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Full Length Research Paper

In vitro investigation of the repellent effects of the essential oil of *Lippia javanica* on adults of *Hyalomma marginatum rufipes*

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A suitable *in vitro* tick climbing repellency bioassay was developed to evaluate the repellent effects of the essential oil (n-hexane extracts of distillate) of *Lippia javanica* (Burm. F.) Spreng (Verbernaceae) on adults of *Hyalomma marginatum rufipes* Koch, 1844. The GC-MS analysis of the distillate of the essential oil of *Lippia javanica* revealed that myrcene (13.4%), 1,8-cineole (8%), dyhydrotagetone (7.9%), ipsenone (9.6%), bicyclo (3.1.1) heptanes-2-one (20.8%) and 2-butanone (13.3%) were the major constituents. A significant ($P < 0.05$) dose dependent repellency response was observed for the essential oil of *L. javanica* on adults of *H. marginatum rufipes* when data were subjected to Kruskal Wallis analysis.

Key words: *Hyalomma marginatum rufipes*, *Lippia javanica*, repellency bioassay, essential oil, GC-MS.

INTRODUCTION

The biology of ticks and the problems they cause to man and other animals are well documented (de Castro, 1997; Gonzalez et al., 1998). However, in spite of the use of synthetic acaricides such as chlorinated hydrocarbons and organophosphates (Rajput et al., 2006) as the main weapons for tick-control, ticks continue to be amongst the leading ectoparasites and vectors of disease-causing agents. In addition, over-reliance on synthetic acaricides for tick control has led to problems including the emergence of tick strains that are resistant to acaricides (Li et al., 2003), environmental pollution (Bhattacrya et al., 2003) and acaricide residues in products that are destined for human consumption (Karraliede et al., 2003).These problems highlight the need for alternative tick control methods that are environmental friendly yet effective against ticks.

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Interest in plant-based products that can be used as alternatives for the control of arthropods is growing rapidly among researchers (e.g. Pascual-Villabos and Robledo, 1998; Lwande et al., 1999; Abdel-Shafy and Zayed, 2002; Fields et al., 2002; Thembo et al., 2010). The basis for this interest is three-fold: firstly, some plant species, for example Neem (Handule et al., 2002; Liang et al., 2003) have been shown to have anti-arthropod properties; secondly, plant-based products are biodegradable and as a result pose lesser problems to the environment; and thirdly, plant-based products may not be susceptible to the existing mechanisms of resistance to arthropocides.

In this study, we investigated the repellent effects of the essential oil of *Lippia javanica* on *Hyalomma marginatum rufipes* ticks. *L. javanica* is widely distributed in southern Africa (van Wyk et al., 1997) and the indigenous people of southern Africa believe that its strong infusions have arthropocidal properties (Matlebyane et al., 2010). *H. m. rufipes* is widely distributed in southern Africa (Walker et al., 2003) and transmits Crimean-Congo haemorrhagic

fever virus to humans (Hoogstraal, 1979).

MATERIALS AND METHODS

Ticks

Host-seeking (3 to 4 weeks old) *H. m. rufipes* adult ticks used in this study were obtained from the laboratory colonies maintained on rabbits, in the Department of Biology at University of Limpopo, Medunsa campus. These ticks were maintained in glass humidity chambers at 25 ± 1°C, 75 ± 5% RH and natural day/night regime.

Plant material and extraction of essential oil

Leaves, branches and flowers of *L. javanica* were collected in April 2004 from a maize farm managed by the Department of Plant Production and Soil Sciences, at the University of Pretoria.

Fresh leaves, softer branches and inflorescences were sliced into smaller pieces and together were hydrodistilled using a Clevenger–type apparatus with slight modifications (Evans, 1989). Heat supply was provided by heating-mantle equipped with a thermostat and heat was maintained at 90°C. 200 g of plant materials in 400 ml of distilled water were introduced into the round bottom flask and hydrodistilled for 2 h. The distillate was collected as the essential oil band above water. The yield of the essential oil of *L. javanica* obtained was 0.08 ml / 200 g x 100 = 0.04% (v/w). The essential oil obtained was stored in a refrigerator at 4°C until used.

