Ars Pharmaceutica

Synergistic effect of *persica* mouthwash and Iranian ethanolic extract of propolis against biofilm formation of oral pathogens (in vitro study)

Efecto sinérgico del enjuague bucal *persica* y del extracto etanólico iraní de propolis contra la formación de biofilm de patógenos orales (estudio in vitro)

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Artículo Original
Original Article

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Conflicto de interés
Competing interest

All Authors declare no conflicts of interest

Agradecimientos
Acknowledgment

This study was granted by Drug Applied Research Center, Tabriz University of Medical Sciences and approved by Tabriz University of Medical Sciences. Author Thank all staff of Biomedicine (Medical Biology) Research Institute.

Received: 05.10.2017
Accepted: 26.12.2017

ABSTRACT

http://dx.doi.org/10.30827/ars.v58i4.6764

Aims: Several pathogens are responsible for oral diseases and dental plaque and their main mechanism is biofilm production. Natural products are point of interest for controlling these infections. persica mouthrinse, propolis and honey are some of these products with considerable antibacterial effects. In this study, we aimed to investigate synergy effect of these products on their antibiofilm and antibacterial effect.

Material and Methods: Minimal Inhibitory effect and Minimial Biofilm inhibitory concentration of persica mouthrinse, propolis, honey solely and in combination was calculated against *Streptococcus mutans* ATCC35668, methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis*, *Enterococcus faecalis* ATCC 29212 , *Escherichia coli* ATCC 25922.

Results: Combination of persica and propolis had a better profile in biofilm's inhibition than honey. None of herbal combinations had synergistic effect against studied bacteria; MIC of the persica mouth had the best effect against *Streptococcus mutans*, which causes caries.

Conclusions: There was no synergistic effect of persica and propolis and the best antimicrobial effect was observed on subminimum inhibitory concentration of persica mouthwash. Findings of the present study suggest use of other combination than honey, propolis and persica for improving antimicrobial activity of these components.

Keywords: *Streptococcus mutans, Staphylococcus aureus*, biofilm, plaque, persica, propolis, honey.

RESUMEN

Objetivos: Varios patógenos son responsables de enfermedades orales y la placa dental y su mecanismo principal es la producción de biofilm. Los productos naturales son puntos de interés para controlar estas infecciones. persica mouthrinse, propolis y miel son algunos de estos productos con considerables efectos antibacterianos. En este estudio, se buscó investigar el efecto sinérgico de estos productos sobre su antibiótico y efecto antibacteriano.

Material y Métodos: Se calculó el efecto inhibidor mínimo y la concentración inhibitoria mínima de biofilm de enjuague bucal de persica, propóleos, miel únicamente y en combinación, contra *Staphylococcus aureus* ATCC 29213, *S. epidermidis*, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922.

Resultados: La combinación de persica y propóleo tuvo un mejor perfil en la inhibición de la biopelícula que la miel. Ninguna de las combinaciones de hierbas tuvo efecto sinérgico contra las bacterias estudiadas; La Se calculó el efecto inhibidor mínimo de la boca de la persica tuvo el mejor efecto contra *Streptococcus mutans*, que causa la caries.

Conclusiones: No hubo efectos sinérgicos de la persica y el propóleo y se observó el mejor efecto antimicrobiano en la concentración mínima inhibitoria de enjuague bucal de persica. Los hallazgos del

presente estudio sugieren el uso de otra combinación que la miel, el propóleo y la persica para mejorar la actividad antimicrobiana de estos componentes.

Palabras clave: *Streptococcus mutans*, *Staphylococcus aureus,* biofilm, placa, persica, propóleos, miel.

INTRODUCTION

Dental plaque is one of biofilm's kind that plays a major role in pathogenesis of oral disease[1] such as calculus formation, periodontal disease & gingivitis, and above all caries that is so important and prevalent[2]. Since the disruption of biofilm & killing the bacteria in this structure requires a concentration that is 10-1000 times higher than Minimum inhibitory concentration (MIC) of antimicrobial agents; so in order to control the biofilm or plaque, we should prevent the biofilm formation that is possible even with sub-MIC amounts of proper antimicrobial agents. To control the plaque formation, we can use the antimicrobial and anti-biofilm agents as a mouthwash. Recent approaches are to find natural mouth rinses with higher antimicrobial and antibiofilm effect with less toxicity and tolerable flavor, less irritation and without any changing in the color of teeth[3]. Among the bacteria producing biofilm, the acidogenic bacteria have a greater effect in cariogenic cycle[4,5].

