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COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFECTS OF GREEN TEA EXTRACT WITH CHLORHEXIDINE ON P GINGIVALIS, S.AUREUS AND E.FAECALIS.

Periodontology

Dr. Lipika Gopal* Sr. Lecturer Department Of Periodontology Manav Rachna Dental College, Faridabad Haryana *Corresponding Author

Ms. Shakila Mahesh Reader Department Of Microbiology Manav Rachna Dental College, Faridabad Haryana

Dr. Pooja Palwankar Professor & HOD Department Of Periodontology Manav Rachna Dental College, Faridabad Haryana

ABSTRACT

Tea has been linked to a group of medicaments -Ayurveda, the ancient Indian system of medicine, known as 'Rasayanas' that confer attainment of positive health, resistance to diseases and assured full lifespan of quality living, unlike drugs that cure after disease has struck. It is considered to be one of the most common beverage of the world. Nowadays, tea is becoming the source of interest for research and prevention of oral diseases. Out of various types of tea, Green tea has been extensively studied and has been found to have various beneficial effects. Green tea has received considerable attention because of its numerable scientifically proven health benefits, due to polyphenols Besides polyphenols, green tea contains additional antioxidants such as carotenoids, tocopherols (vitamin E derivatives) and vitamin C. Its extract is considered to be a naturally occurring antimicrobial agent. Green tea being an antimicrobial and an antioxidant has shown inhibition of certain bacteria causing oral diseases. The microorganisms causing oral diseases are the gram positive and gram negative bacteria. This paper elaborates the effect of green tea extract with chlorhexidine on the 3 major oral bacteria namely *P.gingivalis, S.mutans and E.faecalis.*

KEYWORDS

Green Tea extract, Chlorhexidine, P gingivalis

INTRODUCTION

An increasing population all around the world are turning to the nature by using the natural herbal products in both prophylaxis and treatment of different diseases. Tea is the most popular beverage in the world after water[4]. Tea being one of the most popular beverage in the world and is now extensively studied for treatment and prevention of various diseases and bacteria. Out of various types, Green tea has been extensively studied and has received considerable attention because of its numerable scientifically proven benefits. It consists of various polyphenols (catechin) like (+)catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate[18] with anticariogenic, anti-inflammatory, anticollagenolytic properties[6]. Success of any antimicrobial agent depends on its ability to achieve bacteriostatic or bactericidal concentrations to inhibit the growth of bacteria.

The oral cavity possesses a number of features which make it a distinct habitat for a collection of microorganisms. The surfaces in the oral cavity are continuously bathed in saliva at a narrow temperature range (34 to 36°C) and a pH close to neutrality[16]. With such an ideal environment, various classes of microflora are found to be distributed in various ecological niches[19].

In this study the antimicrobial property of microorganisms such as *P.gingivalis, S.aureus and E.faecalis* are considered . ***Porphyromonas gingivalis*** is a nonmotile, Gram-negative, rod-shaped, anaerobic, pathogenic bacterium. It forms black colonies on blood agar. It is found in the oral cavity, where it is implicated in certain forms of periodontal disease, as well as in the upper gastrointestinal tract, the respiratory tract, and the colon. *P. gingivalis* is known to produce a range of virulence factors that could penetrate the gingiva and cause tissue destruction, by induction of inflammation[10]. It is a major periodontopathic organism, which has the virulent factors, cysteine proteinases that are regarded as important virulence determinants as demonstrated by various in vitro assays. Previous *in vitro* studies showed that green tea catechin inhibits the growth of *P. gingivalis, Prevotella intermedia*, and *Prevotella* nigrescens[11]. It inhibits the adherence of *P. gingivalis* onto human buccal epithelial cells[8]. In addition, green tea catechins inhibit the production of toxic metabolites of P. gingivalis. A study showed that green tea catechins, EGCg and Ecg, inhibit the activity of *P. gingivalis*-derived collagenase[15].

Staphylococcus aureus is a gram-positive, facultative anaerobe bacterium and spherical cocci in clusters. Several reviews have highlighted the paucity of both clinical and laboratory data on the role of *S. aureus* in the oral cavity. Some oral infections are caused at least in part by *S. aureus*, for example, angular cheilitis, parotitis and staphylococcal mucositis[21]. In various studies, oral carriage rates of S.aureus have ranged from 17-48%[20]. More recently, workers have revealed that between 94-100% of healthy adults had oral colonisation with staphylococcus species and oral carriage of S.aureus ranged from 24-36%[22]. The presence of prostheitc devices within oral cavity, such as acrylic dentures may encourage carriage of Staphylococcus[27]. Whilst there are few studies on presence of Staphylococcus species in healthy oral cavity, more has been reported from patients demonstrating ill health[8]. In a study of 110 patients attending dental OPD with a range of oral diseases, there was an observed prevalance of S.aureus in saliva of 21% and from gingival swabs of 11%[13].

