Original Article

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Effect of crude extract and essential oil of *Cordia verbenacea* in experimental periodontitis in rats

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Abstract

Aim: To evaluate the effect of crude extract and essential oil of *Cordia verbenacea* (*C.V.),* systemically administered, on ligature-induced periodontitis in rats. **Methods:** Periodontitis was induced in 54 Wistar rats: one of the first mandibular molars was randomly assigned to receive a ligature, whereas the contralateral molar was left unligated. Then, animals were randomly assigned to one of the following groups: non-treatment group (n=18): animals that received 10 mL/day of vehicle; *C.V.* extract group (n=18): animals that received 100 mg/kg/day of crude extract of *C. verbenacea*; and *C.V.* essential oil group (n=18): animals that received 100 mg/kg/day of essential oils free of *C. verbenacea.* All therapies were administered orally 3 times daily, for 11 days. Next, the animals were sacrificed, and the specimens were processed for morphometric analysis. Bone loss was determined on the buccal surface of the lower first molars by the distance of the cementoenamel junction from the alveolar bone. **Results:** Both extract and essential oil of *C. verbenacea* orally administered decreased alveolar bone loss in the ligated teeth when compared with the non-treated group ($p<0.05$). **Conclusions:** The present study demonstrated that systemic administration of both formulations of *Cordia verbenacea* may attenuate the progression of ligature-induced periodontitis.

Keywords: alveolar bone loss, cordia, inflammation, periodontitis.

Introduction

Periodontitis is characterized by an infectious condition leading to inflammation of the periodontal supporting tissues, attachment loss, and alveolar bone destruction. Although the etiological role of microorganisms in the pathogenesis of this disease is clear, the host's immune-inflammatory response can lead to protective and/or destructive effects on periodontal tissues[1]. Thus, an unbalanced host response to periodontopathogens is an essential determinant in the outcome of the disease[1].

Innumerous biological systems have been suggested as alternatives to modulate the host's immune-inflammatory response involved in periodontal disease[2]. Among them, the nonsteroidal anti-inflammatory drugs (NSAIDs) are probably the most studied medications, showing effective outcomes in controlling

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periodontal breakdown in pre-clinical experiments when systemically administered[3-5]. Nevertheless, clinical trials have not observed consistent benefits when NSAIDs are associated with conventional mechanical therapy[5]. Additionally, these drugs, both non-selective inhibitors and selective cyclooxygenase-2 inhibitors, are frequently associated with side effects, impairing patients' compliance to their use[6].

This way, the search for new drugs that effectively interfere with the immune-inflammatory process is currently important. In this context, evidence has shown that the use of medicinal plants is greatly relevant in folk medicine to treat different inflammatory conditions, including periodontitis[7-11]. The species *Cordia verbenacea*, formally classified as *C. curassavica*, is a native Brazilian medicinal plant belonging to the Boraginaceae family, distributed along the Brazilian coastal regions and popularly known as Erva Baleeira. Studies have demonstrated that, in the form of alcoholic extracts, decoctions, and infusions, *C. verbenacea* exhibits important anti-rheumatic, anti-inflammatory, analgesic, and healing properties which are related to a protective effect on the gastric mucosa, as well as very low toxicity when orally or topically administered[12-17]. However, no information has previously been available regarding the effects of *C. verbenacea* on periodontal disease.

Taking into account the extensive presence of *C. verbenacea* in Brazilian folk medicine, and considering the anti-inflammatory activity previously reported[12-17] our group was prompted to assess the impact of *C. verbenacea* in modulating periodontal disease progression. Therefore, the present study investigated the role of systemically administered *C. verbenacea* in induced-ligature periodontitis. Moreover, two formulations of the drug (crude extract and essential oil) were evaluated to determine whether they produce different therapeutic effects when used in a pathological situation, such as periodontitis.

The hypothesis of this investigation was that systemic therapy with both formulations of *C. verbenacea* could positively modify the progression of experimentally induced periodontitis in rats, representing a promising new approach for the management of periodontal diseases.

Material and methods

Animals

Fifty-four male Wistar rats were obtained from the Butantan Institute (São Paulo, SP, Brazil). The rats were 90-day-old and weighed 304 ± 23 g at the beginning of the study. During the acclimatization (5 days) and experimental period (11 days), each animal was housed in a plastic cage with access to food (Labina, Purina, Paulínia, SP, Brazil) and drinking water *ad libitum* in the Animal Care Facility of Paulista University. The protocol was approved by the Paulista University Institutional Animal Care and Use Committee (036/10 CEP/ICS/UNIP).

