

Chapter 5

Evaluation of selected South African plant species for antioxidant, antiplatelet and cytotoxic activity

M.M. Suleiman, V. Bagla, V. Naidoo and J.N. Eloff

Preface

Aspergillosis and most fungal infections are associated with immune depression and causing inflammation. Antioxidants essentially reverse several conditions associated with immune deficiencies and inflammatory diseases. Plant extracts that have antifungal activity in this study will be evaluated for antioxidant activity as an indicator of their ability to boost the immune system. Since acetone extract both polar and intermediate polar compounds where antioxidants are found, it was used as an extractant. We further evaluated the safety of the extracts as a step towards the use of active plant extracts in clinical practice. The text in this chapter was submitted and accepted as manuscript for publication to *Pharmaceutical Biology* to indicate the results obtained.

Abstract

The antioxidant, antiplatelet and cytotoxic effects of seven South African plant extracts, namely *Combretum vendae* A.E. van Wyk (Combretaceae), *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Kirkia wilmsii* Engl. (Kirkiaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae), *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) and *Protorhus longifolia* (Anacardiaceae) were evaluated using established *in vitro* assays. All the extracts had comparably low toxicity except for the extract of *C. harveyi* that had a high haemagglutination assay titre value, which indicates toxicity. The extracts of *P. longifolia*, *K. wilmsii*, *O. natalitia*, *L. alata*, *C. harveyi* and *C. vendae* had antioxidant properties in the qualitative assay using DPPH. In the quantification of antioxidant activity using ABTS, only the extracts of *P. longifolia*, *L. alata*, and *C. vendae* had antioxidant activity with TEAC values of 1.39, 1.94 and 2.08 respectively. In the quantitative DPPH assay, *L. alata* (EC₅₀, 3.6 ± 0.2 µg/ml) and *K. wilmsii* (EC₅₀, 3.6 ± 0.4 µg/ml) did not differ significantly ($p \leq 0.05$) from the control. *K. anthotheca* had a higher EC₅₀ (176 ± 27 µg/ml) value, and differed significantly ($p \leq 0.05$) from all the other extracts and control. In addition, the extract of *C. vendae* and *C. harveyi* had antiplatelet activity that were significantly ($p \leq 0.05$) better than the control (aspirin) with EC₅₀ of 0.06 ± 0.01 µg/ml, 0.19 ± 0.00 µg/ml, respectively. Lower EC₅₀ values in the antioxidant and antiplatelet studies indicate superior activity of the plant extract against oxidation and platelet aggregation.

Keywords: South African plants, antioxidant assay, antiplatelet activity, cytotoxicity effect.

5.1. Introduction

Bioactive compounds commonly found in plants have possible health benefits partly due to their antioxidative properties (Cao and Cao, 1999). It is well known that reactive oxygen species (ROS) are involved in a diversity of important processes in medicine, including among others: inflammation, atherosclerosis, cancer and reperfusion injury (Kehrer, 1993). One of the major fundamental tissue-destructive mechanisms is oxidative stress through an excessive release of reactive oxygen metabolites (ROM) (McCord, 2000). Although the generation by phagocytes (and to a lesser extent by eosinophils, lymphocytes and fibroblasts) is essential for an effective host defence against bacterial infection, continuous overproduction during inflammatory processes may also cause extensive tissue destruction (Weiss, 1989). One way by which a substance can interfere with these processes is by acting as an antioxidant or free radical scavenger. Antioxidants abate inflammation and protect tissues from oxidative damage caused by free radicals. Inflammation has been described as the release of chemicals from tissues and migrating cells. Agents such as prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF), 1 and interleukin 1 (IL 1) play some part in the inflammatory processes. In addition, prostaglandins and other inflammatory mediators produce oxidant products that play an important role in causing tissue oxidation (Pekoe et al., 1982). Many anti-inflammatory drugs are able to react with oxidants *in vitro*, so considerable interest has been expressed in the possibility that oxidant scavenging contributes to the action of these drugs *in vivo* (Halliwell et al., 1988). Despite the presence of wide arrays of anti-inflammatory drugs (steroidal and nonsteroidal), they still present a wide range of side effects for which the major reason is nonselective inhibition of cyclooxygenase I (COX I) and cyclooxygenase II (COX II) (Vane and Botling, 1995). Prostaglandins, thromboxanes and platelet-activating factor (PAF) contribute to platelet aggregation and, 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). Furthermore, thromboxane A₂ (TXA₂), an AA product formed via the COX pathway in platelets has been reported to be a potent vasoconstrictor and pro-aggregatory agent (Page et al., 1984). COX inhibitors, such as aspirin, are known to inhibit platelet aggregation (Saeedu et al., 1997).

A large number of naturally occurring compounds such as flavonoids, catechins, lignans, and phenolic acids contained in plants and herb remedies have antioxidant properties (Dall'Acqua et al., 2008). These reasons have recently prompted research into natural antioxidants. The aim of this study was to test the antioxidant, anti-platelet and cytotoxicity effects of acetone extracts of leaves of seven South African tree species which were selected based on their antifungal activity in an effort to develop a product that could protect poultry against aspergillosis. .

5.2. Materials and methods

5.2.1. Plant collection

The leaves of *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Combretum vendae* A.E. van Wyk (Combretaceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae) and *Protorhus longifolia* (Bernh. Ex C. Krauss) Engl. (Anacardiaceae) were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* Engl. (Kirkiaceae) and *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plants were collected in November 2006 and were identified and authenticated by Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

5.2.2. Plant storage

Immediately after collection and transportation to our laboratory, leaves were separated from stems and dried at room temperature with good ventilation. The dried plants 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.

5.2.3. Plant extraction

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting with 10 ml of acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 1 h using a Labotec model 20.2 shaking machine at a moderate speed. Extracting at lower speed for a longer period allows the solvent to penetrate more into the plant tissues; allowing the extraction of more of the compounds contained in the plant species (Silva et al., 1998). After centrifuging at 3500 x *g* for 10 min, the supernatant was decanted into pre-weighed labelled containers. The whole process was repeated three times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature to quantify the extraction.

5.2.4. Evaluation of antioxidant activity

Qualitative antioxidant screening to determine the number of antioxidant compounds was employed using 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Takao et al., 1994). TLC plates loaded with 100 µg of each extract were developed in chloroform/ethyl acetate/formic acid (5:4:2) [CEF] solvent system and sprayed with 0.2% DPPH in methanol. Compounds with antioxidant activity were visualized as yellow bands against a purple background (Bors et al., 1992). Quantification of antioxidant activity was by spectrophotometric means using two radicals, ABTS and DPPH.

