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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INTRODUCTION

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.
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. 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.

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. However, some infusions, as tea, the beverage most largely consumed in the world, are valuable sources of fluoride and tannin (polyphenols) with antibiofilm and anticariogenic activity. 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°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°C, under anaerobiosis (90% N₂ + 10% CO₂) 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_ ), aniseed ( _Pimpinella anixem_ ), chamomile ( _Matricaria chamomilla_ ), Japanese green tea ( _Camelia sinensis_ , Ban-cha variety), 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°C, for one hour at 55°C and overnight at 25°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 in 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. 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, 1/16, and 1/32 of the original preparation.

   In the test, microbial inoculum (10⁵ 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°C for 48 hours. The higher antimicrobial dilution was defined as the higher dilution of plant infusion that inhibited completely microbial growth on agar.

   In 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°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°C 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 37°C in anaerobiosis (90% N₂ + 10% CO₂), at 37°C, 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. 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% RH.

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 3000xg 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 600xg 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 37°C 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>
<thead>
<tr>
<th>Plant extract</th>
<th>Higher dilution with antimicrobial activity</th>
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<tbody>
<tr>
<td></td>
<td>RANGE</td>
</tr>
<tr>
<td>Boldo (P. boldus)</td>
<td>1/4 - &gt; 1&quot;</td>
</tr>
<tr>
<td>Camomile (M. chamomilla)</td>
<td>1/4 - &gt; 1&quot;</td>
</tr>
<tr>
<td>Black tea (C. sinensis)</td>
<td>1/4 - 1</td>
</tr>
<tr>
<td>Green tea (Variety Banchá)</td>
<td>1/8 - 1</td>
</tr>
<tr>
<td>Balm mint (M. officinalis)</td>
<td>1/4 - 1</td>
</tr>
<tr>
<td>Aniseed (P. anisum)</td>
<td>1/4 - 1</td>
</tr>
<tr>
<td>Mint (M. piperita)</td>
<td>1/4 - 1</td>
</tr>
<tr>
<td>Mate (I. paraguayensis)</td>
<td>1/8 - 1</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Plant extract</th>
<th>Isolates (%)</th>
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<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>Boldo (P. boldus)</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>Camomile (M. chamomilla)</td>
<td>27 (84.4)</td>
</tr>
<tr>
<td>Black tea (C. sinensis)</td>
<td>32 (100.0)</td>
</tr>
<tr>
<td>Green tea (variety Banchá)</td>
<td>32 (100.0)</td>
</tr>
<tr>
<td>Balm mint (M. officinalis)</td>
<td>27 (84.4)</td>
</tr>
<tr>
<td>Aniseed (P. anisum)</td>
<td>26 (81.3)</td>
</tr>
<tr>
<td>Mint (M. piperita)</td>
<td>30 (93.8)</td>
</tr>
<tr>
<td>Mate (I. paraguayensis)</td>
<td>32 (100.0)</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Inhibition of hemagglutination N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boldo (<em>P. boldus</em>)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Camomille (<em>M. chamomilla</em>)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Black tea (<em>C. sinensis</em>)</td>
<td>28 (87.5)</td>
</tr>
<tr>
<td>Green tea (variety Banchá)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Balm mint (<em>M. officinalis</em>)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Aniseed (<em>P. anisum</em>)</td>
<td>13 (40.6)</td>
</tr>
<tr>
<td>Mint (<em>M. piperita</em>)</td>
<td>22 (68.8)</td>
</tr>
<tr>
<td>Mate (<em>I. paraguayensis</em>)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

**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*, *Treponema* ssp., *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* among others. This ability might be involved in the initiation and progression of mixed infections.

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. However, infusions prepared with traditional plants of Brazilian savannah evidenced noticeable inhibitory activity on anaerobes. However, Nwaokorie et al. 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 in similar concentrations to those found in beverages. Epigalocatechin 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. These phenolic compounds (polyphenols, flavonols, epicatechin, epigalocatechin) can further inhibit the transcriptase reverse enzyme of the HIV
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\(^2\).}

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 este estudo, avaliar a atividade inibitória *in vitro* de alguns chás normalmente consumidos na dieta, sobre *Fusobacterium nucleatum* e na sua capacidade de aderir às 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 anisum (erva doce) adicionadas à mistura bactéria/eritrócito, reduziram significativamente a capacidade de adesão dos microorganismos.

**Palavras chave:** Placa Dentária; Periodontite; Prevenção de Doenças; Extratos Vegetais; Hemaglutininação; 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 inhibidoras y bactericidas se establecieron y prueba haemagglutinativa en micropalas 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 anisum (anís) añadidos a las bacterias / compuesto de eritrocitos redujo significativamente la adherencia de microorganismos.

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

**REFERENCES**


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