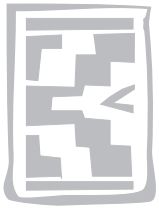

Determination and quantification of the *in vitro* activity of *Aloe marlothii* (A. Berger) subsp. *marlothii* and *Elephantorrhiza elephantina* (Burch.) skeels acetone extracts against *Ehrlichia ruminantium*

V. NAIDOO[1], E. ZWEYGARTH[2] and G.E. SWAN[1]

ABSTRACT

NAIDOO V., ZWEYGARTH, E. & SWAN, G.E. 2006. Determination and quantification of the *in vitro* activity of *Aloe marlothii* (A. Berger) subsp. *marlothii* and *Elephantorrhiza elephantina* (Burch.) skeels acetone extracts against *Ehrlichia ruminantium. Onderstepoort Journal of Veterinary Research*, 73: 175–178

An *Ehrlichia ruminantium* culture system was utilized for the anti-rickettsial evaluation of two ethnoveterinary plants, *Elephantorrhiza elephantina* and *Aloe marlothii.* Well-established *E. ruminantium* cultures were incubated with the plant leaf acetone extracts and compared to oxytetracycline and untreated controls. Effectivity was established by comparing the percentage parasitised cells and the calculation of both EC_{50} and extrapolated EC_{90} in µg/mℓ. The plant extracts were also screened for antibacterial activity using bioautography. *Elephantorrhiza elephantina* and *A. marlothii* demonstrated anti-ehrlichial activity with an EC_{50} of 111.4 and 64.5 µg/mℓ and EC_{90} of 228.9 and 129.9 µg/mℓ, respectively. The corresponding EC_{50} and EC_{90} for oxytetracycline was 0.29 and 0.08 µg/mℓ. Both plants appeared to produce their inhibitory activity by a similar mechanism, unrelated to that of the tetracyclines. Both the plant acetone extracts demonstrated antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (ATCC strains).

Keywords: *Aloe marlothii*, antibacterial, anti-ehrlichial, *Ehrlichia ruminantium*, *Elephantorrhiza elephantina*, ethnoveterinary, herbal remedy, screening

INTRODUCTION

Ehrlichia ruminantium is the cause of a tick-borne rickettsial disease known as heartwater, which affects both domestic and wild ruminants in South Africa (Du Plessis, De Waal & Stoltz 1994). The disease, which is characterised by severe non-specific neurological signs such as ataxia, chewing movements, twitching of the eyelids, circling, aggression, apparent blindness, recumbency, convulsions and in many cases death (Prozesky 1987; Yunker 1996), represents a major economic burden to the informal small-stock farming industry. In endemic heartwater areas of South Africa it is believed that the mortalities due to heartwater are three times greater than the combined deaths caused by babesiosis and anaplasmosis (Bezuidenhout, Prozesky, Du Plessis & Van Ster 1994).

Currently available drugs for treatment are either the tetracycline antibiotics or sulphonamide group of antimicrobials (Van Amstel & Oberem 1987). In addition to these commercially available compounds, in the more rural areas of the country where the disease is believed to be more prevalent, many local

[1] Section of Pharmacology and Toxicology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[2] Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110 South Africa

Accepted for publication 5 April 2006—Editor

farming communities are using herbal remedies to treat infected animals (Van der Merwe, Swan & Botha 2001)

Seventeen plants have been documented as being possibly effective against the parasites causing redwater, heartwater and anaplasmosis (Van der Merwe *et al.* 2001). *Aloe marlothii* (A. Berger) subspecies *marlothii* (Asphodelaceae) and *Elephantorrhiza elephantina* (Burch.) Skeels (Fabaceae) were selected for *in vitro* evaluation of anti-ehrlichial activity in infected endothelial cells in a pure culture system. This selection was based on the ease of accessibility of naturally growing plant material in the Pretoria area, South Africa. In addition, since the currently available therapeutic agents that are used for the treatment of heartwater are also antibacterial in nature these plants extracts were also tested for their antibacterial activity against a single isolate of nosocomial Gram-positive (*Staphylococcus aureus*) and Gram-negative bacterial (*Escherichia coli*) species.

