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RESEARCH ARTICLE

AN EVALUATIVE COMPARISON OF COMMONLY USED MOUTHRINSES, ESSENTIAL OILS AND POMEGRANATE JUICE AGAINST MICRO-ORGANISMS ISOLATED FROM THE DENTAL PLAQUE

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ABSTRACT

Dental plaque is a structurally- and functionally-organized biofilm that is formed in an ordered way and has a diverse microbial composition that, in health, remains relatively stable over time (microbial homeostasis). The predominant species from diseased sites are different from those found in healthy sites, although the putative pathogens can often be detected in low numbers at normal sites. In dental plaque, there is a shift toward community dominance by acidogenic and acid tolerating species such as *Streptococcus mutans* and lactobacilli, although other species with relevant traits may be involved. Strategies to control plaque could include inhibition of biofilm initiation by various chemical and natural substances. In this study, various products both natural and commercial were used to evaluate its ability to control plaque formation. Essential oils such as Eugenol, Eucalyptus, Thyme, Peppermint, Cinnamon have been well known since centuries for their anti-microbial, anti-inflammatory properties and have been used to prevent and cure dental plaque formation. Along with traditional use of essential oils, recent trends of commercial mouth rinses such as Listerine, Colgate Plax, Chlorhexidine etc have also been used widely for prevention of dental plaque prevention. The diet we consume also plays a major role in the formation of dental plaque, different kinds of food that we consume daily contribute differently to plaque formation, previous reports suggest that cranberry juice, pomegranate juice have the ability to eradicate dental plaque. Hence in this study, the activity of pomegranate juice was also assessed for inhibition of plaque.

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INTRODUCTION

Dental plaque is primarily responsible for the initiation of gingivitis, which if left untreated can eventually progress to periodontitis and undermine the entire periodontium. Regular plaque is essential for controlling the progression of gingival and periodontal diseases (Listgarten, 1988). Mechanical plaque control remains the gold standard of periodontal therapy (Hays, 1975). However, mechanical plaque control by means of tooth brushing and flossing is not always completely effective as it is based on the dexterity and motivational level of individual (Hays, 1975). Moreover, bacteria present in the soft tissues can re-colonize the tooth surfaces even after mechanical plaque control. Adjunctive use of chemical plaque control agents has demonstrated better efficacy in the control of plaque and gingival inflammation (Keyes and Shern, 1971; Van der Weijden *et al*., 2015). Chlorhexidine mouth-rinses have long been known for their efficacy against oral plaque development owing to its superior antiplaque effect and its substantivity for a period of 10–12 h (Hesselgren *et al*., 1972; Kobylanska, 1972). However, the side effects of chlorhexidine such as tooth and tongue staining, taste alterations, and mucosal erosions limit patient compliance, which has led to the emergence and exploration of newer chemical anti-plaque agents in recent years (Graziani *et al*., 2015). In addition, Listerine is an essential oil containing mouthwash, well known for its bactericidal properties, that is available over the counter and has been in use since the 1890s (Fornell *et al*., 1975). Short-term and long-term clinical studies have indicated that