In oral medicine, propolis has been used as a remedy for surgical traumas, root canal irrigation solution, cariogenic process inhibitor, periodontitis treatment, dentin hypersensitivity and as the antibacterial and antifungal substance in root canals and showed good results in most of these experiences[2]. The role of propolis in inhibitory mechanisms of plaque formation by the main accused bacterium for caries namely *S. mutans* have been approved[6]. In addition to antibacterial & anti-biofilm effect of propolis and its synergic effect with other antibiotics, its anti-inflammatory and anesthetic effects makes it as good choice for developing new mouthwashes[2,4].

Persica mouthwash includes three herbal extracts (*Salvadora persica*, *achillea millefolium*, *mentha spicata*) and among them *Salvadora persica* has a great history in oral health but studies revealed that antimicrobial effect of persica mouthwash is higher than persica extract[6]. Also the prophylactic effect of persica mouthrinse against caries, periodontal disease has been approved [3]. So due to improving the effective bacterial spectrum and tendency to use natural substances and possibility of getting the synergic effect and afterward decreasing the toxic effects and gaining the other beneficial effects we aimed to evaluate synergy effect of persica mouthrinse with honey and propolis on their antibiofilm and antibacterial effect.

MATERIAL AND METHODS

In order to assess the MIC & MBIC (minimum biofilm inhibitory concentration) of persica mouth rinse, propolis, honey solely and in combination in this in-vitro study, we used the standard suspension of *Streptococcus mutans* ATCC35668, methicillin-resistant *Staphylococcus aureus* ATCC 33591, *S. aureus* ATCC 29213, *Staphylococcus epidermidis*, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922. To evaluate the synergic effect, MIC should be determined for the pure and combinational form of substances. For calculating the MICs in combinational form, we did the same way in TSB (Tryptic Soy Broth medium, Merck, Germany) with subMIC concentration of the substance. For biofilm experience, biofilm inhibition was evaluated as described previously [7,8].

All isolates were obtained from Iranian Research Organization for Sciences and Technology, Tehran. The bacterial inoculums have been prepared in 0.9 % sodium chloride from fresh cultures after 24 hours. The turbidity of the suspension has been adjusted to the McFarland 0.5 turbidity standard [9].

Extraction of propolis

Extraction was conducted by method of maceration in ethyl alcohol. The crude propolis was collected in Shabestar located in the north-west area of East-Azarbayjan in Iran in October 2015. Hand-collected propolis was kept desiccated and in the dark place before it's processing. The sample was frizzed and chopped into small blender and dissolved in 70% ethyl alcohol with a ratio of 3:10 (30 g of propolis in 100 ml of 70 % ethyl alcohol). Then, the propolis samples were kept for one week at room temperature in laboratory shaker in a dark place. The ethanolic extract of propolis (EEP) was filtered by Whatman paper no. 4 (Whatman, UK). The EEP was then concentrated in a rotary vaccume evaporator to obtain the pure propolis extract in powder form. That powder was weighted and dissolved in TSB (tryptic soy broth + 10% ethyl alcohol) to make a concentration of 25 mg/ml [1,2]. Other concentrations were obtained by serial dilution with TSB (25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195, 0.0975, 0.0487, 0.0243, 0.0121, 0.006, 0.003 mg/ml).

Preparation of persica

For preparing the concentrations, we used persica mouthwash (poursina, Tehran, Iran) and after homogenizing the contents made the stoke solutions with TSB and finally did the serial dilution as: 60-50-40-30-25-20-15-10-7.5-5-2.5-1.25-0.625-0.312%v/v.

