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Assessment of the response of *Rhipicephalus annulatus* and *Rhipicephalus microplus* to the synthetic analogues of assembly pheromone by Petri-dish bioassay[#]

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Abstract

Semiochemical assisted tick control is a novel, promising alternate tick control perspective which can be utilised for controlling tick populations both on and off the host. In the present study, optimal concentrations of synthetic analogues of assembly pheromone (AP) viz., guanine, adenine and xanthine were encapsulated in calcium alginate microparticles. Qualitative analysis of encapsulation of AP within the beads was performed by Fourier Transform Infra-Red Spectroscopy (FTIR). Test beads with AP-deltamethrin combination in alginate microparticles, pheromone control, acaricide control and polymer control beads were prepared. In vitro Petri-dish bioassay was performed for evaluating the responses of unfed larvae and partially fed adults of Rhipicephalus annulatus and Rhipicephalus microplus ticks to the test and control microparticles. Behavioural responses like attraction, cessation of kinetic activity, clustering, sluggishness and mortality of ticks were recorded at 10 min, 30 min, 2h and 24 h post exposure intervals. Larvae and adult stages of both Rhipicephalus spp. exhibited attraction and clustering on exposure to AP microparticles. Statistical analysis revealed a highly significant difference in the number of ticks that were attracted to pheromone control as compared to polymer control. In test plates more than

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70 per cent ticks were found dead within 30 min post exposure and cent per cent mortality of ticks was recorded at 2 h post exposure period with test microparticles. There was highly significant difference in the number of ticks that were found dead with test beads as compared to acaricide control. Slow and steady increase in mortality was recorded with acaricide control beads while none of the ticks were found dead on exposure to pheromone control beads. Assembly pheromone-acaricide beads were very effective in controlling ticks in vitro as compared to using only AP or only acaricide.

Keywords: *Rhipicephalus annulatus*, *Rhipicephalus microplus*, *assembly pheromone, Petri-dish bioassay*

Ticks are often regarded as the most efficient and adaptable vectors of a broad spectrum of pathogens affecting livestock globally. *Rhipicephalus (R.) annulatus* and *Rhipicephalus (R.) microplus* are small, brevirostrate ixodid ticks of the genus *Rhipicephalus*, sub genus *Boophilus*, belonging to the *R. microplus* complex, which are the closely related one-host ticks of livestock that preferentially feed on cattle and are widely distributed in tropical and subtropical regions. They are the notorious vectors of *Babesia (B.) bigemina*, *Babesia (B.) bovis* and *Anaplasma (A.) marginale*. *Rhipicephalus microplus* is the predominant tick species infesting cattle and buffaloes in India (Singh *et al*., 2021) while *R. annulatus* is the widely prevalent tick of bovines in Kerala (Akhil *et al*., 2021). Theileriosis, anaplasmosis and babesiosis are the most prevalent economically important tick-borne diseases of domestic livestock in India. Ticks and tick-borne diseases are creating a deeply disquieting effect on the farming community and are having a greater impact on the growing economy of the developing nation (Ghosh and Nagar, 2014). The presence of *Anaplasma (A.) bovis*, *Theileria (T.) orientalis* (Nimisha *et al*., 2019), *B. bigemina*, *A. marginale*, *Anaplasma (A.) phagocytophilum* and *Rickettsia* spp. (Hembram *et al*., 2022) have been confirmed in *R. annulatus* ticks from Kerala. Unlike the two-host and three-host ticks, the comparatively short life cycle (3 to 4 weeks) of one-host *Rhipicephalus* spp. can result in the faster development of resistance to single or multiple acaricides.

