Efficacy of a *Kalanchoe gastonis-bonnieri* extract to control bacterial biofilms and dental calculus in dogs

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**ABSTRACT.** Abdalla S.L., Costa S.S., Gioso M.A., Casanova L.M., Coutinho M.A.S., Silva M.F.A., Botelho M.C.S.N. & Dias R.S.G. 2017. Efficacy of a *Kalanchoe gastonis-bonnieri* extract to control bacterial biofilms and dental calculus in dogs. *Pesquisa Veterinária Brasileira* 37(8):859-865. Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: pgcir@usp.br

An aqueous leaf extract of the medicinal species *Kalanchoe gastonis-bonnieri* (here denominated KGB) has been found to be effective as an antimicrobial agent against canine oral cavity bacteria in *in vitro* assays. In this study, we investigated the effect of topical oral administration of KGB on the development of dental biofilm in Beagle dogs. The experiments were performed with an experimental group (0.2% of KGB extract), a negative control group (0.9% of saline solution) and a positive control group (0.12% chlorhexidine). Each treatment was sprayed into the oral cavity daily for 28 days. Thirty Beagle dogs with similar characteristics and kept under the same management and diet were used. The measurement of dental plaque and calculus was performed using a computerized analytical method. The phenolic profile of KGB extract was analyzed by HPLC-DAD. KGB extract at 0.2% showed efficacy in controlling the formation of plaque compared to the negative control group, and dental calculus in relation to the negative and positive control groups. A significant difference was observed among these three groups. Peaks attributed to flavonoids and phenolic acids were identified in the HPLC-DAD chromatogram of the KGB extract. The presence of these substances could be related to the activity observed. Our findings demonstrate that treatment with KGB is effective in controlling periodontal disease in dogs, providing new insights into the medicinal properties of this plant. KGB extract has a potential use as a supplemental agent in pharmaceutical products for the prevention of periodontal disease.

**INDEX TERMS:** *Kalanchoe gastonis-bonnieri*, plant extract, bacterial biofilm, dental calculus, dogs, periodontal disease, dental plaque, flavonoids.

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Eficácia do extrato de *Kalanchoe gastonis-bonnieri* no controle do biofilme bacteriano e cálculo dentário em cães. Um extrato aquoso de folhas da espécie medicinal *Kalanchoe gastonis-bonnieri* (aqui denominado como KGB) foi efetivo como um agente antimicrobiano contra as bactérias da cavidade oral de cães em testes *in vitro*. Neste estudo, investigou-se o efeito da administração oral tópica de KGB sobre o desenvolvimento do biofilme dental em cães da raça Beagle. Os experimentos foram realizados com um grupo experimental (0,2% de extrato de KGB), um grupo controle negativo (0,9% de solução salina) e um grupo controle positivo (0,12% de gluconato de clorexidina). Cada tratamento foi aplicado no interior da ca-
Periodontal disease is caused by dental biofilm accumulation on teeth and adjacent structures (Lindhe et al. 1975). It has been identified as the most frequently occurring clinical condition in domestic dogs (Kyllar & Witter 2005).

Human beings have used plants empirically since ancient times to treat health problems (Cragg et al. 2009). The World Health Organization (WHO) has estimated that in some Asian and African countries 80% of primary health care depends on plant-derived traditional medicines. Plant products are also consumed as medicines by people in developed countries (World Health Organization 2013). Traditional medicines need to be evaluated for safety and effectiveness, as well as scientific explanations concerning any collateral effects and their mechanisms of action (Booker et al. 2012, Luo et al. 2012, Shaw et al. 2012).

*Kalanchoe* species (Crassulaceae family) are widely used in traditional medicine in many regions of the world, especially India, Africa, China and Brazil due to their beneficial effects on inflammations, wounds and abscesses (Yadav & Dixit 2003, Nguelefack et al. 2004). Flavonoids seem to be the most important compounds in the plant. KGB extract has also shown the ability to affect spermatozoa motility, viability and sperm density, and to significantly decrease carmine and sialic acid content in the caudal epididymal plasma (Beltrán et al. 2003). The KGB extract has also exhibited in vitro antimicrobial activity on oral cavity bacteria (Menezes 2006) and antifungal activity (Costa et al. 2015).

From the standpoint of dentistry, medicinal plants have been poorly studied. They could be a source of phytopreparations for the treatment and prevention of periodontal disease, or even for post-operative therapy in classic surgical approach. These natural alternatives could reduce the need for surgical treatment, and cost of therapy. Also they could reduce the emergence of bacterial strains resistant to antibiotics and their side effects.

