Acaricidal effects of *Corymbia citriodora* oil containing *para*-menthane-3,8-diol against nymphs of *Ixodes ricinus* (Acari: Ixodidae)

Fawzeia H. Elmhalli · Katinka Pålsson · Jan Örberg ·
Thomas G. T. Jaenson

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Abstract The toxicity of *para*-menthane-3,8-diol (PMD), the main arthropod-repellent compound in the oil of the lemon eucalyptus, *Corymbia citriodora*, was evaluated against nymphs of *Ixodes ricinus* using five methods (A–E) of a contact toxicity bioassay. Mortality rates were estimated by recording numbers of dead nymphs at 30 min intervals during the first 5 h after the start of exposure and at longer intervals thereafter. The mortality rate increased with increasing concentration of PMD and duration of exposure with a distinct effect after 3.5 h. From the results obtained by methods A, C and E, the LC₅₀ range was 0.035–0.037 mg PMD/cm² and the LC₉₅ range was 0.095–0.097 mg PMD/cm² at 4 h of exposure; the LT₅₀ range was 2.1–2.8 h and the LT₉₅ range was 3.9–4.2 h at 0.1 mg PMD/cm². To determine the duration of toxic activity of PMD, different concentrations (0.002, 0.01, 0.1 mg PMD/cm²) were tested and mortality was recorded at each concentration after 1 h; thereafter new ticks were tested. This test revealed that the lethal activity of PMD remained for 24 h but appeared absent after 48 h. The overall results show that PMD is toxic to nymphs of *I. ricinus* and may be useful for tick control.

Keywords *Corymbia citriodora* · *Ixodes ricinus* · Acaricides · *Para*-menthane-3,8-diol · Ticks · Toxicity

Introduction

Ticks comprise a group of exclusively blood-feeding ectoparasites with a worldwide distribution. They are the most important arthropod vectors of disease agents to humans and animals in the northern hemisphere (Sonenshine 2003). Ticks may cause skin

F. H. Elmhalli · K. Pålsson · T. G. T. Jaenson (✉)
Medical Entomology Unit, Department of Systematic Biology, Evolutionary Biology Centre,
Uppsala University, Norbyvägen 18d, 752 36 Uppsala, Sweden
e-mail: Thomas.Jaenson@ebc.uu.se

J. Örberg
Department of Environmental Toxicology, Evolutionary Biology Centre,
Uppsala University, Uppsala, Sweden

irritation, allergic reactions, severe—sometimes fatal—anaphylactic reactions, and, indirectly, severe infections due to transmission of viruses, bacteria, protozoa or nematodes (Estrada-Peña and Jongejan 1999; Sonenshine 2003). Lyme borreliosis (Lyme disease) and tick-borne encephalitis (TBE) are two tick-borne infections of major importance. Both occur in Europe and Asia, with Lyme borreliosis also extending into North America (Sonenshine 2003). The main vector of these infections in Europe is the common tick *Ixodes ricinus* (Acari: Ixodidae), which also occurs in parts of North Africa (Sarih et al. 2003; Zhioua et al. 1999). *I. ricinus* also transmits other infections, including anaplasmoses (ehrlichioses), tularaemia and babesiosis, to humans and domestic animals (Sonenshine 2003). *I. ricinus* seems to have increased its geographical distribution and density-activity in many areas of Europe during the last decades, possibly partly due to the changing climate. This has led to an increase in the number of reported cases of tick-borne diseases (Lindgren and Jaenson 2006). Examples from Scandinavia include changes in tick distribution or abundance (Tälleklint and Jaenson 1998; Lindgren et al. 2000; Lindgren and Jaenson 2006) and incidence of LB or TBE (Lindgren and Gustafson 2001; Bormane et al. 2004; Jensen and Jespersen 2005; Bennet et al. 2006; Kampen et al. 2004). Furthermore, studies from the Czech Republic indicate that changed occurrence of *I. ricinus* and TBE cases toward higher altitudes in recent decades are due to a prolonged vegetation period, particularly milder autumns (Daniel et al. 2003; 2004; Materna et al. 2005).

