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In vitro growth-inhibitory effect of Brazilian plants extracts against *Paenibacillus larvae* and toxicity in bees

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ABSTRACT

American foulbrood (AFB) is a serious worldwide spreading disease in bees caused by *Paenibacillus larvae*. Plants extracts are known to decrease or inhibit the growth of these bacteria. The purpose of this study was to evaluate the antimicrobial activity of *Calendula. officinalis, Cariniana domestica,* and *Nasturtium officinale* extracts against the *P. larvae* and to evaluate the toxicity of the extracts in bees. *In vitro* activity against *P. larvae* of the extracts was evaluated by micro dilution method and the minimal inhibitory concentrations (MICs) were also determined. The concentrations used in the toxicity test were established based on the MIC values and by the spraying application method. The *P. larvae* was susceptible to the evaluated crude extract of *C. officinalis* and *N. officinale.* To *C. domestica,* only the ethyl acetate (EtAc) fraction and *n*-butanol (BuOH) fractions had activity against *P. larvae*. Toxicity analysis in bees showed no toxicity for *N. officinale* crude extract and for *C. domestica* BuOH fraction during 15 days of treatment, however, some deaths of bees occurred during the first three days of treatment with *C. officinalis* and *C. domestica* EtAc fraction. The results with these species were firstly described and showed that *N. officinale* crude extract and *C. domestica* BuOH fraction both presented not toxic effects in the concentration tested by the spraying application method, and can be a useful alternative for treatment or prevention of AFB.

Key words: Antibacterial activity, American foulbrood, Brazilian plants, *Paenibacillus larvae*.

INTRODUCTION

American foulbrood (AFB) is a serious worldwide spreading disease of honey bee (*Apis mellifera*) caused by the spore-forming, Gram-positive bacterium *Paenibacillus larvae* (Eguaras et al. 2005, Flesar et al. 2010). AFB is highly infectious and can be fatal to colonies. At a minimum it decreases honey productivity of colonies and increases labor and treatment costs to beekeepers to control disease transmission (Hansen and Brødsgaard 1999, Bastos et al. 2008).

The popular approach in treatment of AFB in some countries, such as the US, is to suppress the

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clinical phase of the disease with supplemented antibiotics, however, this practice has been shown to lead to bacterial resistance (Eguaras et al. 2005, Flesar et al. 2010) such as tetracycline in which vegetative cells of *P. larvae* have become increasingly resistant to treatments (Kochansky et al. 2001, Cox et al. 2005). Furthermore, antibiotic residues are found in honey and its use in the colony can also contaminate royal jelly (Bogdanov 2006).

This situation calls for an alternative and effective control of the disease with either therapeutics or prophylactic feed additives that do not contribute to the phenomenon of bacterial resistance (Lewis and Ausubel 2006, Flesar et al. 2010).

Plant extracts, herbs, spices, essential oils and isolated compounds are known to delay or inhibit the growth of bacteria, yeast and moulds (Hayouni et al. 2008, Boligon et al. 2013). Brazil has in its flora, species with antimicrobial activity such as *Calendula officinalis* L. known in Brazil as calendula (Efstratiou et al. 2012), *Cariniana domestica* (Mart.) Miers. known as jequitibá-roxo (Janovik et al. 2009), and *Nasturtium officinale* commonly called agrião (Antúnez et al. 2008). Studies conducted by Boligon et al. (2013) showed positive results of *Scutia buxifolia* extract against *P. larvae* as well as low toxicity to bees. In addition *Copaifera officinalis* (copaiba) oil in studies by Santos et al. (2012), have also presented similar results.

The aim of the present work was to evaluate the antimicrobial activity of *C. officinalis, C. domestica* and *N. officinale* extracts against the etiologic agent of American Foulbroad Disease, the *P. larvae*. Furthermore, the toxicity of the extracts in honey bees *A. Mellifera* was also evaluated.