In the search for a new natural product against periodontal disease, this study aimed to evaluate the effect of an aqueous extract of *Kalanchoe gastonis-bonnieri* leaves on the dental plaque and calculus of dogs. Measurements were carried out using a computerized analytical method.

**MATERIALS AND METHODS**

The Bioethics Committee of the Veterinary Medical and Animal Science Faculty at the University of São Paulo (Protocol Number 1782/2009), and the Ethics Committee of the Veterinary Institute, at the Federal Rural University of Rio de Janeiro (Protocol Number 23083.000380/2008-71) duly authorized this experimental study.

**Plant material.** *Kalanchoe gastonis-bonnieri* specimens were cultivated in a residential garden in Rio de Janeiro, Brazil. Leaves from various specimens without any flowers were collected early in the morning in May, 2009. A voucher of a flowering specimen (RGA 31592) was deposited at the herbarium of the Botanical Department of the Institute of Biology, at the Federal University of Rio de Janeiro.

**Plant extraction.** Fresh leaves (448.57 g) of KGB were triturated in a blender with distilled water at 10% (w/v). After filtration on cotton, a light-yellow aqueous preparation (KGB extract) was obtained. An aliquot corresponding to 100 mL (0.2 g) of this extract was separated and lyophilized for chemical analysis. The KGB extract was prepared at the Laboratory of Chemistry of Bioactive Natural Products - IPPN, Bio (Institute of Research on Natural Products - IPPN, UFRJ).

**HPLC-DAD analysis.** HPLC-DAD analysis was performed on a Shimadzu liquid chromatograph LC-10AD with a diode-array wavelength SPD-M10VP detector, using a Merck reverse-phase column C-18 (5 μm, 250 mm, 2.5 mm). The mobile phase consisted of water containing 0.1% acetic acid (eluent A) and acetonitrile (eluent B). The solvent gradient used was: 0–5 min (100-80% A), 5–10 min (80-78% A), 10–25 min (78-75% A), 25–30 min (75-70% A), 30–35 min (70-40% A), 35–40 min (40-30% A), 40–50 min (30-20% A), 50–60 min (20-0% A); 60 min of total run time. The HPLC-DAD analysis was performed after dilution of 10 mg of the extract in 1 mL of deionized water. 20 μL of the extract were injected and the flow elution was 1 mL/min. The absorbance was monitored between 200 and 500 nm. HPLC-DAD analyses were carried out at the Institute of Research on Natural Products, UFRJ, Brazil.

**Animals.** Thirty male and female Beagle dogs, from 12 to 60 months old and weighing between 10 to 15 kg were selected from the experimentation kennel of the Laboratory of Experimental Chemotherapy in Veterinarian Parasitology, Department of Animal Parasitology at the Federal Rural University of Rio de Janeiro.

All dogs in the study presented normal mesencephalic occlusion, full dentition, and presence of periodontal disease until sta-
Efficacy of a Kalanchoe gastonis-bonnieri extract to control bacterial biofilms and dental calculus in dogs

Biochemical analyses. To assess any possible toxic effects of the KGB extract, biochemical analyses were performed comparing the experimental group before and after treatment with the KGB extract. Genesi Laboratory (Rio de Janeiro, RJ, Brazil) carried out the analyses of creatinine, urea, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase of the dogs (Melo et al. 2006). The statistical analyses were performed using the Student’s t-test as described above (Shaw et al. 2012).

RESULTS

Chemical profile by HPLC-DAD

Four major peaks attributed to phenolic substances were identified in the HPLC-DAD chromatogram of the KGB extract, as shown in Figure 1. Based on the retention times and UV absorbance spectra of each peak, the chemical classes of these phenolic substances were identified as shown in Table 2. The two flavonoids previously isolated by our group from KGB extract were identified based on retention time and confirmed by co-injection with these reference standards.

Table 2. Major phenolic compounds detected in the KGB leaf extract

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>λmax (nm)</th>
<th>Area (%)</th>
<th>Chemical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.5</td>
<td>265</td>
<td>21.7</td>
<td>Hydroxybenzoic acid derivative</td>
</tr>
<tr>
<td>2</td>
<td>12.0</td>
<td>290</td>
<td>10.7</td>
<td>Hydroxybenzoic acid derivative</td>
</tr>
<tr>
<td>3</td>
<td>13.6</td>
<td>271,335</td>
<td>11.4</td>
<td>Vicenin-2</td>
</tr>
<tr>
<td>4</td>
<td>16.8</td>
<td>255,349</td>
<td>2.2</td>
<td>Quercetin 3-O-a-rhamnoside-7-O-β-D-glucosyl-(1→3)-α-L-rhamnoside</td>
</tr>
</tbody>
</table>

