CHAPTER 11

In vivo antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats

11.1. Introduction

Wound healing consists of an orderly progression of events that establishes the integrity of the damaged tissue. The process of wound healing is essential to prevent the invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue (Sumitra *et al*., 2005). The process of wound healing is promoted by several plant products (Suguma *et al*., 1999), containing active principles like triterpenes, alkaloids, flavonoids (Sharma *et al*., 1990) and biomolecules (Chithra *et al*., 1995). These agents usually influence one or more phases of healing processes. Wound healing properties of two tropical plants *Centella asiatica* (Suguma *et al*., 1996) and *Terminalia chebula* (Suguma *et al*., 2002) have been demonstrated on dermal wound healing in rats.

In earlier studies we found *Combretum* and *Terminalia* extracts had remarkable antifungal activity against *Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus* having minimum inhibitory concentration (MIC) as low as 0.02 and 0.04 mg/ml (Masoko *et al*., 2005 and 2006). The next stage is to evaluate the *in vivo* topical antifungal activity of some of these plant extracts against fungal infections in an animal model. Cytotoxicity using cell lines and the brine shrimp mortality assay were determined (**Chapter 9**) and extracts were not toxic based on these assays. We assume that the extracts applied topically will not have systemic activity, but this has to be confirmed.

A member of the Phytomedicine Programme (Kruger, 2004) had developed a method to test crude extracts and an isolated compound on rats infected with *Staphylococcus aureus*. The animal experiment was carried out at the Onderstepoort Veterinary Institute (OVI) of the Agricultural Research Centre (ARC) in 2002. This work led to a patent and licensed product. Several improvements based on his method were used in this fungal infection study.

11.1.1. Aim

Previous experiments on extracts *of Combretum nelsonii, Combretum imberbe, Combretum albopunctactum* and *Terminalia sericea* (Masoko *et al*., 2005 and Masoko *et al*., 2006), indicated

excellent *in vitro* activity against *C. albicans, C. neoformans, M. canis, S. schenckii* and *A. fumigatus.* The next stage in the potential use of leaf extracts or isolated compounds from these species was to determine *in vivo* activity of *Combretum* and *Terminalia* extracts. A non-infected and fungal infected skin wound model in rats had to be developed to test irritation and effectivity. The study was divided into two pilot studies and main study, each with its aims as follows:

Pilot study I (Local irritancy and wound healing study)

Aim: To establish whether an aqueous cream used as vehicle has any irritant effect.
To determine irritant/ tolerance effect of 10% and 20% crude extracts in cream based on rats.
To determine the degree of wound healing within three weeks.

Pilot study II (Infection with different pathogens)

Aim: To determine the effects of plant extracts in aqueous creams on irritation and the infection and wound healing.
To determine the rate and extent of infection from different pathogens.
To investigate possibility of systemic infection.

Main study (Confirmation study)

Aim: To determine if suppressing the immune system of the rats would make them more susceptible to fungal infection.
To determine healing activity of the extracts and isolated compound under these condition.
To determine antifungal activity of extracts on infected wounds.

11.1.2. Objective

To investigate the effects of *Combretum nelsonii*, *Combretum imberbe*, *Combretum albopunctactum* and *Terminalia sericea* extracts applied topically on skin wounds in control and rat skin wounds infected with fungal pathogens. Wound irritancy and wound healing will be evaluated by physical and histological methods. Aspects evaluated will include wound healing, erythema, exudate formation, crust formation, possible toxic effects of the extracts and histopathology.

11.2. Materials and methods

The research was approved by the Research and Animal Use and Care Committee of the University of Pretoria (VI 010/05 approval number).

11.2.1. Selection of rats

Healthy male Wistar rats weighing 150-200 g were used. The test was conducted using a single gender as a way of reducing variability and to minimize 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).

11.2.2. Housing and feeding conditions

Rats were kept at the University of Pretoria Biomedical Research Centre at Onderstepoort and housed in separate cages at a temperature of 22 ^{o}C (± 2 ^{o}C) and 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). Environmental enrichment e.g. bedding (wood wool), 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, is an effective way to improve their welfare and to distract them from tampering with dressings (Zhu *et al*., 2006).

11.2.3. Preparation of animals

Cages of the rats were labeled with numbers to facilitate identification. Rats were kept in their cages for at least 5 days prior to treatment to allow for acclimatization to the laboratory conditions (Spielmann *et al*., 1999). They were also handled daily in this period. The rats used in pilot studies were not immunosuppressed but the 24 used for the experiment were immunosuppressed 4 days before challenge by subcutaneous injection of 500 µg of estradiol valerate. Estradiol pretreatment is known to inhibit innate and acquired immune defenses (Carlsten *et al*., 1991).

11.2.4. Wound creation

The hair on the back area was removed by cutting it with electrical clippers. The area was disinfected using 0.5% chlorhexidine in 70% alcohol and allowed to dry. Rats were anaesthesized with isoflurane (0.01- 0.05 µg/kg). Six evenly spaced circular wounds were made on each rat using 6 mm diameter punch biopsies (Simosen *et al*., 2002). The whole process was carried out in a biosafety class II cabinet to limit infection and prevent infection of people.

11.2.5. Induced fungal infections

The fungi (*Candida albicans, Cryptococcus neoformans, Microsporum canis* and *Sporothrix schenckii*) were grown for 5-7 days on Sabouraud agar slant at 30°C. Thereafter the fungal material was scraped aseptically from slants, and pooled in 30 ml of sterile water and briefly homogenized. Volumes of 100 µl of the fungal suspension was introduced 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 test products were introduced and the resultant inhibition of growth or healing quantified on the basis of erythema, exudate and physical size of the lesion on a Monday, Wednesday and Friday for 3 weeks. Infection by fungi was clinically detected by the presence or absence of the swelling, erythema, pain and ulceration of the inoculation sites. Rise in body temperature, not eating for 24 hours and weight loss were also notes as clinical signs.

11.2.6. Preparation of extracts

C. nelsonii, C. imberbe, C. albopunctactum and *T. sericea* powdered leaves were extracted with acetone. Extracts were dried at room temperature under a stream of cold air and ground with mortar and pestle and then mixed with aqueous cream consisting of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol to a concentration of 10% (1 g/10 g cream) and 20% (2 g/ 10 g) and kept at 4 ^{o}C until use.

11.3. Pilot studies

11.3.1. Exploratory studies

Procedure: In Pilot study 1 the treatments were applied as shown in **Table 11.1** on sites of the rats.

Table 11.1. Treatment in topical to study skin tolerance.

Sites on the rat	Treatment
A	No treatment
B	Cream only
C	10% *C. nelsonii* crude extract
D	20% *C. nelsonii* crude extract

These sites were randomly allocated when the treatment was repeated. Treatment shown above was repeated using *C. imberbe*, *C. albopunctactum* and *T. sericea* extracts. Two rats were used for each plant extract *i.e*. eight rats were used. Rats were weighed and new creams and creams with crude extracts were applied, every Monday, Wednesday and Friday. The lesions were also measured. All rats were observed daily for any indication of interference with the wound dressing. Severe irritation and enlargement of the wound lead to the termination of that specific treatment. If there are no signs of irritation the experiment was terminated after three weeks when wounds had completely healed. Treatment with best effect was used in subsequent experiments if no adverse effects are found. The lesions were evaluated, temperature and weight measured.

11.3.2. Infection with different pathogens

Procedure: In Pilot study 2 different infections were treated as follows on **Table 11.2**. The study was double blinded

Table 11.2. Treatment of different rats in efficacy experiment.

Site on the rat	Cream	*C. albicans*	Extracts	Amphotericin B
A	-	-	-	-
B	√	√	-	-
C	√	√	-	√
D	√	√	I	-
E	√	√	N	-
F	√	√	P	-
G	√	√	T	-

T= *T. sericea* crude extract, N= *C. nelsonii* crude extract, I= *C. imberbe* crude extract, P= *C. albopunctactum* crude extract, concentration with best effect of crude dried acetone extract in cream, √ = Added, - = Not added.

Wounds were created in the same fashion as pilot study I. Twenty percent of the extracts was chosen from first pilot. Once the wounds were created on each rat, 6 of the 7 wounds were infected with 0.1 ml of the fungal suspension. Three rats were allocated to each fungus, namely *C. albicans, C. neoformans, M. canis* and *S. schenckii*. Treatments as laid down in **Table 11.2** were initiated after 48 hours. The rats were inspected three times a week for any signs of systemic infection by determining mass, food intake, and temperature by means of a microchip.

11.3.2.1. Parameters of infection/recovery

The erythema and exudate were evaluated three times a week using the score provided in **table 11.3.**

Table 11.3. 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

11.3.3. Confirmation study (Treatment experiment)

Once all the necessary precautions were taken, the experiment was carried out with treatments shown in **Table 11.4**. Pilot study 2 was repeated with the following amendments:

Rats were immunocompromised.

Isolated compound was added as part of treatment.

Rats were infected by pads soaked in cultures

Table 11.4. Treatment of different rats in efficacy experiment.

Site on the rat	Cream	*C. albicans*	Extracts	Isolated compound	Amphotericin B	No. of rats
A	√	√	-	-	-	01 – 06
B	√	√	-	-	√	01 – 06
C	√	√	I	-	-	01 – 06
D	√	√	N	-	-	01 – 06

E	√	√	P	-	-	01 – 06
F	√	√	T	-	-	01 – 06
G	√	√	-	√	-	01 – 06
Site on the rat	**Cream**	***C. neoformans***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	07 – 12
B	√	√	-	-	√	07 – 12
C	√	√	I	-	-	07 – 12
D	√	√	N	-	-	07 – 12
E	√	√	P	-	-	07 -12
F	√	√	T	-	-	07 – 12
G	√	√	-	√	-	07 – 12
Site on the rat	**Cream**	***M. canis***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	13 – 18
B	√	√	-	-	√	13 - 18
C	√	√	I	-	-	13 – 18
D	√	√	N	-	-	13 – 18
E	√	√	P	-	-	13 – 18
F	√	√	T	-	-	13 – 18
G	√	√	-	√	-	13 – 18
Site on the rat	**Cream**	***S. schenckii***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	19-24
B	√	√	-	-	√	19-24
C	√	√	I	-	-	19-24
D	√	√	N	-	-	19-24
E	√	√	P	-	-	19-24
F	√	√	T	-	-	19-24
G	√	√	-	√	-	19-24

T= *T. sericea* crude extract, N= *C. nelsonii* crude extract, I= *C. imberbe* crude extract, P= *C. albopunctactum* crude extract in all cases 20 % of crude dried acetone extract in cream, √ = Added, - = Not added.

11.3.3.1. Treatment of different sites on individual rats

Aqueous cream consisting of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol was used to prepare different mixtures.

Site A: Treated with aqueous cream only (negative control),
Site B: Treated with antibiotics e.g. amphotericin B mixed with aqueous cream (positive control),
Site C: Treated with *C. imberbe* extracts mixed with aqueous cream
Site D: Treated with *C. nelsonii* extracts mixed with aqueous cream
Site E: Treated with *C. albopunctactum* extracts mixed with aqueous cream
Site F: Treated with *T. sericea* extracts mixed with aqueous cream
Site G: Treated with isolated compound mixed with aqueous cream

Wounds were medicated with a local application (enough to cover the wound) of each cream. This study was randomised, and blinded by mixing cream with different compounds/extracts, as well as changing the sites on the rats. The person applying the treatments did not know which treatment was being used so that bias in evaluation was removed.

11.3.3.2. Administration of doses

The test material was formulated into topical creams by blending in a mortar and pestle. A 1% concentration in aqueous cream of the isolated compounds were used while a 20% of the crude extract (Kruger and Eloff, 2004), also in emulsifying cream was used. The positive control was 0.1% amphotericin B. The wound was cleaned every 48 hours with clean cotton-wool and the creams were applied to the wounds for 3 weeks, or until the positive control (wound treated with amphotericin B) has 100 % healed or until the untreated controls have healed, whatever was the latest.

11.3.3.3. Observations

Each animal served as its own control with five test sites for the crude and isolated compound, one as a positive control with Amphotericin B and one site as a negative control. The presence of factors such as erythema, exudate, swelling, ulceration, crust formation, healing and infection were checked. The measuring of the size of the lesion relative to that of 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, each rat was taken out of the cage, given anaesthetic, the dressing removed and the different parameters were measured. Thereafter the test samples and control treatment were applied and new dressings were applied to each test rat and it was placed in its cage.

11.3.3.4. Daily observations on weekdays

Observations were systematically recorded with individual records kept for each rat. Rats were observed individually at least once during the first 30 minutes after dosing and periodically during the first 24 hours (with special attention given during the first four hours). The rats were observed daily for up to 3 weeks. The time at which any abnormalities were observed as well as when they disappear were noted. Observations included changes in skin and fur, diarrhoea, lethargy, unusual sleepiness, weight loss and coma. After the completion of the experiment rats were euthanased by CO_2 inhalation and a necropsy was done. The liver, heart, lungs, intestine, lymph nodes and kidney of the rats were checked by the pathologist for gross abnormalities.

11.4. Evaluation of lesions

The mass of each rat, body temperature and lesion characteristics were recorded three times a week. The study was blinded (person doing the evaluation did not know the treatment).

11.4.1. Lesion size measured

The lesion sizes of each rat were measured with the same callipers three times a week using the horizontal and vertical diameters.

11.4.2. Recording of data

The data for each rat was recorded on a single form for the three weeks period. The recording sheets and results are shown on Tables 11.5 and 11.6 (appendix)

11.5. Pathological and histopathological studies

Histopathological studies were done with the help of a pathologist (**Dr Joshua Dabwroski**) 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 and Mowry, 1965). Sections were qualitatively assessed under the light microscope and graded in respect of congestion, oedema, infiltration of polymornuclear leukocytes (PMNLs) and monocytes, necrosis, fibroblast proliferation, collagen formation, angiogenesis and epithelisation (Shukla *et al*., 1999). Necropsies were performed and the presence of fungi were determined using the PAS stain.

Schematic presentation of the methods is presented from slide 1 to slide 31 (Figure 11.1 to 11.3)

11.6. RESULTS

11.6.1. Pilot 1 (Local irritancy and wound healing study)

Results for first Pilot study are presented in the paper: **Evaluation of the wound healing activity of selected *Combretum* and *Terminalia* species (Combretaceae)** (*Submitted to Onderstepoort Journal of Veterinary Research)*

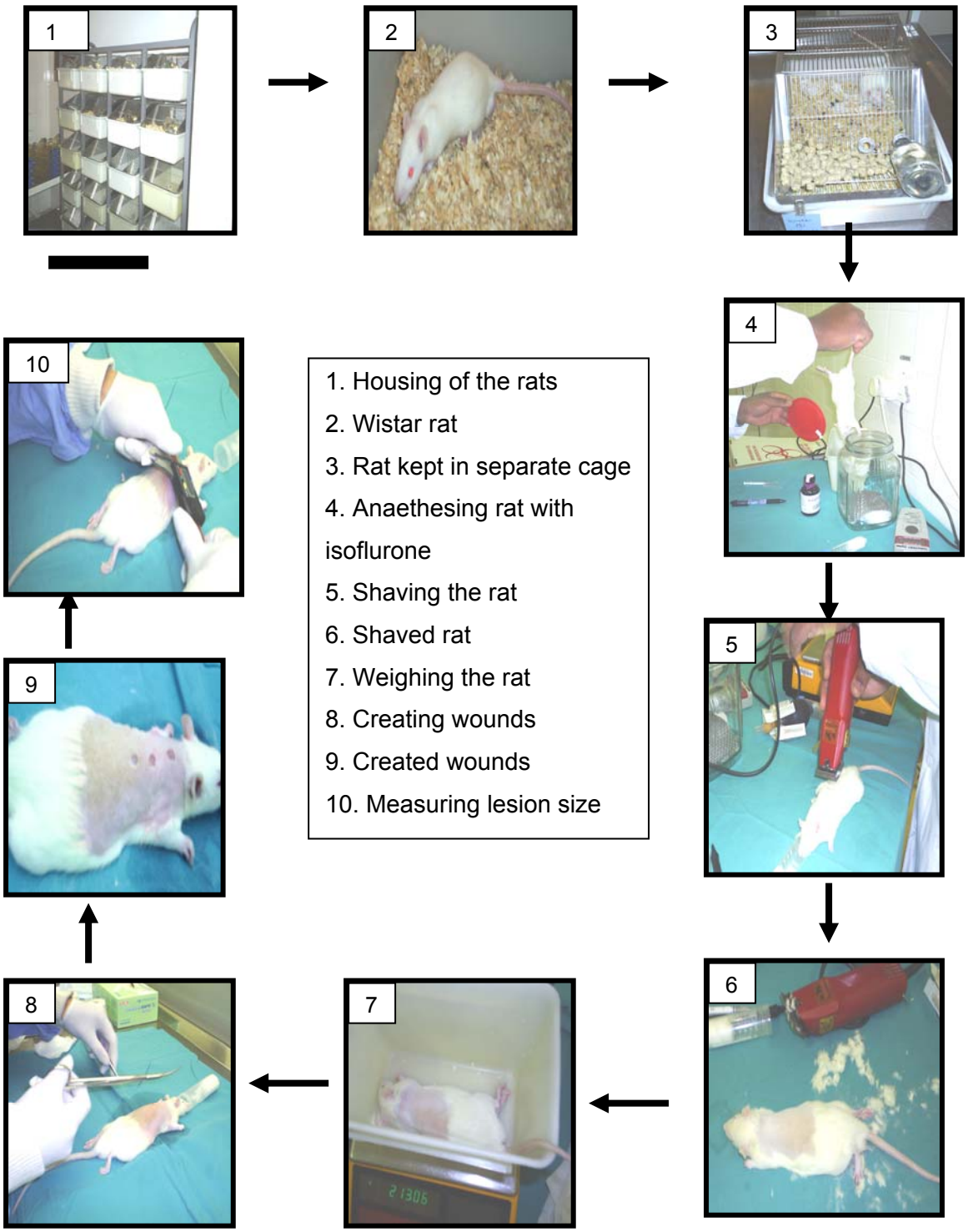


Figure 11.1. Wounds creation

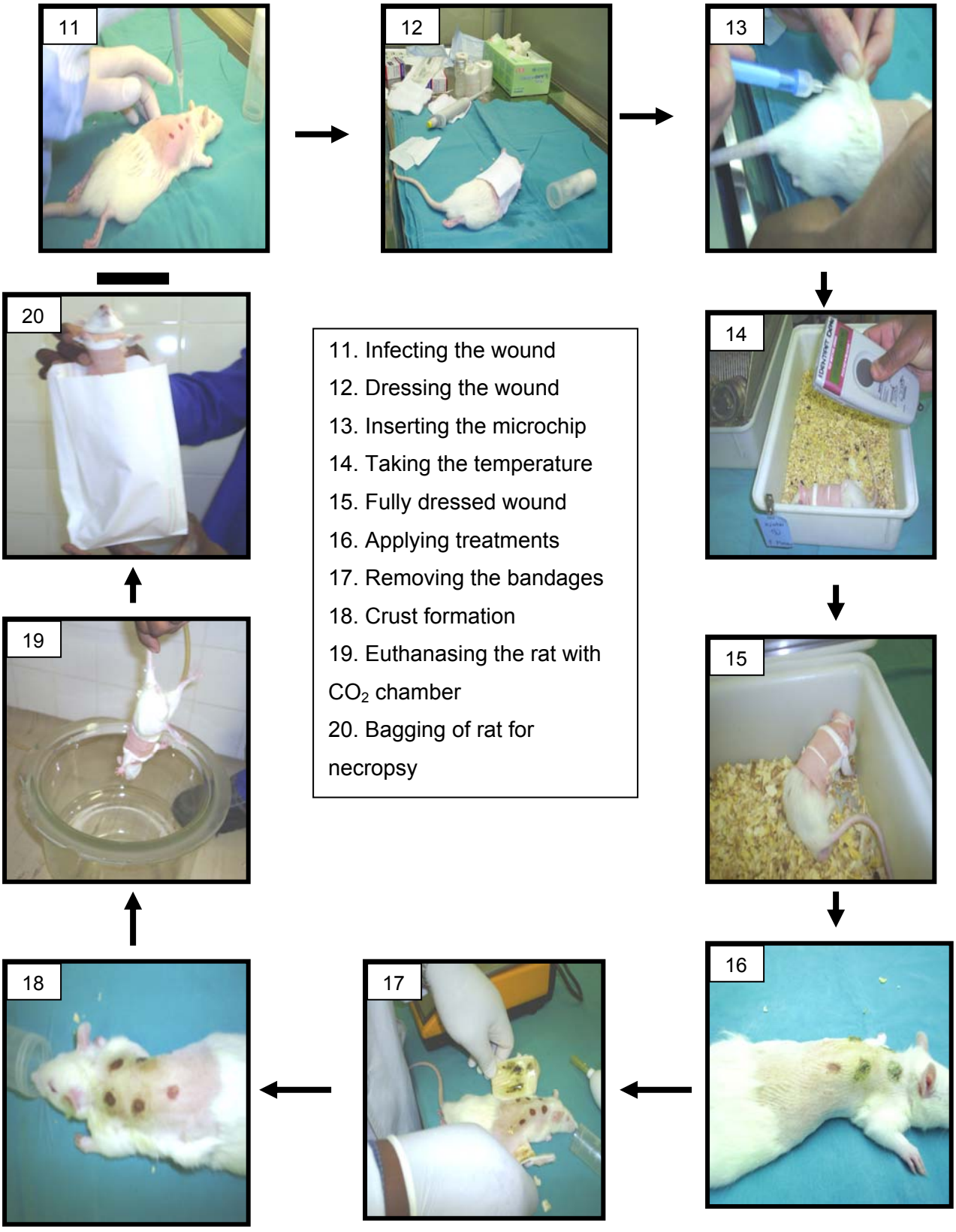


Figure 11.2. Wound treating and dressing.