In the ABTS method, the Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (Re et al., 1999) was determined. This was based on the scavenging of the ABTS radical into a colourless product by antioxidant substances. The blue/green chromophore ABTS⁺ was produced through the reaction between ABTS and potassium persulfate. The absorbance was read at 734 nm using a Versamax microplate reader (Molecular Devices). Trolox is a Vitamin-E analogue and was used as a standard in this assay. Percentage change in absorbency was calculated by the formula below:

$$\text{Initial absorbency of ABTS}^+ - \text{New Initial absorbency of ABTS}^+ / \text{Initial absorbency of ABTS}^+ \times 100$$

The curves were plotted with the dependent variable being the percentage change in absorbency and the independent variable being the different concentrations at which test substances were analysed. Mathematical comparison of antioxidant activity of different plant extracts was done by dividing the slope obtained for extract to that of Trolox to get the Trolox equivalent antioxidant capacity (TEAC). An extract with a TEAC value of 1 indicates an equivalent antioxidant value of Trolox. Decrease or increase of antioxidant activity is depicted by a lower or higher value of TEAC, respectively.

The DPPH free radical assay was conducted as described by Mensor et al (2001). Briefly, 10 µL of 0.3 mM DPPH in ethanol was added to 25 µL of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Appropriate blank and negative control solutions were prepared for each test. L-ascorbic acid (Vitamin C) was used as positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AA %) using the formula:

$$\text{AA\%} = 100 - \{[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100] / \text{Abs}_{\text{control}}\}$$

Where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and Abs_{control} is the absorbance of the control. The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test extracts ($\mu\text{g/ml}$) against the mean percentage of the antioxidant activity obtained from three replicate assays using Microsoft Office Excel. EC_{50} values obtained from the regression lines had coefficient of determination $r^2 \geq 95\%$. A lower EC_{50} value indicates high antioxidant activity.

5.2.5. *In vitro* platelet aggregation assay

The modified method of Fratantoni and Poindexter (1990) was used to determine the inhibitory effect of the plant extracts on platelet aggregation. Briefly, fresh equine (*Equus caballus*) blood was collected from healthy representative breeds in the Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria by Mrs. Stellet de Villiers into sterile 5 ml glass tubes containing 3.8% trisodium citrate solution as anticoagulant at a ratio of 1:9 volume of anticoagulant to blood.

The platelet rich plasma (PRP) and platelet poor plasma (PPP) were separated by centrifugation at 160 g for 10 min and 1600 g for 15-30 min at 20-25°C, respectively, using a Beckman^(R) GS-15R centrifuge. PRP was later centrifuged at 1000 g for 15 min at 20-25°C to sediment the platelets. The platelet count of the PRP was adjusted to 300,000/pl by adding PPP. Both PRP and PPP were then stored at room temperature. The cell suspension was adjusted to approximately 3.0×10^8 platelets per ml using phosphate buffered saline (PBS). Different concentrations of the extracts and aspirin as the reference drug were added and incubated at 37°C for 3 min. After incubation, platelet aggregation was induced by the addition of 50 μl of adrenaline. The degree of platelet aggregation was determined spectrophotometrically at 600 nm after 30 min. Percentage platelet aggregation inhibition was calculated using the following equation.

$$X (\%) = \frac{A-B}{A} \times 100$$

Where A= maximal aggregation of the control and B= maximal aggregation of sample-treated PRP.

The EC_{50} values were calculated by linear regression of plots using Microsoft Office Excel. The abscissa represents the concentration of tested plant extracts and the ordinate the average percent of antioxidant activity from three separate tests.

5.2.6. Cytotoxicity assay

5.2.6.1. MTT assay

The plant extracts 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% CO₂ incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts were prepared by reconstitution to a concentration of 100 mg/ml in 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 120 h incubation with plant extracts. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was used as an indicator for cell growth. The absorbance was measured at 570 nm. Berberine chloride (Sigma) was used as a positive control. Tests were carried out in quadruplicate and each experiment was done in triplicate.

5.2.6.2. Haemagglutination assay

Fresh equine (*Equus caballus*) blood was collected from a representative breed in the Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria into sterile 5 ml glass tubes containing 3.8% trisodium citrate solution. Erythrocytes were fixed with formalin and prepared according to method of Sadique et al. (1989) as modified by Iwalewa et al. (2005). Briefly, 20 ml of the mixed blood was centrifuged at 4000 rpm for 10 min, using a Beckman^(R) GS-15R centrifuge. The packed red blood cells were washed with 10 mM phosphate buffer saline (PBS) pH 7.2 until a clear supernatant was obtained. The washed packed RBCs were suspended in 5% (v/v) formaldehyde-phosphate buffer saline (1:12.3 v/v) solution. The mixture was left at room temperature for 24 h. The final fixed RBC were washed and centrifuged with PBS 3 times, and preserved with 1 ml (50 mg/ml) gentamicin containing 0.1% methyl paraben to prevent microbial growth and stored at 4°C.

PBS (100 µL) was added to wells of 96-well microtitre plates. The first row was used as a control without extracts. The extracts (100 µl) were added into the first well of the second row, and a 2-fold serial dilution was made until the last well (well 12). Then 50 µl of equine RBC was added to all the wells. They were incubated at room temperature for 1 h. The presence of buttons in the centre of the well indicated no agglutination and the haemagglutination titre values of the extracts were read as the reciprocal of the last dilution having agglutination

5.2.7. Statistical analysis

Antioxidant and antiplatelet experiments were done in triplicate. The results are presented as mean \pm standard error of mean (SEM). A one-way analysis of variance (ANOVA) was used for comparison of means using Microsoft Office Excel 2003. A difference was considered statistically significant when $p \leq 0.05$.

5.3. Results

The extraction yields of acetone extracts of plants used in this study are presented in Table 1. *C. vendae* gave the highest yield of 15.7%, while *K. wilmsii* possess the lowest yield (6.9 %). The TLC DPPH method of qualitative antioxidant detection showed that the acetone extracts of *P. longifolia*, *K. wilmsii*, *O. natalitia*, *L. alata*, *C. harveyi* and *C. vendae* displayed antioxidant compounds due to their DPPH free radical scavenging activity. Antioxidant compounds were seen as yellow bands against a purple background. The *L. alata* extract had the most antioxidant compounds. The acetone extract of *K. anthotheca*, however, did not show any antioxidant compound on the TLC plate (Figure 5.1).

