A Clinical Trial and Oral Wound Treated by Essential Oil of *Lippia sidoides* Mouthrinse in Horses

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**ABSTRACT**

**Background:** Frequently the equine oral cavity is target of dental alterations, irregular wear of the dentition, tartar accumulation, gums inflammation, and other oral mucosa lesions. Therapeutically, active plants have been proposed to act preventively against oral infections. The objective of this study was to evaluate a mouthrinse prepared with *Lippia sidoides* Cham. essential oil in equine dental diseases and oral wound healing treatment.

**Materials, Methods & Results:** The experimental protocol was approved by the Ethics Committee of the Use of Animals (CEUA) of the State University of Ceara (UECE), n° 1158255-0/75. Mouthrinse was prepared with essential oil extracted from leaves of *L. sidoides* (EOLS). Thymol was the main constituent of the oil, up to 70%. Horses were chosen based on clinical and oral examination. Dental calculus and gingivitis grade from incisor and canine teeth were scored during evaluation. Two groups were used; OELS group (n = 10), and control group (n = 6), treated with mouthwash without EO (MR). A surgical scar (0.5 x 2 cm) was made near tooth 106 with the animals sedated. The lesions were observed on days 0 (D0), 3 (D3), 7 (D7), and 14 (D14) after treatment that was applied once in 48 h for 14 days. Recovery of oral mucosa was, blindly, evaluated in tissue samples. EOLS treatment reduced teeth calculus scores from D0 to D14 (*P* ≤ 0.05). In relation to histological parameters, EOLS treatment induced discreet proliferation of fibroblasts and a moderated inflammation on D3; moderated proliferation of fibroblasts and moderated inflammation, intense angiogenesis and re-epithelization on D7, and intense fibroblast proliferation, intense angiogenesis, and reduced inflammation with complete epithelialization of the tissue on D14. On the other hand, control group differed from the EOLS group in that it showed moderate angiogenesis and moderate tissue regrowth (*P* ≤ 0.05).

**Discussion:** EOLS mouthrinse used in horses reduced the accumulation of calculus triggering release of masses of tartar. Canines and incisors calculus accumulation is common in horses, especially the lower incisors, where the calculus is in contact with the gums and soft tissue leading to inflammation and causing ulcers in the cheeks and the in periodontal area. An EOLS mouthrinse used in dogs reduced the clinical scores as well as the histological parameters used to evaluate the gingival inflammation. Furthermore, the equines did not present any side effect to the treatment, such as bad breath or irritation in the oral cavity. These data confirms the possible benefit of this EOLS in controlling oral illness. In our study, EOLS mouthrinse induced an inflammatory response showing reconstitution of collagen, favoring the oral wound healing. EOLS mouthrinse did not delay the mucosa healing process. Mucous and skin are epithelial tissues associated to a protective barrier that defends the individuals and are structural and functionally similar with particular differences. It is worth noting that oral soft tissue wounds heal in an accelerated fashion than the dermal wounds. Mechanisms of oral healing scarless are yet to be revealed. In conclusion, the use of EOLS was shown to be highly promising to treat wounds in oral equine mucosa.

**Keywords:** equine dentistry, oral mucosa, wound healing, mouthrinse, *Lippia sidoides*.
INTRODUCTION

Frequently, the equine oral cavity is the target of dental abnormalities that can lead to inefficient chewing, discomfort and lesions of mouth soft tissues that can, in turn, provoke inflammations and culminate with oral mucosa disease [26]. Enamel tooth points are frequently seen in equine odontology [10,15,16] and can cause injury to cheek, lips and tongue, with consequent pain [37]. In addition, another source of oral scars is the use of bits that can lead to periosisitis of the jaw [7].

Corrective odontological treatment has been shown to be an important tool to permit a good quality of life, maintenance of health, and to improve athletic ability. Dental examination and treatment should be periodic and regular [8] to avoid the development of serious conditions or to retard its progress [5,17,38,39].

