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Original Article

POTENTIAL USE OF *BUDDLEJA THYRSOIDES* FOR THE CONTROL AND PREVENTION OF AMERICAN FOULBROOD DISEASE IN HONEY BEES

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Abstract

***Paenibacillus larvae* is the causative agent of American Foulbrood (AFB), a severe disease that affects the larvae of the honeybees. The use of plant extracts are considered to be an alternative way of controlling the disease. In this study, the in vitro antimicrobial activity of *Buddleja thyrsoides* Lam. against the *Paenibacillus* species, including *P. larvae*, was evaluated. In Mueller-Hinton broth, the minimal inhibitory concentration (MIC) was assessed using the microdilution method. All *Paenibacillus* species were sensitive to the crude extract and the fractions of *B. thyrsoides.* The ethyl acetate (EA) fraction showed a better result with MIC values of 1.68 – 3.36 mg/mL, followed by butanolic (BU) (MIC = 2.18 - 6.54 mg/mL), dichloromethane (DCM) (7.40 - 14.80 mg/mL), and crude extract (CE) (7.51 – 16.90 mg/mL). The toxic effect of the CE and fractions of *B. thyrsoides* against bees were also evaluated using the spraying application method with the same concentrations of MICs. Bee mortality was evident in treatment with DCM fractions only, while CE, EA, and BU extracts showed no toxic effects after 15 days of observation. Furthermore, phenolic acids, tannins, and flavonoids were identified and quantified by high-performance liquid chromatography (HPLC), and may be partially responsible for the antimicrobial properties observed. These results show, for the first time, that *B. thyrsoides* might be a natural alternative for the prevention/control of AFB.**

Keywords: Antibacterial activity, bee survival, *Buddleja thyrsoides*, HPLC, *Paenibacillus larvae.*

INTRODUCTION

The medicinal properties of plants have been investigated in recent scientific developments throughout the world, due to their potent biological activities and economic viability (Dasari et al., 2012). Several studies describe such biological activities of plants as antibacterial, antifungal, and antiviral. These activities are related to the presence of a diverse group of chemical compounds including phenolics, flavonoids, glycosides, steroids, and alkaloids (Boligon et al., 2012a; 2012b; 2013b).

American foulbrood (AFB) is among the most severe bacterial diseases affecting honeybee larvae (*Apis mellifera* L.). This disease causes the decline of the population. American foulbrood can also lead to the death of the colony (Genersch et al., 2005; Santos et al., 2012). The causative agent is *Paenibacillus larvae* (White), a gram positive and sporeforming bacterium that is distributed worldwide (Spivak and Reuter, 2001; Genersch et al., 2006). American foulbrood is considered a global threat to apiculture because the etiologic agent produces environmentally stable spores which are very virulent, and resistant to

heat, to desiccation, and to disinfectants (Genersch, 2010; Boligon et al., 2013a). Products derived from plants, such as propolis (Bastos et al., 2008; Mihai et al., 2012), essential oils (Albo et al., 2003; Santos et al., 2012); and extracts of *Myrtus communis* L., *Eucalyptus dunnii* (Maiden), *Rosmarinus officinalis* L., *Zingiber officinale* (Roscoe), *Scutia buxifolia* (Reissek), among other species (Flesar et al., 2010; Boligon et al., 2013a) were previously investigated and exhibited a growth-inhibitory effect against AFB.

Buddleja is a genus with pan tropical distribution that occurs in South Asia, Africa, and America (Mahlke et al., 2009). Species belonging to the *Buddleja* genus exhibit antibacterial, antihepatotoxic, pesticidal, anti-inflammatory, sedative, analgesic, and diuretic actions (Houghton et al., 2003; Mahlke et al., 2012). *Buddleja thyrsoides* Lam. (Scrophulariaceae) is commonly known as "Barbasco" or "Cambara-do-campo", in Brazilian folk medicine, its leaves and flowers are taken by drinking an infusion made with hot water for the treatment of bronchitis and a cough (Mahlke et al., 2013). Some previously published work by Mahlke et al. (2009; 2012; 2013) describes the essential oil composition, antimicrobial, and antioxidant activities, as well as the antiplatelet and acetylcholinesterase inhibition, of *B. thyrsoides*. Our work is the first time that antimicrobial activity of *B. thyrsoides* crude extract and fractions against the *Paenibacillus* species have been evaluated. Toxicity against the honey bee, *Apis mellifera*, was also investigated. Furthermore, phenols and flavonoids compounds were identified and quantified by high-performance liquid chromatography (HPLC/DAD).

