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Acaricidal properties of ylang-ylang oil and star anise oil against nymphs of *Ixodes ricinus* (Acari: Ixodidae)

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Abstract
Ylang-ylang oil (YYO) from *Cananga odorata* (Lam.) Hook.f. & Thomson and star anise oil (SAO) from *Illicium verum* Hook.f. were tested at four concentrations 0.05, 0.1, 0.2, 0.4 µl/cm^2. Mortality rates were obtained by counting dead nymphs at 30-min intervals during the first 5 h after the start of exposure and then at 24, 48 and 72 h. Mortality increased with increasing oil concentration and time of exposure. The two highest concentrations of YYO (0.2, 0.4 µl/cm^2) gave maximum lethal concentrations (LC) of 50 and 95% mortality after 4.5 h exposure. Mortality of 95% was obtained after 24 h with the next highest dose (0.1 µl/cm^2), whereas LC_{95} required 3 days with the lowest YYO (0.05 µl/cm^2). The lethal effect time (LT) was correlated with the duration of exposure, with a significant effect at 0.4 µl YYO/cm^2 after 3 h' (LT_{50} = 3.2 h, LT_{95} = 4.3 h). In contrast, only the highest concentration of SAO, 0.4 µl SAO/cm^2, showed increasing mortality with time of exposure. This reached LT_{50} after 10 h and LT_{95} after 24 h. However, with the lower concentration (0.2 µl/cm^2) 50% mortality was reached after 24 h and 100% at 72 h. At to the lowest concentration of SAO (0.1 µl/cm^2), 67% mortality after 48 h. The study indicates that YYO and SAO exhibit strong acaricidal properties against nymphs of *I. ricinus* and suggest that both YYO and SAO should be evaluated as potentially useful in the control of ticks.

Keywords Acaricide · *Cananga odorata* · *Illicium verum* · *Ixodes ricinus*

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Introduction

Ticks (Acari: Ixodoidea) are obligatory blood-sucking arthropods and are among the most harmful ectoparasites and most important disease vectors of domestic and wild animals (Randolph 1998). These arachnids can directly cause tick paralysis, physical irritation and allergic reactions. Most importantly, ticks are major vectors of human pathogens (Estrada-Peña and Jongejan 1999; Sonenshine 2003).

One of the medically most important tick species is *Ixodes ricinus* L., which is common throughout most of Europe and maybe established locally in parts of North Africa (Zhioua et al. 1999; Sarih et al. 2003). Due to its widespread distribution, catholic feeding habits and because of its willingness to bite humans, *I. ricinus* is the most important European vector of many pathogens, such as tick-borne encephalitis virus (TBEV), the protozoan parasite *Babesia divergens* (M'Fadyean and Stockman 1911) and several bacteria, e.g. *Anaplasma phagocytophilum*, several rickettsiae and the Lyme borreliosis spirochetes.

These and other tick-transmitted infections might be controlled and/or reduced by minimizing human contact with high-risk tick-infested habitats. However, this is often not practical. One widely used method for controlling tick populations affecting domesticated animals is based on the limited application of synthetic acaricides, such as pyrethroids, onto the animals or in their habitat. However, these substances often cause toxic reactions when applied on domestic animals and can potentially harm the environment. Consequently, a simple and cheap method of protecting humans and domestic animals against ticks is needed.

Plant extracts have been used for millennia to attempt to control arthropod pests, so there is a potential for discovery of effective plant products for tick control (Silva-Aguayo 2004; Katinka et al. 2008). Many natural substances have a relatively low mammalian toxicity and degrade rapidly in the environment, properties that make them attractive alternatives to many synthetic acaricides currently in use (Papachristos et al. 2004; Kim et al. 2007; De Souza Chagas et al 2012). This study investigates the potential utility for control of ticks of two naturally occurring essential oils, ylang-ylang oil (YYO) from *Cananga odorata* (Lam.) Hook.f. & Thomson (Annonaceae) and of star anise oil (SAO) obtained from *Illicium verum* Hook.f. (Illiciaceae). The toxic properties of these essential oils have, to our knowledge, not previously been investigated as acaricides against *I. ricinus.* We evaluate the toxicity of these oils against nymphs of *I. ricinus*, including their maximal lethal concentrations.

