CHAPTER 7

IN VIVO ANTIFUNGAL AND WOUND HEALING ACTIVITY OF *CURTISIA DENTATA* EXTRACTS, LUPEOL AND BETULINIC ACID

7.1. BACKGROUND

Recently, fungal infections, including *Candida albicans*, have increased owing to immunosuppressive therapy, chemotherapeutic treatments to cancer patients and an increase in HIV/AIDS cases. (Denning *et al*., 1994). *C. albicans* is the most commonly detected and isolated fungal species in AIDS patients (Fisher-Hoch & Hutwanger, 1995). Non-*Candida albicans* candidiasis, which occurs at increasing frequencies, further compromises the health of the effected patients (Parkinson, 1995).

Amphotericin B (AMB) is used to treat fungal infections with some degree of success. Treatment with AMB is, however, associated with a number of severe side effects. Recently, various new formulations of AMB have been introduced into clinical use. The ability of these new formulations to decrease these side effects at therapeutic doses is still a subject of intense scrutiny. Furthermore, the cost of treatment with such formulations is a major drawback especially in poor communities in the Third World countries (Tang & Bowler, 1997). Fluconazole (FLC) resistance has also been reported. The emergence of resistance to FLC (Nolte *et al*., 1997), the lack of efficacy of FLC and the limited efficacy of intraconazole (ITC) against fungal infections have highlighted the need for new broad-spectrum antifungal agents.

Resistance to azoles, especially among the *Candida* species, has been the subject of intensive research investigations (Tritz & Woods, 1993). As a consequence of the increasing AIDS epidemic, the past decade has seen an overwhelming increase in mucosal infections caused by *Candida* species associated with emergence of resistance to azoles (Denning *et al.,* 1994). Resistance to AMB emerges associated with an increase in the number of opportunistic and invasive infections due to the so-called emerging fungi. Many of these fungi show resistance to AMB and other antifungal drugs, and may cause invasive infections, usually accompanied by a high mortality rate (Tritz & Woods, 1993). Other *Candida* species have also shown resistance

to AMB (Rex, *et al*., 2000). In light of these and other problems, an alternative source of cheap yet effective antifungals may provide relief in poor communities.

Many plants species are known to produce a variety of secondary metabolites with known therapeutic properties. Compounds that exhibit either fungistatic or fungicidal activity with low toxicity to host cells are considered good candidates for developing new antimicrobial drugs. In recent years, antimicrobial properties of medicinal plants have been extensively published from different continents of the world (Cowan, 1999). Higher plants are still regarded as potential sources of new medicinal compounds or lead compounds for synthesis of drugs. Throughout the world, plants are used traditionally to treat or control many ailments, particularly infectious diseases (Mitscher *et al.,* 1987). Traditional preparations of some medicinal plants are used in wound healing. It is an entirely exhaustive process to determine if the administration of a plant preparation to a fungal infection site works through antifungal activity and/or wound healing (Masoko, 2006).

Wound healing is a well known physiological process that consists of a cascade of events that re-establish the integrity of the damaged tissue. This process protects damaged tissues from infection with pathogens, especially bacteria and fungi, and promotes the sealing of the damaged tissue (Sumitra *et al*., 2005). Wound healing is promoted by several plant products (Suguma *et al*., 1999), which contain several different active compounds (Sharma *et al*., 1990) and biomolecules (Chithra *et al*., 1995).

In our previous studies, *Curtisia dentata* extracts had remarkable activity against *Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus,* with MIC values as low as 0.02 and 0.04 mg/ml (**Chapter 3**). The next step in the investigation was to evaluate the antifungal activity of extracts of *Curtisia dentata* against fungal infections in an animal model. It was assumed that the extract applied topically would not result in systemic effects. An ethnobotanical study by Grierson and Afolayan (1999) indicated that, in the Eastern Cape, infusions of *Curtisia dentata* bark are used to treat pimples which are characterized by lesions on the skin, suggesting a role for this plant species in the treatment of wounds in this region.

Masoko (2006), a member of the Phytomedicine Programme developed a method to test crude extracts and isolated compounds on rats infected with different fungal pathogens, including *Candida albicans.* Several modifications were introduced and adapted into this method by

Masoko (2006) (Protocol number 1/010/05). The method of Masoko was followed using extracts and isolated compounds from *Curtisia dentata*.

Previous experiments on extracts of *Curtisia dentata* (**Chapter 3**) indicated excellent *in vitro* activity against *Candida albicans.* The aim of the study was to determine *in vivo* activity of the crude extract and compounds isolated in sufficient mass from the leaves of *Curtisia dentata* in a non-infected and *Candida* infected skin wound model in rats.

The objective of the study was to investigate the wound irritancy and efficacy of *C. dentata* extracts and pure compounds applied topically to skin wounds in a *Candida albicans* infected rat model. Wound irritancy and wound healing was evaluated using microscopic, physical and histological methods. Evaluated parameters included wound healing, erythrema, exudate formation and possible toxic effects of the extracts.

7.2. MATERIALS AND METHODS

Ethical approval was obtained from the Animal Use and Care Committee (AUCC), University of Pretoria as well as the Research Committee of the Faculty of Veterinary Science, University of Pretoria. The health care of all rats was under the supervision of a veterinarian and trained laboratory technologist throughout the trial period

The study was conducted in a separate room in a Biosafety II cabinet with restricted access. Only people involved in the study were allowed access to the study site. Protective clothing (coats, gloves and mask) were worn at all times and kept on site. All animal waste and disposable materials were incinerated. All non-disposable clothing was autoclaved after every experiment.

7.2.1. Rats

Healthy female Wistar rats weighing 150-200 g were purchased from the National Health Laboratory Services in Edenvale. The test was conducted using a single gender (female) to minimize variability and the numbers required (OECD, 2000). At the commencement of the study, each rat was 8 – 12 weeks old and the weight variation of animals used did not exceed ± 20 % of the mean weight of all previously dosed animals (National Institute of Environmental Health Sciences, 2001).

7.2.2. Housing and feeding conditions

Rats were kept at the University of Pretoria's Biomedical Research Centre (UPBRC) in cages at a temperature of 22 ^{o}C (± 2^{o}C) and controlled relative humidity (50 % - 60 %) in a light/dark cycle of 12 hours. The rats were fed conventional rodent diets with an unlimited supply of drinking water (National Institute of Environmental Health Sciences, 2001). Pieces of paper towel were provided to keep rats busy. Previous work suggests that the provision of enrichment items, which give laboratory rats the opportunity to perform exploratory and gnawing activities, improves their welfare and distracts them from tampering with dressings.

7.2.3. Preparation of animals

Rats were moved into single cages marked rat 1 – 12 for at least 5 days prior to treatment to allow for acclimatization to the laboratory conditions (Spielmann *et al*., 1999). The rats were immunosuppressed, 4 days before challenge with *Candida albicans*, by subcutaneous injection of 500 µg of estradiol valerate (50 µl from 10 mg/ml ampoule). Estradiol pretreatment inhibits innate and acquired immune defenses (Carlsten *et al.,* 1991).

7.2.4. Wound creation

The hair on the back area of each rat was shaved using an electrical clipper. The area was disinfected using 70% alcohol. Rats were anaesthesized with isoflurane (0.01- 0.05 µg/kg) in a closed chamber. Eight evenly spaced wounds were made on each rat. The outline of the wound was marked using 6 mm diameter biopsy punch (Simosen *et al*., 1992) and the skin removed from the marked area using surgical scissors. The procedure was conducted in a biosafety class II cabinet to limit infection. The lesions were traced onto a transparent paper using a black permanent marker. The area of the circular lesions was calculated as follows:

$A = \frac{\pi D_1 D_2}{4}$, where A is the area of the lesion, D_1 and D_2 were the horizontal and vertical diameters of the lesion, respectively, and $\pi = 3.142$

7.2.5. Infection with *C. albicans*

Candida albicans was cultured for 24 hours prior to infection on Sabouraud agar in Petri dishes at 30°C. The fungal cultures were scraped aseptically from agar, pooled into 30 ml of sterile water and briefly homogenized. Small pieces (5 mm x 5 mm) of autoclaved dressing were suspended into *Candida albicans* culture in sterile water and placed in the wound area. The area was covered with an occlusive wrapping (Transpore^R) and left to incubate for 48 hours. After 48 hours the test products were introduced and the resultant inhibition of growth or healing quantified on the basis of erythema, exudates and physical size of the lesion on a daily basis for 2 weeks. Infection by fungi was clinically detected by the presence or absence of swelling, erythema, pain and ulceration of the inoculation sites. Clinical signs that were also observed were a rise in body temperature, loss of appetite for 24 hours and weight loss.

7.2.6. Preparation of extracts

Curtisia dentata was extracted with water and acetone as described in Chapter 2. Acetone and water were selected as the extracting solvents to allow *in vivo* testing of an organic and an aqueous extract. The acetone extract had excellent antifungal activity in previous studies (Chapter 3). Extracts were dried at room temperature under a stream of air and ground to powder using mortar and pestle. The extracts were mixed with aqueous cream composed of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol to concentrations of 10 % (1 g/10 g cream) and 20 % (2 g/ 10 g), and kept at 4^{o}C until use. The isolated compound preparations were adjusted to concentrations of 1% (0.1 g/10 g cream) and 2% (0.2 g/g cream). Amphotericin B (0.1%) was used as a positive control. Water extracts of *Buddleja* spp. were used as positive controls as these are known to have wound healing properties (Eloff and Houghton, personal communication). An outline of the treatments applied to each rat was as follows:

Rats 1 – 6:

Site A: treated with aqueous cream only (negative control),

Site B: treated with amphotericin B mixed with aqueous cream (positive control),

Site C: 1% lupeol in aqueous cream,

Site D: 2% lupeol in aqueous cream,
Site E: 1% betulinic acid in aqueous cream,
Site F: 2% betulinic acid in aqueous cream,
Site G: 10% water extract of *Buddleja* spp. aqueous cream and
Site H: 20% water extract of *Buddleja* spp. aqueous cream (positive control).

Rats 7 – 12:
Site A: treated with aqueous cream only (negative control),
Site B: treated with amphotericin B mixed with aqueous cream (positive control),
Site C: 10% acetone extract of *C. dentata* in aqueous cream,
Site D: 20% acetone extract of *C. dentata* in aqueous cream,
Site E: 10% water extract of *C. dentata* in aqueous cream,
Site F: 20% water extract of *C. dentata* in aqueous cream,
Site G: 10% water extract of *Buddleja* spp. aqueous cream and
Site H: 20% water extract of *Buddleja* spp. aqueous cream (positive control).

The diagrammatic representation of wound sites is displayed in Fig. 7.1.

7.2.7. Observations

The rats were inspected three times a week for signs of systemic infection by determining mass and food intake of each rat, as well as temperature using a microchip inserted subcutaneously above the tail region (Fig. 7.1). At the completion of the experiment, *post mortem* and pathological investigations were conducted to determine the emergence of systemic infection with *Candida albicans*.

Each animal served as its own control with four test sites for the crude extracts (acetone and water extracts) and isolated compounds, one site as a positive control with Amphotericin B, one site as a negative control and two sites treated with 10 and 20% water extract of *Buddleja* spp. The presence of factors such as muscle necrosis, foreign bodies and skin contamination were recorded. The measuring of the sizes of the lesions relative to the negative and positive controls or the complete healing of the lesion served as the means of measurement of the antimicrobial activity. Every Monday, Wednesday and Friday at the same time, the dressings on each rat were removed and the different parameters measured. Treatments were reapplied to wounds and fresh dressing used to cover the wounds.

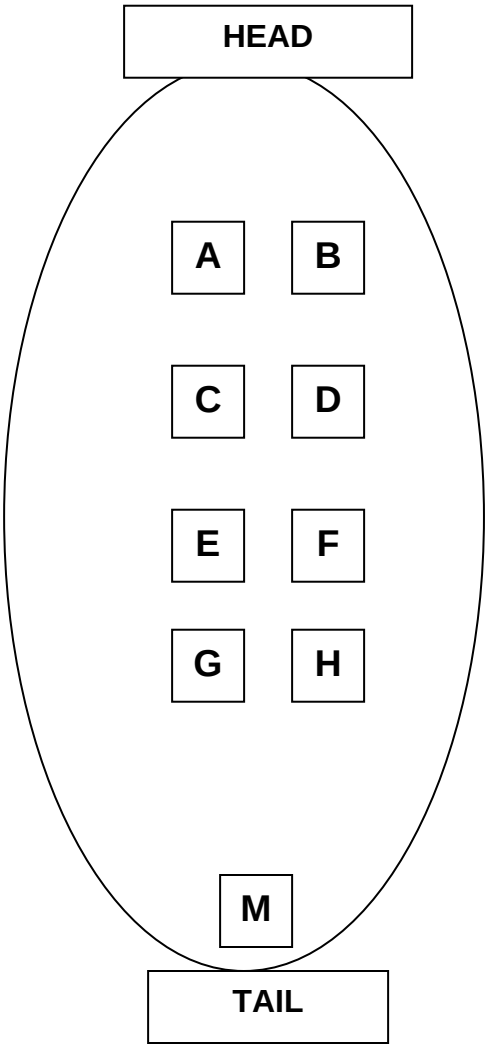


Figure 7.1. A diagrammatic representation of wound positions on the back of each rat. Samples as outlined in **Section 7.2.6.** were applied on the specific wound sites. Site M represents the position where the temperature microchip was inserted.

A photographic record of the overall procedure performed on rats is outlined in Figs. 7.2. and 7.3. The rats were observed daily for two weeks until wounds were about 80% healed. Observations noted included changes to skin and fur, diarrhoea, lethargy, sleep, weight loss and coma. After the completion of the experiment, rats were euthanized using increased doses of isoflurane in closed glass chambers and the *post mortem* performed. Liver, heart and kidney were analysed by the pathologist for different parameters. Sterile swabs were used to collect innocula from internal organs to allow culturing on SB agar (Fig. 7.4).

