Chapter 5

Biological activity of the extracts in test animals

5.1 Problem statement and aim of the exercise

As seen in the results thus far, *Terminalia sericea* had reasonable antibacterial activity against *Staphylococcus aureus*, so it was decided to test both the crude extract as discussed in 4.2.3 and terminoic acid isolated from the leaves of *T. sericea* in an animal model. A method had to be developed for efficacy testing against skin infections caused by *S. aureus.*

5.1.1 Introduction

5.1.1.1 Development of the animal model: criteria for investigation

Animal models may be developed in several ways to study antimicrobial agents (Hobson *et al.,* 1968), Sanford, M. *et al* (1967). A few of the methods used thus far are discussed below.

5.1.1.1.1 Expanded flora test

This method can be used to evaluate broad-spectrum antimicrobial activity against large numbers of Gram-positive and Gram-negative organisms introduced by pre-treatment

occlusion i.e. by closing the treated area to inhibit infection by external pathogens. The expansion of flora that normally occurs on the skin of the animal when an occlusive wrap is applied to the skin is inhibited by the introduced antibiotic. A plastic wrap is applied for 48 hours prior to the application of an antimicrobial agent. To be considered effective, a test material should destroy 99 percent of microorganisms. Bacterial counts will be low only if the antibiotic is active against both Gram-positive and Gram-negative organisms.

5.1.1.1.2 Reduction of expanded flora produced by occlusive wrapping of the site

The inhibition of the expanded flora can be observed by application of an antimicrobial agent after a sufficient time-period of occlusion with the wrap. Prevention of expansion of the flora can also be used to test antibacterial activity. The antibacterial agent is applied to the wound or test site and after continued occlusion, the inhibition of expansion of the flora is observed. The antimicrobial agent should eliminate the organism(s) from most lesions cultured within 18 hours after the agent is applied.

5.1.1.1.3 Persistence test

This test determines the reservoir effect of the antimicrobial agent, or its ability to bind to the stratum corneum to give a prolonged effect. In this test, the antimicrobial agent is usually applied three times a day for 3 days. After three days, these areas are occluded for 24 hours and then sampled for bacteria. Occlusion allows bacterial growth in the presence of the antimicrobial agent being tested. The persistence of antimicrobial activity after application on the skin extends the time over which an antibiotic can exert

its effect on the bacterial cell. This test is one way of measuring this characteristic on an animal model.

5.1.1.1.4 Occlusion test

This test primarily estimates the bacteriostatic activity of an antimicrobial agent against Gram-positive microorganisms found on normal skin, showing how well the agent prevents a small number of bacteria from rapidly proliferating. Lesions are produced either by stripping with cellophane tape or by application of ammonium hydroxide, a skin irritant that increases susceptibility to infections, followed by inoculation with pathogenic organisms (usually *staphylococci*) and are then covered by an occlusive wrap. Lesions may be treated with a test compound after inoculation and then inhibition of growth is observed or rapidity of healing is judged.

We decided to use the occlusion test for our *in vivo* experiments. To limit the use of animals as well as to prevent inter animal differential resistance we decided to use each animal as its own positive and negative control as described below.

5.2 Materials and Methods

5.2.1 *In vitro* testing

The sensitivity of four different strains of *S. aureus* was tested as an initial step in order to find a strain that was sensitive to the gentamycin (Garamycin^{R}) standard cream that

was to be used as a positive control. The National Committee for Clinical Laboratory Standards (NCCLS) strains ATCC 25923 (Cowan A), ATCC 25913 and two other available local laboratory strains were tested. The Cowan A strain was selected because it showed the greatest sensitivity in an *in vitro* test by Dr Maryke Henton from the microbiology laboratory at the Agricultural Research Council at Onderstepoort.