Materials and methods

Unfed nymphs of *I. ricinus* were collected in a woodland area 6–8 km south of Uppsala city, east-central Sweden, during April-September 2006. Ticks were collected by dragging a 1-m^2 light-coloured flannel cloth over the ground vegetation (Mejlon and Jaenson 1993). The cloth was inspected at every 10 m step when all nymphs adhering to the cloth were collected. Nymphs were maintained at 85–95% relative humidity (RH) and ≈4 °C in complete darkness for 2 months. Before testing, the nymphs were allowed to adapt to the test environment (21–23°, 85–95% RH) for 4 h.

Substances tested

Star anise oil and ylang-ylang oil were bought from www.crearome.se. Star anise oil was obtained by stem distillation from the fruits and seeds of the Chinese star anise *I. verum*, which is grown almost exclusively in southern China and Japan. The dried fruits may contain 5–8% of essential oil, consisting predominately (85–90%) of anethole, i.e., trans-1-methoxy-4-(prop-1-enyl) benzene. Phellandrene, safrole, 1,8-cineole, α-terpineol, estragole, limonene, linalool, methyl-chavicol, para-anisaldehyde and trepinen-4-ol are known to be toxic to certain arthropods (Duke 2008).

Ylang-ylang oil is 100% oil from *C. odorata* cultivated on Madagascar, obtained by steam distillation of the flowers of *C. odorata*. The main constituents of the oil are linalool, β-caryophyllene, γ-muurolene and α-trans, trans-farnesene (Baratta et al. 1998). Benzyl acetate, linalool, p-cresyl methyl ether and methyl benzoate give the oil its characteristic odor (Manner and Craig 2006).

Assay 1: Open filter paper method (A)

As shown in assay 1 the acaricidal effects of YYO and SAO against nymphs of *I. ricinus* were tested in a bioassay based on the "open filter paper method" as described in WHO (1996). Oils were tested in the laboratory at 21–23 °C and 85–95% RH. The number of dead ticks was counted using a dissection microscope at 25–50X magnification. Four concentrations of the two oils, YYO and SAO, were prepared by dissolving in 1 ml of acetone. The resulting solutions were applied separately onto round Whatman filter paper no.1 to final concentrations of 0.05, 0.1, 0.2 and 0.4 µl oil/cm^2. Filter papers impregnated with acetone were used as controls. The filter papers were placed at the bottom of plastic cups (122 cm^3 volume), where the solvent evaporated completely within 20 min. Ten randomly-selected tick nymphs were introduced into each plastic cup, which were then covered by fine-meshed cloth secured with rubber bands around the top to prevent the ticks' escape. Each cup was put separately into a closed plastic container with wet tissue paper at the bottom to maintain high humidity. Dead ticks were recorded every 30 min for the first 5 h, and then at 24, 48 and 72 h. The tests of each concentration were repeated five times.

Concentration-response curves were obtained by plotting the number of dead ticks, expressed as a percentage of the total number of individuals for each time of exposure versus the two oils' concentrations. LC_{50} and LC_{95} for the different exposure times were then determined from these concentration-response curves. Time-response curves were obtained by plotting the number of dead ticks, expressed as a percentage of the total number of individuals for each concentration, versus time of exposure. LT_{50} and LT_{95} were then determined from these time-response curves (Johnson et al. 1984).

