Comparative in vitro anti-tick efficacy of commercially available products and newly developed phyto-formulations against field collected and resistant tick lines of *Rhipicephalus (Boophilus) microplus*

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Abstract *Rhipicephalus (Boophilus) microplus* is considered as one of the most widely distributed tick species ecto-parasitizing on livestock and causes fatal diseases with significant production loss. To address the problem of controlling acaricide resistant tick infestations on animals, attention has been paid to develop eco-friendly phyto-acaricides. The present study was undertaken to evaluate the comparative anti-tick activities of commercially available herbal and chemical products with chemically characterized phyto-formulations developed recently against field ticks and resistant tick lines of *R. (B.) microplus*. The chemical product Butox[®] Vet was found nearly passive against all the tested resistant tick lines. However, one of the commercial polyherbal product, Zerokeet[®] showed an efficacy (E%) of 41.8–75.4 % ($\bar{X} = 63.5 \pm 7.4$ %) using recommended dilution (1:2) against field ticks and resistant tick lines. However, the other commercial product, Erina[®] EP has very limited efficacy against all the tested tick. In comparison, the newly developed phyto-formulation, NBA/13/B/2 and NAC-01 conferred an E% of 82.4–91.3 % ($\bar{X} = 85.9 \pm 2.1$ %) and 62.3–94.6 % ($\bar{X} = 80.0 \pm 8.1$ %), respectively, against tested resistant ticks. Results indicated higher marketing potentiality of newly developed formulation in the existing tick problem scenario.

Keywords *Rhipicephalus (Boophilus) microplus* · Resistance · Zerokeet[®] · Erina[®]-EP · Butox[®] Vet · Phyto-formulation

Introduction

The cattle tick, *Rhipicephalus (Boophilus) microplus*, is posing major threat on economical maintenance of the cattle industry in tropical and sub tropical countries including India. It causes severe losses by blood feeding, transmitting many fatal pathogens and by direct damage to cattle skins and hides for the leather industries (Ghosh et al. 2006). Today, tick control has become a challenge to researchers exploring a sustainable way to mitigate the problem of tick infestations.

Chemicals groups commercially available in the market viz., synthetic pyrethroids, organophosphates, formamidines and macrocyclic lactones are repeatedly used for the control of ticks infesting livestock. These chemicals kill non-target organisms and threaten human health due to the toxic residues in milk and meat (Graf et al. 2004). Intensive use of these chemical acaricides results in the development of resistant tick populations which causes the failure of the chemical-based tick control program (Rosario-Cruz et al. 2005; Rodriguez-Vivas et al. 2006a, b).

Efforts have been made to develop sustainable immunological means for controlling ticks and tick-borne diseases in order to check the fast development of resistance against acaricides. Two commercial vaccines against *R. (B.) microplus* were developed and marketed (Willadsen 2004; de la Fuente et al. 1998, 1999). The vaccine efficacy was found highly variable and farmers' expectation has not been met (Canales et al. 2009). Moreover, none of the vaccine is giving significant protection against multi-

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species tick infestations, a common problem faced by the livestock owners of Asia and Africa. Similarly, fungal biopesticides and entomopathogenic nematodes proved unsuccessful (Benjamin et al. 2002; Polar et al. 2005).

Extensive work has been done on plant based natural products having acaricidal activities against many species of ticks (Borges et al. 2003; Pereira and Famadas 2006; Fernandes and Freitas 2007; Kamaraj et al. 2010; de Souza Chagas et al. 2012; Juliet et al. 2012; Sunil et al. 2013) including acaricide resistant species (Ghosh et al. 2011, 2013) as the natural products can delay the development of resistance due to the presence of a number of active molecules having different mode of action (Wang et al. 2007).

