Chapter 7
Biological activities of two antifungal compounds isolated from *Loxostylis alata* (Anacardiaceae)

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Preface

The carbon tetrachloride fraction of *Loxostylis alata* was subjected to column chromatography to isolate the active compound(s) present. We further characterize the isolated compounds both chemically and biologically. Characterisation of the compounds will give an insight into ways to design and enhance the action of the isolated compounds. This chapter was prepared for submission to the **South African Journal of Botany** for publication.

Abstract

Loxostylis alata (Spreng.) f. ex Reichb is used in southern African traditional medicine to control labour pain and to boost the immune system. Extracts and compounds isolated from leaves of *Loxostylis alata* were evaluated for their *in vitro* antimicrobial, anti-inflammatory (Cyclooxygenase-1 and -2) activities and evaluated for their potential toxic effects using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and *Salmonella typhimurium* tester strains TA98 and TA100. Antimicrobial activity was evaluated using the micro-dilution and bioautographic assays. The bacterial strains used were *Staphylococcus aureus* (ATCC29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). While the fungal strains used were *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporum canis* and *Candida albicans*. A bioassay guided fractionation of the crude extract yielded two antimicrobial compounds namely, Lupeol **1** and β-sitosterol **2.** In addition β-sitosterol exhibited selective inhibition of COX-1 (IC_{50} = 55.3 ± 2) None of the compounds isolated were toxic in the *Salmonella typhimurium*/microsome assay and MTT cytotoxicity test. The isolation of these two compounds is reported for the first time from *Loxostylis alata*.

Keywords: *Loxostylis alata*; Antimicrobial activity; Anti-inflammatory activity; Cytotoxicity; Genotoxicity

7.1. Introduction

About 60% of the world's population relies almost entirely on herbal medicines to treat different ailments (Rates, 2001). Plant derived drugs have for ages been regarded as an essential source of therapeutically effective medicines and still remain enormously important with about 25% of the drugs prescribed worldwide being herbal formulations (Rates, 2001). Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). In 1997, the world market for phytomedicine products was estimated at US$10 billion (Soldati, 1997). Recent development of natural products into chemotherapeutic armamentarium include the antimalarial drug artemisinin and the anticancer agents taxol, docetaxel and camptothecin In some cases, the use of natural products has been the single most successful strategy for the discovery of new medicines (Harvey and Waterman, 1998). Where natural compounds do not have pharmacological activity, they may serve as lead compounds, allowing the design and rational planning of new drugs (Hamburger and Hostettmann, 1991).

As plants produce an array of diverse chemical compounds, the separation and determination of their active compounds will provide an insight into their pharmacological, pharmacokinetic and toxicological properties (Sun and Sheng, 1998).

Loxostylis alata A.Spreng. ex Rchb is a member of the family Anacardiaceae (Coates-Palgrave, 2002). The bark and leaves of *Loxostylis alata* are used in South African traditional medicine during childbirth to relieve pain during labour (Pooley 1993) and also to stimulate the immune system (Pell, 2004). Ginkgol (3-(8Z-pentadecenyl) phenol) and ginkgolic acid (6-(8Z-pentadecenyl) salicylic acid) were previously isolated from the plant (Drewes *et al*., 1998). To date no studies have been carried out on the species to determine its pharmacologically active constituents.

In a study of seven South African plant species active against the fugal pathogen *Cryptococcus neoformans*, *Loxostylis alata* had the highest activity. The primary objective of this study was to isolate the compounds active *Aspergillus fumigatus* (an important fungus in the poultry industry) from *Loxostylis alata* leaf extracts.. The isolated compounds were investigated further for their anti-inflammatory (Cyclooxygenase-1 and -2), mutagenic (*Salmonella*/microsome assay) and cytotoxic (MTT assay) activities to evaluate other activities and the safety.

7.2. Materials and methods

7.2.1. Plant collection

Leaves of *Loxostylis alata* A.Spreng. ex Rchb were collected at the Marie van der Schijff Botanical Garden of the University of Pretoria, South Africa. Samples of the plant were identified and authenticated by Lorraine Middleton and Magda Nel of the Botanical Garden of the University of Pretoria. Voucher specimen of the plant (number; PRU PRU96508) was deposited at the Schweikert Herbarium of the Department of Plant Science, University of Pretoria, South Africa.

7.2.2. Extraction, isolation and identification of constituents

Leaves of *Loxostylis alata* were dried at room temperature, milled to a fine powder and stored at room temperature in closed containers in the dark until used. The ground plant material (500 g) was extracted with acetone (5 litres × 3). The solvent of the combined extracts was removed *in vacuo*.
The same compounds that showed activity against *A. fumigatus* on bioautogram were also active against *S. aureus* because of their similar R_f values (Figures 7.2 and 7.4). Since it is difficult to work with *A. fumigatus* which is a spore forming organism and carries greater health risk to the researcher and the environment, we decided to use *S. aureus* as a model for isolating the active compounds. The acetone extract (70 g) was subjected to solvent fractionation using carbon tetrachloride, hexane, chloroform, aqueous methanol, butanol and water (Stuffiness and Douros, 1979).Column chromatography (37 × 5 cm, silica gel 60) of the CCl_4 fraction (10 g) using a hexane: ethyl acetate step gradient followed by ethyl acetate: methanol step gradient was performed. Initially, 100% hexane was used, and then reduced to 0% hexane by the addition of 10% ethyl acetate in succession. This followed by ethyl acetate: methanol gradient where ethyl acetate was reduced to 0% by the addition of 10% methanol in success increments. Thirteen fractions were collected and each tested for activity against *S. aureus* using the bioautographic method (Begue and Klein, 1972). Based on the bioautography profile, fractions containing active compounds with the same R_f value were combined and were further fractionated by column chromatography eluted isocratically with hexane: ethylacetate (7:3) to yield the pure compounds. Structures of the two isolated compounds were elucidated using ^{1}H NMR and ^{13}C NMR spectral analysis using Variant Unit Innova 300 MHz system (Oxford instruments) and Brüker DRX instrument at the Medical University of South Africa (MEDUNSA). The spectra were confirmed by Mass spectroscopy (MS) at the Department of Chemistry, University of Botswana, Gaborone.

7.2.3. Antimicrobial activity

7.2.3.1. Fungal and bacterial cultures

Bacterial strains used for antibacterial testing were the Gram-positive *Staphylococcus aureus* (ATCC29213), *Enterococcus faecalis* (ATCC 29212), and the Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). Pathogenic fungal isolates used were *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporum canis* and *Candida albicans* (obtained from the Microbiology Unit, Department of Veterinary Tropical Diseases, University of Pretoria). Bacterial cells were inoculated into fresh Müller-Hinton (MH) broth (Fluka, Switzerland) and incubated at 37 °C for 14 h prior to the screening procedures. Fungal cultures were grown in Sabouraud dextrose (SD) broth at 37 °C and maintained on SD agar at 4 °C.

7.2.3.2. Bioautography

The antibacterial and antifungal bioautographic assays were carried out according to the method described by Begue and Kline (1972) with slight modification by Masoko and Eloff (2005). Briefly, thin layer chromatography (TLC) plates were loaded with 100 µg of each fraction or 10 µg of pure compound, and dried before developing in chloroform/ethyl acetate/formic acid (5:4:1): [CEF] and hexane/ethyl acetate (7:3) [HE] mobile phases for the fractions and the pure compounds, respectively. The solvent was allowed to evaporate from the plates under a stream of fast moving air for 2-5 days. Plates were then sprayed with concentrated cultures of bacteria or fungal species until completely moist. The moist plates were incubated at 37 °C for 24 h. Thereafter, the plates were then sprayed with 2 mg/ml of *p*-iodonitrotetrazolium violet (INT) and incubated for a further 1 h in case of bacteria and 24 h for fungi (Begue and Kline, 1972). White areas over a purple background on the TLC plate indicate the non-reduction of INT to coloured formazan and therefore an indication of microbial inhibition by the compounds present.

7.2.3.3. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of extracts, column fractions and isolated compounds against bacteria and fungi were determined using the serial microdilution assays (Eloff, 1998a; Masoko et al, 2005). In brief, two-fold serial dilutions of the samples were prepared in wells of 96-well microtitre plates. Bacterial or fungal culture (100 µl of an overnight culture) was then added to each well before incubation for 24 h for bacteria or 48 h in case of fungi at 37 °C. *p*-iodonitrotetrazolium chloride (INT, Sigma) was added to each well as indicator of bacterial or fungal growth. The minimum inhibitory concentration (MIC) was read as the concentration of sample that inhibited microbial growth, as indicated by a visible reduction in the red colour

of the INT formazan. In each assay, negative solvent controls, growth controls and a positive control were included. Gentamicin and amphotericin B (Sigma) were used as the antibacterial and antifungal controls, respectively. The samples were tested in triplicate and the assays were repeated twice to confirm results.

7.2.4. Cytotoxicity assay

The isolated compounds were tested for cytotoxicity against the Vero monkey kidney cell line. The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% Gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37 °C in a 5% CO_2 incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the compounds were prepared by reconstitution to a concentration of 10 mg/ml in dimethylsulphoxide (DMSO). Serial 10-fold dilutions of each extract were prepared in growth medium (1-1000 µg /ml). The method described by Mosmann (1983) was used to determine the viability of cell growth after 5 days incubation with the compounds. MTT was used as an indicator for cell growth. The absorbance was measured at 570 nm. Berberine chloride (Sigma) and DMSO were used as positive and negative controls, respectively. Tests were carried out in quadruplicate and each experiment was done in triplicate. Furthermore, the selectivity index of each fraction was calculated as follows (Shai et al., 2008):

Selectivity index (SI) = Lethal concentration 50 (LC_{50})/Minimum inhibitory concentration (MIC)

This ratio gives the relative safety of each fraction.

7.2.5. Genotoxicity test

The potential mutagenic effects of the investigated plant compounds were detected using the Ames test. The Ames assay was performed with *Salmonella typhimurium* (TA98 and TA100) strains using the plate incorporation procedure described by Maron and Ames (1983). One hundred microliters of bacterial stock solution was incubated in 20 ml of Oxoid Nutrient broth for 16 h at 37 °C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (plant extract, solvent control or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 h at 37 °C. After incubation, the number of revertant colonies (mutants) was counted. All cultures were made in triplicate (except the solvent control where five replicates were made) for each

assay. The assays were repeated twice. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/ml.

7.2.6. Anti-inflammatory assay

Infection with *Aspergillus* organisms causes excessive production of prostaglandins by the host tissue. Prostaglandin production during fungal infection is an important factor in promoting fungal colonization, inflammation and chronic infection (Noverr et al., 2002). Inhibition of prostaglandin biosynthesis by the plant extract and isolated compounds was investigated using both the COX-1 and COX-2 assays (Jäger et al., 1996, Noreen et al. 1998). The COX-1 enzyme (from ram seminal vesicles, Sigma Aldrich) and COX-2 (human recombinant, Sigma-Aldrich) were activated with co-factor solution and pre-incubated on ice for 5 min. Sixty microliters of this enzyme/co-factor solution was added to 20 µl of crude extract of *Loxostylis alata* extract (20 µl of extract solution) or 20 µl of compound and pre-incubated for 5 min at room temperature. Twenty microliters of ^{14}C-arachidonic acid was added to the tested samples and incubated at 37 °C for 10 min. After incubation, the reaction was terminated by adding 10 µl of 2N HCl. Four microliters of a 0.2 mg/ml carrier solution of unlabelled prostaglandins was added. In each assay, four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of ^{14}C-arachidonic acid, and two were solvent blanks. Indomethacin was included in each test assay as a standard. Percentage inhibition of plant extracts was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank. IC_{50} was calculated from at least 5 concentrations. Results are presented as mean ± S.E.M. of two experiments carried out in duplicate.