Assay 2: Limited exposure time method (B)

Four concentrations of YYO and SAO corresponding to 0.05, 0.1, 0.2 and 0.4 µl/cm^2 in four replicates of each oil were prepared using the procedure described in method A. The effect on nymphal mortality of different exposure times was investigated as follows: six different batches of 10 nymphs each were placed in plastic cups with oil-impregnated filter paper and covered with fine-meshed cloth secured with rubber bands as in procedure A (above). Nymphs were kept on the oil-impregnated filter papers for 1.5, 2.0, 2.5, 3.0, 3.5 or 4.0 h. After exposure, the nymphs were moved to un-impregnated filter paper, and

mortality was checked by observing the ticks under a dissection microscope at 25–50X magnification every 30 min for 4.5 h and then at 24, 48 and 72 h. These tests were repeated three times. Time-response curves were obtained for each oil concentration by plotting the number of dead ticks, expressed as a percentage of the total number of individuals, versus time after the end of exposure. LT50 and LT95 were then determined from these time-response curves.

Statistical analysis

The data are expressed as the percentage mortality. Groups were compared using a two-way ANOVA for repeated measurements, and multiple regression test. Levene's test was used for equality of variances, and *t*-test for equality of means. The Multiple Range test (LDS) was used for post hoc analysis. A value of $p < 0.05$ was considered significant.

Results

Open filter paper method

Ylang-ylang oil

For YYO, nymph mortality of nymphs increased with increasing oil concentration and time, reaching 100% mortality after 4.5 h for the group exposed to the highest YYO concentration (0.4 µl/cm^2). With the lowest concentration (0.05 µl YYO/cm^2), 100% mortality was reached after 72 h. The analysis of variance shows a highly significant difference in mortality rate among the controls and exposed nymphs ($F = 109.2$, $p < 0.0001$), and also among different exposure times (ET) for nymphs exposed to the same YYO concentration. After the nymphs were exposed to YYO for 2 h the p value was 0.005 which is considered significant, moreover with the longer ETs (2.5–5 h) it gave more significant toxicity results ($p < 0.0001$).

The lethal concentrations at different exposure times to YYO were calculated at 4.5 h $LC_{95} = 0.385$–0.4 µl/cm^2 of YYO (Table 1). Lethal times at different concentrations of YYO were calculated. The result was at 0.4 µl/cm^2 of YYO $LT_{95} = 4.4$–4.5 h (Table 1). Moreover, it was observed that nymphs exposed to 0.2–0.4 µl YYO/cm^2 were not able to climb the wall of the cup, whereas nymphs exposed to 0.1 µl YYO/cm^2 started to climb as soon as they were placed in the cup. Then they stayed on the upper edge of the cup, as far away as possible from the impregnated filter papers, until they dropped down and became motionless.

Star anise oil

For SAO, only the ticks subjected to the highest concentration, 0.4 µl SAO/cm^2, showed increased mortality with increased time (Fig. 1c). This reached 50% mortality after 10 h and a maximum of 99% mortality after 24 h. However, at the lower concentration (0.2 µl SAO/cm^2), 50% mortality was only reached after 24 h, 90% after 48 h, and 100% at 72 h. With the group exposed to the lowest concentration of SAO (0.1 µl/cm^2), 67% mortality occurred after 48 h, reaching 100% mortality by the third day (Table 2). The analysis of

Table 1 The lethal concentrations at different exposure times to ylang-ylang oil (YYO), and lethal times at different concentrations of YYO, in method A

Lethal concentrations (LC)			Lethal time (LT)		
ET to YYO (h)	LC_{50} (µl/cm^2)	LC_{95} (µl/cm^2)	Conc. of YYO (µl/cm^2)	LT_{50} (h)	LT_{95} (h)
3	0.4	–	0.05	24	66
3.5	0.31	–	0.1	22	47
4	0.19	–	0.2	3.9	21
4.5*	0.185	0.385	0.4*	2.8	4.4
5	0.16	0.36			
24	0.049	0.29			
48	0.03	0.09			
72	0.026	0.048			

*At 4.5 h LC_{95} = 0.385 µl/cm^2 of YYO ≈ 0.4 µl/cm^2 of YYO, and at 0.4 µl/cm^2 of YYO LT_{95} = 4.4–4.5 h. The data were calculated from the curves for different exposure times shown in Fig. 1b. and the curves of different concentrations shown in Fig. 1a

variance shows a significant difference between control and all substance concentrations ($F = 2.40$, $p < 0.0001$).