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the daily use of Listerine, a mouthwash that contains phenolics such as thymol, eucalyptol, menthol, and methyl salicylate, may retard plaque buildup and reduce gingivitis. Phenolic compounds in the listerine, however, are also known to interfere with the inflammatory process (Fornell *et al*., 1975). Therefore, it was observed that when it was used as a mouthwash two times daily for 6 weeks, it had limited influence on plaque, but was effective in reducing gingival inflammation similar to chlorhexidine. Other studies with a similar study design showed that the regular use of it failed to retard plaque-associated gingivitis (Fine *et al*., 1985; Stoeken *et al*., 2007). With the continuous need to counter the adverse effects, improve the antiplaque and antigingivitis potential, and to reduce the increasing microbial resistance to conventional antiseptics and antibiotics, attention is now turning to the use of natural antimicrobial compounds (herbal extracts) (Gupta *et al*., 2015). Herbal products are being used in India since ancient times for the treatment of various ailments. Of late, the commercial use of these products in toothpaste and for oral irrigation delivery has increased manifold (Gupta *et al*., 2015). Some of the natural or herbal products and their extracts, such as guava, pomegranate, neem, propolis, tulsi, green tea, cranberry, and grapefruit, when used in mouthwashes have shown significant advantages over the chemical ones. Essential oils such as eucalyptus, thyme, have demonstrated to be effective in preventing gingivitis and reducing dental plaque (Elias-Boneta *et al*., 2015). They have also been considered to be safe by the Food and Drug Administration (FDA) to be safe for human use (Elias-Boneta *et al*., 2015). Furthermore, essential oils have the ability to destroy the cell membrane and inhibit bacterial enzymatic activity as well as to prevent bacteria adhesion to the existing biofilm and reduce bacteria multiplication (Sharma *et al*., 2002). Moreover, recent studies have also effectively demonstrated the use of pomegranate juice in the control of dental plaque micro-organisms. Pomegranate juice contains anthocyanins, glucose, ascorbic acid, ellagic acid, gallic acid, caffeic acid, catechin, epigallocatechin, quercetin, rutin, iron and amino acids possessing anti-atherosclerotic, antihypertensive, antiaging and potent antioxidative properties (Kote *et al*., 2011). Hence, the aim of this study was to compare the anti-microbial activity of chlorhexidine, essential oils and pomegranate juice against micro-organisms from the dental plaque in vitro.

MATERIALS AND METHODS

1.Morphological characterization of the bacteria in the sample: Plaque samples were obtained from 12 healthy individuals by rubbing the cotton swab against the teeth and sub gingival region. Selective media used included Actinomycetes agar (HiMedia Laboratories Pvt. Ltd, India), MacConkey's agar (HiMedia Laboratories Pvt. Ltd, India), Mannitol Salt Agar (HiMedia Laboratories Pvt. Ltd, India), Blood agar (blood was procured from Kanitkar pathology laboratory, Modern Colony), EMB agar, Potato Dextrose agar and Chocolate agar. 100 mL of all the different media were sterilized at 121^{o}C for 15 minutes (please refer to supplemental section 1 for details). Loopful of each swab was first enriched in 100 mL of Nutrient broth (HiMedia Laboratories Pvt. Ltd, India) and Luria Bertani broth (HiMedia Laboratories Pvt. Ltd, India) for 24h at 37^{o}C. The swabs were then streaked on3 plates each of different selective media, so as to obtain as many different isolates as possible and incubated for 24h at 37^{o}C. For Blood agar and Chocolate agar, the plate was incubated in the dessicator so as to provide microaerophilic conditions for the isolation of microaerophilic organisms. The others were incubated at 37^{o}C for 24 hours. The isolated colonies were then identified macroscopically and the morphological characteristics of the colony were noted down.

2. Biochemical tests: The bacteria from the dental plaque was evaluated by the sugar fermentation test with the help of five isomers of sucrose (Glucose, Mannitol, Maltose, Lactose, Sucrose) and the IMViC test (Indole, Methyl red, VogesProskauer and citrate utilization). (Please refer to supplemental section 2 for details)

