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Acaricidal action of destruxins produced by a marine-derived *Beauveria felina* on the bovine tick *Rhipicephalus* (*Boophilus*) *microplus*

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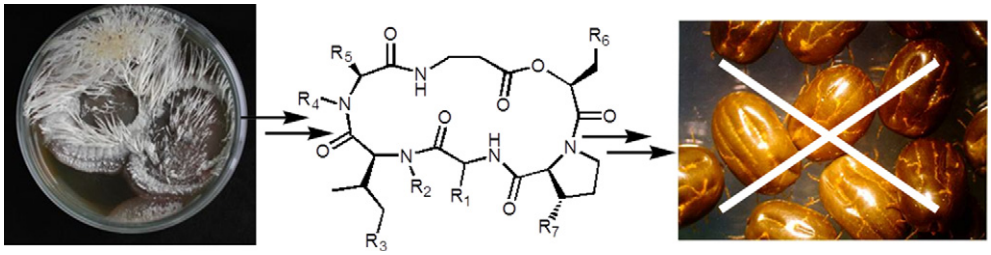
HIGHLIGHTS

- The tick *Rhipicephalus* (*Boophilus*) *microplus* is a major cattle pest.
- The enthomopathogenic fungus *Beauveria felina* produces destruxins.
- Destruxins were obtained from *B. felina* growth media.
- Destruxins displayed 30% inhibition of *R.* (*B.*) *microplus* engorged females development.
- Destruxins have potential to be developed as ticks' biological control.

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing resistance of *Rhipicephalus* (*Boophilus*) *microplus* tick to commercial insecticides requires alternative methods for the control of this cattle plague. The enthomopathogenic fungus *Beauveria felina* produces destruxins in culture media, cyclic depsipeptides which display an array of biological activities. The present investigation aimed to evaluate the acaricide action of destruxins isolated from *B. felina* culture media on *R.* (*B.*) *microplus* engorged females. *B. felina* was grown in MF medium under 19 different growth conditions. HPLC-PDA analysis of chromatographic fractions obtained from the 19 different growth media extracts indicated the presence of destruxins in all lipophylic fractions. Such fractions were combined and subjected to separation by HPLC. Fractions containing distinct destruxins composition were tested against *R.* (*B.*) *microplus*. Two fractions, composed of destruxin Ed_1 and pseudodestruxin B and/or pseudodestruxin C (fraction **P1**) as well as by hydroxyhomodestruxin B and/or destruxin D and/or roseotoxin C (fraction **P7**), displayed 30% and 28.7% acaricidal efficacy, respectively. This activity profile in such low concentration is adequate to consider destruxins as potential leading compounds to be developed for tick biological control.

1. Introduction

The bovine tick *Rhipicephalus* (*Boophilus*) *microplus* Canestrini, 1888 (Murrell and Barker, 2003) is considered the major cattle parasite and accounts for productivity losses which amount to US$ 2 billion/year. Chemical acaricides are usually employed in the control of ticks. However, acaricide-resistant ticks are of increasing occurrence, and, therefore, new and effective alternative tick control methods are required (Grisi et al., 2002; Borges et al., 2011).

Metarhizium anisopliae (s.l., Metchnikoff, 1879) and *Beauveria bassiana* (Bals.-Criv.) Vuill. 1912 fungi strains have demonstrated acaricidal effects on *R.* (*B.*) *microplus* (Fernandes et al., 2011; Sousa et al., 2011; Bahiense et al., 2007, 2008). Among sixty fungi strains tested against *R.* (*B.*) *microplus*, five *Beauveria* spp. strains caused 30% mortality of this tick after 10 days of treatment (Fernandes et al., 2011). An extract composed of 0.25% of immature *Melia azedarach* (L.) fruits and a suspension of 2.4×10^8 *B. bassiana* conidia resulted in less engorged females of *R.* (*B.*) *microplus*, indicating a

