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ORIGINAL PAPER

Acaricide activity in vitro of *Acmella oleracea* against *Rhipicephalus microplus*

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Abstract Cattle tick control has been limited by the resistance of these parasites to synthetic acaricides. Natural products are a possible alternative as they have different mechanisms of action. *Acmella oleracea* is a native plant with a large cultivated area in the Amazon region and could be easily used for large-scale preparation of a commercial product. This study evaluated the in vitro action of the hexane extract of the aerial parts of *A. oleracea* on larvae and engorged females of the cattle tick *Rhipicephalus microplus*. Spilanthol was the major constituent with a content of 14.8 % in the extract. The hexane extract of *A. oleracea* was highly effective against larvae of *R. microplus* with an LC_{50} of 0.8 mg mL^{-1}. Against engorged females, hexane extract of *A. oleracea* reduced oviposition and hatchability of eggs with an LC_{50} of 79.7 mg mL^{-1}. Larvae and engorged females were killed by the hexane extract with high efficiency (>95 %) at concentrations of 3.1 and 150.0 mg mL^{-1}, respectively. These results demonstrate that the hexane extract of *A. oleracea* has significant activity against *R. microplus* and has potential to be developed into formulations for tick control.

Keywords Cattle · Control · Extract · Tick

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Introduction

Rhipicephalus microplus is the most economically important cattle ectoparasite in tropical and subtropical regions (Furlong et al. 2007). Synthetic acaricides have been used indiscriminately to control this parasite over the years. As a result, the selection of resistant tick populations is increasing (Mendes et al. 2011). Natural plant products are an alternative for the control of ticks (Andreotti et al. 2013). Plant natural products degrade quickly in the environment, and the development of resistance to natural products is slower than the development of resistance to synthetic acaricides (Hernández et al. 1987; Gupta et al. 2000; Ismail et al. 2002; Borges et al. 2003). In the process of choosing a plant to control arthropods, the ease of obtaining plants and economic viability must be considered for future commercial product development (Viegas Junior 2003).

Acmella oleracea is a typical herb from the Amazon region. Known in Brazil as "Jambu," this herb is used in local cuisine. In folk medicine, plants from the genus *Acmella* and *Spilanthes* are used as analgesics, local anesthetics, and insecticides (Ramsewak et al. 1999; Torres and Chavez 2001). Additionally, insecticide and acaricide activities have been described for oils and extracts originating from this same genus (Kadir et al. 1989; Chungsamarnyart et al. 1991a; Oya and Tsukada 2002; Pandey et al. 2007). However, the efficacy of hexanic extracts from this genus has not been reported. Insecticidal activity of these extracts has been related the presence of several bioactive compounds, which includes spilanthol and a group of other isobutylamides (Saraf and

Dixit 2002; Pandey et al. 2007). Spilanthol, isolated from extracts of *A. oleracea,* has greater efficiency than synthetic insecticides (Kadir et al. 1989; Sharma et al. 2012).

In this study, we aimed to evaluate acaricide activity of plant extract from *A. oleracea* on a large-scale against larvae and engorged female of *R. microplus*.

Materials and methods

Collection and identification of plant

Aerial parts of *A. oleracea* were collected in Parnaíba City, Piauí State, Brazil. A voucher specimen of *A. oleracea* was deposited in the Herbarium CEN from Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, Brazil, under the number 81102.

Preparation of extracts

The plant material was dried at 40 °C, ground in a hammer mill (1 mm sieve), and used for three consecutive extractions one per hour with hexane solvent, being kept under constant stirring and heating at 40 °C for 2 h. The hexane was evaporated under reduced pressure using a rotary evaporator until complete removal of the solvent and formation of the hexane extract.

Chemical analysis

The hexane extract of *A. oleracea* was analyzed by isocratic high-performance liquid chromatography using a Hitachi Lachron Elite L-2000 (Merck) with a Lichrospher C18 5 μm (125×4 mm) column (Merck), acetonitrile-water (30:70), a flow rate of 1.0 mL/min., and a UV detector at 229 nm, with a running time of 10 min and a 20-μL injection volume (Internal Development). The external standard used was 95.9 ppm of spilanthol.

Tick preparation

Engorged *R. microplus* females (≥4.5 mm) were collected from naturally infested calves, washed with water, and dried with a paper towel. The engorged females were selected morphologically and used in the adult immersion test (AIT) or maintained in the laboratory at 27 °C and relative humidity ≥80 % for 16 days until the eggs were laid. These eggs provided the larvae used for the larval packet test (LPT).

