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Repellency to ticks (Acari: Ixodidae) of extracts of *Nigella sativa* (Ranunculaceae) and the anti-inflammatory DogsBestFriend™

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Abstract Motivated by observations that the canine anti-inflammatory cream DogsBestFriend™ (DBF) appeared to deter flies, mosquitoes, and ticks from treated animals, repellent efficacy bioassays using four species of ticks were conducted with three extracts of *Nigella sativa* L. (Ranunculaceae), a constituent of DBF. The DBF cream was tested against nymphs of lone star tick, *Amblyomma americanum* (L.). In vertical filter paper assays, the three extracts applied at 0.413 mg extract/cm^2 filter paper repelled 96.7–100 % of brown dog tick, *Rhipicephalus sanguineus* (Latreille) nymphs, whereas, at the same rate, only one extract repelled >90 % *A. americanum* nymphs. Adult (mixed sexes) American dog ticks, *Dermacentor variabilis* (Say), required a higher concentration to be repelled effectively; two extracts, applied at 0.827 mg extract/cm^2 filter paper, repelled ≥90 % of the *D. variabilis*. In contrast, all extracts applied at much lower concentration (0.206 mg extract/cm^2 filter paper) repelled 100 % adult blacklegged ticks, *Ixodes scapularis* Say (only females tested). Of the two more repellent extracts, one lost most of its activity against *A. americanum* nymphs in <4 h when applied at 0.827 mg extract/cm^2 filter paper, whereas the other repelled 66.7 % of the nymphs at 192 h after application. At 0.206 mg extract/cm^2 filter paper, one extract was as repellent as deet against *A. americanum* nymphs. In a vertical bioassay in which nylon organdy was substituted for filter paper, DBF, at the rates of 1.67 and 0.835 mg cream/cm^2, repelled 76.7 and 30.0 % *A. americanum* nymphs, respectively. These findings indicate that when applied appropriately DBF should afford some protection to canines against tick bites.

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Introduction

Throughout much of the habitable world, ticks and the pathogens they transmit harm humans and domestic animals. Recently, the US Centers for Disease Control and Prevention (CDC) revised the estimated number of human cases of Lyme disease diagnosed annually to 300,000 (CDC 2013). Furthermore, Adrion et al. (2015) have shown the comparative costliness of post-treatment Lyme disease. In North America, there are additional risks of tick-borne ehrlichioses, Rocky Mountain spotted fever, babesiosis, and tularemia (Stafford 2007). Lyme disease and some ehrlichioses are also particularly troublesome for canines. Although technologies developed in last two decades have shown some promise for controlling tick populations, they are not in wide use (Piesman and Eisen 2008), lending a greater reliance on arthropod repellent products. Repellents are an important means of personal protection and their use is recommended to the public by the CDC (2002). Deet (*N*,*N*-diethyl-3-methyl benzamide) has been the principal arthropod repellent for use on human skin since the 1950s (Debboun et al. 2007) and permethrin has long proven to be effective for use on clothing (Schreck et al. 1982). Although other synthetic repellents, such as picaridin, are now available, there is considerable public interest in repellents derived from natural sources (Bissinger and Roe 2010; Pages et al. 2014). Recent studies have revealed tick-repellent activity in a variety of plant sources (Bissinger and Roe 2010).

The blacklegged tick, *Ixodes scapularis* Say, is vector of the spirochete that causes Lyme disease (Spielman et al. 1985), and the lone star tick, *Amblyomma americanum* L., transmits the rickettsial pathogens *Ehrlichia chaffeensis* and *E. ewingi* (Childs and Paddock 2003). Both tick species feed on a wide variety of host species, with larvae, nymphs and adults biting humans and dogs (Sonenshine 1993; Mixson et al. 2006; Goddard and Varela-Stokes 2009). The larval and nymphal stages of the American dog tick, *Dermacentor variabilis* (Say), feed on small mammals, whereas the adults feed on dogs, medium-sized mammals and humans. American dog ticks can transmit the pathogens that cause Rocky Mountain spotted fever and tularemia (Sonenshine 1993). The brown dog tick, *Rhipicephalus sanguineus* (Latreille), although principally a canine feeder, is known to bite other hosts, including humans and is distributed widely around the world. The brown dog tick is a vector for canine ehrlichiosis and canine babesiosis (Stafford 2007). All four species are three-host ticks, i.e., they must acquire hosts in the larval, nymphal, and adult stages.

