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Acaricidal activity of alkaloid fractions of *Leucas indica* Spreng against *Rhipicephalus (Boophilus) annulatus tick*

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Abstract. The acaricidal activity from alkaloid and non-alkaloid fractions of *Leucas indica* were studied against *Rhipicephalus (Boophilus) annulatus* tick using adult immersion test under laboratory conditions. For this purpose, the engorged female *R.(B.) annulatus* tick were exposed to two fold serial dilutions of alkaloid extract (50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6 mg/ml and 3 mg/ml) using 'dipping method' *in vitro*. The efficacy was assessed by measuring the percentage of adult mortality, inhibition of fecundity and hatching rate. The alkaloid fraction of the extract produced concentration dependent delayed adult tick mortality. The extract at a concentration of 50 mg/ml demonstrated 66.67 per cent mortality and 55.16 per cent inhibition of fecundity. Nicotine was identified as one of the compounds of alkaloid fraction. However, it did not reveal any acaricidal activity when tested *in vitro* at concentrations ranging from 62.5-1000 µg/mL. Hence, the acaricidal action of *L. indica* is not due to nicotine. Non alkaloid fraction also did not reveal any acaricidal effects against *R. (B.) annulatus* tick.

INTRODUCTION

Ticks, obligatory blood sucking arthropods are among the most harmful ectoparasites of domestic animals (Samish & Rehacek, 1999). They cause huge economic loss by sucking blood from host animals and acting as important vector for bacterial, viral and protozoal infections of man and animals (Wall & Shearer, 1997; Roberts & Janovy, 2005).

At present tick and tick borne diseases (TTBD) are mainly controlled by chemical acaricides. Resistance developed to these acaricides by the ticks and the deleterious effects on the environment in terms of residues are matters of serious concern. In addition, these chemical acaricides have a considerable genotoxic and cytotoxic effect on human target cells (Undeger & Basaran, 2005). Hence, it is necessary to look for alternative methods which are adaptable, safer and cheaper than chemical substances.

Leucas indica is a common weed seen on wastelands and roadsides all over India. The plant is used as an insecticide and indicated in traditional medicine for cough, colds, painful swellings and chronic eruptions (Chopra *et al.*, 2002). The plant was previously evaluated pharmacologically for its anti-inflammatory, analgesic and protective effects against cobra venom

poisoning (Reddy *et al*., 1993). The compounds isolated from the plant include triterpene (leucolactone), sterols (sitosterol, campesterol, stigmasterol) and a novel phenolic compound (Pradhan *et al*., 1990; Misra *et al*., 1992; 1993; 1995).

Recently, Ravindran *et al*. (2011) reported that ethanolic extract of *L. aspera* at very low concentrations inhibited the hatching of eggs laid by the treated *R. (B.) annulatus* ticks. Mangathayaru *et al*. (2006) isolated nicotine from *L. aspera*. The present study evaluates the potential of alkaloid and non-alkaloid fractions of *L. indica* for their acaricidal activity against *R.(B.)annulatus* tick. The study also evaluates acaricidal properties of the pure compound ' nicotine.'

MATERIALS AND METHODS

Ticks

Fully engorged adult female ticks were collected from infested calves of Instructional Cattle Farm, College of Veterinary and Animal Sciences, Pookode. The ticks were washed in tap water and dried with an absorbent paper. These females were used for the adult immersion test.

Plant material

The plant *L. indica* popularly known as *Thumba* in local language was collected from Wayanad district of Kerala in September 2011. The plant was identified and authenticated by a botanist and deposited at Calicut University Herbarium (Accession number, CALI :6640), Calicut, Kerala.

Preparation of alkaloid fraction

The plant leaves were dried in shade at room temperature. They were pulverized using a plant sample grinder. The powdered plant material was used for ethanolic extraction in a soxhlet extraction apparatus attached with a rotary vacuum evaporator (Rotavac, Buchi, Switzerland). Solvents were evaporated off by rotavac (140 mbar pressure at 34ºC for 40 minutes) and then kept at room temperature (27ºC).

