Experimental and Applied Acarology (2022) 87:351–363
https://doi.org/10.1007/s10493-022-00736-5

The association of extracts of *Achyrocline satureioides* and the fungus *Beauveria bassiana* against the tick *Rhipicephalus microplus*

Rafaela Regina Fantatto[1] · Yousmel Alemán Gainza[2] · Amanda Figueiredo[2] · Rodrigo Sorrechia[1] · Ana Carolina de Souza Chagas[2] · Rosemeire Cristina Linhari Rodrigues Pietro[1]

Received: 12 October 2021 / Accepted: 16 July 2022 / Published online: 24 August 2022

Abstract
The tick *Rhipicephalus microplus* (Canestrini) is a major economic hurdle to the global livestock industry. The incorrect and indiscriminate control carried out by synthetic chemical compounds has contributed to the emergence of parasite resistance present today to many products available in the veterinary market. Effective, economically viable and potentially safe alternatives need to be investigated, including herbal medicine and biological control by entomopathogenic fungi. In this study, extracts from *Achyrocline satureioides* (Lam.) DC. obtained by extraction with solvents of different polarities associated and not associated with the entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin were evaluated on different stages of the life cycle of *R. microplus*. The activity of plant extracts, fungus and their associations in ticks was analyzed in vitro through the adult immersion test, larval packet test and larval immersion test. The highest efficacy on engorged females (43.3%) was reached by the hexanic partition (ASh) at 5 mg/mL + fungus, reducing larval hatchability. ASh associated with *B. bassiana* also caused high mortality of larvae, reaching LC_{50} of 6.23 and 2.65 mg/mL in the packet and immersion tests, respectively. The results obtained showed that extracts, when used in combination with the fungus, have their action enhanced, reaching 100% mortality of larvae. Among the evaluated methodologies, it was observed that the larval immersion test allows better assessment, due to the time of contact with the larvae. The results obtained with *A. satureioides* associated with the fungus are considered promising and open new perspectives for future product development.

Keywords *Rhipicephalus microplus* · *Achyrocline satureioides* · *Beauveria bassiana* · Synergistic effect

Extended author information available on the last page of the article

Introduction

The tick species *Rhipicephalus microplus* (Canestrini) (Acari: Ixodidae) parasitizes cattle throughout the world. Due to its high prevalence and wide distribution, *R. microplus* is responsible for large losses related to animal mortality, in the levels of reduction in cattle breeding, reproductive disorders and transmission of disease-causing pathogens as babesia (*Babesia bovis* and *Babesia bigemina*) and anaplasma (*Anaplasma marginale*), responsible for the Bovine parasite sadness (Rodríguez-Vivas et al. 2014; Singh et al. 2014).

Currently, the control of *R. microplus* is predominantly done with the use of chemical acaricides, composed of active ingredients belonging to the classes of macrocyclic lactones, organophosphates, formamidines, synthetic pyrethroids, phenylpyrazoles and growth inhibitors (Reck et al. 2014). However, constant use against ticks, sometimes without adequate technical criteria, has resulted in the selection of resistant tick populations (Klafke et al. 2017). The development of new synthetic compounds is a long and expensive process, which reinforces the need for alternatives to control tick infestations (Chagas et al. 2012; Rashid et al. 2019).

Studies of sustainable alternatives have generated great interest from commercial, medical and scientific communities in various countries, as phytotherapy and biological control have the potential to reduce the use of chemotherapy agents currently used to control ectoparasite infestations (George et al. 2014; Politi et al. 2012). *Achyrocline satureioides* (Lam.) DC. is a species in the Asteraceae family, common in Brazil, occurring mainly from Minas Gerais to Rio Grande do Sul, growing spontaneously in pastures (De Souza et al. 2002; Retta et al. 2012). This species already presents activity against several insect species such as simuliids (borrachudo) reported by Dal Magro et al. (1998), *Triatoma infestans* (barber) (Rojas de Arias et al. 1995) and *Myzus persicae* (peach green aphid) (Saupe 2003) besides larvicidal activity against *Aedes fluviatilis* (Macêdo et al. 1997).

Another important strategy to control parasites involves biocontrol through entomopathogenic fungi. Combined with its high capacity to control pests, it also has the advantage of causing less environmental impact than chemical pesticides, being efficient and safe (Zimmermann 2007; Fernandes et al. 2008; Lacey et al. 2015). The mode of action of fungi on arthropods is the adhesion of conidia to the cuticle of terrestrial hosts (ticks, cockroaches, triatomines) and the formation of hyphae that penetrate the integument with the aid of secreted enzymes such as proteases, chitinases and lipases (Moraes et al. 2003; Santi et al. 2019). Toxins and organ destruction are what kills the ticks also with fungi.

Several studies have demonstrated the action of entomopathogenic fungi on the tick *R. microplus* in vitro particularly using *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin [Hypocreales: Cordycipitaceae]. Strains of *B. bassiana* are among the most promising agents for use against ectoparasites of veterinary importance, such as the tick *Amblyomma cajennense* (Reis et al. 2008), *Rhipicephalus sanguineus* (Prette et al. 2005), and *R. microplus* (Sun et al. 2013; Fernandes et al. 2008, 2012).

