Antimicrobial Activity of Plants Infusions on Oral Fusobacteria and their Adherence to Human Erythrocytes

Atividade antimicrobiana de infusões de vegetais sobre fusobactérias bucais e sua capacidade de adesão a eritrócitos humanos

Actividad antimicrobiana de infusiones vegetales en fusobacterias orales y su capacidad de adhesión a los eritrocitos humanos

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Periodontal disease is the result of the interrelationship between microbiotic aggression and the host's organic defence. Amongst the microorganisms involved in periodontopathies, Fusobacterium nucleatum is conspicuous by establishing a link between the initial and final colonizers, besides producing toxic compounds and adhering to the host's cells. Control of bacterial biofilm can be achieved by use of chemical agents, many of which extracted from plants. Thus the object of this study was to evaluate the inhibitory activity in vitro of some teas, generally taken in a normal diet, on Fusobacterium nucleatum and your adherence to host's cells. Minimum inhibitory and bactericidal concentrations were established and haemagglutinative test in microplaques was effected. It was ascertained that all plant extracts have inhibitory activity and that infusions of Camellia sinensis (black tea and green tea), Mentha piperita (mint) and Pimpinella anixem (aniseed) added to the bacteria/erythrocyte compound reduced significantly the adherence of microorganisms.

Keywords: Dental Plaque; Periodontitis; Disease Prevention; Plant Extracts; Hemagglutination; Cell Adhesion.

INTRODUCTION

The oral fusobacteria are one of the most relevant microorganism in the biofilm, representing a bridge between the early and late colonizers of the biofilm[1]. The association of oral *Fusobacterium* species with gingivitis and periodontitis has received studies[2,3] and their ability to co-aggregate with other oral anaerobes seems to be involved in mixed infections[4].

Periodontitis represent serious health problems as they involve, to a greater or lesser extension, all ethnic groups, regardless gender or socio-economic levels[2,3]. However, inflammation of periodontal tissues might be minimized or even blocked, in a significant part of the

population, by establishing means for bacterial biofilm control.

Fusobacterium nucleatum is able to adhere and colonize a large variety of human cells, particularly erythrocytes, lymphocytes, neutrophils, fibroblasts, epithelial cells, and HeLa cells[5]. Studies of microbial adherence to the host‘s cells are important instruments for understanding the colonization of the periodontal environment by resident microorganisms. In addition, the adherence of oral fusobacteria to erythrocytes was closely related to their adhesion to epithelial cells and other human cell lines[6].

There is a cultural tendency in the population, in considering the use of medicinal plants in infusions as adequate pharmaceutical resources in the treatment of oral problems, and there is the false belief that these compounds have no collateral effects[7]. However, some infusions, as tea, the beverage most largely consumed in the world[8], are valuable sources of fluoride and tannin (polyphenols) with antibiofilm and anticariogenic activity[9]. However, it is opportune to check whether compounds of the human diet have an antimicrobial effect on oral fusobacteria or if they might inhibit the microbial capacity of hemagglutination. Thus, this study aimed to evaluate the inhibitory activity of several plant infusions used in Brazilian diet on fusobacteria and the effects of such infusions on capacity of adherence of these anaerobes on human erythrocytes.

MATERIAL AND METHODS

1. MICROORGANISMS

All tested isolates of *Fusobacterium nucleatum* were maintained at -80^{o}C and were recovered from 1993 to 1997 from lesions of chronic periodontitis. Reactivation of selected strains was performed in peptone yeast extract broth incubated at 37^{o}C, under anaerobiosis (90% N_2 + 10% CO_2) for 48 hours. *F. nucleatum* ATCC 10953 was used as control.

2. PLANT INFUSIONS

Aqueous extracts of mint (*Mentha piperita)*, mate (*Ilex paraguayensis*) , black tea (*Camelia sinensis*), chamomile (*Matricaria chamomilla*), Japanese green tea (*Camellia sinensis*, Ban-cha variety), aniseed (*Pimpinella anixem*), balm mint (*Melissa officinalis*) and boldo (*Peumus boldus*) were prepared. The different plants were acquired at the shops and represent the main trademarks available.

