African Journal of Biotechnology Vol. 7 (4), pp. 476-481, 19 February, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

The anti-tick properties of the root extracts of *Senna italica* subsp. *arachoides*

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Accepted 15 November, 2007

This study examined the anti-tick properties of the root extracts of *Senna italica* subsp. *arachoides* against adults of *Hyalomma marginatum rufipes*. Of the hexane, chloroform, dichloromethane, ethyl acetate and methanol extracts tested, only ethyl acetate extracts proved to be potent against adults of *H. marginatum rufipes*. The acaricidal activity of the ethyl acetate root extract of *S. italica* subsp. *arachoides* increased significantly ($P < 0.05$) with concentration when tested against *H. marginatum rufipes*. The potency of the extract persisted to the second day. The LC_{50} of the ethyl acetate root extract of *S. italica* subsp. *arachoides* in 24 h was 8.66% (w/v) while in 48 h was 3.59% (w/v). Chemical characterization of the extracts revealed 1,2-benzenedicarboxylic acid, dibutyl ester, 1,8-dihydroxy-3-methylanthraquinone, 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, hexadecanoic acid, 9-hexadecanoic acid as components.

Key words: *Senna italica* subsp. *arachoides*, *Hyalomma marginatum rufipes*, contact toxicity, root extract.

INTRODUCTION

The use of synthetic acaricides for control of tick populations is marred by problems which include environmental pollution and the demise of non-targeted species (Boeke et al., 2004). This situation creates the need for alternative tick control methods with lesser problems to the environment. Scientific research on plant-based products that are toxic to ticks is intensifying. This is primarily a result of the recognition of plants as the potential sources of anti-tick agents by many scientists (e.g. Abdel-Shafy and Zayed, 2002; Franscisco et al., 2003). One of the commonly cited advantages that may result from the use of botanicals for tick control is their biodegradability (Liang et al., 2003). This would make botanical acaricides to be less toxic to the environment and non-targeted species. So far, promising results have been obtained from some plants screened for anti-tick properties. For example, Wilson and Surthest (1990) reported on the sticky secretions of some tropical pasture legumes of the genus *Stylosanthes*, which immobilize and kill ticks. Also, Nchu et al. (2005) demonstrated the toxic effects of dichloromethane extracts of garlic (*Allium sativum*) bulb on adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus*. Most recently, Thorshell et al. (2006) demonstrated that oils of citronella, lavender, lily of the valley and peppermint have similar repellent effects as the commercially traded DEET (N, N-diethyl-m-tuluamide).

However, despite the promising results indicated in the foregoing paragraph many plants are still scientifically untested for anti-tick properties. In an attempt to contribute towards this need, we examined the effects of the root extracts of *Senna italica* subsp. *arachoides* (Family Caesalpiniaceae), commonly known as Sebete amongst the Batswana people of South Africa and Botswana, against adults of *H. marginatum* rufipes. Traditional claims by the Batswana people are that the root extracts of *S. italica* subsp. *arachoides* have among others, anti-tick properties. The tick *H. marginatum rufipes* used in this study is indigenous to Southern Africa and transmits Crimean-Congo Haemorrhagic Fever virus to man and animals (Horak et al., 2001).

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Table 1. Mean weights (g) of the root extracts of *Senna italica* subsp. *arachoides* residues obtained following evaporation of the solvents. The weighed extracts were ready for use in direct contact toxicity bioassay

Crude mixture	Chloroform	Ethyl acetate	DCM	Hexane	Methanol
5 g/50 ml	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.01	0.01 ± 0.02	0.05 ± 0.06
10 g/50 ml	0.04 ± 0.02	0.02 ± 0.01	0.04 ± 0.02	0.01 ± 0.02	0.09 ± 0.13
15 g/50 ml	0.05 ± 0.02	0.16 ± 0.05	0.04 ± 0.02	0.02 ± 0.01	0.11 ± 0.02

MATERIALS AND METHODS

Ticks

Unfed 3 weeks old adult ticks of *H. marginatum rufipes* from a pathogen free laboratory colony bred on rabbits were used in this study. Prior to and after infestation, these ticks were maintained in the laboratory at 25 ± 2ºC and 75 ± 5% RH in the Department of Biology at the University of Limpopo (Medunsa Campus). The relative humidity (RH) was attained by using saturated sodium chloride solution in glass humidity chambers according to methods described by Winston and Bates (1960).

