Research, Society and Development, v. 10, n. 9, e16310917907, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i9.17907

# Antibacterial activity of crude extract of *Tabernaemontana catharinensis* latex (A. DC) against *Alicyclobacillus* spp.

**Atividade Antimicrobiana do extrato bruto do látex de *Tabernaemontana catharinensis* (A. DC) em *Alicyclobacillus* spp.**

**Actividad antimicrobiana del extrato crudo de látex de *Tabernaemontana catharinensis* (A. DC) en *Alicyclobacillus* spp.**

Received: 07/05/2021 | Reviewed: 07/10/2021 | Accept: 07/13/2021 | Published: 07/23/2021

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**Abstract**
*Alicyclobacillus* spp. is composed of Gram-positive, aerobic, thermoacidophilic, endospore-forming bacteria that cause food and beverage spoilage. The presence of *Alicyclobacillus* spp. may result in the production of guaiacol, which leads to sensory changes in the odour and taste of citrus juices and acidic foods. *Tabernaemontana catharinensis* (A. DC) is a plant belonging to the family *Apocynaceae* that produces milky latex with several biological activities described as antioxidant, antiviral, antimicrobial, trypanocidal and anti-leishmanicidal. Therefore, this study aims to evaluate the antimicrobial activity of the crude latex of *T. catharinensis* (A. DC) against microorganisms of the genus *Alicyclobacillus* spp. The minimum inhibitory concentration of latex was 7.81 µg/ml for the five *Alicyclobacillus* species analysed. The minimum bactericidal concentration for the species *Alicyclobacillus acidoterrestris* 0244$^T$, *A. hesperidum* 0298$^T$, *A. acidiphilus* 0247$^T$ and *A. cycloheptanicus* 0297$^T$ was 250 µg/ml. Cytotoxicity analysis demonstrated that latex was toxic to Vero cells at concentrations greater than 84.67 µg/ml. Scanning electron microscopy revealed changes in the cell wall of *A. acidoterrestris* 0244$^T$ present in orange juice when treated with crude latex. The results obtained suggest that the crude latex of *T. catharinensis* (A. DC) displays an antimicrobial effect against *Alicyclobacillus*, with potential for application in the food industry.
**Keywords:** Antibacterial activity; *Tabernaemontana catharinensis* (A. DC); Latex; *Alicyclobacillus* spp.; Orange juice.

**Resumo**
O gênero *Alicyclobacillus* spp. é composto por bactérias Gram-positivas, aeróbicas, na forma de bacilos, ácido termoresistentes, com capacidade de formar esporos e atuarem como deteriorante de bebidas e alimentos. O desenvolvimento dessas bactérias pode resultar na produção do composto Guaiacol que leva a alterações sensoriais. A planta *Tabernaemontana catharinensis* (A.DC) pertence à família *Apocynaceae* é produtora de uma substância chamada látex com atividade biológica descrita. O estudo do látex bruto de *T. catharinensis* (A. DC) frente aos microrganismos do gênero *Alicyclobacillus* tem como objetivo investigar sua atividade antimicrobiana. As metodologias desenvolvidas foram: determinação da concentração inibitória mínima (MIC) e concentração bactericida mínima (MBC), citotoxicidade e microscopia eletrônica de varredura. Os resultados encontrados foram MIC de 7,81 µg/mL para todas as bactérias analisadas e o resultado mais eficaz de MBC ocorreu para as cepas *A. acidoterrestris* 0244$^T$, *A. hesperidum* 0298$^T$, *A. acidiphilus* 0247$^T$ e *A. cycloheptanicus* 0297$^T$ que apresentaram MBC de 250 µg/mL. Já para a cepa *A. acidocaldarius* 0299$^T$ o MBC foi de 500 µg/mL. A análise de citotoxicidade demonstrou que foi tóxico em células Vero em concentrações superiores a 84,67 ± 8,14 µg/mL. No microscópio eletrônico de varredura foi possível observar alterações na integridade da parede de *Alicyclobacillus acidoterrestris* 0244$^T$ em suco de laranja, quando tratado com látex bruto em diferentes concentrações. Podemos concluir que o látex bruto de *Tabernaemontana catharinensis* (A. DC) apresenta efeito antimicrobiano frente a *Alicyclobacillus acidoterrestris* 0244$^T$.
**Palavras-chave:** Atividade antibacteriana; *Tabernaemontana catharinensis* (A. DC); Látex; *Alicyclobacillus* spp.; Suco de Laranja.