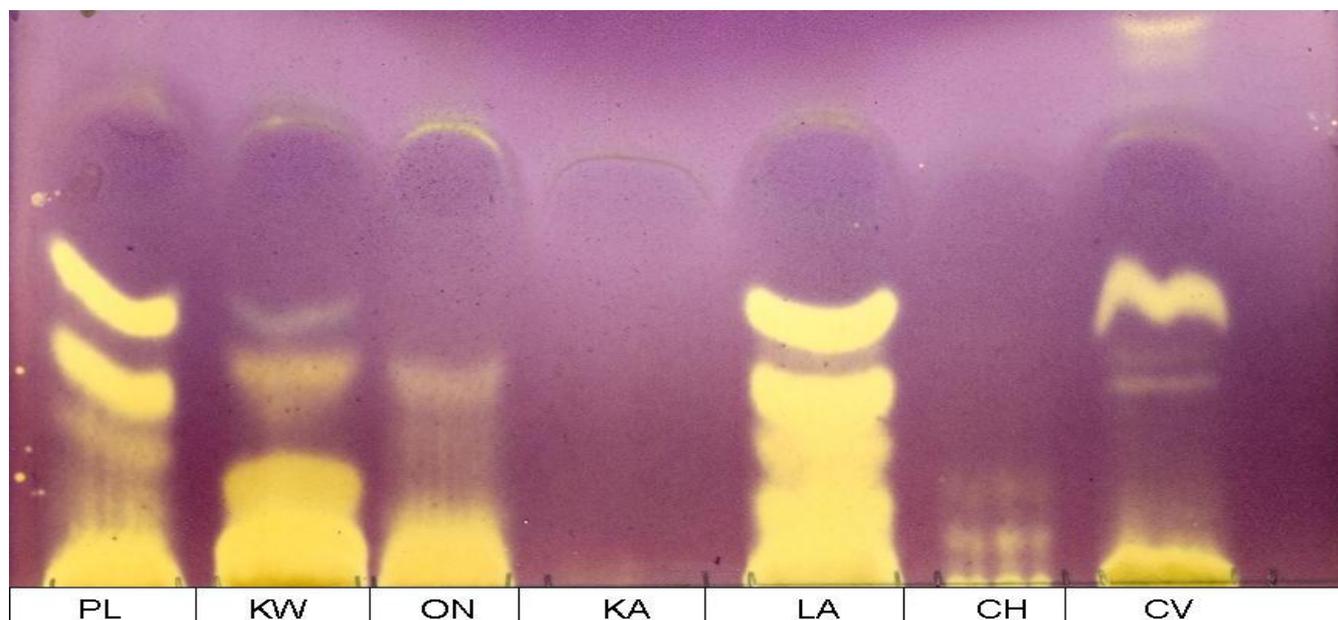


Figure 5.1. Chromatogram of 100 μ g acetone extracts of the leaves of *P. longifolia* (PL), *K. wilmsii* (KW), *O. natalitia* (ON), *K. anthotheca* (KA), *L. alata* (LA), *C. harveyi* (CH) and *C. vendae* (CV), separated with CEF mobile phase and sprayed with 0.2% DPPH. Antioxidant compounds are indicated by yellow areas.

In the TEAC antioxidant assay, extracts of *P. longifolia*, *L. alata*, and *C. vendae* had superior free radical scavenging activity compared to the other extracts and the standard antioxidant (Trolox) used in this study

with respective TEAC value of 1.39, 1.94, and 2.08 (Table 5.1). The results corroborate that of the qualitative assay where the extracts showed the presence of antioxidant compounds. Extracts of *O. natalitia*, *K. wilmsii*, *C. harveyi* and *K. anthotheca* had lower TEAC values of 0.79, 0.67, 0.15 and 0.10, respectively.

In the quantitative DPPH assay (Table 1), L-ascorbic acid had the lowest EC₅₀ (1.59 ± 0.80 µg/ml) value than all the extracts (i.e. the highest antioxidant activity), however, L-ascorbic acid no significant ($p \leq 0.05$) difference in its free radical scavenging effect when compared with *L. alata* (EC₅₀, 3.58 ± 0.23 µg/ml) and *K. wilmsii* (EC₅₀, 3.57 ± 0.41 µg/ml). *K. anthotheca* had a higher EC₅₀ (176.40 ± 26.56 µg/ml) value, and differed significantly ($p \leq 0.05$) from all the other extracts and L-ascorbic acid. Extracts of *P. longifolia*, *C. vendae*, *C. harveyi*, and *O. natalitia* had EC₅₀ of 6.57 ± 0.23 , 4.41 ± 0.14 , 10.47 ± 1.96 and 7.50 ± 0.13 , respectively. The lower the EC₅₀ of a substance, the more effective is its free radical scavenging effect.

Table 5.1. Antioxidant and antiplatelet activity of acetone extracts of seven South African plants.

Plant	Voucher specimen number	Extract yield (%)	Antioxidant values		Antiplatelet activity (EC ₅₀ ± SEM, µg/ml)
			TEAC	DPPH (EC ₅₀ ± SEM, µg/ml)	
<i>C. vendae</i>	PRU96507	15.7	2.08	4.41 ± 0.14 ^b	0.06 ± 0.01 ^a
<i>C. harveyi</i>	PRU96506	14.2	0.15	10.47 ± 1.96 ^b	0.19 ± 0.00 ^a
<i>K. anthotheca</i>	PRU96509	8.9	0.10	176.40 ± 26.56 ^c	0.97 ± 0.03
<i>K. wilmsii</i>	PRU96503	6.9	0.67	3.57 ± 0.41 ^a	0.22 ± 0.02
<i>L. alata</i>	PRU96508	13.7	1.94	3.58 ± 0.23 ^a	0.35 ± 0.03
<i>O. natalitia</i>	PRU96504	12.6	0.79	7.50 ± 0.13 ^b	0.39 ± 0.03
<i>P. longifolia</i>	PRU96505	15.3	1.39	6.57 ± 0.23 ^b	0.23 ± 0.02
L-ascorbic acid	-	N/A	N/A	1.59 ± 0.80 ^a	N/A
Aspirin	-	N/A	N/A	N/A	0.04 ± 0.00 ^a

N/A = Not available

Means within the same column and with different superscript letters (a, b or c) differ significantly ($p \leq 0.05$)

The antiplatelet actions of *C. vendae* and *C. harveyi* did not differ significantly ($p \leq 0.05$) from that of aspirin (standard agent) (Table 5.1). The low EC_{50} by *C. vendae* ($0.06 \pm 0.01 \mu\text{g/ml}$) and *C. harveyi* ($0.19 \pm 0.00 \mu\text{g/ml}$) depicts good antiplatelet activity when compared with that of aspirin ($0.04 \pm 0.00 \mu\text{g/ml}$).

The cytotoxic activities of the extracts in the Vero Monkey kidney cell line assay are provided in Figure 5.2. In the assay the extracts of *O. natalitia*, *K. anotheca*, *L. alata*, *C. harveyi* and *C. vendae* at the highest concentration used were relatively toxic; they caused complete death of the Vero cells. However, at lower concentrations all the extracts had relatively lower toxicity when compared with the reference agent berberine (cytotoxic agent). Over 90% of Vero cells treated with berberine at the concentration of $10 \mu\text{g/ml}$ were not viable. The effects of the extracts on fixed equine erythrocytes are presented in Table 5.2. All the plant extracts exhibited very low HA titre values with a wide range of concentrations at which agglutination occurred on. However, the acetone extract of *C. harveyi* had a very high HA titre value and a very low concentration at which agglutination occurred.