The great potential of plant extracts (Minho et al. 2020) and entomopathogenic fungi (Santi et al. 2019) on ectoparasites has been demonstrated, but in most of these studies the extraction yields are low and the concentrations required to achieve this effect are high, which often makes its use on a large scale unfeasible. An alternative for this would be the joint use aiming at a synergistic effect; however, some natural pesticides can act negatively on the fungi, inhibiting their growth and causing genetic mutations, factors that can lead to

reduced effectiveness (Islam and Omar 2012; Mohan et al. 2007; Ribeiro et al. 2012). To avoid such factors, testing is recommended to determine the minimum inhibitory concentration. Compatibility studies between pesticides and entomopathogenic fungi have been carried out almost entirely involving chemicals (Soares 2011), with a very small number of studies dealing with the compatibility of these microorganisms with natural pesticides (Luckmann 2013). Therefore, the objective of this study was to evaluate the efficacy of extracts from *A. satureioides* associated or not with the entomopathogenic fungus *B. bassiana* on different stages of the tick *R. microplus*.

Materials and methods

Plant material

The plant material used was obtained through a scientific cooperation agreement signed between the School of Pharmaceutical Sciences of Araraquara (FCFAr-UNESP), Araraquara, SP, Brazil, and the Pluridisciplinary Center for Chemical, Biological and Agronomic Research (CPQBA- UNICAMP), Campinas, SP, Brazil. The species *A. satureioides* obtained from the CPQBA Medicinal Plants Collection, whose cultivation was under the responsibility of Prof. Dr. Ílio Montanari Jr., was collected in the city of Campinas-SP (22°48'S, 47°07'W), and have been registered and authorized for use by SISGEN under registration number AD73F75.

Production and fractionation of plant extracts

Plant material was dried in an oven (Fanem, Guarulhos, SP, Brazil) with air circulation at 40 °C and later ground in a knife mill (Fabbe, São Paulo, Brazil). Ethanol extracts (ASb) were obtained from aerial parts of *A. satureioides* by the method of maceration 1:10 in absolute ethanol (Merck, Darmstadt, Germany). After 7 days, the contents were filtered, rotoevaporated (Rotary Evaporator Fisatom, São Paulo, Brazil) and placed in an amber bottle. Fractionation was carried out by the liquid-liquid partition method with high, medium and low polarity solvents, generating the partitions ethyl acetate (ASac), hexane (ASh) and water (ASwater). For fractionation 2 g of ethanolic extract was solubilized in 200 mL of ethyl acetate (Merck), centrifuged and its supernatant placed in a separating funnel together with 100 mL of distilled water, remaining for 45 min with stirring every 15 min, removing and adding water 3×. Then the acetate fraction was rotoevaporated and resuspended in ethyl acetate, which was placed in a separating funnel together with 50 mL of hexane (Merck). Both fractions were rotoevaporated and stored in a beaker at a temperature of − 80 °C.

Fungus growth conditions

Beauveria bassiana strain 487 used in the trials was obtained from the Embrapa Cenargen Collection and registered in the ARSEF database (Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, USA). Cultivation for maintenance and production of conidia was carried out in Petri dishes (10×2 cm) with Potato Dextrose Agar (PDA; Merck) for 7 days, kept in an oven (Fanem, Guarulhos, SP, Brazil) at 28 ºC.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC determination was performed according to the Clinical Laboratory and Standards Institute M38A2 (CLSI, 2008), with modifications, adapted in 96-well microplates and RPMI medium (Sigma Aldrich, Saint Louis, MO, USA) with 2% glucose buffered in MOPS (Sigma Aldrich) pH 7.2 and inoculum of 10^8 CFU/mL. Extracts and fractions were diluted in sterile water at a concentration of 40 mg/mL and 100 µL was added to the first well of the microplate containing 100 µL of RPMI medium (Sigma Aldrich). Serial dilution of the samples was then carried out, also including positive and negative controls. The inoculum was obtained by suspending the fungal cells in an aqueous solution of 0.1% Tween (Sigma Aldrich) for counting in a Neubauer chamber and the number of cells was adjusted to the cell concentration 10^8 CFU/mL. To test the acaricide effect of the fungal strains, they were added to microplates containing 100 mL of medium and 100 µL of fungal cell suspension. The microplate was kept for 7 days at 28 ºC in an oven with agitation at 120 rpm. Visual reading was performed for MIC determinations. After reading, a sample from each well was collected and inoculated in a Petri dish containing PDA medium, with the aid of a sterile toothpick. The Petri dish was kept in an oven at 28 °C for 5 days, and then a visual reading of growth inhibition was performed to determine the minimum fungicidal concentration (MFC). The MFC obtained supported the use of concentrations of crude extracts and fractions that were associated with the *B. bassiana* fungus without inhibiting it.

Preparation of fungal suspension for testing with *Rhipicephalus microplus*

To prepare the fungal suspension, the spores were scraped from the plates with the medium in which they were cultured and placed in 0.09% saline solution, after which, counting was performed in a Neubauer chamber to determine the volume needed to reach 10^6 spores/mL. In the suspension 10% of RPMI medium (Sigma Aldrich) was added so that the fungus had a source of nutrients to support its initial growth. The viability of the conidia was monitored by growing a sample of conidia inoculum placed in Petri dishes containing PDA medium (Merck), for 7 days, in an oven (Fanem) at 28 ºC.

Immersion test of females of *Rhipicephalus microplus*

Rhipicephalus microplus engorged females were collected from naturally infested cattle at Embrapa Pecuária Sudeste, São Carlos, SP, Brazil (21°57’42"S, 47°50’28"W). According to a resistance test carried out in 2019, the ticks in this herd were resistant to pyrethroids, organophosphates and amidines. The tests were conducted following the biocarrapaticidogram technique as described by Drummond et al. (1973), in which three homogeneous groups of 10 females per treatment/concentration were used.