**Resumen**
El género *Alicyclobacillus* spp. és compuesto por bactérias Gram positivas, aerobias, en forma de bacilos termorresistentes, que tienen capacidad de formar esporas que funcionan como deterioro de alimentos y bebidas. El desarollo de estas bacterias puede resultar en la producción del compuesto Guaiacol, que tiene como consecuencia modificaciones sensoriales. La planta *Tabernaemontana catharinensis* (A.DC), perteneciente a la familia *Apocynaceae*, produce una sustancia llamada látex con actividad biológica descrita. El estudio del látex crudo de *T. catharinensis* (A. DC) frente a microorganismos del género *Alicyclobacillus*, tiene como objetivo investigar su actividad antimicrobiana. Las metodologías aplicadas fueron: determinación de concentración mínima inhibitoria (MIC) y concentración mínima bactericida (MBC), citotoxicidad y microscopía electrónica de barrido. Los resultados encontrados fueron: una MIC de 7.81 µg/mL para todas las bacterias analizadas; y un resultado de MBC más efectivo que ocurrió para las cepas *A. acidoterrestris* 0244$^T$, *A. hesperidum* 0298$^T$, *A. acidiphilus* 0247$^T$ y *A. cycloheptanicus* 0297$^T$ que presentaron MBC de 250 µg/mL. En cuanto a la cepa *A. acidocaldarius* 0299$^T$, el MBC fue de 500 µg/mL. El análisis de citotoxicidad demostró que era tóxico para las células VERO en concentraciones superiores a 84,67 ± 8,14 µg/mL. En el microscopio electrónico de barrido fue posible observar alteraciones en la integridad de la pared de *Alicyclobacillus acidoterrestris* 0244$^T$ en jugo de naranja, cuando expuesto a látex crudo en diferentes concentraciones. Podemos concluir que el látex crudo de *Tabernaemontana catharinensis* (A. DC) p efecto antimicrobiano frente a *Alicyclobacillus acidoterrestris* 0244$^T$.
**Palabras clave:** Actividad antibacteriana*; Tabernaemontana catharinensis* (A.DC); Látex; *Alicyclobacillus* spp.; Jugo de naranja.

## 1. Introduction

The juice deterioration process caused by *Alicyclobacillus acidoterrestris* was first described in 1984 in apple juice, with changes in taste and odour resulting from the production of 2-methoxyphenol (guaiacol) and 2,6-dibromophenol by the microorganism (Chang and Kang, 2005; Yamazaki et al., 1996).

The cell wall of species of the genus *Alicyclobacillus* is composed of cyclic fatty acids that confer resistance at temperatures of 25 to 70 ºC and low pH (2.0 to 6.5), which together with the potential to form spores, causes problems in the citrus juice and acidic foods industry, with consequent economic losses (Cai et al., 2015; Chang and Kang, 2005).

Brazil is responsible for 34% of the production of oranges and more than half of the production of concentrated orange juice (66°Brix) worldwide, and, consequently, juice exports contribute significantly to the country's trade balance (Neves and Trombin, 2017). Therefore, ensuring the quality of concentrated juice, increasing its shelf life and finding natural substances to prevent the development of spoilage species of this product is critical to the industry (Neves and Trombin, 2017).

*Tabernaemontana catharinensis* (A. DC) is a lactescent tree belonging to the family *Apocynaceae*, found in Brazil, Argentina, Paraguay and Uruguay, used in folk medicine as an antidote for snake bites, toothache and vermifuge relief. It presents biological action, such as antioxidant, antiviral, antimicrobial, trypanocidal and antileishmanial (Janning et al., 2011; da Silva Brum et al., 2016; Soares et al., 2007).

This plant has lactiferous cells that secrete latex, an aqueous emulsion with various chemical substances and varied bioactive compounds, such as polyphenols, flavonoids, sugars, amino acids, *n*-alkanes, alkaloids and proteins that are secreted in response to tissue damage in the plant (Boligon et al., 2015; Dussourd, 2017; Lewinsohn, 1991; da Silva Menecucci et al., 2019).

The chemical composition of *T. catharinensis* latex used in the current study presents 52.5% protein, 21.2% free amino acids, and a strong proteolytic activity in addition to other properties as described by Menecucci et al., 2019.