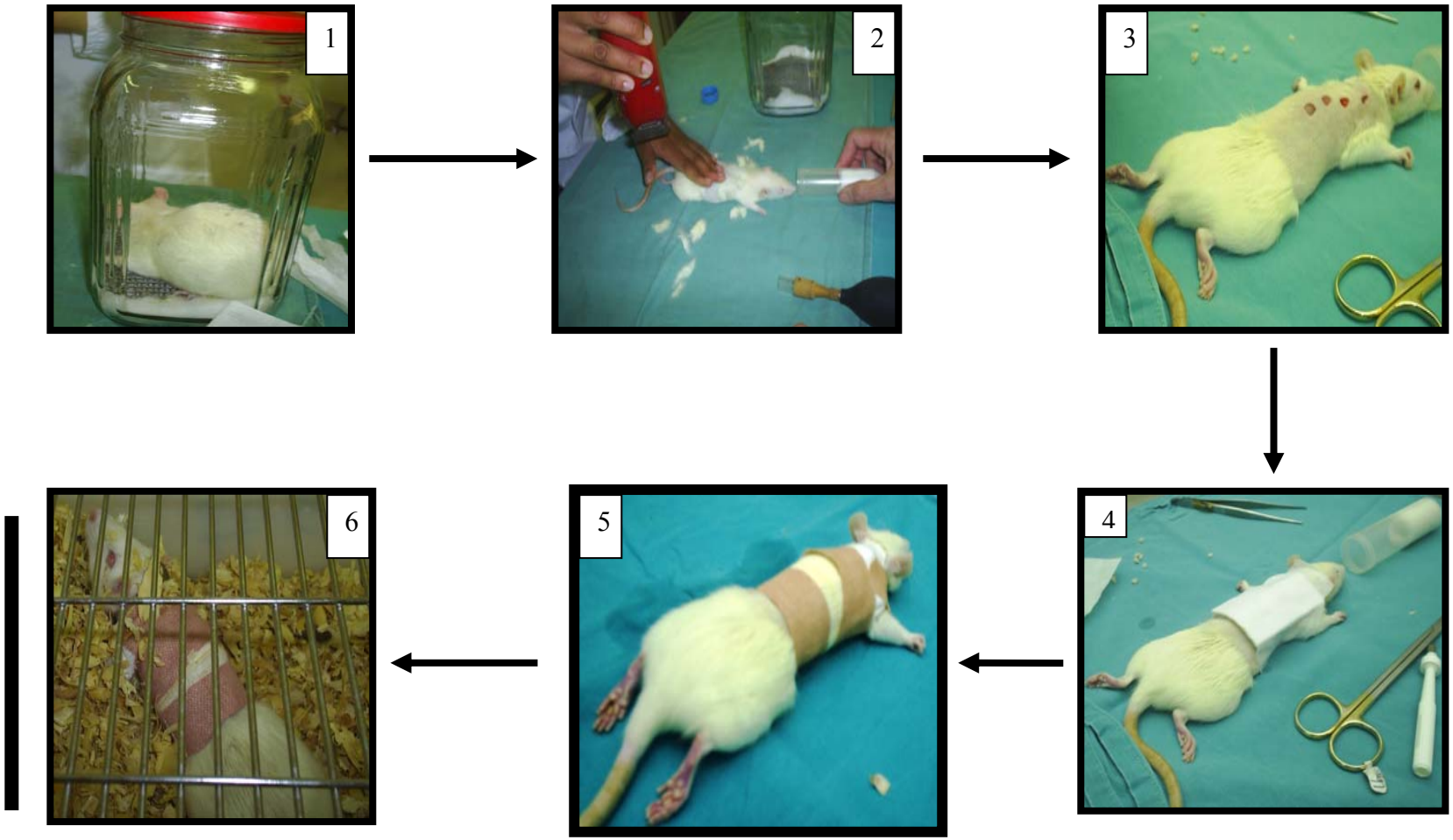


Figure 7.2. The diagrammatic representation of surgical wound creation and dressing of excisional wounds on rats. 1, rat subjected to anaesthesia in a closed glass chamber; 2, shaving of the back with an electric clipper; 3, creation of wounds using scissors and infection with a concentrated overnight culture of *Candida albicans*; 4,5,6, dressing of wounds and housing in separate cages.

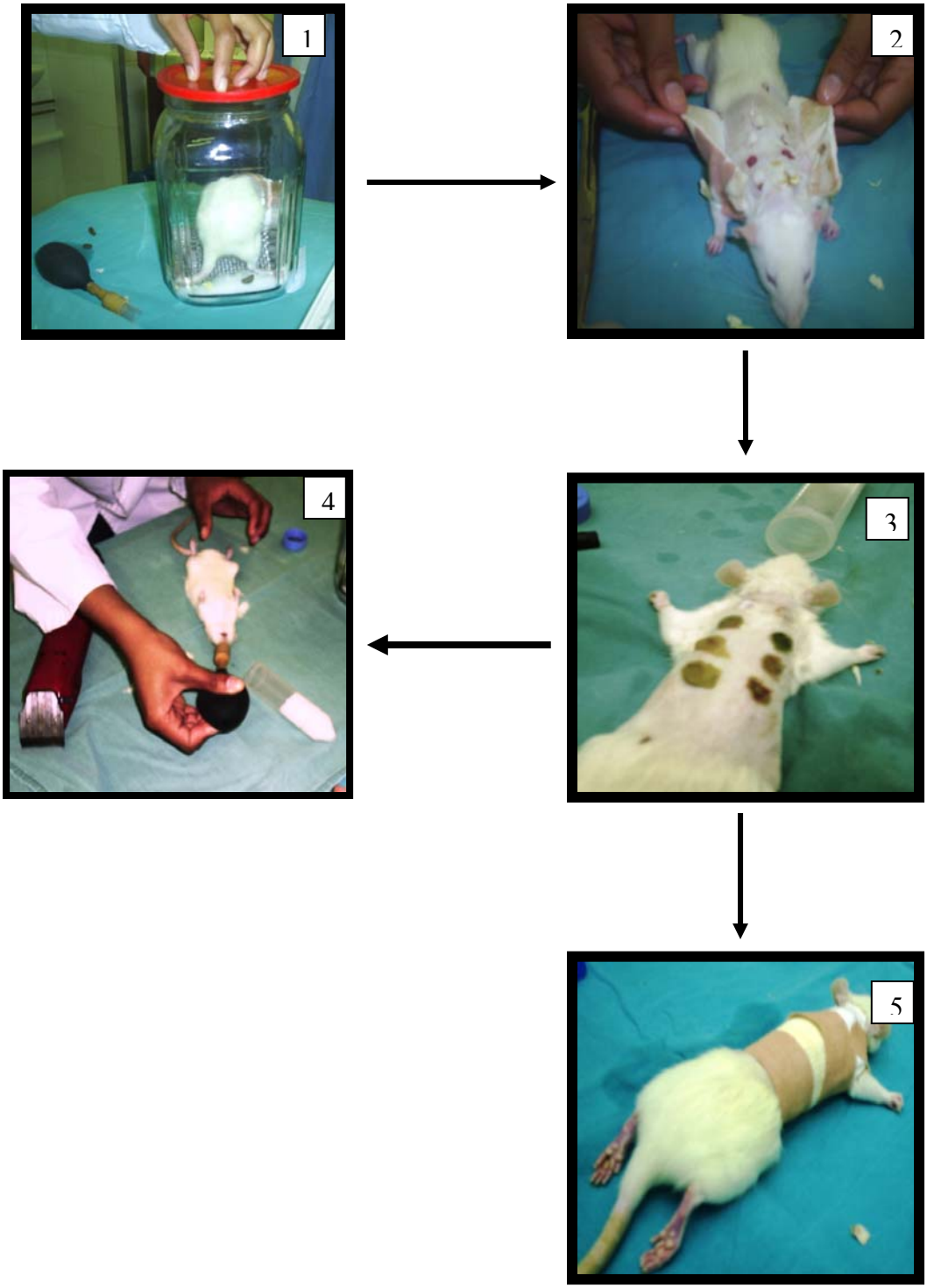


Figure 7.3. Representation of changing of wound dressing and treatment with varying products. 1, rat subjected to anaesthesia; 2, removal of bandages; 3, application of treatment on wounds using sterilized spatulas; 4, resuscitation of non-breathing rat; 5, redressing of wounds.

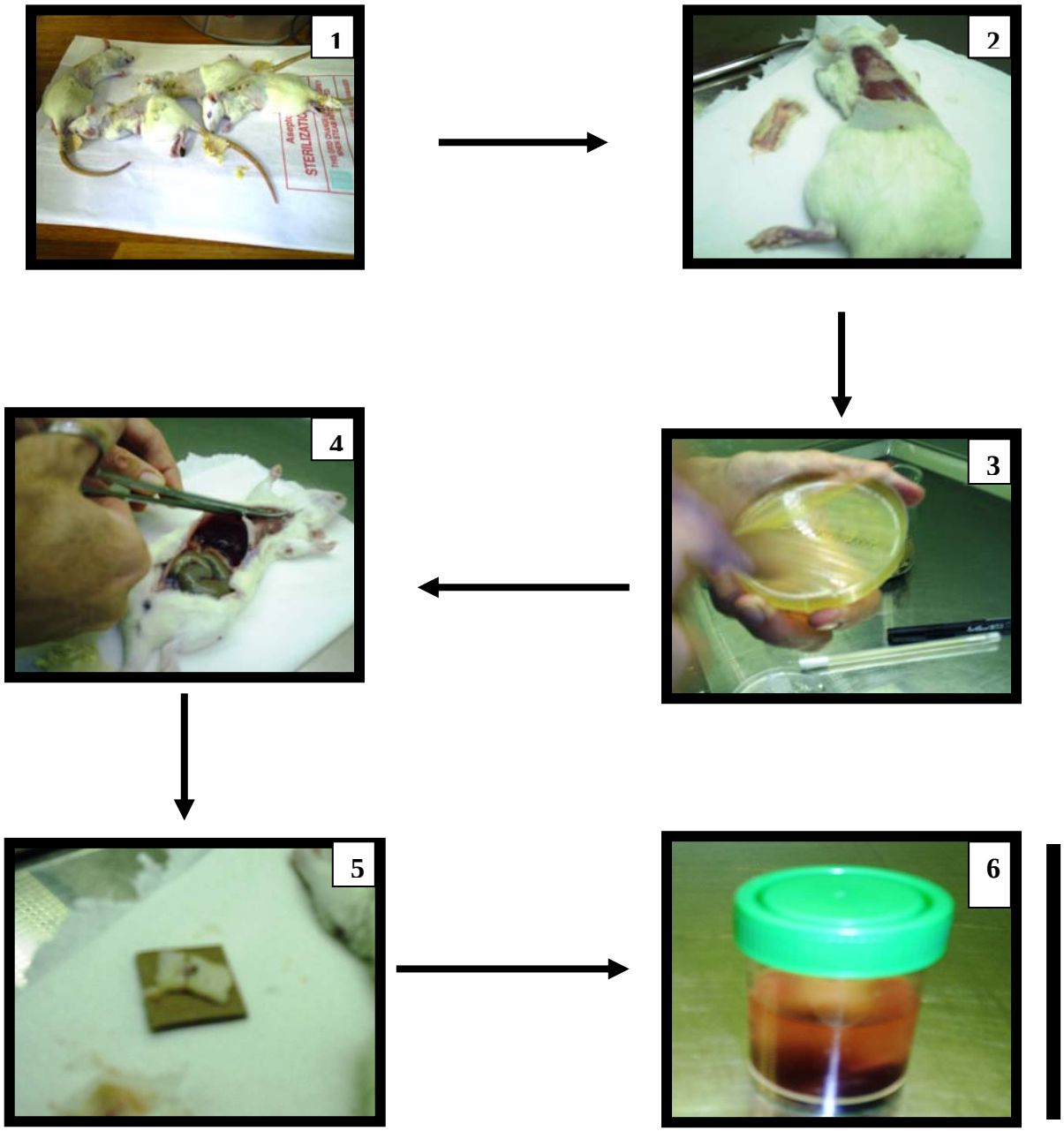


Figure 7.4: Preparation of tissue specimens for histopathological examinations. 1, rats eutheniased in excess isoflurane; wound areas removed from skin; 3, inocula collected from underskin surfaces using sterile swabs and streaked on SD agar; 4, liver, kidneys, lungs and spleen removed and preserved in buffered formalin, and swabs used to collect fungi from organs; 5, 6, wound areas anchored to pieces of cardboard and preserved in formalin.

7.2.8. Evaluation of lesions

The mass of each rat and lesion characteristics, including lesion severity scores, were recorded three times a week and general observations noted. The study was blinded (the person doing evaluation did not know the treatment). The lesion sizes were measured using a caliper on each routine observation day. Erythema and exudate formation were evaluated during routine observation and scored as in Table 7.1.

Table 7.1. Scoring used in the evaluation of erythema and exudate

Score	Erythema	Exudate
0	No red colour at all	No exudate
1	Light red just visible	Exudate just visible
2	Clearly red	Easily visible
3	Dark red, not whole area	Substantial quantity
4	Dark red wide spread	Large quantity

7.2.9. Recording of data

The data for each rat was recorded on a single form for the 2 week period (Table 7.2.). Day 0 data was also collected as for the other days in Table 7.2.

7.2.10. Statistical analysis

Results were represented as general linear model (GLM) with repeated measure ANOVA. One way ANOVA was used to identify differences between groups and $P< 0.05$ was considered significant. Student t-test was used to determine differences between control and treatments.

7.2.11. Pathological and histopathological studies

Histopathological studies were performed with the help of a pathologist at the end of the experiment. Wound tissue specimens from treated and non-treated rats were collected in 10 % buffered formalin and after processing 6 µm thick sections were cut and stained with haematoxylin and eosin (McManus & Mowry, 1965). Sections were qualitatively assessed under the light

microscope and graded in respect of congestion, oedema, infiltration of polymorphonuclear leukocytes (PMNLs) and monocytes, necrosis, fibroblast proliferation, collagen formation, angiogenesis and epithelisation (Shukla *et al*., 1999). Table 7.3. was used as a template for recording histopathological results.

7.3. RESULTS

The rats lost weight after the surgical procedure until day 6. During the next routine observation on day 10 the weight of the rats increased substantially through day 11. The rats acclimatized to the handling in the study as indicated by the overall weight gain after day 9 (Table 7.4. and Fig. 7.5.). The average weight of the rats, for reasons not yet understood, dropped on observation day 13. A similar trend also occurred with the temperatures of the rats. Following initial anaesthesia the temperatures of the rats dropped to between 31.8 and 35.5 ^{o}C. The temperature only increased with the days of treatment, peaking at around 38^{o}C (Table 7.5 and Fig. 7.6).

The area of the lesions was measured by tracing the boundary of open wounds on a semi-transparent plastic sheet and the sizes measured using an electronic calliper. Statistical calculations using ANOVA single factor revealed statistically significant differences in lesion sizes between groups (treatments) on day 6 with a P-value of 0.0054 at 95% confidence interval. T-tests were calculated for each treatment and compared with cream only control and amphotericin B control. The lesions treated with 20% acetone averaged about 76% of the original lesion sizes and were significantly larger than lesions treated with the cream only and AMB (*P*-value less than 0.05) on day 6.