MATERIALS AND METHODS

Plant extraction

Plant material of *A. marlothii* and *E. elephantina* were collected in November and December, respectively, identified by the South African National Biodiversity Institute, Pretoria and voucher specimens were deposited in the Herbarium of the Onderstepoort Veterinary Institute (OVI) (N01 and N02). The remaining plant material was air dried at room temperature to constant mass and then soaked in 1:5 (m/v) ratio of plant material to acetone (Merck) on a shaker platform for 30 min. The supernatant was collected, filtered and dried to a powder in a rotary evaporator under vacuum at 60 °C. Extraction was thereafter twice repeated using the same plant material and method (Eloff 1998). All samples were pooled and stored after the third extraction.

In vitro model system

Endothelial cell line

The BA 886 bovine endothelial cell line established by Yunker, Byrom & Semu (1988) were used as host cells for *E. ruminantium* (Zweygarth & Josemans 2001). These were propagated in a medium consisting of Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12)(Sigma, St. Louis, MO, USA; D 0547) containing 15 mM HEPES and 1.2 g/ℓ sodium bicarbonate. It was further supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 2 mM/mℓ L-glutamine, 100 IU/mℓ penicillin and 100 µg/mℓ streptomycin. This medium was used for infected and uninfected cell cultures. The cells were cultured as monolayers at 37 °C in a humidified atmosphere of 5 % CO_2 in air and were used at passage 100 to 125.

Infection of cell cultures

A strain of *E. ruminantium* (Welgevonden stock), isolated in a tissue culture from a tick homogenate was used in the experiment (Zweygarth, Josemans & Horn 1998). Endothelial cell cultures heavily infected with *E. ruminantium,* after a 48-h incubation period, were harvested by scraping the cell monolayer into the above medium. The cell suspension was centrifuged (800 *g* for 10 min at room temperature) and 200 µℓ volume of the supernatant, containing elementary bodies, were distributed into individual 25 cm² culture flasks containing monolayers BA 886 cells and 2 mℓ of medium. These were incubated for approximately 3 h, prior to replacing the medium with 5 mℓ of fresh medium and adding 10 µℓ of test compound. Cultures were thereafter incubated for 48 h at 37 °C.

The dried plant extract was reconstituted in di-methylsulphoxide (DMSO) (Merck) and acetone in a ratio of 1:4 (v/v) to a final concentration of 50 mg/mℓ. Prior to the commencement of each experiment the plant extract was further diluted with the DMSO: acetone diluent to 10, 5, 2.5 and 1.25 mg/mℓ. The DMSO: acetone diluent and one untreated flask was used as the negative control while oxytetracycline 10 % m/v (Intervet, SA) was used as the positive control.

All plant extracts were tested at a final concentration of 100, 50, 25 and 10 µg/mℓ, while the oxytetracycline was tested at 1, 0.1, 0.01 and 0,001 µg/mℓ due to the further dilution within the culture medium. Each plant extract and the oxytetracycline, at their various dilutions, were tested twice 7 days apart to obtain the average effective concentration.

Quantification of anti-ehrlichial activity

After 48 h the adherent cells in the culture flask were air-dried, methanol-fixed, stained with Diff Quick (Kyron, SA) and evaluated by light microscopy. The mean number of parasitized cells (MP) was determined by manually counting the number of infected cells in five microscope fields (300 X magnification) of 100 cells each. This figure was subsequently converted to a percentage (PP), by dividing the MP of the test substances with the MP of the negative control. This allowed for a comparison between the

effects of the four plant extracts and the oxytetracycline.

Significant differences between the cell counts of the various treatments were determined by analysis of variance (ANOVA) in Excel (Microsoft Office 2000).

Semi-logarithmic dose-response curves (natural log (ln) dose versus PP) were used to determine the concentration effective against 50 % and 90 % of the organism.

