In vitro investigation of the toxic effects of extracts of *Allium sativum* bulbs on adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus*

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ABSTRACT

The toxic effects of the extracts of *Allium sativum* (Garlic) were evaluated against adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus* using three types (Types A, B and C) of contact toxicity bioassays. *A. sativum* bulbs were extracted with acetone, ethanol and dichloromethane (DCM) solvents. Among these three solvents, it is the DCM extract of *A. sativum* that appears to have anti-tick activity. In the Type A contact toxicity bioassay, DCM extracts of *A. sativum* demonstrated a high acaricidal bioactivity against *H. m. rufipes* with 100 % of ticks killed in less than an hour, and toxicity persisted to the second day. A weak acaricidal activity of aqueous extracts of *A. sativum* was observed in the Type B contact toxicity bioassay. In the Type C contact toxicity bioassay, a concentration of 24 % w/v of DCM extracts of garlic in sunflower oil (*Helianthus annuus*) had killed 100 % of *H. m. rufipes* (LC_{50} = 5.9 % w/v) and *R. pulchellus* (LC_{50} = 10.3 % w/v) by 24 hours post-treatment of ticks. The results obtained from this study suggest that DCM extract of *A. sativum* is a potential source of novel acaricidal agents.

Key words: *Allium sativum,* contact toxicity bioassay, *Hyalomma marginatum rufipes, Rhipicephalus pulchellus.*

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INTRODUCTION

Ticks are haematophagous ectoparasites of vertebrates[27] and they play a very important role in the transmission of many disease-causing agents to humans and livestock[5,11,25]. Tick infestation has been linked to production losses in livestock[22] and also to the increase of life-threatening diseases in humans[23].

The current methods used to control ticks rely largely on the use of acaricides, but, despite their efficacy[7,15], extensive usage has been associated with increased resistance to them[20]. Furthermore, their uncontrolled use has led to environmental toxicity[2].

As a result, scientific research on plant-based products that are toxic to arthropod pests of economic and medical importance is intensifying[1,4,18]. Such, botanical pesticides are said to be easily biodegradable[16], making them less toxic to the environment and non-targeted species[6].

So far, encouraging results from laboratory experiments on anti-tick plant-based products have been obtained. For example, plant extracts derived from *Azadirachta indica* and *Adenium obesum* have been shown to be toxic to *Boophilus* species and *R. pulchellus,* respectively[12,21]. Also, oil-based formulations consisting of fungal conidia in 15 % peanut oil, 1 % emulsifier and 84 % water were shown to be more effective against *R. appendiculatus* than an aqueous formulation without the peanut oil[19]. In a related study, maize, groundnut, sunflower and sesame oils reduced oviposition in bruchid species[24]. *Allium sativum* (garlic), a member of the family Alliaceae, formerly placed in Liliaceae, has been shown to possess arthropocidal properties[14,28]. These reports demonstrate the recognition by many researchers that plants are a potential source of anti-arthropod agents, for example pyrethroids derived from *Chrysanthemum pyrethrum*[9]. However, despite this recognition, knowledge about botanicals as anti-arthropod agents is still sparse. In this study the toxic effects of the extracts of garlic (*A. sativum*) on *H. m. rufipes* and *R. pulchellus* were examined using suitable *in vitro* bioassays. These 2 tick species occur naturally in Africa and are vectors of Crimean-Congo haemorrhagic fever virus and *Trypanosoma theileria,* respectively[5,13].

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MATERIALS AND METHODS

Ticks

Only adult *H. m. rufipes* and *R. pulchellus* ticks (1–2 months old) were used in this study. These ticks were obtained from laboratory colonies maintained on Himalayan rabbits in the Department of Biology at the University of Limpopo. Both tick species were kept in a temperature controlled room at 25 ± 1 °C and a natural photoperiodic regime but in separate containers with different relative humidities (75 ± 5 % RH for *H. m. rufipes* and 85 ± 5 % RH for *R. pulchellus)* prior to the start of the experiment. All experiments during this study were performed under ambient conditions (25 ± 5 °C, RH 40 ± 10 % and a natural photoperiodic regime).

