Comparative analysis of various antimicrobial agents present in locally available mouthwashes against oral pathogens

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Abstract: Oral cavity exhibits general health of the body and affects the quality of life. Poor hygienic conditions are associated with chronic and systemic diseases. There are almost 750 species of various microorganisms which exist in oral cavity and most of these are involved in oral diseases. Among these, *Streptococcus mutans* and *Candida albicans* are the most common opportunistic pathogens. This study was conducted to find out the effects of various mouthwashes available in Pakistan, against oral pathogens. It was focused on the determination of most effective products through their antimicrobial activity using agar well diffusion and Minimum Inhibitory Concentration (MIC) assays. Out of selected (n=10) mouthwash (containing benzydamine hydrochloride, chlorhexidine gluconate, cetylpyridinium chloride, triclosan and hydrogen peroxide etc. as antimicrobial agents) formulations / brands 2, 7 and 9 inhibited the selected pathogens. Comparatively, formulations 4, 6 and 8 exhibited moderate level inhibition with some exceptions. Rest of the oral products showed less inhibitory activity against oral pathogens. Results of different mouthwashes containing various antimicrobial agents in association with other inactive ingredients, varied. Various formulations are responsible for different (as some showed higher while others lower) antimicrobial activities. ANOVA was performed and highly significant ($P<0.01$) values referring to different oral care products, observed.

Keywords: Antimicrobial agent, mouthwash, oral pathogens, agar well diffusion assay, minimum inhibitory concentration.

INTRODUCTION

The oral cavity can act as an area for the dissemination of pathogens to far-off body parts, especially among the immuno-compromised hosts such as patients suffering from diabetes, HIV/AIDS, Rheumatoid arthritis, undergoing corticosteroid, or other immunosuppressive treatments (Mealey and Oates, 2006). Numerous epidemiological studies have showed that oral infection can easily lead into systematic infection, especially marginal and apical periodontitis. An oral disease commences with the accumulation of microorganisms on the tooth surface where they secrete polysaccharides and glycoproteins; known as dental plaque.

Among numerous identified species of bacteria from human oral cavity, *Lactobacillus* and *Streptococcus* spp. were found to be dominant. S*treptococcus mutans* is the most common opportunistic pathogen (Burton *et al*., 2011; Gamboa *et al*., 2004). However, *Staphylococcus aureus* is an important pathogen, able to colonize several anatomical sites in human body, but mouth and hands are the main reservoirs for its propagation (Tanomaru *et al*., 2008). *Candida albicans* is the most common yeast present in oral cavity. Although more than 20 species of *Candida* have reportedly been isolated from oral cavity but *C. albicans* has been the most common opportunistic pathogen isolated from oral infections caused by *Candida* spp. in infected and immuno-compromised individuals (Öztan *et al*., 2006).

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A number of physicochemical methods are used to remove dental plaque (Al Sadhan and Almas, 1999), mouth-rinse is a counter step against foul smelled breath or oral malodor. The mouthwashes particularly are well accepted by patients and consumers owing to their convenience (Parkar and Janu, 2011). They contain active ingredients, like sodium monofluorophosphates and sodium fluoride (also act as anti-caries agents). While tetrasodium pyrophosphate, sodium hexametaphosphate, triclosan /copolymer, chlrohexidine and chlorine dioxide carry importance as anti-calculus and antiplaque effects and may also promote the tissue regeneration (Iqbal *et al*., 2011).

Among the mouth-rinse formulations, chlorhexidine was found to be more effective (as triclosan) against *S. mutans*, *Escherichia coli* and *C. albicans*. Mouthwashes containing chlorhexidine gluconate, sodium fluoride and zinc chloride showed greater efficacy against above mentioned organisms (Prasanth, 2011). There is an evidence that plant extracts, essential oils (EO) and purified phytochemicals added in mouthwashes have been used as preventive or treatment therapies for oral diseases (Palombo, 2011).

