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Antibacterial activity of *Schinopsis brasiliensis* against phytopathogens of agricultural interest

Atividade antibacteriana de *Schinopsis brasiliensis* contra fitopatógenos de interesse na agricultura

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Resumo

O objetivo deste trabalho foi avaliar a atividade antimicrobiana *in vitro* da planta *Schinopsis brasiliensis* contra alguns fitopatógenos de interesse na agricultura. Extratos etanólicos foram obtidos e ensaiados contra o fungo *Fusarium solani* e bactérias do gênero *Xanthomonas*, *Pectobacterium*, *Ralstonia* e *Acidovorax*. Os resultados mostraram, em geral, um bom desempenho destes extratos contra as bactérias ensaiadas, com valores de concentração inibitória mínima entre 312,5 e 10.000µg.mL^{-1}. Além disso, um triterpeno (glutinol) e um pirocatecol são relatados como constituintes químicos desta espécie. Conclui-se que *S. brasiliensis* apresenta potencial para ser usado no controle de doenças de plantas causadas pelas bactérias pertencentes aos gêneros *Xanthomonas*, *Ralstonia* e *Acidovorax*.

Palavras-chave: fitopatógenos; *Schinopsis brasiliensis*; glutinol; pirocatecol; *Artemia salina*; agricultura.

Abstract

The aim of this work was to evaluate the *in vitro* antimicrobial activity of *Schinopsis brasiliensis* against certain phytopathogens of agricultural interest. Ethanolic crude extracts were obtained and assayed against *Fusarium solani* and bacteria pertaining to the genera *Xanthomonas*, *Pectobacterium*, *Ralstonia* and *Acidovorax* using the broth micro-dilution technique. The results demonstrated a good aptitude of *S. brasiliensis* extracts to inhibit growth of the majority of the evaluated phytopathogenic bacteria, with MIC values in the range from 312.5 to 10,000µg.mL^{-1}. Moreover, triterpene glutinol and a pyrocatechol are shown to be the chemical constituents of *S. brasiliensis*. The results suggest that *S. brasiliensis* has the potential for agricultural use to control plant diseases

caused by phytopathogenic bacteria belonging to the genera *Xanthomonas*, *Ralstonia* and *Acidovorax*.

1. Introdução

Recent estimates indicate that the world's population is expected to expand from the current 6.7 billion to 9 billion by 2050 (Ronald, 2011). Global food production is not increasing fast enough to support this rapidly growing population; therefore, it is believed that world agricultural production needs to increase 50% by 2030 to accommodate this higher demand for food (Ronald, 2011; Davies et al., 2009). Diseases of crop plants are among the most important constraints in the production of adequate quantities of food, committing up to a third of the world production (Khan, 2008; Agrios, 2005). Therefore, a reduction in the losses of crops to pests and phytopathogens is understood to be equivalent to creating more land and more water to increase real agricultural production (Ronald, 2011).

Pesticides are widely used to control plant diseases around the world (Khamna, Yokota and Lumyong, 2009). Although the use of such products has a positive short-term effect for the producer, their long-term employment has many negative effects for society and the environment, such as the pollution of soil and water, deposition of agrochemical residues on food and the emergence of resistant pathogens, among others (Khamna, Yokota and Lumyong, 2009; Stangarlin et al., 1999; Kimati et al., 1997).

To reduce the negative effects of pesticides, natural alternatives are being searched for the control of phytopathogens. Within this context, there is an intense and increasing current search for new antimicrobial agents from plants as a result of the growing resistance of pathogenic microorganisms to synthetic products (Amaral and Bara, 2005).

According to Di Piero, Novaes and Pascholati (2010) one valid alternative is to exploit the biological activity of secondary metabolites available in crude plant extracts and essential oils from medicinal plants of native flora. In this connection, some examples can be found in the literature, from which we select three ones: 1. *In vitro* control of the fungi *Colletotrichum gloeosporioides*, *C. musae* and *Fusarium subglutinans* f. sp. *ananas* by the essential oils of *Cymbopogon nardus, C. citratus* and *Eucalyptus citriodora* (Alves et al., 2003); 2. Control of anthracnose in guava fruit (*Psidium guajava* L.) by the aqueous extract and essential oil of cloves (*Syzygium aromaticum*) (Rozwalka et al., 2008); 3. Control of common bacterial blight in bean plants by bushy matgrass (*Lippia Alba*) and pepper-rosmarin (*Lippia sidoides*) tinctures and the essential oils of rosemary (*Rosmarinus officinalis*) and cinnamon (*Cinnamomum zeylanicum*) (Vigo et al., 2009).