This research aimed to evaluate the antimicrobial activity of the crude latex of *T. catharinensis* against microbial species of the genus *Alicyclobacillus*.

## 2. Methodology

### *2.1 Bacterial strains and culture media*

The bacterial reference strains used in this study (*A. acidoterrestris* 0244$^T$, *A. hesperidum* 0298$^T$, *A. acidiphilus* 0247$^T$, *A. cycloheptanicus* 0297$^T$ and *A. acidocaldarius* 0299$^T$) originated from the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), located at the Centre for Chemical, Biological and Agricultural Research (CPQBA/UNICAMP). The strains were stored in cryotubes containing *Bacillus acidoterrestris* BAT medium (Deinhard et al., 1987) with 30% glycerol (Thermo Fisher Scientific, Waltham, MA, USA) at –20 °C at the Laboratory of Microbiology of Water, Environment and Food of the State University of Maringá (Maringá, Brazil).

The culture medium used in the assays was the BAT with final pH adjusted to 4.0 with 1 M NaOH or 1 M HCl solutions. Concentrated orange juice (66°Brix, pH 4.0) used in the assays was provided by a citrus juice industry in the north-western state of Paraná, Brazil and reconstituted to 11°Brix in sterile distilled water under aseptic conditions and free of *Alicyclobacillus* spp., as analysed in the laboratory.

### *2.2 Collection and preparation of the crude latex*

Samples of the crude extract of *T. catharinensis* (A. DC) were obtained in 2016 from plants grown on the campus of the State University of Maringá (approximate latitude and longitude of –23.4253 and –51.9386, respectively, based on the

geodetic datum WGS84). Identification of the botanical material was carried out at the Herbarium (Department of Biology) at the State University of Maringá. A voucher has been registered under code 29012.

Latex was obtained through superficial incisions in the stem of *T. catharinensis* (A. DC), collected in an equal volume of water. Samples were centrifuged (5,000*g*) at 10 °C for 25 min. The precipitate was discarded, and the resulting supernatant was lyophilised and maintained at –20 °C until assays were performed. The plot comprising almost all compounds of water-soluble latex was named latex of *T. catharinensis* (A. DC) (Mousinho et al., 2011; da Silva Menecucci et al., 2019).

### *2.3 Chemical characterisation of the crude latex of Tabernaemontana catharinensis*

The biochemical composition of the crude latex of *T. catharinensis* presented 52.5% protein, 21.2% free amino acids, and potent proteolytic activity, as described by da Silva Menecucci et al. (2019). The analysis also revealed that crude latex contains 26.3% of total sugars.

Latex was also chemically analysed to determine the presence of bioactive secondary metabolites (da Silva Menecucci et al., 2019), which demonstrated that *T. catharinensis* latex is also a renewable non-wood source of biologically active monoterpenoid indole alkaloids.

### *2.4 Determination of minimum inhibitory concentration and minimum bactericidal concentration*

The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) for crude latex were determined by the 96-well plate microdilution technique (TPP Techno Plastic Products AG, Trasadingen, Switzerland), according to the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines, using BAT medium for culture (Deinhard et al., 1987). The crude latex was serially diluted in the wells, obtaining concentrations ranging from 0.49 to 1000 µg/ml. Next, 5 µl of vegetative cells of the microorganism previously standardised to 0.5 McFarland ($10^8$ CFU/ml) was added and re-diluted 1:10 (v/v) to obtain the standard concentration used ($10^4$ CFU/ml). Each well contained 100 µl of the culture. The assays were performed individually for each species. The 96-well plate was incubated at 45 °C for 24 h, and then the turbidity of the well was evaluated visually. The MIC was considered the lowest concentration resulting in inhibition of growth, as visually assessed. The MBC was determined by sub-culturing 20 µl of each negative well to the surface of a BAT agar plate that was incubated at 45 °C for 24 h. The assays were performed in triplicate, according to the methodology described by (Anjos et al. 2016).