The essential oil of *L. javanica* was analyzed using QP 20-10 Shimadzu GC-MS equipment. The gas chromatograph column temperature was programmed to rise from 50 to 300°C at 10°C/min. The injector temperature was 250°C. The total flow rate was 24 ml/min and column flow rate was 1 ml/min. Supelco equity 1 column with a film thickness of 30 m x 0.25 microns was used. 1 µl of each of the sample (essential oil) was used. Ultra high purity Helium was used as the carrier gas with injector split ratio of 20:1. The ion source and interphase temperatures were 200 and 250°C respectively. The solvent cut time of 4 min and detector gain was 0.70 kv. A Wiley 229 library search was conducted on major peaks of each sample in order to identify the components of the sample. The relative percentage of each compound was determined by calculation of the area under the peak (width at ½ height × height) (Houghton and Raman, 1998).

Anti-tick activity

The repellency bioassay described here below, is a modification of that described by Carroll (1998). However, this bioassay is sufficiently different to merit re-description. This bioassay was based on the climbing behaviour of host-seeking ticks. Except for the genus *Amblyomma*, most ticks climb to vantage positions on grass waiting for the vertebrate hosts. In this bioassay, two glass-rods (L = 21.5 cm and diameter = 0.5) were vertically and firmly inserted on a single rectangular polystyrene platform (L = 19.5 cm, W = 6 cm, and H = 3.5 cm). The two glass-rods were positioned at the opposite ends of the platform (15 cm apart). Water was added into the container to completely surround the platform and to almost reach its height. This was done in order to discourage ticks from moving away from the platform and to stabilize humidity (Carroll et al., 1995). Each of the first (bottom) and the last (top) 5 cm of the one glass-rod were covered with a Whatman No. 1 filter paper strip (2.5 cm x 5 cm). On each test filter paper strips (bottom and top), 100 µl of the distillate was released on the filter papers, which were used to cover the test glass-rods. The rational of fixing filter papers on the first and last 5 cm of the glass rods was to expose ticks to the essential oil before they started to climb and also when they were questing at the top of the glass rod. Only the hexane solvent (100 µl) was released on the filter paper strips applied on the control glass-rods. The filter paper strips were dried prior to been applied on the glass rods. Surgical gloves were used to handle glass rods and filter paper strips. To develop different concentrations, a mixture of n-hexane solvent and the distillate were prepared by making 1.5 ml of the solution with 40, 80 and 160 µl quantities of essential oils in varied amounts of the hexane solvent using an adjustable pipette. The concentrations of essential oil of *L. javanica* used were 2.7, 5.3 and 10.7% (v/v), respectively. There were five replications for n-hexane extracts of the essential oil of *L. javanica* with 15 ticks (at least 5 males and 8 females) per replication. Each tick was placed midway between the glass rods on the platform in a direction that was perpendicular to the imaginary straight line joining the two glass rods. The locations of ticks were recorded at 30 min intervals during a 3 h testing period. The repellent indexes were calculated using the formula:

$$R = [(Nc - Nt) / (Nc + Nt)] \times 100$$

Where, Nc = number of ticks on the control glass rod and Nt = number of ticks on the treatment glass rod (Lwande et al., 1999).

Data analysis

Data collected at each time interval in each replicate were grouped together and tested for significance using the non-parametric Mann-Whitney U test. The non-parametric Mann-Whitney U test was used because the number of ticks in each replication varied and transformation of data into Log X+1 did not yield normal distribution in some of the data, when analyzed with Shapiro-Wilk test for normality. Also the number of ticks recorded varied from one replication to the other. To determine the effect of increasing concentration on repellency at different time intervals, non-parametric Kruskal-Wallis test (Hammer et al., 2001) was used.

RESULTS AND DISCUSSION

Essential oil composition

Among the constituents of the essential oil of *L. javanica* identified by GC/MS analysis, myrcene (13.4%), 1,8-cineole (8%), dyhydrotagetone (7.9%), ipsenone (9.6%), bicyclo (3.1.1) heptanes-2-one (20.8%) and 2-butanone (13.3%) occurred in large quantities (Figure 1 and Table 1).

Tick repellency

The repellent indexes increased with increasing concentrations of *L. javanica* (Tables 2, 3 and 4). A significant dose-dependent response was observed between the essential oil of *L. javanica* and adults of *H. m. rufipes* at all the time intervals. The median repellent indexes increased with increasing concentration. However, for each concentration of the essential oil of *L. javanica*, differences in repellent indexes at different time intervals were not statistically significant (Tables 2, 3 and 4). A significant difference ($P < 0.05$) was observed at all