The two *T. sericea* test preparations (crude extract (4.2.3) and terminoic acid) as well as a gentamycin cream and gentamycin in an injectable form (Fermentycin^{R}) were evaluated. The cream as well as the injectable form was evaluated to have an alternative dosage form available if one should be unsuitable. Mueller-Hinton agar was used according to the NCCLS guidelines for a disk diffusion method. A form of an *in vivo* antibiogram test, based on NCCLS guidelines was employed. A lawn of *S. aureus* was prepared on the Mueller-Hinton agar. The various preparations (gentamycin cream and injectable form, *T. sericea* crude extract, and terminoic acid) were each placed in a 6 mm well. Amounts of 0.1 ml of each preparation were used in the test. The sensitivity or resistance of *S. aureus* to the preparations was then evaluated. The test was purely meant as a screening test and was performed by Dr Maryke Henton. In all cases the test organisms were sensitive to the different formulations. We consequently decided to use a cream as carrier base for the test material as the positive commercial control was in a cream, as well as the fact that creams are the most practical application for applying topical treatments. A 1% concentration of terminoic acid and a 20% concentration of the crude extract in emulsifying cream (British Pharmacopoeia) were prepared. The test substances were formulated into topical creams by blending using a mortar and pestle.

The positive control was a commercial 0.1% gentamycin cream (Garamycin^{R}) by Schering-Plough (SA).

5.2.2 *In vivo* testing

Rats were used for testing the antimicrobial effect of the *Terminalia* extracts. After promising results with the *in vitro* testing of antibacterial compounds from various *Terminalia* species and *Terminalia sericea* in particular (Eloff, 1999b; Chapter 4), it was decided to conduct an *in vivo* evaluation of the isolated compound as well as a crude extract of the plant. After a few different procedures were tested in preliminary experiments, a procedure was developed. The ethics committee of the Onderstepoort Veterinary Institute (OVI) approved the method and the procedure was carried out under the supervision of veterinarian, Dr. Johan Joubert – head of Toxicology.

The test method was applied to 11 rats supplied, fed and maintained at the Onderstepoort Veterinary Institute of the Agricultural Research Council's (ARC) Department of Toxicology.

The rat hair on the test area was removed by first cutting it with scissors and then shaving the skin with a blade. The area was then sterilised by cleaning it with 70% alcohol. The rats were sedated with a benzodiazepine derivative (Comelin^{R}) and left for 15 minutes for the drug to take effect. Lesions were produced by cutting four roughly circular areas (marked A = crude, B = terminoic acid, C = negative control (no treatment) and D = Positive control; gentamycin) of skin on the back of the rat on both sides with a pair of scissors and introducing the test organism (*Staphylococcus aureus*) onto the test area. The area was covered with an occlusive wrapping (Transpore^{R}) and left to

incubate for 48 hours. After 48 hours, the antimicrobial agents and gentamycin were introduced. The crude extract, terminoic acid (sample JK 3-2) and gentamycin control were applied to the marked sites A, B and C respectively, while D was left to serve as a negative control. Each animal therefore served as a control in itself by having two test sites for the crude and isolated compound, one for a positive control with gentamycin and one site as a negative control. Figure 5.1 shows a photograph of a rat prepared for an experiment. The wound sites were covered after initial digital images were recorded.

The resulting inhibition of growth or healing was quantified on the basis of erythema (red discoloration of wound), exudate (puss) formation and physical size of the lesion on a daily basis for 5 days. Factors such as muscle necrosis, foreign body, skin contamination and variation in number of organisms were taken into consideration as controls. Measuring the size of the lesion or the degree of healing of the lesion was used as one way to determine the antimicrobial activity. On each day, at 08:00 from day 1 to day 5, each infected site on each rat was inspected. The dressing was removed and the different parameters were measured and tabled. Thereafter, the test samples, positive control and new dressings were re-applied to each test rat and the animals were then replaced in their cages.

An arbitrary figure was allocated, as it was difficult to measure the degree of erythema as well as the quantification of the exudate that formed. Subsequently a scale from 1 to 5 was used with one being the lowest degree of erythema or exudate formed and 5 the highest degree of erythema or exudate formed. Quantification was by comparing initial erythema and exudate after incubation and before each treatment.