Under the world bank funded National Agricultural innovation Project intensive search on plant having anti-tick properties has resulted in the identification of two solvent guided plant extracts. The data on chemotypic variation in different accessions of the identified plants, active molecules present in the most potential accession, safety profile, stability at different stress conditions, in vivo efficacy in experimental challenge conditions and in multilocational clinical trial were generated and two phyto-formulations were developed, applied for patent (Patent Application Nos. 2452/DEL/2010 and 2196/DEL/2011) and are under process of commercialization. While assessing the market potentiality of the newly developed phyto-formulations, a study was conducted to assess the comparative efficacy of two commercial herbal products, Zerokeet[®], a product of Ayurved Ltd., Solan, India and Erina-EP, a product of The Himalaya Drug Company, Bangalore, India, the most aggressively marketed anti-tick chemical, “Butox[®] Vet” a product of Intervet India Pvt. Ltd. and the phyto-formulations generated from plants. Although, the markedly available anti-tick natural products are polyherbal and expected to provide significantly higher efficacy, in the present study, the efficacy of monoherbal phyto-formulations was compared and elaborated.

Materials and methods

Animals

For maintaining different resistant tick lines (IVRI-3, IVRI-4 and IVRI-5) separate male cross-bred calves (*Bos taurus* × *B. indicus*) of 6–8 months old were used. Calves were procured at the age of 3–4 months from the Livestock Management Division of the Indian Veterinary Research Institute and were maintained in animal sheds of the Division of Parasitology following standard feeding and management conditions. The experimental animals were maintained according to the approved guidelines laid down

by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory Indian body.

Tick lines

Three resistant tick lines, diazinon-resistant IVRI-3, deltamethrin-resistant IVRI-4, and multi-acaricide resistant IVRI-5 of *R. (B.) microplus* are continuously maintained at the Entomology Laboratory of the Indian Veterinary Research Institute (IVRI). The IVRI line-3 tick was originally collected from Raebareilly of Uttar Pradesh, India while line 4 and 5 were collected from Danapur and Muzaffarpur areas, respectively, of Bihar, India. To maintain the resistance level, each generation of tick lines-3 and 4 was exposed to diazinon and deltamethrin, respectively, while ticks of IVRI-5 line were exposed to diazinon, deltamethrin and cypermethrin. Resistance status of each resistant line was determined using different discriminating concentrations of technical grade pure diazinon, deltamethrin and cypermethrin. The resistance factor was calculated as 24.3 for line 3 against diazinon, 194.5 for line 4 against deltamethrin and 16.7, 20.04 and 23.2 of line 5 against diazinon, cypermethrin and deltamethrin, respectively.

Field ticks

Rhipicephalus (Boophilus) microplus females were collected from an area in and around Bareilly, Uttar Pradesh, India and were used on the day of collection for in vitro bio assay using the different test formulations.

Phyto-formulations

For development of formulation, plant materials for the preparation of NBA/13/B/2 formulation was initially collected from the herbal garden of National Botanical Research Institute, Lucknow, UP., located at an altitude of 123 m, latitude of 26°55'N and longitude of 80°59'E., while for other formulation, NAC-01, plant material was collected from the green campus of Indian Veterinary Research Institute, Bareilly, UP., located at an altitude of 178 m, latitude of 28°25'7.37"N and longitude of 79°23'0.11"E. Both the formulations were chemically characterized and quality control markers were identified. Multi-locational clinical trial of the formulations conferred 70–80 % protection against natural tick infestations during tick active season of the year.

Commercially available herbal products

Erina[®]-EP, a product of Himalaya Drug company, Tumkur, India, an ecto-parasitocidal dusting powder containing

extracts of *Melia azadirachta*, *Tephrosia purpurea* and *Psoralea corylifolia* and marketed for topical use on dogs against fleas and tick infestation and Zerokeet[®], a commercial product of Ayurved Ltd., Solan, India, in liquid form containing extracts of *Cedrus deodara*, *Pongamia glabra*, *Azadirachta indica*, *Eucalyptus globulus* and *Acorus calamus* recommended for the control of ticks, fleas lice and mites.

Commercially available chemical acaricide

Commercially available synthetic pyrethroid product, Butox[®] Vet (Intervet India Pvt. Ltd.) was used. Butox[®] Vet is a hydro dispersible emulsion concentrate (EC) containing 1.25 % deltamethrin for spray or dip charging/toping up to prevent and control ectoparasitic infestations on animals.