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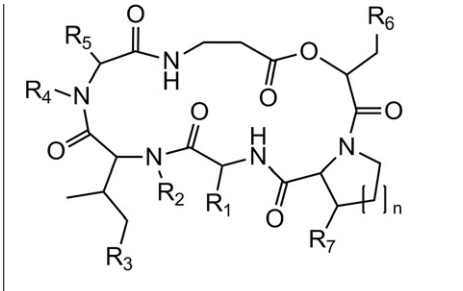
possible synergistic effect of the plant extract with the fungal mycelia (Sousa et al., 2011). *M. anisopliae* was evaluated against different stages of *R.* (*B.*) *microplus* using the stall test for 28 days. Treatments were performed in the bovines applying *M. anisopliae* conidal suspension by aspersion. The egg production index presented reduction in the second day post treatment. The treated group showed mortality percentage 33% higher than the control group. Larvae was more susceptible to the fungus, presenting highest mortality after 17 days of treatment (Bahiense et al., 2007). In another study, *M. anisopliae* and its combination with deltamethrin were evaluated against *R.* (*B.*) *microplus* in calves. The mean mortality rate was 32.57% in the groups treated with *M. anisopliae*, 38.58% in those groups treated with deltamethrin, and 30.92% in those treated with fungus and combined with deltamethrin. Additionally, ticks that dropped off from calves after 2 days had a lower nutrient index (NI) and egg production index (EPI) than those that dropped off at a later day after treatment (Bahiense et al., 2008).

The fungi *M. anisopliae*, *B. bassiana* and *Beauveria felina* (DC.) J.W. Carmich. 1980 produce destruxins (Fig. 1), cyclic hexadepsipeptides which display activities such as insecticidal, phytotoxic, cytotoxic, anti-viral and inhibition of ATPases (Pedras et al., 2002; Vázquez et al., 2005). Destruxins also affect the fertility and viability of arthropod incubated eggs (Ojeda-Chi et al., 2011). However, to the best of our knowledge there are no studies on the acaricidal activity of destruxins against *R.* (*B.*) *microplus*. The present investigation aimed to evaluate the acaricidal activity of these substances on *R.* (*B.*) *microplus* engorged females.

2. Material and methods

2.1. B. felina growth and destruxins production

The fungus *B. felina* was isolated from the marine alga *Caulerpa* sp., collected at the Cabelo Gordo beach, (São Sebastião, SP, Brazil), in April 2002. The fungus identification has been previously reported (Vita-Marques et al., 2008). *B. felina* was cultivated in MF medium, composed of of 2% glucose (Acumedia), 1% starch (Synth), 2% soytone (Acumedia), 0.5% peptone (Acumedia), 0.03% meat extract (Acumedia) and 0.5% yeast extract (Acumedia) (Kakeya et al., 1995). The *B. felina* strain CBMAI 738 used in this work is deposited at Coleção Brasileira de Micro-organismos de Ambiente e Indústria – CBMAI (CPQBA, Universidade Estadual de Campinas).



R_1 = CH(Me)CH_2Me or CH_2Ph or $CHMe_2$
R_2 = Me or H
R_3 = Me or H
R_4 = Me
R_5 = Me or $CHMe_2$ or CH2$CHMe_2$
R_6 = $CHOHCH_2OH$ or $CHOHCH_2Cl$
or $CH{=}CH_2$ or $CHMe_2$ or $CHMeCH_2OH$
or CHOHMe or CMeMeOH or $CHMeCO_2H$
R_7 = Me or H
n = 1 or 2

Fig. 1. Destruxins isolated from entomopathogenic fungi.

Growth conditions were tested using a previously described experimental design procedure (Pimenta et al., 2010), with distinct concentration of nutrients, of saline concentration in artificial sea water (ASW), temperature, time of growth and medium pH. As described by Pimenta et al. (2010), 16 different conditions were tested, in addition to three additional growth experiments with average growth conditions for each growth parameter (see Table 3 in Supportive Material).