To date, the use of synthetic acaricides have been the most common method for tick control. This has led to problems with environmental pollution, development of resistant tick populations and increasing costs. Therefore, one or more environmentally friendly and cost-effective methods for protecting humans and domestic animals against ticks would be welcome. Plant-derived products are usually biodegradable, environmentally less contaminating than synthetic alternatives and often—but not always—exhibit a lower risk to non-target organisms. Many plants have evolved protection mechanisms such as repellents and insecticides/acaricides against arthropods (Silva-Aguayo 2004). The principal active component of the essential oil of the lemon eucalyptus (*Corymbia citriodora*. Hook) is *cis*- and *trans*-*p*-menthane-3,8-diol (PMD) (Curtis et al. 1991). The aim of this study is to evaluate the toxic activity, including the optimal lethal concentrations, of PMD against *I. ricinus* nymphs.

Materials and methods

Tick collection

Non-blood-fed nymphs of *I. ricinus* were collected near Uppsala, east-central Sweden during April–September 2006, by dragging a cloth over the ground vegetation (Mejlon and Jaenson 1993). The 1 m² cotton flannel cloths were inspected every 10 m to collect all nymphs found. Collections were performed during daytime between 10 a.m. and 3 p.m. Nymphs were maintained at 80–95% RH and $\approx 4^{\circ}\text{C}$ in complete darkness. Before the test, they had been adapted to the test environment (21–23°C and 85–95% RH) for 4 h.

Substance tested

PMD is the main active component of the essential oil of *C. citriodora* (family Myrtaceae). The oil had been obtained by steam distillation of the leaves of *C. citriodora*. The weight of mass of the oil is 0.92 kg/l and PMD constitutes approximately 50% w/w of the oil.

Open filter paper method

In all tests the toxic effect of PMD-containing *C. citriodora* oil was evaluated in the laboratory at 21–23°C and 85–95% RH and any dead ticks were recorded by observation under a dissection microscope at 25–50× magnification. After preliminary tests, the acaricidal effect of four PMD concentrations was tested in a bioassay design based on “The open filter paper method” as described in WHO (1996). Four doses of *C. citriodora* oil 3.5, 7, 14, 28 µl oil corresponding to 0.025, 0.05, 0.1, 0.2 mg PMD/cm² in four replicates were used, prepared by diluting the oil in 1 ml of acetone. Each concentration was uniformly spread with the aid of a micropipette on a round Whatman filter paper no.1. After 20 min the solvent had evaporated completely. Filter papers impregnated with acetone were used as controls. Each of the five batches consisted of ten nymphs of ticks selected randomly and introduced into a plastic cup (122 cm³) with filter paper at the base. The plastic cups were covered by fine meshed cloth with rubber bands around the top of the cup to prevent ticks to 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 after 24 h. The tests 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 PMD concentration. LC₅₀ and LC₉₅ for the different exposure times were then determined from these concentration–response curves. Time–response curves were obtained by plotting number of dead ticks expressed as a percentage of the total number of individuals for each concentration versus time of exposure. LT₅₀ and LT₉₅ were then determined from these time–response curves.

Limited exposure time method

This test was carried out in the laboratory with a concentration of 0.1 mg PMD/cm² prepared using 14 µl of *C. citriodora* oil and followed the same procedure as used in method A. The effect on nymphal mortality of different exposure times was investigated as follows: seven different batches of nymphs (ten nymphs per batch) were kept on the PMD impregnated filter papers for 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 h, respectively. After the exposure period the nymphs were kept in fresh air on unimpregnated filter papers and mortality was checked every 30 min for 4.5 h and at 24, 48 and 72 h. Each test period was repeated three times to find out when the effect of the substance declined. Time–response curves were obtained for 0.1 mg PMD/cm² by plotting number of dead ticks expressed as a percentage of the total number of individuals versus time spent in fresh air after the end of exposure. LT₅₀ and LT₉₅ were then determined from these time–response curves.

Folded filter paper method

This test was based on the “Folded filter paper method” as described by Al-Rajhy et al. (2003). Whatman filter papers no.1 were impregnated with three doses of *C. citriodora* oil (1.4, 14, 140 µl) corresponding to the concentrations 0.01, 0.1 and 1.0 mg PMD/cm². The filter papers were folded into “envelopes” using metal clips. For each concentration, ten nymphs were put into each envelope and the four envelopes were put, separately, into Petri dishes (17 cm³), which were kept in one larger Petri dish (308 cm³). Thus, 40 nymphs were subjected simultaneously to each concentration, and the humidity level was maintained by using a wet tissue paper at the bottom of the biggest Petri dish, with a glass cover

on the top. Any dead nymphs were recorded at 4, 6, 12 and 24 h. For each PMD concentration the test was repeated three times. Time–response curves were obtained for each concentration by plotting number of dead ticks expressed as a percentage of the total number of individuals versus time of exposure. LT_{50} and LT_{95} for the different concentrations were then determined from these time–response curves.