MATERIAL AND METHODS

Plant collection

Leaves of *Buddleja thyrsoides* were collected in São Luiz Gonzaga, State of Rio Grande do Sul, Brazil, on July of 2006. For future reference, exsiccate was archived as voucher specimens in the herbarium of the Department of Biology at the Federal University of Santa Maria by the register number SMDB 10125.

Extraction and partition of the leaves

Air dried and powdered leaves of *Buddleja thyrsoides* (420 g) were extracted using ethanol (70% v/v) at room temperature, and daily agitation which was done for 7 days. After filtration, the extract was evaporated under reduced pressure to remove the ethanol in order to obtain a water suspension that was sequentially extracted at room temperature with dichloromethane, ethyl acetate, and *n*-butanol (3 x 100 mL for each solvent). The yield of the dichloromethane fraction (DCM) was 13.06 g or 3.11%; ethyl acetate fraction (EA) was 12.85 g our 3.06%; and *n*-butanol fraction (BU) was 21.55 g our 5.13%. A new amount of the same batch of plant material (100 g) was extracted with ethanol (70%) at room temperature for seven days to afford the crude extract (CE) which was used in this study, with a yield of 11.7 g our 11.7%.

Microorganisms tested

In this study, six isolates of the *Paenibacillus* species from the collection of The Ministry of Agriculture (LANAGRO/RS), Brazil, were used. The test organisms included isolates of *P. alginolyticus, P. pabuli, P. azotofixans, P. borealis, P. validus,* and *P. larvae* (ATCC 9545). The microorganisms were grown in Mueller-Hinton broth (Difco, Sparks, Maryland, USA) at 37°C for 24 h, and maintained on slopes of nutrient agar (Difco).

Determination of the minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of crude extract, fractions, and compounds identified in *B. thyrsoides*, were determined by microdilution techniques in Mueller-Hinton broth (Difco) for the *Paenibacillus* species (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. The MICs were recorded after 24 h of incubation. The minimum inhibitory concentration was defined as the lowest concentration of compounds that inhibits bacterial growth. This test was performed in triplicate on separate occasions. As an indicator of bacterial growth, 2,3,5-triphenyltetrazolium chloride was used. The solution of DMSO (5 %, v/v) was simultaneously assayed as the negative control. Analyses were carried out in duplicate and one blank for each concentration of samples was used.

Toxicity assay

The crude extract and fractions of *B. thyrsoides* were dissolved in DMSO to reach the final concentrations of 11.27, 7.40, 2.18, and 1.68 mg/mL for CE, DCM, BU, and EA, respectively. The final concentration of DMSO was 5% v/v; a value that did

not cause interference in the antimicrobial activity. These concentrations used in the toxicity test were established from the determination of MIC values (Tab. 1). The spraying application method was performed according to Santos et al. (2012). Petri dishes (150 × 15 mm) padded with absorbent filter paper on the inner bottom and with an extra lid of plastic mesh were used. Six adult worker bees were placed in every modified Petri dish. Then, one mL of each concentration (crude extract and fractions) was individually sprayed on the bees through the plastic lid using a hand sprayer. A device with a water solution of sugar (approximate concentration) was placed inside each unit as food for the bees. Six bees in a modified Petri dish sprayed with DMSO (5%, v/v) were included as the negative control. Six bees in a modified Petri dish sprayed with 0.07% Deltamethrin (DTT) (Pirisa-Piretro Industrial Ltd, Brazil) were included as the 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. Bee mortality was evaluated every day, for 15 days.