Table 7.2. Form used for measuring different parameters on the 6 rats (in a group) on the different sites. The exudates and erythema were measured on an arbitrary scale 0-5 with one being the best rate of healing and five the worst. Lesion size was be measured in mm^2 and mass in grams (g), temperature in ^oC. Swelling and ulcerations were marked as follows: 1, slight; 2, moderate; 3, marked; 4, extensive; 0, absent. A-H represent wound sites on each rat.

Day	Rat No.	Exudate								Erythema								Lesion size (mm^2)								Weight (g)	Temp. (^oC)	Swelling	Ulceration
		A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H				
M1																													
W1																													
F1																													
M2																													
W2																													
F2																													

Table 7.3. Form used for quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams**.** *Neut's= Neutrophils; Mac's= Macrophages

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
	A											
	B											
	C											
	D											
	E											
	F											
	G											
	H											

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

Table 7.4: Mass of rats 1 – 12 measured during specified time periods.

DAYS	RAT NUMBERS AND MASS IN GRAMS											
	1	2	3	4	5	6	7	8	9	10	11	12
0	169.1	183.7	200.2	200.1	190.1	211.7	205.9	184.1	185.0	199.7	177.6	185.3
2	168.5	182.7	200.3	196.0	187.9	214.2	206.7	182.0	182.4	198.2	177.5	183.6
4	170.0	181.2	201.2	199.1	187.3	208.4	208.8	182.7	188.4	198.9	185.7	181.5
6	169.9	179.3	203.3	194.8	187.3	207.0	210.9	167.3	182.7	201.9	187.7	169.8
9	175.4	182.5	206.8	196.4	190.2	216.1	208.6	180.3	191.2	208.3	185.3	181.2
11	174.0	177.9	208.8	200.0	190.7	212.4	212.2	185.8	194.0	213.4	189.9	178.1
13	174.2	181.6	193.2	184.4	193.4	212.2	209.0	188.8	194.2	210.8	187.1	179.8
Average	171.6	181.3	202.0	195.8	189.6	211.7	208.9	181.6	188.3	204.5	184.4	179.9

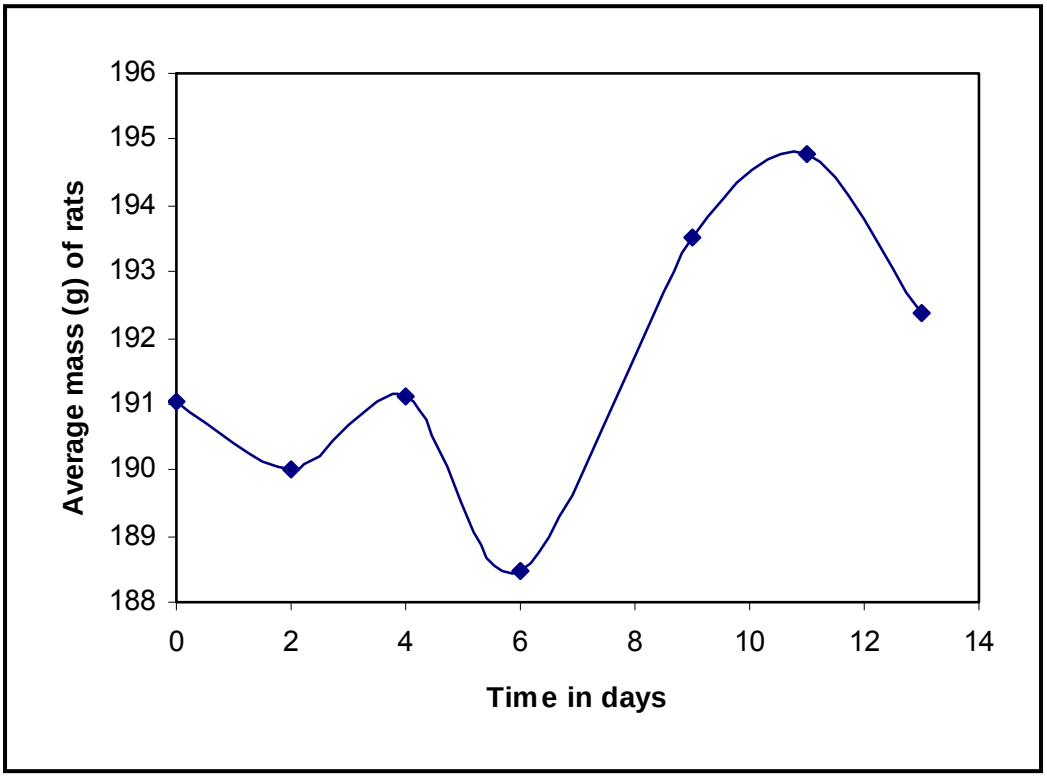


Figure 7.5: Average mass of the rats on different treatment days.

Table 7.5: Temperatures (°C) of rats from day 0 to day 13. **CF,** microchip missing.

DAYS	RAT NUMBERS and temperature in °C											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0	33.6	34.4	35.5	33.6	34.9	34.6	35.4	34.6	33.7	34.4	33.3	31.8
2	35.1	36.2	38.0	36.5	36.1	36.5	35.7	36.7	38.0	37.1	36.1	39.1
4	37.7	37.0	37.1	36.7	36.1	37.4	35.8	36.5	35.8	37.1	35.8	36.7
6	36.7	35.6	36.3	36.9	36.9	38.3	35.6	36.2	36.7	37.0	37.7	36.1
9	37.3	36.0	37.6	37.3	36.6	36.3	35.3	37.2	35.6	37.3	35.8	37.9
11	35.7	36.3	37.0	37.0	36.0	35.1	35.2	35.7	36.4	37.9	37.2	36.2
13	36.0	35.8	36.2	36.3	37.9	CF	CF	36.6	35.4	38.2	36.5	35.5

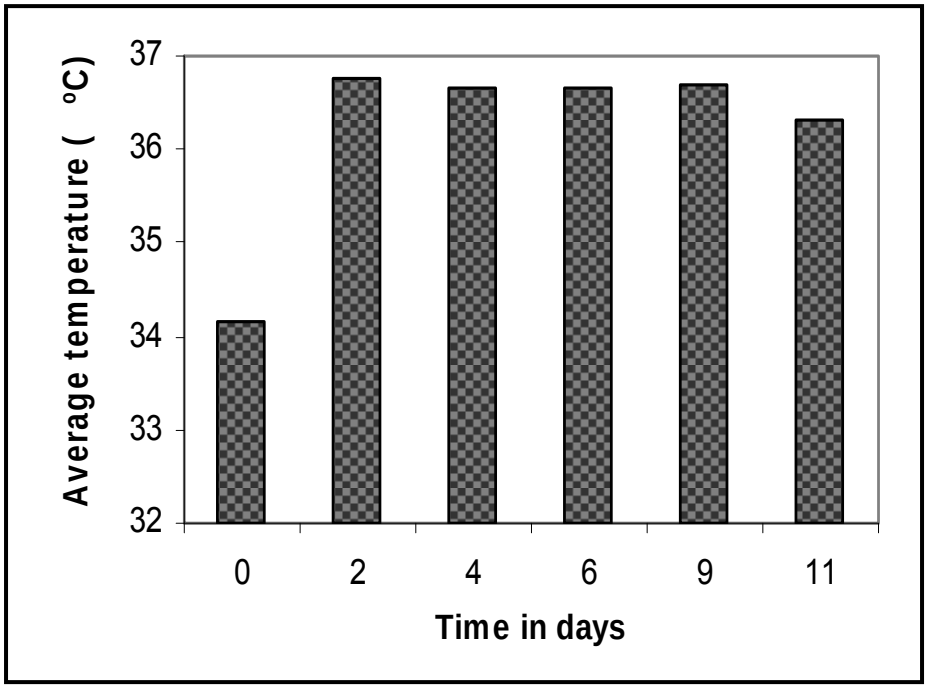


Figure 7.6. Average temperature (°C) of rats up until 11 days of treatment

The wounds treated with 20% acetone extract of *Curtisia dentata* had significantly higher lesion sizes than both the cream only control and amphotericin B-treated control on treatment day 4, averaging at 120% of the original lesion sizes (*P*-value less than 0.05 at 95% confidence interval). This suggested that the acetone extracts did not result in any observable decrease in lesion sizes compared with the cream only controls and amphotericin B controls. Generally, all lesions

decreased in size with the days of treatment. There was no marked acceleration of reduction of lesion sizes with any single treatment (Figs. 7.7 and 7.8). Statistical data analysis is shown on Tables 7.6 and 7.7).

No swelling was observed in any of the wounds. The excision wounds were initially round or almost round. As they healed all the wounds contracted into an oval shape and gradually healed in a straight line. In literature, it is reported that open wounds contract as if margins are pulled into the centre. Though round wounds are slower to heal than square wounds, the healed scar is linear (Majno, 1975).

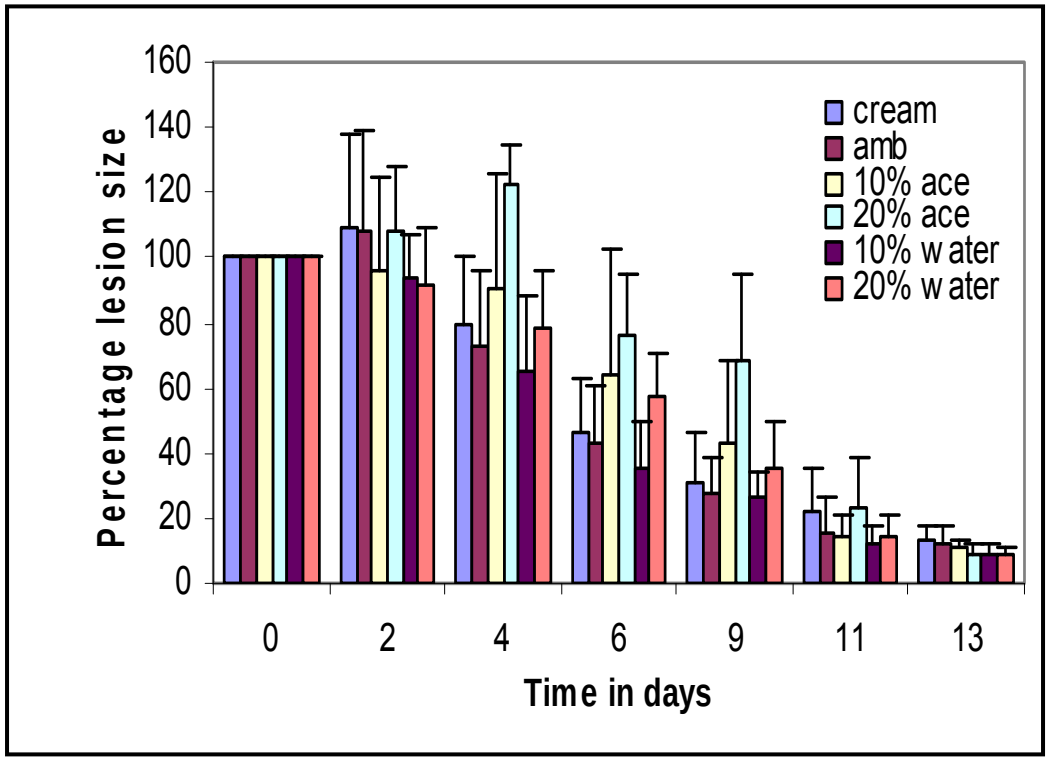
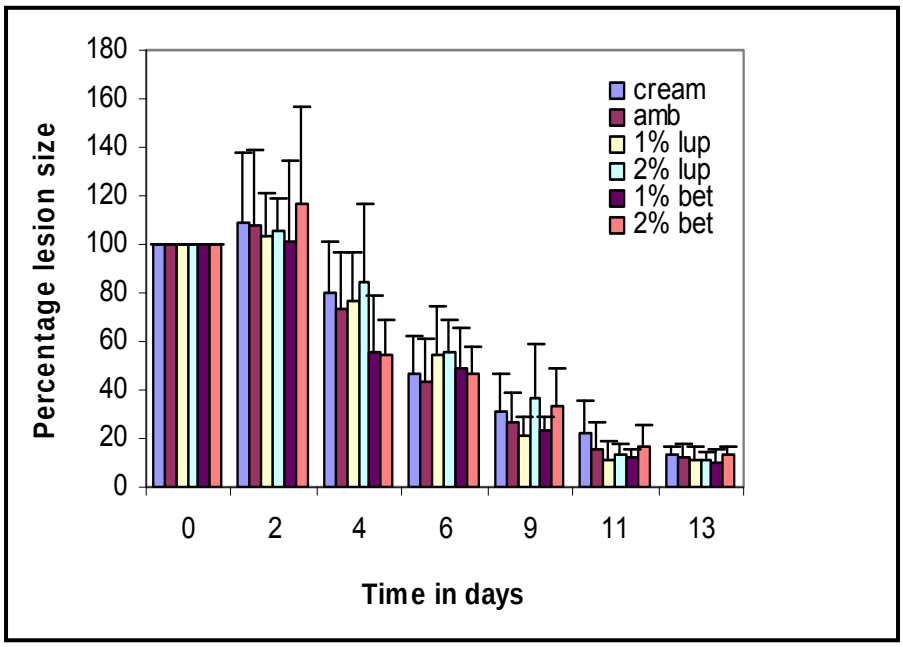


Figure 7.7. Average sizes of lesions treated with different extracts of *C. dentata*. Lesions were calculated as percentages of the original lesion sizes. Amb, amphotericin B; ace, acetone extracts.



igure 7.8. Average sizes of lesions treated with different compounds isolated from *C. dentata*. Lesions were calculated as percentages of the original lesion sizes. Amb, amphotericin B; lup, lupeol; bet, betulinic acid.

Table 7.6. Treatment day 4 statistical analysis of lesion sizes

Treatments	Count	Sum	Average	Variance
Cream only	12	955.3	79.61	437.08
Amphotericin B	12	878.6	73.22	536.22
1% lupeol	6	461.9	76.98	390.59
2% lupeol	6	504.5	84.08	1071.94
1% betulinic acid	6	331.6	55.27	567.72
2% betulinic acid	6	325.2	54.20	218.51
10% acetone	6	540.8	90.13	1234.16
20% acetone	6	732.6	122.10	149.80
10% water	6	392.0	65.33	510.01
20% water	6	468.4	78.07	335.29
10% Buddleja	12	937.0	78.08	697.04
20% Buddleja	12	1108.9	92.41	645.38

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	22808.82	11	2073.529	3.639065	0.00031	1.904539
Within Groups	47862.96	84	569.7972			
Total	70671.78	95				

Table 7.7. Treatment day 6 statistical analysis of lesion sizes

Treatment	*Count*	*Sum*	*Average*	*Variance*
Cream only	12	560.4	46.70	256.55
Amphotericin B	12	522.1	43.51	292.01
1% Lupeol	6	328.5	54.75	397.36
2% Lupeol	6	335.3	55.88	165.95
1% betulinic acid	6	291.6	48.60	294.86
2% betulinic acid	6	280.0	46.67	121.18
10% acetone	6	387.1	64.52	1475.54
20% acetone	6	456.2	76.03	366.87
10% water	6	213.2	35.53	213.02
20% water	6	342.3	57.05	187.10
10% Buddleja	12	597.8	49.82	499.07
20% Buddleja	12	812.2	67.68	233.30

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10583.60	11	962.15	2.676194	0.005392	1.904539
Within Groups	30199.69	84	359.52			
Total	40783.29	95				

The average exudate release on wounds was less in wounds treated with 2% betulinic acid than the cream only-treated controls. Treatment with 1% and 2% lupeol resulted in the highest exudate formation (Fig. 7.9). Treatment with 20% water extract formulation resulted in more exudate release than the control wounds treated with cream only, whereas treatment with amphotericin B inhibited exudate release from wounds (Fig. 7.10).