Plant material and extraction of crude extract and essential oil

Fresh leaves and stems of *C. verbenacea* were collected from Multidisciplinary Center for Chemical, Biological and Agricultural Research (CPQBA) of the University of Campinas (UNICAMP). A voucher specimen (UEC 112744) is deposited at the UNICAMP's Biological Institute. To obtain the plant crude extract, the material was allowed to dry under circulating air (40 ºC) and ground prior to use. The powder was submitted to dynamic maceration with ethanol for 4-h periods. This procedure was repeated 3 times. Concentration of the extract under reduced pressure yielded extracts which were denoted as ethanol crude extracts. The essential oil was extracted from fresh chopped leaves by hydrodistillation for 4 h, using a Clevenger-type apparatus. Under these conditions, the yield of essential oil was 0.37% (considering 80% humidity). Both the crude ethanol extract and volatile oil were analyzed by GC/MS (HP 6890/mass detector HP 5975 / automatic injector 7673 Agilent Technologies, Palo Alto, CA) using a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm / stationary phase 5% methyl silicone). Helium was used as the carrier gas (1.0mL.min^{-1}). The detector was acquired by electron impact (scan mode) using an ionization energy of 70 eV. One microliter of sample was injected in the splitless mode. The column was initially heated at 60ºC and then heated at 3ºC min^{-1} to 240ºC. Injector and detector temperature were 220ºC and 250ºC, respectively. The compounds were identified by comparing their mass spectra with the system data bank NIST-2005. A homologous series of n-hydrocarbons C-9-C18 and C-20 was co-injected with the ample in order to calculate the retention index and co-injection of authentic standards to provide additional criteria for identification. The essential oil components were therefore identified crossing their retention index, with comparison of their mass spectrums compared with those of authentic samples.

For the quantitative determination of α-humulene calibration was carried out using a standard solution of α-humulene in acetone (25–127 µg/mL) containing dibutylphthalate (200 µg/mL) as the internal standard. The correlation between the peak area ratio and the concentrations of the compound was linear over the range tested. In order to determine the contents of α-humulene, oil samples (100.00 ± 0.1 mg) were dissolved in acetone (10 mL) containing the internal standard (200 µg/mL) and aliquots (1.0 µl) injected into the GC/MS. All chemical analyses were performed in triplicate. Purity of α-humulene was 99%.

Ligature placement

General anesthesia was obtained by intramuscular administration of ketamine hydrochloride (10 mg/kg) (Dopalen®, Agribrands Brasil Ltda., Paulínia, SP, Brazil) and xylazine hydrochloride (10 mg/kg) (Rompun®, Bayer S.A., São Paulo, SP, Brazil). One of the mandibular first molars of each animal was randomly assigned to receive a cotton ligature (Corrente Algodão no 10; Coats Corrente, São Paulo, SP, Brazil) in a cervical position. Briefly, the thread was introduced in the proximal space between the first and second molars and the ligatures were kept in position in order to

allow biofilm accumulation over 11 days[5,9-10]. The contralateral tooth was left unligated to be used as a control.

Treatment

After ligature placement, animals were randomly assigned to one of the following groups, according to a computer-generated code: Non-treatment group (n = 18): animals received orally 10 mL/day of vehicle; *C.V.* extract group (n = 18): animals received orally 100 mg/kg/day of crude extract of *C. verbenacea*; and *C.V.* essential oil group (n = 18): animals received orally 100 mg/kg/day of essential oils isolated from *C. verbenacea of flow rate).* Treatments were administered by oral gavage with a 1 mL syringe, using 0.3 mL of the respective substances, 3 times daily (7 a.m., 1 p.m., and 8 p.m.) for 11 days.

The animals were evaluated at each of these moments (7 a.m., 1 p.m., and 8 p.m.) throughout the experiment, to assess possible clinical or toxicological symptoms. At the conclusion of the experiment, the animals' weights were monitored and compared with the baseline weight.

Morphometric analysis

The animals were sacrificed by CO_2 inhalation on the 12th d of periodontitis induction. Subsequently, the mandibles were excised and defleshed after immersion in 8% sodium hypochlorite for 4 h. The specimens were washed in running water and immediately dried with compressed air. To outline the cementum enamel junction (CEJ), 1% methylene blue (Sigma-Aldrich®, Saint Louis, MO, USA) was applied to the specimens for 1 min and then washed in running water. Photographs were obtained with a 6.1-megapixel digital camera (Canon® 40D) on a tripod to keep the camera parallel to the ground at the minimal focal distance. The specimens were fixed in wax with their occlusal planes parallel to the ground and long axes perpendicular to the camera. Photographs of the buccal aspects were made both in test and control sides. To validate measurement conversions, a millimeter ruler was photographed with all specimens[18]. Alveolar bone loss was determined on the buccal surface of the lower first molars, by the distance of the CEJ from the alveolar bone crest (ABC), measured at 3 equally distant sites. Measurements were made along the axis of each root in 3 regions of the first molar (3 roots) (Figure 1). The total alveolar bone loss was obtained by taking the sum of the linear recordings from the buccal tooth surface of the end of the root and dividing by 3.

