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Green Tea and *Salvadora persica L* Synergistic Combination Effect against *Staphylococcus aureus* Activity on Soft Liner Acrylic Denture Base

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ABSTRACT

One of the most serious problems associated with acrylic based soft lining materials was microbial colonization such as Staphylococcus aureus bacteria which may end with infection, discomfort and, failure of the soft liner.

Aim: The aim of the current study is to investigate the anti-bacterial efficiency of a synergistic mixture of green tea and Salvadora persica L. aqueous extracts incorporated into acrylic based heat cured soft liner against S. aureus bacterial infection.

Materials and Methods: Fifty specimens with dimensions of 10 mm in diameter and 3 mm in thickness were prepared by addition of various concentrations of Salvadora persica L., green tea and combination of both into heat cured acrylic based soft lining material. Disc diffusion method was utilized to determine the anti-microbial effect against Staphylococcus aureus bacteria. The samples were divided into 5 groups (10 samples for each group); the first group (control group) include samples contain no additive. For the second group, green tea extracts were added to the soft liner. While for the third group, Salvadora persica L. extracts were added to the soft liner. Regarding the fourth and fifth groups, both Salvadora persica L. and green tea mixture were added in different proportion based on minimum inhibition concentration methods. Fourier transform infrared analysis was conducted to determine if there is any chemical reaction between soft denture liner and additives.

Results: The results showed no inhibition zone around the control samples. High significant increase in the mean values of the inhibition zone around the synergistic mixture of Salvadora persica L. and green tea extracts (P<0.001). While, a non-significant differences in the mean values of the inhibition zone around Salvadora persica L. and green tea extracts (p=0.060). The lowest values were obtained in green tea extracts samples.

Conclusion: Combination between Salvadora persica L. and green tea aqueous extracts into heat cured acrylic based soft liner exhibited synergistic anti-bacterial activity against Staphylococcus aureus, and could be utilized as a useful active agent to produce oral health care products.

Key words: *Salvadora persica L.*, Green tea, *Staphylococcus aureus*, Disc diffusion method, Denture soft liner

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INTRODUCTION

Natural rubbers have been utilized since 1869 by Twichell to construct the first soft lining material in a dental field [1]. From that point forward, a clear evolution has involved dental materials result in the development of different types of soft lining materials each has its own advantages and drawbacks [2].

Poor adaptation of the denture base to the underlining mucosa mostly due to alveolar bone resorption may lead to discomfort of the patients; this problem can be managed by using relined dental prosthesis [3]. In general, soft denture liners are applied over the hard denture bearing surface to assist patients whose suffering from annoyance during wearing of complete and partial dentures to act as a cushion which absorbs the load generated by the masticatory process, reduces its traumatic effects of occlusal forces and spread it equally and also makes the denture wearer more comfortable [4,5].

Staphylococcus aureus appears to be as the main causative factor in denture stomatitis, angular cheilitis, oral mucositis especially in elderly persons and patients with parotid gland infection [6]. Traditional medical plants are widely used nowadays for preparation of new chemical medicine as they provide a new active biological compound [7].

Green tea is one of the most popular beverages worldwide. As well it is considered as a source of several natural beneficial health impacts in human due to its different biologically active composition. Green tea extracted from

fresh plants leaves of the shrub *Camellia sinensis* (L.) Kuntze. Following which, the fresh leaves were steamed and dried by the heat [8]. A study done by Yoda et al. [9] revealed that the green tea extracts exhibits an inhibitory effect against the development of *Streptococcus pyogenes* and *Staphylococcus aureus* when increasing concentrations of the green tea extracts.

Salvadora persica L. is traditionally natural chewing sticks were used for many years by the people in rural areas, predominantly young, and still to our time most common than commercial toothbrushes and toothpastes. The main benefit of these chewing sticks is maintaining their teeth healthy with whiter aesthetic appearance [10]. An aqueous extracts of *Salvadora persica L.* exhibits antibacterial activity against several oral bacteria such as *S. mutans, S. mitis,* and *S. aureus* [11].

Regarding phytotherapy, it is a science based on the treatment by the medicinal plants or herbs, therefore the synergistic interactions of plant extracts may have many advantages such as decrease undesirable effects, increase the efficiency and bioavailability of the free agents, as well the need of small dose of medicine can give an adequate therapeutic effect when compared to each synthetic material separately [12].

