RESEARCH ARTICLE

Antimicrobial Efficacy of Two Shrubs (*Punica granatum* and *Camellia sinensis*) and an Herb (*Mentha piperita*) on Two Human Pathogens, *Streptococcus mutans* and *Streptococcus sanguinis*

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Abstract

The antimicrobial activities of *Punica Granatum*, *Camellia Sinensis* and *Mentha Piperita* alone and in combination was studied on two human pathogens, *Streptococcus mutans* ATCC 35668 and *Streptococcus sanguinis* ATCC 10556 by macro- and micro-broth dilution alone and in combination with two and three extracts. The minimum inhibitory concentrations (MIC) for *P.Granatum*, *C.Sinensis* and *M.piperita* extracts in *S.mutans* were found as 3.125, 6.25 & 1.56 mg/ml respectively, and for *S.sanguinis* they were 1.56, 3.125 & 1.56 mg/ml respectively. MIC of *P.Granatum* and *C.Sinensis* simultaneously in *S.mutans* were 3.125 mg/ml; *S.sanguinis* 1.56 mg/ml, *P.Granatum* and *M.Piperita* simultaneously in *S.mutans* were 6.25 mg/ml and *S.sanguinis* 3.125 mg/ml; and *C.Sinensis* and *M.Piperita* simultaneously in *S.mutans* were 3.125 and *S.sanguinis* 1.56 mg/ml, respectively. MIC of *P.Granatum*, *C.sinensis* and *M.piperita* extracts simultaneously in *S.mutans* were 0.39 and *S. sanguinis* 0.78 mg/ml, respectively. Conclusively, *P.granatum*, *C.sinensis* and *M.piperita* revealed satisfactorily antibacterial effects on *S.mutans* and *S.sanguinis*, using a combination of the three extract makes the synergism antimicrobial effect and had the lowest MIC.

Introduction:

Dental caries is the most common oral disease which is caused by poor oral hygiene and proceeding cariogenic microorganism accumulation (Holbrook & Magnúsdóttir, 2012; Ghapanchi *et al.*, 2015; Lavaee *et al.*, 2016; 2018). Oral streptococci in oral cavity are one of the most important cariogenic species in dental plaque (Semyari *et al.*, 2011; Rezazadeh *et al.*, 2016; Ghapanchi *et al.*, 2016). *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacilli* are important acidogenic microorganism in dental caries induction. In the early biofilm *Streptococci* colonizes primarily (Chhour *et al.*, 2005). Physical and chemical interventions (tooth brushing, dental flossing, and mouth rinses), can decrease supragingival plaque and gingivitis (Palmer *et al.*, 2012). Chlorhexidine is one of the most widely used antimicrobial mouthwashes with some side effects including taste change, teeth and restoration staining (Gunsolley, 2010).

Nowadays many studies focused on herbal substitution instead of the chemical mouthwash to avoid

the microbial resistance and other side effects (Vahabi *et al.*, 2011; Palombo, 2011; Hillman *et al.*, 1985). A fruit, Pomegranate (*Punica granatum*) is known historically to cure cancer, cardiovascular diseases, diabetes mellitus, dental diseases, and also bacterial infection. The Asian tree *P.granatum* Linn is the family of Punicae and it grows well in Iran and northern India, Asian Mediterranean, Europe and Africa. Several parts of pomegranate such as its flowers, trunk skin, fruits, roots and seeds can be used for medical purposes (Jurenka, 2008; Bagri *et al.*, 2009). Recently the antimicrobial properties of *P.granatum* have been recognised (Vasconcelos *et al.*, 2006; Duman *et al.*, 2009). Various tree parts of *P.granatum* acts as antimicrobial agent against various microorganisms (Dastjerdi *et al.*, 2014). The antibacterial activity of *P.granatum* in the oral cavity was well documented by Chinsebu (2016). *Camellia sinensis* is a shrub of the Theaceae family usually, its leaves are used in preparing three different types of tea, Green tea (non-fermented), Oolong tea (semi-fermented) and Black tea (fermented) (Goenka

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et al., 2013; Voravuthikunchai *et al.*, 2004). Green tea, the non-fermented type with the greatest amount of catechin having antimicrobial property, acts as an antioxidant and also able to decrease cholesterol level (Tahir & Moeen, 2011; Mitscher *et al.*, 1997)

Peppermint (*Mentha piperita* L.), an aromatic plant of the Labiatae family possess various medicinal properties among which the antibacterial activity result from materials such as menthol, menthone, and their derivatives (Rodrigues *et al.*, 2004). Till date the studies which have been carried out in it are inadequate, lacking proper microbiological approach.