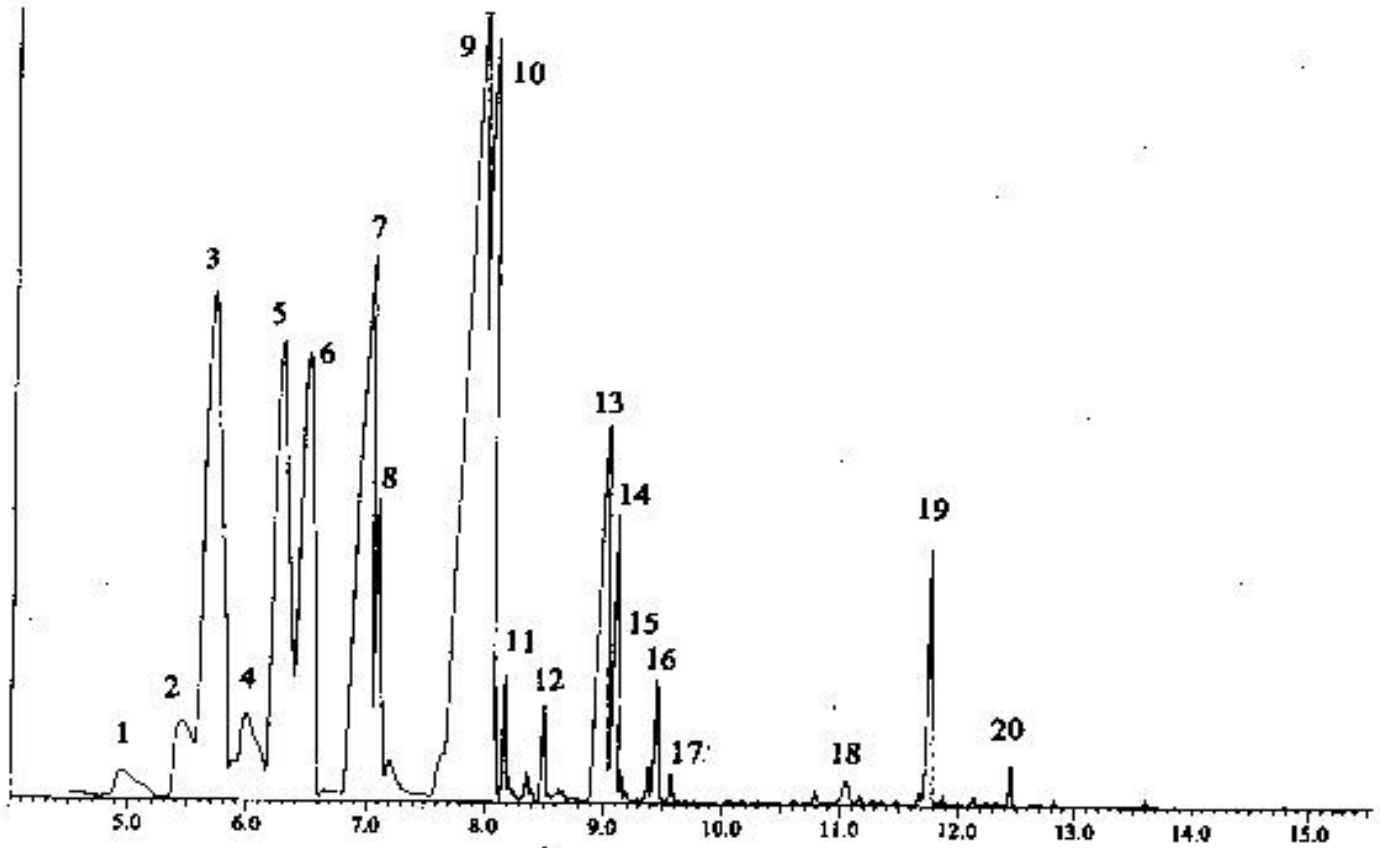

Figure 1. GC-MS chromatogram of the essential oil of *L. javanica*.

Table 1. Constituents of the essential oil of *L. javanica* and the relative amount in a GC-MS analysis.

Compound	Relative percentage
α-Pinene	5.33
Sabinene	2
Myrcene	13.4
Cyclooctene	2.2
1,8-Cineole	8
Dehydro tagetone	7.9
Ipsenone	9.6
Z and E epoxy-ocimene	2.7
Bicyclo(3.1.1)heptan-2-one	20.8
2-Butanone	13.3
Unknown	0.57
B-Fenchyl alcohol	0.4
3-mythyl-2-(2-methyl-2-butenyl)-furan	3.3
3-methyl-2-(2-methyl-2-butenyl)-furan	2.5
Thiophene	1.7
2-Cyclohexen-1-one	0.5
Unknown	0.01
Unknown	0.02
Carryophellene	1.13
Germacrene-D	0.17

Table 2. Tick repellent indexes (RI) at a concentration of 2.7% (v/v) of the essential oil of *L. javanica* at different time intervals.