Furthermore, researchers found that green tea and *Salvadora persica L*. extracts contain ingredients that enhance intra oral health state and these compositions may be biologically and synergistically active more efficient when mixed together [13].

To our knowledge, this is the first study that investigates the synergistic effects of the green tea and *Salvadora persica L*. against the *Staphylococcus aureus* activity on heat-cured acrylic base soft liner. The present study hypothesize that this combination exhibits a synergistic anti-bacterial effect.

MATERIALS AND METHODS

Extraction of green tea

First, 100 g of green tea powder (Great British Tea Company, Sri Lanka) was placed in 1000 mL of non-ionized distilled water for the maceration process. The final mixing ratio was 1 g plant/10 mL solvent. Then, the mixture was left for 3 days at room temperature. Following which, the green tea aqueous extracts were filtered with a filter paper (JIAO JIE, China) to exclude coarse plant material and centrifuged for 10 min at 10000 rpm, then filtered again with a filter paper. The filtered extracts were dried by the hot oven (GCA Corp., USA) at 100°C to get a dry powder of extracts green tea. The green tea extracts powder was stored in a glass container at room temperature to be ready for the experiment.

Extraction of *Salvadora persica L.*

Firstly, *Salvadora persica L.* (TYBAH SEWAK, Saudi Arabia) root sticks were cut into small pieces (about 1 cm) using sharp scissors. Then, the pieces were broken down into smaller pieces and grounded into the fine particles by using a grinder machine.

Secondly, 100 g *Salvadora persica L.* fine powder was placed into 1000 mL of non-ionized distilled water for maceration process. The final mixing ratio was 1 g plant/10 mL solvent. Following which, the *Salvadora persica L.* aqueous extracts were filtered with a filter paper to exclude coarse plant particles, and centrifuged for 10 minutes at 10000 rpm then filtered again with a filter paper. The filtered extracts were dried by hot oven at 100°C to get *Salvadora persica L.* dry powder. The *Salvadora persica L.* extracts powder was stored in a glass container at room temperature to be ready for the experiment.

Isolation of *Staphylococcus aureus*

At Department of Educational Laboratories in the medical city of Baghdad and by using of sterile cotton swabs sixteen isolates of *S. aureus* were separated from 70 samples taken from patients' throat and saliva, then inoculated into blood agar (HiMedia Lab., India) (a media for *S. aureus* growth) which prepared according to the instructions listed by manufacturer. Then, agar plates contain *S. aureus* were incubated in an aerobic condition for 48 hours at 37°C.

Synergistic estimation of the green tea and *Salvadora persica L.* extracts mixture

Five test mixture combination of green tea (Gt) and *Salvadora persica L.* (Sp) aqueous extracts were prepared according to the minimal inhibitory concentration (MIC) values as follows:

- First, 4 MICgt was mixed with a concentration of 8.192 mg/mL and 4 MICsp with a concentration of 4.096 mg/mL.
- Second, 2 MICgt was mixed with a concentration of 4.096 mg/mL and 4 MICsp with a concentration of 4.096 mg/mL.
- Third, 4 MICgt was mixed with a concentration of 8.192 mg/mL and 2 MICsp with a concentration of 2.048 mg/mL.
- Fourth, 4 MICgt was mixed with a concentration of 8.192 mg/mL and 1 MICsp with a concentration of 1.024 mg/mL.
- Fifth, 1 MICgt was mixed with a concentration of 2.048 mg/mL and 4 MICsp with a concentration of 8.192 mg/mL.

Disc diffusion test

Pilot study: The aim of the pilot study was to estimate the synergistic effect of green tea and *Salvadora persica L*. mixture against bacterial activity of heat cured soft liner denture base material with minimum effect on other properties when compared to control group (without any addition). The pilot study was done using the disc diffusion method (three samples were selected for the test) with a concentration of 2.048 mg/mL of green tea and 1.024 mg/mL of *Salvadora persica L.*

Preparation of samples: Fifty plastic disc models with dimensions of 10 mm in diameter and 3 mm in thickness were prepared and invested into freshly mixed extra hard dental stone (Durux, Spain) to simulate the final shape of soft liner samples [14]. Heat cure soft lining materials (Vertex, Netherlands) was proportionate as directed by manufacturer instructions (1 mL of monomer/1.2 g of powder) and mixed with a combination of green tea and *Salvadora persica L.* to evaluate the antibacterial properties of soft liners denture base materials.