Preparation of crude plant extracts

S. italica subsp. *arachoides* was collected from Moruleng village (a village of the Batswana people) of Rusternburg. A garden fork was used to dig the plant and its roots out of the ground. The plant was first identified by a plant taxonomist in the Department of Biology at the University of Limpopo prior to being used in the experiment.

Dried roots of *S. italica* subsp. *arachoides* were crushed into finer particles using iron pestle and mortar. The particles were then weighed into 5, 10 and 15 g placed into different glass beakers. For each weight category, five different extracts were prepared using 50 ml of each of the following solvents: hexane, chloroform, dichloromethane, ethyl acetate and methanol to give crude mixtures. The solvents were selected based on the differences in their polarity. The crude mixtures were allowed to stand overnight prior to filtration. About 10 ml of the supernatant from each beaker was filtered out using Whatman no. 1 filter paper into separate vials of known weights. For each solvent type, a control was prepared by adding the same volume of the solvent in control vials as in the treatment set-up. The solvents in both the treatment and control set-ups were then allowed to evaporate completely overnight in a fume chamber. Following evaporation of solvents, thin layers of residues were obtained in the treatment vials only (Table 1).

Bioassay procedures

The extracts of *S. italica* subsp. *arachoides* derived as detailed in the foregoing paragraphs were tested for anti-tick properties using free contact and topical application. These bioassays have been successfully used previously by other researchers (Pascual-Villalobos and Robledo, 1998; Nchu et al., 2005) in screening plants for anti-tick properties.

Free contact

Ten unsexed ticks (*H. marginatum rufipes*) were included in each vial of the treatment and control groups. The vials were then closed with a mesh and rubber bands to prevent ticks from crawling out. Tick mortality was recorded after every 24 h for two weeks. In order to assess the stability of the potent extract (i.e. extract that killed ticks in the initial 24 h), dead ticks were replaced by fresh ones. Four replications were done only for the ethyl-acetate extract because it is the only one that showed some potency against ticks.

Topical Application

In this bioassay only ethyl acetate extracts of *S. italica* subsp. *arachoides* were used because they showed some potency against ticks in the previous bioassay. *S. italica* subsp. *arachoides* extracts were prepared in the same way as in free contact bioassay. However, for each treatment 10 ml of each solution was filtered out and the solvent completely evaporated. The following amounts of residues were obtained: 5 g yielded 0.151 g, 10 g yielded 0.248 g and 15 g yielded 0.317 g. The residues obtained were then mixed with 2.5 , 2.1 and 1.3 ml of sunflower oil (Garol 100% Pure Sunflower oil) to yield 0.151 g/ 2.5 ml (6%, w/v), 0.248 g/ 2.1 ml (12%, w/v), and 0.317 g/ 1.3 ml (24%, w/v) respectively. Each mixture was carefully stirred for 3 minutes and left standing overnight to ensure homogeneity. Fresh ticks were randomly divided into treatment and control groups, each with 10 ticks. For each tick in the treatment group, 10 µl of a mixture of ethyl-acetate extract of *S. italica* subsp. *arachoides* and sunflower oil was topically applied on the dorsal surface of the Idiosoma using a micropipette (Nichipet EX, Nichiryo 4-10 Iwamoto, 2-chome). Similar procedures were carried for ticks in the control group except that only sunflower oil was applied on ticks. Four replications were done for each concentration indicated above. Tick mortality was recorded after every 24 h in 48 h. Data are presented as percentage mortality.

Fractionation and identification of extracts

In this study, the chemical components of the root extracts of *S. italica* subsp. *arachoides* were separated and identified using Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance (NMR) and Gas Chromatography-Mass Spectrometry (GC-MS). These techniques are respectively used to fractionate, identify and quantify components of a mixture of substances (Houghton and Raman 1998).