Bioautography

The dried plant extract was weighed, and made up into a stock solution of 20 mg/mℓ with acetone. Five microlitres of the extracts (equivalent to 100 µg dry mass) was loaded on Merck TLC F_{254} plates and eluted in either ethyl acetate: methanol: water (EMW) (10:1.3:1) or chloroform: ethyl acetate: formic acid (CEF) (5:4:1) in triplicate. Separated components were marked under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). One set of the eluted chromatograms was subsequently sprayed with 0.35 % vanillin (in 5 % H_2SO_4 in methanol) and developed at 105 °C to optimal colour development (Stahl 1969).

The second set of chromatograms was developed according to the bioautographic procedure described by Begue & Kline (1972). The dried TLC plates were sprayed with an actively growing strain of *S. aureus* (ATCC 29213) or *E. coli* (ATCC 25922), incubated overnight at 100 % humidity and subsequently sprayed with a 2 mg/mℓ solution of *p*-iodonitrotetrazolium violet (INT, Sigma). Any clear zones seen after a further 1 h of incubation indicated inhibition of growth.

RESULTS AND DISCUSSION

Anti-ehrlichial activity

The DMSO:acetone control had no inhibitory activity on the growth of *E. ruminantium*. This lack of activity is most likely linked to the second dilution that resulted upon addition of the culture medium, i.e. the original concentration of 1:4 was therefore reduced to below 1:400 (v/v).

Both *E. elephantina* and *A. marlothii* leaf extracts significantly reduced parasitized endothelial cells in culture ($P < 0.05$) (Table 1). The *A. marlothii* extract was twice as effective compared to *E. elephantina*. In addition to interfering with the growth of the parasites, the *E. elephantina* extract appeared to have an effect on the size of the *Ehrlichia* colonies that did grow, even at lower concentrations. These colonies were small and were particularly difficult to visualize. From this it may be concluded that this plant extract only slowed down the growth and proliferation of the colonies instead of killing the parasite. This tends to indicate that the plant extract has an inhibitory effect on the growth of the parasite instead of killing the parasite.

The EC_{50} and extrapolated EC_{90} values for the plants and the oxytetracycline control are listed in Table 2. When comparing the semi-logarithmic graphs (ln dose vs PP) (Fig. 1) of *E. elephantina* and *A. marlothii*, it was noted that the slope of the linear portions of the curves were parallel (55.8 and 57.2 on linear regression, respectively). It would appear that the mechanism of action is similar. The differences in potency obtained between the two plant extracts may be due to differences in concentration of active compounds within the plants.

TABLE 1 Percentage parasites for the plant extracts at the serial dilutions

Conc. (µg/mℓ)	*E. elephantina* (%)	*A. marlothii* (%)
100	56.19	20.66
50	94.86	73.23
25	98.50	103.00
10	101.28	102.36

TABLE 2 Effective concentrations (µg/mℓ) of *E. elephantina* and *A. marlothii* leaf extracts which suppress 50 % and 90 % of *E. ruminantium* in cell cultures compared to pure oxytetracycline derived by linear regression from the logarithmic dose-response curves

	E. elephantina	*A. marlothii*	Oxytetracycline
EC_{50}	111.398	64.548	0.290
EC_{90}	228.920	129.877	0.800

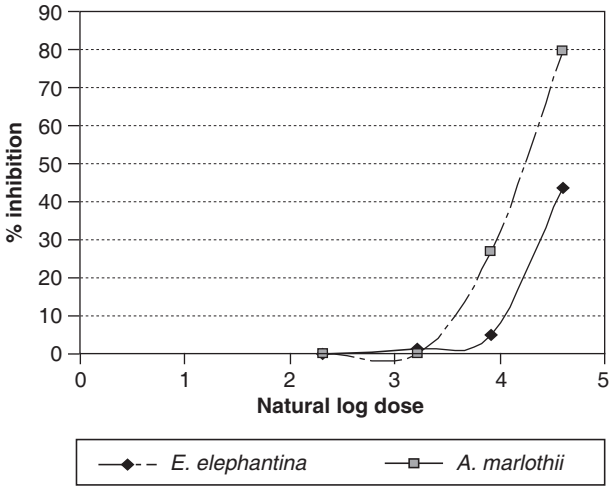

FIG. 1 Semi-logarithmic dose-response curves for *E. elephantina* and *A. marlothii* leaf extracts against *E. ruminantium*