### *2.5 Cytotoxicity analysis*

The cytotoxic activity of *T. catharinensis* latex crude (A. DC) was evaluated by the colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described by Mosmann (1983). Accordingly, 96-well plates were prepared with a monolayer of Vero cells and incubated until confluence. For the cytotoxicity assays, serial dilutions (31.25, 62.50, 125, 250, 500 and 1000 µg/ml) of the crude latex were prepared, and 200 µl/well of each concentration was added, followed by incubation of the plates at 37 °C and 5% $CO_2$ for 72 h. After incubation, the old medium was removed, the wells were washed with 100 µl of phosphate-buffered saline (PBS), and then 50 µl/well of MTT (2 mg/ml) was added, and the plates incubated in an oven at 37 °C for 4 h under light. The reagent was then removed, 150 µl/well of dimethyl sulfoxide was added, and the reading was carried out at 570 nm. The cytotoxicity was determined according to Eq. (1): Cellular destruction (%) = 1 - ($OD_t/OD_{cc}$) where $OD_t$ is the optical density of the treated sample and $OD_{cc}$ is the optical density of the control cells. The selectivity index (SI) was obtained through the formula: SI = $LC_{50}$/MIC (Makhafola et al., 2014) where $LC_{50}$ denotes the lethal concentration at which 50% of the cells are killed. The experiment was performed in triplicate, and the result expressed

as the average of three independent experiments. Positive and negative controls of the test were analysed simultaneously as the samples.

### *2.6 Scanning electron microscopy*

*Alicyclobacillus acidoterrestris* 0244$^T$ cells were treated with the crude latex of *T. catharinensis* (A. DC) at concentrations of 1 x MIC, 4 x MIC and 8 x MIC in orange juice reconstituted at 11°Brix and pH 4.0. The microtubes were incubated at 45 °C for 24 h. Afterwards, the cells were washed with PBS (pH 7.2) and fixed with 2.5% glutaraldehyde (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 h at room temperature. Once removed from the fixative solution and washed twice with 0.1 M cacodylate buffer, the cells were transferred to a poly-L-lysine-coated coverslip for 1 h. The cells were then washed three times with a cacodylate buffer and dehydrated using increasing concentrations of ethanol (50, 70, 80, 90 and 100%), followed by critical point drying in $CO_2$. The specimens were covered with gold for observation under a Quanta 250 scanning electron microscope (FEI Company, Hillsboro, OR, USA) (Haddad et al., 2007). All the assays were performed in triplicate.

## 3. Results and Discussion

### *3.1 Minimum inhibitory concentration and minimum bactericidal concentration*

The MIC and MBC of the crude latex are shown in Table 1. For all strains examined, the MIC was 7.81 µg/ml, and the most effective MBC result of 250 µg/ml occurred for strains *A. acidoterrestris* 0244$^T$, *A. hesperidum* 0298$^T$, *A. acidophilus* 0247$^T$ and *A. cycloheptanicus* 0297$^T$. For the strain *A. acidocaldarius* 0299$^T$, the MBC was 500 µg/ml.

**Table 1 -** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Tabernaemontana catharinensis* (A. DC) crude latex in *Alicyclobacillus* spp.

| Species | MIC (µg/ml) | MBC (µg/ml) |
|---|---|---|
| *A. acidoterrestris* 0244$^T$ | 7.81 | 250 |
| *A. hesperidum* 0298$^T$ | 7.81 | 250 |
| *A. acidophilus* 0247$^T$ | 7.81 | 250 |
| *A. cycloheptanicus* 0297$^T$ | 7.81 | 250 |
| *A. acidocaldarius* 0299 $^T$ | 7.81 | 500 |

Sourse: Authors (2021).

According to the study by Holetz et al. (2002), which evaluated the antimicrobial activity of several plants against various bacteria, a MIC below 100 µg/ml is classified as good antimicrobial activity, 100–500 µg/ml is described as weak activity, and a MIC above 1000 µg/ml is considered inactive. In this context, the MIC result of <100 µg/ml obtained in the current study, demonstrated that the crude latex of *T. catharinensis* could be considered to exert good activity against the *Alicyclobacillus* species evaluated.

Gindri et al. (2011) showed the *n*-butanol fraction of the leaves of *T. catharinensis* (A DC) exerted a positive antibacterial activity against *Micrococcus* (MIC 31.25 µg/ml), *Aeromonas* sp. (MIC 250 µg/ml), *Proteus mirabilis* and *Enterococcus faecalis* (MIC 62.50 µg/ml). The authors suggested that some components present in this extract may act as antibacterial agents through mechanisms of inhibition of nucleic acid synthesis, bacterial membrane rupture and/or inhibition

of energy metabolism. In the present study, the crude latex was used, and therefore, it is likely to contain the same compounds plus others not extracted by *n*-butanol.

