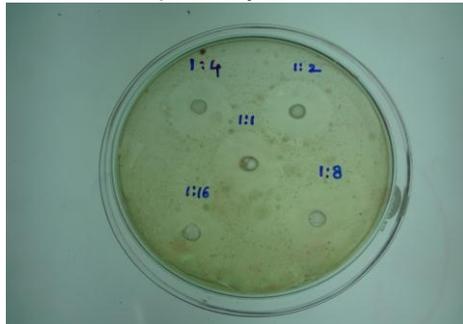
a) Control



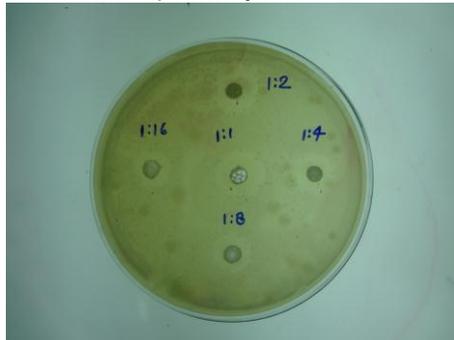
b). Toothpaste A



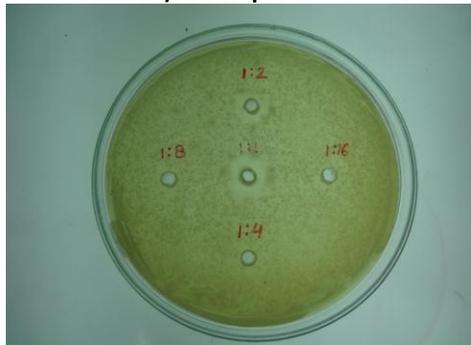
c). Toothpaste B



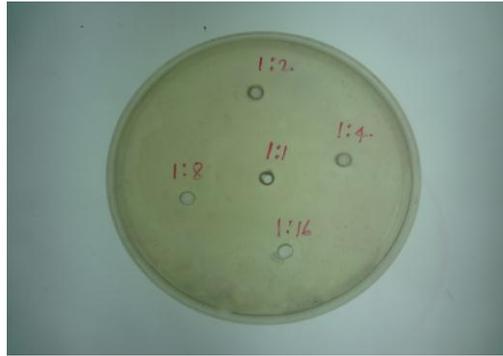
d). Toothpaste C



e). Toothpaste D



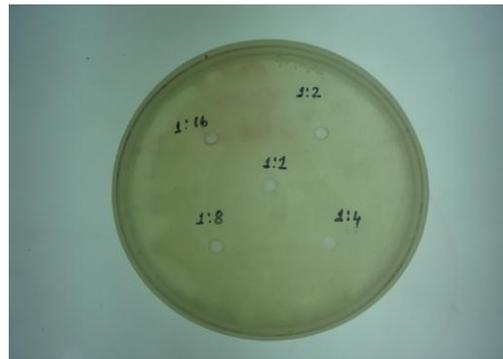
f). Toothpaste E



g). Toothpaste F



h). Toothpaste G



i). Mouthrinse H



j). Mouthrinse I

DISCUSSION

Neglect of oral hygiene is the key to the development of dental diseases. The primary etiological factor for dental diseases is dental plaque. This dental plaque is formed due to accumulation of oral pathogenic microflora, thus tooth surface get coated with a dense complex micro community that ends up in the destruction of hard enamel tissue.

Microflora like *Escherichia coli* and *Candida* are associated with active caries lesions. The oral microorganisms should be kept at a consistent level with oral health by antimicrobial agents.

These substances are added to oral products they kill microorganism by disrupting their cell walls & inhibiting their enzymatic activity. They also prevent bacterial aggregation, slow multiplication & release of endotoxins [12]. Data from our studies are in support of this assertion as all the investigated dental care products exhibited wide variations in their effectiveness against the two test microorganism. Results from previous study like have shown that triclosan containing toothpaste formulations were more effective in controlling the oral microflora compared to non-triclosan containing synthetic toothpastes [7] however, according to our study, among all the investigated toothpastes, formulation A, which is Non-triclosan based toothpaste, emerged as the most effective in 1:1 dilution for both the used micro-organisms. The antibacterial activity of formulation A is less in comparison to formulation B at higher dilutions.

In case of *C. albicans*, formulation A appears to be most effective. This might be due to presence of following compositions:

Anacyclus pyrethrum(akarakya) contain pyrethrine.

Azadirachata indica(neem) contain azadiractin, alkanoid, glycosides, flavanoids and saponins.

Acacia arabica(babul) contain tannins, saponins & glycosides.

Xanthoxylum alatum(tomar) contain bornylacetate, a-terpineol, b-pinene, caryophyllene, limonene, linodokol

Syzygium aromaticum(lavang) contain eugenol (2-methoxy-4-alkyl phenol).

Barleri aprionitis(bajrdanti) contain benzofuran, phenol, cyclohexane, benzoic acid, cyclopropane, carboxylic acid.

Curcuma longa(haldi) contain curcumin, demethoxycurcumin, bisdemethoxy curcumin

Piper sylvatium(pepal) contain lignoides amides bearing isobutyl pyrodine, pyrones.

The antimicrobial activity of formulation B is due to the presence of triclosan and sodium monofluorophosphate.

Triclosan shows antifungal and antibacterial property while fluoride helps in prevention of tooth decay and dental caries.

Next to these best formulations other toothpastes C, D, E, F, G also contain statistically significant data and this may be due to the ingredients present in their formulations.

Formulation I and H are mouth-rinses and exhibited very less effectiveness compared to other the other test formulations.

This may be due to the ingredients present such as benzoic acid, thymol, eucalyptol, sodium benzoate and povidone iodine

There is no other way of knowing their real clinical effects without a randomized clinical trial.

In the present study, the herbal formulations studied appeared to be equally effective as the flourides containing formulations.

CONCLUSION

The present study was based on *in-vitro* experiments. Results from this study have shown that toothpaste formulation containing natural antimicrobial agents were more effective in controlling the oral microflora compared to toothpastes containing synthetic antimicrobial agents like triclosan. It cannot be assumed that the results of antimicrobial efficacy could be proportional or transferable to the oral cavity and translated into clinical effectiveness. This was an *in-vitro* study therefore it is not necessary that the results that we obtained shows the same effects on *in-vivo* experiments hence it is needed to proceed this study under *in-vivo* conditions. The study can further be taken as an approach to evaluate the antimicrobial efficacy of various herbal tooth pastes and compare them with conventional dentifrices of known antibacterial effect. HPLC technique can further be performed for extracting pure molecular form of antibacterial components to increase the efficacy of dentifrices. It has been concluded from our results that certain dentifrices has shown less zone of inhibition due to less solubility of antibacterial components. So, it is required to study further and perform certain experiments which could increase their solubility.

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