In preparation of the infusions, 10 g of leaves were added to 100ml of 0,1M buffered phosphate saline solution (PBS) and left for 5 minutes at 100^{O}C, for one hour at 55ºC and overnight at 25^{o}C. The aqueous extracts were then submitted to fractioned filtration in cellulose membrane with 0.65 µm and sterilized through filtration in membranes of 0.22 µm (Millipore®). The infusions were prepared immediately before use the tests.

3. EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF PLANT INFUSIONS

In order to determine the higher inhibitory dilution of the infusions, the agar dilution method was employed as previously described[10]. It was used Wilkins-Chalgren agar supplemented with yeast extract (0.5%). In the preparation of different dilutions of the infusions in agar, water originally used in preparation of the culture medium was partially substituted by plant infusions in order to obtain final dilutions representing 1/2, 1/4, 1/8, 1/16, and 1/32 of the original preparation.

In the test, microbial inoculum (10^5 colony forming units-CFU) was transferred using a Steers’ replicator onto Wilkins-Chalgren agar plates in triplicate. The screening and control plates (without plant infusions) were incubated in anaerobiosis, at 37^{o}C for 48 hours. The higher antimicrobial dilution was defined as the higher dilution of plant infusion that inhibited completely microbial growth on agar.

The contact inhibitory activity was evaluated as described hereinafter. Bacterial cells were cultivated in peptone yeast extract broth (Difco, Rochester, NY, USA) with glucose (1%) in anaerobiosis, at 37^{0}C for 48 hours. Aliquots of 0,1 ml were transferred to Eppendorf tubes and rinsed three times in PBS, pH 7,2, by centrifuging at 3000xg per 8 minutes. Then PBS was

removed and 1,5ml of plant infusion was added to the inoculum (10^7 CFU) and incubated at 37^0C in anaerobiosis. In the tests, phosphate buffered saline solution was used as control.

After 30 min. aliquots of 0,1ml of these mixtures were removed and submitted to serial ten-fold dilutions in PBS and inoculated onto brain heart infusion (BHI) agar plates supplemented with yeast extract (0.5%) and defibrinated horse blood (5%). The plates were incubated at 37ºC in anaerobiosis (90% N_2 + 10% CO_2), at 37ºC, for 72 hours. The results were expressed as the plant infusion that reduced 90% of the microbial inoculum, in relation of control (PBS).

4. INHIBITION OF HEMAGGLUTINATION

This test was performed through methodology described by Falker Jr., Hawley[6]. The tests was carried out on microtitration plates, using human blood (type A, B, O and AB with the corresponding RH-positive and RH-negative) collected in Alsever solution at 10%[24].

The bacterial cells were cultivated in BHI broth supplemented with yeast extract (0.5%) for 48 hours. The cells were then submitted to centrifugation at 3000x*g* per 8 minutes, in PBS, and rinsed three times to eliminated residues of the culture medium and resuspended using plant infusions until reaching concentration of 10^8 CFU/ml. The erythrocytes were rinsed twice by centrifugation at 600x*g* per 5 minutes in PBS and resuspended in plant infusions until reaching the concentration of 1%.

Initially 50µl of the bacterial suspension was added to the microplates followed by serial dilutions on base 2, using plant infusion in the preparation of serial dilutions. This was followed by the addition of 50µl of the erythrocyte suspension at each dilution. The mixtures on the microplates were homogenized slightly for 1 minute and maintained at 37ºC for 30 min., and at room temperature for 2 hours, the title of hemagglutination being then defined and evaluated as reciprocal of the higher bacterial dilution presenting agglutination of erythrocytes. The tests were performed in duplicate.

It was considered that inhibition of the hemagglutination occurred when the hemagglutination title of the microorganism was reduced to at least 50% in the in the presence of plant infusion, in comparison to control (PBS).

RESULTS

The values of higher inhibitory dilutions of plant infusions on oral *F. nucleatum* are presented in Table 1, whereas Table 2 showed the contact inhibitory activity of these infusions on targeted anaerobes.