Schinopsis brasiliensis Engl. (Anacardiaceae) is the most important species representing the genus *Schinopsis* native to Brazil (Saraiva et al., 2011; Albuquerque et al., 2007). An endemic tree popularly known as "braúna" or "baraúna" (Albuquerque et al., 2007; Cardoso, David and David, 2005), it is typical of the Caatinga and of high economic value to the Northeast region (Albuquerque et al., 2007). The antibacterial activity of baraúna extracts against resistant strains of *Staphylococcus aureus* has been reported (Saraiva et al., 2011). Furthermore, few phytochemical studies have been conducted with this

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plant, of which we highlight the isolation of a few alkyl and alkenyl phenols, among other components (Cardoso, David and David, 2005).

Schinopsis brasiliensis extracts have shown antibacterial action against microorganisms pathogenic to human beings and are considered to be a plant species with a potential use in phytopathogen control. As no research until this moment has described the antimicrobial effect of this plant against phytopathogenic microorganisms, the purpose of this work was to assess the antimicrobial activity of *Schinopsis brasiliensis* extracts against some phytopathogenic agents with important economic value for agriculture. Therefore, the *S. brasiliensis* extracts were tested against the fungus *Fusarium solani*, a causative agent of root rot of potatoes and beans; as well as against the phytopathogenic *Xanthomonas* bacteria: *Xanthomonas campestris* PV (pv). *Campestris* and *Xanthomonas campestris* pv. v*iticola,* which causes black rot in crucifers and bacterial canker in grapevines, respectively. In addition, the phytobacteria *Pectobacterium carotovorum* subsp*. carotovorum*, which causes soft rot in greenery; *Ralstonia solanacearum*, which causes bacterial wilt in banana trees and green peppers; and *Acidovorax avenae* subsp. *citrulli*, responsible for bacterial spot in melons were also assayed. At last, the ecotoxity potential of *S. brasiliensis* extracts was assessed by means of brine shrimp lethality (BSL) bioassay against *Artemia salina*.

2. Material and Methods

2.1. Plant material collection and extract preparation

The leaves, bark and secondary roots of *Schinopsis brasiliensis* were collected at the Caatinga Grande ranch, municipal area of Malhada de Pedras, southwest region of Bahia, during the month of June,

2011. An exsiccate was deposited at the UESB herbarium, in Vitória da Conquista city, Bahia state, Brazil, under the code HUESBVC 4124. The different parts were dried separately in a forced air circulation oven at 50ºC for 48 hours. The dried plant material – leaves, bark and secondary roots – was milled and exhaustively extracted with 96% ethanol by maceration to give, after concentration in a rotary evaporator, the corresponding ethanolic extracts of the leaves (EEL, 12.90g), stems (EES, 11.26g) and roots (EER, 5.58g).

2.2. Isolation of compounds 1 and 2 from EEL

A part of the crude ethanolic leaf extract of *Schinopsis brasiliensis* (EEL, 9.0g) was chromatographed on a silica gel column and eluted with increasingly polar solvent systems, resulting in eleven fractions of 500mL each: two from hexane (Frs. 1-2), four from dichloromethane (Frs. 3-6), four from ethyl acetate (Frs. 7-10) and one from methanol (Fr. 11). Fraction 3 (294mg) was rechromatographed on the silica gel column and eluted with increasingly polar mixtures of hexane and ethyl acetate, resulting in forty-six sub-fractions of 10mL each. Sub-fraction 11, eluted with hexane-ethyl acetate (8:2), afforded substance **1** (10mg), which was pure under TLC and spectroscopic analysis. This substance was identified as triterpene glutinol on the basis of the IR, ^{1}H and ^{13}C NMR data that were identical to those described in the literature (Tuan, Hung and Chien, 2005).

The initial fractionation of EEL was repeated to obtain a larger quantity of the dichlorometanic fractions, which were grouped (1.0g) and submitted again to column chromatography on silica gel, employing increasingly polar mixtures of hexane and ethyl acetate as eluents to afford seventy-seven fractions 30mL each. Fraction 54, eluted with hexane-ethyl acetate (7:3), provided compound **2** (44mg), which was identified as a pyrocatechol whose IR and NMR data were very similar to those reported for *Z,Z*-4-

(heptadeca-8,11-diennyl)-benzene-1,2-diol (Sargent, Wangchareontrakul and Jefferson, 1989).

