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Effect of a Commercial Extract of Green Tea and a Pure Catechin on Two *Veillonella strains*

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Abstract

Abstract: The catechin Epigallocatechin-3-O-Gallate (EGCG) which is found in of Green Tea extracts (GTE), displays a variety of microbicidal properties. It is largely believed that EGCG inhibits the growth of cariogenic and periodontopathic bacteria. **Objective:** In this paper we compared the inhibitory activity of EGCG and a commercial GTE on the growth of *Veillonella parvula*. Chlorhexidine was used as positive control. **Methodology:** *V. parvula* ATCC 10790 and a clinical isolate obtained from a periodontal disease patient were cultured in the presence of EGCG or a commercial GTE, and the measurements of bacterial growth inhibition were compared to the values obtained with 0.12 and 0.2% chlorhexidine. **Results:** Chlorhexidine inhibited bacterial growth, however in contrast to a previous report, neither EGCG nor the GTE showed any effect on bacterial growth. **Conclusions:** The data show and confirm that chlorhexidine is a growth inhibitor of *V. parvula* while EGCG and GTE do not display such effect.

Keywords

Veillonella Parvulla, Chlorhexidine, Green Tea, Catechin

1. Introduction

Green tea has been largely consumed in Asian countries like China for centuries to preserve and improve health. Due to its medical properties green tea is a widely used dietary agent for prophylaxis and to treatseveral diseases (Cabrera *et al.* [1]). The benefits of green tea in health are attributed to an individual and mixtures of catechins contained in green tea extracts, a reason for which they are used in food to promote human health (Vuong *et al.* [2]). Catechins are considered natural antioxidants due to their properties of radical scavenging (Yilmaz *et al.* [3]).

Catechins are flavonoids (flavan-3-ols) and the main catechins present in green tea include a group of functional health compounds, especially catechin, epicatechin, and epicatechin gallates. EGCG represents almost 59% of the total catechins, Epigallocatechin (EGC) 19%, other catechins are approximately 20%. Other components of green tea are gallic acid (AG), chlorogenic acid and caffeic acid, and flavonols (Cabrera *et al.* [1]). The properties of EGCG as anti-inflammatory, antioxidant, anti-cancer, anti-collagenase, and anti-fibrosis compound, increase its potential use and the need for additional studies in this field (Chu [4]).

The mechanism by which green tea may have such effects has not been elucidated. Human clinical studies demonstrate that single doses of up to 1.6 grams of green tea extract are well tolerated. The maximum tolerated dose in humans is reported to be 9.9 grams per day; a dose equivalent to 24 cups of green tea. Side effects of high doses of green tea extract are usually mild and include headache, dizziness, and nausea. The safety and tolerability of long-term use of green tea extracts has not been well defined [5]. However, the amount of EGCG in a daily dose of green tea extract can range from 5 mg to 1000 mg. Based on safety assessment of green tea products, the European Food Safety Authority recently found that green tea supplements providing more than 800 mg of EGCG per day are linked with a greater risk of liver injury [6]. As consumption of green tea has increased in recent years, so too have reports of its adverse effects. Hepatotoxicity is apparently caused by enzymatic interaction that leads to cellular damage and interference with biological response systems and metabolic reactions. Examples are the chemical interactions with enzymes such as UDPGT, alcohol dehydrogenase and cytochrome P450 and interactions with the mitochondrial enzyme and immune systems. This analysis finds that even though the mechanisms by which green tea causes hepatic toxicity are still a mystery, certain catechins of camellia sinensis and interactions at the cellular and mitochondrial levels may be responsible for this toxicity [7].

