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Full Length Research Paper

Variation in the repellency effects of the leaves of *Mentha piperita* against adults of *Amblyomma hebraeum*

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The repellency effects of *Mentha piperita* leaves, collected from two different locations in South Africa, (Ga-Rankuwa and Malelane) on adults of *Amblyomma hebreaum* were studied. Leaves extraction was carried out using two different solvents (dichloromethane (DCM) and ethylacetate) to make concentrations of 0.24 g/10 ml, 0.24 g/15 ml and 0.24 g/20 ml (w/v). The extracts were tested for repellency on the adults of *A. hebraeum* using glass plate repellency bioassay. The separation and qualitative analysis of plant extracts (0.24 g/10 ml (w/v)) extracted with DCM were investigated using TLC. Trace elements concentration from the soil and leaves samples from each study areas were determined by ICP – OES. The result shows that plant extract of both DCM and ethylacetate solvents from Ga-Rankuwa plants were more effective at all concentrations in repelling adults' *A. hebraeum* when compared with extracts of the plant collected from Malelane. Significantly, higher concentrations of trace metals were reported from the soil and plant collected from Malelane ($p < 0.05$). TLC plates showed that there are differences in the chemical composition of the DCM extracts of *M. piperita* from Ga-Rankuwa and Malelane. The study shows that plant species collected from different geographical areas can produce contrasting results.

Key words: *Mentha piperita,* repellency bioassay, *Amblyomma hebraeum.*

INTRODUCTION

Many studies have been done in which synthetic acaricides were used to control the spread of the hard ticks (Dipeolu and Ndungu, 1991). However, the use of these acaricides have been realized to have several drawbacks, such as their effectiveness over time due to resistance from the ticks over a prolonged period of time (Sonenshine, 1991; Rajput et al., 2006), their cost (de Castro and Newson, 1993) and the threat they pose to the environment (Bhattacharya et al., 2003). A recent economic control method which involves the use of plants for the tick diseases had been put in place and its effectiveness is still under research (Mkolo and Magano, 2007). However, studies have shown that the plant-based anti-arthropod products are very effective as a control method (Lwande et al., 1999; Mkolo and Magano, 2007). The advantages of using these products include bio-degradability, having to move away from contaminating the environment and in most cases accessible and affordable (Lwande et al., 1999). However, Carroll et al. (2007) reported that same plant species used on the same tick species collected from different geographical areas can provide contradicting results. This shows that geographical factors may affect the chemical composition of these plants. It is therefore important to examine and get knowledge on the factors that may influence different chemical composition from the same plant. Some researchers have suggested factors such as genetic variation, geographic origin, seasonal variation, growth stage, plant parts utilized, post harvest drying, length of the exposure to sunlight, availability of water, plant density and presence of fungi diseases and insects

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Figure 1. The glass plate repellency bioassay. A, Petri-dish; B, filter paper treated with organic solvent; C, filter paper treated with plant extract.

played important role in determining the effectiveness of the plant (Burbott and Lamis, 1957; Galambosi and Peara, 1996; Graven et al., 1990; Margina and Zheljazkov, 1994).

Contamination of soil by heavy metals has become a major environmental concern due to their potential adverse ecological effects. Plant can take up trace metals from the soil and store it in their tissues. Several studies have reported on the toxicity behavior of trace metals in plants. Zn toxicity is known to inhibit many plant metabolic functions; results in retarded growth and cause senescence (Choi et al., 1996). Exposure of plants to excess Cu may generates oxidative stress in plants (Stadtman and Oliver, 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus et al., 2001). High level of Pb also causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005). Pb inhibits the activity of enzymes at cellular level by reacting with their sulfhydril groups. High Pb concentration also induces oxidative stress by increasing the production of ROS in plants (Reddy et al., 2005). Shanker et al. (2005) reported an increased production of metabolites such as glutathione and ascorbic acid due to an increased concentration of chromium. At concentrations of >20 to 30 µg/g, copper becomes toxic to plants and alters membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthesis and respiratory processes and may induce senescence (Fernandes and Henriques, 1991).

