REPELLENT EFFICACY OF AZADIRACHTIN ON *IXODES RICINUS* TICKS (ACARI, IXODIDAE)

Aleksandra Petrović[1], Vojislava Bursić[1], Ivana Ivanović[1], Dušan Marinković[1], Nikola Puvača[2], Tijana Stojanović[1], Gorica Vuković[3]

[1]*Department for Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia*
[2]*Department of Engineering Management in Biotechnology, Faculty of Economics and Engineering Management in Novi Sad, University Business Academy in Novi Sad, Cvećarska 2, 21000 Novi Sad, Serbia*
[3]*Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia*
e-mail: aleksandra.petrovic@polj.uns.ac.rs

Abstract
The repellent efficacy testing, as well as the search for the most efficient and economically most justified preventive measures, emerge from the fact that in the last few years a zoogeographic expansion of certain tick species has been observed, in addition to an increased frequency of previously registered and emergent tick-borne diseases. The aim of this study was to compare the repellent efficacy of different azadirachtin essential oil concentrations as a function of time on *Ixodes ricinus* ticks. Azadirachtin essential oil had more than 50% repellent efficacy on *I. ricinus* three minutes after the application, and it decreased with time when all tested concentrations were concerned. Eight hours after repellent administration, concentrations of 50% and 75% have had repellent effects of 50%, while pure essential oil (100%) showed a repellency of 63.33%. In order for people to spend more relaxed and free time in nature, it is necessary to apply protective measures against ticks and one of the recommended ways is to use the substances that could be applied to the skin or clothes, and which have a repellent effect, such as azadirachtin essential oil.

Introduction

Urban ecosystems and rural areas are usually covered with fragmented green areas (parks, lawns, picnic areas, sports and recreation areas, meadows, forests, river banks), consisted of different vegetation types and structures. The specific floristic composition, the presence of diverse animal species suitable as the potential ticks' hosts, and the appropriate microclimatic conditions make these areas optimal habitats for the appearance, maintenance and increase of different tick species populations. Therefore, in order to predict the potential risk of tick-borne diseases (TBDs), both for humans and animals, it is necessary to distinguish the effects of a large number of abiotic and biotic factors in a certain habitat.

The lifespan of certain tick species can be more than three years [1]. During the warm months, from mid-June to the end of August, due to high temperatures and low relative humidity, ticks have reduced activity, and thus lower population density at some localities, which still does not decrease the risk of potential contact with these haematophagous ectoparasites. In average, the tick temperature optimum is 20-25°C, when more than 40% adults and 30% nymphs are active. The optimal value for relative air humidity is from 45 to 80%. The photoperiod is also an important environmental factor for tick activity [2]. Considering that, it can be concluded that the seasonal peaks of ticks increased activity, their occurrence in certain habitats, and their quest for an adequate host coincide with increased human activity in nature (sports, recreation, leisure, agricultural activities). The longer people spend their time in nature, the higher is the probability that direct contact with ticks will occur, and therefore, the possibility of various pathogens transmission from ticks to humans. In the last ten years, more than 15 zoonotic pathogens and vector-borne diseases have been registered in Europe, of which, at least six are solely tick-borne: *Rickettsia* sp., *Anaplasma phagocytophilum*, *Borrelia burgdorferi* s.l., *Francisella tularensis*, Crimean-Congo hemorrhagic virus, and tick-borne encephalitis virus [3]. TBDs are very common in Europe and the United States. According to Mysterud et al. [4] about 300,000 people in the United States and 65,000 people in Europe are infected annually with tick-borne Lyme borreliosis. The registered TBDs in Serbia are: Lyme disease, human granulocytic anaplasmosis, tularemia, Q-fever, babesiosis, and rickettsial infections. However, it could be assumed that the real epidemiological situation is underestimated, because autochthonous cases of these diseases are registered sporadically, and the unified registration system at the national level has not still been established.