2.2. Isolation of fractions enriched in destruxins

At the end of each growth experiment, liquid media were filtered through glass wool. Liquid media were then subjected to solid-phase extraction (SPE) on a C_{18} reversed-phase silica gel column (10 g), eluted with 100% H_2O (discarded), 75:25 H_2O/MeOH (fraction F1), 50:50 H_2O/MeOH (fraction F2), 25:75 H_2O/MeOH (fraction F3) and 100% MeOH (fraction F4), and the fractions were evaporated to dryness. Aliquots of fractions F1–F4 were analyzed by HPLC, using a C_{18} reversed-phase silica gel analytical/semi-preparative column (Phenomenex Synergi Fusion RP80 C_{18}, 250 × 4.6 mm, 4 μm pore) with a gradient of MeOH in H_2O (95% H_2O during 1 min, then a linear gradient from 95% H_2O to 95% MeOH during 25 min, then 10 min at 95% MeOH, then back to 95% H_2O during 5 min to re-equilibrate to the initial gradient conditions), with a flow rate of 1 mL/min and detection at λ_{max} 220 nm. Standards of the destruxin derivatives pseudodestruxin C, roseotoxin B and [Phe^3-*N*-Me-Val^5] destruxin B (Lira et al., 2006) were used to check the presence of destruxins. Destruxins were observed in fractions F3 and F4 obtained by SPE of the growth media of each of the 19 growth experiments. Fractions F3 were all pooled and renamed as BfMF-F3, as well as all fractions F4 (BfMF-F4), since their compositions were very similar.

The pool of F3 fractions, BfMF-F3, was separated by HPLC, using a C_{18} reversed-phase silica-gel analytical column (CSC-Inertsil 150A/ODS, 2.25 × 0.94 mm, 5 μm pore) using a photo-diode array detector and 38:37:21 MeOH/MeCN/(0.1 HCO_2H solution in H_2O) as eluent, during 40 min and 1 mL/min flow rate, monitoring at λ_{max} 220 nm. The HPLC separation yielded thirteen fractions (BfMF-F3-**P1** to BfMF-F3-**P13**), each of which was analyzed by RMN-^{1}H (DMSO-d_6, 400 MHz). Seven of these fractions were subjected to assays against *R.* (*B.*) *microplus* tick: BfMF-F3-**P1** (12.0 mg) BfMF-F3-**P2** (4.7 mg), BfMF-F3-**P4** (2.2 mg), BfMF-F3-**P5** (9.4 mg), BfMF-F3-**P6** (2.7 mg), BfMF-F3-**P7** (9.3 mg) and BfMF-F3-**P8** (3.3 mg) (Fig. 2).

2.3. Immersion test of engorged females

In vitro tests were performed at the Animal Health Laboratory of the Embrapa Southeast Livestock unit (Embrapa Pecuária Sudeste – CPPSE), in São Carlos, São Paulo. The immersion test of engorged females was performed in following the procedures reported by Drummond et al. (1973). *R.* (*B.*) *microplus* engorged females, measuring more than 4.5 mm, were collected from naturally infested cattle at the CPPSE farm. In the laboratory, ticks were washed with water and dried on paper towels, then selected according to their integrity, motility, and maximum engorgement. After this selection, ticks were divided in groups of ten and weighed to establish their uniformity (group average weight of 2.2 g) and placed in disposable cups. Solutions of destruxin fractions were prepared in 2% Tween 80 and distilled H_2O. Toxicity limits have been determined previously on *R.* (*B.*) *microplus* (Chagas et al., 2003). Each group of ten females was immersed in 0.2 mg/mL solutions of the fractions BfMF-F3-**P1**, BfMF-F3-**P2**, BfMF-F3-**P5**, BfMF-F3-**P6**, BfMF-F3-**P7** and BfMF-F3-**P8** and in a 0.1 mg/mL solution of fraction BfMF-F3-**P4**, during

