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In *vitro* assessment of assembly and sex pheromones in the control of larval stages of brown dog ticks#

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Abstract

The increasing incidence of tick-borne diseases and the growing resistance to conventional acaricides underscore the urgent need for innovative and sustainable tick management strategies. This study investigates the potential of assembly and sex pheromones for the control of larval stages of Rhipicephalus sanguineus, the brown dog tick, which poses significant health risks to dogs. The ticks were collected from various canine hosts in Thrissur, Kerala and subjected to Petri-dish bioassays using vapour patches impregnated with synthetic assembly pheromone (AP) and sex pheromone (SP), both individually and in combination. The efficacy of these semiochemicals was assessed based on the attraction and clustering behaviour of unfed larvae and partially fed adult ticks over a 24-hour period. Results demonstrated a significantly higher attraction to AP-impregnated patches, with notable efficacy observed in causing tick aggregation, particularly beyond 30 min of exposure. While SP also showed potential in attracting ticks, the combination of SP and AP did not result in a synergistic effect. The study highlights the potential of using semiochemicals as an environmentally sustainable alternative to traditional acaricides, highlighting their role in integrated pest management strategies.

Keywords: *Rhipicephalus sanguineus, adenine, guanine, xanthine, 2,6-DCP, Petri-dish bioassay*

Ticks are significant vectors for a multitude of infectious agents that are capable of inducing diseases in humans, livestock and companion animals. Among these, the brown dog tick, *Rhipicephalus sanguineus*, is notably prevalent among canine populations in various states of India (Abd rani *et al*., 2011; Balakrishnan *et al*. 2019) and Kerala is not an exception (Reena, 2023). This tick species are vectors of pathogens such as *Babesia canis*, *Ehrlichia canis* and *Hepatozoon canis*, which present considerable health threats to affected hosts without timely intervention (Jain *et al*., 2018).

While acaricides have demonstrated efficacy in tick management both on hosts and in the environment, their extensive and non-targeted application has precipitated resistance among tick populations. Deltamethrin and amitraz resistance is widespread among brown dog tick population in Kerala (Amrutha *et al*., 2021; Reena *et al*., 2023).

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Moreover, the pervasive employment of acaricides has been implicated in environmental degradation and poses potential health hazards to public. Contemporary strategies in pest management have shifted towards the integration of lure and kill techniques, which amalgamate attractants with acaricides to engineer formulations or devices with controlled release properties. Specifically, the employment of pheromones as attractants has emerged as a focal point in the management of ticks, underscoring its growing significance in innovative pest control methodologies (Latha, 2012). Semiochemicals constitute chemical signalling compounds synthesised by either the host or the tick, which are emitted into their surroundings and may modulate tick behaviour (Sonenshine, 1985). Assembly pheromones, originating from the nitrogenous by-products of tick metabolism found in excreta and exuviae, trigger a behavioural response termed 'arrestment', wherein ticks cease movement and aggregate, potentially facilitating host detection and mitigation of stress. Sex pheromones, integral to the mating process, are delineated into three categories: attractant sex pheromones (ASPs), mounting sex pheromones (MSPs) and genital sex pheromones (GSPs) (Sonenshine, 2004). Although sex pheromones like 2,6-dichlorophenol (2,6-DCP) exhibit volatility and instability, components of AP such as guanine, adenine and xanthine demonstrate lesser volatility, indicating varied stability and dispersal characteristics among tick pheromones (Otieno *et al*., 1985).

In the background of the high prevalence of tick-borne infections in the dog population of the state and the widespread prevalence of deltamethrin and amitraz resistance in *R. sanguineus*, there is a critical need for more targeted, efficient and environmentally sustainable approaches to tick control. In this background, a preliminary study was conducted on the *in vitro* assessment of assembly and sex pheromones in the control of *R. sanguineus.*

Material and methods

Sample collection

During the time frame from January 2023 to January 2024, a total of 462 ticks were collected from canines that were brought to veterinary dispensaries, housed in privately operated kennels and maintained within animal shelters by non-governmental organizations located in Thrissur. To ascertain the ticks' gross morphological characteristics, such as the body profile, the proportional length and width of the mouthparts relative to the basis capitulum, the configuration of the basis capitulum, the presence of spurs on the pedipalp segments and coxae, the contour of the scutum's posterior margin and the existence and form of grooves, plates, caudal appendage and festoons, a stereo zoom microscope (Leica Microsystems GmbH, Germany) was employed, guided by the morphological keys outlined by Sen and Fletcher (1962).