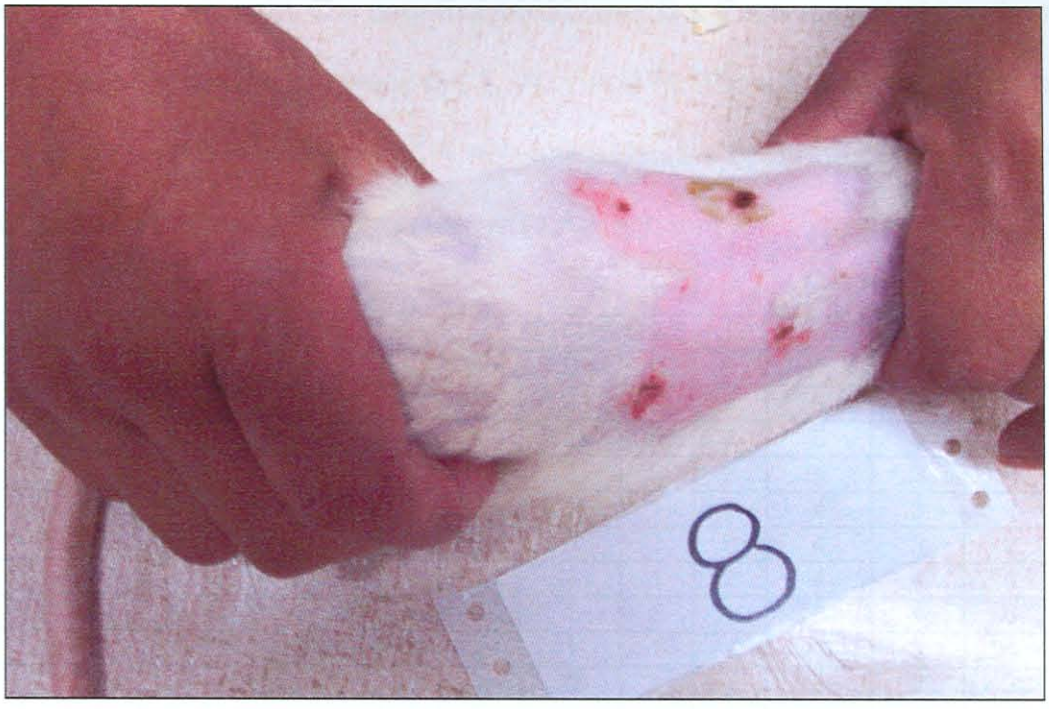

Fig. 5.1. Photograph of back of rat during experiment indicating treatment areas

5.3 Results

In the initial in vitro experiments on the sensitivity of *S. aureus* to gentamycin preparations, *T. sericea* crude extract and terminoic acid, the *S. aureus* strain used was sensitive to all the treatments and formulations.

The results of the *in vivo* rat model experiment are recorded in Table 5.2 and in Figures 5.1 to 5.5.

Table 5.1. Different parameters measured on the 11 rats on the different test sites (A = crude, B = Terminoic acid, C = negative control and D = Positive control (gentamycin)) on 5 consecutive days of the week (M, T, W, T and F). The exudate and erythema were measured on an arbitrary scale of 1-5 with one being the best rate of healing and five the worst while the lesion diameter was measured in mm