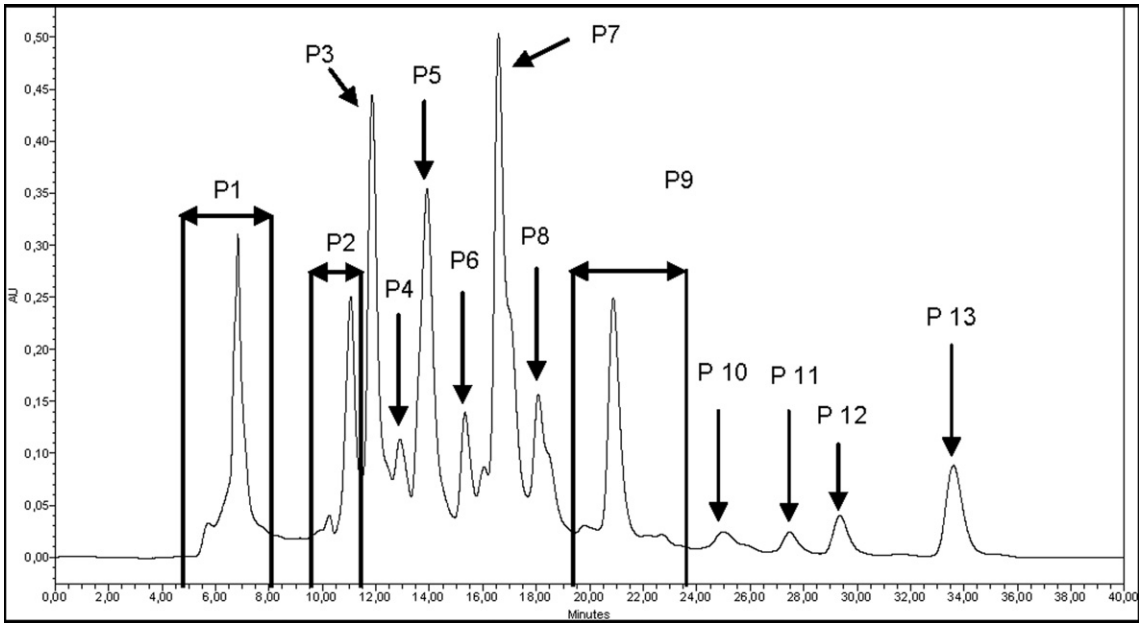


Fig. 2. HPLC chromatogram of the extract BfMF-F3 obtained from *Beauveria felina* culture media. Column: C_{18} reversed-phase silica-gel (CSC-Inertsil 150A/ODS, 2.25 × 0.94 mm, 5 μm pore); eluent: 38:37:21 MeOH/MeCN/(0.1 HCO_2H solution in H_2O).

5 min. A control solution of 2% Tween 80 and distilled H_2O was also prepared to the control group. The tests were performed in duplicate for all fractions and control group. After immersion, the females were removed, dried and placed in an incubator (±27 °C, RH > 80%) for oviposition. After 18 days, the eggs from each group were weighed and placed back in the incubator under the same conditions for larval hatching, which was read visually per group after 16 days of incubation. The same assay was performed for fractions BfMF-F3-**P1** and BfMF-F3-**P7**, as well as for the pooled fractions, in order to check for a possible synergistic activity of fractions obtained from *B. felina* growth medium.

The estimated production (ER) of the engorged female and the acaricide efficacy percentage (E) of each of the *B. felina* SPE fraction tested were calculated according to the equations indicated below (Drummond et al., 1973).

$$ER = \frac{\text{egg mass weight} \times \% \text{ hatching} \times 20.0000}{\text{weight of engorged females}} \quad (1)$$

$$E = \frac{\text{control ER} - \text{treatment ER} \times 100}{\text{control ER}} \quad (2)$$

2.4. Statistical data analysis

Means and standard errors were calculated for female and egg weights, egg hatching and female estimated reproduction. The average comparisons of theses parameters were performed by LSMEANS and the Tukey test at 5% significance. Microsoft Excel (2010) was used for statistical analysis.

