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Scientific Research

Antibacterial Effect of Aqueous Extracts and Bioactive Chemical Compounds of *Coffea canephora* against Microorganisms Involved in Dental Caries and Periodontal Disease

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Abstract

This study aimed to evaluate the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Coffea canephora* aqueous extract (Cc), trigonelline (Tg) and 5-caffeoylquinic acid (5-CQA) against cariogenic microorganisms: *Streptococcus parasanguinis* ATCC 903 (SP), *Lactobacillus rhamnosus* ATCC 9595 (LR); and pathogens related to periodontal disease: *Porphyromonas gingivalis* ATCC 33277 (PG), *Fusobacterium nucleatum* ATCC 25586 (FN), *Prevotella intermedia* ATCC 49046 (PI) and *Prevotella nigrescens* ATCC 33563 (PN). Different concentrations of Cc (0.15625 to 10 mg/mL), Tg and 5-CQA (0.005 to 10.24 mg/mL) were tested. Chlorhexidine (0.05%) was used as positive control and the substances without the inoculum comprised the blank control. The Cc showed bacteriostatic action against SP (MIC = 5 mg/mL) and LR (MIC = 10 mg/mL). Tg showed bacteriostatic action against SP (MIC = 2.56 to 1.28 mg/mL), LR (MIC = 2.56 mg/mL), PG (MIC = 2.56 to 1.28 mg/mL), FN (MIC = 5.12 mg/mL), PN (MIC = 2.56 mg/mL), and PI

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(MIC = 2.56 to 1.28 mg/mL). Also, Tg showed bactericide properties against SP (MBC = 2.56 mg/mL), PG (MBC = 2.56 mg/mL), FN (MBC = 10.24 mg/mL), PN (MBC = 5.12 mg/mL), and PI (MBC = 2.56 mg/mL). Although 5-CQA has previously shown activity against *Streptococcus mutans*, in the present study, it showed no activity against all tested microorganisms. *C. canephora* extract only showed antibacterial activity against cariogenic microorganisms, not presenting action against periodontal pathogens. It was concluded that trigonelline presented the best effect against all pathogens tested, therefore coffee extracts with higher trigonelline content should be tested against these specific pathogens.

Keywords

Oral Pathogens, Dental Caries, Periodontal Disease, Coffee, Trigonelline, Chorogenic Acid

1. Introduction

Oral health is essential to general health and quality of life. It implies being free from mouth and facial pain, oral and throat cancers, oral infection and sores, periodontal disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing [1]. Despite great achievements in oral health of populations globally, problems still remain in many communities all over the world, since dental caries and periodontal diseases have been considered the most important global oral health burdens [2].

These two most prevalent oral diseases are dental biofilm dependable [3] [4]. In the early stages of development, dental biofilm comprises predominantly oral streptococci and actinomyces, and usually exists in commensal harmony with the host. However, later population shifts lead to overrepresentation of acidophiles, such as *Lactobacilli* species or of Gram negative obligate anaerobes in subgingival plaque, which, respectively, contribute to the onset and progression of the dental caries and periodontal diseases [5]-[7]. Thus, dental biofilm control represents a basic procedure to prevent these oral diseases.

Mechanical removal of the biofilm, performed by appropriate use of toothbrush and dental floss, has been the main tool in oral hygiene care [8]. However, despite recognition of the effectiveness of mechanical biofilm control, the process may be potentialized by chemical control, especially in individuals at high risk to develop oral diseases [8] [9]. The foremost chemical agents currently available are chlorhexidine, triclosan, cetylpyridinium chloride, and natural products [10] [11]. In this respect, natural products have been proposed in attempt to minimize undesirable effects caused by synthetic agents such as imbalance of the resident microbiota, and altered taste, besides representing a new antimicrobial possibility that might be used to face a situation of microbial resistance [12].

According to Newman and Cragg [13], medicinal plants have been investigated as a natural resource to treat microbial infections and also subsidize the development of new drugs with specific therapeutic properties. Among several plant species presenting antibacterial properties, coffee is the most popular in terms of consumption [14] [15]. The two most commercialized coffee species are *Coffea arabica* and *Coffea canephora*, and in a recent study [16], the authors reported that *C. canephora* extracts exerted better performance in relation to inhibition of a *Streptococcus mutans* biofilm compared to *C. arabica*. Additionally, the same research group affirmed that trigonelline and chlorogenic acids, which are bioactive coffee chemical compounds, largely found in *C. canephora*, showed antibacterial activity against these cariogenic bacteria [16] [17].