Direct contact with non-absorbing surface

To test the toxicity of the oil on a non-absorbing surface, three doses of *C. citriodora* oil (0.14, 1.4, 14 μ l oil) were evenly distributed to the walls and bottom of 50 ml cylindrical glass jars containing treated filter paper; these doses, corresponding to three concentrations of PMD (0.002, 0.01 and 0.1 mg PMD/cm²), were prepared by diluting the oil in 1 ml acetone, then covering the whole area of the cylindrical glass. Complete evaporation of the acetone took place in a fume hood for 1 h. A fourth jar, treated with only acetone, was used as a control. Batches of ten nymphs were then placed in each cylinder, the top of which was covered by fine meshed cloth. Each jar was put separately in a closed container with wet tissue paper on the bottom to maintain high humidity. The number of dead nymphs, expressed as a percentage of the total number of individuals, was recorded after 60 min. To find the duration of toxic activity of the PMD-containing oil, new nymphs were introduced to the same oil-treated cylinders after 24 and 48 h and the procedure repeated as described above. This test was repeated four times.

Topical application method

The acaricidal effect of the PMD-containing oil was tested by pipetting 0.1 μ l of diluted oil on the nymphs of *I. ricinus*. Four doses of *C. citriodora* oil (5.4, 10, 21.5, 43 μ l) diluted in 100 μ l of 98% iso-propanol (1,2-propanediol), corresponding to 0.025, 0.05, 0.1 and 0.2 mg PMD/cm² nymphal surface area were tested. The mean surface area of an *I. ricinus* nymph, 0.0815 cm², was estimated from ten nymphs which were selected randomly and whose surface area was calculated using ImageJ program measurement (<http://rsb.info.nih.gov/ij>). For each oil concentration each nymph was covered completely with 0.1 μ l solution for 1 min. The nymphs were then dried with filter paper, and kept at 21°C and 85% RH in 50 ml Falcon vials (116 × 29 mm, made of transparent plastic), and with moistened tissue paper in a plastic bag. Ten nymphs were used in each treatment. Dead nymphs were recorded every 30 min. The test was repeated three times. Concentration–response curves were obtained by plotting number of dead ticks expressed as a percentage of the total number of individuals for each time of exposure versus the PMD concentration. LC_{50} and LC_{95} for the different times after topical application of PMD were then determined from these concentration–response curves. Time–response curves were obtained by plotting number of dead ticks expressed as a percentage of the total number of individuals for each concentration versus time of application. LT_{50} and LT_{95} for the different doses were then determined from these time–response curves.

Statistical analysis

The data are expressed as percentage mortality. Groups were compared using a two-way ANOVA for repeated measurements and a multiple regression test. Levene's test was used for equality of variances and the *t*-test for equality of means. Multiple range test (LDS) was

used for post hoc analysis in method A and B. Friedman's ANOVA and Kendall's concordance test, and ANOVA Chi Square test were used in method C. *t*-Test for dependent samples was used in method D. Multiple regression test for analysis of variances was used in method E.

Results

Open filter paper method

In method A the effect on the ticks was clearly visible: 1 min after the nymphs were introduced into the treated cups, the nymphs did not climb the walls of cups covered with the two highest PMD concentrations. In contrast, nymphs climbed the wall directly with lower concentrations (0.05, 0.025, 0.00 mg/cm² PMD). From observation during the first hour with the two highest PMD concentrations, the ticks seem to be “confused” and displayed “unstable” movements. After 1.5 h the ticks tended to be slower in movement, and turned upside down. After 2 h the ticks stopped all movement unless they had been disturbed, in which case they started to move their legs or to tremble or shake. This observation has led us to think that PMD has a neurotoxic effect on the nymphs. Mortality increased with increasing concentration and time, reaching a maximum mortality at 4.5 h with the group on the highest and the next highest concentrations (0.2 and 0.1 mg PMD/cm²). With the two lowest concentrations (0.05, 0.025 mg PMD/cm²) maximum mortality was reached between 5 and 24 h (Fig. 1). The analysis of variance showed a significantly increased mortality with increasing concentration of PMD ($F = 24.498$, $P = 0.0006$). The LT₅₀, LT₉₅, LC₅₀ and LC₉₅ are shown in Table 1. At 0.1 mg PMD/cm² LT₉₅ = 4 h, and at 4 h LC₉₅ = 0.0975 mg PMD/cm² $\approx$ 0.1 mg PMD/cm².

