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In vitro acaricidal activity of ethanolic and aqueous floral extracts of *Calendula officinalis* against synthetic pyrethroid resistant *Rhipicephalus* (*Boophilus*) *microplus*

R. Godara[1] · R. Katoch[1] · A. Yadav[1] · R. R. Ahanger[1] · A. D. S. Bhutyal[1] · P. K. Verma[2] · M. Katoch[3] · S. Dutta[1] · F. Nisa[1] · N. K. Singh[4]

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Abstract Detection of resistance levels against deltamethrin and cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* collected from Jammu (India) was carried out using larval packet test (LPT). The results showed the presence of resistance level II and I against deltamethrin and cypermethrin, respectively. Adult immersion test (AIT) and LPT were used to evaluate the in vitro efficacy of ethanolic and aqueous floral extracts of *Calendula officinalis* against synthetic pyrethroid resistant adults and larvae of *R.* (*B.*) *microplus*. Four concentrations (1.25, 2.5, 5 and 10 %) of each extract with four replications for each concentration were used in both the bioassays. A concentration dependent mortality was observed and it was more marked with ethanolic extract. In AIT, the LC_{50} values for ethanolic and aqueous extracts were calculated as 9.9 and 12.9 %, respectively. The egg weight of the live ticks treated with different concentrations of the ethanolic and aqueous extracts was significantly lower than that of control ticks; consequently, the reproductive index and the percent inhibition of oviposition values of the treated ticks were reduced. The complete inhibition of hatching was recorded at 10 % of ethanolic extract. The 10 % extracts caused 100 % mortality of larvae after 24 h. In LPT, the LC_{50} values for ethanolic and aqueous extracts were determined to be 2.6 and 3.2 %, respectively. It can be concluded that the ethanolic extract of *C. officinalis* had better acaricidal properties against adults and larvae of *R.* (*B.*) *microplus* than the aqueous extract.

✉ R. Katoch
parasitologyskuastjammu@gmail.com

[1] Division of Veterinary Parasitology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology, R.S. Pura, Jammu 181 102, India

[2] Division of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology, R.S. Pura, Jammu 181 102, India

[3] Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180 001, India

[4] Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141 004, India

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Introduction

Rhipicephalus (*Boophilus*) *microplus*, 'the tropical cattle tick' is the major tick species infesting dairy animals in tropical and subtropical regions of the world. In India, *R.* (*B.*) *microplus* is widely distributed and infesting several host species (Ghosh et al. 2007). It causes huge production losses in the form of reduced weight gain and milk production, anaemia, hide damage, and even mortalities (Ram et al. 2004), besides it transmits several disease causing pathogens such as *Babesia bovis*, *B. bigemina* and *Anaplasma marginale* (Soulsby 1982). Further the direct effect of tick infestation has tremendous effect on the availability of good quality hides for Indian leather industry, which is suffering from huge shortfall of 3000 million pieces of hides and skin per year. Economic losses due to tick infestation in livestock of India have estimated as high as US $498.7 million per annum (Minjauw and McLeod 2003).

The currently available tools for tick control consist of chemical acaricides used with different application methods and various formulations, tick-resistant animals, tick vaccines, and rotations between livestock and crops. Among these, the chemical acaricides form the centre of control and eradication efforts because they offer relatively quick and cost-effective suppression of tick populations. Long-term use, however, has generated acaricide resistance in many tick species (Sharma et al. 2012; Khajuria et al. 2014; Singh and Rath 2014), thereby reducing the ability to control them. The progressive evolution of resistance of ticks affecting cattle to almost all of the available acaricides has frustrated the efforts of cattle producers to manage tick and tick-borne diseases (TTBDs) affecting their animals. It is therefore necessary to look for alternative measures which are adaptable and less expensive, especially for the subsistence farmers with limited capital who constitutes the majority of animal rearers in the developing countries, including India.

