

Evaluation of the effect of an organic extract obtained from *Ipomoea alba* L. on experimental periodontitis in rats

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Abstract: The aim of this study was to evaluate the effect of an organic extract obtained from *Ipomoea alba* L. (Convolvulaceae or OE 1493), on experimental periodontal disease in rats. Periodontitis was induced in thirty six Wistar rats: a first mandibular molar was randomly assigned to receive a ligature, whereas the contralateral molar was left unligated. Animals were randomly assigned to two groups and treated topically, three times a day, for 11 days, as follows: Control Group - vehicle-treated (n = 18), and Test Group - OE 1493-treated (n = 18). The rats were sacrificed on the 12th day. Morphometrical measurements from the cementoenamel junction to the bone crest were performed to determine alveolar bone loss, using standardized photographs. Single- and multi-dose acute toxicity assays were carried out after OE 1493 treatment. Morphometrical analysis demonstrated that topically-administered OE 1493 showed no effect on reducing bone loss when compared with the control group (p > 0.05). In addition, OE 1493 did not present toxicity. Within the limits of this investigation, it may be concluded that OE 1493 did not show any positive influence on the progression of ligature-induced periodontitis in rats, when administered according to the regimen used in the present study.

Descriptors: Drug Toxicity; Periodontitis; Rats; Administration, Topical; Anti-Bacterial Agents.

Introduction

The presence of bacterial plaque is the main etiologic factor involved in the initiation and progression of periodontitis.¹ The aim of periodontal therapy is to remove periodontal pathogens by providing patients with adequate oral hygiene instructions combined with professional mechanical plaque control.² However, this conventional treatment strategy is not always successful and the addition of chemical agents to dentifrices and mouth rinses has been suggested to enhance their efficacy as adjuncts to the therapeutic approach for achieving better oral health.³

Natural products represent a significant source of substances for use in the control of oral diseases, especially in managing plaque-related diseases such as gingivitis.⁴⁻⁸ Brazil is said to be the richest country in the world in terms of its biodiversity, and few data are available about the pharmacological and chemical potential of its flora as a source of new medicines. In previous investigations developed by our research group,

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an organic extract obtained from *Ipomoea alba* L. (OE 1493 or Convolvulaceae) showed significant *in vitro* activity against *Streptococcus mutans*, *S. sanguinis* and *Enterococcus faecalis* (unpublished data). However, there is no information available as regards the effect of OE 1493 on reducing bone resorption in experimental periodontitis.

Thus, the purpose of this study was to make a morphometric evaluation of OE 1493 with respect to its ability to reduce the development of periodontitis in rats.

Methodology

Plant collection and plant extract preparation

Ipomoea alba L. (OE 1493 or Convolvulaceae) was collected in *igapó* forests, Amazon rain forest, Manaus, AM, under permit from the agency controlling environmental and natural resources, “*Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis*” (IBAMA). Access to its genetic patrimony was obtained under a permit from the Genetic Patrimony Management Council, “*Conselho de Gestão do Patrimônio Genético*”.^{9,10}

Plants were dried by air circulation at a temperature of up to 40°C. After that, dried plant material was ground in a hammer mill (Holmes) before being macerated with a mixture of dichloromethane and methanol (1:1) for 24 hours. The organic extract was evaporated under reduced pressure (Büchi) and was lyophilized. The extract was kept in a freezer until use. The maceration process used to obtain the extract is well known¹¹ and does not alter chemical or physical properties of the natural components of the extract. Extracts were prepared at a concentration of 100 mg/mL, using 10% Tween 80 in water as vehicle.

Experimental periodontal disease

Thirty six male Wistar rats (210–320 g), nine weeks old, were used for the induced periodontitis experiment in the present study. The animals were acclimatized to the housing conditions during the course of two weeks, and a 12-hour light and dark cycle was applied. They were housed six per cage at a permanent temperature of 21°C. Standard rat

chow pellets and water *ad libitum* were available. The sample size of this investigation was based on previous published studies.¹²⁻¹⁴

General anesthesia was obtained by intra-muscular administration of ketamine hydrochloride (10 mg/kg) (Dopalen®, Agribands Brasil Ltda., Paulínia, Brazil) and xylazine hydrochloride (10 mg/kg) (Rompun®, Bayer S.A., São Paulo, 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, Brazil) in a cervical position. Briefly, the thread was introduced into the proximal space between the first and second molars and two knots were tied on the mesial face of the first molar. The ligatures were kept in position in order to allow biofilm accumulation for 11 days.^{15,16} The contralateral tooth was left unligated for use as a control.

