Original Article

Comparison of Time-kill Assay to Evaluate the Antimicrobial Efficacy of Garlic (*Allium sativum*) and Guava (*Psidium guajava*) Extracts on Periodontal Pathogens

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Abstract

Background: The role of Gram-negative anaerobic periodontal pathogens in periodontal diseases has led to the loss of tooth-supporting structures. These diseases can be prevented by the inhibition of bacterial biofilm on the tooth surfaces. Many treatment modalities have been tried to prevent periodontal diseases. With the rise in resistance to synthetic antimicrobials, there is a requirement to develop natural antimicrobials for the control of periodontitis. **Aim:** The aim of the study was to evaluate and compare the efficacy of garlic (*Allium sativum*) and guava (*Psidium guajava*) extracts on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* using time-kill assay. **Materials and Methods:** Aqueous garlic extract (AGaE), ethanolic garlic extract (EGaE), aqueous guava extract (AGuE), and ethanolic guava extract (EGuE) were prepared. Time-kill assays were performed on *P. gingivalis* and *A. actinomycetemcomitans.* The aqueous and ethanolic extracts of guava and garlic were compared to assess the maximum bactericidal potency. **Results:** The comparison of time-kill assay of AGaE and AGuE on *P. gingivalis* showed a statistically significant difference at 2 h ($t = 5.29$, $P < 0.01$), 4 h ($t = -4.867$, $P < 0.01$), and 6 h ($t = -3.647$, $P < 0.001$). The comparison of time-kill assay of EGaE and EGuE on *A. actinomycetemcomitans* showed a statistically significant difference at 2 h ($t = 4.54$, $P < 0.01$) and highly significant difference at 4 h ($t = 6.57$, $P < 0.001$). **Conclusions:** The, judicious use of these phytomedicinal products could be cost-effective and also the adverse effects caused due to the long-term usage of synthetic antimicrobials can be avoided.

Keywords: *Aggregatibacter actinomycetemcomitans, Allium sativum, antimicrobial, garlic, guava, Porphyromonas gingivalis, Psidium guajava, time-kill assay*

Introduction

The role of Gram-negative anaerobic periodontal pathogens in periodontal diseases has been well-documented.[1] The Gram-negative anaerobes mainly *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are found to be linked with the onset and/or development of periodontitis. These organisms express a number of virulence factors which have been implicated in causing periodontal attachment loss.[2] Among these virulence factors, cysteine proteases including arg-gingipains play a major role in *P. gingivalis* virulence by degrading the host tissue and activating the host pro-inflammatory mediators, thus neutralizing the host immune systems.[3] Leukotoxin of *A. actinomycetemcomitans* is one of the major endotoxins causing periodontal disease.[4] Long-standing exposure of the periodontal tissues to these microbial toxins results in the loss of supporting structures such as periodontal ligament and alveolar bone, ultimately leading to the loss of teeth.[5]

Developing a better diagnosis and a cost-effective way of curing periodontal disease is required. These diseases can be prevented by the inhibition of bacterial biofilm on the tooth surfaces. This includes the mechanical debridement and the use of antimicrobial agents.[6]

Various researches have shown the inhibitory effect of various antimicrobial agents on the oral biofilm. Nevertheless, these antimicrobials might have some ill effects such as staining of teeth, gastrointestinal disturbance, and risk of developing

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antibacterial resistance. Hence, a naturally available herbal antimicrobial that interferes with the development of dental plaque is the need of the hour.[7]

Garlic (*Allium sativum*) is considered as an ancient medicine and known to have antimicrobial properties.[8,9] It has been observed that the resistance to allicin that is one of the active compounds in garlic is thousand times less compared to certain synthetic antimicrobials.[10] Garlic exhibits antimicrobial effect against a variety of oral microorganisms, including Gram-negative periodontal pathogens.[11]

Psidium guajava is a phytotherapic plant commonly known as guava. Guava has been demonstrated for its antimicrobial, antiparasitic, antioxidant, antigenotoxic, anticancer, and antihyperglycemic effects.[12] The leaves of guava have been reported to be used for the maintenance of oral hygiene.[13] The antibacterial activity of guava extract against cariogenic bacteria *Lactobacillus acidophilus* is reported to be similar to that of chlorhexidine mouthrinse.[14]

Even though guava and garlic are naturally available medicinal plants with proven antimicrobial property, the literature on its effect on periodontal pathogens and their virulence factors and enzymes are scanty. Very few studies have evaluated the efficacy of garlic and guava as phytomedicines against periodontal pathogens.[11,15-17] However, there is no literature found regarding comparison of the effect of garlic and guava on oral Gram-negative microbes. Thus, the present study aimed to evaluate and compare the efficacy of guava (*P. guajava*) and garlic (*A. sativum*) on *P. gingivalis* and *A. actinomycetemcomitans* using time-kill assay.