Calendula officinalis (Asteraceae), commonly known as pot marigold, is widely distributed throughout the world as an ornamental plant (Verma et al. 2014). It has long history of usage by the folk systems because of its rich ethanomedicinal values. The plant contains saponins, flavonoids, carotenes, mucilage, resin bitter glycosides and steroidal compounds (Anna et al. 2002). It is also rich source of free and esterified triterpenic alcohols and polyunsaturated fatty acids such as calendic acid (Neukiron et al. 2004; Muley et al. 2009). Because of these phytochemical ingredients plant have potent anti-inflammatory (Hamberger et al. 2003), anti-tumor (Jimenez-Medina et al. 2006), antioxidant (Fonseca et al. 2011), anti-HIV (Kalvatchev et al. 1997), immunomodulatory (Barbour et al. 2004) and hepatoprotective properties (Rusu et al. 2005). These phytochemical ingredients have been found in various parts of the plant (Liu et al. 2010) and the amounts of active ingredients vary with the plant maturity and the time of harvesting (Kasperzyk et al. 1970). The plants with known biological activity such as *C. officinalis,* merit research to explore it as an alternative for the control of parasitic diseases, and a potential source of new active ingredients. To the best of our knowledge, no study was conducted to investigate the acaricidal activity of *C. officinalis*, although preceding publication suggested its possible insecticidal effects (Lans et al. 2008). The present study was therefore designed to evaluate the in vitro acaricidal effect of ethanolic and aqueous extracts obtained from the

flowers of *C. officinalis* against synthetic pyrethroid (SP) resistant *R.* (*B.*) *microplus* of cattle at Jammu, India.

Materials and methods

Rhipicephalus (*Boophilus*) *microplus* ticks

The dropped engorged-adult female ticks were collected from the cattle sheds (care was taken that the animals were not treated with acaricides in the previous 2 weeks) located at R.S. Pura, Jammu (India) and brought to the laboratory in wide mouthed glass jars sealed with muslin cloth. The ticks were thoroughly washed with tap water and dried on filter paper towel. The identification of ticks was made under stereomicroscope according to keys and descriptions given elsewhere (Soulsby 1982). These ticks were used in the adult immersion test (AIT) within 24 h of collection or were incubated at a temperature of 27 ± 2 °C and relative humidity of 80 ± 5 % until the eggs were laid. These eggs provided the larvae used for larval packet test (LPT).

Acaricides

Technical grade (100 % pure) cypermethrin and deltamethrin (AccuStandard®, USA) were used for conducting LPT. The acaricides were dissolved in methanol to prepare stock solutions, and different test concentrations were prepared in distilled water from the stock solutions.

Larval packet test

The LPT was conducted according to FAO (2004) guidelines with minor modifications. Briefly, 0.5 mL of different concentrations of cypermethrin (100, 200, 400 and 800 ppm) and deltamethrin (25, 50, 100 and 200 ppm) in water were used to impregnate 7.5 × 9.0 cm filter paper (Whatman No. 1, Maidstone, UK). These filter papers were dried for at least 1 h in incubator at 37 °C. The treated papers were folded in half and sealed on the sides using clamps. About 100, 14 days old larvae were dropped into impregnated filter packets before finally sealing the packet with clamp at the top. The packets were than incubated at a temperature of 27 ± 2 °C and relative humidity of 80 ± 5 % and subsequent mortality of larvae was quantified after 24 h. Each testing dose was tested four times. The larvae in the control group were treated with distilled water and four replications were maintained.

The mortality rate was obtained according to the following formula (Godara et al. 2014a): Mortality (%) = (Number of dead larvae / Total number of larvae) × 100 %

Plant material and extraction

The flowers of *C. officinalis* were collected from Jammu, India. The plant sample was taxonomically identified at Department of Botany, University of Jammu and voucher sample was deposited with the curator of the museum. After collection, the flowers were air dried under shade at a well-ventilated place (temperature not exceeding 40 °C) for 4–5 weeks. The air dried flowers were pre-crushed and later pulverized to powder form

using electric blender. The aqueous and ethanolic floral extracts were prepared as per described by Verma et al. (2014). The extracts were transferred to an air-tight container and stored in a freezer at −20 °C till subsequent uses.

Bioassay

The ethanolic and aqueous extracts of *C. officinalis* were dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions. Serial dilutions of the extracts used in the AIT and LPT were made in distilled water, in order to obtain the concentrations of 1.25, 2.5, 5 and 10 %.

