Original Research Paper

University J Dent Scie 2025; Vol. 11, Issue 4

Comparison of Antimicrobial Efficacy of Chlorhexidine, Green Coffee Bean Extract and Green Tea Leaf Extract On Anaerobic Periodontal Pathogens: An In Vitro Study

Abstract:

Objective: Numerous aerobic and anaerobic microorganisms such as Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia, and Aggregatibacter actinomycetemcomitans are responsible for periodontal diseases. Chemotherapeutic agents are used to prevent periodontal diseases but overzealous use of this agents may lead to bacterial resistance, staining, mucositis etc. Therefore, this study was performed to find a safe anti-plaque agent. Recent studies on green tea and coffee extracts have shown to have positive effects on periodontal health owing to their anti-inflammatory, antimicrobial, and antioxidant property.

Methods: In this study, samples were collected from sub gingival region of indexed teeth from systemically healthy periodontitis patients and transported in to a freshly prepared transport media. Then samples were inoculated onto their respective culture plates and incubated in an anaerobic jar. Finally, the zone of inhibitions were measured to find out the antibiotic susceptibility of green tea and green coffee beans in comparison to 0.2% chlorhexidine.

Results: Green coffee beans shown lowest zone of inhibition in an alcohol base and 0.2% chlorhexidine gluconate shown highest zone of inhibition. As the concentration of the solution increases, the efficacy of the solution also increases towards the anaerobic microorganisms.

Conclusion: To conclude this, green tea leaf and green coffee bean has still showed some amount of positive antimicrobial capability when mixed with different solvent and when used in higher concentrations

Key-words: Chlorhexidine, Green tea, Green coffee beans, Porphyromonus. gingivalis, Culture media, Blood agar,

Introduction:

Oral cavity harbors many aerobic and anaerobic microorganisms which causes a broad spectrum of oral diseases.The majority of the microorganisms are either facultative anaerobic or obligatory anaerobic despite the accessibility of the oral cavity to air containing about 20% oxygen. In addition, there are some capnophilic and microaerophilic species present. Periodontal diseases are among the most widespread oral bacterial diseases of humanity that affect 15–20% of the world's population, including Asia, eventually leading to tooth loss, if left untreated. Dental plaque is the main etiological factor for the initiation and progression of the disease. It comprises of polymicrobial community of different species with increasing prevalence of anaerobic microorganisms such as Porphyromonasgingivalis, Fusobacteriumnucleatum, Prevotella intermedia and Aggregati bacteractinomy cetemcomitans. Non-surgical periodontal therapy is considered as the gold standard as it aims at eradication of microbial biofilm for reduction of bacterial load and

[1]MINGKIAM GAO, [2]RAJASHREE GANGULY, [3]AMRITA DUTTA, [4]SANJUKTA DATTA, [5]AMIT De, [6]SHIBENDU BISWAS[6]

[1]Clinical Practitioner, India

[2-5]Department of Periodontics, Kusum Devi Sunderlal Dugar Jain Dental College & Hospital

[6]Department of Microbiology, Guru Nanak Institute of Dental Sciences and Research

Address for Correspondence: Dr. Rajashree Ganguly
Bd-391 Sector-I, Saltlake
Kolkata: 700064
Email : Rgbhaduri@gmail.com

Received : 6 May, 2025, **Published :** 31 Dec, 2025

How To Cite This Article: Amit De, Ganguly, P., Amrita Dutta, Mingkiam Gao, Sanjukta Datta, & Shibendu Biswas. (2025). Comparison of Antimicrobial Efficacy of Chlorhexidine, Green Coffee Bean Extract And Green Tea Leaf Extract On Anaerobic Periodontal Pathogens: An In Vitro Study . University Journal Of Dental Sciences, 11(4).

Access this article online

Website: www.ujds.in

Quick Response Code

DOI: https://doi.org/10.21276/ujds.2025.11.4.3

modification of environmental risk factors, achieved by both mechanical and chemotherapeutic approaches. Chemotherapeutic agents have added advantages over mechanical therapy as it not only overcomes the limitations of mechanical therapy but also modulates host tissue response.Chlorhexidine gluconate is the gold standard agent for chemical plaque control due to its broad antibacterial activity, and it reduces the level of microorganism up to 90% for several hours but the disadvantages of chlorhexidine are staining, altered taste sensation and mucosal erosion. So the search of safe anti plaque agent is still going on to overcome the disadvantages of chlorhexidine. Recent studies on green tea and coffee extracts have shown to have positive effects on periodontal health owing to their anti-inflammatory, antimicrobial, and antioxidant property.The most abundant components in green tea are **polyphenols,** in particular flavonoids such as **catechins**, **catechism gallates(cg)** and **proanthocyanidins**. Green tea polyphenolic catechins can inhibit the growth of a wide range of gram negative bacterial species with moderate potency. Evidence suggests that these molecules may be useful in the control of common oral infections, such as dental caries and periodontal disease.