Materials and Methods

Preparation of guava and garlic extract

Aqueous garlic extract (AGaE), ethanolic garlic extract (EGaE), aqueous guava extract (AGuE), and ethanolic guava extract (EGuE) were prepared equivalent to the previously reported studies in the literature.[15,16]

Microbes and growth condition

Periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* were utilized from the stock culture for the present study. Kanamycin blood agar was used to isolate and Oxoid anaerobic jar was used for cultivating *P. gingivalis.* Dentaid agar was used to isolate and candle jar technique was used to cultivate *A. actinomycetemcomitans*.[15,16]

Inoculum preparations

The colonies were transferred to the brain heart infusion (BHI) broth with a sterile straight wire. The turbidity of the suspensions of the bacteria was calibrated with a photometric device to 0.5 McFarland turbidity standards.

Growth kill assay

Serial dilutions of garlic/guava (test extracts) were made to estimate the time-kill assay. A set of 10 tubes were taken and numbered from 1 to 10. One milliliter of the extract to be tested was taken in the first tube. BHI broth (0.5 ml) was added to the remaining tubes numbered from 2 to 10. 0.5 ml of the first tube extract was transferred to the second tube which consisted of BHI broth and it was mixed thoroughly. 0.5 ml from the second tube was serially transferred to the third tube until the 9th tube. 0.5 ml was discarded from the 9th tube and the 10th tube, i.e., the last tube, acted as a control [Figure 1].

The solutions of garlic extract were thus serially diluted and concentrations at 500, 250, 125, 62.5, 31.25, 16.6, 8.3, 4, and 2 mg/ml for EGaE and concentrations at 500, 250, 125, 62.5, 31.25, 16.6, 8.3, 4, and 2 µl/ml for AGaE were obtained. Similarly, the guava extract solutions were serial diluted and concentrations at 500, 250, 125, 62.5, 31.25, 16.6, 8.3, 4, and 2 mg/ml for EGuE and concentrations at 500, 250, 125, 62.5, 31.25, 16.6, 8.3, 4, and 2 µl/ml for AGuE were obtained. 0.1 ml of culture cells (10^7 cells) were then inoculated into the tube containing test extract. Then directly, it was plated and colonies were observed at 0 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the extract that completely inhibited the growth of the organisms.

The anaerobic jar for *P. gingivalis* and CO_2 jar for *A. actinomycetemcomitans* were used for culturing. At the end of 2 h again, the first tube was plated. The same procedure was repeated after every 2 h, i.e., after 4 h, 6 h, and 24 h. Then, the plates were incubated in either CO_2 jar or anaerobic jar as per the requirement. After 48 h of incubation at 37°C, the plates were taken out and the colonies were calculated.

Statistical analysis

One-way ANOVA was applied to analyze the significance of difference of colonies at various time intervals. *Post hoc* Bonferronis test was used to determine the pairwise significance of difference in the means of colony formed if the differences across the time intervals were statistically significant. The comparisons of the extracts were analyzed by unpaired *t*-test. All the analyses were carried out using SPSS version 20.0 (SPSS Version 20.0. Armonk, NY: IBM Corp.) and the statistical significance was tested at a 5% level.

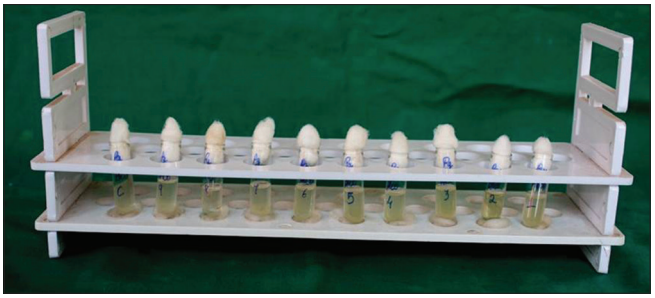

Figure 1: Serial dilutions performed for time-kill assay

Results

Minimal inhibitory concentration of garlic and guava extracts

The AGaE exhibited MIC at 16.6 µl/ml and EGaE exhibited MIC at 62.5 mg/ml on *P. gingivalis.* The MIC for the AGaE aqueous extract was determined at 62.5 µl/ml on *A. actinomycetemcomitans*, whereas *A. actinomycetemcomitans* was completely resistant to all the concentrations of the EGaEs [Table 1].

