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Acaricidal activity of *Ocimum basilicum* and *Spilanthes acmella* against the ectoparasitic tick, *Rhipicephalus (Boophilus) microplus* (Arachinida: Ixodidae)

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Abstract. The ectoparasitic tick, *Rhipicephalus* (*Boophilus*) *microplus* collected at various cattle farms in and around Chennai was subjected to treatment of different crude solvent extracts of leaves of *Ocimum basilicum* and *Spilanthes acmella* for acaricidal activity. Among various solvent extracts of leaves of *O. basilicum* and *S. acmella* used, chloroform extract of *O. basilicum* at concentrations between 6% and 10% exhibited 70% and 100% mortality of ticks when compared to control. The LC_{50} and LC_{90} values of the chloroform extract of leaves of *O. basilicum* treatment on the ticks after 24 h were observed as 5.46% and 7.69%. Quantitative and qualitative analysis of α- and β- carboxylesterase enzymes in the whole gut homogenate of cattle tick, *R. microplus* treated with chloroform extract of leaves of *O. basilicum* revealed higher level of activities for the enzymes. This indicated that there was an induced response in the tick, *R. microplus* against the toxic effects of the extract of *O. basilicum*.

INTRODUCTION

Ticks, *Rhipicephalus (Boophilus) microplus*, the ectoparasite of livestock animals including human are considered as vectors of many infectious diseases all over the world. It has been reported that approximately 75% of livestock animals are at risk for ticks and tick-borne diseases causing a global annual loss of 7000 million American dollars (Van den Broek *et al.*, 2003; Wall, 2007; Ghosh *et al.*, 2007). *Rhipicephalus microplus* is an important pest of cattle in subtropical and tropical regions of the world (Estrada *et al.*, 2006). All species of *Boophilus* including *B. microplus* have been reclassified into the genus *Rhipicephalus* (Murrell & Barker 2003). *R. microplus* transmits the protozoa *Babesia bigemina*, *Babesia argentina*, *Anaplasma marginale*; the rickettsia *Coxiella burnetii* and spirochaetes *Borrelia theileri* to animals and humans in different parts of the world (Neitz, 1956). Babesiosis caused by *B. bigemina* and anaplasmosis by *A. marginale* are found in cattle. Besides, the parasites cause damage to the skin of cattle directly affecting its quality (Ducornez *et al.*, 2005).

Control measures of *R. microplus* in tropical and sub-tropical countries are usually practiced by regular chemical acaricide applications including synthetic pyrethroids, organophosphates and amitraz. Most of these acaricides possess harmful effects on animal health, beneficial organisms and environment (Mansour *et al.*, 2004). Furthermore, cattle ticks have been reported to develop resistance to synthetic acaricides (Olivo *et al.*, 2009). Their use also has caused great concern in society and government, by harming the animals themselves and humans who consume the products from these animals (Chagas *et al.*, 2003). These concerns have spurred the

search for alternative methods which are adaptable, safer and cheaper than chemical substances. There are reports of many plant extracts with acaricidal properties. The acaricidal effects of various plants have been studied in many parts of the world (Chagas *et al.*, 2002, Fernandes *et al.*, 2005; Fernandes & Freitas, 2007; Zahir *et al.*, 2010). The present study was therefore intended to explore the acaricidal properties of two traditional medicinal plants namely, *Ocimum basilicum* and *Spilanthes acmella* against *R. microplus*.

MATERIALS AND METHODS

Collection of ectoparasitic tick, *R. microplus*

Adult ticks were collected from various cattle farms located in and around Chennai. They were collected from the body of cows by hand picking or with the aid of blunt forceps and brush to avoid any harm to host animals and ticks. The collected ticks were placed in small glass vials with sufficient aeration by covering the top of the vial with muslin cloth and brought to the laboratory for the studies. They were identified using their morphological characteristics according to the standard descriptions provided by Pratt (1956) and Walker *et al.* (2003).