In vitro and clinical studies support the usefulness of *Nigella sativa* L. (Ranunculaceae) fixed and essential oils for topical anti-inflammatory (Amin and Hosseinzadeh 2016; Majdalawieh and Fayyad 2015) and anti-microbial (Mahmoudvand, et al. 2014) applications. DogsBestFriend™ (DBF) was formulated with *N. sativa* fixed and essential oils to provide rapid relief for inflammation resulting from insect bites and skin infections (Yousefi et al. 2013; Al-Abu-Al-Basal 2009; Hannan, et al. 2008), and to speed wound healing (Al-Mutheffer 2010). During a routine walk with his three dogs, the co-author (jgb) noticed that flies and mosquitoes would not approach the head of the dog that was being treated with DBF for a deer fly (Tabanidae: Diptera) bite over his left eye. Rather, the

insects would not approach closer than 30 cm from the dog's head before flying away, while continuing to bother the two untreated dogs. Our interest was further piqued by observations that when adult *I. scapularis* were placed on the untreated center of a C-shaped application of DBF on paper, they appeared to avoid the DBF before exiting the C on untreated paper. A search of the literature revealed studies describing the mosquito repelling activity of the seed and essential oil of *N. sativa* (Bulugahapitiya and Arachchige 2007) and novel insecticidal activity on adult mosquitoes (McAllister and Adams 2010). During the fall tick season it was observed that an application of DBF to the top of the head and along the back of a dog dramatically reduced the tick count on the treated dog. There were, however, no published reports in the scientific literature on the tick repellent activity of *N. sativa* seeds or oils.

Nigella sativa is an herbaceous annual and is native to southwestern Asia, so its use would satisfy demands for a natural repellent. Its seeds, which have been traditionally used as a spice, have been found to have medicinal properties (Ali and Blunden 2003). According to Owen (1805), *Nigella* is reported to have been burned to repel gnats possibly as long ago as the time of the Roman Empire. Because so little is known about the efficacy of *N. sativa* as an arthropod repellent, we evaluated three *N. sativa* extracts and DBF as repellents against four species of ticks (*I. scapularis*, *A. americanum*, *R. sanguineus*, and *D. variabilis*) that bite dogs and humans.

Materials and methods

Ticks

Nymphal *A. americanum* and *R. sanguineus* and adult *D. variabilis* were obtained from colonies at Oklahoma State University, and held at 23–24 °C, 97 % RH, and a photoperiod of 16:8 h (L:D). Host-seeking adult *I. scapularis* were captured by flagging at the USGS Patuxent Wildlife Research Center, Laurel, MD, USA, and held at 16–18 °C, 97 % RH, and a photoperiod of 9:15 h (L:D). At the time of testing the *A. americanum* were 1–6 months, the *R. sanguineus* 1–2 months, and *D. variabilis* 2–3 months since their previous molt. Adult *I. scapularis* were field-collected in November, 2012 and tested 2 months later. Bioassays with *D. variabilis* adults used both sexes. However, with *I. scapularis*, only females were tested and were stored in separate vials from males.

Chemicals

Deet was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nigella sativa extracts

Three supercritical CO_2 extracts were prepared according to the procedure described in US patent 8.535.740 in two batches (Pacioretty et al. 2013). An essential oil and oleoresin extract from the first batch, extracts #1 and #2, respectively, and an essential oil extract (#4) from the second batch were used in this study. The thymoquinone content of these fractions was determined according to the method of Ghosheh et al. (1999). Essential oil extracts #1 (EO35) and #4 (EO 24) contained 35 and 24 % thymoquinone, respectively.