DAY	RAT NO	EXUDATE/5				ERYTHEMA/5				LESION SIZE (mm)			
		A	B	C	D	A	B	C	D	A	B	C	D
M	3	2	3	1	2	4	2	2	2	5	3	3	2
T		3	3	3	2	4	2	2	2	5	3	3	2
W		2	3	1	2	4	2	2	2	4	3	2	2
T		1	1	1	2	2	1	1	1	4	3	2	2
F		0	0	0	0	1	0	1	0	4	3	2	2
M	4	3	1	1	1	1	1	1	2	4	4	4	4
T		2	1	3	1	2	1	2	1	5	4	6	7
W		1	1	1	1	2	1	1	1	4	3	4	5
T		1	1	1	1	1	1	1	1	3	2	3	4
F		0	0	0	1	0	0	0	1	3	2	3	4
M	5	3	1	5	3	4	1	5	3	3	2	6	3
T		3	1	5	2	3	1	5	2	4	3	7	2
W		2	1	3	2	2	1	4	2	3	3	6	2
T		1	0	2	1	1	1	1	1	3	2	4	2
F		0	0	1	1	1	1	1	1	3	2	4	2
M	6	2	3	1	5	3	3	2	4	3	5	3	7
T		3	2	1	3	3	2	2	3	5	5	3	4
W		2	1	1	2	2	2	2	2	4	3	2	3
T		1	0	0	0	1	1	1	1	3	2	2	2
F		0	0	0	0	0	0	1	1	2	2	2	2
M	7	2	3	2	4	2	2	2	3	3	3	3	5
T		1	2	2	4	1	1	1	4	2	2	3	6
W		0	0	2	3	0	0	1	3	0	0	3	6
T		0	0	2	3	0	0	1	3	0	0	3	6
F		0	0	1	2	0	0	1	2	0	0	2	4
M	8	4	3	2	2	2	2	3	2	4	4	4	5
T		3	2	2	2	1	2	3	2	4	3	2	3
W		1	1	2	2	1	1	3	2	4	3	2	3
T		1	1	2	1	1	0	2	2	4	3	2	3
F		0	0	1	1	0	0	2	2	3	2	2	2

DAY	RAT NO	EXUDATE/5				ERYTHEMA/5				LESION SIZE (MM)			
		A	B	C	D	A	B	C	D	A	B	C	D
M	9	1	3	4	5	1	3	3	3	3	4	5	7
T		2	2	4	4	1	2	4	2	5	4	6	5
W		1	1	4	3	1	1	3	3	5	3	6	4
T		0	0	4	2	0	0	3	2	5	3	5	4
F		0	0	3	2	0	0	2	2	4	3	4	4
M	10	4	3	3	3	3	4	4	4	5	3	4	4
T		3	2	2	2	2	3	3	3	5	3	5	6
W		2	2	2	1	1	2	2	2	3	3	4	5
T		1	1	2	1	1	1	1	2	3	3	3	4
F		1	1	1	1	0	1	1	1	2	3	3	4
M	11	1	4	4	3	2	1	2	4	3	6	5	4
T		1	2	4	2	2	1	3	3	3	4	4	3
W		1	1	3	2	1	1	2	2	2	3	4	3
T		1	1	2	1	0	0	2	1	2	2	2	3
F		1	2	1	1	0	1	2	0	2	5	3	2
M	12	2	4	4	2	1	2	4	4	2	5	5	5
T		1	3	4	2	1	3	4	3	5	6	5	3
W		1	3	4	1	1	3	4	2	2	5	3	3
T		1	2	2	1	0	1	3	1	2	3	2	3
F		0	1	2	0	0	1	2	0	2	3	2	3
M	13	1	1	3	1	2	2	3	2	2	2	4	2
T		1	1	2	1	1	1	2	2	2	2	4	2
W		1	1	1	1	0	0	1	1	2	2	3	2
T		1	1	1	1	0	0	1	1	2	2	2	2
F		0	0	1	1	0	0	1	0	2	2	2	2
Av		1.29	1.4	2.1	1.8	1.27	1.2	2.15	1.96	3.14	2.96	3.49	3.54

Table 5.2. Average effect of four treatments on lesion size, erythema and exudate (From Table 5.1) Same letters indicate where no significant differences were found. P=0.05.