3. Results and discussion

Destruxins are cyclic depsipeptides which display an array of biological activities, including antibiotic, antitumoral and insecticidal (Liu and Tzeng, 2012). The potent insecticidal activity of destruxins is responsible for the entomopathogenicity of *M. anisopliae* and *B. bassiana*. Conidia of these fungi are currently used in biological control of different insect pests (Liu and Tzeng, 2012). However, the acaricidal activity of destruxins has not been previously reported in the literature.

We have tested 19 distinct growth conditions for destruxin production by *B. felina*. However, after SPE separation of liquid media of each growth experiment and HPLC analysis, we observed that SPE fractions F3 and F4 from the liquid media of each growth experiment presented almost identical composition in destruxins. The results obtained suggested that *B. felina* growth and destruxin production in MF medium are practically not sensible to the growth conditions. Fractions BfMF-F3-**P1** and BfMF-F3-**P7** displayed the highest efficacy against *R.* (*B.*) *microplus* engorged females as well as on the inhibition of their reproductive efficiency. HPLC–UV–MS analyses of these fractions indicated the presence of destruxin Ed_1 (**1**) and pseudodestruxin B (**2**) and/or pseudodestruxin C (**3**) (both of which are isobaric) in fraction BfMF-F3-**P1**, and of destruxins hydroxyhomodestruxin B (**4**) and/or destruxin D (**5**) and/or roseotoxin C (**6**) (the three are isobaric) in fraction BfMF-F3-**P7** (Table 1).

Data in Table 2 indicate that fractions BfMF-F3-**P1** and BfMF-F3-**P7** displayed the most potent inhibitory activity on *R.* (*B.*) *microplus* females reproduction, at 65.36 and 66.61, respectively. The same fractions displayed the best acaricidal efficacy, of 30% and 28.7%, respectively. However, when tested together, in order to check for a possible synergistic action, fractions BfMF-F3-**P1** and BfMF-F3-**P7** presented lower efficacy, of only 1.5%. The whole extract of pooled SPE fractions BfMF-F3 displayed no activity at all, indicating that either the concentration of the active destruxin(s) is too low or that the presence of inactive destruxins may mask the activity of the active ones.

Fractions BfMF-F3-**P1** and BfMF-F3-**P5** contain destruxin Ed_1 (**1**). Since fraction BfMF-F3-**P1** was active against *R.* (*B.*) *microplus* but not fraction BfMF-F3-**P5** (Table 2), it seems logical that destruxin Ed_1 (**1**) should not be active, but rather **2** or **3**. Considering that the biological activities of destruxins correlate with the nature of the α-hydroxy acid group ($-R_6$), the results we observed are not unexpected. In fact, destruxins with less polar α-hydroxy acid chains are usually more potently bioactive than those destruxins with more polar α-hydroxy acid chains (Pedras et al., 2002).

Table 1
Destruxins present in the active fractions BfMF-F3-**P1** and BfMF-F3-**P7** isolated from *Beauveria felina* culture media.

Compound	Name	MW (Da)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	*n*
1	Destruxin Ed_1	625	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHOHCH_2OH$	H	2
2	Pseudodestruxin B	669	CH_2Ph	Me	H	Me	CH_2CHMe_2	$CHMe_2$	H	1
3	Pseudodestruxin C	669	CH_2Ph	Me	H	Me	$CHMe_2$	$CHMe_2$	Me	1
4	Hydroxyhomodestruxin B	623	$CH(Me)CH_2Me$	Me	Me	Me	Me	$C(Me)_2OH$	H	1
5	Destruxin D	623	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMeCO_2H$	H	1
6	Roseotoxin C	623	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMeCH_2OH$	Me	1

Table 2
Means of the females weight (FW), egg mass weight (EW), percentage of egg hatching (EH), estimated reproduction (ER), and efficacy of the BfMF-F3 fractions (E) in engorged females of *Rhipicephalus* (*Boophilus*) *microplus*, tested by means of immersion test.