The general use of mouthwashes as an oral hygiene support is relatively less practiced in developing countries like Pakistan, as present scenario of dental healthcare practices is less satisfactory. Each product (carrying different chemical composition) claims to have anti-plaque, anti-cavity and antimicrobial properties. However,

scientific data available (in this regard) is rather scanty. Keeping in view of the prevailing situation, present study was designed for the first time in Pakistan to determine the antimicrobial activities of various mouthwashes against common oral pathogens such as, *Streptococcus* spp., *Staphylococcus aureus* and *Candida albicans*.

MATERIAL AND METHODS

Microorganisms

The oral samples were collected from almost 200 patients from different hospitals of Faisalabad (Pakistan) and were tested for the presence of pathogenic strains. Nutrient agar and Sabouraud dextrose agar media (Difco, USA) were used for isolation and identification of bacterial and fungal cultures respectively. Primary isolates were further inoculated on blood and Staph 110 agar media (Oxoid) for differentiation and purification of cultures. The identification of bacteria was performed by conventional cultural, microscopic and biochemical characterization (Liberio *et al*., 2011), as well as with the use of API kit system (Biomerieux, France). The fungal culture was identified on the basis of phenotypic and microscopic features (Alghalibi *et al*. 2011; Alaghehbandan *et al*. 2012).

Mouthwash sampling

The selected mouthwashes (table 1) were two-fold serially diluted with sterile distilled water (v/v), in order to assess the antimicrobial activities of their different dilutions (1:1-1:128) against oral pathogens (Panacek *et al*., 2009).

Agar well diffusion assay

The antimicrobial susceptibility of microorganisms against different dilutions of mouthwashes was determined by agar well diffusion assay (Da-Silva *et al*., 2012) with slight modification. Nutrient and Sabouraud dextrose agar were inoculated with respective microorganisms (bacteria and the fungus) with the turbidity set at 0.5 McFarland index. Wells of (10mm in diameter) were punched using sterile borer, followed by addition of 0.1ml (100µl) mouthwash sample (of different dilutions) in each well respectively. After refrigeration for 30 min, the bacterial plates were incubated at 37°C for 24 hrs. and fungal plates at 28°C for 48 hrs. and the diameters (mm) of inhibitory zones were measured thereafter.

Minimum inhibitory concentration (MIC)

The MIC was determined using Micro broth dilution assay (Riaz *et al*., 2009). Culture was prepared with turbidity equivalent to 0.5 McFarland index. Nutrient broth (50µl) was transferred to all the wells of a row of 96-well micro-titration plate (Titertek, UK). Undiluted mouthwash (50µl) was added to the first well (containing nutrient broth), mixed well and transferred 50µl to the next well and so on till 10^{th} well. For positive and negative control, 11^{th} and 12^{th} (last) wells were used respectively. Culture (50µl) was added in all except last well. Mouthwash (50µl) was placed in last well of each row. Micro-titration bacterial plate was incubated for 24 hours at 37°C and fungal plate at 28°C for 48 hrs. The last well without any growth/turbidity was considered as MIC (Shokri, 2011; Unnisa *et al*., 2012).

STATISTICAL ANALYSIS

Observed values were expressed as means ± SD. The significance of differences among means and standard deviation of different mouthwashes and their dilutions were assessed. The statistical analysis was done using analysis of variance (ANOVA) with the help of SPSS software version 16, as statistical tool (Adejumo *et al*., 2008).