Adult immersion test

The AIT was performed as described by Sharma et al. (2012) with minor modifications. The ticks were weighed and assigned to groups randomly (five ticks per group). The different groups of ticks were immersed in 10 mL of the respective concentrations of *C. officinalis* by placing them directly into containers and stirred with glass rod before and after adding ticks. After 5 min, the acaricide was poured off through a sieve and the ticks were transferred to the tissue paper towel for drying and kept separately in glass tubes and sealed with muslin cloth. For each concentration four replications were maintained. Simultaneously, the ticks in the control group were treated with 10 % DMSO and four replications were maintained. The treated ticks were kept in desiccator which was kept in BOD incubator at a temperature of 27 ± 2 °C and relative humidity of 80 ± 5 % for oviposition. The mortality was observed on day 14 post treatment (PT). The ticks which did not oviposit even after 14 days were considered as dead (Sharma et al. 2012). The eggs laid by these ticks were collected, weighed and observed separately at the same condition of incubation for the next 30 days for visual estimation of hatching rate.

The reproductive index (RI) and the percentage inhibition of oviposition (IO) were calculated as follows:

$$\text{RI} = \text{Average weight of eggs laid (mg)} / \text{Average weight of live ticks (mg)}$$

$$\text{IO \%} = [(\text{RI of control ticks} - \text{RI of treated ticks}) / \text{RI of control ticks}] \times 100$$

The LPT for ethanolic and aqueous extracts was conducted as described above using concentrations of 1.25, 2.5, 5 and 10 %. In the control group, 10 % DMSO was used. Four replications were made for each testing dose.

Statistical analyses

The dose–response data were analysed by probit method (Finney 1962) using Graph Pad Prism 4 software. The lethal concentrations (LC_{50}) and their respective 95 % confidence intervals (CI) were determined by applying regression equation analysis to the probit transformed data of mortality. The resistance factors (RF) were calculated as per Sharma et al. (2012). On the basis of RF, the resistance levels (RL) were classified as susceptible (RF < 1.4), level I resistance (RF = 1.5–5.0), level II resistance (RF = 5.1–25.0), level III resistance (RF = 25.1–40) and level IV resistance (RF > 40) (Sharma et al. 2012). The between group significance of entomological data was calculated by one-way ANOVA test and Duncan's test was used for post hoc analysis ($\alpha = 0.05$).

Results

Resistance status in *R.* (*B.*) *microplus* against deltamethrin and cypermethrin

The data on slope value, goodness of fit (R^2), LC_{50}, RF and RL against deltamethrin and cypermethrin are shown in Table 1. The regression graph of mean mortality of larval ticks plotted against values of progressively increasing concentrations of deltamethrin and cypermethrin is shown in Fig. 1. The results indicated the presence of resistance of level II and I against deltamethrin (RF = 8.78) and cypermethrin (RF = 1.98), respectively.

In vitro efficacy of ethanolic extract against adults of SP resistant *R.* (*B.*) *microplus*

The results of AIT using ethanolic extract of *C. officinalis* are presented in Table 2. The efficacy of ethanolic extract against SP resistant *R.* (*B.*) *microplus* was assessed by estimating the adult mortality, RI, IO and hatching rate. Mortality varied from 0.0 to 60.0 %, when tested at concentrations ranging from 1.25 to 10.0 %. At 10 % concentration, the mortality rate was significantly different in comparison to the control group. The regression equation derived after comparing probit mortality versus log values of concentrations of the extract ($y = 6.3116x - 26.518$, $R^2 = 0.9$) revealed that 90 % of correlation with log concentration in probit mortality could be assigned to the concentration of the extract (Fig. 2). From the regression equation, the LC_{50} (CI) value was calculated as 9.85 % (9.54–10.18 %). The slope values and R^2 values of mortality, egg mass, RI and IO are given in Table 3. The extract induced a significant decrease in egg masses produced by the treated ticks, resulting in significant decrease in RI in all concentrations in comparison to control ticks. The IO ranged from 12.8 to 83.3 % (Table 2). The hatching of eggs laid by the treated ticks was completely blocked at 10 % concentration of the extract (Table 2).