3. Induction, Formation and Quantification of Biofilm by crystal violet assay: The products evaluated in this study were Listerine (Phenolicessential oil, Warner- Lamber), Colgate plax, Chlorhexidine mouthwash, Thyme oil, Peppermint oil, Eugenol oil (Oswal pharmaceuticals), Eucalyptus oil (Oswal pharmaceuticals), Organic pomegranate juice without any preservatives, Streptomycin and Penicillin (Kwality Pharmaceuticals LTD, India) were used as the positive control. The essential oils were prepared according to the steam distillation process. 30 g of fresh and partly dried flowering tops and leaves of *Thymus vulgaricus* was used to extract thyme oil and 30 g of peppermint leaves were used to extract peppermint oil (please refer to supplemental section 3 for details). The final isolates obtained were induced to form biofilm in :i)Tryptic Soy Broth (HiMedia Laboratories Pvt. Ltd, India) with 0.9% NaCl and 5% Glucose ii)Tryptic Soy Broth (HiMedia Laboratories Pvt. Ltd, India) with 0.9% NaCl and 10% Glucose iii)Minimal medium (M9) (HiMedia Laboratories Pvt. Ltd, India) with 0.5% NaCl. Biofilm formation was carried out in a 96-well microtitreplate. 100µL Listerine, Eugenol oil, Eucalyptus oil, Pomegranate juice were tested for their potential to prevent biofilm formation of two isolates. A 24 h culture of the isolate was grown on Tryptic soy broth and M9 medium and 100µLof the medium was added in each micro titrewell. The products were added to the growth medium at the time of inoculation to check for inhibition, as well as after biofilm formation so as to check biofilm destruction. The cells were allowed to form biofilms on incubation at 37^{o}C for 48 h. The medium without these products was used as the non-treated well and the medium was set as control. Following incubation at 37°C for 48 h, culture supernatants from each well were decanted and planktonic cells were removed by washing three times with Sterile Distilled water. Cells in biofilm were fixed with methanol for 15 minutes, air dried and stained with 1% crystal violet. Biofilm formation was quantified by measuring the absorbance at 595 nm*

4.Determination of antibacterial activity by Agar diffusion method: The disk diffusion method for antimicrobial susceptibility method was carried out as per CLSI guidelines to assess the presence of antibacterial activities of the products in use. 100µL of 24 h old culture was spread on sterile Mueller-Hinton agar plate. Wells were bored in the plate at equidistance with the help of a cork borer, 50µL of the products were poured in each well, where one well consisted of the positive control i.e. Streptomycin or Penicillin with respect to the organism that is to be assessed, and the negative control was 50µL of pure culture in one well. The plates were incubated at 37^{o}C for 24 h

Minimum inhibitory concentration: Turbidimetric assay was performed for the extracts which showed a zone of inhibition on the Muller Hinton agar plates. This method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antimicrobial agent in a fluid medium that is favorable to its rapid growth in the absence of the agent (please refer to supplemental section 4 for details).

RESULTS

1.Dental plaque is primarily composed of both gram positive and negative bacteria:

The swabs were collected the next day for isolation of the organisms. They were streaked on 3 plates each of different selective media as per mentioned in the materials and methods, incubated at 37^{o}C for 24 h. Referring to Bergey's manual of determinative bacteriology, biochemical tests and gram staining procedures were performed for the isolates obtained and results reported the probable presence of the following organisms; 1: *Staphylococcus aureus,* 2: *Escherichia coli and* 3: *Streptococcus mutans* (See Table 1, 2 and Figure 1).

15 minutes. After solubilisation, quantification was carried out by colorimetrically at 545 nm. Clove and Eucalyptus oil significantly inhibited biofilm formation as compared to Listerine and pomegranate juice ($p<0.01$ as represented by *).

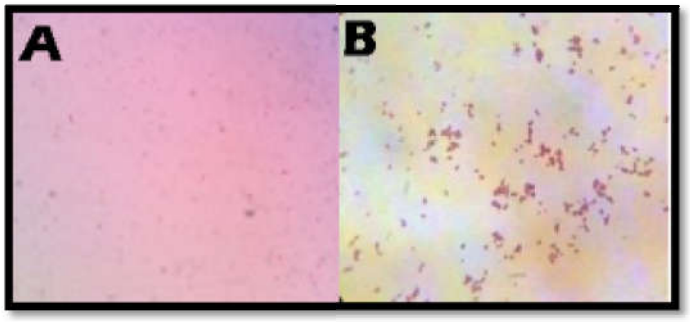

Figure 1. Gram stain (A and B)

There was no difference in the inhibition of biofilm formation between Listerine and pomegranate juice ($p>0.01$) (See table 3 and Figure 2). Furthermore, the antibacterial activity of the mouthrinses, oils and pomegranate juice was determined using

Table 1. Morphological characteristics of different colonies obtained from dental plaque