The study evaluated the impact of geographical location on the repellency activities of *Mentha piperita L.* collected from two different geographical locations in South Africa. The study also checked in part the effect of trace metals on the repellency ability of this plant.

M. piperita also known as mint or peppermint and classified as Lamiaceae (Lorenzi and Matos, 2002) was used for this study. The insecticidal activities of essential oil of *M. piperita* L. emend. Huds. against mosquitoes as disease vectors was recognized by Samarasekera et al., (2008). However, factors that can influence the anti-tick activities of this plant are not well known. Thus, the main goal of this study was to conduct a laboratory evaluation of the tick repellency effects of the *M. piperita* leaves collected from two different locations in South Africa against *Amblyomma hebraeum*.

MATERIALS AND METHODS

Tick, host and infestation

Colonies of *A. hebraeum* used for this study were bred on the Himalayan rabbits situated at the Animal Production Unit, Department of Biology (University of Limpopo, Medunsa campus). The ticks were kept in glass humidity chambers, under natural day and night regimens at 25 ± 1°C (temperature) and 75 ± 5% (relative humidity).

Plant collection and extraction of crude extract

Fresh leaves of *M. piperita* were collected from Ga-Rankuwa, Medunsa campus (North-West Province) with geographical co-ordinates: 25°37' 8" South and 28°1' 22" East and Malelane (Mpumalanga Province) geographic co-ordinates: 25°29' 0" South and 31°31' 0" East. 70 ml of dichloromethane (non polar) and ethylacetate (mid polar) were used to extract 25 g of the crushed fresh leaves, separately. The mixture was left for 24 h, following which the supernatant was filtered using Whatman no. 1 filter paper. The solvent was evaporated completely at room temperature. The extract residues obtained were re-dissolved in varied volume of relevant solvent in order to obtain the following concentrations: (0.24 g/10ml, 0.24 g/15ml and 0.24 g/20ml) w/v. These extracts were tested for tick repellency.

The glass plate repellency bioassay

The glass plate repellency bioassay was used for this study. The Whatman filter paper was trimmed to fit a 10 cm diameter glass plate and divided into two equal halves (Figure 1). Half of each filter paper was applied with 10 ml of the plant extract concentration prepared, while the other half was used as control using either dichloromethane or ethylacetate. The whole setup was covered with a transparent 8 cm diameter plastic Petridish (with diminutive openings) to prevent the tick from escaping.

Experimental procedure

The unsexed *A. hebraeum* adult ticks (n =10) were placed at the center of every filter paper. Prior to the experiment procedure, ticks were allowed to have 10 minutes acclimatization period, following which their position on the glass plate was noted at 5 min interval for 1 h. After each 5 min interval, the ticks were moved back to the centre. Ticks found on the treatment were considered not repelled,