Limited exposure time method

Ylang-ylang oil

Nymph mortality was also measured by exposing them to (0.1, 0.2 and 0.4) µl/cm^2 YYO for varying lengths of time (1.5, 2.0, 2.5, 3.0, 3.5 or 4.0 h). An analysis of variance of nymph death rate (%) versus exposure time (ET) showed a highly significant difference in mortality rate between control versus exposed nymphs for different concentrations of YYO ($F = 24.9$, $p < 0.0001$). Figure 2a shows that at a concentration of 0.4 µl/cm^2 there was a significant variation in mortality rate between nymphs for different ETs ($F = 30.48$, $p < 0.0001$). After 1.5 h of exposure to 0.4 µl/cm^2 YYO the nymphs that had been moved away from the impregnated filter paper appeared weak and inactive. However, these nymphs recovered in fresh air and more than 75% were active and still alive even after 48 h, and their mortality after 72 h in fresh air was less than 40%. Even after 4 days, mortality among the exposed nymphs did not reach 50%. On the other hand, 50% of nymphs which were exposed to 0.4 µl/cm^2 YYO for 2 h were dead after 4 h in fresh air, and all were dead after 24 h. 50% of the nymphs exposed to 0.4 µl/cm^2 YYO for 2.5 h were dead after 2.5 h in fresh air, and all were dead after 3.9 h. After 3.0 h' exposure, 100% mortality occurred after 1.5 h in fresh air, which means that the total time after the nymphs were exposed and moved out was 4.5 h, whether they had been moved to fresh air or not. In contrast, Fig. 2b. shows that at 0.2 µl/cm^2 of YYO there was no significant difference in mortality rate between different ETs. Only nymphs exposed to YYO for 3.0 h or longer reached 50% mortality after 2.5 and 2 h in fresh air, and 100% mortality between 4.5 and 24 h. With 0.1 µl YYO/cm^2 mortality of the nymphs with exposure time of 2.5, 3 and 3.5 h, show LT_{50} of 4.8, 24 and 26.4 h, respectively (Fig. 2c). an LT_{95} was only reached with the