In vitro efficacy

Engorged females of resistant tick lines were collected from ear bags of the animals, cleaned while field ticks were collected from surrounding areas and were tested in vitro either by Adult Immersion Test (AIT) format or as product use guidelines provided by the company. For in vitro bioassay a dilution of 1:2 of Zerokeet[®] in water, the developed phyto-formulations at 8 and 10 % concentration for NBA/13/B/2 and NAC-01, respectively, while deltamethrin at a concentration of 0.003 % (30 ppm) in water were prepared. The Erina[®]-EP, being a ready to use topical dusting powder, no separate concentration was prepared. For AIT, the laboratory standardized protocol was used (Sharma et al. 2012). Briefly, the thoroughly cleaned and weighed fully engorged female ticks were immersed in the prepared recommended concentrations of different products for 2 min and after draining the excess fluid, ticks were transferred to the plastic Petri dishes padded with Whatman filter paper no.1 while control ticks were immersed in the solvents used for each product. For Erina[®]-EP, ticks were placed in Petri dishes (5 cm in diameter) and were dusted with 50 mg of powder. All the Petri dishes containing treated and control ticks were kept at 28 °C and 85 ± 2 % RH in desiccators in B.O.D incubators for 15 days. For each group, four replications and in each replication five ticks were maintained. Daily observations were done on every day and death of ticks was confirmed by observing loss of motility and pedal reflex after exposing to light. The egg masses were weighed for the determination of percentage inhibition of oviposition on 15 day after treatment. The inhibition of oviposition was evaluated after weighing the egg masses using the formula of Stendel (1980):

$$\begin{aligned} \text{Reproductive Index (RI)} &= \text{Egg masses/Engorged tick weight} \\ \% \text{ Inhibition of oviposition (\% IO)} \\ &= \frac{\text{RI}(\text{control}) - \text{RI}(\text{treated}) \times 100}{\text{RI}(\text{control})} \end{aligned}$$

Efficacy of different formulation was determined by adopting the formula of Azhahianambi et al. (2009)

$$E\% = 100[1 - (\text{CRT} \times \text{CRO})],$$

where, E% is the efficacy of phyto or chemical acaricide, CRT = NLTA/NLTC, is the reduction in the number of adult live females; NLTA = number of live ticks on 15th day in acaricides treated group; NLTC = number of live ticks on 15th day in control group; CRO = MEWA/MEWC is the reduction in egg laying capacity, MEWA, the mean egg masses of female ticks on 15th day in acaricides treated group; MEWC, the mean egg masses of females ticks on 15th day in the control group.

Results

The details of the efficacy of the different formulations are given in Table 1. Mortality of adult ticks of different tick lines and field ticks treated with commercially available formulations were in the range from 0 to 50.0 ± 5.7 % while the commercially available deltamethrin preparation induced a very low mortality ranging from nil to 5.0 ± 5.0 % only. In comparison to commercially available products, the monoherbal phyto-formulations (NBA/13/B/2 and NAC-01) conferred mortality in the range of 35 ± 2.9–90.0 ± 5.0 %. The inhibition of oviposition in different group of ticks was highly variable viz., in the range of 27.2–50.7, 4.7–18.9, 1.5–7.7, 56.2–65.0 and 25.2–63.6 %, respectively, in ticks treated with Zerokeet[®], Erina[®]-EP, Butox[®] Vet, NBA/13/B/2 and NAC-01. Analysing the mortality data it was observed that there was no significant differences in the mortality pattern of ticks of IVRI-4 and 5 lines treated with monoherbal formulations. But the differences was significant in field ticks and in IVRI-3 line. Although, the mortality pattern of Zerokeet[®] is comparatively higher in comparison to other two commercial products, the value was lower in comparison to monoherbal phytoformulation.

Efficacy (E%) of acaricide was calculated in terms of reduction in tick number due to mortality and reduction in egg laying capacity. Maximum efficacy was observed in NBA/13/B/2 (82.4–91.3 %), followed by NAC-01 (62.3–94.6 %), Zerokeet[®] (41.8–75.4 %), Erina[®]-EP (4.7–14.8 %) with least efficacy of 1.5–6.2 % was observed in Butox[®] Vet treated ticks.

