SUPPLEMENTARY MATERIAL

Susceptibly of *Alphitobius diaperinus* to *Beauveria bassiana* extracts

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Alphitobius diaperinus is an important pathogen with worldwide distribution that causes severe economic loss of efficiency in broilers. This study evaluates the potential of organic extracts of two strains entomopathogenic fungus *Beauveria bassiana* (CG71 and UNI40) as a biocontrol agent on *A. diaperinus* and promotes the phytochemical investigation. The effective percentages of mortalities were 95.97% (UNI40 methanolic extract), 69.23, 64.64, and 50.39% (CG 71 methanolic, ethyl acetate and butanol extracts). However, there was a decrease in the lesser mealworms susceptibility in relation to the use of insecticides and extracts. The metabolites 5-hydroxymethyl-2-furanoic acid, dipicolinic acid and monomethyl dipicolinate were isolated from ethyl acetate extract, and *β*-adenosine of butanolic extract of B. bassiana CG 71. In addition, the cyclodepsipeptides were identified in methanolic extracts of the two strains. The insecticide activity results indicated that the *B. bassiana* extracts are an alternative to *A. diaperinus* control.

Origin and Preservation of Beauveria bassiana

The entomopathogenic fungal strains are preserved in the culture collections of Embrapa Genetic Resources and Biotechnology (CG 71), and the State University of Cascavel (CG 71 and Unioeste - UNI 40), and the State University of Londrina (CG 71 and UNI 40). Both strains are also preserved at -80 °C.

Morphological Characterization

The strains were preliminarily identified as *Beauveria bassiana* based on the macro- and micromorphological characteristics of colonies, conidia and conidiophores after cultivation for 7 days at room temperature on Malt Extract Agar (Himedia, Mumbai, India). The CG 71 and Unioeste 40 strains were previously identified as *B. bassiana* by DNA sequencing and phylogenetic analysis (Daniel et al. 2017).

Culture of Beauveria bassiana

B. bassiana strains were cultivated on potato-dextrose-agar (PDA; Himedia, Mumbai, India) at 25 °C for 7 days. Afterwards, three agar disks (8-mm diam.) were aseptically pre-inoculated into four Erlenmeyer flasks of 250 mL containing 60 mL of a supplemented liquid medium (ML): 30.0 g glucose; 20.0 g malt extract; 2.0 g bactopeptone; 1.0 g yeast extract; 0.5 g KCl; 0.5 g $MgSO_4.7H_2O$; 0.5g KH_2PO_4; 1 L water. The flasks were incubated in a rotary shaker (200 rpm) for 8 days. These seed cultures (240 mL of each strain) were used to aseptically inoculate into ten litres ML medium, by sterile pipette of 50 mL, 24 mL for each seed culture strain into fifty 1-L Erlenmeyer flasks containing 200 mL of medium. The fungal cultures were left stationary at 25°C for 21 days under a photoperiod of 12h. One flask of each culture medium was not inoculated and used as a control.

Fungal cultures in ML were harvested by filtration. The filtrated liquid was extracted with ethyl acetate to liquid-liquid extraction (3 × 300 ml for each 1-L Erlenmeyer flasks), room temperature producing the ethyl acetate extract. After evaporation under reduced pressure at 40°C, the yield of these extracts was CG71A (4.7600g) and UNI40A (4.9623g). After lyophilization, the mycelial masses were

entirely extracted at room temperature with methanol (MeOH, 3 × 200 ml for each biomass contained in the 1-L Erlenmeyer flasks). The yield of these extracts was CG71M (18.8054g) and UNI40M (14.6802g). The methanol extract CG71M was partitioned with butanol (6 × 500 ml) at room temperature yielding CG71B (5.0358g) for liquid-liquid fractionation. The organic solvents were removed by vacuum distillation at 55 and 60 °C using a rotor evaporator.

Extraction and Chromatographic fractionation

The crude extracts of the CG 71 strain were fractionated through a Sephadex LH-20 (1.75 mm x 0.3 Ø) column, eluting with methanol (MeOH), yielded 94, 106 and 104 fractions from ethyl acetate, methanolic and butanolic extracts, respectively. The fractions analysis was accompanied by silica gel thin-layer chromatography (TLC) visualized under UV light at 254 and 365 nm and vanillin reagent, followed by heating the plate to 100 to 110 ºC for 5 min. The fraction 62-67 (700 mg) obtained from the ethyl acetate extract (CG71A) was rechromatographed in Sephadex LH-20 (MeOH), resulting in two more purify subfractions 11-42 (190 mg) and 49 (430 mg). The subfraction 11-42 was eluted in Sephadex LH-20 (MeOH) yielded 5-(hydroxymethyl)furan-2-carboxylic acid or Sumiki's acid, (1, 26.2 mg). Subfraction 49 was submitted to recrystallization with 15 mL of the methanol at 60 ºC and freezing for 12 hours at room temperature, yielding the mixture of dipicolinic acid and monomethyl dipicolinate in ration 5:1 (2 + 3; 44.6 mg).