The concentrations of the extract and fungus associations to be evaluated were determined using MFC values, which was found to be safe to start at 10 mg/mL. The treatments also had a negative control (water), solvent control (Tween 2%, Sigma Aldrich) and fungus control. The females of the test groups were immersed in the treatments for 5 min. Then they were gently dried and placed in Petri dishes that were placed in a biochemical oxygen demand (BOD) incubator (Eletrolab 2023) with an average temperature of 27 °C and > 80%

RH for 18 days. The humidity kept high inside the incubator was obtained by the presence of 2 L of sterile water inside the BOD tray, monitored daily and replaced when necessary. After 18 days, the egg laying was evaluated, with the eggs being weighed and placed in adapted disposable syringes and kept in the incubator for another 18 days. Reproductive parameters were provided for calculation of the percentage of posture reduction (=oviposition index) and performed according to the equations (Drummond et al. 1973; Politi et al. 2012):

$$Posture\,reduction\ (\%) = \frac{M_{eggs}}{M_{females}} \times 100,\ and$$

$$Hatchability\ reduction\ (\%) = \frac{Hatchability_{control} - Hatchability_{treated}}{Hatchability_{control}} \times 100,$$

where M_{eggs} is the mass of the eggs laid (g), $M_{females}$ is the mass of engorged females (g), $Hatchability_{control}$ is the oviposition index of the control group, and $Hatchability_{treated}$ is the oviposition index of the treated group.

The reproductive efficiency (%) and the product effectiveness (%) were calculated according to equations (Drummond et al. 1973; Politi et al. 2012):

$$Reproductive\ efficiency\ (\%) = \frac{M_{eggs} + \%\,hatching \times 20,000}{M_{females}},\ and$$

$$Product\ efficiency\ (\%) = \frac{reproductive\ efficiency_{control} - reproductive\ efficiency_{treated}}{reproductive\ efficiency_{control}} \times 100.$$

The results are expressed as mean ± SD.

Sensitivity of larvae in contact test on impregnated paper

The larval packet test (LPT) of *R. microplus* larvae was performed as recommended by FAO (1971). In this methodology approximately 100 larvae from engorged females collected at Embrapa Pecuária Sudeste were placed on filter paper measuring approximately 10 · 8 cm, impregnated with different concentrations for samples, negative controls (water + Tween 2% and fungus) and positive control [cypermethrin (15%), chlorpyrifos (25%) and citronella (0.1%)]. Each of these papers impregnated with extracts and/or *B. bassiana* at a concentration of 10^6 spores/mL was folded to form a 'sandwich' and sealed with plastic pegs. The envelopes were conditioned in an incubator at 27 °C and 80% RH. Three replicates were performed for each concentration, as well as for the control groups. After 7 days the test was read differentiating live from dead larvae with the aid of a vacuum compressor.

Larvae immersion test

In the larval immersion test (LIT), 300 larvae were added to 1 mL of the various solution concentrations to be tested that contained plant extracts and/or *B. bassiana* fungus. In this solution, 0.02% Triton was added to ensure immersion of the larvae. These larvae were immersed for 10 min. After this period, the larvae were placed in filter paper envelopes, sealed and conditioned in an Eletrolab EL2023 BOD incubator at 27 °C and 80% RH. After 48 h the test was read differentiating live from dead larvae with the aid of a vacuum compressor (Sabatini et al. 2001). Three replicates were performed for each concentration, as well as for the control groups: negative controls (water + 0.02% Triton and fungus) and positive control [cypermethrin (15%), chlorpyrifos (25%) and citronella (0.1%)].

Statistical analysis

Statistical analyses were performed using ANOVA followed by comparison of means using Tukey's honestly significant difference test ($\alpha = 0.05$) using ASSISTAT free software (https://assistat.software.informer.com/). The Probit procedure (SAS Institute, Cary, NC, USA) was used to calculate the lethal concentrations of 50% (LC_{50}) to reflect the treatment concentration that resulted in 50% parasite mortality and their respective 95% confidence intervals (95% CI).

Results

Immersion test of females of *Rhipicephalus microplus*

The immersion test of engorged females evaluated the effectiveness of extracts and associations on the female, egg laying and egg hatching. An increase in *A. satureioides* efficacy was observed when associated with entomopathogenic fungus (Table 1). This was mainly observed in the highest percentages. The negative control composed by 2% Tween was significantly different from most of the treatments, presenting 0% efficacy. The hexanic partition (ASh) at 10 and 5 mg/mL + fungus reached 40.6 and 43.5% efficacy, respectively, causing reduction of larval hatching and reproductive efficiency. For ASb, ASac and ASwater, the efficacy percentages were slightly but significantly higher when associated with the fungus *B. bassiana* strain 487. Reduction of posture was not so significant, reaching a maximum of 28.1% for ASac at 10 mg/mL + fungus. When the larval hatching parameter was evaluated, the extract/fungus association was also not highly effective, highlighting again ASh at 10 and 5 mg/mL + fungus.

Sensitivity of larvae in contact test on impregnated paper

In the larval packet test, the extracts of *A. satureioides* at all concentrations, when associated with the fungus *B. bassiana* strain 487, showed strong effects (Table 2), with the highest mortalities being found in the association ASb at 20 mg/mL + fungus (100%) and ASh at 20 mg/mL + fungus (71.5%). All concentrations of ASb associated with fungus were as lethal as the positive control [cypermethrin (15%), chlorpyrifos (25%), citronella (0.1%)].