The oleoresin extract used contained 1.24 % thymoquinone (OR 1.24) and the commercial sample of DBF was formulated as 10 % oleoresin and 0.5 % essential oil fraction.

Bioassays

The tendency of many species of host-seeking ticks to climb when they encounter a vertical surface was exploited to expose the ticks to repellent treatments. The bioassay described by Carroll et al. (2004) used a 4 × 7 cm rectangle of Whatman No. 4 filter paper that was marked with a pencil into three zones (two 1 × 4 cm zones at the far ends and a central 4 × 5 cm zone). Using a pipettor, 165 µl of test solution (solvent ethanol) was applied evenly to both sides of the central zone of the filter paper. After allowing 10–15 min for the test solution to dry, the filter paper was suspended from a bulldog clip hung from a slender horizontal dowel held by an Aptex No. 10 double clip work holder (Aptex, Bethel, CT, USA). A Petri dish (9 cm diameter) glued centrally in a 15 cm Petri dish created a circular moat when water was added between the dishes' walls (1.5 cm high). The moated Petri dishes were centered directly below the vertically suspended filter paper strip. When *A. americanum* and *R. sanguineus* nymphs climbed to the rim of an open storage vial centered in a second set of moated petri dishes, the bulldog clip holding the filter paper strip was removed from the dowel and positioned so that ticks could transfer from the vial to the lower untreated zone of the filter paper. When the tenth tick had climbed onto the filter paper, the paper was reaffixed to the work holder. Vials containing adult *D. variabilis* or adult *I. scapularis* were opened in the central dish of moated Petri dish unit and allowed to disperse in the inner Petri dish for 20-30 min before testing. Due the larger size of adult *I. scapularis* females and *D. variabilis* males and females, an individual tick was allowed to crawl onto the lower end of a section of a handheld wooden barbecue skewer (10 cm long). The skewer was then positioned so that the tick could climb it and transfer to the lower untreated zone of the filter paper which remained attached to the work holder. The locations of the ticks were recorded at 1, 3, 5, 10, and 15 min after the tenth tick grasped the lower untreated zone of the filter paper. Ticks were considered repelled if they were in the lower untreated zone at 15 min after the tenth tick had crawled onto the filter paper or if they dropped from the filter paper without having crossed the upper boundary of the treated zone.

For testing DBF cream, nylon organdy was substituted for filter paper as the substrate, because the small amounts of cream could be applied more evenly on the cloth than on filter paper. When the desired amount of cream was placed on slick-surfaced weighing paper, it was removed with a narrow spatula and applied to the cloth. The paper was reweighed to ascertain the weight of DBF applied. The spatula was also used to spread the cream on the cloth. The DBF bioassay was otherwise the same as the filter paper bioassay except that as a control the nylon organdy was untreated.

Experimental design

An ethanol control was tested against 10 ticks each day that an extract or deet was tested against that species. Ticks were tested in replicates of ten ticks per combination of concentration of extract or deet. Nymphal *A. americanum* were tested at 0 (ethanol control), 0.413, 0.206, and 0.103 mg extract/cm^2 filter paper and 0, 0.413, 0.206, 0.103, and 0.063 mg deet/cm^2 filter paper. Nymphal *R. sanguineus* were tested at 0, 0.413, 0.206, and 0.103 mg extract/cm^2 filter paper. Adult *D. variabilis* were tested at 0, 0.827 and

0.413 mg extract/cm^2 filter paper, and adult *I. scapularis* were tested at 0.206 mg extract/cm^2 filter paper.