The fraction 50-73 (250 mg) from the butanolic extract (CG71b) was rechromatographed in Sephadex LH-20 (MeOH), yielding 9-*β*-Adenosine (4; 22.3 mg). Furthermore, methanolic extract was detected in the mixtures of cyclodepsipeptides (Daniel et al. 2017): beauvericin (5), beauvericin A or F (6 and 6a), beauvericin E (7) and bassianolide (8).

The crude ethyl acetate and methanol extracts of UNI 40 strain were passed through a Sephadex LH-20 (MeOH) to obtain 115 and 102 fractions, respectively. Some fractions of ethyl acetate extract contained peptides with a mass of above 1000 ppm (data not shown), and fractions of methanolic extracts contained also the same cyclodepsipeptides reported above for analysis of mass spectra (Daniel et al. 2017).

For TLC analyses plates of silica gel 60 (Macherey-Nagel®, Germany) and SephadexTM LH-20 (GE Healthcare®, Sweden) were used. Mass analyses used for

elucidation of 1-4 compounds were realized in Thermo Scientific, Ion Trap, LTQ-XL and all the solutions were filtered through a 0.22 m membrane. The elucidation of the compounds was achieved by 1H and 2D (HSQC and HMBC) NMR spectroscopy in Bruker® Ascend600 (600 MHz for H, 125 MHz for C) and compared with published data. For NMR analyses methanol-d_4 (Cambridge Isotope Laboratories, USA) were used.

Insect culture

The *A. diaperinus* adults were collected from a unique poultry house (23°23'32.5"S 51°13'43.2"W) with a history of pyrethroids and organophosphates use, located in Londrina, Brazil, on October 2011, March 2012, April 2012 and 09 May 2013.

On the day prior to the start of each experiment, the beetles were extracted from samples of broiler-house litter by sieving through 0,2 mm screens. After they were transported to the laboratory, separated from the substrate, fed with bird feed and maintained at room conditions until the experiments were carried out.

Bioassay to control Alphitobius diaperinus adults

Insects used in the bioassays were collected on dates related above due to the lack of *A. diaperinus* in the cleaning and application of insecticides.

The CG 71 and Unioeste 40 extracts were tested using 1.0 % (g/mL). Adult insects were submerged for 30 s in 5 mL of extracts prepared with sterilized distilled water, dimethyl sulfoxide DMSO (10%) and Tween 20 (0.02%). The solution containing only the DMSO and Tween 20 was used as a negative control. Furthermore, pyrethroids group insecticide lambdacyhalothrin 0.03% Icon Vet – Syngenta® (Matias 1992) and neem oil 1.0 % NATUNEEM - Natural Rural® (Marcomini et al. 2009) were used as positive controls. Five replicates containing 30 insects were prepared for each concentration, totaling 150 insects per treatment. After immersion, the insects were placed into plastic Petri dishes (9-12 cm diameter) on sterilized humidified filter paper and bird feed. The insects were maintained in an incubator (25 ± 1ºC and a 12-hour photoperiod) and evaluated daily over a period of fourteen days. The dead insects were placed in another petri dish for mortality confirmation. The bioassays methods were adapted from those previously described by Santoro et al. (2008).

Insect susceptibly or mortality percentage (%) was calculated from the equation:

[(average live in negative control – average live in test)/average live in negative control X 100)]

Statistical analysis

The results were analyzed from the statistical analysis (Montgomery 2001). Mortality, day of test and dish factor data were all analyzed with one-way analysis of variance (ANOVA). P values <0.05 proofs that the means of the variable response differs between factors (time and treatments). The data was analyzed by R software Core Team 2013 (version 3.2.0).

