



Antimicrobial efficacy of grape seed extract in terminating the ramifications of plaque microorganisms: a randomized control study

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Abstract

Background. Grape seed extract contains Proanthocyanin, which reduces collagen degradation by inhibiting interstitial and extracellular collagenase, thus having the potential to reduce the progression of periodontitis. Here we compare and evaluate the aerobic and anaerobic microorganism's CFUs (colony forming units) in plaque samples of Group A, Group B, Group C and severity of periodontal disease on day 0 and 7.

Methods. Forty-five subjects in age range 18-30 years were selected among undergraduate students and randomly divided into Group A: 15, 2% grape seed extract mouthwash (GSE), Group B: 15, 0.2% chlorhexidine mouthwash (CHX) and Group C: 15, distilled water (control). The supragingival plaque was collected into transport media. Kruskal Wallis test followed by Mann Whitney test was used to compare the mean CFUs ($\times 10^3$) of microorganisms and severity of periodontal disease was compared, by clinical parameters among all groups on day 0 and 7.

Results. There was a significant difference concerning mean scores of all clinical parameters ($P < 0.001$) and mean CFUs of microorganisms between 3 study groups ($P = 0.005$) at 7 days post-intervention period. Intragroup comparison, mean scores were significantly reduced on day 7 as compared to day 0 ($P < 0.001$) in Group A and B, but no significant difference was noted with Group C.

Conclusion. Intervention with GSE mouthwash showed a positive effect on reducing CFUs in the plaque when compared with the control group. GSE group also showed similar results in reducing CFUs in plaque when compared to CHX group, thereby demonstrating the agent's antimicrobial efficacy, therapeutic effect and its potential usefulness in controlling plaque and periodontal diseases.

Keywords: grape seed extract, colony forming unit, plaque, randomized control study, mouthwash

Introduction

Dental plaque biofilm majorly consists of microorganisms encased within an extracellular matrix [1]. Its pathogenicity is governed by a dynamic, constantly changing equilibrium between the oral microbiota and numerous elements that differentially foster or impede the survival of its microbial contents. About 1000 different bacterial species are identified in the dental biofilm by modern molecular

biological techniques, which are twice as many as can be cultured [2].

Periodontitis is a chronic multifaceted inflammatory disease linked with dysbiotic plaque biofilms and characterized by the continuous dismantling of the tooth-supporting structures [3]. A few of the microorganisms detected in periodontal diseases are *Porphyromonas gingivalis*, *Streptococcus species*, *Aggregatibacter actinomycetemcomitans*, etc [2].

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Therefore we need a potent agent that reduces periodontal disease causing organisms, thus preventing the incidence and prevalence of plaque microorganisms in the oral cavity.

Based on potential problems associated with the extended use of systemic antibiotics for managing dental plaque [4], an immense range of studies have been directed towards topical antimicrobial agents these days. Mouthwashes have the potentiality to provide therapeutic ingredients and its benefits to all accessible surfaces in the mouth, including inter-proximal hard and soft tissue, and based on their composition, remain active for enhanced periods. But in clinical practice, antiseptic mouthwashes are either used as an adjunct to improve the efficacy of mechanical oral hygiene or as the only measure of plaque control [5]. Therefore we have introduced 2% grape seed extract mouthwash (GSE) with a natural ingredient as a topical antimicrobial agent in our study.

Chlorhexidine digluconate (CHX) has been used for over 40 years and is still contemplated as the most effective oral hygiene agent in plaque inhibition. To date, no microbial resistance or a shift in the oral microflora has been noticed in conjunction with its use. However, CHX has a finite use period because of its known side effects, such as discoloration of the tooth, mucosal irritation, and taste alteration. Additionally, CHX may cause allergic reactions and in very rare cases it may lead to anaphylaxis.

Given the widening evidence of the link between oral health and systemic health, dental practitioners should respond to their patients' oral hygiene needs by providing research based products with naturally occurring herbal active ingredients that bring off the desired antimicrobial, antioxidant, and anti-inflammatory effects [6]. Grape seed extract is a natural substance recommended in the traditional system of medicine "*Ayurveda*" for treating various oral and systemic diseases, perceived to be a rich source of polyphenolic compounds, largely monomeric catechin, epicatechin, gallic acid, polymeric and oligomeric procyanidins. All of which manifest biological effects such as anti-oxidant, anti-inflammatory, and antimicrobial activity [7]. It has revealed its effectiveness against several bacteria, yeasts, and viral strains, counting a wide range of gram-positive and gram-negative organisms [8,9].

