An Acad Bras Cienc (2020) 92(4): e20200562 DOI 10.1590/0001-3765202020200562
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Chitosan gels for buccal delivery of *Schinus molle L.* essential oil in dogs: characterization and antimicrobial activity *in vitro*

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Abstract: Periodontal disease is considered the main oral cavity disorder in dogs. Essential oils have the potential for use in the prevention and treatment of oral diseases. The antimicrobial activity of *Schinus molle* L. essential oil (SMEO) has already been reported. Chitosan, a natural product with antimicrobial activity and good biocompatibility has potential in biodental applications. In this study, we evaluated the *in vitro* antimicrobial activity of SMEO against bacteria associated with periodontal disease in dogs, developed and evaluated the physicochemical properties of a novel chitosan-based buccal delivery system containing SMEO. SMEO showed antimicrobial activity against Gram positive and Gram negative bacteria associated with canine periodontitis, with MIC values of 750 $\mu g.mL^{-1}$ for *Staphylococcus* spp. and *Streptococcus* spp, 1000 $\mu g.mL^{-1}$ for *Corynebacterium* spp. and 1250 $\mu g.mL^{-1}$ for *Pseudomonas* spp. All formulations evaluated presented adequate physicochemical properties, good stability, and pH values close to buccal pH (5.0–7.0). Chitosan gel loaded with SMEO showed potential as a SMEO delivery system, having the ideal physicochemical and rheological properties (pseudoplastic and apparent viscosities) required for application on buccal tissue. Thus, we can conclude that formulation has the potential to be used for buccal mucosa delivery in the prevention and treatment of periodontal disease in dogs.

Key words: essential oils, gel mucoadhesive, periodontitis canine, *Schinus molle L.*

INTRODUCTION

Periodontal disease is an oral disease caused by the agglomeration of biofilms composed of bacteria and their products on the surface of the teeth and gums, which promote the progressive inflammatory process of the periodontium (Perchyonok 2018). It commonly occurs in dogs, being their main oral cavity disease (Kačírová et al. 2019).

Plant extracts, essential oils and purified phytochemicals have the potential for use in the prevention and treatment of oral diseases (Palombo 2011). The antimicrobial activity against oral pathogens in humans of some essential oils, such as *Melaleuca alternifolia* (Hammer et al. 2003), *Artemisia lavandulaefolia* and *Artemisia scoparia* (Cha et al. 2005), *Lavandula officinalis* (Takarada et al. 2004), *Lippia sidoides* (Botelho 2007), and *Ocimum basilicum* (Besra & Kumar 2018), have already been reported. Several essential oils, such as *Thymus vulgaris, Rosmarinus officinalis L., Origanum vulgare, Syzygium aromaticum*, have been used as nutraceuticals in the treatment and prevention of canine periodontitis (Gupta et al. 2019).

Essential oils (EOs) from *Schinus molle* L. (Anacardiaceae) has shown antioxidant (Martins et al. 2014), ectoparasiticidal (Batista et al. 2016) and antihemostatic properties (Siqueira et al. 2020). Their antimicrobial activity has also been reported (Marino et al. 2001, Deveci et al. 2010, Martins et al. 2014, Eryigit et al. 2017).

The development of buccal delivery systems for treating periodontitis is necessary to obtain prolonged local effect in the oral cavity. Due to its antimicrobial activity and biocompatibility, chitosan (CHT) is a potential material for biodental applications (Husain et al. 2017). Furthermore, its adhesion properties (Brannigan & Khutoryanskiy 2019) make chitosan-based formulations, such as films or gels, suitable for delivery systems in the prevention and treatment of periodontal disease (Gupta et al. 2019).

We evaluated the *in vitro* antimicrobial activity of *Schinus molle* L. essential oil (SMEO) against bacteria associated with periodontal disease in dogs. Moreover, we developed a novel chitosan-based buccal delivery system containing SMEO and evaluated the influence of both dimethyl sulfoxide (DMSO) and EO concentrations on gels' properties (pH, rheology, physical stability) to gauge the potential of these formulations as buccal delivery systems for the treatment and prophylaxis of canine periodontal disease.

MATERIALS AND METHODS

Plant material

Leaves of *S. molle* were collected during the summer of 2017 in the city of Volta Redonda, Rio de Janeiro, Brazil (GPS 22°31'36·23S; 44°04'31·62W). A voucher specimen was deposited with the herbarium of the Institute of Botany (UFRRJ, Brazil) under the code RBR 35791. Authorization to collect botanical material was obtained from the National Genetic Heritage and Associated Traditional Knowledge Management System (A85E6DF).