After this, animals were randomly assigned to one of the experimental groups:

- Control Group - vehicle-treated (n = 18) and
- Test Group - OE 1493-treated (n = 18).

Treatments were topically applied with a 1-mL syringe, using 0.3 mL of the respective substances, 3 times a day (7 a.m.; 1 p.m. and 8 p.m.) for 11 days.

To apply the drug, the animals were immobilized by one researcher, while another person applied the substance, enabling the molar area to be visualized to ensure adequate administration.

The protocol was approved by the Paulista University Institutional Animal Care and Use Committee (036/10 CEP/ICS/UNIP).

Morphometric analysis

The animals were sacrificed on the 12th day of periodontitis induction and were euthanized by CO₂ inhalation. The mandibles were excised and defleshed after immersion in 8% sodium hypochlorite for 4 hours. The specimens were washed in running water and dried with compressed air. To outline the cemento-enamel junction (CEJ), 1% methylene blue (Sigma-Aldrich®, Saint Louis, USA) was applied to the specimens for 1 minute and then washed in running water. Photographs were obtained with a 6.1-megapixel digital camera (40D Canon® Tokyo,

Japan) fixed 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 plane kept parallel to the ground and their long axis perpendicular to the camera. Photographs were taken of the buccal aspects. To validate measurement conversions, a millimetric ruler was photographed together with all specimens.¹⁷ Alveolar bone loss was determined on the buccal surface of the mandibular first molars by the distance of the CEJ from the alveolar bone crest (ABC). Measurements were made along the axis of each root in three regions of the first molar (three 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 roots and dividing by three.

The measurements were taken by the same calibrated masked examiner, after intraexaminer calibration, during which 8 non-study images presenting alveolar bone loss similar to those in the present study were evaluated. The examiner took the linear measurements of all photographs twice within 24 hours. The Intra Class correlation showed 97% reproducibility.

Toxicity assay

OE 1493 single-dose acute toxicity assay

Nine mice weighing 25–30 g, 6 to 9 weeks old, were used in single-dose acute toxicity assays. Mice

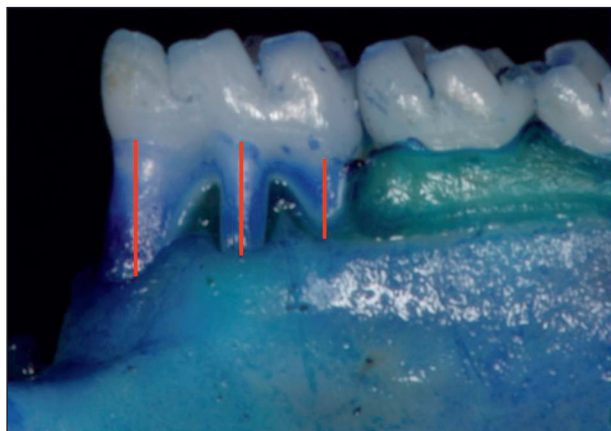


Figure 1 - Schematic image illustrating the morphometrical parameter evaluated: White lines represent the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC).

were brought to the vivarium at least two days in advance to become acclimatized to the housing conditions. A 12-hour light and dark cycle was applied and water and food were offered *ad libitum*. Five animals were kept in one cage and four in another. At the end of the experiments, the animals were sacrificed in a CO₂ gas chamber.

In order to prospect toxicity and obtain lethal dose tendency, three mice per group were used to assess lethality in each dose.¹⁸ Doses of 5.0 g/kg, 2.5 g/kg and 1.25 g/kg of OE 1493 were intraperitoneally administered. The occurrence of massive toxic reactions and/or death was observed in the first 15, 30, 60, 120 and 180 minutes after administration of treatments and every 24 hours in the following 14 days. The corresponding volume of vehicle was administered to the control group. Animals were weighed three times during the observation period, at days 1, 7 and 14. At the end of the period of observation, necropsy was performed on each animal to look for macroscopic reactions in the liver, gut, heart and kidneys. Latency for death was assessed.

