

Research Paper

The sensitivity of bacterial foodborne pathogens to *Croton blanchetianus* Baill essential oil

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Submitted: October 13, 2011; Approved: April 4, 2013.

Abstract

The aim of this study was to assess the activity of essential oil extracted from the leaves of *C. blanchetianus* Baill, popularly known as “marmeleiro”, in inhibiting the growth and survival of pathogenic microorganisms in food by determining their survival *in vitro* and by observing the behaviour of *Listeria monocytogenes* inoculated into a food model (meat cubes) that was stored at refrigeration temperature (7 ± 1 °C) for 4 days. The results indicated a bactericidal effect against *Aeromonas hydrophila* and *Listeria monocytogenes* and bacteriostatic action against *Salmonella* Enteritidis. A bacteriostatic effect on meat contaminated with *L. monocytogenes* was found for all concentrations of essential oils tested. These results showed that essential oil from the leaves of *C. blanchetianus* Baill represents an alternative source of potentially natural antimicrobial agents that may be used as a food preservative.

Key words: essential oil, *Croton blanchetianus* Baill, antimicrobial activity, foodborne microorganisms.

Introduction

Food preservation techniques are used to provide food with high nutritional quality and microbial stability, which is achieved by controlling the growth/survival of spoilage-associated and foodborne microorganisms (Baidar *et al.*, 2004; Benkeblia, 2004). Historically, several physical and/or chemical procedures have been used to ensure the microbiological safety of foods (Daferera *et al.*, 2003; Marino *et al.*, 2001). Recently, the use of chemical food preservatives has been questioned, particularly by consumers who demand more natural foods (Mendoza-Yepey *et al.*, 1997; Radhrakrishanan-Sridhar *et al.*, 2003). More than 1,340 plants are known as potential sources of

antimicrobial compounds, but very few of these have been studied scientifically (Seidil, 2000).

This scenario has encouraged research aimed at the possible development and use of vegetable products with antimicrobial properties (Souza *et al.*, 2005; Valero and Salmerón, 2003). Species from the genus *Croton* are frequently used in popular medicine (as infusions, teas and poultices) to relieve pain (Abreu *et al.*, 2001), constipation, diarrhea and other digestive symptoms, diabetes, wounds, inflammation, fever, and hypertension (Salatino *et al.*, 2007). Studies conducted in certain species have demonstrated several pharmacological properties of plants within this genus, such as antidiabetic (Barbosa-Filho *et al.*, 2005), anti-inflammatory, antiulcerogenic, analgesic and

anti-hypertensive activities (Palmeira-Junior *et al.*, 2006), among others. *C. blanchetianus* Baill (synonym *Croton sonderianus* Müll. Arg.) (Govaerts *et al.*, 2000), known as “black marmeleiro”, is a widely disseminated shrub found in northeast Brazil. Its leaves and bark are used as popular medicines for treating gastrointestinal disturbances, rheumatism and cephalalgia (Chaves and Reinhard, 2003). It has a high essential oil content, and its yield may range from 0.5% to 1.5% (Chaves and Reinhard, 2003). Furthermore, this plant is rich in diterpenes with different biological activities, including anti-inflammatory, gastroprotective and antimicrobial properties (Marino *et al.*, 2001).

The aim of this study was to evaluate the efficacy of essential oil from the leaves of *C. blanchetianus* Baill in inhibiting the growth of gram-positive and gram-negative pathogenic bacteria and to analyse the antibacterial effectiveness of *C. blanchetianus* Baill essential oil in meat cubes stored under refrigeration.

Materials and Methods

Essential oil

Fresh leaves of *C. blanchetianus* were collected in the rural municipality of Patos (7°05'10" S, 37°15'45" W), located in the semiarid central region of Paraíba, Brazil, from February–March 2011. The species was identified by comparison with a herbarium specimen, and a dried sample (no. 1462) was deposited in the Brazil Herbarium, Federal University of Campina Grande, Patos, Paraíba.

