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First report on evaluation of *in vitro* anti-tick efficacy of commercially available poly-herbal acaricide preparation against cattle tick *Hyalomma anatolicum*

Shaheen Samma, Bhavana Rathore, Kuldeep Fageria and Avinash Chouhan

Abstract
The present study was undertaken to evaluate the anti-tick activity of commercially available poly-herbal acaricide preparation containing extracts of *Eucalyptus globulus*, *Cedrus deodara*, *Acorus calamus*, *Pangomia glabra* and *Azadirachta indica* in a fixed concentration against egg and adult stages of *Hyalomma anatolicum* ticks. The ticks and their eggs were treated with various concentrations of herbal acaricide (1ml/100ml, 1.5ml/100ml, 2ml/100ml, 3ml/100ml and 5ml/100ml). Poly-herbal product yielded unsatisfactory results with nearly no mortality (0.00) in adult ticks treated with all five dilutions. However, there was slight reduction in egg laying capacity of treated female ticks; the reduction brought by the product at highest dilution of 5ml/100ml was 89.20 mg of egg mass. The hatchability of eggs laid by treated females represented a declining effect, with 34.07 eggs hatched at highest concentration of 5ml/100ml. A good efficiency was reported in hatchability of eggs directly treated with herbal acaricide, at 1ml/100ml concentration only 30 eggs hatched whereas no hatching was observed at 5ml/100ml. The product was found moderately efficacious as well as safe and eco-friendly. Besides the immediate effect on adult ticks, the egg-laying property of the ticks was reduced along with tremendous effect on decreasing the hatchability of eggs treated directly with it.

Keywords: *Hyalomma anatolicum*, herbal acaricide, *Eucalyptus globulus*, *Acorus calamus*, *Pangomia glabra*

Introduction
Livestock is an integral part of the agricultural production system in India and plays an important role in the national economy and socio-economic development of millions of rural households. However, almost of all dairy and meat animals in India are suffering from tick infestation (Ghosh *et al*., 2006) [12] and causes significant economic loss. When economic significance of ticks is taken into account it proves that these ticks have a debilitating action; production losses by acting as a vector to haemoprotozoan diseases, losses to the tanning industry and dairy industry (Rao *et al*., 2018) [25]. Control of tick and tick-borne diseases (TTBDs) is mainly focused on vector control, which is a very difficult proposition. The multi host-tick, *Hyalomma*, is the commonest tick species in India. Among the 10 species of this genus reported from India, *Hyalomma anatolicum* is the most dominant one (Geeverghese and Dhanda 1987) [11] and cattle serves as its main host. They have assumed noticeable importance because of their role as the major vector of the haemoprotozoan parasite, *Theileria annulata* in cattle (Bhattacharyulu *et al*., 1975) [5] and in transmitting the Crimean-Congo Hemorrhagic Fever (CCHF) virus in humans (Gordon *et al*., 1993; Karti *et al*., 2004) [14, 19].
Presently, tick control is focused on the use of chemical acaricides. Unlimited use of chemical acaricides for their control has resulted in problems related to environmental pollution, milk contamination and resistance development in the target species (Onofre *et al*. 2001) [23]. To tackle the problem of resistance, focus has been directed towards the development of alternative approaches involving the utilization of eco-friendly sustainable methods in a strategic integrated manner, one out of which is use of herbal acaricides which are safe for animal use with fewer chances of development of resistance against them. Plant extracts and essential oils have shown significant activity against all the stages of economically important tick species (Iori *et al*., 2005; Ribeiro *et al*., 2010, 2011; Rosado-Aguilar *et al*., 2010; Ghosh *et al*., 2011) [16, 26, 27, 28, 13]. India possesses 45,000 plant species of which 15,000-20,000 have proven medicinal value (Ghosh *et al*., 2011) [13]. Plant extracts kill and repel pests, affect insect

growth and development, have anti feeding, arresting effects, as well as antifungal, antiviral and antibacterial properties against pathogens.

Literature suggests most of the studies remained centered towards testing the acaricidal and ectoparasiticidal activity of individual herbal extracts instead of evaluating the combined potential of herbal formulations. Thus the present study was envisaged to promote the practice of herbalism by assessing a commercially available herbal acaricide preparation against cattle tick *Hyalomma anatolicum*. The product comprises of extracts of herbs *viz*. *Eucalyptus globulus*, *Cedrus deodara*, *Acorus calamus*, *Pangomia glabra* and *Azadirachta indica* in a fixed concentration.

Materials and methods

A commercially available poly-herbal acaricide combination (by AYURVET Ltd., Solan, India) was evaluated against *Hyalomma anatolicum*.