Vitis vinifera (grape) extract as an active ingredient has been explored and validated as an effective antimicrobial and antiplaque agent. A few studies have also expressed that there is decreased collagen degradation by inhibitory interstitial and extracellular collagenase and they could have the potentiality to reduce the origin of several periodontal diseases and their progression [10]. Therefore we designed a study to evaluate the antimicrobial efficacy of 2% grape seed extract mouthwash (GSE) and 0.2% chlorhexidine mouth wash (CHX) with distilled water as control, followed by a short-term clinical trial to investigate the effectiveness of the mouthwash onto gingival index (GI), simplified - oral hygiene index (OHI-S), plaque index

(PI) and bleeding on probing (BOP).

The null hypothesis was that 2% GSE mouthwash would make no significant difference in the plaque microorganisms and tested parameters when compared with CHX and control after 7 days intervention.

Methods

A total of 45 subjects age ranging 18-30 years were selected from the undergraduates of P.M.N.M. Dental College and hospital, Bagalkot, Karnataka, India. Ethical committee approval was obtained from the institutional review board of P.M.N.M. Dental College and Hospital (PMNMDCH/1943/2019-20) before conducting the study.

All potential participants were clearly explained regarding the need and design of the study. A written duly signed informed consent was obtained from all the subjects. Among them who had no history of any dental treatment, antibiotic or anti-inflammatory drug therapy for the past 3 months and good general health were included in the study. Subjects with any history of systemic diseases/ conditions, those who had used antibiotics or mouthwash for 5 consecutive days or corticosteroids in the past 30 days, and who had a habit of smoking were excluded from the study. Those subjects, who had a history of sensitivity to any mouthwash, used any removable prostheses or an orthodontic appliance and who had undergone professional measures to remove plaque & calculus in the past 15 days were excluded from the study.

Preparation of 2% grape seed extract mouthwash

Two percent GSE mouthwash was prepared based on the minimum inhibitory concentration (MIC) and minimum toxic concentration found by Furiga et al., 100ml of distilled water was mixed with 0.2 g of grape seed extract (Bulksupplements, USA) in a sterile beaker and then the beaker was placed on hot plate magnetic stirrer at 60°C to dissolve the extract in solvent until a homogenous solution was obtained and then was mixed with 900 ml of distilled water to make a final volume of 1000 ml in a clean sterile measuring cylinder. The solution was then bottled into a sterile amber color glass bottle, labeled, and was stored under refrigeration until further use [11].

Collection of data

Sample size calculation and methodology

The sample size was determined (N=15) at a power of 80% with the level of significance set at $P < 0.05$ using a formula [12]. The study was carried out on 45 subjects who were randomly divided into Groups- A, B, C, containing 15 subjects each out of which 9 samples were lost due to follow-up during the COVID -19 pandemic. Group A: 13, 2% grape seed extract mouthwash (GSE), Group B: 12, 0.2% chlorhexidine mouthwash (CHX), Group C: 11, distilled water (Control).

All three study groups followed the same oral hygiene instructions, except for the use of allocated mouthwash. Clinical parameters were recorded at baseline and after 7 days.

During the 7 days of the trial, the subjects continued to exercise their regular, self-performed plaque control measures. All subjects were given a personal kit containing the same type of toothbrush and toothpaste to decrease the bias. No oral prophylaxis was done before or after the study. The subjects were instructed to rinse 10 ml of their assigned mouthwash twice a day. They were asked to retain the mouthwash for 1 minute before expectorating it and not to consume any food or drink for 30 minutes after the use of mouthwash.

Plaque sampling, CFU count, and analysis

On day 0 and 7 supragingival plaque was collected using sterile curette from the buccal surface of the first molar in each quadrant; plaque was mixed in sterile eppendorf tubes containing 500 µl of reduced transport fluid (RTF). The sample was inoculated in sheep blood agar plates to obtain isolated colonies and incubated for 24hrs at 37°C aerobically. Colony-forming units (CFUs) in culture plates were counted and analyzed. Sample collection was done by a single investigator and analysis of plaque sample was done by a microbiologist, who was double-blinded.