Despite all the evidence concerning the antibacterial activity of *C. canephora* against *S. mutans* [16]-[18], studies clarifying the role of coffee extract and isolated compounds on the growth of cariogenic microorganisms different from *S. mutans* are still needed. Furthermore, the effect of coffee species and its bioactive chemical compounds against oral pathogens related to periodontal disease has not yet been investigated. Exploring these themes would result in a more realistic perspective of the use of *C. canephora* as an oral disease preventable product.

Therefore, in the present study, we evaluated the antibacterial effect of a *C. canephora* aqueous extract against planktonic forms of both: cariogenic bacteria such as *Streptococcus parasanguinis* and *Lactobacillus rhamnosus*; and some of the main bacteria involved in periodontal disease—*Porphyromonas gingivalis*, *Fusobacterium*

nucleatum, *Prevotella intermedia* and *Prevotella nigrescens*.

2. Materials and Methods

2.1. *Coffea canephora* Extract

Regular *Coffea canephora* (Cc) cv. Conillon beans (voucher: Brasil, Estado do Espírito Santo, Distrito de Cachoeiro de Itapemirim, Fazenda Experimental de Pacotuba, INCAPER, A.G. Antonio, s/n—RFA 37915) were roasted in a commercial spouted bed roaster (I-Roast-2, Gumee, IL, USA), operatingat a max temperature of 220˚C, for 6 min, to produce a moderately light roasting degree (SCAA, USA). Roasted coffee beans were ground in a laboratory-scale mill to pass through a 0.46 mm sieve. An aqueous coffee extract at 20% was obtained by a coffee brewing procedure, percolating 100 mL of pre-boiling (95˚C) Milli-Q purified water through 20 g of ground roast coffee.

2.2. Coffee Chemical Compounds

Chlorogenic acid (5-caffeoylquinic acid-5-CQA) and trigonelline (Sigma-Aldrich, St. Louis, MO, USA) solutions at 5 mg/mL were prepared using Milli-Q purified water (Millipore Corp., Billerica, Ma, USA). All standard solutions were filtered through sterile filter paper disks (TPP, 0.22 µm membrane, Zurich, Switzerland) prior to microbiological tests.

2.3. Characterization of Trigonelline and Chlorogenic Acid Compounds from *Coffea canephora* Extract

The contents of chlorogenic acids were determined by gradient LC-DAD-ESI-MS according to Farah *et al*. [19], using a Magic C30 column (150 × 2.0 mm, 5 µm, 100 Å, Michrom Bioresources, Inc., Auburn, CA); whereas the contents of trigonelline (Tg) were determined by LC-ESI-MS according to Perrone, Donangelo and Farah [20], using a Spherisorb® S5 ODS2 Microbore column (150 × 2.0 mm, 5 µm, Waters, Milford, MA, USA). The LC equipment (Shimadzu, Kyoto, Japan) comprised a LC-10AD*vp* quaternary pump, a CTO-10AS*vp* column oven, an 8125 manual injector (Rheodyne) with a 5 µL loop and a SPD-M10A*vp* diode array detector. This LC system was interfaced with a LC-MS 2010 mass spectrometer (Shimadzu, Kyoto, Japan).

For sample clarification prior to the chromatographic analyses of the phenolic compound (5-CQA), 500 µL of the *C. canephora* extract were mixed with 500 µL of each Carrez solution and the volume was made up to 50 mL with Milli-Q water. For the determination of trigonelline, Carrez solutions were replaced by 1 mL of saturated basic lead acetate solution [19]. The mixtures were shaken, let rest for 10 min and filtered through a Whatman #1 qualitative filter paper and a 0.22 µm membrane (Millipore). The filtrates were used directly for chromatography. Chemical analyses were performed in triplicate.

