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Dr. Greeshma Rao UB
MVSc Scholar, Department of Veterinary Parasitology, COVAS Parbhani, Maharashtra, India

Dr. BW Narladkar
Associate Professor, Department of Veterinary Parasitology, COVAS Parbhani, Maharashtra, India

Dr. SR Rajurkar
Associate Professor and Head, Department of Veterinary Pharmacology, COVAS Parbhani, Maharashtra, India

Correspondence
Dr. Greeshma Rao UB
MVSc Scholar, Department of Veterinary Parasitology, COVAS Parbhani, Maharashtra, India

In-vitro evaluation of the herbal acaricide product against the cattle tick *Rhipicephalus* (*B.*) *microplus* (Acarina: Ixodidiae)

Dr. Greeshma Rao UB, Dr. BW Narladkar and Dr. SR Rajurkar

Abstract
The present research was conducted at Parbhani district of Maharashtra to evaluate the *Anti-tick* efficacy of the herbal acaricide product containing Neem oil, Karanj oil, Eucalyptus oil, Rohit Gawash and Karpura against egg and adult stages of *Rhipicephalus (B.) microplus* 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. The treated female *Rhipicephalus (B.) microplus* ticks which were not died were separately maintained for eggs collection and waited for their hatchability. In the concentration above 1.5ml/100ml, all the treated ticks in 10 tubes died indicating 100% mortality. Hence no question about the recording the egg laying capacity and was considered as nil, while control group laid eggs to the extent of 934.29 per female. The treated females laid eggs very meager in number and amongst them very few have hatched. Herbal product had good efficacy against egg and adult stage of *R. (B.) microplus* and can form an alternative against chemical acaricide in the integrated tick management programmes.

Keywords: *Azadirachta indica, Cinnamomum camphora, Cymbopogon martinii, Eucalyptus globulus, Pongamia glabra, Rhipicephalus (B.) microplus*

1. Introduction
Parasitic diseases are a global problem and considered as a major obstacle in the health and product performance of animals [1]. Amongst many parasites infesting livestock; ticks are obligate, blood-feeding ectoparasites of vertebrates belonging to the class Arachnida, Order Acari [2].

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, also losses to the tanning industry and dairy industry [3]. India suffers losses of about 57.2 million US dollars annually due to Babesiosis in livestock [4]. The tick *B. microplus* is a native to Asia and now migrating to many countries. *R. (B.) microplus* has been proved as an important tick in the tropical areas like India. Its infection results in retarded development of affected animals causing reduced milk and meat production [5]. Unlimited use chemical acaricides for control of this tick has resulted in problems related to environmental pollution, milk contamination and resistance development in the target species [6]. Thus scientists are concentrationg on non-chemical alternatives, one of them is herbal. India is one of the world's 12 regions having the largest biodiversity and possesses 45,000 plant species of which 15,000–20,000 have proven medicinal value [7]. The application of botanicals to livestock in order to control the ectoparasites of veterinary importance is widespread in the developing countries. Thus the present study was undertaken with the objective of evaluating the efficacy of user friendly herbal acaricide combination containing Neem oil, Karanj oil, Eucalyptus oil, Rohit Gawash and Karpura against egg and adult stages of *Rhipicephalus (B.) microplus* ticks.

2. Materials and Methods
Study area: The research work was undertaken at Department of Veterinary Parasitology and College of Veterinary and Animal Sciences, (MAFSU) Parbhani, Maharashtra, India.
Duration of the study: November 2016-May 2017
Parasitic species studied: *Rhipicephalus* (*B.*) *microplus* (Adults and eggs).
Herbal acaricide combination evaluated against *Rhipicephalus* (*B.*) *microplus*:
100 ml of herbal acaricide contains (water soluble herbal solution)

a) *Cymbopogonmartinii* (RohitGawash): 5 ml
b) *Eucalyptus globulus* (Nilgiri): 5 ml
c) *Azadirachtaindica* (Neem): 3 ml
d) *Pongamiaglabra* (Karanj): 3 ml
e) *Cinnamomumcamphora* (Karpura): 2 ml
f) Base: q.s.

Collection of ticks and identification

Requisite number of blood engorged female ticks was collected from cattle (cows/bullock) body using forceps at the junction of skin and ticks. Ticks were identified as female *Rhipicephalus* (*B.*) *microplus* under zoom stereoscopic microscope as *Rhipicephalus (B.) microplus* before being introduced in the experiment [8].