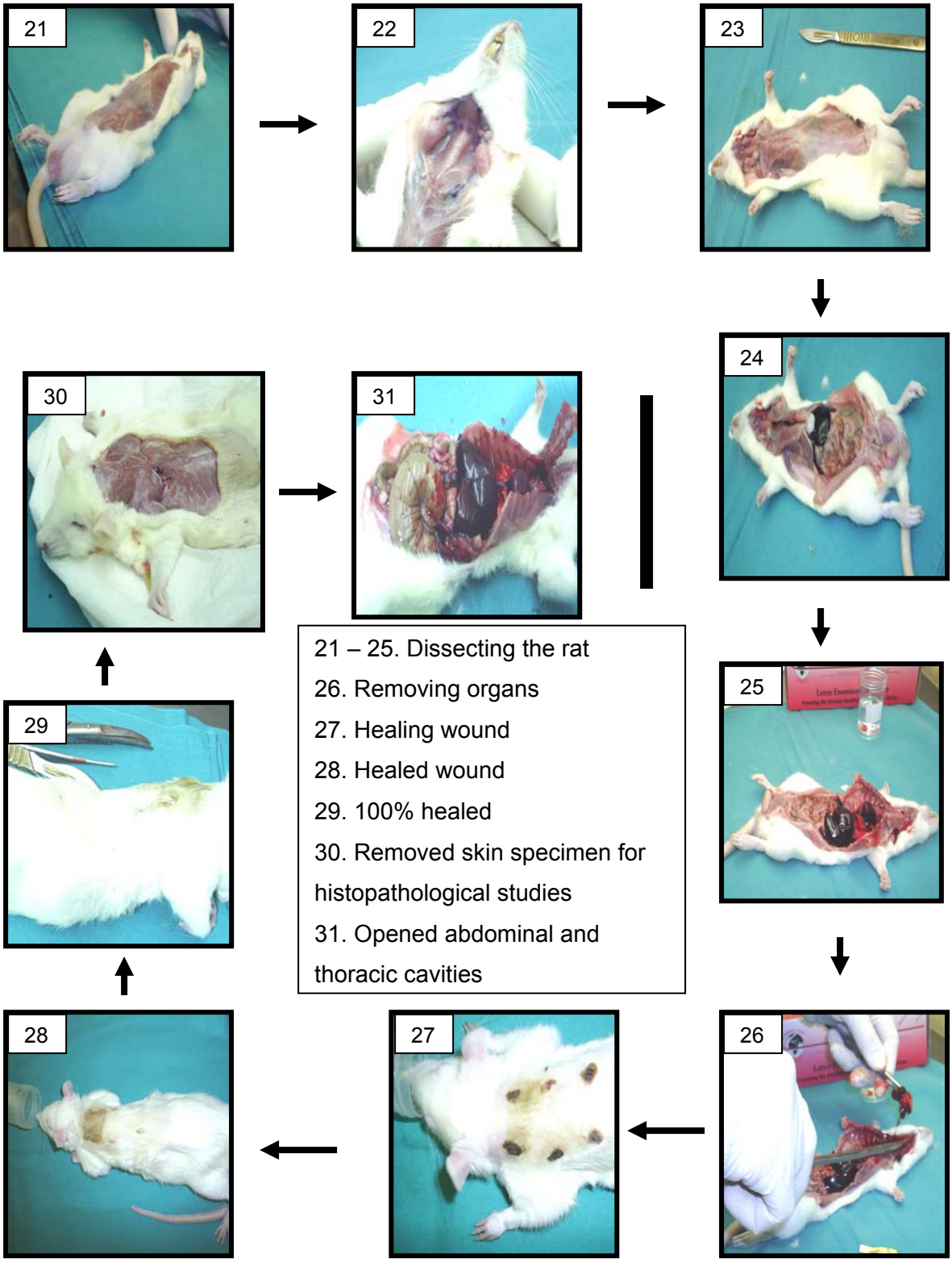


Figure 11.3. Wound healing and necropsy.

11.6.2 Pilot study 2 (Infection with different fungal pathogens)

The aim of pilot study II was to determine the effects of plant extracts in aqueous creams on the infection and wound healing and to determine the rate and extent of infection from different pathogens. Results were recorded in **Table 11.2** (**Appendix**). Rats 1 to 3 were infected with *C. albicans*, 4 to 6 with *C. neoformans*, 7 to 9 with *M. canis* and 10 to 12 with *S. schenckii*.

Although there was an initial weight loss in all the rats, all except Rat 11 gradually recovered the weight lost (**Figure 11.10a-b**). As Rat 11 was suffering from weight loss and pneumonia it was terminated and a necropsy was done on it.

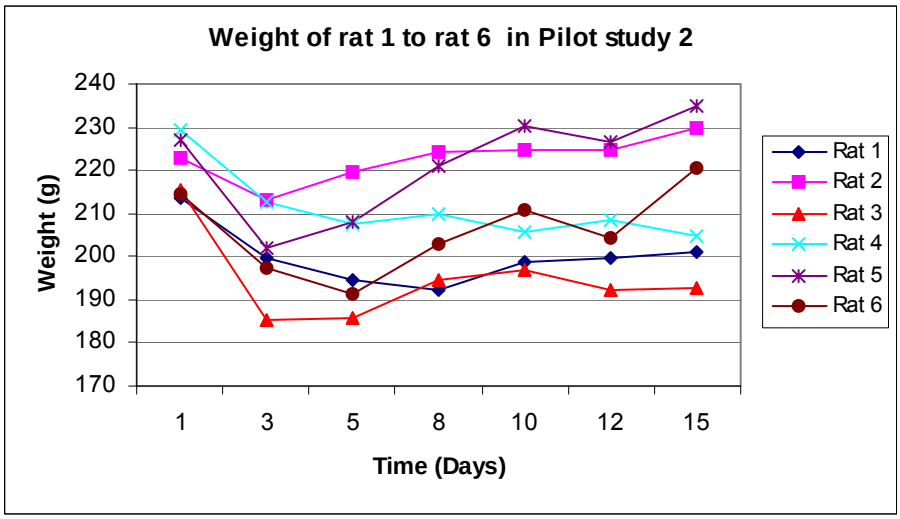


Figure 11.10a. Weights of rats 1 to 6 in pilot study 2

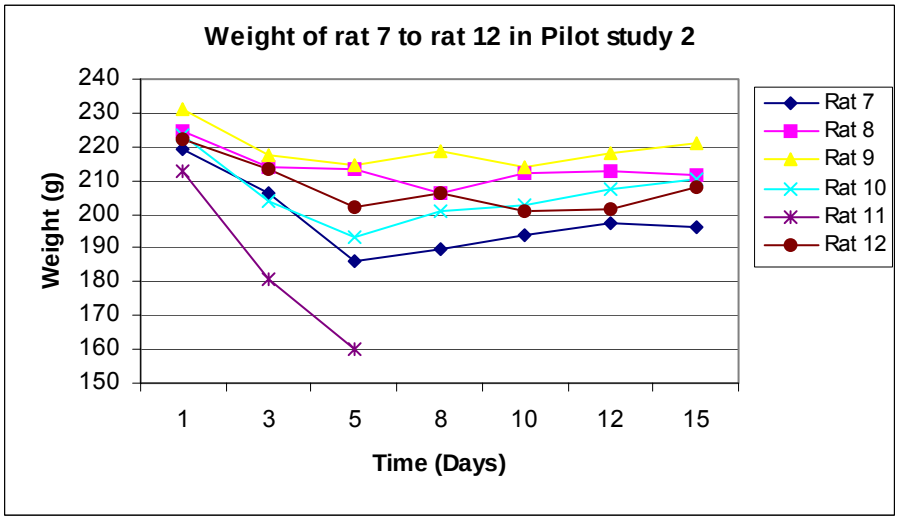


Figure 11.10b. Weights of rats 7 to 12 in pilot study 2

Temperatures of all 12 rats were within the normal parameters, even of rat 11, which was terminated. In all instances the initial temperature was low. That was because of temperatures were measured immediately after inserting the microchips and prolonged anaesthesia (**Figure 11.11a-b**).

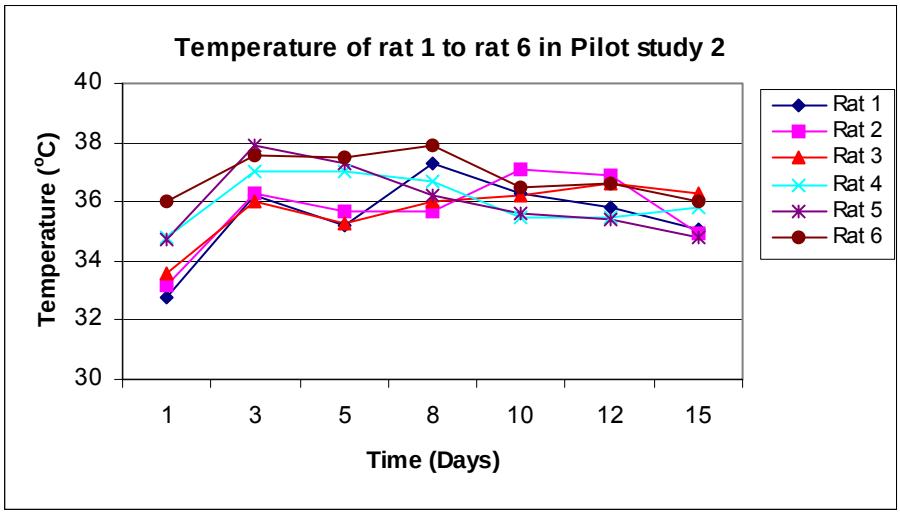


Figure 11.11a. Temperatures of rats 1 to 6 in pilot study 2

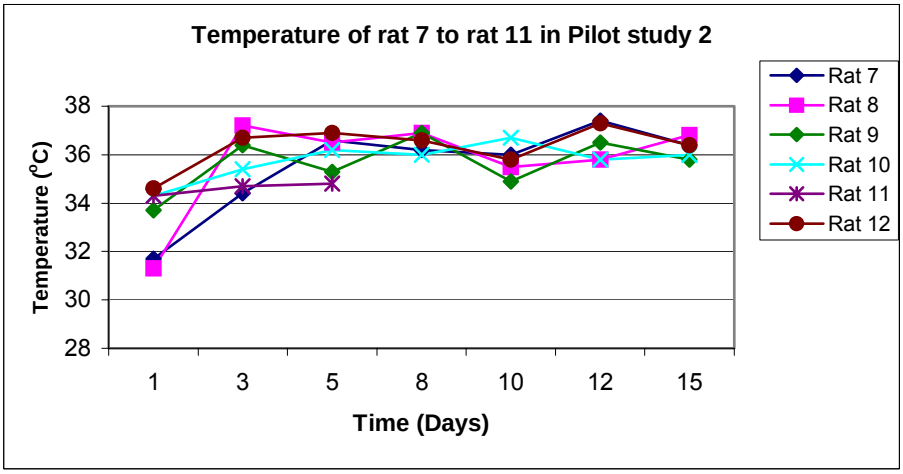


Figure 11.11b. Temperatures of rats 7 to 12 in pilot study 2

Lesion sizes were measured. The rats were group according to the fungal pathogens they were infected with, *C. albicans* (**Figure 11.12a**), *C. neoformans* (**Figure 11.12b**), *M. canis* (**Figure 11.12c)** and *S. schenckii* (**Figure 11.12d**). The lesion sizes were calculated the same way as in pilot study I. Amphotericin B was used as the positive control and the negative control was the untreated lesion but infected. In rats infected with *C. albicans* amphotericin B had the best activity

and *C. albopunctactum* had worst activity, but after Day 8 it was within the same activity as other treatments.

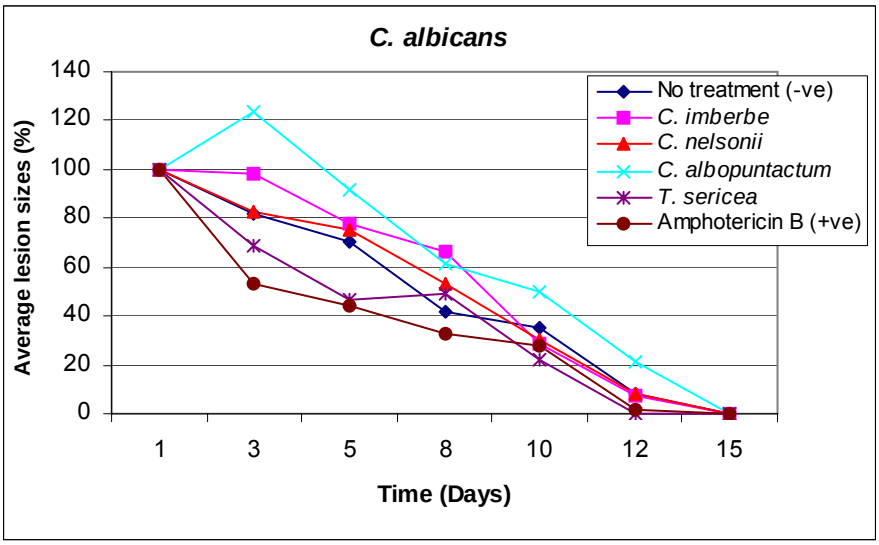


Figure 11.12a. The average lesion size of lesions infected with *C. albicans* and treated with four extracts and Amphotericin B (positive control).

Lesion sizes in rats infected with *C. neoformans* followed the same format of healing, although *T. sericea* had better activity and C. imberbe had the least activity. After Day 10 wounds without treatment were the ones with higher lesion sizes (**Figure 11.12b**).

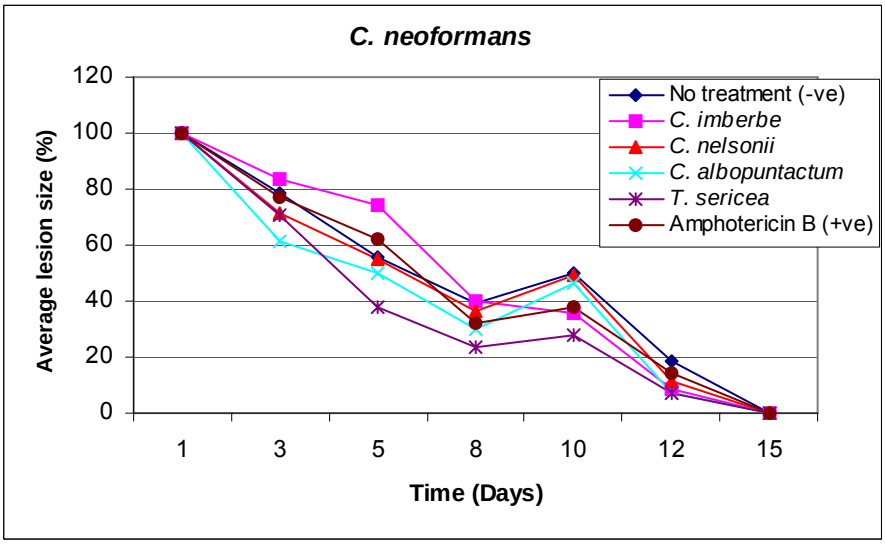


Figure 11.12b. The average lesion size of lesions infected with *C. neoformans* and treated with four extracts and Amphotericin B (positive control).

Lesion sizes in rats infected with *M. canis* (**Figure 11.12c**), wounds treated with *C. nelsonii* increased in sizes at Day 3 and at Day 10 they were at the same healing range as others. After Day 12 they were the best as expected based on *in vitro* studies. In rats infected with *S. schenckii* (**Figure 11.12d**) wound treated with *T. sericea* took longer time to reduce size.

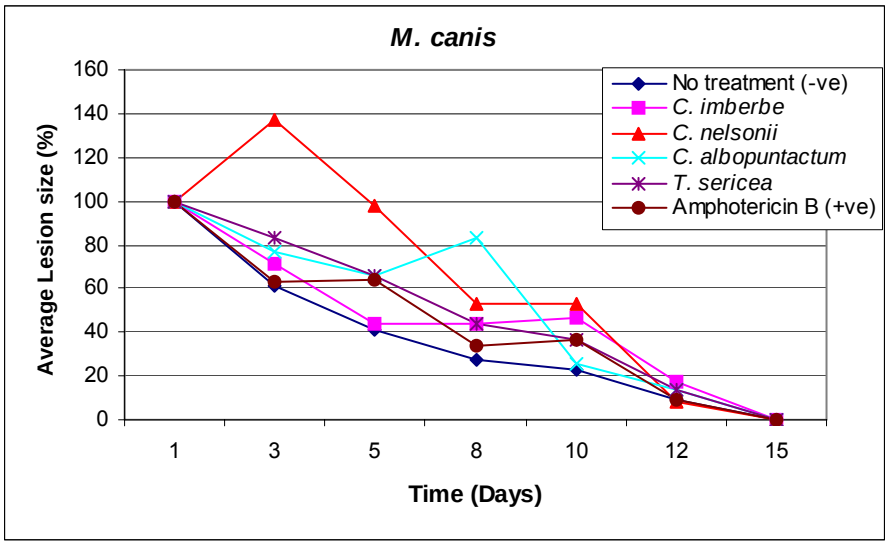


Figure 11.12c. The average lesion size of lesions infected with *M. canis* and treated with four extracts and Amphotericin B (positive control).

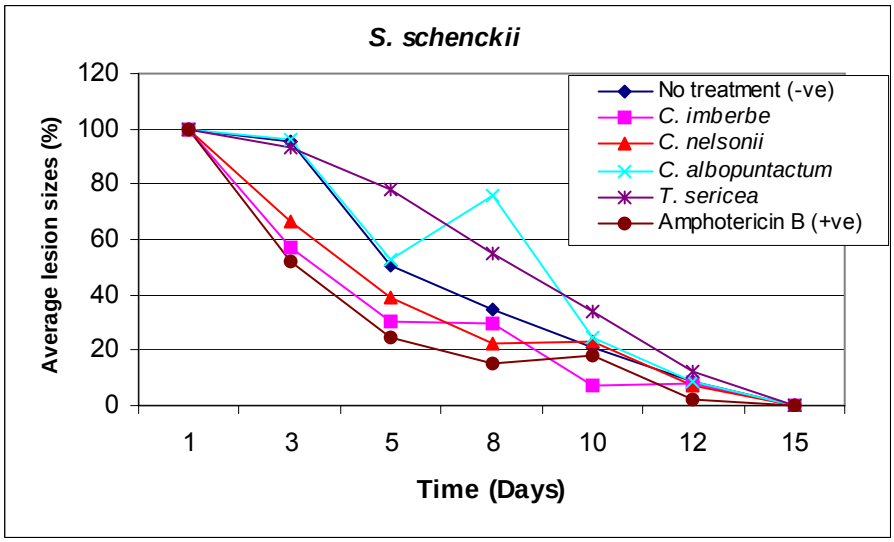


Figure 11.12d. The average lesion size of lesions infected with *S. schenckii* and treated with four extracts and Amphotericin B (positive control).

Again the resulting healing was quantified on the basis of erythema (**Figure 11.13a**), exudate (**Figure 11.14a**) and crust formation (**Figure 11.15a**). An arbitrary value was allocated, as it was difficult to measure the degree of erythema as well as the quantification of the exudate and crust formation formed. Subsequently a scale of 1 to 5 was used, 1 being the lowest and 5 being the highest formed. Averages of all 12 rats were used. Error bars were also drawn to show the confidence level of data or the deviation along a curve, erythema (**Figure 11.13b**), exudate (**Figure 11.14b**) and crust formation (**Figure 11.15b**).