Fresh leaves (150 g) were submitted to hydrodistillation for 3 h using a Clevenger type apparatus according to the method recommended in the European Pharmacopoeia (2004). The obtained oils were allowed to dry over anhydrous sodium sulphate. The oils were filtered and then stored at 4 °C prior to testing and analysis. The yield of the oil was 0.7%. The essential oil was tested at concentrations ranging from 80–0.6 µg.mL⁻¹. Essential oil solutions were prepared in Mueller Hinton broth (MH) (HiMed[®], Mumbai, India) using bacteriological agar (0.15 g.100 g⁻¹) as a stabilising agent (Bennis *et al.*, 2004; Mann and Markham, 1998).

Bacterial strains

Strains of *Aeromonas hydrophila* INCQS 7966, *Listeria monocytogenes* ATCC 7644 and *Salmonella* Enteritidis CDC 49812 were used as test microorganisms. Inocula used in antimicrobial assays were obtained from overnight cultures grown on MH agar slants at an optimum growth temperature (28 °C for *A. hydrophila* and 37 °C for the other bacteria). A loopful of the culture was diluted in sterile saline solution (0.85 g.100 mL⁻¹) to a final concentration of approximately 10⁸ colony-forming units per mL (cfu.mL⁻¹) adjusted according to the turbidity of 0.5 McFarland standards. The final concentration of the inoculum in the medium used for the antimicrobial assays was approximately 10⁶ cfu.mL⁻¹.

Tests of cell viability

Our previous experiments with *C. blanchetianus* Baill essential oil against the tested pathogens using disc diffusion revealed clearance zones of 10–12 mm and minimum inhibitory concentrations (MICs) of 20 µL.mL⁻¹, 1.25 µL.mL⁻¹ and 40 µL.mL⁻¹ for *Aeromonas hydrophila*, *Listeria monocytogenes* and *Salmonella* Enteritidis, respectively (unpublished data). Cell counts were performed to test cell viability at concentrations of MIC/2, MIC and 2x MIC. For this purpose, 5 mL of double concentrated MH broth was inoculated with 1 mL of bacterial suspension. Next, 4 mL of *C. blanchetianus* Baill essential oil solution was added to obtain an appropriate final concentration, and the system was incubated at 37 °C under aerobiosis. At intervals of 0 min., 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 10 h after incubation, 1.0 mL aliquots of the suspension were diluted (10⁻¹–10⁻⁵) in sterile peptone water and uniformly seeded onto Petri plates containing MH agar using the plate spreading technique. After the incubation period, the viable cells were counted, and the resulting value was expressed in log cfu.mL⁻¹. Control samples without essential oil were tested in parallel (Barros *et al.*, 2009).

Antimicrobial activity in a food model

Bovine meat knuckles were trimmed of all external fat and cut into pieces of uniform sizes (3x3x3 cm). The meat pieces were placed in glass flasks and sterilised using an autoclave (121 °C for 15 min, 121 atm). Then, meat pieces were inoculated with a microbial suspension by individual submersion in 50 mL of the bacterial inoculum (*L. monocytogenes* containing approximately 10⁸ cfu.mL⁻¹, prepared in sterile 0.85 g.100 mL⁻¹ saline solution) with rotation with a sterile glass rod for 1 min to ensure even inoculation. The inoculated pieces were air dried for 30 min in a bio-safety cabinet and finally washed with the essential oil (Oliveira *et al.*, 2010). The inoculated pieces were randomly divided into five groups and treated for 30 s (1:4 w/v) as follows: (I) control, dipped in sterile distilled water; (II) dipped in 1x MIC essential oil solution; (III) dipped in 2x MIC essential oil solution; (IV) dipped in 5x MIC essential oil solution; and (V) dipped in 10x MIC essential oil solution. The pieces were then placed in sterile sealed polypropylene cups and stored under refrigeration (7 °C ± 1 °C). At 0, 24, 48, 72 and 96 h of storage, the meat samples were submitted to an *L. monocytogenes* count according to the procedure described by AOAC (1995). The results were presented in log cfu per gram of meat (log cfu.g⁻¹ of meat) (Oliveira *et al.*, 2010). All antimicrobial assays were carried out in triplicate, and the results are expressed as an average of the three parallel assays.

Statistical analysis

Statistical analysis was performed to determine the significance of differences between groups ($p < 0.05$) using

the Tukey test to estimate the time of inactivation. Statistica software version 7.0 (StatSoft Inc., USA) was used for the data analysis.