MATERIALS AND METHODS

Plant Collection and Extraction

The *C. officinalis* (flowers) and *N. officinale* produced in a hydroponic system (leaves and branches) used in this study were purchased in supermarkets in the city of Santa Maria-RS, Brazil. The *C. domestica* (leaves) were collected in Tangará-da-Serra, Mato Grosso do Sul, Brazil. Exsiccate of *C. domestica* was archived as voucher specimen in the herbarium of the Department of Pharmacology at UFSM (SMDB 11818). The vegetables were cleaned and dried at room temperature. The dried plants were powdered in a knife mill and the powder was macerated with 70% ethanol for a week with daily shake-up. The extract was filtered and concentrated under reduced pressure in rotary evaporator (temperature ± 40°C) in order to eliminate the ethanol, then the aqueous extract was dried, thus obtaining the crude extract.

For the *C. domestica*, an equal part of aqueous extract was partitioned with solvents of increasing polarity dichloromethane (DCM), ethyl acetate (EtAc) and *n*-butanol (BuOH) and were also dried to provide each corresponding fraction.

Microorganism Tested

In this study, *P. larvae* (ATCC 9545) from the collection of the Ministry of Agriculture (LANAGRO/RS) Brazil was used. The microorganism was grown in Mueller–Hinton broth (Difco, Sparks, Maryland, USA) at 37°C for 24h and maintained on slopes of nutrient agar (Difco).

Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) of *C. officinalis, N. officinale* and *C. domestica* crude extracts and *C. domestica* fractions were determined by microdilution techniques in Mueller–Hinton broth (Difco) for *P. larvae* (CLSI 2008). The assay was carried out in 96-well microtitre plates. Each sample was mixed with an inoculum prepared in the same medium at a density adjusted per tube to 0.5 of the McFarland scale (1.5×10^8 CFU/mL) and diluted 1:10 for the broth microdilution procedure. Microtitre trays were incubated at 37°C and the MICs were recorded after 24h of incubation. The

MIC was defined as the lowest concentration of compounds that inhibit bacterial growth. This test was performed in triplicate on separate occasions. The 2,3,5-triphenyltetrazolium chloride was used as an indicator of bacterial growth.

Toxicity Assay

The *C. officinalis* and *N. officinale* crude extracts, and EtAc and BuOH fractions of *C. domestica* were dissolved in DMSO. The concentrations used in the toxicity assay were determined based on the MIC values. The spraying application method was performed according to Santos et al. (2012). Petri dishes (150 x 15 mm) padded with absorbent filter paper on the inner bottom and with an extra lid of plastic mesh were used. Fifteen adult worker bees were placed in every modified Petri dish. Then, 1 mL of each concentration (crude extracts or fractions) was individually sprayed on the bees throughout the plastic lid using a hand sprayer. A device with candy and water was placed inside each unit as food for the bees. Fifteen bees in a modified Petri dish sprayed with DMSO were included as negative control, and six bees in a modified Petri dish sprayed with 0.07% Deltamethrin (DTT) (Pirisa-PiretroIndustrial Ltda, Brazil) were included as positive death control. Four replicates for each experimental group were run. Bioassay dishes were placed in incubators at 28 ± 1°C and 60% relative humidity. Mortality of bees was evaluated daily, by visual inspection for a period of 15 days.

Statistical Analysis

Differences in survival after 15 days of observation were assessed by Kaplan–Meier analysis followed by the Logrank test. A p value < 0.05 was considered statistically significant. All statistical analyses were performed with the software package GraphPadPrism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

P. larvae Susceptibility Test and Determination of Mics

The *P. larva was* susceptible to the assessed crude extract of the *C. officinalis* and *N. officinale*. For the *C. domestica,* only the EtAc and BuOH fractions indicated activity against *P. larvae*. The MICs of these samples ranged from 0.98–30.51 mg/mL, and the crude extract and DCM fraction of *C. domestica* did not show activity against *P. larvae* (Table I).