Chemical control has been the most preferred and widely followed strategy adopted by farmers in India for years in managing ticks. However, the indiscriminate dependence on chemical acaricides for controlling ticks on and off the host has likely led to the development of acaricide resistant tick fauna, the presence of chemical residues in the environment and incidental effects on non-target organisms. The rapid emergence of resistant ticks to commonly used acaricides, the longer duration and higher cost associated with the development of new acaricides, anti-parasitic agents and the continuous emergence of tick-borne diseases warrant the need for developing innovative and more effective tick control strategies (Ranju *et al*., 2012)

Semiochemical assisted tick control is a novel, promising alternate tick control perspective. Semiochemicals are chemical signal compounds of host or tick origin which are secreted into the external environment that mediate tick behaviour. Assembly pheromones are a group of semiochemicals which are the nitrogenous by products of blood meal metabolism of ticks (Hamilton, 1992), released in tick excreta and also been identified in tick exuvia. The perception of AP by ticks leads to the formation of arrested tick clusters in their shelter microenvironments (Leahy *et al*., 1973).

In the present study *in vitro* Petri-dish bioassay has been utilised for evaluating the response of tick to the test compound. Laboratory bioassays are the initial experiments for identifying attractants and evaluating their significance in acarine biology, as well as for determining their potential utility in pest control (Carr and Roe, 2016). To effectively control ticks, it is essential to use pheromones in combination with a suitable acaricide, as relying solely on pheromones is inadequate (Sonenshine, 2004). Since AP have been proven to evoke the clustering of various species of hard and soft ticks, this study is aimed at evaluating the response of *R. annulatus* and *R. microplus* ticks to synthetic analogues of AP encapsulated in porous calcium alginate microparticles in combination with acaricide.

Materials and methods

Collection and identification of ticks and maintaining tick colonies in laboratory

Fully engorged females, partially fed male and female ticks were collected from cattle reared in organised and unorganised farms in different regions of Thrissur and Palakkad districts. Ticks were morphologically identified as per the taxonomic keys of Walker *et al.* (2013). Partially fed male and female ticks of both *R. annulatus* and *R. microplus* were used immediately after collection and identification for Petri-dish bioassays while fully engorged female ticks were maintained in laboratory for oviposition at a temperature of about 28°C and relative humidity (RH) of ≥85 per cent in a desiccator containing 10 per cent KOH solution. Ticks were left undisturbed, allowing the laying of eggs and hatching of eggs to larvae. Unfed larvae after seven to 14 days of hatching were used for Petri-dish bioassays.

Optimal concentration of AP and acaricide used

The synthetic analogues of AP *viz.,* guanine, adenine and xanthine (Sigma-Aldrich, USA) were used in 25:1:1 ratio as indicated by Sonenshine (2004). A total of 102.6mg of AP was dissolved in 4 ml of millipore water and 200µL of the solution and 250 µL of the commercial preparation of the synthetic pyrethroid, deltamethrin (12.5 mg/mL) (BUTOX® VET, MSD Animal Health Pvt. Ltd.) was encapsulated in alginate microparticles.

Preparation of AP microparticles

Test beads were prepared by dispersing optimal concentrations of AP and acaricide in alginate matrix followed by cross linking with calcium chloride in acetic acid by extrusion method (Anwar *et al*., 2009). Pheromone control, acaricide control and polymer control beads were also prepared. Beads were washed in millipore water and dried in room temperature for 24 h. Dried beads were stored in sterile plastic containers at room temperature and were subsequently used for performing Petri-dish bioassays.

Characterisation of calcium alginate beads

The shape and surface morphology of beads were assessed by stereo zoom microscopy (Leica microsystems GmbH, Germany). Encapsulation of AP within the beads was confirmed by Fourier transform infra-red spectroscopy (FTIR) before performing Petri-dish bioassays.

Petri-dish bioassays

The Petri-dish bioassay method reported by Yoder and Stevens (2000) was modified and employed in the present study. Glass Petri-dish (Borosil, India) of nine cm diameter was used, on which four quadrants were marked outside. For each assay, test, pheromone control, acaricide control and polymer control plates were prepared. Two milligram of test beads containing AP-deltamethrin combination were placed at one quadrant of the test plate. Similarly, pheromone control, acaricide control and polymer control plates were prepared by placing AP beads, deltamethrin beads and alginate beads, respectively. The unfed larvae or partially fed adults of a single species of tick were introduced 10 numbers each at a time to each Petri-dish, in the opposite quadrant to which the beads were placed. Each Petri-dish was then covered with another Petri-dish having the same diameter and sealed with laboratory grade parafilm. The entire procedure was carried out in room temperature and the behavioural responses and mortality of ticks were recorded at 10 min, 30 min, 2 h and 24 h post exposure intervals. The trials were replicated until n=100 for unfed larvae and n=50 for adult ticks of each species were assayed. For each trial, unconditioned ticks, freshly prepared beads and clean Petri-dishes were used.