Preparation of plant extracts

The fresh leaves of *O. basilicum* (Lamiaceae) and *S. acmella* Murr. (Asteraceae) were collected from local medicinal farms. They were chosen and used based on their traditional medicinal applications. The leaves of *O. basilicum* are particularly known for its antimicrobial and insecticidal properties. The leaves of *S. acmella* are well known for its use in treatment of skin diseases. The collected leaves of these plants were shade-dried at room temperature and finely pulverized using grinder machine. Two hundred grams of each plant powder were extracted sequentially with increasing polarity of solvents such as pure hexane, chloroform and ethyl acetate at room temperature for one week with occasional shaking in an aspirator bottle. The extracts were filtered and vacuum evaporated and these crude extracts were used for screening of acaricidal activity.

Ticks Bioassay

Groups of ten *R. microplus* of both sexes of uniform size were chosen during collection. They were starved for 24 hrs immediately after collection and then used for bioassay studies. They were immersed for 5 min in 50 ml glass beaker containing 10 ml of the respective dilutions of plant extracts of *O. basilicum* and *S. acmella*. Various concentrations of extracts (2%–10%) were prepared by using 9 ml of 1% Tween-20 and 1 ml of acetone. A 10 ml of the same solution was used as control. After treatment, ticks were taken out and excess solution on ticks was removed by blotting with tissue paper. They were then placed individually in petri plates, incubated at 27-28ºC and 70-80% RH. The mortality of ticks was counted after 24 hrs and percentage of mortality [(percent mortality of test group – percent mortality of control group) / (100 – percent mortality of control group) x 100] was calculated. Lethal concentrations (LC) to kill 50% and 90% of ticks and their respective 95% confidence intervals (CI) were calculated by probit analysis.

Assay on carboxylesterases

The activity of carboxylesterase enzymes (against both alpha and beta naphthyl acetates as substrates) were estimated in the gut homogenate of both control and plant extract treated ticks. After 24 hrs of treatment, ticks were removed from petri plates and washed with double distilled water; the adhering water was completely removed from the body by blotting with tissue paper. The gut region of ticks was dissected out (10 individuals) from each of the treatments and gut contents were carefully removed by washing thoroughly with saline followed by extraction buffer. They were then transferred separately to 2 ml micro-centrifuge tubes and homogenized using a teflon hand homogenizer in 150 µl of ice-cold 20 mM phosphate buffer, pH 7.0 for extraction of esterases. The homogenates were centrifuged at 10000 rpm for 20 min at 4ºC

and the clear supernatants were preserved for esterase assays.

The protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used to obtain the standard curve. The carboxylesterase activity in the gut homogenates was measured by the modified method of Van Asperen (1962) as described by Argentine & James (1995). Aliquots of 2 µl of naphthol solutions at various concentrations (100 to 500 µM) were taken in a microtitre plate with 198 µl of 20 mM sodium phosphate buffer (pH 7.0) and 50 µl of freshly prepared 0.3% Fast blue B salt in 3.3% sodium dodecyl sulphate (SDS) were successively added to each tube and mixed well. After 15 min incubation at RT, the optical density of colour developed in each standard solution was read at 430 nm against a reagent blank (consisting of phosphate buffer and Fast blue B in 3.3% SDS solution) in microplate reader (PowerWave Xs, BioTek). A standard graph was prepared by plotting various test concentrations of α- and β- naphthol against their respective optical density. Ten µl of tick gut homogenate of *R. microplus* was incubated with 190 µl of 20 mM sodium phosphate buffer (pH 7.0) containing 250 µM of α- or β-naphthyl acetates for 15 min at RT. After incubation, 50 µl of freshly prepared 0.3% Fast blue B salt in 3.3% SDS were added to stop the enzymatic reaction and allowed to develop colour for 15 min at RT. The optical density of samples was read at 430 nm against the blank consisting of same reagents and buffer substituted for the homogenate. The level of α- and β-carboxylesterase activities was expressed as µM α- and β-naphthol released per minute/mg protein.