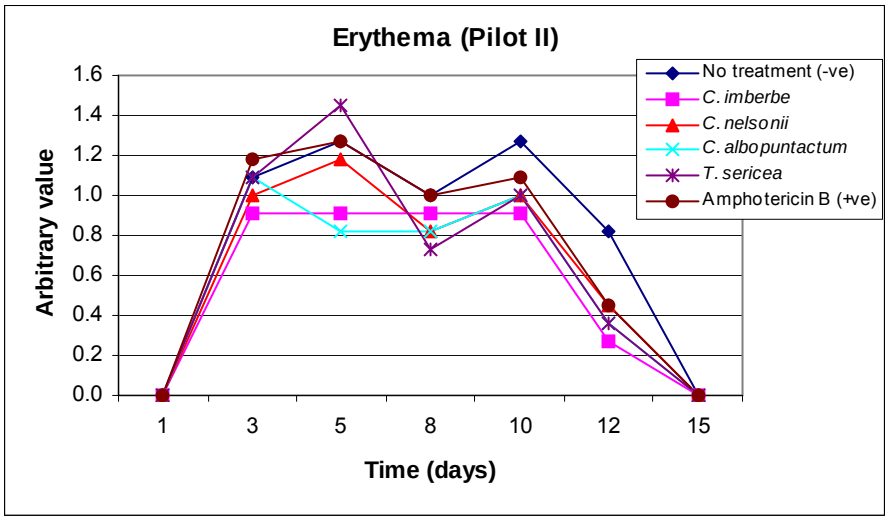


Figure 11.13a. The influence of the crude extracts and Amphotericin B (positive control) on the wound erythema of rat in pilot study 2

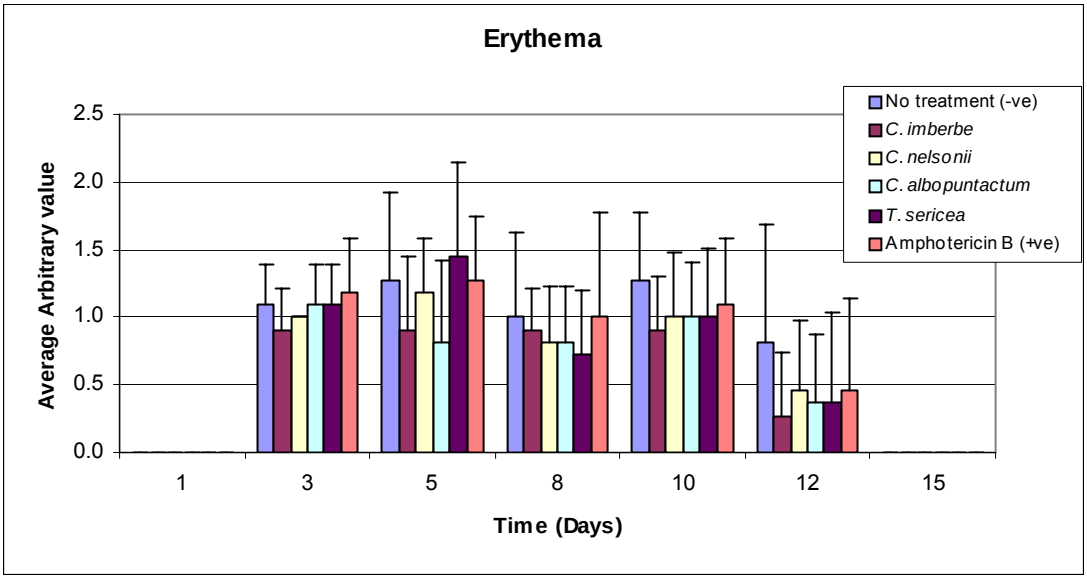


Figure 11.13b. Average arbitrary values of erythema of rats in pilot study 2 with error bars

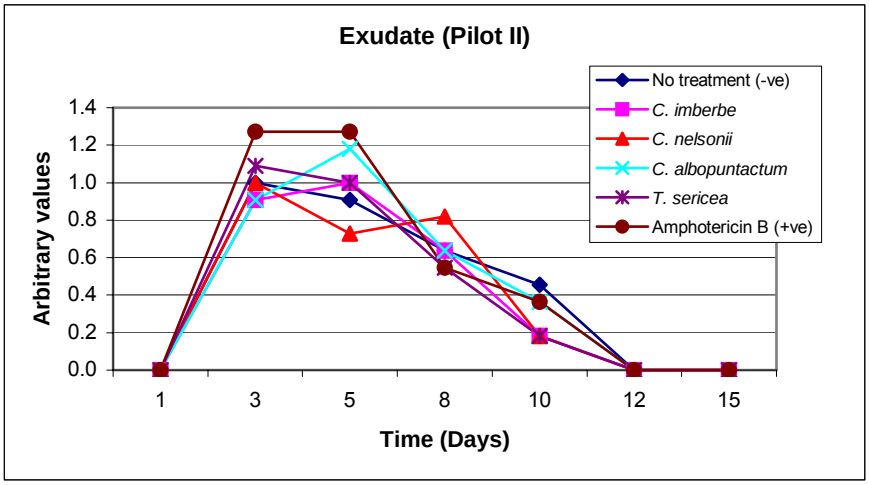


Figure 11.14a. The influence of the crude extracts and Amphotericin B (positive control) on the exudate formed of rats in pilot study 2

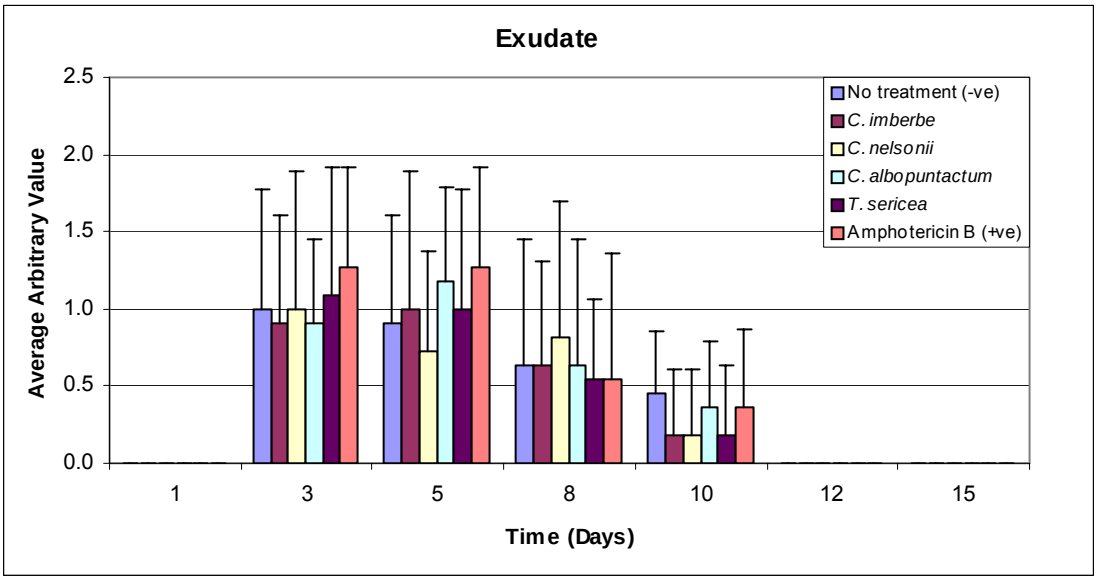


Figure 11.14b. Average arbitrary values of exudate of rats in pilot study 2 with error bars

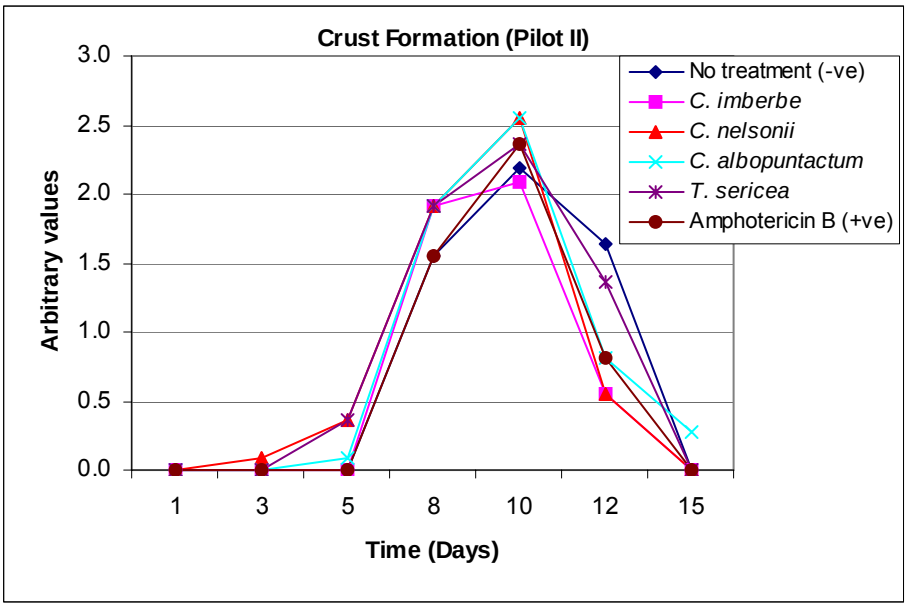


Figure 11.15a. The influence of the crude extracts and Amphotericin B (positive control) on the crust formed of rats in pilot study 2

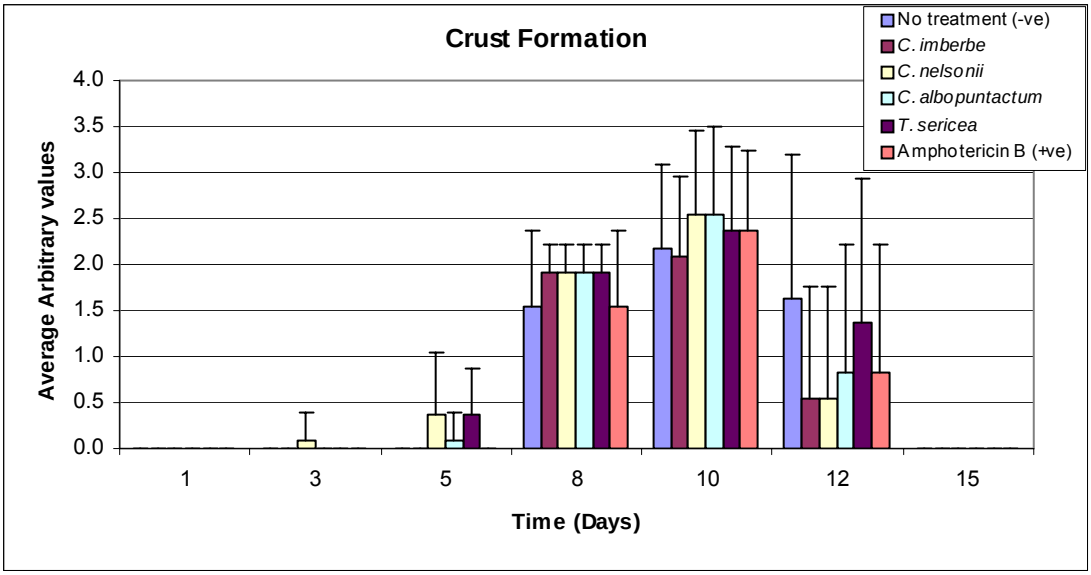
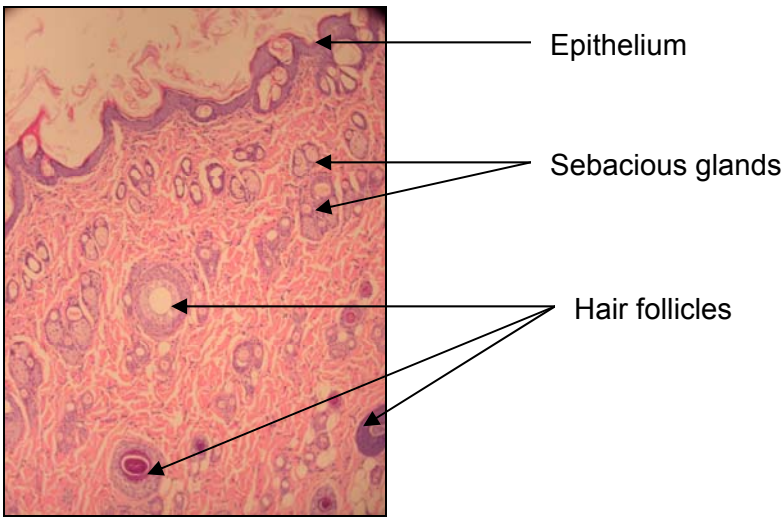


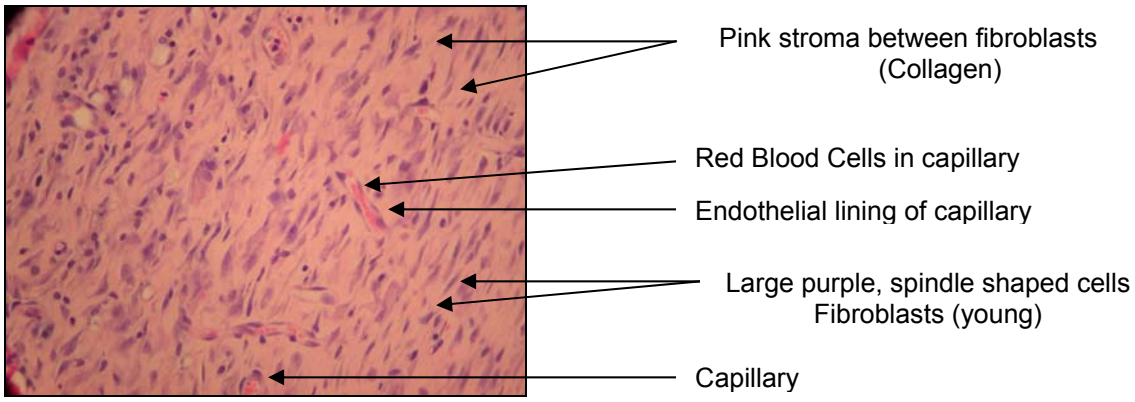
Figure 11.15b. Average arbitrary values of crust formation of rats in pilot study 2 with error bars

All 12 rats were euthanased after 3 weeks. The following observations were made, all lesions were properly 100% healed, there was no sign of inflammation underneath the skin and plant extracts in aqueous creams had wound healing properties on the infected wounds

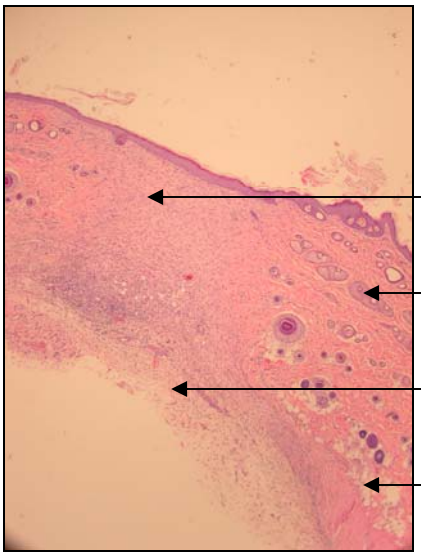
Figure 11.16 showed normal skin and the other histological observations which were fully presented in **table 11.6a** to **11.6d**. All lesions showed wound healing properties and few had fungal hyphae which indicate the treatments used had antifungal activities.



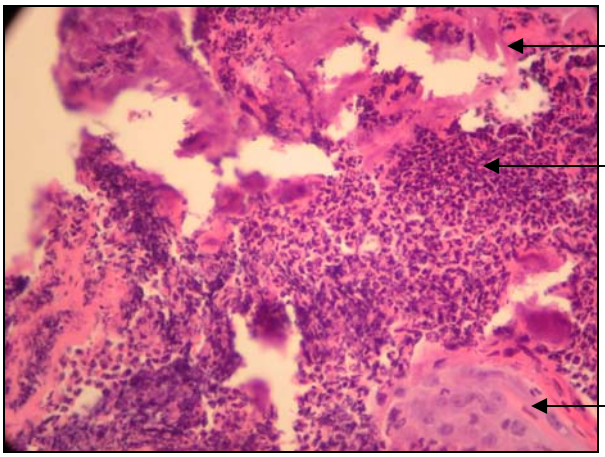
(A) Normal rat skin



(B) Fibrosis/Fibroplasia and Angiogenesis



(C) Fibrosis



(D) Degeneration of cells

Table 11.5a. Quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams (A= *T. sericea* crude extract, B= *C. nelsonii* crude extract, C= *C. imberbe* crude extract, D= *C. albopunctactum* crude extract, E= Negative control and F= Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	1(++)	1(+)	-	-	2(++)	2(+)	2(+)	2(++)	2(+)	2	-
	B	2(++)	1(+)	-	2(±)	2(++)	2(+)	2(+)	2(++)	2(+)	2	-
2	A	2(++)	-	-	-	2(++)	-	1(++)	2(+)	2(++)	2	++
	B	3(++)	1(+)	+	2(+)	2(++)	2(+)	2(++)	2(++)	3(+++)	2	++
	C	1(++)	1(+)	-	-	2(++)	1(±)	2(+)	2(++)	1(++)	2	+
	D	1(++)	1(+)	-	-	1(+++)	1(±)	2(+)	1(++)	2(++)	2	+
	E	1(+)	1(+)	-	-	1(+)	-	±	1(++)	1(++)	2	+
	F	**Unable to evaluate accurately**										±
3	A	2(++)	-	-	-	2(++)	±	2(+)	2(+)	2(++)	2	-
	B	2(+)	2(+)	-	-	2(+)	-	+	2(+)	2(++)	2	-

*Neut's= Neutrophils; Mac's= Macrophages

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

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5b. Quantitative histopathological findings of wounds of rats infected with *C. neoformans* after topical application of different creams (A= Negative control, B= *C. nelsonii* crude extract, C= Positive control (Amphotericin B), D= *C. albopunctactum* crude extract, E= *T. sericea* crude extract and F= *C. imberbe* crude extract)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
4	A	2(++)	2(+)	-	-	2(++)	1(+)	1(+)	2(+++)	2(++)	2	+
	B	3(++)	2(+)	-	1(±)	2(++)	-	2(±)	2(+++)	2(++)	2	+
	C	2(++)	1(+)	-	1(±)	2(+)	2(+)	2(+)	2(++)	3(++)	2	+
	D	1(+)	1(+)	-	1(+)	1(++)	1(+)	1(±)	1(++)	1(+)	2	+
	E	2(++)	2(+)	-	-	2(++)	2(+)	2(++)	2(++)	2(++)	2	+
	F	3(+++)	-	-	2(+)	2(+)	2(+)	1(+)	2(+)	3(++)	2	+
5	A	2(++)	2(±)	-	1(±)	2(+++)	2(+)	2(++)	2(++)	2(++)	2	-
	B	3(++)	1(+)	+	-	3(++)	3(+)	3(+)	3(++)	2(+)	2	-

*Neut's= Neutrophils; Mac's= Macrophages

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

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5c. Quantitative histopathological findings of wounds of rats infected with *M. canis* after topical application of different creams (A= *T. sericea* crude extract, B= *C. albopunctactum* crude extract, C= *C. imberbe* crude extract, D= *C. nelsonii* crude extract, E= Positive control (Amphotericin B) and F= Negative control)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
7	A	2(+)	-	+	2(±)	2(++)	2(+)	-	2(++)	2(+)	2	-
	B	3(++)	2(+)	-	-	2(+++)	2(++)	2(++)		2(++)	2	-
9	A	3(++)	2(+)	-	-	3(++)	-	-	3(++)	3(++)	2	+
	B	3(+++)	-	-	+++	3(++)	3(++)	3(++)	3(++)	3(++)	2	+
	C	2(++)	2(+)	+	-	2(++)	1(±)	2(++)	2(++)	2(++)	2	+
	D	2(++)	2(±)	±	2(±)	2(++)	-	2(+)	2(++)	2(++)	2	+
	E	1(++)	1(++)	+	1(±)	1(++)	1(+)	1(+)	1(+++)	1(++)	2	+
	F	1(++)	1(+)	±	-	2(++)	-	-	1(++)	1(++)	2	+

*Neut's= Neutrophils; Mac's= Macrophages

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

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5d. Quantitative histopathological findings of wounds of rats infected with *S. schenckii* after topical application of different creams (A= *T. sericea* crude extract, B= *C. albopunctactum* crude extract, C= *C. imberbe* crude extract, D= *C. nelsonii* crude extract, E= Positive control (Amphotericin B) and F= Negative control)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
10	A	3(++)	1(+)	+	-	2(++)	2(±)	2(+)	3(++)	3(++)	2	+
	B	3(++)	-	-	-	2(++)	2(±)	2(+)	2(+)	3(++)	2	+
	C	1(+)	-	-	1(±)	1(+)	1(±)	1(±)	1(+)	1(+)	2	±
	D	1(+)	1(+)	-	-	1(+)	1(++)	1(+)	1(++)	1(+)	2	±
	E	2(+)	2(+)	-	-	2(++)	-	2(+)	2(++)	2(++)	2	±
	F	2(+)	1	-	-	2(+)	-	2(+)	2(+)	2(++)	2	-
11	A	3(+++)	1(++)	-	1(+++)	3(+++)	3(+++)	3(++)	3(++)	2(++)	2	++
	B	3(+++)	1(++)	-	1(+++)	3(+++)	3(++)	3(++)	3(++)	3(++)	2	+
	C	2(++)	1(++)	-	1(+++)	2(++)	-	2(+)	2(++)	2(++)	2	+
	D	1(++)	1(++)	+	1(+++)	1(+++)	1(±)	1(+)	1(++)	1(+)	2	++
	E	3(++)	1(++)	-	1(+++)	2(+++)	1(±)	2(+)	2(++)	2(++)	2	++
	F	2(++)	1(++)	+	1(+++)	2(++)	-	1(±)	2(++)	2(++)	2	++

*Neut's= Neutrophils; Mac's= Macrophages

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

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

There was no evidence of systemic infection and there was no differences in treatments This formed the basis of starting with the main study, where the isolated compound was added as part of the treatment.