Type A contact toxicity bioassay

Allium sativum bulbs used in this study were obtained from a greengrocer in Pretoria North. Extracts of *A. sativum* were prepared by crushing 100 g of the bulb manually for 15 minutes followed by extraction with 250 mℓ dichloromethane (DCM) (a non-polar solvent). This process was repeated using acetone and ethanol as extractants. In each case, the extract was allowed to stand for 90 minutes prior to the removal of insoluble matter by filtration using Whatman no. 1 filter paper. About 30 mℓ of each solution (5 replications for each solvent) was poured into separate 100 mℓ beakers of known weights and allowed to evaporate in a fume chamber with the aid of a fan until the solvent was completely removed. The rate of evaporation of DCM was rapid, requiring only 4 hours to evaporate completely, while acetone and ethanol took 14 hours. Following evaporation of DCM, thin layers of extracts weighing approximately 0.04 g were obtained. A larger yield of oily to jelly-like material resulted from ethanol (0.16 g) and acetone (0.21 g) extracts of *A. sativum.*

Extracts of *A. sativum,* which were prepared according to the procedures described above, were tested for toxicity on unsexed *H. m. rufipes* adults. The control in each case was established by adding the same volume of the appropri-

ate solvent into a beaker. *H. m. rufipes* ticks were randomly divided into treatment (5 ticks) and control (5 ticks) groups. Solvents were allowed to evaporate completely before unsexed ticks were introduced into both the control and treatment beakers. A mesh was used to cover the opening of the beakers in order to prevent ticks from escaping. Five replications were done. Mortality was recorded after 1 and 24 hours following preparation of the extracts. The stability of the extract that caused 100 % mortality in 1 hour was tested by exposing new ticks to it 24 hours after it was prepared. Ticks that did not respond to human breath (CO_2) and a tactile stimulus were considered dead. Data are presented as mean percentage mortality.

Type B contact toxicity bioassay (dipping)

In this bioassay only aqueous extracts of *Allium sativum* were used. Aqueous extracts of *A. sativum,* were prepared by crushing 100 g of the bulb for 15 minutes followed by extraction with 250 mℓ of distilled water. The crude mixture of the plant material and distilled water was allowed to stand for 90 minutes after which the insoluble matter was filtered out using Whatman no. 1 filter paper.

Adult *H. m. rufipes* ticks were immersed in water extract of *A. sativum* for 1 minute and thereafter placed on filter paper for 30 seconds to dry. These ticks were then placed in glass vials whose open ends were covered with a mesh to allow ventilation. For the control, ticks were dipped in distilled water only. The percentage mortality was recorded after 1 and 24 hours. Ticks that did not respond to human breath (CO_2) and tactile stimulus were considered dead. The experiment was replicated 5 times with 5 adult ticks per replication. Data are presented as mean percentage mortality.

Type C contact toxicity bioassay (topical application)

DCM extracts of garlic were used in this bioassay. Different quantities of crushed garlic bulbs were subjected to extraction for 90 minutes according to the following concentrations: 20 g/50 mℓ, 40 g/50 mℓ, 80 g/100 mℓ and 120 g/150 mℓ. After complete evaporation of the solvent, the following amounts of crude extracts were obtained: 20 g/50 mℓ yielded 0.06 g, 40 g/50 mℓ yielded 0.134 g, 80 g/100 mℓ yielded 0.25 g and 120 g/150 mℓ yielded 0.49 g. The yield per gram was closely similar in all cases (0.3–0.34 %). Each of the extracts obtained was subsequently mixed with a volume of commercially available sunflower oil (*Helianthus annuus*) to provide concentrations of 0.06 g/ 2 mℓ (3 % w/v), 0.134 g/2.24 mℓ (6 % w/v), 0.25 g/ 2.1 mℓ (12 % w/v) and 0.49 g/2.04 mℓ (24 % w/v). The dissolved mixture was carefully stirred for 3 minutes to ensure homogeneity.

H. m. rufipes and *R. pulchellus* ticks were each divided into treatment (10 ticks) and control (10 ticks) groups. Each tick in the treatment group was treated with a topical application of 5 μℓ of the mixture using a micropipette. Ticks in the control group were treated with a topical application consisting of 5 μℓ of sunflower oil only. In order to reduce movement, legs of ticks were stuck onto a double-sided sticky tape in a glass Petri dish prior to treatment. Five replications were carried out for each concentration. Tick mortality was recorded after 24 hours. Ticks that did not respond to human breath (CO_2) and tactile stimulus were considered dead. In order to test for the stability of the mixture, unexposed ticks were treated as above with a 24 hour old DCM extract of garlic. Tick mortality was recorded 24 hours later (that is 48 hours after preparation of garlic extract/ sunflower oil mixture).