Plant extract

Four beakers, each containing 20 g of finely crushed root material were prepared. Each 20 g of plant material was extracted with 60 ml of each of the following solvents: dichloromethane, chloroform, hexane and ethyl acetate. The mixtures were left standing overnight prior to filtration. About 20 ml of the supernatant from each mixture was filtered out using Whatman no. 1 filter paper on Buchner funnel. The solvents were subsequently evaporated completely in a fume chamber, yielding residues indicated in Table 2.

Chromatography

TLC using silica gel plate was done to determine the number of components in each extract. Each yielded extract indicated in Table

Table 2. Quantity extracted from *S. italica* subsp. *arachoides* roots.

Solvent (60 ml)	Mass extracted (g)	Yielded weight (g)
Dichloromethane	20	0.025
Chloroform	20	0.020
Hexane	20	0.015
Ethyl acetate	20	0.022

2 was dissolved in 2 ml of dichloromethane (DCM) and 10 µl of each mixture was spotted (loaded) with a micropipette 1 cm from the bottom of a silica gel plate. The plate was then introduced into a TLC tank containing the standardized mobile phase. The mobile phase used consisted of standardized solvent mixture of hexane: ethyl acetate (7:1), routinely used in the Department of Chemistry and Biochemistry at the University of Limpopo, Medunsa Campus, while the stationary phases used were silica gel plate. The plate was run until the mobile phase was approximately 1 cm from the top. The silica gel plate was subsequently removed from the tank and air-dried. Separated components were visualized under UV light (F_{254} and F_{365}, Camal Universal UV lamp TL600). To improve detection of compounds, the TLC chromatograms were sprayed with vanillin-sulphuric acid. The plate was heated at 100°C for 4 min for optimal colour development.

Chloroform extract was selected for TLC analysis using glass silica gel (preparative) plate (1000 microns, Silica gel GF) because it extracted more clearly defined components than other solvents (see Figure 4). About 40 µl of the mixture was spotted on a glass silica gel (preparative) plate (1000 microns) and developed as described in the case of TLC using ordinary silica gel plates described above. Once air-dried, each chromatogram together with the silica gel, were scrapped into separate beakers. Chloroform was added to each beaker and filtered using cotton wool on Buchner funnel to remove the silica gel. Chloroform was also evaporated, leaving residues that were subsequently analyzed using GC-MS and NMR.

GC-MS analysis

The samples were analyzed using QP 20 -10 Shimadzu GC-MS equipment. Supelco equity 1 column with a film thickness of 30 m x 0.25 microns was used. The total flow rate was 24 ml/min and column flow rate was 1 ml/min. 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°C 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 x height) (Houghton and Raman, 1998).

NMR analysis

NMR was used to elucidate the structure of each of the six isolated compounds. Samples were dissolved in appropriate deuterated solvent (chloroform) for NMR analysis on a 300 MHz Varian NMR spectrometer, following methods used in the Chemistry Department of the University of Limpopo, Medunsa Campus. NMR spectra for ^{1}H and ^{13}C were recorded on Varian Mercury 300 MHz spectrometer with $CDCl_3$ as solvent. Chemical shifts were relative to tetramethylsilane (TMS). GC-MS results were verified by those of NMR.

Statistical analysis

Data are presented as mean percentage mortality in all toxicity bioassays. An association between percentage mortality and concentration of the extracts was determined using simple linear regression analysis. Probit analysis (a free software provided in the EPA website; http://www.epa.gov/nerleerd/stat2.htm) was used to determine LC_{50}.