Clinical parameters

Baseline measurement for all subjects was recorded for gingival index (GI) by (Silness and Loe 1963), plaque Index (PI) by (Silness and Loe 1964), simplified - oral hygiene index (OHI-S) by (Greene and Vermilion, 1964), and bleeding on probing (BOP) by (Muhlemann and Son, 1971) at day 0 and all subjects were recalled for a reevaluation of the same clinical parameters at day 7.

Statistical analysis

Statistical package for social sciences (SPSS) for windows version 22.0 released 2013 was used for statistical analysis. Descriptive analysis of all the explanatory and outcome parameters was done using frequency and proportions for categorical variables, whereas in mean & standard deviation for continuous variables. Inferential statistics was obtained by a One-way ANOVA test followed by Tukey's Post hoc Test, to compare the mean values of clinical parameters between 3 groups at baseline and 7 days post-intervention period. Kruskal Wallis Test followed by the Mann Whitney Post hoc Test was used to compare the mean CFUs ($\times 10^3$) of aerobic and anaerobic microorganisms, between 3 groups at baseline and 7 days post-intervention period. Student Paired t-Test was used to compare the mean values of different clinical parameters between the baseline and 7 days post-intervention period in each study group.

Results

According to demographic data of 36 study subjects, the mean age of the subjects was 22.5 years and gender distribution was 12 males and 24 females.

The mean scores demonstrate that there are no significant differences observed concerning the clinical parameters between 3 study groups at baseline but it showed that there exists a significant difference for mean scores of all clinical parameters between 3 study groups ($P < 0.001$) at 7 days post-intervention period. Multiple comparisons between 3 study groups revealed Group C showed significantly higher mean scores of all clinical parameters as compared to Group A and Group B at $P = 0.001$ and $P < 0.001$ respectively, however, no significant difference was noted between Group A and B ($P = 0.52$) as depicted in (Table I).

Table I. Comparison of mean values of clinical parameters between 3 groups at 7 days post intervention period using one-way ANOVA test followed by Tukey's post hoc test.

Parameters	Groups	N	Mean	SD	Min	Max	P-Value ^a	Sig. Diff	P-Value ^b
Gingival index (GI)	Group A	13	0.99	0.13	0.8	1.2	<0.001*	A vs B	0.52
	Group B	12	0.93	0.11	0.8	1.1		A vs C	0.001*
	Group C	11	1.24	0.22	0.8	1.5		B vs C	<0.001*
Simplified - Oral hygiene index (OHIs)	Group A	13	1.62	0.43	1.0	2.4	<0.001*	A vs B	0.36
	Group B	12	1.35	0.39	0.9	2.4		A vs C	0.007*
	Group C	11	2.28	0.64	1.4	3.2		B vs C	<0.001*
Bleeding on probing (BOP)	Group A	13	1.38	0.42	0.7	1.9	0.002*	A vs B	0.57
	Group B	12	1.19	0.38	0.8	1.7		A vs C	0.02*
	Group C	11	1.89	0.55	1.1	2.9		B vs C	0.002*
Plaque index (PI)	Group A	13	1.02	0.25	0.7	1.7	0.001*	A vs B	0.25
	Group B	12	0.88	0.19	0.7	1.4		A vs C	0.04*
	Group C	11	1.23	0.15	1.0	1.5		B vs C	0.001*

* - Statistically Significant

Note: a. P-Value derived by one-way ANOVA test; b. P-value derived by Bonferroni's post hoc test.

The mean GI, OHIs, BI & PI scores significantly reduced at day 7 as compared to the baseline period at P<0.001 in Group A and B but no significant difference was noted in Group C (Figure 1 and 2). The microbial test results demonstrate that there exists a significant difference concerning mean CFUs of aerobic and anaerobic microorganisms scores between 3 study groups [P=0.005] at 7 days post-intervention period. Multiple comparisons between 3 study groups revealed that Group C had a significantly higher mean of aerobic and anaerobic organism's CFUs as compared to Group A and Group B at P=0.002 and P=0.01 respectively, however, no significant difference was noted between Group A and B (P=0.46) (Table II).

The test results demonstrate that mean CFUs of aerobic, anaerobic organisms, significantly reduced at day 7 as compared to the baseline period in Group A and B but the non-significant reduction was noted in mean CFUs of Group C (Table III).