Polyphenols obtained from green tea participate in transduction events due to EGCG-induced cellular signals that appear to have implications as inhibitors of cellular events of induction of apoptosis of cells preneoplastic and neoplasticstage, angiogenesis, and metastasis (Khan *et al.* [8]). It is well known that the interaction of EGCG within several diseases including cancer, neurological and cardiovascular diseases [9]. Also, EGCG has shown used in diseases like Parkinson's, disease, stroke, Alzheime's disease, obesity, diabetes, and high antioxidant activity. It has also been used as an anti-inflammatory treatment (Mata-Bilbao *et al.* [10]). Various authors have studied the inhibitory effects of catechin contained in green tea on periodontal pathogens, which may provide the basis for beneficial effect of daily intake of green tea on periodontal health [11]. Green tea catechins with steric structures of 3-galloyl radial, EGCG, ECG and gallocatechin gallate, which are major tea polyphenols, inhibit production of toxic end metabolites of *P. gingivalis*. A study showed that green tea catechin, EGCG and ECG inhibit the activity of *P. gingivalis*-derived collagenase [12]. Hirasawa *et al.* showed that, Epigallocatechin gallate (EGCG) and epicatechin gallate inhibited lactate dehydrogenase activity

much more efficiently than epigallocatechin, epicatechin, catechin or gallocatechin. These results suggest that EGCG is effective in reducing acid production in dental plaque and mutans streptococci [13]. The amounts of catechins were always higher in green tea. EGCG and EGC were major catechins present with average contents of 7.358% and 3.955%, respectively; ECG and EC values are 0.910 and 3.556% respectively [1]. Catechins are flavonoids (flavan-3-ols) and the main catechins present in green tea include a group of functional health compounds, especially catechin, epicatechin, and epicatechin gallates [14].

Polyphenols are found in green tea leaves, within polyphenols are catechins that are naturally found in various foods and medicines, such as legumes, Rubiaceae, buckwheat, stem bark of Fabaceae species, cocoa beans, grapes, lychees, and apples (Cabrera [1]). The highest antimicrobial activity of tea is due to presence of catechins polyphenols which damage the bacterial cell membrane [15]. The bactericidal action of catechin is due to its hydrogen peroxide generation [16]. Previous studies of GTE have shown the inhibitory activity of cariogenic and periodontophatic bacteria and could be used in mouthwashes to prevent periodontal diseases and dental caries (Araghizadeh *et al.* [17], Awadalla *et al.* [18]). Evidence shows that EGCG has the potential to decrease bone loss in conditions that occur in osteoporosis and periodontal disease (Vargas-Sanchez *et al.* [19]).

In a recent trial it was found that purified EGCG improves the results of non-surgical treatment of periodontal lesions (Wang *et al.* [20]). Gadê-Neto CR et al, analyzed in their study that the predominant bacteria in periodontal pockets are: *Porphyromonas gingivalis, Veillonella parvula*, *Fusobacterium necrophorum, Prevotella loescheii* (Gadê-Neto *et al.* [21]). Chronic inflammatory diseases of the gingiva could lead to affect the supporting and protective tissues of the tooth. Periodontitis is more significant because it can cause tooth loss; however, all periodontitis begins with the initial presence of a gingivitis. The main cause of periodontal disease is the deposit of bacteria and their products on oral surfaces;thus, the use of antimicrobial drugsis a suitable choice in the treatment of periodontitis (Cabrera *et al.* [1]).

Conventional mechanical and/or surgical treatment is the most important aspect of periodontal treatment, although in some cases the use of antibiotics as a complement to periodontal therapy, however this implies side effects on the patient. The ideal antibiotic for the treatment of periodontal disease should be specific for periodontal pathogens, non-toxic and inexpensive, however that are available do not meet all the requirements. Having a natural antibiotic that does not cause allergies and whose action in the body is not aggressive, does not cause discomfort and is economical, would be a good option in periodontal treatments, since the mode of action is different in natural products than in the drugs. Based on this, it is important to identify natural alternatives that are effective to treat periodontal disease. Various investigations have studied the positive effects of EGCG in the field of dentistry. It is considered important to evaluate its effects on bacterial inhibition and compare its effects with a commercially used GTE applying it specifically on the periodontopathogenic bacteria such as *Veillonella* [21].