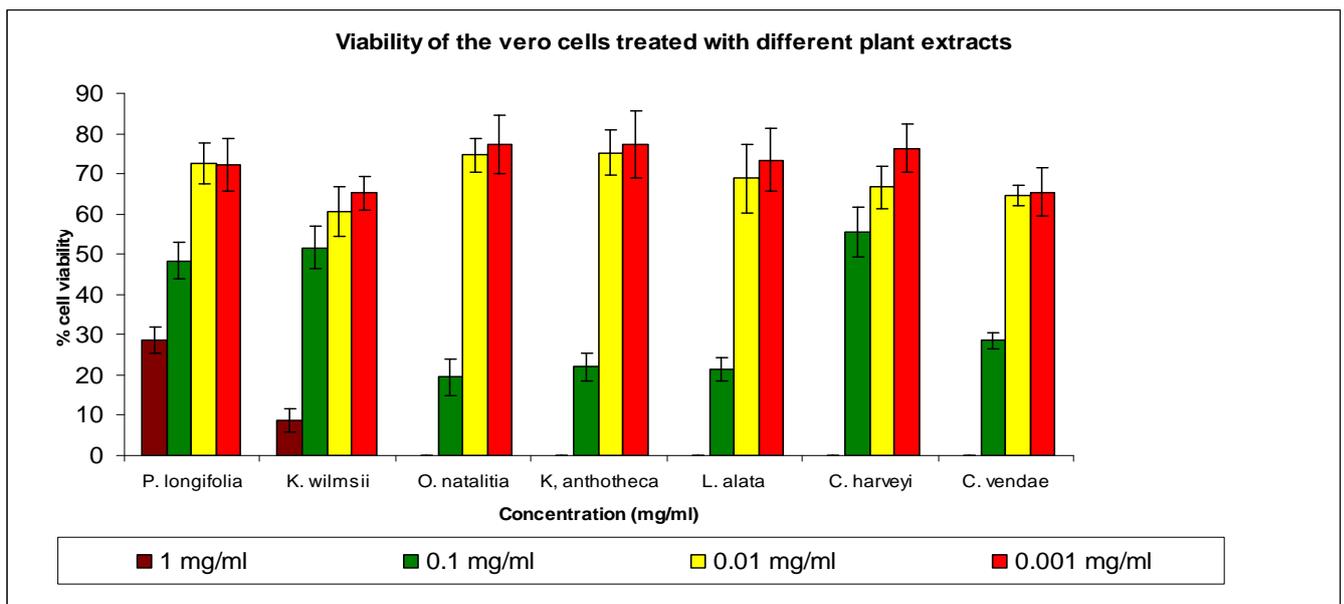


Figure 5.2. Viability of Vero monkey kidney cell line treated with different concentrations of acetone extracts of seven South African plants. Values are mean \pm S.E.M.

Table 5.2. Cytotoxic effect of acetone extracts of seven South African plants on formaldehyde-fixed equine erythrocytes.

Plant extract	Concentration value where agglutination occurred (mg/ml)	Haemagglutination assay titre value
<i>C. vendae</i>	1.25	0.80
<i>C. harveyi</i>	0.31	3.23
<i>K. anthotheca</i>	1.25	0.80
<i>K. wilmsii</i>	1.25	0.80
<i>L. alata</i>	1.25	0.80
<i>O. natalitia</i>	2.5	0.40
<i>P. longifolia</i>	1.25	0.80

5.4. Discussion

In this study, we have applied established *in vitro* assays in order to evaluate the antioxidant, antiplatelet and cytotoxic action of extracts of leaves of seven South African tree species. Extracts of these species had promising antifungal and bacterial activities in previous studies. This study is the first to report the antioxidant, antiplatelet and cytotoxicity activities of these plants. These natural products were able to scavenge free radicals in a concentration-dependent fashion in two separate antioxidant assays. DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants (Mensor et al., 2001). The extracts that had antioxidant activity separated poorly from the origin when applied and eluted on TLC. This could have been the result of overloading the TLC plates or due to presence of polyphenolic compounds (Naidoo et al., 2006). Polyphenols do not move well from the origin due to their high polarity leading to tight binding with normal phase silica (Davidson, 1964).

The ABTS⁺ assay described here involves the direct production of the blue/green ABTS⁺ chromophore through the reaction between ABTS and potassium sulphate, which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm (Miller et al., 1993; Re et al., 1999).

Both Trolox and L-ascorbic acid have been used as standards in quantification of antioxidant activity (Fukomoto and Mazza, 2000). The EC₅₀ of the *P. longifolia*, *K. wilmsii*, *O. natalitia*, *L. alata*, *C. harveyi* and *C. vendae* were lower than that of *Ginkgo biloba* extract (EGb 761) whose EC₅₀ is 40.72 µg/ml (Mensor et al., 2001; Bridi et al., 2001; Aderogba et al., 2004). The extract of *Ginkgo biloba* (EGb 761) has been widely

employed for its significant benefit as an antioxidant for the prevention of neurodegenerative disorders (Bridi et al., 2001). Similarly, these extracts scavenge free radicals in the DPPH TLC assay.

It has been suggested that more than one method of antioxidant testing should be used to obtain detailed knowledge of antioxidant activity of test substances, and in addition, the extrapolation of *in vitro* data to *in vivo* situations is often difficult (Aruoma, 2003). The TEAC assay is used commonly for screening compounds, food products and extracts for antioxidant activity, and is particularly useful in providing a ranking order of antioxidants (van den Berg et al., 1999) despite the limitations it carries.

The agonist adrenaline (epinephrine) induced platelet aggregation in equine platelets. The extracts of *P. longifolia*, *K. wilmsii*, *O. natalitia*, *K. anthotheca*, *L. alata*, *C. harveyi*, and *C. vendae* inhibited platelet aggregation induced by this agonist with different potencies. However, only the extract of *C. vendae* had a statistically significant ($p < 0.05$) platelet inhibitory effect.

Platelet activation is usually accompanied by a rise in cytosolic Ca^{2+} levels and this occurs through stimulation of the enzymes that are not fully functional at the low Ca^{2+} concentration present in the resting platelets (Berridge, 1993; Heemskerk and Sage, 1994). In platelets, either the stimulation of phospholipase C (PLC) or the activation of inhibitory G-protein (G_i)-linked receptors elevates the cytosolic Ca^{2+} levels (Puri et al., 1995). This takes place through the release of Ca^{2+} from internal stores or through the entry of Ca^{2+} across the plasma membrane from external medium (Berven et al., 1995; Obberghen-Schilling and Pouyssegur, 1993).

An alternate pathway of increasing the Ca^{2+} influx is through activation of the G_i -linked pathway. Agonist like adrenaline is known to inhibit adenylyl cyclase activity in platelets, leading to a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels (Puri et al., 1995). Multiple studies revealed that agents that decrease cAMP levels stimulate platelet aggregation (Niewland et al., 1994; Siess et al., 1993). This occurs through activation of α_2 -adrenergic receptors (Siess and Lapetina, 1989). α_2 -Adrenoceptors in platelets are known to be coupled to the guanine-nucleotide-binding protein G_i , which mediates inhibition of adenylyl cyclase. This mainly takes place either through an increase in Ca^{2+} influx (Shah et al., 1996) or as a result of activation of some other proteins (Musgrave and Seifert, 1995). It is possible that the extracts in this study may contain components which block these Ca^{2+} channels or act via an unknown mechanism to cause increase level of intracellular cAMP in platelets, as demonstrated through inhibition of platelet aggregation.

Anti-inflammatory drugs could affect oxidant damage in several ways. First, they might directly scavenge such reactive oxidants as $\cdot\text{OH}$ and HOCl . Most, if not all, anti-inflammatory drugs are capable of reacting quickly with OH . Hence drugs with good anti-inflammatory activity could be of use as free radical scavengers (Halliwell et al., 1988).

Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, prevention of platelet aggregation by drugs should provide effective prophylactic and/or therapeutic treatments for such diseases (Hsiao et al., 2003).

Studies have demonstrated that botany and medicine are related. Free radicals and lipid peroxidation have been suggested as potentially important causative agents of several diseases in animals and humans (Nair et al., 2003). It will be worthwhile to explore and find safer and efficacious remedies from natural sources, particularly from the relatively undiscovered and unexplored rich flora of South Africa.

The antioxidant and antiplatelet effects of these extracts, and the additional benefit of their low cytotoxicity, provide strong motivation for the development of these plants as possible drugs for the control of diseases in animals and humans. Most considerably, these properties substantiate the use of plant screening exercises for detecting disease control agents.

In conclusion, these plant species appeared to be potential sources of antioxidant and anti-inflammatory agents. Moreover, they are also good candidates as antifungal agents. From these results *L. alata* was selected for bioassay-guided fractionation procedure to isolate and characterise antifungal compounds in a continuation of this study.

Postscript

Loxostylis alata had promising pharmacological action and was therefore selected for further research work. In the next chapter the possibility of using different extractants to facilitate the isolation of the antimicrobial and anti oxidative compounds will be discussed.

Chapter 6

Preliminary screening of some fractions of *Loxostylis alata* (Anacardiaceae) for antimicrobial and antioxidant activities

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Preface

To simplify the isolation of the antifungal compounds from the *L. alata* fractions the extract was first fractionated into different fractions based on polarity in a mild solvent-solvent fractionation process. The pharmacological activities of the different fractions were determined to facilitate the isolation of the antifungal compounds. Because stimulation of the immune system could also aid in an organism combating the infections, antioxidant activity of the different fractions was also determined.

The text presented in this chapter was prepared for submission to the *African Journal of Biotechnology* for publication.

Abstract

An acetone extract of *Loxostylis alata* was separated into six fractions based on polarity by a solvent-solvent fractionation procedure and the different fractions were screened for antimicrobial and antioxidant activities. The number of antimicrobial compounds in the carbon tetrachloride (CCl₄), chloroform (CC), aqueous methanol (AM) and butanol (BT) fractions was determined by bioautography. Each fraction was loaded onto TLC plates and eluted using the two different mobile solvent systems, namely chloroform/ethyl acetate/formic acid (5:4:1) [CEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW]. Relative front (R_f) values of active compounds against bacteria ranged from 0.4-0.9 in CEF and EMW solvent system. Similarly, the R_f values of compounds active against fungi are in the range of 0.7-0.9. Hexane, carbon tetrachloride, chloroform, aqueous methanol and butanol fractions showed areas of inhibition against bacterial organisms, while only hexane and carbon tetrachloride fractions depicted areas of fungal growth inhibition on their chromatograms. The CCl₄ extract was active against six out of the 9 microbial strains used and was particularly active against *S. aureus*, *E. faecalis*, *A. fumigatus*, *C. albicans*, *C. neoformans* and *M. canis* with MIC of 0.04, 0.04, 0.1, 0.1, 0.06 and 0.03 mg/ml, respectively. *Microsporium canis* was the most sensitive organism with the lowest average MIC of 0.16 mg/ml. Qualitative antioxidation using DPPH and qualitative assay using both ABTS and DPPH radicals revealed the presence of three antioxidant compounds in the AM and BT fractions, while the water fraction had only one antioxidant compound. However, the concentration of the antioxidant compounds is more in the AM and BT fractions as revealed by their pronounced colour intensity on the TLC plates. The water fraction had more free radical

scavenging effect against DPPH with EC₅₀ value of 0.62 ± 0.03 µg/ml. The lower the EC₅₀ value of an extract, the more effective its antioxidant activity. In a similar manner, TEAC value of water fraction was 2.97, which further indicates superior free radical scavenging effect of the fraction against ABTS radical. The greatest reduction of reactive oxygen species (ROS) production induced by diclofenac was also achieved by the water fraction which on the overall shows that the water fraction contains more antioxidants.

Keywords: Medicinal plant, antibacterial, antifungal, serial microdilution, minimum inhibitory concentration, antioxidants

6.1. Introduction

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants have been assayed for medical activity. It is estimated that there are about 250,000 species of plants on Earth (Verpoorte, 1998). Such a wealth of identified species, which have not been thoroughly investigated, constitutes an enormous potential source of plant-derived chemicals useful to man (Cowan, 1999). Plants have a long history of use on the African continent for the treatment of various diseases and complaints. In certain African countries, up to 90% of the population still relies exclusively on plants as a source of medicines (Hostettmann et al., 2000). In the global context, natural products and their derivatives form about 50% of drugs in clinical use with about 25% coming from higher plants (Farnsworth and Morris, 1976; Buwa and Staden, 2006; O'Neill and Lewis, 1993).

In a tree screening project of the Phytomedicine Programme (University of Pretoria), plant species with activity against the animal pathogenic fungus *Cryptococcus neoformans* were selected for further testing against *Aspergillus fumigatus* which is an important fungus in the poultry industry. The crude extract of *Loxostylis alata* was one of the plants tested and found to have activity against the tested pathogen.

Loxostylis alata A.Spreng. ex Rchb belongs to the family Anacardiaceae (Coates-Palgrave, 2002). In South Africa, the bark and leaves of *L. alata* are used in traditional medicine during childbirth (Pooley, 1993) and also to stimulate the immune system (Pell, 2004). Compounds like 3-(8Z-pentadecenyl) phenol (ginkgol) and 6-(8Z-pentadecenyl) salicylic acid (ginkgolic acid) were isolated from the leaves of *L. alata* (Drewes, et al., 1998).

The aim of the present study was to further assess the antimicrobial properties of fractions of *L. alata* against some important animal pathogenic fungi and bacteria, and also to test the antioxidant activity of the fractions. This is done with a view of isolating compound(s) that are active.

6.2. Materials and methods

6.2.1. Plant collection and processing

Loxostylis alata leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. The plant leaves were collected in April 2007. 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; PRU PRU96508 was deposited at the Schweikert Herbarium of the Department of

Plant Sciences, University of Pretoria, South Africa. 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 (technical grade-Merck) in a macerating bottle. The bottle was shaken for 1 hour in Labotec model 20.2, shaking machine at low speed. Lower speed extraction method was employed for about 24 hours in order to allow the solvent penetrate deeper into the plant tissues so as to extract more of the plant compounds (Silva et al., 1998). After allowing the mixture to settle for 15 min the supernatant was filtered into clean, labelled containers. The extraction process was repeated three times to exhaustively extract the same plant material, and the fractions were combined. The solvent was removed under reduced pressure using a rotary evaporator (Büchi Rotavapor R-114, Switzerland). Seventy grams of the extract was fractionated using solvents of varying polarities (Suffness and Douros, 1979).

6.2.2. Microorganisms and medium

Fungal organisms used were *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporium canis* and *Sporothrix schenckii*. All fungal organisms were isolated from animal clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth. The bacteria used were the Gram-positive bacteria: *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 29213), and the Gram-negative bacteria: *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4°C and cultured in MH broth at 37 °C.