Some components in coffee such as caffeine, volatile and nonvolatile organic acids, phenols and aromatic compounds are reported to have antimicrobial activity. **Chlorogenic acid (CGA)** and **caffeic acid**, which are nonvolatile organic acids found in coffee, inhibit the growth of some Gram-positive microorganisms such as Staphylococcus aureus, Bacillus cereus, Lactobacillusbulgaricus, Streptococuslactis and Streptococcus faecalis and Gram-negative bacteria like Escherichia coli, Salmonella typhi and Pseudomonas auerginosa. Green (or raw) coffee is a major source of CGA in nature (5–12 g/100 g).

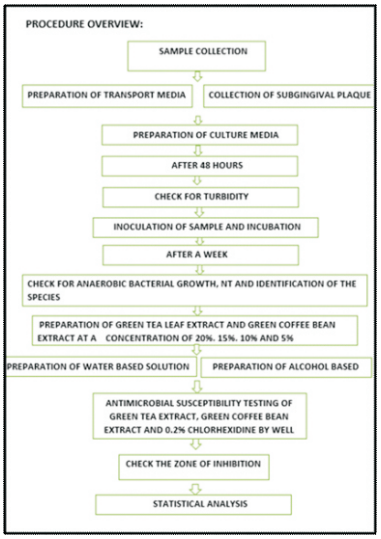
This study has been conducted to assess the minimum inhibitory concentrations (MICs) of pure green coffee bean extract and green tea leaf extract that can be safely and effectively administered as local drug delivery system on specific anaerobic periodontal pathogens in comparison with standard 0.2% chlorhexidine.

Materials and Methods:

The samples were collected from sub gingival region of indexed teeth (maxillary first and second premolar) from systemically healthy periodontitis patients, who had not taken any antibiotic or antisepticmedicines, attending theOPD of GNIDSR after taking permission from the institutional ethical committee.

Preparation of Transport Media:

The broth was prepared following manufacturer's instructions. 3.7g of Brain-Heart infusion broth powder as described in ***Ricky P Singh et al*** (2017) was measured and dissolved in 100ml distilled water in a conical flask and autoclaved. After cooling the broth to about 40-45^{0}C, 0.5mg of hemin and 0.1ml stock solution of Vit k_1 was added and mixed.



Collection of Subgingival Plaque:

The materials for microbiological examination were collected in dry field conditions by inserting two sterile paper points (ISO 45) carefully into the deepest part of the gingival sulcus parallel to the vertical axis of the tooth for 60 seconds. The paper points were then dropped into a conical flask containing 50 ml of transport media and immediately transported to the research laboratory for further analysis. Continuous stream of nitrogen gas was blown onto the area of collection during the procedure to ensure minimal exposure to oxygen. The pH of the BHI broth was 7.20 - 7.60 before incubating in the anaerobic jar.

A)Probing depth measurement; B)Sample collection with paper point;C) Paper point immersed in the stock media; D)Transfer into the anaerobic jar E) Incubating F) Turbidity appears

Preparation oof Culture Media

Preparation of Anaerobic Blood Agar Base With 5mg Hemin/l, 10mg/ml of Vit K_1 Supplement And 5% Blood:

The media was prepared according to the manufacturer's instruction, 4.40 gm of anaerobic blood agar base in 100ml of distilled water in a conical flask and autoclaved at 121°C at 15 psi for 15 minutes. After the Media was cooled to about 45°C, 0.5mg of hemin, 0.1ml of Vit K_1 supplement and 5% of goat blood was added, mixed and poured into pre-sterilized petri dish plates and it was allowed to solidify. Mouth of the conical flask was flamed before and after pouring of the media to ensure sterility.

Inoculation of Sample

0.50 µl from the microbial sample was drawn from the stock culture using a micropipette and inoculated onto their respective plates of culture media and was spread using a sterile loop.