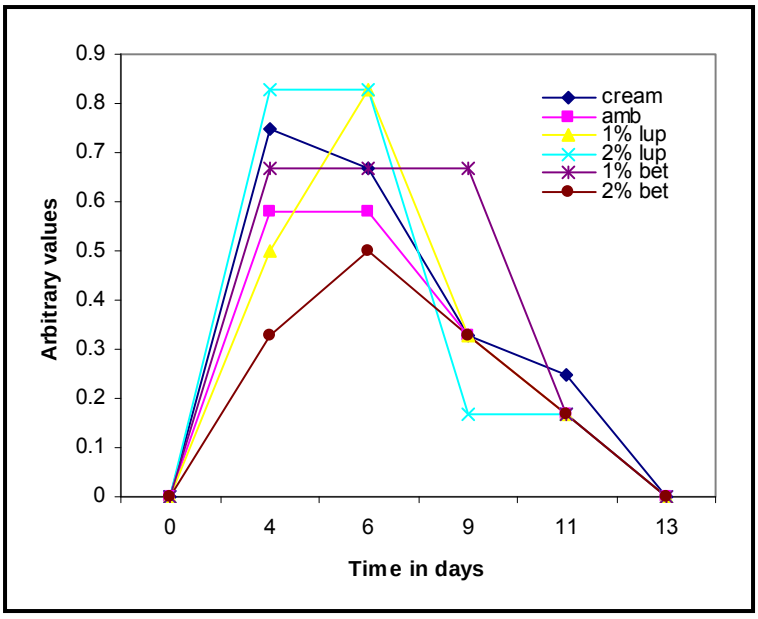


Figure 7.9. Exudate release from lesions treated with different *C. dentata*-derived compounds

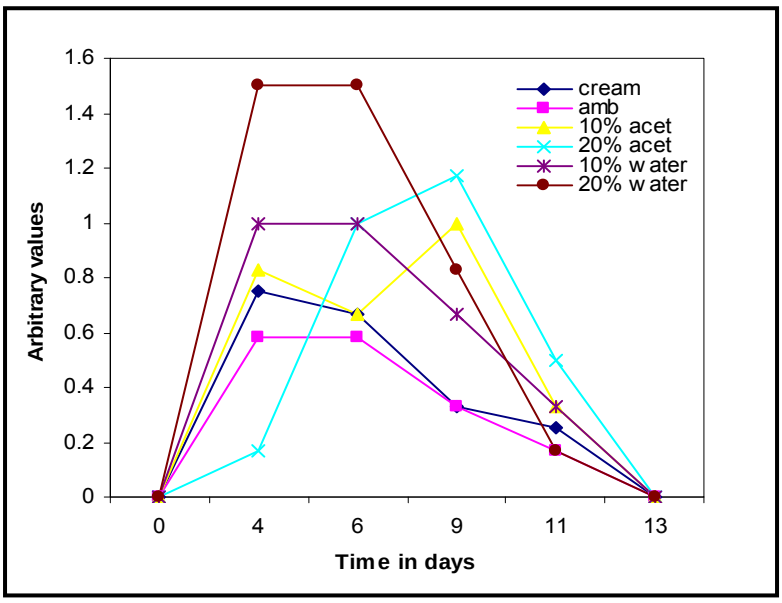


Figure 7.10. Exudate release from lesions treated with different *C. dentata*-derived extracts (acetone and water extracts).

Erythema was consistently high in wounds treated with 10% and 20% acetone extracts. Wounds treated with 10% and 20% acetone extracts had a larger surface area and lower wound contraction capacity. Perhaps there is a correlation between pronounced eythema and wound contraction capacity (Fig. 7.11 & 7.12).

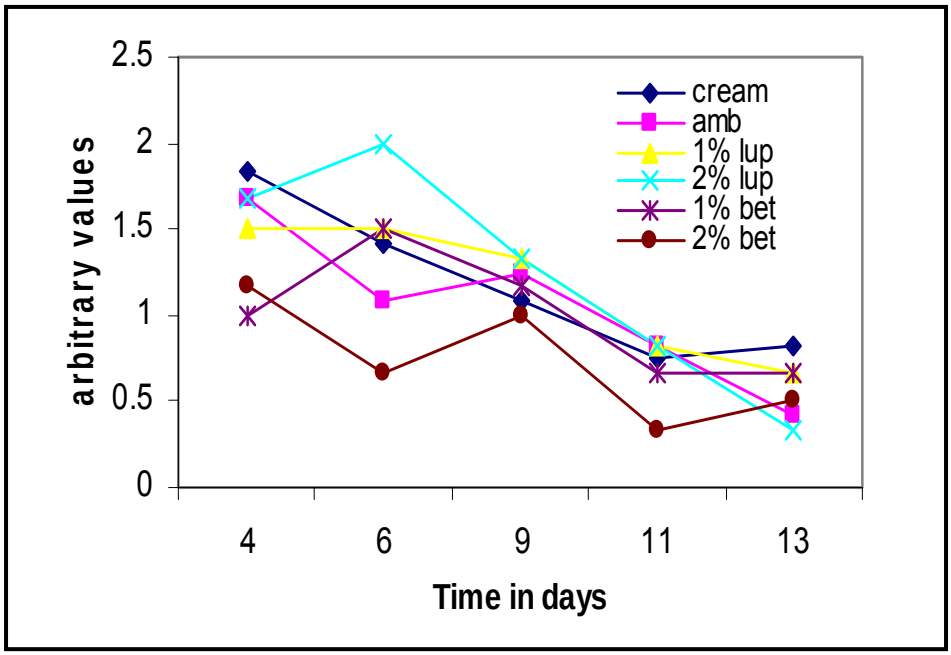


Figure 7.11. Erythema (arbitrary values) in wounds treated with compounds isolated from *C. dentata*.

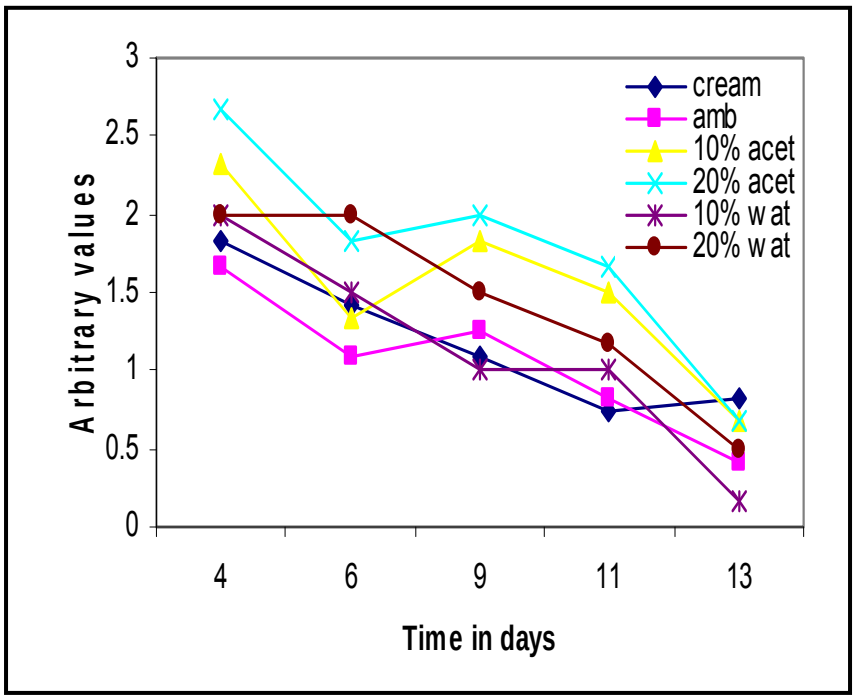


Figure 7.12. Erythema (arbitrary values) in wounds treated with extracts (acetone and water extracts) derived from *C. dentata*.

Crust formation was also determined on all wounds treated with various *Curtisia dentata*-derived products. Presence of crust on wounds was scored as 1 (one) while absence thereof was represented by zero (0). In all cases on day 4 minimal crust formation was observed, and it peaked on day 9 for most treatments. The maximal crust formation on wounds treated with 2% betulinic acid was the highest together with 10 and 20% acetone extract and 20% water extract treatments. Wounds treated with 1% lupeol displayed the least overall crust formation with just over 60% of the wounds positive for crust formation on day 9. On day 13, crust formation dropped in all wounds to levels as low as 0 (arbitrary values) (Figs. 7.13 and 7.14).

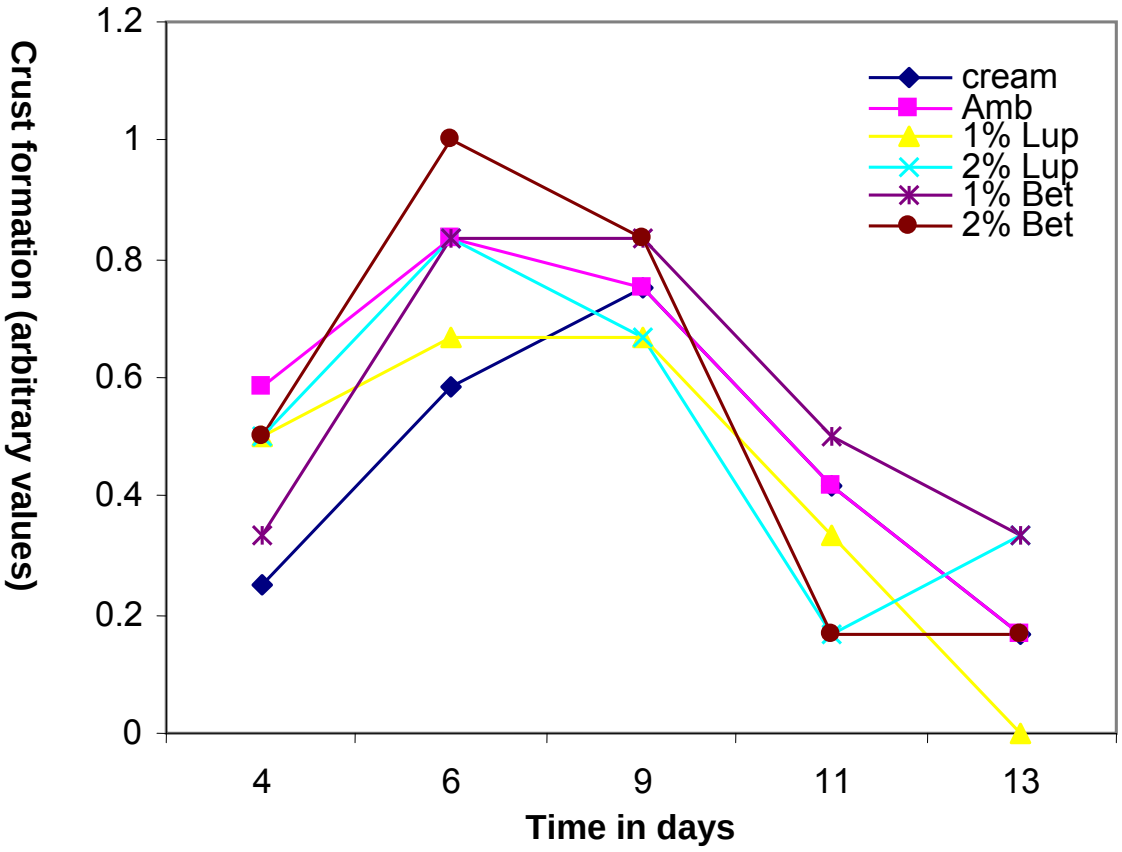


Figure 7.13: Crust formation in wounds treated with various concentrations of compounds during specified days of treatment. Amb, amphotericin B; Lup, lupeol; Bet, betulinic acid.

After 13 days swabs of the subcutaneous skin surfaces of rats 1, 5 and 11 produced viable colonies of *Candida albicans* on SD agar. The skin surface of all the other rats did not have any viable colonies as shown by absence of fungal growth on agar plates. The organs of rats 1, 4, 6, 7 and 11 were infected as indicated by the presence of colonies on agar plates (inoculated with samples of the internal organs). The identity of the infecting microbial culture was not confirmed, though *Candida albicans*, the organism used to infect the rats during the initial stages of the study remains the likeliest candidate (Table 7.8).

Fungal organisms or any fungal fragment were not detected in all haematoxylin-eosin stained skin sections. These skin sections were originally treated with various formulations containing either plant extracts or isolated compounds. The skin lesions were completely healed. Furthermore, no specific pathology could be demonstrated in the parenchymal organs examined.

In most of the kidney specimens, mild or moderate cortical or medullary congestion were present. About half of the liver specimens showed no signs of abnormality, while the rest had mild sinusoidal dilation and congestion. In general, the summary histopathological report suggest that there were no specific pathological abnormalities observed in the skin and parenchymal organ specimens.