RESULTS

Antimicrobial activities of different mouthwashes in terms of inhibitory zones and relative mean ± standard deviation against isolated bacteria and fungus are shown in tables 2-4 and figs. 2-3. For undiluted samples of mouthwashes, analysis of variance (ANOVA) showed highly significant values ($P<0.01$). Two test formulations 9 and 2 exhibited consistent antimicrobial activity against all the five test organisms i.e. *Staph. aureus*, *S. salivarius*, *S. mutans*, *S. intermedius* and *C. albicans*. Undiluted samples of formulation 9 gave maximum zones of inhibition *in vitro* against *Staph. aureus* (39.75±2.50) and *S. mutans* (38.00±1.00), whereas, the formulation 2 against *S. salivarius* (30.25±0.957), *S. intermedius* (42.25±1.708) and *C. albicans* (33.25±1.708), followed by formulations 7, 4 and 8, which gave bigger zones of inhibition respectively. Formulation 6 was effective against *S. intermedius*, *Staph. aureus*, and *C. albicans* (38.50±1.291, 16.50±1.291, 16.50±1.291; table 4; figs. 2 & 3) respectively and less effective against rest of the pathogens while, formulations 10, 5, 3 and 1 showed moderate to low level of effectiveness against all the selected pathogens (tables 2-4; figs. 1-3). Serially diluted samples of all formulations showed decrease in inhibitory activity whereas, CFU/ml remains constant. As regards *S. aureus*, formulation 7 exhibited highest MIC value (1:32) while, 2 and 9 showed higher MIC value (1:16). Formulation 9 showed higher MIC values (1:32 and 1:16) against all the pathogens (*S. intermedius*, *S. salivarius*, *C. albicans* and *S. mutans*, *Staph. aureus*, respectively) while formulation 2 exhibited highest MIC value (1:64) against *C. albicans* (table 5).

DISCUSSION

Different techniques were used to test the antimicrobial effectiveness of ten mouthwashes and to rank them

Table 1: Selected mouthwash formulation codes and their compositions

Formulation codes of mouthwashes	Active ingredients as listed on packages
1	Sodium Fluoride 0.05% w/w, Potassium Chloride 0.5% w/w
2	Benzydamine Hydrochloride 0.15% w/v, Chlorhexidine Gluconate 0.2% w/v
3	Povidone Iodine 1.0 g
4	Triclosan 0.01% w/v, Sodium Monofluorophosphate 0.055% w/v
5	Thymol USP 0.063% w/v, Eucalyptol N.F 0.091% w/v, Menthol USP 0.042% w/v, Methyl salicylate 0.06%
6	Benzydamine Hydrochloride 0.15% w/v
7	Chlorhexidine Gluconate 0.2%
8	Cetylpyridinium chloride 0.05%
9	Triclosan 0.05% w/v, Hydrogen peroxide 1.5% w/v
10	Potassium Nitrate 1% w/w

Table 2: Comparison of Mean ± SD of various dilutions of different mouthwash formulations against s*trept. Salivarius*

Formulation codes for mouthwashes	1:1 Mean ± SD	1:2 Mean ± SD	1:4 Mean ± SD	1:8 Mean ± SD	1:16 Mean ± SD	1:32 Mean ± SD	1:64 Mean ± SD	1:128 Mean ± SD
1	17.500± 1.732*	12.25± 2.99	8.75± 6.08	5.50± 6.40	00±00	00±00	00±00	00±00
2	30.250± 0.957*	23.50± 3.42	21.50± 3.42	16.75± 2.75	13.00± 2.58	7.50± 5.26	00±00	00±00
3	19.000± 0.816*	14.33± 2.52	12.67± 2.52	7.00± 6.24	00±00	00±00	00±00	00±00
4	21.25± 2.50*	15.67± 2.52	11.67± 2.52	00±00	00±00	00±00	00±00	00±00
5	15.000± 0.816*	8.50± 6.03	5.25± 6.18	00±00	00±00	00±00	00±00	00±00
6	10.500± 1.915*	8.00± 5.89	5.00± 6.00	00±00	00±00	00±00	00±00	00±00
7	28.00± 2.16*	20.75± 2.50	18.25± 2.63	15.75± 3.30	9.75± 6.95	00±00	00±00	00±00
8	22.500± 1.291*	21.00± 5.16	20.75± 2.50	18.25± 2.63	15.75± 3.30	9.75± 6.95	00±00	00±00
9	27.00± 2.58*	22.50± 5.51	16.75± 3.77	14.00± 3.65	00±00	00±00	00±00	00±00
10	13.000± 0.816*	6.75± 4.79	4.00± 4.69	00±00	00±00	00±00	00±00	00±00