2.4. Bacterial Strains and Culture

The bacterial strains used were from the American Type Culture Collection (ATCC): 1) cariogenic bacteria—*S. parasanginis* ATCC 903 and *Lactobacillus rhamnosus* ATCC 9595; 2) bacteria involved in periodontal disease —*Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 25586, *Prevotella nigrescens* ATCC 33563 and *Prevotella intermedia* ATCC 49046. Bacteria were kept at –20˚C in Tryptic Soy Broth (TSB) (Oxoid, Hampshire, England) with 20% glycerol and activated by transfer into blood agar (Plast Labor, Rio de Janeiro, Brazil), and incubation at 37˚C for 48 h, with 5% CO_2 for cariogenic bacteria, and anaerobic condition for those periodontal pathogens. Bacterial cells were suspended, according to the 0.5 McFarland scale [21], in saline solution to produce a suspension of about 1.5×10^8 CFU/mL. 300 µL of this suspension was mixed with 9.7 mL of Mueller-Hinton bacterial broth medium (Difco, Sparks, USA) for cariogenic bacteria and for bacteria involved in periodontal disease (Gram-negative species), the medium was the Bruscella PRAS. These procedures resulted in inoculuns with 4 - 5 × 10^6 CFU/mL.

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

The antibacterial activity of Cc, Tg and 5-CQA was examined by determining the minimum inhibitory concen-

tration (MIC) and minimum bactericidal concentration (MBC), in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. MIC was performed in 96-well microplates, inoculated with 4 - 5 × 10^5 CFU/mL (final concentration), using Mueller-Hinton (Difco, Franklin Lakes, NJ, USA), when cariogenic bacteria were tested; and Bruscella PRAS medium in the case of periodontal pathogens. The concentrations of Cc and chemical compounds ranged from 0.15625 to 10 mg/mL and 0.005 to 10.24 mg/mL, respectively. The positive control was 0.05% chlorhexidine digluconate and the substances without the inoculum comprised the blank control. An inoculated Mueller-Hinton Broth medium (Difco) and Bruscella PRAS medium without the test compounds was the negative control.

The plates with cariogenic bacteria were incubated at 37˚C with 5% CO_2 for 48 h, and those with periodontal pathogens were incubated in anaerobic condition (37˚C) for 48 h. MIC was defined as the lowest concentration of Cc or chemical compounds that allowed no visible growth, confirmed by optical density (absorbance of 600 nanometers) of 0.4 in a microplate reader (spectra max-Molecular Devices, USA). All experiments were performed in triplicate. MBC was determined by sub-culturing on blood agar 50-µL aliquots of each incubated well that presented concentration higher than the MIC [23].

2.6. Statistical Analysis

SSPS software, version 20.0 was used for statistical analysis. The Kruskal-Wallis test was used for statistical comparison of susceptibility assays results, among the tested substances. A 5% significance level was considered.

3. Results and Discussion

The tests with antimicrobial agents on oral bacteria may qualify an agent for the treatment of biofilm-dependent oral infections or disqualify it, if these tests prove negative results [24]. Therefore, *C. canephora* aqueous extract and two of its bioactive compounds, 5-CQA and trigonelline, were tested for some cariogenic and periodontal pathogens growth inhibition. For those compounds and extract showing growth inhibition in any of the tested concentrations, MIC and MBC were determined. The results obtained on the inhibitory activity of the different concentrations of *C. canephora* extract, 5-CQA and Trigonelline against the tested strains, can be observed in **Table 1**. As expected, the positive control (0.05% chlorhexidine digluconate) showed bactericide activity against all oral pathogens tested.

Regarding results from incubation with 5-CQA, despite the fact that a few studies have demonstrated that this compound exerts a strong inhibitory performance against several microorganisms such as *S. mutans* [16] [18], *Leigionella pneumophila* [25], *Serratia marcescens* and *Enterobacter* cloacae [26], in the present study, it was not able to inhibit the growth of all pathogens tested here, even at concentrations higher than those tested by the researches cited above.

It is known that one of the mechanisms for the antimicrobial action of phenolic compounds is related to inactivation of cellular enzymes, which depends on the rate of penetration of the substance into the cell or caused by membrane permeability changes [27] [28]. Increased membrane permeability is a major factor in the mechanism of antimicrobial action, where compounds may disrupt membranes and cause a loss of cellular integrity and

Table 1. Antibacterial (MIC) and Bactericidal (MBC) activities of *C. canephora aqueous* extract, 5-CQA and trigonelline.