The required quantity of the extract (200 g) was weighed and transferred to a separating funnel and was successively defatted with petroleum ether (200 ml) and filtered. The air dried residue was washed successively with methanol (60 ml) and filtered. The residue was transferred to a beaker in which water (10 ml) and HCl (2N, 10 ml) were added. It was stirred and then ether (100 ml) was added to extract tannins and other impurities. The aqueous layer was neutralized by adding 10 per cent sodium carbonate (10 ml) solution and then kept for 5 days in a freezer and filtered. Then, two fractions namely, solid and ethereal fractions were obtained (Goudgaon *et al*., 2003). Both these fractions were kept in a desiccator and stored in a freezer bath at -20ºC.

Phytochemical analysis

The extracts were tested for plant secondary metabolites such as tannins, saponins, steroids, alkaloids and glycosides (Harbone, 1991).

High performance thin layer chromatography (HPTLC)

HPTLC analysis was carried out on a HPTLC (Camag, Switzerland) system with nicotine (99.33% purity) as standard. Nicotine standard was run along with solid fraction and got similar peaks. Chromatographic separation was performed on Merck TLC plates precoated with silica gel 60 F254 (20cm x 10 cm with 200 µm layer thickness) from E. Merck, Germany. Standard solution (0.5 µl and 3 µl) was applied onto the plates as a band with 8 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) using Camag Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (20 x 10 cm) with the mobile phase hexane: chloroform (30:70) and chloroform: methanol: ammonia (90:10:2) for nicotine standard. Scanning was performed using Camag TLC scanner 3 at 254 nm, 366 nm and 550 nm through fluorescence mode and operated by winCATS software (version 1.4.1, Camag). Plates are visualized under UV 254

nm, UV 366 nm and in visible light after derivatizing with Anisaldehyde – sulfuric acid (ANS) reagent.

Adult Immersion Test

Solid and ethereal fractions of *L. indica* and nicotine (Sigma Aldrich, India) were prepared in distilled water and tested on ticks through adult immersion test as Drummond *et al.* (1973). Different dilutions of fractions (50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6 mg/ml and 3 mg/ml) and nicotine standard (0.1%, 0.05%, 0.025%, 0.0125% and 0.00625%) were prepared in distilled water and tested using the test mentioned above. Deltamethrin (30 ppm) and distilled water were used as positive and negative controls. A total of 24 ticks (four replicates of six ticks) were used for each dilution of fractions/nicotine standard/ deltamethrin. Ticks were weighed prior to the experiment and immersed for 2 minutes in the respective dilution (10 ml) in a 50 ml beaker with gentle agitations. Ticks were recovered from the solution, dried using tissue paper towels and placed in separate plastic specimen tubes (25 x 50). The tubes were incubated at 28 ± 2ºC and 80 per cent relative humidity in a BOD incubator. These ticks were observed for oviposition and death up to 15 days. Adult tick mortality, per cent inhibition of fecundity and hatching of eggs laid by treated ticks were evaluated (Gonçalves *et al.*, 2007).

Adult tick mortality

The specimen tubes were observed and the number of ticks died within 15 days after incubation was identified. The per cent adult tick mortality was determined in comparison to the control.

Per cent inhibition of fecundity and hatching

The eggs laid by the ticks of each tube were collected, weighed and kept under the same conditions of incubation for the next 30 days for visual estimation of hatching. Ticks under different treatments were compared with that of the controls.

The percentage inhibition of fecundity was calculated as follows:

Index of fecundity/ egg laying (IE) = weight of eggs laid (mg) / weight of females (mg)

Percentage inhibition of fecundity (IF) = [IE (control group) – IE (treated group)] x 100 / IE (control group).