Table 1- Susceptibility of 32 isolates of *Fusobacterium nucleatum* to different plant infusions.

Plant extract	Higher dilution with antimicrobial activity		
	[1]RANGE	[2]MDI_{50}	[3]MDI_{90}
Boldo (*P. boldus*)	1/4 - > 1[a]	1/2	1
Camomile (*M. chamomilla*)	1/4 - > 1[d]	1/2	1
Black tea (*C. sinensis*)	1/4 -1	1/4	1
Green tea (Variety Banchá)	1/8 -1	1/8	1
Balm mint (*M. officinalis*)	1/4 - > 1	1/2	1
Aniseed (*P. anixem*)	1/4 - > 1	1/2	1
Mint (*M. piperita*)	1/4 - > 1	1/4	1
Mate (*I. paraguayensis*)	1/8 -1	1/8	1

These data evidenced a significant heterogeneity among clinical isolates of fusobacteria. The aqueous extracts of black tea, green tea and mate were able to inhibit all tested strains. Only the infusions of black tea and green tea (*C. sinensis*) and mate (*I. paraguayensis*) presented bactericide effect after 2 hours in contact with the fusobacteria.

Table 2- Contact inhibitory activity of plant infusions on oral *Fusobacterium nucleatum*

Plant extract	Isolates (%)	
	Susceptible	Resistant
Boldo (*P. boldus*)	25 (78.1)	7 (21.9)
Camomile (*M. chamomilla*)	27 (84.4)	5 (15.6)
Black tea (*C. sinensis*)	32 (100.0)	0 (0.0)
Green tea (variety Banchá)	32 (100.0)	0 (0.0)
Balm mint (*M. officinalis*)	27 (84.4)	5 (15.6)
Aniseed (*P. anixem*)	26 (81.3)	6 (18.8)
Mint (*M. piperita*)	30 (93.8)	2 (6.3)
Mate (*I. paraguayensis*)	32 (100.0)	0 (0.0)

The present study evidenced that all the tested plant extracts had a antimicrobial effect on tested isolates strains of *F. nucleatum* (Tables 1 and 2). However only the extracts of black tea and green tea (C. *sinensis*) and of mate (*I. paraguayensis*) had generalized bactericidal activity in the concentration used.

The hemagglutination titles and the effect of plant infusions on the adherence of *F. nucleatum* to erythrocytes are presented in Table 3. All the isolated were able to haemagglutination human blood whereas extracts of black tea, mint and aniseed infusions inhibited, to a higher or lesser degree, this phenomenon.

Table 3- Effects of plant infusions on capacity of hemagglutination of oral *Fusobacterium nucleatum* isolates.

Plant extract	Inhibition of hemagglutination N (%)[1]
Boldo (*P. boldus*)	0 (0.0)[2]
Camomile (*M. chamomilla*)	0 (0.0)[2]
Black tea (*C. sinensis*)	28 (87.5)
Green tea (variety Banchá)	0 (0.0)[3]
Balm mint (*M. officinalis*)	0 (0.0)[2]
Aniseed (*P. anixem*)	13 (40.6)
Mint (*M. piperita*)	22 (68.8)
Mate (*I. paraguayensis*)	0 (0.0)[3]

DISCUSSION

Prevention of periodontal diseases is one of Dentistry's greatest challenges particularly in view of the complexity of the elements involved in their epidemiology. The importance of some bacterial species' participation in periodontal disease has been continuously elucidated in recent years. Amongst the periodontopathogens, special importance is attributed to *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Fusobacterium nucleatum*, which produce a number of virulence factors capable of harming the host or depleting its defenses, not only in oral cavity but also in other anatomical sites[11].

The most studied mechanism of virulence is related to the microbial adherence to the host's cells and tissular structures, since the microorganism that do not adhere to these surfaces is quickly removed by saliva, consequently will not colonize the host and will be unable to express its virulence factors or to cause any damage[12]. *Fusobacterium nucleatum* would act as a link between the initial colonizers of bacterial biofilm, represented mainly by streptococcus, and the final colonizers such as *Selenomonas flueggei*, *Eubacterium* ssp., *Treponema* ssp., *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* among others[1]. This ability might be involved in the initiation and progression of mixed infections[4].