According to the results obtained from the pilot study, five groups of the tested samples (10 from each group) were prepared:

- First group, control samples which contain no additive material to monomer liquid.
- Second group, green tea samples were prepared by adding 2.048 mg/mL of aqueous extracts of green tea for each 1 mL of monomer.
- Third group, *Salvadora persica L.* samples were prepared by adding 1.024 mg/mL of aqueous extracts of *Salvadora persica L.* for each 1 mL of monomer.
- Fourth group, samples were prepared by adding an aqueous extracts mixture of 2.048 mg/mL of green tea and 1.024 mg/mL of *Salvadora persica L.* for each 1 mL of monomer.
- Fifth groups, samples were prepared by adding an aqueous extracts mixture of 1.024 mg/mL of green tea and 2.048 mg/mL of *Salvadora persica L.* for each 1 mL of monomer.

The mixture was placed in dry, clean, glass suitable container then mixed by probe sonication apparatus (Soniprep 150, England) which vibrated at 120 W and 60 KHz for 3 minutes to break them into small size crystals and to spread the materials to all monomer solution parts [15].

Preparation of Mueller-Hinton agar: According to the manufacturer's instructions, Mueller-Hinton agar (HiMedia Lab., India) was prepared by dissolving 38 g of the medium in 1000 mL of distilled water. Then, the solution was boiled for 1 min to obtain the entirely dissolve medium. Following which, the medium was autoclaved (Tuttnauer, USA) at 121°C for 15 min and cooled at room temperature, then pour the cooled Mueller-Hinton Agar into sterile Petri-dishes on a horizontal level surface to give uniform depth. The final pH was 7.3 ± 0.1 at 25°C. Finally, the plates were stored at 2°C-8°C.

Disc diffusion method: Kirby-Bauer method was used as recommended by World Health Organization [16] to determine anti-microbial effect of the green tea, *Salvadora persica L*. and synergistic combination of green tea and *Salvadora persica L.* after incorporation of each one into soft lining material. The bacterial suspension prepared by selected 1-2 isolated colonies of *S. aureus* from the incubated culture and placed into a test tube which contains 4 mL of normal saline to get a bacterial suspension of turbidity approximately equals to 1.5 × 108 CFU/mL.

Sterile cotton was utilized to swab a small portion of the bacterial suspension, carefully carried and evenly spread on Mueller Hinton Agar medium by streaking on the surface of the media, and then it was left for 10 min. Thereafter, with a sterile forceps the samples discs were inserted on the agar and pressed incorrect manner to ensure firm contact with the agar. Finally, the plates were incubated for 18 h-24 h at 37°C. The inhibition zone (the shortest distance from the outer margin of the well to the initial point of microbial growth) was measured by millimeter according to Clinical Laboratories Standards Institute using a metric ruler (CLSI, 2011), the results were recorded from the average of the two measurements.

Statistical analysis

The results of this research were analysed using SPSS (statistical package for social science–version 24) computer software. Descriptive statistics were made which include Means, Standard deviation and Graphical presentation by bar-chart. The homogeneity of variances was confirmed and also inferential statistics includes; one way ANOVA (analysis of variance) was used to compare means among all groups and Bonferroni multiple comparisons test was used to show the significance among different groups.

RESULTS

The results of disc diffusion method of 1 MIC green tea and 1 MIC *Salvadora persica L.* incorporated samples exhibited the highest mean value of the inhibition zone (19.90 mm), followed by 0.5 MIC green tea and 2 MIC *Salvadora persica L.* incorporated samples (18.55 mm) and *Salvadora persica L.* incorporated samples (11.70 mm), while the lowest value (4.35 mm) was obtained in green tea incorporated with heat cured soft lining samples as presented in Figure 1 and Table 1. Regarding the control group (without additives), no inhibition zone was obtained (Figure 2).