TABLE I
MICs of *C. officinalis, N. officinale* and *C. domestica* on *P. larvae.*

Extract or fraction	MICs on *P. larvae* (mg/mL)
C. officinalis crude extract	12.76
N. officinale crude extract	30.51
C. domestica crude extract	-
C. domestica DCM fraction	-
C. domestica EtAc fraction	4.06
C. domestica BuOH fraction	0.98

Toxicity Assay in Bees

Toxicity analysis in bees, evaluated by the spraying application method of *C. officinalis, N. officinale* crude extracts, and EtAc, BuOH fractions of *C. domestica*, showed no toxicity for *N. officinale* crude extract and *C. domestica* BuOH fraction during 15 days of treatment, however, some deaths of bees occurred during the first three days of treatment with *C. officinalis* and *C. domestica* EtAc fraction (Fig. 1). Bee mortality was evident in treatment with DTT (positive death control group).

DISCUSSION

The search for the discovery of drugs, as well as the determination of the prudent use of antimicrobial agents, are the basis to solve the global problem of microbial resistance; and the resources for the discovery of drugs are the natural products (Santos et al. 2012).

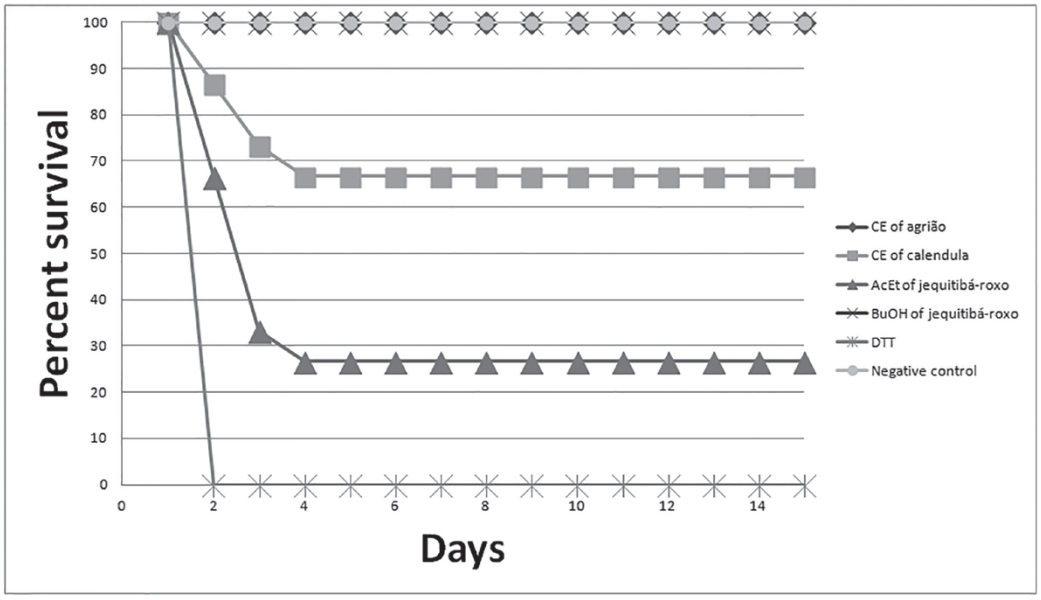

Figure 1 - Percentage of survival of bees subjected to spraying with *C. officinalis* (calendula) crude extract, *N. officinale* (agrião) crude extract, and *C. domestica* (jequitibá-roxo) EtAc and BuOH fractions. CE (crude extract).

The crude extracts of three Brazilian species were investigated: *N. officinale, C. officinalis* and *C. domestica*. The determination of the MIC through the method of broth micro dilution showed that only the crude extract of *N. officinale* and *C. officinalis* (Table I) exhibited an antibacterial effect against *P. larvae,* with respective MICs of 12.76 mg/mL and 30.51mg/mL. Therefore these two species were not fractionated. The *C. domestica* crude extract did not show such activity, for this reason, its aqueous extract was fractionated using solvents of increasing polarity, with the objective of concentrating molecules with similar polarity in the same fractions and, consequently, promote better activities, as observed in Table I, in which low MIC values were found for AcEt and BuOH fractions of *C. domestica.*

One of the most usual problems in the use of medicinal plants is the amount of extract used. In this case the MIC values were between 0.98 and 30.51 mg/mL, however this is not a serious problem, since the herbal products are relatively safer than synthetic drugs (Abubakar 2010). The MIC values obtained for the crude plant extracts were high when compared with the MIC values of 0.01 - 10 μg/mL, frequently recorded for conventional antibiotics. The differences can be due to the fact that synthetic antibiotics are in a pure form, plants extracts don't act the same way as synthetic antibiotics, that's why were found high MIC values (Abubakar 2010).