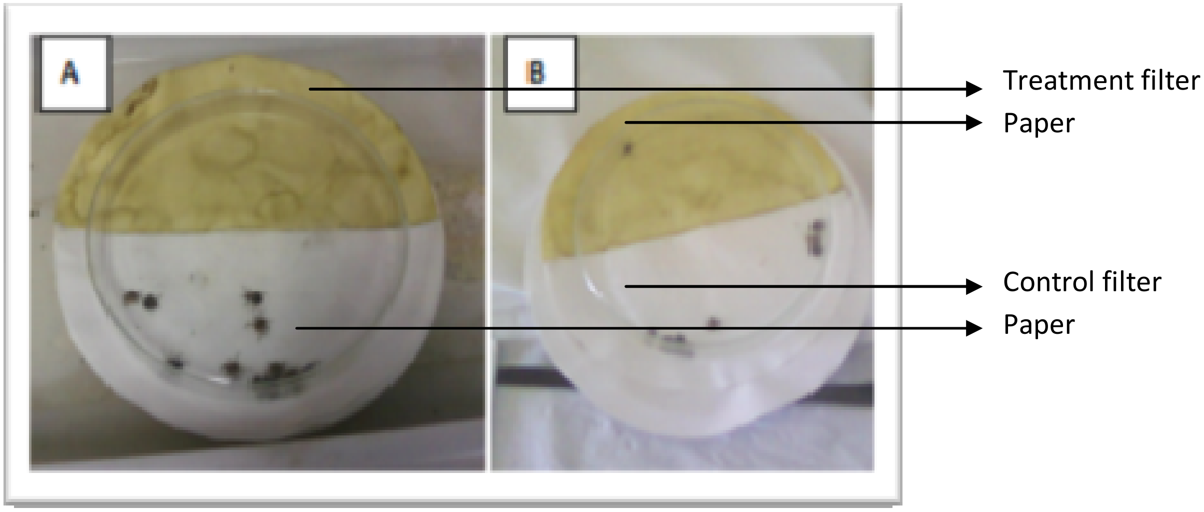

Figure 2. Example of the tick repellency effects of *M. piperita* from (A) Ga-Rankuwa; (B) Malelane.

while those found on the control were considered repelled. Three replications were done for each concentration of the plant extract. To prove that solvent itself was not a repellent, similar procedure was followed. However, half of each filter paper was applied with 10 ml of either dichloromethane or ethylacetate while the other half was used as control without any solvent applied on it. The effective concentration to repel 50% (EC_{50}) of the ticks was then calculated.

Plant sampling and analysis

Leaves samples from each study area were obtained by removing leaves from five different plants in the area and combining them. Leaves were washed so as to exclude the effect of surface pollutant as a result of aerial fallout and later dried. Approximately 0.5 g of the fine powdered leaf samples were used for the analysis of trace metal contents. The analysis for trace metal content was carried out using inductively coupled plasma optical emission spectrometer (ICP – OES) (Olowoyo et al., 2010).

Soil sampling and analysis

The soil samples were collected at depth of 0 to 15 cm with the aid of an auger of 7.0 cm in diameter and were placed in different polythene bags prior to laboratory analysis. The soil samples were allowed to air dry for two days at room temperature and were crushed into fine particles using a mortar and a pestle. After sieving each soil sample with a 2 mm mesh, a 5 g of each sample was measured and transferred to 100 ml volumetric flasks containing 5 ml of perchloric acid and 12 ml of nitric acid were used for acid digestion. The resulting soil solutions were allowed to boil for two hours and were then analyzed for trace metals content with the aid of ICP – OES. The soil pH was determined at 0.01 M $CaCl_2$ using pH meter.

Thin layer chromatography (TLC) analysis

The plant (from Malelane or Ga-Rankuwa) extracted with dichloromethane (highest concentration: (Conc.: 0.24 g/10 ml w/v)) and air dried, were dissolved in dichloromethane to yield 10 mg/ml stock solution. The extracts were analyzed by TLC on Merck TLC F_{254} 10 × 20 cm in one solvent systems, namely: DCM: Ethylacetate (1:1 v/v). Aliquots of 10 µl of the extracts were loaded with a micropipette at 1 cm from the bottom of a labeled TLC plate of the extracts were solvents were allowed to run up to 2 cm from the edge of the plate. The developed plates were air dried in the fume cupboard and thereafter visualized under a UV light (254 and 360 nm Camac Universal UV lamp). The pictures were taken using a Nikon camera to show the separated bands on a developed TLC plate.

Analysis of data

Data analysis of the repellency bioassay

Probit analysis, a free software package (US Environmental Protection Agency; http://www.epa.gov/nerleerd/stat2.htm 2010) aided in determining the effective concentration that repelled 50% (EC_{50}) of the ticks. With percentage repellency, a formula: Percentage repellency = 100 – (mean number of ticks on test)/ (mean number of ticks on control) × 100. The significance of the differences between the treatment and the control was determined using the Mann-Whitney u-test (two-tailed).