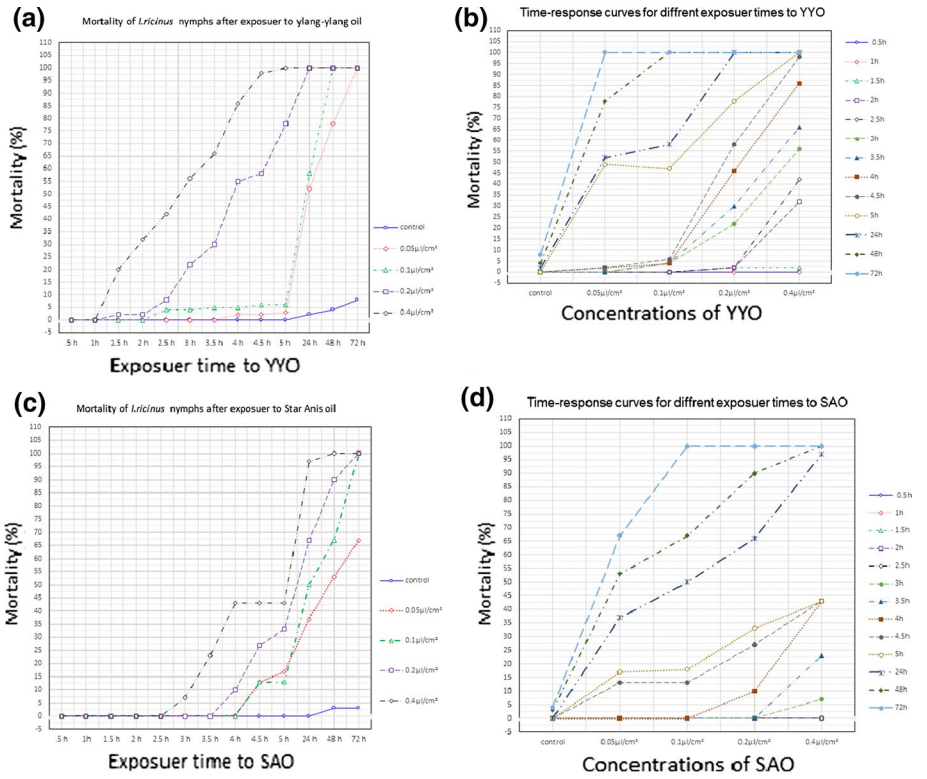

Fig. 1 **a** Mortality (%) of *Ixodes ricinus* nymphs exposed to different concentrations of ylang-ylang oil (YYO) (µl/cm^2) and different exposure times (hours) based on method A. From this graph the LT_{50} and LT_{95} were calculated (Table 1). **b** The LC_{50} and LC_{95} of YYO, based on method A, were calculated from the curves for different exposure times shown in this graph. (Table 1). **c** Mortality (%) of *I. ricinus* nymphs exposed to different concentrations of star anise oil (SAO) (µl/cm^2) and different exposure times (hours) based on method A. From this graph the LT_{50} and LT_{95} were calculated (Table 2). **d** The LC_{50} and LC_{95} of SAO, based on method A, were calculated from the curves for different exposure times shown in this graph. (Table 2)

Table 2 The lethal concentrations at different exposure times to star anise oil (SAO), and lethal times at different concentrations of SAO, in method A

Lethal concentrations (LC)			Lethal time (LT)		
ET to SAO (h)	LC_{50} (µl/cm^2)	LC_{95} (µl/cm^2)	Conc. of SAO (µl/cm^2)	LT_{50} (h)	LT_{95} (h)
24*	0.1	0.39	0.05	42	–
48	0.048	0.09	0.1	24	68
72	0.034	0.048	0.2	18	60
			0.4*	14	24

*At 24 h LC_{95} = 0.39 µl/cm^2 of SAO ≈ 0.4 µl/cm^2 of SAO, these results were calculated from the curves for different exposure times shown in Fig. 1c. * At 0.4 µl/cm^2 of SAO LT_{95} = 24 h, these results were calculated from the curves for different concentrations shown in Fig. 1d

