

Minimum Inhibitory Concentration of Adherence of *Punica granatum* Linn (pomegranate) Gel Against *S. mutans*, *S. mitis* and *C. albicans*

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The purpose of this study was to investigate the antimicrobial effect of a *Punica granatum* Linn (pomegranate) phytotherapeutic gel and miconazole (Daktarin® oral gel) against three standard streptococci strains (*mutans* ATCC 25175, *sanguis* ATCC 10577 and *mitis* ATCC 9811), *S. mutans* clinically isolated and *Candida albicans* either alone or in association. The effect of minimum inhibitory concentrations of the gels on the adherence of these microorganisms to glass was assessed in the presence of 5% sucrose, using increasing and doubled concentrations of the diluted solution of the gels ranging from 1:1 to 1:1024. The minimum inhibitory concentrations of adherence of *Punica granatum* L. gel against the test organisms were: 1:16 for *S. mutans* (ATCC), *S. mutans* (CI) and *S. sanguis*; 1:128 for *S. mitis* and 1:64 for *C. albicans*. The minimum inhibitory concentrations of adherence of miconazole against the same organisms were: 1:512, 1:64, 1:4, 1:128 and 1:16, respectively. In experiments with three and four associated microorganisms, the *Punica granatum* L. gel had greater efficiency in inhibiting microbial adherence than the miconazole. The results of this study suggest that this phytotherapeutic agent might be used in the control of adherence of different microorganisms in the oral cavity.

Key Words: *Punica granatum* Linn, *Streptococcus mutans*, *Candida albicans*.

INTRODUCTION

Homeostasis is a characteristic of the oral microbiota in healthy individuals. The microorganisms are capable of cohabiting in saprophytism in the different sites of the mouth, depending directly on pH, availability of nutrients and mucous surface (1). Factors related to adhesion mechanisms can, however, modify this homeostasis and lead to microbial colonization and biofilm formation, which constitute the primary etiologic agents of oral diseases (2).

The establishment and maintenance of oral microbiota is related not only to interbacterial

coaggregations but also to interactions of these bacteria with yeasts such as *Candida albicans* (3). Fungi are frequently isolated in several oral sites, including the tongue, jugal mucosa, palate, dental biofilm, subgingival microbiota, carious lesions and prosthetic appliances (4). Studies (5,6) have suggested a possible relation between *C. albicans* and periodontal disease, dentin and/or root caries. These studies showed that *C. albicans* has similar capacity of colonizing hydroxyapatite as that of *S. mutans*, however using different mechanisms.

Enamel and dentin demineralization produced by fungal organic acids, as well as the presence of cells with *C. albicans* hyphas invading dentinal tubules, prove this

ability of fungi to invade and destroy organic and inorganic dental tissues (7). This microorganism adheres to hydroxyapatite, especially through electrostatic interactions and at smaller numbers. *Candida albicans* has also the ability to dissolve hydroxyapatite at a larger rate when compared to *Streptococcus mutans* (6).

Nostro et al. (8) investigated whether sublethal concentrations of *Helichrysum italicum* extract would affect the cariogenic properties of *S. mutans*. The antibacterial activity of the ethanolic extract against oral streptococci (*S. mutans*, *S. salivarius* and *S. sanguis*) was evaluated *in vitro* to determine whether this would influence cell surface hydrophobicity regarding glass surface adherence and *S. mutans* aggregation. All streptococci were sensitive to the minimum inhibitory concentrations of the extract. Concentrations of minimum inhibitory subconcentrations of *H. italicum* reduced the hydrophobicity and adherence (approximately 90%) of *S. mutans* to glass surface.

The antimicrobial activity of *Punica granatum Linn* has been widely investigated (9,10). The findings of several studies, including some relating to inhibition of adherence, suggest that the phytotherapeutic use of this plant might be a viable option in controlling different microbial species. The largest components of the *Punica granatum L.* fruit extract are tannin and polyphenolics (11).

There is a growing interest in using tannins as antimicrobial agents in caries prevention (12). The action of tannins against bacteria and yeasts can be established by a relation between their molecular structure and their toxicity, astringent properties or other mechanisms. The effect of tannins on microbial metabolism can be measured by their action on membranes. They can cross the cell wall, composed of several polysaccharides and proteins, and bind to its surface. This adhesion can also help determining minimum inhibitory concentrations for yeasts and bacteria.