Enterococcus faecalis is a Gram-positive, facultative anaerobic coccus. Although enterococci were initially regarded as non-virulent, they are now recognized as one of the major causes of nosocomial infections worldwide. In dentistry, *Enterococcus* species, in particular *Enterococcus faecalis*, have been found to be associated with chronic periodontitis[26] and failed root canal treatments involving chronic apical periodontitis[3]. Various studies have shown that most oral *E. faecalis* possess virulence factors related to adhesion and biofilm formation[15,9]. Moreover, some strains can also produce an anti-phagocytic capsule which evade the immune system and sustain successful long-term infection. In heavily infected sites, these virulence factors may contribute to the pathogenesis of post-treatment apical and marginal periodontitis[25].

In this study the antimicrobial property of the 3 oral microorganisms *P.gingivalis, S.aureus and E.faecalis* are considered and an attempt has been made to concentrate on the antimicrobial activity of green tea extract compared with chlorhexidine on these oral bacteria.

MATERIALS & METHOD

Nutrigreen tea extract (80%) was provided by Anthem Cellutions Pvt Ltd, Delhi. It is a brown coloured powder with bulk density of 0.5g/ml consisting polyphenols of 92.59%.

Dispensing of extract.

The drug formulation was dispensed in syringe. The entire operation was performed under aseptic conditions, and were sterilized by gamma irradiation at 2.5 Mrad Shriram Centre For Industrial Research, Delhi University .

Materials

1. Mckintosh & Fildes anaerobic jar

2. Anaerobic Pouches with colour indicating tablet
3. P.gingivalis ATCC 33277 strain
4. Inoculation wire loop
5. Mcfarlands turbidity tube
6. McConkeys Agar
7. BHI broth
8. Thioglycollate broth
9. Sterile Cotton swab stick
10. Blood Agar culture plates

Study was done using ATCC stock cultures available from Department of Microbiology, Manav Rachna Dental College.

Subculture of stock culture & Preparation of Inoculum

P.gingivalis

a. Subculture & Isolation of P.gingivalis from Vial -ATCC 33277 strain purchased from Himedia and was subcultured in Thioglycolate broth and further cultured on blood agar under anaerobic conditions in Mckintosh & Fildes anerobic jar for confirmation of *P gingivalis*. Turbidity was seen in thioglycolate broth after 10 days. Subculture on blood agar showed black pigmentation after 7-8 days of incubation. Gram staining was performed from the broth which showed varied morphology of coco-bacillary forms and Gram negative bacilli. Biochemical test was done to confirm the presence of P gingivalis. Biochemical tests- Indole test and hydrogen sulphide test showed positive results and motility test was negative.

b.Preparation of Inoculum for performing Agar well diffusion method- *P.gingivalis* was inoculated in Thioglycolate broth and turbidity was measured according to Mcfarlands turbidity tube. The inoculum was incubated for 30mins.

E faecalis

a. *E. faecalis* was cultured on McConkeys and Blood Agar medium . Single colony was inoculated in BHI broth. Broth subculture was done on blood and McConkeys Agar. The bacteria were grown under Aerobic conditions at 37°C for 18-24hrs. *E faecalis* showed lactose fermentation and on blood Agar, it showed non-hemolytic colonies. Gram staining was performed which showed Gram positive ovoid cocci in short chains. Biochemical test was performed to confirm the result. Heat Tolerance Test and Bile Esculin hydrolysis test showed positivity.

b. Preparation of BHI broth Inoculum - E faecalis was inoculated in BHI broth and turbidity was measured according to Mcfarlands turbidity tube. The inoculum was incubated for 30mins.

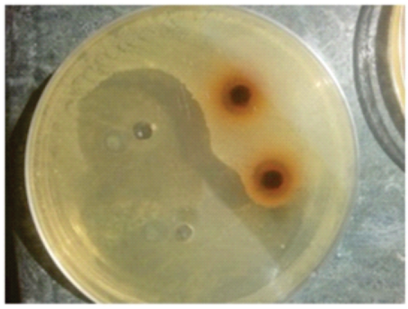
Staph aureus - Pure strains were subcultured on Blood agar medium. Inoculum was prepared using peptone water. β hemolysis with golden yellow pigmentation. Gram staining showed G+ve rods in clusters and confirmed with biochemical test which showed positivity for Mannitol fermentation, Tellurite reduction test and coagulase test.

b. Preparation of Inoculum - *Staph aureus* was subcultured in peptone water and turbidity was matched according to Mcfarland's tube. The bacteria were grown under Aerobic conditions at 37°C for 18-24hrs.