The AGuE exhibited MIC at 4 µl/ml, whereas EGuE exhibited MIC at 2 µl/ml for *P. gingivalis. A. actinomycetemcomitans* showed lesser resistance to ethanolic extracts. The MIC for the AGuE was determined at 16.6 µl/ml, whereas *A. actinomycetemcomitans* was completely susceptible to all the concentrations of the EGuE [Table 1].

Time-kill assay of garlic extracts

The garlic extracts exhibited only bacteriostatic activity for both the organisms. Bactericidal effect on *P. gingivalis* was not evident over the first 2 h of incubation; however, bacteriostatic activity was noticed between 2 and 6 h. The aqueous extract showed greater bacteriostatic activity when compared to the ethanolic extract, followed by a gradual increase in the colony-forming units and bacteriostatic activity was not observed at 24 h [Table 2 and Figure 2]. The control tube showed no drop in colony count during the same period.

The *A. actinomycetemcomitans* showed resistance to the bactericidal activity of the garlic extracts. Bacteriostatic activity was noticed between 0 and 2 h incubation period for aqueous and ethanolic extracts. Later on, a gradual increase in the colonies was observed up to 24 h [Table 3 and Figure 3].

Time-kill assay of AGaE and EGaE was compared for both the microbes used in the study [Tables 2 and 3]. The comparisons analyzed by unpaired *t*-test showed statistical significance with *P. gingivalis* at 2 h ($t = -9.205$, $P < 0.001$), 4 h ($t = -8.962$, $P < 0.001$), and 6 h ($t = -7.046$, $P < 0.001$).

However, statistical significance was seen only at the beginning at 0 h between AGaE and EGaE on *A. actinomycetemcomitans.*

Time-kill assay of guava extracts

A statistically significant decrease in the colonies of *P. gingivalis* was seen up to 6 h in both AGuE and EGuE. Bacteriostatic activity was seen during 4–6 h in case of both the type of guava extracts where there was no statistically significant difference in the colonies count from 4 to 6 h. There was a raise in the colony-forming units with no bacteriostatic activity observed at 24 h [Table 4 and Figure 4].

Table 1: Minimal inhibitory concentration of garlic and guava extracts

	500	250	125	62.5	31.25	16.6	8.3	4	2	Control
MIC of garlic on *P. gingivalis*										
AGaE	S	S	S	S	S	R	R	R	R	R
EGaE	S	S	S	R	R	R	R	R	R	R
MIC of garlic on *A. actinomycetemcomitans*										
AGaE	S	S	S	R	R	R	R	R	R	R
EGaE	R	R	R	R	R	R	R	R	R	R
MIC of guava on *P. gingivalis*										
AGuE	S	S	S	S	S	S	S	R	R	R
EGuE	S	S	S	S	S	S	S	S	R	R
MIC of Guava on *A. actinomycetemcomitans*										
AGuE	S	S	S	S	S	R	R	R	R	R
EGuE	S	S	S	S	S	S	S	S	S	R

S: Susceptible; R: Resistant; MIC: Minimal inhibitory concentration; AGaE: Aqueous garlic extract; EGaE: Ethanolic garlic extract; AGuE: Aqueous guava extract; EGuE: Ethanolic guava extract; *P. gingivalis: Porphyromonas gingivalis, A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans*

Table 2: Time-kill assay for *Porphyromonas gingivalis* by garlic extracts

Extracts	Time (h)					Significance	
	0	2	4	6	24	*F*	*P*
AGaE	130.8	70.6	111.4	112.0	500	843.84	<0.001*
EGaE	141.4	177.6	199.8	177.4	500	1031.58	<0.001*
t	−0.69	−9.20	−8.96	−7.04			
P	0.50	<0.001**	<0.001**	<0.001**	NS		

Post hoc Bonferronis test: AGaE 0 h versus 4 h and 6 h; 2 h versus 4 h and 6 h; 4 h versus 6 h – Nonsignificant. EGaE 0 h versus 2 h, 2 h versus 4 h and 6 h; 4 h versus 6 h – Nonsignificant. *Significant, **Highly significant; NS: Nonsignificant, AGaE: Aqueous garlic extract, EGaE: Ethanolic garlic extract

Table 3: Time-kill assay for *Aggregatibacter actinomycetemcomitans* by garlic extracts

Extracts	Time (h)					Significance	
	0	2	4	6	24	*F*	*P*
AGaE	73.6	38.4	47.6	68.4	500	419.29	<0.001**
EGaE	215.2	58.6	60.6	56.4	500	91.11	<0.001**
t	−3.02	−2.12	−1.07	0.72			
P	<0.05*	0.06	0.31	0.48	NS		