6.2.3. Minimum inhibitory concentration (MIC) determination

The MIC was determined using a serial microdilution assay (Eloff, 1998a). The fractions were individually dissolved in acetone to a concentration of 10 mg/ml. One hundred µl of each plant extract were serially diluted 2-fold with autoclaved distilled water in 96-well microtitre plates. Two millilitres of concentrated fungal or bacterial cultures grown overnight at 37 °C were transferred to 100 ml of fresh SD or MH broths for fungi and bacteria, respectively, and 100 µl of the resultant culture was added to each well. Densities of bacterial cultures used for the screening were as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml and that of fungi were *A. fumigatus*, 8.1×10^4 cfu/ml; *C. albicans*, 2.5×10^4 cfu/ml; *C. neoformans*, 2.6×10^4 cfu/ml; *M. canis*, 1.5×10^5 cfu/ml; *S. schenckii*, 1.4×10^5 cfu/ml. Amphotericin B and Gentamicin (Virbac®) were used as positive controls for

fungi and bacteria, respectively. Acetone was used as the negative control agent in all assays. After incubating bacteria overnight at 37°C, *p*-iodonitrotetrazolium violet (INT) at a concentration of 0.2 mg/ml was used as an indicator of bacterial growth. Forty µl of INT was added to each of the microtitre wells. Thereafter, the plates were incubated at 37°C for 1 hour. For the fungal assays, INT was added immediately after the serial dilution and plates were incubated as described earlier. MIC values were assessed after 1 and 2 hour periods for bacteria and 24 and 48 hours for fungi. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of fraction that inhibits bacterial growth as indicated by INT formazan formation.

6.2.4. Bioautography

Ten µl (10 mg/ml) of each fraction were loaded onto TLC plates and eluted using the two different mobile solvent systems, namely chloroform/ethyl acetate/formic acid (5:4:1): [CEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW] (Kotze and Eloff, 2002). The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of fungal organisms grown on SD agar were each transferred into 250 ml of freshly prepared SD broth using a sterile swab so as to contain approximately 1×10^9 organisms per ml of actively growing fungi. In the case of bacteria, overnight cultures grown on MH broth were used. The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out using a biosafety Class II cabinet (Labotec, SA) for fungi, and a laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) (Begue and Klein, 1972) and further incubated overnight or longer in the case of *S. schenckii* and *M. canis*. White areas or spots indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms.

6.2.5. Evaluation of antioxidant activity

Qualitative antioxidant screening was by using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (Takao et al., 1994). TLC plate loaded with 100 µg of each of the 6 fractions of *L. alata* was developed in CEF solvent system and sprayed with 0.2% DPPH in methanol. Compounds with antioxidant activity were visualized as yellow bands against a purple background (Bors et al., 1992).

The antioxidant activity was quantified by spectrophotometry using two radicals, ABTS and DPPH. In the ABTS method, the TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (Re et al., 1999) was determined. This was based on the scavenging of

the ABTS radical into a colourless product by antioxidant substances. The blue/green chromophore ABTS⁺ was produced through the reaction between ABTS and potassium sulphate. The absorbance was read at 734 nm using a Versamax microplate reader (Molecular Devices). TROLOX is a Vitamin-E analogue and was used as a standard in this assay. An extract with a TEAC value of 1 indicates an equivalent antioxidant value of TROLOX. Decrease or increase in antioxidant activity is depicted by a lower or upper value of TEAC, respectively. The DPPH free radical assay (Mensor et al., 2001) was also employed to quantify antioxidant compounds in the fractions. Briefly, 10 µl of 0.3 mM DPPH in ethanol was added to 25 µl of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Appropriate blank and negative control solutions were prepared for each test. L-ascorbic acid (Vitamin C) was used as positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AA %) using the formula:

$$AA \% = 100 - \left\{ \frac{(Abs_{\text{sample}} - Abs_{\text{blank}}) \times 100}{Abs_{\text{control}}} \right\}$$

Where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and Abs_{control} is the absorbance of the control. The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test fractions (µg/ml) against the mean percentage of the antioxidant activity obtained from three replicate assays using Microsoft Office Excel. EC_{50} values obtained from the regression lines had coefficient of determination $r^2 \geq 95\%$. A lower EC_{50} value indicates high antioxidant activity.

6.2.6. ROS studies

Vero monkey kidney cells were grown in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). The cells were incubated for 12 hours with diclofenac (50 µM) per well in the presence or absence of different concentrations of the fractions. Diclofenac is a potent stimulant for ROS production in kidney cells (Naidoo and Swan, 2009). Thereafter, the cell cultures were incubated with dichlorofluorescein diacetate (DCFH-DA) as an indicator for intracellular ROS production, for 30 min and washed with PBS. The absorbance was measured at 504 nm (Somogyi et al., 2007). The degree of ROS production was evaluated as a percentage of ROS production of treated to untreated wells on the same plate. Tests were carried out in quadruplicate and each experiment was done in triplicate.

6.3. Results and discussion

6.3.1. Quantitative yield obtained after solvent-solvent fractionation

The acetone extract of *L. alata* was resolved into 6 different fractions using chloroform, carbon tetrachloride, hexane, aqueous methanol, butanol and water (Suffness and Douros, 1979). A schematic representation of the solvent-solvent resolution is shown in Figure 6.1. The butanol fraction had the greatest quantity of material from the crude acetone extract with a percentage yield of 47%, while chloroform extracted the least material with a yield of 1.30%. The recovery of the fractions from the original crude extract was 85%. It therefore indicates that 15% of the total mass of the crude extract was lost during fractionation. In some cases a pellicle is formed between different phases and the pellicle was discarded probably explaining the loss.