Statistical analysis of soil and plant results

SPSS 17.0 for window was used to carry out the statistical analyses. Differences between the elemental contents of the soils and plants were tested for significance using Student's t-test and a *p* value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Figure 2 shows the typical repellency effects of *M. piperita* against adult ticks of *A. hebraeum*. The summary of the repellency effect of *M. piperita* against adult ticks of *A. hebraeum* are presented in Tables 1 to 4. The study showed that plant extracts of both DCM and ethylacetate solvents from plants collected from Ga-Rankuwa,

Table 1. Mean percentage repellency of the DCM extracts of *M. piperita* leaves collected from Ga-Rankuwa against *A. hebraeum.*

Concentration (w/v)		Time							
		5 min	10 min	15 min	20 min	25 min	30 min	1 h	2 h
0.24 g/10ml	PR	100	98	100	99	98	92	98	99
	Range of PR	100	97-100	100	97-100	97-100	75-100	97-100	97-100
	P-value	*	*	*	*	*	*	*	*
0.24 g/15ml	PR	100	98	100	90	100	100	99	98
	Range of PR	100	97-100	100	97-100	100	100	97-100	97-100
	P-value	*	*	*	*	*	*	*	*
0.24 g/20ml	PR	69	90	69	76	49	69	77	63
	Range of PR	57-75	75-97	57-75	57-97	33-57	57-75	33-100	33-75
	P-value	*	*	*	*	**	*	*	*
	$EC_{50(\%)}$	-	-	-	-	1.246	-	1.013	1.194

PR: Percentage repellency; Conc.: concentration; w/v: weight over volume; *significant difference between the treatment and the control, P < 0.05; **no significant difference between the treatment and the control, P > 0.05; - not determined.

Table 2. Mean percentage repellency of the ethylacetate extracts of *M. piperita* leaves collected from Ga-Rankuwa against *A. hebraeum.*

;Concentration (w/v)		Time							
		5 min	10 min	15 min	20 min	25 min	30 min	1 h	2 h
0.24 g/10ml	PR	100	90	97	97	100	97	97	93
	Range of PR	100	90	90-100	90-100	100	90-100	90-100	90-100
	P-value	*	*	*	*	*	*	*	*
0.24 g/15ml	PR	100	97	97	97	90	91	98	93
	Range of PR	100	90-100	90-100	90-100	75-97	75-100	90-100	90-100
	P-value	*	*	*	*	*	*	*	*
0.24 g/20ml	PR	82	90	57	73	90	61	63	63
	Range of PR	75-97	75-97	57	57-97	75-97	33-75	57-75	57-75
	P-value	*	*	*	*	*	*	*	*
	$EC_{50(\%)}$	-	-	-	-	-	1.308	-	-

PR: Percentage repellency; Conc.: concentration; w/v: weight over volume; *significant difference between the treatment and the control, P < 0.05; **no significant difference between the treatment and the control, P > 0.05; - not determined.

Pretoria were more effective at all concentrations in repelling adults of *A. hebraeum* when compared with the plant extracts of the plant collected from Malelane, Mpumalanga. Furthermore, there was no significant difference (p > 0.05) in the repellency between solvents (DCM or ethylacetate) treated filter paper and untreated filter paper. This proved that solvent itself is not repellent. However, the repellency seemed to be solvent dependent, since DCM extracts for both plant collected from Malelane and Ga-Rankuwa were more effective in repelling adults of *A. hebraeum* when compared with ethylacetate extract. This result consolidate previous findings of Borges et al. (2003), where in less polar extract of *Melia azadarach,* ripe fruit were more effective against larvae and engorged female *Boophilus microplus* ticks than polar extract of the same plant.