Post hoc Bonferronis test: AGaE 0 h versus 2 h, 4 h, and 6 h; 2 h versus 4 h and 6 h; 4 h versus 6 h – Nonsignificant. EGaE 0 h versus 2 h, 4 h,6 h, and 24 h, 2 h versus 4 h and 6 h; 4 h versus 6 h – Nonsignificant. *Significant, **Highly significant. NS: Nonsignificant, AGaE: Aqueous garlic extract; EGaE: Ethanolic garlic extract

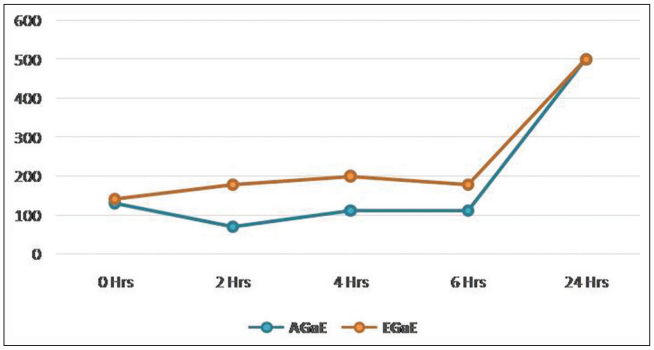

Figure 2: Time-kill assay for *Porphyromonas gingivalis* by garlic extracts

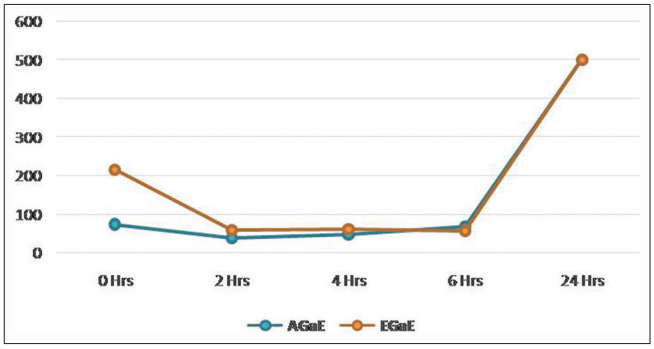

Figure 3: Time-kill assay for *Aggregatibacter actinomycetemcomitans* by garlic extracts

The *A. actinomycetemcomitans* colonies count decreased statistically up to 4 h with aqueous extract and there was an increase in the colony-forming units with no bacteriostatic activity at 6 h and 24 h. However, with ethanolic extract, there was a statistically significant decrease in the colonies count up to 6 h with no significant difference from 2 h to 6 h, indicating bacteriostatic activity between 2 and 6 h [Table 5 and Figure 5]. Control cell suspensions without guava extract showed no drop in viability over the same period.

Time-kill assay of AGuE and EGuE was compared for *P. gingivalis* and *A. actinomycetemcomitans* [Tables 4 and 5]. The comparisons analyzed by unpaired *t*-test showed statistical significance with *P. gingivalis* at 0 h (t = 6.485, $P < 0.001$), 2 h (t = 19.901, $P < 0.001$), 4 h (t = 10.346, $P < 0.001$), and 6 h (t = 11.926, $P < 0.001$) and with *A. actinomycetemcomitans* at 2 h (t = 2.701, $P < 0.05$), 4 h (t = −10.1, $P < 0.001$), and 6 h (t = 10.42, $P < 0.001$).

Comparison of aqueous extracts of guava and garlic on *Porphyromonas gingivalis*

Time-kill assay of AGuE and AGaE was compared for *P. gingivalis*. The comparisons showed statistically significant difference at 2 h (t = 5.29, $P < 0.01$), 4 h (t = −4.867, $P < 0.01$), and 6 h (t = −3.647, $P < 0.001$) [Table 6].

Comparison of ethanolic extracts of guava and garlic on *Porphyromonas gingivalis*

Time-kill assay of EGuE and EGaE was compared for *P. gingivalis*. The comparisons showed statistically high significant difference at 2 h (t = −32.85, $P < 0.001$), 4 h (t = −12.45, $P < 0.001$), and 6 h (t = −11.95, $P < 0.001$) [Table 7].

Comparison of aqueous extracts of guava and garlic on *Aggregatibacter actinomycetemcomitans*

Time-kill assay of AGuE and AGaE was compared for *A. actinomycetemcomitans*. The comparisons showed statistically high significant difference at 2 h (t = 9.62, $P < 0.01$) and significant difference at 0 h (t = 3.64, $P < 0.01$) and 6 h (t = 4.59, $P < 0.01$) [Table 8].