Results and discussion

Gross morphology of Rhipicephalus annulatus

It is a small brevirostrate tick. Body was oval to rectangular in outline (Fig. 1). Palps were very short. The internal margin of first palpal article was long and slightly concave without any protuberance on ventral view and

Fig. 1. Morphology of *R. annulatus* male-ventral view

the hypostomal teeth were in 4+4 columns. Short paired spurs were present on first coxae ventrally and well-developed ventral plates were observed in male ticks. In females, indistinct paired spurs were present on first coxae. Spurs on adanal and accessory adanal plates were indistinct ventrally and were not visible dorsally. Caudal appendage was absent in male ticks. Spiracular plate was oval. Festoons were absent in both sexes.

Gross morphology of Rhipicephalus microplus

Internal margin of first palpal article was short and more concave without any bristle bearing protuberance. In males, spurs on first palpal article was long and the cleft between spurs was distinct. The anterior spurs on the first coxae were visible from the dorsal side and a narrow caudal appendage was visible from both dorsal and ventral sides. In females, short spurs were present in the second coxae and those on the first coxae were distinct (Fig. 2 and 3).

Characters of calcium alginate microparticles

Stereo zoom microscopy of beads revealed almost spherical shape with rough or irregular surface. Fourier transform spectroscopy analysis revealed the individual peak at wave number 2600 cm^{-1} region corresponding to AP, thus confirming the encapsulation of AP with in calcium alginate.

Response of unfed larvae of R. annulatus

The response of larvae to test and control beads are represented in Table 1. No peculiar behaviour response was exhibited in polymer control plates. With pheromone control beads, the larvae exhibited directional movement towards beads, came in contact with the beads and became akinetic. The per cent of larvae that became akinetic continually increased for up to 30 min. Only a slight increase in the per cent of akinetic larvae could be recorded at the end of 2 h post exposure period, after which there was no further increase. Statistical analysis gave highly significant difference in the

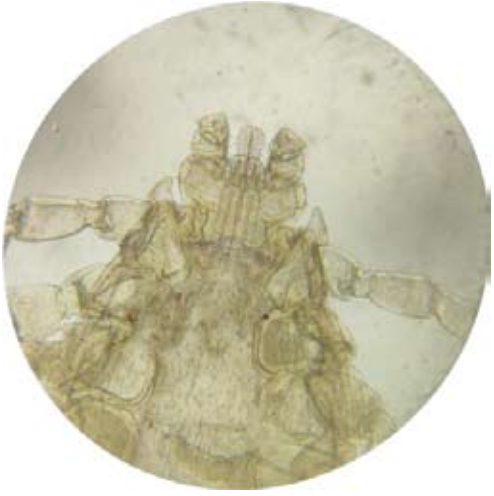

Fig. 2. *R. microplus* male anterior half ventral view

Fig. 3. *R. microplus* posterior half ventral view

attraction of larvae between polymer control and pheromone control.

In test plates, larvae rapidly moved towards test beads and crawled on to the beads and in the narrow space between beads, became sluggish and dead in between the beads. Per cent mortality during 10 and 30 minutes were 38 and 75 per cent, respectively. Cent per cent larval mortality was recorded at the end of 2 h. In acaricide control plates, a gradual increase in sluggishness and death were recorded. The mortality and sluggishness of larvae was found highly significant during each observation period in test plates as compared to acaricide control plates. In acaricide control plates, dead larvae were not found in between and around the beads but distributed in all the four quadrants.