Media	Isolates	Size	Shape	Colour	Margin	Consistency	Elevation	Opacity	motility	Gram character
Blood agar	colony 1	1mm	Circular	Pink	Entire	Smooth	Concave	Opaque	Non-motile	Gram positive cocci in clusters
	colony 2	1mm	Circular	White	Entire	Smooth	Concave	Opaque	Non-motile	Gram positive cocci in chain
Mannitol salt agar	colony 3	1mm	Circular	Yellow	Entire	Smooth	Concave	Opaque	Non- motile	Gram positive cocci in clusters
MacConkey's agar	colony 4	1mm	Circular	Pink	Entire	Smooth	Concave	Translucent	Non-motile	Gram negative short rods
EMB agar	colony 5	1mm	Circular	Green sheen	Entire	Smooth	Concave	Opaque	Non-motile	Gram negative short rods
Nutrient agar	colony 6	1mm	Circular	Yellow	Entire	Smooth	Concave	Opaque	Non-motile	Gram positive cocci in clusters
	colony 7	1mm	Circular	White	Entire	Smooth	Concave	Translucent	Non-motile	Gram negative short rods

Table 2. Biochemical tests

Table 2.1. Biochemical test results for isolate 1

Sugar fermentation					Growth on MSA	NA+7.5% NaCl	Nitrate reduction	Coagulase test
Dex	Lac	Mann	Mal	Suc				
+	+	+	+	+	+	+	+	+

Table 2.2. Biochemical test results for isolate 2

Sugar fermentation					Growth on MacConkey's agar	NA+ 7.5% NaCl	Nitrate reduction	IMViC			
Dex	Lac	Mann	Mal	Suc				Indole	MR	VP	Citrate utilization
+	+	+	-	+	+	-	+	+	+	-	-

Table 2.3. Biochemical test results for isolate 3

Sugar fermentation					Growth on Blood agar	NA+ 7.5% NaCl	Nitrate reduction	Arginine hydrolysis
Dex	Lac	Mann	Mal	Suc				
-	+	+	-	-	α haemolysis	+	-	+

2.Essential oils can significantly inhibit dental plaque micro-organisms biofilm formation:

After 48 h, the plate was observed for biofilm formation. Biofilm was quantified by adding 0.1% crystal violet in each well for 15 minutes, washed with distilled water and dried for 24 h. The next day 150µL of 3% Acetic acid was added in each well so as to solubilise the crystal violet and incubated for

the agar diffusion method. After incubation for 24 h, the plates were observed to determine the antibacterial effect of the products by estimating the zone of inhibitions formed for each well. The results reported greater antibacterial activity with the use of essentials oils as compared to the Listerine and pomegranate juice (See Figure 3). Moreover, in order to determine the precise minimum concentration at which the essential oils inhibit the bacteria growth i.e. minimum

inhibitory concentration, an optimisation test constituting of concentrations of 10,20 30,40 50 and 60 ul was performed. The results demonstrated inhibition of the *Staphylococcus aureus* and *Escherichia coli* at 20ul and 40ul for eucalyptus oil and 10ul and 20ul for clove oil respectively (See Table 4).

Table 4. Minimum Inhibitory Concentration

Organisms	Eucalyptus oil	Clove oil
Staphylococcus aureus	20 µl	20 µl
Escherichia coli	40 µl	10 µl

Table 3. Biofilm formation by *E.coli* and *S.aureus*

	Control		Listerine		Eugenol oil		Eucalyptus oil		Pomegranate	
	5% (glucose containing media)	10% (glucose containing media)	5% (glucose containing media)	10% (glucose containing media)	5% (glucose containing media)	10% (glucose containing media)	5% (glucose containing media)	10% (glucose containing media)	5% (glucose containing media)	10% (glucose containing media)
Colony 1	0.58	0.70	0.96	0.90	0.26	0.30	0.55	0.65	0.62	0.77
	Control		Listerine		Eugenol oil		Eucalyptus oil		Pomegranate	
Colony 2	0.90		1.1		0.65		0.73		0.98	

