

## Inhibitory effect of *Persea cordata* Mez. (pau-andrade) bark extracts against *Clostridium perfringens* causing gangrenous mastitis

### Efecto inhibitorio de extractos de corteza de *Persea cordata* Mez. (pau-andrade) contra *Clostridium perfringens* causa de mastitis gangrenosa

Valfredo Schlemper, Susana Regina de Mello Schlemper, Denise Maria Sousa de Mello

Universidade Federal da Fronteira Sul (UFFS), Campus Realeza, PR, Brazil.

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#### ABSTRACT

**Introduction:** Gangrenous mastitis is a special clinical presentation of mastitis in cattle and small ruminants. The bark of the tree *Persea cordata* Mez. is used in Brazilian ethnoveterinary medicine to treat wounds in farm animals.

**Objectives:** Examine in vitro antimicrobial action of apolar fractions of *P. cordata* bark against a wild strain of *C. perfringens* isolated from the udder of a cow with gangrenous mastitis, and against a reference strain.

**Methods:** A milk sample was collected from the udder, aliquots were diluted and Gram-stained smears were performed. The aliquots were inoculated in broth and planted in blood agar, and then incubated in anaerobiosis at 37°C / 24h. Biochemical identification was based on bacterial isolation. In vitro inhibitory activity of apolar fractions of *P. cordata* was evaluated by agar diffusion and MIC (minimum inhibitory concentration) using the agar dilution method.

**Results:** In both tests the plant extracts displayed significant in vitro inhibitory activity against the clinical and reference strains of *C. perfringens* assayed.

**Conclusion:** The study is the first demonstration of the inhibitory effect of *P. cordata* on *C. perfringens*, due to its antimicrobial properties, which serves as evidence supporting its folk use. The extracts could be used as adjuvants in the treatment of gangrenous mastitis.

**Keywords:** medicinal plants; *Persea cordata*; antimicrobial; *Clostridium perfringens*; mastitis.

## RESUMEN

**Introducción:** la mastitis gangrenosa es una presentación clínica especial de mastitis en el ganado y pequeños rumiantes. *Persea cordata* Mez., es un árbol conocido en la etnoveterinaria brasileña, cuya corteza se utiliza en la curación de heridas en animales de granja.

**Objetivos:** investigar el efecto antimicrobiano *in vitro* de fracciones apolares de la corteza de *P. cordata* contra una cepa salvaje de *C. perfringens*, aislada de la ubre de una vaca con mastitis gangrenosa y una cepa de referencia.

**Métodos:** Se recogió una muestra de leche de la ubre, se diluyeron alícuotas, y se realizaron frotis teñidos por Gram. Las alícuotas fueron inoculadas en caldo y sembradas en agar sangre, y posteriormente incubados en anaerobiosis a 37 °C/24h. La identificación bioquímica fue realizada a partir del aislamiento bacteriano. La actividad inhibitoria *in vitro* de las fracciones apolares de *P. cordata* fue evaluada utilizando la técnica de difusión en agar y la CMI (concentración mínima inhibitoria) mediante el método de dilución en agar.

**Resultados:** los extractos de la planta, en ambas pruebas, presentaron significativa actividad inhibitoria *in vitro* contra las cepas clínica y de referencia de *C. perfringens* ensayadas.

**Conclusión:** se concluyó que, por primera vez, se demuestra un efecto inhibitorio de *P. cordata* sobre *C. perfringens*, reforzando el uso popular, debido a sus propiedades antimicrobianas. Los extractos podrán ser utilizados como coadyuvantes en el tratamiento de la mastitis gangrenosa.