HPLC analysis

High-performance liquid chromatography (HPLC) was used to analyse CE and DCM, EA and BU fractions of *B. thyrsoides* leaves. Reverse-phase chromatographic analyses were carried out in isocratic conditions using C-18 column (4.6 x 250 mm) packed with 5-μm in diameter particles; the mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45-lm membrane filter and then degassed by an ultrasonic bath, prior to use. Stock solution of gallic acid, caffeic acid, chlorogenic acid, ellagic acid, catechin, epicatechin, quercetin, isoquercitrin, rutin, and kaempferol standard reference were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.250 mg/mL (Boligon et al., 2013b). Quantification was carried out by the integration of the peak using the external standard method. The flow rate was 0.8 mL/min, injection volume was 40 μL, and detection was done at 257 nm for gallic acid, 280 nm for catechin and epicatechin, 325 nm for caffeic, ellagic, and chlorogenic acids, and 365 nm for quercetin, isoquercitrin, kaempferol, and rutin. The chromatographic peaks were confirmed by comparing their retention time and UV spectra with those of the reference standards and by spiking the isolated compounds in the plant sample. All chromatographic operations were performed at room temperature and in triplicate. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. The limit of detection and the limit of quantification were calculated as 3.3 and 10 r/S, respectively, where r is the standard deviation of the response and S is the slope of the calibration curve (Boligon et al., 2012c).

Statistical analysis

Differences in survival after 15 days of observation were assessed by Kaplan-Meier analysis followed by the Logrank test. All statistical analyses were performed with the software package GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Results of the HPLC-DAD quantification were considered statistically significant when $p<0.001$ by Tukey test.

RESULTS

Antimicrobial susceptibility test and determination of MIC

All *Paenibacillus* species were susceptible to the assessed CE, DCM, EA, and BU of the *B. thyrsoides*. In addition, caffeic acid, ellagic acid, and quercetin also showed antimicrobial activity against the *Paenibacillus* species. The MICs of these samples ranged from 1.68 to 16.90 mg/mL (Tab. 1).

Lethal concentration on bees

The bees treated with CE, EA, and BU fractions of *B. thyrsoides* showed similar results to the results observed in the negative control group, throughout the whole observation time. No mortality of bees was observed for the 15 days of treatment (Fig. 1). Bee mortality was evident only in the treatment with the DCM fraction and DTT (the positive control group). Within 24 hrs after the treatment, all bees were dead in the positive control group. Bees treated with the DCM fraction showed a mortality index of about 10% per day until day 3, with 70% survival at the end of the experiment (Fig. 1).

Phytochemical analysis

HPLC profile of CE, DCM, EA, and BU fractions of the *B. thyrsoides* were obtained (Fig. 2). These regions showed typical patterns of UV absorption, supporting the presence of gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, epicatechin, rutin, isoquercitrin, quercetin, and kaempferol. Retention time and quantifications of these compounds is showed in Table 2. The EA, BU, and DCM fractions

Table 1.

Minimum inhibitory concentration of *Buddleja thyrsoides* crude extract and fractions on the *Paenibacillus* species

Microorganisms	*Buddleja thyrsoides* (mg/mL)				Compounds (mg/mL)		
	CE	DCM	EA	BU	CA	EL	QU
P. larvae (ATCC 9545)	11.27	7.40	1.68	2.18	6.54	2.18	3.36
P. borealis	16.90	14.80	3.36	6.54	9.93	2.18	6.54
P. validus	11.27	7.40	1.68	2.18	16.90	11.27	14.80
P. pabuli	7.51	7.40	1.68	2.18	16.90	6.54	11.27
P. alginolyticus	11.27	7.40	3.36	2.18	7.51	16.90	16.90
P. azotofixans	11.27	7.40	1.68	2.18	11.27	16.90	9.93

Crude extract (CE), dichloromethane fraction (DCM), ethyl acetate fraction (EA), butanolic fraction (BU), caffeic acid (CA), ellagic acid (EL), and quercetin (QU).

Table 2.