Treatment	Lesion size	Erythema	Exudate
Crude extract	3.14 a	1.27 d	1.29 h
Terminoic acid	2.96 b	1.20 e	1.40 l
Negative control	3.49 a, b	2.15 f	2.10 j
Gentamycin	3.54 c	1.96 g	1.80 k

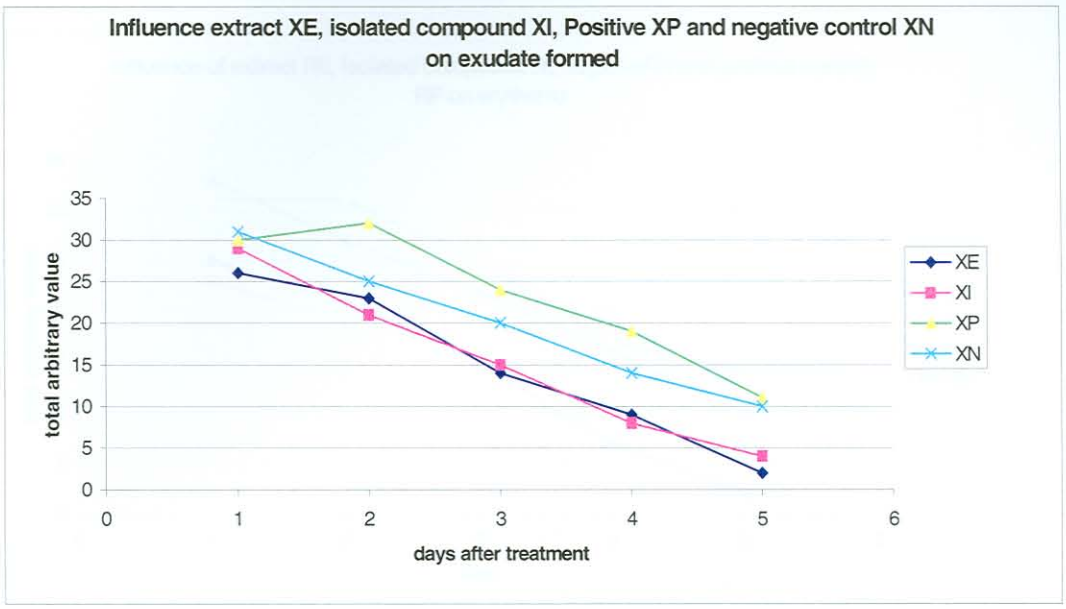

Fig. 5.2. The influence of the crude extract XE, the isolated terminoic acid XI, the positive control XP and the negative control XN on the exudate formed

5.3.1 Effects of extracts on exudate

On average there was more exudate formed from the control (2.1), followed by the gentamycin treatment (1.8) then terminoic acid (1.4) and then the crude extract (1.29) The difference over a time-period is presented in Fig 5.2. The crude extract and terminoic acid appeared to decrease the exudate formation, but the gentamycin treatment initially led to more exudate formation than the negative control.

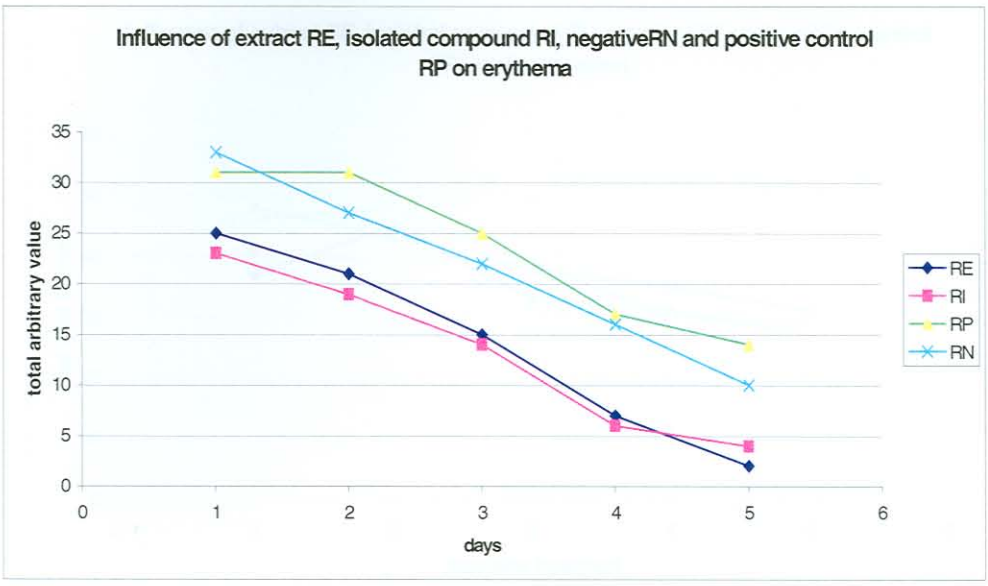

Fig. 5.3. The influence of the crude extract RE, isolated terminoic acid RI, positive RP as well as negative RN controls on the wound erythema

