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## Evaluation of the antimicrobial activity of pecan nut [*Carya illinoensis* (Wangenh) C. Koch] shell aqueous extract on minimally processed lettuce leaves

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### Abstract

Pecan nutshell is a residue from food industry that has potential to be used as biopreservative in foods. The objective of this study was to evaluate the antimicrobial activity of pecan nutshell aqueous extract *in vitro* and its effectiveness to inhibit spoilage microorganisms on lettuce leaves. The results indicate that the aqueous extract presents inhibitory activity against important foodborne pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Bacillus cereus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. Antimicrobial activity was not observed against *Corynebacterium fimi*, *Clostridium perfringens*, *Escherichia coli*, and the phytopathogenic fungi tested. When applied onto lettuce leaves, pecan nutshell extract reduced the counts of mesophilic and psychrotrophic bacteria in 2 and 4 log CFU/g, respectively, during storage of leafy for 5 days at refrigeration temperature (5 °C). The extract was not effective to inhibit yeast on lettuce leaves. Thus, the aqueous extract of pecan shell showed great potential to be used as a natural preservative in foods, acting mainly in the inhibition of spoilage and pathogenic bacteria.

**Keywords:** pecan nutshell; antimicrobial activity; lettuce.

**Practical Application:** Pecan nutshell aqueous extract has antibacterial activity against pathogenic and spoilage bacteria, being affective to inhibit bacterial growth onto lettuce leaves. Thus, pecan nutshell presents great potential to be used as a natural preservative in foods.

## 1 Introduction

The increased demand for minimally processed foods, with extended shelf life and without addition of chemical preservatives has led industries and research groups to search and develop natural alternatives. Agro-industrial byproduct extracts have shown up as trend because they may represent a natural source of bioactive and biopreservatives compounds (Ayala-Zavala et al., 2011; Siqueira et al., 2013; Gyawali & Ibrahim, 2014; Andrade et al., 2015), representing a way of industrial waste management, also potentially reflecting on final product costs, due to their potential as low-cost source (Silveira et al., 2013).

Pecan nut [*Carya illinoensis* (Wangenh) C. Koch], widely produced in southern Brazil, presents approximately 45% of its mass as shell, which is an industrial processing residue. Pecan nutshell is a phenolic rich agro-industrial byproduct, which polyphenolic profile includes high concentration of gallic acid, chlorogenic acid, *p*-hydroxybenzoic acid, epigallocatechin and epicatechin gallate (Prado et al., 2014). Due to the elevated concentration of polyphenols, the consumption of its infusion is associated with antioxidant activity, decreasing liver damage, reduction of total cholesterol and action as natural anxiolytic (Malik et al., 2009; Prado et al., 2014; Reckziegel et al., 2011; Müller et al., 2013). Additionally to the benefits to human health, Prado et al. (2014) found that pecan nutshell infusion presents antibacterial activity against Gram-positive bacteria

such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus*. On the other hand, authors did not observed inhibition when pecan nutshell infusion was tested against Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and fungi such as *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp. *Rhizopus* sp., *Penicillium roquefortii* (Prado et al., 2014).

In addition to *in vitro* antimicrobial analysis, it is essential to evaluate the effectiveness of natural agents on real foods. Minimally processed fruits and vegetables are suitable to spoilage and pathogenic bacteria, indicating the need to improve the sanitary conditions along the food chain (Santos et al., 2010; Maistro et al., 2012; Jamali et al., 2013). Since microorganisms may present different sensibility to preservatives, there is still much to be explored and it is essential to maintain efforts to study plant matrixes as source of natural preservatives. In this sense, the evaluation of the antimicrobial activity of aqueous extract of pecan nutshell against a broad range of pathogenic bacteria and fungi and test its effectiveness to inhibit spoilage microorganisms when applied on foods is an important step to suggest their use in large scale. Thus, the objective of the present study was to evaluate the antimicrobial activity of an aqueous extract of pecan shell and its capability to inhibit spoilage fungi and bacteria when applied onto lettuce leaves.