Compounds determined by HPLC in *Buddleja thyrsoides* (%). Retention time-Rt (minutes)

B. thyrsoides	Rt	CE	DCM	EA	BU
		Stem bark			
Gallic acid	11.45	0.70 ± 0.02^{a}	2.18 ± 0.04^{a}	1.42 ± 0.01^{a}	1.57 ± 0.01^{a}
Catechin	16.03	1.23 ± 0.03^{b}	2.87 ± 0.01^{b}	2.05 ± 0.04^{b}	2.13 ± 0.04^{b}
Chlorogenic acid	22.17	4.81 ± 0.01^{c}	2.30 ± 0.01^{c}	5.03 ± 0.01^{c}	1.61 ± 0.05^{a}
Caffeic acid	25.01	5.17 ± 0.01^{d}	4.91 ± 0.02^{d}	4.65 ± 0.01^{d}	4.58 ± 0.09^{c}
Ellagic acid	30.11	2.92 ± 0.03^{e}	4.25 ± 0.03^{e}	7.54 ± 0.02^{e}	3.11 ± 0.04^{d}
Epicatechin	34.68	2.75 ± 0.03^{e}	2.09 ± 0.01^{c}	4.89 ± 0.03^{d}	1.54 ± 0.01^{a}
Rutin	40.93	2.74 ± 0.02^{e}	-	2.11 ± 0.03^{b}	4.86 ± 0.03^{e}
Quercetin	44.85	4.69 ± 0.01^{c}	-	5.49 ± 0.01^{e}	6.95 ± 0.01^{f}
Isoquercitrin	51.34	0.76 ± 0.03^{a}	-	2.91 ± 0.04^{f}	4.93 ± 0.02^{e}
Kaempferol	60.13	2.35 ± 0.08^{f}	-	1.32 ± 0.02^{a}	2.01 ± 0.01^{b}

Results are expressed as mean ± standard deviation (SD) of three determinations. Different letters in each column represent significant differences using analysis of variance followed by Tukey test (p values<0.001 were considered as significant).
Crude extract (CE), dichloromethane fraction (DCM), ethyl acetate fraction (EA), butanolic fraction (BU).

presented a higher quantity of phenolics compounds (37.87%, 33.29%, and 28.12%, respectively) when compared with CE (18.60%). The calibration curve, limit of detection, and limit of quantification of the standards used in HPLC are shown in Table 3.

DISCUSSION

The present work reports the first study on antimicrobial activity of *Buddleja thyrsoides* crude extract and fractions against various *Paenibacillus* species, showing an important antimicrobial effect in all strains tested. Better results were shown by EA and BU fractions for *P. larvae* with MIC of 1.68 and 2.18 mg/mL, respectively (Tab. 1). These results are in agreement with Boligon et al. (2013a), that also describes the best result for the EA fraction of *S. buxifolia* against the *Paenibacillus* species, using the same methodology. Flesar et al. (2010) described 13 natural compounds and 16 crude extracts that exhibited an antimicrobial effect against *P. larvae* with MICs values ranging from 2 to 256 μg/mL, using the broth microdilution method. In the case of the extracts, *Humulus lupulus* L. and *Myrtus communis* L. exhibited the highest growth-inhibitory effect. Propolis extract was also described as

Table 3.

Calibration curves of the standards used in HPLC.
LOD and LOQ variations for compounds (µg/mL)

Standards used	calibration curve	LOD	LOQ
Gallic acid	Y = 12673x + 1125.6 (r = 0.9999)	0.022	0.067
Catechin	Y = 14037x + 1281.5 (r = 0.9985)	0.008	0.026
Chlorogenic acid	Y = 13582x + 1175.2 (r = 0.9997)	0.019	0.057
Caffeic acid	Y = 13841x + 1197.4 (r = 0.9999)	0.035	0.014
Ellagic acid	Y = 13841x + 1197.4 (r = 0.9999)	0.027	0.082
Epicatechin	Y = 11876x + 1183.7 (r = 0.9991)	0.041	0.135
Rutin	Y = 14739x + 1356.5 (r = 0.9994)	0.034	0.103
Quercetin	Y = 12685x + 1327.1 (r = 0.9998)	0.030	0.091
Isoquercitrin	Y = 14052x + 1285.5 (r = 0.9992)	0.007	0.023
Kaempferol	Y = 13674x + 1189.3 (r = 0.9999)	0.024	0.072

LOD: limit of detection; LOQ: limit of quantification.