Due to the high prevalence of dental caries and new global efforts for introducing herbal substitute, this study was conducted to evaluate the antimicrobial effects of few potent medicinal plants; *Punica granatum*, *Camelia sinensis*, and *Mentha Piperita* in their lone capacity as well as combined effects against *Streptococcus mutans* and *Streptococcus sanguinis*

Materials and methods:

Preparation of the plant extracts: extracting of plants materials were done by using the following methods. The dried form of these plants was powdered; 50g of the plant was soaked in 500ml 96% ethanol at room temperature for 48 hours. The filtrate supernatant was centrifuged (for 10 minutes at 2000 rpm) and concentrated (on a rotary evaporator at 42°C) in order to eliminate ethanol. The extracts were stored in a refrigerator in sterile bottles (Menezes *et al.*, 2006; Taweekaisupapong *et al.*, 2005).

Activation of microorganisms: standard strains of *S. mutans* (ATCC 35668, PTCC 1683), *S. sanguinis* (ATCC 10556, PTCC 1449) in lyophilized form were prepared from the Persian Type Culture Collection center. The brain heart infusion agar (BHIA, Merck, Germany) was used to activate. After incubation at 37°C, a 24-hours culture was used for microbial suspension preparation. Using spectrophotometer (625nm) the microorganism concentration in the microbial suspension was adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) (Jorgensen & Turnidge, 2015).

Minimal Inhibitory Concentration (MIC)

Broth Macro dilution Method: a standard technique of clinical and laboratory standards institute (CLSI, 2012) was used for MIC determination by the microdilution method. The 0.5 McFarland standard suspensions were diluted from 1 to 150 by serial dilution in BHI broth medium to have 10^6 CFU/mL of bacteria. 1ml of a diluted microbial suspension was added to the tubes of serially diluted extract. The tubes with only culture medium and extract and tubes with culture medium and microbial suspension were considered as a negative and positive control.

The MIC value of extracts for each bacterial species was measured after 24 hours of incubation at 37°C. The evaluation was repeated for 3 times against each bacteria.

Broth Microdilution Method: the concentration of each bacteria (*S. mutans* and *S. sanguinis*) suspension in Muller Hinton broth was adjusted to 10^7 CFU/ml. Twofold dilution of each herbal extract was prepared from the stock solution. Aliquots (200 µl) of each dilution were dispensed in 96-well culture plates after adding 100 microliters of each bacterial suspension to each well and 48 hours incubation at 37°C, the absorbance was then measured at 595nm. MIC was considered the highest dilution of the extracts at which no growth (OD = 0.05) was detected.

Minimum Bactericidal Concentration (MBC): determining the MIC, 20 µL of the tube with MIC of each extract and the tube with no bacterial growth was cultured in the plates containing BHIA. After 24 hours of incubation at 37°C, the plates were evaluated for the growth of microorganisms. MBC was considered as the concentration with no bacterial growth. This test was repeated 3 times for each bacteria.

Statistical analysis: data analysis by using two-way analysis of variance (two way ANOVA) and post hoc tests were performed.

Results:

While using the macro- and micro-dilution broth method, the extracts of *P. granatum*, *C. sinensis*, and *M. piperita* showed inhibitory effects on the growth and proliferation of *S. mutans* and *S. sanguinis*. MIC and MBC values of these extracts for different microorganisms by using macro-dilution tests are presented in Tables-1.

Table -1: MIC and MBC of the extracts by macro-dilution test (Mean ± SE are of triplicates)

Bacteria	Extracts	MIC + SE	MBC + SE
<i>S. mutans</i>	<i>P. granatum</i>	3.125 ± 0.01	6.25 ± 0.01
	<i>C. sinensis</i>	6.25 ± 0.01	12.5 ± 0.01
	<i>M. piperita</i>	1.56 ± 0.01	3.125 ± 0.01
<i>S. sanguinis</i>	<i>P. granatum</i>	1.56 ± 0.01	3.125 ± 0.01
	<i>C. sinensis</i>	3.125 ± 0.01	6.25 ± 0.01
	<i>M. piperita</i>	1.56 ± 0.01	1.56 ± 0.01

M. piperita and *P. granatum* had the highest antibacterial effect on *S. mutans* with MIC and MBC values of 1.56 and 3.125 mg/ml, respectively. MIC and MBC values of this extract for *S. mutans* and *S. sanguinis* by using micro-dilution tests are presented in Table 2.