**Palabras clave:** plantas medicinales; *Persea cordata*; antimicrobiano; *Clostridium perfringens*; mastitis.

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## INTRODUCTION

Mastitis is the inflammation of breast tissue caused by bacteria, viruses, algae and fungi,<sup>1</sup> enzootic in bovine what causes important loss to the milk-production chain. Gangrenous mastitis is a special clinic feature of mastitis in bovine, caused mainly by *Staphylococcus aureus*, *Mannheimia haemolytica*, *Escherichia coli*, *Clostridium perfringens* and *Arcanobacterium pyogenes*.<sup>2</sup> Published reports about the occurrence of gangrenous mastitis by *C. perfringens* are relatively old.<sup>3-5</sup> In Brazil, the descriptions of gangrenous mastitis by *C. perfringens* in bovine are rare. Most of the studies have described the illness in small ruminants.<sup>6-8</sup> Baldassi<sup>9</sup> related the occurrence of gangrenous mastitis in bovine caused by *Clostridium species* and Gonçalves<sup>10</sup> published the first gangrenous mastitis clinical case in Brazil caused *C. perfringens* in bovine. Although most of bacterial mastitis are caused by aerobic ones,<sup>11</sup> there is growing concern of the involvement of anaerobic organisms in those pathological processes. The main reason for the limited number of published studies on the involvement of *Clostridium sp* in mastitis is due to the difficulty of microorganisms growing.<sup>12</sup> Gangrenous mastitis by *C. perfringens* is caused, predominantly, by the upward contamination of the teat canal, because a bacterium is frequently isolated of faeces from healthy animals<sup>13</sup> and the environmental contamination supported by weather conditions and organic matter accumulation in areas where animals live can propitiate upward contamination of breast papillae. Moreover, before the estimated date of birth, cows show weakened immune system

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due to colostrogenesis and the release of endogenous corticoids,<sup>14</sup> features which can support contamination and installment of the infectious process. Local alterations, as lack of oxygen in injuries and digestive tract, so propitiating tissues invasions and their proliferation.<sup>15</sup> Clinically, it is characterized by mammary gland redness and necrosis of parenchyma accompanied with bloody, foul-smelling milk.

In searching for therapeutic alternatives against bacteria, medicinal plants have been standing out as potential sources of active ingredients able to control infectious processes. To that end, given the occurrence of gangrenous mastitis in bovine in Southwest Paraná, this study was based on the search for plants with ethnopharmacological indicatives for infections in animals.

*Persea cordata* (Vell.) Mez. (Lauraceae) a common big tree in Araucaria Forest, also known as "pau-andrade", "abacateiro do mato", "maçaranduba" or "canela rosada"<sup>16</sup> in popular medicine, has its bark used as plaster for wound healing.<sup>17-18</sup> It is common, in farms in the South Brazil, to find *P. cordata* with their barks removed carefully in order to treat some diseases in animals. Some biological activities attributed to *P. cordata* barks have already been proved experimentally *in vivo* and *in vitro* such, antibacterial and antifungal,<sup>19,17</sup> antispasmodic.<sup>20,21</sup> neuroprotector<sup>22</sup> and as antiedematogenic<sup>18</sup> actions. *P. cordata* healing action was described from the use of an ointment made of its barks,<sup>23</sup> so indicating a beneficial effect on granulation tissue evolution, facilitating the newly epithelial tissue and reducing time of wounds mobilization. Possibly, contributing with its healing action, it was found its polar fraction of ethyl acetate showed antimicrobial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*, which corroborate with its popular use in rural areas for treating infected wounds of animals.<sup>19,23</sup>

Antibacterial activity of the plant was related against Gram-positive *S. aureus*, *S. epidermidis* and *Bacillus cereus* bacteria, and against Gram-negative *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Shigella dysenteriae*, *Proteus vulgaris* and *Salmonella typhimurium*.<sup>19,23</sup> The objective of this study was to evaluate the inhibitory activity of *P. cordata* semi-purified extracts *in vitro* on the growing of *C. perfringens*, an anaerobic bacteria causing gangrenous mastitis.

## METHODS

### Botanical material

*P. cordata* barks were collected in Fazenda Leny, Bom Retiro, Santa Catarina, according to the agreement of the farmer and where it was inserted the legal forest reserve. Intervention in the natural environment was minimal and did not cause risk for the integrity of the trees. For the barks collection, the project was registered in Plataforma Chico Mendes (SISBIO) under the number 34821-1. The botanical material was identified by Dr. Roseli Botoluzzi (Agronomy Graduation Course/ CAV/ UDESC, Lages - SC) and a dried specimen was set in the herbarium of the Centro Agroveterinário, Lages, under the number 045.

### Extract and fractions collection

Barks were dried at room temperature (22-28 °C), grinded and macerated in 50 % methanol during 10 days, in order to obtain the hydroalcoholic extract. With the evaporation of the solvent under vacuum using a rotary evaporator at 50 °C, it

resulted a crude extract which was partitioned with hexane, dichloromethane, ethyl acetate and butanol solvents, generating different solid semi-purified fractions. All fractions were dissolved in 5 % dimethyl sulfoxide (DMSO) and subsequently diluted in phosphate buffer solution (PBS) until the desired concentration for using.