Comparison of ethanolic extracts of guava and garlic on *Aggregatibacter actinomycetemcomitans*

Time-kill assay of EGuE and EGaE was compared for *A. actinomycetemcomitans*. The comparisons showed statistically significant difference at 2 h (t = 4.54, $P < 0.01$) and highly significant difference at 4 h (t = 6.57, $P < 0.001$) [Table 9].

Discussion

Periodontal disease is known as an immune modulatory disease which results in the destruction of periodontal tissue, loss of attachment, and alveolar bone resorption. *P. gingivalis* and *A. actinomycetemcomitans* are known to be associated with periodontitis.[18] *P. gingivalis* is one of the most important bacteria causing periodontal disease, and it can colonize in the subgingival area, which may begin the process of periodontal disease, thereby activating other Gram-negative bacteria species to colonize and further infect periodontal tissue. Inhibition or elimination of the subgingival periodontopathogens like *P. gingivalis* and *A. actinomycetemcomitans* is the key for the successful treatment of periodontitis.[18-20]

Table 4: Time-kill assay for *Porphyromonas gingivalis* by guava extracts

Extracts	Time (h)					Significance	
	0	2	4	6	24	*F*	*P*
AGuE	142.2	131.4	102.4	99.6	500	71318.96	<0.001*
EGuE	126.0	95.8	74.6	70.0	500	28879.90	<0.001*
T	6.48	19.90	10.34	11.92			
P	<0.001**	<0.001**	<0.001**	<0.001**	NS		

Post hoc Bonferronis test: Both AGuE and EGuE for 4 h versus 6 h – NS. *Significant, NS: Nonsignificant; AGuE: Aqueous guava extract; EGuE: Ethanolic guava extract; **Highly Significant

Table 5: Time-kill assay for *Aggregatibacter actinomycetemcomitans* by guava extracts

Extracts	Time (h)					Significance*	
	0	2	4	6	24	*F*	*P*
AGuE	132.0	98.2	72.4	101.6	500	85394.20	<0.001*
EGuE	128.2	92.6	86.4	84.4	500	23372.97	<0.001*
T	1.182	2.701	−10.1	10.42			
P	0.271	<0.05*	<0.001**	<0.001**	NS		

Post hoc Bonferronis test: AGuE 2 h versus 6 h NS. EGuE 2 h versus 4 h, 2 h versus 6 h, 4 h versus 6 h NS. *Significant. NS: Nonsignificant; AGuE: Aqueous guava extract; EGuE: Ethanolic guava extract; **Highly Significant

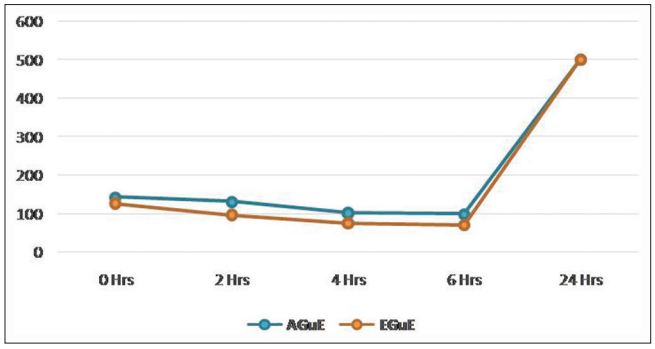

Figure 4: Time-kill assay for *Porphyromonas gingivalis* by guava extracts

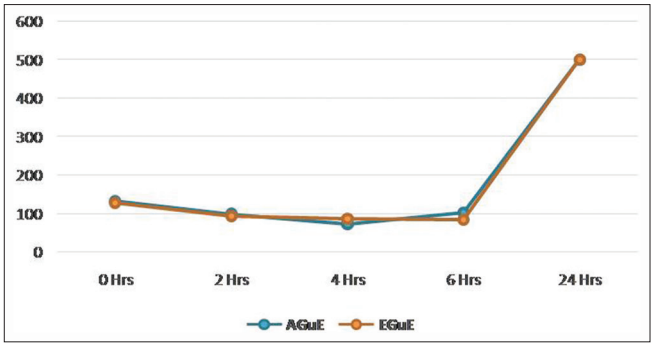

Figure 5: Time-kill assay for *Aggregatibacter actinomycetemcomitans* by guava extracts

In the present study, the inhibitory effect of aqueous and ethanolic extracts of garlic and guava on *P. gingivalis* and *A. actinomycetemcomitans* was evaluated using the time-kill assay. The efficacy of aqueous extracts of guava was compared with that of garlic; similarly, the ethanolic extracts of guava were compared with that of garlic.