Results are expressed as mean percentage mortality. A simple linear regression analysis of untransformed percentage mortality data and concentration was used to determine if there was significance of dose–mortality relationship at 95 % confidence interval[10]. The *t*-statistic for the slope was considered significant at $P < 0.05$ (2-tailed *t*-test). The lethal concentration needed to kill 50 % of the ticks (LC_{50}) was determined. Confidence intervals of LC_{50} obtained by probit analysis (free software on EPA website; http://www.epa.gov/nerleerd/stat2. htm)[29] were used to test for significance between responses of *H. m. rufipes* and *R. pulchellus* in Type C contact toxicity bioassay.

Table 1: **Percentage mortality of *Hyalomma marginatum rufipes* caused by contact with crude extracts of *Allium sativum* in Type A contact toxicity bioassay** ($n = 5$ ticks/exposure × 5 replications).

Plant species	Solvent	Time			
		1 hour		24 hours	
		Control	Treatment	Control	Treatment
A. sativum	Ethanol[1]	0	0	0	13
	Acetone[1]	0	0	0	100
	DCM	0	100		

[1]Ticks became submerged in the oil extract.

Table 2: **Percentage mortality of *Hyalomma marginatum rufipes* caused on contact with aqueous crude extracts of *Allium sativum* in Type B contact toxicity bioassay** ($n = 5$ ticks/exposure × 5 replications).

Plant species	Solvent	Time			
		1 hour		24 hours	
		Control	Treatment	Control	Treatment
A. sativum	Distilled water	0	0	0	27

RESULTS

Type A contact toxicity bioassay

DCM extract of garlic killed 100 % of *H. m. rufipes* adults in less than 1 hour. This potency of the DCM extract of garlic to kill 100 % of *H. m. rufipes* adults in less than an hour, persisted for 24 hours. However, for ethanol and acetone extracts of *A. sativum,* mortality was recorded only after 24 hours following exposure of ticks to these extracts (Table 1). Those ticks were submerged in the oily extracts, in other words the ticks were suffocated.

Type B contact toxicity bioassay

Tick mortality resulting from the aqueous extracts of *A. sativum* used in this study was generally low with only 27 % mean mortality (Table 2).

Type C contact toxicity bioassay

Data recorded 24 hours following the start of the experiment shows that the mortality of *R. pulchellus* increased significantly ($P < 0.05$) with an increase in the concentration of DCM extract of garlic in sunflower oil (Figs 1, 2 ; Table 3). All the *R. pulchellus* ticks which were treated with sunflower oil only survived. The results of this study show that the toxicity of the DCM extract of garlic decreased with time and fewer ticks were killed at 48 hours post-preparation of DCM extract (Table 3).

The mortality of *H. m. rufipes* ticks also increased significantly ($P < 0.05$) with increasing concentrations of DCM extracts

Table 3: **Mean percentage mortality of DCM extract of garlic in sunflower oil on contact with *Rhipicephalus pulchellus* adults in Type B contact toxicity bioassay. Control had sunflower oil only.**

Conc % w/v	Mean % mortality of ticks after 24 hours	±SE	*t*-statistics for slope *P*	Mean % mortality of ticks after48 hours	±SE	*t*-statistics for slope *P*
3	0	0	Rs	0	0	Rs
6	12	0.2		0	0	
12	58	0.2		26	0.245	
24	100	0		68	0.489	
0 (control)	0	0		0	0	

Rs, indicates significance ($P < 0.05$) in concentration–mortality response relationship following regression analysis. Where 24 hours regression coefficient (r) = 0.9820 and P (2-tailed) = 0.0029, and 48 hours regression coefficient (r) = 0.9736 and P (2-tailed) = 0.005. w/v = weight/volume, Conc. = concentration.

of garlic in sunflower oil (Figs 3, 4; Table 4). All the *H. m. rufipes* ticks which were treated only with sunflower oil survived. The results show that the toxicity of DCM extract of garlic decreased with time and fewer ticks were killed at 48 hours post-preparation of DCM extract (Table 4). A more acute slope (4.017) in percentage mortality against concentration (g/mℓ) was obtained after 24 hours compared with 48 hours (1.467).

The LC_{50} (DCM extract of garlic) for *R. pulchellus* was significantly ($P < 0.05$) higher (10.3 % w/v) compared with the 5.9 w/v for *H. m. rufipes* at 24 hours post-treatment (Table 5). This is because the lower and the upper confidence intervals for both LC_{50}s do not intersect. For the 24-hour-old mixture, the LC_{50} values were 18.1 % w/v and 30 % w/v for *R. pulchellus* and *H. m. rufipes* respectively.