### Phytochemical screening

Chromatographic profile of *P. cordata* methanolic extract was evaluated through thin-layer chromatography (TLC) using aluminum chromatographic plates, pre-coated with thin layers of silica (Merck, 200 µm thickness) with different solvent systems. The research of bioactive secondary metabolites was performed through Liebermann-Buchard reaction for terpenes and steroids, Keller-Kelianni and Baljet reactions for aglycone and total sugars detection, Mayer and Bertrand, Bouchardat and Dragendorff reactions for alkaloids.<sup>24,25</sup>

### Microorganisms, isolation and identification

Bacterial samples used in the study were: a strain of the material collected from the udder of a Holstein cow, 4 years-old, with clinical history of gangrenous mastitis; and a standard *C. perfringens* strain (ATCC 13124) used as reference.

At clinical examination, the veterinarian who attended the animal related that it showed sudden lactation drop, anorexia, hyperthermia, continuous decubitus and melena. The front right quarter of the udder was swollen, touch-sensitive and blackened, and the milk secretion showed serous fluid with yellowish flocculation and bloody striations. Milk samples from the affected udder quarter were aseptically collected in sterile tubes with screw caps. At laboratory, aliquots were diluted in 0.5 mL of 0.9 % saline solution and from those it was performed stained smears by Gram technique.

Both samples (collected material and standard one) were inoculated in tubes with Cooked Meat Medium (CMM, Difco). Tubes were previously heated at 80 °C during 15 min in water bath and quickly cooled in tap water, and they were incubated at 37 °C for 24-48 h. An aliquot of each sample was inoculated by pouring into petri dishes with 5 % sheep blood agar, and incubated under aerobiosis and strict anaerobiosis in McIntosh & Fields jars at 37 °C during 24-48 h. After incubation, the colonies were observed in appearance, coloring and haemolysis. Samples showing doubled-halo, smooth, rounded and umbilical hemolytic colonies were submitted to Gram stain, and in sequence they were inoculated in CMM and incubated at 37 °C for 24-48 h.

With the resulting growth of the broth it was performed standard biochemical assays in order to identify the species: sugars fermentation (glucose, lactose, sucrose, maltose, salicin and mannitol), lipase and urease activity, indole production, hydrolysis gelatin, casein digestion, nitrate reduction and iron-milk reaction,<sup>8,26,27</sup> Additionally, it was made subcultures into blood agar plates and agar base tryptose sulfite cycloserin (TSC) plus 5 % egg-yolk selective-indicator media to demonstrate double zone of beta hemolysis and lecithinase reaction, respectively, and incubated at 37 °C for 18-24 h.

## ANTIBACTERIAL ACTIVITY

### Preliminary screening

The inhibitory activity of semi-purified hexane and dichloromethane fractions from *P. cordata* barks was tested against standard and collected strains through agar diffusion method.<sup>8</sup> Strains were activated into thioglycollate broth and a 10<sup>6</sup> CFU/mL final inoculum had its density adjusted by 530 nm spectrophotometer. In the sequence, they were inoculated into 5 % sheep blood agar plates where previously it was made wells at 6 mm diameter into a circular layout parallel to the edge of the plates with a cylindrical metal device, sterilized and with sharp edges. Wells were filled with 50 µL *P. cordata* extracts in 500, 1000 and 2000 µg/mL concentrations. Then, plates were incubated at 37 °C for 48 h in bacteriological incubator under anaerobiosis. After that, plates were examined for the increase of colonies. Results were collected according to Bauer *et al.*,<sup>28</sup> and growth inhibition was analyzed through measurement of halos with pachymeter. Antibacterial activity was expressed through concentration-response curves of bacterial growth inhibition. As a positive control it was used commercial antibiotics in liquid form, as penicillin (10 µg/mL). Plates were incubated for 24-48 h at 37 °C into anaerobic chamber.

### Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of *P. cordata* crude extract and semi-purified fractions on *C. perfringens* growing was determined through broth macrodilution method protocol<sup>29</sup> in tubes with Reinforced Clostridial Medium (RCM) (Labor M443). MIC was defined as the smallest concentration of each extract which visibly inhibited bacterial growing. To this end, a battery of tests using 9 tubes with different concentrations of each extract fraction and crude extract (1 a 256 µg/mL) was prepared in final volume from 1 to 2 mL per tube. An inoculum-control tube was prepared as well. Tubes with extract fractions were inoculated with a 5 × 10<sup>6</sup> UFC/mL standard bacterial medium. Final cell concentration was adjusted using spectrophotometer. After 24-48 hours of incubation in anaerobiosis chamber, it was performed the turbidimetry process according to Daguet and Chabbert<sup>30</sup> technique. The determination of penicillin MIC for the reference strain was done as collateral control for the validation of the methodology.

### Statistical analysis

The results were contained with the average of the experiments ± standard error of the averages, except IC<sub>50s</sub> (i.e. the concentration of the extracts requested to inhibit the bacteria growth 50 % in relation to answer controls), which are presented as geometric means accompanied by their respective 95 % confidence intervals. The statistical analyses were obtained by the ANOVA test, followed by the Dunnett's test when necessary.  $p < 0.05$  or  $p < 0.01$  was considered significant. The IC<sub>50s</sub> were obtained using the GraphPad Prism 7.0 program.

## RESULTS

Milk aliquots smear from collected samples showed short gram-positive bacilli. On blood-agar under anaerobiosis, colonies were gray-white color, big, smooth, rounded, regular convex, surrounded by a typical zone of β-haemolysis, and stained smear

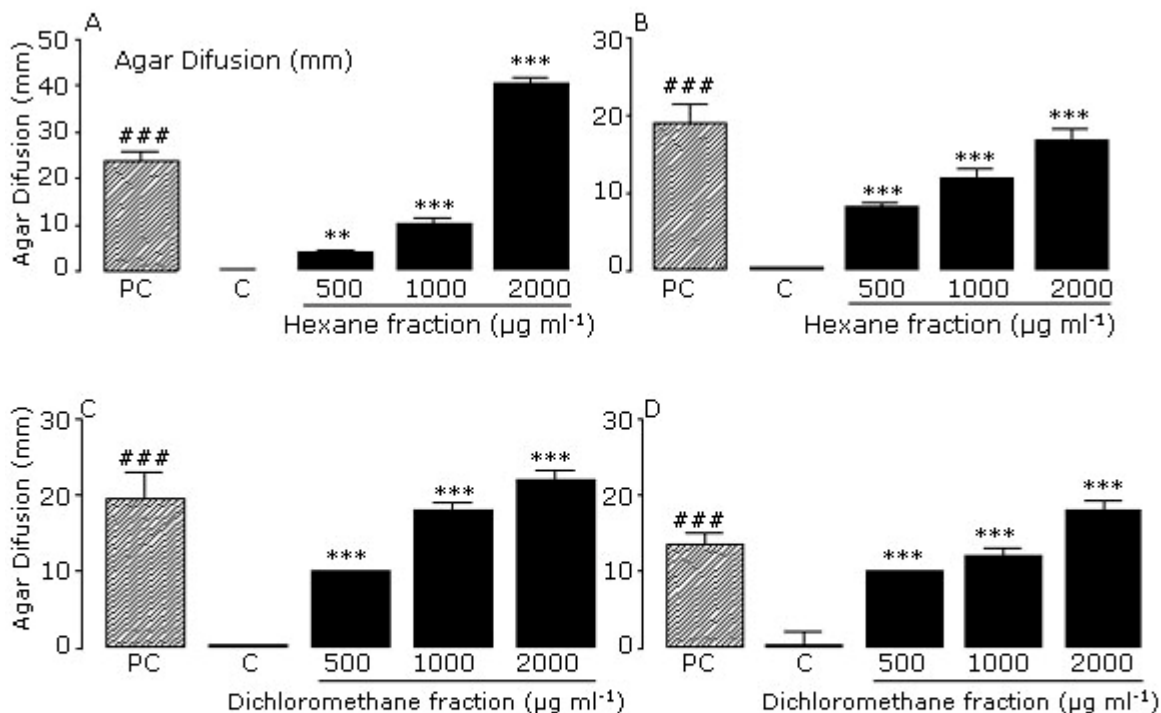
showed gram-positive bacillus. In biochemical tests were founded: fermentation of sugars (positive for glucose, lactose, sucrose, maltose and salicin; negative for mannitol, a negative activity on lipase and urease, no indol production, gelatin hydrolysis, casein digestion, reduction of nitrates in nitrites and promotion of stormy clot.<sup>8</sup> Additional cultivation in blood agar and TSC agar showed double haemolysis zone and lecithinase positive reaction, respectively. Based on phenotypic and biochemical characteristics, the isolated microorganism was identified as *C. perfringens*.