Response of partially fed adults of R. annulatus

Polymer control beads failed to evoke any peculiar behavioural response in adult ticks. On releasing to pheromone control plates,

Table 1. Response of unfed larvae of *R. annulatus* in Petri-dish bioassay

Observation time	Response of the larvae	Polymer control (Alginate)	Pheromone control (Alginate + AP)	Test (Alginate +AP + Deltamethrin)	Acaricide control (Alginate + Deltamethrin)	Chi-square value
10 min	Active	100^{a} (100)	85^{c} (85)	28^{d} (28)	95^{b} (95)	187.35**
	Akinetic	0^{b}	15^{a} (15)	0^{b}	0^{b}	16.22**
	Sluggish	0^{c}	0^{c}	34^{a} (34)	5^{b} (5)	59.59**
	Dead	0^{b}	0^{b}	38^{a} (38)	0^{a} (0)	46.91**
30 min	Active	100^{a} (100)	65^{c} (65)	0^{d}	85^{b} (85)	248.53**
	Akinetic	0^{b}	35^{a} (35)	0^{b}	0^{b}	42.42**
	Sluggish	0^{c}	0^{c}	25^{a} (25)	9^{b} (9)	31.91**
	Dead	0^{c}	0^{c}	75^{a} (75)	6^{b} (6)	176.26**
2 h	Active	100^{a} (100)	63^{b} (63)	0^{c}	72^{b} (72)	220.63**
	Akinetic	0^{b}	37^{a} (37)	0^{b}	0^{b}	45.40**
	Sluggish	0^{b}	0^{b}	0^{b}	15^{a} (15)	16.21**
	Dead	0^{c}	0^{c}	100^{a} (100)	13^{b} (13)	251.83**
24 h	Active	100^{a} (100)	63^{b} (63)	0^{c}	50^{b} (50)	205.94**
	Akinetic	0^{b}	37^{a} (37)	0^{b}	0^{b}	45.40**
	Sluggish	0^{b}	0^{b}	0^{b}	24^{a} (24)	27.27**
	Dead	0^{c}	0^{c}	100^{a} (100)	26^{b} (26)	221.02**

** Significant at 0.01 level ($p<0.01$). Numbers having different letter as superscript differ significantly within a row. Figures in parenthesis indicate percentage.

ticks exhibited intense questing and movement towards the pheromone beads. Momentary cessation of kinetic activity was noticed on contacting the beads. The per cent of ticks that were akinetic during 10 min was 12 per cent which greatly increased to 40 per cent in 30 min after which a slight increase of four per cent was recorded in 2 h. There was no increase in the per cent of akinetic ticks after 2 h post exposure. The per cent attraction with pheromone control was statistically highly significant as compared to polymer control (Table 2).

On exposing the adult ticks to test beads, initial intense questing and movement towards test beads were noticed. On reaching the test quadrant, some ticks exhibited a momentary cessation of kinetic activity. After which they crawled on to the beads, became sluggish and subsequently died. Per cent mortality was 12 per cent in 10 min which escalated up to 76 per cent in 30 min. All the ticks were found dead by 2 h post exposure. In acaricide control plates the per cent mortality recorded was 0 in 10 min, which raised to 10

Table 2. Response of partially fed adults of *R. annulatus* in Petri-dish bioassay

Observation time	Response of partially fed adults	Polymer control (Alginate)	Pheromone control (Alginate + AP)	Test (Alginate + AP + Deltamethrin)	Acaricide control (Alginate + Deltamethrin)	Chi-square value
10 min	Active	50^{a} (100)	44^{b} (88)	26^{c} (52)	42^{b} (84)	40.94**
	Akinetic	0^{b}	6^{a} (12)	0^{b}	0^{b}	6.38*
	Sluggish	0^{c}	0^{c}	18^{a} (36)	8^{b} (16)	22.71**
	Dead	0^{b}	0^{b}	6^{a} (12)	0^{b}	6.38*
30 min	Active	50^{a} (100)	30^{b} (60)	0^{c}	33^{b} (66)	105.52**
	Akinetic	0^{b}	20^{a} (40)	0^{b}	0^{b}	25.00**
	Sluggish	0^{b}	0^{b}	12^{a} (24)	12^{a} (24)	14.29**
	Dead	0^{c}	0^{c}	38^{a} (76)	5^{b} (10)	71.32**
2 h	Active	50^{a} (100)	28^{b} (56)	0^{c}	25^{b} (50)	100.63**
	Akinetic	0^{b}	22^{a} (44)	0^{b}	0^{b}	28.20**
	Sluggish	0^{b}	0^{b}	0^{b}	15^{a} (30)	17.65**
	Dead	0^{c}	0^{c}	50^{a} (100)	10^{b} (20)	116.67**
24 h	Active	50^{a} (100)	28^{b} (56)	0^{d}	17^{c} (34)	105.60**
	Akinetic	0^{b}	22^{a} (44)	0^{b}	0^{b}	28.20**
	Sluggish	0^{b}	0^{b}	0^{b}	17^{a} (34)	20.48**
	Dead	0^{c}	0^{c}	50^{a} (100)	16^{b} (32)	105.84**