Fig. 1 Mortality (%) of *Ixodes ricinus* nymphs exposed by method A to different concentrations of PMD (mg PMD/cm²) and different exposure times (hours). From this graph the LT₅₀ and LT₉₅ were calculated (Table 1)

Table 1 The lethal concentrations of PMD for different exposure times, and lethal time at different concentrations of PMD (based on method A)

Lethal concentrations (LC)			Lethal time (LT)		
Exposure time to PMD (h)	LC ₅₀ (mg PMD/cm ²)	LC ₉₅ (mg PMD/cm ²)	Concentrations of PMD (mg/cm ²)	LT ₅₀ (h)	LT ₉₅ (h)
3	0.09	—	0.025	4.7	—
3.5	0.045	—	0.05	3.3	—
4 ^a	0.037 ^a	0.0975 ^a	0.1 ^a	2.8 ^a	4 ^a
4.5	0.034	0.09	0.2	2.8	4.1
5	0.018	0.08			
24	0.013	0.025			

^a At 0.1 mg PMD/cm² LT₉₅ = 4 h; at 4 h LC₉₅ = 0.0975 mg PMD/cm² $\approx$ 0.1 mg PMD/cm²

Limited exposure time method

Nymphs that had been exposed to a concentration of 0.1 mg/cm² of PMD for 1.5 h and then removed from the exposure area appeared weak and inactive but resumed activity when exposed to fresh air. However, increased mortality was evident at 24 h, reaching 50% at 48 h and 100% at 72 h. In contrast, with nymphs which were exposed to 0.1 mg PMD/cm² for 2 h, mortality appeared at 30 min (from the time they had been moved out to fresh air), increased clearly at 2 h, reached 50% mortality at 24 h and 100% mortality at 72 h. In the groups that were moved out to fresh air after 3.5, 4 and 4.5 h of exposure all nymphs were dead at the same time after transfer to fresh air; 1.45, 0.85, 0.2 h, respectively. When exposed to 0.1 mg PMD, the nymphs reached LT₉₅ mortality between 4 and 6 h irrespective of whether they had been moved out to fresh air or not. The analysis of variance showed a significant difference in mortality between the times of exposure ($F = 36.83$, $P < 0.0001$).

Folded filter paper method

At 0.1 mg/cm² the LT₅₀ was 2.1 h and the LT₉₅ was 4.25 h. There was no significant difference in mortality between nymphs exposed to a concentration of 0.1 mg PMD/cm² ($t = 20.75$, $df = 21$, $P < 0.000001$) and those exposed to a ten times higher concentration ($t = 20.79$, $df = 21$, $P < 0.000001$). However, when we decreased the concentration by a factor from 10 to 0.01 mg PMD/cm², mortality reached 60% after 12 h and 100% after 24 h ($t = 2.95$, $df = 4$, $P = 0.042$). The analysis of variance showed a significant difference in mortality among times of exposure to PMD [$(N = 4, df = 10) F = 22.126$, $P = 0.014$] (Figs. 2, 3, 4).

Direct contact with non-absorbing surface

100% mortality was reached after 1 h with all concentrations (0.002, 0.01, 0.1 mg/cm²) of PMD during the first day with the first group of nymphs ($t = 6.71$, $df = 15$, $P < 0.0001$). The same result (100% mortality within 1 h) was obtained after 24 h when new nymphs were placed in the same cylinder. In contrast, after 48 h and using the same impregnated cylinder, all nymphs in the third group survived after 1 h, showing that the toxic activity had started to decrease and did not give the same level of mortality after 1 h. Equivalent results were obtained for all concentrations of PMD in this test; the analysis of variance showed no differences ($P = 0.88$) among the concentrations of PMD.