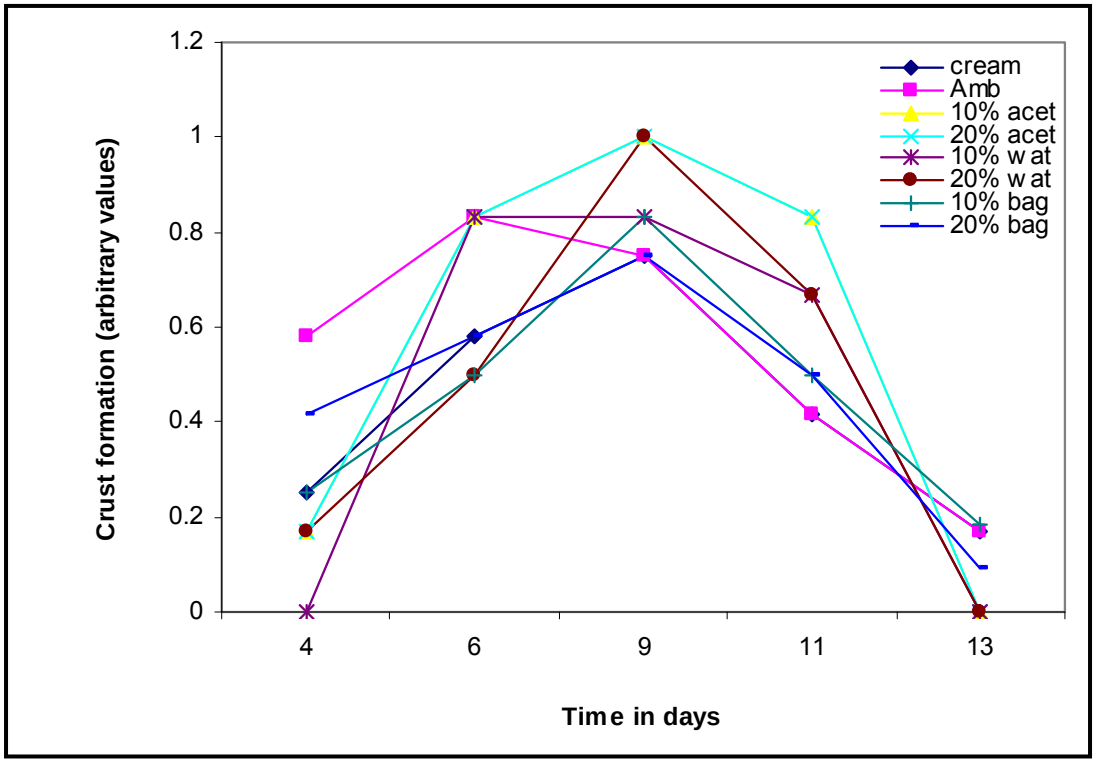


Figure 7.14: Crust formation in wounds treated with various concentrations of compounds during specified days of treatment. Amb, amphotericin B; ace, acetone extract; wat, water extract; bag, *Buddleja*.

Table 7.8. Number of colonies derived from inoculate of the subcutaneous tissue (skin under the wounds) and the internal organs. Key: - no colonies observed

Rat	Skin tissue	Internal organs
1	100	20
2	-	-
3	-	-
4	-	115
5	7	-
6	-	3
7	-	5
8	-	-
9	-	-
10	-	-
11	30	24
12	-	-

Granulomatous dermatitis was less in both amphotericin and cream treated controls. In all the other wounds granulomatous dermatitis was pronounced, recorded above 0.6 (arbitrary values). Wounds treated with 10% acetone extract of *Curtisia dentata* had the highest granulomatous dermatitis (Fig. 7.15).

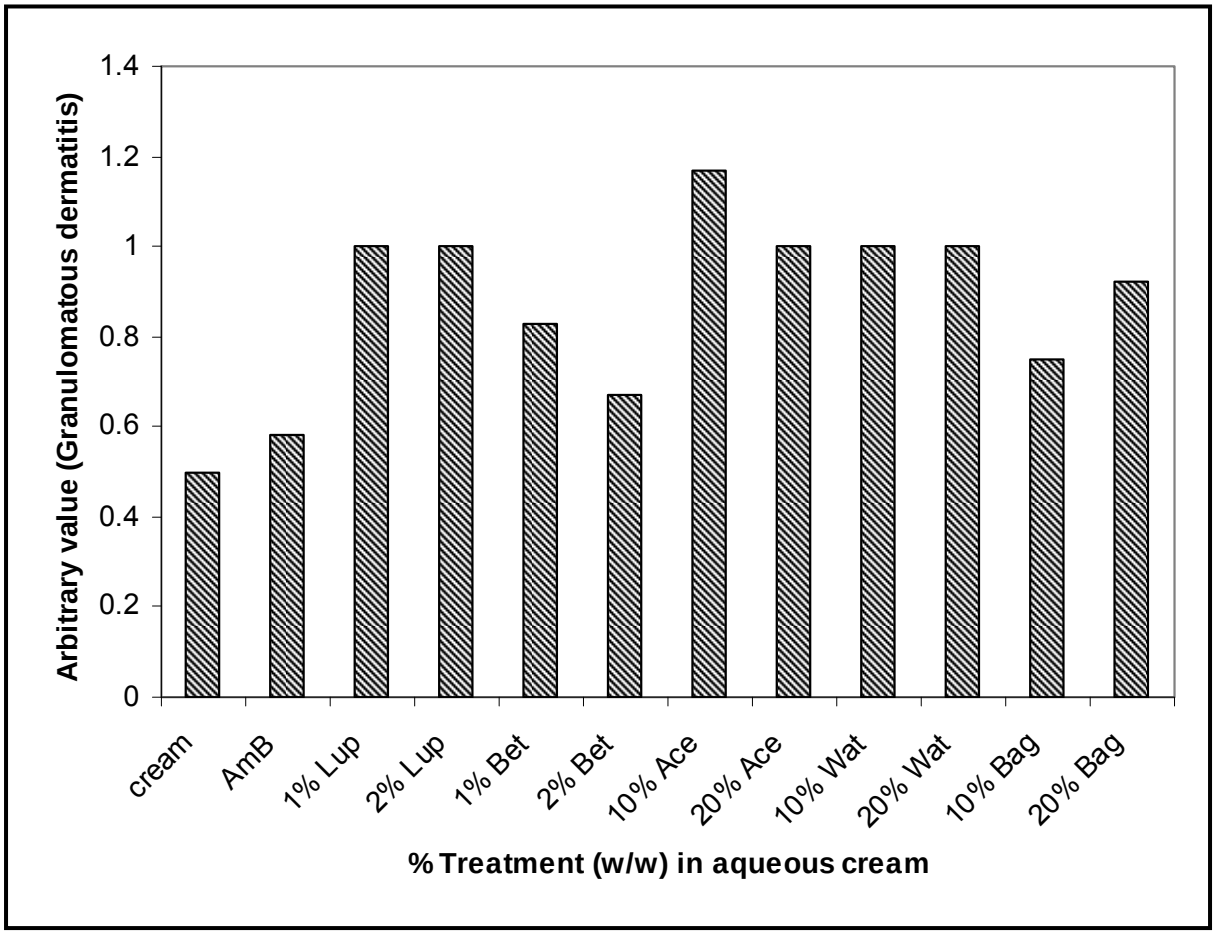


Figure 7.15: Histopathology for granulomatous dermatitis. Amb, amphotericin B; ace, acetone extract; wat, water extract; bag, *Buddleja* spp.; Lup, lupeol; Bet, betulinic acid.

7.4. DISCUSSION AND CONCLUSIONS

7.4.1. Weight loss and temperature

The loss of weight may have been due to the administered anaesthetic and shock from handling, including shaving and surgical creation of wounds. Furthermore, the infection of wounds with *Candida albicans* could have contributed to the overall weight loss. On observation day 9 the average weight of the rats increased, possibly resulting from the animals regaining functioning of their immune system following immunosuppression with estradiol valerate. Perhaps this allowed the animals to fight the infections, hence the increase in weight.

7.4.2. Lesion sizes

Lesions treated with 10 and 20% acetone had significantly larger sizes than the controls at the completion of the experiment. This may emanate from inhibition of tissue regeneration by these extracts. Since it was shown in the earlier chapters that the acetone extracts had *in vitro* antifungal activity against *Candida albicans* it is unlikely that the larger lesion sizes after 6 days resulted from increased infection of the wounds with the pathogen.

Wound healing involves an ordered series of events, namely, coagulation to prevent blood loss, inflammation of wound area, epithelial repair that includes proliferation, mobilization, migration and differentiation, and tissue remodelling and collagen deposition (Rashed *et al.,* 2003). Wound contraction was not markedly improved by any of the treatments applied to wounds since the lesion sizes remained comparable throughout the treatments. However, these extracts, due to their antimicrobial activity, may be used in wound healing mixtures to ward off microbial infection.

This study may be repeated with one excision wound per rat and one plant product applied to minimize interference resulting from systemic absorption of active contituents when more than one extract is applied on the same rat. In the present study, the assumption was made that systemic absorption of the applied extract, compound or commercial antifungal drug did not occur. This was necessary to limit as much as possible the number of rats needed in the study.

7.4.3. Exudate and erythema

Pronounced erythema was observed on wounds treated with 10 and 20% acetone extracts. The lesions had bigger surface areas than other treated wounds, suggesting that a relationship exists between wound contraction and erythema. These same wounds, together with those treated with 10 and 20% water extracts had higher exudation than other treated wounds, further correlating wound contraction to parameters such as exudation and erythema. Exudation is a direct result of the inflammatory response to injury, and includes recruitment of leukocytes to the site of injury (Majno & Joris, 1996). The presence of infection in the wound recruits more white blood cells resulting in pus (Majno & Joris, 1996). Treatment of wounds with amphotericin B and 2% betulinic acid resulted in the slightest release of exudates, suggesting that in these wounds *Candida albicans* load was kept at a minimum. Betulinic acid was shown in earlier chapters to have good *in vitro* antifungal activity.

Lupeol, at concentrations of 1 and 2% led to a pronounced release of exudate. It would seem that the increased exudate was a direct result of accumulation of leukocytes at the infected wound. In earlier chapters it was shown that lupeol did not greatly inhibit the growth of *Candida albicans* and other fungal species. Resulting from the failure of lupeol to sufficiently deal with *Candida albicans* in wounds, inflammation may have increased to combat the infection, hence the explicit exudation.

As the exudate seeps out onto the surface of the wound, it clots and eventually dries out into a scab or crust (Majno & Joris, 1996). The scab's function is to seal the wound from the environment, hence it is sometimes referred to as natural dressing (Grillo, 1964). It was also observed that crust formation on wounds treated with 2% betulinic acid formulation, 10 and 20% acetone and 20% water extracts had pronounced crust formation. Wounds treated with 2% betulinic acid still healed like those treated with amphotericin B if lesion sizes are considered. Wounds treated with both acetone extracts and 20% water extract had higher lesion sizes, indicating decreased wound contraction. However, these wounds still showed signs of healing, hence the pronounced crust formation. Treatment of wounds led to less scab formation for unknown reasons. However, the failure of lupeol to adequately inhibit *Candida albicans* infections might be responsible.

7.4.4. Presence of infection on skin tissue and internal organs

Skin tissues and internal organs of some of the rats harboured some fungal microorganism(s) as shown by the presence of colonies on agar plates inoculated with the respective samples. Though the identity of the infecting microrganism(s) was not confirmed or elucidated, *Candida albicans* remains the likeliest candidate. The masses and the temperatures of the rats that showed infection on the different sites remained unaffected. Furthermore, the organs did not show any abnormalities, whether or not the presence of microorganisms was detected. The wounds on these rats still healed similarly to those on the other rats, suggesting that the presence of infection on skin tissue or internal organs did little to hinder the healing process.

In evaluating the lack of correlation between *in vitro* and *in vivo* results one should keep in mind that *C. albicans* usually infects moist membranes or moist areas and that the model used may not be applicable to test the efficacy of compounds against *C. albicans* infections.

CHAPTER 8

ANTHELMINTHIC ACTIVITY OF *CURTISIA DENTATA* EXTRACTS, LUPEOL, URSOLIC ACID AND BETULINIC ACID

8.1. INTRODUCTION

The increasing emergence of resistance of gastrointestinal nematodes to conventional anthelminthics and resulting economic losses demonstrate an urgent need for intervention (Hertzberg & Bauer, 2000). *Trichostrongylus colubriformis* causes parasitic enteritis that predisposes sheep to diarrhoea, weakness and death. This parasite is frequently found in cattle and sheep in South Africa causing loss in production (Horak, 2003; Horak *et al.,* 2004). *Haemonchus contortus* is one of the most important nematodes due to its high prevalence and pathogenicity (Hounzagbe-Adote *et al.,* 2005). For instance, in southern Benin, the prevalence of *H. contortus* was estimated at 92.5% in goats and sheep (Salifou, 1996).

Control of infection with parasitic nematodes such as *T. colubriformis* and *H. contortus* involves the utilization of commercial anthelminthics. However, resistance to most of the commercialized anthelminthics is threatening productivity (van Wyk *et al.,* 1997). Furthermore, these chemical products are often highly priced so that subsistence and small-scale livestock farmers in developing countries cannot access them (Hounzangbe-Adote *et al.,* 2005). In these countries small-scale and subsistence farmers rely on traditional methods of deworming that include preparation of remedies from plants (Hammond *et al.,* 1997; Waller *et al.,* 2001; Akhtar *et al.,* 2000). The scientific evidence to support employing plant-based remedies as anthelminthics is lacking (Hammond *et al.,* 1997).

Extracts of several African plant species are active against parasitic and free-living nematodes (Enwerem *et al.,* 2001, Hammond *et al.,* 1997; McGaw *et al.,* 2000; Bizimenyera *et al.,* 2006). Extracts of *Artemisia* species have shown activity against *Haemonchus* species (Idris *et al.,* 1982; Iqbal *et al.*, 2004), *Trichostrongylus* species (Sharma, 1993) and other parasitic nematodes. Furthermore, Hördegen *et al.* (2003) demonstrated anthelminthic activity of ethanol extracts of several plant species in an *in vivo* sheep model.

The motivation for investigating antihelmintic activity is based on the activity of betulinic acid against *Caenorhabditis elegans* at 500 µg/ml after seven days of incubation (Enwerem *et al.,* 2001). Betulinic acid is one of the compounds isolated from *Curtisia dentata* leaves. The dichloromethane and acetone extracts of *Curtisia dentata* leaves, which are enriched with betulinic acid, were selected for investigation of anthelminthic activity as they were the most active against *Candida albicans.* Furthermore the azole antifungal agents also have anthelminthic activity. If the extracts with antifungal activity have the same target as the azole agents, they may also have anthelminthic activity.

In this study, extracts of *Curtisia dentata* were tested for *in vitro* anthelminthic activity against *Caenorhabditis elegans, Trichostrongylus colubriformis* and *Haemonchus contortus.* In the previous chapters the acetone and dichloromethane extracts of *Curtisia dentata* leaves were active *in vitro* against *Candida albicans* and several other fungal species. Betulinic acid, ursolic acid and lupeol, isolated from the leaves of *Curtisia dentata*, were also tested for anthelminthic activity.

8.2. MATERIALS AND METHODS

8.2.1. Plant extracts and compounds

Powdered leaves of *Curtisia dentata* were serially extracted as described in Chapter 2. Concentrations of 100 mg/ml of the dichloromethane and acetone extracts were dried and then dissolved in DMSO. Stock solutions of 2 mg/ml of betulinic acid, lupeol and ursolic acid were prepared in DMSO.