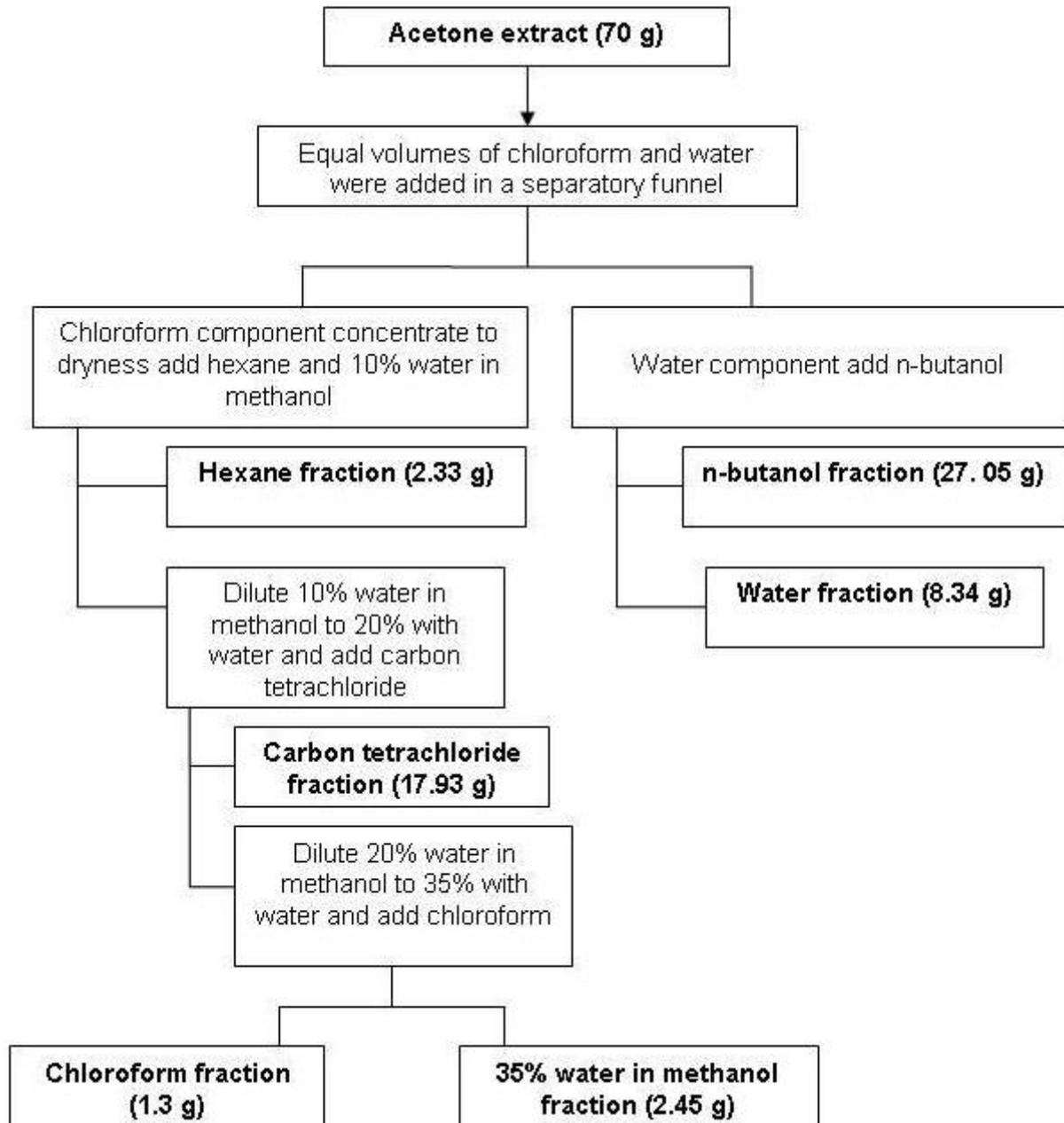


Figure 6.1. Stepwise procedure for the fractionation of *L. alata* acetone extract. Text in bold represents final collected fraction.

6.3.2. Bioautography

The bioautographic results against *S. aureus* and *A. fumigatus* are shown in Figure 6.2. Hexane, carbon tetrachloride, chloroform, aqueous methanol and butanol fractions showed areas of inhibition against bacterial organisms, while only hexane and carbon tetrachloride fractions had areas of fungal growth inhibition on their chromatograms. R_f values of active compounds against bacteria ranged from 0.4-0.9 in CEF and EMW solvent system. Similarly, the R_f values of compounds active against fungi are in the range of 0.7-0.9. Compounds that are active against bacteria in the hexane and carbon tetrachloride fractions also had activity against fungi as shown by their same R_f values. Inhibition of microbial growth was only seen clearly on TLC plates separated by CEF and EMW.

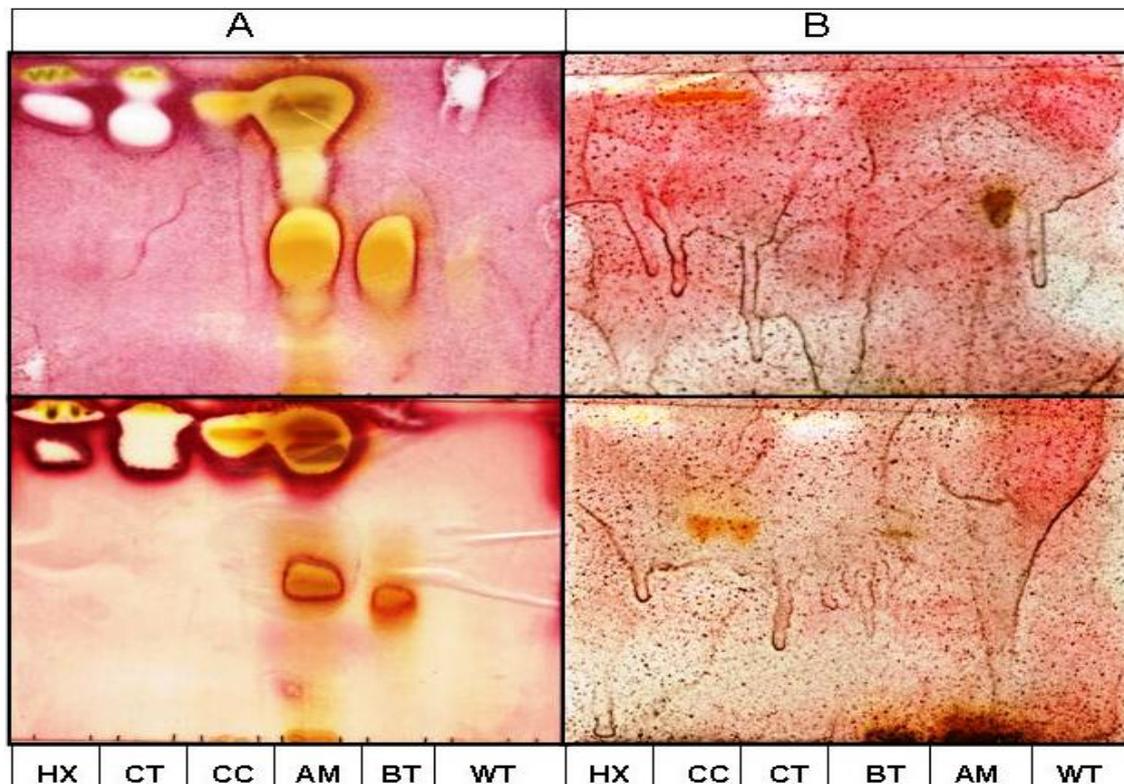


Figure 6.2. Hexane (HX), carbon tetrachloride (CT), chloroform (CC), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *L. alata* separated on TLC plates using CEF and EMW and sprayed with *S. aureus* (A) and *A. fumigatus* (B) and 24 hours later by INT. White areas indicate inhibition of microbial growth after 60 minutes of incubation at 37°C.