Maintenance of tick colony

Fully engorged female ticks collected from dogs were individually allocated to dry plastic specimen tubes (dimensions: 37x100 mm), each distinctly labelled and subsequently sealed with a permeable fabric. These specimens were reared within a laboratory setting, regulated at an ambient temperature of approximately 28°C and a relative humidity (RH) of 85%, utilising a desiccator, as detailed in the methodology by Reena (2023).

Petri-dish bioassay using vapour patches

Synthetic tick pheromones, specifically the sex pheromone (2,6-dichlorophenol, D70201) and assembly pheromones (Adenine - A8626, Guanine - G11950, and Xanthine - X0626), with a purity of 99 per cent, were acquired from Sigma Aldrich, Germany. The assembly pheromone (AP) composition included 95 mg of guanine, 3.8 mg of adenine and 3.8 mg of xanthine in a 25:1:1 ratio, dissolved in 4 ml of Type 1 ultrapure water. A 200 µl aliquot of this solution was determined as the optimal concentration for application (Sonenshine, 2004; Ranju, 2011; Boopathy and Latha, 2017; Anish *et al.,* 2017). Similarly, the sex pheromone (SP), 2,6-DCP, was prepared by dissolving 8.23 mg in 500 µl of acetone, utilised at an optimal concentration based on the findings of Louly *et al*. (2008) and Ranju *et al*. (2012a). A mixture of SP and AP was formulated by combining the respective solutions and employed at an identified optimal concentration. Vapour patches, consisting of a 150 gsm non-woven fabric with a 350 mm diameter, were supplied by the Department of Veterinary Parasitology, Madras Veterinary College, TANUVAS. These patches were designed to steadily release the active ingredients through sustained entrapment. The impregnation on the patches involved an optimal concentration of 500 µl SP, 200 µl AP and 250 µl of deltamethrin (Gowrishankar *et al*., 2021a), alongside suitable controls. Fourier transform infrared spectroscopy (FTIR) analysis was conducted at the Central Instruments Laboratory (CIL), College of Veterinary and Animal Sciences, Mannuthy, Thrissur, to qualitatively assess the impregnation and entrapment of pheromones within the vapour patches, which were utilised within seven days of impregnation.

A Petri-dish bioassay, as described by Yoder and Stevens (2000) and Gowrishankar *et al.* (2021a), was conducted to evaluate tick attraction to the vapour patches, recording ticks drawn to the treated quadrant versus those in the remaining quadrants. These assays were carried out at ambient room temperature, with behavioural responses noted at intervals of 10 minutes, 30 minutes, 2 hours, and 24 hours, including attraction, clustering and feeding posture.

Statistical analysis was performed using SPSS version 24.0, employing a Z-test for two independent

proportions to compare the percentage of ticks attracted between unfed and partially fed states. Additionally, the chi-square test for multiple proportions was utilized to compare attraction rates over time within each group and among the four different treatment groups.

Results and discussion

Ticks

The ticks harvested from naturally infested canines were identified as *Rhipicephalus sanguineus sensu lato*, distinguished by a hexagonally shaped basis capitulum, short mouthparts, a pronouncedly bifurcated first coxa, presence of festoons and comma-shaped spiracular plates, as described by Dantas-Torres (2008). The incidence of tick infestation within the canine population was documented as 34.41 percent. Nikhat (2022), in a study encompassing 1000 ticks collected

Table 1. Comparison of percentage attraction of unfed larvae to SP, AP, SP+AP and control vapour patch