Plants with antimicrobial and anti-inflammatory properties are used in animals and humans [11] to control bacterial plaque growth [28], treat gingivitis [40,43] and periodontitis [34]. *L. sidoides* essential oil has been used as an oral antiseptic [41] to treat infections of the mouth and throat, periodontitis [9,33], and by its antibacterial [29] and anti-fungal [19] properties and, also, as a mouthrinse to treat gingivitis in dogs [20].

Therefore, the use of natural products as dental preventive treatment in horses, could reduce the incidence of diseases that affect the oral mucosa, favoring healing. Thus, this work aims to evaluate the use of a mouthrinse that contains essential oil of *Lippia sidoides* Cham. to treat dental affections and healing of wounds experimentally induced in oral mucosa of horses.

MATERIALS AND METHODS

Obtaining *L. sidoides* essential oil

The essential oil extracted from the leaves of *L. sidoides* grown in the state of Ceará was obtained by steam distillation of water technique [12], and was purchased commercially1.

Chemical analysis of the essential oil of *L. sidoides*

The chemical composition of essential oil of *L. sidoides* (EOLS) was determined by gas chromatography associated with mass spectrometry, GCMS. EOLS was analyzed using Shimadzu spectrometer model GCMS-QP 5050 with a capillary column of silicone founded by W Scientific DB5MS (50 m X 0.25 mm) distilling gas: He (1 mL/min); temperature of the injector: 200°C, temperature of the column: 35-180°C a 4°C and then 250°C/15 min; spectrum of mass by electronic impact at 70 eV. The times of retention and visual comparison of the spectra obtained with the data in the library of archived mass spectra on computer make possible identification of the constituents of the essential oil utilized in this study [1,4].

Mouthrinse preparation

The mouthrinse was prepared with six milliliters of *L. sidoides* essential oil that was mixed with 20 g of sodium saccharin, 30 g of citric acid, 25 g of glycerol and 2.5 g of sodium fluoride. Then, distilled water was added to bring the solution up to 1 L. This mouthrinse was calling EOLS. Another mouthrinse was prepared with the same reagents excluding the EOLS that was used in the control group [20, modified], and was calling MR.

Animals

Sixteen horses of undetermined breed were used, with an average weight of 400 Kg, ages varying from six to 10 years, with an average of 8 years and an average body score of five, according to the scale proposed by Henneke et al. [24] ranging from one to nine, where one is the score given to an emaciated animal and nine to an extremely obese animal. All the animals were provided by the Esquadrão da Polícia Montada do Estado do Ceará Coronel Moura (EPMONT, Brazil) from Fortaleza metropolitan region. The animals were randomly distributed into two groups and kept individually in stalls, and fed with commercial feed in a fixed amount of 4 kg and about 4 kg of hay Tifton 85 and 5 kg of elephant grass, with free access to water and mineral salt for horses during the experiment.

The horses were selected on the basis of clinical and odontological examination performed by a qualified veterinarian a screened by blood tests and biochemical analysis to exclude the pathological conditions (data not presented). All the animals had a history of treatment for worms and routine vaccinations and were all kept in the same conditions. All the animals had previously undergone dental examination to assure the absence of any wounds in the mucosa and around the mouth.

Clinical evaluation

Animals were scored on calculus and gingivitis of the incisor and the canine teeth. Calculus was evalu-
ated by the Warrick-Gorrel method adapted. A probe was used to verify the visual impression of cover and measured thickness of calculus on the tooth of the second right upper premolar tooth using the 0-4 scale according to Gorrel et al. [22] and Hennet [25] adapted as follows: 0, no detectable calculus; 1, scattered calculus covering ≤ 25% of the buccal tooth surface; 2, calculus covering between 25 and 49% of the buccal tooth surface; 3, calculus covering between 50 and 74% of the buccal tooth surface; 4, calculus covering ≥ 75% of the buccal tooth surface. Gingivitis (G) was scored according to intensity of redness, edema and bleeding on probing of the third right upper premolar tooth using the 0-4 scale according to Löe [30] and Gorrel et al. [22] adapted, as follows: 0, no gingivitis; 1, incipient or very mild gingivitis (red, swollen but no bleeding when probed); 2, mild gingivitis (red, swollen and delayed bleeding when probed); 3, moderate gingivitis (red, swollen and immediate bleeding when probed); 4, severe gingivitis (ulceration, spontaneous hemorrhage and profuse bleeding when probed).