Incubation in Anaerobic Jar:

The gas pack acts by chromous principle. The chromium powder was dispensed into the petri plate with holder for charge. 60ml of previously prepared 25% by volume sulphuric acid was added to the powder to start the reaction. The petri plates were then enclosed in the jar with the activated charge. The lid was greased at the brim and the clips were applied. The adjoining tube for safety release of excess hydrogen produced was filled with saturated solution of sodium carbonate up to three quarters of its height.The reaction which occurs was

$$Cr + H_2SO_4 \longrightarrow CrSO_4 + H_2$$

Chromium reacts with sulphuric acid, forming chromous sulphate with vigorous evolution of hydrogen.

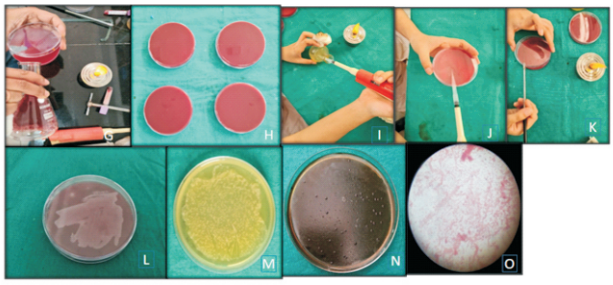
$$4CrSO_4 + 2H_2SO_4 + O_2 \longrightarrow 2Cr_2(SO_2)_3 + 2H_2O$$

The chromous sulphate in the presence of sulphuric acid absorbs oxygen and is readily oxidized to chromic sulphate.

Two steps reaction involving two reagents serves two purposes:

a) Displacement of air by the generated hydrogen
b) Absorption of the residual oxygen by the chromous compound.

The inoculated petri plates were placed in the anaerobic gas jar with the activated gas pack and the jar was sealed and placed in an incubator with temperature between 37°C. The incubation lasted for 7 days under anaerobic conditions generated by the gas pack system.



In Fig: G) blood mixed with anaerobic agar base;H)solidified agar base, I)& J) Ejecting and transferring the sample into agar base respectively; L) Petri dish after inoculation; M) Anaerobic periodontal pathogens in agar base after 1 wk, N) Produces black pigmentation after 2 wks;O) : Photo micrograph (x1000) of gram staining of anaerobic periodontal pathogens

Identification Of Bacterial Species:

After 7 days of incubation period the Petri dishes were observed for bacterial growth. Anaerobic blood agar was the selective medium for isolation of anaerobic bacteria. The colonies of bacteria were provisionally identified by colony characteristics described by ***X Zhou***, ***Y Li et al***(2015) and ***Condorelliet al***. (1998)**.** Hereafter, gram staining was done for gram negative anaerobic bacteria, observation was done under bright field compound microscope.

Preparation Of Green Tea Leaf Extract And Green Coffee Bean Extract Alcohol Based Solution At Concentration 20%, 15%, 10% And 5%

Commercially available Green tea leaf and green coffee bean was obtained and were grinded to obtain powder form, then they are weighed for 20 mg each.Preparation of 20% of alcohol extract was done by percolating 100ml of ethanol onto 20 mg of ground coffee and green tea leaf powder, it was left for 10 mins for the extract to release. Filter paper on funnel was used to filter out the extract. After preparation of 20% alcohol extract of green coffee bean powder and green tea leaf powder, further dilution was done using sterile water to obtain the concentration of 15%, 10% and 5%. 3 wells of 5mm diameter were created on 4 petri dishes for different concentrations, 20%, 15%, 10%, and 5% of green tea extract, green coffee extract and 0.2% chlorhexidine. Using the micropipette 0.1µl of green coffee bean extract and green tea leaf extract solutions were added in each wells of different concentration keeping 0.2% of chlorhexidine constant in its assigned wells. Within 10 mints, the agar plates were shifted to anaerobic jar with gas pack which was kept in incubator for 72 hrs. The diameter of zones of inhibition was measured for all wells using a divider. The mean scores of zones of inhibition were calculated for each solution respectively.