Observation time	SP (n=100)	AP (n=100)	SP+AP (n=100)	Control (n=100)	χ^2 value (P-value)
10 min	24 (24)^{a}	36(36)^{a}	28(28)^{a}	3(3)^{b}	33.842** (<0.001)
30 min	38 (38)^{b}	52(52)^{a}	44(44)^{ab}	4(4)^{c}	59.254** (<0.001)
2 hrs	51 (51)^{b}	65(65)^{a}	59(59)^{ab}	7(7)^{c}	83.678** (<0.001)
24 hrs	56 (56)^{b}	79(79)^{a}	64(64)^{b}	11(11)^{c}	103.018** (<0.001)

Values in the brackets are percentages. ** Significant at 0.01 level; Percentage having different letter as superscript differ significantly within a row.

Table 2. Comparison of percentage attraction of partially fed adult to SP, AP, SP+AP and control vapour patch

Observation time	SP (n=50)	AP (n=50)	SP+AP (n=50)	Control (n=50)	χ^2 value (P-value)
10 min	5(10)^{c}	19(38)^{a}	9(18)^{b}	1(2)^{c}	25.372** (<0.001)
30 min	13(26)^{b}	30(60)^{a}	15(30)^{b}	3(6)^{c}	61.451** (<0.001)
2 hrs	20(40)^{b}	34(68)^{a}	19(38)^{b}	3(6)^{c}	40.917** (<0.001)
24 hrs	23(46)^{b}	39(78)^{a}	23(46)^{b}	5(10)^{c}	46.788** (<0.001)

Values in the brackets are percentages. ** Significant at 0.01 level; Percentage having different letter as superscript differ significantly within a row.

Table 3. Comparison of percentage of ticks attracted to SP impregnated vapour patch with exposure times

Exposure time	Unfed larva (n=100)	Partially fed adult (n=50)	Z-value (P-value)
10 min	24 (24)^{c}	5(10)^{c}	2.326* (0.020)
30 min	38 (38)^{b}	13(26)^{b}	1.524^{ns} (0.128)
2 h	51 (51)^{ab}	20(40)^{ab}	1.288^{ns} (0.198)
24 h	56 (56)^{a}	23(46)^{a}	1.160^{ns} (0.246)
χ^2 value (P-value)	25.277** (<0.001)	18.186** (<0.001)	

Values in the brackets are percentages. ** Significant at 0.01 level; * Significant at 0.05 level; ns non-significant. Percentage having different letter as superscript differ significantly within a column.

Table 4. Comparison of percentage of ticks attracted to AP impregnated vapour patch with exposure times

Observation time	Unfed larva (n=100)	Partially fed adult (n=50)	Z-value (P-value)
10 min	36(36)^{c}	19(38)^{c}	0.239^{ns} (0.811)
30 min	52(52)^{b}	30(60)^{b}	0.937^{ns} (0.349)
2 h	65(65)^{b}	34(68)^{ab}	0.369^{ns} (0.712)
24 h	79(79)^{a}	39(78)^{a}	0.140^{ns} (0.889)
χ^2 value (P-value)	41.461** (<0.001)	18.243** (<0.001)	

Values in the brackets are percentages. ** Significant at 0.01 level; ns non-significant. Percentage having different letter as superscript differ significantly within a column.

Table 5. Comparison of percentage of ticks attracted to SP+AP impregnated vapour patch with exposure times

Observation time	Unfed (n=100)	Partially fed (n=50)	Z-value (P-value)
10 min	28(28)^{c}	9(18)^{b}	1.419^{ns} (0.156)
30 min	44(44)^{b}	15(30)^{ab}	1.715^{ns} (0.086)
2 h	59(59)^{a}	19(38)^{a}	2.487* (0.013)
24 h	64(64)^{a}	23(46)^{a}	2.111* (0.035)
χ^2 value (P-value)	31.650** (<0.001)	9.679* (0.022)	

Values in the brackets are percentages. ** Significant at 0.01 level; ns non-significant. Percentage having different letter as superscript differ significantly within a column.

from Thrissur, Kerala, observed that 800 specimens were classified as *Rhipicephalus sanguineus s.l.* Moreover, Abd Rani *et al.* (2011) reported a prevalence rate of *Rhipicephalus* infestation in canines as being 100 percent across Mumbai, Delhi and Ladakh.