Preparation of honey samples:

Natural, un-treated and unpasteurized honey samples were obtained directly from amateur beekeepers in Shabestar .first concentration (50%) was made by adding equal amount of honey to equal amount of TSB. after mixing by stirring with vortex different concentrations were obtained by serial dilution with TSB. Further equal volumes of first solutions and more TSB were used to obtain dilution up to 1.56%.

Antimicrobial Effect of propolis and persica

Antimicrobial activity of propolis extracts and persica was evaluated by using broth micro dilution method to measuring MIC and MBC as recommended by the Clinical and Laboratory Standards Institute [10]. In order to determine MIC value, bacterial strains were incubated in 96 cell microplates in broth containing different concentrations of propolis (25mg/ml – 0.003mg/ml) or of persica (0.312-60 %v/v) for 24 hours, at the temperature of 37°C. MIC value was estimated by visual and spectroscopic method by absorbance measurement at 600nm (OD600—optical density reading at 600 nm) [3]. Control tubes with the TSB (without bacterium) and concentration of any substance or both concentrations in combinational condition as positive controls and TSB with bacterium as negative controls were used [11].

In vitro synergism assays were carried out after evaluating the MIC of EEP and persica alone. One-fourth of MIC 90% was considered as sub-inhibitory concentration of EEP and persica in the synergism assays [12].

After determination of MICs of persica and propolis, various concentrations of persica and propolis below their MIC were prepared. Various mixtures of persica in sub inhibitory concentration of propolis which prepared in TSB (the original growth medium) and various mixtures of propolis in sub inhibitory concentration of persica were prepared to study the interactions like synergism in antibacterial or anti biofilm aspect.

Antibacterial synergism was identified when the MIC of persica or propolis in combination was lower than the MIC of persica or propolis alone [1].

Anti-biofilm synergistic effect

The method for evaluating the anti-biofilm and antibacterial synergistic effect was the same, but to reach the biofilm inhibition or formation percentage, we need an extra step to color the biofilms and to read the absorbance in 620 nm in spectrophotometer.

The coloring method we chose was crystal violet method with these steps: decantation of planktonic phase right after 24 h from incubation and adding the phosphate buffer 0.1 M. Color fixation of biofilms with methanol and adding the crystal violet. Finally pouring the acetic acid into micro tubes and finally the OD measurement in 620 nm.

The mean and standard deviation of MIC and biofilm's growth percentage have been used to express the descriptive statistics.

Statistical analysis

The analytical statistics was included a comparison of mean MIC±SD and mean of biofilm growth percentage ±SD between different bacteria for each substance which has been conducted by one way ANOVA. Furthermore, for investigating the antibacterial synergistic effect, the difference of mean MIC ± SD for each of two independent groups (alone and combination) was calculated and to assess significant difference between them, the two independent T-test was used (significance level of the test is 0.05). Also in the synergism of anti-biofilm effect, the 2-way ANOVA was used (significance level of the test is 0.05).

In order to achieve the pure absorbance (OD) of biofilm's growth we had to clear the back grounds caused by sedimentations of substances by eliminating the positive control (TSB medium + concentrations) from them.

Biofilm's growth percentage = pure OD or growth of any concentration/maximum growth (negative control) ×100

Biofilm's inhibitory percentage =100- biofilm's growth percentage

To evaluate the synergistic effect in addition to judgments based on graphs and statistical analysis we can refer to FBIC indexes; and if

$\sum$ FBIC (fractional biofilm inhibitory concentration) ≤ 0.5 describes the synergistic effect, $\sum$FBIC (0.5- 4) shows no interactions and if $\sum$ FBIC ≥ 4 it defines antagonistic effect.

1) FBIC (substance A) =MBIC (minimum biofilm inhibitory concentration) (substance A in combination)/MBIC (substance A alone)

FBIC (substance B) =MBIC (substance B in combination) / MBIC (substance B alone)

2) FBIC index or $\sum$FBIC= FBIC (substance A) + FBIC (substance B)

RESULTS

MIC results for propolis and persica and honey are presented in Table 1. Propolis in combination with persica had highest efficiency against *E. faecalis* and *S. epidermidis*. Table 2 and 3 indicates two-way analysis of variance test to compare the biofilm formation for different combinations. Figure 1 shows effect of propolis, persica, honey and their combinations against all studied strains. Results of synergy among all combinations are presented in Table 4. Honey was not a specific biofilm inhibitor for *S. mutans*, Methicillin resistant *S. aureus* and *E. faecalis*.