Table 1 Mean (±SD) parameters (%) obtained in the immersion test of engorged *Rhipicephalus microplus* females in various extracts of *Achyrocline satureioides* associated or not with the fungus *Beauveria bassiana* (10^6 cells/mL)

Treatment	Concentration (mg/mL)	Posture reduction	Hatchability reduction	Reproductive efficiency	Product efficacy
ASb	10	0±0Aa	8.75±1.50Gb	89.56±6.40Db	11.26±1.50Da
	10+fungus	16.79±8.70Eb	5.56±2.90Ea	84.24±12.10Ba	14.14±12.30Fb
	5	0±0Aa	7.07±1.50Fb	93.70±17.00Eb	7.16±1.50Ba
	5+fungus	16.54±3.90Eb	4.44±10.70Da	85.36±4.70Ca	13.00±4.80Eb
	2.5	0±0Aa	7.07±1.10Fb	93.70±2.80Ea	7.16±0.50Bb
	2.5+fungus	7.52±6.01Bb	0±0Aa	100±0Fb	0±0Aa
	1.25	0±0Aa	2.69±1.50Ca	93.32±11.60Eb	7.53±3.70Ba
	1.25+fungus	14.04±7.10Db	2.22±1.90Ca	89.04±9.40Da	9.25±9.60Cb
Control		12.78±8.70Cb	10.00±2.90Hb	77.02±2.20Aa	21.50±12.00Gb
Negative control		0±0Aa	1.00±2.20Ba	100±0Fb	0±0Aa
ASac	10	0±0Aa	4.71±1.00Da	96.59±1.60Hb	4.30±1.60Ca
	10+fungus	28.07±9.06Hb	5.19±3.30Eb	73.11±10.60Aa	25.49±10.80Jb
	5	4.69±3.00Ca	2.02±1.10Cb	94.10±2.00Gb	6.76±0.50Da
	5+fungus	20.30±7.40Gb	0±0Aa	89.73±11.70Ea	8.55±11.90Fb
	2.5	8.50±6.30Da	5.05±1.50Eb	87.81±6.50Da	13.00±6.40Gb
	2.5+fungus	8.02±9.70Da	1.85±4.00BCa	96.92±10.30Hb	1.22±10.50Ba
	1.25	1.76±12.30Ba	4.71±2.10Da	93.63±13.40Fb	7.22±13.30DEa
	1.25+fungus	14.29±6.01Fb	9.26±1.60Fb	83.75±9.60Ca	14.64±9.70Hb
Control		12.78±8.70Eb	10.00±2.90Gb	77.02±2.20Ba	21.50±12.00Ib
Negative control		0±0Aa	1.00±2.20Ba	100±0Ib	0±0Aa
ASh	10	0±0Aa	1.68±3.00Ba	100±0Ib	0±0Aa
	10+fungus	25.06±2.40Gb	28.15±13.80Eb	58.24±13.30Ba	40.64±16.00Hb
	5	11.29±10.90Ca	0.67±3.60Aa	84.99±1.50Eb	15.79±6.30Ea
	5+fungus	25.81±4.10Gb	30.74±7.20Fb	55.41±6.20Aa	43.53±6.30Ib
	2.5	9.18±6.30Ba	0.34±3.10Aa	86.81±0.50Fa	13.98±2.80Db
	2.5+fungus	14.04±7.50Eb	0±0Aa	94.00±7.80Hb	4.20±7.90Ba
	1.25	12.64±16.20Da	3.37±3.60Cb	81.08±3.70Da	19.66±11.00Fb
	1.25+fungus	18.55±10.50Fb	0±0Aa	90.41±15.30Gb	7.85±15.60Ca
Control		12.78±8.70Db	10.00±2.90Db	77.02±2.20Ca	21.50±12.00Gb
Negative control		0±0Aa	1.00±2.20Ba	100±0Ib	0±0Aa
ASwater	10	0±0Aa	1.35±2.50Ba	100±0Ib	0±0Aa
	10+fungus	17.54±12.80Gb	2.59±2.70Cb	90.41±12.20Ea	7.85±12.40Db
	5	4.69±8.30Ca	2.02±3.00Cb	94.10±6.50Gb	6.76±6.50Ca
	5+fungus	20.30±11.00Hb	0±0Aa	89.73±14.40Da	8.55±14.60Ea
	2.5	8.50±27.4Da	5.05±1.70Eb	87.81±27.2Ca	13.00±26.90Fb
	2.5+fungus	8.02±4.59Da	1.85±6.10BCa	96.92±8.90Hb	1.22±9.10Ba
	1.25	1.76±9.40Ba	4.71±2.10Da	93.63±5.70Fb	7.22±5.60Da
	1.25+fungus	14.29±6.51Fb	9.26±4.40Fb	83.75±11.40Ba	14.64±11.00Gb
Control		12.78±8.70Eb	10.00±2.90Gb	77.02±2.20Aa	21.50±12.00Hb
Negative control		0±0Aa	1.00±2.20Ba	100±0Ib	0±0Aa

*Means within a column followed by different upper-case letters (comparing different concentrations) or lower-case letters (comparing treatments with the same concentration) are significantly different (Tukey's test: $p<0.05$)

Achyrocline satureioides extracts: ASb, crude ethanol extract; ASac, acetate partition; ASh, hexanic partition; ASwater, aqueous partition of *A. satureioides*. Control: fungus only; negative control: water+2% Tween

Table 2 Larval packet test: mean (±SD) mortality (%) of *Rhipicephalus microplus* larvae in contact with papers impregnated with *Achyrocline satureioides* extracts of different polarities associated or not with the fungus *Beauveria bassiana*