2. Preparation of lawn culture - The inoculum was used to make lawn culture using sterile cotton swab on blood agar plate for *P. gingivalis* and *E. faecalis* while the inoculum of *Staph. aureus* was taken and streaked on Muller Hinton agar plate.

A total of 4 wells were punched in the plates using a sterile punch and the test materials of Green tea extract solutions was placed in 2 wells using a sterile micropipette & and 2 % chlorhexidine solution taken as positive control was placed in the remaining 2 wells. Normal saline was taken as negative control. The depth of well was measured to be 4mm. The plates were incubated at 37°C for 18- 24hrs for aerobic culture of *E.faecalis* & *Staphylococcus aureus* and 7-10 days for anaerobic culture of *P.gingivalis*. Triplicates were done for all the organisms to observe the results.

The diameter of the zone of inhibition was observed in the culture plates and recorded to the nearest size in mm, using the measuring scale.(Fig 4)



3. Dilution of Green tea Extract- Concentration of GTE was serially diluted using sterile saline at 3 different concentrations namely 10^{-5}, 10^{-6} and 10^{-7}.

RESULTS

(Table 1)

Table 1 – Amount of inhibition zone shown by all 3 micro-organisms

	Serial Dilution	Microorganisms					
		E faecalis		P gingivalis		S aureus	
		GTE	Chlx	GTE	Chlx	GTE	Chlx
1.	Undiluted	10	20	15	20	16	20
2.	10-5	08	18	13	18	14	23
3.	10-6	06	16	11	16	12	21
4.	10-7	00	14	09	14	10	19
5.	10-8	00	10	06	12	07	15

a. *E faecalis* - The zone of inhibition was 10mm around GTE undiluted while 20mm around chlorhexidine solution.On increasing the dilution to 10^{-5},10^{-6} , $10^{-7}$and 10^{-8} the inhibition zone decreased to 8mm,6mm,0mm and 0mm respectively with GTE and 18mm,16mm,14mm and 10mm with chlorhexidine.(Fig1) Mean of inhibition zone for GTE was 4.8mm and for chlorhexidine was 15.6mm.

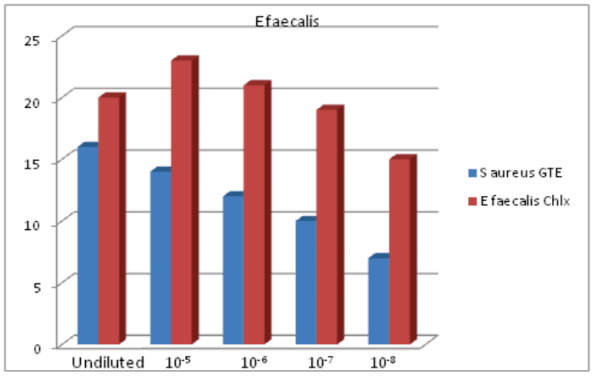


Fig 1 – Bar graph showing effect of catechin and chlorhexidine on E faecalis

b. *P gingivalis* - Undiluted solution of GTE showed 15mm inhibition zone while with chlorhexidine it was 20mm. On increasing the dilution to 10^{-5},10^{-6} , $10^{-7}$and 10^{-8} the inhibition zone decreased to 13mm,11mm,9mm and 6mm respectively with GTE and 18mm,16mm,14mm And 12mm with chlorhexidine. (Fig 2) Mean of inhibition zone for GTE was 10.8mm and for chlorhexidine was 16mm.

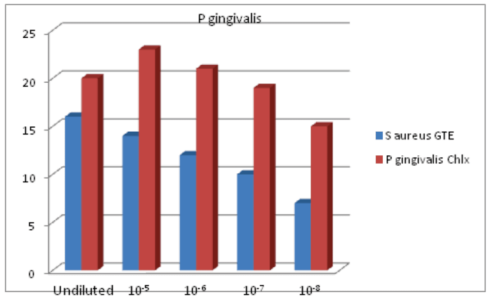


Fig 2- Bar graph showing effect of catechin and chlorhexidine on P gingivalis

c. *Staph aureus* - The zone of inhibition was 16mm with GTE and chlorhexidine was 20mm. On increasing the dilution to 10^{-5},10^{-6} , $10^{-7}$and 10^{-8} the inhibition zone decreased to 14mm,12mm,10mm and 7mm respectively with GTE and 23mm,21mm,19mm and 15mm with chlorhexidine.(Fig 3) Mean of inhibition zone for GTE was 11.8mm and for chlorhexidine was 19.6mm.