Extraction, content (% w/w) and chemical characterization of the essential oil

S. molle leaves were dried at room temperature, protected from light and moisture. Subsequently they were manually ground, subjected to the extraction process by hydrodistillation (50 g of dry leaves) and characterized by GC-FID and GC-MS as described by Cavalcanti et al. (2015) and Batista et al. (2016). To separate, detect and quantify the constituents, 1 µL of the essential oil (10 µL/mL) was injected into the gas chromatograph (GC). A Hewlett-Packard 5890 Series II (Palo Alto, USA), equipped with flame ionization detection and a split/splitless injector, in a split ratio of 1:20 was used to separate and detect the constituents in the essential oil. The compounds were separated on a non-polar fused silica capillary column, similar to DB5 with 30 m × 0.25 mm (i.d.) × 0.25 µm (film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The column temperature was programmed as follows: 60°C for 2 min followed by heating at 5°C min–1 to 110°C, followed by heating at 3°C min–1 to 150°C and finally by heating at 15°C min–1 until 290°C and holding constant for 15 min. The injector temperature was 220°C and the detector temperature was 290°C. For GC/MS analysis, 1 µL of essential oil was injected in the gas chromatograph coupled to mass spectrometer (GC-MS) QP-2010 Plus (Shimadzu, Japan). The flow of the helium gas carrier, the capillary column and the temperature conditions for the GC-MS analysis were the same as described for the GC. The temperature of the injector was 220°C and the temperature of the interface was 250°C. Mass spectra were obtained with a quadrupole detector operating at 70 eV, with 40–400 *m/z* mass range and scanning rate equal to 0.5 scan s^{-1}.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the broth microdilution technique in the concentration range of 0.625-20,000 $\mu g.mL^{-1}$ in DMSO. The analyses were carried out in triplicate. The oral bacterial strains used in this study were *Corynebacterium* spp, *Pseudomonas* spp, *Staphylococcus aureus* and *Streptococcus*. spp, isolated from animal samples belonging to the bacterial stock of the Veterinary Bacteriology Laboratory-UFRRJ, according to CLSI (2018).

Preparation of gels

Chitosan (degree of deacetylation of 76%) was purchased from Sigma-Aldrich and gels were prepared based on previous studies (Cid et al. 2012). Pure CHT gels (chitosan gels without active ingredients or adjuvants) were obtained by dispersion of appropriate amounts of CHT in 1% aqueous lactic acid (stirred mechanically until homogenization), yielding 3.0% (w/w) of gels. Gels were loaded with weighed amounts of DMSO, the penetration enhancer (1.0%, 2.0%, and 3.0% w/w), and SMEO to final concentrations of 0.125%, 0.25% and 0.5% (w/w). Concentrations of CHT, SMEO and DMSO reported in Table I are expressed as weight/weight percentages (% w/w). No insoluble particles were observed after preparation of the gels.

Evaluation of gel formulations

Physical appearance of gel formulations

The gels were subjected to visual analysis for opacity, consistency and presence of particles.

Determination of pH of gel formulations

The pH values were determined with a potentiometer fitted with a DME-CV4 electrode. One gram of each formulation was weighed and homogenized in 10.0 ml of purified water in a glass container. Measurements were performed in triplicate and means and standard deviations were calculated.

Centrifugation test

The centrifugation test was performed under refrigeration, where 1.0 gram of each formulation was transferred to an Eppendorf tube and centrifuged at 3,000 rpm for 30 minutes and at 3800 rpm for 5 hours, each formulation was checked in terms of sedimentation (Aslani et al. 2018).

Cooling and heating test

In order to evaluate the thermal stability, the formulations were submitted to freezing and thawing cycles. In this case, 1.0 gram of each formulation was transferred to an Eppendorf tube and subjected to three 48-hour cycles at 45°C and 4°C (Aslani et al. 2018).

Rheological measurements

Oscillatory measurements were carried out at 25°C with a Thermo Scientific HAAKE RheoStress 1 rotary rheometer with cone-plate geometry and #CP52 spindle. Samples were placed in the cylinder and the internal rotating spindle was set to rotate at rising angular velocity (1 rpm to 20 rpm), to initially disrupt the system, which was then reorganized by decreasing the angular velocity. All measurements were performed at room temperature. CHT gels containing SMEO and DMSO in different concentrations were evaluated using the power law rheological model to determine the effects of flow index, consistency index and viscosity (apparent viscosity).

