DEVELOPMENT AND IN VITRO EVALUATION OF BIOPOLYMERS AS A DELIVERY SYSTEM AGAINST PERIODONTOPATHOGEN MICROORGANISMS

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ABSTRACT

Periodontal disease is the major cause of tooth loss in adults. Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans are considered key pathogens in periodontitis. The treatment consists of oral hygiene education, instrumentation for removal of calculus (scaling), chemotherapy and periodontal surgery. Several agents are commercially available; these chemicals can alter oral microbiota and have undesirable side-effects such as vomiting, diarrhea and tooth staining. Hence, the search for alternative products continues and natural phytochemicals isolated from plants used as traditional medicine and the use of biomaterials are considered good alternatives. Chitosan and pullulan are polymers that have been proposed due to their favorable properties such as biocompatibility, biodegradability, and adhesion ability. They can be used as local delivery systems of active principles of plant extracts.

Thymus vulgaris, Matricaria chamomilla, Croton lechleri, Calendula officinalis L. and Juliana adstringens Schl. are known to have medicinal activity, and they are used in Mexican traditional medicine. Their extracts were tested in vitro for antimicrobial activity against P. gingivalis and A. actinomycetemcomitans, using agar diffusion and microdilution methods. The antimicrobial activity of films from biopolymers with plant extracts was evaluated by measuring the zones of inhibition against the tested organisms.

The aim of this study was to develop bioadhesive films from chitosan and pullulan with added plant extracts and determine the antimicrobial activity of films against periodontal pathogens.

Key words: biopolymers, plant extracts, delivery system, antimicrobial activity, periodontopathogen microorganisms.

DESARROLLO Y EVALUACIÓN IN VITRO DE BIOPOLÍMEROS COMO UN SISTEMA DE LIBERACIÓN CONTRA MICROORGANISMOS PERIODONTOPATÓGENOS

RESUMEN

La enfermedad periodontal es la principal causa de pérdida de dientes en los adultos. Los agentes causales comunmente identificados con la enfermedad son Porphyromonas gingivalis y Aggregatibacter actinomycetemcomitans. El tratamiento de la enfermedad consiste en educación sobre higiene oral, remoción de cálculos por medio de instrumentación (raspado y alisado de la raíz), la administración de medicamentos y cirugía. Hay múltiples agentes químicos disponibles comercialmente; éstos pueden alterar la microflora oral y tener efectos secundarios indeseables como vómito, diarrea y pigmentación dental. Por lo tanto, los productos naturales como los fitoquímicos aislados de plantas que son usadas como medicinas tradicionales y los biomateriales, son considerados buenas alternativas. El quitosan y el pululan son polímeros que han sido propuestos debido a sus propiedades de biocompatibilidad, biodegradabilidad, habilidad de adhesión y que pueden ser usados como sistemas de liberación de los principios activos de extractos de plantas.

Los extractos de Thymus vulgaris, Matricaria chamomilla, Croton lechleri, Calendula officinalis L. y Juliana adstringens Schl. son conocidos por tener actividad medicinal y se usan en la medicina tradicional Mexicana. La actividad antimicrobiana de sus extractos fue probada in vitro contra P. gingivalis y A. actinomycetemcomitans usando los métodos de difusión en agar y de microdilución. La actividad antimicrobiana de películas a base de biopolímeros con extractos de plantas fue evaluada midiendo las zonas de inhibición de crecimiento de los organismos probados.

El propósito de este estudio fue desarrollar películas bioadhesivas de quitosan y pululan adicionadas con extractos de plantas y evaluar su actividad antimicrobiana contra periodontopatógenos.

Palabras clave: biopolímeros, extractos de plantas, sistema de liberación, actividad antimicrobiana, microorganismos periodontopatógenos.

INTRODUCTION

Periodontal disease is an undesirable condition which has widespread occurrence. It is an inflammatory disease that affects the periodontum, which consists of the investing and supporting tissues surrounding a tooth (i.e., the periodontal ligament, the gingiva, and the alveolar bone[1].

Aggregatibacter actinomycetemcomitans, Tannerella forsythensis and *Porphyromonas gingivalis,* are among the principal microorganisms associated with

the process of periodontal diseases[2,3]. Microorganisms contribute to both the initiation and progression of gingivitis, plaque, and periodontal disease. Therapeutic approaches include oral hygiene instruction, surgical techniques and mechanical debridement of the root surfaces, scaling and/or treatment of the infection with systemic or local antibiotics[4].