11.6.3. Confirmation study with immunocompromised rats (Main experiment)

The data of all parameters measured were recorded in **Table 11.7** (*C. albicans*), *C. neoformans* (**Table 11.8**), *M. canis* (**Table 11.9**) and *S. schenckii* (**Table 11.10**). All the tables are placed in the **Appendix** because they were big and they had too much data.

11.6.3.1. Weight

All the rats infected with different fungal pathogens i.e. *C. albicans* (**Figure 11.16**), *C. neoformans* (**Figure 11.17**), *M. canis* (**Figure 11.18**) and *S. schenckii* (**Figure 11.19**) gradually lost weight, which was not the case in pilot study (2) and this was attributed to immunosuppressive reaction and the additional treatment (isolated compound). On Day 15 all the rats gained weight except Rat 3. We decide to leave the rats for 6 additional days without handling them and an increase in weight was observed. Rat 22 lost more weight after Day 5 but gradually recovered the weight lost after Day 8

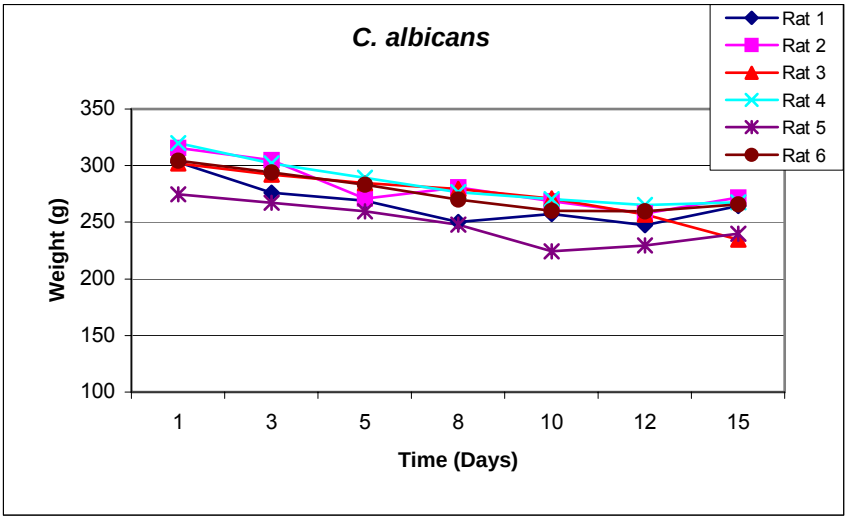


Figure 11.16. Weights of rats (1 to 6) infected with *C. albicans*.

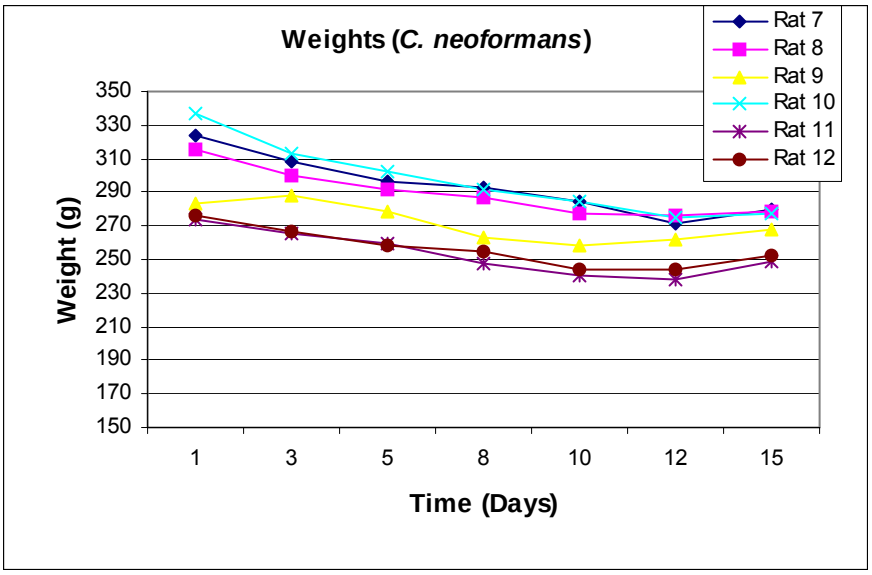


Figure 11.17. Weights of rats (7 to 12) infected with *C. neoformans.*

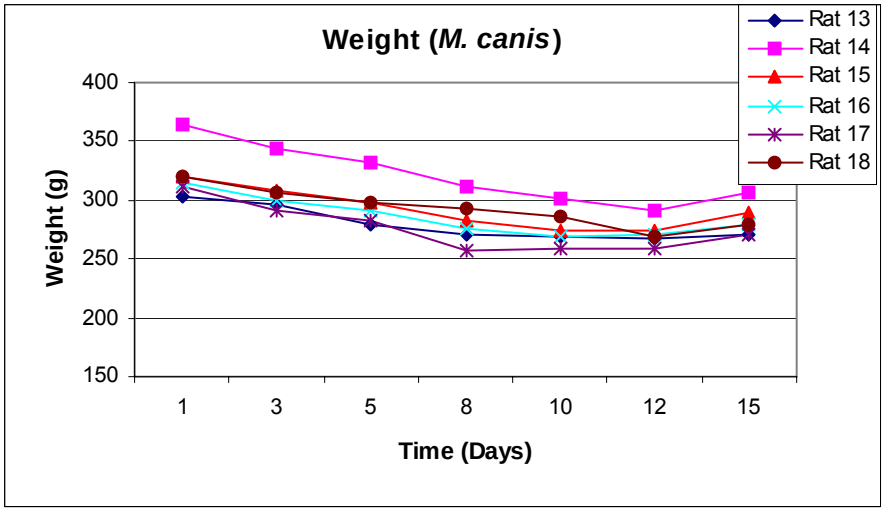


Figure 11.18. Weights of rats (13 to 18) infected with *M. canis.*

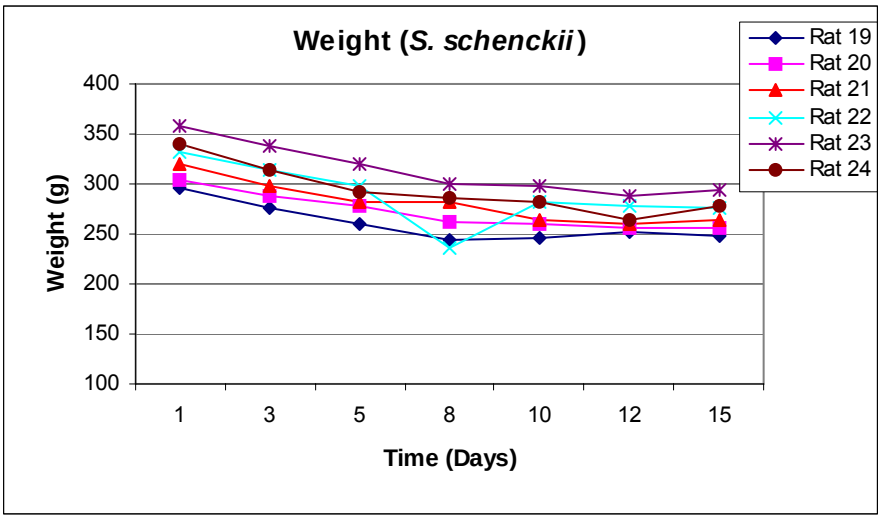


Figure 11.19. Weights of rats (19 to 24) infected with *S. schenckii*.

11.6.3.2. Temperature

The temperatures of rats infected with *C. albicans* (**Figure 11.20**), *C. neoformans* (**Figure 11.21**), *M. canis* (**Figure 11.22**) and *S. schenckii* (**Figure 11.23**) were within the expected range (35-37 ^{o}C) at the end of the experiment (Day 15). Temperature above normal were recorded for rat 24 on Day 3, rat 12 on Day 10 and rats 3 and 16 on Day 12.

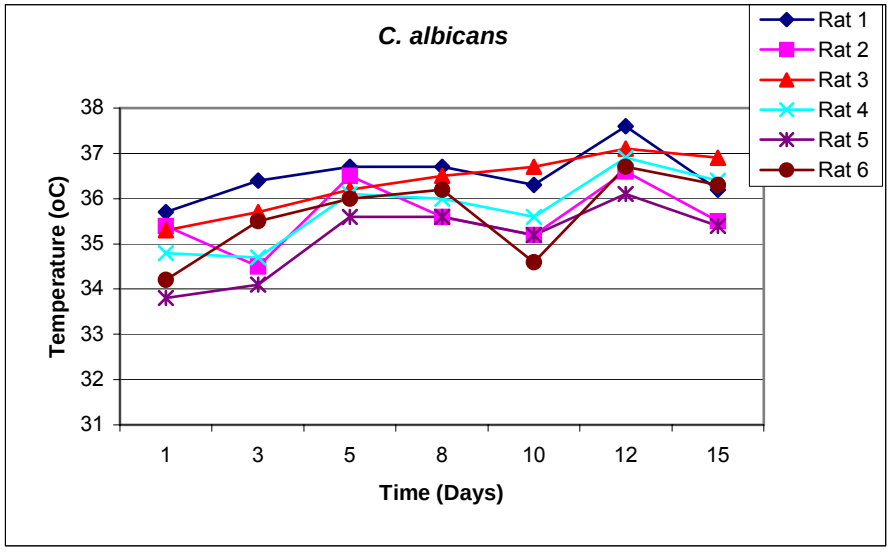


Figure 11.20. Temperatures of rats (1 to 6) infected with *C. albicans*.

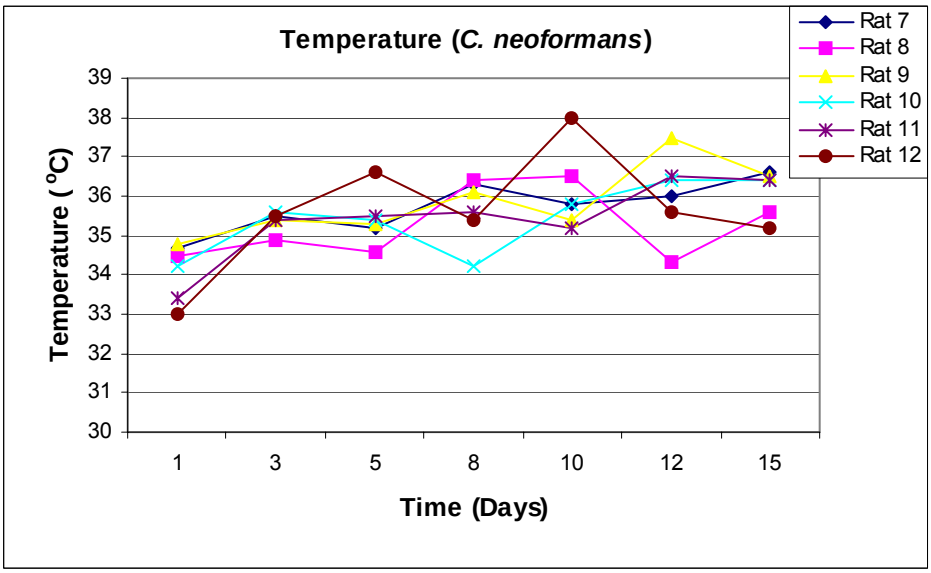


Figure 11.21. Temperatures of rats (7 to 12) infected with *C. neoformans*.

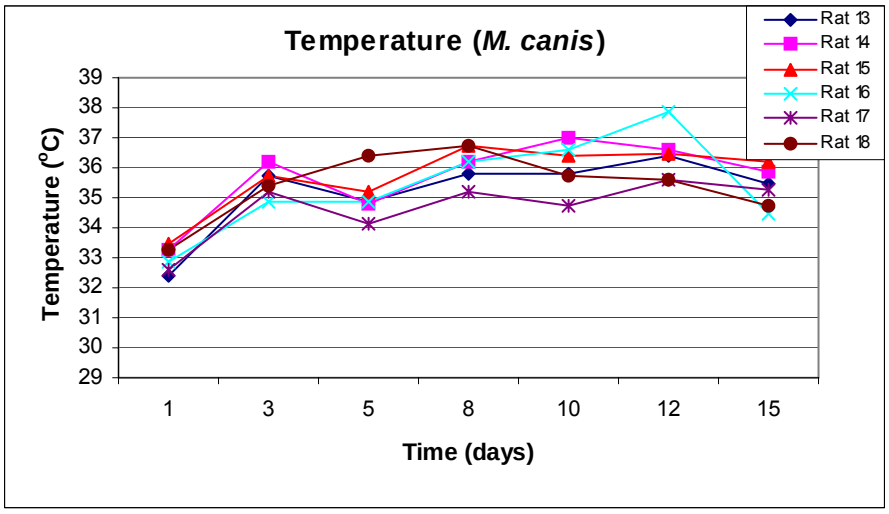


Figure 11.22. Temperatures of rats (13 to 18) infected with *M. canis.*

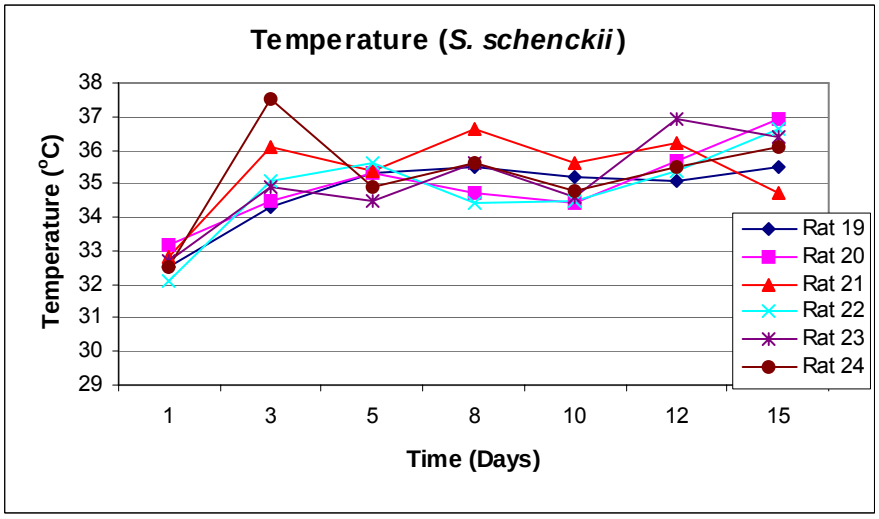


Figure 11.23. Temperatures of rats (19 to 24) infected with *S. schenckii*.

11.6.3.3. Lesion size

A circular full-thickness lesions were created on the back of rats. Lesion sizes were measured, *C. albicans* (**Figure 11.24**), *C. neoformans* (**Figure 11.25**), *M. canis* (**Figure 11.26**) and *S. schenckii* (**Figure 11.27**). The lesion sizes were calculated the same way as in pilot study I. The open lesion was healed by the process of wound contraction. The epithelial closure in all rats occurred by 17 days. The transient formation of granulation tissue was vigorous on day 12 after wounding. There was no significant difference in the contraction of lesion area treated with different extracts. In all the experiments, lesions treated with isolated compound healed faster than the extracts and amphotericin B. However, contraction was fastest in the untreated wounds.

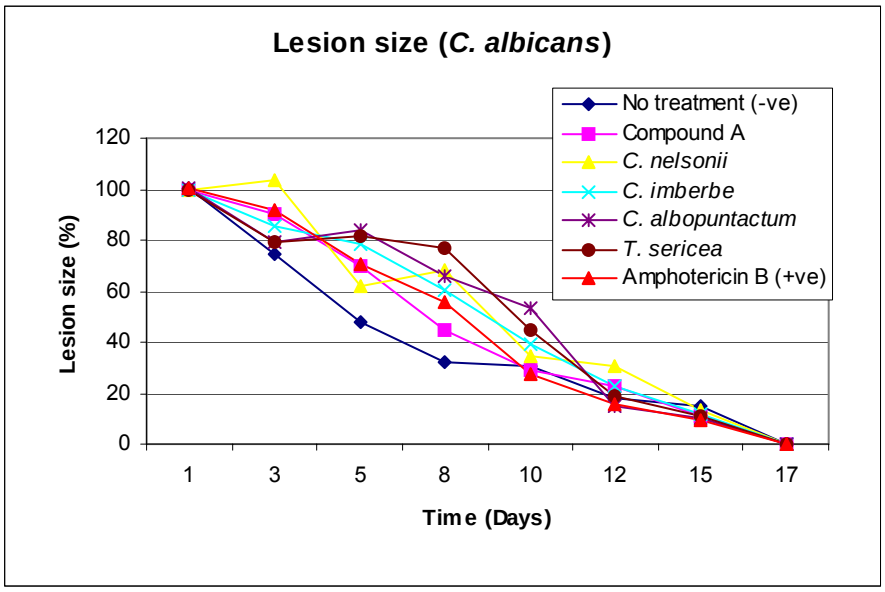


Figure 11.24. The average lesion size of lesions infected with *C. albicans* and treated with four extracts, isolated compound and Amphotericin B (positive control).

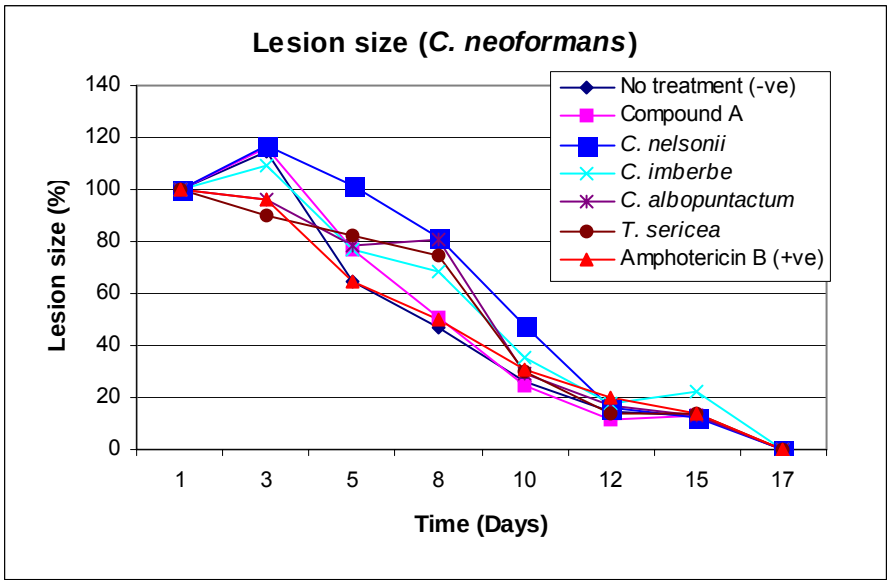


Figure 11.25. The average lesion size of lesions infected with *C. neoformans* and treated with four extracts, isolated compound and Amphotericin B (positive control).

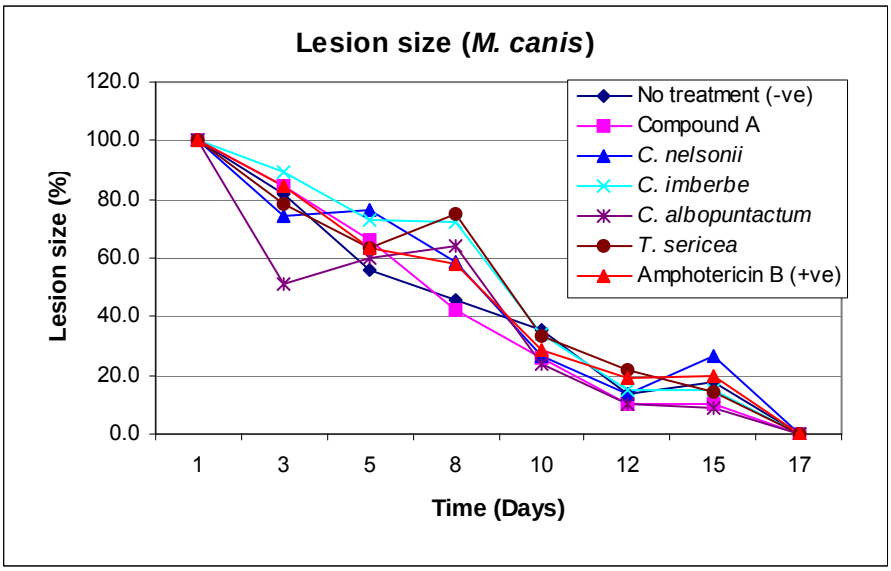


Figure 11.26. The average lesion size of lesions infected with *M. canis* and treated with four extracts, isolated compound and Amphotericin B (positive control).