Fig. 2 The LC_{50} and LC_{95} of PMD were calculated from the curves for different exposure times shown in this graph. Results from method A

Fig. 3 Mortality of nymphs of *Ixodes ricinus* exposed to several limited periods of a fixed concentration, 0.1 mg PMD/cm^2 . After exposure to PMD for 3.5 h the $LT_{95} = 1.45 \text{ h}$, and after exposure to PMD for 4.5 h the $LT_{95} = 0.2 \text{ h}$. Results from method B

Topical application method

Mortality started after 30 min for all concentrations, but increased at different rates dependent on concentration. The analysis of variance showed a significant difference among the concentrations of PMD ($F = 21.43$, $P < 0.001$). As shown in Fig. 5, at 0.2 mg PMD/cm^2 mortality reached 100% at 3.5 h, ($t = 6.29$, $df = 13$, $P < 0.0001$). At 0.1 mg

Fig. 4 Mortality (%) of *Ixodes ricinus* nymphs exposed to different PMD concentrations 0.01–1 mg/cm² against exposure time. At 0.1 mg PMD/cm² the LT₅₀ = 2.1 h and LT₉₅ = 4.25 h. Results based on method C

PMD/cm² 100% mortality was reached at 4 h, ($t = 6.78$, $df = 13$, $P = 0.000013$). However at 0.05 mg PMD/cm² 100% mortality was reached after 24 h, ($t = 7.4$, $df = 13$, $P = 0.000005$). A similar result was obtained with nymphs subjected to 0.025 mg PMD/cm², ($t = 5.22$, $df = 13$, $P = 0.00016$). The LT₅₀, LT₉₅, LC₅₀ and LC₉₅ are shown in Table 2. At 0.1 mg/cm² of PMD LT₉₅ = 3.9 h, and at 4 h LC₉₅ = 0.095 mg/cm² of PMD $\approx$ 0.1 mg/cm² of PMD.

Discussion

Many studies have shown that *Eucalyptus* (Myrtaceae) oils have the potential to control arthropod pests on stored products. The oils of *Eucalyptus* exhibit a lethal effect on pests such as the rice weevil (*Sitophilus oryzae* L., Coleoptera: Curculionidae), the drugstore beetle (*Stegobium paniceum* L., Coleoptera: Anobiidae), the southern cowpea weevil (*Callosobruchus chinensis* L., Coleoptera: Bruchidae), the house fly (*Musca domestica* L., Diptera: Muscidae) (Ahmed and Eapen 1986) and adult *Tetranychus urticae* L. (Acari: Tetranychidae) (Choi et al. 2004). Additionally, in a recent field study in Sweden, the protective effect against *I. ricinus* of a lemon eucalyptus extract (Citriodiol) was evaluated; the number of reported bites of *I. ricinus* on 111 persons decreased significantly from a medium of 1.5 for unprotected persons to 0.5 for persons who used PMD applied to the legs (Gardulf et al. 2004). Another recent study in Sweden showed that PMD and a product based on *C. citriodora* oil repel to a significantly high degree *I. ricinus* nymphs (Jaenson et al. 2006; Garbouei et al. 2006). These data suggested to us that PMD may be toxic to *I. ricinus* nymphs. Our results also indicate that PMD is neurotoxic to the ticks.

We chose to test PMD against nymphs of *I. ricinus*, which is the tick stage of greatest importance as a vector of microorganisms causing disease in humans. One reason for this phenomenon is that the nymphs are far more abundant than the adult ticks; the latter are

Fig. 5 Mortality of nymphs of *Ixodes ricinus* after topical application (method E) of different concentrations of PMD (mg/cm²) against exposure time (hours)

Table 2 The lethal concentrations of PMD after topical application and lethal time at different concentrations of PMD (based on method E)

Lethal concentrations (LC)			Lethal time (LT)		
Time after topical application of PMD (h)	LC ₅₀ (mg PMD/cm ²)	LC ₉₅ (mg PMD/cm ²)	Concentrations of PMD (mg/cm ²)	LT ₅₀ (h)	LT ₉₅ (h)
2.5	0.085	—	0.025	5	6.3
3	0.039	—	0.05	2.6	6.3
3.5	0.037	0.18	0.1 ^a	2.3 ^a	3.9 ^a
4 ^a	0.035 ^a	0.095 ^a	0.2	2.8	3.4
4.5 ^a	0.03 ^a	0.093 ^a			
5	0.025	0.093			
5.5	0.017	0.091			
6	0.015	0.085			
24	0.01	0.024			

^a At 0.1 mg/cm² of PMD LT₉₅ = 3.9 h; at 4 h LC₉₅ = 0.095 mg/cm² of PMD $\approx$ 0.1 mg/cm² of PMD

also much more easily detected due to their larger size and are therefore usually removed more rapidly.