Duration tests of extracts 1 and 4 (0.827 mg extract/cm^2 filter paper) were conducted against *A. americanum* nymphs. Three tests of 10 nymphs each were conducted at 10 min, 2 and 4 h after application and a single test each at 6 and 24 h after application. Against extract 4, initially four tests (10 ticks each) were conducted at 10 min, 2, 4, and 6 h after application and three tests 24 h after application. A series of duration tests was conducted with extract 4: four tests at 10 min, 2, and 24 h, three tests at 4 h, and one test at 6 h after application. Based on the results of the aforementioned duration tests, a more extended series of tests of extract 4 and deet were conducted against *A. americanum* nymphs, with bioassays at 24 (four tests), 48 (three tests), 72 (three tests), 96 (four tests), 192 (three tests), 240 (one test), and 360 h (one test) after application. An ethanol control was conducted each day of duration testing. DBF was tested at 33.3 and 16.7 mg cream/cm^2 nylon organdy. The controls for DBF tests were untreated cloth.

Statistical analysis

Since the analyses are based on repelled/not repelled (binary) data, we used a generalized linear model, assuming the data are samples from over-dispersed binomial distributions. Parameter estimates and statistical tests were performed using the R software (R Core Team 2014), a posteriori means comparisons were made using the R MultComp package (Hothorn et al. 2008).

Results

All four species of ticks (*A. americanum*, *D. variabilis*, *I. scapularis*, and *R. sanguineus*), representing four genera, were repelled by extracts of *N. sativa*. Repellency varied among the species (Fig. 1). Adult (female) *I. scapularis* were the most easily repelled, with 100 % of the ticks repelled by extracts #1 and #4 applied at the rate of 0.206 mg extract/cm^2 filter paper ($n = 30$, $n = 20$, respectively). In contrast, 0.827 mg extract/cm^2 filter paper of extracts #1 and #4 were needed to repel 100 and 90 %, respectively, adult (both sexes) *D. variabilis* ($n = 30$). High proportions (>87 %) of nymphs of *A. americanum* and *R. sanguineus* were repelled by 0.413 mg extract/cm^2 filter paper, whereas that concentration of extract #1 and #4 repelled just 60 and 30 %, respectively, of *D. variabilis* adults.

Extracts #1 and #4 were more repellent to *A. americanum* and *D. variabilis* than extract #2. All three extracts were similar in repellency to *R. sanguineus* at the concentrations tested (Fig. 1).

At 0.413 mg extract or compound/cm^2 filter paper, extracts #1 and #4 did not differ significantly from deet in their repellency against *A. americanum*, nor did extract #1 and deet differ significantly in repellency at 0.206 mg extract or compound/cm^2 filter paper (Fig. 1). Extracts #1 and #4 and deet were tested for the longevity of their repellency against *A. americanum* nymphs. Extract 4 afforded protection over a considerably longer time (Table 1). Although the repellency of extract #4 diminished to ~60 % by the sixth hour after application (Fig. 2), it remained at about that level for 8 days, with 66.7 % of *A. americanum* nymphs repelled at 192 h after application (Fig. 2). At 96, 192, 240, and 360 h post application, extract #4 did not differ significantly (Fig. 2) in efficacy from deet, which repelled <70 % of the ticks at those times (Fig. 2).