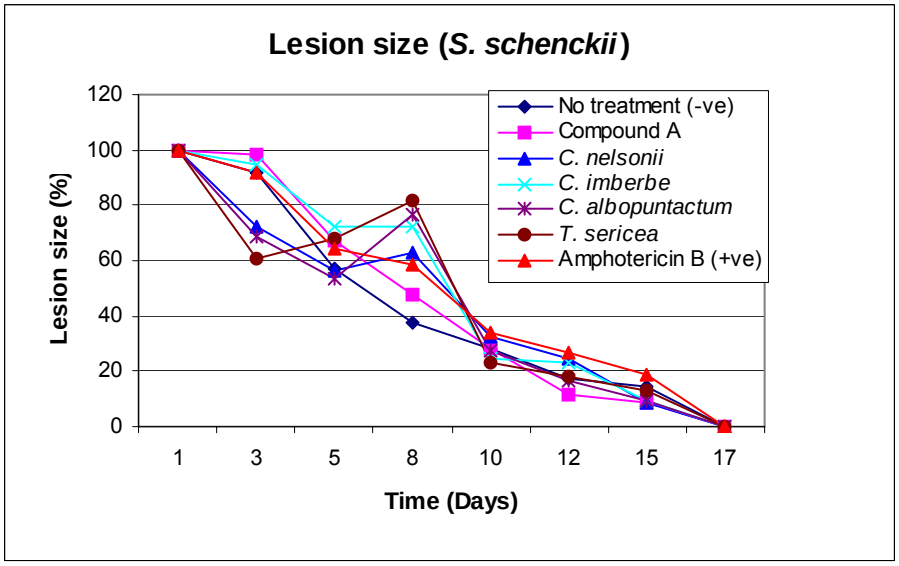


Figure 11.27. The average lesion size of lesions infected with *S. schenckii* and treated with four extracts, isolated compound and Amphotericin B (positive control).

11.6.3.4. Erythema

One fundamental property of the skin is its ability to respond to treatment. In rats populations these responses are clearly adaptive where the first response, erythema (redness) is a sign that the immune system is active and the healing process has begun. The resulting healing was quantified on the basis of erythema, *C. albicans* (**Figure 11.28a**), *C. neoformans* (**Figure 11.29a**),

M. canis (**Figure 11.30a**) and *S. schenckii* (**Figure 11.31a**). As described in Section 11.6.2. a scoring system was used to determine the degree of erythema. Subsequently a scale of 1 to 5 was used, 1 being the lowest and 5 being the highest formed. Averages of all 6 rats were used in all groups infected with different pathogens. Error bars were also drawn to show the confidence level of data or the deviation along a curve, *C. albicans* (**Figure 11.28b**), *C. neoformans* (**Figure 11.29b**), *M. canis* (**Figure 11.30b**) and *S. schenckii* (**Figure 11.31b**). The variability in the results of erythema at each lesion in rats infected with different fungal pathogens differs between the treatments used, lesion without treatment took longer time to heal in all cases. Although the differences were not statistically significant, the plant extracts tended to decrease erythema in practically all cases.

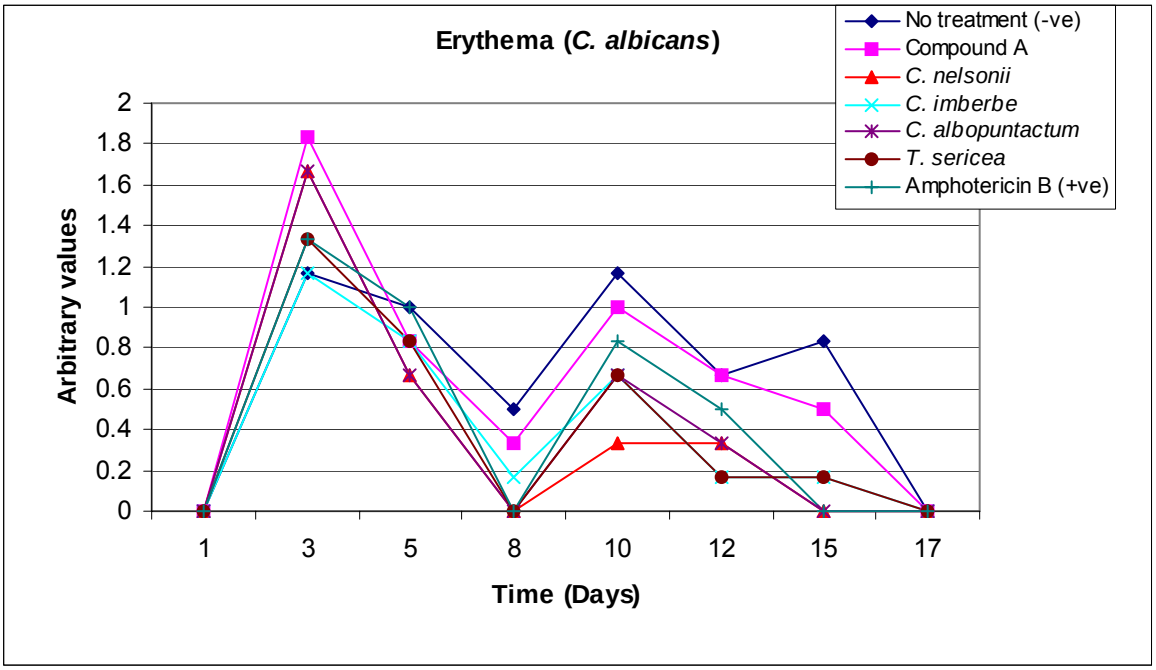


Figure 11.28a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *C. albicans*.

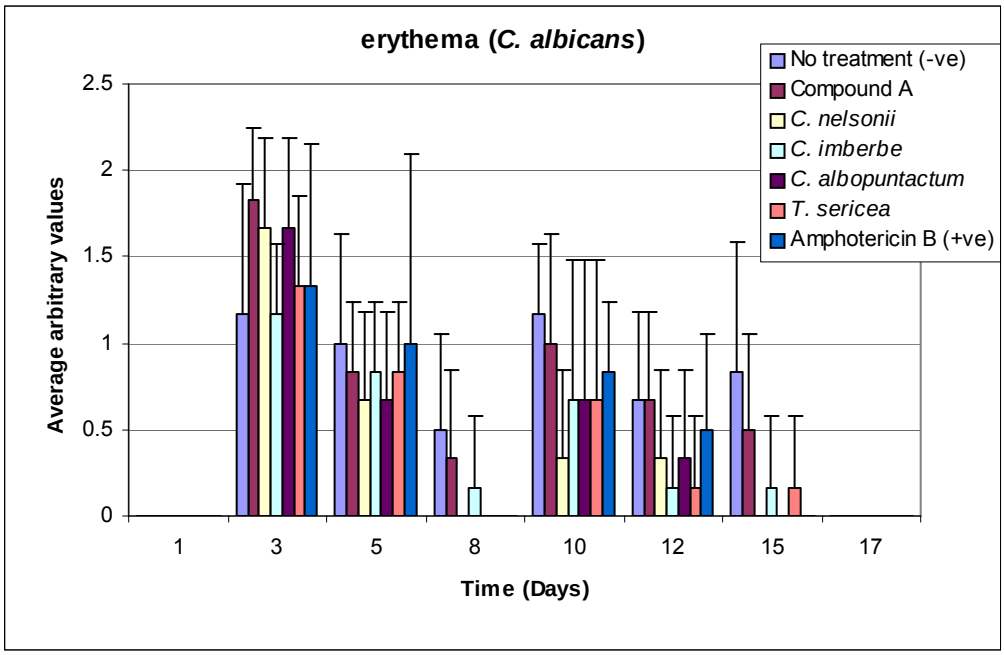


Figure 11.28b. Average arbitrary values of erythema of rats infected with *C. albicans* with error bars

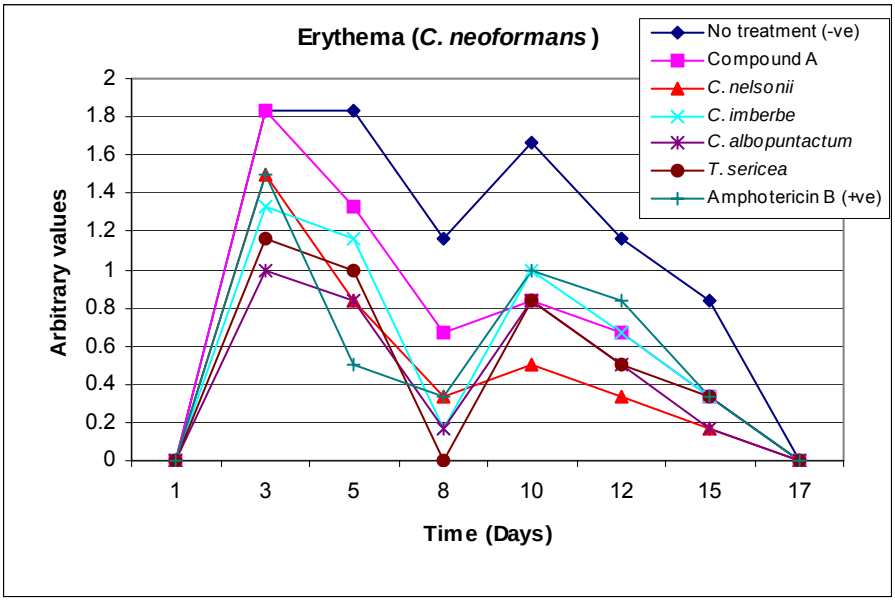


Figure 11.29a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *C. neoformans*.

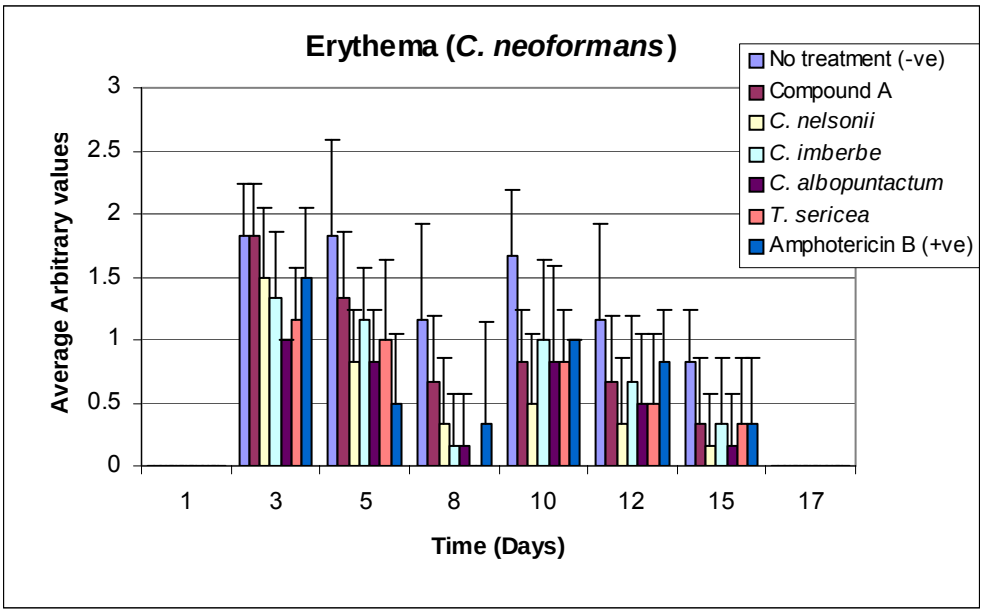


Figure 11.29b. Average arbitrary values of erythema of rats infected with *C. neoformans* with error bars

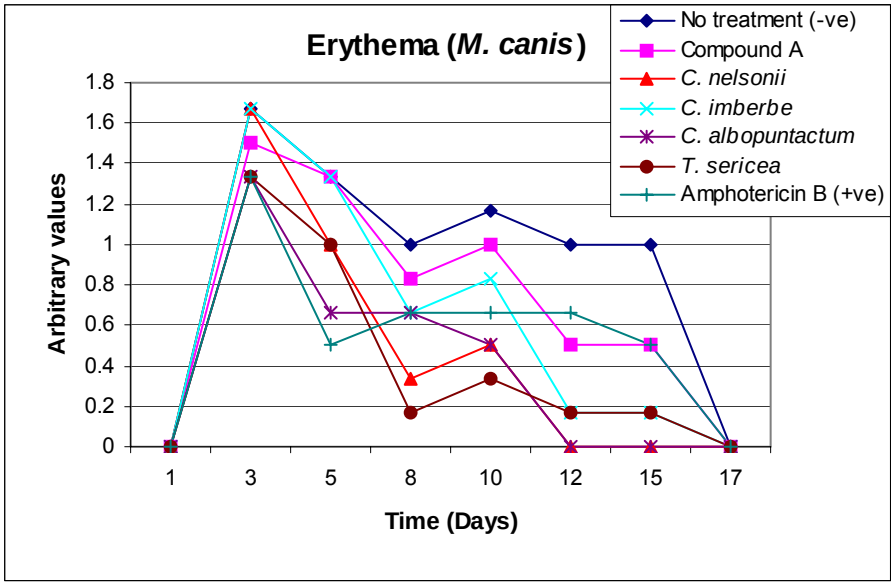


Figure 11.30a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *M. canis*.

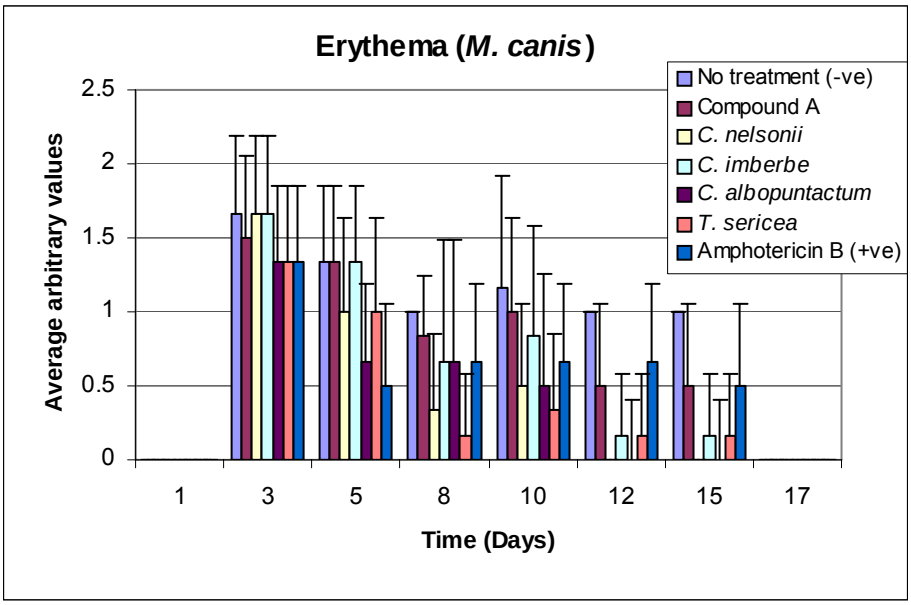


Figure 11.30b. Average arbitrary values of erythema of rats infected with *M. canis* with error bars

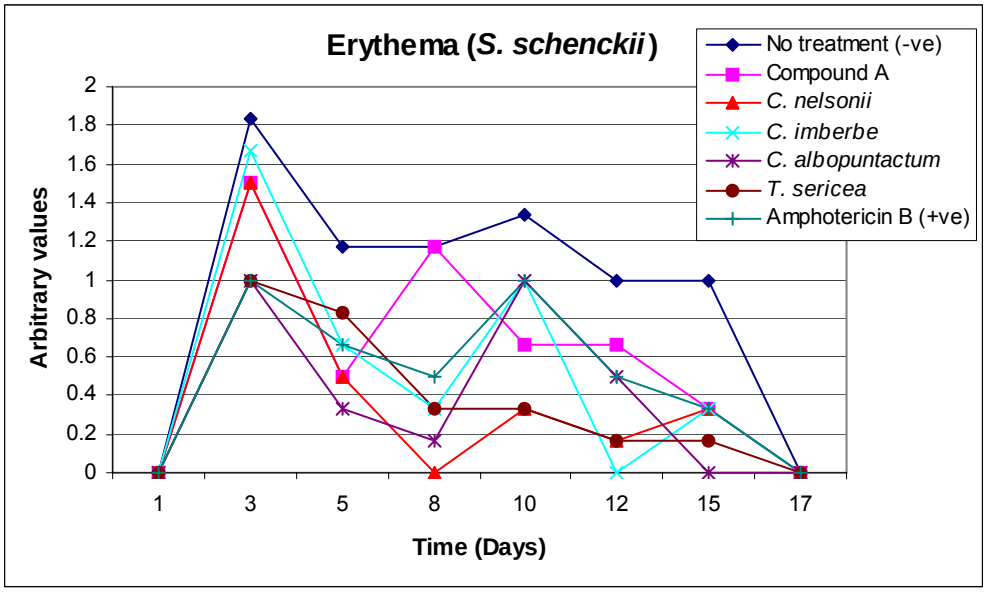


Figure 11.31a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *S. schenckii*.

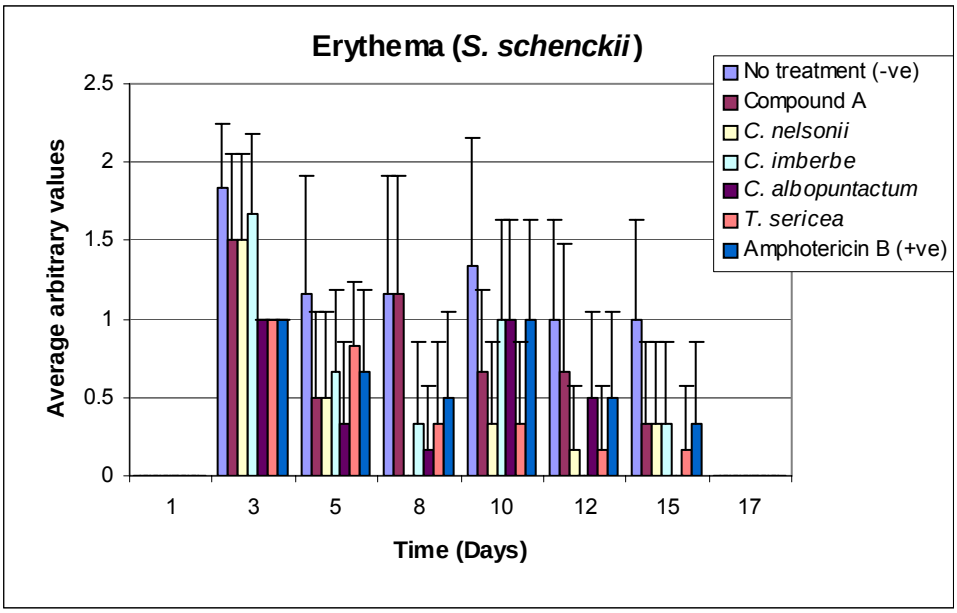


Figure 11.31b. Average arbitrary values of erythema of rats infected with *S. schenckii* with error bars

11.6.3.5. Exudate

Exudate formation was one of the parameters used to quantify the healing process, *C. albicans* (**Figure 11.32a**), *C. neoformans* (**Figure 11.33a**), *M. canis* (**Figure 11.34a**) and *S. schenckii* (**Figure 11.35a**). The same scale used in erythema was used. Error bars were also drawn to show the confidence level of data or the deviation along curves, *C. albicans* (**Figure 11.32b**), *C. neoformans* (**Figure 11.33b**), *M. canis* (**Figure 11.34b**) and *S. schenckii* (**Figure 11.35b**). Exudate formation was observed until Day 12 in rats infected with *C. albicans*, Day 8 in rats infected with *C. neoformans*, except the lesions, which were not treated. In lesions infected with *M. canis* and *S. schenckii*, exudate formation was observed until Day 10. There was less exudate formation in lesions not treated.

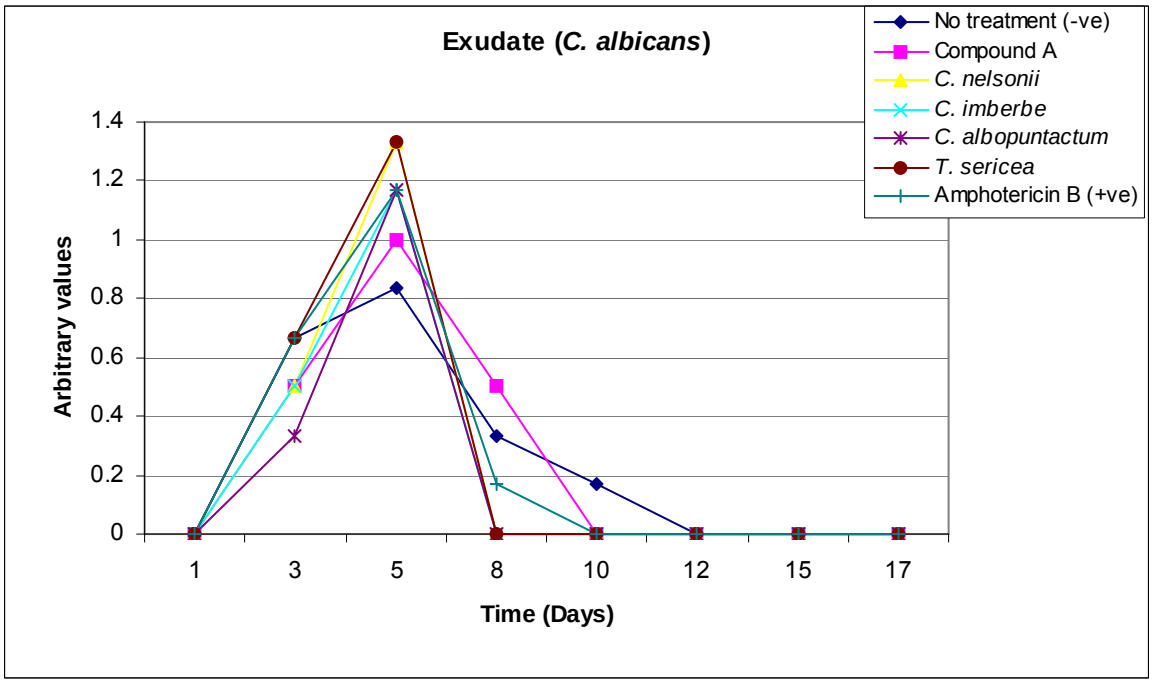


Figure 11.32a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *C. albicans*.