RESULTS

Free contact bioassay

Data on percentage mortality of *H. marginatum rufipes* caused by the extracts of *S. italica* subsp. *arachoides* in free contact bioassay are summarized in Table 2. Of all the extracts tested, only the ethyl acetate extract of *S. italica* subsp. *arachoides* obtained from the 15 g/50 ml crude mixture produced 100% mortality of ticks in 24 h. Residues obtained from other solvents showed no anti-tick activity

Topical application bioassay

Data obtained in this study indicate that an increase in the concentration of the ethyl-acetate extracts of *S. italica* subsp. *arachoides* dissolved in sunflower oil, resulted in an increase in tick mortality (Table 3, Figures 1 and 2). The toxicity of the extract persisted to the second day. No mortality was recorded for *H. marginatum rufipes* ticks on which only sunflower oil was topically applied.

Probit – analysis

It was observed that the acaricidal activity of *S. italica* subsp. *arachoides* increased significantly ($P < 0.05$) with concentration when tested against *H. marginatum rufipes*. The potency of the extract persisted to the second day. The LC_{50} decreased with an increase in the duration of tick exposure to the mixture of ethyl acetate extract of *S. italica* subsp. *arachoides* and sunflower oil (Tables 4 and 5). The LC_{50} of the ethyl acetate extract of *S. italica* subsp. *arachoides* and sunflower oil in 24 h was 8.66% (w/v) while in 48 h was 3.59% (w/v).

Table 3. Percentage (%) mortality of *H. marginatum* rufipes caused by contact with crude extracts of root of *S. italica* subsp. *arachoides* (n = 10 ticks/experiment X 4 replications)

Solvent	5 g /50 ml				10 g /50 ml				15 g /50 ml			
	24 h		48 h		24 h		48 h		24 h		48 h	
	C	T	C	T	C	T	C	T	C	T	C	T
Chloroform*												
Ethyl acetate	0	0	0	0	0	0	0	0	0	100	0	100
DCM*												
Hexane*												
Methanol*												

C = Control; t = Treatment; * = no mortality recorded.

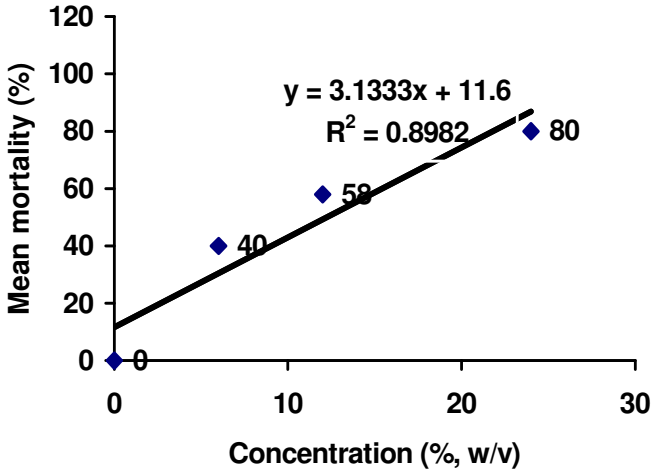

Figure 1. Association between concentrations of ethyl acetate extract of *S. italica* subsp. *arachoides* in sunflower oil and mortality of *H. marginatum* rufipes for 24 h during topical application.

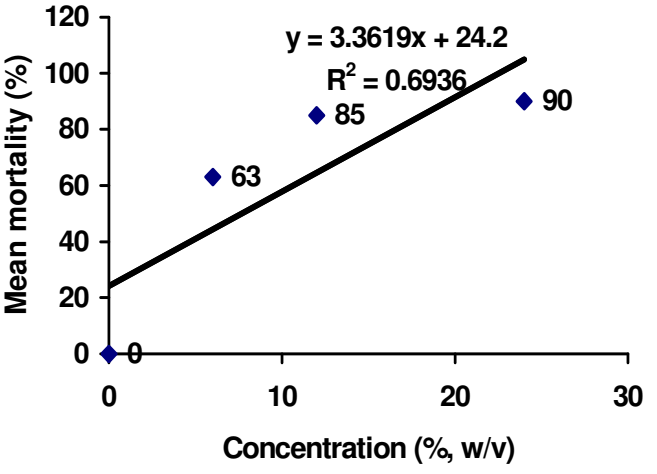

Figure 2. Association between concentrations of ethyl-acetate extract of *S. italica* subsp. *arachoides* in sunflower oil and mortality of *H. marginatum rufipes* for 48 h during topical application