Time-kill assay of AGaE and EGaE was compared for both the microorganisms revealed statistical significance for *P. gingivalis* at 2 h, 4 h, and 6 h. However, statistical significance was seen only at the beginning at 0 h between aqueous and EGaEs on *A. actinomycetemcomitans.*

Thus, garlic extract elicited its antimicrobial activity in a time-dependent manner exhibiting distinct time-kill assay, suggesting differences in the growth inhibitory response in tested isolates to it. Similar responses were observed by Yin *et al*.[21] and Iwalokun *et al*.[22] However, the exceptionality of time-kill assay on Gram-negative pathogens in the present study may be due to the structural variability between these two microorganisms.

The *A. actinomycetemcomitans* colonies count decreased statistically up to 4 h with aqueous extract and there was a raise in the colony-forming units with no bacteriostatic activity at 6 h and 24 h. However, with ethanolic extract, there was statistically significant decrease in the colonies count up to 6 h with no significant difference from 2 h to 6 h, indicating bacteriostatic activity between 2 and 6 h. Time-kill assay of AGuE and EGuE was compared for both the microorganisms. The comparisons revealed statistically significant results for *P. gingivalis* at 0 h, 2 h, 4 h, and 6 h, whereas *A. actinomycetemcomitans* showed statistically significant difference at 2 h, 4 h, and 6 h.

These observations interpreted that the guava extract showed its antimicrobial activity in a time-dependent fashion producing distinct time-kill assay, suggestive of differences in the growth inhibitory response of the tested microorganisms to guava extracts.

The guava extracts showed a better time-kill profile on *A. actinomycetemcomitans* compared to garlic extracts. Similar findings were reported by Kwamin *et al*.[4] stating that extracts of guava leaves and twigs contain components that efficiently neutralize the leukotoxicity of *A. actinomycetemcomitans.* Toma and Genet[23] showed

Table 6: Comparison of aqueous extracts of guava and garlic on *Porphyromonas gingivalis*

Micro-organism	Time	Extract	*n*	Mean±SD	Significance	
					t	*P*
P. gingivalis	0 h	AGuE	5	142.2±2.2	0.88	0.401
		AGaE	5	130.8±28.6		
	2 h	AGuE	5	131.4±1.9	5.29	<0.01*
		AGaE	5	70.6±25.6		
	4 h	AGuE	5	102.4±3.2	−4.86	<0.01*
		AGaE	5	111.4±2.5		
	6 h	AGuE	5	99.6±2.9	−3.64	<0.01*
		AGaE	5	112.0±7.0		
	24 h	AGuE	5	500.0±0.0	0.00	NS
		AGaE	5	500.0±0.0		

*Significant. NS: Nonsignificant; AGaE: Aqueous garlic extract; AGuE: Aqueous guava extract; SD: Standard deviation; *P. gingivalis: Porphyromonas gingivalis*

Table 7: Comparison of ethanolic extracts of guava and garlic on *Porphyromonas gingivalis*

Micro-organism	Time	Extract	*n*	Mean±SD	Significance	
					t	*P*
P. gingivalis	0 h	EGuE	5	126.0±5.0	−1.76	0.116
		EGaE	5	141.4±18.8		
	2 h	EGuE	5	95.8±3.4	−32.85	<0.001**
		EGaE	5	177.6±4.3		
	4 h	EGuE	5	74.6±5.0	−12.45	<0.001**
		EGaE	5	199.8±21.9		
	6 h	EGuE	5	70.0±4.6	−11.95	<0.001**
		EGaE	5	177.4±19.5		
	24 h	EGuE	5	500.0±0.0	0.00	NS
		EGaE	5	500.0±0.0		

**Highly significant. NS: Nonsignificant; EGuE: Ethanolic guava extract; EGaE: Ethanolic garlic extract; SD: Standard deviation; *P. gingivalis: Porphyromonas gingivalis*

that this leukotoxin-neutralizing activity of guava extract is stable and persist for at least 24 h; probably, this would have been one of the reasons for guava extract showing better time-kill profile on *A. actinomycetemcomitans* than garlic. However, in the present study, the time-kill potency of *A. actinomycetemcomitans* was between 2 and 6 h.