In this regard the great majority of mouth rinses available in the market present serious disadvantages when used for long periods, such pigmentation of the teeth, or hot and burning sensation. Thus the evaluation of antimicrobial activity and antiadherence properties of plant infusions should be estimated since they are largely used in popular medicine and are found in normal diet, such as black tea, mate and green tea.

In the literature, data about antimicrobial activity of plant infusions are scarce and they are almost limited to inhibitory activity on *mutans streptococci* or the effect on tooth decay in the population[13,14]. However, infusions prepared with traditional plants of Brazilian savannah evidenced noticeable inhibitory activity on anaerobes[15]. However, Nwaokorie et al.[16] demonstrated that infusions of koula, a Nigerian plant largely used in oral hygiene, are highly active against fusobacteria, producing significant reduction in microbial populations in vitro.

Polyphenols and the tannic acid found in teas and other natural products might inhibit bacterial enzymes or lead to precipitation of bacterial proteins[13] in similar concentrations to those found in beverages[17]. Epigaloctechin one of the phenolic compounds found in tea, can lead to perforation of the membrane, which accounts for the higher sensitivity of Gram-negative bacteria[18]. These phenolic compounds (polyphenols, flavonols, epicatechin, epigalocatechin) can further inhibit the transcriptase reverse enzyme of the HIV

virus[19], and hemolysines of *S. aureus* and *Vibrio parahaemolyticus*[20]. However, since mouth rinses generally are left in oral cavity for short periods in inhibitory concentrations but could remain for longer periods in subinhibitory concentrations, the residual concentrations could affect the determinants of microbial virulence, the same may occur with residues of these phenolic compounds carried by tea and plant infusions.

Among these virulence traits of *Fusobacterium nucleatum*, special attention is given to the hemagglutination activity that represent a valuable instrument for the study of adherence of this bacterium to the host's cells and to other microorganisms, being important in the colonization of the gingival sulcus and/or the periodontal pocket. In this study all the isolated from *F. nucleatum* agglutinated erythrocytes of all blood groups, which is in accordance with literature available[21]. The hemagglutinating activity regarding the different blood groups was similar, evidencing similarities among hemagglutinins[5]. The hemagglutination process was strongly inhibited by the addition of *C. sinensis* extract and, to a lesser extent by extracts of *M. piperita* and *P. anixem.*

Black tea, Oolong (Taiwanese tea) and, to a lesser extent, green tea (*C. sinensis*, Ban-cha variety) are capable to inhibit adherence of *F. nucleatum* with *P. gingivalis* possibly by the same mechanism that inhibited hemagglutination since, apparently, the same proteins that mediate the hemagglutination by *F. nucleatum* cells are involved in the coaggregation process with *Streptococcus* ssp., *P. gingivalis*, *Bacteroides* ssp.[21].

Since the coaggregation and bacterial adherence to the host's cells depend on the interaction of adhesins (proteins) found on the surface of the bacterium and the host's receptors, the phenolic compounds in tea extracts could produce denaturation of proteins[22] on the bacterial surface, inhibiting the adhesin/receptor interaction.

Some of the tested infusions could also induce spontaneous haemagglutination, which would occur by alterations to the membrane structures of the erythrocytes. Other extracts induced hemolysis, which could be related to these phenolic compounds' capacity to alter the structure of membranes of live cells.

The results of this study suggest that aqueous plant extracts found in the normal diet of the Brazilian people and, particularly, of edentulous children or with deciduous dentition may have a bacteriostatic or even bactericidal effect on *Fusobacterium nucleatum*, one of the main periodontopathogenic bacteria that frequently colonize the oral cavity of these children[23].

Other studies must be carried out in order to evaluate, *in vivo*, the effects of tea drinking and other extracts of medicinal plants on the composition of subgingival microbiota and the preventive effects of these compounds on periodontopathies.