Table -2: MIC and MBC of the extracts by micro-dilution test (Mean ± SE are of triplicates)

Bacteria	Extracts	MIC + SE	MBC + SE
<i>S. mutans</i>	<i>P. granatum</i>	1.56 ± 0.01	3.125 ± 0.01
	<i>C. sinensis</i>	3.125 ± 0.01	6.25 ± 0.01
	<i>M. piperita</i>	1.56 ± 0.01	3.125 ± 0.01
<i>S. sanguinis</i>	<i>P. granatum</i>	1.56 ± 0.01	3.125 ± 0.01
	<i>C. sinensis</i>	3.125 ± 0.01	6.25 ± 0.01
	<i>M. piperita</i>	1.56 ± 0.01	1.56 ± 0.01

Table-3: MIC and MBC of the combination extract by macro-dilution test (Mean $\pm$ SE are of triplicates)

Bacteria	Extracts	MIC + SE	MBC + SE
<i>S.mutans</i>	<i>P.granatum</i> + <i>C.sinensis</i>	3.125 + 0.01	6.25 + 0.01
	<i>C.sinensis</i> + <i>M.piperita</i>	3.125 + 0.01	6.25 + 0.01
	<i>M.piperita</i> + <i>P.granatum</i>	6.25 + 0.01	6.25 + 0.01
<i>S.sanguinis</i>	<i>P.granatum</i> + <i>C.sinensis</i>	1.56 + 0.01	3.125 + 0.01
	<i>C.sinensis</i> + <i>M.piperita</i>	1.56 + 0.01	3.125 + 0.01
	<i>M.piperita</i> + <i>P.granatum</i>	3.125 + 0.01	6.25 + 0.01

The combination of *P.granatum* and *C.sinensis* similar to *C.sinensis* and *M.piperita* revealed the highest effect on *S.sanguinis* with MIC and MBC values of 1.56 and 3.125 mg/ml respectively by using macro-dilution test, as presented in Table 3.

Table-4 shows that the combination of *C.sinensis* and *M.piperita* the same as *P.granatum* and *C.sinensis* had the highest effect of *S.mutans* with MIC values of 1.56mg/ml, but the MBC was different. All the results of Microdilution test for combination extracts on *S.mutans* and *S.sanguinis* are presented in Table-4.

Table-3: MIC and MBC of the combination extract by micro-dilution test (Mean $\pm$ SE are of triplicates)

Bacteria	Extracts	MIC + SE	MBC + SE
<i>S. mutans</i>	<i>P. granatum</i> + <i>C. sinensis</i>	1.56 + 0.01	3.125 + 0.01
	<i>C. sinensis</i> + <i>M. piperita</i>	1.56 + 0.01	1.56 + 0.01
	<i>M. piperita</i> + <i>P. granatum</i>	3.125 + 0.01	6.25 + 0.01
<i>S. sanguinis</i>	<i>P. granatum</i> + <i>C. sinensis</i>	1.56 + 0.01	3.125 + 0.01
	<i>C. sinensis</i> + <i>M. piperita</i>	3.125 + 0.01	6.25 + 0.01
	<i>M. piperita</i> + <i>P. granatum</i>	3.125 + 0.01	6.25 + 0.01

The best solution containing three extracts of *P.granatum*, *C.sinensis* and *M.piperita* showed the highest antibacterial effect on *S.mutans* and *S.sanguinis* in all extracts used in this study by macro- and micro-dilution test. MIC and MBC values for *S.mutans* were 0.39 and 0.78 mg/ml respectively, and for *S.sanguinis* they were 0.78 and 1.56 mg/ml, respectively. (Figure-1)

Figures-1 & 2 show the comparison of the MIC of the combinational extract by the macro-dilution test for

Figure-1: Comparative analysis of mixed extracts on *S.mutans* by macro-dilution test (+SE = 0.01 for all assays) MIC Vs MBS, Bar having similar alphabets do not differ from each Other at $p < 0.05$ Figure-3: Comparative analysis of mixed extracts on *S.sanguinis* by macro-dilution test (+SE = 0.01 for all assays) MIC Vs MBS, Bar having similar alphabets do not differ from each Other at $p < 0.05$ Figure-3: Comparative analysis of mixed extracts on *S.mutans* by micro-dilution test (+SE = 0.01 for all assays) MIC Vs MBS, Bar having similar alphabets do not differ from each Other at $p < 0.05$ Figure-4: Comparative analysis of mixed extracts on *S.sanguinis* by micro-dilution test (+SE = 0.01 for all assays) MIC Vs MBS, Bar having similar alphabets do not differ from each Other at $p < 0.05$

Discussion:

Dental infections are one of the crucial causes of head and neck infections. Risk of serious consequences resulting from the extension of a dental abscess is the most common cause of hospitalization for sepsis caused by oral infections.