Results and Discussion

Figures 1, 2 and 3 show the effects of MIC/2, MIC and 2xMIC of *C. blanchetianus* Baill essential oil on the viability of the bacteria of interest in food. The survival curve is presented as a way to measure the capacity of a compound to act on the viability of a microorganism. Furthermore, it may be inferred that the estimated mortality of a microbial population (determined by the number of viable cells on the plates) at a given concentration of an antimicrobial compound reflects the speed of the bactericidal effect or the duration of a bacteriostatic effect (Burt, 2004).

Three bacterial strains were selected for this test (*Aeromonas hydrophila*, *Listeria monocytogenes* and *Salmonella* Enteritidis), and the inhibition results of antimicrobial tests were used as the inclusion criteria. All of the growth curves obtained showed different activities for the different values of MIC. MIC and 2x MIC exhibited statistically significant inhibition of pathogen growth ($p < 0.05$) compared with the control sample.

Figure 1 shows that the microbial population size found at the last time interval analysed (10 hours) was approximately 10^3 cfu.mL⁻¹, representing a reduction of 3-4 orders of magnitude in the number of viable cells relative to the initial inoculum. In turn, the microbial population size of the control sample was between 10^9 and 10^{10} cfu.mL⁻¹ at time interval of 10 h, an approximately 100- to 1000-fold increase from the value of the initial inoculum. MIC was more effective in inhibiting the growth of bacterial strains after 8 hours of interaction, MIC/2 after at least 10 hours

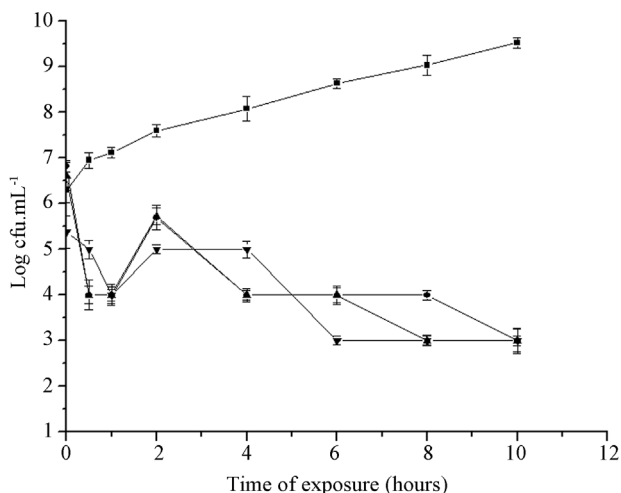


Figure 1 - Effect of the *Croton blanchetianus* Baill essential oil on the cell viability of *A. hydrophila*: (■) control (0 $\mu\text{g mL}^{-1}$); (●) MIC/2 (10 $\mu\text{g mL}^{-1}$); (▲) MIC (20 $\mu\text{g mL}^{-1}$); (▼) 2x MIC (40 $\mu\text{g mL}^{-1}$) of essential oil.

and 2x MIC after only 6 hours. A compound is recognised as having a strong bactericidal effect when it is capable of causing a 1000-fold reduction (3 orders of magnitude, or 99.9%) of the initial inoculum (LaPlante, 2007).

Figure 2 shows evidence of the bacteriostatic action (reduction of growth rate) of *C. blanchetianus* Baill essential oil against the bacterial strain studied. This bacteriostatic action was evident throughout the entire period of interaction (10 hours). The bacteriostatic activity is characterised by the ability of a substance to render a bacterium incapable of growing/multiplying in the broth but still capable of growth when removed from the broth and plated on appropriate agar (Smith-Palmer *et al.*, 1998).

Some foodborne pathogens may show an initial exponential decrease in growth capacity followed by a subsequent increase in antimicrobial resistance upon exposure to compounds with antimicrobial properties (Rowan, 1999). This phenomenon occurs when the resistant microbial cells that represent only a small proportion of the initial microbial population are selected for and ultimately contribute a greater proportion of the total final population (Rowan, 1999).

Evidence that this phenomenon occurred in our experiments may be observed in Figure 2, in which an exponential reduction of the microbial population is observed from 0-8 hours of interaction, followed by a smaller reduction effect and an ascending growth curve up to 10 hours of interaction.