Bacterial strain (ATCC)	*C. canephora* extract		5-CQA		Trigonelline	
	MIC	MBC	MIC	MBC	MIC	MBC
	mg/mL					
S. parasanguinis	5.0	-	*ND*	-	2.56 to 1.28	2.56
L. rhamnosus	10.0	-	*ND*	-	2.56	-
P. gengivalis	*ND*	-	*ND*	-	2.56 to 1.28	2.56
P. intermedia	*ND*	-	*ND*	-	2.56 to 1.28	2.56
P. nigrences	*ND*	-	*ND*	-	2.56	5.12
F. nucleatum	*ND*	-	*ND*	-	5.12	10.24

All experiments were performed in triplicate. Results are expressed in mg/mL. *ND*, Not determined. (-), Not detected. Among the tested substances, trigonelline showed the highest antibacterial properties ($p < 0.05$, Kruskall Wallis test).

eventual cell death [29]. Despite this information, the antimicrobial potential of the phenolic acids may be different against several microorganisms due to the number and position of substitutions in the benzene ring of phenolic acids and the saturated side-chain and length [30]. Therefore, statistical analyses have been applied to confirm similarities and differences among phenolic acids based on their antimicrobial potency [31]. In general, variations in antimicrobial activities among bacteria may reflect differences in cell surface structures between Gram-negative and Gram-positive species. Although Gram-positive species appeared to be more susceptible to the action of phenolic acids than Gram-negative bacteria in previous works [31], our present results are partially in accordance with these information, since not only Gram-negative bacteria (*P. gingivalis*, *F. nucleatum*, *P. intermedia* and *P. nigrescens*) presented resistance to 5-CQA, but also the Gram-positive species tested (*S. parasanguinis* and *L. rhamnosus*).

Gury *et al*. [32] also affirmed that phenolic acids are toxic for numerous Gram-positive bacteria under acidic condition. These authors stated that phenolic acid decarboxylase activity (PAD) in these bacteria is a detoxifying system specifically and strongly induced by these chemicals. Two genes involved in the phenolic acid stress response have been characterized in *Bacillus subtilis* (also a Gram-positive bacterium): *padA* and *padR*. The *padA* gene (named *padC* in *B. subtilis*) encodes the PadA enzyme and *padR* encodes the PadR transcriptional repressor. Deletion of *padA* leads to growth inhibition in the presence of phenolic acids [33], while deletion of *padR* leads to constitutive over expression of *padA* and, consequently, to high resistance to phenolic acids. So, we hypothesize that the Gram-positive bacteria tested in the present study could present some similar genetic characteristics to the mentioned *B. subtilis*, resulting in an over expression of some gene responsible for its resistance to 5-CQA. However, this matter should be investigated in future studies.

Despite the negative inhibitory effect of 5-CQA on *S. parasanguinis* and *L. rhamnosus* (Gram-positive bacteria), we observed that the *C. canephora* aqueous extract, which is rich in phenolic compounds (2.47 ± 0.734 mg/mL; **Table 2**), showed bacteriostatic activity against these microorganisms (MIC = 5 mg/mL for *S. parasanguinis* and MIC = 10 mg/mL for *L. rhamnosus*). Like 5-CQA (responsible for about 60% of total chlorogenic acids in coffee extract), other CQA and diCQA isomers are also formed from caffeic and quinic acids, and it is very possible that these compounds in *C. canephora* extract, jointly exert antibacterial activity, contributing to a possible synergistic antibacterial action. In addition, trigonelline contents, and of its derivatives, in *C. canephora* extract may also have contributed to its positive antibacterial properties against the tested cariogenic bacteria, since this substance showed a strong antibacterial activity (**Table 1**) for all pathogens. Moreover, the authors speculated that other non-bacteriostatic compounds such as polysaccharides, aminoacids, etc. that are also part of coffee composition may have contributed to increase the inhibitory properties of coffee extract on bacteria cell-growth compared to those of the isolated 5-CQA.

Trigonelline was bacteriostatic for all tested oral bacteria and also bactericidal, except for *L. rhamnosus*. Selected vitamins such as d-biotin, pyridoxine, p-aminobenzoic acid, nicotinic acid, thiamine, pantothenic acid and riboflavin enhance the lactic acid production by *L. rhamnosus* [34]. Thus, the present authors hypothesize that

Table 2. Contents of cinnamic acid derivatives (chlorogenic acids) and trigonelline in *Coffea canephora aqueous* extract at 20%[a,b].