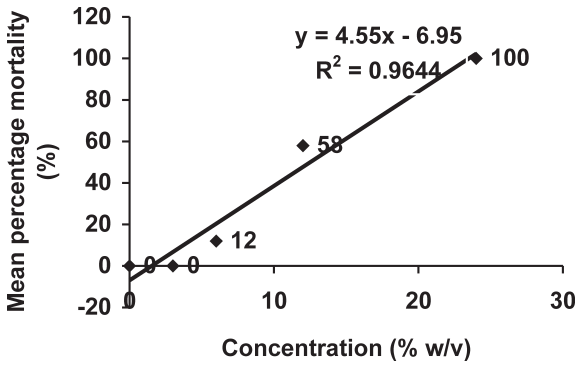

Fig. 1: **Relationship between different concentrations of DCM extract of garlic in sunflower oil and mortality of *Rhipicephalus pulchellus* after 24 hours of exposure.**

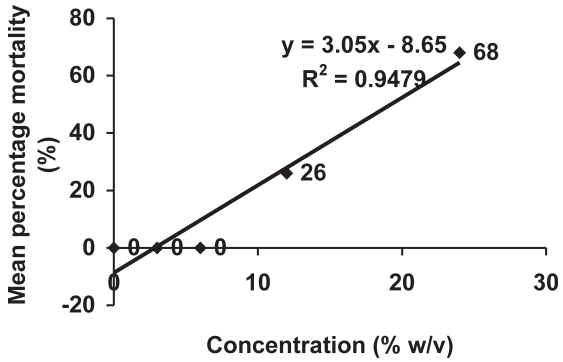

Fig. 2: **Relationship between different concentrations of DCM extract of garlic in sunflower oil (24 hours old) and mortality of *Rhipicephalus pulchellus* after 24 hours of exposure.**

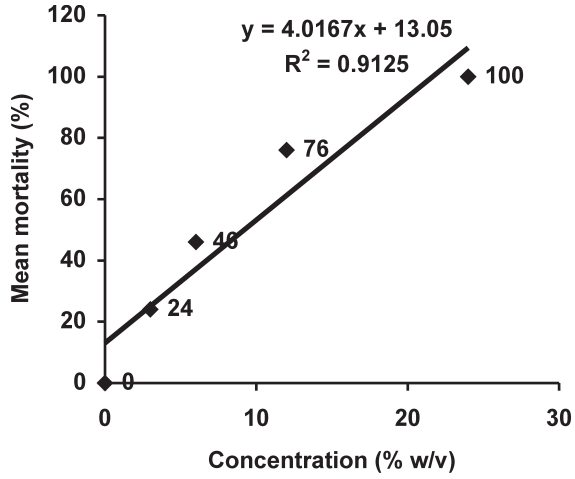

Fig. 3: **Relationship between concentrations of DCM extract of garlic in sunflower oil and mortality of *Hyalomma marginatum rufipes* after 24 hours of exposure.**

DISCUSSION

The results obtained in this study indicate that non-polar extracts of garlic (DCM extracts) were more toxic to both *H. m. rufipes* and *R. pulchellus* ticks than mid-polar (acetone) and polar (ethanol) extracts. This strengthens previous findings wherein less polar extracts of *Melia azedarach* ripe fruits were more effective against larvae and engorged female *B. microplus* ticks than polar extracts of the same plant[3]. In the Type A contact toxicity bioassay, DCM extract of garlic killed 100 % *H. m. rufipes* within an hour. This finding coupled with the fact that the potency of the extract persisted to the 2nd day presents *A. sativum* as a possible source of agents that can be used to control ticks. The tick mortality results obtained with acetone and ethanol extracts of *A. sativum* should be cautiously viewed. Because the acetone and ethanol extracts were viscous, ticks became submerged in these extracts. Consequently, the observed mortality of ticks that resulted from ethanol and acetone extracts of *A. sativum* in this study may be due to suffocation. We therefore suggest that future investigations on the toxicity of acetone and ethanol extracts of *A. sativum* to ticks should use alternative bioassays which will avoid ticks becoming submerged in these extracts. In the Type B bioassay, aqueous extracts of garlic showed weak bioactivity against adults of *H. m. rufipes.*

In the Type C contact toxicity bioassay, the toxicity of garlic significantly ($P < 0.05$) increased with concentration when

Table 4: **Mean percentage mortality of DCM extract of garlic in sunflower oil on contact with *Hyalomma marginatum rufipes* adults in Type B contact toxicity bioassay. Control had sunflower oil only.**

Conc. % w/v	Mean % mortality of ticks after 24 hours	±SE	*t*-statistic for slope *P*	Mean % mortality of ticks after 48 hours	±SE	*t*-statistic for slope *P*
3	24	0.244	Rs	0	0	Rs
6	46	0.4		0	0	
12	76	0.245		6	0.4	
24	100	0		34	0.245	
0 (control)	0	0		0	0	

Rs, indicates significance ($P < 0.05$) in concentration-mortality response relationship following regression analysis. Where 24 hours regression coefficient (r) = 0.9553 and P (2-tailed) = 0.0113, and 48 hours regression coefficient (r) = 0.9424 and P (2-tailed) = 0.0165. w/v = weight/volume, Conc. = concentration.