7.3. Results and discussions

7.3.1. Biological activity of the extract

In a preliminary screening, the acetone extract of *Loxostylis alata* had promising activity against *Cryptococcus neoformans*. In this study, an investigation on the antifungal activity of the acetone extracts of *Loxostylis alata* against *Aspergillus fumigatus* confirmed earlier findings. The yield, MIC value together with the total activity of the crude extract and the different fractions resulted from solvent - solvent fractionation of the acetone extract (70 g) are presented in Table 7.1. Total activity (TA) is calculated by dividing the quantity extracted in mg with the MIC value in mg/ml. This value indicates the volume to which the active constituent present in one gram of the fraction can be diluted and still inhibit the growth of the test organism (Eloff, 2004). A higher value of total activity indicates increased usefulness and economic value of the plant material and is of benefit in enabling rural use of the plant. The CCl_4 fraction was the most active fraction with MIC and TA value of 0.08 mg/ml and 3201.79 ml/g, respectively. It therefore

means that 1 gram of CCl_4 fraction can be diluted in 3201.79 ml of the solvent used and still inhibit the growth of *A. fumigatus*. Similarly, the CCl_4 fraction showed greater zone of inhibition against all the tested microorganisms. Hexane and aqueous methanol fractions showed little zone of inhibition, while butanol and water fractions were not active when the TLC bioautographic method was used (Figure 7.1). In addition, column fractions of CCl_4 were separated on TLC plates using CEF and sprayed with *S. aureus*. All the fractions showed activity against the tested pathogen (Figure 7.2)

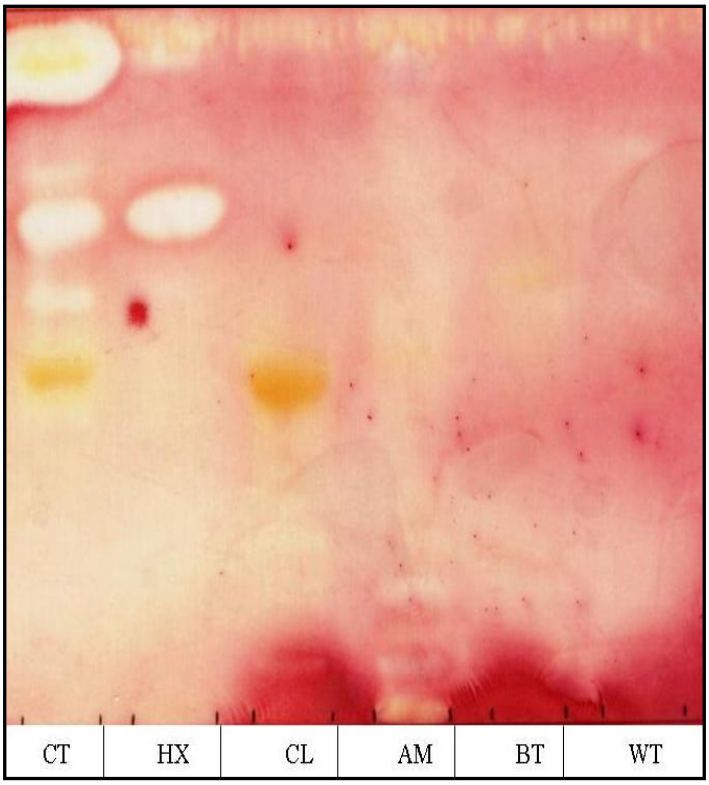

Figure 7.1. Carbon tetrachloride (CT), hexane (HX), chloroform (CL), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *Loxostylis alata* separated on TLC plates using CEF and sprayed with *S. aureus* and 24 h later by INT. White areas indicate inhibition of microbial growth after 60 minutes of incubation at 37 ^{o}C.

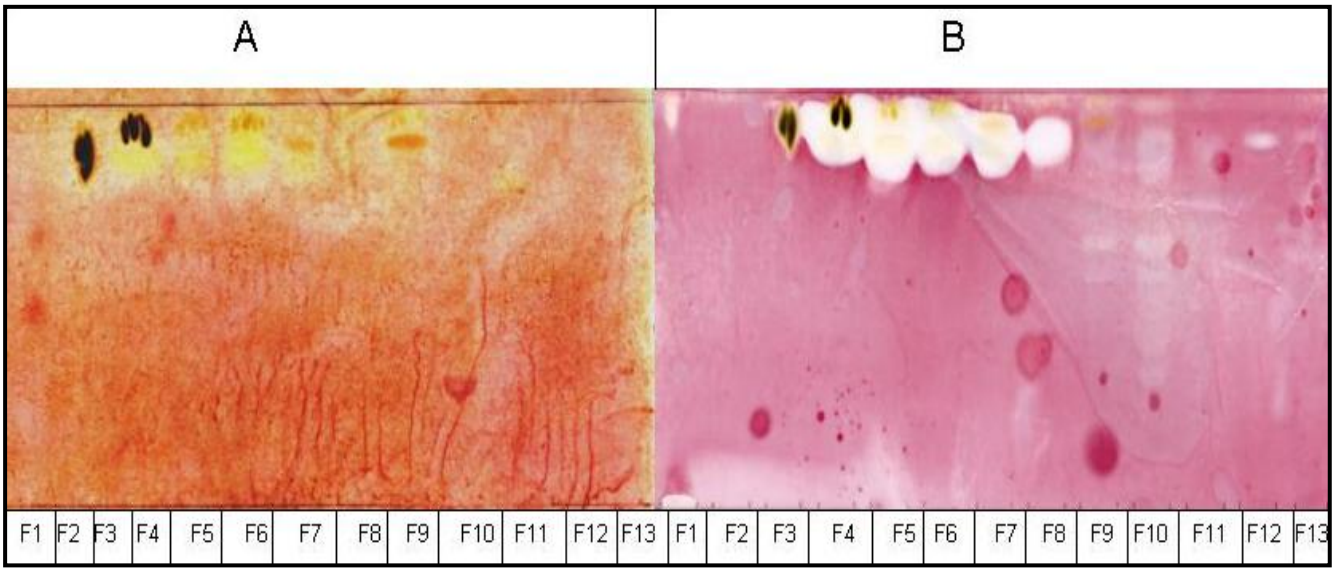

Figure 7.2. F1-F13 fractions of CCl_4 separated on TLC plates using CEF and sprayed with *A. fumigatus* (A) and *S. aureus* (B) and 24 h later by INT. White or yellow areas on the bioautogram indicate inhibition of microbial growth after 60 minutes of incubation at 37 °C.

Table 7.1. Minimal inhibitory concentrations and safety evaluation of various fractions of *Loxostylis alata* against *Aspergillus fumigatus*.

Fraction	Fraction yield (%)	MIC (mg/ml)	Total activity (ml/g)	Cytotoxicity (LC_{50} ± S.E.M in mg/ml)	Selectivity index
Hexane	3.33	0.24	138.69	0.41 ± 0.003	1.71
Carbon tetrachloride	25.61	0.08	3201.79	0.23 ±0.001	2.88
Chloroform	1.86	0.94	19.76	0.67 ±0.002	0.71
Aqueous methanol	3.50	1.88	18.62	NT	NA
Butanol	38.64	2.5	15.46	NT	NA
Water	11.91	2.5	47.66	NT	NA
Amphotericin B	-	0.091	NA	NT	NA
DMSO	-	NT	NA	NDT	NA

NT = not tested; NA= not available; NDT= no detectable toxicity

7.3.2. Chemistry of isolated compounds

Bioactivity guided fractionation of the CCl_4 fraction led to the isolation of two compounds **1** and **2**, which are lupeol and β-sitosterol, respectively (Figure 7.3.). The ^{1}H NMR and ^{13}C NMR spectral data of compound 1 exhibited characteristics spectra features of pentacyclic triterpene. The presence of olefinic protons (4.68 broad signal at H-29a, 4,56 broad signal at H-29b), 3.18 dd, with seven methyl signals are due to lupeol type triterpene. Signals were readily characterised by comparison with signals of lupeol from previous reports. Mass spectrum of the compound with M+426, and prominent signals at 218 and 207 confirmed that the compound is lupeol. (Sholichin et al., 1980; Mahato and Kundu, 1994; Tomosaka et al 2001; Imam et

al., 2007). Analysis and interpretation of the spectroscopic data obtained with previously reported data (Table 7.2) led to the proposed structure for the compound as lupeol (Figure 7.3 (1)) with a molecular formula $C_{30}H_{50}O$. Lupeol, although a compound commonly found in higher plants, is been reported for the first time in *L. alata*.

The characteristic signal of compound 2 is the chemical shift of the 4-6 olefinic signal (5.35) and multiplet at 3.55 due to H-3. This confirmed the isolated compound to be a 24-steroid derivative. Comparison of the carbon spectral data for compound 2 with previously compiled data (Table 7.3) led to the proposed structure of the compound to be β-sitosterol (Figure 7.3 (2)). Mass spectroscopy with molecular ion of 414 and prominent peaks at 396 and 105 served to confirmed the compound to be β-sitosterol with a molecular formula $C_{30}H_{50}O$ (Rubinstein et al., 1976; Chaurasia and Wichtl, 1987; Lopes et al., 1999).

Table 7.2. ^{13}C NMR spectra data for compound 1 (C1).

Carbon	C1	Lupeol (Sholichin et al. 1980)
1	38.7	38.7
2	27.5	27.5
3	79.4	79.0
4	38.9	38.9
5	55.6	55.3
6	18.3	18.3
7	34.3	34.3
8	40.9	40.9
9	50.5	50.5
10	37.2	37.2
11	21.0	21.0
12	25.2	25.2
13	38.4	38.1
14	42.9	42.9
15	27.5	27.5
16	35.1	35.6
17	43.0	43.0
18	48.0	48.0
19	48.3	48.3
20	151	150.9
21	29.9	29.9
22	40.0	40.0
23	28.0	28.0
24	15.3	15.3
25	16.1	16.1
26	16.0	16.0
27	14.6	14.6
28	18.0	18.0
29	19.3	19.3
30	109.3	109.3

Table 7.3. ^{13}C NMR spectra data for compound 2 (C2).

Carbon	C2	β-sitosterol (Shameel et al., 1996)
1	37.2	37.3
2	32.0	31.8
3	72.0	71.9
4	42.3	42.4
5	140.1	140.9
6	122	121.9
7	31.7	32.1
8	31.9	32.1
9	50.1	50.8
10	36.5	36.6
11	21.1	21.1
12	40.0	40.0
13	42.3	42.6
14	56.8	56.8
15	24.3	24.3
16	28.2	28.2
17	56.0	56.2
18	12.0	11.9
19	19.4	19.4
20	36.5	36.2
21	19.1	19.1
22	34.0	34.0
23	29.1	29.3
24	45.9	45.8
25	29.6	29.2
26	19.4	19.8
27	19.1	19.8
28	23.2	23.1
29	11.7	11.9

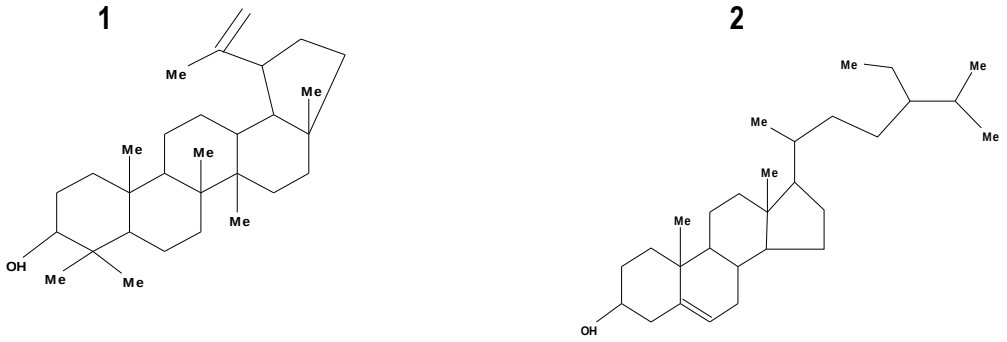

Figure 7.3. Structure of lupeol (**1**) and β-sitosterol (**2**) isolated from *Loxostylis alata* leaves.