Concentration (mg/mL)	ASb	ASac	ASh
20	2.43 ± 1.74 c	0.42 ± 0.72 d	1.33 ± 0.93 d
20 + fungus	100 ± 0 a	65.47 ± 4.43 b	71.50 ± 1.12 b
10	1.07 ± 0.33 c	0 ± 0 d	0.29 ± 0.51 d
10 + fungus	99.55 ± 0.78 a	47.56 ± 5.88 c	49.96 ± 5.44 c
5	0.18 ± 0.30 c	0 ± 0 d	0 ± 0 d
5 + fungus	92.53 ± 2.26 a	51.03 ± 1.58 c	40.76 ± 7.48 c
2.5	0 ± 0 c	0 ± 0 d	0 ± 0 d
2.5 + fungus	93.31 ± 8.66 a	40.76 ± 8.12 c	44.19 ± 4.71 c
Negative control	0 ± 0 c	0 ± 0 d	0 ± 0 d
Positive control	100 ± 0 a	100 ± 0 a	100 ± 0 a
Fungus control	41.65 ± 3.7 b	41.65 ± 3.7 c	41.65 ± 3.7 c

Means within a column followed by the same letter do not differ significantly (Tukey's test: $P > 0.05$)

Achyrocline satureioides extracts: ASb, crude ethanol extract; ASac, acetate partition; ASh, hexanic partition. Negative control: water + 2% Tween. Positive control: cypermethrin (15%), chlorpyrifos (25%) and citronella (0.1%). Fungus control: only *B. bassiana* cells

No statistical difference was found among ASac and ASh at 2.5–10 mg/mL + fungus; larval mortality of these combinations was the same as that of the fungus-only control (41.7%). These results indicate increased activity against tick larvae of the fungus in association with *A. satureioides*, especially of ASb + fungus. The LC_{50} calculated (with 95% CI in parentheses) were ASb + fungus 0.30 (0.17–0.53) mg/mL, ASac + fungus 5.80 (3.53–9.52) mg/mL, and ASh + fungus 6.23 (4.19–9.27) mg/mL.

Larvae immersion test

In the larval immersion test, the ASh partition showed the highest mortality from 20 to 5 mg/mL + fungus (Table 3). In association with the fungus *B. bassiana*, all extracts at the higher concentration caused 100% mortality of the larvae, equal to the positive control and significantly higher than the fungus control. The latter caused 64.5% mortality, demonstrating again the potential of the association. The LC_{50} calculated in this test (with 95% CI) were ASb + fungus 2.86 (2.21–3.70) mg/mL, ASac + fungus 3.61 (3.13–4.16) mg/mL, and ASh + fungus 2.65 (2.51–2.79) mg/mL.

Discussion

This is the first study involving the biological effect of *A. satureioides* extracts in association with *B. bassiana* strain 487 on *R. microplus* ticks. The compatibility of the extract and partitions with the fungus was evaluated by the minimum inhibitory concentration assay. The tick life cycle consists of several stages, in which the behavior and morphological constitution present remarkable differences, which may alter the efficacy of the compounds evaluated for tick control. The results presented here demonstrated that the extracts, from the concentration of 20 mg/mL (10^6 spores/mL) down, would be viable for the association

Table 3 Larval immension test: mean (±SD) mortality (%) of *Rhipicephalus microplus* larvae immersed in extracts of *Achyrocline satureioides* of different polarities associated or not with the fungus *Beauveria bassiana*

Concentration (mg/mL)	ASb	ASac	ASh
20	9.03±36.42 d	4.25±3.96 d	48.37±19.92 c
20+fungus	100±0 a	100±0 a	100±0 a
10	9.28±5.26 d	4.63±3.10 d	6.47±5.90 d
10+fungus	68.08±8.85 b	100±0 a	100±0 a
5	5.71±1.18 d	0.54±0.93 d	4.04±1.93 d
5+fungus	48.49±1.44 c	34.81±1.58 c	90.78±8.84 ab
2.5	2.79±9.76 bc	1±15.6 d	2.30±7.3 d
2.5+fungus	59.63±9.30 bc	57.82±10.18 b	46.66±5.53 c
Negative control	5.24±0.31 d	5.24±0.31 d	5.24±0.31 d
Positive control	100±0 a	100±0 a	100±0 a
Fungus control	64.49±1.21 b	64.49±1.21 b	64.49±1.21 b

Means within a column followed by the same letter do not differ significantly (Tukey's test: $P > 0.05$)

Achyrocline satureioides extracts: ASb, crude ethanol extract; ASac, acetate partition; ASh, hexanic partition. Negative control: water+0.02% Triton. Positive control: cypermethrin (15%), chlorpyrifos (25%), citronella (0.1%). Fungus control: only *Beauveria bassiana* cells

with *B. bassiana*, causing no damage to the growth of the fungus and showing a positive interaction.

In the immersion test of engorged females, the highest concentrations of ASh+fungus presented the best results, decreasing larval hatching and reproductive efficiency. In the moment of oviposition, females not only produce an egg mass, they also secrete a 'protective wax' by Gene's organ that in nature serves to protect eggs from solar radiation and attack by fungi and bacteria (Vasconcellos et al. 2018; Matos et al. 2019) – possibly this wax did not allow the growth of *B. bassiana*.