** Significant at 0.01 level ($p<0.01$); * Significant at 0.05 level ($p<0.05$). Numbers having different letter as superscript differ significantly within a row. Figures in parenthesis indicate percentage.

per cent in 30 min and gradually increased to 20 per cent and 32 per cent in 2 h and 24 h, respectively. Statistically significant difference was recorded in mortality between the test and acaricide control plates in 10 min post exposure, while a highly significant difference was recorded at 30 min, 2 h and 24 h post exposure.

Response of unfed larvae of R. microplus

The per cent of larvae that became akinetic with pheromone beads was 13 in 10 min, which greatly increased to 33 in 30 min. By 2 h time, only one per cent increase in akinetic larvae was recorded after which no increase in the per cent of akinetic larvae was recorded.

The response of larvae with test beads were very similar as that exhibited by *R. annulatus* larvae to test beads. Per cent mortality was 40 in 10 min which shot up to 77 in 30 min. Cent per cent of the released larvae were found dead in 2 h (Table 3).

Table 3. Response of unfed larvae of *R. microplus* in Petri-dish bioassay

Observation time	Response of the larvae	Polymer control (Alginate)	Pheromone control (Alginate + AP)	Test (Alginate + AP + Deltamethrin)	Acaricide control (Alginate + Deltamethrin)	Chi-square value
10 min	Active	100^{a} (100)	87^{b} (87)	30^{c} (30)	94^{b} (94)	180.63**
	Akinetic	0^{b}	13^{a} (13)	0^{b}	0^{b}	13.94**
	Sluggish	0^{c}	0^{c}	30^{a} (30)	6^{b} (6)	47.73**
	Dead	0^{b}	0^{b}	40^{a} (40)	0^{b} (0)	50.00**
30 min	Active	100^{a} (100)	67^{c} (67)	0^{d}	82^{b} (82)	243.10**
	Akinetic	0^{b}	33^{a} (33)	0^{b}	0^{b}	39.52**
	Sluggish	0^{c}	0^{c}	23^{a} (23)	12^{b} (12)	25.68
	Dead	0^{c}	0^{c}	77^{a} (77)	6^{b} (6)	183.32**
2 h	Active	100^{a} (100)	66^{b} (66)	0^{c}	72^{b} (72)	223.22**
	Akinetic	0^{b}	34^{a} (34)	0^{b}	0^{b}	40.96**
	Sluggish	0^{b}	0^{b}	0^{b}	15^{a} (15)	16.21**
	Dead	0^{c}	0^{c}	100^{a} (100)	17^{b} (17)	240.69**
24 h	Active	100^{a} (100)	66^{b} (66)	0^{c}	53^{b} (53)	208.88**
	Akinetic	0^{b}	34^{a} (34)	0^{b}	0^{b}	40.96**
	Sluggish	0^{b}	0^{b}	0^{b}	22^{a} (22)	24.72**
	Dead	0^{c}	0^{c}	100^{a} (100)	25^{b} (25)	222.86**

** Significant at 0.01 level ($p<0.01$). Numbers having different letter as superscript differ significantly within a row. Figures in parenthesis indicate percentage.