Statistical analysis

Statistical analysis of data was performed (Snedecor & Cochran, 1994). Data were expressed as the mean ± SEM. Groups were compared using one-way ANOVA for repeated measurements using SPSS software. Duncan's test was used for post-hoc analysis. A value of $P<0.05$ was considered significant.

RESULTS

On phytochemical analysis, the solid fraction gave positive result for alkaloids but the ethereal fraction was negative for them. HPTLC results confirmed the presence of three alkaloids in solid fraction (Fig. 1–3). Further, HPTLC profiling confirmed the presence of nicotine as one of the alkaloids (Fig. 4). The results of adult immersion test using crude alkaloid fraction of *L. indica* against *R. (B.) annulatus* tick are presented in table 1. The per cent adult tick mortality caused by the alkaloid fraction of the *L. indica* was concentration dependent and varied from 0 to 66.66 per cent, when tested at concentrations ranging from 3 to 50 mg/ ml. Adult mortality observed was statistically significant at concentrations from 25 mg/ml and 50 mg/ml. The inhibition of fecundity ranged from 5.75 to 55.16 per cent on the treated ticks. Alkaloid fraction showed concentration dependent adult tick mortality and inhibition of fecundity. The adult tick mortality of the nonalkaloid fraction varied from 0–12.49 per cent only. The per cent inhibition of fecundity in ticks treated with ethareal fraction at concentrations ranging from 6 to 50 mg/ml varied significantly from the negative and positive controls (Table 2). Nicotine at concentrations ranging from 62.5–1000 µg/ml did not exhibit acaricidal activity. Moreover, the inhibition of fecundity varied from 5.81–16.35 (Table 3) for nicotine.

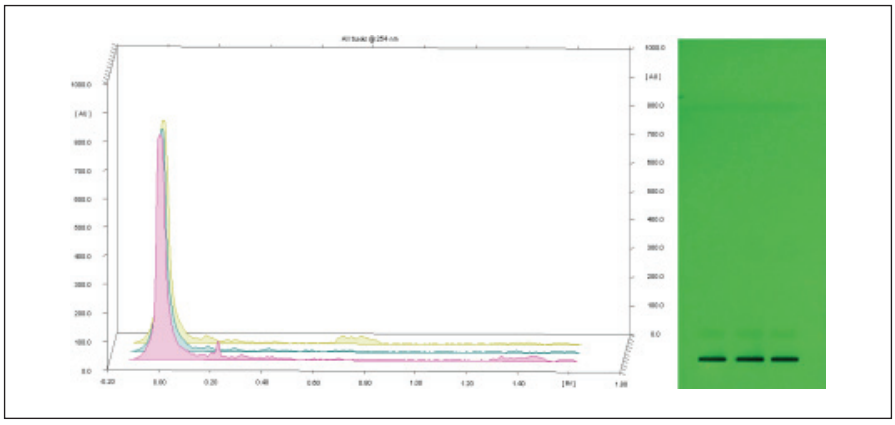

Figure 1. General HPTLC finger print profile of alkaloid fraction of aerial parts of *L. indica* at wavelength 254 nm with hexane: chloroform (30:70)