Figure 3 demonstrates a strong bactericidal effect in that the microbial population found in the last time interval analysed (10 hours) present values of approximately 10^3 cfu.mL⁻¹, a decrease of 3-5 orders of magnitude in the number of viable cells relative to the initial inoculum. In turn, the control sample showed a microbial population of between 10^8 and 10^9 cfu.mL⁻¹ at 10 h, that is, an increase of

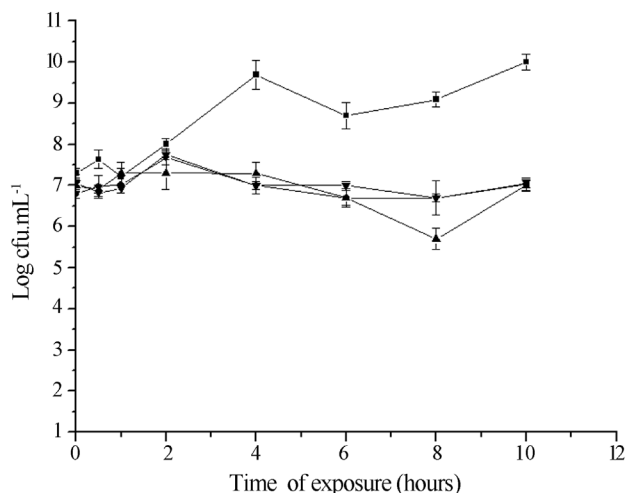


Figure 2 - Effect of the *Croton blanchetianus* Baill essential oil on the cell viability of *Salmonella* Enteritidis: (■) control (0 $\mu\text{g mL}^{-1}$); (●) MIC/2 (20 $\mu\text{g mL}^{-1}$); (▲) MIC (40 $\mu\text{g mL}^{-1}$); (▼) 2x MIC (80 $\mu\text{g mL}^{-1}$) of essential oil.

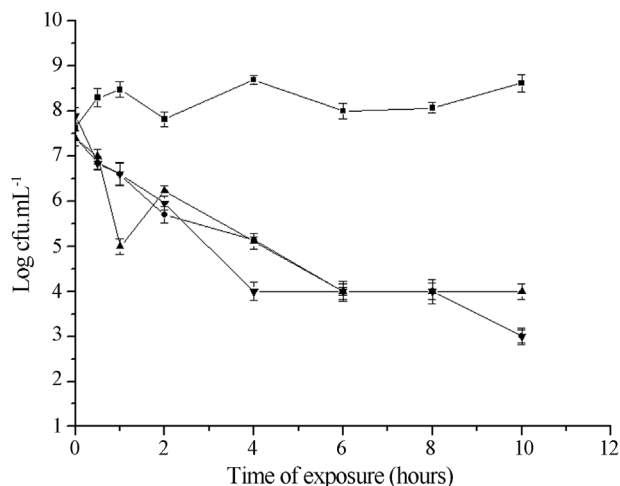


Figure 3 - Effect of the *Croton blanchetianus* Baill essential oil on the cell viability of *L. monocytogenes*: (■) control (0 $\mu\text{g mL}^{-1}$); (●) MIC/2 (0.6 $\mu\text{g mL}^{-1}$); (▲) MIC (1.25 $\mu\text{g mL}^{-1}$); (▼) 2x MIC (2.5 $\mu\text{g mL}^{-1}$) of essential oil.

approximately 10 to 100 times the value found in the initial inoculum. The greatest efficacy of essential oil in inhibiting the growth of bacterial strains was observed after 4 hours of exposure to the oil in 2.5 $\mu\text{g mL}^{-1}$, and 6 hours in 0.6 and 1.25 $\mu\text{g mL}^{-1}$ of essential oil. Thus, a significant reduction ($p < 0.05$) in bacteria count was promoted in comparison with the control sample. We also observed a prolonged lag phase (stationary phase) of the bacterial growth curve. The lag phase is typically considered an indicator of the time required to adapt to a new environment; therefore, the longer the lag phase, the more difficult it is for the microorganism to adapt to the new environment and establish exponential growth (Souza *et al.*, 2005).

Even though a satisfactory antimicrobial efficacy was found for the MIC of *C. blanchetianus* Baill essential oil, particularly against *L. monocytogenes*, Figure 4 shows when applied to food model, it did not decrease the number of microbes relative to the initial inoculum, as observed *in vitro*; however, there was a bacteriostatic effect at all concentrations tested. Growth inhibition was more effective at MIC and 10x MIC, which maintained the initial contamination level of 10^5 cfu.mL $^{-1}$ until the end of 4 days.