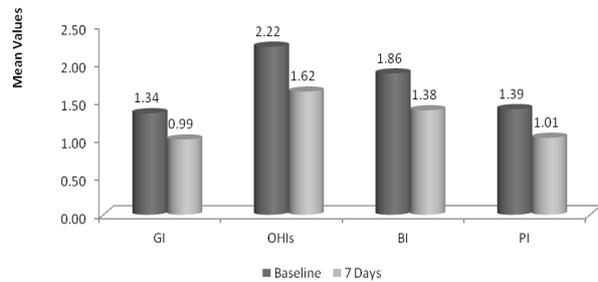


Figure 1. Mean values of different clinical parameters between baseline and 7 days post intervention period in Group A.

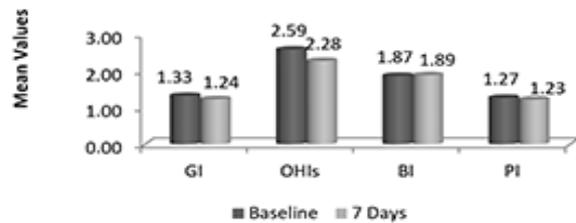


Figure 2. Mean values of different clinical parameters between baseline and 7 days post intervention period in Group C.

Table II. Comparison of mean CFUs [$\times 10^3$] of aerobic and anaerobic organisms between 3 groups at 7 days post intervention period using Kruskal Wallis test followed by Mann Whitney post hoc test.

Parameters	Groups	N	Mean	SD	Min	Max	P-Value ^a	Sig. Diff	P-Value ^b
Aerobic	Group A	13	101.00	46.45	3	160	0.005*	A vs B	0.46
	Group B	12	92.67	63.14	2	220		A vs C	0.002*
	Group C	11	169.09	50.69	120	290		B vs C	0.01*
Anerobic	Group A	13	78.15	40.34	6	150	0.002*	A vs B	0.57
	Group B	12	71.67	59.52	0	160		A vs C	0.001*
	Group C	11	151.82	48.13	30	200		B vs C	0.005*

* - Statistically Significant

Note: a. P-Value derived by Kruskal Wallis test; b. P-value derived by Mann Whitney post hoc test.

Table III. Comparison of mean CFUs of aerobic and anaerobic organisms between baseline and 7 days post intervention period in Group A, B, C using Wilcoxon Signed Rank test.

Group A	Time	N	Mean	SD	Mean Diff	P-Value
Aerobic	Baseline	13	161.38	53.99	60.38	0.001*
	7 days	13	101.00	46.45		
Anerobic	Baseline	13	151.54	47.93	73.39	0.001*
	7 days	13	78.15	40.34		
Group B	Time	N	Mean	SD	Mean Diff	P-Value
Aerobic	Baseline	12	209.58	123.44	116.91	0.002*
	7 days	12	92.67	63.14		
Anerobic	Baseline	12	150.00	99.00	78.33	0.005*
	7 days	12	71.67	59.52		
Group C	Time	N	Mean	SD	Mean Diff	P-Value
Aerobic	Baseline	11	182.73	47.77	13.64	0.06
	7 days	11	169.09	50.69		
Anerobic	Baseline	11	166.82	55.33	15.00	0.07
	7 days	11	151.82	48.13		

* - Statistically Significant

Discussion

Biofilm is made up of a lower density layer containing microbes, other organic or inorganic matter bound together by a polysaccharide matrix and outside this is a loose layer that is irregular in form. This fluid layer binding the film has a stationary 'viscous sub-layer' between which solutes penetrate by molecular diffusion [13]. Inadequate literature and substantial studies on such biofilms are observed, therefore in our study, we compared antimicrobial activity of grape seed extract mouthwash (GSE) and Chlorhexidine mouthwash (CHX) with distilled water as a control group on dental plaque, which can be considered as a footmark study of its kind. At the concentration of 2000 mg/ml, GSE shows the best antibiofilm activity against multispecies biofilm producer (including *P. gingivalis*, *F. nucleatum*, *Streptococcus sobrinus*, *Lactobacillus rhamnosus*, and *Actinomyces viscosus*). Higher concentrations of GSE led to a fall of its effectiveness, principally caused by its poor dissolution in water [14]. Therefore we have considered preparing a GSE mouthwash of 2% in our study.