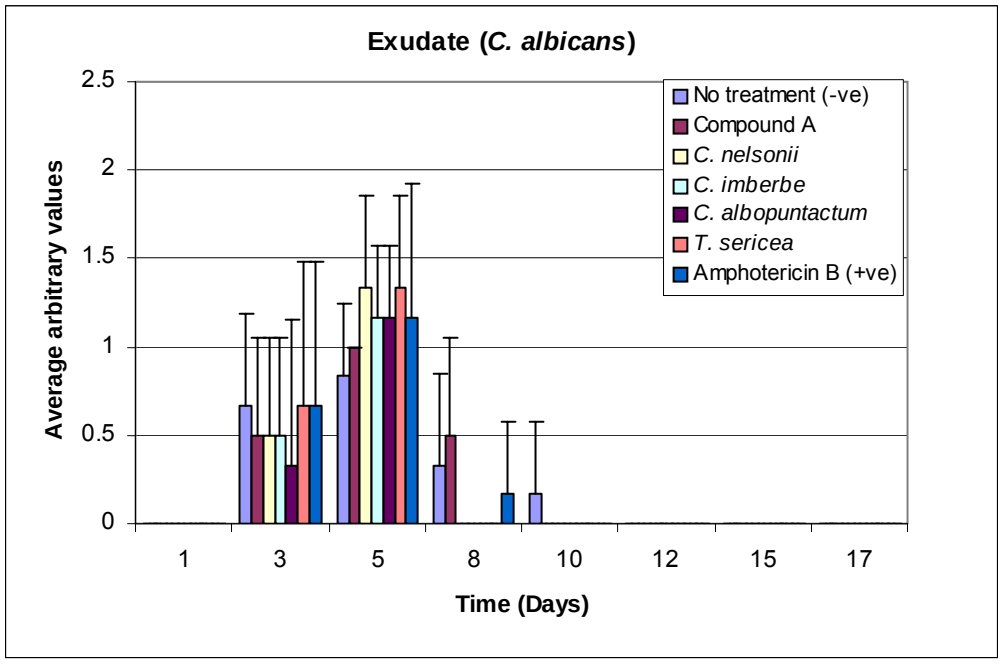


Figure 11.32b. Average arbitrary values of exudate of rats infected with *C. albicans* with error bars

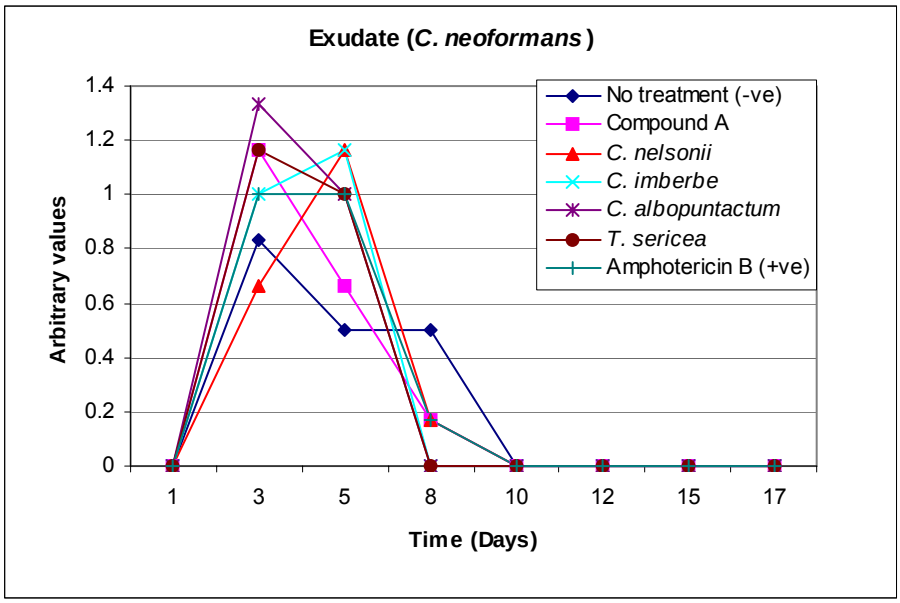


Figure 11.33a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *C. neoformans*.

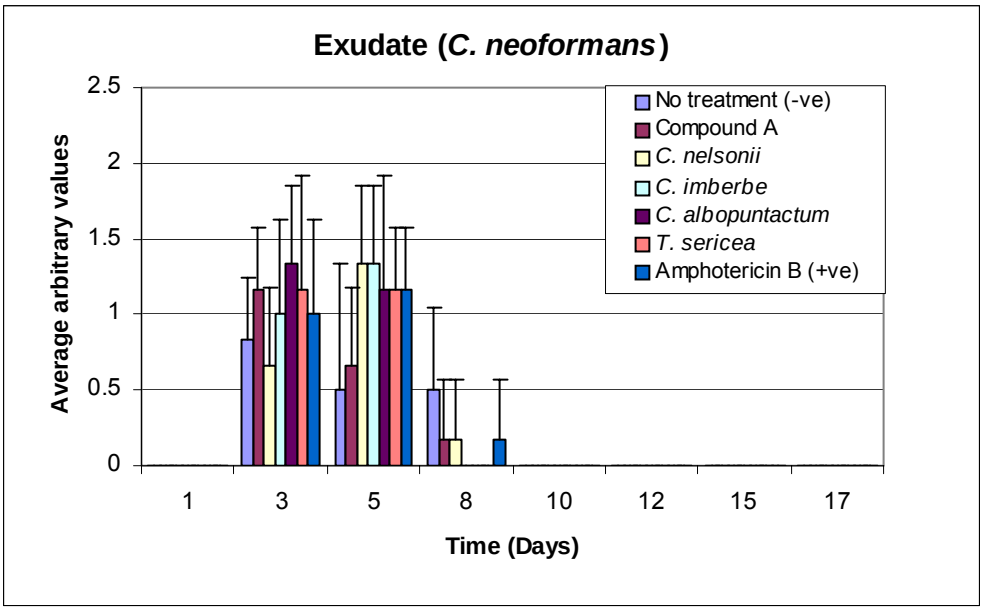


Figure 11.33b. Average arbitrary values of exudate of rats infected with *C. neoformans* with error bars

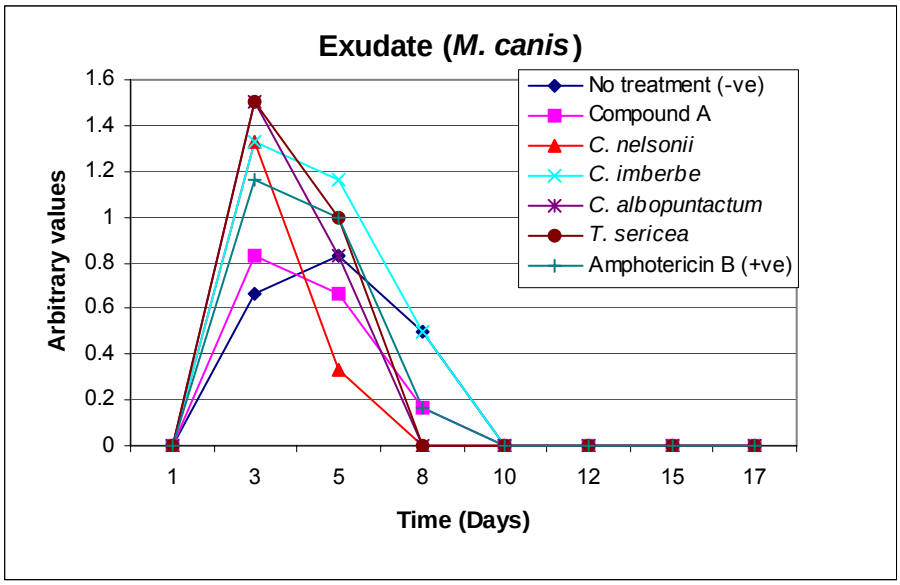


Figure 11.34a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *M. canis*.

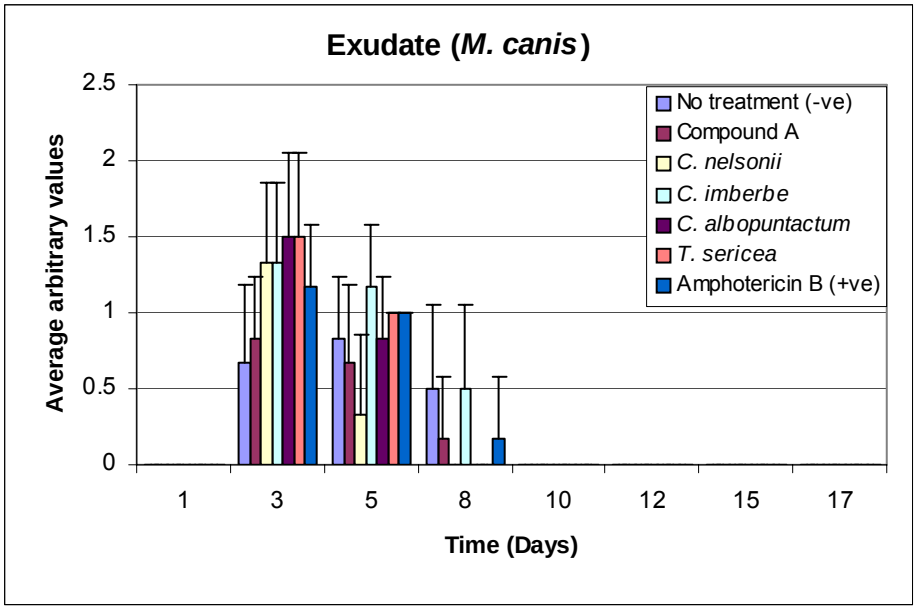


Figure 11.34b. Average arbitrary values of exudate of rats infected with *M. canis* with error bars

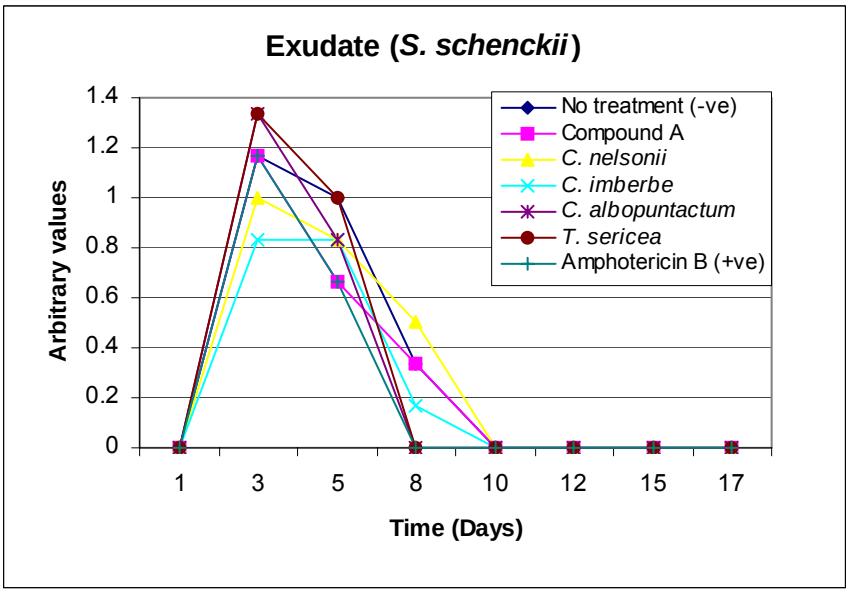


Figure 11.35a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *S. schenckii*.

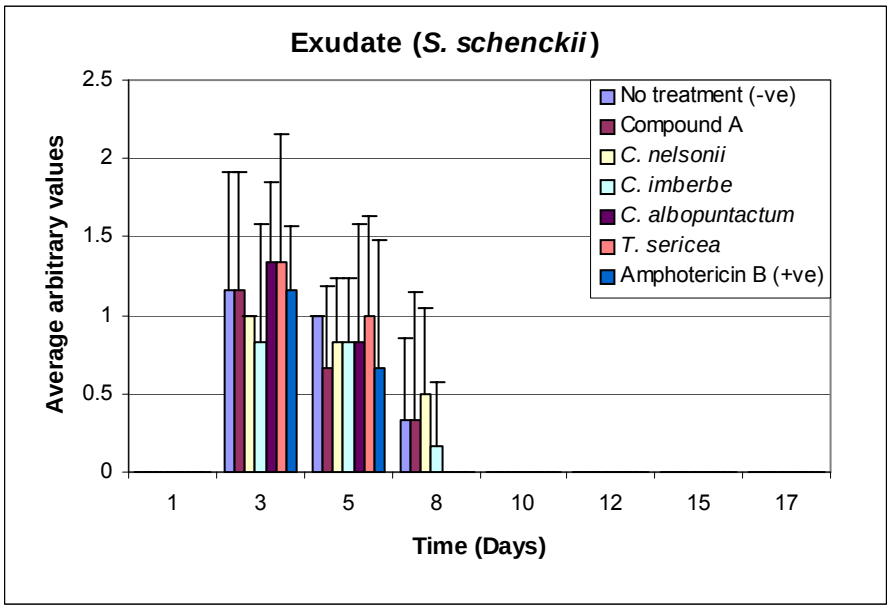


Figure 11.35b. Average arbitrary values of exudate of rats infected with *S. schenckii* with error bars

11.6.3.6. Crust Formation

Wound healing process was also quantified by crust formation, *C. albicans* (**Figure 11.36a**), *C. neoformans* (**Figure 11.37a**), *M. canis* (**Figure 11.38a**) and *S. schenckii* (**Figure 11.39a**). The

same scale used in erythema was used. Error bars were also drawn to show the confidence level of data or the deviation along curves, *C. albicans* (**Figure 11.36b**), *C. neoformans* (**Figure 11.37b**), *M. canis* (**Figure 11.38b**) and *S. schenckii* (**Figure 11.39b**). The treated group presented a rigid, dark and thick crust. It is probably due to proteins and wound exudates interconnected with the extract constituents favouring the local homeostasis and protecting the new tissue by forming an external cover that furnished mechanic protection. The crust formation in all infected rats follow the same patter. i.e. crust start forming after Day 3 until Day 15. There was no marked difference in crust formation of all the treatments.

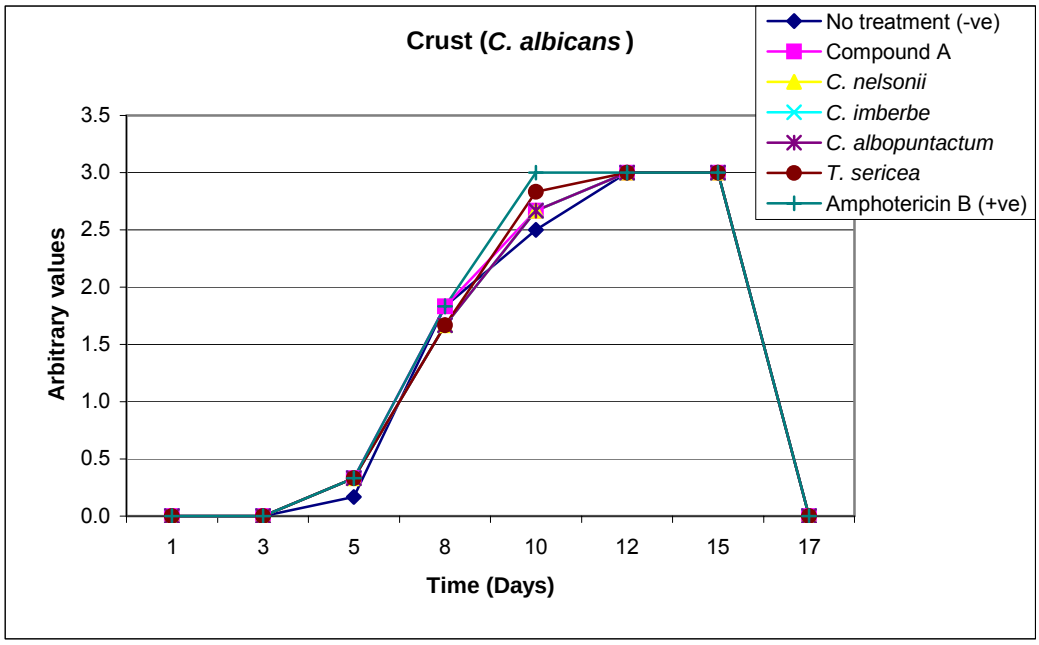


Figure 11.36a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *C. albicans*.

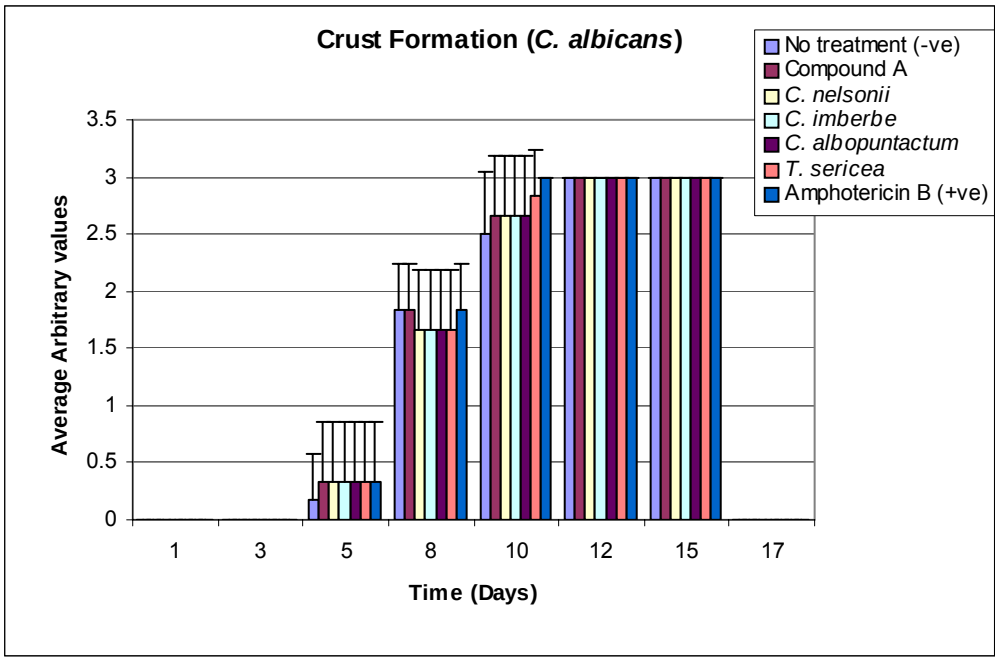


Figure 11.36b. Average arbitrary values of crust formation of rats infected with *C. albicans* with error bars

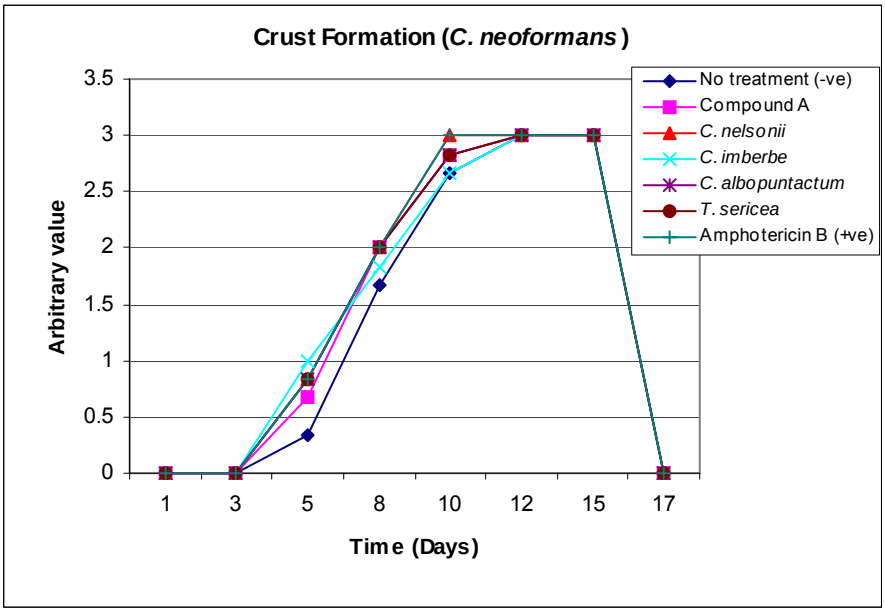


Figure 11.37a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *C. neoformans*.