Chemical compounds	Mean ± SD
Cinnamic acid derivatives	2.47 ± 0.734
3-CQA	0.53 ± 0.035
4-CQA	0.60 ± 0.036
5-CQA	1.00 ± 0.042
4 + 5-FQA	0.24 ± 0.024
3,4-diCQA	0.03 ± 0.002
3,5-diCQA	0.02 ± 0.001
4,5-diCQA	0.05 ± 0.003
Trigonelline	0.69 ± 0.014

[a]Results are shown as mean of triplicate analysis, expressed in mg/mL ± Standard Deviation; [b]CQA = cafeoylquinic acids, FQA = feruloylquinic acids, diCQA = dicaffeoylquinic acids, SD = standard deviation.

these vitamins could consequently improve the survival of these microorganisms. Following this reasoning and considering that trigonelline is a *N*-metyl-betaine, which is demethylated to form nicotinic acid or degraded into low molecular weight compounds during the roasting of coffee, we suggest that own trigonelline or some unknown mechanism during the experiment could also demethylated the referred substance in niacin (nicotinic acid), which could favored the *L. rhamnosus* resistance to this substance. Even so, the presence of trigonelline resistance determinants in the bacteria genome must be systematically screened.

In the present study, for the first time, the effects of different concentrations of *C. canephora* extract and some of its bioactive compounds 5-CQA and trigonelline were tested on bacteria related to periodontal disease and also cariogenic bacteria different from *S. mutans*. Our results showed that among all substances studied, trigonelline was the most effective against the growth of the oral pathogens tested. These findings could be of interest because like chlorogenic acids and caffeine, trigonelline is one of the most abundant bioactive compounds in raw coffee beans [35] and coffee by-products, and it is a natural ingredient that may be obtained in large quantities at low cost relative to drugs. Corroborating to this fact, Anila Namboodiripad and Kori [36] observed a reduction of teeth caries in a population with the habit of coffee consumption compared to other without the same custom.

In contrast, coffee consumption may not be effective on periodontal diseases, since the evaluated extract showed only inhibitory properties against tested cariogenic bacteria and not the bacteria involved in periodontal diseases. However, other important microorganisms also implicated in such illness such as *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis* and *Treponema denticola*, which were not investigated in the present study, could be non-resistant to coffee extract. Moreover, arabica coffee extract richier in trigonelline and derivatives should still be tested. Therefore, as the *in vitro* tests employed in this study are essentially preliminary screenings, further investigation should be performed to verify the effect of coffee extracts and their components on these bacteria.

An important topic that cannot fail to be mentioned is that some studies have suggested that consumption of coffee is associated with a significant increase in risk of fracture, osteoporosis and also periodontal disease [37] [38]. Hypotheses to explain these associations have centered on the caffeine content of coffee [39]. However, this is controversial in the literature, since *in vivo* studies have shown that coffee does not stimulate the bone loss [40] [41]. Furthermore, Duarte *et al*. [42] described the positive correlation between caffeine consumption in the early stages of bone healing and the bone loss in rats, but they emphasized that it only occurred when a high daily caffeine intake is administrated. In this case, decaffeinated coffee could be an alternative. Nevertheless, Antonio *et al*. [16] and Almeida *et al*. [18] showed that caffeine enhances the inhibitory effect of coffee extracts against of *S. mutans*'s growth. Bearing in mind that only trigonelline showed a strong antibacterial effect on periodontal pathogens, the consumption of coffee beverage as a preventable ingredient for periodontal disease may be an insubstantial prescription. Even so, the authors suggest that the antibacterial activity of other bioactive coffee chemical compounds such as ferulic acid and caffeic acid should be investigated against the oral pathogens related to pediodontal diseases.

4. Conclusion

The present study showed that trigonelline exerts inhibitory activity against the growth of *S. parasanguinis*, *L. rhamnosus*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *F. nucleatum*, that such activity is stronger on period onto pathogens than the cariogenic ones, and that *C. canephora* extract seems to exert better performance in relation to inhibition of cariogenic bacteria as opposed to those involved in periodontal diseases.

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