When investigating the antibacterial activity of *Piperaceae* extracts and nisin against *A. acidoterrestris* 0244$^T$, Ruiz et al. (2013) reported *Piper aduncum* as having the lowest MIC of 15.6 μg/ml although nisin also exhibited strong antibacterial activity. Nisin is an antibacterial polypeptide produced by *Lactococcus lactis* sp., used as a food preservative, which has a high production cost. Therefore, when compared with the current results, the crude latex of *T. catharinensis* shows a great potential application in the inhibition of *Alicyclobacillus* spp.

Previously, dos Anjos et al. (2016) suggested the antimicrobial activity of papain and bromelain against *Alicyclobacillus* spp. could be associated with the high proteolytic capacity of these enzymes. According to da Silva Menecucci et al. (2019), the crude latex of *T. catharinensis* has a strong proteolytic activity, and this might explain its good antibacterial action against *Alicyclobacillus* spp.

### *3.2 Cytotoxicity*

The *in vitro* cytotoxic effect of the latex of *T. catharinensis* (A. DC) was evaluated by the MTT assay in Vero cells. The $LC_{50}$ value found for the crude latex was 84.67 ± 8.14 μg/ml. At this concentration, the crude latex was able to reduce 50% of the viable Vero cells.

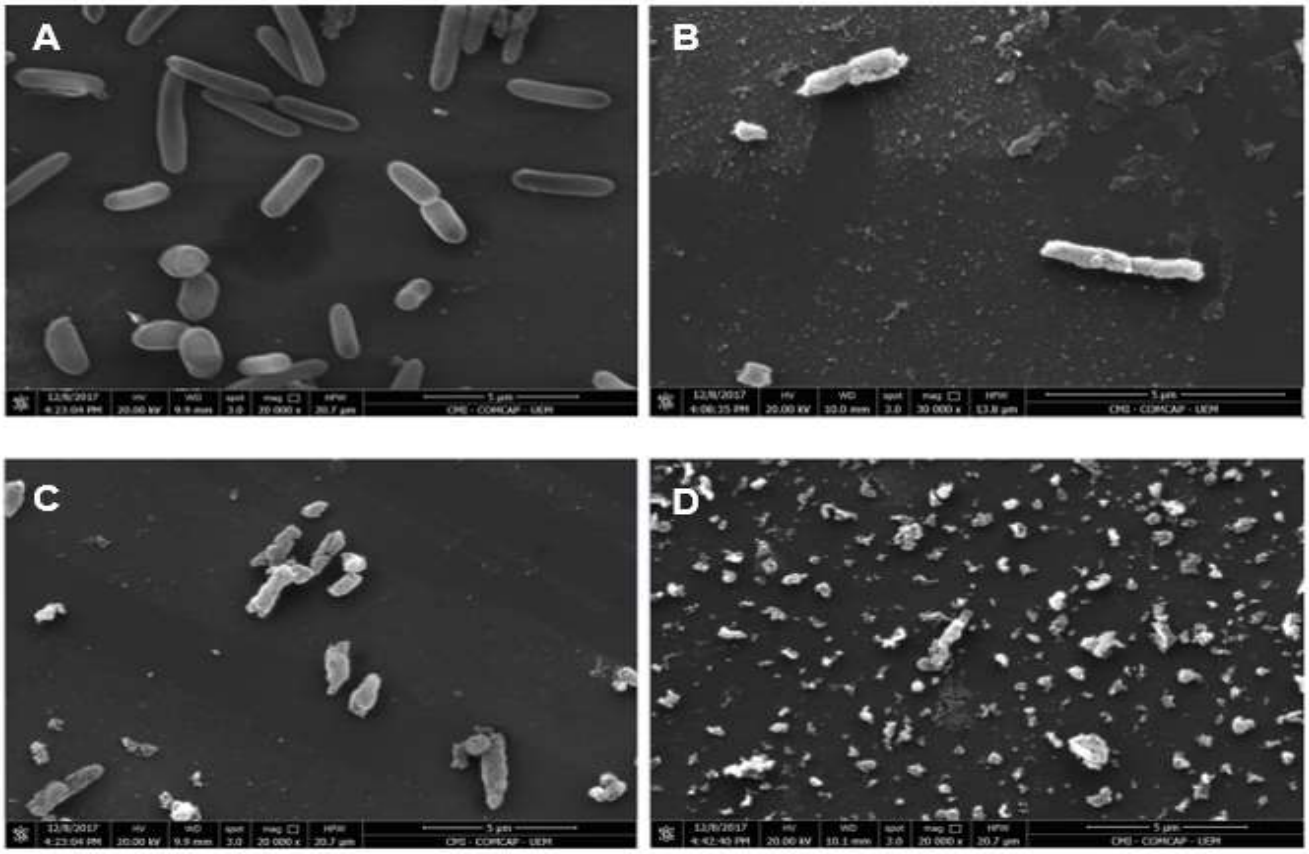
The SI indicates the relative safety of the plant extract according to its toxicity–activity, which presented a result equivalent to 10.84. High SI values (>1.0) indicate a large safety margin between the crude latex concentration capable of eliminating the bacterium and the toxic concentration for mammalian cells (Makhafola et al., 2014). SI values lower than 1.0 are considered to be less selective for microorganisms, indicating that the sample tested is more toxic (Santos et al., 2008).