Table 1: Each 10 ml of herbal formulation contains (water soluble herbal solution) following contents (in grams)

S. No.	Contents	Concentration in grams
1.	*Cedrus deodara* (Devdar)	2 gms
2.	*Pangomia glabra* (Karanj)	0.5 gm
3.	*Acorus calamus* (Vacha)	0.2 gm
4.	*Eucalyptus globulus* (Nilgiri)	0.2 gm
5.	*Azadirachta indica* (Neem)	0.3 gm
6.	Emulsifier Base	q.s.

Collection of ticks

Blood engorged female *Hyalomma anatolicum* ticks were collected from cattle sheds of both organized and unorganized farms. The ticks were collected in plastic specimen tubes bearing the mesh window ventilator which allow air and moisture exchange.

In vitro evaluation of herbal formulation against *Hyalomma anatolicum* adult female ticks

Test procedures as described by Srivastava *et al*., (2008) [29], Bagherwal *et al*., (1994) [3], Maske *et al*., (2000) [21], Kaaya and Hassan (2000) [18], Narladkar and Shivpurje (2015) [22], Bharkad *et al*., (2018) [4], Rao *et al*., (2018) [25] with slight modifications were conducted. For *in vitro* trials working concentrations were prepared in soap water as an emulsifier at the rate of 2 grams per litre of water. In each diluted concentration, 15 engorged female ticks were dipped for 1 minute, dried on filter paper and placed in test tubes as single tick per tube. These tubes were covered with a piece of muslin cloth. The mortality of the ticks was monitored at 24 hrs till 96 hrs. All the surviving ticks were further observed for oviposition until the period egg laying was completed in control group. The individual ticks were considered as dead when no motility was shown by them, even after pricking with a pin. The mortality data were recorded and tabulated. Efficacy was calculated in terms of per cent mortality. To judge the egg laying capacity, live treated ticks were monitored for amount of egg mass laid as compared with ticks of control group.

***In vitro* trials of herbal preparations on eggs from treated females:** The treated female *Hyalomma anatolicum* ticks which were not dead were maintained for egg collection. Eggs collected from these ticks were counted in petri dishes and separated in batches of 100 eggs in number. These batches of 100 eggs were transferred in test tubes, closed with piece of muslin cloth and labelled as per the treated concentration of adult ticks from which these eggs were collected. These tubes were kept in desiccators with humidity levels maintained @ 75%. The eggs were observed for hatching process till the period hatching was completed in eggs of control group.

In vitro trials of herbal preparations on eggs from untreated females

Few female *Hyalomma anatolicum* ticks were separately maintained for egg collection. Collected eggs were counted into batches of 100 in petri dishes, treated with different concentrations of herbal agents and dried on filter paper. Further, these treated batches of 100 eggs were transferred separately in individual test tubes, labelled, subsequently, covered with piece of muslin cloth and placed in desiccators maintained @ 75% RH. The eggs were monitored for hatching, until the period hatching process of eggs was completed in the control group.

Criteria's for assessment of efficacy of Herbal Control Agents

- Mortality of adult ticks.
- Reduction in egg laying capacity.
- Hatchability of eggs laid by treated female ticks.
- Hatchability of treated eggs.

Statistical analysis

The data obtained from various parameters was analyzed by employing two factor factorial experiment and completely randomized design using computer application, SPSS version 20.

Results and Discussion

Mortality of adult ticks

The poly-herbal acaricide at both lower and higher working concentrations (from 1ml/100ml to 5ml/100ml) failed to cause mortality of treated adult female ticks. The tick replicates in all the 15 test tubes were motile, showing pedal reflexes, indicating no mortality occurred within a span of 48 to 72 hrs and even started egg laying after 96 hours of monitoring.

Table 2: Mean mortality of *Hyalomma anatolicum* female ticks after treatment with Herbal product at various concentrations

S. No.	Total No. of ticks	Treatment	Mean ± SE	Range
1.	15	1ml/100ml	0.00 ± 0.0	0
2.	15	1.5ml/100ml	0.00 ± 0.0	0
3.	15	2ml/100ml	0.00 ± 0.0	0
4.	15	3ml/100ml	0.07 ± 0.67	0-1
5.	15	5ml/100ml	0.00 ± 0.0	0
6.	15	control	0.00 ± 0.0	0
Significance		Non-significant (NS)		
Critical Difference				
At 5% = 0.063		At 1% = 0.090		

Reduction in egg laying capacity

The poly-herbal acaricide was ineffectual on adult ticks, all ticks laid a decent amount of eggs, it was however, in lesser quantity as compared to control group. At higher concentrations i.e., 3ml/100ml and 5ml/100ml, the egg mass laid was even subsidiary. This stipulates that herbal preparation have some reducing effect on laying capacity on treated adult ticks.