As a public health concern, the notion of periodontal disease being a risk factor for cardiovascular disease, stroke and premature birth brings increased urgency to the need for controlling and preventing the disease in a cost-efficient manner[5].

Chitosan a (1→4)-linked 2-amino-2-deoxy-s-D-glucan, is prepared by N-deacetylation of chitin, which is the second most abundant polysaccharide found in nature[6] and distributed widely in nature, especially in marine invertebrates, insects, yeast, and fungi. There are numerous works on the use of chitosan in a number of biomedical applications, including drug-delivery systems, tissue engineering, and orthopedics[7].

Pullulan is a neutral linear polyssacharide composed of (1→6)-linked α-D-maltotriose residues synthesized from starch or sugar by *Aureobasidium pullulan*, which has adhesive properties and can be used in wound-healing compositions[8].

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years in many parts of the world. Mexico has a great wealth of medicinal plants and it has been popular tradition to use plants for scientific investigation, many of which deal with the antimicrobial properties of the plant extracts and their potential as a clinically relevant antimicrobial therapy.

Thymus vulgaris (thyme), *Matricaria chamomilla* (chamomile), *Croton lechleri* (sangre de drago), *Calendula officinalis* L. (marigold) and *Juliana adstringens* Schl. (cuachalalate) are known to have antimicrobial, antiviral, anti-inflammatory and antifungal properties[9-11].

Many compounds derived from plants used in traditional medicinal systems have been recorded in pharmacopeias as agents used to treat infections, and a number of these have been recently investigated for their efficacy against oral microbial pathogens.

Essential oil and extracts from fresh leaves and flowers of *T. vulgaris* can be used as aromatic additives in foods, pharmaceuticals, and cosmetics. Its primary constituents are thymol and carvacrol.

The constituents of *M. chamomilla* thought to have antimicrobial properties include alpha-bisabolol, luteolin, quercetin, and apigenin[11].

The chemical composition of *C. lechleri* includes a considerable number of compounds, including several simple phenols and diterpenes, proanthocyanidins, and phytosterols[12].

Marigold is a phytotherapeutic plant rich in biologically active metabolites, such as sesquiterpens, triterpens, flavonoids, carotenoids and tannins. These components confer antiseptic action, anti-inflammatory, anti-edematous, immunomodulatory activity and antimicrobial effects[13].

J. adstringens Schl. is an endemic plant of Mexico. Its principal compounds are masticadienonic acid; 3alpha-hydroxymasticadienonic acid; 3-epi-oleanolic; as well as the sterol beta-sitosterol[14,15].

Due to the safety risk of systemic uptake of drugs administered by local delivery[16,17], the use of natural, safe plant medication[11] provides an attractive alternative for treating oral diseases with potentially high compliance. The plant extracts mentioned in this work are effective in treating various oral disorders such as plaque reduction, candidiasis, canker sores and alveolitis[15,18,19].

The aim of this work was to provide a self-bioadhesive film for a topical application that adheres to the oral mucosal tissues and investigate the *in vitro* antimicrobial effects against two pathogenic microorganisms that have been associated with periodontal diseases, in order to assess the potential for developing them into agents that can be used as preventive or treatment therapies, either alone or in combination with conventional treatments.

MATERIAL AND METHODS

Bacteria Growth conditions

Porphyromonas gingivalis ATCC 33277 and *Aggregatibacter actinomycetemcomitans* ATCC 43718 were activated in trypticase soy broth and brain heart infusion in the anaerobic chamber and incubated at 35°C for 48 hours in anaerobic and 5% CO_2 atmosphere conditions respectively. For long-term storage, strains were made on glycerol stocks.

Plant material

Different parts of *Thymus vulgaris*, *Matricaria chamomilla*, *Croton lechleri*, *Calendula officinalis* L. and *Julliana adstringens* Schl. were collected

and extracted with hydroalcoholic solvents to obtain aqueous and organic extracts.
The extracts were filtered with filters role of Whatman Ltd., sterilized by filtration by 0.22 µm Millipore filters Durapore ® GV and stored in sterile amber bottles.
To evaluate the antimicrobial activity of each extract, 1:2 and 1:3 dilutions were prepared and the Minimum Inhibitory Concentration (MIC) was determined by microdilution technique NCCLS (National Committee for Clinical Laboratory Standards, 1997).