Table 1: Descriptive statistics analysis of disc diffusion method of experimental groups

					95% Confidence Interval for Mean	
Group	N	Mean (mm)	Std. Deviation	Std. Error	Lower Bound	Upper Bound
1	10	4.35	0.914	0.289	3.7	5
2	10	11.7	1.085	0.343	10.92	12.48

3	10	19.9	1.37	0.433	18.92	20.88
4	10	18.55	1.012	0.32	17.83	19.27

Where: Group 1: Green tea samples; Group 2: *Salvadora persica L.* samples; Group 3: 1 MIC green tea and 1 MIC *Salvadora persica L.* samples; Group 4: 0.5 MIC green tea and 2 MIC *Salvadora persica L.* samples

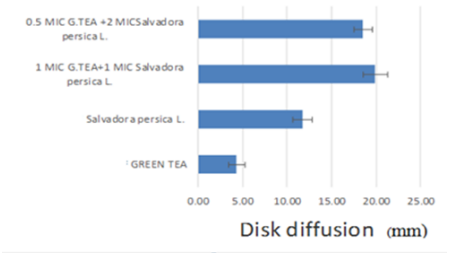

Figure 1: Mean values of disc diffusion method of the experimental groups

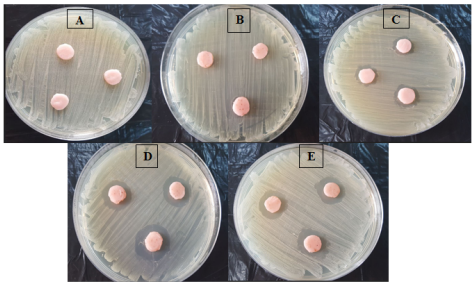

Figure 2: The result of disc diffusion method of (A) Control samples; (B) Green tea samples; (C) *Salvadora persica L.* samples; (D) 1 MIC green tea and 1 MIC *Salvadora persica L.* samples; (E) 0.5 MIC green tea and 2 MIC *Salvadora persica L.* samples

Test of homogeneity of variances showed non-significant difference among the experimental groups (P>0.05) (Table 2). One-way ANOVA-test was used to compare the mean values of disc diffusion of different groups, the results showed a highly significant difference among the experimental groups (P<0.001) (Table 3).

Bonferroni test used in multiple comparisons revealed a non-significant difference between group 3 and 4 (P=0.060), while high significant differences were observed among other groups (P<0.001) (Table 4).

Fourier transform infrared spectroscopy

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR tensor 27, Bruker, Germany) was employed to determine if there is any chemical reaction between soft denture liner and additives. The results of FTIR analysis demonstrates no difference in the spectra among acrylic soft lining samples and acrylic soft lining material with additives since the pattern and the alignment of the absorption peaks didn't show any change. This indicates that there is no chemical reaction occurred between green tea, *Salvadora persica L.,* green tea and *Salvadora persica L.* (synergistic mixture) with acrylic based soft lining material (Figure 3).

Table 2: Test of homogeneity of variances

Levene Statistic	*P-value	df2	*P value
0.754	71.473	36	0.527
*P<0.05 Highly Significant			

Table 3: One-way ANOVA-test to compare the mean value of disc diffusion of experimental groups

Source of variation	Sum of Squares	df	Mean Square	F-test	*P value
Between Groups	1533.625	3	511.208		
Within Groups	44.25	36	1.229	415.898	0
Total	1577.875	39			
*P<0.001 Highly Significant					

Table 4: Multiple comparisons between the mean values of disc diffusion method of experimental groups using Bonferroni test

Groups		Mean Difference	P value	Sig.
1	2	-7.35	0	HS
	3	-15.55	0	HS
	4	-14.2	0	HS
2	3	-8.2	0	HS

	4	-6.85	0	HS
3	4	1.35	0.06	NS
N.S: No statistically significant difference between groups at p>0.05				
HS: Highly significant difference between groups at p<0.001				

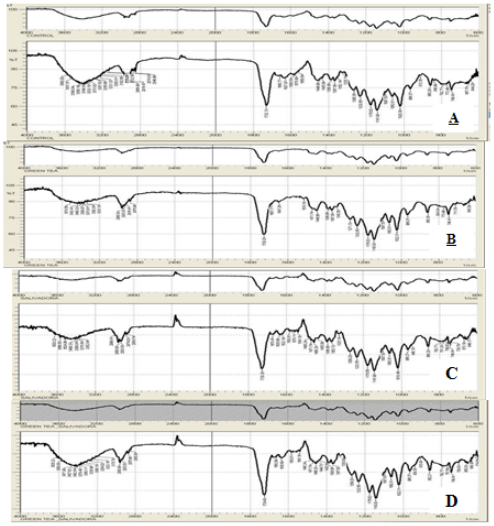

Figure 3: A) FTIR of acrylic soft lining material; B) FTIR of acrylic soft liner/green tea; C) FTIR of acrylic soft liner/*Salvadora persica L.*; D) FTIR of acrylic soft liner/*Salvadora persica L.* and green tea.