Table 1 Anti-tick activity of developed phyto-formulations and commercially available herbal and chemical products against field ticks and tick lines

Products Concentration	Zerokeet® 1:2	Erina®-EP Powder	Butox® Vet 0.003 %	NBA/13/B/2 8 %	NAC-01 10 %	Control Water
<i>Field ticks*</i>						
Mortality% (mean ± SE)	45.0 ± 5.0 ^a	5.0 ± 5.0 ^b	5.0 ± 5.0 ^b	60.0 ± 14.1 ^a	82.5 ± 7.0 ^c	0.0 ± 0 ^{bd}
I.O.	48.1	18.9	7.7	60.4	63.6	0.0
R.I	0.261	0.408	0.464	0.199	0.183	0.503
Efficacy%	68.3	14.8	2.7	82.4	92.9	0.0
<i>IVRI-5 line**</i>						
Mortality% (mean ± SE)	50.0 ± 5.7 ^a	5.0 ± 2.9 ^b	5.0 ± 2.9 ^b	75.0 ± 9.6 ^c	90.0 ± 5.0 ^{cd}	0.0 ± 0.0 ^{be}
I.O.	50.7	10.9	6.2	65.0	49.1	0.0
R.I	0.254	0.459	0.483	0.180	0.262	0.515
Efficacy%	75.4	10.9	6.2	91.3	94.6	0.0
<i>IVRI-3 line***</i>						
Mortality% (mean ± SE)	20.0 ± 0.0 ^a	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	70.0 ± 0.0 ^c	35.0 ± 2.9 ^d	0.0 ± 0.0 ^{be}
I.O.	27.2	4.7	1.5	57.6	50.0	0.0
R.I	0.390	0.511	0.528	0.227	0.311	0.536
Efficacy%	41.8	4.7	1.5	87.3	62.3	0.0
<i>IVRI-4 line****</i>						
Mortality% (mean ± SE)	40.0 ± 8.2 ^a	5.0 ± 3.3 ^b	0.0 ± 0.0 ^b	60.0 ± 11.5 ^c	60.0 ± 14.1 ^{ac}	0.0 ± 0.0 ^d
I.O.	47.3	13.7	1.5	56.2	25.2	0.0
R.I	0.238	0.390	0.445	0.198	0.338	0.452
Efficacy%	68.4	10.1	2.4	82.5	70.1	0.0

* Zerokeet® versus Erina®-EP, Butox® Vet, NAC-01 and control = $P < 0.01$

** Zerokeet® versus NBA/13/B/2 $P < 0.05$; between other products- $P < 0.001$

*** Zerokeet® versus Erina®-EP, Butox® Vet and control = $P < 0.05$

**** Zerokeet® versus NBA/13/B/2, and Erina®-EP versus NAC-01 = $P < 0.01$, Between other products = $P < 0.001$

a,b,c,d Values with different superscript in a column differ significantly

Comparing the efficacy of different products against individual tick line it was observed that Zerokeet® provided maximum efficacy of 75.4 % against multi acaricide resistant IVRI-5 line followed by similar comparative activity against IVRI line-4 and against field ticks, the efficacy against diazinon resistant ticks was minimum of 41.8 %. In comparison to Zerokeet®, the activity of other markedly available natural and synthetic products conferred insignificant protection against all the tick lines and field ticks (Table 1). However, monoherbal formulation, NBA/13/B/2 conferred maximum protection of 91.3 % against IVRI-5 line and more than 80 % against all other ticks tested. The other formulation, NAC-01 conferred more than 90 % protection against IVRI line-5 and against field ticks while against IVRI lines 3 and 4 the efficacy was within 60–70 %. The overall mean efficacy of all tested products were 63.5 ± 7.4 , 10.1 ± 2.1 , 3.2 ± 1.0 , 85.9 ± 2.1 and 80.0 ± 8.1 %, respectively, against Zerokeet®, Erina®-EP, Butox® Vet, NBA/13/B/2 and NAC-01.