Antimicrobial activity

In vitro antimicrobial activities of aqueous extracts of *T. vulgaris*, *Matricaria chamomilla, C. lechleri*, *Calendula officinalis* L., *J. adstringens* Schl and Chlorhexidine 0.12% (as positive control) were evaluated against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.
The minimum inhibitory concentrations (MIC) were determined by the microdilution method using 96-well plates. The inoculum was prepared and adjusted to 0.5 McFarland standard turbidity.
The correct turbidity density was measured at 540nm using a spectrophotometer.
All the extracts were dissolved in trypticase and brain heart infusion broth (for *P. gingivalis* and *A. actinomycetemcomitans* respectively) and then serial two-three fold dilutions were made. The 96-well plates were prepared by dispensing 100 µL of each dilution into double well and adding 100 µL of the inoculum. The final volume in each well was 200 µL. Two wells were left with 200 µL of media, two more with 200 µL of inoculum and two with 200 µL of chlorhexidine (as positive control). The plates were incubated at 35°C for 24 hours according to the requirements of each strain. The MIC was determined from the lowest concentration of extracts to inhibit the growth of microorganisms. Inhibition of proliferation was assessed by optical density measurements (625 nm).
For minimum bactericidal concentrations (MBC), inoculum from clear wells was resuspended in PBS to give a standard concentration of cells ($1x10^{-1}$) and 10 µL were inoculated into Petri dish pouring molten trypticase or brain heart infusion agar to 45-50°C and swirled to distribute the medium homogeneously. The inoculated plates were incubated at 37°C for 24 hours in anaerobiosis and 5% CO_2 atmosphere conditions. Absence of bacterial growth noted on the solid medium was considered to be indicative of bactericidal activity of the extracts. Petri dishes containing media and 10 µL of chlorhexidine from the control wells were used as control.
All assays and controls were made three times in triplicate.

Preparation of the polymers

Chitosan with deacetylation degree 85% was purchased from Sigma Chemical CO. St. Louis MO. USA.
Pullulan (molecular weight=200,000) was donated by Hayashibara Company (Okayama, Japan).
Three different formulations of each biopolymer were tested to obtain the best properties (thickness and solubility) for the production of films. 1.0, 1.2 and 0.8 g of chitosan powder were dissolved in 100mL 1% (vol/vol) acetic acid aqueous solution for final concentrations of 1%, 1.2% and 0.8%.
Pullulan was used at 10%, 12% and 10%, plus sorbitol (as plasticizer) dissolved in bidistilled water.
The average pH was 3.9 for chitosan and 5.7 for pullulan. From the three formulations tested, the final selected concentration for chitosan was 1% and 10% plus 0.3% of sorbitol for pullulan. These formulations of biopolymer were added to aqueous extracts in concentrations of about 10% of the MBC of the plants that showed the best antimicrobial activity (*T. vulgaris*, *J. adstringens* Schl and *C. lechleri)* and stirred until the mixture became homogeneous.
The method used was "casting", pouring the formulation into acrylic moulds, spreading the mixture with a spoon and drying at room temperature. Films were recovered manually after 24 hours. Once recovered, the films were cut with a 6 mm diameter hole puncher and stored at 25°C and 25% relative humidity until they were used.

Antimicrobial activity by disc diffusion assay

The discs (6 mm in diameter) of biopolymers with the added extracts where applied by pressing slightly on the solid trypticase and brain heart infusion agar medium inoculated with 1 µL of bacterial suspension (10^8 CFU/mL) of *P. gingivalis* and *A. actinomycetemcomitans*. One disc impregnated with chlorhexidine was used as positive reference in each plate. The inoculated plates were

incubated at 37°C for 24 hours in anaerobiosis and 5% CO_2 atmosphere conditions respectively. Antimicrobial activity was evaluated by measuring the inhibition zones formed on the media in millimeters with a ruler. All inhibition assays and controls were repeated three times with each test conducted in duplicate. The collected data were analyzed by descriptive statistics ANOVA test using SPSS 17.0 software. The results were presented as mean ±S.D. Statistical significance was accepted at the $p<0.05$ level.

RESULTS AND DISCUSSION

Periodontal diseases are the most frequent infections in humans. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are among the microorganisms most commonly identified with the process[1]. Increasing resistance to drugs and the side effects of some antibiotics and mouthwashes such a chlorhexidine, used in the treatment of periodontitis, justify the development of novel options as alternative of dental treatments.

Plant extracts used in different countries as traditional medicines have been studied extensively -due to their properties as antimicrobials[20]. In this work, we tested the antimicrobial activity of aqueous extracts of *T. vulgaris*, *M. chamomilla*, *C. lechleri*, *C. officinalis* and *J. adstringens* Schl. by microdilution and agar diffusion method. The antimicrobial effect of the two biopolymers (chitosan and pullulan) with added aqueous extract of the plants on cultures of *P. gingivalis* and *A. actinomycetemcomitans* was tested using the disc diffusion method.