Carboxylesterase enzymes in the whole gut homogenates of ticks were also analyzed using discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions following Maurer (1971). This was performed using 3% stacking gel (pH 6.7) and 7% resolving gel (pH 8.9) in tris-glycine buffer (pH 8.3). Samples of the whole body homogenates (each 80 µg of protein) of control and experimental ticks were electrophoresed at a constant current of 3 mA per sample at 10ºC on a slab gel (170 x 150 x 1.5 mm). After electrophoresis, gels were suitably stained for detection of esterase activity. The gel was first incubated with phosphate buffer (20 mM, pH 7.0) for 15 min at RT. After decanting the buffer, the gel was then re-incubated for 30 min at RT with freshly prepared α-naphthyl acetate and Fast blue B solution for detection of α-carboxylesterase or β-naphthyl acetate and Fast blue B solution for detection of β-carboxylesterase. The gels were washed with distilled water, stored in 7% acetic acid and relative mobility (Rm = distance migrated by an esterase fraction / dye front) of each enzyme fraction was calculated.

RESULTS

Different crude solvent plant extracts were screened against the cattle ticks, *R. microplus* for acaricidal activity. In the initial screening, 5% of solvent extracts (hexane, chloroform and ethyl acetate) of *O. basilicum* and *S. acmella* were used for the bioassay on the cattle tick, *R. microplus*. Among three solvent extracts of both the plants tested, the chloroform extract of *O. basilicum* showed maximum mortality of ticks when compared to the control and other solvent extracts (Table 1). Then the chloroform extracts of leaves of *O. basilicum* alone was subjected to acaricidal activity at different concentrations. Among various concentrations used, 6%, 8% and 10% crude chloroform extracts of leaves of *O. basilicum* treatment produced 70%, 80% and 100% mortality respectively on the treated ectoparasites (Table 2). This mortality data was then subjected to the determination of LC_{50} and LC_{90} values for the cattle pest, *R. microplus*. The LC_{50} and LC_{90} values of the extracts after 24 h treatments on the ticks were observed as 5.46% and 7.69% (Table 3).

The quantity of α- and β-carboxylesterases exhibited higher levels in all the treatments in the whole gut homogenate of cattle tick, *R. micoplus* exposed to the solvent extracts of *O. basilicum* when compared to control. In control, the quantities of α- and β-

Table 1. Initial screening of three different solvent extracts of leaves (5%) of *Ocimum basilicum* and *Spilanthes acmella* on cattle tick, *Rhipicephalus microplus* for acaricidal activity

	Mortality (%)[@]	
	Ocimum basilicum	*Spilanthes acmella*
Hexane	46.7 ± 0.58	30.0 ± 1.00
Chloroform	73.3 ± 0.58	26.7 ± 0.58
Ethyl acetate	40.0 ± 1.00	20.0 ± 1.00

Each value represents Mean ± SD of three replicates
No mortality was observed in the negative control (9 ml 1% Tween-20 + 1 ml acetone)
[@] Ten numbers of *R. microplus* irrespective of sex were chosen with uniform size for bioassay studies

Table 2. Bioassay of chloroform extracts of leaves of *Ocimum basilicum* on cattle tick, *Rhipicephalus microplus*

Dose (%)	No. of ticks exposed[@]	Tick mortality after 24 hrs				Mortality (%)
		[*]R1	[*]R2	[*]R3	Mean	
10	10	10	10	10	10.00	100.00
8	10	09	08	07	08.00	80.00
6	10	07	07	07	07.00	70.00
4	10	02	01	01	01.33	10.00
2	10	01	00	00	00.67	10.00
Control[#]	10	01	00	00	00.67	10.00

[*] Replicate
[@] Ten numbers of *R. microplus* irrespective of sex were chosen with uniform size for bioassay studies
[#] Negative control (9 ml 1% Tween-20 + 1 ml acetone)

Table 3. LC_{50} and LC_{90} values of chloroform extracts of leaves of *Ocimum basilicum* on the cattle tick, *Rhipicephalus microplus*