**Effect of essential oil of L. sidoides mouthrinse (EOLS) on oral wound healing**

To evaluate the effect of EOLS on healing, surgical incisions were made in the jugal mucosa. The horses were divided in two groups, one group (n = 10) were treated with EOLS and the control group (n = 6) were treated with MR.

For the purpose of the procedures of evaluation and the scarring surgery, the animals were confined and sedated with 0.02 mg/kg of detomidine hydrochloride. Immediately after, the oral cavity was washed with clean running water to remove any food and an oral speculum for horses was used to keep the mouth opened. A visual and palpation inspection of the teeth and surrounding soft tissue was conducted by headlight. An experimental wound of a depth to reach the muscular fascia (2.0 x 0.5 cm) was induced at jugal mucosa in area corresponding to tooth 106 [31 adapted]. The scars were observed on D0, D3, D7, and D14, once treatment began. The mouthrinse was administered once in 48 h, for 14 days with the help of a metal syringe with a total capacity of 300 mL with the objective of creating turbulence in the oral cavity.

**Histological evaluation**

For histopathological analysis fragments of 5 mm diameter of the wounds jugal mucosa were collected on D0, D3, D7 and D14. The fragments obtained were initially fixed in 10% neutral buffered formalin, processed by conventional histological techniques. Sections of five micrometers were cut and stained by hematoxylin-eosin [27]. The tissues were evaluated under optical microscope, with 400x magnification and the scores were classified as absent (-) light, (+) moderate (+++) and intense (++++) for the mucosa recovery. The follows parameters were evaluated: inflammatory infiltrate, interstitial edema, vascular congestion, formation of granular tissue, fibroplasia, abscesses, and necrosis [3]. The histological examinations were realized on blind.

**Statistical analysis**

It was used the one-tailed Wilcoxon signed-rank test for the differences in paired measurements for each subject within control group and EOLS group between day 0 and day 14 (P ≤ 0.05). The two-tailed Mann–Whitney test was used to compare control and EOLS groups at day 0, and to compare control and EOLS groups at day 14 (P ≤ 0.05).

**RESULTS**

The chemical constituents of L. sidoides EO are displayed in Table 1 according to their elution order from a non-polar column. This oil was characterized by a high content of Thymol (70.97%).

All animals selected showed early gingivitis by the dentistry exam at baseline. Equine teeth calculus scores decreased significantly from day 0 to day 14 in the EOLS group, but was not changed significantly in the control group (P ≤ 0.05). At baseline and after the treatment, both groups were similar (Table 2).

Gums retraction was absent during the experiment. This fact may be attributed to the reduced time of observation to evaluate a chronic process; however, it is believed that the reduction of tartar buildup achieved is related to the use of an EOLS which prevents periodontal inflammation and gingival retraction progression.

After surgical scarring, the mucosa wounds exhibited bleeding that was easily controlled. In this study, no edema or crusting nor oozing were observed, and the wounds did not show any particular inflammation focus, which could be associated to oral mucosa characteristics. The presence of any food adhering to the wound was neither observed in none of the examinations during the experiments.
Results of histological analyses from lesions were presented in Table 3 and Figure 1. The animals treated with EOLS presented a light proliferation of fibroblasts and a moderate inflammation showing also the beginning of epithelium recovery, differing from the control group on D3, and light proliferation of fibroblasts with moderate inflammation with light angiogenesis and intense epithelium recovery on D7.

In this phase of the evaluation, the control group still exhibited only light epithelium regrowth and moderate inflammation on D7. The treatment with EOLS induced intense proliferation of fibroblasts, intense angiogenesis, with light inflammation and complete epithelium recovery on D14. The control group differed from the EOLS group, shown only moderate angiogenesis and moderate epithelial tissue recovery (Table 3).