Table 1: MIC*s of propolis with persica in synergy combination and honey alone

MIC: Bacteria:	honey (mg/ml)	propolis in combination with persica (mg/ml)	honey in combination with persica (mg/ml)
S. mutans	7.81±2.73	0.28±0.09	7.55±2.50
MRSA#	8.75±3.04	0.78±0.023	10.0±0.33
S. aureus	7.55±2.56	0.195±0.045	8.75±2.56
S. epidermidis	12.03±0.98	0.34±0.12	12.5±4.97
E. faecalis	7.88±2.73	0.34±0.12	6.25±2.55
E. Coli	24.22±17.56	> 25	> 30

* Minimal Inhibitory concentration
# Methicillin resistant *S. aureus*

Table 2: The results of p-value in Two-way analysis of variance test to compare the biofilm formation percentage in propolis alone and propolis in combination with persica.

Bacteria: Source of change:	*E.coli*	*E. faecalis*	*S. epidermidis*	*S.aureus*	*MRSA**	*S.mutans*
Type of substance	0.019	0.145	0.047	0.183	0.058	P<0.001
concentration	0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
Interaction between both	0.068	0.913	0.845	0.227	0.622	P<0.001

P value<0.05
* Methicillin resistant S. aureus

Table 3: The results of Two-way analysis of variance test to compare the biofilm formation percentage in persica alone and persica in combination with propolis.

Bacteria: Source of change:	*E.coli*	*E.faecalis*	*S.epidermidis*	*S. aureus*	*MRSA*	*S.mutans*
Type of substance	0.485	0.004	0.002	0.004	P<0.001	0.230
concentration	0.573	0.033	0.063	0.046	0.001	0.965
Interaction between both	0.752	0.254	0.367	0.467	0.032	0.959

P value<0.05
* Methicillin resistant S. aureus

Table 4: Results for synergy effect of propolis and persica in Combination for all studied strains.

Bacteria	regression in combinational form for propolis*	regression in combinational form for persica	Total response to synergy test
S. mutans	+	-	-
MRSA#	-	+	-
S. aureus	-	+	-
S. epidermidis	-	+	-
E. faecalis	-	+	-
E. coli	+	-	-

* "-" means no significant difference
# MRSA = Methicili resistance S.aureus

Figure 1. Effect of Propolis and Persica in combination and honey against biofilm formation of *S. mutans*, Methicilin resistant *S. aureus, S. aureus, S. epidermidis, E. faecalis* and *E.coli.*

DISCUSSION

Microbial biofilms are a structural matrix consisting of microbial cells; attached to an inert or living surface & polysaccharides, enzymes & virulence factors secreted by them [8,12] and dental plaque is one of biofilm's examples that includes layers of growing microorganisms, epithelial cells, macrophage & leucocytes that gathered by adhesive organic matrix. This Plaque grows quickly in absence of any controlling agent and damages the dental and tissues [2]. With dental plaque grows, bacterial composition changes and micro colonies of biofilm connects to each other through aqueous canals and causes ability to physiological absorbance & resistance to antimicrobial agents [1,13]. Dental plaque is the main cause of calculus formation, periodontal disease & gingivitis and above all caries that is so important & prevalent [2,14].

There are some mechanisms to control the biofilm or plaque formation, including prevention of formation and growth, destruction of biofilm structure and killing the bacteria living in biofilm's structure. Among these mechanisms, prevention of biofilm formation and growth and colonization by using the antibacterial agents is the most effective method [15]. For this purpose, mouth rinses are qualified by these features: lack of any systemic or local allergic reactions, lack of changing in dental and mucosal color or changing in bacterial flora towards some harmful strains, minimum toxicity, tolerable flavor, anesthetic effect for oral wounds, appropriate antibacterial & antibiofilm effect [3].