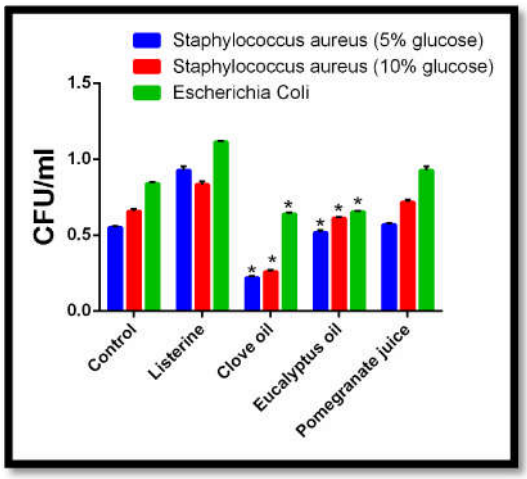

Figure 2. Colorimetric analysis for the inhibition of the biofilm formed incubated with listerine, essential oils and pomegranate juice

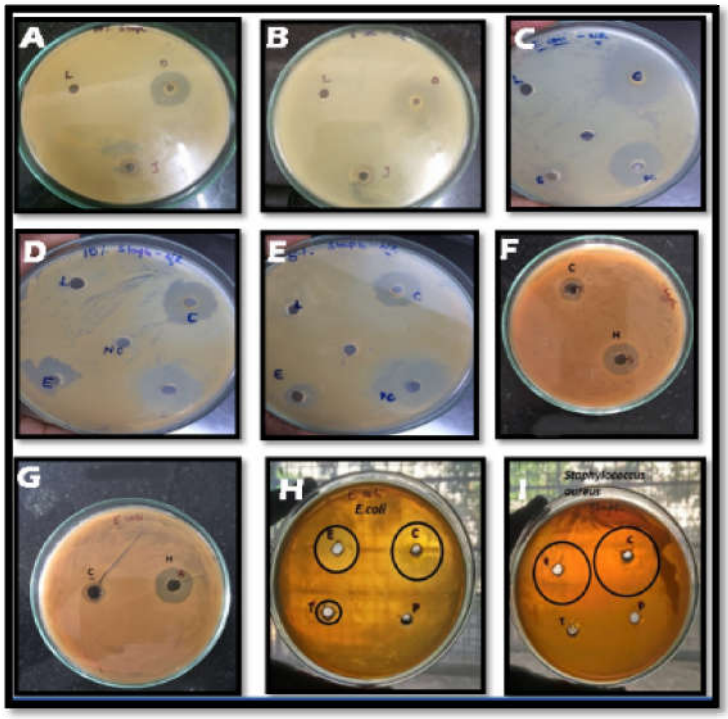

Figure 3. A: antimicrobial activity of clove oil (O), Listerine (L) and pomegranate juice (J) on S.aureus; B: antimicrobial activity of clove oil, Listerine, pomegranate juice on E.coli; C: antimicrobial activity of Listerine (L), clove oil (C), eucalyptus oil (E) on E.coli; D: antimicrobial activity of Listerine, clove oil, eucalyptus oil on S.aureus grown in 10% glucose; E: antimicrobial activity of clove oil, Listerine and eucalyptus oil on S.aureus grown in 5% glucose. PC: positive control (streptomycin antibiotic used for S.aureus and penicillin antibiotic used for E.coli) NC: negative control (distilled water); Figure F: antimicrobial activity of Chlorhexidine (H) and Colgate Plax (C) on Staphylococcus aureus; G: antimicrobial activity of Chlorhexidine (H) and Colgate Plax (C) on Escherichia coli Figure H: antimicrobial activity of eucalyptus oil (E), clove oil (C), thyme oil (T), peppermint oil (P) on E.coli; I: antimicrobial activity of eucalyptus oil (E), clove oil (C), thyme oil (T), peppermint oil (P) on S.aureus