Impregnation into vapour patches

Vapour patches, impregnated with synthetic pheromones (SP), assembly pheromones (AP) and a combination of SP and AP, with and without the addition of acaricides, were explored as potential transdermal delivery systems. These patches, designed for pharmaceutical use, facilitate the direct dermal absorption of active substances. Kulkarni and Vandana (2018) posited that such impregnated vapour patches exhibit potential as transdermal drug delivery systems (TDDS), capable of releasing essential oils over a period of up to six hours. A formulation incorporating synthetic analogues of guanine, xanthine and adenine in a ratio of 25:1:1 elicited responses comparable to those triggered by natural pheromones, as evidenced in studies by Anish *et al.* (2017) and Gowrishankar *et al.* (2021a, 2021b) and was thus selected for use in the current investigation.

Gowrishankar *et al.* (2019a, 2019b, 2021a, 2021b) demonstrated that SP, prepared by dissolving 8.23 mg of 2,6-dichlorophenol (2,6-DCP) in 500 µl of acetone, attracted all developmental stages of *Rhipicephalus sanguineus* in field trials, leading to the adoption of this concentration for the current study. However, the addition of deltamethrin to the vapour patches was observed to detrimentally alter their physical properties, such as increased moisture retention and separation from the adhesive backing. Hence acaricide-impregnated patches were excluded from bioassay trials. This aligns with the findings of Kulkarni and Vandana (2018), who noted that only potent drugs requiring lower dosages are suitable for vapour patch impregnation, as the patches' capacity might be insufficient for acaricide incorporation without compromising their physical integrity. Similarly, Gowrishankar *et al.* (2019b) also omitted acaricides in their vapour patch studies.

Bioassays employing Petri-dishes utilised vapour patches impregnated with SP, AP, and their combination, alongside control patches, to assess efficacy. The impregnated patches exhibited additional spectral peaks at 2800 cm^{-1} and 2600 cm^{-1}, indicative of SP and AP, respectively. Carr and Roe (2016) have affirmed that the Petri-dish bioassay serves as an effective *in vitro* methodology for gauging the attractiveness of test compounds to ticks, facilitating the evaluation of their significance in tick biology and the preliminary assessment of these compounds before their integration into field-based tick control strategies

Table 6. Comparison of overall attraction of ticks to SP, AP, SP+AP and control vapour patch after 24 h

Type of vapour patch	Ticks attracted	
	No	Per cent
SP	79	52.67^{b}
AP	118	78.67^{a}
SP+AP	87	58.00^{b}
Control	16	10.60^{c}
χ^2 value (P-value)	146.40** (<0.001)	

Values in the brackets are percentages. ** Significant at 0.01 level; Percentage having different letter as superscript differ significantly within a row.

Petri-dish bioassay

There was a significant increase in attraction of unfed larvae and partially fed adults between SP, AP and SP+AP compared to the control vapour patch (Table 1,2). Both unfed larvae and partially fed adults exhibited a significantly higher level of attraction to AP impregnated vapour patches beyond 30 min of exposure when compared to SP, SP+AP and control patches. Clustering was more evident in AP impregnated vapour patches. Higher percentage of tick attraction to AP was also reported by Anish *et al.* (2017), who conducted an assessment of the reactivity of unfed stages of brown dog ticks to gold nanoparticles encapsulated with AP through a Petri-dish bioassay. The unfed stages demonstrated a 100 per cent attraction response when exposed to gold nanoparticles.