To do the spraying on *A. mellifera* extracts or fractions that have shown activity against *P. larvae* were used in exactly the same concentration, in order to verify, by the percentage of survival, the possible toxic effects (Fig. 1).

N. officinale crude extract and *C. domestica* BuOH fraction showed 100% survival in all periods analyzed, demonstrating no toxic effects at the concentrations tested.

The *C. officinalis* crude extract and *C. domestica* EtAc fraction showed, respectively, 66.68% and 26.64 % of survival in the concentration tested at the end of the experiment.

The *N. officinale* that showed good results have triterpenes, steroids, flavonoids, phenyl propanoids and saponins (Carvalho et al. 2009). Studies by Boligon et al. (2013) found in the crude extract of *N. officinale* 104.41 mg/g of phenols, 71.83 mg/g of flavonoids,

and analyzed by HPLC-DAD showed chlorogenic acid (1.25%), caffeic acid (5.08%), and rutin (1.92%). The antimicrobial activity of *N. officinale* has also been shown by Abu-Zinadah (2008).

In a similar study performed by Janovik et al. (2009), the *C. domestica* showed that in the BuOH fractions had 486.22 mg/g of polyphenols and 15.26mg/g of flavonoids, and in the EtAc fraction had 510 mg/g of polyphenols and 39.92 mg/g of flavonoids. In this same study the EtAc fraction also presented: gallic acid (2.5 mg/g), caffeic acid 12.82 (mg/g), chlorogenic acid (15.27 mg/g), rutin (11.45), and kanferol (0.85 mg/g).

For *C. officinalis*, the chemical constituents include some triterpene saponins, triterpene alcohols, triterpene esters, carotenoids such as flavoxanthin, lutein, rubixanthin, β-carotene, and lycopene (Pintea et al. 2003, Fonseca et al. 2010), flavonoids (quercetin, rutin, narcison, isorhamnetin and kaempferol) and coumarins (Fonseca et al. 2010).

A study performed by Ducat et al. (2011) showed that this species contains, 92.35 mg/g of phenolic acid and 13.55 mg/g of flavonoids in a 70% ethanolic extract, and also showed an antimicrobial activity for *Staphylococcus aureus* and *Klebsiella pneumoniae.* Other study by Efstratios et al. (2012) showed antifungal and antibacterial activity against *Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa and Klebsiella pneumoniae.*

These substances found in these species may be related to activities against *P. larvae*. The antimicrobial action of polyphenolics, including flavonoids, aromatic and phenolic acids, is frequently reported (Banskota et al. 2001). Specifically, Flesar et al. (2010) confirmed the antimicrobial activity against *P. larvae* of the naringenin (MIC 64 microgram/mL), caffeic acid (>128 microgram/mL), quercetin dihydrate (>128) catechin (>128) among other phenolic compounds.

It has been proposed that the antimicrobial activity could be due to synergism between the various components. It was observed that none of the single component showed a higher activity than the total extract (Bonvehi et al. 1994, Flesar et al. 2010, Boligon et al. 2013).

Similarly, the antibacterial activity of the propolis extract could be related with its chemical composition, which includes phenolic compounds (flavonoids and aromatic acids), terpenes and essential oils among others (Sforcin 2007, Antúnez et al. 2008).