* P<0.01

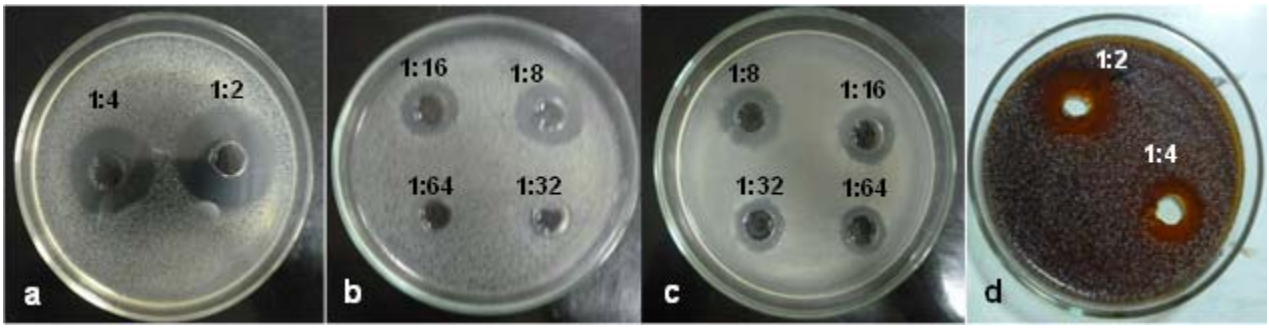

Fig. 1: Antimicrobial activity of different mouthwash formulations against (a & b) *Streptococcus mutans*, (c) *Streptococcus salivarius*, (d) *Candida albicans* by agar well diffusion assay

Table 3: Comparison of Mean ± SD of various dilutions of different mouthwash formulations against *Strept. Mutans*

Formulation codes for mouthwashes	1:1 Mean ± SD	1:2 Mean ± SD	1:4 Mean ± SD	1:8 Mean ± SD	1:16 Mean ± SD	1:32 Mean ± SD	1:64 Mean ± SD	1:128 Mean ± SD
1	8.00± 7.00*	7.67± 6.66	00±00	00±00	00±00	00±00	00±00	00±00
2	30.67± 2.52*	27.33± 3.06	24.00± 2.00	18.00± 2.00	14.00± 2.00	8.67± 7.57	00±00	00±00
3	13.667± 1.528*	10.333± 1.528	00±00	00±00	00±00	00±00	00±00	00±00
4	29.667± 1.528*	26.00± 2.00	23.67± 1.528	17.33± 3.06	12.67± 3.06	12.00± 2.00	00±00	00±00
5	13.333± 1.528*	7.67± 6.66	00±00	00±00	00±00	00±00	00±00	00±00
6	7.00± 6.24*	6.24±4.24	00±00	00±00	00±00	00±00	00±00	00±00
7	32.67± 2.52*	24.67± 3.06	23.00± 3.00	20.33± 3.51	17.67± 4.51	17.00± 2.65	15.00± 3.61	11.00± 2.65
8	24.000± 1.000*	23.667± 1.528	17.33± 3.06	12.67± 3.06	12.00± 2.00	00±00	00±00	00±00
9	38.000± 1.000*	32.67± 2.52	24.67± 3.06	23.00± 3.00	15.00 ± 3.61	00±00	00±00	00±00
10	7.67± 6.66*	7.00± 6.24	00±00	00±00	00±00	00±00	00±00	00±00

P<0.01

Table 4: Comparison of Mean ± SD of various dilutions of different mouthwash formulations against *Strept. Intermedius*