When method B was used, 0.1 mg PMD/cm² was the only concentration chosen to test, since this concentration gave almost as strong results as the higher concentration (0.2 mg PMD/cm²) in method A. The results from method B demonstrated that mortality was correlated with the duration of exposure of ticks to PMD, with a distinct effect after at least 3.5 h exposure. Variability in tick feeding rates in relation to spirochete transmission among different tick hosts has not been determined, so it is not possible to know if the findings from these animal studies are applicable to humans (Yeh et al. 1995). However, animal studies suggest that the risk of *B. burgdorferi* transmission is low within the first

24 h of attachment but increases thereafter (Piesman 1993). On the other hand, with the TBE virus infection, transmission of infective virions may occur almost immediately when the tick is attaching and has begun to inject saliva. In general, ixodids take some time to locate an appropriate site to bite so 3–4 h might be a sufficient time for acceptable protection against pathogen-infected ixodids.

From the results of method C we conclude that there is no need to increase the concentration above 0.1 mg PMD/cm², since this concentration produced as high mortality as the higher concentrations. However, different results were obtained when we decreased the concentration below 0.1 mg PMD/cm². Also, the results obtained from method C (when *I. ricinus* nymphs were kept in folded treated filter papers and surrounded with the test substance) were virtually the same as those produced from method A (where *I. ricinus* nymphs walked freely on the treated filter papers).

In method D the duration of toxic activity of PMD seems to decrease against *I. ricinus* after 48 h. In the natural environment this essential oil could behave in a similar way; a previous study (Jaenson et al. 2006) has shown that repellency of *C. citriodora* oil containing PMD on treated blankets in field experiments declined slightly after 24 h, but showed a more rapid decline after 48 h. The results from method D also demonstrated strong mortality (100%) after just 1 h. A possible explanation could be that there is direct contact between the oil drops which remain on the cylinder's walls, after the solvent has evaporated, and the nymphal bodies. In method E there is also a direct contact with the substance, but in this case the *I. ricinus* nymphs were exposed to the oil by topical applications for just 1 min. In most methods the mortality increased with increasing concentration and exposure time (Fig. 6).

A comparison of the results from methods A, C, D and E at one fixed concentration (0.1 mg PMD/cm²) is shown in Fig. 7. From the results obtained using methods A, C and E, the LC₅₀ range was 0.035–0.037 mg PMD/cm², and the LC₉₅ range was 0.095–0.0975 mg PMD/cm² at 4 h of exposure time. Also the LT₅₀ range was 2.1–2.8 h and the LT₉₅ range was 3.9–4.2 h at 0.1 mg PMD/cm². A previous study from Sweden showed that

Fig. 6 LC₅₀ and LC₉₅ of PMD were calculated from the curves for different exposure times in this graph. Results based on method E

Fig. 7 Comparison of methods A, C, D and E at a fixed concentration, 0.1 mg/cm², of PMD. The LT₅₀ range was 2.1–2.8 h and the LT₉₅ range was 3.9–4.2 h in methods A, C and E

PMD and products based on *C. citriodora* oil had a high degree of repellency for *I. ricinus* nymphs at 0.1 mg PMD/cm² concentration (Jaenson et al. 2006). Thus, this concentration may be used as a tick repellent and an acaricide at the same time. However, more research in the natural environment is needed so that comparisons could be drawn between results obtained in the field and in the laboratory. Yet, all the results obtained so far from the different bioassays indicate a potentially high toxicity of PMD on *I. ricinus* and suggest that this substance could be useful for tick control. Recent advances have been made in converting common essential oil constituents, such as citronellal, to PMD, making the production of PMD relatively cheap on an industrial scale (Strickman 2006; Kenmochi et al. 2008). Recent enquires indicate that PMD (>95% purity) may be purchased in 10 kg drums at a price about 60% of that of DEET (>95%).

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