DISCUSSION

One of the most serious problems associated with the application of the soft lining materials is the microbial colonization (bacteria, fungi, and plaque) on the surface of the denture liners which end with infection and/or inflammation of the alveolar mucosa [17,18].

In this study, the result of disc diffusion test revealed a highly significant increase in the inhibition zone around heat cure soft liner samples incorporated with *Salvadora persica L.* extracts compared to samples incorporated with green tea extracts. This outcome may be attributed to the presence of phytochemical ingredients (i.e. saponins, cardiac glycosides, flavonoids, alkaloids, benzy lisothiocyanate, nitrate, sulphur, tannis, trimethylamine, salvadorine, various phenolic compounds, chloride, and steroids) in *Salvadora persica L.* extracts which considered as bioactive constituents with high anti-bacterial activity. This finding agrees with earlier studies by Akhtar et al. and Darout et al. [19,20]. On the other hand, saponins and alkaloids widely exist in *Salvadora persica L.* plants which contain anti-bacterial compounds may interfere with the microbial DNA [21].

Studies have shown that an aqueous extracts of green tea exhibited anti-bactericidal activity against *S. aureus* [22] and have a significant effect on inhibition multidrug-resistant *S. aureus* infections [23,24]. Furthermore, the result of disc diffusion test revealed a significant an increase in the inhibition zone around heat cure soft liner samples incorporated with green tea extracts compared to control samples and this due to phytochemical ingredients (i.e. saponins, glycosides, terpenoids, tannins, and flavonoids such as polyphenolic catechins) in green tea extracts which contain many bioactive constituents with high anti-bacterial activity [25].

In the present study, the disc diffusion observations of green tea incorporated soft lining samples agree with the previous study which revealed that Catechins ingredient had higher anti-bacterial activity against Gram-positive bacteria than that of Gram-negative bacteria because the outer membrane of Gram-positive bacteria consists of a thick peptidoglycan layer [26]. Another explanation of inhibition zone around green tea incorporated samples with may be due to epigallocatechin gallate activity which may initiate damage in bacterial gram positive cell wall or changing the cell osmotic pressure and interrupt cytoplasmic membrane of the bacteria leading to micro-molecules leakage [27,28].

In the previous study, the synergistic effect between green tea and *Salvadora persica L.* aqueous extracts was studied and found that this mixture revealed anti-bacterial activity against *S. mitis, S. sanguinis,* and *A. viscosus* which responsible for the primary colonization of dental plaque [29]. Synergistic effect of *Salvadora persica L*. and green tea aqueous extracts mixture showed high inhibition activity against *S. aureus* through increase the inhibition zone in disc inhibition technique. This synergistic effect may be attributed to ambitious inhibition effect of ingredients which found in both of *Salvadora persica L.* and green tea such as kaempferol, quercetin and tannic acid. These results agree with the study that kaempferol, quercetin and tannic acid have anti-biofilm activity against *S. aureus* which reduces infections also they have the ability to decrease haemolysis activity of *S. aureus* [30].

Although the mechanism of synergy is still deficient, but many researched were investigated the effect different herbs on many species of bacteria and discovered that different active phytocompounds may responsible for increase synergistic effect (synergistic enhancers) which have better antimicrobial effect rather than one herbs use separately.

CONCLUSION

Within the limitations of the present study; the following conclusions can be obtained:

1. An aqueous extracts mixture of *Salvadora persica L.* and green tea was used as a potent anti-bacterial herbal medicament and successfully incorporated into heat cured acrylic-based denture soft lining to obtain a material with a continuous natural drug

delivery system against *Staphylococcus aureus* bacteria growth.

2. The combination of 1 MIC green tea and 1 MIC *Salvadora persica L.* samples exhibited substantial synergistic anti-bacterial efficiency. Thus, this combination could be applied as a useful active agent for the development of oral health products.

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CONFLICT OF INTEREST

The authors declared no conflicts of interests.

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