Evaluation of plaque and calculus scores

The results of the dogs in the experimental group (KGB) had statistically significantly lower plaque ($P=0.01$) and calculus ($P=0.0164$) scores when compared to the negative control group, on 7th day and 28th day, respectively. Also the dogs treated with KGB showed lower calculus scores ($P=0.026$) when compared with dogs of the positive control group. The means of plaque and calculus index score were 20% and 19% inferior for the experimental group compared with the negative control group, and 6.1% and 22% lower than the positive control group, respectively (Table 3).
Table 3. Comparison of plaque and calculus scores of the computerized assessment (Abdalla et al. 2009) of areas of buccal surface with modified Logan and Boyce scores (1994) after use of the treatment protocols

<table>
<thead>
<tr>
<th>Groups</th>
<th>Positive control</th>
<th>Negative control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dogs</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>1 Week Mean Plaque</td>
<td>2.082 (0.16)</td>
<td>2.436 (0.5)</td>
<td>1.955 (0.1)</td>
</tr>
<tr>
<td>4 Weeks Mean Calculus</td>
<td>2.25 (0.2)</td>
<td>2.178 (0.48)</td>
<td>1.76 (0.09)</td>
</tr>
<tr>
<td>Calculus reduction (%)</td>
<td>-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plaque reduction (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

比较 vs. Negativo Controle  
Comparison vs. Positive Control

Table 4. Results of Student t-tests comparing serum hepatic and renal enzymes, before and after treatment with Kalanchoe gastonis-bonnieri extract

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Zero day average</th>
<th>28 day average</th>
<th>Zero day SD</th>
<th>28 days SD</th>
<th>Student’s t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP-AMP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>68.4</td>
<td>59.2</td>
<td>60.96</td>
<td>65.77</td>
<td>P=0.3439</td>
</tr>
<tr>
<td>UI/L</td>
<td>60.9</td>
<td>74.1</td>
<td>50.19</td>
<td>81.23</td>
<td>P=0.5160</td>
</tr>
<tr>
<td>ALT&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22.2</td>
<td>26.7</td>
<td>6.32</td>
<td>11.48</td>
<td>P=0.1311</td>
</tr>
<tr>
<td>AST&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.84</td>
<td>0.81</td>
<td>0.21</td>
<td>0.21</td>
<td>P=0.3434</td>
</tr>
<tr>
<td>CREATININE mg/dL</td>
<td>30.6</td>
<td>34.5</td>
<td>13.03</td>
<td>22.12</td>
<td>P=0.3630</td>
</tr>
</tbody>
</table>

<sup>+</sup> Alkaline phosphatase, <sup>+</sup> Alanine aminotransferase, <sup>+</sup> Aspartate aminotransferase.

Table 4. Results of Student t-tests comparing serum hepatic and renal enzymes, before and after treatment with Kalanchoe gastonis-bonnieri extract

Biochemical analyses

The values of creatinine, urea, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase of the experimental group were compared between before and after treatment with KGB extract. There was no significant difference in the biochemical analyses for dogs before and after treatment with the KGB extract (Table 4).

DISCUSSION

Plants have been used empirically by humans since ancient times to treat health problems (Cragg et al. 2009, World Health Organization 2013). Based on the well-known pharmacological properties of natural compounds from plant origin we looked for a natural anti-plaque agent to prevent periodontal disease in dogs.

The most frequently occurring pathological condition in domestic dogs is periodontal disease. Our study aimed to find an effective agent against plaque formation since this is the etiological agent of periodontal disease (Löe 1967, Varoni et al. 2012). Preventing plaque formation is the key factor in thwarting periodontal disease (Lindhe et al. 1975, Klukowska et al. 2012). As KGB has been shown to have antimicrobial activity against canine oral cavity bacteria in in vitro assays we assessed the effect of this extract in in vivo trials, with the participation of 30 Beagle dogs distributed into three groups of 10 animals.

In this study, the staining of bacterial plaque was made by using an aqueous solution of 2% eosin. We opted for the eosin in this study because it does not prevent antimicrobial activity and is easily removed from the tooth surface (Silva et al. 2002). These features make eosin an advantageous option for assessing specific products with antiplaque action. Furthermore, it was found that rinsing the oral cavity to remove the aqueous eosin 2% did not influence the results, since only materia alba and food debris (Schwartz et al. 1971) can be removed by mechanical action of a water jet. The plaque (Mandel 1966) as well as dental calculus were not affected (Harvey & Emily 1993).