An investigation into the cytotoxic activity of the crude extract of leaves and stem bark of *T. catharinensis* (A. DC) conducted by Boligon et al. (2015), determined $LC_{50}$ values of 59.53 ± 4.56 and 62.82 ± 4.75 μg/ml, respectively. In comparison, the crude latex of the present study presented a higher $LC_{50}$, allowing the use up to nine times the MIC, remaining within the limits of cytotoxicity.

### *3.3 Scanning electron microscopy*

Morphological changes were visualised by scanning electron microscopy of *A. acidoterrestris* 0244$^T$ vegetative cells treated with the crude latex of *T. catharinensis* (A. DC) in reconstituted orange juice. It was observed that the untreated cells of *A. acidoterrestris* 0244$^T$ in orange juice presented an intact cell wall, and a smooth and rod-like membrane, characteristic of this bacterium, with no visible changes (Figure 1A). In contrast, when the cells were treated with the crude latex of *T. catharinensis* at concentrations of 1 x MIC, 4 x MIC and 8 x MIC, as shown in Fig. 1B, C and D, respectively, significant morphological changes were apparent, with damage to the cell wall and the membrane. These modifications exacerbated as the concentration of the crude latex in contact with the bacteria was increased, demonstrating that the combination of latex with this microorganism greatly affects the biological structure of *A. acidoterrestris* 0244$^T$.

**Figure 1** - *Alicyclobacillus acidoterrestris* 0244$^{T}$ scanning electron microscopy treated with *Tabernaemontana catharinensis* crude latex (A. DC) minimum inhibitory concentration (MIC): (a) untreated cells, (b) treated cells 1 x MIC, (c) treated cells with 4 x MIC, and (d) treated cells with 8 x MIC.




Sourse: Authors (2021).

Similar characteristics regarding the morphological alterations were reported by Molva and Baysal (2015) when examining *Alicyclobacillus acidoterrestris* bacterial and spore cells in apple juice treated with pomegranate fruit extract and grape seed, Likewise, de Pascoli et al. (2018) noted morphological changes to *A. acidoterrestris* 0244$^{T}$ cells exposed to *Piperaceae* extracts that also corroborated our observations.

## 4. Conclusion

The results of the present study showed that *T. catharinensis* latex displays good antimicrobial activity against *A. acidoterrestris* bacteria. The images captured by scanning electron microscopy allowed the visualisation of the morphological changes caused to the cells by the crude latex. Therefore, the crude latex of *T. catharinensis* is a promising alternative for the concentrated orange juice industry. The cytotoxicity test presented satisfactory results, allowing the use of crude latex at up to nine times the MIC without reaching the limits of cytotoxicity, although further studies of *in vivo* toxicity for the application of this compound in food are still necessary. This study is the first to evaluate the antimicrobial activity of *T. catharinensis* against *Alicyclobacillus* spp. and the use of this compound as a food preservative is a sustainable alternative.

The proteolytic enzymes isolated from the latex of *Tabernaemontana catharinensis* are of great interest mainly because they are non-wood products that avoid destructive harvesting of plant sources. The appropriate processing of the crude latex and isolating of protein and/or peptides is very important to identify the major component responsible for antibacterial activity. We suggest as future studies the verification of antimicrobial activity of latex in other food matrices.

**Acknowledgments**

The authors are grateful to the Coordination of Improvement of Higher Education Personnel (CAPES), Post-graduate Program in Food Science of the State University of Maringá, Brazil; the Complex of Research Support Centres of the State University of Maringá (COMCAP- EMU).

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