The purpose of this study was to investigate the antimicrobial effect of a *Punica granatum Linn.* (pomegranate) phytotherapeutic gel and Miconazole (Daktarin® oral gel) against three standard streptococci strains (*mutans* ATCC 25175, *sanguis* ATCC 10577 and *mitis* ATCC 9811), *S. mutans* clinically isolated and *Candida albicans* either alone or associated with other microorganisms. The effect of minimum inhibitory concentrations of the gels on the adherence of these microorganisms to glass was assessed.

MATERIAL AND METHODS

The *Punica granatum L.* fruits used in this study were obtained at a public market in the city of João Pessoa, PB, Brazil. Botanical identification was conducted at the Pharmaceutical Technology Laboratory of the Federal University of Pernambuco. After washing, the peel was separated from the mesocarp, dried in an incubator at 33°C for 7 days. The material was thereafter ground in an electric grinder to produce a powder. The active principles were isolated and a concentrated extract was obtained. At this stage, a basic gel consisting of carbopol, water and triethanolamine was prepared. Next, 0.5 mL of the brute extract, equivalent to 540 mg of the plant powder, was incorporated, thus resulting in the *Punica granatum L.* gel.

Standard *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguis* (ATCC 10557), *Streptococcus mitis* (ATCC 9811) strains were used. These species were obtained from “André Tozello” Tropical Research and Technology Foundation (Campinas, SP, Brazil). Clinical isolates of *S. mutans* obtained from a patient and *Candida albicans* strains were also used. The latter were further reactivated at the Microorganism Genetics Laboratory of the Department of Molecular Biology, CLEN, Federal University of Paraíba, Brazil).

The minimum inhibitory concentration of adherence of the organisms to glass was determined in the presence of 5% sucrose, using increasing and doubled concentrations of the diluted gel ranging from 1:1 up to 1:1024. After overnight growth, the strains were subcultured (30 µL of growth) in 30 mL of sucrose-containing Mueller-Hinton Broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 1 h to obtain an inoculate. A gel scale was then prepared by solubilization of 5 g of the pomegranate gel in every 5 mL of sterile distilled water. Distribution was made of 1.6 mL saline and 0.2 mL of the subculture in hemolysis tubes, where 0.2 mL of gel scale was further added. Incubation was done at 37°C for 24 h in microaerophilia, with the tubes inclined at a 30° angle. Reading of the results was done by visual inspection of microbial adherence to tube walls after shaking. Minimum inhibitory concentration of adherence was defined as the smallest concentration of the agent in sucrose that prevented adherence to the glass.

The minimum inhibitory concentration of adherence of the microorganisms in pairs or groups was also determined. *S. mutans* (ATCC), *S. sanguis*, *S. mitis* and

Candida albicans were used. The microorganisms were placed in hemolysis tubes with 1.4 mL of the sucrose-containing solution in addition to 0.2 mL of the bacterial subculture and 0.2 mL of the pomegranate gel scale. Incubation and reading of the results were then performed in the same way as described above.

To compare the results, the experiment was also performed using a scale prepared with pure pomegranate extract and another with miconazole (Daktarin® gel oral, Janssen Farmacêutica Ltda, São Paulo, SP, Brazil).

RESULTS

Punica granatum L. gel was effective in inhibiting the adherence of the bacterial strains and *C. albicans*, in the presence of sucrose. The minimum inhibitory concentrations of adherence of this phytotherapeutic agent are shown in Table 1. The antifungal agent (miconazole) also showed a significant adherence inhibition effect against the tested strains (Table 1).

The etiology of oral diseases is related to various local factors including the simultaneous participation of bacteria and yeasts. In this study, the minimum inhibitory concentrations of the *Punica granatum L.* gel were also evaluated against microbial pools. There was an increase in microbial adherence when different bacteria were associated, or when bacteria were associated with *C. albicans* (Table 2). In this case, the minimum inhibitory concentrations of miconazole were lower than those of *Punica granatum L.* gel.

In the assays with three and four associated organisms, the *Punica granatum L.* gel had greater efficiency than miconazole. The *Punica granatum L.* gel was effective in inhibiting the adherence of the following associations: *S. mutans* (ATCC) + *S. mitis* + *S. sanguis*; *S. mutans* (ATCC) + *S. mitis* + *S. sanguis* + *C. albicans* and *S. mutans* (IC) + *S. mitis* + *S. sanguis*. Miconazole did not show inhibitory activity against these associations (Table 3). This suggests that phytotherapy seems to have a greater astringent strength than antifungal allopathy.