When examined in agar-diffusion test, it was observed both hexane (fig. 1A and 1B) and dichloromethane (fig. 1C and 1D) fractions of *P. cordata* caused significant and dependent-concentration inhibition halos as for control strains as for the collected ones in relation to control groups. It was calculated fractions potencies based on two triplicate samples for each experimental protocol. IC<sub>50</sub>s obtained from hexane fraction were 1560 (1270/1866) µg/mL for each reference strain (fig. 1A) and 467(444/509) µg/mL for collected strain (fig. 1B). For dichloromethane fraction, IC<sub>50</sub>s potencies ranged 648 (591/689) µg/mL for reference strain (fig. 1C) and 482 (433/499) µg/mL for collected strain (fig. 1D). For experiments using hexane and dichloromethane fractions for reference strain, the greatest tested concentration (2000 µg/mL) had inhibition efficacy up to the concentration of positive control (G penicillin, 10 µg/mL), and for the collected strain there was a greater efficacy for dichloromethane fraction in relation to positive control.

In broth macrodilution test, the semi-purified fraction of hexane showed moderate bacteriostatic activity with MIC values in an interval of 0.5 to 2 µg/mL against reference and collected strains (table). For dichloromethane fraction, MIC values ranged around 16 µg/mL for both strains. But the crude extract was less potent than semi-purified fractions showing MIC values of 32 µg/mL for clinical strain and 64 µg/mL for reference one.

**Table.** Minimal inhibitory concentration (MIC) of *P. cordata* barks crude extract, dichloromethane fraction, hexane fraction and penicillin for the reference and clinical strains.

Strains tested	MICs (µg . mL <sup>-1</sup> )			
	CE	DCM	HEX	PEN
Reference strain	64	16	< 1	< 1
Clinical strain	32	16	< 2	< 1



**Fig.** Average inhibitory effect of hexane and dichloromethane fractions of *P. cordata* on reference (A and C) and clinical origin (B and D) strains of *Clostridium perfringens* in agar-diffusion test in relation to control group (C, saline solution) and positive control group (PC, G penicillin 10 µg/mL). Columns represent the average of 6 experiments and vertical bars show S.E.M.s. Differing significantly: \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , relative treated versus negative control; ###  $p < 0.001$  relative positive control versus negative control.

## DISCUSSION

Infectious diseases caused by *Clostridium* sp. have a broad range of clinical severity that varies greatly. Among clostridium, *C. perfringens* has been associated with sudden death, toxicity, bleeding and gastrointestinal diseases.<sup>31,32,33</sup> However, mastitis caused by *C. perfringens* is a disease that generally shows a dramatic clinical condition with irreversible damage to mammary gland of female bovine and ovine in high dairy production.<sup>24,34</sup> There is a huge quantity of publications about antibacterial activity of medicinal plants around the world,<sup>35-37</sup> and a lot of plants have been researched due their biological activity against *C. perfringens*,<sup>38-41</sup> including clinical strains from isolated infections of hospitalized humans<sup>42,43</sup> or *C. perfringens* isolated from commercial broiler chickens diagnosed with necrotic enteritis,<sup>44,45</sup> but not with clinical pathogenic strains isolated from udder infection of lactating dairy cows. Researches performed with crude extracts of several plants and fungi showed, in a sorting of 238 extracts, 25 ones showing antimicrobial activity against *C. perfringens*.<sup>40</sup> Bento *et al.*<sup>45</sup> analyzed the synergism of essential oils of oregano, rosemary and thyme and its bactericidal effect to confront *C. perfringens* from standard strains. Jimoh *et al.*<sup>46</sup> obtained important results with garlic against *C. perfringens* from caeca of broiler chickens. Until now, there is no reference in the literature regarding the use of *P. cordata* extracts or fractions against *C. perfringens*.