***In-vitro* trials of herbal acaricide against *Rhipicephalus (B.) microplus* adult female ticks**

For all *in- vitro* trials, working concentration were prepared in soap water as emulsifier @ 2g per liter of water. Standard test procedure was followed with slight modification [9-12]. In each diluted concentration 10 female ticks were dipped for 1 minute, were dried on filter paper and then placed in test tube as single tick per tube, followed by closure of tube with the piece of muslin cloth and rubber band. The mortality of ticks was observed at 24 hrs till 96 hrs. All survived ticks and ticks from control were observed further for the egg laying process, till the period control group ticks have completed the egg laying process. Similarly if not dead, to judge the egg laying capacity, they were observed for number of eggs laid and compared with control ticks which were only treated with water.

***In-vitro* trial of herbal acaricide on eggs from treated females**

The treated female *Rhipicephalus (B.) microplus* ticks which were not died, were separately maintained for eggs collection. Eggs collected from such female ticks were counted in petri dishes in the batches of 100 numbers, were transferred in tubes which were closed with piece of muslin cloth tied with rubber band. These tubes were maintained in desiccators in which humidity levels were maintained @ 75 %. The eggs were observed for hatching, till the period hatching process of eggs in the control group were completed.

***In-vitro* trial of herbal acaricide on eggs**

Few female *Rhipicephalus (B.) microplus* ticks were separately maintained for egg collection. Collected eggs were counted in petri dishes in the batches of 100 numbers were firstly treated with liquid solution of herbal oils, dried on filter paper and then transferred in tubes which were closed with piece of muslin cloth tied with rubber band. The eggs were observed for hatching, till the period hatching process of eggs in the control group were completed

3. Statistical Analysis

The data obtained from various parameters was analyzed by employing two factor Factorial Experiment and Completely Randomized Design using computer application, WASP.

4. Results and Discussion

Mortality of adult ticks and eggs

At the concentration of 1ml/100ml and 1.5ml/100ml of herbal acaricide product, mortality count was average 0.20 and 0.90, respectively. With rising concentration of above 1.5ml/100ml, i.e. 2ml/100ml, 3ml/100ml, 5ml/100ml all the treated ticks in 10 tubes died indicating 100% mortality while all ticks of control group survived and laid eggs. The mortality occurred within a span 48hr to 72 hrs (Table 1).

Table 1: Mean mortality of *Rhipicephalus (B.) microplus* female ticks after treatment with herbal acaricide product at various concentrations.

S. No	Treatment	Mean± SE	Range
1	1ml/100ml water	^{b}11.43±7.69	0-50
2	1.5ml/100ml water	^{b}4.29± 4.29	0-30
3	2ml/100ml water	^{b}0.00±0.00	0-0
4	3ml/100ml water	^{b}0.00±0.00	0-0
5	5ml/100ml water	^{b}0.00±0.00	0-0
6	Control	^{a}934.29 ±405.78	10-2515
Significant	HIGHLY SIGNIFICANT		
Critical Difference			
	At 5 % = 475.228	At 1 % = 637.157	

Reduction in egg laying capacity

The herbal acaricide at the concentration of 1ml/100ml and 1.5ml/100ml had failed to cause mortality of all treated eggs and ticks. However, ticks which were live laid eggs which were very meager in number as 11.425 and 4.287, respectively as against 934.287 eggs per female in control group. At the concentration of 2ml/100ml, 3ml/100ml and 5ml/100ml exerted a knock down effect on treated ticks which resulted in total mortality of ticks, thus no question about the recording the egg laying capacity and was considered as nil, while female ticks from control group laid eggs to the extent of 934.29 per female. The results from this table indicate that herbal acaricide has almost *at par* knock down effect with that of like chemical acaricides (Tables 2).

Table 2: Mean egg laying capacity of *Rhipicephalus (B.) microplus* female ticks after treatment with herbal acaricide product at various concentrations.

S. No	Treatment	Mean± SE	Range
1	1ml/100ml water	^{b}11.43±7.69	0-50
2	1.5ml/100ml water	^{b}4.29± 4.29	0-30
3	2ml/100ml water	^{b}0.00±0.00	0-0
4	3ml/100ml water	^{b}0.00±0.00	0-0
5	5ml/100ml water	^{b}0.00±0.00	0-0
6	Control	^{a}934.29 ±405.78	10-2515
Significant	HIGHLY SIGNIFICANT		
Critical Difference			
	At 5 % = 475.228	At 1 % = 637.157	

Hatchability of eggs laid by treated female ticks

After the treatment with herbal acaricide product only the ticks from the groups which were treated with the concentration of 1ml/100ml and 1.5ml/100ml laid eggs. The numbers of eggs laid were very meager in number and amongst them very few have hatched. Number of eggs hatched at the concentration 1ml/100ml and 1.5ml/100ml were 3.33 and 0.954, respectively, as against 97.57 eggs have hatched from the control group (Table 3).