Formulation codes for mouthwashes	1:1 Mean ± SD	1:2 Mean ± SD	1:4 Mean ± SD	1:8 Mean ± SD	1:16 Mean ± SD	1:32 Mean ± SD	1:64 Mean ± SD	1:128 Mean ± SD
1	11.750± 1.500*	10.05± 5.00	00±00	00±00	00±00	00±00	00±00	00±00
2	42.250± 1.708*	26.00± 3.61	20.67± 3.51	15.67± 2.08	13.33± 3.06	12.00± 2.00	00±00	00±00
3	12.250± 0.957*	12.00± 1.000	6.33± 5.51	00±00	00±00	00±00	00±00	00±00
4	21.50± 2.52*	11.66± 1.528	6.33± 5.51	00±00	00±00	00±00	00±00	00±00
5	11.75± 1.258*	10.05± 5.00	00±00	00±00	00±00	00±00	00±00	00±00
6	38.50± 1.291*	30.00± 5.29	27.33± 5.03	22.67± 3.06	18.00± 4.00	14.67± 3.06	00±00	00±00
7	28.75± 2.22*	26.00± 3.6	20.67± 3.5	15.67± 2.08	00±00	00±00	00±00	00±00
8	17.50± 1.291*	9.33± 8.33	00±00	00±00	00±00	00±00	00±00	00±00
9	33.50± 2.65*	22.00± 6.00	19.00± 2.65	14.667± 1.528	7.33± 6.43	00±00	00±00	00±00
10	12.75± 2.22*	11.67± 1.528	00±00	00±00	00±00	00±00	00±00	00±00

P<0.01

according to their antibiotic effectiveness. The results exhibited significant ($p<0.01$) variation in their effectiveness. This may be due to different concentrations of antimicrobial active ingredients present in various mouthwashes. Among all tested mouthwashes, formulation 9 exhibited maximum zones of inhibition *in vitro*, might be due to the presence of triclosan along with hydrogen peroxide (H_2O_2; containing cytotoxic property, beneficial for microbial inhibition but could be harmful for oral environment) in its composition. The current work is affirmed by other workers who discovered, the ability of triclosan was higher even at dilution 1:16, to inhibit the five tested microorganisms (Prasanth, 2011). Triclosan; a broad spectrum, immediate and persistent antimicrobial agent, also utilized in clinical health care settings and being used as a common antifungal and antibacterial agent for more than 40 years; widely accepted due to its safety and effective nature (Jones, 1997).

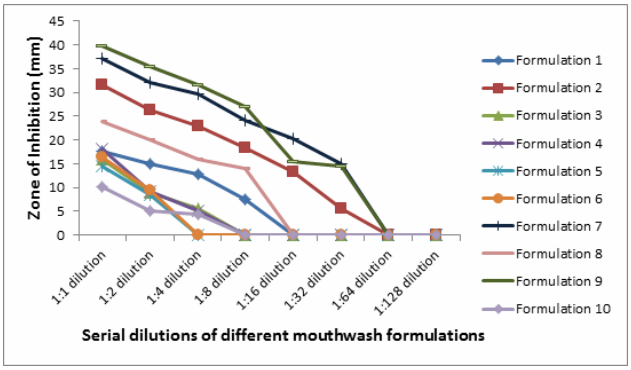

Fig. 2: Antimicrobial activity of various dilutions of different mouthwash formulations against *Staph. aureus*

Followed by formulation 9, 2 and 7 gave higher zones of inhibition against all tested microorganisms, both contain chlorhexidine (CHX) as an active ingredient. The current findings are in agreement with Lorenz *et al*. (2006) and Ghapanchi *et al*. (2015), who reported CHX as a gold standard antibacterial agent against *Streptococcus* spp. Mouthwashes containing chlorhexidine gluconate as an active ingredient showed higher inhibitory activity, as having broad-spectrum of antimicrobial activity and effective against gingivitis and plaque.