Veillonella microorganisms are strictly anaerobic non-fermentative Gram-negative cocci. They are members of the normal microbiota of the mouth, respiratory, genitourinary tracts, and gut of humans. There are eight species as a part of genus Veillonella (*Matera et al.* [22]). *Veillonella species of the mouth are well known as early colonizers in the in the in vivo biofilm formation of composed of multiple species and to facilitate the succession of species in the development of oral biofilms in vivo. There are six species that inhabit dental biofilms on the mouth mucosa* (*Mashima et al.* [23]).

Periodontal disease, preterm labor, intravenous drug use, and immunodeficiency are some of the risk factors for infection due to *Veillonella. V. parvula* is the main etiological agent implicated in periodontitis and other anaerobic infections such as neck infections and chronic sinusitis infections. They have been also reports the osteomyelitis, meningitis, and other acute and severe infections like chronic pleuropulmonary infection (Matera *et al.* [22]). Several species of-*Veillonella* spp, including *V. parvula* have been isolated frommouth of patients with severe cases of caries in toddlers, in intraradicular infections (including abscesses). They can produce sulfur compounds that are responsible for oral malodour (Mashima *et al.* [23]).

In addition, some phyllotypes, such as *Eikenella corrodens* and *Fusobacterium nucleatum*, are significantly more often found in patients with diabetes than in non-diabetic persons (Casarin *et al.* [24]). *Veillonella* also can produce vitamin K, which it is known that could stimulate the growth of *Porphyromonas gingivalis*, a typical periodontal pathogen. The isolation in the periodontal pockets of *V. parvula* is significantly higher than in the sulcus of the gingiva. Therefore, *V. parvula* is a pathogen associated with chronic periodontitis onset (Mashima *et al.* [23]).

Objective:

To compare the inhibitory activity of EGCG and a commercial GTE on the growth of *Veillonella parvula.*

2. Materials and Methods

We used *V. parvula* ATCC 10790 (reference strain) and a clinical *V. Parvula* isolate obtained from a chronic periodontitis patient at the School of Stomatology.

The selection criteria were the following:

Inclusion: Cultures of *Veillonella parvula* ATCC 10790 with the appropriate amount and dilution of the components used.

Exclusion: Cultures of *Veillonella parvula* ATCC 10790 contaminated with other bacteria.

Elimination: Cultures in which a colonial morphology different from that of the *Veillonella parvula* strain ATCC 10790 is observed.

Description of Procedures

The present work did not develop a new procedure that should be standardized since there is already a reference to a previously standardized procedure by Dong *et al.* [25], Shumi *et al.* [26]. Therefore, this previously standardized tech-

nique was used. The procedure is briefly described below.

To guarantee an adequate performance of the technique, each test was carried out in triplicate at the same time carried out by the expert researcher and the thesis student and the result was the average of the triplicate test. The test was considered adequately performed when there was consistency in the results obtained.

Growth inhibition: It was considered that there was growth inhibition, when there was a lack of bacterial growth around a paper disk containing some growth inhibitory substance.

The way in which growth inhibition was evaluated was by measuring a zone of absence of bacterial growth measured in millimeters around the perimeter of the paper disk; any zone of absence of bacterial growth around the paper disk was considered as "zone of inhibition of growth".

Positive control: A positive control was used in the present study. Chlorhexidine at 0.12% and 0.2% was used as a positive control, because according to the literature this substance inhibits bacterial growth, since these substances have been used for a long time in periodontal treatments to control biofilm.

Therefore, any zone of inhibition of bacterial growth around the paper impregnated with the different concentrations of chlorhexidine was considered operationally during the present work as a positive control.

The growth inhibitory effect on *Veillonella parvula* quantified in millimeters due to EGCG and commercial green tea extract was compared with the growth inhibition produced by chlorhexidine.

Techniques and Procedures.

For this study, 2 strains of *Veillonella parvula*, strain ATCC 10790 and wild strain, and a positive control (chlorhexidine 0.12% and 0.2%) were used.