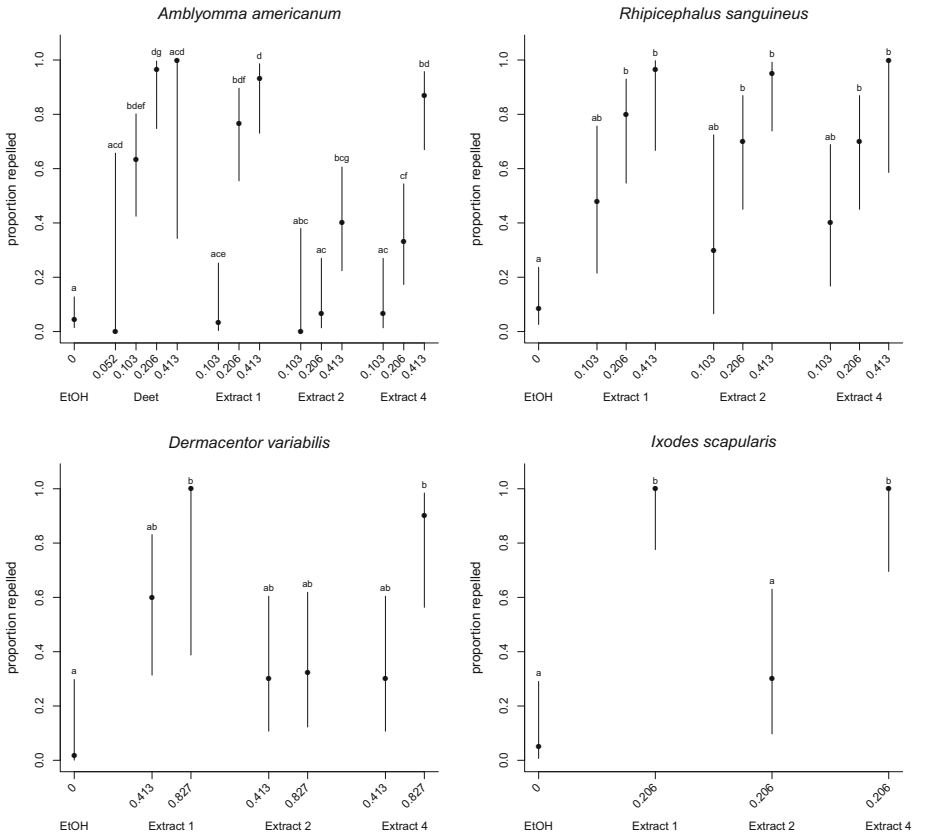

Fig. 1 Responses of *A. americanum* nymphs to extracts of *Nigella sativa* and deet and an ethanol control (concentration zero), and *R. sanguineus* nymphs, adult *D. variabilis* (mixed sexes), and adult *I. scapularis* (females) to extracts of *N. sativa* and an ethanol control in vertical filter paper bioassays

Table 1 Repellency (% ± SD) of extracts 1 and 4 applied at 0.827 mg extract/cm^2 filter paper against *Amblyomma americanum* nymphs at 10 min, 2, 4, 6, and 24 h after application

Ticks repelled					
Time after application	10 min	2 h	4 h	6 h	24 h
Treatment					
Extract 1[a]	93.3 ± 0.66	83.3 ± 1.20	26.7 ± 1.67	20.0	10.0
Extract 4[b]	97.5 ± 0.25	75.0 ± 1.56	60.0 ± 0.58	62.3 ± 1.11	67.1 ± 1.09
Control (ethanol)[c]	0				4.3 ± 0.20

[a] Three replications of 10 ticks each for each combination of extract 1 except 6 and 24 h for which 10 ticks each were tested

[b] Four replications of 10 ticks each for each combination of extract 4 except 24 h for which 7 replications of 10 ticks were tested

[c] Four replications of 10 ticks were tested for the ethanol control at 10 min and 7 replications for the 24 h tests

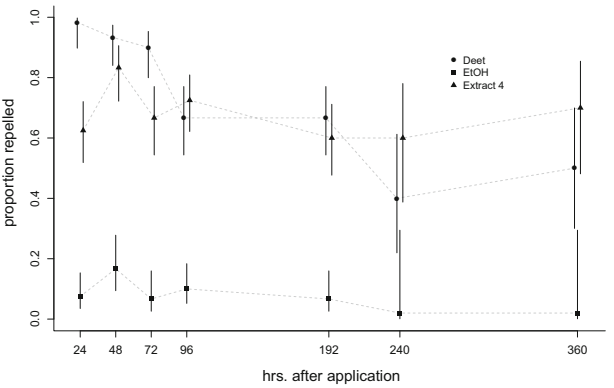

Fig. 2 Responses of *Amblyomma americanum* nymphs to extract 4 of *Nigella sativa*, deet (0.827 mg extract/cm^2 filter paper), and an ethanol control (concentration zero) at 24, 28, 72, 192, 240, and 360 h after application in vertical filter paper bioassays