Table 4. Mean percentage mortality (±SD) of *H. marginatum rufipes* subjected to topical application with ethyl acetate extract of *S. italica* subsp. *arachoides* dissolved in sunflower oil (n = 10 ticks/ experiment X 4 replications)

Duration	Concentration		
	6%	12%	24%
24 h	40 ± 4.08[a]	57.5 ± 2.5[b]	80 ± 8.16[c]
48 h	62.5 ± 6.29[a]	85 ± 5[b*]	90 ± 4.08[b]
Control	0	0	0

Similar superscript in a row indicates lack of significant differences

Table 5. LC_{50} for ethyl acetate extract of *S. italica* subsp.*arachoides* in sunflower oil on adult *H. marginatum rufipes*

Time	LC_{50} (%)	Lower CI	Upper CI
24 h	8.66	5.08	11.68
48 h	3.59	0.40	5.99

Chromatography

Dichloromethane and chloroform extracts showed six spots when hexane and ethyl-acetate extracts showed fewer spots (Figure 3).

GC-MS and NMR analysis

The components identified following GC-MS and NMR analysis are indicated in Table 6. However, no conclusive result could be arrived at for component 1.

DISCUSSION

Data obtained from the direct contact bioassay used in this study show that ethyl- acetate extracts of *S italica* subsp*. arachoides* have anti-tick properties compared to the residues of other extracts. The residue obtained from the crude mixture (15 g/50 ml) of ethyl-acetate extracts of

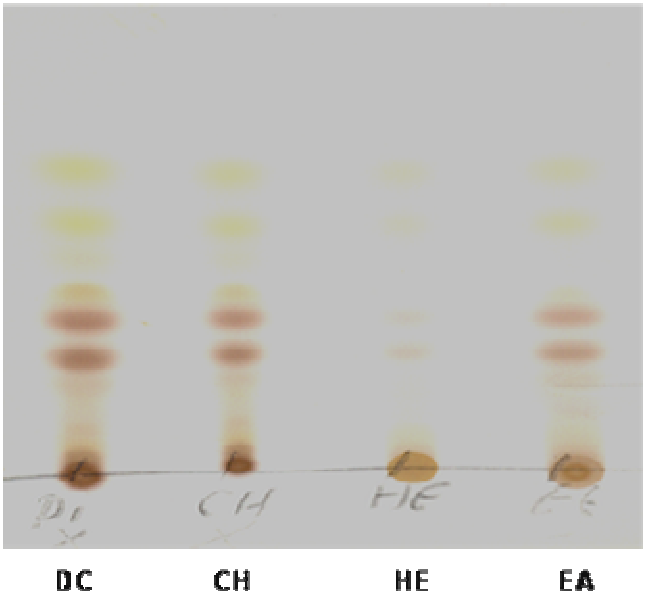

Figure 3. TLC chromatograms DC =Dichloromethane, CH = Chloroform, HE = Hexane and EA = Ethyl acetate

Figure 4. TLC chromatogram of chloroform extract on TLC preparative plate.

S. italica subsp. *arachoides* produced 100% mortality of ticks in 24 h. In addition, the potency of this extract against ticks persisted to the second day. No tick mortality was recorded for the control group. These findings undoubtedly present *S. italica* subsp. *arachoides* as a possible source of anti-tick agents. Furthermore, the results obtained in this study suggest that the active compound of *S. italica* subsp. *arachoides* dissolved in mid-polar solvent, ethyl acetate, than other polar or non-polar solvents. These results substantiate the findings by Mi-Kyeong et al. (2004), in which mid-polar extracts of 28 medicinal plants were found to be more effective against larvae of *Attagenus unicolor japonicus* (Japanese black carpet beetle) than less polar extracts of the same plants.