8.2.2. Anthelminthic activity

8.2.2.1. Recovery and preparation of eggs

The parasite eggs were prepared using guidelines by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles *et al.,* 1992). Faecal pellets were collected from lambs, infected with either *T. colubriformis* or *H. contortus* or both, using sterilized harnesses and collecting bags. Water was added to the faecal pellets to facilitate homogenization in a blender. The liquid slurry was filtered through a sieve of 400 µm mesh size to remove coarse plant debris. The suspension was serially filtered through sieves of 250, 150, 90 and 63 µm mesh sizes. The eggs

were then trapped on a sieve of 38 µm mesh size and washed into 50 ml centrifuge tubes that were filled with distilled water. These tubes were centrifuged at 3 000 x g for 5 min. The supernatant was collected into a 1 L conical cylinder filled with distilled water to allow the eggs to sediment for 2 hours. The eggs were siphoned from the bottom of the conical flask into a beaker subjected to electromagnetic stirring. The suspension was adjusted to a final concentration of 100 eggs per 10 µl. The suspension was used within 1 hr (Bizimenyera *et al.,* 2006).

8.2.2.2. Egg hatch inhibition assay

Approximately 100 eggs in 200 µl aliquots were pipetted into each well of a 48-well microtitre plate. In the test wells, 200 µl of the appropriate plant extract in concentrations of 16.7, 8.3, 4.17, 2.1, 1.03, 0.53 and 0.27 mg/ml or isolated compounds at 333, 167, 83, 42 and 21 µg/ml were added. Triplicates were prepared for each concentration. The eggs were incubated at room temperature in a humidified chamber for 48 hours prior to counting of eggs that failed to hatch to L1 stage of larval development (Bizimenyera *et al.,* 2006).

8.2.2.3. Anthelminthic activity against L3 stage larvae

The eggs (1 ml) were added into Petri dishes in the presence of 50 µl of a suspension of lyophilised *E. coli* (ATCC 9637) for the development of nematode larvae (Hubert & Kerboeuf, 1992) and 10 µl of amphotericin B (Sigma®) to control fungal growth. The dishes were incubated for five days to develop larvae into L3 stage of development. The L3 larvae were frozen until needed. For the anthelminthic assay, the larvae were washed off the base of dish with M9 buffer and treated with various concentrations of compounds and extracts of *Curtisia dentata* in wells and incubated for 48 hrs. Motile and non-motile larvae were counted to obtain percentage inhibition of motility. Larvae that did not display signs of motility were regarded as dead larvae.

8.2.2.4. Anthelminthic activity against developing larvae

The eggs hatched in **8.2.2.2.** above were used in this assay procedure. The emerging larvae were incubated under humidified conditions for 24 hours prior to treatment with various concentrations of isolated compounds and extracts of *Curtisia dentata* leaves. The larval suspensions were incubated for a further 5 days before

counting of motile and immotile larvae. The experiments and controls were performed in triplicate.

8.2.2.5. *C. elegans*

Caenorhabditis elegans was maintained in Petri-dishes on Nematode Growth agar (composition/litre: 3 g NaCl, 2.5 g peptone and 17 g agar, 5 mg cholesterol, 0.246 g $MgSO_4.7H_2O$, 0.147 g $CaCl_2.2H_2O$, 2.7 g KH_2PO_4, 0.9 g K_2HPO_4) (Brenner, 1974) seeded with *E. coli*. The dishes were incubated at 20°C in the dark for 6 days prior to anthelminthic activity assay procedures. The plates were washed with 1 ml M9 buffer (composition/litre: 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$ (Brenner, 1974). Several plates were washed into a beaker to establish a density of 100 nematode/10 µl. Into 25-well plates 2 ml of plant extracts and compounds in M9 buffer were added. Nematodes (10 µl) were added into the wells and plates incubated for two hours in the dark. Larvae that did not display signs of motility were regarded as dead larvae. Numbers of dead and viable larvae were counted and percentage of dead cells calculated using the formula:

$$\% \text{ immotile larvae} = \frac{\text{number of immotile larvae}}{\text{(Number of immotile + motile larvae)}} \times 100\ \%$$

Levamisole (5 and 10 µg/ml) was used as a positive control. Untreated larvae were used as negative controls.

8.2.2.6. Calculation of LC_{50} values

The LC_{50} (lethal concentration that resulted in 50% inhibition of larval motility) values were estimated from direct extrapolation of a plot of concentration of either compound or extract (X-axis) against the inhibition of larval motility (% of the control). An example of the type of graph drawn is displayed in Fig. 8.1.

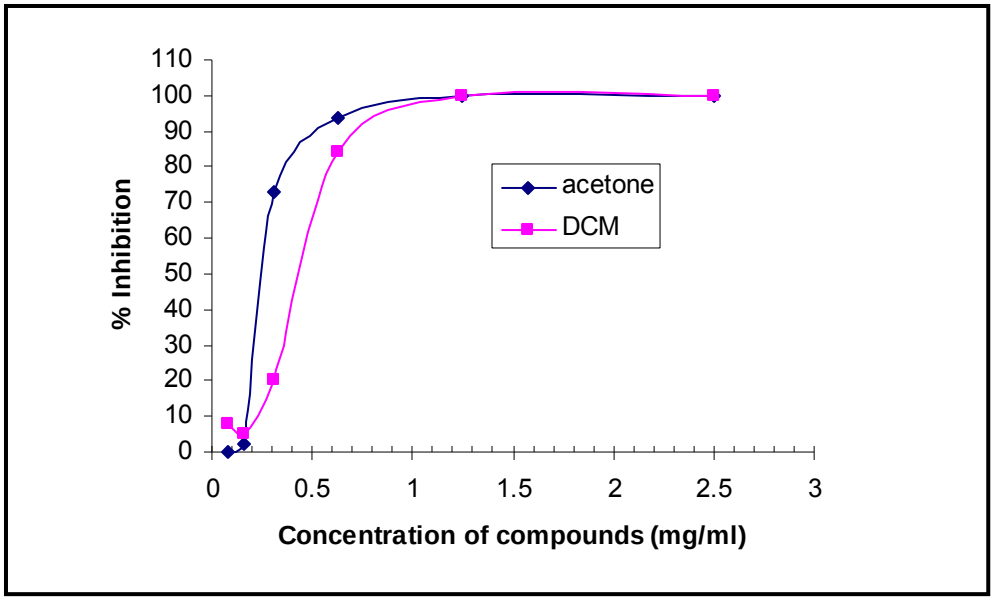


Figure 8.1. An example of a graph drawn to estimate the LC_{50} values of compounds and bulk fractions against parasitic and free-living nematodes.

8.3. RESULTS

8.3.1. Egg hatching

T. colubriformis and *H. contortus* were treated with various concentrations of isolated compounds and extracts of *C. dentata* and incubated for five days. The larvae were treated a day after egg hatching. The percentages of motile larvae in high concentrations of compounds (333 and 167 µg/ml) were low. At these concentrations there were virtually no motile larvae. The number of motile larvae only increased as low concentrations of isolated compounds (Fig. 8.2). Similar results were obtained with crude extracts of *C. dentata* leaves, where high concentrations led to little or no observable evidence of motile larvae after 5 days of incubation. At low concentrations the number of motile larvae increased (Fig. 8.3). In solvent-treated controls about 91% of larvae were motile.

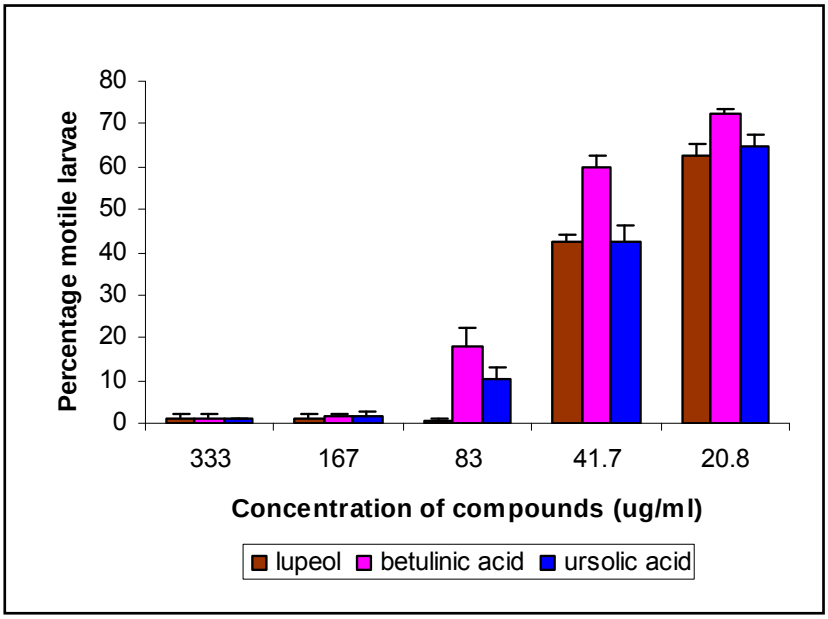


Figure 8.2. Percentage of motile larvae (mixture of *Haemonchus contortus* and *Trichostrongylus colubriformis*) treated with isolated compounds after five days of incubation. The LC_{50} values were 35 µg/ml for lupeol, 65 µg/ml for betulinic acid and 35 µg/ml for Ursolic acid.

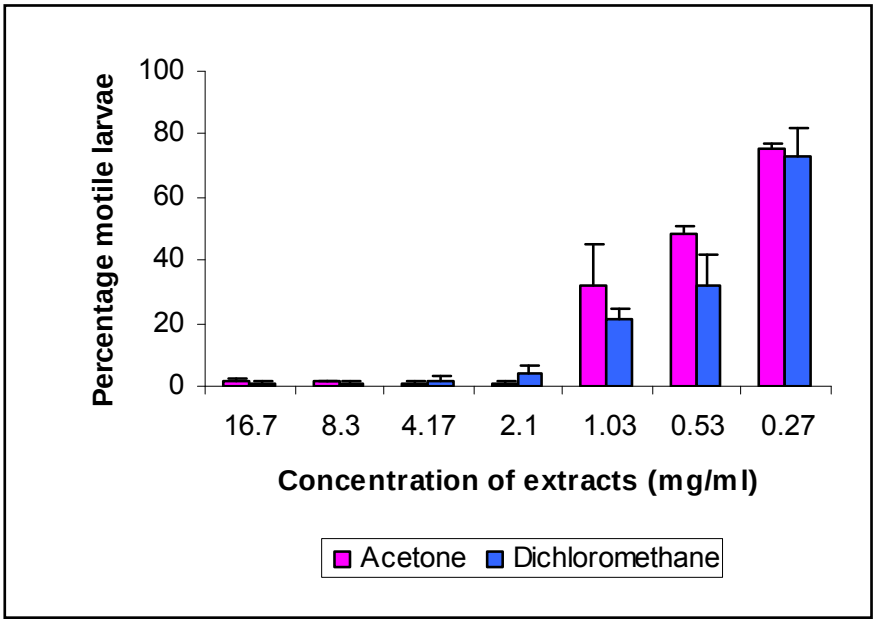


Figure 8.3. Percentage of motile larvae (mixture of *Haemonchus contortus* and *Trichostrongylus colubriformis*) treated with acetone and dichloromethane extracts of *Curtisia dentata* after five days of incubation. The LC_{50} values were estimated at 0.4 mg/ml for both fractions.

The compounds and extracts did not affect egg hatching of *H. contortus* and *T. colubriformis.* In the controls the number of unhatched eggs was comparable to eggs treated with plant extracts and compounds. It was observed that at high concentrations (16.7, 8.3, 4.17 and 2.1 mg/ml) of plant extracts no eggs or larvae were present, suggesting that lysis occurred. In concentrations where the numbers of motile larvae were low the eggs still hatched prior to paralysis.

8.3.2. Inhibition of motility of adult parasitic nematodes

The acetone and dichloromethane extracts of *Curtisia dentata* inhibited motility of *Trichostrongylus colubriformis* at a concentration range of 0.16 – 2.5 mg/ml. The lowest toxic concentration of both the acetone and dichloromethane extracts, 0.16 mg/ml, led to over 60% paralysis of the larvae. The lowest tested concentration of both extracts, 0.08 mg/ml resulted in less than 10% of non-motile larvae. The larval motility percentages resulting from acetone extracts were very similar to those due to the dichloromethane extracts. The average survival in controls was 99% (Fig. 8.4).

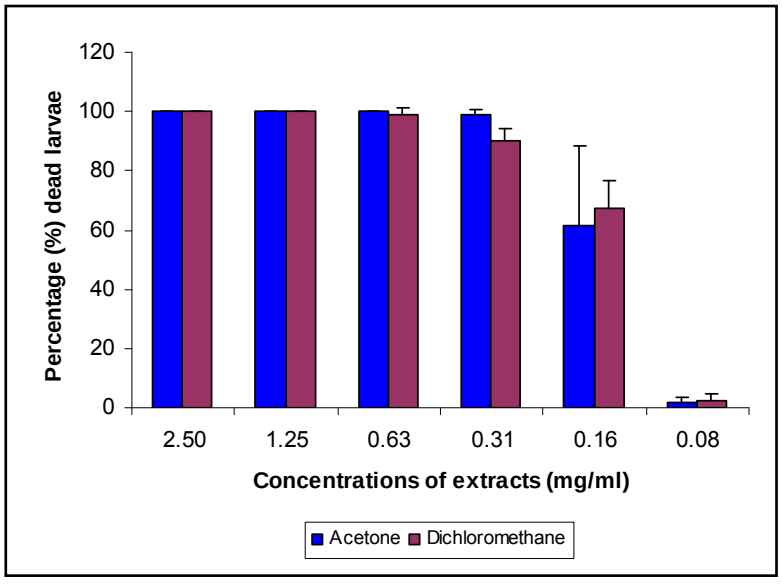


Figure 8.4. Percentage of dead *Trichostrongylus colubriformis* larvae after 48 hours of incubation in the presence of varying concentrations of the acetone and dichloromethane extracts of *Curtisia dentata* leaves. The estimated LC_{50} value was calculated as 0.15 mg/ml for both acetone and DCM bulk fractions.

The dichloromethane extract was active against *Haemonchus contortus* at a concentration range of 0.63 – 2.5 mg/ml. About 20% average inhibition of motility of

larvae resulted from a concentration of 0.63 mg/ml. The acetone extract was active at concentration range 0.31 to 2.5 mg/ml. Over 70% of the larvae were not motile after 48 hours of incubation in the presence of 0.31 mg/ml of the acetone extract. At this concentration (0.31 mg/ml) the percentage of immotile larvae resulting from the acetone extract was significantly higher than that resulting from the dichloromethane extract. Concentrations of 2.5 mg/ml and 1.25 mg/ml of both acetone and dichloromethane resulted in no motile larvae after 48 hours of incubation. Concentrations of 0.16 and 0.08 mg/ml of both extracts only managed to inhibit motion of less than 10% of the larvae (Fig. 8.5).