Table 3: Mean hatchability of eggs harvested from *Rhipicephalus (B.) microplus* female ticks treated with herbal acaricide product at various concentrations.

S. No	Treatment	Mean± SE	Range
1	1ml/100ml water	[b] 3.33±2.19	0-13
2	1.5ml/100ml water	[bc]0.954±0.954	0-6
3	2ml/100ml water	[c] 0.00±0.00	0-0
4	3ml/100ml water	[c] 0.00±0.00	0-0
5	5ml/100ml water	[c]0.00±0.00	0-0
6	Control	[a]97.57±0.71	95-100
Significant	Highly Significant		
Critical Difference			
	At 5 % = 2.913	At 1 % = 3.903	

Hatchability of treated eggs

The herbal acaricide product containing different herbal oils when tested against eggs of *R. (B.) microplus* showed the best efficacy and succeeded in 100% ovicidal activity. At the concentration 1ml/100ml, only 1 egg has hatched. In the concentration of 1.5ml/100ml and above 5ml all the treated eggs died and could not hatch to lay 1st instar larvae indicating its enormous ovicidal activity (Table 4).

Table 4: Mean hatchability of *Rhipicephalus (B.) microplus* female tick eggs treated with herbal acaricide product at various concentrations.

Sr. No	Treatment	Mean± SE	Range
1	1ml/100ml water	[b] 1±0.634	0-3
2	1.5ml/100ml water	[b]0.00±0.00	0-0
3	2ml/100ml water	[b] 0.00±0.00	0-0
4	3ml/100ml water	[b] 0.00±0.00	0-0
5	5ml/100ml water	[b]0.00±0.00	0-0
6	Control	[a]96.20±1.15	93-100
Significant	Highly Significant		
Critical Difference			
	At 5 % = 1.578	At 1 % = 2.131	

Principally, natural products can fill up the gap, if properly exploited. 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 [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 by tick populations [14]. The anti-tick activity of a significantly high number of plant extracts were tested *in-vitro*, however, in most of the cases neither the activity has been tested *in-vivo* nor further progress on chemical analysis of the identified extracts were made.

In the present study a product containing four herbs were evaluated and it yielded excellent results to the extent of 100% mortality and 100% reduction in egg laying capacity. Contrary to our observation, monoherbal products were found more efficacies as compared to polyherbal products [15]. However there are several problems in maintaining monoherbal natural formulation because there are chances of losing the efficacy and impact of season. Therefore a comprehensive polyherbal product can be a better choice compared to single herb.

The herbal acaricide product was evaluated in the present study with triple aim

a) To evaluate an alternative to the chemical acaricide and promising bio-pesticide.
b) Each constituent of this product particularly Lemon grass, Neem, Eucalyptus and Karanj have had insecticidal properties is well documented [12]. However by testing the product containing combination of all these four, can it will yield synergistic effect?
c) Herbal product containing neutral base can help in uniform dissolution and will it facilitate effective spraying on the target?

In the present study excellent results obtained in terms of mortality of ticks and ovicidal effect have replied to above three questions-cum-aims and answers ascertained were affirmative and of excellent nature.

Review of early work witnesses the efficacy and need of developing the herbal products against ticks and other pests of livestock. By employing recently developed molecular arrays for testing efficacy and resistance of anti-tick chemical acaricides, the acaricide resistant status in ticks of seven states of India was worked out and found alarmingly high [16, 17]. Overall, most of the tick isolates developed resistant to multiacaricides and in many areas the efficacy of the commonly used acaricides has lost efficacy completely at optimum dose. It is well known that the development of new anti-tick compound is highly cost and time intensive, as a result, the pace of development and marketing of new acaricides has taken backseat and focus has been shifted to the development of eco-friendly product having multiple mode of action. In this context, the herbal acaricide product evaluated in the present study can be a one step ahead in this direction. Therefore the product tested under present study can form an alternative to chemical acaricide and can be inducted in the integrated management programme.

The mixture of neem (*Azadirachta indica*) and Karanj (*Pongamia glabra*) was found less efficacious than when Karanj (*Pongamia glabra*) alone was used [18, 19]. Contrast to these observations, present study noted the 100 percent efficacy of herbal acaricide product in comparison to individual constituent, it could be due to 1) product contained a base which helped for homogenous mixing of herbal oils in water, and 2) it contained a Camphor and Rohit gawash, which might have helped in synergizing the accruing the effect.