Response of partially fed adults of R. microplus

The response of adult ticks to test and control beads were similar to that recorded with *R. annulatus* (Table 4). In pheromone control plates, 10 per cent became akinetic in 10 min, which rose to 38 per cent in 30 min after which only a slight increase of four per cent was recorded in 2 h post exposure period. The number of akinetic ticks on exposure to pheromone control was found highly significant statistically as compared to polymer control.

In test plates, the per cent mortality was 14 in 10 min, 78 in 30 min and 100 in 2 h. Mortality of ticks at different time intervals in test plates were highly significant statistically as compared to acaricide control.

In the present study statistical analysis revealed a highly significant difference in the number of larvae and adults of both the tick species that became akinetic with pheromone control as compared to acaricide control. There was also a highly significant difference in the number of ticks that were found dead with test

Table 4. Response of partially fed adults of *R. microplus* in Petri-dish bioassay

Observation time	Response of partially fed adults	Polymer control (Alginate)	Pheromone control (Alginate + AP)	Test (Alginate + AP + Deltamethrin)	Acaricide control (Alginate + Deltamethrin)	Chi-square value
10 min	Active	50^{a} (100)	45^{b} (90)	25^{c} (50)	42^{b} (84)	45.87**
	Akinetic	0^{b}	5^{a} (10)	0^{b}	0^{b}	5.26*
	Sluggish	0^{c}	0^{c}	18^{a} (36)	8^{b} (16)	22.71**
	Dead	0^{b}	0^{b}	7^{a} (14)	0^{b}	7.53**
30 min	Active	50^{a} (100)	31^{b} (62)	0^{c}	31^{b} (62)	104.38**
	Akinetic	0^{b}	19^{a} (38)	0^{b}	0^{b}	23.46**
	Sluggish	0^{b}	0^{b}	11^{a} (22)	13^{a} (26)	14.58**
	Dead	0^{c}	0^{c}	39^{a} (78)	6^{b} (12)	84.00**
2 h	Active	50^{a} (100)	29^{b} (58)	0^{c}	25^{b} (50)	101.12**
	Akinetic	0^{b}	21^{a} (42)	0^{b}	0^{b}	26.58**
	Sluggish	0^{b}	0^{b}	0^{b}	16^{a} (32)	19.05**
	Dead	0^{c}	0^{c}	50^{a} (100)	9^{b} (18)	119.07**
24 h	Active	50^{a} (100)	29^{b} (58)	0^{d}	16^{c} (32)	107.53**
	Akinetic	0^{b}	21^{a} (42)	0^{b}	0^{b}	26.58**
	Sluggish	0^{b}	0^{b}	0^{b}	18^{a} (36)	21.95**
	Dead	0^{c}	0^{c}	50^{a} (100)	16^{b} (32)	105.84**

**Significant at 0.01 level ($p<0.01$); * Significant at 0.05 level ($p<0.05$). Numbers having different letter as superscript differ significantly within a row. Figures in parenthesis indicate percentage.

beads in comparison with the acaricide control. A remarkable observation was that a very high per cent of ticks became akinetic in 30 min post exposure period which could be correlated with the high per cent mortality of ticks in test plates where more than 70 per cent ticks were found dead in 30 min. In test plates, the attraction and assembly of ticks to the test beads resulted in acquiring lethal doses of acaricide, hence a steep rise in mortality was recorded within a short time period while a slow and steady rise in sluggishness and mortality was recorded with acaricide control beads, owing to the absence of the attractive component, the roaming ticks barely came in contact with the lethal dose of deltamethrin needed to elicit an immediate knock down response. Since pheromone control beads were devoid of acaricide, the attracted ticks after their desperate attempts to probe and feed on the beads, exhibited a tendency to move away from the beads as time progressed which was also observed in the study by Dhivya (2013).

Petri-dish bioassay is an effective method for identifying an attractant chemical and assessing its persistent activity. In Petri-dish bioassay, ticks can come into direct contact with the test chemical and can perceive the test compound through both olfactory and gustatory stimuli (Carr and Roe, 2016).