Testing the repellent and acaricidal efficacy of already registered and newly developed preparations, as well as finding the most efficient and economically most effective measures, emerge from the fact that in the last few years a zoogeographic expansion of certain tick species has been observed, as well as an increasing frequency of previously registered and emergent TBDs, all with the aim to protect humans and animals and prevent the possible pathogens transmission. Each step in the tick-host interaction can be interrupted, but only certain activities and processes can be counted as *sensu stricto* repellency which implies complete tick repellent from the host and/or its abandonment [5]. Therefore, the studies on the efficacy and repellency duration of certain compounds is of high importance and requires constant studying and methodology improvement, in order to monitor trends in the new synthetic and natural repellents development, the adequate tests application to assess repellency and finally, introduction and application of economically viable and widely available commercial preparations that are not harmful to human and animal health. Azadirachtin, the main bioactive ingredient of neem seeds (*Azadirachta indica* A. Juss., 1830 neem tree), is a very potent insecticide that affects the nutrition, development and molting of various insect larvae at very low concentrations, however low concentrations of this oil either have no effect or are negligible when it comes to ticks [6]. Therefore, the aim of this study was to compare the repellent efficacy of different azadirachtin essential oil concentrations as a function of time on *Ixodes ricinus* ticks.

Experimental

Ticks were collected using the flag-hour method [7]. Collected specimens were placed in plastic containers with perforated lids to provide sufficient ventilation, with a piece of cotton wool moistened with water to prevent the ticks' desiccation. Collected ticks were determined up to species level according to the identification keys [8].

The repellent efficacy of azadirachtin essential oil was determined according to the methods proposed by Kröber et al. [9] and Adenubi et al. [10]. Azadirachtin essential oil was administered in the form of Azadiroko Neem Cake® (derivative of *Azadirachta indica* seed), manufactured by BioGenesis. For the purposes of the experiment, the preparation was diluted with distilled water in five concentrations: 10, 25, 50, 75 and 100% and applied to ticks in three replicates. The control group was treated with distilled water. The bottom of Petri dishes (90 x 14.5 mm) was covered with filter paper on which three fields were drawn: zone without repellent, neutral - initial zone and the zone with repellent. Each tick was exposed to the repellent for 3 minutes. The repellent efficacy was determined by ticks movement away or in the direction of the administrated preparations, and calculated according to Thorsell et al. [11] and Tunón et al. [12], immediately after placing the ticks in the Petri dishes, and then after 1, 2, 4 and 8 hours. After each test cycle of 3 minutes, ticks were removed, and Petri dishes were stored in the laboratory, at a constant temperature of 20-22°C and a relative humidity of 40-

43%. The obtained results were statistically analyzed by ANOVA and Fisher's LSD test using Statistica 14.0.0 (TIBCO, University license).

Results and discussion

The average repellent efficacy was calculated for five different concentrations, three replicates, and five time intervals and presented in Table 1.

Table 1. The average repellent efficacy

Concentrations	Time intervals				
	3 minutes	1 hour	2 hours	4 hours	8 hours
10%	53.33	50.00	43.33	30.00	13.33
25%	63.33	60.00	50.00	50.00	40.00
50%	70.00	60.00	53.33	53.33	50.00
75%	83.33	73.33	63.33	60.00	50.00
100%	93.33	86.67	80.00	70.00	63.33

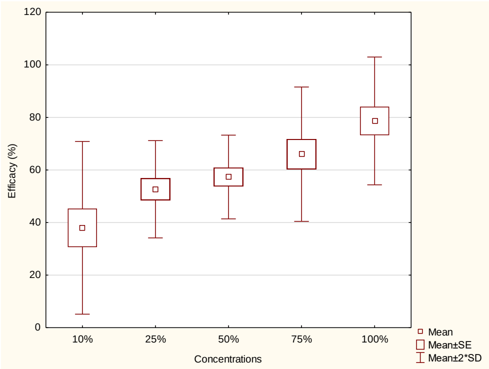
The obtained results are in accordance with the previously published data, where neem seed essential oil in a concentration of 40% was the most effective and with the highest toxicity, while a concentration of 10% showed the lowest toxicity [13]. The same study emphasized that higher concentrations showed a higher mortality rate of *Rhipicephalus pulchellus* ticks compared to the lower concentrations, which is similar to our results, where higher concentrations had higher repellent effects on *I. ricinus* ticks. The same authors recommended the concentration of 30% of neem seed essential oil as the most efficient and economically justified [13], although our results highlighted concentrations over 50%.
Based on the obtained results, it can be concluded that azadirachtin essential oil had more than 50% repellent efficacy on *I. ricinus* after three minutes of application, and it has decreased with time when all tested concentrations were concerned. Eight hours after repellent administration, concentrations of 50% and 75% have had repellent effects of 50%, while pure essential oil (100%) showed a repellency of 63.33% (Table 1). Application of pure azadirachtin essential oil to the rabbits provided them complete protection against *Amblyomma variegatum* bites for 2 to 4 days [14]. Statistical analyses of the obtained results indicated that different azadirachtin essential oil concentrations had high statistical differences in relation to the repellent efficacy as a dependent variable ($p=0.000559$ for $p<0.01$) (Graph 1.). Contrary, the time intervals were not statistically significant (Graph 2). The application of Fisher's LSD test defined the statistical significances among the applied concentrations (Table 2).