Glutinol (1). IR, v_{max} (cm^{-1}): 3445, 3391, 2930, 2850, 1454, 1361, 1180. ^{1}H NMR (CDCl$_3$, 500 MHz, δ): 5.63 (*m*, 1H), 3.47 (*s*, 1H), 1.16 (*s*, 3H), 1.14 (*s*, 3H), 1.04 (*s*, 3H), 1.09 (*s*, 3H), 1.00 (*s*, 3H), 0.99 (*s*, 3H), 0.95 (*s*, 3H), 0.85. (*s*, 3H). ^{13}C NMR (CDCl$_3$, 125 MHz, δ): 18.2 (C-1), 27.8 (C-2), 76.3 (C-3), 40.8 (C-4), 141.6 (C-5), 122.0 (C-6), 23.6 (C-7), 47.4 (C-8), 34.8 (C-9), 49.7 (C-10), 34.6 (C-11), 30.3 (C-12), 39.3 (C-13), 37.8 (C-14), 32.1 (C-15), 36.0 (C-16), 30.1 (C-17), 43.1 (C-18), 35.1 (C-19), 28.2 (C-20), 33.1 (C-21), 38.9 (C-22), 28.9 (C-23), 25.4 (C-24), 16.2 (C-25), 19.6 (C-26), 18.4 (C-27), 32.0 (C-28), 34.5 (C-29), 32.3 (C-30).

Pyrocathecol 2. IR, v_{max} (cm^{-1}): 3375, 3008, 2924, 2854, 1604, 1518, 1462, 1373, 1280, 1192, 1114, 956, 864, 786, 721. ^{1}H NMR (CDCl$_3$, 500 MHz, δ): 6.76 (*d, J*=8.0, 1H), 6.69 (*t, J*= 2.0, 1H), 6.59 (*dd, J* =2.0, 8.0; 1H), 5.35-5.14 (*m*, 4H), 2.77 (*m,* 2H), 2.48 (*t, J*=8.0, 2H), 2.06-2.01 (*m,* 4H), 1.55 (*m,* 2H), 0.89 (*t, J*=7.0, 3H). ^{13}C NMR (CDCl$_3$, 125 MHz, δ): 141.31 (C-1'), 143.43 (C-2'), 115.44 (C-3'), 136.10 (C-4'), 120.61 (C-5'), 115.16 (C-6'), 35.23 (C-1), 31.88 (C-2), 29.68 (CH$_2$), 29.40 (CH$_2$), 29.30 (CH$_2$), 27.20 (CH$_2$), 130.11 (CH), 127.92 (CH), 25.62 (CH$_2$), 128.00 (CH), 130.20 (CH), 29.23 (CH$_2$), 31.57 (CH$_2$), 22.66 (CH$_2$), 14.09 (CH$_3$).

2.3. Antifungal assay

For the antifungal evaluation of the extracts, the fungus *Fusarium solani* isolated from passion fruit trees and kept at the UESB Phytopathology Laboratory at the Vitória da Conquista campus was used. *Fusarium solani* colonies were grown on 90-mm Petri dishes containing potato-dextrose-agar (PDA) culture medium (Merck). The dishes were placed in Biochemical Oxygen Demand (BOD)

incubation at 25ºC for seven days to allow the fungus to colonize the entire diameter of the dish.

Extracts EEL, EES and EER (200mg each) were separately diluted in acetone (5mL) and topped with off distilled, autoclaved water (20mL), resulting in solutions each with a 10mg.mL^{-1} concentration. To arrive at the desired concentrations of 0.001; 0.005; 0.01; 0.02; 0.05; 0.1; 0.25; 0.5 and 1mg.mL^{-1}, the solutions obtained from the extracts in PDA were proportionally diluted and placed on Petri dishes. For the negative control without extracts (0mg.mL^{-1}), only PDA medium, distilled and autoclaved water (20mL) and acetone (5mL) were used.

Following solidification of the culture medium on the Petri dishes containing the concentrations mentioned above, 5-mm discs containing 8-day-old *Fusarium solani* mycelium were minced to the center of each dish, which were then sealed with plastic film and placed randomly into the incubation chamber at 25ºC. The lowest concentration of extract that inhibited the growth of the test microorganisms was determined to be the Minimum Inhibitory Concentration (MIC) (Ostrosky et al., 2008). This analysis was conducted by measuring the diameters of the colonies (the mean out of two diametrically opposed measurements) every two days, beginning after 48 hours of incubation and lasting for seven days, i.e., until the moment when the control treatment fungus had colonized the entire surface of the culture medium. Potassium phosphite (K$_2$HPO$_3$) was used as the positive control.