OE 1493 multi-dose acute toxicity assay

The multi-dose acute toxicity assay was performed using the rats included in the induced periodontitis. OE 1493 was diluted in 10% Tween 80/water to a concentration of 100 mg/mL, and 0.3 mL of this solution was administered three times a day, totaling 910 mg of extract a day. Animals placed in glass cages were observed for toxic reactions in general activity, central nervous system, autonomous nervous system, psychomotricity and in sensorial reactions.

Statistical analyses

To test the null hypothesis that OE 1493 had no influence on alveolar bone loss, an intergroup analysis was performed using Student's-*t* test. In addition, Student's-*t* test was used for intragroup comparisons between ligated and unligated teeth. The significance level established for all analyses was 5% ($p < 0.05$). Linear regression was used to obtain the tendency of lethal dose in the toxicity assay (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, USA).

Results

Clinically, at the time of sacrifice, signs of gingival inflammation were observed in the ligated teeth of all the groups. No signs of gingival inflammation were observed in the unligated teeth.

Morphometric analysis

Intragroup analysis showed that ligatures placed around the teeth were able to promote bone loss when compared with unligated teeth ($p < 0.05$) (Control Group: 1.32 ± 0.11 mm and 1.73 ± 0.16 mm, for unligated and ligated teeth, respectively; and Test Group: 1.39 ± 0.13 mm and 1.69 ± 0.08 mm, for unligated and ligated teeth, respectively). Intergroup analysis comparing ligated teeth revealed no differences in bone loss between groups (1.73 ± 0.16 mm and 1.69 ± 0.08 mm for Control and Test Group, respectively) ($p > 0.05$) (Figure 2).

Toxicity assay

Results obtained from the single-dose acute toxicity assay performed with mice that received OE 1493 intraperitoneally and were under observation for 14 days showed a tendency of $LD50 = 2.19$ g/kg, after linear regression (correlation coefficient $r = 0.7559$; $r^2 = 0.5714$; $F = 1.333$). Latency for death was obtained for three doses tested to obtain $LD50$; the 5 g/kg dose showed latency for death of 95.67 ± 15.31 h; the 2.5 g/kg dose showed latency

for death of 300.00 ± 519.62 h and the 1.25 g/kg dose did not cause the mice to die in a period of 14 days. The standard deviation observed in the latency for death after administration of the 2.5 g/kg dose was noted to be extremely high, and this was caused by the death of only one mouse in the group of three mice. Multi-dose acute toxicity tests showed that OE 1493 did not show any tendency towards toxicity when 0.3 mL was locally administered three times a day for 11 days. No signs of toxicity were observed when compared with the control group.

Discussion

Over the last few years, new natural medicinal products have been described as an important source of substances for use in the treatment of oral inflammatory conditions.^{13,19-22} In this context, it has been evidenced that OB 1493 presented effective anti-bacterial activity *in vitro* (unpublished data). However, until now, there has been no information available in the literature as regards the effect of OB 1493 on experimental periodontitis. Therefore, this investigation was designed to determine the effect of OB 1493 on attenuating alveolar bone loss in ligature-induced periodontitis in rats. The morphometrical assays of the present study demonstrated that topically-administered OB 1493 had no influence on reducing alveolar bone resorption in experimental periodontitis, presenting bone loss comparable with that of the Control group.

Although very few studies have examined the role of natural products in controlling periodontitis, Botelho *et al.*⁸ recently tested a locally-applied carvacrol gel (*Lippia sidoides* derivatives) and determined its efficacy in controlling bone loss in experimental periodontitis in rats. The authors showed evidence that this natural chemical agent preserved alveolar bone resorption and showed anti-inflammatory and antibacterial activities in periodontitis. In contrast, the treatment using a plant extract derived from *Ipomoea alba*, evaluated in the present study, was not able to reduce the alveolar bone loss promoted by ligature-induced periodontitis. To the best of our knowledge, no pre-clinical or clinical investigations have studied the impact of OB 1493 on periodontitis, hampering comparisons with the re-