Fractions	FW (g)	EW (g)	EH (%)	ER	E (%)
Control	2.23 ± 0.03	1.10 ± 0.13a	97.50 ± 3.54a,b	93.35 ± 9,40a	—
BfMF-F3-**P1**	2.22 ± 0.01	0.85 ± 0.00b	85.00 ± 0.00b	65.36 ± 0,04c	30
BfMF-F3-**P2**	2.22 ± 0.00	0.90 ± 0.05a,b	95.00 ± 0.00a,b	77.37 ± 3,88b,c	17.1
BfMF-F3-**P4**	2.21 ± 0.00	0.99 ± 0.04a,b	87.5 ± 3.54a,b	78.48 ± 0.30a,b,c	15.9
BfMF-F3-**P5**	2.23 ± 0.01	1.00 ± 0.01a,b	97.5 ± 3.54a,b	87.78 ± 2.13a,b	6
BfMF-F3-**P6**	2.25 ± 0.01	0.93 ± 0.02a,b	92.5 ± 3.54a,b	76.44 ± 1.30b,c	18.2
BfMF-F3-**P7**	2.23 ± 0.01	0.78 ± 0.08b	95.00 ± 7.07a,b	66.61 ± 2.12c	28.7
BfMF-F3-**P8**	2.25 ± 0.03	0.88 ± 0.00a,b	100 ± 0.00a	78.01 ± 1.29b,c	16.5

Different letters in the same column represent a difference ($p \leqslant 0.05$) within the same parameter.

Pedras et al. (2002) discussed the insecticide action of destruxins on different insects (*Bombix mori, Cetonia aurata, Epilachna sparsa, Galleria mellonella, Heliothis virescens, Musca domestica, Oryctes rhinoceros, Phaedon cochleariae, Plutella xylostella* and others), causing muscle paralysis to death. Destruxins bearing hydrophylic α-hydroxyacids are less active. In the present investigation, we observed that fractions containing the polar destruxins Ed_1 (**1**) and destruxin E diol were not active, as previously observed for the insecticidal activity (Pedras et al., 2002). On the other hand, the double bond-containing α-hydroxyacid destruxin A is potently insecticidal (Pedras et al., 2002). However, destruxin A containing fraction BfMF-**P8** was inactive against the tick *R.* (*B.*) *microplus* in the present work (Table 2). This result is in agreement to those observed by Gôlo et al. (2011), who tested the toxicity of destruxin A in concentrations of 5, 10 and 20 μg/mL on *R.* (*B.*) *microplus* engorged females, but observed no activity at all. Therefore, the activity profile of destruxins varies depending on the parasites on which they are assayed (Strasser et al., 2000). Destruxins were evaluated at concentrations of 0.2 mg/mL, below the concentrations tested for commercial acaricides such as ivermectin, doramectin, moxidectin and abamectin, used in their preparations at concentrations of 10 mg/mL (Oliveira et al., 2006). Our results indicate that some destruxins may be more potent than some of the commonly used acaricides, such as ectoban, ciperthion, alatox, M3 ecto, suppocade and cipermetrine (Neto and Toledo-Pinto, 2006; Spagnol et al., 2010).

4. Conclusion

In conclusion, we have observed that fractions from the growth culture medium of the marine-derived fungus *B. felina* containing destruxins Ed_1 (**1**) and pseudodestruxin B (**2**) and/or pseudodestruxin C (**3**), and hydroxyhomodestruxin B (**4**) and/or destruxin D (**5**) and/or roseotoxin C (**6**) displayed an acaricidal activity of about 30% against the tick *R.* (*B.*) *microplus*. Since the concentrations at which the destruxins were evaluated were low, further studies to evaluate the acaricidal activity of pure destruxins are necessary to establish their actual activity potency. This will be possible after a scale-up of destruxin production, in order to better evaluate the potential of destruxins as acaricides against *R.* (*B.*) *microplus*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2012.08.011.

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