Table I. Rheological parameters and pH values in formulations containing chitosan gel 3% and different concentrations of *Schinus molle* essential oil and dimethyl sulphoxide.

Formulation		Flow index (n)	Consistency index	Apparent viscosity (mPA)	pH value*
1	CHT pure gel 3%	0.4033	276.6	4282	5.01 ± 0.03[a]
2	CHT gel 3% + DMSO 1%	0.4093	249.5	3893	5.02 ± 0.01[a]
3	CHT gel 3% + DMSO 2%	0.4118	263.6	4185	5.00 ± 0.04[a]
4	CHT gel 3% + DMSO 3%	0.4201	272.0	4889	5.00 ± 0.05[a]
5	CHT gel 3% + SMEO 0.125%	0.4755	376.2	7233	5.01 ± 0.03[a]
6	CHT gel 3% + SMEO 0.125% + DMSO 1%	0.5083	314.3	6667	5.01 ± 0.04[a]
7	CHT gel 3% + SMEO 0.125% + DMSO 2%	0.5186	318.9	6983	5.05 ± 0.03[a]
8	CHT gel 3% + SMEO 0.125% + DMSO 3%	0.4946	379.6	7724	5.08 ± 0.02[a]
9	CHT gel 3% + SMEO 0.250%	0.4338	464.5	7848	5.12 ± 0.01[b]
10	CHT gel 3% + SMEO 0.250% + DMSO 1%	0.4580	412.3	7414	5.13 ± 0.01[b]
11	CHT gel 3% + SMEO 0.250% + DMSO 2%	0.3977	649.0	9799	5.12 ± 0.01[b]
12	CHT gel 3% + SMEO 0.250% + DMSO 3%	0.4509	427.3	7636	5.14 ± 0.01[b]
13	CHT gel 3% + SMEO 0.500%	0.4337	253.5	4286	5.17 ± 0.01[c]
14	CHT gel 3% + SMEO 0.500% + DMSO 1%	0.4308	267.6	4479	5.18 ± 0.02[c]
15	CHT gel 3% + SMEO 0.500% + DMSO 2%	0.3870	323.3	4766	5.23 ± 0.02[d]
16	CHT gel 3% + SMEO 0.500% + DMSO 3%	0.3986	309.2	4722	5.27 ± 0.01[e]

CHT: chitosan, SMEO: *Schinus molle* essential oil, DMSO: dimethyl sulphoxide.
***pH values ± sd.**
Different letters differ significantly P <0,05.
Equal letters do not differ significantly from each other P > 0,05.

RESULTS

Chemical composition of *Schinus molle*

Our research group has previously published a number of papers on *S. molle* extracts and analysis of its essential oil. These papers have shown SMEO to be rich in monoterpenes (1 – *ß*-pinene – 6.7%; 2 – trans-pinocarveol – 6.2%) and sesquiterpenes (3 – spathulenol - 11.7%, 4 – cubenol – 127.1% and 5 - caryophyllene oxide – 15.3%) (Batista et al. 2016, Cavalcanti et al. 2015, Siqueira et al. 2020). These are reported in Figure 1.

Minimum inhibitory concentration (MIC) determination

The broth microdilution technique revealed that *Staphylococcus* spp. and *Streptococcus* spp. presented the lowest MIC value (750 $\mu g.mL^{-1}$), followed by *Corynebacterium* spp., which presented 1000 $\mu g.mL^{-1}$, and *Pseudomonas* spp., with the highest value, 1250 $\mu g.mL^{-1}$.

Evaluation of gel formulations

The formulations submitted to centrifugal and thermal tests showed good stability, with no phase separation under the experimental conditions tested.

The organoleptic characteristics were influenced by the addition of DMSO and SMEO. Formulations #1 to #4 showed yellowish color and opacity, with characteristic chitosan odor and taste. The incorporation of the SMEO in different concentrations (#5 to #16) altered the odor, which became that characteristic of the essential oil of *S. molle*. In addition, formulations with a higher concentration of essential oil (0.5%) showed increased opacity and consistency. No precipitation or dispersed particles were observed in the gel, demonstrating the compatibility of the formulation components.