Table 5: **LC_1 and LC_{50} for DCM extract of garlic in sunflower oil on adults of *Rhipicephalus pulchellus* and *Hyalomma marginatum rufipes*.**

Tick species	Time (h)	LC_1 (%)	Lower CI	Upper CI	LC_{50} (%)	Lower CI	Upper CI
H. m. rufipes	24	0.9	0.48	1.44	5.9	4.9	6.99
	48	8	3.58	10.9	30	24.5	47.87
R. pulchellus	24	3.99	2.87	4.94	10.3	9.24	11.58
	48	5.46	3.44	7.12	18.1	15.91	21.26

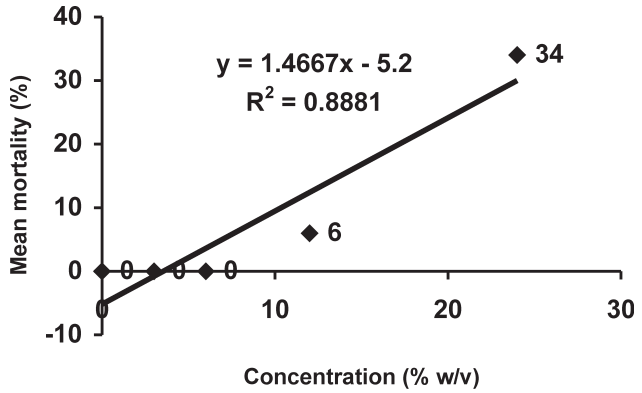

Fig. 4: **Relationship between concentrations of DCM extract of garlic in sunflower oil (24 hours old) and mortality of *Hyalomma marginatum rufipes* after 24 hours of exposure.**

tested against *H. m. rufipes* and *R. pulchellus* adults, respectively. The good correlation between concentration and mean mortality indicates that either the higher concentrations dissolved effectively in the sunflower oil or that suspended particles also had an effect.

There was a significant ($P < 0.05$) difference between LC_{50} values of the DCM extract of garlic and sunflower oil on *R. pulchellus* (10.3 % w/v) and *H. m. rufipes* (5.9 % w/v) 24 hours after preparation of extracts. It is important to note that the potency of the mixture persisted to the 2nd day. The LC_{50} of *H. m. rufipes* after 48 hours could not be determined because less than 50 % of the ticks died at the highest concentration (24 % w/v). However, the LC_{50} of DCM extracts of garlic in sunflower oil on *R. pulchellus* was lower (18.1 % w/v) when ticks were tested 24 hours after preparation of the extract. These results suggest that the mixture of sunflower oil and DCM extract of garlic is less toxic to *R. pulchellus* than *H. m. rufipes* and as time progressed *R. pulchellus* became more susceptible to the toxic effect of the mixture compared with *H. m. rufipes.* It should be noted that as time progressed the compounds in garlic such as allicin (S-allyl-cysteine) probably decomposed into dially disulphide (DADS)[26] and may have become more or less potent to any of the 2 species. A similar observation was made with the LC_1 values on *R. pulchellus* and *H. m. rufipes.* The LC_1 is an important indication of the concentration at which mortality begins.

The idea of investigating the effects of combined plant products has been exploited by many authors. For example, Rajapakse and Van Emden[24] investigated the arthropocidal effects of botanical oils as well as the combined effects with other plant products. Pregnant Karen women preferred DEET or permethrin mixed with "Thanaka" (a root paste from the medicinal plant *Limonia acidissima)* and the combination increased bioactivity of the chemical repellents by providing protection against mosquito bites for up to 10 hours[17]. In a related study, a ground mixture of dried tobacco leaves and a mineral called 'Magadi soda' proved to be effective as an acaricide against all stages of *R. appendiculatus*[8].

The development of effective acaricidal formulations that are inexpensive and less toxic to the environment should receive urgent attention. The use of *in vitro* bioassays in the screening of plants with potential acaricidal properties is a useful tool to achieve this objective.

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