In vitro efficacy of aqueous extract against adults of SP resistant *R.* (*B.*) *microplus*

The results of AIT using aqueous extract are presented in Table 2. The mortality varied from 0.0 to 20.0 %, when tested at concentrations ranging from 1.25 to 10.0 %. At 10 % concentration, the mortality rate was significantly different in comparison to the control group. The regression equation derived after comparing probit mortality versus log values of concentrations of the extract ($y = 5.341x - 22.291$, $R^2 = 0.84$) revealed that 83.8 % of correlation with log concentration in probit mortality could be assigned to the concentration of the extract (Fig. 2). Based on regression equation, the LC_{50} (CI) value was

Table 1 Dose-response data of larvae of *Rhipicephalus* (*B.*) *microplus* against deltamethrin and cypermethrin

Acaricide	Slope ± SE	R^2	LC_{50} (ppm) (95 % CI)	RF	RL
Deltamethrin	4.181 ± 0.9283	0.8712	117.6 (115.1–120.2)	8.78	II
Cypermethrin	2.678 ± 0.2664	0.9712	274.9 (265.6–284.5)	1.98	I
IVRI-1	3.42 ± 0.498	0.871	11.8 (11.6–12.0)	1.00	S

R^2 Goodness of fit, *RF* resistance factor, *RL* resistance level, *CI* confidence interval, *S* susceptible

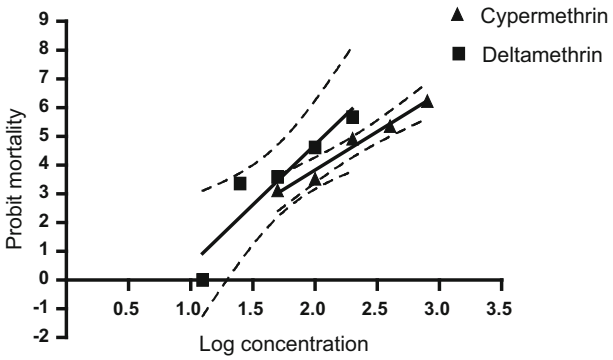

Fig. 1 Dose–mortality curve of larvae of *Rhipicephalus* (*B.*) *microplus* against log concentrations of deltamethrin and cypermethrin

determined as 12.87 % (12.4–13.36 %). The slope values and R^2 values of mortality, egg mass, RI and IO are given in Table 3.

It is important to note that the live ticks which were treated with different concentrations of aqueous extract laid egg masses which were significantly lower in weight than the egg masses of control ticks, despite the low adult mortality in the treated ticks. Further, a significant decrease in RI was recorded in all concentrations in comparison to control ticks. The IO observed in AIT varied from 17.5 to 58.3 % (Table 2). The hatchability of eggs of treated ticks was decreased up to 50 % at the 10 % concentration of the extract (Table 2).

In vitro efficacy of extracts against larvae of SP resistant *R.* (*B.*) *microplus*

Both ethanolic and aqueous extracts of *C. officinalis* evaluated in the study showed acaricidal activity against the larvae of SP resistant *R.* (*B.*) *microplus* ($p < 0.05$). The extracts were lethal to the larvae in the concentration of 10 % after 24 h (Table 3; Fig. 3). In the control group, the survival rate was 100 %. The slope, R^2 and the LC_{50} (CI) values for ethanolic and aqueous extracts are given in Table 3.

Discussion

In India, the most widely used method for the control of ticks is the direct application of acaricides to host animals and thus, the consumption of acaricides has been increased manifold during last decades. The SPs (deltamethrin and cypermethrin) are commercially available in India and at present are two predominant acaricides marketed aggressively by many companies for tick control in the country. These SPs are also used extensively for the control of mosquitoes further leading to increased and indiscriminate usage (Tiwari et al. 2010). The resistance recorded in field isolates of *R.* (*B.*) *microplus* from Jammu, India is relatively less pronounced to cypermethrin as compared to deltamethrin at present but it is very likely that higher resistance to cypermethrin will be acquired by these ticks in near future. Recently, the presence of wide spread resistance to SPs in *R.* (*B.*) *microplus* has been reported from the different parts of the country (Vatsya and Yadav 2011; Sharma et al. 2012; Abdullah et al. 2013; Kumar et al. 2013; Khajuria et al. 2014; Singh and Rath 2014).