The Kirby-Bauer disk approximation method (KBDA) was used, the interpretation of susceptibility to the 3 substances was based on the sizes of the inhibitory zone [27]. Briefly, we took around 5 colonies of each Veillonella strain used in this work, with a wire loop from the original culture plate and introduced into a test tube containing 4 ml of trypticase soy broth. These tubes were incubated for around 4 hr., to produce a bacterial suspension, then it was diluted, to match the 0.5 McFarland standard which was prepared as describe below. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/liter H2SO4 (1% vol/vol) with constant stirring to maintain a suspension. Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy. Use this suspension within 15 minutes of preparation. Organisms to be tested must be in the log phase of growth. It is recommended that subcultures of the organisms to be tested be made the previous day. Dip a sterile swab into the inoculum tube. Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. We used thioglycolate agar plates enriched with 5% Sheep Blood. Plates were dried for about 30 min. before inoculation and are used within 4 days of preparation [27] [28].

The swab should not be dripping wet. Inoculate the plate with the test organism

by streaking the swab in a back-and-forth motion very close together as you move across and down the plate. Rotate the plate 60˚ and repeat this action. Rotate the plate once more and repeat the streaking action. This ensures an even distribution of inoculum that will result in a confluent lawn of growth. After the inoculum has dried, the disks were placed on the agar with flamed forceps to ensure contact. After overnight incubation at temperature range of 35˚C ± 2˚C, the zone diameters were measured with a ruler on the undersurface of the Petri dish or with calipers near the agar surface. All measurements were made with the unaided eye while viewing the back of the petri dish. It was holding the plate a few inches above a black, nonreflecting surface illuminated with reflected light. We viewed the plate using a direct, vertical line of sight to avoid any parallax that may result in misreading. The data was recorded the zone size on a recording sheet [27] [28].

7 Petri dishes were labeled with the following names:

Chlorhexidine 0.12%;

Chlorhexidine 0.2%;

GTE 62.5 mg/mL;

GTE 100 mg/mL;

EGCG 2.5 mg/mL;

EGCG 12.5 μg/mL;

EGCG 125 μg/mL.

The paper discs were placed inside the boxes, in each box there are 20 discs, each tube is opened with the reagent and the automatic micropipette is calibrated to 10 μl and is applied to each disc.

Bacterial strains (type strain and wild strain) were harvested in the Petri dishes, the bacteria are needed in an exponential phase since they need to be actively growing in the periodontal pockets.

Each box containing the culture medium was inoculated, some with a Type strain and others with a wild strain 3 previously prepared discs were placed in each one.

The Petri dishes were incubated at 37˚C elsius for 48 hrs. to observe the inhibition halos.

After carrying out the inoculation, the 3 discs were placed per plate, allowing to rest for 48 h to observe the inhibition halos.

The Petri dishes of both strains of *V. parvula* are shown below and inhibition was observed in them according to the substance used.

Disk diffusion zone determinations were made using a modified Kirby-Bauer technique. Each one of the Petri dishes was opened and the inhibition halo was measured with a vernier caliper.

3. Results

In the plates with the *V. parvula* ATTC 10790 strain and *V. parvula* wild strain in which the following reagents were used; GTE 62.5 mg/mL, GTE 100 mg/mL, EGCG 2.5 mg/mL, EGCG 12.5 μg/mL, EGCG 125 μg/mL, no inhibition was observed. In the positive control (Chlorhexidine 0.12% and Chlorhexidine 0.2%)

Table 1. Values of inhibition halo diameters.

	Inhibition halo diameter (mm)		Inhibition halo diameter (mm)
VpTs + GTE 62.5 M*	0 (no inhibition)	VPWs + GTE 62.5 M*	0 (no inhibition)
VpTs + GTE 100 M*	0 (no inhibition)	VPWs + GTE 100 M*	0 (no inhibition)
VpTs + EGCG 2.5 M*	0 (no inhibition)	VPWs + EGCG 2.5 M*	0 (no inhibition)
VpTs + EGCG 31.25 μ*	0 (no inhibition)	VPWs + EGCG 31.25 μ*	0 (no inhibition)
VpTs + EGCG 125 μ*	0 (no inhibition)	VPWs + EGCG 125 μ*	0 (no inhibition)
VpTs + Chlorhexidine 0.12%	16	VPWs + Chlorhexidine 0.12%	14
VpTs Chlorhexidine 0.2%	16	VPWs + Chlorhexidine 0.2%	15

VpTs = *Veillonella parvula* ATTC10790;

VPWs = *Veillonella parvula* Wild strain;

M* = mg/mL;

μ* = μg/mL.

the inhibition was 16 mm and in the plate with wild *V. parvula* Chlorhexidine 0.2% the inhibition was 16 mm, while in the plate with *V. parvula* wild strain Chlorhexidine 0.12% the inhibition was 14 mm (Table 1).