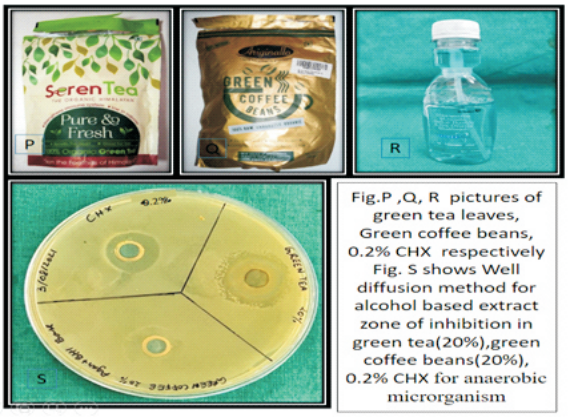


Fig.P ,Q, R pictures of green tea leaves, Green coffee beans, 0.2% CHX respectively Fig. S shows Well diffusion method for alcohol based extract zone of inhibition in green tea(20%),green coffee beans(20%), 0.2% CHX for anaerobic microrganism

Result And Statistical Analysis:

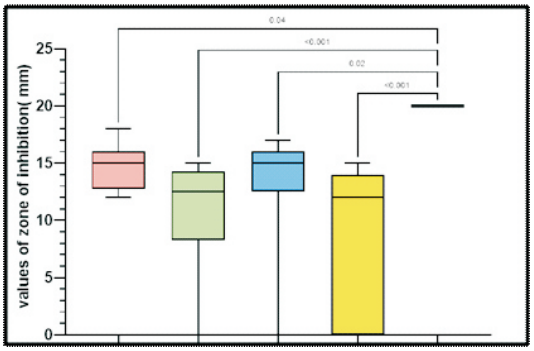
The collected data was tabulated in a spreadsheet using Microsoft Excel 2019 and then statistical analysis was carried out using IBM SPSS Statistics for Windows, Version 26.0. A Shapiro-Wilk's test and a visual inspection of the histograms, normal Q-Q plots and box plots showed that the collected data were not normally distributed the study groups. Non-Parametric test (Kruskal-Wallis Test)was carried out to compare the data between the study groups for quantifying the zone of inhibition formed at various concentrations. The P-value of 0.05 was considered as the level of significance. 0.2% chlorhexidine mouthwash showed the greatest zone of inhibition against all the samples of anaerobic pathogens. Green tea leaf extract at concentrations of 20%, 15%, 10%, 5% has showed antimicrobial activity. Green coffee bean extract at concentrations of 20%, 15%, 10% has showed antimicrobial activity. Green coffee has showed lowest zone of inhibition.

Concentration of green tea leaf alcohol extract

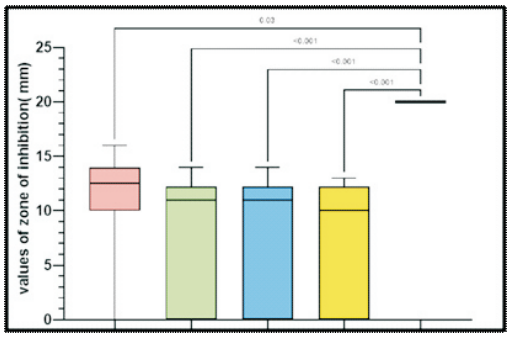
Green tea leaf 20 mg	Sterile water	Ethanol extract Concentration
2 ml	-	20 %
1.5 ml	0.5 ml	15 %
1 ml	1 ml	10 %
0.5 ml	1.5 ml	5 %

Concentration of green coffee bean alcohol extract

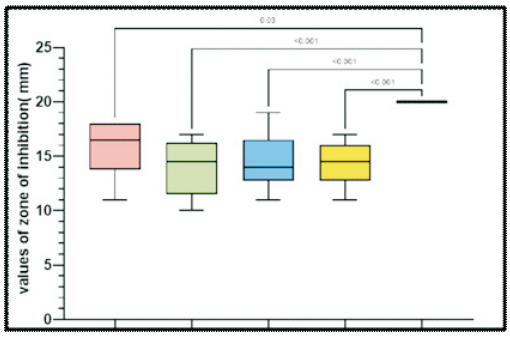
Green coffee bean 20 mg	Sterile water	Alcohol extract Concentration
2 ml	-	20 %
1.5 ml	0.5 ml	15 %
1 ml	1 ml	10 %
0.5 ml	1.5 ml	5 %



Comparison of zone of inhibition for 5% conc of green tea leaves, green coffee beans and 0.2% CHX