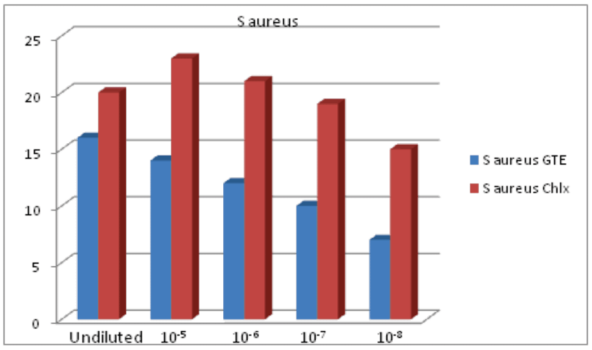


Fig 3 – Bar graph showing effect of catechin and chlorhexidine on S aureus

According to the statistical analysis (Table 2), significant difference was observed in the inhibition zone by green tea extract and chlorhexidine in all 3 microorganisms. While P gingivalis and S aureus showed better inhibition with the extract than chlorhexidine when compared with E faecalis.

DISCUSSION

The major advantage of using herbal alternatives are easy availability, cost-effectiveness, increased shelf-life, low toxicity, lack of microbial resistance. Catechins possess the property of preventing the attachment of oral streptococcal pathogens to surfaces[28]. They are reported to exert antibacterial activity against many pathogens like *E.coli, Streptococcus mutans, Vibrio cholera, Shigella dysenteriae* etc[2]. Catechins in the green tea extract have excellent antioxidant, anti-inflammatory and radical scavenging property[2]. The active antibacterial component has been nailed to be EGCG. EGCG has received much attention for its effect on the inhibition of HIV infection and multi drug resistant Stap aureus infection. The bactericidal action of catechins is due to its hydrogen peroxide generation. a number of membrane-dependent cellular processes such as cell signaling and cell cycle[7] arachidonic acid metabolism and cell proliferation[1] and apoptosis and mitochondrial function[23] may be influenced by interaction of catechins with cellular phospholipid palisade[5].

In vitro, the antimicrobial activity of tea suggested for many years by anecdotal evidence was first demonstrated almost 100years ago in a laboratory by Mc Naught (1906), a Major in the British Army Medical Corps and showed that brewed black tea killed *S typhi* and *B melitensis*. However, systematic research of antimicrobial activity of green tea did not begin until late 1980's.

E.faecalis, facultative anaerobic gram positive cocci is highly prevalent and can facilitate the adherence of host cells and extracellular matrix, tissue invasions, immunomodulation effect and cause toxin mediated damage.
Huber B et al[12] have evidenced that polyphenolic compounds can interfere with bacterial quorum sensing. Epigallocatechin is reported to bind to the ATP site of DNA gyrase b subunit of bacteria and inhibit the activity of gyrase enzyme. Tea catechins, including EGCG, inhibit the growth of planktonic P.gingivalis.

According to study by Nakayama M et al[17] Combined Green Tea Extract / NaCl treatment caused greater cellular damage in *S. aureus* NBRC 13276. Green Tea Extract attached to proteins on the surface of the bacteria to form high-molecular weight complexes.

Su P et al in 2008[24] demonstrated the potential for combined therapy using the green tea extract plus probiotics on microbial infections caused by *Staph. aureus* and *Strep. pyogenes*. As probiotics and the green tea extract are derived from natural products, treatment with these agents may represent important adjuncts to, or alternatives to, conventional antibiotic therapy.

Mageed et.,al in 2015[14] showed that alcoholic green tea extract was able to inhibit and kill Porphyromonas gingivalis and had higher concentration of EGCG than the aqueous extract.

Chlorhexidine at low concentration (0.2%) causes the leakage of potassium and Phosphorus out of the cell and act as a bacteriostatic agent but at higher concentration (2%), chlorhexidine is bactericidal as precipitation of cytoplasmic contents occurs, which results in cell death. Molecular docking calculations highest that the benzopyran ring of EGCG penetrates deep into the active site and the galloyl moiety anchors it to the cleft through.

CONCLUSION

The use of natural herbal products presents a great alternative to chemicals used for treatment. The present study was designed to check the antibacterial property of green tea extract on the 3 oral microorganisms. *P gingivalis* and *S aureus* showed better antibacterial property with green tea extract when compared with *E faecalis*. The fact that green tea extract have shown similar bacterial inhibition to the 3 pathogenic microorganisms studied as chlorhexidine, this leads to the fact that further research should focus on using green tea extract as antibacterial agent. This will further help us to design natural chemotherapy against the disease.

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