Discussion

Employing recently standardized discriminating concentration of commonly used anti-tick chemicals and resistant monitoring tools, the acaricide resistant status in ticks of seven states of India was worked out and found alarmingly high (Kumar et al. 2011, 2014; Sharma et al. 2012; Shyma et al. 2012; Singh and Rath 2014; Singh et al. 2014a, b; Jyothimol et al. 2014). 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. As the development of new anti-tick compound is highly cost and time intensive, the pace of development and marketing of new acaricides has taken backseat and focus has been shifted to the development of ecofriendly product having multiple mode of action. 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 (Habeeb 2010; Zaman et al. 2012). 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). 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. Amongst the different plant extracts having anti-tick activities, extracts of *Melia azedarach* (Morgan and Thorton 1973; Nakatani et al. 1985) *Annona squamosa* (Kawazu et al. 1989; Hopp et al. 1998; Parvin et al. 2003), peel oil of citrus spp. (Chungsamarnyart and Jansawan 1996) *Azadirachta indica* (Abdel-Shafy and Zayed 2002), *Ageratum houstonianum* (Tedonkeng Pamo et al. 2005), *Cunila angustifolia*, *C. spicata*, *C. microcephala* (Apel et al. 2009), *Eucalyptus globules* (Pirali-Kheirabadi et al. 2009), *Petiveria alliacea* (Rosado-Aguilar et al. 2000), *Acacia pennatula*, *Piscidia piscipula*, *Leucaena leucocephala* and *Lysiloma latisiliquum* (Fernández-Salas et al. 2011), *Acorus calamus* (Ghosh et al. 2011) and *Ricinus communis* (Ghosh et al. 2013) were studied extensively to establish its potential as acaricide against various tick species.

Various workers have reported the tickicidal activity of plants used in Zerokeet[®] and Erina[®]-EP. The crude extract of *Acorus calamus* rhizome showed toxicity to nymph and adult brown dog tick (Leenuwongphun et al. 2008), larvae and adults of *R. (B.) microplus* (Chungsamarnyart et al. 1988; Ghosh et al. 2011). 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), treatment of *R. (B.) microplus* with *Pongamia glabra* extract and seed oil resulted in lower egg production and hatchability (Bisen et al. 2010, 2011), neem seed oil/extracts (*Azadirachta indica*) elicited acaricidal effects on egg, immature, and adult stages of *Hyalomma anatolicum excavatum* (Abdel-Shafy and Zayed 2002) and *R.(B.) microplus* (Srivastava et al. 2008). Different species from the genus *Tephrosia* were studied for larvicidal, insecticidal, and antifeedant activities. The whole plant extract of *Tephrosia purpurea* shown 100 % larvicidal activity in very small doses against the larvae of *Culex quinquefasciatus* (Kumar et al. 2012). Even though anti tick activity of *T. vogell* was reported, there is little documentary evidence demonstrating the potency of *T. purpurea* in tick control.

To develop natural formulations suitable to existing complicated tick problem which is expected to be complicated further in coming years, two formulations were developed, characterized, both safety and stability data were generated and found effective in clinical trial (Ghosh et al. 2014). In the present study, it was observed that although both the natural products are commercially

available for the control of ticks, one product, Erina[®]-EP, is not effective against any of the resistant tick lines. The other product, Zerokeet[®], is showing comparatively better efficacy than Erina[®]-EP but overall protection can reached up to 63.5 % which is lower than the efficacy obtained using monoherbal formulations which corroborates with the lower efficacy, high percent hatchability and higher mean egg masses in ticks treated with mixture of neem (*Azadirachta indica*) and karanj (*Pongamia glabra*) than when karanj (*Pongamia glabra*) alone was used (Bisen et al. 2010 and Bisen et al. 2011).

One of the major problems 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 (s) is crucial to maintain the quality of the finished product. In a recent study conducted by the present multidisciplinary group it was observed that the anti-tick activity of the same plant extracts prepared from 64 accessions collected from 15 Indian states varied from 10 to 90 % (Ghosh, Unpublished data). The comparatively higher anti-tick activity of the newly developed formulations need to be exploited for large scale commercial production and marketing in economical way for the control of multiacaricide resistant ticks infesting animals of India and other tropical countries.

Conclusion

The high anti-tick activity of the monoherbal characterized phyto-formulation against the ticks resistant to chemical acaricides, reported in the present study, might help to develop a commercial product for tackling the multi-acaricide resistant tick infestation problem on animals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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