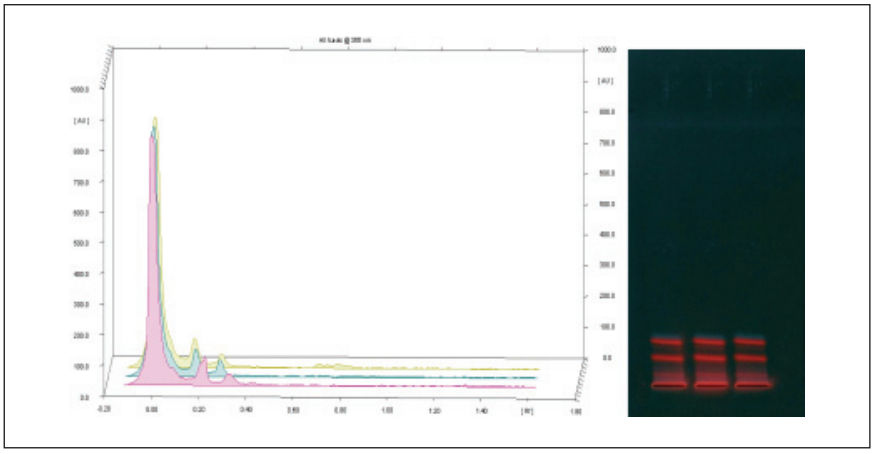

Figure 2. General HPTLC finger print profile of alkaloid fraction of aerial parts of *L. indica* at wavelength 366 nm with hexane: chloroform (30:70)

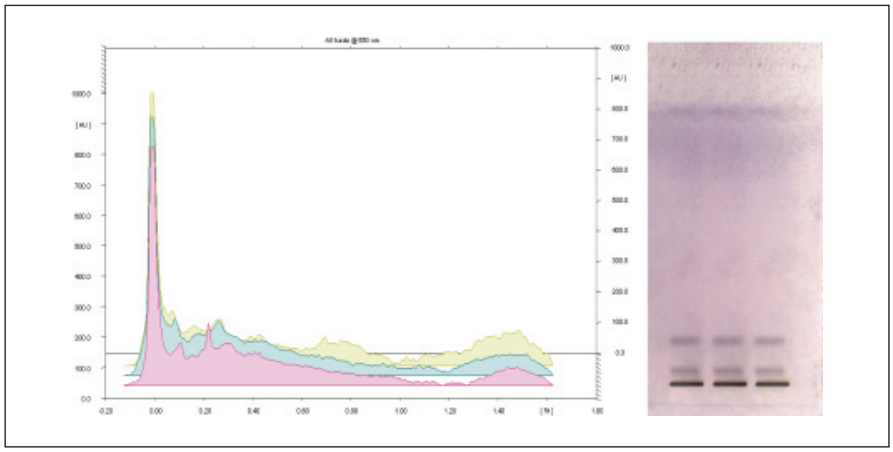

Figure 3. General HPTLC finger print profile of alkaloid fraction of aerial parts of *L. indica* at wavelength 550 nm with hexane: chloroform (30:70)

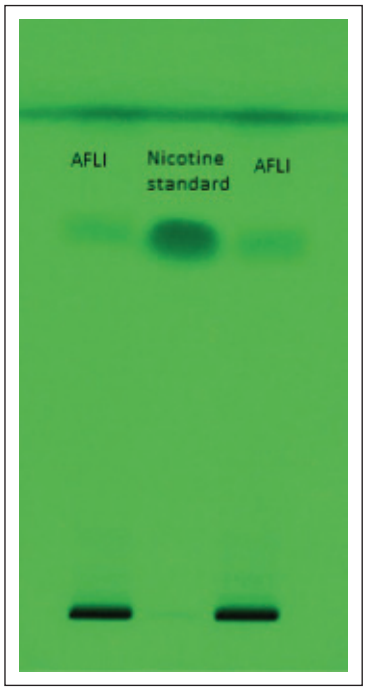

Figure 4. General HPTLC finger print profile of nicotine standard with alkaloid fraction of *L. indica* (AFLI) at wavelength 254 nm with chloroform: methanol: ammonia (90:10:2)

Hence, the acaricidal activity of *L. indica* is mainly with the alkaloid fraction. The nonalkaloid fraction and the nicotine pure compound did not produce any significant acaricidal effects.