The plants used in this work showed antimicrobial activity against the two oral pathogens tested. Figure 1 shows the results obtained on the inhibitory activity of the different concentrations of *T. vulgaris, M. chamomilla, C. lechleri, C. officinalis* L. and *J. adstringens* Schl. against the tested strains. Our results show that minimal inhibitory concentration (MIC) of *J. adstringens* Schl. was 37 mg/mL for *P. gingivalis* and the same concentration of *C. lechleri* for *A. actinomycetemcomitans*. MIC of *T. vulgaris* and *M. chamomilla* were 62.5 mg/mL for both strains, since *C. officinalis* L. was 62.5 mg/mL for *P. gingivalis* and 250 mg/mL for *A. actinomycetemcomitans* and *C. lechleri* 111 mg/mL for *P. gingivalis*.

1.2 mg/mL of chlorhexidine was effective for both strains.

The MBC value of *J. adstringens* and *C. lechleri* was 37 mg/mL for *P. gingivalis* and *A. actinomycetemcomitans* respectively. Concentrations of 62.5 mg/mL of *T. vulgaris* for *P. gingivalis* and *J. adstringens* for *A. actinomycetemcomitans* were needed. MBC of *T. vulgaris* were 111 mg/mL for *A. actinomycetemcomitans* and *C. lechleri* for *P. gingivalis*. *M. chamomilla* required higher concentrations (250 mg/mL) for both microorganisms. MBC of *C. officinalis* was 250 mg/mL for *A. actinomycetemcomitans* and 111 mg/mL for *P. gingivalis*. For chlorhexidine, 1.2 mg/mL was effective for both strains tested (Fig. 2).

Previous works indicate that chitosan and pullulan have certain *in vitro* antibacterial activity[21].

Other studies show that the exposure of *A. actinomycetemcomitans* to chitosan resulted in the disruption of cell membranes and that it could be considered for the treatment of periodontal diseases[22]. In this study, we tested the antimicrobial activity of two biopolymers (chitosan and pullulan) with added aqueous extract of *T. vulgaris*, *J. adstringens* Schl. and *C. lechleri* on cultures of *P. gingivalis* and *A. actinomycetemcomitans* using the disc diffusion method.

Thickness of chitosan films was an average of 0.03 mm and for pullulan 0.07 mm.

Figure 3 shows the inhibition zones formed by the biopolymers chitosan and pullulan with added aqueous extracts of *T. vulgaris*, *J. adstringens* Schl. and *C. lechleri*, against *A. actinomycetemcomitans* cultures.

For chitosan, the inhibition zones were 4.5 mm without extract (CONTROL), 4 mm with *T. vulgaris*, 4.3 mm with *J. adstringens* Schl., and 6 mm with *C. lechleri*.

For pullulan, the inhibition zones were 9.3 mm without extract (CONTROL), 9.6 mm with *T. vulgaris*, and 7.3 mm with *J. adstringens* Schl.

Figure 4 shows the inhibition zones formed by the biopolymers with plant extracts against *P. gingivalis*.

For chitosan, the inhibition zones were 4 mm without extract (CONTROL), 3 mm with *T. vulgaris*, 3.6 mm with *J. adstringens* Schl. and 4.5 mm with *C. lechleri*.

For pullulan, the inhibition zones were 4 mm without extract (CONTROL), 6 mm with *T. vulgaris* and *C. lechleri*, and 5 mm for *J. adstringens* Schl.

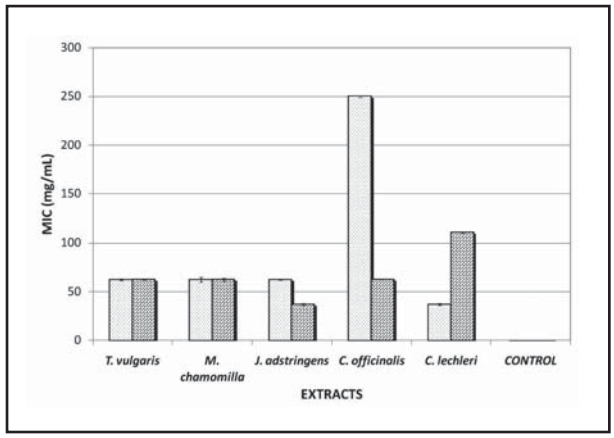

Fig. 1: Minimum inhibitory concentration (MIC) of the plant extracts on cultures of periodontopathogen microorganisms tested. ⁞⁞ A. actinomycetemcomitans; ▨ P. gingivalis. CONTROL (chlorhexidine 0.12%).