**Table 1.** Percentage composition of *L. sidoides* essential oil obtained by gas chromatography/mass spectrometry.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrcene</td>
<td>2.12</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>0.50</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>7.51</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>0.80</td>
</tr>
<tr>
<td>Thymol methyl ether</td>
<td>1.45</td>
</tr>
<tr>
<td>Thymol</td>
<td>70.97</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>0.30</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.11</td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>8.30</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>1.59</td>
</tr>
<tr>
<td>Others constituents</td>
<td>6.35</td>
</tr>
</tbody>
</table>

**Figure 1.** Photomicrograph of histological transverse sections of wounds in jugal equine mucosa, in a clinical trial of an EOLS mouthrinse. A minimum of two sections from each animal for each treatment were analyzed. HE staining. Original magnification: 100x. [Scale bar: 200 μm].
Table 2. Before-and-after comparisons of oral-health scores for equines, in a trial of a mouth-rinse containing essential oil from *L. sidoides*.

<table>
<thead>
<tr>
<th>Score</th>
<th>Control group (n = 6)</th>
<th>EOLS group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0; mean (± SE)</td>
<td>Day 14; change from day 0; mean (± SE)</td>
</tr>
<tr>
<td>Clinical score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incisors Calculus</td>
<td>1.67 (±0.82)</td>
<td>1.67 (±0.52)</td>
</tr>
<tr>
<td>Canines Calculus</td>
<td>1.83 (±0.98)</td>
<td>1.83 (±0.75)</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>1.33 (±1.21)</td>
<td>1.17 (±0.89)</td>
</tr>
<tr>
<td>Gingival Retraction</td>
<td>1.00 (±0.89)</td>
<td>1.00 (±0.89)</td>
</tr>
</tbody>
</table>

EOLS mouthrinse was administered once in 48 h, for 14 days with the help of a metal syringe with a total capacity of 300 mL with the objective of creating turbulence in the oral cavity. Animals were scored on calculus and gingivitis of the incisor and the canine teeth. *Score on day 14 minus score on day 0 for each horse. So that negative score implies a decrease. †One-tailed *P* value from Wilcoxon paired-sample (matched-rank) test. ‡Two-tailed *P* value from Mann-Whitney rank-sum test comparing control horse to EOLS on day 0. §Two-tailed *P* value from Mann-Whitney rank-sum test comparing control horse to EOLS on day 14.

Table 3. Before-and-after comparisons of histological scores for wounds in jugal equine mucosa, in a trial of an EOLS mouthrinse

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>EOLS</th>
<th>Control</th>
<th>EOLS</th>
<th>Control</th>
<th>EOLS</th>
<th>Control</th>
<th>EOLS</th>
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<th>Control</th>
<th>EOLS</th>
<th>Control</th>
<th>EOLS</th>
</tr>
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<tbody>
<tr>
<td>Histological score</td>
<td>D3</td>
<td>D7</td>
<td>D14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>++a</td>
<td>+, ns</td>
<td>+a</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>c</td>
<td>+b</td>
<td>&lt;0.05</td>
<td></td>
<td>+c</td>
<td></td>
<td>++</td>
<td></td>
<td>+++</td>
<td></td>
<td>++</td>
<td></td>
<td>+++</td>
<td></td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>c</td>
<td>c</td>
<td>&lt;0.05</td>
<td></td>
<td>+c</td>
<td></td>
<td>+</td>
<td></td>
<td>+++c</td>
<td></td>
<td>+++c</td>
<td></td>
<td>+++c</td>
<td></td>
<td>+++c</td>
<td></td>
</tr>
<tr>
<td>Reepithelization</td>
<td>c</td>
<td>+b</td>
<td>&lt;0.05</td>
<td></td>
<td>+c</td>
<td></td>
<td>+++++</td>
<td></td>
<td>+++++</td>
<td></td>
<td>++++</td>
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<td>++++</td>
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<td>++++</td>
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</tbody>
</table>