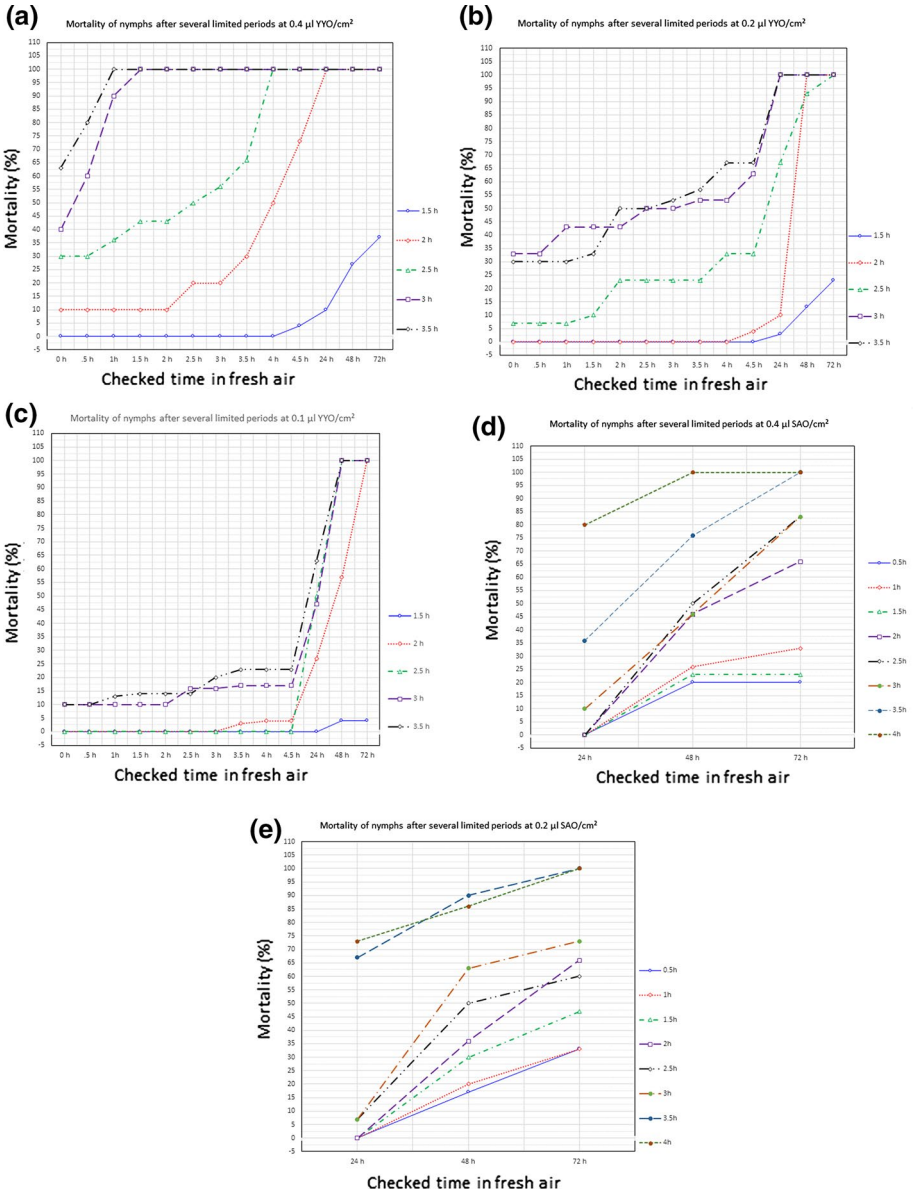

Fig. 2 **a** Mortality of Nymphs of *Ixodes ricinus* after several limited exposure times to 0.4 µl ylang-ylang oil (YYO)/cm^2 shown after 3 h exposure to YYO $LT_{50}=3.2$ h and $LT_{95}=4.3$ h. And after 3.5 h exposure to YYO $LT_{95}=4.4$ h. **b** Mortality of nymphs of *I. ricinus* exposed to several limited periods of one fixed concentration (0.2 µl YYO/cm^2). After exposure to YYO for 3 and 3.5 h the $LT_{50}=2.5$, 2 h and $LT_{95}=4.9$ for both (based on method B). **c** Mortality of nymphs of *I. ricinus* exposed to several limited periods of one fixed concentration (0.1 µl YYO/cm^2). After exposure to YYO for 2.5, 3 and 3.5 h the $LT_{50}=4.8$, 24 and 26.4 h, and the $LT_{95}=43.2$ h (based on method B). **d** Mortality of nymphs of *I. ricinus* exposed to several limited periods of one fixed concentration (0.4 µl star anise oil (SAO)/cm^2). After exposure to SAO for 2, 2.5, 3 and 3.5 h the $LT_{50}=54$, 48, 52 and 32 h, respectively. And after exposure to SAO for 3.5 and 4 h the $LT_{95}=67$ and 42 h (based on method B). **e** Mortality of nymphs of *I. ricinus* exposed to several limited periods of one fixed concentration (0.2 µl SAO/cm^2). After exposure to SAO for 2, 2.5 and 3 h the $LT_{50}=58$, 48 and 42 h, and after exposure to SAO for 3.5 and 4 h the $LT_{95}=60$ and 62 h (based on method B)

nymphs exposed to 2.5 h, while the nymphs that exposed to 3 and 3.5 h never reached LT_{95} even after 72 h (Fig. 2c).