In topical application bioassay, it was found that tick mortality increased significantly ($p < 0.05$) with an increase in concentration of ethyl-acetate extract of *S. italica* subsp. *arachoides*. The good correlation between concentration and mean mortality indicates that either the higher concentrations dissolved effectively in sunflower oil or that the suspended particles had direct effect on ticks. The LC_{50} decreased with an increase in the duration of exposure to the ethyl-acetate extract of *S. italica* subsp. *arachoides* and sunflower oil concentration. The LC_{50} at 48 h (3.59%, w/v) is lower than that at 24 h (8.66% w/v), suggesting that prolonged periods are required in order to have an effect at lower concentrations. These results consolidate findings by Zhao and Newman (2004), in which the effect of exposure duration and latent mortality was investigated. These authors found that the higher the concentration of the extracts, the lesser the time needed for a certain proportion of arthropod pests to die.

The idea of investigating the effects of combined plant products has been exploited by many researchers. For example, Rajapakse and Van Emden (1997), investigated the arthropocidal effects of botanical oils as well as combined effects with other plant products. Lindsay et al. (1998) found that pregnant women preferred DEET or permethrin mixed with “Thanaka” (a root paste from the medicinal plant *Limonia acidissima*) and the combination increased the bioactivity of the chemical repellents by providing protection against mosquito bites for up to 10 hrs. Also Kéïta et al. (2003), investigated the effect of a mixture of Kaolin (fine clay powder) and essential oil, and observed that there was an increase in mortality of arthropod pests.

The results obtained in dipping bioassay suggest that polar aqueous extracts of *S. italica* subsp. *arachoides* did not have any activity against *H. marginatum* rufipes since no mortality was observed in this bioassay.

In order to fully understand the biological activity of the root extracts of *S. italica* subsp. *arachoides*, it became necessary to separate and identify their components. According to Vuorela et al. (2004) naturally derived compounds play a major role as lead structures for the development of synthetic analogues. Furthermore, the characterization of components in plant extracts may lead to the identification of novel compounds with anti-tick properties. Of the five compounds of *S. italica* subsp. *arachoides*, chrysophanic acid (1,8-dihydroxy-3-methylanthraquinone) and hexadecanoic acids have been previously identified in plant-based material with arthropocidal activities. Members of the genus *Senna* with high levels of chrysophanic acid are known to have anti-fungal, anti-bacterial and anti-mite properties (Phongpaichit et al., 2004; http://www.mehandi.com/hair/cassiaobavata.html). Also, hexadecanoic acid was identified in a fecal shield of su-

Table 6. Compounds that were identified from extracts of *Senna italica* subsp. *arachoides*

Component	Name of compound	Relative amount
1	Unknown	-
2	1,2-Benzenedicarboxylic acid, dibutyl ester	2.32
3	1,8-dihydroxy-3-methylanthraquinone	76.41
4	1,2 -Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	20.19
5	Hexadecanoic acid	51.55
6	9-Hexadecanoic acid	11.84

mac flea beetle, *Blepharida rhois* which serves as a very effective chemical defense against a naturally relevant, generalist predatory ant (Vencl and Morton, 1998). These authors found that the original source of the compounds identified in the fecal shield was the host plant of sumac flea beetle. Given the ant-tick properties of *S. italica* subsp. *arachoides* demonstrated in this study, it is reasonable to attribute these findings, at least in part, to the presence of chrysophanic and hexadecanoic acids in the extracts of this plant.

The possible role of other compounds identified in this study cannot be accounted for particularly with regard to the results obtained. However, it is important to note that the results obtained in this study may be due to the additive and synergistic combinations of most of the compounds contained in the roots of *S. italica* subsp. *archoides*. Liu (2003) suggested that isolated compounds may either loose their bioactivity or may not behave the same way as the compound in a whole mixture.

In summary, the present study demonstrated that the root extract of *S. italica* subsp. *archoides* may be a source of anti-tick agents. However, further studies are needed to further elucidate the efficacy of the identified compounds.

ACKNOWLEDGEMENTS

We appreciate the University of Johannesburg for GCMS analysis. Also thanks are due to the University of Limpopo for providing space and equipment for the study.

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