The formulations showed pH values around 5.0, close to buccal pH (5.0–7.0) (Mangilal et al. 2019), thus suitable for buccal application (Table I).

The incorporation of DMSO (penetration enhancer) at different concentrations did not influence the pH values, since there was no statistical difference between loaded gels (#2, #3, #4) and pure gel (#1) (Table I). The incorporation of SMEO at the lowest concentration (#5) did not influence the pH either, but the increase of EO concentration led to an increase of pH values (#9, #13).

The gel loaded with DMSO at the highest concentration (#4, 4889 mPa) had higher apparent viscosity than the pure gel (#1, 4282 mPa). The addition of OE at the highest concentration (#13) did not affect the apparent viscosity (4286 mPa). However, at lower concentrations (0.125% and 0.250 %), the apparent viscosity increased, reaching 7233 mPa (#5) and 7848 mPa (#9). Gel loaded with 0.250% SMEO had higher apparent viscosity with the addition of DMSO, reaching 9799 mPa at a concentration of 2% (#11).

The rheograms showed concave curves for shear stress in relation to shear rate. An inversely

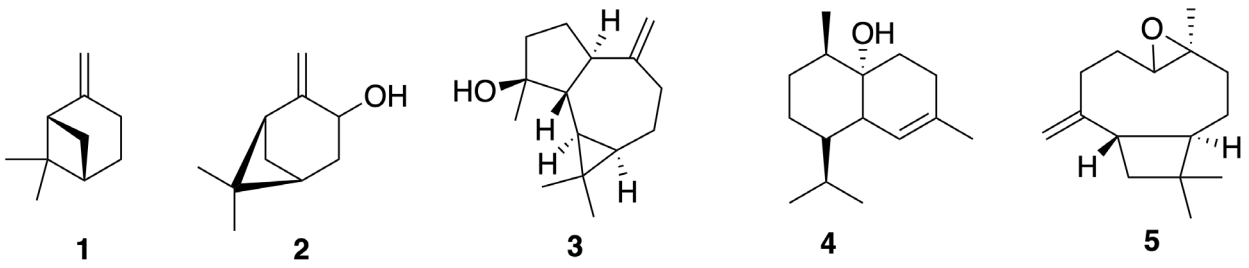

Figure 1. Chemical structures of the major compounds from *Schinus molle* essential oil.

proportional relationship between these two parameters was observed (Figure 2). This curve profile is characteristic of non-Newtonian fluids and represents pseudoplastic behavior (Rapp 2017). This behavior was confirmed by the flow index (n) evaluation, where all formulations presented n value less than 1, the characteristic value of fluids classified as pseudoplastic (Table I) (Macossko et al. 1994). Decreasing apparent viscosity with increasing shear rate also characterizes pseudoplastic materials (Schott 1995).

DISCUSSION

Although the etiology of periodontal disease is poorly studied, it is known that bacteria play an important role (Williams et al. 2011). Among the organisms most often associated with this disease are *Bacteroides fragilis, Porphyromonas salivosa, Prevotella intermedia* (Gupta et al. 2019), *P. gingivalis* and *P. intermedia* (Ramseier et al. 2009), among others. Anaerobic Gram positive bacteria including *Streptococcus spp.* and *Staphylococcus spp.* are predominant at the beginning of plaque formation, while Gram negative bacteria become predominant with increased thickness and maturation of the biofilm (Gupta et al. 2019). $Gram^+$ (*Streptococcus spp.* and *Staphylococcus spp.*) and $Gram^-$ (*Corynebacterium spp.* and *Pseudomonas spp.*) bacteria used in this study have already been associated with periodontitis in dogs (Pieri et al. 2014, 2016, Williams et al. 2011).

The results of our antibacterial activity assays showed that the SMEO exhibits greater antimicrobial activity against $Gram^+$ strains of *Staphylococcus spp.* and *Streptococcus* spp. (750 $\mu g.mL^{-1}$) compared with the activity against $Gram^-$ strains *Corynebacterium* spp. (1000 $\mu g.mL^{-1}$) and *Pseudomonas* spp. (1250 $\mu g.mL^{-1}$), corroborating the results reported by Martins et al. (2014). However, the values found in this study for *Staphylococcus* are higher than those reported by Martins et al. (2014) (MIC = 125 $\mu g.mL^{-1}$) and lower than those reported by Deveci et al. (2010) (MIC = 2000 $\mu g.mL^{-1}$). The different responses to antimicrobial activity can be explained by the diversity of the composition and concentration of each component in the SMEO (Marino et al. 2001). It is important to highlight the strong activity against *Streptococcus* spp., since they are described as the most important in the initial adhesion of dental plaque in humans (Katsura et al. 2001).