Table 3: Mean egg laying capacity of *Hyalomma anatolicum* female ticks after treatment with Herbal product at various concentrations

S. No.	Treatments	Mean ± SE	Range
1.	1ml/100ml	168.20 ± 13.633[c]	82-261
2.	1.5ml/100ml	144.60 ± 9.218[bc]	83-187
3.	2ml/100ml	143.67 ± 8.668[bc]	95-201
4.	3ml/100ml	114.73 ± 4.304[ab]	84-140
5.	5ml/100ml	89.20 ± 4.699[a]	57-124
6.	Control	213 ± 8.373[d]	178-280
Significance	Highly significant		
Critical difference			
At 5% = 20.51		At 1% = 29.26	

Hatchability of eggs laid by treated female ticks

The hatchability of eggs harvested from females treated with poly-herbal acaricide decreased slightly but was nearly constant at all working concentrations. Number of eggs hatched for females treated with 1ml/100ml dilution was 39.07 while that for 5ml/100ml dilution was 34.07, which are nearly same range values.

Table 4: Mean hatchability of eggs harvested from treated *Hyalomma anatolicum* female ticks with Herbal product at various concentrations

S. No.	Treatments	Mean ± SE	Range
1.	1ml/100ml	39.07 ± 2.632[a]	22-59
2.	1.5ml/100ml	43.60 ± 3.612[a]	11-65
3.	2ml/100ml	38.93 ± 3.304[a]	14-64
4.	3ml/100ml	43.87 ± 3.688[a]	0-58
5.	5ml/100ml	34.07 ± 3.510[a]	17-103
6.	Control	96.80 ± 0.895[b]	91-103
Significance	Highly significant		
Critical difference			
At 5% = 7.28		At 1% = 10.39	

Hatchability of treated eggs

The poly-herbal acaricide product containing different herbal extracts when tested against eggs of *H. anatolicum* showed a fair efficacy and inhibited some ovicidal activity. At the concentration of 1ml/100ml concentration 30 eggs hatched. With increasing concentration, the ovicidal efficiency increased; at highest 5ml/100ml concentration no hatching was observed.

Table 5: Mean hatchability of *Hyalomma anatolicum* tick eggs after treatment with Herbal product at various concentrations

S. No.	Treatments	Mean ± SE	Range
1.	1ml/100ml	30.00 ± 3.257[b]	14-61
2.	1.5ml/100ml	33.67 ± 3.286[b]	14-58
3.	2ml/100ml	26.47 ± 3.075[b]	10-52
4.	3ml/100ml	3.20 ± 0.829[a]	0-10
5.	5ml/100ml	0.00 ± 0.0[a]	0
6.	Control	98.13 ± 1.133[c]	93-107
Significance	Highly significant		
Critical difference			
At 5% = 5.5		At 1% = 7.84	

Plants provide a number of natural compounds which can intervene in all biological processes of insects interrupting their life cycle and are considered as an important part of ethno-veterinary practices (Habeeb 2010, Zaman *et al*., 2012) [15, 31]. Plant based acaricides have been found to exhibit adulticidal effects on ticks (Ribeiro *et al*., 2011; Elango and Rahuman, 2011; Ghosh *et al*., 2011) [27, 10, 13]. In comparison to synthetic acaricides, the botanicals are usually less toxic to mammals, have no residual effects and have less chance of development of resistant tick populations (Chungsamarnyart *et al*., 1991) [8]. Amongst the different plant extracts having anti-tick activities, extracts of *Eucalyptus globules* (Pirali-Kheirabadi *et al*., 2009) [24], *Acorus calamus* (Ghosh *et al*., 2011) [13] *Azadirachta indica* (Abdel-Shafy and Zayed 2002) [1], *Pongamia glabra* (Bisen *et al*., 2010 and Bisen *et al*., 2011) [6, 7] were studied extensively to establish its potential as acaricide against various tick species.