DISCUSSION

The use of mouthrinses reportedly augments oral hygiene regimens based on regular brushing and is believed to offer enhanced protection against dental caries, particularly when recommended oral healthcare regimens are not strictly adhered to (Brady *et al*., 1975). Various chemical mouthwashes have been in use since the last century, but are associated with side-effects such as immediate hypersensitivity reactions, toxicity, tooth staining, etc (van der Bijl and Dreyer, 1982). Alternative medicines may be developed from medicinal plants as these plants contain natural phytochemicals, and hence, can replace synthetic drugs. In this paper, to our knowledge, for the first time in literature, we have assessed the activity of conventional mouthrinses, essential oils and pomegranate juice in the inhibition of dental plaque bacteria activity. Dental plaque biofilms support the view that this ecological niche is a highly selective environment as we observed only two distinct genera at an appreciable abundance (Marsh and Bradshaw, 1995). *Streptococcus* species is generally found to be the dominant (Pasquantonio *et al*., 2008). The presence of a relatively small number of genera representing a much larger number of species is thought to enhance the "biochemical repertoire" and complementarity of functions encoded within the dental plaque community. The sharing of ''biochemical burden'' amongst community members creates webs of mutualistic relationships. After collecting the samples and characterizing them, the isolates were identified to be *Staphylococcus aureus, Escherichia coli* and *Streptococcus sp.* But *Streptococcus* couldn't be further sub cultured and hence we couldn't proceed further with the isolate.

After checking for the inhibitory effect of Listerine on *S. aureus* and *E.coli,* it was concluded that Listerine showed no inhibitory effect. It instead increased the biofilm forming ability of the organism. In an in vivo study as well it was observed that Listerine alone showed no effect on the bacterial load but the mouthwash which contained essential oils (EO) showed inhibitory effect against the oral pathogens. Sorbitol solution is one of the inactive ingredients in Listerine (Fornell *et al*., 1975). Sorbitol does not share the same effectiveness in preventing tooth decay as xylitol. Sorbitol has certain limitations like, carcinogenic bacteria can digest it, and acidic wastes by products are still produced (Ben Slama, 2006). The probable reason why Listerine showed no antimicrobial activity could be because of the use of sorbitol instead of xylitol. Because sorbitol is cheaper than xylitol, a number of studies have evaluated products that have been formulated with both. Another probable reason might be attributed to the presence of sodium saccharin in Listerine, which is often employed as a sweetener used in the food industry. Some studies have reported the growth of dental plaque organisms with its use (Fine, 2010). Thus by decreasing the percentage or using another sweetener in the formulation of Listerine might give us better results. Other mouthrinses that were used to check for the inhibitory effects were Colgate Plax and Chlorhexidine. In the case of Colgate Plax, it showed very poor results which weren't significant. This could be due to the presence of sodium saccharin and sorbitol. Chlorhexidine on the other hand showed a zone of inhibition against *S.aureus* and *E.coli*. One study investigated antimicrobial activities of herbal mouthrinse, Chlorhexidine 0.12% and essential oil mouthwash (McKenzie *et al*., 1992). Chlorhexidine was the most potent mouthwash. Thus the results that were obtained were similar to the earlier findings. From the above results obtained it can be concluded that Chlorhexidine mouthwash might be better than Listerine and Colgate Plax.

The inhibitory effect of some essential oils were also evaluated. Clove oil has been used since centuries to relieve tooth ache. It is an ancient home remedy for dental care due to its germicidal properties (Chaieb *et al*., 2007). It is also very effective in relieving mouth ulcers, sore gums. Its strong smell also removes bad breath and eases throat pain (Chaieb *et al*., 2007). Previous studies have reported biological activities of eugenol including antifungal, anti‐carcinogenic, anti‐allergic, anti‐mutagenic activity, antioxidant and insecticidal properties (Chaieb *et al*., 2007). In the present study, Clove oil showed antimicrobial activity against *S.aureus* and *E.coli*. It also showed significant inhibition of biofilm formation. Clove is a spice that is used in regular food preparations in India. Hence it can easily be made available. Another essential oil that was tested was eucalyptus oil, Eucalyptus Essential Oil is a germicide that has been reported to fight plaque build-up, gingivitis and prevent cavities (Low *et al*., 1974; Whitman and Ghazizadeh, 1994). In the present study, eucalyptus oil showed antimicrobial activity against *E.coli* and *S.aureus.* It also showed inhibition of biofilm forming ability of the organisms (table 5). However clove oil reported better results than eucalyptus oil in inhibiting biofilm formation. Other essential oils that were extracted were peppermint oil and thyme oil. Thyme oil showed a very small zone of inhibition against *E.coli* but no inhibition was observed with respect to *S.aureus.* Peppermint oil on the other hand showed no inhibition against *S.aureus* and *E.coli*. Previous studies showed that peppermint oil and thyme oil have antimicrobial activity against dental plaque forming organisms. The difference in result might be because of some error during extracting the oils. For future prospects, different methods of extractions should be tried for extracting the essential oils.