7.3.3. Antimicrobial activity of isolated compounds

The two compounds isolated from CCl_4 fraction of *Loxostylis alata* were active against *S. aureus* and *E. coli* with R_f values in hexane: ethyl acetate (7:3) solvent system of 0.47 for lupeol and 0.81 for β-sitosterol. Figure 7.4 represents the biautogram of the 2 isolated compounds. Lupeol showed the most pronounced zone of inhibition against *S. aureus* and *A. fumigatus*. Similarly, when MICs of the 2 compounds were determined, only lupeol had relatively good activity with MIC ≤ 100 µg/ml against 8 out of 10 of the tested pathogens (Table 7.2). However, β-sitosterol had activity against *S. aureus* and *E. coli* with MICs of 90 and 110 µg/ml, respectively. In a similar study, lupeol had low activity against *Candida albicans* with MIC values of more than 250 µg/ml but had high activity against *Sporothrix schenckii* and *Microsporum canis* (MIC values of 12 and 16 µg/ml, respectively); this is in agreement with what Shai et al., 2008, reported. Lupeol and β-sitosterol, previously isolated from the stem bark of *Buchholzia coriacea*, had activity against some species of pathogenic bacteria and fungi (Ajaiyeoba et al., 2003). Similarly, β-sitosterol was found to have good inhibitory activity against the fungi *Aspergillus niger*, *C. cladosporioides*, and *Phytophthora sp*. (Lall et al., 2006). The isolated compounds from *L. alata* leaves are widely distributed in most plants and hence are not novel in character. We observed also that the activity of the compounds against *A. fumigatus* is lower than that produced by the crude extract of *L. alata*. Unless these compounds are destroyed during chemical elution by chromatographic means or bioautography, it strongly appears that synergism plays an important role in the plants activity against *A. fumigatus*. The antifungal compounds involved in synergism appear not to be active on their own.

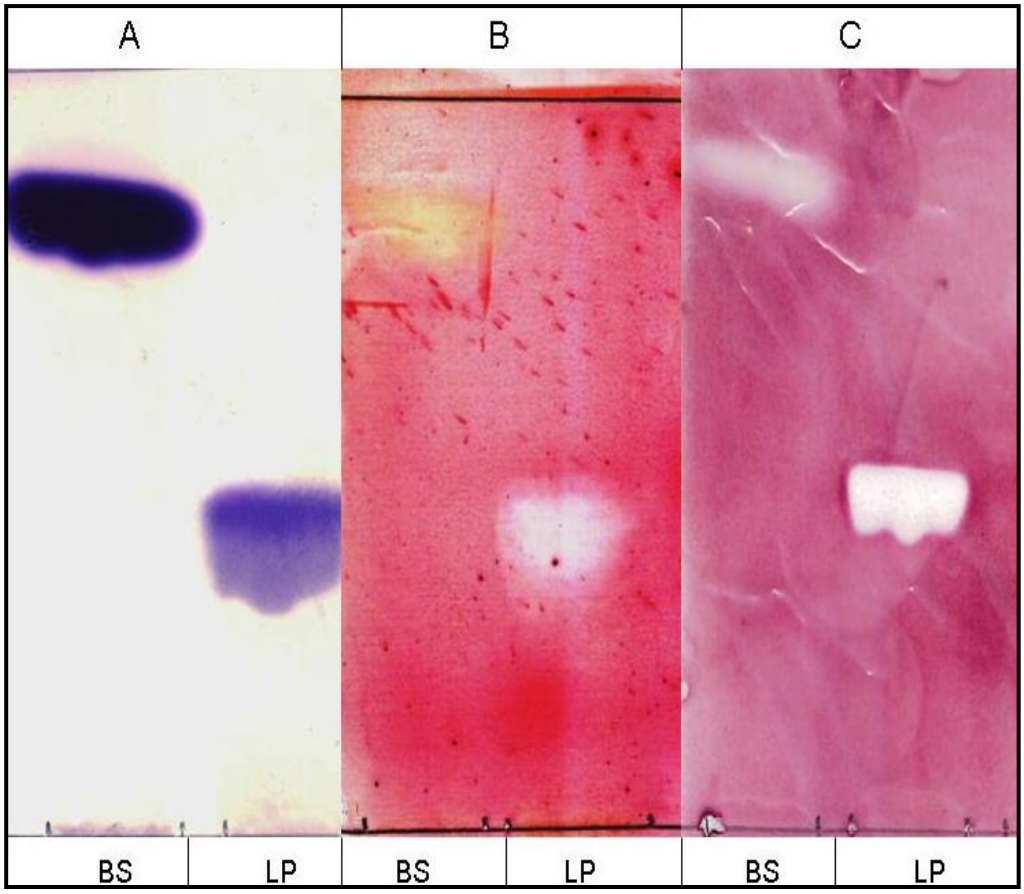

Figure 7.4. Isolated compounds of *Loxostylis alata* spotted on TLC plates and eluted in hexane: ethylacetate (7:3) solvent system. The compounds (spotted left to right) are β-sitosterol (BS) and lupeol (LP). The plates were sprayed with acidified vanillin (A), *A. fumigatus* (B) or *S. aureus* culture (C). White or yellow areas on plate B and C indicate inhibition of microbial growth after 60 minutes of incubation at 37 °C.

Table 7.4. Minimal inhibitory concentrations and safety evaluation of compounds isolated from *Loxostylis alata*.

Compound	MIC values against the tested pathogens (µg/ml)									**Cytotoxicity (LC_{50} ± S.E.M in µg/ml)**
	SA	EF	EC	PA	AF	CA	CN	MC	SS	
Lupeol	29	67	83	150	92	120	47	63	57	76.66 ± 4.1
β-sitosterol	90	>250	110	>250	>250	>250	>250	>250	>250	136.60 ± 7.2
Gentamicin	6.7	4.2	15.2	12.67	NA	NA	NA	NA	NA	NA
Amphotericin B	NA	NA	NA	NA	6.4	0.81	0.32	0.41	0.45	NA
Berberine	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.36 ± 0.8

Staphylococcus aureus (SA), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Aspergillus fumigatus* (AF), *Candida albicans* (CA), *Cryptococcus neoformans* (CN), *Microsporum canis* (MC), *Sporothrix schenckii* (SS)
NA = not available

7.3.4. Anti-inflammatory assay

The crude extract of *Loxostylis alata* and lupeol inhibited COX-1 in a concentration dependent manner (IC_{50} = 92.5 ± 1.6 and 134.0 ± 5.1 μM, respectively). Indomethacin had an IC_{50} of 3.30 ± 0.006 and 122.5 μM against COX-1 and COX-2, respectively. Neither the crude extract nor lupeol or 2 had any inhibitory effect on COX-2. Although lupeol did not have activity against COX-2, it did produce a dose-dependant inhibition of carrageenan induced paw oedema in rats (Agarwal and Rangari, 2003). The *in vivo* action exhibited by lupeol may probably be ascribed to the fact that when administered into the body, it undergoes biotransformation to an active moiety that confers its anti-inflammatory action. In some cases a drug becomes pharmacologically active only after it has been metabolized in the body (Rang et al., 2003). Both COX -1 and -2 regulate the biosynthesis of prostaglandins from arachidonic acid. COX-1 is a constitutive form and has a clear physiological function while COX-2 is mainly induced by inflammatory mediators. It is the inhibition of prostaglandin synthesis by COX-1 and -2 that is responsible for the anti-inflammatory action of NSAIDs (Rang et al., 2003). In addition, COX-1 selective inhibitors have beneficial anti-thrombotic effect. Inhibition of COX-1 by both the crude extract and lupeol may exert beneficial anti-thrombotic effect and protect from heart diseases. Further supporting evidence is that the crude extract inhibited equine platelet aggregation (Suleiman et al., 2009). Moreover, lupeol could form a base for the development of new semi-synthetic drugs for the management of thrombolic disorders.

7.3.5. *In vitro* safety test

The cytotoxicity result of the two compounds is also summarized on Table 7.2. Compounds **1** and **2** were relatively non toxic with LC_{50} of 76.66 ± 4.13 and 136.60 ± 7.20 μg/ml, respectively as compared with the reference compound berberine with LC_{50} of 6.36 ± 0.81 μg/ml. β-sitosterol which occurs as an sterol in many plants is potentially useful in improving human health such as their action as anti-inflammatories, antipyretics, immunomodulators, and antineoplastics effects (Gupta et al. 1980; Bouic 2001).

Results obtained from the mutagenicity test of the 2 compounds using *Salmonella* TA98 and TA100 strains are expressed as mean ± S.E.M (Table 7.3) and are based on number of induced revertant colonies. Substances are considered active if the number of induced revertant colonies is twice the revertant colonies of the negative control (blank) (Maron and Ames., 1983). None of the compounds under investigation were mutagenic in the Salmonella/ microsome tester strains TA98 and TA 100. The lack of cytotoxic or mutagenic effects of these compounds does not guarantee their safe use as traditional medicines. Detailed laboratory and clinical evaluations are needed to justify their use as medicines (Debnath et al., 1991).

Table 7.5. Mutagenic activity expressed as the mean and standard error of mean of the number of revertants/plate in *Salmonella typhimurium* strains TA98 and TA100 exposed to extract and compounds of *Loxostylis alata*, at different concentration.

Treatment	Revertant/plate in *Salmonella typhimurium* strains					
	TA98 (µg/plate ± S.E.M)			TA100 (µg/plate ± S.E.M)		
	2000	200	20	200	20	2
Lupeol	29.30 ± 1.5	29.67 ± 3.6	27.33 ± 1.8	161.33 ± 7.9	167.00 ± 20.1	158.50 ± 8.5
B-sitosterol	22.30 ± 2.2	28.00 ± 2.0	26.67 ± 2.7	179.00 ± 5.9	170.70 ± 1.1	163.30 ± 1.1
Crude extract	NT	NT	NT	NT	NT	NT

Negative control for the *Salmonella typhimurium* test is DMSO (100 µl/plate; TA98: 19.30 ± 2.89; TA100: 152.60 ± 7.07), while the positive control is 4-nitroquinoline 1-oxide (4-NQO) (10 µg/plate; TA98: 170.33 ± 14.14; TA100: 960 ± 24.89). All values quoted are mean ± S.E.M

7.4. Conclusion

The extract of *Loxostylis alata* had varying degrees of antimicrobial and cyclooxygenase inhibitory activities. However, detailed toxicity studies using sophisticated means and also clinical trial in both laboratory and target animal species are required to justify their use in clinical practice. Moreover, there is also need for proper investigation into the mechanism underlying the antimicrobial actions of these compounds from *Loxostylis alata*.

Postscript

Since the extract of *Loxostylis alata* had very promising results in our earlier investigations, we decided to test its activity *in vivo* using target animal species with a view of finding out if what obtained *in vitro* will translate to in vivo activity. It is also a step towards establishing clinical usage of the plant extract.

Chapter 8
A leaf extract of *Loxostylis alata* (Anacardiaceae) prevents experimental aspergillosis in chicks

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Preface

It was disappointing that the two antifungal compounds isolated from *L. alata* had such a low activity against *Aspergillus fumigatus*. This inhibits the development of a single compound that can be used therapeutically. Because the crude extract had very good activity we decided to investigate the safety and potential use of this extract in an animal experiment.

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Abstract

A crude acetone extract of *Loxostylis alata* leaves was evaluated for toxicity and for antifungal activity in experimental aspergillosis of broiler chicks based on the excellent *in vitro* activity against this pathogen. At a dose of 300 mg/kg, the extract had some toxicity. Consequently, lower concentrations were used. Chicks were infected intraperitoneally with *A. fumigatus*. Antifungal activity was assessed by comparing the degree and severity of clinical signs, lesion scores, fungal re-isolation and a series of biochemical and haematological indices observed between from chicks treated and not treated with the extract. The extract at the dose of 100 and 200 mg/kg reduced the lesions due to aspergillosis and the amount of *Aspergillus* organism isolated from infected chicks significantly ($p \leq 0.05$) in a dose dependent fashion. The extract was as active as the positive control ketoconazole at a dosage of 60 mg/kg the highest allowable dose to prevent toxicity. The results indicate that a crude extract of *L. alata* leaves has potential as an antifungal agent to protect poultry against avian aspergillosis.

Keywords: *Loxostylis alata*; Aspergillosis; Broiler chicks; Safety evaluation; Lung tissues.

8.1. Introduction

The poultry industry which is an important component in world agricultural economy faces heavy economic losses due to many health hazards caused by the fungus *Aspergillus fumigatus*. Losses caused low productivity, mortality and carcass condemnations at slaughter (Morris and Fletcher, 1988; Richard, 1997; Kunkle, 2003). About US$11 million is the reported as average yearly lost due to aspergillosis alone in the USA (Kunkle, 2003). The disease affects mainly the respiratory tract of birds and has a worldwide distribution, having been reported in almost every farmed bird as well as in wild species (Akan et al., 2002; Chang Reissig et al., 2002).