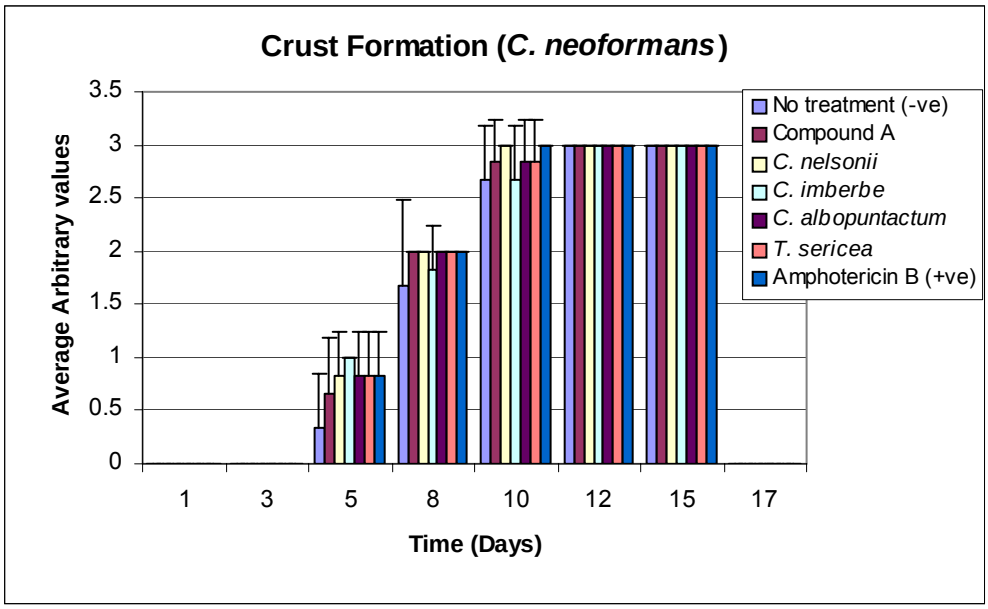


Figure 11.37b. Average arbitrary values of crust formation of rats infected with *C. neoformans* with error bars

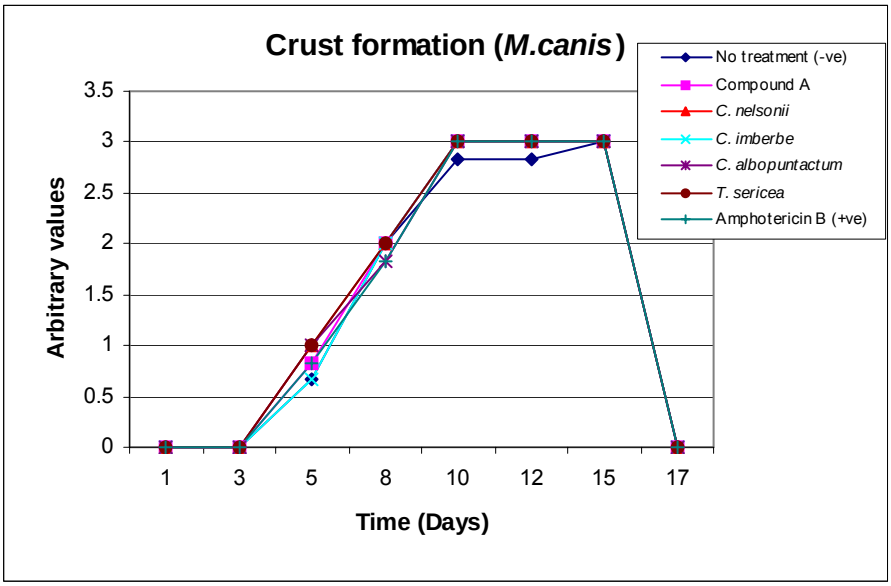


Figure 11.38a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *M. canis*.

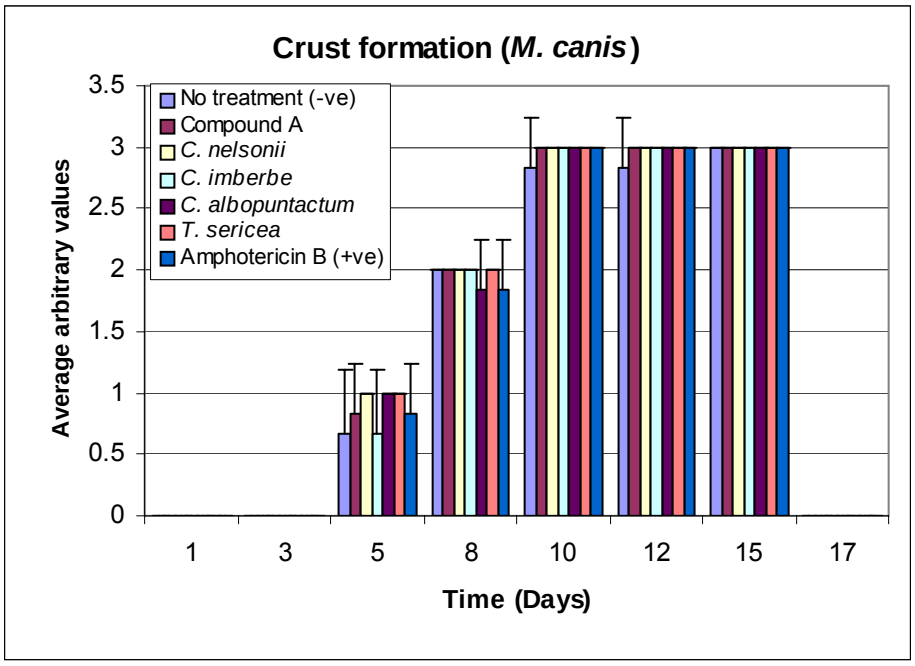


Figure 11.38b. Average arbitrary values of crust formation of rats infected with *M. canis* with error bars

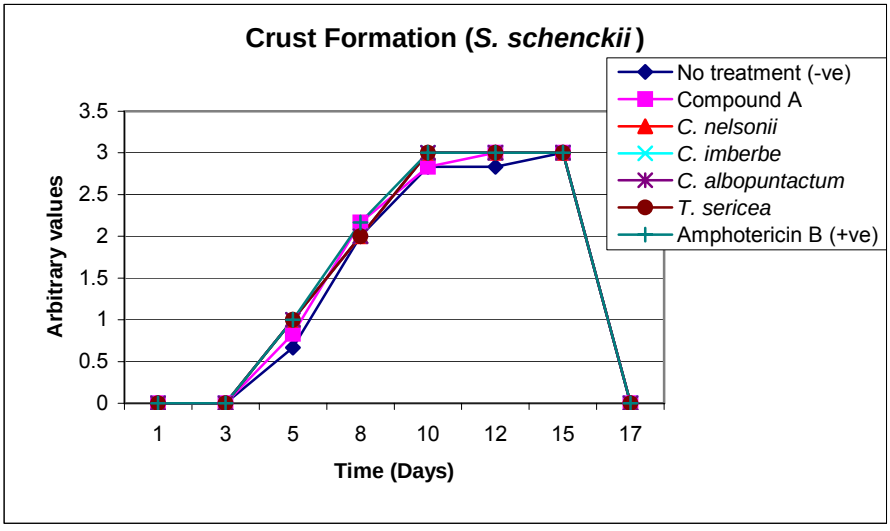


Figure 11.39a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *S. schenckii*.

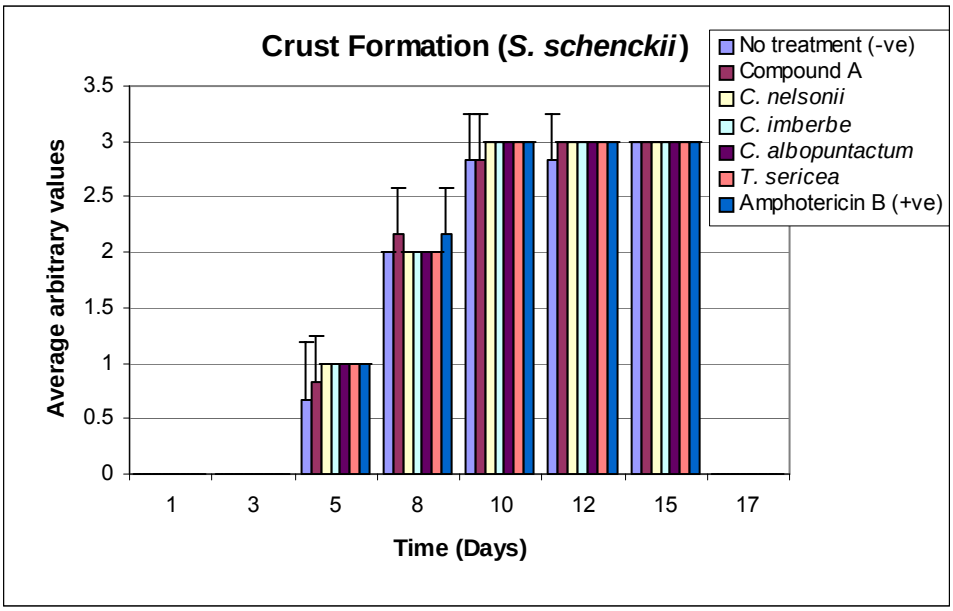


Figure 11.39b. Average arbitrary values of crust formation of rats infected with *S. schenckii* with error bars

11.6.4. Comparisons of lesions sizes

Comparison of lesion sizes was done to check the effect of treatments on fungal pathogens. Isolated compound (**Figure 11.40**) was very active against *M. canis* and *S. schenckii*. Amphotericin B (**Figure 11.41**) had almost similar activity against tested pathogens. All extracts were very active against *M. canis* and *S. schenckii* and least active against *C. albicans* and *C. neoformans.* (**Figure 11.42 – 11.45**).

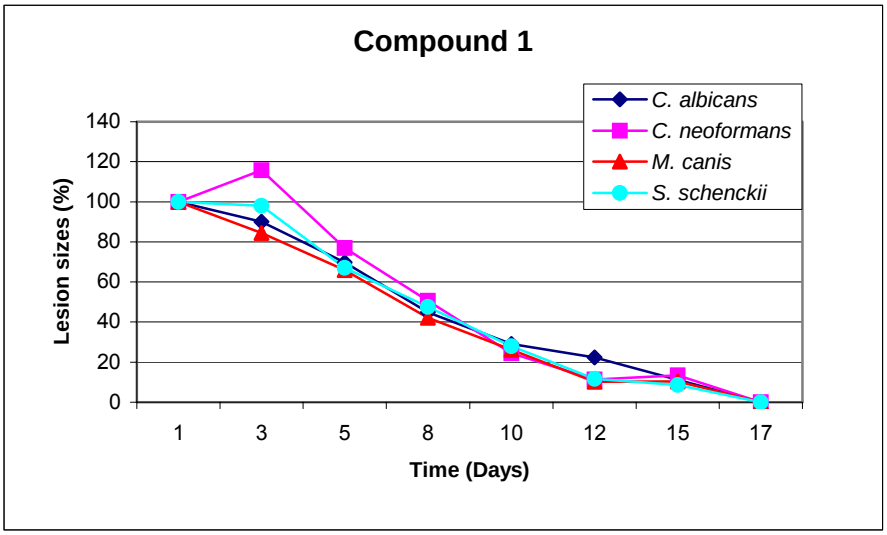


Figure 11.40. Effect of compound 1 on fungal pathogens.

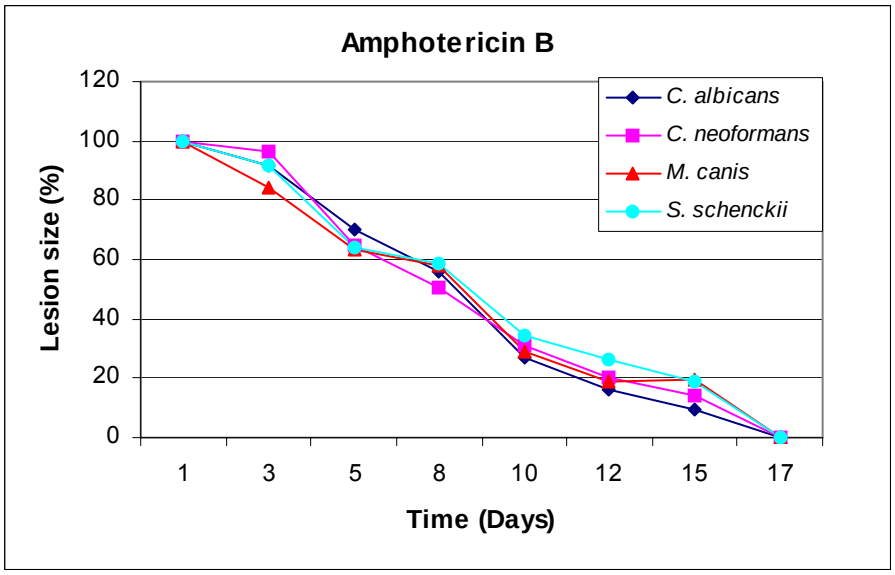


Figure 11.41. Effect of amphotericin B on fungal pathogens.

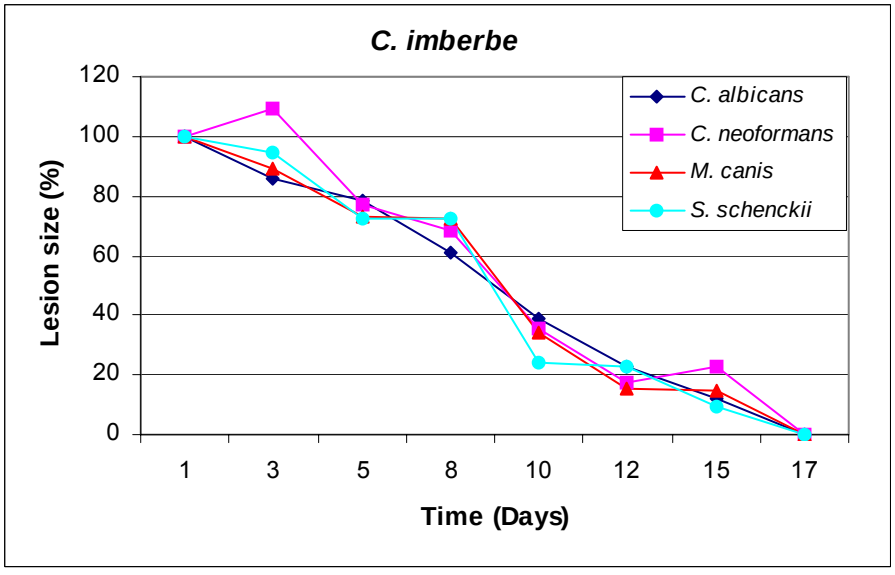


Figure 11.42. Effect of *C. imberbe* acetone extract on fungal pathogens.

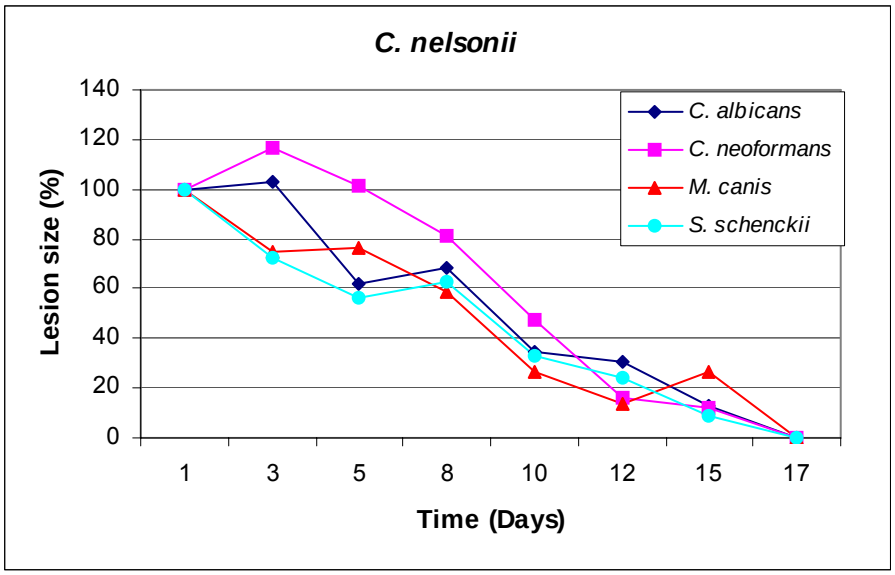


Figure 11.43. Effect of *C. nelsonii* acetone extract on fungal pathogens.

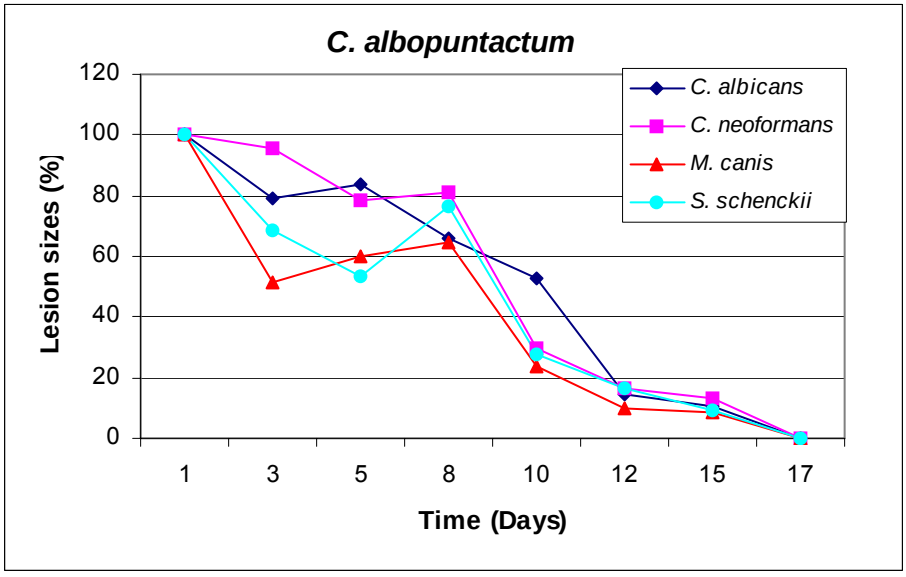


Figure 11.44. Effect of *C. albopunctactum* acetone extract on fungal pathogens.

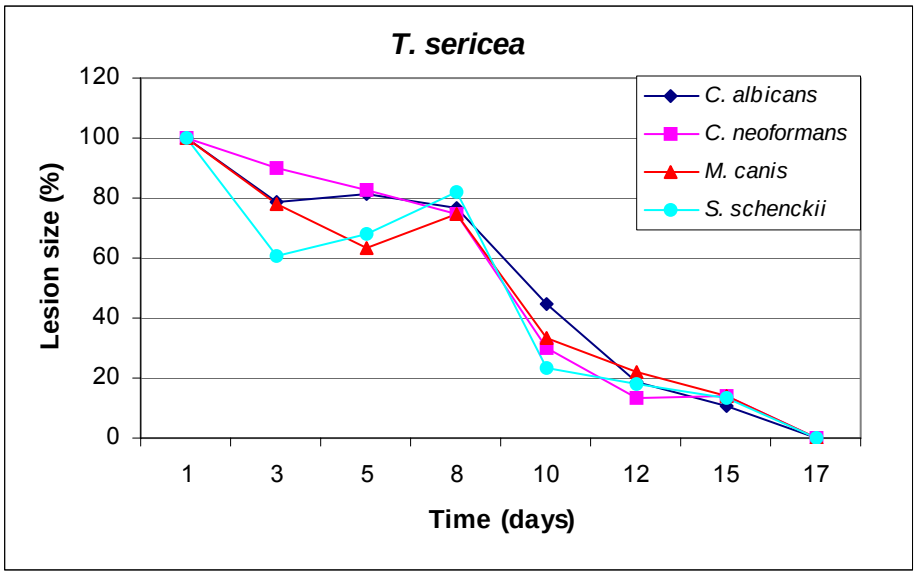


Figure 11.45. Effect of *T. sericea* acetone extract on fungal pathogens.

11.6.5. Histopathological findings

Quantitative histopathological findings were determined on 4 rats, each rat representing the group i.*e.* first six rat were infected with *C. albicans,* rat 7 to rat 12 with *C. neoformans,* rat 13 to 18 with *M. canis* and rat 19 to rat 24 with *S. schenckii* (**Table 11.6a – d**). The clumps of fungi were observed in all treatment and controls in all infected rats with different fungal pathogens. Epithelialisation was observed in the dermus except for the rats infected with *C. neoformans,* whereby it was observed on the epidermus on the wound treated with *C. nelsonii* and the untreated wound. Clumps of degenerating neutrophils, necrotic changes in the upper dermis with loss of epidermis were also observed up to day 17. Scant fungi were noted in all the wounds indicating that infection had occurred, but had generally cleared. Exceptions were treatments with isolated compound and *T. sericea* on the *S. schenckii* infected wounds where there were high numbers of fungi.