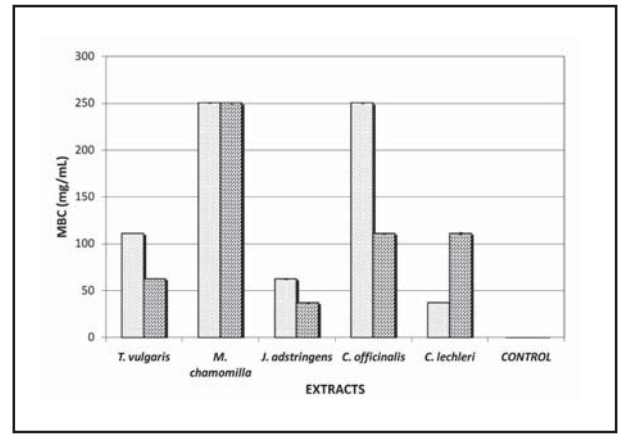

Fig. 2: Minimum bactericidal concentration (MBC) of the plant extracts on cultures of periodontopathogen microorganisms. ⁞⁞ A. actinomycetemcomitans; ▨ P. gingivalis. CONTROL (chlorhexidine 0.12%).

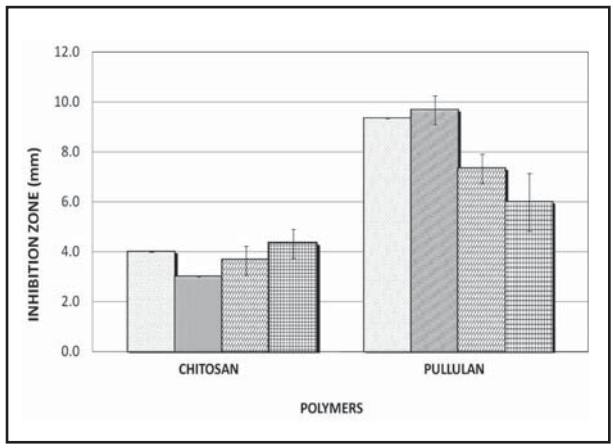

Fig. 3: A histogram plotting the width of the inhibition zones of the biopolymers with aqueous extracts on A. actinomycetemcomitans.

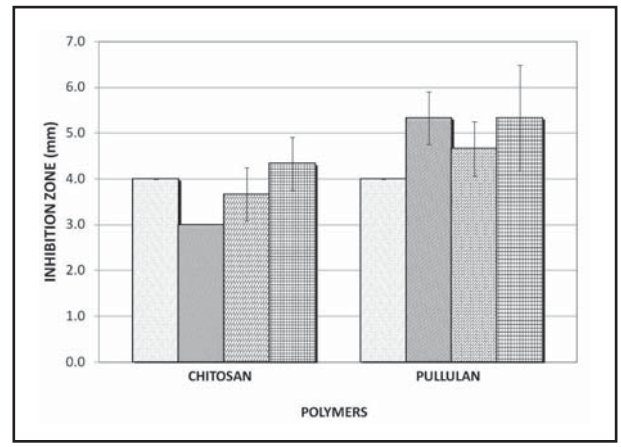

Fig. 4: A histogram plotting the width of the inhibition zones of the biopolymers with aqueous extracts on P. gingivalis.

⁞⁞ *Polymer without extract (CONTROL)*
\\\ *Polymer with T. vulgaris extract*
▨ *Polymer with J. adstringens extract*
Polymer with C. lechleri extract

⁞⁞ *Polymer without extract (CONTROL)*
\\\ *Polymer with T. vulgaris extract*
▨ *Polymer with J. adstringens extract*
Polymer with C. lechleri extract

CONCLUSIONS

The findings suggest that the use of pullulan with plant extracts had synergistic effects, enhancing the antimicrobial activity of the films. Chitosan films without extracts showed inhibitory effect against the growth of both strains.

Our results reveal that both biopolymers with added plant extracts have antibacterial activity and can be used as bio-adhesive film against the periodontopathogens tested. These *in vitro* data still need to be validated as natural treatment for periodontal diseases by assessing clinical performance.

Data on the antimicrobial activity on pathogens are important for the future application of these polymers and plant extracts in medicine and dentistry, especially for *J. adstringens*, which showed the best result. This is an endemic plant of Mexico which is in danger of extinction.

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