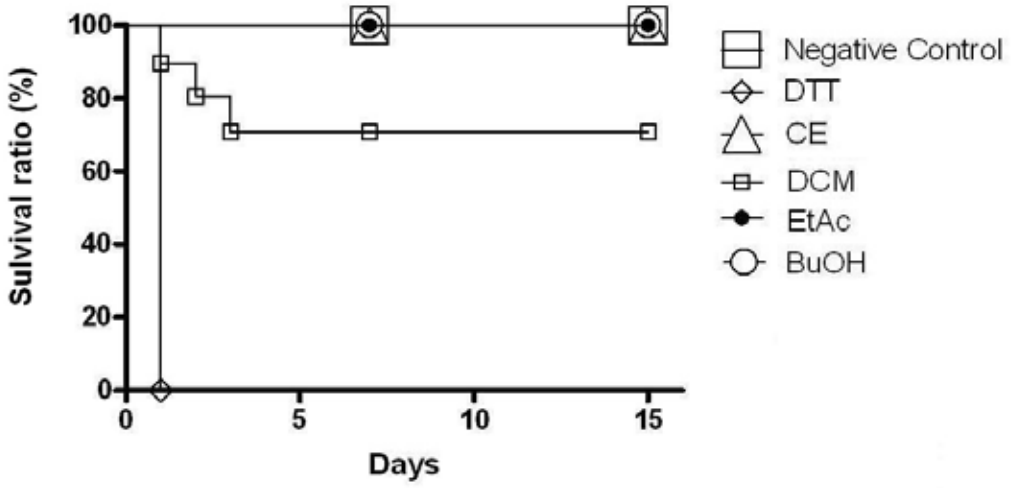

Fig. 1. Effects of *Buddleja thyrsoides* Lam. spraying applications on *Apis mellifera* L.

a natural alternative for the control of AFB (Bastos et al., 2008; Finstrom and Spivak, 2010).

In addition, previous works reported the antibacterial properties of *B. thyrsoides* against such diverse pathogens as *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa*, *Candida glabrata*, and *Prototheca zopfii* (Mahlke et al., 2009). Based on earlier works and the present experiments with different fractions, an obvious antibacterial activity could be related to the chemical composition of the plant species. It is known that EA and BU fractions are rich in phenolic acids, tannins and flavonoid compounds, such as, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, catechin, epicatechin, rutin, quercetin, isoquercitrin, and kaempferol (Fig. 2; Tab. 2). Therefore, flavonoids and phenolic compounds identified in *B thyrsoides* were also tested against the *Paenibacillus* species. However only, caffeic acid, ellagic acid, and quercetin inhibited the growth of the *Paenibacillus* species, with MICs values ranging from 2.18 to 16.90 mg/mL (Tab. 1). These compounds may contribute in part to the good antimicrobial activity found in this study, since they are present in large quantities in the most effective fractions of *B. thyrsoides*. It has been suggested that the antimicrobial activity could be due to the additional effect or may be synergism of several chemical compounds, since various plant extracts and fractions show higher antimicrobial activity than any of the single compounds separately tested (Flesar et al., 2010; Boligon et al., 2013a). The inhibitory effect of a propolis extract and *S. buxifolia* fractions against *P. larvae* was attributed to a possible synergism of flavonoids and phenols (Mihai et al., 2012; Boligon et al., 2013a).