As a disease, aspergillosis affects birds whether in captive or free-ranging environments, young and mature, and whether immunocompetent or immunosuppressed. However, young birds appear to be much more susceptible than adults. The lower respiratory tract is where *Aspergillus* spp. tends to initially colonize (Tell, 2005) but blood infection with subsequent dissemination to other organs frequently occurs, leading to macroscopic lesions in a wide range of organs or tissues. In spontaneous cases, lesions range from miliary to larger granulomatous foci (Singh et al., 1994). These white lesions are protrusive to the surface of the internal organ. Thickening of the walls of the air sacs frequently occurs (Perelman and Kuttin, 1992). Lesions in avian species are commonly confined to the lungs and air sacs, although infections also occurs in oral mucosa, trachea, brain, eye, skin, bone, liver, kidney (Richard, 1997), and nasal passages (Fitzgerald and Moisan 1995) have also been described. Typical lesions are characterized by granulomatous inflammation with necrosis, haemorrhage, and intralesional fungal elements that are locally invasive. The pathogenesis of aspergillosis appears to be complicated. In recent years, aspergillosis has also emerged as a significant disease in humans that are immunocompromised by HIV-AIDS, neoplasia, or chemotherapy (Denning et al., 1991).

Although there are commercial drugs available for the treatment of systemic and superficial mycoses (Uno et al., 1982; Lyman and Walsh, 1992; Orosz, 2000), none of them are ideal in terms of efficacy, safety and antifungal spectrum (Di Domenico 1998; Ablordeppey et al, 1999). There is therefore a need to explore new remedies to treat the disease.

In a preliminary study, the extracts of *Loxostylis alata* A. Spreng, ex Rchb. (common name tarwood) had good *in vitro* activity against *Aspergillus fumigatus* and other pathogenic animal fungi with minimum inhibitory concentration (MIC) as low as 0.07 mg/ml. Moreover, bioautography of *L. alata* extracts with *A. fumigatus* revealed good antifungal activity (results not shown). This study aims at evaluating the *in vivo* effect of *L. alata* leave extract against experimental aspergillosis in poultry.

8.2. Materials and methods

8.2.1. Plant collection, extraction and processing

Leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. Samples of the plant were identified and authenticated by Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimen of the plant with number; PRU96508 was deposited at the Schweicker Herbarium, University of Pretoria, South Africa. After collection and transportation to the laboratory, leaves were separated from stems and dried at room temperature with good ventilation. The dried leaves were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eriez®, Bramley) and stored at room temperature in closed containers in the dark until used. Five hundred grams of finely ground plant material was extracted with 5 litres of acetone. The extraction process was repeated three times to exhaustively extract the same plant material, and the extracts were combined. The solvent was removed *in vacuo* to yield 75 grams of dark greenish solid. The extract was dissolved in 0.2% aqueous dimethylsulphoxide (DMSO) at a concentration of 200 mg/ml.

8.2.2. Animals and management

Three day old broiler chicks were used to evaluate the safety of the extract in a toxicity trial (n=3) and its efficacy (n=8) on birds experimentally infected with *Aspergillus fumigatus*. The birds were purchased from a healthy breeding flock at Eagles Pride Hatchery, Pretoria. At the start of the study, the weight variation of the birds did not exceed 20% of the mean species average weight (National Institute of Environmental Health Sciences, 2001). Throughout the experiment, broiler chicks were kept at the Poultry Reference Centre, Faculty of Veterinary Science, University of Pretoria in an enclosed temperature-controlled house with adequate ventilation, an artificial light at the recommended light intervals source. Clean wood shaving was used as bedding on wood shavings (North, 1981). Feed intake and weight gain for each group were determined every other day. All experimental protocols described in this study were approved by the Animal Use and Care Committee of the University of Pretoria, South Africa (V036/08) in accordance with the international guidelines for use of animals in experimentation.

8.2.3. Safety evaluation of the extract

A modified version of the guidelines for Organisation for Economic Cooperation and Development (OECD) for determining the toxic nature of the chemical (Annex 2b; starting dose of 50 mg/kg) was used to determine the dose of the extract that will produce toxic signs and possibly death in treated birds. Toxic signs exhibited such as ruffled feathers, diarrhoea, depression, off-feed, etc rather than death was used as an end point in determining the safety of the extract. Chicks were randomly assigned into 4 groups of 3

birds each. The first group received the extract at the dose of 300 mg/kg recommended by OECD, while the second group was dosed with 0.2% aqueous dimethylsulphoxide at the dose of 0.2 ml/100 g body weight in water and served as control. All birds were monitored for 12 days. Due to the toxic effect of the starting dose (300 mg/kg), lower doses of 50 and 200 mg/kg of the extract were administered to groups 3 and 4, respectively. These chicks were also examined for signs of toxicity. The dose that did not produce any toxic sign was used as the maximum tolerated dose (MTD) for the chemotherapeutic trial. All treatments were given intraperitoneally with a 23½ G needle attached to a 1ml syringe (TERUMO Medical Corporation, Elkton, MD 21921, USA).

8.2.4. Experimental inoculum

The *Aspergillus fumigatus* used was isolated from an infected chick airsac on a broiler farm in Gauteng, South Africa by Dr J. Picard and maintained on Sabouroud dextrose agar 6.5% supplemented with 50 mg/ml gentamicin at the Department of Veterinary Tropical Diseases, University of Pretoria. Asexual spores (conidia) were obtained from 3 day-old culture by flooding the plates with sterile distilled water, pelleted by centrifugation at 3500 x g for 10 min washed in phosphate-buffered saline (0.15 M) and quantified by determining turbidity with a spectrophotometer (CECIL CE 1011, 1000 series) at a wavelength of 540 nm (Delap et al., 1989).

8.2.5. Chemotherapeutic trial

Five chicks were randomly selected from the control group and sacrificed at the beginning of the experiment, their lungs were aseptically removed and contamination by *A. fumigatus* was evaluated by placing lung sections onto Sabouraud dextrose agar. The plates were incubated at 37 °C and the presence of *A. fumigatus* colonies was checked every day for 1 week (Femenia et al., 2007). The chicks (n=8) except the neutral group (n=10) were inoculated by transcutaneous injection into the right caudal thoracic air sac with 100 µl spore suspension of a 3-day-old *A. fumigatus* culture containing 10^8 spores. Birds were observed at least twice a day for the appearance of clinical signs of aspergillosis (Femenia et al., 2007). Clinical sign of infection (dyspnoea) was evident 3 days post infection. Group 1 served as neutral control and was neither infected nor treated with any substance. Birds in groups 2, 3, and 4 were treated with the extract at 50, 100 and 200 mg/kg, respectively; while groups 5 and 6 were dosed with ketoconazole (60 mg/kg) the positive control and 0.2% DMSO in water (0.2 ml/100 g) the vehicle respectively. All birds were marked or tagged for identification and were fed non-medicated feed, and clean water was provided freely. All treatments were instituted 3 days post infection and were given intraperitoneally once daily for 3 consecutive days.

8.2.6. Biochemical and haematological analysis

At the end of toxicity and during chemotherapeutic trials, blood samples from all birds were collected from the wing vein or by jugular venipuncture in vacutainer tubes with or without heparin as anticoagulant. Blood samples were centrifuged at 1200 *x g* for 15 minutes in a refrigerated centrifuge (4 °C) to separate serum. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) were measured using Alfa Wassermann. Total protein, albumin, calcium and phosphorous concentrations were measured using NExCT™ Total Protein reagent, NExCT™ Albumin reagent, ACE™ Calcium-Arsenazo reagent and Alfa Wassermann Inorganic Phosphorus reagent, respectively. Serum globulin levels were deduced by subtracting the albumin levels from the total protein levels (Gildersleeve et al., 1983), and the albumin/globulin (A/G) ratio calculated. The analyses were performed using the ACE TM and NExT TM Clinical Chemistry Systems (ALFA Wassermann, Bayer Health Care, and Johannesburg). Heparinized blood samples were analysed for haemoglobin (Hb), red cell count (RCC), haematocrit (HT) , mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW) using ADVIA 2120 haematology analyser (ADVIA, , Bayer Health Care, and Johannesburg). White cell count (WCC), absolute neutrophil (total), absolute neutrophil (matured), absolute neutrophil (immatured), absolute lymphocyte, absolute monocyte, absolute eosinophil, absolute basophil analyses were done using manual counter.

8.2.7. Mycological and pathologic analyses

Birds that died during the toxicity trial were examined by examining the lungs, airsacs, liver, heart, kidneys, spleen and brain observed for gross lesions. The organs were later fixed in 10% formaldehyde. Sections of the organs were embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin-eosin stain for histopathological examination (Femenia et al., 2007). Birds that survived were euthanized in a CO_2 chamber and similarly examined.

The lungs, air sacs and liver of birds that died during the chemotherapeutic trial were specifically evaluated for aspergillosis related lesions. The lungs were aseptically removed and individually plated on SDA using sterile swabs. Plates were incubated for 48 hours at 37 °C. When fungal colonies developed, species identification was done by microscopic examination of conidiophores and conidia, in addition to the observation of colony morphology. *A. fumigatus* is characterized by green echinulate conidia, 2 to 3 mm in diameter, produced in chains from greenish phialids, 6 to 8 mm by 2 to 3 mm in size (de Hoog et al ., 2000). A portion of the lungs was also fixed for histopathology as for the toxicity study. The lungs were,

however, specifically stained with Grocott methenamine (hexamine) silver for further examination of *Aspergillus fumigatus* hyphae in the tissue section (Bancroft and Gamble, 2003).

8.2.8. Evaluation of healing effect

At the end of the chemotherapeutic trial, the organism was recovered from infected chicks was and the severity of infection was graded. Microbiological severity of infection evaluated by adding up the total area covered by individual colony of *Aspergillus fumigatus* on the SDA plate and was graded as follows: no growth: grade 0; colonies < 2 mm^2: grade 1; colonies 2 -10 mm^2: grade 2; colonies 10-20 mm^2: grade 3, colonies > 20 mm^2: grade 4. Lesion scores were based on gross examination. The grading system proposed by Delap et al. (1989) used. Briefly, the grading is done as follows: grade 0: negative (no lesion), grade 1: localized plaque, grade 2: discrete plaque-moderate, grade 3: discrete plaque that is extensive, grade 4: confluent plaques.

8.2.9. Statistical analysis

Data obtained from the re-isolation steps of *A. fumigatus* and the sum of area of growth in infected and treated groups were expressed as mean ± S.E.M. Difference between the groups was analyzed using one way analysis of variance (ANOVA). Results were considered significant if $p \leq 0.05$. A post-hoc Dunnett test was used to test for differences to the control for which ANOVA indicated a significant ($p \leq 0.05$) F-ratio.

8.3. Results

8.3.1. Safety evaluation of the extract

Chicks treated with 50 and 200 mg/kg of the extract did not exhibit any clinical sign of toxicity and remained apparently healthy throughout the experimental period. However, at the dose of 300 mg/kg (starting dose), depression, decrease feed intake, diarrhoea, marked weight loss were noticed. One chick died 4 days post treatment (p.t.). In addition, there was marked increased in weight in the DMSO (control) group compared with chicks treated with 50 and 200 mg/kg of the extract. The effect of the extract on weight changes of the birds is presented in Figure 8.1. The extract caused decreased weight gain when administered at 300 mg/kg. Results pertinent for the serum biochemical profile and haematological analysis conducted at the end of the toxicity trial are presented in Table 8.1 and 8.2, respectively. Only the globulin concentration of chicks treated with 300 mg/kg of the extract differ significantly ($p \leq 0.05$) from other groups. Haematologically, the white cell count (WCC), absolute neutrophil (total and immatured) and absolute lymphocyte of chicks treated with the extract at 200 mg/kg differed significantly ($p \leq 0.05$).