LPT

The LPT was performed according to the method described by Stone and Haydock (1962) and modified by FAO (1971) and Leite (1988), as described below. Approximately 100 tick larvae were placed on one sheet and then covered with another sheet, forming a sandwich. The sandwiched filter papers and larvae were then placed in an envelope of folded non-impregnated filter paper (72.25 cm^2) and sealed with a plastic clothes pin. The envelope was placed in an incubator and maintained at 27±1 °C with relative humidity (RH) ≥80 % for 24 h. After this time, alive and dead larvae were counted. Ticks showing no movement were considered dead. Each treatment was performed in triplicate. The tests were performed at concentrations ranging from 0.4 to 100.00 mg mL^{-1}, utilizing solutions of 50 % ethanol and 1 % DMSO as solvents. Furthermore, a solution of 50 % ethanol and 1 % DMSO was used as negative control. The lethal concentrations (LC_{50}) were calculated by Probit using GraphPad Prism 5.0. The differences among the concentrations were analyzed by Tukey test ($p<0.05$).

AIT

The sensitivity of engorged *R. microplus* females was determined using the AIT described by Drummond et al. (1973). Engorged *R. microplus* females were collected from artificially infested calves. In the groups of ten engorged female ticks, each was individually weighed in order to obtain groups with similar weights.

Each tick group was submerged for 5 min in one of six concentrations ranging from 12.5 to 200.0 mg mL^{-1}, utilizing a solution with 50 % ethanol and 1 % DMSO as the solvent. A solution with 50 % ethanol and 1 % DMSO was used as the negative control group. After submersion, the engorged females were dried on a paper towel, placed in Petri dishes, and maintained in a biochemical oxygen demand (BOD) incubator at 27±1 °C and RH ≥80 % for 16 days to further evaluate oviposition. The egg mass from each group was transferred to suitable syringes, sealed, and placed in the incubator. After 20 days, the percentage of hatchability was evaluated by using a stereomicroscope with visual verification was applied as parameter for this test (Drummond et al. 1973). The experiment was performed with three replicates for each treatment.

The egg production index (EPI), the reduction in oviposition (RO), and the efficiency of the extract (EP) were calculated according to the following formulas: EPI=(weight of eggs/weight of engorged female)×100 (Bennett 1974), RO=(EPI control group−EPI experimental group/EPI control group)×100 (Roulston et al. 1968), reproduction efficiency index (REI)=(egg mass weight× eggs hatched (percentage)/engorged females weight)× 20,000, and EP=(REI control−REI treated)/(REI control×100) (Drummond et al. 1973). Lethal concentrations (LC_{50}) were calculated by Probit using GraphPad Prism 5.0. Significant differences among the concentrations were analyzed by Tukey test ($p<0.05$).

Results

Spilanthol was the major compound in the hexane extract of *A. oleracea*, representing 14.8 % of total mass. This extract was highly effective against the larvae of the tick *R. microplus* at concentrations above 1.6 mg mL^{-1} (Table 1). A low LC_{50} (0.8 mg mL^{-1}, CL 95 % 0.7–0.8) confirmed the high efficiency of this extract (Table 2).

There was a significant reduction ($p<0.05$) in oviposition for engorged females of *R. microplus* treated with hexane extract of *A. oleracea* at concentrations above 100 mg mL^{-1}. This same concentration showed a significant reduction ($p<0.05$) in the hatchability of eggs from these ticks. A high efficiency (>95 %) of the hexane extract of *A. oleracea* against the engorged *R. microplus* females was found at concentrations above 150.0 mg mL^{-1} (Table 2). The LC_{50} of hexane extract of *A. oleracea* for engorged females was 79.7 mg mL^{-1} (95 % CL 69.7–91.3) (Table 3).

Discussion

A. oleracea is a medicinal plant from the Asteraceae family, with many therapeutic purposes and a growing demand worldwide (Prachayasittikul et al. 2013). The antifungal (Sharma et al. 2012), larvicidal (Amer and Mehlhorn 2006), and antimalarial activity (Pandey and Agrawal 2009) of *A. oleracea* has been well documented. Several species of this family are also described as having acaricidal activity (Ribeiro et al. 2008; Garcia et al. 2012). *A. oleracea* is considered the most important native vegetable of the Amazon region, with the largest cultivated area (Rebello and Homma 2005). That gives this species a real potential for industrial and commercial scale production. The cultivation of this species is also conducted in the northeast region of Brazil and can be a source of alternative income for the rural population. Suspension cultures of *A. oleracea* for the production of spilanthol, its main compound, are already available (Singh and Chaturvedi 2012a; Singh and Chaturvedi 2012b). This provides for large-scale production and standardization of these compounds (Prachayasittikul et al. 2013) and the potential for use in the control of *R. microplus*.

Hexane extract of *A. oleracea* was highly effective against larvae of the tick *R. microplus*, with high mortality at concentrations above 1.6 mg mL^{-1} and a low LC_{50} (0.8 mg mL^{-1}, CL 95 % 0.7–0.8). These results demonstrate greater efficacy of this extract compared to other plant extracts of the family Asteraceae, such as *Calea serrata* and *Artemisia annua* (Ribeiro et al. 2008; Chagas et al. 2011).

Interference of plant extracts with the reproduction of female ticks has been reported (Borges et al. 2003; Vendramini et al. 2012a; Vendramini et al. 2012b). Interfering with reproduction is extremely important for controlling infestations due to the high biotic potential of these parasites (Oliver 1989). The hexane extract of *A. oleracea* caused a reduction in the posture and hatchability of eggs in *R. microplus*, indicating its ability to interfere in the reproductive cycle of this tick.