Oxytetracycline exhibited a different dose response relationship compared to that of the plant extract. The slope was two log scales to the left and had a flatter best-fit slope (40 % ln dose^{-1}). The non-parallel nature of the graphs for the plant extracts and the oxytetracycline seems to indicate that of the mechanism of action induced by the plant extracts are different to that of oxytetracycline, i.e. inhibition of protein synthesis. However, Barrantes & Guinea (2003) described the presence of aloin in *A. barbadensis,* a compound with a similar structure to doxycycline, a tetracycline antibiotic. It is currently not known whether aloin is present in *A. marlothii.* Even though the plant extracts were less effective than oxytetracycline, possibly due to the crude nature of the extracts the activity observed in the case of the plant extracts is still considered to be significant.

Bioautography

Elephantorrhiza elephantina and *A. marlothii* leaf acetone extracts demonstrated one band with inhibitory activity against both *E. coli* and *S. aureus*. The degree of activity was, however, not quantified. From the chromatograms, the active compound in both plants had a similar retardation factor (*Rf*) of 0.9 when eluted in EMW, thereby suggesting that the active ingredient may be the same.

CONCLUSION

The *E. elephantina* and *A. marlothii* leaf extracts demonstrated good anti-ehrlichial and antibacterial activity. Based on dual anti-ehrlichial and antibacterial activity of existing commercial products it is suggested that the antibacterial compounds identified in the plant extracts may be also be responsible for the anti-ehrlichial activity. With the mechanism of action of the plant extract appearing to be different to that for the oxytetracycline it is possible that these plants may contain a novel antibacterial compound. Further research is required to isolate and characterise the active ingredients present in the plant extract.

ACKNOWLEDGEMENT

This study was supported by a National Research Foundation Grant on Indigenous Knowledge Systems (Gun 2053499).

REFERENCES

BARRANTES, E. & GUINEA, M. 2003. Inhibition of collagenase and metalloproteinases by aloins and aloe gel. *Life Science*, 72:843–850.

BEGUE, W.J. & KLINE, R.M. 1972. The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*, 64:182–184.

BEZUIDENHOUT, J.D., PROZESKY, L., DU PLESSIS, J.L. & VAN STER, S.R. 1994. Heartwater, in *Infectious Diseases of Livestock*, edited by J.AW. Coetzer, G.R. Thomson, R.C. Tustin & N.P.J Kriek. Cape Town: Oxford University Press.

DU PLESSIS, J.L., DE WAAL, D.T. & STOLTSZ, W.H. 1994. A survey of the incidence and importance of the tick-borne diseases heartwater, redwater and anaplasmosis in the heartwater-endemic regions of South Africa. *Onderstepoort Journal of Veterinary Research*, 4:295–301.

ELOFF, J.N. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, 60:1–8.

PROZESKY, L. 1987. The pathology of heartwater. III. A review. *Onderstepoort Journal of Veterinary Research*, 54:281–286.

STAHL, E. 1969. *Thin-layer chromatography: a laboratory handbook.* Berlin: Springer.

VAN AMSTEL, S.R. & OBEREM, P.T. 1987. The treatment of heartwater. *Onderstepoort Journal of Veterinary Research*, 54:475–479.

VAN DER MERWE, D., SWAN, G.E. & BOTHA, C.J. 2001. Use of ethnoveterinary medicinal plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. *Journal of the South African Veterinary Association*, 72:189–96.

YUNKER, C.E. 1996. Heartwater in sheep and goats: a review. *Onderstepoort Journal of Veterinary Research*, 63:159–170.

YUNKER, C.E., BYROM, B., SEMU, S. 1988. Cultivation of *Cowdria ruminantium* in bovine vascular endothelial cells. *The Kenya Veterinarian*, 12:12–16

ZWEYGARTH, E. & JOSEMANS, A.I. 2001. A chemically defined medium for the growth of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 68:37–40.

ZWEYGARTH, E., JOSEMANS, A.I. & HORN, E. 1998. Serum-free media for the *in vitro* cultivation of *Cowdria ruminantium*. *Annals of the New York Academy of Science*, 849:307–312.