2.4. Antibacterial assay

To evaluate the antibacterial activity of the *S. brasiliensis* extracts through the broth micro-dilution technique, the following standard bacterium strains were utilized, which were supplied by the Cultures Collection of the Phytopathology Laboratory, Rural Federal University of Pernambuco, with the respective cultures from where they were isolated:

Xanthomonas campestris pv. *campestris* (Xcc56) from cabbage leaves; *Xanthomonas campestris* pv. *viticola* (Xcv112) from vine branches; *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc23) from lettuce leaves; *Ralstonia solanacearum* (Race 1) (CGH12) from fresh pepper stems; *Ralstonia solanacearum* (Race 2) (B19) from bananas; and *Acidovorax avenae* subsp. *citrulli* (Aac1.12) from yellow melon.

2.4.1. Preparation of inoculum

The inoculum was prepared by emulsifying the overnight colonies from an agar *Mueller Hinton* by diluting a broth culture (Suffredini, Varella and Younes, 2007). A 0.5 McFarland standard (Andrews, 2001) was used for visual comparison to adjust the suspension to a density equivalent to approximately 10^8 colony-forming units per mL (CFU/mL). Alternatively, the inoculum could be adjusted photometrically. The suspensions of the organisms were diluted in 0.85% saline or broth to give 10^8CFU/mL. The plates were inoculated within 30min of standardizing the inoculum to avoid changes in the inoculum density.

2.4.2. Microdilution broth assay

The extracts were tested using the microdilution broth assay (MDBA) in sterile conditions, according to the Clinical and Laboratory Standards Institute (2009) (CLSI, 8th Ed.). The assay was adapted for high-throughput conditions using Müeller-Hinton broth (MHB) medium (Difco™) in 96-well microplates. The inoculum was adjusted to 1×10^8CFU/mL with fresh colonies grown on sterilized Müeller-Hinton Agar (MHA) medium (Difco™). The bacterial suspensions at the different concentrations were prepared in MHB from the 1×10^8CFU/mL stock. A 10μL aliquot of the bacterial suspension was dispensed into the wells containing MHB medium (45μL) and a 45-μL aliquot of the extract at different concentrations (20; 10; 5;

2.5; 1.25; 0.63; 0.31 and 0.16mg.mL^{-1}). The microplates were incubated at 25°C for 24h. The inhibition of bacterial growth was assessed using 0.01% Resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide, Sigma-Aldrich®) solution to verify the presence of microbial growth (Suffredini et al., 2004). A pink or red color after the addition of the Resazurin solution indicated the presence of growing viable cells, and a blue color indicated a lack of microbial growth. MIC was determined to be the lowest concentration visually analyzed to effect total growth inhibition. Gentamicin was used as the positive control.

2.5. Brine Shrimp Lethality Bioassay

The toxicity bioassay against *Artemia salina* was conducted according to the procedure described recently (Batista et al., 2009). Brine shrimp eggs were hatched in artificial seawater (40g/L, sea salt). After 24 hours of incubation at warm room temperature (27-30 ºC) under artificial light, the nauplii were collected with a Pasteur pipette and maintained for an additional 24 hours under the same conditions to reach the metanauplii stage. We prepared the solutions by diluting 50mg of each extract in 1mL of dimethylsulphoxide (DMSO). Working in triplicate 12.5, 25 and 50μL aliquots of these solutions were diluted in 5mL of previously prepared sea salt solution containing *A. salina* larvae (10-20 metanauplii), thereby obtaining concentrations of 125, 250 and 500μg/mL of EEL, EES and EER extracts. A negative control was prepared with DMSO sea salt solution with only *A. salina* larvae (10-20 metanauplii), and lapachol was used as the positive control. Twenty four hours later, the number of survivors was counted, and the lethal concentration 50% (LC$_{50}$), defined as the sample concentration that causes the death of 50% of the larvae, was calculated using Probit analysis with 95% confidence intervals (Finney, 1971).

3. Results and Discussion

The antifungal, antibacterial and brine shrimp lethality activities of *Schinopsis brasiliensis* extracts EER, EES and EEL were evaluated, and their corresponding results are shown in **TABLE 1**. As noted, the EER, EES and EEL extracts were inactive against *Fusarium solani,* displaying MIC values above 1mg/mL.