In the present study, the AGaE was more potent than the ethanolic extract, similar to observations of Roy *et al*.,[24] Jaber *et al*.,[25] and El-Mahmood and Amey[26] but in contrast with that of Debnath.[27] One of the probable explanations for this could be the evaporation of volatile components of EGaE when it was heated at 800°C.

The efficacy of ethanolic guava leaf extract was found to be better than aqueous guava leaf extract. Ethanolic extract contains tannins as well as flavonoids, whereas aqueous extract contains tannins but not flavonoids. This difference in composition of ethanolic and aqueous extract can be attributed to the difference in the solubility of various components of guava leaves in water and organic solvents.[28]

Table 8: Comparison of aqueous extracts of guava and garlic on *Aggregatibacter actinomycetemcomitans*

Micro-organism	Time	Extract	*n*	Mean±SD	Significance	
					t	*P*
A. actinomycetem comitans	0 h	AGuE	5	132.0±2.4	3.64	<0.01*
		AGaE	5	73.6±35.7		
	2 h	AGuE	5	98.2±2.8	9.62	<0.001**
		AGaE	5	38.4±13.5		
	4 h	AGuE	5	72.4±2.0	2.14	0.097
		AGaE	5	47.6±25.7		
	6 h	AGuE	5	101.6±2.6	4.59	<0.01*
		AGaE	5	68.4±15.9		
	24 h	AGuE	5	500.0±0.0	0.00	NS
		AGaE	5	500.0±0.0		

*Significant, **Highly significant. NS: Nonsignificant; SD: Standard deviation; *A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans*

Table 9: Comparison of ethanolic extracts of guava and garlic on *Aggregatibacter actinomycetemcomitans*

Micro-organism	Time	Extract	*n*	Mean±SD	Significance	
					t	*P*
A. actinomycetem comitans	0 h	EGuE	5	128.2±6.7	−1.97	0.084
		EGaE	5	215.2±98.2		
	2 h	EGuE	5	92.6±3.6	4.54	<0.01*
		EGaE	5	58.6±16.3		
	4 h	EGuE	5	86.4±2.3	6.57	<0.001**
		EGaE	5	60.6±8.4		
	6 h	EGuE	5	84.4±2.6	1.87	0.098
		EGaE	5	56.4±33.3		
	24 h	EGuE	5	500.0±0.0	0.00	NS
		EGaE	5	500.0±0.0		

*Significant, **Highly significant. NS: Nonsignificant; EGuE: Ethanolic guava extract; EGaE: Ethanolic garlic extract; *A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans*

Thus, AGaE showed significant antimicrobial activity against *P. gingivalis* and EGuE showed maximum bactericidal activity on *A. actinomycetemcomitans*. However, the combination of both garlic and guava extract has to be tried out in future. Further studies and clinical trials need to be undertaken to explore the efficacy of guava and garlic in humans. Mouthwashes can be prepared and the effects can be compared with the synthetic mouthwashes available in the market. Combination of guava and garlic extract should be tried and evaluated. Garlic and guava extracts could be used as mouthwashes, local drug delivery agents in the form of gel; chip and threads could treat and manage both localized and generalized periodontitis. Hence, using these phytomedicinal extracts as an adjunct with surgical and nonsurgical therapy might result in the elimination of periodontal pathogens.

Limitations of the study

This present study is an *in-vitro* microbiological study; there still lies a void in research with respect to the clinical trials of these phytomedicinal agents in periodontal diseases. A second limitation is regarding the therapeutic usefulness of these phytomedicinal extracts that the constituents of garlic/guava might form a complex with blood proteins and its efficacy in the presence of bleeding at a periodontal site was not evaluated. Future targeted long-term clinical trials of these phytomedicinal remedies are required.

Conclusions

Garlic and guava extract displayed a significant antimicrobial effect on *P. gingivalis* and *A. actinomycetemcomitans*. Garlic was found to be most effective against *P. gingivalis*, whereas guava showed the highest efficacy on *A. actinomycetemcomitans*. Time-kill assay results revealed probable use of garlic and guava as a suitable adjuvant to synthetic antimicrobials. Thus, judicious use of these naturally occurring phytomedicinal products could be cost-effective and also the adverse effects caused due to the long-term usage of synthetic antimicrobials can be avoided.

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Conflicts of interest

There are no conflicts of interest.