Comparatively, large zones of inhibition were exhibited by formulation 4. It contains 0.01% triclosan and might give low results due to less percentage of triclosan in its composition as compared to formulation 9. Formulation 6 was effective against *S. salivarius* and *S. intermedius* pathogen respectively and less effective against all other pathogens. The mouthwash contain benzydamine hydrochloride as active ingredient, showed low antimicrobial activity as compared to triclosan, CHX and CPC. These outcomes are supported by Cheng & Yuen (2006), who explained that benzydamine hydrochloride exhibited lesser efficacies against caries.

Formulation 8 showed good antimicrobial activity against all oral pathogens and it contains cetylpyridinium chloride (CPC), which is a good antimicrobial agent. These results are confirmed by other workers; Almas *et al*. (2005) reported that CPC is among one of widely used antiseptic in dentistry. It gave moderate zones of inhibition. Witt *et al*. (2006) estimated that CPC (a broad spectrum antimicrobial agent inhibited the growth of microorganism at very low concentration) was being used as an antiseptic in oral hygiene products since last 50 years. CPC might kill almost all microorganisms, related with plaque and gingivitis. Low concentration (0.07%) showed inhibitory activity in clinical trials.

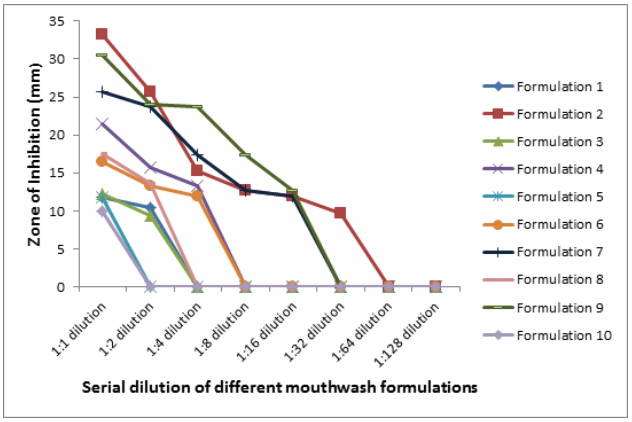

Fig. 3: Antimicrobial activity of various dilutions of different mouthwash formulations against *C. albicans*

Formulations 3, 5, 10 and 1 exhibited moderate to small zones of inhibition. These results are supported by other workers; Seguin *et al*. (2014) found no single evidence to acclaim formulation 3 containing povidone iodine, with oral care, Aneja *et al*. (2010) observed that formulation 5 displayed very little antimicrobial activity. Formulation 10 contains potassium nitrate as an active ingredient. Hooper *et al*. (2014) assessed that Potassium nitrate can play important role to protect enamel and avoid tooth erosion. On the other hand, formulation 1 contains sodium fluoride and potassium chloride. Zero (2006) described, concentration of fluoride, time of exposure, method of delivery and frequency of use may affect effectiveness of fluoride. Daily use of fluoride mouthwashes were found more effective as compared to biweekly use of fluoride mouthwashes in high concentration. Different mouthwash solutions have variable antibacterial activity. Bacteria in their biofilm state, pose a challenge to dental hygiene/care where bacteria become resistant to majority of available mouthwash solutions (Masedeh *et al*. 2013). The above mentioned reports confirmed the recent work presented in this research.

CONCLUSION

From the above mentioned facts and figs. it was concluded that triclosan along with H_2O_2 exhibited

highest antimicrobial activity among all selected oral care products. In effectiveness, triclosan is followed by CHX and CPC containing mouthwashes (w.p.r. to size of zone of inhibition). This is probably due to the difference in formulations of various mouthwashes in association with other ingredients. Different formulations may be responsible for different inhibitory activities. Selected oral pathogenic strains exhibited resistance against mouthwashes, containing different salts (sodium fluoride, potassium nitrate, potassium chloride etc.) in their composition. It is thus, recommended that triclosan, CHX, and CPC are effective antimicrobial agents and could be used further in oral care products. However, instead of remarkable antimicrobial potential and safe use of triclosan, long-term use of formulation 9 is not recommended, as could be harmful for oral cavity due to presence of cytotoxic H_2O_2.

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