It must be noted that higher concentrations of essential oil are generally needed in food to achieve antimicrobial effectiveness similar to that obtained in laboratory experiments. In studies with food models, the required concentration values were two times higher in semi-skim milk (Karatzas *et al.*, 2001), ten times in pork sausages (Pandit and Shelef, 1994), fifty times in a soup (Ultee and Smid, 2001) and twenty-five to one hundred times in cheese (Mendoza-Yepes *et al.*, 1997). Our results showed that the effective inhibition of *Listeria monocytogenes* growth in meat required a concentration ten times higher than that used in the *in vitro* experiment.

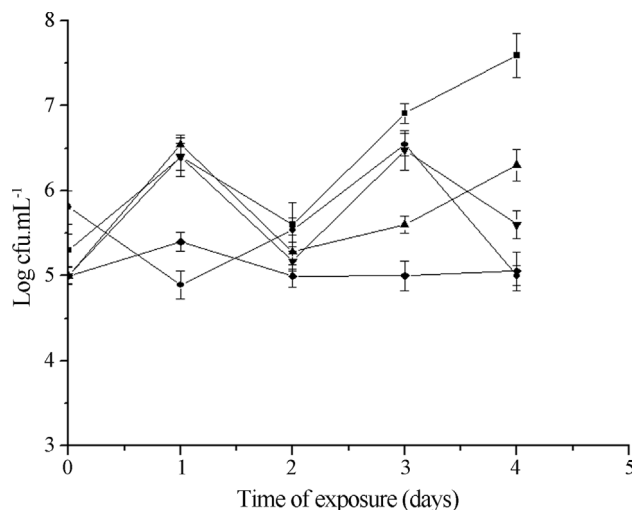


Figure 4 - Effect of the *Croton blanchetianus* Baill essential oil on the cell viability of *L. monocytogenes* on pieces of meat during storage under refrigeration for 5 days: (■) control (0 $\mu\text{g g}^{-1}$); (●) MIC (1.25 $\mu\text{g g}^{-1}$); (▲) 2x MIC (2.5 $\mu\text{g g}^{-1}$); (▼) 5x MIC (6.2 $\mu\text{g g}^{-1}$); (◆) 10x MIC (12.5 $\mu\text{g g}^{-1}$) of essential oil.

The mechanism responsible for the lower antimicrobial effectiveness of essential oils when used in foods is still not well established (Gil *et al.*, 2002). It has been suggested that the great variety of nutrients available in foods compared with culture medium could allow the microbial cell to recover more quickly from cell damage (Gil *et al.*, 2002). It is also possible that the essential oil is dissolved in the lipid phase of the food, making it less capable of acting against the microorganisms present in the aqueous phase (Mejholm and Dalgaard, 2002).

In food model systems, both the intrinsic properties of the food and extrinsic determinants may influence the antimicrobial effectiveness of essential oils. According to reports from Gutierrez, Barry-Ryan and Bourke (Gutierrez *et al.*, 2008; Gutierrez *et al.*, 2009), various essential oils applied alone or in combination were more effective against pathogenic bacteria when applied in a medium with high protein content and high acidity, low levels of fats and carbohydrates, and moderate levels of simple sugars. Therefore, the Figure 3 demonstrates the model meat food used in the present experiment should not have interfered substantially in the antimicrobial effectiveness of the essential oil. Parallel results suggested that a longer period of exposure of the microorganism to the essential oil might be necessary to obtain a better understanding of the antibacterial kinetics.

Conclusion

It was concluded that the essential oil of *C. blanchetianus* Baill is effective as an antimicrobial agent *in vitro*; however, in a model system using fresh meat, its efficacy was reduced, and consequently a higher concentration of essential oil was required to inhibit bacterial growth. A

major challenge for the practical application of essential oils as food preservatives is to optimise the use of essential oil at low concentrations in conjunction with other conservation techniques that enable food security and longer shelf lives.

Acknowledgments

We thank CAPES (Brazilian Federal Agency for Support and Evaluation of Graduate Education) for the financial support.

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