Among the main bacteria, producing biofilm which play a major role in oral diseases *S. mutans*, *S. aureus*, *Lactobacillus spp.* and *E. faecalis* plays the main role. *S. mutans* is the primary reason of caries because of its colonization & production of insoluble glucan with high molecular weight[3,16]. *Lactobacillus spp.* as a secondary & coexisting factor plays important role in this procedure [14].

Propolis is a complex of organic resin (50-70%) that produced by *Apis mellifera* from tree gum and bud exudates that combined with bee wax (30-50%) and pollens (5-10%) and about 5% is variable by the time and place of collection. propolis contains other compounds like phenolic and aromatic compounds that have antimicrobial effects. Amounts of the antimicrobial components depend on geographical region and harvest season. In the beehive, propolis is used to seal the pores & protect from cold weather, humidity, invaders, microbes and generally as a disinfectant in hive.

Aromatic compounds such as coffeic acid cause antibacterial effect against anaerobic oral pathogens like *lactobacillus acidophilus*. The growth inhibitory mechanism of propolis against *S. mutans* and *Streptococcus sabrinus* and *Streptococcus sanguis* has been defined [9]. Because of antigens and receptors, *S. mutans* can stick to the primary film of salivary mucin glycoproteins and colonize in it. *S. mutans* feeds from sucrose which can form the insoluble glucan polymer from the monomers of glucose that released by glucosyltransferase [17]. This polymers gathers other local *S. mutans* on the dental surface and with cooperation of other bacteria, develop the biofilm matrix [15]. Propolis has a certain mechanism of inhibition against glycosyltransferase of *S. mutans* [16]. Nowadays, there are many forms of propolis like: toothpaste, mouth rinse, gel, lozenge, tablet, soap and all of these are due to its numerous benefits such as antibacterial effect against main cariogenic bacteria, antiviral, antifungal antiplaque, anti-inflammatory, anesthetic, antioxidant, sealing effect. These effects have been used in several investigations such as remedy for surgical traumas, root canal irrigation solution, cariogenic process inhibitor, periodontitis treatment, dentin hypersensitivity and as an antibacterial and antifungal substance in root canals.

Since the propolis in combination with antibiotics leads to 10-100 times greater effect and due to increasing ratio of the antibiotic resistance, tendency to use the natural products and utilizing other benefits such as anti-inflammation and improving effects of other herbal mouth rinses w synergistic effect of propolis and persica's combination can be helpful.

The efficiency of ethanol in extraction of propolis, especially about bioflavonoids as an important ingredient of propolis made to choose the ethanolic extraction for propolis.

Persica mouthwash manufactured by poursina company includes three herbal extracts (*Salvadora persica* 30 %, *Achillea millefolium* 25 %, Mentha spicata 40%) that mentha spicata has antimicrobial & antibiofilm[18] and anesthetic effects and flavoring role[18] and achillea millefolium has analgesic and anti-inflammatory effects [19]. Overall Salvadora persica has antimicrobial and antibiofilm effects and with the abilities to increase the saliva with its astringent effect and glycosyltransferase inhibitingand fluoride releasing[20] can prevent from gingivitis and caries [21]. *Salvadora persica* has been used since 7000 years ago as the miswak stakes. Through a docking study, *Salvadora persica* had a specified connection to biofilm[1]. Persica's extract has antibacterial effect against *S. mutans*, *Lactobacillus spp.* Therefore, it can be used to reduce the microbial load as a root canal irrigant [20]. Despite these pros miswak stakes releasing components with minor reproductive toxicity in rats after 24 h [22].

According to previous studies, persica mouth rinse has a better antibacterial activity than *Salvadora persica* extract [22]; therefore, we chose persica mouthwash for this study.

Comparing antimicrobial effect of persica and chlorhexidine mouthwashes showed persica had less antibacterial effect and considerable toxicity [21,22]. Findings of the present study indicated that persica, honey and propolis had higher antimicrobial activity solely and their combinations had no significant effect on increasing anti microbial properties of these components.

CONCLUSION

There was no synergistic effect of persica and propolis and the best antimicrobial effect was observed on subMIC concentration of persica mouthwash. Findings of the presents suggest use of other combination than honey, propolis and persica for improving antimicrobial activity of these components.

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