The repellency appeared to be dose-dependent since high repellency persisted for longer periods in higher

Table 3. Mean percentage repellency of the DCM extracts of *M. piperita* leaves collected from Malelane against *A. hebraeum.*

Concentration (w/v %)		Time							
		5 min	10 min	15 min	20 min	25 min	30 min	1 h	2 h
0.24 g/10ml	PR	100	100	91	100	91	91	96	91
	Range of PR	100	100	86-100	100	86-100	86-100	86-100	86-100
	P- value	*	*	*	*	*	*	*	*
0.24 g/15ml	PR	98	91	100	91	98	100	99	91
	Range of PR	97-100	86-100	100	86-100	97-100	100	97-100	86-100
	P-value	*	*	*	*	*	*	*	*
0.24 g/20ml	PR	90	69	71	57	69	90	63	69
	Range of PR	75-97	57-75	40-86	40-66	57-75	75-97	33-75	57-75
	P- value	*	*	*	*	*	*	*	*
	$EC_{50(\%)}$	-	-	1.196	1.198	-	-	1.210	-

PR: Percentage repellency; Conc.: concentration; w/v: weight over volume; *significant difference between the treatment and the control, P < 0.05; **no significant difference between the treatment and the control, P > 0.05; - not determined.

Table 4. Mean percentage repellency of the ethylacetate extracts of *M. piperita* leaves collected from Malelane against *A. hebraeum.*

Concentration (w/v %)		Time							
		5 min	10 min	15 min	20 min	25 min	30 min	1 h	2 h
0.24 g/10ml	PR	79	91	73	79	64	73	86	79
	Range of PR	66-86	86-100	66-86	66-86	40-86	66-86	86	66-86
	P-value	*	*	*	*	*	*	*	*
0.24 g/15ml	PR	66	73	49	57	49	49	40	49
	Range of PR	66	66-86	40-66	40-66	40-66	40-66	40	40-66
	P-value	*	*	**	*	**	**	**	**
0.24 g/20ml	PR	57	49	57	57	71	64	73	49
	Range of PR	40-66	40-66	40-66	40-66	40-86	40-86	66-86	40-66
	P-value	*	**	*	*	*	*	*	**
	$EC_{50(\%)}$	1.675	1.171	1.534	1.452	1.572	1.580	1.987	1.984

PR: Percentage repellency; Conc.: concentration; w/v: weight over volume; *significant difference between the treatment and the control, P < 0.05; **no significant difference between the treatment and the control, P > 0.05; - not determined.

concentration than lower concentration. Moreover, TLC plates (Figure 3) showed that there may be differences in the chemical composition of the DCM extracts of *M. piperita* from Ga-Rankuwa and Malelane, since the two DCM plant extracts consisted of different bands on TLC plates. In a separate study, Iscan et al. (2002) found that menthol and menthone of *M. piperita* may be the major compounds shown on TLC plates.

The soil pH from both sites is in the range 4.6 to 7.9. Significantly higher concentrations of trace metals were recorded for the soil collected from Malelane in Mpumalanga ($p > 0.05$). Tables 5 and 6 present the results obtained for the concentrations of trace metals in plants and soil, respectively. Concentrations of trace metals such as Cr, Pb, Zn and Cu were significantly higher in plants and soil collected from Malelane (Tables 5 and 6). No significant difference was reported for the concentrations of Fe from both study sides ($p > 0.05$). From all the analyzed trace metals, the concentrations of Ni, Zn and Fe were clearly more than the other elements and the differences in concentrations were significant ($p > 0.01$). Variations in the concentrations of trace metals observed both from the soil and leaves of the plants might have accounted for the observed differences in the ability of the plants to repel ticks. For example, Cakmak and Marshner (1993) reported that the toxicity of Zn may