11.7. Discussion

These experiments were designed to afford a simple *in vivo* method for comparing the relative effectiveness of various plant extracts against fungal pathogen wound infectious. The duration of therapy and the dosage employed determined the end point of the experiment, and gave an index of their relative effectiveness. The preliminary survey of therapy reveals that a considerable

number of plant extracts are effective locally in the prevention of fungal infection. The choice depended upon consideration of toxicity that was determined in previous chapters.

Table 11.6a. Quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

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*			
1	A	2(++)	0	0	1&2(+++)	3(+)	3(±)	3(±)	3(++)	3(++)	2	1(±)
	B	2(++)	0	0	2(±)	3(++)	3(±)	3(±)	3(+++)	3(++)	2	1(±)
	C	2(+)	2(+)	0	1(+++);2(±)	2(+)	2(±)	2(±)	2(+++)	2(+)	2	1(±)
	D	2(++)	0	0	2(±)	2(++)	2(±)	2(±)	2(+++)	2(++)	2	1(±)
	E	2(++)	0	0	2(±)	2(+++)	2(±)	0	2(+++)	2(+)	2	1(±)
	F	2(+)	2(±)	0	2(±)	2(+)	2(++)	0	2(++)	2(+)	2	1(±)
	G	2(+)	0	0	0	2(++)	0	0	2(+++)	2(+)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

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 11.6b. Quantitative histopathological findings of wounds of rats infected with *C. neoformans* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

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*			
1	A	3(++)	2(+)	0	1&2(++)	3(++)	3(±)	0	3(++)	3(++)	1	1(±)
	B	2(++)	0	0	0	2(+)	2(±)	0	2(+)	2(++)	2	1(±)
	C	2(+++)	1(++)	0	1(++);2(+)	2(+++)	2(+)	2(+)	2(+++)	2(++)	1	1(±)
	D	2(++)	2(±)	0	0	2(+++)	2(+)	2(±)	2(++)	2(+)	2	1(±)
	E	3(++)	2(±)	0	2(+)	3(++)	3(+)	3(+)	3(++)	3(++)	2	1(±)
	F	2(++)	2(±)	+	0	2(+)	3(±)	0	3(++)	2(++)	2	1(±)
	G	2(++)	2(±)	±	2(±)	2(++) & 3(+)	2(+)	0	2(+)	2(+)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

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 11.6c. Quantitative histopathological findings of wounds of rats infected with *M. canis* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

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*			
1	A	3(++)	3(±)	0	0	3(++)	3(±)	3(±)	3(++)	3(++)	2	1(±)
	B	3(++)	0	0	3(±)	3(+)	3(±)	0	3(++)	3(++)	2	1(±)
	C	2(++)	2(±)	±	2(±)	2(+++)	2(±)	0	2(++)	2(++)	2	1(±)
	D	2(++)	2(±)	0	2(±)	2(++)	2(+)	3(+)	2(++)	2(++)	2	1(±)
	E	2(++)	2(++)	+	2(+)	2(+++)	2(±)	0	2(+++)	2(++)	2	1(±)
	F	2(++)	0	0	0	2(++)	2(+)	0	2(+)	2(++)	2	1(±)
	G	3(+++)	0	0	2(±)	3(++)	2(±)	0	3(+++)	2(++)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

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 11.6d. Quantitative histopathological findings of wounds of rats infected with *S. schenckii* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

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*			
1	A	4(++)	4(±)	0	1(+++)	4(+++)	4(+)	4(+)	4(++)	3(++)	1	1(±)
	B	4(++)	1(++)	0	1(+++)	4(+++)	4(+)	4(±)	4(++)	3(++)	0	1(±)
	C	2(+++)	0	0	2(+)	2(+++)	2(+)	0	2(++)	2(+++)	1	1(±)
	D	2(+++)	2(±)	0	0	2(+++)	2(+)	0	2(++)	2(+++)	2	2(±)
	E	2(+++)	2(+)	0	2(+)	2(+++)	2(+)	0	2(+++)	2(+++)	2	1(±)
	F	2(++)	2(+)	0	0	2(+++)	2(+)	0	2(+)	2(++)	2	2(+++)
	G	4(+++)	4(++)	0	2(++)	4(+++)	4(±)	0	3(+++)	4(++)	1	4(+++)

*Neut's= Neutrophils; Mac's= Macrophages

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

During the study, I realised that the treatments used had wound healing properties as well. Inngjerdingen *et al*. (2004) reported that some Combretaceae species had wound healing activities when the plant powder was applied directly on the wound. The treatments were usually repeated every day until the wounds were healed. In this study I treated the rats with the selected leaf extracted every second day. Wound healing is a multifactorial process where microbial infections and the formation of free radicals may contribute to retard or inhibit its resolution. Free radicals can oxidise the endogenous inhibitors or proteases, this reduces their ability to inhibit elastase and the proteases responsible for the deterioration of the extra-cellular matrix (Kudi *et al*., 1999). The possibility of the wound healing due to free radicals was eliminated in previous chapters, where the antioxidant activity of the selected plants was studied. The selected plant extracts and compound **1** did not have antioxidant activity based on the DPPH assay.

The main focus of the chapter was to investigate the antifungal activities of the four selected acetone extracts (*C. nelsonii*, *C. imberbe*, *C. albopunctactum* and *T. sericea*) and compound **1** on wounds infected with *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii*. The clinical treatment of skin infected with fungi has become a major problem especially in immuno-compromised patients. Therapeutic agents selected for the treatment of infected wounds had ideally shown antifungal activity on *in vitro* studies. I also checked whether these agents would improve phases of wound healing without producing deleterious side effects.

This study describes some unique features with respect to the therapeutic effect of leaf extracts of selected plants on dermal wound of rats infected with fungal pathogens. Plants products are potential agents for wound healing and the treatment of fungal infections (Masoko *et al*., 2005 and Masoko *et al*., 2006) and largely preferred because of their widespread availability, low toxicity and their effectiveness as crude preparations. We have reported that *Combretum* and *Terminalia* species have antifungal activity (Masoko *et al*., 2005 and 2006 and Masoko and Eloff, 2005 and 2006). These findings prompted us to further investigate *in vivo* activity of the four most active extracts. The study was divided into 2 pilot studies and the main experiment.

Pilot study I (Local irritancy and wound healing study)

In this pilot study the created wound were not infected. I wanted to establish whether an aqueous cream used as vehicle had no irritant effect on the rat, to determine irritant/ tolerance effect of 10 % and 20 % crude extracts in cream based on rats and to determine degree of wound healing within three weeks. Rats did not show any sign of irritancy to any of the treatments. They

maintained their normal temperature and often an initial weight loss gained, indicating that they tolerated the procedures well and that systemic infection did not occur. There was no swelling and ulcerations as well. The wounds were 100% healed after three weeks.

I found that the extracts of *C.imberbe* and *C. nelsonii* were superior in wound healing abilities. The wound treated with *C. imberbe* extracts healed faster than the control. Wound without treatment lesion size increased after day 3 but it started decreasing after day 5, at day 10 it was within the same range as others. The wound without treatment and cream only lesion size increased after Day 10. *C. nelsonii* extract healed the wound faster but the controls lesion sizes increased after Day 8. In the wound treated with *T. sericea*, the cream only healed the wound faster, before Day 8 the extract lesion size decreased faster than the cream only but after Day 8 wound treated with cream only had smaller wound size. From these results the use of 20% extracts was recommended as they ought to have a better antifungal effect based on MIC values and did not have a negative effect on wound healing as was the case of *C. albopunctactum* where the 10% extract was found to have a slightly negative effect on wound healing. Crude extracts were better in reducing the erythema of the wounds. The average erythema of wounds treated with *C. imberbe* was lower than the other extracts. After Day 10 the wound without treatment had high average erythema formation. The crude extracts and had lower average erythema than the untreated wounds. Exudate formation was more formed from extracts used. All the treatments decreased the exudate formation after 12 days. Crust formation took longer in the untreated wounds.

Pilot study II (Infection with different pathogens)

The aim of this part of the study was to determine the effect of plant extracts in aqueous cream on fungal infection of wounds and subsequent healing. Since three of the four fungi tested are known to cause deep or systemic infection, it was also necessary to determine whether this occurred. From the four acetone extracts used, amphotericin B was added as the positive control and the negative control was the infected wound but not treated. The rats were left for 48 hours after infection before topical treatment was applied. This was done to encourage infection. All 12 rats lost weight (**Figure 11.10**) within 48 hours of infection and started picking up weight after the first treatment, with exception of Rat 11 which continued to lose weight. Therefore Rat 11, infected with *S. schenckii* was euthanased. All rats infected with *S. schenckii* had a nasal discharge. Histopathological results of the lymph nodes of Rat 10 showed moderate lympoid hyperplasia with infiltration of moderate numbers of lymphocytes, plasma cells, mild number of mast cells and

eosinophils. Lymph node was large (4x2 mm). (MD: Moderate lymph node hyperplasia with lymphodenitis, mild, subacute).

After Rat 11 was terminated, necropsy was performed and the following observations were made: stomach and intestine were empty which means the rat was not eating and that led to weight loss; lymph nodes were enlarged, and lungs showed a diffuse pneumonia, other organs (lliver, thymus, heart, spleen, kidney and pancreas) were normal. Specimens were sent to Pathology Section for histopathological examination and some to Bacteriology Section. After bacteriological examination it was found that the nasal discharge was not due to fungi but bacterial infection. It was then concluded that the rat had pneumonia. Histopathological results of the lungs showed moderate congestion, moderate to severe interstitial pneumonia with infiltration of polymorphonuclear leukocytes and moderate alveolar oedema. No significant lesions in other organs examined other than moderate generalised congestion were observed.

Temperature of the remaining 11 rats was within the expected range of 34 to 37 ^{o}C (**Figure 11.11**).

While all rats survived for the duration of the experiment, technical difficulties reduced the sample size to 11. Wound sites were evaluated for erythema, exudate and crust formation during the period of the study. There were no signs of erythema surrounding any wound sites throughout the experiment. All of the dressings applied during the experiment did not adhere to the wound bed. Since the experimental dressings remained securely fastened, all wounds were therefore included in the calculations.

Crude extracts and amphotericin B reduced the erythema (**Figure 11.13**) of the wounds. The average erythema of wounds treated with *C. imberbe* was lower than the other extracts including amphotericin B. The average erythema of amphotericin B increased on Day 5 and again on Day 10 but went down after Day 10. The crude extracts and amphotericin B had lower average erythema than the untreated, therefore they can be regarded as being effective in reducing erythema caused by fungal infection.

More exudate was formed (**Figure 11.14**) from treatment with amphotericin B followed by *C. albopunctactum*. All the treatment decreased the exudate formation after 12 days. This results correlate with the one of erythema. Untreated wounds were the last to form crust (**Figure 11.15**), which was expected. Wounds treated with *C. nelsonii* were the first ones to form crust.

There was difference in lesion size of wounds infected with different fungal pathogens. In wounds infected with *C. albicans* (**Figure 11.12a**) and in *S. schenckii* (**Figure 11.12d**), amphotericin B decreased lesion size quicker compared to crude extracts. Wounds treated with *C. albopunctactum* had the least effect in reducing the lesion sizet. In *C. neoformans* (**Figure 11.12b**) *T. sericea* was the best in reducing lesion size, and in *M. canis* (**Figure 11.12c**), *C. nelsonii* was the best.

All these parameters showed that the crude extracts and amphotericin B were effective in decreasing formation of the exudate, increasing crust formation and that they have antifungal activities even when use in *in vivo* studies.

A specimen sample of skin tissues of each group of rats were taken out from the healed wounds of the animals in the above excision wound model for histopathological examination. All lesions showed wound healing activity with few or no fungal hyphae. Lesions of Rat 11 which was euthanased before the end of the experiments had high presence of fungal hyphae because it didn't finish treatment.

In pilot study 2 all the wounds healed almost at the same time even the untreated, therefore it was concluded that it was due to the strong immune system of the rats and wound contraction being the primary means of wound closure.

Confirmation study (Main study)

There was no evidence of systemic infection caused by the irritant effects in the second pilot study, therefore I continued with the main study where 24 rats were used. The rats were divided in four groups based on fungal pathogens. All the treatments used in the second pilot study were used but the compound 1 a mixture of asiatic acid and arjunolic acid was added. In the main study rats were immunocompromised by subcutaneous injection of 500 µg of estradiol valerate. Estradiol pretreatment is known to inhibit innate and acquired immune defenses (Carlsten *et al*., 1991).

Acetone extracts of leaves of *C. nelsonii*, *C*. *albopunctactum*, *C. imberbe* and *T. sericea* possess remarkable growth inhibitory activities against fungal pathogens (**Chapters 5** and **6**). Acetone extracts of leaves and mixture of asiatic acid and arjunolic acid demonstrated wound healing properties comparable with that of antibiotic powder (amphotericin B). Even the untreated wounds

healed but not at the same rate as the treated wounds. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither showed any signs of restlessness nor scratching/biting of wound site when the extracts were applied.

All the rats lost weight in this study until Day 12 and started increasing weight from Day 15. It was assumed that it was due to immunocompromising the rats. After 3 weeks of the experiment, bandages were removed and all rats were kept for another week. Thereafter it was found that all have gained weight except Rat 6 where the mass remained constant. The temperatures were also within the normal ranges. All other parameters measured were the same as explained in second pilot study.

Mixture of asiatic acid and arjunolic acid (**Figure 11.40**) and *C. nelsonii* (**Figure 11.42**) were very active against *M. canis* and *S. schenckii*. Wounds infected with *C. neoformans* took longer time to reduce size. Amphotericin B (**Figure 11.41**) had almost same activity against all tested pathogens. *C. imberbe* (**Figure 11.42**) and *T. sericea* (**Figure 11.45**) had similar impact on the pathogens. *C. albopunctactum* (**Figure 11.44**) was very active against *M. canis*.

After 3 weeks all 24 rats were euthanized with CO_2 and necropsies performed. From rats infected with *C. albicans* (Rat 1 to 6) organs were taken from Rat 1 for histopathological studies. The lungs of Rat 3 lungs had block specks and the intestines were blue. The lungs were taken to Bacteriology lab for culturing. Organs of Rat 7 together with lungs of Rat 10 were taken for histopathological studies. Organs in group 2 (rats infected with *C. neoformans*) (Rats 7 to 12) were normal. Again organs of Rat 13 in group 3 (rats infected with *S. schenckii*) (Rats 13 to 18) and liver of Rat 16 were taken to Pathology section, together with organs of Rat 19 and the left lymph node of Rat 23 in group 4 (rats infected with *M. canis*) (Rats 19 –24).

Unfortunately some of the results of histopathological studies are not reported here due a delay in evaluation of the samples by the pathologists at Onderstepoort. Only results of four rats were discussed and some of the results will be included in a publication to be written. The following comparisons were made from histopathology results: generally more fibrosis with crude extracts, but an exception is Amphotericin B treatment of *S. schenckii*. The other parameters of healing i.e. angiogenesis and epithelisation were present or complete with the exception of S. schenckii infections where the extracts performed better. Possibly a synergistic effect. No noticeable differences in wound necrosis. Neutrophils were evident and in deeper levels in the untreated.

Plasma cells, lymphocytes and macrophages were the most predominant cell types. These are the most predominant cells in fungal infections and are also the more common ones in chronic infections. Macrophages are also the most active cell in wound healing acting as potent wound debriders.

A close examination of tissue sections revealed that there was marked infiltration of lymphocytes, eosinophils, neutrophils, mast cells and macrophages and enhanced proliferation of fibroblasts as a result of treatments. Increased cellular infiltration observed from hematoxylin and eosin (H&E) staining in treated rats may be a result of chemotactic effect enhanced by the extract, which might have attracted inflammatory cells toward the wound site. Increased cellular proliferation may be a result of the mitogenic activity of the plant extract, which might have significantly contributed to healing process. Early dermal and epidermal regeneration in treated rats also confirmed that the extract had a positive effect toward cellular proliferation, granular tissue formation and epithelialisation.

Histopathological studies of the wounds bring prominent aspects, that both the antibiotic and the plant extracts individually are capable of healing the wound. What needs to be further worked out is the relative quantities of plant extracts that are necessary for optimal effect, the maximum period for which the extract can be kept stable. Whether such preparations should be and could be sterilized is also an aspect, which may be pertinent. And finally the most important question that arises from the study is “which constituent of extracts evokes wound healing effect”. Bioactivity may also be associated with some other components such as prostaglandin precursors or some other molecule; an identification and isolation of such molecule may also be desirable. Until such a possibility is brought to reality, plant extracts in their natural form may be our only choice. The isolated mixture of asiatic acid and arjunolic acid from *C. nelsonii* which showed high activity in *in vitro* studies did not have the same effect in *in vivo* studies. Rat infected with *M. canis* and treated with the mixture, formation of the fibroblast and infiltration of the cells occurred in deeper tissues. Same with rat infected with *S. schenckii*, wound treated with mixture, formation of fibroblast occurred in extensive deeper tissues and there was delay of epithelisation.

In some instances there were more traces of fungal hyphae in wound treated with amphotericin B compared to extracts i.e. rat infected with *S. schenckii*. Maybe this antibiotic was not the right choice, as the control and the presumption is that any other antibiotic could have behaved in a manner similar to amphotericin B but certainly that needs to be experimentally confirmed. Amphotericin B was selected as it is the most potent broad spectrum antifungal that is available.

However, it is not usually used as a topical treatment, the inidazoles are better for that. These aspects if at all considered important will have to be studied separately.

Some organ samples were also studied. In most instances the lungs showed diffuse, sub-acute mono-morphonuclear (mainly lympho-plasmacytic, with lesser numbers of macrophages) interstitial pneumonia with moderate to severe, diffuse haemorrhage. Spleen showed mild red pulp hyperplasia with many haemosiderin laden macrophages. Small intestine showed mild to moderately increased numbers of eosinophils within the lamina proprium of the small intestine wall. Some fungal spores were seen within the lumen, but no signs of any reaction were visible. Prescopular lymph nodes of Rat 23 infected with *S. schenckii* showed moderate cortical hyperplasia. A handful (4 or 5) of fungal spores was seen in two foci just below the capsule, two of them within a macrophage phagosome. This is a common mode of spread of this fungus and as was exhibited rodents are particularly susceptible to *S. schenckii* infections.

The pulmonary lesions are commonly seen in experimental rodents, and are possibly as a results of various environmental factors/stressors. The moderate amounts of haemosiderin within splenic macrophages may possibly be a result of the pulmonary haemorrhage or the wounds created for the experiment. The few fungal spores within the lymph nodes of rat 23 are most likely a result of lymphatic drainage from the experimental wounds as opposed to direct infection, as there were no signs of inflammation in the tissues surrounding the lymph nodes. The occasional fungal spores seen on all of the epidermal surface of the skin samples can be regarded as incidental. These spores were always in association with normal skin, hardly ever would they be seen over the lesion itself.

Lung tissues of Rats 3 and 10 were cultured on blood agar and MacConkey agar. After Gram staining, wet preparation and haemolytic tests, the conclusion was reached to the effect that the lungs were infected with a Streptococcus sp an opportunistic pathogen.

Healing is a physiological process and does not normally require much help but still wounds cause discomfort and are prone to infection and other complications. Therefore, use of agents expediting healing is indicated. Further, some diseases like diabetes, immunocompromised conditions, ischaemia and conditions like malnourishment, ageing, local infection, local tissue damage due to burn or gun shot wounds lead to delay in healing. Such conditions often require the use of agents, which can facilitate the healing process (Mensah *et al*., 2001).

The rat model descripted in these studies was used for the first time to test for fungal pathogens in our group. I have observed that rats must be immunocompromised to ensure a localized fungal infection. I have demonstrated that all the extracts used have antifungal activity. Although amphotericin B gave better results the isolated mixture of asiatic acid and arjunolic acid gave promising results and thus can be considered for future treatment due to the toxicity of amphotericin B. Exudate formation, erythema and lesion size are good parameters to consider for wound healing. Ulcerations did not occur indicating that wound healing progressed normally. Generally the technique works and it can be used as the model for future studies. The main objective was to test plant extracts activity on infected animals. I didn't experienced any systemic infection except in one rat and the infection was not from the fungal pathogens used.

11.8. Conclusion

The results of this study have confirmed the antifungal potentials of crude extracts and wound healing properties of selected plants and mixture of asiatic acid and arjunolic acid on rat model. The extracts of these plants may possibly further be developed into phytomedicines for the management of septic wounds, because they did not show any signs of irritancy to rats. The model used was successful as there were no systemic infections in all the rats and the wounds healed within three weeks.

Rat models infected have often been used for the determination of the wound healing properties of various dressings and topical formulations, it is generally acknowledged that these models may not reflect accurately the biological processes occurring in humans during wound healing, likely due to significant inter-species skin differences in morphology and function (Dorsett-Martin, 2004). Still there is a potential to consider using it for animal and human infections.

In conclusion, treatment from the leaves of selected plants exhibited significant pro-healing activity in the infected wound when topically applied on rats by affecting various stages of healing process.