Some studies have already reported the activity of plant extracts, essential oils and purified phytochemicals against periodontitis-related bacteria in dogs. Girão et al. (2003) reported significantly reduced histological and clinical aspects of the oral mucosa in treated with mouthwash of essential oil of *Lippia menosides*. Pieri et al. (2014) highlighted the potential of *Copaifera officinalis* oil for the treatment and prevention of canine periodontitis. The oil showed activity against *Streptococcus* spp. and *Staphylococcus* spp., but only at a high concentration (10%). In another study, Pieri et al. (2016) demonstrated the activity of the compound β-caryophyllene as a natural alternative for the treatment and prophylaxis of periodontitis in dogs, but in much higher concentrations than in the present study, with MIC values in the range of 6.25 to 50 $mg.mL^{-1}$ against *Streptococcus* spp., and from 25 to 100 $mg.mL^{-1}$ against *Staphylococcus* spp.

All formulations evaluated presented adequate physical properties and good stability when submitted to centrifugal and thermal tests, and pH values around 5.0, close to buccal pH (5.0–7.0) (Teelavath & Patnaik 2019), meaning they are suitable for buccal application. The formulations also presented adequate rheological behavior,

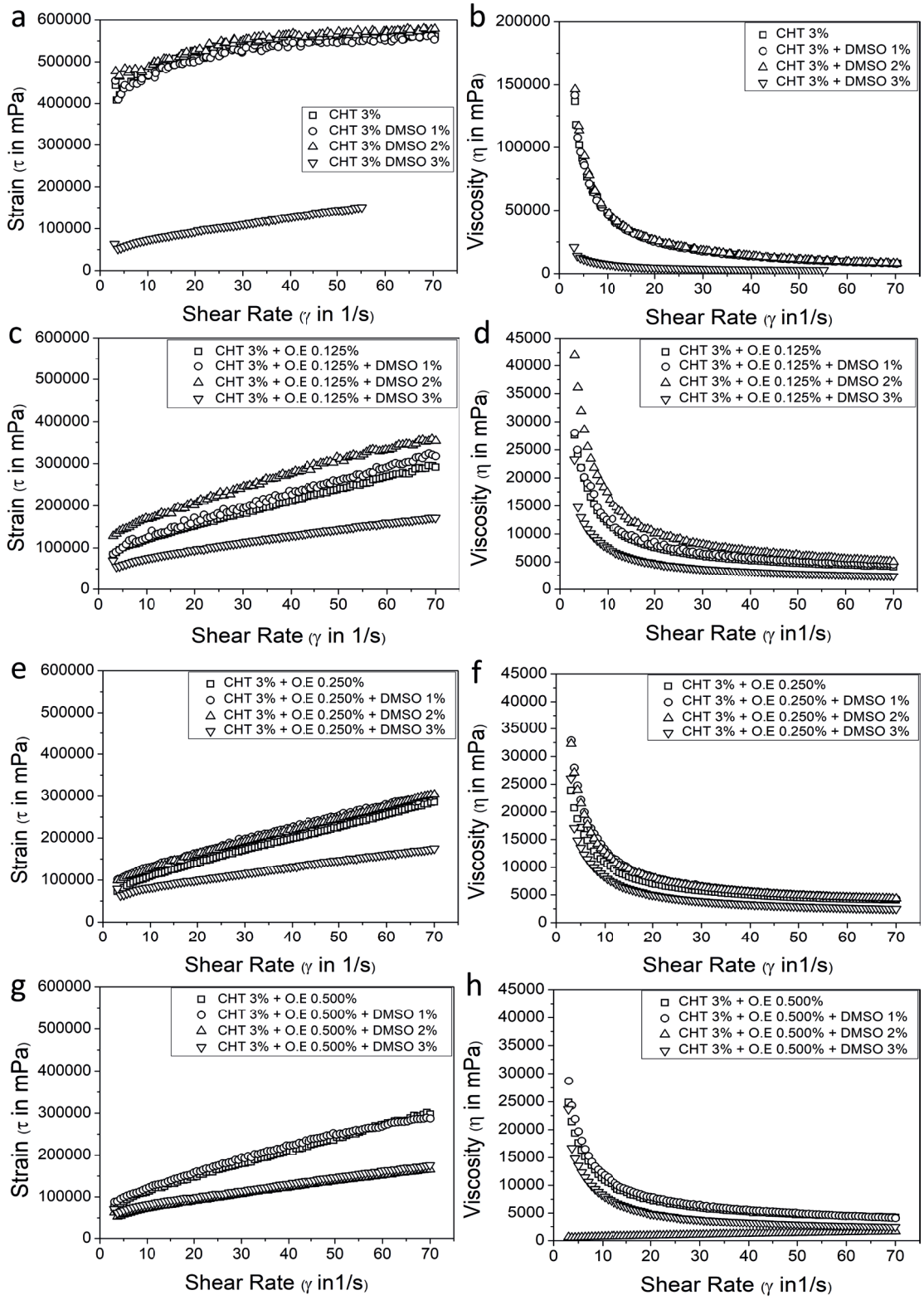

Figure 2. Stress curves and viscosity (a, b): formulations F1-F4 in the absence of *Schinus molle* essential oil; (c, d): formulations F5-F8 with *Schinus molle* essential oil 0.0125%; (e, f): formulations F9-F12 with *Schinus molle* essential oil 0.250%; (g, h): formulations F13-F16 with *Schinus molle* essential oil 0.500%.

presenting pseudoplastic properties with a flow index (n) less than 1 (Macossko et al. 1994, El-Hefian & Yahaya 2010). The flow index of pure chitosan gel (#1) (0.4033) corroborates the value of 0.39 reported by Cid et al. (2012). In general, the pseudoplastic properties favor the local action of drugs, which remain longer in the free form, have increased bioavailability, and consequently have stronger local effect. This pseudoplastic behavior of chitosan hydrogels has already been reported in other studies (e.g. Perioli et al. 2008). Although all the formulations evaluated showed adequate physicohemical and rheological properties, the formulation containing 0.250% SMEO and 2% DMSO (#11) presented the highest values of apparent viscosity when compared to the other formulations. For buccal application, more viscous pharmaceutical forms have the advantage of a slow flow index, which minimizes the risks of poisoning by accidental swallowing, in addition to ensuring good adhesion and greater contact with the mucosa (Wróblewska et al. 2020).