Treatment for tick control is usually based on eliminating the animal parasites; however, one of the major problems is the high prevalence of larvae in pastures, representing high chances of reinfestation, perpetuating the parasitic cycle. To evaluate the efficacy of extracts and partitions against larvae, two methodologies were used, the larval packet test (FAO 1971) and the larval immersion test (Sabatini et al. 2001). In the larval packet test, ASb+fungus demonstrated high potential against *R. microplus* larvae, whereas ASac and ASh from 10 mg/mL down, had their effect mainly due to the fungus presence – mortality was quite similar (40.8–51.0%) to the fungus control (41.7%). The study by Barci et al. (2009) with the fungal strain of *B. bassiana* IBCB66 demonstrated that the fungus at a concentration of 10^7 CFU/mL presented 38.1% of larval mortality on *R. microplus*, whereas 100% mortality was achieved only at 10^9 CFU/mL. This comparison demonstrates that the fungal strain used in our study had almost twice the mortality at a lower concentration, as we used 10^6 CFU/mL, reinforcing the hypothesis that the fungus associated with the extract significantly increases the mortality rate.

In the larval immersion test, ASac+fungus until 10 mg/mL and ASh+fungus until 5 mg/mL presented the best biological effect instead of ASb. All the other results seem to be an effect of the fungus alone. The difference among tests may be due to better contact of the treatment with the parasite in the larval immersion test, as it does not allow fast volatilization of the compounds, as in the larval packet test. Moreover, it seems that compounds

extracted by the hexanic solvent may present better effect on *R. microplus* in immersion tests, although in the adult immersion test the ASb at highest concentration also showed a small effect. Studies with associations among extract/fungus to control the cattle tick are rare, but a study using an association of *Melia azedarach* (Meliaceae) with *B. bassiana* in an emulsion showed an apparent synergistic effect in the control of infestations of *R. microplus* in cattle (Sousa et al. 2011). In the present study, the efficacy of the association on larvae can be explained by the adherence of extracts to the cuticle of the larvae. At higher concentrations, the solution tends to be thicker, thus facilitating adherence to the larvae, offering a higher possibility of the fungus spores to penetrate, whereas when placed in more fluid solutions, the spores end up not adhering properly.

In the in vitro tests in the present study, it is noted that lower concentrations of the association of *A. satureioides* extracts + fungus cause higher percentages of mortality than *A. satureioides* extracts alone, even at higher concentrations. This was true mainly for *R. microplus* larvae. One explanation for this curious finding may be a hormesis effect (Southam et al. 1943). Hormesis – i.e., a stronger effect by a lower dose – has been found in various groups of organisms, ranging from bacteria and fungi to higher plants and animals (Calabrese 2005). This effect becomes interesting, as it allows a decrease in the concentration used.

The results showed that the association between the ASb and ASh + fungus presents potential use against *R. microplus* larvae, suggesting a sustainable alternative for the cattle tick control. These results open new perspectives for studies that will allow the development of acaricide formulations with those compounds for future evaluation in vivo.

Acknowledgements We thank to the National Council for Scientific and Technological Development (CNPq) for the scholarship granted (RRF), the School of Pharmaceutical Sciences of Araraquara (UNESP) and Embrapa Southeastern Livestock Unit for keeping the host donors by the project 20.18.03.017.00.02.002.

Author's contributions RRF: conceived and designed the experiments; performed the experiments; prepared the manuscript; YAG: provided support in collecting and identifying ticks, analyzing the data; AF: provided support in collecting and identifying ticks; RS: performed the experiment; ACSC: provided support in collecting and identifying ticks, analyzing the data, prepared the manuscript. RCLRP: conceived and designed the experiments; analyzing and interpreting data, prepared the manuscript, supervised the study. All authors read, agreed and approved the final manuscript.

Funding This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil), Process FAPESP N° 2018/14116-4 and in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Data Availability The data sets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate All procedures of the present study were approved by the Embrapa Pecuária Sudeste Ethics Committee on Animal Experimentation (CEUA process nº. 01/2019).

Competing interests The authors declare that they have no competing interests.

Consent for publication Not applicable.

References

Barci LAG, Almeida JEM, Nogueira AHC, Prado AP (2009) Determination of LC 90 and LT 90 of IBCB66 *Beauveria bassiana* (Ascomycetes: Clavicipitaceae) isolate for *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) control. Rev Bras Parasitol Vet 18:34–39. https://doi.org/10.4322/rbpv.018e1006

Calabrese EJ (2005) Paradigm lost, paradigm found: The reemergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ Pollut 138:378–411. https://doi.org/10.1016/j.envpol.2004.10.001

Chagas ACS, Barros LD, Cotinguiba F, Furlan M, Giglioti R, Oliveira MCS, Bizzo HR (2012) *In vitro* efficacy of plant extracts and synthesized substances on *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). Parasitol Res 110:295–303. https://doi.org/10.1007/s00436-011-2488-z

Clinical and Laboratory Standards Institute (CLSI) (2008) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard, 2nd ed.; CLSI document M38-A2; CLSI: Wayne, PA, USA. https://clsi.org/media/1455/m38a2_sample.pdf

Dal Magro J, Renk MH, Garcia FRM, Yunes RA, Magro D (1998) Eficiência de repelentes à base de extratos de *Achyrocline satureioides* e Cucurbita pepo sobre simulídeos. XV Simpósio de Plantas Medicinais do Brasil, Águas de Lindóia. Anais do Congresso p.163

De Souza KCB, Schapoval EES, Bassani VL (2002) LC determination of favonoids: separation of quercetin, luteolin and 3-O-methylquercetin in *Achyrocline satureioides* preparations. J Pharmacol Biomed 28:771–777. https://doi.org/10.1016/s0731-7085(01)00693-8