References

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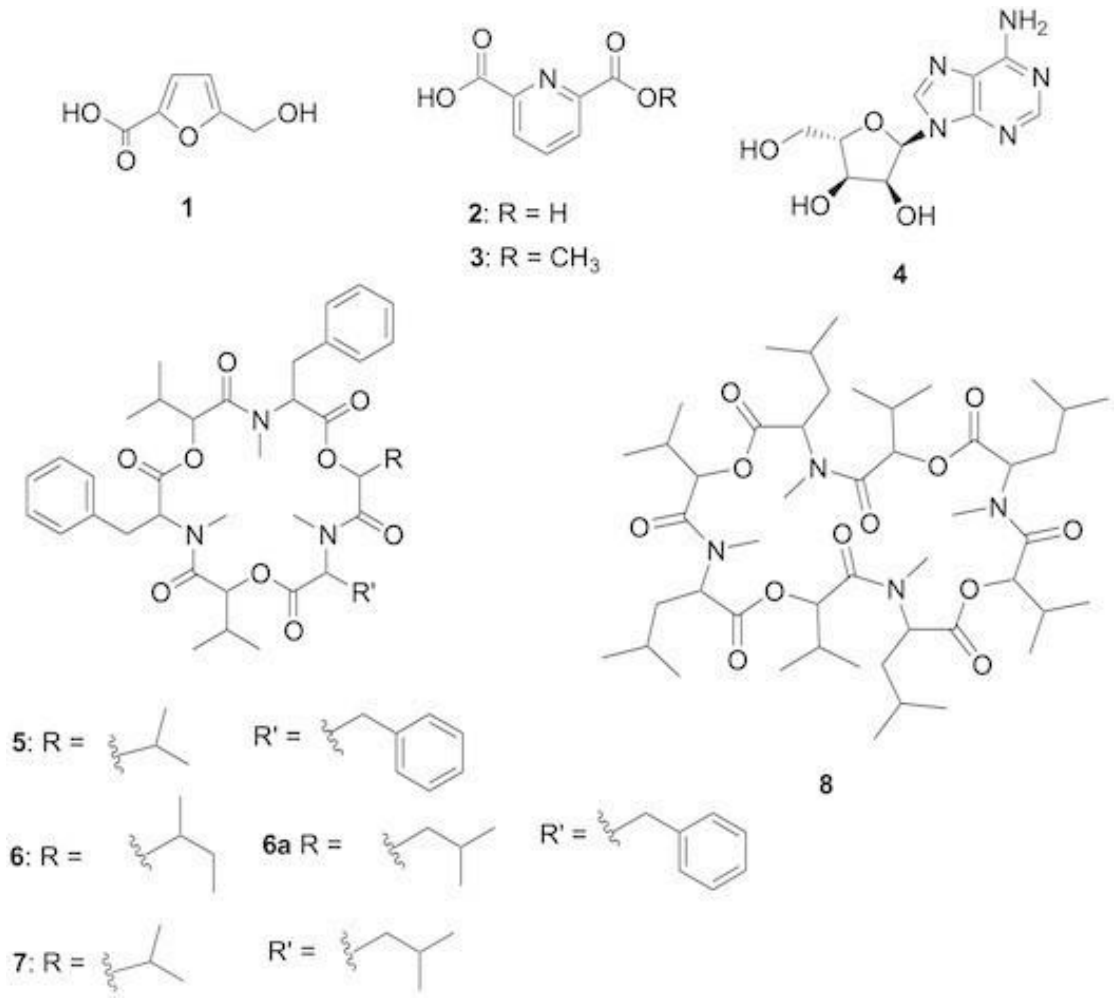

Figure S1: Structures of compounds isolated from ethyl acetate (1-3), from butanolic (4) of CG71 extracts, and identified (5-8) of *Beauveria bassiana* CG71 and UNI40 methanolic extracts.

Table S1: Percentage mortality (%) *Beauveria bassiana* (CG71 and UNI40) extracts (concentrations of 1.0 %) on *Alphitobius diaperinus*.

	Date			
Extracts[1]	**03/10/11**	**03/22/12**	**05/26/12**	**05/09/13**
Percentage mortality (%)				
***Beauveria bassiana* (CG71 and UNI40) extracts (concentrations of 1.0 %)**				
CG71M	32.88^{a,A} (65.79)	69.23^{a,B} (46.44)	-	5.26^{a,A} (13.81)
CG71A	33.02^{a,AB} (53.25)	64.64^{a,A} (28.36)	39.56^{a,AB} (13.04)	6.58^{a,B} (12.69)
CG71B	-	-	50.39^{ac,AB} (7.12)	1.04^{a,B} (9.41)
UNI40M	44.96^{a,A} (36.45)	-	95.97^{b,B} (2,35)	13.20^{a,A} (10.72)
NEEM OIL[2]	-	37.88^{a,A} (13.84)	74.86^{ab,B} (15.56)	14.22^{a,A} (12.87)
INSECTICIDE[3]	68.97^{b,A} (35.79)	37.88^{a,AC} (13.84)	82.38^{b,A} (5.50)	8.27^{a,BC} (7.36)

Notes: - assay not realized; Values followed by the same uppercase letter in the line, lowercase in the column and superscripts do not differ statistically according to the Scott-Knott ($p \leq 0.05$). [1]Extracts – CG71M and UNI40: methanolic extracts; CG71A: ethyl acetate extract; CG71B: butanol extract. [2]Neem oil: concentration of 1%; [3]Lambdacyhalothrin: concentration of 0.03%.