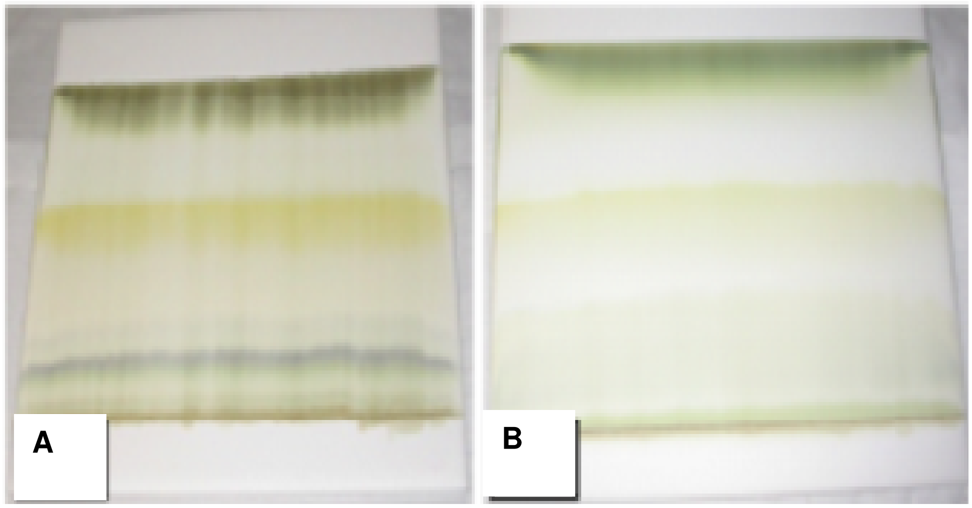

Figure 3. TLC profile of the plant collected from (A) Ga-Rankuwa; (B) Malelane.

Table 5. The concentrations of trace metals analyzed from the leaves of *M. piperita* collected from Ga-Rankuwa and Malelane.

Location	Metal (µg/g)						
	Cu	Cr	Pb	Zn	Ni	Fe	Mn
Garankuwa	10.62±0.21	13.31±0.31	1.32±0.04	45.34±1.23	6.95±0.23	36.89±0.21	24.45±00.78
Melalane	12.32±1.11	18.32±1.01	5.38±0.02	62.45±1.15	8.32±0.11	44.14±0.21	38.56±0.02
T-test	*	*	*	*	NS	NS	*

*Significant difference in values; NS: no significant difference in values.

Table 6. The concentrations of trace metals analyzed from the soils collected from Ga-Rankuwa and Malelane.

Location	Metal (µg/g)						
	Cu	Cr	Pb	Zn	Ni	Fe	Mn
Garankuwa	13.57± 0.12	36.49±0.89	10.41±0.15	28.21±0.54	34.02±0.84	57.72±1.31	64.07±0.65
Melalane	18.32±0.14	54.21±0.32	35.31±0.25	58.38±0.68	49.35±0.64	65.14±0.57	72.32±0.55
T- Test	*	NS	*	*	*	NS	NS

*Significant difference in values; NS: no significant difference in values.

affect the plant development, metabolism and an induction of oxidative damage in various plants. Exposure of plants to excess copper may generates oxidative stress which in turn may lead to the disturbance of metabolic pathways and damage to macromolecules (Singh and Tewari, 2003; Seregin and Kozhevnikor, 2005). Shanker et al. (2005) reported an increased production of metabolites such as glutathione and ascorbic acid due to increased concentration of chromium. Sharma and Dubey (2005) stated that high level of lead may inhibit enzyme activities, cause water imbalance, alterations in membrane permeability and disturbs mineral nutritions. Moreover, the repellency of *M. piperta* appeared to be associated with the presence of monoterpenoids and sesquiterpenes (Jaenson et al., 2006; Olennikov and Tankhaeva, 2010). Santiago et al. (2000) found that the phenolic compounds increase in *Phyllantus tenellus* leaves after being sprayed with copper sulphate. In addition, Dixon and Paiva (1995) showed that the induction of phenolic metabolism is a plant response to multiple stresses such as pathogen attack, wounding, herbivore feeding, UV light or high visible light level.

Conclusion

The study shows that the same plant species collected from different geographical areas can produce contrasting results. Moreover, it may be vital to know the soil composition before harvesting *M. piperta* for tick repellency. However, in spite of the positive results obtained in this study regarding the repellency properties of *M. piperita* on *A. hebraeum,* more studies are needed in order to further explain the anti-tick properties of this plant on other tick species. It is recommended that further research be done on: isolation and characterizing the compounds of plant using NMR and GC-MS and to explore the level of genetic diversity within the plant population.

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