CONCLUSIONS

The results presented in this investigation evidenced that all the plant infusions produced inhibitory effects on tested isolates of *Fusobacterium nucleatum*, but only infusions of black tea, green tea and mate had bactericidal effect on all the microorganisms. Moreover, infusions of black tea, aniseed and mint were able to inhibit the bacterium compound inhibited bacterial adherence erythrocyte.

RESUMO

A doença periodontal é o resultado da inter-relação entre a capacidade de agressão da microbiota e a defesa orgânica do hospedeiro. Dentre os microrganismos envolvidos nas periodontopatias, Fusobacterium nucleatum se destaca por constituir uma ponte entre os colonizadores iniciais e finais, além de produzir compostos tóxicos e aderir às células do hospedeiro. O controle da placa bacteriana pode ser realizado por meio da utilização de agentes químicos, muitos dos quais extraídos de vegetais. Desta forma, foi objetivo deste estudo, avaliar a atividade inibitória in vitro de alguns chás normalmente consumidos na dieta, sobre Fusobacterium nucleatum e na sua capacidade de aderir a células do hospedeiro. Foram determinadas as concentrações inibitórias e bactericidas mínimas, além da realização do teste de hemaglutinação em microplacas. Verificou-se que todos os extratos vegetais possuem alguma atividade inibitória, e as infusões de Camellia sinensis

(chá preto e chá verde), Mentha piperita (hortelã) e Pimpinella anixem (erva doce) adicionadas à mistura bactéria/eritrócito, reduziram significativamente a capacidade de adesão dos microrganismos.

Palavras chave: Placa Dentária; Periodontite; Prevenção de Doenças; Extratos Vegetais; Hemaglutinação; Adesão Celular.

RESUMEN

La enfermedad periodontal es el resultado de la interrelación entre la agresión microbiana y la defensa ecológica del huésped. Entre los microorganismos implicados en periodontopatías, Fusobacterium nucleatum *es visible mediante el establecimiento de un enlace entre los colonizadores iniciales y finales, además de producir compuestos tóxicos y de adherirse a las células del huésped. Control de la biopelícula bacteriana se puede lograr mediante el uso de agentes químicos, muchos de los cuales se extraen de plantas. Así, el objeto de este estudio fue evaluar la actividad inhibidora in vitro de algunos tipos de té, generalmente adoptadas en una dieta normal, por* Fusobacterium nucleatum *y su adherencia a las células huésped. Las concentraciones mínimas inhibitorias y bactericidas se establecieron y prueba haemagglutinative en microplacas fue efectuada. Se comprobó que todos los extractos de plantas tienen actividad inhibidora y que las infusiones de* Camellia sinensis *(té negro y té verde),* Mentha piperita *(menta) y* Pimpinella anixem *(anís) añadido a las bacterias / compuesto de eritrocitos redujo significativamente la adherencia de microorganismos.*

Palabras clave: Placa dental; periodontitis, prevención de enfermidades; extractos vegetales; hemaglutinación; adhesión celular.