TABLE 1 also presents the Minimum Inhibitory Concentrations (MIC) of the *S. brasiliensis* ethanolic extracts obtained by the broth micro-dilution method against six phytopathogenic bacteria, displaying MIC values in the range from 312.5 to 10,000µg.mL^{-1}. The leaf extract exhibited a lower MIC against three of the microorganisms tested: *Ralstonia solanacearum* (Race 2, B19), *A. avenae* subsp. *citrulli* (Aac1.12) and *X. campestris* pv. *campestris* (Xcc56). The stem extract was efficient against only the bacteria *R. solanacearum* (Race 1, CGH12), having a MIC of 312.5µg.mL^{-1}. The leaf, stem and root extracts were equally efficient against *X. campestris* pv. *campestris* (Xcc56). From all the bacterias tested, *P. carotovorum* subsp. *carotovorum* (Pcc23) was the most resistant, having a MIC between 5,000 and 10,000µg.mL^{-1} for all three of the *S. brasiliensis* extracts assayed. This result allowed us to consider EER, EES and EEL to be inactive against this bacterium according to the criteria established by Tegos and collaborators (2002). The root extract displayed a MIC of 625µg.mL^{-1} against the bacteria *X. campestris* pv. *campestris* (Xcc56) and *A. avenae* subsp. *citrulli* (Aac1.12), and of 1,250µg.mL^{-1} against the bacteria *X. campestris* pv. *viticola* (Xcv112) and *R. solanacearum* (Race 1, CGH12). We also noted that the stem extract presented the highest MIC values against the majority of the assayed bacteria, with the exception of *X. campestris* pv. *campestris* (Xcc56) and *R. solanacearum* (Race 1, CGH12), against which the extract reached a MIC value of 625 and 312.5µg.mL^{-1}, respectively. Additionally, the *S. brasiliensis* leaf extract presented the best performance, being capable of inhibiting the growth of the microorganism *A. avenae* subsp. *citrulli* (Aac1.12), with a MIC value of 312.5µg.mL^{-1}. In general, these results demonstrate the good aptitude of *S. brasiliensis* extracts to inhibit the growth of the evaluated phytopathogenic bacteria.

EEL was the most active extract against the phytopathogenic bacteria assayed. It was submitted to a phytochemical study aiming to identify the chemical entity(ies) responsible for its antibacterial activity. This extract was fractionated on a silica gel column, which provided triterpene glutinol (**1**) and a pyrocatechol **2** (**FIGURE 1**), whose structures were determined by comparison of their IR, ^{1}H and ^{13}C NMR data with those available in the literature (Tuan, Hung and Chien 2005; Sargent, Wangchareontrakul and Jefferson, 1989). To the best of our knowledge, this paper is the first to report the occurrence of both natural products **1** and **2** in *Schinopsis brasiliensis*. When compounds **1** and **2** were assayed against the same phytopathogenic bacteria, neither was found to be active, with MIC values above 5,000µg.mL^{-1} (**TABLE 1**). These results indicate that the most potent antibacterial activity observed for EEL is not attributed to these isolated compounds, although they may take part in a likely synergism or potentiation when present in this extract, and thus deserve further studies to clarify this possibility. Furthermore, Saraiva and collaborators (2011) and Chaves and collaborators (2011) attributed the antimicrobial activity of *Schinopsis brasiliensis* to the high concentration of phenolic compounds, such as tannins and flavonoids, which could not be isolated from EEL in our attempts described in this present paper.
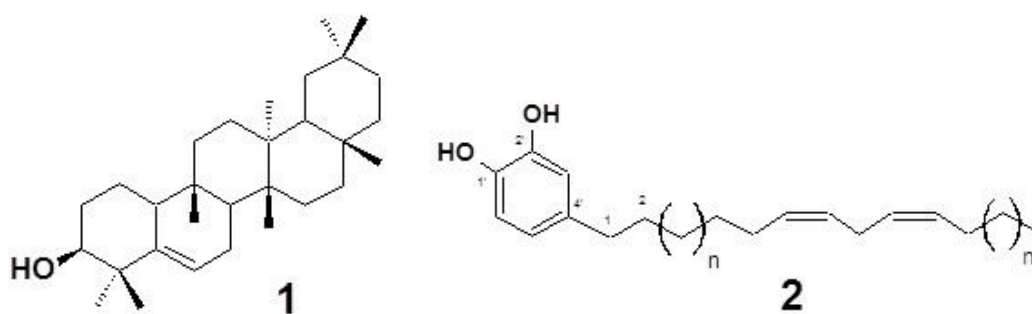
FIGURE 1. Chemical structures of compounds glutinol (**1**) and pyrocatechol **2** isolated from *Schinopsis brasiliensis* leaves.