Table 2 Mean (± SE) tick weight, mortality, egg weight, reproductive index (RI) and inhibition of oviposition (IO) of SP resistant *Rhipicephalus* (*B.*) *microplus* against ethanolic and aqueous floral extracts of *Calendula officinalis*

Extract	Conc. (%)	Live tick wt. (mg)	Mortality (%)	Egg wt. (mg)	RI	IO (%)	Hatching (%)
Ethanolic	Control	103.8 ± 4.8	0.0 ± 0.0^{a}	60.6 ± 2.8^{a}	0.587 ± 0.02^{a}	–	100
	1.25	92.3 ± 3.6	0.0 ± 0.0^{a}	44.9 ± 3.5^{b}	0.472 ± 0.02^{b}	19.3 ± 4.7^{a}	95
	2.5	99.3 ± 4.3	0.0 ± 0.0^{a}	51.1 ± 2.5^{bc}	0.509 ± 0.01^{bc}	12.8 ± 1.5^{ab}	95
	5.0	90.4 ± 3.4	5.0 ± 5.0^{a}	38.5 ± 3.7^{bd}	0.418 ± 0.03^{bd}	28.6 ± 5.8^{ac}	90
	10.0	89.9 ± 2.1	60.0 ± 11.2^{b}	8.7 ± 2.9^{e}	0.098 ± 0.03^{e}	83.3 ± 5.7^{d}	00
Aqueous	Control	103.8 ± 4.8	0.0 ± 0.0^{a}	60.6 ± 2.8^{a}	0.587 ± 0.02^{a}	–	100
	1.25	92.4 ± 3.9	0.0 ± 0.0^{a}	45.3 ± 2.9^{b}	0.485 ± 0.02^{b}	17.5 ± 2.7^{a}	95
	2.5	90.8 ± 3.2	0.0 ± 0.0^{a}	43.2 ± 2.5^{b}	0.468 ± 0.02^{b}	20.2 ± 2.8^{a}	90
	5.0	93.4 ± 3.2	10.0 ± 6.9^{ab}	40.9 ± 3.8^{b}	0.428 ± 0.04^{b}	26.3 ± 6.1^{a}	90
	10.0	92.8 ± 3.8	20.0 ± 9.2^{b}	22.8 ± 4.2^{c}	0.246 ± 0.04^{c}	58.3 ± 6.9^{b}	50

Means within an extract type followed by different letters are significantly different (ANOVA followed by Duncan's test: $p < 0.05$)

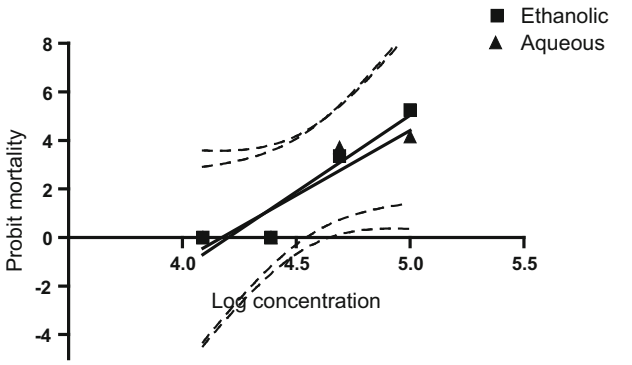

Fig. 2 Regression line of probit mortality of adults of SP resistant *Rhipicephalus* (*B.*) *microplus* against log concentrations of ethanolic and aqueous floral extracts of *Calendula officinalis*

Table 3 Dose-response data of adults and larvae of SP resistant *Rhipicephalus* (*B.*) *microplus* against ethanolic and aqueous floral extracts of *Calendula officinalis*