Name of the plant	LC_{50} (%)[*]	LC_{90} (%)[*]
Ocimum basilicum	5.46 (3.8 – 6.4)	7.69 (6.5 – 11.91)

[*] 95% confidence level

carboxylesterases were 3.82 and 0.22 µM.min^{-1}.mg protein^{-1}. The enzyme quantities of α-carboxylesterase in all the treatments were in the range of 2.20 to 6.77 µM.min^{-1}.mg protein^{-1}. Likewise, β- carboxylesterase were in the range between 2.95 to 5.67 µM.min^{-1}.mg protein^{-1} (Table 4). Esterase enzyme gel electrophoresis was carried out with α- and β-naphthyl acetates as substrates for whole gut homogenate of *R. microplus* treated with chloroform extract of *O. basilicum*. In control, there were three different enzyme fractions of α-carboxylesterases resolved in the gel with the mobility ranging between 0.547 and 0.397. Upon exposure, the intensity of staining of these three enzyme fractions

Table 4. Quantity of α- and β- carboxylesterase in the cattle ticks *Rhipicephalus microplus* treated against chloroform extract of leaves of *Ocimum basilicum*

Substrate	Esterase activity (μM.min^{-1}.mg protein^{-1})					
	Control	2%	4%	6%	8%	10%
α- napthylacetate	3.82	2.20	3.82	6.28*	6.16*	6.77*
β- napthylacetate	0.22	2.95	3.03	4.89*	5.25*	5.67*

* Significant at $P < 0.05$

Table 5. Relative mobility of α- and β-carboxylesterase isozyme profile of cattle ticks *Rhipicephalus microplus* treated against chloroform extract of leaves of *Ocimum basilicum*

Enzyme fractions	Control	2%	4%	6%	8%	10%
α-est1	0.616	0.602	0.602	0.589	0.575	0.547
α-est2	0.561	0.534	0.534	0.534	0.520	0.479
α-est3	0.397	–	0.397	0.397	0.397	0.383
β-est1	0.661	0.676	0.691	–	0.691	0.676
β-est2	0.588	0.617	0.617	0.632	0.617	0.602
β-est3	0.455	–	0.455	0.485	0.470	0.470

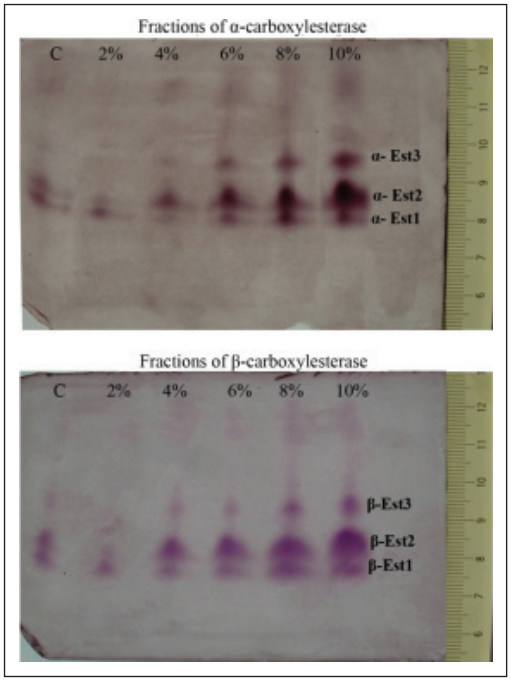

Figure 1. α- and β-carboxylesterase fractions in the gut of the adult cattle tick, *Rhipicephalus microplus* upon exposure to chloroform extracts of leaves of *Ocimum basilicum*

was gradually and significantly increased with increasing concentrations of extracts when compared to control. There was also a disappearance of enzyme fraction 1 with the mobility of 0.547 in 2% extract treatment (Table 5 and Figure 1). Similarly, the profile of β-carboxylesterases on native gel showed three bands in both control and treated ticks with relative mobility ranging between 0.661 and 0.455 with disappearance of esterase enzyme fraction 1 in the treatment. Upon exposure, the intensity of staining of these three enzyme fractions was significantly increased with increasing concentrations of extracts over control (Table 5 and Figure 1).