The response of unfed larvae and partially fed adults to the SP-impregnated vapour patch with respect

to exposure time is shown in Table 3. The unfed larvae or partially fed adults were introduced into one quadrant of Petri-dish opposite the SP-impregnated vapour patch. The unfed larvae and adults moved towards the SP-impregnated vapour patch on exposure and showed questing behaviour. The attraction of unfed larvae was significantly higher to the vapour patch impregnated with SP when compared to that of adults in the first 10 min. The unfed larvae or partially fed adults, upon contact with impregnated SP, exhibited a feeding posture by lowering their mouthparts and raising their posterior end, more evident in partially fed adults. Similar response not observed in Petri-dish bioassay with control vapour patch. Ranju *et al.* (2012a) and Gowrishankar *et al.* (2019b) also recorded similar behavioural modifications. In the present study, behavioural reactions were more evident in the case of SP, which agrees with the observations of Norval *et al*. (1991) who documented that 2,6-DCP is not solely an SP and may have an added role as an attractant stimulant. With the increase in time of exposure, a significant increase in attraction was noted in the case of unfed larvae and partially fed adults at 10 min, 30 min, 24h. However, there was no significant difference between 30 min and 2 h of exposure.

The present study revealed that for unfed larvae and partially fed adults, a significant increase in attraction to AP was observed with time at 10 min, 30 min and 24 h. Clustering was more evident in AP impregnated vapour patches. At 10 min, 30 min and 24 h, there was a significant increase in attraction of unfed larvae and partially fed adults and no significant increase was observed between 30 min and 2 h (Table 4). Ranju *et al*. (2018) evaluated the effectiveness of AP with deltamethrin by impregnating it in filter paper and recorded that the effectiveness of AP decreases over time, causing the attracted ticks to gradually move away from the pheromone source.

Responses of unfed larvae and partially fed adults to vapour patches impregnated with both SP and AP were recorded. Behavioural responses such as attraction, clustering, feeding posture were not observed for the combination of pheromones. For unfed larvae, a significant increase in attraction was observed with time at 10 min, 30 min and 2 h. However, beyond 2 h there was no significant difference in attraction. In partially fed adult ticks, a significant difference in attraction was observed between 10 min and 2 h exposure. The attraction was more till 2h and further the change in attraction was not significant (Table 5).

Overall, after 24 hours of exposure, a significantly higher number of ticks (78.67 per cent) showed attraction to the AP impregnated vapour patch compared to the SP, SP+AP and control patches. There was no significant difference in tick attraction between SP and SP+AP, however the percentage of attraction was higher in SP+AP than in SP (Table 6). According to Gowrishankar *et al*. (2021b), when unfed larvae were introduced to vapour patches impregnated with SP, AP, or SP+AP, they were attracted at rates of 63.6 per cent, 85.2 per cent, and 63.6 per cent, respectively after 24 hours and only 9.2 per cent attraction observed for control vapour patches, which showed a similar trend as in our study. The use of SP+AP impregnation in a vapour patch resulted in a lower attraction rate (58 per cent) compared to AP alone (78.67 per cent) and was not significantly higher than that of SP alone (52.67 per cent). This suggests that there was no synergistic response between SP and AP.

The results of Petri-dish bioassay suggested the high efficacy of AP impregnated vapour patches to attract brown dog ticks. Further investigations are needed to validate the result under field conditions. The sustainability of vapour patches in field conditions along with the incorporation of eco-friendly acaricide preparations can be explored in future for designing effective integrated pest management strategies. The promising results from this preliminary study pave the way for multidisciplinary research endeavours, involving entomology, veterinary sciences, and environmental sciences, to harness the full potential of semiochemicals in pest control.

Conclusion

Rhipicephalus sanguineus is the most prevalent tick species on dogs in Thrissur. Semiochemical assisted tick control strategies were investigated using Petri-dish bioassay with vapour patches impregnated with synthetic assembly pheromone (AP) and sex pheromone (SP), both individually and in combination. The efficacy of these semiochemicals was assessed based on the attraction and clustering behaviour of unfed larvae and partially fed adult ticks over a 24-hour period. The findings underscore the high efficacy of assembly pheromone (AP) impregnated vapour patches in attracting brown dog ticks, highlighting a promising avenue for the integration of semiochemicals into tick management practices. As we move forward, it is imperative to foster collaborations among researchers, practitioners, and industry stakeholders to translate these findings into practical, scalable solutions for tick management, thus reinforcing the role of innovative strategies in the fight against tick-borne diseases.

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Conflict of Interest

There are no potential conflicts of interest

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