The measurements were performed by the same calibrated masked examiner after intraexaminer calibration, by evaluating 10 non-study images presenting alveolar bone loss similar to the present study. The examiner measured the linear measurements of all photographs twice within 24 h. The intraclass correlation showed 93% reproducibility.

Statistical Analyses

To test the null hypothesis that both crude extract and essential oil of *C. verbenacea* had no influence on alveolar bone loss, an intergroup analysis was performed by ANOVA.

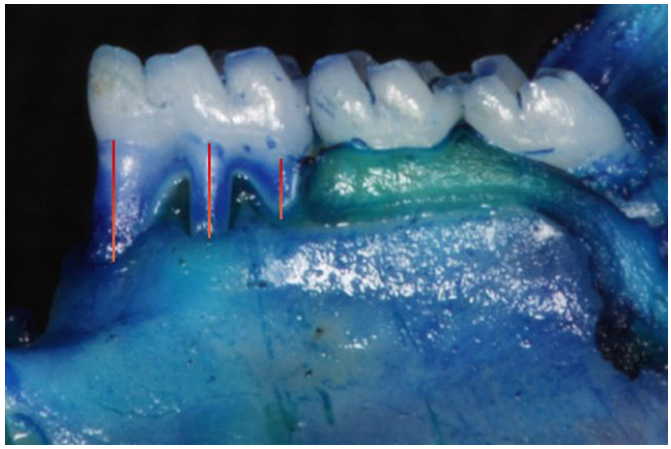

Fig. 1 - Morphometric parameter evaluated: red lines represent the distance to the cementoenamel junction from the alveolar bone crest

When statistical difference was found, analysis of the difference was determined using the Tukey's test. In addition, the paired Student's t-test was used for intragroup comparisons between ligated and unligated teeth. The significance level set for all analyses was 5% ($p < 0.05$).

Results

The essential oil was obtained in 1% yield from freshly collected plants. The main component identified in the essential oil was α-humulene, which represented 80% of the total oil's content, whereas the ethanol crude extract was obtained by reflux in a soxhlet system. The α-humulene yield content of this extract represented 0.047%. The animals did not lose weight throughout the experimental period. Indeed, the tested therapies did not promote side effects or alterations in the animals' behavior and in their general activity related to the toxicity. Deaths were not observed.

Morphometric results

A significant difference in the alveolar bone loss between unligated and ligated teeth was observed for all experimental groups ($p < 0.05$), showing that the cotton ligatures around the teeth were able to promote bone loss (Table 1). Both groups treated with *C. verbenacea* - crude extract and essential oil - presented a significant reduction of the alveolar bone loss in ligated teeth when compared with the non-treated

Table 1. Means and standard deviation of alveolar bone loss [mm] for ligated and unligated teeth in all experimental groups.

Groups	Ligated teeth	Unligated teeth
Non-treatment	1.71 ± 0.11 Aa	1.21 ± 0.08 Ab
C.V. extract	1.53 ± 0.15 Ba	1.16 ± 0.11 Ab
C.V. essential oil	1.59 ± 0.10 Ba	1.12 ± 0.15 Ab

Means followed by different capital letters in a column represent significant inter-group differences by ANOVA/Tukey test, $p<0.05$. Means followed by different non-capital letters in a line represent significant inter-group differences by student's t-test, $p<0.05$.

group ($p < 0.05$), and no difference was observed between crude extract and essential oil formulations ($p > 0.05$) (Table 1). Figure 2 illustrates the morphometric findings.

Fig. 2 - Representative photographs illustrating the morphologic findings of non-treatment **[A]**, *C.V.* extract **[B]**, and *C.V.* essential oil **[C]** groups.

Discussion

Studies have demonstrated that *C. verbenacea* extracts or essential oil display marked anti-inflammatory effects in several models of inflammation and currently this substance has been used in the therapy of inflammatory conditions, such as tendinitis and muscular or articular pain[12-16]. However, to date, no study has investigated the impact of *C. verbenacea* in periodontitis. In this investigation, the effect of *C. verbenacea* orally administered on ligature-induced periodontitis in rats was assessed. Indeed, two formulations of *C. verbenacea* [crude extract and essential oil] were evaluated, in order to determine whether they possess different effects. The outcomes indicated that the *C. verbenacea* therapy was effective for reducing alveolar bone loss in experimentally induced periodontitis, independent of the plant formulation.

To the best of our knowledge, there have been no clinical or pre-clinical studies that have previously evaluated the impact of *C. verbenacea* in periodontitis, hampering a more direct comparison with the outcomes of the present investigation. However, the results of this study corroborate some previously published data on other inflammatory conditions[16], supporting the view that this medicinal plant can control chronic inflammation.