Two compounds isolated from *Curtisia dentata* leaves, namely, betulinic acid and lupeol were tested to evaluate their ability to paralyse *Trichostrongylus colubriformis* and *Haemonchus contortus* larvae. The compounds were tested at the concentration range 0.32 – 1000 µg/ml. Both compounds inhibited larval motility at high concentrations. Low concentrations of the compounds did not result in appreciable numbers of motionless larvae. In the presence of compounds at the tested concentration range only less than 80% of the larvae of both nematode species were motionless after 48 hours of incubation. Lupeol and betulinic acid suffer from poor solubility in aqueous media (Figs. 8.6 & 8.7). Upon mixing with water during dilution the precipitation resulted in a milky suspension, making these compounds possibly unavailable to act on the test nematode larvae. Modifications of the structures may result in more soluble derivatives of these lupane triterpenes.

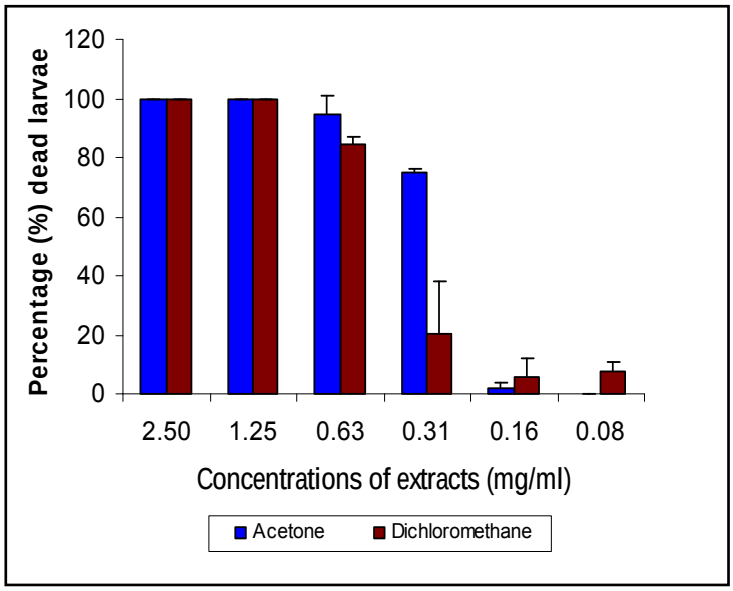


Figure 8.5. Percentage of dead *Haemonchus contortus* larvae after 48 hours of incubation in the presence of varying concentrations of the acetone and dichloromethane extracts of *Curtisia dentata* leaves. The estimated LC_{50} values were 0.2 mg/ml for acetone bulk fraction and 0.45 mg/ml for the DCM fraction.

The extracts showed more activity against the larvae than the isolated compounds. It would seem that, apart from lupeol and betulinic acid, other components in the extract contribute to the activity against larval motility. This emanates from the fact that betulinic acid and lupeol were, by themselves not very active against the tested nematodes. The compounds only managed less than 80% inhibition of larval motility at all tested concentrations.

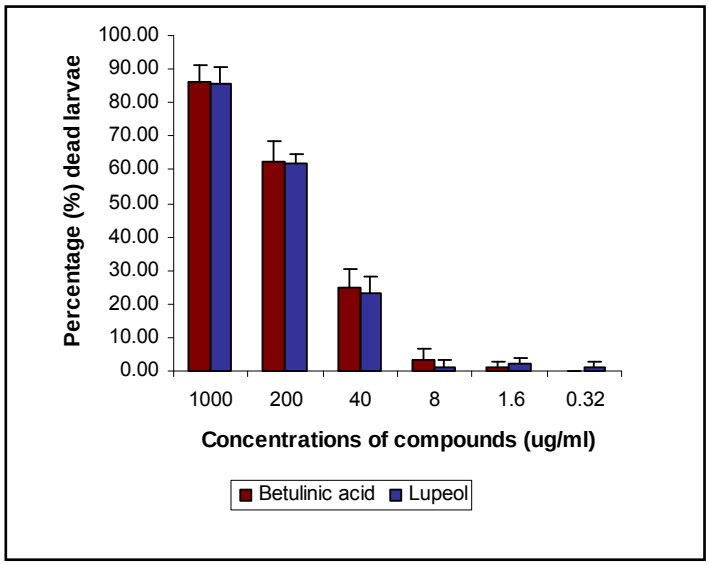


Figure 8.6. Percentage of dead *Trichostrongylus colubriformis* larvae after 48 hours of incubation in the presence of varying concentrations of lupeol and betulinic acid. The estimated LC_{50} value was 80 µg/ml for both lupeol and betulinic acid. Nonmotile larvae were considered dead.

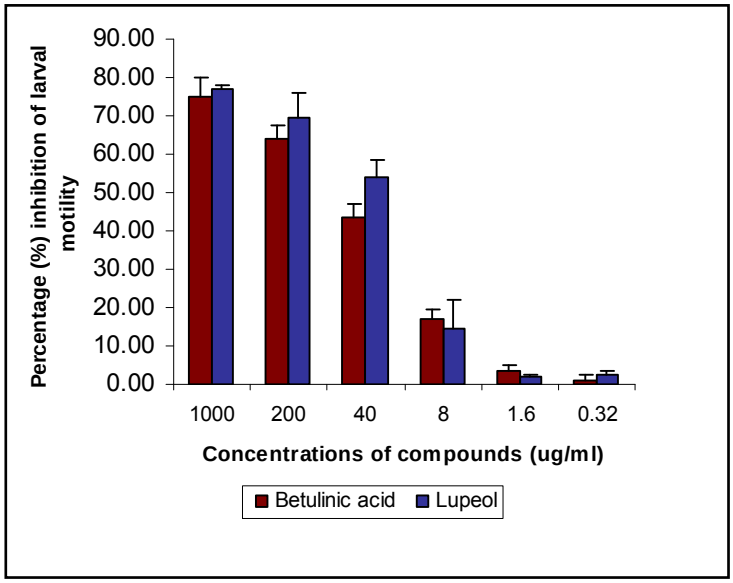


Figure 8.7. Percentage of dead *H. contortus* larvae after 48 hours of incubation in the presence of varying concentrations of lupeol and betulinic acid. LC_{50} values were 20 µg/ml for lupeol and about 50 µg/ml for betulinic acid.

8.3.3. Inhibition of motility of free-living nematodes

Three compounds isolated from the dichloromethane extract of *C. dentata,* namely, lupeol, betulinic acid and ursolic acid, together with the acetone and dichloromethane extracts were assessed for ability to inhibit the motility of the free-living nematode *Caenorhabditis elegans.* After 2 hours of incubation, the highest concentrations of isolated compounds (200 µg/ml) inhibited up to 90% of larval motility. Concentrations ranging from 0.16 to 40 µg/ml resulted in about 20% inhibition of larval motility after 2 hours of incubation at 25 ^{o}C (Fig. 8.8). The highest concentrations of extracts (2.5 mg/ml) resulted in about 35% inhibition of larval motility whereas lower concentration ranging from 1.25 to 0.08 mg/ml resulted in less than 20% inhibition of larval motility (Fig. 8.9). In the untreated control 4% motionless larvae were observed after 2 hrs of incubation. In the levamisole-treated (10 µg/ml) controls average inhibition of larval motility was at 25%.

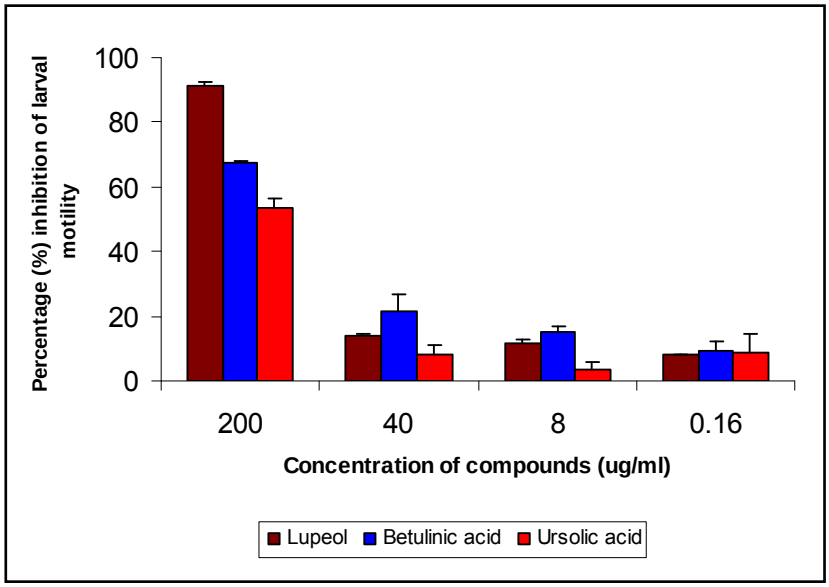


Figure 8.8. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with compounds isolated from *Curtisia dentata* leaves. After treatment with compounds the nematodes were incubated for 2 hrs before counting motionless worms. The LC_{50} values were estimated at 120 µg/ml for lupeol, 140 µg/ml for betulinic acid and 180 µg/ml for Ursolic acid.

After 7 days of incubation the highest concentrations of all tested compounds resulted in 100% inhibition of larval motility. Lupeol was observed as the most active of the isolated compounds. A concentration of 40 µg/ml of lupeol resulted in over 80% inhibition of larval motility while betulinic acid led to 40% inhibition. Ursolic acid,

though less active than lupeol led to higher inhibition of motility than betulinic acid at most concentrations tested (Fig. 8.10). After 7 days of incubation in the untreated controls approximately 85% motility was observed. In 5 and 10 µg/ml levamisole-treated controls 65% and 48% motility were observed, respectively.

Both the acetone and dichloromethane extracts of *Curtisia dentata* leaves were similar in their inhibition of larval motility after 7 days of incubation, displaying a concentration-dependent inhibition of larval motility. Concentrations ranging from 2.5 to 0.63 mg/ml led to over 80% inhibition of larval motility after 7 days of incubation. At 0.31 mg/ml, both extracts caused over 60% inhibition of larval motility while lower concentrations were less effective (Fig.8.11).

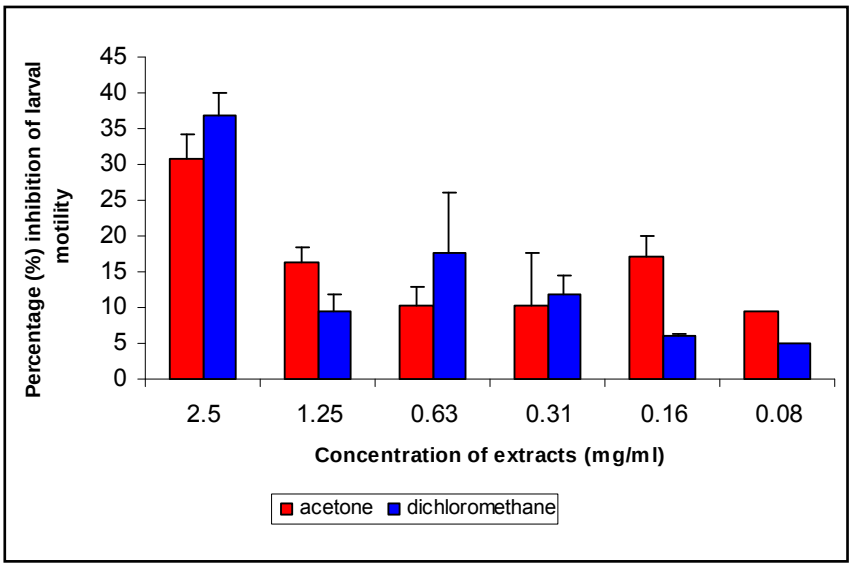


Figure 8.9. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with acetone and dichloromethane of *Curtisia dentata* leaves. After treatment with plant extracts the nematodes were incubated for 2 hrs before counting motionless nematodes.

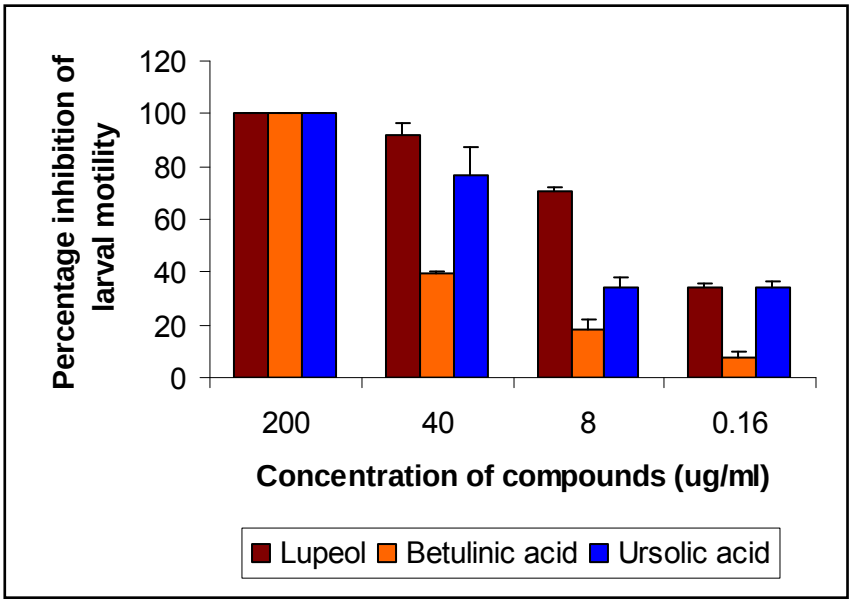


Figure 8.10. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with isolated compounds from leaves of *Curtisia dentata*. After treatment with the pure compounds, the nematodes were incubated for 7 days before counting motionless and motile worms. The LC50 values were 2 µg/ml for lupeol, 70 µg/ml for betulinic acid and about 12 µg/ml for Ursolic acid.