Star anise oil

The analysis of variance showed that there was no significant difference in tick mortality between the SAO concentrations of 0.4 and 0.2 µl/cm^2 that were tested ($F=0.129$, $p=0.72$). However, at a concentration of 0.4 µl SAO/cm^2 (Fig. 2d) significant differences in mortality was found for different times of exposure ($F=8.26$, $p<0.0001$). After an ET of 0.5–1.5 h, ticks that moved out from the impregnated area appeared weak and inactive but regained activity when exposed to fresh air, and after 1 week, and even after 1 week more than 50% were still alive. Conversely, the ticks that were exposed to SAO for 2.0, 2.5, 3.0 and 3.5 h reached 50% mortality (LT_{50}) with post-exposure times of 52, 50, 48, and 32 h, respectively. In addition, the ticks that were exposed to SAO for 3.5 or 4.0 h reached 95% mortality (LT_{95}) after, respectively, 42 and 68 h in fresh air. Thus, for SAO at the concentrations tested, significant mortality ($p<0.001$) was found only at 72 h post-exposure. On the other hand, results from the groups of ticks that were exposed to 0.2 µl SAO/cm^2 were not significant ($p>0.05$). The trends in mortality for these nymphs that were exposed to 0.2 µl SAO/cm^2 for more limited periods were ambiguous (Fig. 2e). After exposure for 2.0, 2.5 and 3.0 h the LT_{50}'s were 58, 48 and 42 h, respectively, while exposures of 3.5 and 4.0 h gave an LT_{95} of 60 and 62 h, respectively.

Discussion

Nymphs of *I. ricinus* are more abundant but less noticeable than adults and will therefore, pose more of a risk in disease transmission. In addition, nymphal ticks' non-specific host preference gives them considerable opportunities to acquire and transmit pathogens among numerous host species, including humans (Jensen and Jespersen 2005). This is why we focused on the nymphal stage in this study. Furthermore, *I. ricinus* seems to have increased in abundance in many locations in Northern Europe during the last decades (Gray 1984, 1991; Mejlon and Jaenson 1993; Jaenson et al. 2012a, b), which has also led to an increase in the number of human cases of tick-borne diseases (Lindgren and Jaenson 2006; Jaenson et al 2012a, b). Examples from Northern Europe include changes in tick distribution or abundance (Tälleklint and Jaenson 1998; Lindgren et al. 2000; Lindgren and Jaenson 2006) and incidences of Lyme borreliosis and TBE (Lindgren and Gustafson 2001; Bormane et al. 2004; Kampen et al. 2004; Jensen and Jespersen 2005; Bennett et al. 2006).

YYO and SAO are known to possess bacteriostatic and germicidal properties and are used to cure infections of the skin (Duke 2008; Kim et al 2007). The insecticidal property of YYO has also been tested on some human ectoparasites such as the human head louse *Pediculus humanus capitis* (Yang et al. 2004) on which YYO induced a high level of mortality (Heukelbach et al. 2006). In addition, YYO has a lethal effect on the mites *Dermatophagoides farinae* and *D. pteronyssinus* (Rim and Jee 2006), and linalool, which is a major constituent of YYO, has a significant and wide spectrum of acaricidal activity (Hink and Duffey 1990). SAO is toxic to adults of *Blattella germanica*, and it seems that one of SAO's components, (*E*)-anethole, is responsible for this effect (Chang and Ahn 2002). In addition, SAO has an ovicidal action against *Tribolium castaneum* and *Sitophilus zeamais* (Ho et al. 1995).