Table 3 also shows that 10% *Punica granatum L.* gel (1:2 concentration) inhibited glass adherence in the associations of *C. albicans* and *S. mitis*, *S. sanguis*

and *S. mutans* strains. However, the phytotherapeutic gel was not able to inhibit glass adherence of the association of *C. albicans* with *S. mitis*, *S. sanguis* and *S. mutans* (CI). This indicates a possible strengthening of adherence promoted by clinically isolated microorganisms.

Table 1. Minimum inhibitory concentration of adherence of *Punica granatum Linn gel* and miconazole against the bacterial and yeast strains alone.

Microbial Strains	<i>Punica granatum L.</i> gel	Miconazole
<i>S. mutans</i>	1:16	1:64
<i>S. mutans</i> (CI)	1:16	1:4
<i>S. mitis</i>	1:128	1:16
<i>S. sanguis</i>	1:16	1:512
<i>C. albicans</i>	1:64	1:128

CI = Clinical isolate.

Table 2. Minimum inhibitory concentration of adherence of *Punica granatum Linn gel* and miconazole against the associations of bacterial strains and yeast (pairs).

Microbial strains	<i>Punica granatum L.</i> gel	Miconazole
<i>S. mutans</i> + <i>C. albicans</i>	1:8	1:128
<i>S. mutans</i> + <i>S. mutans</i> (CI)	1:2	1:32
<i>S. mutans</i> (CI) + <i>C. albicans</i>	1:8	1:16
<i>S. mitis</i> + <i>C. albicans</i>	1:4	1:32
<i>S. mitis</i> + <i>S. mutans</i> (CI)	1:8	1:64
<i>S. mitis</i> + <i>S. mutans</i>	1:8	1:32
<i>S. sanguis</i> + <i>C. albicans</i>	1:16	1:16
<i>S. sanguis</i> + <i>S. mutans</i> (CI)	1:2	1:16
<i>S. sanguis</i> + <i>S. mutans</i>	1:4	1:16

CI = Clinical isolate.

Table 3. Minimum inhibitory concentration of adherence of *Punica granatum Linn gel* and miconazole against the associations of several bacterial strains and yeast (groups).

Microorganism associations	<i>Punica granatum L.</i> gel	Miconazole
<i>S. mutans</i> + <i>S. mitis</i> + <i>S. sanguis</i>	1:4	-
<i>S. mutans</i> + <i>S. mitis</i> + <i>S. sanguis</i> + <i>C. albicans</i>	1:2	-
<i>S. mutans</i> + <i>S. mitis</i> + <i>S. sanguis</i>	1:8	-
<i>S. mutans</i> + <i>S. mitis</i> + <i>S. sanguis</i> + <i>C. albicans</i>	-	-

DISCUSSION

S. mutans are directly involved in the etiopathogenesis of caries and periodontal diseases, denture-associated stomatitis and other infections because they can contribute to alter the equilibrium of oral microbiota by creating favorable conditions to adherence of opportunistic organisms, such as fungi, to the surfaces of teeth, oral tissues and prosthetic appliances (6,13,14,16).

This study did not have the aim of investigating the action mechanisms by which streptococci increase the adherence of yeasts. It is believed that the quantification of involved species, the association of different organisms and the possible relations with clinically observed characteristics are of great diagnostic and therapeutic importance for several oral infections.

Few studies have addressed interactions between commensal bacteria and yeasts (17). However, the effect of these bacteria interacting with *Candida albicans* can be seen with the use of antibiotic therapy and in the increase of candidosis (18).

C. albicans is a fungus frequently found on dental biofilm and its ability to secrete organic acids and collagenolytic enzymes can determine its role in the onset of caries disease (13). Demineralization produced by fungal organic acids, as well as the presence of cells with *C. albicans* hyphas invading dentinal tubules, would prove the capacity of this yeast to invade and destroy organic and inorganic dental tissues.

As the participation of oral bacteria in fungal adhesion to prosthetic surfaces has been demonstrated (16), it may be speculated that denture wearers are not only susceptible to candidiasis, but also at higher risks of caries and periodontal disease progression due to the presence of cariogenic bacteria like *S. mutans*. The clinical implications of these fungal-bacterial interactions emphasize the importance of patient oral hygiene in controlling denture-associated stomatitis. Supposing that the microbial adherence to glass observed in this study is similar to that occurring on prosthetic surfaces, the use of prosthetic plate samples as test specimens in future experiments would be of interest.

In vivo studies have demonstrated the antibacterial (9) and antifungal (10) effects of phytotherapeutic agents derived from *Punica granatum Linn* extract. This study investigated *in vitro* the therapeutic potential of this agent against bacteria and yeasts either alone or pairs and groups. *In vitro* studies using biofilm models

are those that comes closer to a clinical situation and more precisely reflect *in vivo* conditions (17).