SMEO showed antimicrobial activity against Gram positive and Gram negative bacteria associated with periodontitis in dogs. The 3% chitosan gel loaded with 0.250% SMEO and 2.0% DMSO (# 11) showed potential as a SMEO delivery system, having the ideal physicochemical and rheological properties (pH, pseudoplastic and apparent viscosities) required for application on buccal tissue, so it has promise for administration of SMEO by buccal mucosa delivery for the prevention or treatment of periodontal disease in dogs. Our results are encouraging, however, additional *in vitro* and *in vivo* studies should be performed, such as the *in vitro* antimicrobial activity of the formulations, since chitosan is also antimicrobial and a possible synergistic effect can be observed, as well as *in vivo* assessments of efficacy and safety.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Apoio à Pesquisa Tecnológica da Universidade Federal Rural Rio de Janeiro (FAPUR) also supported the study.

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How to cite
ALVES MCC ET AL. 2020. Chitosan gels for buccal delivery of *Schinus molle L.* essential oil in dogs: characterization and antimicrobial activity *in vitro*. An Acad Bras Cienc 92: e20200562. DOI 10.1590/0001-3765202020200562.

Manuscript received on April 22, 2020
accepted for publication on June 4, 2020

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Author contributions

The development of the study and manuscript was carried out in collaboration with multidisciplinary professionals, aiming to meet all the demands of the project. The development and characterization of the gels were carried out by Melina C.C. Alves, Byanca R. Benevenuto, Thais P. Ferreira, Geraldo A. Pereira, Gabriela C.M dos Santos and Leandra O. Moreira, supervised by Prof. Yara Peluso Cid. The minimum inhibitory concentration test was carried out by Beatriz O. de Farias under the supervision of Prof. Shana M.O. Coelho. The plant material of *Schinus molle L.*, the extraction and elucidation of major components were under the responsibility of Juliana Pereira Freitas under the supervision of Prof. Douglas S. A. Chaves.