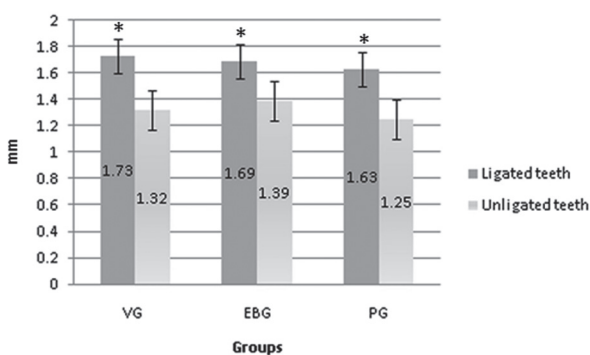


Figure 2 - Mean values and standard deviation (mm) for the alveolar bone loss in unligated and ligated teeth in both groups. In ligated teeth, * signifies that the mean bone loss was significantly lower than that observed for unligated teeth using the paired Student's-*t* test (intragroup analysis) ($p < 0.05$). Intergroup analysis showed no statistical differences between groups using Student's-*t* test ($p > 0.05$).

sults of the present research.

In the present study, the ligature was placed around the first molar teeth for 11 days. The rationale for using this experimental period is based on previous investigations.^{13,15,23} According to Lima *et al.*,²³ placement of a ligature around the molar teeth of rats leads to the presence of inflammatory cells, including osteoclasts, beneath the ligature. These authors reported that the substantial alveolar bone loss originated on day 3 of periodontitis induction reached a maximum between days 7 and 11. In line with this data, Kuhr *et al.*²⁴ revealed that the application of this model of periodontitis induction can only be recommended for short periods (less than 15 days). According to these authors, who evaluated the periodontal destruction following experimentally induced periodontitis in rats over a 60-day observation period, it was demonstrated that the ligature-induced bone loss increased most from day 1 to day 15, whereas on days 30 and 60 slighter increases in bone loss were observed, supporting the period of periodontitis induction used in the present study.

Previously unpublished data indicated that OE 1493 exerts an important antibacterial activity against *Streptococcus mutans*, *S. sanguinis* and *Enterococcus faecalis*. Recently, Duarte *et al.*,²⁵ in their study in rats, reported that high proportions of the human host-compatible species, such as Streptococcus-like species, were observed around ligated teeth after periodontitis induction. Previous research in human subjects, observing the sequential development of oral biofilm, has established that these species are the early colonizers of teeth, creating an adequate microenvironment for the formation of the late colonizers, such as well-recognized periodontal pathogens.²⁶ In addition, other species belonging to the same genus have also shown antimicrobial activities, such as *I. tyrianthina*²⁷ and *I. leptophylla*.²⁸ Indeed, Locher *et al.*²⁹ demonstrated *in vitro* that a Hawaiian *Ipomoea* sp. produced anti-fungal activity against *Microsporum canis*, *Trichophyton rubrum* and *Epidermophyton floccosum*. Although previous studies have indicated the antimicrobial effect of OB 1493 or of similar genes,^{25,28-30} the possible anti-inflammatory activities of this natural product in bone metabolism and periodontal disease remain

to be investigated to explain, at least partly, the outcomes of the present study. Indeed, future research using different doses or administration regimes of this plant extract would contribute to a better understanding of the impact of OB 1493 on the progression of ligature-induced periodontitis.

In the present investigation, a toxicity assay of OE 1493 was also performed. A single-dose acute toxicity evaluation was done in mice, and for ethical and legal reasons, three animals were used per group, providing sufficient information to obtain the tendency towards toxicity. Our data indicated that OE 1493 did not show any signs of toxicity in the central nervous system, autonomous nervous system, psychomotricity or sensorial systems. For this reason, after being submitted to toxic assays, OE 1493 was selected for evaluation in the present pharmacological study in rats.

The significant antibacterial activity of OE 1493, whose minimal inhibitory concentrations and minimal bactericidal concentrations were lower than 0.04 mg/mL, indicated a low toxicity (unpublished data). For this reason, the present study used local administration of 0.3 mL of 10% OE 1493 in Tween 80 to evaluate the prevention of bone resorption in experimental periodontal diseases in rats.

In summary, although *in vitro* studies have shown a significant antibacterial activity of OE 1493 and no signs of toxicity were observed when compared with the control group, the topical use of this drug was not able to reduce bone resorption in experimental periodontitis. Further investigations using distinct protocols for OE 1493 use may be necessary to determine its therapeutic effects in controlling periodontal breakdown. The biodiversity of the Amazon may be considered a substantial source of new lead compounds to be assessed.

Conclusion

Within the limits of this study, it was demonstrated that OE 1493 did not reduce the progression of ligature-induced periodontitis in rats, when administered according to the regimen used in the present investigation.

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