According to Cotter and Kavanagh (18) therapeutic agents that do not exhibit fungicidal or fungistatic activity can inhibit yeast adherence by other mechanisms and it is possible that these agents represent a new perspective in the combat and control of superficial fungal infection. It is believed that the use of a product containing a component that specifically reduces or inhibit the adherence capacity of *C. albicans* can be used in association with already existing antifungal agents.

As far as bacterial adherence inhibition is concerned, the findings of this study were consistent with those of Pereira (9), who assessed the minimum inhibitory concentrations of adherence of *Punica granatum Linn* extract against *S. mitis* (1:512), *S. mutans* (1:256), *S. sanguis* (1:128) and *C. albicans* (1:64). The antifungal action of this agent was tested in its brute state and diluted at different concentrations.

Recently, natural products have proved to be an alternative to synthetic chemical substances. Nostro et al. (8) demonstrated that the *Helichrysum italicum* extract interfered in the cariogenic properties of *S. mutans* through reductions of superficial hydrophobicity, inhibiting adherence of cell growth to glass in 90 to 93%. Those authors believe that the capacity of this extract to prevent bacterial adherence could be due to the effect of its flavonoid components, which have anti-glycosyltransferase activity. While assessing the minimum inhibitory concentrations of adherence of different vegetable dyes and propolis, Gebara et al. (19) observed that the inhibition of *S. mutans* and *S. sobrinus* adherence was a result of the inhibition of glucan synthesis by these substances. The present study found a similar effect on the inhibition of adherence to glass of *S. mutans*, *S. sanguis*, *S. mitis* and *C. albicans* by the *Punica granatum Linn* in the presence of sucrose.

Kakiuchi et al. (20) and Pereira (9) demonstrated the specific antimicrobial action of *Punica granatum Linn* on dental biofilm bacteria, i.e., disturbance of polyglycan synthesis, thus acting on the adherence mechanisms of these organisms to dental surface. The present study evaluated the inhibitory capacity of adherence of a gel derived from *Punica granatum Linn*, which is a fruit rich in tannin and polyphenolics. The possibility that this component interfered with different mechanisms of toxicity or astringency was investigated on the adherence of one yeast and three bacterial stains

to oral surfaces. The results indicated that the glucan synthesis and its antimicrobial action gave this gel an effective control of the already formed biofilm, which is considered the primary etiologic agent in caries disease and stomatitis.

Gebara et al. (19) reported that dental products containing natural substances have good market perspectives due to popular acceptance of phytotherapy, which represents an alternative to conventional treatments and could be introduced in the dental market as long as they are supported by scientific-based evidence.

The *Punica granatum* Linn (pomegranate) gel presented an inhibitory activity on the adherence of different bacterial strains and one yeast commonly found in the oral cavity. The findings of this study support the possibility that the *Punica granatum* Linn (pomegranate) gel might be used in the control of bacteria and yeasts responsible for oral infections such as caries, periodontal disease and stomatitis.

RESUMO

O propósito deste estudo foi investigar a concentração inibitória mínima de aderência (CIMA) de três linhagens de estreptococos (*mutans* ATCC 25175, *sanguis* ATCC 10557 e *mitis* ATCC 9811), *S. mutans* isolado clinicamente e de cepas de *Candida albicans*, separadamente ou associadas, frente a um gel fitoterápico obtido da *Punica granatum* Linn. (romã) e ao agente antifúngico miconazol (Daktarin® gel oral). A concentração inibitória mínima de aderência das bactérias ao vidro foi determinada na presença de sacarose a 5%, usando-se concentrações crescentes e dobradas da solução diluída do gel variando de 1:1 a 1:1024. Os valores de inibição do gel fitoterápico foram de 1:16 para *Smutans* (ATCC), *S. mutans* (IC) e *S. sanguis*; 1:128 para *S. mitis* e 1:64 para *C. albicans*. Sobre as mesmas linhagens, as concentrações inibitórias mínimas de aderência do miconazol foram: 1:512, 1:64, 1:4, 1:128, 1:16 respectivamente. O gel da romã apresentou maior eficácia sobre associações de três e quatro microrganismos do que o gel do miconazol. Os achados deste estudo sugerem o emprego desse agente fitoterápico pode ser uma opção no controle da aderência dos microrganismos testados na cavidade bucal.

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Accepted April 3, 2006