5.3.2 Effects of extracts on wound erythema

Terminoic acid and the crude extract proved to be better in reducing the erythema of the wounds, which is indicative of the infected state of the wound, than gentamycin as positive control and the negative control. These results agreed well with the results on exudate formation.

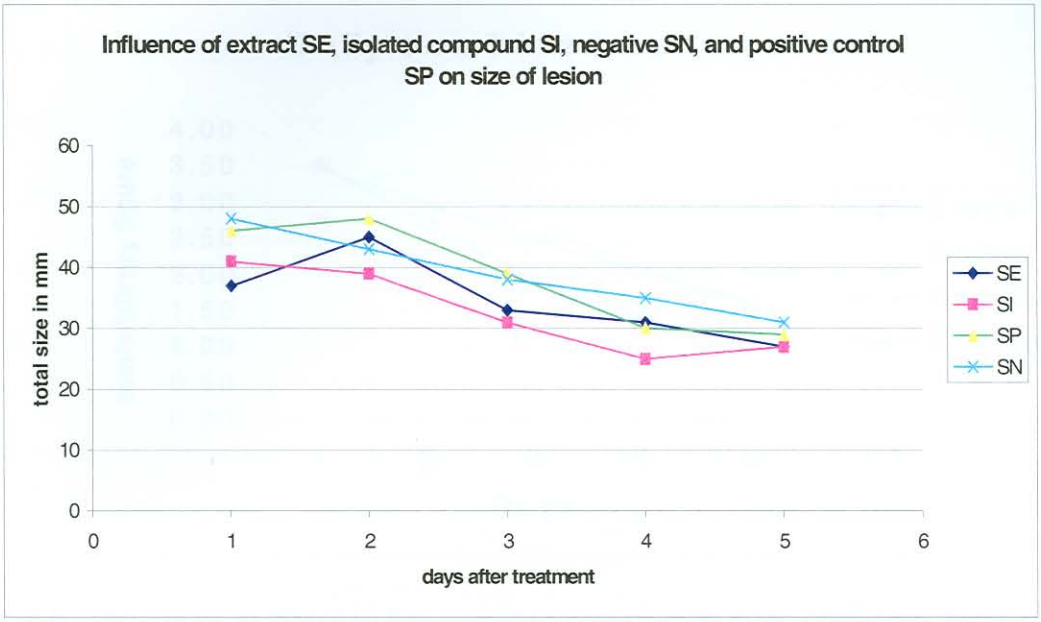

Fig. 5.4. The reduction in total diameter (mm) over a 5-day period of the different test sites using the crude extract, the isolated extract and gentamycin for the different animals.

5.3.3 Effects of extracts on wound size

The four treatments led to a similar result on wound size than the more subjective results obtained with erythema and exudate formation.

It is clear from Figure 5.5 that the crude and the isolated extract were more effective than non-treatment or gentamycin in reducing the size of the lesions.