The literature contains many studies that document the toxicity and acaricidal properties of the chemical components of YYO and SAO against many species of arthropods. Macchioni (2006) showed the acaricidal activity of *Laurus nobilis* oil, whose main components are 1,8-cineole (39.2%) and linalool (7.4%). Additionally, 1,8-cineole in molasses grass oil (*Melinis minutiflora*) produced 100% mortality in the cattle tick *Boophilus microplus* after 5 min' exposure (Prates et al. 1998). An α-terpineol in tea tree oil (*Melaleuca alternifolia*) has acaricidal activity on *Sarcoptes scabiei var. hominis* (Walton et al. 2004). Iori et al. (2005) also showed that tea tree oil has acaricidal effect on *I. ricinus*, and Duke (1992) showed that SAO contains 85–200 ppm of α-terpineol. These studies suggest that chemicals present in YYO and SAO may have a toxic effect on some tick species, which is supported by the results of this study.

Using a filter-paper method (method A), we found that YYO has strong significant acaricidal activity. This is especially obvious for 0.4 µl YYO/cm^2 for which mortality reaches 95% in a short time (4.4 h, Table 1). Even with half this dose (0.2 µl YYO/cm^2), 95% mortality was reached within 24 h, which is considered an acceptable outcome (Piesman 1993). At lower concentrations of YYO such as 0.05 and 0.1 µl/cm^2 the acaricidal effect was present but weak. In fact, a significant repellency effect was observed only at a concentration of 0.2 µl YYO/cm^2 (Fawzeia Elmhalli personal observation).

With SAO the acaricidal activity was not as strong, but remained within the framework of what can be considered acceptable (Piesman 1993) in terms of lethal time (LT_{50} = 14 h, LT_{95} = 24 h at 0.4 µl SAO/cm^2). Piesman (1993) showed that the risk of *B. burgdorferi* transmission is low within the first 24 h of tick attachment but increases thereafter. On the other hand, with the TBE virus, transmission can occur almost immediately when the tick starts feeding (Bröker and Kollaritsch 2007). In general, ixodids take some time to locate an appropriate site to bite, so the first 24 h could be a critical time for acceptable protection against pathogen-infected ixodids. Therefore, concentrations as low as 0.4 µl SAO/cm^2 are acceptable for an acaricide against *I. ricinus* nymphs according to these results (Table 2). However, we also found that lower concentrations have little or no prophylactic effect.

In terms of exposure times, the results of method B show that nymphal mortality is correlated with the duration of exposure to YYO. This shows a distinct effect after 2 h with 0.4 µl YYO/cm^2, which results in 95% mortality within 24 h of exposure (Fig. 2a). This time to mortality is reduced to 4.4 h until LC_{95}, with 3.5 h' exposure. This is irrespective of whether the nymphs are moved to fresh air after their exposure or not. At 0.2 µl YYO/cm^2, 95% mortality was reached within 24 h in fresh air (Fig. 2b), while with the lower concentration of 0.1 µl YYO/cm^2, only 50% mortality was reached after 24 h' post-exposure times (Fig. 2c), which is considered non-significant (Piesman 1993).

With SAO significant lethality (LC_{50}) was only detected after exposure to 0.4 µl SAO/cm^2 for a minimum of 3.5 h (Fig. 2d). This resulted in 50% mortality after 24 h in fresh air and 95% mortality after 24 and 48 h (Fig. 2d). With 0.2 µl SAO/cm^2, high mortality was reached only after 3.5 or more hours' exposure and at 24–48 h' post-exposure (Fig. 2e). This is not an acceptable time frame (Piesman 1993), and suggested that SAO may not possess the same acaricidal potential as YYO.

The results shown here indicate that YYO is an effective acaricide against *I. ricinus*, particularly at a concentration of 0.4 µl YYO/cm^2. Although SAO also affects *I. ricinus* 'negatively', its impact is weaker than that of YYO, since SAO requires higher concentrations of oil and/or longer periods of exposure. Thus, both oils show potential utility as natural acaricides against *I. ricinus*.

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