Pomegranate is currently finding important applications in the field of dental health (Menezes *et al*., 2006). Clinical studies have shown that this popular antioxidant attacks the causes of tooth decay at the biochemical level, with remarkable vigor (Menezes *et al*., 2006). Various studies have already shown that pomegranate's active components, including polyphenolic flavonoids like punicalagins and ellagic acid are believed to prevent gingivitis through a number of mechanisms including reduction of oxidative stress in oral cavity, direct anti-oxidant activity anti-inflammatory effects, antibacterial activity and direct removal of plaque from the teeth (Wiwanitkit and Wiwanitkit, 2012). In the present study, pomegranate juice was procured from a local juice vendor and it didn't significantly show any antimicrobial activity or biofilm inhibition. In a previous study, Pomegranate extract was found to suppress the ability of these microorganisms to adhere to the surface of the tooth (Menezes *et al*., 2006). Plaque may involve four or more different microorganisms combining forces to colonize the surface of the teeth. Remarkably, nature's own pomegranate, fights the organisms ability to adhere by interfering with production of the very chemicals the bacteria use for adhesion. But these pomegranate extracts used in previous studies were extracted from different parts of the fruit. And the pomegranate peel extract gave maximum results. In our study we used the juice of the fruit, so the probable reason why it didn't show any inhibition was because of high sugar content in the juice. This probably enhanced the growth of the organisms.

Conclusion

In conclusion to the study carried out, it was found out that among the commercial mouthwashes used, Listerine exhibited minimum inhibition and Chlorhexidine demonstrated maximum inhibition of dental plaque. On the other hand, out of the essential oils, eugenol and eucalyptus demonstrated maximum inhibition, whereas peppermint and thyme exhibited minimum inhibition. Pomegranate juice showed no inhibition at all and in fact promoted the the growth of microorganisms.

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Supplemental material:

1. Isolation of organisms from Dental plaque:

1.1 Swab preparation:

Swabs were prepared by suspending cotton buds in 2 ml of saline in centrifuge tubes. The tubes were sterilized at 121^{o}C for 15minutes and were distributed for the collection of samples.

1.2 Collection of samples:

Care was taken that the swabs were taken early at dawn, i.e. without the use of any routinely used oral hygiene products from the mouth of 12 healthy individuals. The individuals were asked to rub the swab against their teeth and sub gingival region. These swabs were maintained and stored in sterile saline suspension for further use.

1.3 Media preparation:

100 mL of Nutrient broth and Luria Bertani broth were sterilized at 121^{o}C for 15 minutes. For blood agar, 100 mL of Nutrient agar was sterilized at 121^{o}C for 90 minutes and after sterilization, the agar was allowed to cool down for 5 minutes and 5 ml of anti-coagulated blood was added to the medium and poured in sterile petri plates and was allowed to solidify.
Chocolate agar was prepared by the same procedure as stated above, except that after addition of blood, the flask was kept in a water bath for 5 minutes at 100^{o}C and then poured in sterile plates to solidify.