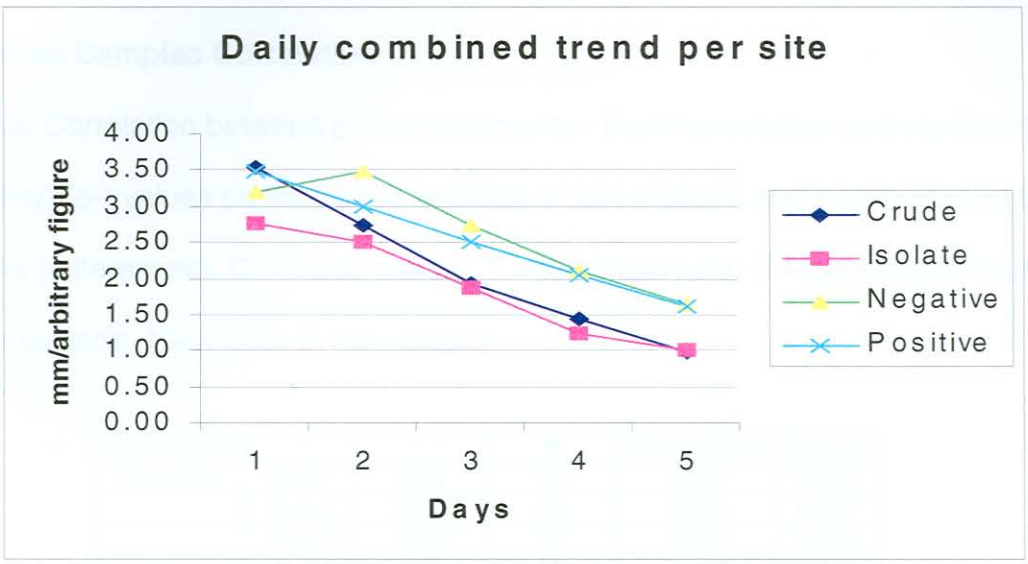

Fig. 5.5. The average effect of the treatments on lesion size, erythema and exudate formed on the test sites of the 11 rats over a five-day period.

5.3.4 Combined effects of extracts and controls

The combined effect of the three parameters on the sites (Figure 5.5) showed that the wound healing ability of the crude compound and terminoic acid was superior to that of the positive and negative controls.

To determine if the differences observed are significant, the data was analyzed using the student-t test with the help of Dr Annemarie Kruger of the Potchefstroom University for CHE. On each outcome (wound size, exudate and erythema), each test application was compared to each other (e.g. Treatment 1(crude extract) with Treatment 2 (terminoic acid) and Treatment 1 with Treatment 3(gentamycin) etc so a total of 6 correlation's were evaluated for each outcome to derive at the mean and p-values on paired and sample statistics.

5.3.6 Paired Samples Correlation

Table 5.3. Correlation between paired treatments - the N correlation and significance (probability) (p-) values on the paired samples of test medium and significance of the outcomes (differences). C = crude extract, T = terminoic acid, G = gentamycin and N = negative controls. (See table 5.1 for detail.)

Outcomes			N	Correlation	Sig.(p)
Exudate	Pair 1	C&T	55	0.609	0.000
	Pair 2	C&G	55	0.299	0.027
	Pair 3	C&N	55	0.350	0.009
	Pair 4	T&G	55	0.375	0.005
	Pair 5	T&N	55	0.474	0.000
	Pair 6	G&N	55	0.427	0.001
Erythema	Pair 1	C&T	55	0.572	0.000
	Pair 2	C&G	55	0.390	0.003
	Pair 3	C&N	55	0.396	0.003
	Pair 4	T&G	55	0.522	0.000
	Pair 5	T&N	55	0.487	0.000
	Pair 6	G&N	55	0.428	0.001
Lesion size	Pair 1	C&T	55	0.507	0.000
	Pair 2	C&G	55	0.344	0.010
	Pair 3	C&N	55	-0.003	0.980
	Pair 4	T&G	55	0.324	0.016
	Pair 5	T&N	55	0.128	0.352
	Pair 6	G&N	55	0.302	0.025

The significance values represent the p-values where > or = 0.05 means a statistically significant value and > or = 0.01 means a highly significant difference (Student t-test).

5.4 Discussion

5.4.1 Exudate

The average exudate formation of the crude extract treatment (1.29)(p=0.000) was highly significantly lower than terminoic acid (1.40) (p=0.000), gentamycin (1.8)(p=0.009) and the control (2.1) (p=0.005) treatment. Furthermore the exudate formed in the crude extract was significantly lower than the exudate formed with terminoic acid (1.4) (p=0.022). The average exudate formed with the gentamycin was highly significantly higher then the control treatment. The crude extract (20%) therefore produced less exudate than terminoic acid (10%) and gentamycin (1%). Gentamycin produced more exudate than the control treatment. There was no significant difference in the lesion size between crude extract and negative control and also between terminoic acid and negative control.