Table 2. The results of the Fisher's LSD post-hoc test

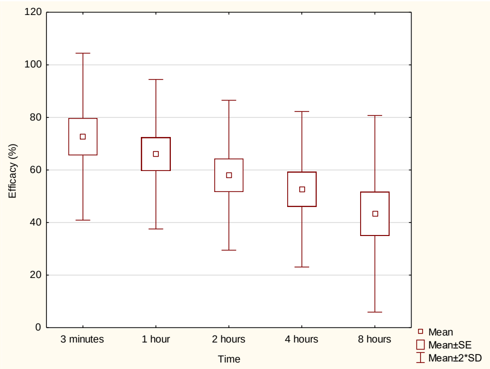
Concentrations	Mean	Statistical significance
10%	37.998	a
25%	52.666	a,b
50%	57.332	b
75%	65.998	b,c
100%	78.666	c

Azadirachtin essential oil had a significant repellent effect on *I. ricinus* if concentrations of 50% and more were used. However, azadirachtin, in addition to its repellent action, has a pronounced acaricidal, ovicidal and larvicidal efficacy on different tick species, as it interferes with the tick metamorphosis [15]. Azadirachtin affects arthropod chemoreceptors, preventing nutrition and oviposition, as well as meiosis, muscle physiology, digestive functions, and biological rhythms

[13]. Its primary mode of action is through prothoracicotropic hormone (PTTH) and allatotropin inhibition. Obstruction of these morphogenetic peptide hormones disrupts the secretion of ecdysone and juvenile hormones in hemolymph, which affects metamorphosis, reproduction, and development.



Graph 1. Repellent efficacy depending on different concentrations



Graph 2. Repellent efficacy depending on time intervals

Higher concentrations of azadirachtin had an ovicidal activity on fertilized eggs of *Hyalomma anatolicum excavatum*, as well as an acaricidal effect on adults who did not adopt a blood meal in the first 24 hours after application [15]. Azadirachtin had a larvicidal effect that depended on the concentration and time of application, with a 100% mortality observed at concentrations of 20, 40, 60, 80 and 100% after 24 hours on *Boophilus decoloratus* larvae [15]. Moreover, azadirachtin had an acaricidal effect on larvae, nymphs, and females of *A. variegatum*, where mortality increased with higher concentrations [14]. In addition, the neem seeds essential oil significantly reduced the number of hatched eggs of *R. appendiculatus* and *B. decoloratus*, where the reduction was higher in eggs treated with undiluted (100%) compared to diluted oil (25%) [14]. Azadirachtin essential oil can be used with certainty to control ticks in animals, as there are no registered side effects [16]. Moreover, neem essential oil has fungicidal and bactericidal properties, which can act on secondary infections caused by tick bites. Neem essential oil with its component azadirachtin can also be used to control ticks in livestock, as it is a cheap and natural acaricide that could also play a significant role in reducing the non-selective use of synthetic chemicals that are potentially dangerous for humans, animals and environmental health [17]. The azadirachtin essential oil can be sprayed on pastures and meadows intended for cattle grazing, which would reduce the ectoparasites infestation of domestic animals and the potential pathogens transmissions that could endanger human and domestic animals health [14].

Conclusion

Azadirachtin essential oil had more than 50% repellent efficacy on *I. ricinus* three minutes after the substance application, but it decreased with time when all tested concentrations were concerned. Eight hours after repellent administration, concentrations of 50% and 75% have had repellent effects of 50%, while pure essential oil (100%) showed a repellency of 63.33%. In order for people to spend more relaxed and free time in nature for rest and various sports and recreational activities, it is necessary to apply preventive protection measures against obligatory haematophagous ectoparasites such as ticks. One of the prevention methods is the use of the substances with confirmed repellent efficacy, which could be applied to the skin or clothes, such as azadirachtin essential oil.

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