Phytochemicals of essential oils from the plants are proved as acaricidal or having action against insect, moths and mites. Their probable mode of action is points a neurotoxic mode action, due to the presence of compounds that may act as acetylcholinesterase inhibitor and can block the octopamine receptor pathway also may act on various targets including g-aminobutyric acid (GABA)-gated chloride channels, octopamine receptors, tyramine receptors, acetylcholine esterase, nicotinic acetylcholine receptors (nAChR), sodium channels, and possibly other targets [20]. Probable mode of action of neem products: a) Insect growth regulation***:*** When the neem components, especially Azadirachtin (one of more than 70 limonoids produced by the neem tree), enter in the body of the larva, the activity of ecdysone is suppressed and the larva fails to moult, remains in the larval stage and ultimately dies. If the concentration of azadirachtin is lower still, the adult emerging from the pupa will be 100% malformed, and absolutely sterile, b) Feeding deterrent***:*** If the surface is treated with a neem product, because of the presence of azadirachtin, salanin and melandriol, there will be an anti-peristaltic wave in the alimentary canal which produces something similar to a vomiting sensation in the insect, the insect does not feed on the neem-treated surface. Ability of the larvae to swallow is also blocked, c) Oviposition deterrent: Neem controls pests is also by way of preventing the females from depositing eggs, d) formation of chitin or the hard part covering the insect exoskeleton is

inhibited; e) 0mating as well as sexual communication are disrupted; c) larvae and adults of insects are repelled; f) sterilization of adults and g) larvae and adults are poisoned.

Essential oils of lemon grass are the mostly widely used natural repellants worldwide. The efficacy of Neem, Karanj and Nilgiri oils, when tested individually against egg and adult stages of *R. microplus* ticks. The mortality of *R. (B) microplus* ticks and reduction in egg laying capacity, similarly shrinkage of embryo and change in shape resulting in reduction in hatchability of *R. (B) microplus* tick eggs.

LC_{50} values in comparison with LD_{50} values in rats for different fungi and herbal acaricide

From the comparison between LC_{50} values and their corresponding LD_{50} values in rats, it can be assessed that LC_{50} values are quite less than LD_{50} values indicating these fungi are safe for use and not harmful to domestic animals and human being (Table 5).

Table 5: LC_{50} values and its corresponding LD_{50} values in rats for herbal acaricide product against adult female *R. (B.) microplus* tick eggs

Herbal acaricide	LC_{50} values for eggs from treated female	LC_{50} values for treated eggs	LD_{50} values in rats	References
Herbal Acaricide	1.16ml/100ml	1ml/100ml	a. Neem oil>1500 mg /kg/day as a basal dose for NOEL (no observed effect level b. Karanj: 2000 mg/kg c. *Cymbopogon martini*:Acute toxicity, Oral >5000mg/kg in rats d. Eucalyptus oil: Acute oral toxicity: 2480 mg/kg e. Camphor: LD50 [oral, mouse]; 1310 mg/kg	a. [21] b. [22] c. [23] d. [24] e. [25]

Overall Effect Rate

The OER (Overall Effect Rate) was calculated using the formula [26]. By using the same formula the OER has been calculated. OER= 100× [1 –WEt/WEc–WLt/WLc], where WEt is the egg laying capacity of infected ticks; WEc the egg laying capacity of non-infected ticks (control); WLt the eggfertility of infected ticks; WLc the egg fertility of non-infected ticks.

OER at 1ml/100ml water 100× (1-0/1578.57×0/96.8)=100%

OER at 5ml/100ml water 100× (1-0/1578.57×1.28/96.8)=100%

All concentrations of herbal acaricide product showed 100% OER against *Rhipicephalus microplus* ticks, potential tick control agents.

5. Conclusion

Herbal acaricide product containing Neem oil, Karanj oil, Eucalyptus oil, RohitGawash and Karpura showed 100% effectiveness as tickicidal and ovicidal, thus can be recommended in the integrated tick control programme. Similarly study also witnesses the combination of 3-4 herbs prepared in a base which act as surface reductant and helps in better dissolution; has the best activity as ovicidal and tickicidal.

From the calculations OER, it can be concluded herbal product has good efficacy against egg and adult stage of *R. (B.) microplus* and shall be inducted in the integrated tick management programmes, subject to the field trials.

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