A very similar study of Boligon et al. (2013) evaluated the antimicrobial susceptibility of the *P. larvae* to the crude extract, dichloromethane , EtAc and BuOH fractions of the *S. buxifolia*, where the MICs were 50, 1.56, 6.25, 25 mg/mL, respectively. The antimicrobial activity was related to the chemical composition of the *S. buxifolia* which is rich in steroids, triterpenes, phenolic compounds, flavonoids and alkaloids (Boligon et al. 2013). In the same study, in the toxicity assay by spraying, *S. buxifolia* showed not be toxic for bees during 15 days.

However, the same did not occur with *C. officinalis*, this species showed an acute toxicity and growth inhibition in milkweed bug, *Oncopeltus fasciatus* Dallas. Also, *Calendula micrantha officinalis* showed molluscicidal activity (Abd-El-Megeed 1999). These important activities can explain the mortality of bees with *C. offinalis* crude extract in this study.

Janovik et al. (2009) isolated lupeol, β-amyrin, β- sitosterol and stigmasterol fraction of *C. domestica* EtAc and also the same compounds of *Inula japonica*, which showed acaricidal effect *in vitro* against carmine spider mite, *Tetranychus cinnabarinus* (Boisduval) in a study performed by Duan et al. (2011), this may explain the reason for the higher mortality in bees, even containing a large amount of polyphenols in this fraction.

Similarly, in a study conducted by Santos et al. (2012) with the sprinkling of Carapa guaianensis (andiroba) oil, only 20% of bee survival was observed after 10 days, the insecticidal effect of andiroba oil was associated with this result.

Plant extracts have a complex blend of different secondary metabolites that are an important source of bioactive molecules, some of them displaying important antibacterial effect for the drug-chemical and the food control (Flesar et al. 2010). Brazil has a rich biodiversity with numerous compounds that can contribute to bee's health; there are many promising species for future research involving alternative natural substances to control AFB.

The elimination of the *P. larvae* in *A. mellifera* colonies involves treatments with acceptable antimicrobial activity that do not present side effects on *A. mellifera* and that minimizes the residues in the honey (Santos et al. 2012).

The three Brazilian species tested and obtained in the collection sites described, showed for the first time, antimicrobial activity. The *N. officinale* crude extract and the *C. domestica* BuOH fraction both presented non toxic effects in the concentration tested.

Therefore, this species can be used in the practice of apiculture by pulverization and may also be a potential and useful alternative for the treatment or prevention of AFB.

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RESUMO

Loque americana é uma grave doença de propagação mundial em abelhas causada pelo *Paenibacillus larvae.* Extratos de plantas são conhecidos por diminuir ou inibir o crescimento dessa bactéria. O objetivo deste estudo foi avaliar a atividade antimicrobiana dos extratos de *Calendula. officinalis, Cariniana domestica,* e *Nasturtium officinale* contra o *P. larvae* e avaliar a toxicidade dos extratos em abelhas. A atividade *in vitro* dos extratos contra *P. larvae* foi avaliada pelo método de microdiluição e as concentrações inibitórias mínimas (MICs) também foram determinadas. As concentrações utilizadas no ensaio de toxicidade foram estabelecidas baseadas nos valores de MIC e pelo método de aplicação por pulverização. O *P. larvae* foi suscetível aos extratos brutos de *C. officinalis* e *N. officinale* avaliados. Para a *C. domestica*, apenas as frações acetato de etila (EtAc) e butanólica (BuOH) tiveram atividade contra *P. larvae*. Análises de toxicidade em abelhas não mostraram toxidade para o extrato bruto de *N. officinale* e fração BuOH de *C. domestica* durante os 15 dias de tratamento, no entanto, ocorreram algumas mortes de abelhas durante os três primeiros dias de tratamento com *C. officinalis* e fração EtAc de *C. domestica*. Os resultados com essas espécies foram pela primeira vez descritos e mostraram que o extrato bruto de *N. officinale* e a fração BuOH de *C. domestica* ambos não apresentaram efeitos tóxicos nas concentrações testadas pelo método de aplicação por pulverização e podem ser uma alternativa útil para o tratamento ou prevenção da loque americana.

Palavras-chave: atividade antibacteriana, loque americana, plantas brasileiras, *Paenibacillus larvae.*

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