2. Biochemical characterization:

2.1 Sugar fermentation test:

- Glucose (Loopful of culture was inoculated in 5 mL Peptone water + 1% Glucose with an inverted Durham's tube, Indicator – Phenol red)
- Mannitol (Loopful of culture was inoculated in 5 mL Peptone water + 1% Mannitol with an inverted Durham's tube, Indicator – Phenol red)
- Maltose (Loopful of culture was inoculated in 5 mL Peptone water + 1% Maltose with an inverted Durham's tube, Indicator – Phenol red)
- Lactose (Loopful of culture was inoculated in 5 mL Peptone water + 1% Lactose with an inverted Durham's tube, Indicator – Phenol red)
- Sucrose (Loopful of culture was inoculated in 5 mL Peptone water + 1% Sucrose with an inverted Durham's tube, Indicator – Phenol red)

2.2 IMViC test :

Indole test- Loopful of culture was inoculated in 5 mL of Peptone water and was incubated for 24 h at 37^{o}C. After incubation, Kovac's reagent was added.
Methyl red-Loopful of culture was inoculated in 5 mL of Peptone water and was incubated for 24 h at 37^{o}C. After incubation, 5 drops of methyl red was added.
VogesProskauer test-Loopful of culture was inoculated in 5 mL of peptone water and was incubated for 24 h at 37^{o}C. After incubation, 1 mL of Potassiumhydroxide and 3mL of alpha naphthol was added.
Citrate utilization-Loopful of culture was streaked on sterile Simmon's citrate agar butt and was incubated at 37^{o}C for 24 h.

1.Catalase test- A drop of 3%H_2O_2 was added on a glass slide, a colony was picked from the nutrient agar plate and tease it with a needle.
2.Oxidase test-Loopful of colony was taken and placed on a moist filter paper containing a drop of reagent (1% tetra-p-phenylenebiaminedihydrochloride)
3.Nitrate reduction test-Loopful of culture was inoculated in 5 ml of sterile Urea broth.
4.Nutrient agar with 7.5% NaCl- Loopful of culture was streaked on the plate to obtain isolates.
5.Arginine hydrolysis-Loopful of culture was inoculated in 5 ml of Nutrient broth with 0.1% arginine and Bromocresol purple.

3. Extraction of Essential Oils Using Steam distillation Method:

Steam distillation is a special type of distillation or a separation process for temperature sensitive materials like oils, resins, hydrocarbons, etc. which are insoluble in water and may decompose at their boiling point. The fundamental nature of steam distillation is that it enables a compound or mixture of compounds to be distilled at a temperature substantially below that of the boiling point (s) of the individual constituent (s). Essential oils contain substances with boiling points up to 200°C or higher temperatures. In the presence of steam or boiling water, however, these substances are volatilized at a temperature close to 100°C, at atmospheric pressure.

- Sample was powdered and soaked in 1:1 water
- Kept in steam distillation unit
- Steam was passed through sample.
- Then steam was condensed in to liquid where aqueous layer and essential oil was collected.
- This aqueous layer and oil were mixed with diethyl ether in 1:1 ration
- There are two separate layers which are separated with separating funnel, where essential oil dissolves in diethyl ether layer.
- Diethyl ether is directly then evaporated with help of rotavapor.
- Pure essential oil extract was collected.

4.Turbidimetric analysis

The term turbidity is used to define any technique that depends upon the change in the bacterial mass after incubation in liquid medium and that is used to quantify the amount of antimicrobial agent in the solution. The change in the bacterial mass is inversely proportional to the amount of antimicrobial agent present. The following protocol was followed for turbidimetric analysis:

- A colony of isolate 1, *S.aureus* was picked from the preserved plate. It was further added to 100mL of Tryptic soy broth. Similarly, a colony of isolate 2, *E.coli* was picked from the preserved plate. It was added to another flask containing 100mL of M9 broth. Both the flasks were kept at 37ºC incubator for 24 h in order to obtain actively growing 24 h old culture.

- After 24h, both the cultures were standardized using McFarland's standard (0.06 O.D) at 630nm using peptone water.
- 50µL of each of these standardized cultures were added to 20tubes containing 5mL of nutrient broth.
- Increasing concentrations of the oil extracts were added to each tube
- This protocol was performed for Eugenol oil and Eucalyptus oil
- The tubes were mixed thoroughly.
- The tubes were then placed at 37ºC for 24h.
- After 24 h, the minimum inhibitory concentration of each oil extract was observed.

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