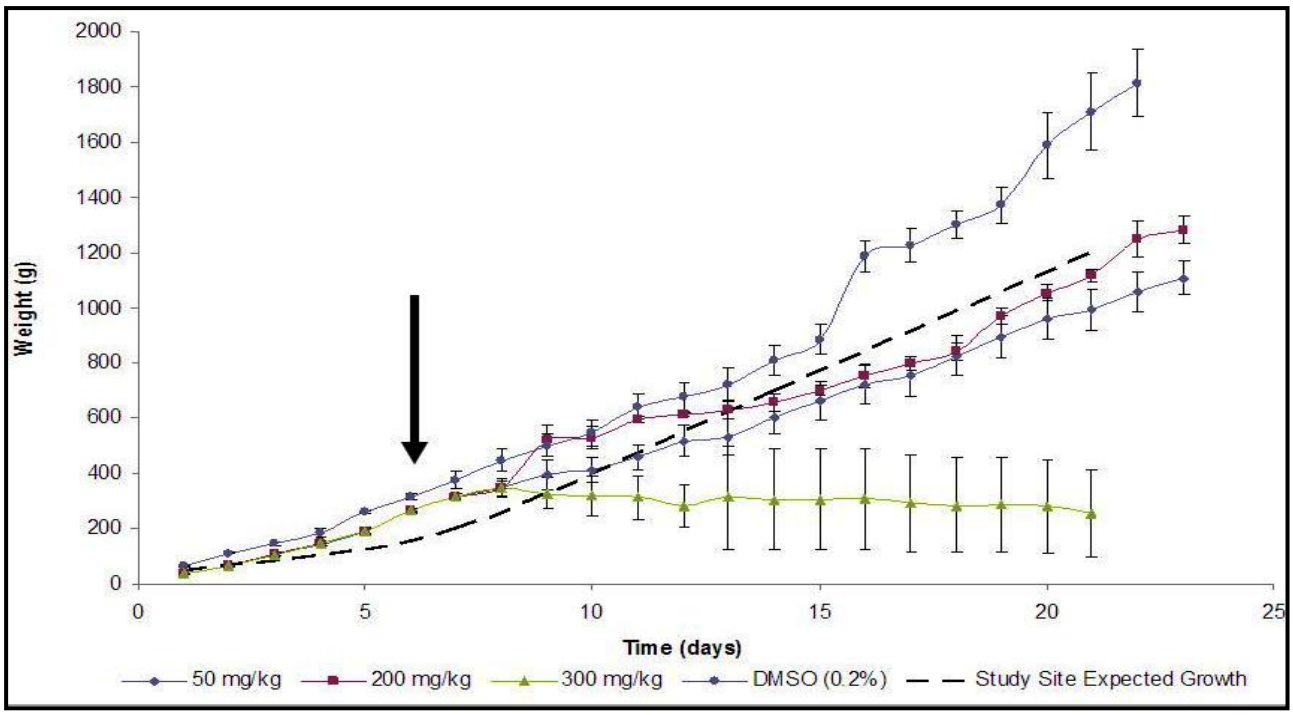


Figure 8.1. The effect of crude extract of *L. alata* on weight of broiler chicks. Arrow indicates time of extract administration.

Table 8.1. Biochemical indices of broiler-chicks given varying doses of crude extract of *Loxostylis alata* in the toxicity study.

Serum biochemistry	Dose administered (mg/kg)			
	Control (DMSO)	50	200	300*
Total serum protein (TSP)	28.53 ± 1.39	27. 13 ± 2.20	34. 43 ± 2.89	27.90 ± 0.35
Albumin (Alb)	16. 67 ± 0.58	15.03 ± 0.61	15.50 ± 1.53	15.50 ± 0.55
Globulin (Glob)	11.87 ± 0.83	12.10 ± 1.59	15.93 ± 3.88	6.70 ± 0.67[a]
Albumin/globulin ratio (A/G)	1.41 ± 0. 05	1.23 ± 0.12	0.96 ± 0.03	2.34 ± 0.01[a]
Calcium (Ca)	2.89 ± 0.03	2.58 ± 0.02	2.62 ± 0.04	2.60 ± 0.13
Serum inorganic phosphate (SIP)	2.45 ± 0.05	2.16 ± 0.07	1.87 ± 0.04	2.00 ± 0.12
Alanine amino transferase (ALT)	2.00 ± 0.82	1.33 ± 1.33	0.00 ± 0.00	2.00 ± 0.00
Aspartate amino transferase(AST)	176.67 ± 4.77	160.67 ± 13.61	148.00 ± 9.88	169.5 ± 9.53
γ-glutamyltransferase (GGT)	18.00 ± 0.47	23.00 ± 4.51	29.00 ± 1.53	23.00 ± 2.01

Means with superscript letter differ significantly ($p \leq 0.05$) from the control.
* Results were from 2 chicks, one of the chicks died 4-days p.t.

Table 8.2. Haematological indices of broiler-chicks given varying doses of crude extract of *Loxostylis alata* in the toxicity study.

Haematology	Dose administered (mg/kg)			
	Control (DMSO ml/kg)	50	200	300*
Haemoglobin (Hb)	143.33 ± 4.7	139.33 ± 0.88	144.67 ± 8.66	135.50 ± 1.50
Red cell count (RCC)	2.62 ± 0.12	2.50 ± 0.11	2.83 ± 0.09	2.40 ± 0.02
HT (haematocrit)	0.36 ± 0.01	0.34 ± 0.01	0.33 ± 0.03	0.30 ± 0.01
Mean cell volume (MCV)	140.00 ± 2.95	135.67 ± 1.77	126.33 ± 6.90	135.00 ± 1.00
Mean cell haemoglobin (MCH)	54.77 ± 0.84	54.17 ± 0.54	51.40 ± 3.22	55.10 ± 0.35
Mean cell haemoglobin concentration (MCHC)	39.27 ± 0.23	39.23 ± 0.84	40.87 ± 0.63	40.90 ± 0.05
Red cell distribution width (RDW)	13.47 ± 0.923	12.47 ± 0.67	14.77 ± 1.76	13.70 ± 1.15
White cell count (WCC)	3.73 ± 0.95	5.33 ± 1.09	19.80 ± 8.06[a]	4.40 ± 0.80
Absolute neutrophil (total)	1.31 ± 0.26	1.94 ± 0.47	10.31 ± 4.77[a]	2.30 ± 0.45
Absolute neutrophil (matured)	1.31 ± 0.26	1.94 ± 0.47	10.31 ± 4.77[a]	2.30 ± 0.45
Absolute neutrophil (immatured)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Absolute lymphocyte	1.99 ± 0.50	2.56 ± 0.49	6.25 ± 1.66[a]	1.50 ± 0.34
Absolute monocyte	0.35 ± 0.15	0.34 ± 0.05	1.83 ± 1.40	0.40 ± 0.16
Absolute eosinophil	0.00 ± 0.00	0.27 ± 0.11	0.88 ± 0.03	0.1 ± 0.03
Absolute basophil	0.09 ± 0.06	0.07 ± 0.04	0.42 ± 0.02	0.00 ± 0.00

Means with superscript letter differ significantly ($p \leq 0.05$) from the control.
Results were from 2 chicks, one of the chicks died 4-days p.t

Gross post-mortem results showed moderate ascites, hydropericardium, lung oedema and, soft and pliable kidneys in the chick that died from group treated with 300 mg/kg. The remaining two chicks that were sacrificed at the end of the experiment from the same group showed similar pathological signs. Birds treated with 50 and 200 mg/kg of the extract showed no gross pathological signs. Birds treated with 300 mg/kg had histological lesions i.e. scattered bile duct proliferation, focal periductular fibrosis and lymphocytes accumulation in the liver tissues (Figure 8.2). The heart and lungs had lympoplasmacytic pericarditis and moderate fibrinopurulent bronchitis (Figures 8.3 and 8.4), respectively. No changes were apparent in the organs of birds treated with 50 and 200 mg/kg of the extract.

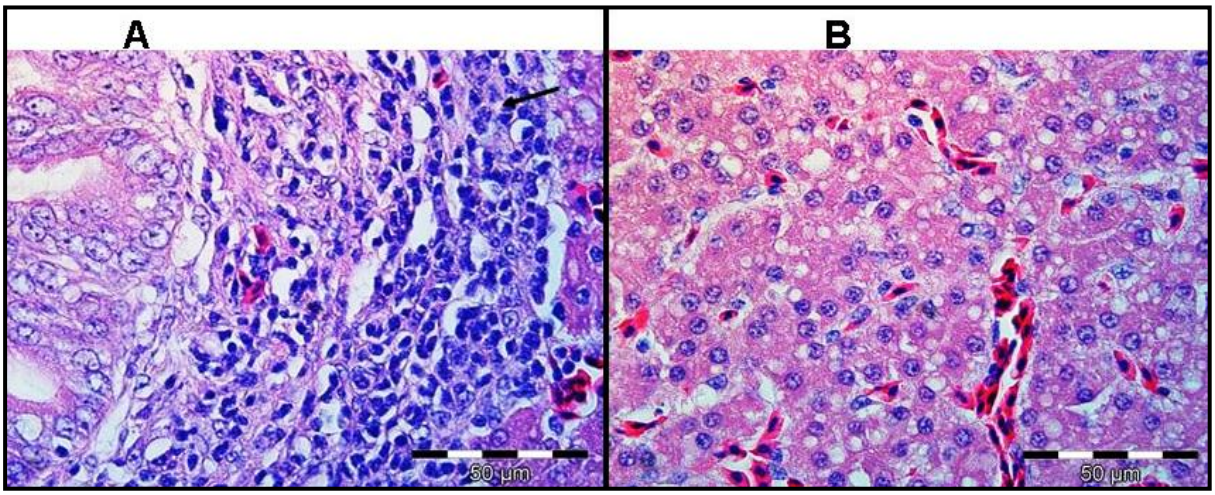


Figure 8.2. Liver tissue of birds treated with 300 mg/kg of extract (A) showing scattered bile duct proliferation, focal periductular fibrosis and lymphocytes accumulation (arrow). Normal liver tissue (B) is shown. H&E

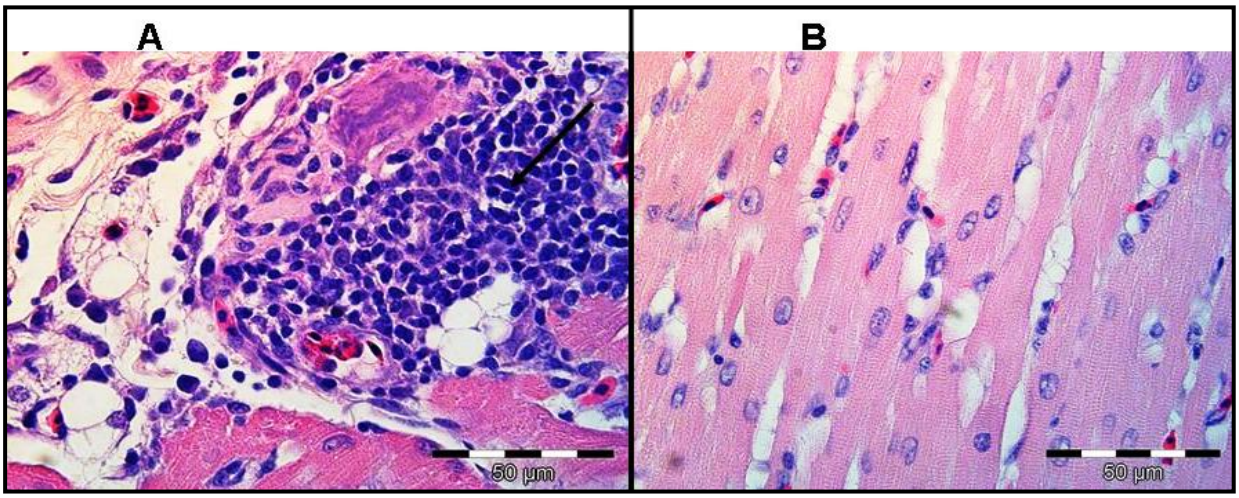


Figure 8.3. The heart tissue of birds treated with 300 mg/kg of extract (A) showing lympoplasmacytic pericarditis (arrow). Normal heart tissue (B) is shown. H&E