Drummond RO, Ernest SE, Trevino JL, Gladney WJ, Graham OH (1973) *Boophilus annulatus* and *Boophilus microplus*: laboratory tests for insecticides. J Econ Entomol 66:130–133. https://doi.org/10.1093/jee/66.1.130

FAO (1971) Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides. Tentative method for larvae of cattle ticks, *Boophilus* spp. FAO Method No. 7. FAO Plant Prot Bull 19:15–18. https://www.cabdirect.org/cabdirect/abstract/19711001927

Fernandes EKK, Bittencourt VREP (2008) Entomopathogenic fungi against South American tick species. Exp Appl Acarol 46:71–93. https://doi.org/10.1007/s10493-008-9161-y

Fernandes EKK, Bittencourt VREP, Roberts DW (2012) Perspectives on the potential of entomopathogenic fungi in biological control of ticks. Exp Parasitol 130:300–305. https://doi.org/10.1016/j.exppara.2011.11.004

George DR, Finn RD, Graham KM, Sparagano OAE (2014) Present and future potential of plant-derived products to control arthropods of veterinary and medical significance. Parasit Vectors 7:1–12. https://doi.org/10.1186/1756-3305-7-28

Islam MT, Omar DB (2012) Combined effect of *Beauvera bassiana* with neem on virulence of insect in case of two application approaches. J Anim Plant Sci 22:77–82. https://parasitesandvectors.biomedcentral.com/articles/https://doi.org/10.1186/1756-3305-7-28

Klafke G, Webster A, Dall Agnol B, Pradel E, Silva J, de La Canal LH et al (2017) Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, Southern Brazil. Ticks Tick Borne Dis 8(1):73–80. https://doi.org/10.1016/j.ttbdis.2016.09.019

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS (2015) Insect pathogens as biological control agents: Back to the future. J Invertebr Pathol 132:1–41. https://doi.org/10.1016/j.jip.2015.07.009

Luckmann D(2013) Compatibilidade de produtos naturais comerciais a fungos entomopatogênicos e seletividade a *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). 2013; 69 f. Dissertação de Mestrado, Universidade Tecnológica Federal do Paraná, Pato Branco, PR, Brasil. http://bdtd.ibict.br/vufind/Record/UTFPR-12_b22857fec46d54c28f090e2e394ca7c7

Macêdo M, Consoli R, Grandi T, Dos Anjos A, De Oliveira A, Mendes N et al (1997) Screening of Asteraceae (Compositae) plant extracts for larvicidal activity against *Aedes fluviatilis* (Diptera: Culicidae). Mem Inst Oswaldo Cruz 92:565–570. https://doi.org/10.1590/S0074-02761997000400024

Matos RS, Oliveira PR, Britto LCM, de Paula LGF, Zeringota CSB, Oliveira Monteiro CM, Daemon E, Camargo-Mathias MI (2019) Thymol: effects on reproductive biology and Gene's organ morphology in *Rhipicephalus sanguineus* sensu lato engorged females (Acari: Ixodidae). Ticks Tick Borne Dis 11(1):101308. https://doi.org/10.1016/j.ttbdis.2019.101308

Minho AP, Domingues LF, Gainza YA, Figueiredo A, Boligon AA, Domingues R, Chagas AC(2020) *In vitro* screening of plant extract on *Haemonchus contortus* and *Rhipicephalus (Boophilus) microplus*. J Essent Oil Res 32(3):269–278. https://doi.org/10.1080/10412905.2020.1746414

Mohan MC, Redoy NP, Devi UK, Kongara R, Sharma HC (2007) Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. Biocontrol Sci Technol 17:1059–1069. https://doi.org/10.1080/09583150701714551

Moraes CK, Schark A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomophatology and acaricid *Methahizium anisopliae*. Curr Microbiol 46:205–2010. https://doi.org/10.1007/s00284-002-3863-x

Politi FAS, Figueira GM, Araújo AM, Sampieri BR, Camargo-Mathias MI, Szabó MPJ, Bechara GH, Santos LC, Vilegas W, Pietro RCLR (2012) Acaricidal activity of ethanolic extract from aerial parts of *Tagetes patula* L. (Asteraceae) against larvae and engorged adult females of *Rhipicephalus sanguineus* (Latreille, 1806). Parasit Vectors 295:1–11. https://doi.org/10.1186/1756-3305-5-295

Prette N, Monteiro AC, Garcia MV, Soares VE (2005) Patogenicidade de isolados de *Beauveria bassiana* para ovos, larvas e ninfas ingurgitadas de *Rhipicephalus sanguineus*. Ciência Rural 35:855–861. https://doi.org/10.1590/S0103-84782005000400017. https://www.scielo.br/j/cr/a/zydpDvtH9Fs

Rashid M, Rashid MI, Akbar H, Ahmad l, Hassan MAA, Ashraf K et al (2019) A systematic review on modelling approaches for economic losses studies caused by parasites and their associated diseases in cattle. Par 146:129–141. https://doi.org/10.1017/S0031182018001282

Reck J, Klafke GM, Webster A, Dall'Agnol B, Scheffer R, Souza UA et al (2014) First report of fluazuron resistance in *Rhipicephalus microplus*: A field tick population resistant to six classes of acaricides. Vet Parasitol 201(1–2):128–136. https://doi.org/10.1016/j.vetpar.2014.01.0127

Reis FS, Barros MC, Fraga EDC, Da Penha TA, Teixeira WC, Dos Santos ACG (2008) Ectoparasitos de pequenos mamíferos silvestres de áreas adjacentes ao rio Itapecuru e área de preservação ambiental do Inhamum, estado do Maranhão. Rev Bras Parasitol Vet 17:69–74