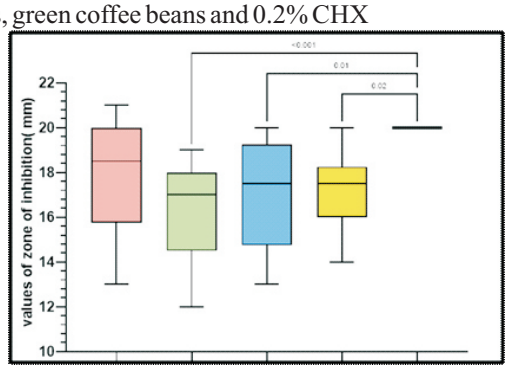


Comparison of zone of inhibitionfor 10% conc ofgreen tea leaves, green coffee beans and 0.2% CHX



15% conc

Comparison of zone of inhibition for 15% conc of green tea leaves, green coffee beans and 0.2% CHX



20% conc

Comparison of zone of inhibition for 20% conc of green tea leaves, green coffee beans and 0.2% CHX

Discussion:

Microbial infections brings significant threat to our periodontal environment, so the maintenance of good oral hygiene both mechanically and chemically is the main key to preventing oral diseases. Microbiological testing may aid the clinician in the selection of the most effective antimicrobial agent or combination of agents. But, the overzealous use of synthetic antimicrobial agent can lead to emergence of antibiotic resistance and superinfection. According to literature, antimicrobial efficacy of green tea leaves and green coffee beans in vitro has been found. The use of green tea and green coffee cannot be disapproved, since, these are commercially available and consumed as daily beverages.

The results of this study confirmed that chlorhexidine, the "gold standard", showed the highest value of zone of inhibition (ZOI), as compared to green tea extract and green coffee bean extract. In the present study 20% green tea had the maximum desired effect when compared to green coffee extract. According to ***Osawa et al***, Green tea catechin has been shown to be bactericidal against *Porphyromonasgingivalis and Prevotella sp*. in vitro. ***Demeule M et al*** reported in their study that tea catechins containing the galloyl radicals possess the ability to inhibit collagenase, an enzyme that plays an important role in the disruption of the collagen component in the gingival tissues of patients with periodontal disease. EGCG (epigallocatechin gallate) has been reported to inhibit production of toxic metabolites of *P. Gingivalis* .EGCG (active at 250−500 μg/ml) inhibited growth and adhesion of *Porphyromonasgingivalis* to buccal epithelial cells. Green tea catechins inhibit enzymatic activities of *P. gingivalis* in a manner similar to that of Chlorhexidine, Doxycycline, and non-antimicrobial chemically modified tetracycline derivatives.

Roasted coffee extract was found to possess antibacterial activity against an inclusive range of microorganisms, including *staphylococcus aureus* and *streptococcus mutans*, whereas green coffee extract displayed no such activity, thus the naturally occurring coffee compounds, such as chlorogenic acids and caffeine, could not therefore be responsible for the substantial antibacterial activity employed by coffee beverages against both bacteria reported by ***Daglia et al.*** in 2007. Green or non-roasted coffee appears to have the highest antimicrobial activity. Furthermore, degree of roasting is inversely proportional to its antimicrobial efficacy.

Different studies have also reported that coffee at very low concentrations of 0.2 μg/ml shows antibacterial properties against *P. gingivalis, P. intermedia and A. actinomycetemcomitans*. After achieving 20% concentration further dilution was done to obtain the concentration 15%,10% and 5% alcohol extract. Overall, it would appear that an increase in the concentration of green tea extract and coffee extract significantly increases the antibacterial activity against periodontal pathogens.

For statistical analysis, the results were divided into 2 groups and the 3rd group was for chlorhexidine which was the +ve control

Group I - Green tea leaf alcohol extract

Group II - Green coffee bean alcohol extract

Group III - Chlorhexidine (+ve control)

Intra-group Comparison Of Zone Of Inhibition

In the present study, the values of zone of inhibition between various concentrations of each group were analyzed with 0.2% Chlorhexidine as Control that showed a high statistically significant difference [$P<0.001$] in all the groups.