Another issue that involves mechanical action on the materia alba and plaque is the dry food chewing. This effect was minimized in this study because dogs had the same breed and showed the same type of occlusion, both factors contributing to the reduction of individual variations. Additionally, dogs mostly chew only a small portion of the feed grain they eat, because of their instinct to swallow large portions as quickly as possible, thus consuming the greatest amount of food in a minor time. Still with the objective of minimizing the mechanical action of the oral mouthwash, the negative control group received a saline 0.9% washing jet, which removed only materia alba and not the plaque, as in the other groups of animals used.

According to the standards set by the Veterinary Oral Health Council (Veterinary Oral Health Council 2012) the minimum difference required for the mean mouth scores (average of all teeth measured for all animals in each group) should be at least 10% reduction for both dental plaque and for calculus when comparing the experimental and negative control groups. In this study, a 20% plaque reduction and a 19% dental calculus reduction was observed when the experimental group was compared with the negative control group. Also dental calculus was reduced by 22% in the experimental group compared with the positive control group, while plaque was only reduced by 6.1% compared with the positive control. The same occurred in an in vivo study with dogs (Pieri et al. 2010) where Copaifera officinalis (Fabaceae) was used in the experimental group, demonstrating the potential of medicinal plants for plaque reduction.

The KGB extract has been shown to be useful in controlling the oral microbiota of dogs. The presence of phenolic acids and flavonoids in the KGB extract (Correa 2010), as shown in Figure 1 and Table 2, could be related to the antibacterial activity and its efficacy on plaque formation when compared with negative control group.

Phenolic-rich plant extracts and fractions have shown...
The safety and collateral effects of KGB extract were also investigated in this work (Booker et al. 2012, Luo et al. 2012, Shaw et al. 2012). No statistically significant differences were observed in the biochemical parameters evaluated in the blood serum of dogs. However, it is important to note that this study duration was short.

In a study with male rats using a KGB aqueous extract, Beltrán et al. (2003) found that 11g/kg was the lethal dose for 50% of animals. This extract produced reversible infertility at doses of 300 and 150 mg/kg (Beltrán et al. 2003). In the present study, dogs with an average weight of 12.5 kg were treated daily with 20 mg of the KGB extract (10ml of a 0.2% solution). This much lower amount of KGB extract administered orally to dogs is very far from the lethal dose observed for rats. When compared to the doses used by these authors, the total amount administered during the treatment is not likely to produce the above mentioned effect, even considering that part of the KGB extract could be absorbed by dog’s oral mucoza. However, further studies are necessary to assess the effects of KGB on dog’s fertility.

Some toxicological data for other medicinal species of Kalanchoe have been reported. An extract from K. pinnata, whose leaves are widely used for healing wounds, was toxicologically evaluated in vivo. Mice orally treated with the K. pinnata aqueous leaf extract for 30 days showed no changes in levels of alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), urea and alkaline phosphatase. Additionally, a 36-year-old man under treatment for leishmaniasis with this plant extract for a period of 14 days showed no changes in serum toxicological parameters or adverse reactions (Torres-Santos et al. 2003).

K. brasiliensis leaf juice produced no acute toxicity in mice at doses from 0.25 to 5 g/kg, administered intraperitoneally (Mourão et al. 1999). Similar results were observed for an aqueous extract of K. integra at an oral dose of 5g/kg, which appeared to be non-toxic to mice (Antwi et al. 2013).

In all cases, we must be careful in the indiscriminate use of medicinal plants because they may present undesirable side effects. Furthermore, the study of safe conditions should be performed for a long-term use of KGB against dental plaque and calculus to guarantee that this treatment does not cause undesirable local and systemic effects (Shaw et al. 2012), as has been described for chlorhexidine and tricosan (Antwi et al. 2013, Lee et al. 2004, Salgado et al. 2006). However, our findings indicate a promising therapeutic application of KGB species in oral pathologies.

**CONCLUSIONS**

A single daily rinse with KGB extract at 0.2% showed efficacy in controlling the formation of dental plaque compared to the negative control group, and dental calculus in relation to negative and positive control groups. There was a significant difference for these three groups.

Thus, the KGB extract has a potential use as a supplemental agent in pharmaceutical products for the prevention of periodontal disease.

Our findings could encourage further studies in view of a potential utilization in human periodontal diseases.
natural medicinal plant that is widely available, with the profile of an anti-calcui and anti-biofilm dental agent is a very promising alternative for the pharmaceutical industry. Further studies are needed to monitor the absence of long-term side effects of a KGB extract, as well as the understanding of the mechanism of this phytopreparation in the impairment of the bacterial biofilm and dental calculus formation.

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