Retta D, Dellacassa E, Villamil J, Suárez SA, Bandoni AL (2012) Marcela, a promising medicinal and aromatic plant from Latin America: A review Ind Crops Prod. 38:27–38. https://doi.org/10.1016/J.INDCROP.2012.01.006

Ribeiro LP, Blume E, Bogorni PC, Dequech STB, Brand SC, Junges E (2012) Compatibility of *Beauveria bassiana* commercial isolate with botanical insecticides utilized in organic crops in southern Brazil. Biol Agric Hort 28:223–240. https://doi.org/10.1080/01448765.2012.735088

Rodríguez-Vivas RI, Pérez-Cogollo LC, Rosado-Aguilar JA, Ojeda-Chi MM, Trinidad-MartinezI, Miller RJ et al (2014) *Rhipicephalus (Boophilus) microplus* resistant to acaricides and ivermectin in cattle farms of Mexico. Rev Bras Parasitol Vet 23:113–122. https://doi.org/10.1590/s1984-29612014044

Rojas de Arias A, Ferro E, Inchausti A, Ascurra M, Acosta N, Rodriguez E et al (1995) Mutagenicity, insecticidal and trypanocidal activity of some Paraguayan Asteraceae. J Ethnopharmacol 45:35–41. https://doi.org/10.1016/0378-8741(94)01193-4

Sabatini GA, Kemp DH, Hughes S, Nari A, Hansen J (2001) Tests to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. Vet Parasitol 95:53–62. https://doi.org/10.1016/s0304-4017(00)00406-4

Santi L, Coutinho-Rodrigues CJB, Berger M, Klein LAS, De Souza EM, Rosa RL et al (2019) Secretomic analysis of *Beauveria bassiana* related to cattle tick, *Rhipicephalus microplus*, infection. Folia Microbiol (Praha) 64:361–372. https://doi.org/10.1007/s12223-018-0659-3

Saupe AC (2003) O chá de macela *Achyrocline satureioides* (Lam) D C no controle do pulgão verde *Myzus persicae* em cultivo protegido: uma alternativa aos agrotóxicos. Florianopolis,50 f. Dissertaçao (Mestrado em Agrossistemas) Centro de Ciências Agrarias, Universidade Federal de Santa Catarina. https://repositorio.ufsc.br/xmlui/handle/123456789/85142

Singh NK, Vemu B, Nandi A, Singh H, Kumar R, Dumka VK(2014) Laboratory assessment of acaricidal activity of *Cymbopogon winterianus*, *Vitex negundo* and *Withania somnifera* extracts against deltamethrin resistant *Hyalomma anatolicum*. Exp Appl Acarol 63:423–430. https://doi.org/10.1007/s10493-014-9791-1

Soares FB(2011) Impactos de inseticidas e fungicidas na densidade populacional de *Beauveria bassiana* no solo sob efeito da microbiota nativa. 82 f. Dissertação de Mestrado, Universidade Estadual Paulista, Jaboticabal, SP, Brasil. https://repositorio.unesp.br/bitstream/handle/11449/94876/soares_fb_me_jabo.pdf

Sousa LAD, Pires Junior HB, Soares SF, Ferri PH, Ribas P et al(2011) Potential synergistic effect of *Melia azedarach* fruit extract and *Beauveria bassiana* in the control of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) in cattle infestations. Vet Parasitol 175:320–324. https://doi.org/10.1016/j.vetpar.2010.10.012

Southam CM, Ehrlich J(1943) Effects of Extract of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathol 33:517–524. https://www.scienceopen.com/document?vid=9c2c5835-b6bf-4e77-8484-4a3ed6dadd6d

Sun M, Ren Q, Guan G, Li Y, Han X, Ma C et al (2013) Effectiveness of *Beauveria bassiana* sensu lato strains for biological control against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) in China. Parasitol Int 62:412–415. https://doi.org/10.1016/j.parint.2013.04.008

Vasconcellos JSP, Fernandes F, Cadore GC, Ratzlaff FR, Felipetto LG et al (2018) Atividade acaricida de diferentes solventes sobre fêmeas ingurgitadas e larvas de *Rhipicephalus microplus*. Pesq Vet Bras 38:1125–1129. https://doi.org/10.1590/1678-5150-PVB-5410

Zimmermann G (2007) Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontrol Sci Technol 17:553–596. https://doi.org/10.1080/09583150701309006

Authors and Affiliations

Rafaela Regina Fantatto[1] · Yousmel Alemán Gainza[2] · Amanda Figueiredo[2] · Rodrigo Sorrechia[1] · Ana Carolina de Souza Chagas[2] · Rosemeire Cristina Linhari Rodrigues Pietro[1]

✉ Rosemeire Cristina Linhari Rodrigues Pietro
rosemeire.pietro@unesp.br

Rafaela Regina Fantatto
rrfbio@hotmail.com

Yousmel Alemán Gainza
yousmel@gmail.com

Amanda Figueiredo
ffigueiredo.amanda@gmail.com

Rodrigo Sorrechia
rodrigo.sorrechia@unesp.br

Ana Carolina de Souza Chagas
carolina.chagas@embrapa.br

[1] Departament of Drugs and Medicines, São Paulo State University UNESP, Rodovia Araraquara-Jaú Km 1, 14800-903 Araraquara, São Paulo, Brazil

[2] Southeastern Livestock Unit, EMBRAPA - Brazilian Agricultural Research Corporation, Rodovia Washington Luiz, km 234, 13560-970 São Carlos, São Paulo, Brazil