4. Discussion

The data reported here indicate that the disk diffusion technique of Kirby-Bauer *et al.* is reliable for the detection of *V. parvula.* The purpose of this research was to measure the inhibitory growth effect of EGCG, as well as a commercial GTE applied on the growth of a Type strain and a wild-type strain of *V. parvula* compared to chlorhexidine. Other studies have suggested that EGCG and GTE inhibit growth of *V. parvula*. The results obtained in this study demonstrate that chlorhexidine has better inhibitory effects on *V. parvula* growth [29]. In contrast, Kaur *et al.* [30], indicated that a mouthwash containing green tea catechin has an antiplaque efficacy comparable to a mouthwash containing chlorhexidine if it used for at least one week, tastes better and has no know any side effects. Similarly, other research work showed that a mouthwash containing ethanol, chlorhexidine, and GTE or chlorhexidine alone, exhibits inhibitory effect of the growth of several species of mouth bacteria, showing greater activity particularly in the case of pathogenic bacteria (Nomura *et al.* [31]).

Emoto *et al.* [21] determined that the consumption of large amount of GTE has been associated with clinical damage due to hepatotoxicity and can induce hepatocellular damage in rodents, even when it seems to have not that effect under normal consumption. Most of these cases who presented a pattern of acute

hepatocellular damage and the most of patients recovered when they stop the use of GTE. For this same reason, higher doses are not proposed to avoid undesirable effect in the patient (Emoto *et al.* [32]).

Although, other studies have shown the capacity of green tea catechins as a preventive and therapeutic potential against periodontal lesions. Also, GTE and particularly EGCG showed inhibition of the adherence and growth of *P. gingivalis*, by its ability to produce a negative expression of genes encoding the factors related to virulence (Fournier-Larente *et al.* [33]).

Different levels of maturation of green tea leaves, (white and black tea) extracts show ability to health for periodontal disease due to its ability to interfere with the growth and inhibit *P. gingivalis* virulence factors, enzymes that can produce damage, and secretion of mediators related with inflammation. These green tea extracts can be incorporated into different products administered orally or locally to diseased periodontal sites (Zhao *et al.* [34], Venkateswara *et al.* [35]).

5. Conclusions

The objective of this study was to assess the use of natural substances that allow the inhibition of *Veillonella parvula* strains, which has been identified as the primary colonizer, viable doses were used to carry out bacterial inhibition, although this was not achieved, without However, the positive effects of 0.12% and 0.2% chlorhexidine were proven, since for a long time it has been considered the gold standard in biofilm control but causes side effects (brown staining in teeth and soft tissues, calculus formation dental and alterations in the sense of taste), for this same reason it is considered important to propose some other agent for a good control of bacterial plaque and use it as an adjuvant agent in periodontal treatment.

It is suggested to continue investigating and testing other effective substances for the control of this bacterium, determining well the dose and the mode of use to achieve effectiveness, in this study higher doses of green tea extract are not proposed since it has been seen that it can cause liver problems in high doses. It is important to remember that a single substance is not capable of inhibiting all the bacteria involved in the development of periodontal disease due to the large number of species that we can find in the biofilm. Therefore, it would be a good option to apply the same protocol but for other bacteria involved in the development of periodontal disease.

Author Contributions

Conceptualization, J.A.Y.S. and J.A.G.; methodology, L.C.R.; validation, L.C.R.; investigation, V.L.A. and E.I.C.R.; resources, L.C.R..; writing—original draft preparation, J.A.Y.S. and J.A.G.; writing—review and editing, J.A.Y.S. and V.L.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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