The production of exudate may be related to inflammation. The crude extract especially and also terminoic acid may have anti-inflammatory activity which would partially explain the decreased exudate. It is not easy to explain why the gentamycin produced more exudate than the control treatment, unless a component in the gentamycin formulation had an irritant effect. It may very well be that an aspect related to the cream may have had an effect because the negative control was not treated with a cream. Nevertheless the crude and terminoic acid formulated with the cream did not increase exudate formation.

The t-test between the crude extract and terminoic acid showed a highly significant difference (p= 0.000), whereas the t-test between the crude and negative was significant (p= 0.027) and between the crude and gentamycin highly significant (p= 0.009). The t-test between the terminoic acid and the positive (p= 0.000) and negative controls (p= 0.005) also proved to be highly significant.

The conclusion can thus be drawn that both the crude extract and terminoic acid were more effective than the positive and negative controls in decreasing formation of the exudate.

5.4.2 Erythema

The average exudate formation for terminoic acid (1.2) was highly significantly lower than values for gentamycin (1.96) (p=0.005) and the control (2.15) (p=0.005) while the crude was lower than terminoic acid treatment (1.2).

The t-test showed a highly significant difference between the crude extract and terminoic acid and the positive and negative controls (p = 0.000 - 0.003). The crude extract and terminoic acid differed significantly enough from the positive and negative controls to be regarded effective in the control of wound erythema.

5.4.3 Lesion size

The interpretation of the results of the lesion size proved to be more complex with highly significant differences occurring between crude (3.14 mm) and terminoic acid (2.96 mm) (p= 0.000) and significant differences between the crude (3.14 mm) and negative (3.54mm) (p= 0.010) to non-significant difference between terminoic acid and negative

control (p= 0.352) and a less significant difference between gentamycin and negative controls (p= 0.025). The explanation may be that the initial lesion sizes differed.

Table 5.4

The average lesion sizes at the start of the experiments.

Lesion	Average lesion size	Average lesion size(start)	Average lesion size (end)
Extract A	3.14	5	2
Terminoic acid B	2.96	3	2
Gentamycin C	3.49	3	2
Negative control D	3.54	2	2

The explanation for the averages in gentamycin and negative controls being higher than the initial values is that the parameter values increased on day 2 and only then started to decrease.

Overall, the statistical analysis verifies the conclusion that the crude *T. sericea* extract and isolated terminoic acid were more effective *in vivo* antibacterial compounds than the commercial gentamycin at the concentrations used.

5.5 Toxicology report

Dr. Johan Joubert, the Head of the Toxicology Department at the Onderstepoort Agricultural Research Institute carried out a post-mortem examination on the rats at the end of the *Terminalia* experiment.

The results indicated that rats numbers one and two used in the tests, number one had no subcutaneous lesions and internally had three pin-point sized abscesses in the lungs.

Rats two to thirteen showed no subcutaneous lesions, not even under the experimental skin lesions. Rat three had three pinpoint sized abscesses in the right lung. Rats two, four, five, nine, ten, eleven and twelve showed no internal abscesses or lesions. A single pea sized abscess was seen in the liver of rats six, seven, eight and thirteen.

Not one of the rats developed lesions or abscesses subcutaneously in the vicinity of the treated areas. This indicates that the liver and lung abscesses seen in a few of them most probably resulted from an infection in their brooder cages.

One can thus conclude that terminoic acid and the crude extract, as well as the controls had no toxic effects on the test animals and their antibacterial efficacy and possibly their anti-inflammatory effects, outweighs the possible toxic effects they might have.

A possible mistake with this study was that it was not blinded. I should have used codes for the different treatments assigned by someone else to ensure that unintentional bias did not occur when scoring the results.