Time (min)	Median RI (%)	Range of RI from lowest to highest value	P-value Man-Whitney U test
30	33.3	9.1 – 81.8	0.0122
60	33.3	7.7 – 85.7	0.0122
90	33.3	0 – 85.7	0.0126
120	33.3	0 – 85.7	0.0126
150	33.6	7.7 – 85.7	0.0122
180	33.3	7.7 – 85.7	0.0122

Table 3. Tick repellent indexes (RI) at a concentration of 5.3% (v/v) of the essential oil of *L. javanica* at different time intervals.

Time (min)	Median RI (%)	Range of RI from lowest to highest value	P-value Man-Whitney U test
30	66.6	60 – 100	0.0122
60	63.36	50 – 100	0.0122
90	63.63	50 – 100	0.0122
120	50	50 – 84.6	0.0122
150	69.2	50 – 86.6	0.0122
180	69.2	50 – 86.6	0.0122

Table 4. Tick repellent indexes (RI) at a concentration of 10.7% (v/v) of the essential oil of *L. javanica* at different time intervals.

Time (min)	Median RI (%)	Range of RI from lowest to highest value	P-value Man-Whitney U test
30	100	85.7 – 100	0.0122
60	100	85.7 – 100	0.0122
90	100	86.7 – 100	0.0122
120	100	85.7 – 100	0.0122
150	100	100	0.0122
180	100	86.7 – 100	0.0122

intervals between the number of ticks in the control and test replications for *L. javanica*'s essential oil (Table 5). Also, the results showed that the range of RI became smaller as concentration was increased from the lowest at 2.7% (v/v) (0 to 85.7) to 10.7% (v/v) (85.7 to 100).

The main goal of this study was to investigate the repellent effects of the essential oil of *L. javanica* on *H. m. rufipes* ticks. Data obtained from this study suggest that the essential oil of *L. javanica* has repellent properties against *H. m. rufipes*. The repellency properties of the essential oil of *L. javanica* have been shown to be effective also against non-tick arthropods (Lukna et al., 1998).

The potency of the essential oil of *L. javanica* to repel ticks in this study, persisted for 3 h, a duration longer than those established for most botanicals against mosquitoes (Frandin and Day, 2002). Considering the design of the bioassay used in this study, the bioactive compounds in the essential oil of *L. javanica* should have been volatile in nature since ticks avoided coming into contact with the treatment filter papers. In addition, hexane which was used as a carrier of the essential oil in this study is known to extract non-polar volatile compounds such as essential oils (Houghton and Raman, 1998).

The major constituents of *L. javanica* in this study were bicyclo (3.1.1) heptan-2-one, mycrcene and 2-butanone. Amongst these, only myrcene has been identified as a major constituent of *L. javanica* by other studies. The variation in chemical composition of *L. javanica* has been noted by other authors. According to Viljoen et al. (2005), *L. javanica* displays quantitative and qualitative variations both within and between natural plant populations. Other chemical constituents of the essential oil of *L. javanica*

Table 5. Dose dependent tick repellency response at each time interval for increased concentrations of the essential oil of *L. javanica.*

Time	Concentrations (% v/v)	Kruskal Wallis at intervals (min) 95% CI
30	2.7, 5.3 and 10.7	s
60	2.7, 5.3 and 10.7	s
90	2.7, 5.3 and 10.7	s
120	2.7, 5.3 and 10.7	s
150	2.7, 5.3 and 10.7	s
180	2.7, 5.3 and 10.7	s

's' Indicates significant difference ($P < 0.05$) of concentration-response relationship for each time interval.

such as alpha-pinene, sabinene, myrcene and 1,8 cineole determined in this study, have been identified in the essential oils of *Eucalyptus saligna* and *Cupressa sempervirens* which were shown to have repellent properties against arthropod pests (Taponndjou et al., 2005). Although the need to know the effect of each individual chemical constituent on ticks exists, Lwande et al. (1999) showed that the crude essential oil possesses increased bioactivity compared to its individual constituents. It is therefore reasonable to suspect that the repellent effects of the essential oil of *L. javanica* observed in this study were a result of the synergy among the chemical constituents.

In summary, the results of this study, further strengthens the view that *L. javanica* is a potential source of anti-arthropod agents and also to some extend validates the traditional use of the plant for insect pest control by the people of southern Africa (Matlebyane et al., 2010). However, it is important that future studies should include field trials in order to confirm laboratory results.

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