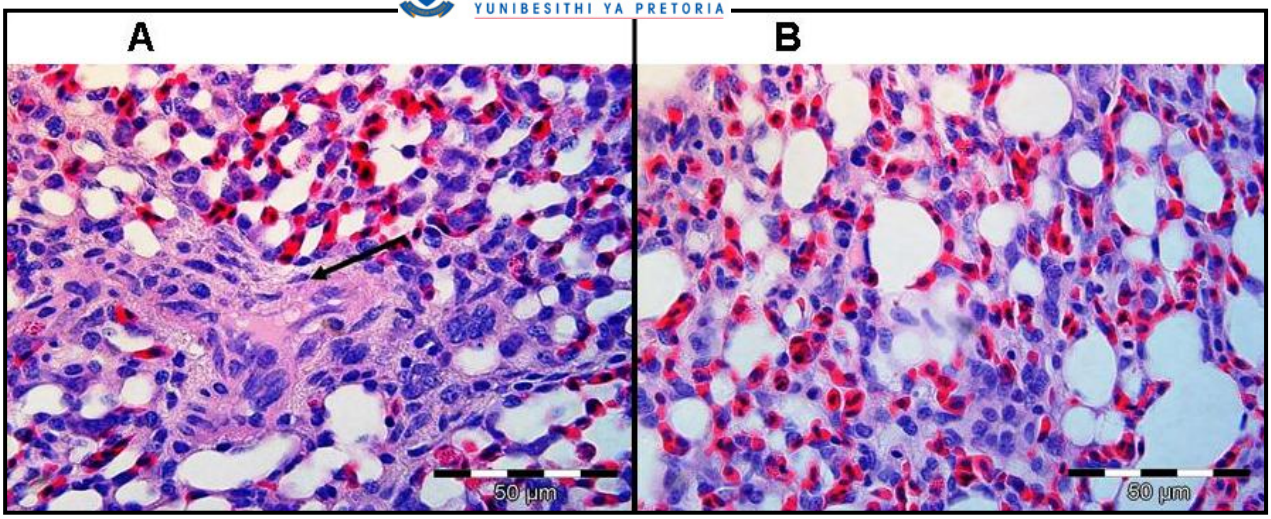


Figure 8.4. The lungs tissue of birds treated with 300 mg/kg of extract (A) showing moderate fibrinopurulent bronchitis (arrow). Normal lung tissue (B) is shown. H&E

8.3.2. Chemotherapeutic trial

8.3.2.1. Clinical signs and survivability

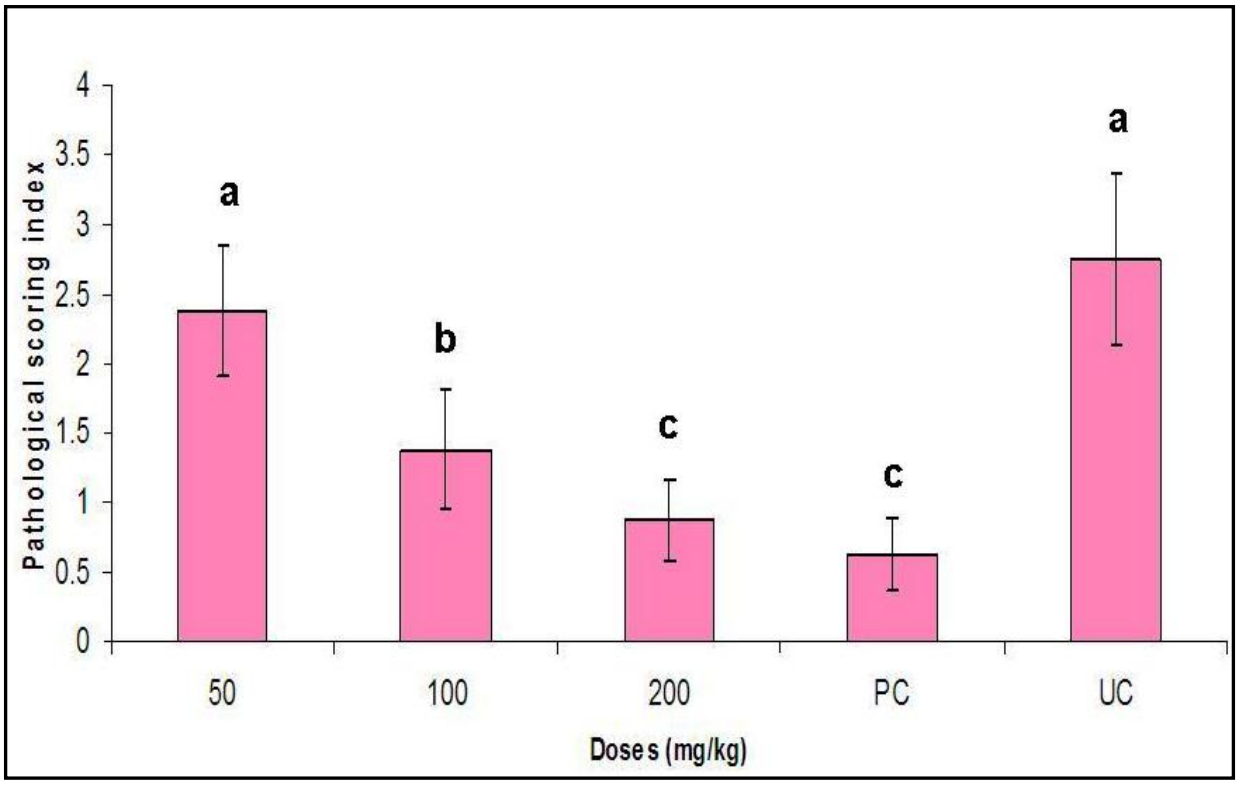
Chicks in the infected groups had clinical signs 3 days post infection (p.i), when one chick (out of eight) in the non-treated control group exhibited mild respiratory distress, ruffled feathers, weakness and diarrhoea. The birds in the non-infected and non-treated (negative) group remained apparently normal throughout the experimental period. The effect of the extract on survival of chicks infected with *Aspergillus fumigatus* is presented on Table 8.3. The extract produced a dose-dependant protection against death in chicks infected with *Aspergillus fumigatus*.

Table 8.3. The effect of the extract dose on survival of chicks infected with *Aspergillus fumigatus*.

Dose (mg/kg)	No. of chicks	No. survived	No. death	% Mortality
0	8	2	6	75
50	8	3	5	62.5
100	8	3	5	62.5
200	8	6	2	25
Ketaconazole (60 mg/ml)	8	7	1	12.5
Neutral	10	8	0	0

8.3.2.2. Macroscopic lesions

Gross lesions were detected at necropsy in all the infected-treated birds. Lesions consisted of small (1 to 3 mm) white nodules on the surface of the lungs and liver. Cloudiness of the thoracic air sacs was also noticed. However, the severity of the lesions was lower in chicks treated with the extract and also in the positive control group (ketaconazole treated chicks). The extract, therefore reduced the severity of the lesions in a dose-dependent fashion (Figure 8.5).

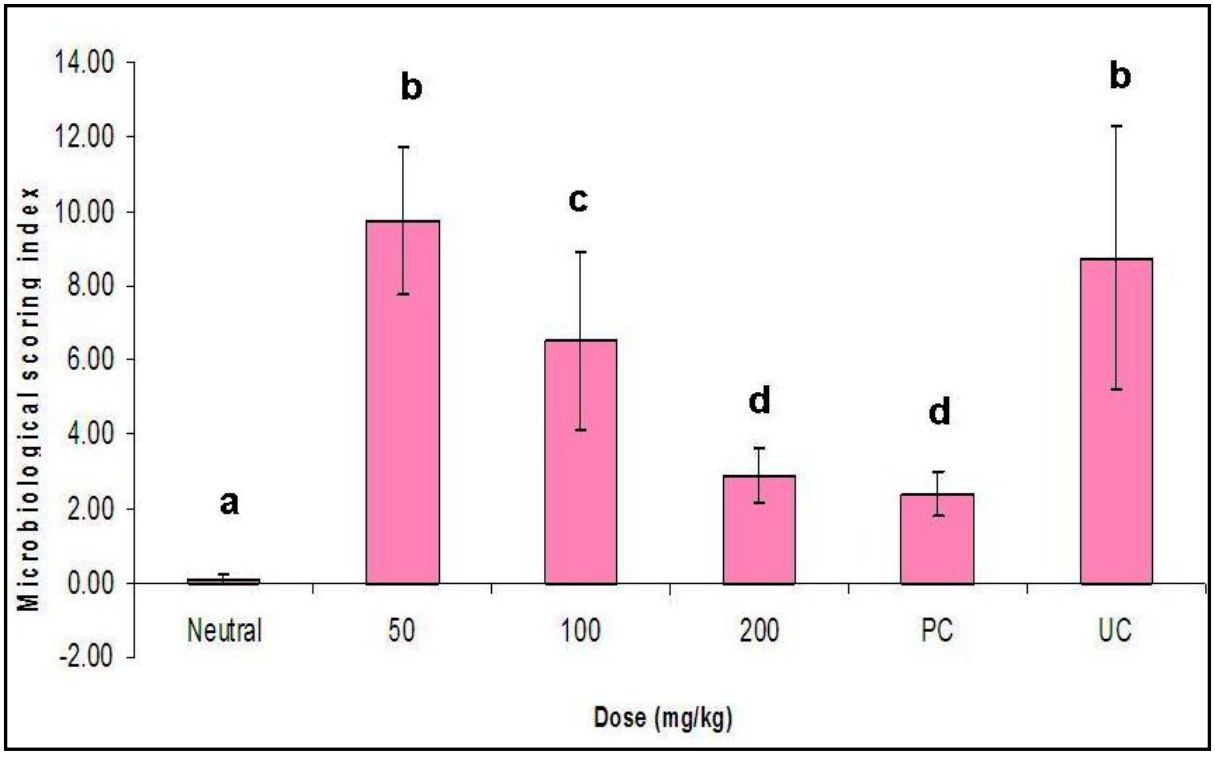


PC = positive control (ketoconazole); UC = untreated control (DMSO)

Figure 8.5. Effect of different doses of *L. alata* extract on pathological lesions in the lungs, airsac and liver of broiler chicks caused by *A. fumigatus*. Values are mean ± SEM. Means with different letter alphabet differ significantly with control and the other treatment groups ($p \leq 0.05$). No pathological lesion was recorded in the uninfected and untreated control chicks.

8.3.2.3. Mycological cultures

No *Aspergillus fumigatus* was isolated from the 5 chicks sacrificed at the start of chemotherapeutic trial. In the infected and treated groups varying amounts of *A. fumigatus* colonies were isolated from the airsac, lungs and liver tissue from days 3 to 7 days p.i. In addition, *A. fumigatus* was also isolated from the lung sample of one of the chicks in the neutral control group (Figure 8.6) indicating a subsequent contamination.



PC = positive control (ketaconazole); UC = untreated control (DMSO)

Figure 8.6. Re-isolation of *A. fumigatus* from the lung, airsac and liver of infected broiler chicks treated with different doses of *L. alata*. Values are mean ± SEM. Means with different letter alphabet differ significantly ($p \leq 0.05$) for the control and the other treatment groups.

8.3.2.4. Special histological evaluation of lungs

Sections of lungs from infected chicks showed the presence of fungal hyphae when special staining method was used. However, no fungal hyphae were noted in the lung samples of chicks in the neutral control group. Figure 8.7 showed the presence of small hyphae of *A. fumigatus* radiating in a pulmonary lobule.

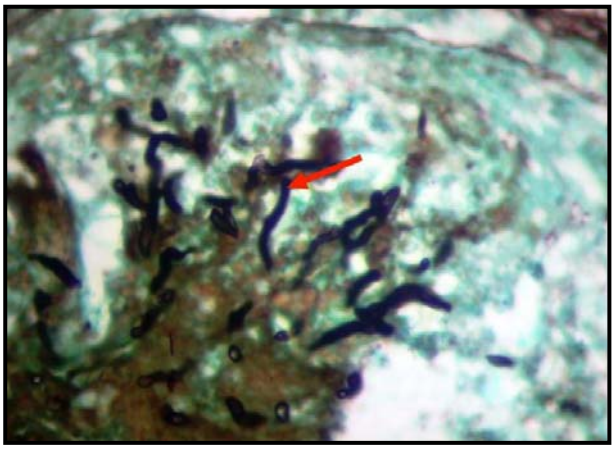


Figure 8.7. *Aspergillus fumigatus* hyphae (arrow) radiating in a pulmonary lobule of one of the infected chicks (X1000).