Dental abscesses, if left untreated, can cause brain abscesses and necrotizing inflammation. In most cases, the appropriate diagnosis and treatment of these infections can be prevented. Almost a number of microorganisms including a variety of *Streptococcus* such as *S.mutans*, *S.sanguinis*, *S.viridans* in addition to *Streptococcus* anaerobic, cocci anaerobic, *Prevotella*, Gram-negative bacilli, etc. are involved in the infection of the tooth (Dziedzic *et al.*, 2013).

The reduction of oral microorganisms prior to oral surgery can play a significant role in reducing the incidence of postoperative oral infections (Munro & Grap, 2004; Taheri *et al.*, 2011). Treating with antimicrobial agents with minimal side effects such as filling restoration and teeth pigmentation and unwanted tissue toxicity is very necessary.

Although we found that *Punica Granatum*, *Camelia Sinensis* and *Mentha Piperita* had high antibacterial effects on *S.mutans* and *S.sanguinis*, using the three extract combination reveal the synergistic antimicrobial effect and had the highest effect. Lalwani *et al.* (2014) reported that the extract of *P.granatum* had an antimicrobial effect against *S.mutans*, which is in the same line with those of our study. Hajifattahi *et al.* (2016) reported that *P.granatum* extract had the greatest influence on the *S.mutans* and *S.sanguinis*, their results are very nearer to our study.

In our study, MIC and MBC against green tea extract on *S.mutans* and *S.sanguinis* revealed an antimicrobial effect on oral bacteria which resembles the findings of Araghizadeh *et al.* (2013). Our MIC and MCB results of *M.piperita* extract on *S.mutans*, *S.sanguinis* is also compromised with Shayegh *et al.* (2008).

The combined effects of herbal extracts has not been well studied before. In the presenting study, MIC and MBC of *P.granatum*. The extract combination of *C.sinensis* and *M.piperita* against *S.mutans* and *S.sanguinis* were lower than these values for each extract alone.

These results showed that the combination of these 3 extracts is the most effective antimicrobial extract and significantly reduces the MIC and MBC values.

P.granatum has different ingredients such as tannin, flavonoids, polyphenol compounds, ellagitannins, punicalin, alkanoids, punicalagin, organic acid and polyphenolic compositions (Voravuthikunchai *et al.*, 2004; Bassiri-Jahromi *et al.*, 2015; Anibal *et al.*, 2013; Al-Zoreky, 2009).

The effective antimicrobial composition of *P.granatum* is punicalin, punicalagin tannins (Yadav & Khan, 2012). These compositions induce protein precipitation in the bacterial cell wall and cause damage (Endo *et al.*, 2010). Phenolic ingredients disrupt bacterial cell membrane, interfere with bacterial DNA, cellular enzymatic activation, bacterial adhesion and induce deprivation induction (Voravuthikunchai *et al.*, 2004; Bassiri-Jahromi *et al.*, 2015; Anibal *et al.*, 2013; Al-Zoreky, 2009; Endo *et al.*, 2010).

As per Golestannejad *et al.* (2017), *M.piperita* have a high concentration of menthol and menthone and tannin. The antibacterial effect of it is considerably related to high concentration of menthol, pulegone, isomethone, piperitone, carvone and dehydro carvone (Sivropoulou *et al.*, 1996; Chami *et al.*, 2004).

Hydrophobic properties of herbal extracts make the penetration of these compositions into the cell membrane, more feasible (Mathur *et al.*, 2011).

Regarding the antibacterial properties, Hassan *et al.* (2018) shown more potency in *C.sinensis* (green tea) in comparison to a mouthwash showed against *S.mutans*. It is assumed that polyphenolic compounds of green tea are responsible for inhibition of bacterial attachment to the dental plaque by changing the bacterial phenotype. On the other hand, glycosyl transferase inhibition which can be induced by catechin can decrease plaque formation more potently (Hassan *et al.*, 2018; Tehrani *et al.*, 2011).

Green tea leaves contain fluoride and polyphenols (catechin) which induce dental caries resistance (Fournier-Larente *et al.*, 2017; Raju *et al.*, 2017;). As it has been proved previously, fluoride cause bacterial growth inhibition and teeth remineralization (Zafar & Ahmed, 2015; Zafar, 2013).

In our study, both microbroth and macrobroth dilution methods were used to assess the antibacterial properties of the *P.granatum*, *C.sinensis* and *M.piperita* extracts in their respective lone capacity and as combined. Introducing a new combination of herbal extracts with noticeable antibacterial effects develops a new insight for improving the antimicrobial strength of them. These herbal extracts can be used as mouthwashes and tooth-pastes. Natural products usually have fewer side effects than chemical products. Designing more researches on different extracts of plants and other oral pathologic microbiota can be suggested for future studies.

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