Assembly pheromones trigger the cessation of movement in ticks, leading to the formation of stationary tick clusters within microenvironments. This clustering promotes mating and improves tick's ability to locate hosts, while also reducing dehydration of ticks, minimizing the chances of predation, and lowering the risk of physical injury (Sonenshine, 2006). In the present study, both *R. annulatus* and *R. microplus* larvae and adults exhibited attraction, cessation of kinetic activity and clustering on exposure to AP microparticles. Akinetic response of ticks observed in the present study has been reported previously in other species of ticks (Carde and Baker, 1984; Grenacher *et al*., 2001; Ranju *et al*., 2018; Bhoopathy and Latha, 2018).

The current study employed the use of synthetic analogues of AP *viz*., guanine, adenine and xanthine in the ratio 25:1:1 for evaluating the response of ticks *in vitro*. The use of different combinations of AP in *in vitro* trials have been previously reported. Allan and Sonenshine (2002) opined that AP components like guanine, xanthine and hematin when mixed in 25:1:1 ratio enhanced the assembly of *I. scapularis* ticks equivalent to that recorded with the cast skins. Bhoopathy *et al*. (2015) reported that AP combination without hematin was more effective in attracting the unfed larvae of *R. sanguineus* while the addition of hematin had no effect in attracting the other developmental stages. *In vitro* trials with AP-acaricide impregnated Whatman filter papers revealed that guanine, xanthine, adenine and hematin in 25:1:1:1 ratio was effective in evoking varying levels of attractive response in ixodid ticks like *R. sanguineus, R. microplus, R. haemaphysaloides, H. bispinosa* and *H. marginatum* (Ranju *et al*., 2018).

Pheromones have a brief duration of effectiveness (Sonenshine, 2004). Even though AP is comparatively less volatile (Otieno *et al.,* 1985), it could be degraded by microenvironmental factors (Bhoopathy *et al*., 2015). Assembly pheromones can remain stable for up to 21 days, after which purine degradation occurs (Grenacher *et al*., 2001). Degradation of AP could be reduced by encapsulating it within natural and synthetic polymer microparticles. In the current study calcium alginate microparticles were used to encapsulate AP and the efficacy of calcium alginate to entrap the pheromone and to release it in a sustained manner was evaluated. Alginate is a biopolymer derived from brown seaweed, consisting of polyuronic acid. It is widely employed in the biomedical field due to its biocompatibility, biodegradability, low toxicity, relatively low cost, and gentle gelation induced by the addition of divalent cations such as calcium (Quong, 2003; Lee and Mooney, 2012). In the present study, after encapsulation, the activity of pheromone was not affected and the porosity of the beads allowed the diffusion of the entrapped AP in a sustained manner for up to two months as reported in earlier studies (Bhoopathy and Latha, 2017; Gowrishankar *et al*., 2019).

Sonenshine (2004) reported that for the effective utilisation of pheromones in tick management, it is recommended to combine pheromones with a suitable acaricide. Deltamethrin was used in the present study at an optimal concentration of 250 µL which was significantly lower than the dose of the same compound employed for routine on and off the host tick control and with the optimal concentration, rapid death of ticks was noticed as reported by Ranju *et al*. (2018). Deltamethrin did not interfere with the attractive response of ticks on combining with AP (Ranju, 2011; Bhoopathy and Latha, 2017).

The larvae and adults of both *R. annulatus* and *R. microplus* revealed a similar pattern of attraction and mortality in Petri dish bioassays. The observations agree with Ranju *et al*. (2018) who recorded a steep rise in per cent mortality of *R. microplus* larvae and adults within 1 h of exposure to AP-deltamethrin impregnated filter paper strips. No published data is available on the response of *R. annulatus* ticks to AP.

Conclusion

The results of the present study highlight the attractive response of *R. annulatus* and *R. microplus* to AP encapsulated calcium alginate beads. Assembly pheromone-deltamethrin combination was found effective in attracting and killing both the larvae and adult stages of *Rhipicephalus* spp. of cattle *in vitro*.

Conflict of interest

The authors declare that they have no conflict of interest.

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