Test	Extract	Variables	Slope ± SE	R^2	LC_{50} (%) (95 % CI)
AIT	Ethanolic	Mortality	6.312 ± 1.49	0.9000	9.85 (9.54–10.18)
		Egg mass	−40.24 ± 18.56	0.7016	
		RI	−0.4024 ± 0.186	0.7015	
		% IO	68.92 ± 31.80	0.7014	
	Aqueous	Mortality	5.341 ± 1.658	0.8384	12.87 (12.4–13.36)
		Egg mass	−23.14 ± 8.976	0.7687	
		RI	−0.2508 ± 0.089	0.7987	
		% IO	42.59 ± 15.92	0.7815	
LPT	Ethanolic	Mortality	4.364 ± 0.966	0.9108	2.633 (2.512–2.761)
	Aqueous	Mortality	4.708 ± 1.385	0.8525	3.185 (3.048–3.327)

R^2 goodness of fit, *CI* confidence interval, *RI* reproductive index, *IO* inhibition of oviposition

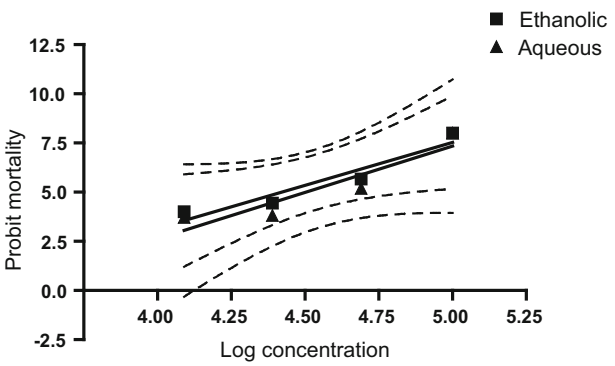

Fig. 3 Regression line of probit mortality of larvae of SP resistant *Rhipicephalus* (*B.*) *microplus* against log concentrations of ethanolic and aqueous floral extracts of *Calendula officinalis*

The results of the study showed that the ethanolic extract induced a much higher concentration dependent increase in the mortality of adult and larval ticks, and a decrease in egg mass production and hatching rate as compared to aqueous extract. Similarly, the IO was also higher in ethanolic extract treated ticks. The variation in the acaricidal activity of ethanolic and aqueous extracts in the current study might be attributed to the extraction process involving ethanol and water, as the ethanolic extract obtained from the floral parts of *C. officinalis* had significantly ($p < 0.05$) higher phenolic, flavonoid and tannin components in comparison to the aqueous extract (Verma et al. 2014).

The slope of RI of the engorged female ticks exposed to various concentrations of the extracts was negative, thus indicating that although the increase in concentration of the extracts could not cause mortality in all the exposed ticks but the egg laying capacity or the efficacy of conversion of live weight into egg mass decreased among the survived female. The population-limiting property of any plant extracts needs to be evaluated to assess the overall efficacy of the extract. In the current study, the effects of *C. officinalis* extracts on the future progeny of the exposed ticks were assessed by estimating the % IO and significant reductions ($p < 0.05$) in the egg mass production in *R.* (*B.*) *microplus* were observed at different levels. Further, the egg mass laid by the surviving ticks at all the concentrations had lost their glossy appearance that occurs due to waxy water proofing when compared to the eggs laid by control ticks. Furthermore, the ethanolic extract caused complete (100 %) blockage of hatching in eggs laid by the ticks treated with 10 % concentration. In the recent past, various plant extracts and essential oils have shown significant activity against all the stages of economically important tick species (Chagas et al. 2012; Ghosh et al. 2011, 2013; Godara et al. 2014a, b, 2015).

The control of ticks presents many great research challenges and prospects for the identification of new, safe and environmentally acceptable acaricides. Use of phytochemical products may be beneficial as acaricides to reduce the problems faced by animal owners, such as resistance and residues. Further, the natural compounds derived from plants are more stable as these are mostly plant secondary metabolites synthesized over a long period of time. Moreover, the natural compounds also provide greater structural diversity than synthetic ones and therefore are a source of low molecular weight structures active against a wide range of target agents and this diversity can preclude the occurrence of resistance. Based on the results of the present study, it can be concluded that the ethanolic and aqueous floral extracts of *C. officinalis* had acaricidal activity against adults and larvae of *R.* (*B.*) *microplus*. Further investigations against different tick species and its actual in vivo application are required to determine the true potential of *C. officinalis* as an effective herbal formulation for the control of tick infestation on animals.

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