EOLS mouthrinse was administered once in 48 h, for 14 days with the help of a metal syringe with a total capacity of 300 mL with objective of creating turbulence in the oral cavity. The lesions were observed on days 0 (D0), 3 (D3), 7 (D7), and 14 (D14) after treatment. *Two-tailed *P* value from Mann-Whitney rank-sum test comparing control horse to EOLS in the same day. Degree of statistical significance calculated by the Kruskal-Wallis test is given in each upper left corner. Statistically significant differences between the three groups were calculated using Dunn’s post hoc test. (*p* < 0.05, †*p* < 0.01, ‡*p* < 0.001; “ns,” not significant)
DISCUSSION

The use of natural products to prevent oral disease has been proposed. In this work, the essential oil was used as a mouthrinse. EOLS reduced the accumulation of calculus causing detachment of tartar masses in horses. The use of a dental gel based in L. sidoides essential oil demonstrated an action against the buildup of plaque and gingivitis in humans [13] and its mouthrinse reduced bacterial plaque and gingivitis in dogs [20]. This effect could be due to the action of thymol, the main active compound of L. sidoides essential oil. This component has demonstrated antimicrobial properties [2,18,19] and its use has been suggested to treat periodontal disease [9]. In horses, the accumulation of calculus on the canines and incisors is common, especially, on the lower canines. The calculus comes in contact with the gums and soft tissue. This action may provoke inflammation and ulcers formation in the cheeks [39,42], and causes a high incidence of tartar on the incisors of horses [36].

In this work, EOLS mouthrinse presented an effect on cellular inflammatory intensity in the EOLS group, which became predominantly composed by polymorph nuclear cells. This EOLS, when used in dogs, reduced both the clinical scores and histological parameters used to assess periodontal inflammation [20]. These results were attributed to the release of chemostatic factors [23] from inflammatory cells that migrate to gingiva induced by EOLS. These cells phagocyte bacteria and release inflammatory mediators in the site of lesions [35]. They also release substances that active the action of fibroblastic factors, which permit the collagen formation to progress [44]. Furthermore, the equines did not present any side effect to the treatment with EOLS, such as bad breath or irritation in the oral cavity. We suggest that EOLS benefit equine oral illness controlling.

EOLS contains thymol and others phenolic compounds, which are biological effective molecules when used in an appropriate manner. Initially, it was explored as antiseptic agent to treat fungal infections skin [32]. More recently, L. sidoides essential oil was used to heal wounds experimentally induced demonstrating a strong inflammatory component without retarding the healing process [14]. This essential oil also demonstrated protective action against gastric mucosa lesions induced by ethanol in an experimental model of gastritis [33]. In our study the EOLS mouthrinse induced an inflammatory response with an acceleration of collagen reorganization, thus favoring the epithelium recovery in wounds induced in the jugal mucosa. The mucosa and the skin are epithelial tissue associated with the protective barrier of the individual and have structural and functional similarities with particular changes [21]. Wound healing is a complex process involving a wide spectrum of cells and molecules that comprises several phases - hemostasis, inflammation, proliferation, and remodeling [6]. Oral mucosal wounds heal in an accelerated fashion when compared to dermal wounds. The exact mechanisms of oral healing scarless are yet to be revealed. Oral wounds contained fewer immune mediators, blood vessels, and profibrotic mediators, more bone marrow–derived cells, a higher re-epithelization rate, and faster proliferation of fibroblasts when compared to dermal wounds [21]. In this respect, the use of EOLS is seen as highly promising in topical therapies that involve the skin and oral mucosa.

CONCLUSIONS

The use of mouthrinse based on essential oil of L. sidoides seems to favor the natural release of tartar and aids in horses jugal mucosa wounds healing. Nevertheless, the molecular mechanism that may modulate oral mucosa healing process should be studied more in depth.

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Acknowledgments. The authors would like to express their appreciation to the Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP, Ceará) for the scholarship granted to the first author, which provided subsidies for the implementation of the project.

Ethical approval. The experimental protocol was approved by the Ethics Committee of the Use of Animals (CEUA) of the State University of Ceara, (UECE) n° 1158255-0/75.

Declaration of interest. The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
REFERENCES