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## 2 Materials and methods

### 2.1 Plant material and obtainment of the aqueous extract

Milled pecan nutshell, from nuts harvest in 2014, was kindly supplied by Agroindústria Pitol (Anta Gorda, RS, Brazil). The material was sieved to 5 mm particle size and stored at  $-18^{\circ}\text{C}$ , protected from light, until use. Aqueous extract was prepared by maintaining 10 g of pecan nutshell with 1 L of boiling distilled water for 30 min, and then filtered through Whatman filter paper nº1.

### 2.2 Antimicrobial activity

Evaluation of antifungal activity was conducted as described by Venturoso et al. (2011). Sporulated cultures of *Bipolaris oryzae*, *Bipolaris curvispora*, *Bipolaris cynodontis*, *Colletotrichum gloeosporioides*, *Aspergillus phoenicis*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium graminearum*, *Monascus purpureus* were transferred into sodium chloride solution 0.85% (w/v), standardized at  $10^4$  spores per mL and added to semisolid Potato Dextrose Agar (PDA). Aliquots of 20  $\mu\text{L}$  of the pecan nutshell extract were added onto the PDA and plates were incubated at  $30^{\circ}\text{C}$  for up to 7 days for evaluation of inhibition zones around the extract drop.

Antibacterial activity was evaluated as described by Kimura et al. (1998). Suspensions of  $10^7$  CFU  $\text{mL}^{-1}$  of *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC14579, *Clostridium perfringens* ATCC3624, *Listeria monocytogenes* ATCC7644, *Listeria innocua*, *Pseudomonas aeruginosa* ATCC15442, *Salmonella* Enteritidis ATCC13076, *Escherichia coli* ATCC25922, *Aeromonas hydrophila* ATCC7966, *Corynebacterium fimi* NCTC7547 were spread onto Plate Count Agar (PCA) with a sterile swab and aliquots of 20  $\mu\text{L}$  of the extract were applied. Plates were incubated at  $37^{\circ}\text{C}$  for 24 h, when the presence of inhibition halos were verified.

### 2.3 Antimicrobial activity on lettuce leaves

To evaluate the antimicrobial activity in minimally processed food, lettuce leaves of 25  $\text{cm}^2$  were aseptically prepared, added of pecan nutshell extract at a ratio of 1 mL per gram of leave and left to dry for 30 min in laminar flow chamber. Samples were stored in sterile polyethylene bags and left for 5 days at  $5^{\circ}\text{C}$ . Microbiological analysis was performed at pre-determined times by homogenization of 10 g samples with 90 mL of 0.1% (w/v) peptone water and further decimal dilutions. Viable spoilage microorganism cells were determined as described by Motta & Brandelli (2002), incubating mesophilic bacteria on Plate Count Agar (PCA) at  $37^{\circ}\text{C}$  for 24 h and psychrotrophic bacteria on PCA at  $7^{\circ}\text{C}$  for up to 10 days. Yeasts were evaluated in PDA by incubation at  $30^{\circ}\text{C}$  for 5 days. Results were expressed as Colony Forming Unit per gram of lettuce leaves (CFU  $\text{g}^{-1}$ ).

### 2.4 Statistical analysis

All experiments were conducted in triplicate and averages of two independent tests were calculated. Values were compared by Tukey's test using the software Statistica 10.0 (StatSoft, Tulsa, OK, USA), and differences were considered statistically significant when  $p < 0.05$ . Graphical plots were performed using Microsoft Excel 2000 (MapInfo Corporation, Troy, NY, USA).