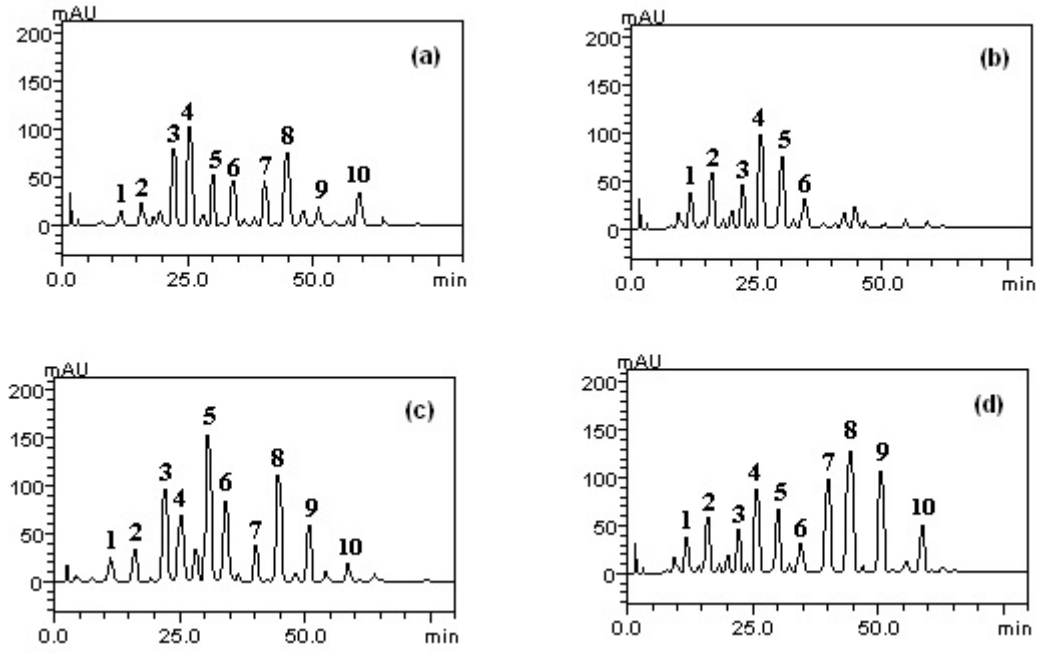

Fig. 2. Representative high performance liquid chromatography profile of *Buddleja thyrsoides*. Crude extract (a), dichloromethane fraction (b), ethyl acetate fraction (c), and butanolic fraction (d). Gallic acid (1), catechin (2), chlorogenic acid (3), caffeic acid (4), ellagic acid (5), epicatechin (6), rutin (7), isoquercitrin (8), quercetin (9), and kaempferol (10).

In order to verify the possible toxic effects, the CE, DCM, EA, and BU fractions of *B. thyrsoides* were sprayed on *A. mellifera*. The results when using CE, EA, and BU fractions were similar to the control group. No toxic effects or animal deaths were caused. However, DCM fraction caused the death of bees in the concentration of 7.4 mg/mL, resulting in a survival rate of approximately 70% after 15 days of observation. The extracts of *S. buxifolia* and copaiba oil showed no toxic effects after 15 and 10 days following the treatment, respectively, using the same method described in the present article (Santos et al., 2012; Boligon et al., 2013a). Brazil has a very rich biological diversity. There are numerous medicinal and pharmacological properties in this vast diversity that should be explored. In the area of honeybee health, the application of alternative natural substances might be a novel method to control AFB. An optimal elimination of *P. larvae* in *A. mellifera* colonies would involve treatments with acceptable antimicrobial activity with no side effects on *A. mellifera*. This treatment would mean a further minimising of residues in honey and could act as a viable alternative to reduce antimicrobial resistance.

CONCLUSION

Brazil has a very rich biological diversity. The numerous medicinal and pharmacological properties should be explored. In the area of honeybee health, Buddleja extracts might be an unexplored way for future research involving alternative natural substances used to control the AFB. Elimination of *P. larvae* in *A. mellifera* colonies involves treatments with acceptable antimicrobial activity with no side effects on *A. mellifera*. Residues in honey and wax would be minimised. These treatments offer a viable alternative to reduce antimicrobial resistance. In this context, *B. thyrsoides* is a potentially useful alternative for suppressing bacterial diseases that affect the honeybee.

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