8.3.2.5. Biochemical and haematological profile

Results for the serum and blood analysis of chicks infected with *A. fumigatus* are presented in Tables 8.4 and 8.5, respectively. There was a significant ($p \leq 0.05$) increase in the concentration of aspartate amino transferase and globulin in chicks that received DMSO. Likewise, serum gamma glutamyl transferase (GGT) were markedly ($p \leq 0.05$) increased in chicks treated with ketaconazole and DMSO. Haematologically, the white cell count (WCC), absolute neutrophil (total and matured) and absolute monocytes in chicks treated with the extract at 200 mg/kg and ketaconazole do not differ significantly ($p \leq 0.05$), however, they increase markedly ($p \leq 0.05$) in untreated control groups.

Table 8.4. Biochemical indices of broiler-chicks infected with *A. fumigatus* and treated varying doses of crude extract of *Loxostylis alata*.

Serum biochemistry	Dose administered (mg/kg)					
	Neutral	50	100	200	PC (60)	UC
Total serum protein (TSP)	25.52 ± 0.48	26.67 ± 0.90	28.98 ± 0.74	30.07 ± 2.26	30.90 ± 2.03	36.05 ± 9.15
Albumin (Alb)	14.33 ± 0.26	14.43 ± 0.12	15.98 ± 0.62	15.33 ± 0.39	15.90 ± 0.17	15.60 ± 0.00
Globulin (Glob)	11.19 ± 0.30	12.23 ± 0.87	13.00 ± 0.35	14.74 ± 2.34	15.00 ± 1.93	24.50 ± 9.20[a]
Albumin/globulin ratio (A/G)	1.29 ± 0.03	1.19 ± 0.09	1.23 ± 0.06	1.13 ± 0.10	1.15 ± 0.12	0.61 ± 0.43[a]
Calcium (Ca)	3.29 ± 0.10	3.42 ± 0.10	3.53 ± 0.14	3.37 ± 0.16	3.40 ± 0.10	2.92 ± 0.07
Serum inorganic phosphate (SIP)	1.43 ± 0.08	1.59 ± 0.10	1.60 ± 0.10	1.66 ± 0.10	1.65 ± 0.11	2.38 ± 0.96
Alanine amino transferase (ALT)	3.00 ± 0.61	3.33 ± 1.67	2.80 ± 0.97	4.86 ± 2.58	1.71 ± 0.57	1.50 ± 1.50
Aspartate amino transferase(AST)	149.20 ± 4.08	153.00 ± 8.54	138.60 ± 7.26	155.00 ± 6.24	129.43 ± 9.86	171.50 ± 22.20[a]
γ-glutamyltransferase (GGT)	19.60 ± 0.50	22.67 ± 0.88	23.00 ± 2.45	24.14 ± 4.12	26.57 ± 3.09	36.50 ± 17.50[a]

Neutral group are chicks that were not infected with *A. fumigatus*, and were also not treated. Positive control (PC) (ketoconazole); Untreated control (UC).
Means with superscript letter differ significantly ($p \leq 0.05$) from the control.

Table 8.5. Haematological indices of broiler-chicks infected with *A. fumigatus* and treated varying doses of crude extract of *Loxostylis alata*.

Serum haematology	Dose administered (mg/kg)					
	Neutral	50	100	200	PC (60)	UC
Haemoglobin (Hb)	131.60 ± 1.93	134.33 ± 4.10	140.00 ± 2.92	133.14 ± 2.38	141.71 ± 5.97	138.00 ± 1.00
Red cell count (RCC)	2.43 ± 0.40	2.48 ± 0.11	2.60 ± 0.06	2.56 ± 09	2.70 ± 0.18	2.77 ± 0.10
HT (haematocrit)	0.34 ± 0.10	0.34 ± 01	0.35 ± 0.01	0.34 ± 01	0.36 ± 02	0.35 ± 0.01
Mean cell volume (MCV)	138.10 ± 1.06	139.00 ± 1.15	136.40 ± 3.75	134.43 ± 2.10	134.71 ± 3.31	125.50 ± 6.50
Mean cell haemoglobin (MCH)	54.14 ± 0.45	54.33 ± 0.94	53.80 ±0.86	52.29 ± 1.06	52.93 ± 1.40	50.00 ± 2.30
Mean cell haemoglobin concentration (MCHC)	39.17 ± 0.28	39.10 ± 0.53	39.52 ± 0.52	38.90 ± 0.35	39.33 ± 0.29	39.95 ± 0.25
(Red cell distribution width) RDW	12.83 ± 0.13	13.53 ± 0.38	13.40 ± 0.57	14.07 ± 0.41	13.93 ± 1.04	14.30 ± 1.40
White cell count (WCC)	15.54 ± 2.13	18.80 ± 1.50	16.60 ± 3. 18	28.29 ± 6.60^{a}	29.43 ± 10.32^{a}	49.00 ± 13.21^{b}
Absolute neutrophil (total)	4.71 ± 0.96	8.82 ± 1.04	6.71 ± 2.58	13.04 ± 4.52^{a}	17.79 ± 7.65^{a}	26.28 ± 16.36^{b}
Absolute neutrophil (mature)	4.71 ± 0. 96	8.82 ± 1.04	6.71 ± 2.58	13.04 ± 4.52^{a}	17.79 ± 7.65^{a}	26.28 ± 16.36^{b}
Absolute neutrophil (immature)	0.00 ± 00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Neutral group are chicks that were not infected with *A. fumigatus*, and were also not treated. Positive control (TC); Untreated control (UC)
Means with differing superscript letters in the same row are significantly different ($p \leq 0.05$).

8.4. Discussion and conclusions

Generally, aspergillosis in chickens is an acute disease causing high mortality in the first few days of life. The disease in some cases can be chronic, with lesions in the lungs, air sacs, and joints (Cutsem, 1983). Evidence of clinical signs associated with *A. fumigatus* infection was quite evident in this study, which is in agreement with was reported in literature.

Our data indicated that the crude extract of *L. alata* has certain level of activity against aspergillosis in broiler chicks and that these birds could serve as model for the study of agents intended for development into safer and effective anti-aspergillus agents. Plant drugs have been assumed to be safe. However, in recent times, there is evidence that many plants used as food or as medicine can be potentially toxic (Kassie et al., 1996; De Sã Ferrira and Ferrão Vargas, 1999). All drugs can produce harmful as well as beneficial effects, hence the need for toxicity testing of any substance intended for development into drug for human or animal use (Rang et al., 2003). Investigation of the acute toxicity is the first step in the toxicological investigations of an unknown substance (Lorke, 1983). The index of the acute toxicity is the lethal dose 50 (LD_{50}). However, the LD_{50} is not regarded as a biological constant, since differing results are obtained on repetition (Lorke, 1983). The OECD guidelines for testing chemicals (2000) for toxicity were adapted in testing the safety of the extract of *Loxostylis alata*. The method is not intended to allow the calculation of a precise LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected since death or appearance of toxic signs of a proportion of the animals is still the major endpoint of this test. The extract was toxic at the dose of 300 mg/kg based on its effects on different parameters measured and clinical signs noticed on the experimental chicks. Dose is an important factor in drug poisoning and most drugs cause clinical signs of poisoning when administered at relatively high dose (Rang et al., 2003). However, at lower doses (200 mg/kg and below) the extract had no apparent toxic effects. Although the chicks treated with 50 and 200 mg/kg had lower weight gain than those in the control group, the weight gain in all the chicks except those treated with 300 mg/kg are all within normal growth rate for broiler chickens (Naidoo et al., 2008; SAPA, 2008).

During preliminary chemotherapeutic trial, we were not able to establish an infection using 10^7 spore suspension of *A. fumigatus* when administered intraperitoneally similar to that reported by Femenia et al (2007). Infection in our study was established using 10^8 spores of *A. fumigatus* suspended in 0.2 ml of sterile distilled water administered intraperitoneally. However, Fadl Elmula et al. (1984) inoculated a lower number of spores (4×10^5 per chick) intraperitoneally to cause infection in 6-day old chicks. Disruption of the *alb1* gene in *A. fumigatus* leads both to virulence attenuation and to an immune response in the form of

increased complement binding and neutrophil-mediated phagocytosis. Hence the mutation led to increased recognition of the conidia by the immune system (Odds et al 2001). Perhaps a similar mechanism of virulence attenuation affected the isolate used for this study, which lowers the infectivity of the organism.

Clinical signs of aspergillosis observed in our study included ruffled feathers, gasping, dyspnoea, dullness, green watery diarrhoea and anorexia. These signs are in agreement and confirmed the clinical signs observed in previous studies (Akan et al., 2002). Although ascites and blindness were observed by Julian and Goryo (1990) and Akan et al (2002), respectively, we did not observe these clinical features in our study. Chicks in this study started dying 3 days p.i. with mortality reaching as up to 75% in the non-treated group. Gűműşsoy et al (2004) inoculated 3-week old quails with *A. fumigatus* intratracheally and recorded mortality of up to 100%. Furthermore, *A. fumigatus* was re-isolated from the lungs and airsacs of infected chicks starting 3 days p.i and up to 12 days p.i. Lesions were seen mainly in the lungs, liver and the airsac. Surprisingly, *A. fumigatus* was isolated from the lung sample of one chick in the neutral group, which was not infected and also not treated. Since systemic infection with *Aspergillus fumigatus* results naturally through the inhalation of spores, (Julian and Goryo, 1990) it is possible that the chicks in the neutral group were infected naturally from fungal spores inhaled in the air. However, since, we use a specific histological staining technique to confirm the presence of an infection, we are certain that the positive result was due to contamination during post mortem examination.

Increase AST and GGT activity as well as A/G ratio are indicative of a liver injury. This was supported by the isolation of the agent from liver. In addition, there was presence of lesions on the liver

Compared with the infected untreated group with a mortality rate of 75%, in the entire infected treated group, the mortality rate reduced and death was stopped on 11th day post-infection. Treatment with extract of *Loxostylis alata* at doses used confer a dose related success in combating infection due to *A. fumigatus* in broiler chicks and that compared favourably with the reference compound (ketoconazole). Compounds administered i.p. are absorbed primarily through the portal circulation and, therefore, must pass through the liver before reaching other organs. This factor can limit the amount of drug reaching its site of action (Lukas et al., 1971). Indeed, there are numerous examples in the literature of drugs that are less effective after i.p. administration than when given by other parenteral routes. Such examples include reserpine (Mueller and Shideman, 1968), phenelzine and phenipramine (Horita, 1961). It will therefore be reasonable to assume that when the extract is administered via another route that bypasses the portal circulation that could result in enhanced action of the extract.

In conclusion, broiler chicks are susceptible to the infection with *A. fumigatus* and the extract of *L. alata* appears to be beneficial in treating and curing it. Additional work is needed on extracts of this species to further establish its detailed safety and efficacy against *A. fumigatus* in broiler chicks and other target species.

Chapter 9
General discussions and conclusions

Aspergillus fumigatus is one of the most common pathogenic fungal species in humans and animals (Rippon, 1982). *A. fumigatus* also causes severe financial problems in the poultry industry. Current treatment of the disease is hampered by drug resistance of the organism to conventional antifungals and also their widespread toxicity to animals. The aim of this study was to find a plant extract or isolated compound that could be used to combat aspergillosis in animals. To attain this aim a number of objectives were identified i.e. to

- Evaluate the antibacterial and antifungal activity of selected South African plant species against a range of pathogenic bacterial and fungal species in order to select the species with the best antimicrobial activity for further investigation.
- Isolate and characterize the compounds active against *Aspergillus fumigatus* from extracts of selected plant species.
- Determine the in *vitro* antimicrobial, antioxidant and anti-inflammatory activities and cytotoxicity of the extracts and isolated compound(s) of selected plant species.
- Evaluate the efficacy of the isolated compound(s) or crude extracts *in vivo* in a poultry model.

The degree to which these objectives were attained is discussed below.