## 3 Results and discussion

The results for the evaluation of antibacterial activity of pecan nutshell aqueous extract are shown in Table 1. The extract showed inhibition against *S. aureus*, *B. cereus*, *L. monocytogenes* and *L. innocua*, although no antimicrobial activity was not observed against *C. fimi* and *C. perfringens*. Similar results were observed by Prado et al. (2014), which verified antimicrobial activity of pecan nutshell infusion against *L. monocytogenes*, *S. aureus*, *B. cereus* and *V. parahaemolyticus*, important Gram-positive foodborne pathogenic bacteria. However, those authors did not observe antimicrobial activity against the Gram-negative bacteria tested: *P. aeruginosa*, *S. enterica* and *E. coli* (Prado et al., 2014). In the present work, aqueous extract was effective to inhibit Gram-negative bacteria such as *S. Enteritidis*, *A. hydrophila* and *P. aeruginosa*, although no inhibition was observed against *E. coli*. Variations in the spectrum of antimicrobial activity may be related to the origin of pecan nutshell and extraction time, which could imply in different composition of the extracts.

Plant extracts possess higher antimicrobial activity compared to pure polyphenols (Puupponen-Pimiä et al., 2001; Prado et al., 2014), suggesting that extracts may exert their action on bacteria due to a combination of different mechanisms. It has been reported that pure polyphenols present antimicrobial activity against Gram-positive and Gram-negative bacteria (Puupponen-Pimiä et al., 2001; Serrano et al., 2009; Prado et al., 2014). Their mechanisms of action are related to oxidation of microbial cell membranes, complexation of essential metal ions, or inhibition of extracellular enzymes (Scalbert, 1991; Serrano et al., 2009). Lou et al. (2012) reported that *p*-coumaric acid acts on altering the permeability of the cell membrane of Gram-negative bacteria. Borges et al. (2013) observed that gallic and ferulic acids are electrophilic products and seem to significantly interact with the bacterial surface component, destabilizing cell cytoplasmic membrane of pathogenic bacteria such as *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. aureus*. Additionally, antibacterial activity of *p*-coumaric acid is related to the polyphenol capacity to bind DNA inhibiting cell function.

**Table 1.** Antibacterial activity of pecan nutshell aqueous extract against Gram-positive and Gram-negative bacteria.

Indicator microorganism	Inhibitory activity*
<b>Gram-positive</b>	
<i>Staphylococcus aureus</i>	+
<i>Corynebacterium fimi</i>	-
<i>Bacillus cereus</i>	++
<i>Listeria monocytogenes</i>	++
<i>Listeria innocua</i>	++
<i>Clostridium perfringens</i>	-
<b>Gram-negative</b>	
<i>Escherichia coli</i>	-
<i>Salmonella</i> Enteritidis	+
<i>Pseudomonas aeruginosa</i>	+
<i>Aeromonas hydrophila</i>	++

\*Average diameter of inhibition zones: +, 7-10 mm; ++, 11-15 mm; -, no inhibition zone.

The analysis of pecan nutshell extract against phytopathogenic fungi showed no capability to inhibit *B. oryzae*, *B. curvispora*, *B. cynodontis*, *C. gloeosporioides*, *A. phoenicis*, *A. flavus*, *F. oxysporum*, *F. graminearum* or *M. purpureus*. Prado et al. (2014) also verified that pecan nutshell infusion was not effective to inhibit *A. niger*, *A. flavus*, *P. roquefortii*, *Rhizopus* sp. and *Fusarium* sp. The absence of inhibitory activity against fungi may be related to a low concentration of compounds with antifungal activity due to the extraction methodology. Additionally, some fungi such as *A. niger* and *Penicillium* sp. can grow in the presence of tannins, using these compounds as a carbon source or due to production of polyphenol hydrolyzing enzymes (Scalbert, 1991; Serrano et al., 2009).

Figure 1 shows the results for antimicrobial activity of pecan nutshell extract against yeast, mesophilic and psychrotrophic bacteria in lettuce leaves stored for 5 days at refrigeration temperature. The control experiment showed that the concentration of mesophilic bacteria (Figure 1A) in lettuce leaves remained constant over the 5 days of storage with viable counts around 6 log CFU/g ( $p > 0.05$ ). When the pecan extract was used, mesophilic bacterial counts were significantly reduced ( $p < 0.05$ ) to 3.5 log CFU/g when samples were stored for 5 days. The analysis of psychrotrophic bacteria (Figure 1B) in the control experiment indicates that the microbial counts increased significantly ( $p < 0.05$ ) during the storage of the lettuce leaves from 4.9 log CFU/g on day zero to 6.3 log CFU/g on the fifth day of storage. The application of the pecan nutshell extract on the samples implied a significant reduction ( $p < 0.05$ ) of psychrotrophic bacteria counts to 2.1 log CFU/g on the third day of storage, which remained constant up to the fifth day of storage ( $p > 0.05$ ). Similar to results observed on *in vitro* antifungal tests, pecan nutshell extract did not inhibit significantly ( $p > 0.05$ ) the yeast growth on lettuce leaves during the storage time (Figure 1C).