DISCUSSION

Plants constitute a rich source of bioactive compounds such as phenolics, terpenoids, coumarins and alkaloids (Harborne, 1993; Ahn *et al*., 1998; Lee *et al*., 2010). Since these compounds which are often active against a limited number of species including specific target insects and are biodegradable to nontoxic products, they are potentially suitable for use in integrated pest management programs, and thereby lead to the development of new classes of safer insect control agents (Park *et al*., 2002; Mansour *et al*., 2004). Different plant preparations including extracts and oils were used for a long time in traditional medicine in India for their pharmacological activity and because of low toxicity. Plant extracts have potential value as tick control agents too (Kayaa, 2000).

Leucas aspera possess repellant activity (Kirtikar & Basu, 1990) and larvicidal activity (Muthukrishnan *et al*., 1997; Maheswaran *et al*., 2008; Vinayagam *et al*., 2008) against mosquitoes. Extracts of leaf, flower and seeds reportedly exhibited larvicidal potential against *Anopheles subpictus* Grassi and *Culex tritaeniorhyncus* Giles (Kamaraj *et al*., 2009). Larvicidal activity was also reported for leaf extract of the plant against *Aedes aegypti* L. and *Culex quinquefasciatus* Say (Bagavan *et al*., 2008). Recently,

Table 1. Acaricidal effects of different dilutions of alkaloid extract of *L. indica* against *R. (B.) annulatus* tick

Sl. No	Alkaloid fraction of *L. indica* (mg/mL)	Mean ticks weight per replicate ± SEM(g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Water	0.8294 ± 0.0142^{b}	0 ± 0^{a}	0.4339 ± 0.0122^{c}	0.5232 ± 0.0129^{c}	0.00	100
2.	3	0.7577 ± 0.0347^{ab}	0 ± 0^{a}	0.3707 ± 0.0108^{bc}	0.4931 ± 0.0304^{c}	5.75	100
3.	6	0.7665 ± 0.0229^{ab}	0 ± 0^{a}	0.3289 ± 0.0320^{b}	0.4287 ± 0.0396^{bc}	18.06	100
4.	12.5	0.7292 ± 0.0379^{a}	4.1650 ± 4.1650^{a}	0.3110 ± 0.0193^{b}	0.4271 ± 0.0189^{bc}	18.37	100
5.	25	0.8198 ± 0.0283^{ab}	37.4950 ± 10.4857^{b}	0.2879 ± 0.0438^{ab}	0.3543 ± 0.0608^{b}	32.28	100
6.	50	0.8268 ± 0.0409^{b}	66.6625 ± 11.7851^{c}	0.1955 ± 0.0503^{a}	0.2346 ± 0.0560^{a}	55.16	100
7.	Deltamethrin (0.03)	0.9653 ± 0.036^{c}	16.6625 ± 6.8035^{a}	0.2081 ± 0.0276^{a}	0.2140 ± 0.0236^{a}	57.3	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c ($P<0.05$), indicate significant difference when compared with the control and recommended concentration of deltamethrin

Table 2. Acaricidal effects of different dilutions of nonalkaloid extract of *L. indica* against *R. (B.) annulatus* tick

Sl. No	Non alkaloid fraction of *L.indica* (mg/mL)	Mean ticks weight per replicate ± SEM (g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Water	0.7919 ± 0.0346^{a}	0 ± 0^{a}	0.4384 ± 0.0124^{c}	0.5570 ± 0.0307^{c}	0.00	100
2.	3	0.7374 ± 0.0404^{a}	0 ± 0^{a}	0.3974 ± 0.0215^{ab}	0.5390 ± 0.0031^{c}	3.23	100
3.	6	0.7332 ± 0.0614^{a}	0 ± 0^{a}	0.3234 ± 0.0401^{b}	0.4363 ± 0.0222^{b}	21.67	100
4.	12.5	0.7438 ± 0.0768^{a}	0 ± 0^{a}	0.3215 ± 0.0418^{b}	0.4324 ± 0.0350^{b}	22.37	100
5.	25	0.7442 ± 0.0216^{a}	4.1650 ± 4.1650^{ab}	0.3182 ± 0.0198^{b}	0.4272 ± 0.0220^{b}	23.30	100
6.	50	0.8033 ± 0.0156^{a}	12.4950 ± 4.1650^{bc}	0.3396 ± 0.0122^{b}	0.4229 ± 0.0140^{b}	24.08	100
7.	Deltamethrin 0.03	0.9653 ± 0.0361^{b}	16.662 ± 6.803^{c}	0.2081 ± 0.0276^{a}	0.2140 ± 0.0236^{a}	57.30	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c *(*$P<0.05$), indicate significant difference when compared with the control and recommended concentration of deltamethrin