*P. cordata* is a Brazilian tree from Araucaria Forest bioma which has ethno-indication as medicinal plant for treating dermatological infection and inflammation, probably due its action against anaerobic and aerobic skin bacteria as *C. perfringens*. While we were performing experiments with *P. cordata* against bacterial strains of veterinary interest, we had the opportunity to receive a clinical material of mammary infection which was confirmed clinically and microbiologically as gangrenous mastitis by *C. perfringens*. In this study, for the first time, it was demonstrated a significant inhibitory effect of *P. cordata* fractions against wild anaerobic microorganisms that usually have high resistance to conventional antibiotics.<sup>47</sup>

There was proposed a classification for the antimicrobial activity of vegetal materials, based on CIM results, as follow: strong inhibitor - CIM until 0.5 mg/mL; moderate inhibitor - CIM between 0.6 and 1.5 mg/mL; light inhibitor - CIM up to 1.6 mg/mL.<sup>48</sup> In other studies the inhibition of vegetal concentrations until 2 mg/mL characterized the extracts as potentially antimicrobial.<sup>49</sup>

In relation to the tests utilized, the hexane extract of *P. cordata* in broth macrodilution test, for all dilutions showed inhibitory effect in concentrations smaller than 1 µg/mL for standard strain and smaller than 2 µg/mL for clinical strain of *C. perfringens*. Dichloromethane extract inhibited bacterial growing significantly, reducing turbidity in the concentration of 16 µg/mL for both samples. Turbidity inhibition with the crude extract was around 32 µg/mL for collected sample and 64 µg/mL for standard sample, so demonstrating less power than semi-purified fractions.

Phytochemical studies by Sieben<sup>50</sup> showed among secondary metabolites obtained through hexane and dichloromethane fractions founded in *P. major* barks (*P. cordata*) are pointed out terpenoids, steroids, aglycons and total sugars, coinciding with we found in our fractions with thin-layer chromatography. Among them, triterpenes and steroids are predominant in the plant and they are highlighted as traditional phytochemical compounds with a well-established antimicrobial activity in other medicinal plants and which are efficient against different gram-positive and gram-negative microorganisms<sup>51,52</sup> including *Clostridium* spp.<sup>53,54</sup> As those steroids and triterpenes are predominant in less polar fractions as hexane and ethyl acetate fraction to a lesser extent, antibacterial actions of the plant could be attributed to those active principles.

Within the classification proposed, hexane extract of *P. cordata* barks showed antimicrobial action with a strong inhibitor against *C. perfringens*, in contrast to the reference antibiotic used (penicillin). The findings have suggested that less polar *P. cordata* fractions show efficient phytochemical compounds for inhibiting the growth of *C. perfringens*, this way justifying the popular use of the plant to treat infirmities caused by that microorganism.

We have demonstrated an important antiedematogenic effect of *P. cordata* polar fractions,<sup>18</sup> that in connection with its antibacterial effect<sup>17</sup> could contribute to the healing actions of the plant<sup>23</sup> according to ethnopharmacological findings from popular medicine. Thus, as a phytotherapeutic product, *P. cordata* barks can be a multifunctional medicine to treat skin wounds of animals, including infected ones. The studied *P. cordata* showed inhibitory activity for the anaerobic bacteria *C. perfringens* which causes several infectious pathologies associated to necrosis and hemorrhagic signs on the udder skin.<sup>55</sup> Despite being a fecal indicator of food contamination, it can be isolated after previous contamination to a distant source due to its ability to transform itself from a vegetative state into a microorganism which produces resistant spores, depending on environmental conditions.<sup>56</sup> Plants with antibacterial activity can potentially be used to treat diseases caused by bacterial infections. In popular medicine, the treatment of infected wounds is an indicative of that activity. Due to the



growing of *C. perfringens* strains more resistant to conventional drugs, new antibiotics will be needed for the therapy of animal infections in the future. There is an ecological rationality that antibacterial cytotoxic products should be synthesized in plants again after microbial attack in order to protect plants from pathogens.<sup>47</sup> Thus, antimicrobial compounds naturally derived from plants can be a new prospect to fight against multi-drug resistant pathogens.

We have concluded that, for the first time, it was demonstrated an inhibitory effect of *P. cordata* extracts on *C. perfringens*, reinforcing the popular use of *P. cordata* due to its antimicrobial properties. Thus the extracts may be used as coadjuvants in the treatment of gangrenous mastitis.

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#### CONFLICT OF INTEREST

The author has no conflicts of interest.

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*Valfredo Schlemper*. Universidade Federal da Fronteira Sul, Brazil. Correo electrónico: [valfredo.schlemper@uffs.edu.br](mailto:valfredo.schlemper@uffs.edu.br)