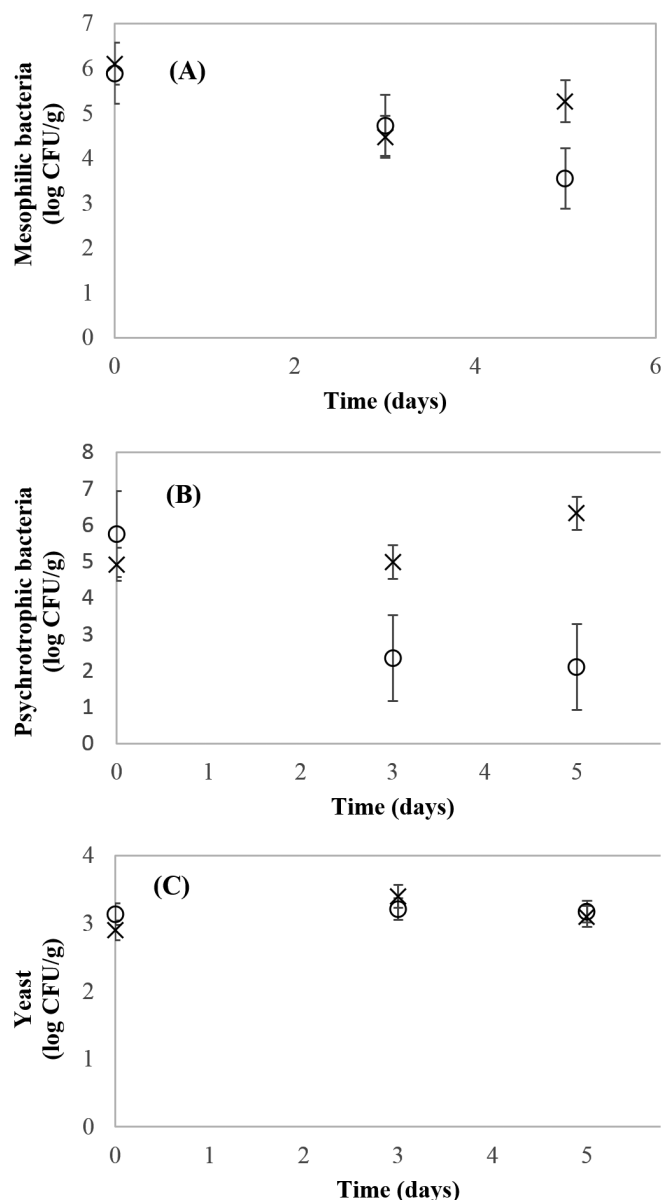
The results of the present work indicate that pecan nutshell extract may support an increased shelf life to lettuce leaves, since mesophilic and psychrotrophic bacteria are directly related to spoilage of foods.

#### 4 Conclusion

The results of the present study show that pecan nutshell extract has antibacterial activity against important pathogenic bacteria, but does not show inhibitory activity against the tested fungi. When applied onto lettuce leaves, the extract was effective in reducing the counts of mesophilic and psychrotrophic bacteria over 5 days of storage of the product at refrigeration temperature. Thus, the results indicate that pecan nutshell extract offers an interesting potential to be used as a natural antimicrobial for food applications.

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**Figure 1.** Mesophilic (A), psychrotrophic (B) and yeast (C) counts on lettuce leaves treated with water (x) or pecan nutshell aqueous extract (o) during 5 days of storage at refrigeration temperature.

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