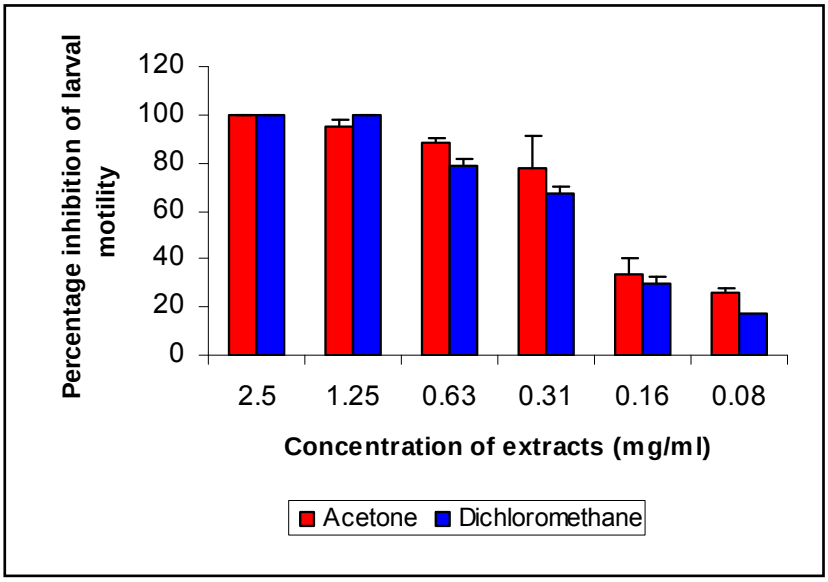


Figure 8.11. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with the acetone and dichloromethane extracts of the leaves of *Curtisia dentata*. After treatment with the pure compounds, the nematodes were incubated for 7 days before counting of motionless and motile worms. The calculated LC50 values were 0.2 mg/ml for both fractions.

8.4. DISCUSSION AND CONCLUSION

Several extracts of different medicinal plants show anthelminthic activity (Raj, 1975; Enwerem *et al.,* 2001; Ademola *et al.,* 2004). Hounzangbe-Adote *et al.* (2005) tested the *in vitro* effects of four tropical plants on activity against parasitic nematode. They reported that concentrations of between 300 and 2400 µg/ml of ethanolic extracts of Fagara, Papaya, Morinda and Newbouldia are active against *H. contortus*. In this study we report that the anthelminthic activity of our extracts were comparable with reported literature (Hounzagbe-Adote *et al.,* 2005). Acetone extracts of *Curtisia dentata* at concentrations ranging from 310-2500 µg/ml were effective against *H. contortus.*

Bizimenyera *et al.* (2006) reported that extracts of *Peltophorum africanum* had *in vitro* activity against egg hatching and larval development of *T. colubriformis.* The ED_{50} values for leaf, bark and root extracts against egg hatching were 0.619, 0.383 and 0.280 mg/ml, respectively. In this study the *in vitro* activity of extracts of *C. dentata* leaves was demonstrated against parasitic and free-living nematodes. These results indicate that the leaves of *C. dentata* may be useful to treat helminthiasis in South African folk medicine.

Despite the indication that some plant extracts are active against several nematodes, the exact mechanism remains unclear. Some compounds such as palasonin, the active principle of *Butea frondosa* inhibit glucose uptake and accelerate glycogen depletion in target nematodes (Kumar *et al.,* 1995). The mechanism of action of many anthelminthic plant extracts may involve inhibition of energy metabolism (Dahanukar *et al.,* 2000).

In this study, betulinic acid induced paralysis of the tested nematodes, *C. elegans*, *H. contortus* and *T. colubriformis* at concentrations of between 200 and 1000 µg/ml. Against *C. elegans* betulinic acid induced 100% paralysis at a concentration of 200 µg/ml. These results confirm the study by Enwerem *et al.* (2001) in which it was reported that betulinic acid isolated from *Berlina grandiflora* had strong activity against *C. elegans* at concentrations of 100 and 500 µg/ml. The mechanism involved in the activity of betulinic acid against parasitic and free-living nematodes is yet to be described. However, its activity against parasitic nematodes *in vivo* and *in vitro* has not been demonstrated. This appears to be the first report of the effects of betulinic acid against parasitic nematodes, *T. colubriformis* and *H. contortus*. Investigations of

the *in vivo* activity of both the extracts of *C. dentata* and the isolated compounds may conclusively indicate their potential as anthelminthics.

CHAPTER 9

GENERAL DISCUSSIONS AND CONCLUSIONS

The aim of this study was to isolate and characterize compounds active against *C. albicans* from leaves of the most promising tree species and investigate the potential use of these compounds or extracts against *C. albicans* infections. The results obtained in addressing the different objectives of the study are discussed below.

9.1. ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF SELECTED PLANT SPECIES

The antifungal and antibacterial activity of acetone extracts of leaves of seven selected trees were determined. Extracts of *Curtisia dentata* had more active compounds on bioautograms against all the bacterial (*Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus*) test organisms. Antifungal compounds with similar R_f values also inhibited bacterial growth, suggesting non-specificity of the antimicrobial activity. The acetone and dichloromethane extracts of *C. dentata* contained more antibacterial and antifungal compounds (4) than the hexane extract. This suggests that the active compounds are of intermediate polarity. The dichloromethane and hexane extracts of six *Terminalia* species had more antifungal compounds (total of 59 and 50) than the acetone extracts (18) (Masoko *et al.* 2005)..

The serial microplate dilution method results showed that the acetone and dichloromethane extracts of most plant species had both antibacterial and antifungal activity. The lowest minimal inhibitory concentration (MIC) recorded was 0.05 mg/ml resulting from the acetone extract of *Curtisia dentata* against *Enterococcus faecalis,* further suggesting that *Curtisia dentata* was the most active species. The acetone extracts of *C. dentata, Terminalia sambesiaca* and *T. phanerophlebia* yielded the lowest MIC values (0.12 – 0.15 mg/ml) against *C. albicans.* This observation further confirmed the antifungal activity of *C. dentata.* However, the low MIC values of the *Terminalia* species were surprising considering that active compounds were not observed on TLC bioautograms against *C. albicans.* Masoko *et al.* (2005) reported low MIC values for both *Terminalia* species with few compounds observed on TLC bioautograms against most fungal species tested. Furthermore, all extracts of both

Terminalia species and acetone extract of *C. dentata* resulted in low MIC values (0.02 – 0.08 mg/ml) against *Cryptococcus neoformans* and *Microsporum canis.* The acetone extracts of the three plant species had low MIC values (0.03 – 0.08 mg/ml) against *Sporothrix schenckii.* These observations, supported by the MIC averages, indicated that these three plant species were the most active against fungal test organisms.

9.2. SELECTION OF THE MOST PROMISING TREE SPECIES FOR ISOLATION OF ACTIVE COMPOUNDS

From a pool of seven plant species, *Curtisia dentata* was selected for further work based on the following reasons, 1) the leaf extracts of the plant species possesed more compounds showing activity against *C. albicans* and other tested microbial organisms on bioautograms, 2) extracts of the leaves resulted in low MIC values against *C. albicans* and 3) its leaf powder was more extractible resulting in higher masses of extracts. *Curtisia dentata* was therefore chosen for isolation of active compounds using bioassay-guided fractionation. The leaves of *C. dentata* were preferred to the stem bark because 1) the leaves were more active than the stem bark and 2) the harvesting of the stem bark may kill the plant and *C. dentata* is a threatened species.

9.3. ISOLATED COMPOUNDS

Using bioassay-guided fractionation four (4) pentacyclic triterpenoids were isolated from the leaves of *Curtisia dentata.* ^{13}C and ^{1}H NMR spectroscopic and mass spectrometric data led to identification of the compounds as the lupane triterpenoids lupeol and betulinic acid, and the ursane triterpenes ursolic acid and colosolic (hydroxyursolic) acid. These compounds are common secondary metabolites present in many plant species (Das and Mahato, 1983). However, these compounds have not been isolated from extracts of *Curtisia dentata,* the only southern African member of the Cornaceae previously. Activities of some of these compounds against infections and cancer progression have however been reported.

Reports concerned with antifungal, antibacterial and anthelminthic activities of betulinic acid and other pentacyclic triterpenoids are scanty. Betulinic acid and its derivatives have potent anti-HIV activity (Aiken & Chen, 2005). Ursolic acid has hepatoprotective, anti-inflammatory, antiviral and antitumor activities (Rao *et al.,*

2001), as well as growth inhibitory activity against *Staphylococcus aureus* (Zeletova *et al.,* 1987) and *Escherichia coli* (Malladavhani *et al.,* 2004). Ursolic acid isolated from *Crataegus pinatifida* is active against HIV-1 protease at 100 µg/ml (Pengsuparp *et al.,* 1994). *Curtisia dentata* is an excellent source of betulinic acid, estimated at approximately 25% in the dichloromethane extract of the leaves. This level of betulinic acid has not been reported anywhere in the Cornaceae family. In *Betula* species the betulinic acid content is estimated at 22% (Yogeeswari and Sriram, 2005).

Betulinic acid, lupeol and ursolic acid resulted in clear zones on TLC bioautograms sprayed with *C. albicans, S. aureus, E. coli* and *Enterococcus faecalis.* Using the serial microplate dilution method, betulinic acid, ursolic acid and colosolic acid resulted in appreciable inhibition of fungal growth with MIC values ranging from 8 to 63 µg/ml. A similar trend was observed with the bacterial test organisms. The extracts were more active than isolated compounds against bacterial test organisms, suggesting that perhaps synergism plays an important role in the activity of the plant species. All the three compounds possess a carboxyl (-COOH) group at position 28. It has been reported that the 28-COOH and the ester functionality at C-3 may be involved in the pharmacological actions of some triterpenoids (Mallavadhan *et al.,* 2004). This suggestion may account for the low antifungal activity of lupeol which lacks the 28-COOH group. This may further suggest that 28-COOH may be important for pharmacological actions of these and other triterpenoids.

9.4. ANTHELMINTHIC ACTIVITY

Some antifungal compounds are also active against nematodes, therefore the anthelminthic activity of the extracts and isolated compounds were investigated. Extracts of different medicinal plants show anthelminthic activity against both parasitic and free-living nematodes in both *in vivo* and *in vitro* experiments (Raj, 1975; Enwerem *et al.,* 2001; Ademola *et al.,* 2004). Recently, Hounzangbe-Adote *et al.* (2005) tested the *in vitro* effects of extracts of four tropical plants on activity against parasitic nematodes. However, there exist no reports regarding anthelminthic activity of *C. dentata* extracts against both parasitic and free-living nematodes.

Lupeol and betulinic acid together with the acetone and dichloromethane extracts of *Curtisia dentata* were investigated for the presence of anthelminthic activity. This appears to be the first report of the anthelminthic activity of extracts of *Curtisia*

dentata. In this study betulinic acid inhibited the motility of *Trichostrongylus colubriformis, Haemonchus contortus* and *Caenorhabditis elegans.* Enwerem *et al.* (2001) reported that betulinic acid inhibited the motility of *C. elegans* at 500 µg/ml (100 µg/ml achieved about 76% inhibition of motility). In our study betulinic acid achieved 100% inhibition of motility at 200 µg/ml. Our results are comparable with those reported by Enwerem *et al.* (2001). These findings further suggest that the activity against *C. elegans* was only observable at high concentrations of betulinic acid. Though *C. elegans* is a valid model organism for the discovery of broad-spectrum anthelminthics, the efficacy of betulinic acid as an anthelminthic has not previously been elevated to include studies on parasitic nematodes. This study demonstrated that betulinic acid has anthelminthic activity against parasitic nematodes at high concentrations (40-200 µg/ml). Ursolic acid and hydroxyursolic acid were isolated in inadequate quantities and these compounds could not be tested for anthelminthic activity.

9.5. CYTOTOXICITY

The cytotoxicity of lupeol and betulinic acid was determined against Vero cells. Toxicity of betulinic acid was comparable to that of berberine with an LC_{50} value of about 10 µg/ml. Lupeol was less toxic to the cells with an LC_{50} value of 90 µg/ml. Some researchers reported that the pharmacological actions of betulinic acid are associated with little cytotoxic activity.

The selectivity index values calculated for betulinic acid were below 1 for all the microorganisms. This could mean that some of the observed activity of betulinic acid against bacteria and fungi emanated from toxicity. Bacteria and fungi were all susceptible to betulinic acid, further suggesting that this compound is non-specific in its antimicrobial activity.

9.6. *IN VIVO* STUDIES

The isolated compounds and extracts were investigated for their *in vivo* wound healing activity and antifungal activity against *C. albicans*. Progression of wound healing in a topical rat model (either enhanced or at a normal rate) was used as measure of antifungal activity. The rate of wound healing of lesions treated with different plant-derived formulations remained comparable with the positive (amphotericin B) and negative (cream only) controls. This model needs to be

validated and optimized. It may require the use of known wound-invading microorganisms in the tests. This model did not yield any evidence of *in vivo* antifungal activity and wound healing activity of the acetone and water extracts of *C. dentata* and isolated compounds (lupeol and betulinic acid).

9.7. CONCLUSIONS

From a pool of seven plant species *Curtisia dentata* contained more antibacterial and antifungal compounds than any of the six species investigated. *C. dentata, Terminalia sambesiaca* and *T. phanerophlebia* were the most active against fungal pathogens based on MIC values. Part of the activity of *C. dentata* could be attributed to the triterpenoids that were isolated. Ursolic acid, betulinic acid and colosolic acid were active against bacterial and fungal test organisms. Surprisingly the extracts were more active against bacterial organisms than the isolated compounds indicating possible synergistic effects.

Lupeol was more active against nematodes. Therefore, this project is further proof that plants contain biologically active compounds. Synthetic chemistry techniques to make derivatives of the isolated compounds may enhance their solubility in water, increase their efficacy and even improve specificity of the compounds. Betulinic acid was isolated in large amounts from the leaves of *Curtisia dentata.* Derivatives of betulinic acid were shown to be more active inhibitors of HIV-1 reverse transcriptase than the parent compound (betulinic acid) (Pengsuparp *et al.,* 1994).

Based on the cytotoxicity data of betulinic acid and the crude extracts, the therapeutic effects of this compound or extracts may be limited only to low concentrations. Further investigation to ascertain the safety of the compounds and extracts to treat candidosis are required. The cytotoxicity data in this study was only obtained with Vero cells (monkey kidney line), and thus studies with several human cell lines may further indicate the safety of the compounds and extracts of *Curtisia dentata* as treatment regiments against candidosis. Furthermore if candidosis is treated by topical application and the toxic components are not absorbed, a useful product may still be developed from *Curtisia dentata* leaves. Because preparations of the extracts, betulinic acid and lupeol in aqueous cream did not result in any observable pathology on rats, topical application may be a viable route to combat *Candida albicans* infections. A total activity of 1072 ml/g for leaf extracts means that the quantity extracted from 1 g can be diluted to more than a litre and still inhibit the

growth of *C. albicans*. Another approach is that derivatization of this compound may provide less toxic and still efficacious derivatives.

It would therefore be worthwhile to invest human and financial resources for intensified screening of medicinal plants with the realistic hope of finding potential antibiotic drugs and cheap alternative medicines. The focus must not only be to isolate compounds, but also to investigate the efficacy of the extracts as these would be cheaper and more freely available.