Table 1 Efficacy of *Acmella oleracea* hexane extract against *Rhipicephalus microplus* larvae

Concentration (mg mL^{-1})	Mortality (%)
Control[a]	0.0±0.0a
0.4	7.8±8.0b
0.8	54.4±16.5c
1.6	93.8±2.8d
3.1	99.4±0.5d
6.2	100.0±0.0d
12.5	100.0±0.0d
25.0	100.0±0.0d
50.0	100.0±0.0d
100.0	100.0±0.0d

Different letters between rows indicate difference statistically significant ($p<0.05$)

[a] 50 % ethanol+1 % DMSO

Table 2 Lethal concentration of *Acmella oleracea* hexane extract against larvae and engorged females of *Rhipicephalus microplus*

Tick	LC_{50}	CL95 %	R^2
Larvae	0.8	0.7–0.8	0.96
Engorged female	79.7	69.7–91.3	0.94

LC_{50} concentration (mg mL^{-1}) at which 50 % of the *R. microplus* died, *CL95 %* confidence limits at 95 % probability, R^2 coefficient of determination

Table 3 Egg production index, reduction in oviposition, eggs hatched, and efficiency of the extract (EP) from *Acmella oleracea* hexane extract in engorged females of *Rhipicephalus microplus*

Concentration (mg mL^{-1})	Egg production Index	Reduction in oviposition (%)	Eggs hatched (%)	EP (%)
Control[a]	61.2±0.9[a]	–	98.6±0.9a	–
12.5	57.9±3.3a	5.5±6.1a	96.6±1.5a, c	7.3±3.8a
25.0	52.2±5.3a	14.7±9.4a	91.6±1.5a, d	20.7±9.3b
50.0	49.6±1.5a	18.9±3.3a	89.3±4.2a, e	26.6±1.7b
100.0	31.6±1.7b	48.3±3.0b	77.9±1.0b	59.2±2.7c
150.0	12.5±5.7c	79.4±9.6c	86.0±3.6b, c, d, e	98.2±0.8d
200.0	13.9±8.4c	77.2±13.9c	83.2±8.7b, d, e	97.9±1.4d

Different letters between rows indicate difference statistically significant ($p<0.05$)

[a] 50 % ethanol+1 % DMSO

A high efficacy of the hexane extract of *A. oleracea* against engorged females (98.2 %) was obtained at a concentration of 150 mg mL^{-1} (≈15 %). *Tagetes minuta* essential oil, another species of the family Asteraceae, required a higher amount of oil (20 %) to obtain a similar efficacy (95 %) against engorged *R. microplus* females. A similar result can be demonstrated against larvae (Garcia et al. 2012).

Chungsamarnyart et al. (1991a) showed partial acaricidal activity (36 % in 48 h) with ethanol extract in *A. oleracea*. However, when an aqueous extract was prepared, with or without warming, no acaricidal activity was observed (Chungsamarnyart and Jansawan 1993). This indicates that the acaricidal substances in this plant have no affinity for solvents of higher polarity and must be extracted by those of lower polarity. Thus, comparing the larvicidal activity (23.5 %) induced by ethanol extract of *A. oleracea* at 10 % (≈100 mg mL^{-1}) (Chungsamarnyart et al. 1991b) to that which was obtained by hexane extract in this study (100.0 %) prepared at much lower concentrations (6.2 mg mL^{-1} or 0.62 %), our results indicate that the lower polarity of hexane facilitates better extraction of the active substances. Extracts of *Melia azedarach* and *Piper tuberculatum* prepared with solvents of low polarity have also shown greater activity against ticks (Borges et al. 2003; Lima et al. 2014).

The major chemical constituent present in the hexane extract of *A. oleracea* was spilanthol, with the concentration of 14.8 %. Differences between species, methods, and solvents used in the extraction, origin of species, soil, climate, stage of growth, and flowering will all influence the concentration of spilanthol present in the *Spilanthes* plant (Cavalcanti 2008). Therefore, a standardization of these variables is necessary. The main observed medicinal effects of *A. oleracea*, such as insecticide and bactericidal activity, are attributed to spilanthol (Ramsewak et al. 1999). Spilanthol is an aliphatic amide described as a burning viscous oil, which produces an anesthetic effect and tingling of the tongue (Molinatorres et al. 1996). This compound has good ability to penetrate the skin (Boonen et al. 2010a; Boonen et al. 2010b; Spiegeleer et al. 2013). This property increases the possibility for the development of pharmaceutical acaricide formulations containing extract of *A. oleracea*, such as pour ons.

The hexane extract of *A. oleracea* has efficacy in vitro on larvae and engorged females. Further studies should be conducted to assess the efficacy of the hexane extract of *A. oleracea* in vivo and assess the potential for the development of pharmaceutical formulations containing this extract.

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