REFERENCES

1. Kolembrander PE, London J. Adhere today, here tomorrow; oral bacterial adherence. J Bacteriol. 1993; 175:3247-52.
2. Khocht A, Yaskell T, Janal M, Turner BF, Rams TE, Haffajee AD, et al. Subgingival microbiota in adult Down syndrome periodontitis. J Periodontal Res. 2012; 47:500-7.
3. Settem RP, El-Hassan AT, Honma K, Stafford GP, Sharma, A. *Fusobacterium nucleatum* and *Tannerella forsythia* induce synergistic alveolar bone loss in a mouse periodontitis model. Infect Immun. 2012; 80:2436-43.
4. Polak D, Shapira L, Weiss EI, Houri-Haddad Y. The role of coaggregation between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* on the host response to mixed infection. J Clin Periodontol. 2012; 39:617-25.
5. Ozaki M, Miyake Y, Shirakawa M, Takemoto T, Okamoto H, Suginaka H. Binding specificity of *Fusobacterium nucleatum* to human erythrocytes, polymorphonuclear leukocytes, fibroblasts, and HeLa cells. J Periodontal Res. 1990; 25:129-34.
6. Falker Jr. WA, Hawley CA. Hemagglutinating activity of *Fusobacterium nucleatum.* Infect Immun. 1977; 15:230-8.
7. Maciel MAM, Pinto AC, Veiga JR VF. Plantas medicinais: a necessidade de estudos multidisciplinares. Quim Nova. 2002; 25:429-38.
8. Park AM, Dong Z. Signal transduction pathways: target for green and black tea polyphenols. J Biochem Mol Biol. 2003; 36:66-77.
9. Ooshima T, Minami T, Aono W, Tamura Y, Mamada S. Reduction of dental biofilm deposition in human by oolong tea extract. Caries Res. 1994; 28:146-9.
10. Sutter VL, Barry AL, Wilkins TD, Zabransky RJ. Collaborative evaluation of a proposed reference dilution method of susceptibility testing of anaerobic bacteria. Antimicrob Agents Chemother. 1979; 16:495-502.
11. Allen-Vercoe E, Strauss J, Chadee K. *Fusobacterium nucleatum.* An emerging gut pathogen? Gut Microbes. 2011; 2:294-8.
12. Sugawara S, Uehara A, Tamai R, Takada H. Innate immune responses in oral mucosa. J Endotoxin Res. 2002; 8:465-8.
13. Brighenti FL, Luppens SB, Delbem AC, Deng DM, Hoogenkamp MA, Gaetti-Jardim Jr E et al. Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production. Caries Res. 2008; 42:148-54.
14. Menezes TEC, Delbem ACB, Brighenti FL, Okamoto AC, Gaetti-Jardim E Jr. Protective efficacy of *Psidium cattleianum* and *Myracrodruon urundeuva* aqueous extracts against caries development in rats. Pharm Biol. 2010; 48:300-5.
15. Gaetti-Jardim JR E, Landucci LF, Arafat OKK, Ranieri RV, Ramos MMB, Ciesielski FIN, et al. Antimicrobial activity of six plant extracts from the Brazilian savanna on periodontal pathogens. Int J Odontostomat. 2011; 5:249-56.
16. Nwaokorie F, Coker A, Ogunsola F, Gaetti-Jardim JR E, Gabriel O, Patricia A, et al. Antimicrobial activities of *Garcinia kola* on oral *Fusobacterium nucleatum* and biofilm. African J Microbiol Res. 2010; 4:509-14.

17. Hamilton-Miller JMT. Antimicrobial properties of tea (*Camellia sinensis L.*). Ant Agent Chemother. 1995; 39:2375-7.
18. Ikigai HT, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. Biochim Biophys Acta. 1992; 1147:132-6.
19. Nakane H, Ono K. Differential inhibitory effects of some catechin derivatives on the activities of human immunodeficiency virus reverse transcriptase and cellular deoxyribonucleic and ribonucleic acid polymerases. Biochem. 1990; 29:2841-5.
20. Toda M, Okubo S, Ikigai H, Shimamura T. Antibacterial and anti-hemolysin activities of tea catechins and their structural relatives. Nippon Saikingaku Zasshi. 1990; 45:561-6.
21. Falkler Jr. WA, Lai R, Vincent JW, Dober L, Spiegel C, Hayduk S. The elisa system for measuring antibody reactive to *Fusobacterium nucleatum* in the sera of individuals with chronic periodontitis. J Periodontol. 1982; 53:762-6.
22. Ooshima T, Minami T, Aono W, Izumitani A, Sobue S, Fujiwara T, et al. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with *mutans* streptococci. Caries Res. 1993; 27:124-9.
23. Könönen E, Asikainen S, Jousemies-Somer H. Relationship between oral Gram- negative anaerobic bacteria in saliva of the mother and the colonization of her edentulous infant. Oral Microbiol Immunol. 1992; 7:273-6.
24. Oyston PC, Handley PS. Surface structures, haemagglutination and cell surface hydrophobicity of Bacteroides fragilis strains. J Gen Microbiol 1990; 136: 941-48

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