References

1. Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: Virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984;63:412-21.
2. Holt SC, Bramanti TE. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit Rev Oral Biol Med 1991;2:177-281.
3. Kuramitsu HK, Yoneda M, Madden T. Proteases and collagenases of *Porphyromonas gingivalis*. Adv Dent Res 1995;9:37-40.
4. Kwamin F, Gref R, Haubek D, Johansson A. Interactions of extracts from selected chewing stick sources with *Aggregatibacter actinomycetemcomitans*. BMC Res Notes 2012;5:203.
5. Laine ML, Crielaard W, Loos BG. Genetic susceptibility to periodontitis. Periodontol 2000 2012;58:37-68.
6. Slots J. Selection of antimicrobial agents in periodontal therapy. J Periodontal Res 2002;37:389-98.
7. Sharma S, Prakash S. To detect the minimum inhibitory concentration and time-kill curve of shiitake mushroom on periodontal pathogens: An *in vitro* study. J Indian Soc Periodontol 2019;23:216-9.
8. Block E. The chemistry of garlic and onions. Sci Am 1985;252:114-9.
9. Ankri S, Mirelman D. Antimicrobial properties of allicin from garlic. Microbes Infect 1999;1:125-9.
10. Gupta KC, Viswanthan R. Combined action of streptomycin and chloramphenicol with plant antibiotics against tubercle bacilli. I. Streptomycin and chloramphenical with cepharanthine. II. Streptomycin and allicin. Antibiot Chemother 1995;5:24-7.
11. Bakri IM, Douglas CW. Inhibitory effect of garlic extract on oral bacteria. Arch Oral Biol 2005;50:645-51.
12. Gupta GK, Chahal J, Arora D. *Psidium guajava* Linn: Current research and future prospects. J Pharm Res 2011;4:42-6.
13. Jebashree HS, Kingsley SJ, Sathish ES, Devapriya D. Antimicrobial activity of few medicinal plants against clinically isolated human cariogenic pathogens – An *in vitro* study. ISRN Dent 2011;2011:541421.
14. Jain D, Dasar P, Nagarajappa S, Kumar S, Airen B, Warhekar S. *In vitro* activity of ethanolic and water extract of guava leaves at various concentrations against *Lactobacillus acidophilus*. J Indian Assoc Public Health Dent 2014;12:232-6.
15. Shetty S, Thomas B, Shetty V, Bhandary R, Shetty RM. An *in-vitro* evaluation of the efficacy of garlic extract as an antimicrobial agent on periodontal pathogens: A microbiological study. Ayu 2013;34:445-51.
16. Shetty YS, Shankarapillai R, Vivekanandan G, Shetty RM, Reddy CS, Reddy H, *et al*. Evaluation of the efficacy of guava extract as an antimicrobial agent on periodontal pathogens. J Contemp Dent Pract 2018;19:690-7.
17. Shetty S, Shetty RM, Rahman B, Vannala V, Desai V, Shetty SR. Efficacy of *Psidium guajava* and *Allium sativum* extracts as antimicrobial agents against periodontal pathogens. J Pharm Bioall Sci 2020;12:S589-94.
18. Ezzo PJ, Cutler CW. Microorganisms as risk indicators for periodontal disease. Periodontol 2000 2003;32:24-35.
19. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. Nat Rev Microbiol 2012;10:717-25.
20. Cohen J. The immunopathogenesis of sepsis. Nature 2002;420:885-91.
21. Yin MC, Chang HC, Tsao SM. Inhibitory effects of aqueous garlic extract, garlic oil and four diallyl sulphides against four enteric pathogens. J Food Drug Anal 2002;10:120-6.
22. Iwalokun BA, Ogunledun A, Ogbolu DO, Bamiro SB, Jimi-Omojola J. *In vitro* antimicrobial properties of aqueous garlic extract against multi drug resistant bacteria and candida species from Nigeria. J Med Food 2004;7:327-33.
23. Toma C, Genet K. The Interaction between Guava-extract and *Aggregatibacter actinomycetemcomitans* Leukotoxin [Internet] [Dissertation]. 2014. Available from: http://urn.kb.se/resolve?urn=urn:nbn:se:umu:diva-102414. [Last accessed on 2020 Oct 22].
24. Roy J, Shakaya DM, Callery PS, Thomas JG. Chemical constituents and antimicrobial activity of a traditional herbal medicine containing garlic and black cumen. Afr J Trad 2006;3:1-7.
25. Jaber MA, Al-Mossawi A. Susceptibility of some multiple resistant bacteria to garlic extract. Afr J Biotechnol 2007;6:771-6.
26. El-Mahmood AM, Amey JM. *In vitro* antibacterial activity of *Parkia biglobosa* (Jacq) root bark extract against some microorganisms associated with urinary infections. Afr J Biotechnol 2007;6:1272-5.
27. Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Steria rebaudiana*. J Med Plants Res 2005;2:48-58.
28. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564-82.