Various workers have reported the tickicidal activity of plants used in herbal acaricide formulation used in present study. The crude extract of *Acorus calamus* rhizome showed toxicity to nymph and adult brown dog tick (Leenuwongphun *et al*., 2008) [20], larvae and adults of *R. (B.) microplus* (Chungsamarnyart *et al*., 1988; Ghosh *et al*., 2011) [9, 13]. The *Eucalyptus globules* essential oils at 5.0% concentration produced 37.5% mortality after six days post treatment in adult stage of *R.* (B.) *annulatus* (Pirali-Kheirabadi *et al*., 2009) [24], treatment of *R.* (B) *microplus* with *Pongamia glabra* extract and seed oil resulted in lower egg production and hatchability (Bisen *et al*., 2010, 2011; Bharkad *et al*., 2018) [6, 7, 4] neem seed oil/extracts (*Azadirachta indica*) elicited acaricidal effects on egg, immature, and adult stages of *Hyalomma anatolicum excavatum* (Abdel Shafy and Zayed 2002) [1] and *R.* (B.) *microplus* (Srivastava *et al*., 2008) [29] and oil of *Cedrus deodara* showing cidal effects on sarcoptic mites (Sharma *et al*., 1997) [30].

In the present analysis, poly-herbal product containing a combination of extracts from five different herbs yielded unsatisfactory results with nearly nil mortality (0.00) in adult *Hyalomma anatolicum* ticks treated with all five dilutions. However, there was slight reduction in egg laying capacity of treated female ticks as compared to the control group. The reduction brought by the product even at highest dilution of 5ml/100ml was 89.20 mg of egg mass. The hatchability of eggs laid by females treated with the herbal acaricide represented a declining effect, at highest concentration of 5ml/100ml, 34.07 eggs hatched. A fair efficacy was reported in decreasing hatchability of eggs directly treated with herbal acaricide, at 1ml/100ml concentration only 30 eggs hatched whereas no hatching was observed at 5ml/100ml. The results of the present study are in slight accordance with results found in experiments conducted on various other herbal alternatives for their efficacy against ectoparasitic infestation. Ajith Kumar *et al*., (2016) [2] compared the efficacy of different plant-based poly-herbal acaricidal products against multi acaricide resistant adult *Rhiphicephalus* (B.) *microplus* tick lines (IVRI line IV and IVRI line V) observing overall mean efficacies of two similar commercially available poly-herbal formulations to be 63.5 ± 7.4 and 10.1 ± 2.1. However, Jumde *et al*., (2013) [17] and Rao *et al*., (2018) [25] reported 100 percent efficacy of poly-herbal acaricide products against cattle tick *Rhiphicephalus* (B.) *microplus* which is inconsistent with the results obtained in present study. This contradiction may be attributed to the variation in the combination of plants used. The methods of extraction, concentration of the herbal extracts used and the level of active principle of the plants of different regions may also have the impact on efficacy levels. The geographical and environmental factors like the soil type, mineral profile of the soil, water levels in the soil and agro climatic conditions may fairly influence the level of active ingredient in the plants of grown in different regions.

The major difference in efficacies reported by various authors

may be attributed to the variation in herbs used in combination; showing either synergistic effect, with having activity complementing each other or antagonistic effect due to differences in bioactive principles and their mode of action. Other reasons of divergence in efficacy could be ascribed to type of emulsifier base used in formulation of herbal alternatives or dissimilar tick species used in the experiments. Most of the studies are done on *Rhiphicephalus microplus* instead of *Hyalomma anatolicum* and thus, the difference in efficacies may be assigned to the fact of differences in nature of both the ticks, since *Hyalomma anatolicum* is hardy multi-host tick while *Rhiphicephalus microplus* is single host tick. Similarly the combination of 3-4 herbs prepared in a base that act as surface reductant and which helps in better dissolution; has better acaricidal properties.

The major problem in developing natural formulations is maintaining efficacy. Since active compounds present in any plant species varies with different stages of growth of plant, season, soil and environmental conditions, identification of quality control marker compound is crucial to maintain the quality of the finished product. Higher anti-tick activity of the newly developed formulations need to be exploited for large scale commercial production and marketing in an economical way for the control of multi-acaricide resistant ticks infesting cattle of India and other tropical countries.

Conclusion

Analyzing the *in vitro* data obtained in the present study, it can be concluded that the poly-herbal acaricidal formulation has low tickicidal but high ovicidal properties, which may provide an effective eco-friendly alternative for the control of multi-acaricide resistant tick infestation on animals, and thus can be recommended in the integrated tick control programme. It promotes the formulation of newer herbal combinations and extracts from different parts of the same plant sources used, resulting in extraordinary acaricidal activity.

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Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

This work was carried out in collaboration among all authors. Author S.S. designed the study, wrote the protocol, conducted the experiments and wrote the first draft of the manuscript. Authors K.F. and A.C. managed the analyses of the study and contributed in conducting experiments. Author B.R. supervised all the authors for conducting this research, guided in methodology and analysis of the study and also reviewed the original draft. All authors read and approved the final manuscript.

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Data availability

The raw data is available on the Rajasthan University of Veterinary and Animal Sciences, Bikaner as part of the M.V.Sc. thesis.

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