The preliminary results (data not shown) suggest that all of the extracts from *Schinopsis brasiliensis* might be acting as bacteriostatic agents. From the point of view of their potential application in agriculture, these findings are important results because apart from controlling the disease caused by the phytopathogens, they also have a lower environmental impact due to the lack of more toxic residues that would rest on the agricultural products. The presence of bactericidal antibiotic substances would damage the local biodiversity and the health of the consumers of these products. As an example, copper sulfate, which is also a bacteriostatic agent, is largely used to control phytopathogens in agriculture and has low toxicity compared to other pesticides (Legaspi and Zenz, 1994).

TABLE 1. Antimicrobial and brine shrimp lethality (BSL) activities of *Schinopsis brasiliensis* extract EER, EES and EEL, and substances **1** and **2** against some phytopathogens and *Artemia salina*.

Sample	Fungus * F. solani (MIC, mg/mL)	Bacteria (MIC, µg/mL) #						A. salina (LC$_{50}$, µg/mL)
		X.c. (I) [a]	X.c. (II) [b]	P.c. [c]	R.s. (I) [d]	R.s. (II) [e]	A.a. [f]	
EER [1]	> 1.0	625	1,250	5,000	1,250	2,500	625	> 1000
EES [2]	> 1.0	625	2,500	10,000	312.5	10,000	2,500	> 1000
EEL [3]	> 1.0	625	1,250	5,000	625	1,250	312.5	> 1000
1			> 5,000					
2		> 5,000	> 5,000	> 5,000	> 5,000	> 5,000	> 5,000	
K$_2$HPO$_3$	0.001							
Gentamicin		0.250	0.031	0.031	0.031	> 4.00	0.031	
Lapachol								68.1

* Using the mycelial growth inhibition method in dishes;   # Using the broth micro-dilution technique.

Phytopathogenic bacteria: [a] *Xanthomonas campestris* pv. campestris (Xcc56); [b] *Xanthomonas campestris* pv. viticola (Xcv112); [c] *Pectobacterium carotovorum* subsp. carotovorum (Pcc23); [d] *Ralstonia solanacearum* (Race 1) (CGH12); [e] *Ralstonia solanacearum* (Race 2) (B19); [f] *Acidovorax avenae* subsp. citrulli (Aac1.12).

[1] Ethanolic extract of roots; [2] Ethanolic extract of stems; [3] Ethanolic extract of leaves.

Papadopoulos and collaborators (2004) reported the presence of glutathione S-transferase (GST) as an internal detoxification system in the three development phases (24h, 48h and adults) of *Artemia salina*, which are affected by a number of internal or external factors, therefore changing the organism's susceptibility to various environmental contaminants. For this reason, this micro-crustacean is a valid model to evaluate the toxicity of xenobiotics against vertebrates and invertebrates (Brophy et al., 1989; Grant and Matsumura, 1988) and can be used as a valid tool to evaluate the ecotoxicity of antimicrobial agents and plant extracts (Nunes et al., 2006). When the ecotoxicity of *S. brasiliensis* extracts was evaluated using the BSL bioassay against *A. salina*, the LC_{50} values were above 1,000μg/mL for all of the extracts and there were no deaths of the microcrustacean's larvae up to the highest concentration assayed, indicating that these extracts are non-toxic for *A. salina* (**TABLE 1**) and potentially non-toxic to the environment.

Our results indicate the potential application of *Schinopsis brasiliensis* for agricultural use as a botanical agent to control plant diseases caused by phytopathogenic bacteria pertaining to the genera *Xanthomonas*, *Ralstonia* and *Acidovorax*. Further studies are still needed to confirm the *in vivo* antibacterial activity of these extracts.

4. Conclusions

This work reports the *in vitro* antibacterial activity of *Schinopsis brasiliensis* extracts against five strains of phytopathogenic bacteria and shows that such extracts are potentially non-toxic to the environment. Moreover, the triterpene glutinol and a pyrocathecol are described here for the first time as the chemical constituents of *S. brasiliensis*. The results suggest that this plant has potential for agricultural use to control plant diseases caused by phytopathogenic

bacteria belonging to the genera *Xanthomonas*, *Ralstonia* and *Acidovorax*.

5. Acknowledgements

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