Table 3. Acaricidal effects of nicotine standard against *R.(B.) annulatus* tick

Sl. No	Nicotine (µg/mL)	Mean ticks weight per replicate ± SEM (g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Water	1.0418 ± 0.0193^{c}	0 ± 0^{a}	0.5164 ± 0.0236^{c}	0.4953 ± 0.0167^{c}	0.00	100
2.	62.5	0.9800 ± 0.0643^{bc}	0 ± 0^{a}	0.4570 ± 0.0298^{bc}	0.4665 ± 0.0667^{bc}	5.81	100
3.	125	0.8618 ± 0.0154^{a}	0 ± 0^{a}	0.3848 ± 0.0232^{b}	0.4476 ± 0.0317^{bc}	9.63	100
4.	250	0.9114 ± 0.0299^{ab}	0 ± 0^{a}	0.4076 ± 0.0137^{b}	0.4474 ± 0.0742^{bc}	9.67	100
5.	500	0.9411 ± 0.0299^{abc}	0 ± 0^{a}	0.4194 ± 0.0318^{b}	0.4442 ± 0.0212^{bc}	10.32	100
6.	1000	0.9781 ± 0.0174^{bc}	0 ± 0^{a}	0.4051 ± 0.0159^{b}	0.4143 ± 0.0154^{b}	16.35	100
7.	Deltamethrin 30	0.9653 ± 0.0361^{abc}	16.662 ± 6.803^{b}	0.2081 ± 0.0276^{a}	0.2140 ± 0.0236^{a}	57.30	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c ($P<0.05$), indicate significant difference when compared with the control and recommended concentration of deltamethrin

Ravindran *et al.* (2011) reported the eclosion blocking effect of ethanolic extract of *L. aspera* against eggs laid by treated *R. (B.) annulatus* ticks. The extract also caused adult tick mortality and inhibition of hatching. Muhammed *et al.* (2012) reported repellent action of *Leucas martinicensis* against adult *Culex* mosquitoes and the phytochemical screening of its leaf extract revealed the presence of alkaloids, flavanoids and volatile oils.

In the present study, it was observed that the alkaloid fraction of *L. indica* exhibited a significant concentration dependent adult tick mortality compared to the nonalkaloid fraction. Further, HPTLC profiling confirmed the presence of the alkaloid 'nicotine' in addition to two other alkaloids in the alkaloid fraction of *L. indica*. Mangathayaru *et al.* (2006) isolated and identified nicotine from *L. aspera* (Willd). Previously, it was speculated that the acaricidal effects of *L. aspera* could be due to the presence of nicotine (Ravindran *et al.*, 2011). However, the present study disproved any acaricidal activity for nicotine. Hence, it is concluded that the acaricidal activity shown by the solid alkaloid fraction of *L. indica* could be due to the presence of other alkaloid compounds present in that extract.

Insecticidal activity of triterpenic alkaloids (Crosby *et al*., 1971a), veratrum alkaloids (Crosby *et al*., 1971b), monoterpenoidindol alkaloids (Trindale *et al*., 2008) and piperidine alkaloids (Kambou & Guisson, 2011) were already reported. Our study revealed the presence of alkaloids other than nicotine with good acaricidal activity in *L. indica*. Further studies are needed to purify and identify the active alkaloid compound responsible for the acaricidal activity in the alkaloid fraction.

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