The possible mechanisms underlying the pronounced effect of *C. verbenacea* in attenuating inflammatory disorders seems to have a relationship, at least in part, with the downregulation of pro-inflammatory mediators, such as TNF-α and IL-1β[16,18]. In fact, alternative therapeutic approaches based on inhibiting TNF-α production have been successfully used in clinical treatment of chronic inflammatory diseases, particularly rheumatoid arthritis[19-20]. On the other hand, Passos et al.[16] (2007) demonstrated that the anti-inflammatory action of *C. verbenacea* was not associated with the reduction of prostaglandin (PG) E2 levels, indicating that the mechanism of action of this medicinal plant seems to be distinct from that of non-steroidal anti-inflammatory drugs[21-22]. Contradictorily, other data have suggested that essential oil from *C. verbenacea*, given orally, greatly reduced the generation of PGE2 in the rat paw[18]. Indeed, this study evidenced that the anti-inflammatory effects of these compounds seem to be closely associated with their ability to inhibit the up-regulation of important inflammatory proteins, such as Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) enzymes[18]. In fact, previous data have indicated that both ethanolic extract and essential oil from *C. verbenacea* leaves have presented a wide variety of constituents, such as flavonoids and sesquiterpenes á-humulene and *trans*-caryophyllene, recognized agents responsible for the anti-inflammatory actions of *C. verbenacea*[13,16,18,23-24]. This supports the promising outcomes obtained in the current investigation by using both formulations of the studied plant.

Studies have shown that this medicinal plant may also present antibacterial activity against some Gram-positive and Gram-negative bacteria[24-25]. Michielin et al.[25] (2009) showed that *C. verbenacea* contains high relative amounts of oxygenated monoterpenes and sesquiterpenes, the main components responsible for its antibacterial activity. Indeed, the antibacterial potential related to *C. verbenacea* could be attributed to the presence of the aromatic compounds in this natural substance[25]. Nevertheless, the antibacterial role of systemic therapy with *C. verbenacea* in periodontitis remains unclear and further investigations are needed to clarify this issue.

Altogether, the data obtained in the current investigation showed for the first time a significant effect of *C. verbenacea* systemically administered, both as crude extract well as essential oil, in controlling bone loss in experimental periodontitis. The results of the present study confirm and extend those of earlier reports, which have demonstrated the potent anti-inflammatory *in vivo* activity of *C. verbenacea*[12-14] and support the systemic use of this natural product as an attractive alternative to prevent further periodontal disease development.

Recently, besides the better understanding of the paradigm of periodontal disease and new information with regard to the role of host immune response in modulating periodontal breakdown, researchers have focused on the development of novel therapeutic strategies and directions of host-modulatory agents for the management of periodontal diseases. In this context, the NSAIDs represent an essential pharmacologic class of agents that has been well studied as modulators of the host response in periodontitis. Pre-clinical evidence has indicated that therapies using either selective (COX-2) or traditional NSAIDs can positively modify the progression of periodontal disease[3-5,26]. Clinical studies have also pointed out that the systemic administration of NSAIDs may provide additional benefits in the periodontal condition when associated with non-surgical periodontal therapy (scaling and root planning) by modulating the host's immunoinflammatory response[27-29], although these findings remain controversial[2]. Nevertheless, chronic treatments using both non-selective and selective inhibitors of COX-2 are related to innumerous adverse effects, such as gastroduodenal problems and renal toxicity, inhibiting patient compliance to their use, especially when prolonged periods of administration are required[6,30]. Therefore, the use of natural plants such as *C. verbenacea* to modulate periodontal breakdown could present advantages when taking into account the well-known systemic side effects attributed to NSAIDs. In this context, previous studies indicated that the extracts of *C. verbenacea* leaves exhibited an anti-inflammatory activity linked to an important protective effect on the gastric

mucosa, and very low toxicity in acute models of experimentation in rats when orally administered[12-14,17]. Indeed, Roldão et al.[17] (2008) found that *C. verbenacea* leaf extract produced an important antiulcer effect, contributing to the maintenance of mucosal integrity. Thus, this therapeutic strategy with a medicinal plant is safe and would be a more practical and viable approach, allowing the possibility of a longer period of drug administration in periodontal disease treatment.

In conclusion, the systemic use of both crude extract and essential oil of *C. verbenacea* could represent a new therapeutic option for the treatment of inflammatory diseases, especially those presenting a chronic profile. The low cost and easy access to *C. verbenacea* justify additional studies on the efficacy of this compound as an adjunct in periodontal therapy in clinical practice, providing new insight for the modulation of periodontal disease progression in individuals suffering from this inflammatory condition. However, additional studies are required to evaluate the molecular mechanism by which these active compounds exert their effects.

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