DISCUSSION

The cattle tick *R. microplus* causes great damage in livestock and is considered as one of the most important tropical ectoparasite. Traditionally they are controlled using synthetic chemical pesticides. However, the indiscriminate use of these compounds has

caused resistance in ticks (Milla *et al.*, 2005). Consequently, development and selection of resistant strains of *R. microplus* in different parts of the world has made several chemical agents ineffective (FAO 2004). Moreover, environmental pollution and contamination of meat and milk are associated with this kind of chemical control (Sonenshine, 1993). Research works on plants and their use in ticks control have been developed to look for extracts with acaricidal properties that can be used in association with or even as replacements for synthetic compounds. One advantage from the use of those compounds is that resistance develops slowly because there is usually a mixture of different active agents with varied mechanisms of action (Chagas *et al.*, 2003; Olivo *et al.*, 2009).

As a preliminary study, the screening of acaricidal activity of different solvent extracts of *O. basilicum* and *S. acmella* was carried out using random collection of adult ticks. The results on bioassay studies revealed that chloroform extracts of leaves of *O. basilicum* were found to be effective as acaricidal agent and 6% to 10% of chloroform extracts of leaves of *O. basilicum* were observed as potential acaricidal concentrations. This kind of acaricidal activity has been observed earlier by Kamaraj *et al.* (2010) in methanol extracts of leaves of *Rhinacanthus nasutus*, methanol extracts of leaves and seeds of *Solanum torvum*, and acetone extracts of seeds of *Terminalia chebula* against the adult of *Haemaphysalis bispinosa*. Likewise, Zahir *et al.* (2010) have reported that the parasite mortality was found in the ethyl acetate extract of leaves of *Achyranthes aspera*, methanol extract of leaves of *Anisomeles malabarica*, methanol extract of flowers of *Gloriosa superba*, and methanol extract of leaves of *Ricinus communis* against the immature stages of *R. microplus*. In those studies, it was observed to be several fold reduction in concentration of extracts required to kill the ectoparasitic ticks when compared to concentrations of extracts of leaves of *O. basilicum* used in the present study. As the mature adult samples of *R. microplus* were used in the present study, it is our assumption that higher doses of chloroform extracts of leaves of *O. basilicum* were necessary to cause significant mortality of ticks.

It has been reported that esterases are associated with acaricide resistance in *R. microplus*. To understand this, it was carried out to analyze qualitative and quantitative profiles of carboxylesterases in the gut samples of the tick, *R. microplus* treated with most effective solvent (chloroform) extract of *O. basilicum*. It was observed from the results that there was a significant increase in activities of both α- and β-carboxylesterases with an indication of toxic influence of the plant extract against the cattle tick, *R. microplus*. Carboxylesterases are found in many tissues including intestine and play a significant role in the metabolism and subsequent detoxification of many agrochemicals (Satoh & Hosokawa, 1998; Potter & Wadkins, 2006). In particular, carboxylesterases hydrolyze pyrethroids (Wheelock *et al.*, 2004) and bind stoichiometrically to carbamates (Sogorb & Vilanova, 2002) and organophosphates (Casida & Quistad, 2004). In *R. microplus*, the resistance mechanisms due to synthetic acaricides associated with esterase detoxification has been investigated by several authors. Our results on increased activities of carboxylesterases in chloroform extract of *O. basilicum* treated ticks are in correlation with earlier works where relationship between resistance to chemicals and enhanced activities of various esterase enzymes was demonstrated (Jamroz *et al.*, 2000; Baxter & Barker, 2002; Hernandez *et al.*, 2002). However, exploration on the actual acaricidal molecules from the extracts of *O. basilicum* and their efficacy on the ticks are necessary to determine the role of these enzymes in detoxification.

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