9.1. Evaluate the antimicrobial activity of selected South African plant species

The antifungal and antibacterial activities of extracts of leaves of seven selected trees were determined. Extracts of *Loxostylis alata* had more active compounds on bioautograms against all the bacteria (*Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus*) used as test organisms and hence were selected as the most promising species to investigate in more detail. It appeared that the same compounds (based on their identical R_f values) inhibited several bacteria and fungi. This non-specificity of activity could be due to a broad antimicrobial action of the plant compounds or the compounds could be general metabolic poisons inhibiting many types of living cells. Acetone is an intermediate polar solvent which extracts both polar and non-polar constituents of plant. The acetone extract of *Loxostylis alata* therefore contained more antibacterial and antifungal compounds than all other extracts. Acetone extracts of *Curtisia dentata* also contained more active compounds against the same bacteria and fungi tested in this work than other extracts used (Shai, 2007). In contrast, the

dichloromethane and hexane extracts of six *Terminalia* species had more antifungal compounds than the acetone extracts (Masoko *et al.* 2005).

All the extracts of the plant species used in this study had antimicrobial activity with MICs generally less than 0.1 mg/ml against at least one pathogen..The lowest MIC of 0.04 mg/ml was of the acetone extract of *Loxostylis alata* against *Sporothrix schenckii*.

9.2. Isolate and characterise the compounds active against *Aspergillus fumigatus* from extracts of selected plant species

9.2.1. Selection of the most promising tree species for further work

From the seven plant species that were screened for antimicrobial activity, *Loxostylis alata* was selected for further work because the powdered leaf of *Loxostylis alata* gave the highest yield of extract (21.8%) and had the highest average total activity (TA) of 1343 and 1414 ml/g against bacteria and fungi, respectively. Total activity is calculated by dividing the quantity extracted from one gram of plant material with the MIC value in mg/ml. This value indicates the volume to which the active constituent present in one gram of the plant material can be diluted and still inhibit the growth of the test organism. Furthermore, leaf extracts of *L. alata* had more compounds active against *Aspergillus fumigatus* and other tested microbial organisms on bioautograms. The acetone extract of the leaves of *Loxostylis alata* had an MIC of 0.07 mg/ml against *Aspergillus fumigatus*. These factors made the plant a potential source of active compounds or extracts for the treatment of *Aspergillus fumigatus* infection. To promote the sustainable use of plant and protect the environment only the leaves were used in this study.

9.2.2. Isolation and characterisation of compounds

The acetone extract (70 g) was subjected to solvent-solvent fractionation using carbon tetrachloride, hexane, chloroform, aqueous methanol, butanol and water. The carbon tetrachloride (CCl_4) fraction had the best antimicrobial activity (MIC and TA value of 0.08 mg/ml and 32012 ml/g, respectively) and therefore, chosen for further isolation of active compounds.

Using bioassay-guided fractionation two active compounds against *A. fumigatus* were isolated from the leaves of *Loxostylis alata.* ^{13}C and ^{1}H NMR spectroscopic and mass spectrometric data led to identification of the compounds as the lupane triterpenoid lupeol and β-sitosterol. Although these compounds are common secondary metabolites present in many plant species (Imam et al., 2007; Lopes et al., 1999) it is reported for the first time from *L. alata* leaves.

9.3. Determine the *in vitro* antimicrobial, antioxidant and anti-inflammatory and cytotoxicity activities of the extracts and isolated compound(s) of selected plant species

9.3.1. Antimicrobial activity

The antifungal activitiy of the fraction of *L. alata* were determined prior to the bioassay guided fractionation. The carbon tetrachloride fraction was the most active with an MIC of 0.08 mg/ml against *Aspergillus fumigatus*. Similarly, the carbon tetrachloride fraction of *L. alata* had a more lines of inhibition against all the tested microorganisms on bioautograms. The hexane and aqueous methanol fractions had few line of inhibition, while the butanol and water fractions did not have any.

Lupeol had reasonable activity against all the tested pathogens with MICs ranging from 29-120 µg/ml. Similarly, lupeol had good activity against *C. albicans, S. aureus, E. coli* and *Enterococcus faecalis* in a similar study (Shai 2007). The isolated compounds from *L. alata* leaves are widely distributed in most plants and hence are not novel in character. The activity of the isolated compounds were lower against *A. fumigatus* than that of the crude extract of *L. alata*. Unless these compounds are destroyed during chemical elution by chromatographic means or bioautography, it appears that synergism plays an important role in the activity of the plant against *A. fumigatus*. The other compounds involved in synergism appear not to be active on their own.

9.3.2. Antioxidant activity

Antioxidants may boost the immune response and by so doing assist the body to combat microbial infections by reversing several conditions associated with immune deficiencies. This prompted us to investigate the antioxidant activity of the plant using both ABTS and DPPH radicals. The extract of *L. alata* had a trolox equivalent antioxidant capacity (TEAC) value of 1.94 and an EC_{50} value of 3.58 ± 0.23 µg/ml, which compares favourably with that of Trolox and L-ascorbic acid (positive controls) of 1 and 1.59 ± 0.80 µg/ml, respectively. The aqueous methanol, butanol and water fractions had antioxidant activities with EC_{50} values of 1.82 ± 0.03, 1.05 ± 0.06 and 0.62 ± 0.03 µg/ml respectively in the DPPH assay. The reference compound (L-ascorbic acid) had an EC_{50} of 0.94 ± 0.11 µg/ml. The lower the EC_{50} value of a substance, the more effective its antioxidant activity. Similarly, the TEAC values of aqueous methanol, butanol, water, chloroform, carbon tetrachloride and hexane fractions were 1.55, 2.21, 2.97, 0.45, 0.12 and 0.56, respectively. A TEAC value greater than 1 indicates better antioxidant activity than that of the reference compound (trolox).

As determined later increased white cell counts (neutrophils and lymphocytes) were recorded in broiler chicks treated with the extract of *L. alata*. Antioxidants in the extract of *L. alata* may have been responsible

for the antifungal action of the extract against *Aspergillus fumigatus* by boosting the host immune response via stimulation of white cell production.

9.3.3. Anti-inflammatory assay

Antioxidants limit the progression of inflammatory diseases by scavenging free radicals produced during inflammation (Knight, 2000). The anti-inflammatory action of *L. alata* was therefore also investigated. Moreover, *L. alata* is used in traditional medicine to treat inflammation and boost the host immune response (Pell, 2004). The action of phospholipase A_2 on cell wall leads to the production of free arachidonic acid (AA). The AA is converted to thromboxane A_2 by cyclooxygenase 1. Thromboxane A_2 is a powerful inducer of platelet aggregation (Vane and Botting, 1987). Inhibition of cyclooxygenase 1 in platelets will limit or stop the production of thromboxane A_2 and prostaglandins (inflammatory mediators). These effects will then prevent platelet aggregation. Platelets therefore serve as a model for *in vitro* anti-inflammatory testing of the plant extracts. Moreover, platelets have an important role in acute inflammation by releasing arachidonic acid (AA) metabolites and PAF (Holmsen et al., 1977; Vincent et al., 1977).

Platelet aggregation inhibition is achieved if the inhibitory agents are able to cross the cell membrane barrier of platelets. If extracts prevented platelet aggregation in this study it means that compounds have the capacity to move across the cell membrane barrier. Lipid soluble drugs are usually non-ionised in solution and have the capacity to diffuse across cellular membranes (Wilkinson, 2006). The intermediate polariry of compounds in some extracts may explain the activiy or inactivity of some of the extracts on platelet aggregation. The extract of *Loxostylis alata* had activity against *in vitro* adrenaline-induced platelet aggregation with EC_{50} of 0.35 ± 0.03 µg/ml which compares favourably with that of aspirin a standard antiplatelet agent. That action could also be beneficial in preventing thromboembolic disorders.

Pathogenic fungi (dermatophytic, subcutaneous, and systemic) have the ability to produce eicosanoids (prostaglandins and leukotrienes) both from host derived arachidonic acid. Host-derived eicosanoids can enhance fungal colonization and the development of inflammation during fungal infections (Noverr et al., 2002).

The crude acetone extract of *L. alata* and the isolated compounds were screened against cyclooxygenase enzymes -1 and -2 (COX-1 and -2).). Both COX -1 and -2 regulate the biosynthesis of prostaglandins from arachidonic acid. COX-1 is a constitutive form and has a clear physiological function while COX-2 is induced by inflammatory mediators. It is the inhibition of prostaglandin synthesis by COX-1 and -2 that is responsible for the anti-inflammatory actions of NSAIDs. The crude extract of *L. alata* and lupeol inhibited COX-1 in a dose dependent manner (IC_{50} = 92.5 ± 1.6 and 134.0 ± 5.1 µM, respectively). Neither the crude extract nor the 2 isolated compounds had an inhibitory effect on COX-2. In addition, COX-1 selective inhibitors have beneficial anti-thrombotic effect. Inhibition of COX-1 by both the crude extract and lupeol

may exert beneficial anti-thrombotic effect and protect from heart diseases. Furthermore, as evidence the crude extract of *Loxostylis alata* inhibited equine platelet aggregation.

9.3.4. Cytotoxic activity

The acetone extracts of the selected plants had comparably low toxicity against Vero Monkey kidney cells and equine red blood cells *in vitro* except for the extract of *C. harveyi* which had a higher haemagluttination assay titre value indicating toxicity of the plant extract. The viability of the cells was between 70-90% when the extract of *L. alata* was used at concentrations of 0.01-0.001 mg/ml. Over 90% of Vero cells treated with berberine (reference compound) at the concentration of 0.01 mg/ml were not viable. Similarly, the extract of *L. alata* had a haemagglutination titre (HA) of 0.8 at a concentration as high as 1.25 mg/ml. These values indicate low toxicity of the extract of *L. alata*. The isolated compounds (lupeol and β-sitosterol) were relatively non-toxic with an LC_{50} of 76.66 ± 4.13 and 136.60 ± 7.20 µg/ml, respectively compared to the reference compound berberine (LC_{50} of 6.36 ± 0.81 µg/ml). β-sitosterol which occurs as sterol in many plants is potentially useful in improving human health as an anti-inflammatoriy, antipyretic, immunomodulator, or antineoplastic agent.The two compounds isolated from *L. alata* had no mutagenic effect on *Salmonella*/ microsome tester strains TA98 and TA 100.

9.4. Evaluate the efficacy of the crude extract *in vivo* in a poultry model

The crude acetone extract of *Loxostylis alata* was investigated for its safety and *in vivo* antifungal activity against experimental aspergillosis in broiler chicks. At a dose of 300 mg/kg, the extract had some toxic effect causing depression, decrease feed intake, diarrhoea, weight loss and death in broiler chicks. The method employed for toxicity testing in this study allows for the determination of defined exposure ranges where lethality is expected since death or appearance of toxic signs of a proportion of the animals is the major endpoint.

Antifungal infection was assessed by comparing the degree and severity of clinical signs, lesion scores and fungal re-isolation observed from treated chicks with those observed from infected chicks not treated with the extract. The extract at a dose of 100 and 200 mg/kg significantly reduced ($p \leq 0.05$) the lesions due to aspergillosis and the quantity of *Aspergillus* organisms isolated from infected chicks in a dose dependent fashion. Moreover, the extract of *L. alata* at a dose of 200 mg/kg enhanced the survival of broiler chicks by 75% since 6 out of the 8 infected chicks survived compared to only 2 out of 8 (25%) in the infected untreated control group. The survivability of chicks treated with ketaconazole (positive control drug) at a

dose of 60 mg/kg was 87.5%. The *L. alata* extract appears to have substantial value in treating and curing aspergillosis, a major health problem facing the poultry industry.

9.5. Conclusions

The crude acetone extract at a dose of 200 mg/kg was as effective as ketoconazole (positive control). The pharmacological action of *Loxostylis alata* could possibly be attributed to the combined action of both antioxidant and antifungal compounds present in the crude extract. It appears likely that the crude acetone extract could be produced at a much lower cost than ketoconazole or other chemical antimicrobial products. If these results can be confirmed in larger studies and if the crude extract does not have a negative effect on the production of the poultry, the crude extract of *L. alata* may be a viable and cost effective alternative to using current antimicrobial products. This study proves that it may be worthwhile to invest human and financial resources in searching for plant related products than can increase animal health and productivity.