Our study results have shown that the mean CFUs ($\times 10^3$) of aerobic and anaerobic microorganisms was significant when GSE was compared with controls ($P=0.002$; $P=0.001$) and CHX was compared with controls ($P=0.01$; $P=0.005$), but no significant difference was observed when GSE and CHX were compared ($P=0.46$; $P=0.57$), suggesting that GSE and CHX have similar and high antimicrobial properties on plaque microorganisms when compared with distilled water. Several antibacterial mechanisms are suggested for GSE. For example, the hydrophobic phenolic components of GSE interact with bacterial surface structures and lipopolysaccharides, which decrease membrane stability leading to bacterial lysis. Motility plays a pivotal role in the initial interaction of the microorganisms with the surface as well as for moving along the surface, which is prevented by GSE in certain species involved in biofilm formation and development. In addition to direct mechanisms of antimicrobial effect, it would be presumable to state that polyphenol of GSE may induce the immune system of a host against microbial pathogens [14].

In the course of the study, we observed that none of the subjects had side effects related to the use of GSE or CHX mouthwash. Both the test groups with GSE and CHX showed a significant reduction in scores of all the clinical parameters (GI, PI, OHI-S, and BOP) when compared on days 0 and 7. The reduction in all the clinical parameters was greater in the GSE group when compared with the control group. These results invalidate our null hypothesis. Multiple comparisons between 3 study groups revealed that the control group showed significantly higher mean in all the scores of the clinical parameter as compared to GSE and CHX Group at $P=0.001$ and $P<0.001$ respectively; however, no significant difference was noted between GSE and CHX Group ($P=0.52$) (Table I), suggesting that both the mouthwashes are equally effective in reducing periodontal disease. These

results of GSE could be because of their antioxidant and anti-inflammatory property. The decrease in the number of microorganisms by GSE may lead to a reduction in the levels of reactive oxygen species (ROS). ROS cause a state called oxidative stress resulting in oxidative damage, leading to cell death, at low concentrations. These free radicals stimulate the growth of fibroblasts and epithelial cells in culture, but at higher concentrations, it results in tissue injury [15]. ROS can lead to tissue destruction by various mechanisms, such as DNA and protein damage, enzyme oxidation, rise in the stimulation of proinflammatory cytokine and lipid peroxidation [16]. Taking into account all of these oxidative properties, there is now considerable interest in research for free-radical scavengers or antioxidants like grape seed extract that inhibit ROS. Proanthocyanidins (PAs) are among the most abundant phenolic compounds in grape seeds (*Vitis vinifera*). These molecules have been associated, at least in part, with the protective effect of red wine for atherogenesis and cardiovascular diseases [17]. Recent studies also have shown that PAs in grape seeds possess antioxidant, anti-inflammatory, antiarthritic, antitumor-promoting activities and prevent heart disease [18]. They also seem to have inhibitory effects on both arachidonic acid cascade and iNOS modulation [19]. As per these studies and some of the newer reviews [15], it may be concluded that ROS may be involved in the development of chronic periodontitis. Therefore, it is of utmost importance to keep ROS at low levels for maintaining a healthy periodontium [20]. The inhibitory effect of these proanthocyanidins on interstitial and extracellular collagenase could inhibit the formation of plaque leading to a pause in the progression of periodontal disease. These results are in contrast with the study by Malhotra R [21] who concluded in his study that herbal mouth rinse is a potent plaque inhibitor, though less effective when compared to chlorhexidine gluconate. This contrast with our study can be justified by the results that we have obtained by evaluating the clinical parameters of Group A&B at day 0 and 7, which has also reduced along with CFUs suggesting that both GSE and CHX may have a similar effect on the initial healing phase of periodontal soft tissue.

Since the recognition of the biological properties of polyphenolic compounds has led to the use of grape seed extract as a dietary supplement [7]. Our study demonstrates that the use of these natural herbal mouthwashes like GSE instead of any other commercially available chemical mouthwashes would result in both periodontal and systemic health benefits that could help confront periodontal diseases and their prevention.

Conclusion

Grape seed extract (GSE) mouthwash, a traditional Indian medicine, is becoming increasingly popular, with many chronic conditions responding well to it. It has shown significant results in reducing aerobic and anaerobic colony

forming units in plaque when compared to chlorhexidine mouthwash (CHX), thereby demonstrating the GSE's antimicrobial efficacy and thus depicting its use as an effective alternative to CHX in the prevention of plaque formation which may have further lead to periodontal diseases. A more definitive conclusion regarding the use of GSE in the treatment of periodontitis can be reached by possibly increasing the concentration of GSE within the limited range, by undertaking a multicentered study involving a larger sample size for assessing its other properties such as anti-inflammatory effect on the periodontium.

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