In the group I, there was a very weak evidence ($P < 0.05$) that values of zone of inhibition was higher at 10% concentration than at 5% concentration ($P>0.05$), at 15% concentration than at 5% concentration ($P>0.05$), at 15% concentration than at 10% concentration($P>0.05$), at 20% concentration than at 10% concentration($P>0.05$), at 20% concentration than at 15% concentration($P>0.05$), at 0.2% Chlorhexidine than at 20% concentration of Green Tea Leaf Alcohol extract ($P>0.05$). There was a very strong evidence that values of zone of inhibition was higher at 0.2% Chlorhexidine than at 5% concentration of Green Tea Leaf Alcohol extract($Z=-5.257, P<0.001$), at 10% concentration of Green Tea Leaf Alcohol extract($Z=-3.894, P=0.001$) at 0.2% Chlorhexidine than at 15% concentration of Green Tea Leaf Alcohol extract($Z=-3.128, P<0.05$) at 0.2% concentration than at 5% concentration($Z=3.6, P<0.01$).

In group II, there was a very weak evidence ($P < 0.05$) that values of zone of inhibition was higher at 10% concentration than at 5% concentration($Z=0.803, P>0.05$), at 15% concentration than at 5% concentration($Z=1.87, P>0.05$), at 15% concentration than at 10% concentration($Z=1.066, P>0.05$), at 20% concentration than at 10% concentration($Z=2.33, P>0.05$), at 20% concentration than at 15% concentration($Z=1.267, P>0.05$), at 20% Chlorhexidine than at 20% concentration of Green Coffee Bean Alcohol extract($Z=-2.179, P>0.05$). There was a strong evidence ($P < 0.05$) that values of zone of inhibition was higher at 20% concentration than at 5% concentration($Z=3.136, P<0.05$), at 0.2% Chlorhexidine than at 5% concentration of Green Coffee Bean Alcohol extract($Z=-5.315, P<0.001$), at 0.2% Chlorhexidine than at 10% concentration of Green Coffee Bean Alcohol extract($Z=-4.512, P<0.001$), at 0.2% Chlorhexidine than at 15% concentration of Green Coffee Bean Alcohol extract($Z=3.445, P<0.01$). There was a very strong evidence ($P < 0.05$) that values of zone of inhibition was higher at 20% concentration than at 5% concentration($Z=3.623, P<0.01$), at 0.2% Chlorhexidine than at 5% concentration of Green Tea Leaf Alcohol extract($Z=-5.582, P<0.001$), at 0.2% Chlorhexidine than at 10% concentration of Green Tea Leaf Alcohol extract($Z=-3.615, P<0.01$), at 0.2% Chlorhexidine than at 15% concentration of Green Tea Leaf Alcohol extract($Z=-3.553, P<0.01$). At 20% concentration, the values of zone of inhibition between the groups at 20% concentration with 0.2% Chlorhexidine as Control was analyzed and it showed a high statistically significant difference[$P=0.001$].

Therefore, based on the findings of this study, we believe that further in vitro and in vivo study with short-term and long-term durations hold promise for assessing the antimicrobial efficacy of green coffee and green tea in the field of periodontics. However, as we have used the stock solution of 20% in three other 15%, 10%, 5% based on a study by **Antonio et al** which could have been modified or increased to see if increased concentrations would increase the antibacterial activity or even show a result near to chlorhexidine. The agar well diffusion method used in this study is capable of measuring varying degrees of antibacterial activity; however, it is not possible to reduce MIC or minimum bactericidal concentration. Hence, the ideal concentration of green tea extract and coffee extract still remains unknown.

Conclusion :

The present study was performed to compare the antimicrobial efficacy of green tea leaf extract, green coffee bean extract with gold standard chlorhexidine on anaerobic periodontal pathogens in an in vitro study. Based on the findings of this study, the following conclusion have been made that commercially available green coffee bean and green tea leaf that is used for daily consumption does not show significant antimicrobial activity towards anaerobic periodontal pathogens in comparison with 0.2% standard chlorhexidine, but both green tea leaf and green coffee bean has still showed some amount of positive antimicrobial capability when mixed with different solvent and when used in higher concentrations. But since both the ingredients are plant based and are readily consumed, it can open a whole new path in the world of mouth rinses and antimicrobials agents. However, further studies on the clinical application and ideal concentration for better efficacy would be beneficial.

Acknowledgment:

I sincerely acknowledge the inspiration from the thoughts and works of the innumerable researchers from the past who had contributed immensely to our knowledge in the field of research in Periodontology .I would like to thanks all the investigators of this research who independently performed different phases of the study including protocol development. Clinical procedure, data analysis, result interpretation and reporting.

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