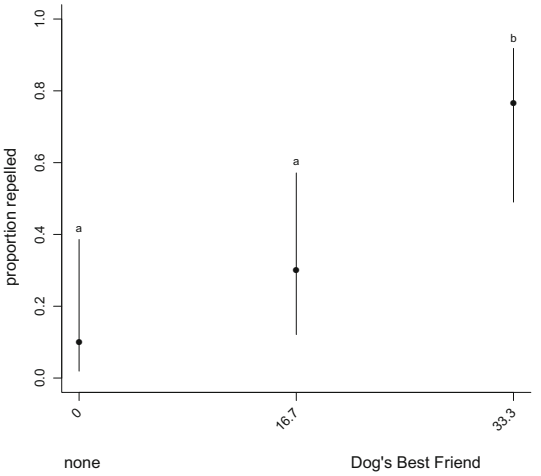

Fig. 3 Responses of *Amblyomma americanum* nymphs to DogsBestFriend™ (DBF) in vertical nylon organdy bioassays (control no treatment)

DBF, applied at the rate of 33.3 and 16.7 mg cream/cm^2 nylon organdy, repelled 76.7 and 30 % of *A. americanum* nymphs, respectively (Fig. 3).

Discussion

The results of the bioassays clearly demonstrated that the four tick species did not respond alike to the extracts; an outcome previously observed for deet and some other repellents tested against more than one species of tick (Schreck et al. 1995; Carroll et al. 2004; Bissinger et al. 2009). Some differences between proportions of adults and nymphs repelled may be related to tick size and the dimensions of the treated area of the filter paper strips. Large adults crossed the treated area in fewer steps and contacts with the treated surface than nymphs. Nevertheless, a major difference was observed between adult *D. variabilis* and adult *I. scapularis*, with the latter repelled by much lower concentrations of

extract (Fig. 1). Interestingly *R. sanguineus* nymphs were repelled similarly by all three extracts, whereas with the other species extract 2 was relatively ineffectual compared extracts 1 and 4.

Extract 1 differed from extract 4 in that the former lost nearly all of its repellency to *A. americanum* nymphs by 6 h post application, whereas extract 4 continued to repel >62 % of nymphs 24–192 h after application (Fig. 2). Further testing is needed to ascertain whether the prolonged efficacy of extract 4 on filter paper under laboratory conditions is indicative of its repellency when applied to skin and pelage and exposed to various environmental conditions.

In previous studies using the same bioassay, 67.7, 46.7, and 90 % of *A. americanum* nymphs were repelled by the essential oils of Chinese weeping cedar, *Cupressus funebris*, and Chinese juniper, *Juniperus chinensis* (Cupressaceae), and deet, respectively, 6 h after 0.827 mg oil/cm^2 were applied to filter paper (Carroll et al. 2011). Similarly amyris, *Amyris balsamifera*, oil repelled 55 % of *A. americanum* nymphs 4 h after application at 0.827 mg oil/cm^2 filter paper (Carroll et al. 2010).

Weldon et al. (2011) showed that ticks exposed for 1 h to several repellent and non-repellent compounds occurring in Citrus peels adversely affected the ticks' ability right themselves when placed on their dorsum and/or their tendency to climb. Further testing of *N. sativa* extracts and DBF is warranted to determine if they have other tick deterrent properties, because typically, once a tick acquires a host it does not attach immediately and commence feeding. It will wander until it finds a suitable attachment site and then feeds for a few days. Thus, the tick is exposed for an extended period to chemicals on the host's integument, pelage or plumage. If an intoxicated tick falls or is dislodged from its host by grooming, it is unlikely to return.

In comparative bioassays, deet repelled *A. americanum* nymphs at lower concentrations than did *Nigella* extracts. However, the concentrations tested were lower than concentrations at which repellents, including deet, are generally applied. *Nigella* based products appear to have promise as safe, lasting, and effective tick repellents.

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