6.3.3. Minimum inhibitory concentration of fractions

The MICs results are presented in Table 6.1. The chloroform (CHCl₃), and carbon tetrachloride (CCl₄) fractions from *L. alata* leaf had interesting activity against both bacteria and fungi. The CCl₄ extract was active against six out of the 9 microbial strains used and was particularly active against *S. aureus*, *E. faecalis*, *A. fumigatus*, *C. albicans*, *C. neoformans* and *M. canis* with MIC of 0.04, 0.04, 0.1, 0.1, 0.06 and 0.03 mg/ml, respectively. Similarly, the CHCl₃ extract had activity against *E. faecalis*, *C. neoformans* and *M. canis* with respective MIC values of 0.08, 0.06 and 0.1 mg/ml. The CCl₄ extract was the most active, with an average MIC of 0.12 mg/ml against all the tested pathogens. *M. canis* was the most sensitive organism with lowest average MIC of 0.16 mg/ml, while *C. albicans* was the least affected with an MIC value of 1.53 mg/ml. The water extract of *L. alata* possessed the least activity against the tested pathogens with average MIC of 1.63 mg/ml. Plant drugs prepared using water as solvent do not usually extract the more lipophilic compounds (Buwa and Staden, 2006). Perhaps the antimicrobial activity of *L. alata* resides in the more lipid-soluble components as was presented in the results of this study. The non-polar fractions possessed the highest antimicrobial effect. Interestingly, the Gram-positive bacteria were more sensitive to the fractions than the Gram-negative bacteria. Gram-negative bacteria are relatively resistant to plant fractions owing to the presence of an outer membrane which is known to present a barrier to penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes, which are capable of breaking down foreign molecules introduced from outside (Nikaido, 1996). The antibacterial and antifungal properties of the fractions of *L. alata* were not as effective as that of the reference drugs gentamicin and amphotericin B (Amp B). However, the reference drugs are pure compounds, which perhaps may be responsible for their high activity. The active compounds when isolated in their pure forms may prove to have comparable or higher activities than the reference compounds.

6.3.4. Total activity and evidence for synergism

The total activity (TA) of the fractions was also calculated (Table 6.1), Total activity is calculated by dividing the quantity present in the extract in mg with the MIC value in mg/ml (Eloff, 2004). This value indicates the volume to which the active constituents present in the fraction can be diluted and still inhibit the growth of the test organism. The TA of the fractions of *L. alata* ranged from 984-597 667 ml. The CCl₄ fraction was the most active with TA value of 597 667 ml against *M. canis*. It therefore implies that if one gram of the CCl₄ fraction is dissolved in 597 667 ml of acetone, the solution obtained will still inhibit the growth of *M. canis*. Furthermore, TA calculation will detect at each step loss in biological activity.

More importantly, this will allow the detection of synergism between the plant compounds if any exist (Eloff, 2004). The crude acetone extract of *L. alata* had TA values of 1 166 666, 437 500, 437 500, 225 807, 1 400 000, 225 807, 333 333, 1 000000 and 1750000 ml against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii*, respectively. The results herein indicated a combined TA of the fractions against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii* to be 509718, 522528, 95027, 109530, 205856, 198982, 355034, 770873 and 160449, respectively. There were lost in total activity in the fractions compared to the crude extract against *S. aureus*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*, *M. canis* and *S. schenckii* of 56%, 92%, 51%, 85%, 12%, 23% and 91%, respectively. Against *E. faecalis* and *C. neoformans* however, there was a slight increase in activity. Because the method used to determine the MIC was based on a two fold serial dilution changes in the activity of c. 50% are probably not significant and may be due to methodological variation.

The results indicate a substantial loss of activity against especially *E. coli*, *A. fumigatus* and *S. schenckii*. In comparing the mass recovered in the different fractions compared to the 70 g of the crude, it represented a loss of about 15%. The loss of > 85% of activity against *E. coli*, *A. fumigatus* and *S. schenckii* is strong evidence for the existence of synergism in the crude acetone extract of *L. alata* against these pathogens.

It also indicates that it may be more efficient to use a crude extract rather than one of the fractions obtained by solvent-solvent fractionation because it would be much cheaper to produce and extract with a higher activity.

Table 6.1. Minimum inhibitory concentrations (MIC) and total activity (TA) of fractions of *Loxostylis alata* against some pathogenic bacteria and fungi (average of three determinations).

Fractions	Mass (mg)	Microorganism																	
		MIC (mg/ml)									TA (ml)								
		SA	EF	EC	PA	AF	CA	CN	MC	SS	SA	EF	EC	PA	AF	CA	CN	MC	SS
CHCl ₃	1300	0.63	0.08	0.31	0.63	0.94	0.63	0.06	0.1	0.63	2063	16250	4194	2063	1383	2063	21667	13000	2063
CCl ₄	17930	0.04	0.04	0.31	0.24	0.1	0.1	0.06	0.03	0.2	448250	448250	57839	74708	179300	179300	298833	597667	89650
Hexane	2330	0.08	0.11	0.63	0.94	0.24	0.94	0.4	0.34	0.71	29125	21182	3698	2479	9708	2479	5825	6853	3282
Butanol	27050	1.25	1.25	1.25	1.25	2.5	2.5	1.25	0.2	0.47	21640	21640	21640	21640	10820	10820	21640	135250	57553
Aq. MeOH	2460	1.25	1.25	2.5	1.25	1.88	2.5	1.4	0.18	0.71	1968	1968	984	1968	1309	984	1757	13667	3465
Water	8340	1.25	0.63	1.25	1.25	2.5	2.5	1.57	1.88	1.88	6672	13238	6672	6672	3336	3336	5312	4436	4436
Amp. B	-	NT	NT	NT	NT	0.00063	0.00031	0.00016	0.00016	0.00016	-	-	-	-	-	-	-	-	-
Gentamicin	-	0.0063	0.0031	0.015	0.015	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	-
Average MIC	-	0.75	0.56	1.04	0.93	1.36	1.53	0.79	0.46	0.77	-	-	-	-	-	-	-	-	-
Sum of TA	-	-	-	-	-	-	-	-	-	-	509718	522528	95027	109530	205856	198982	355034	770873	160449
Crude	70000	0.06	0.16	0.06	0.31	0.05	0.31	0.21	0.07	0.04	1166666	437500	1166666	225807	1400000	225807	333333	1000000	1750000
% loss in activity	-	-	-	-	-	-	-	-	-	-	-	56	-19	91	51	85	-12	7	91

Aq. MeOH = Aqueous methanol

S. aureus (SA); *E. faecalis* (EF); *E. coli* (EC); *P. aeruginosa* (PA); *A. fumigatus* (AF); *C. albicans* (CA); *C. neoformans* (CN); *M. canis* (MC); *S. schenckii* (SS)

NT = not tested

Amp. B= Amphotericin B

6.3.5. Qualitative analysis of antioxidant compounds in different fractions

Antioxidants are substances that assist greatly to stimulate the immune system to combat microbial infection (Knight, 2000). In view of that and based on ethnobotanical information that *L. alata* is used to stimulate immune response (Pell, 2004), the antioxidant activity of the different fractions was also determined. Antioxidants when present at low concentrations compared with that of an oxidizable substrate (carbohydrate, lipid, DNA or protein), significantly delay or prevent the oxidation of that substrate (Halliwell, 1990). Oxygen radicals and lipid peroxides are implicated in the aetiology of many diseases (Halliwell et al., 1988). The potential value of antioxidants has prompted investigators to search for compounds with potent antioxidant activity. The use of the DPPH radical as TLC spray reagent proposed for the first time in 1994 (Takao et al., 1994) for screening antioxidants in marine bacteria, appears to be also well suited for the detection of antioxidants in crude plant fractions or pure compounds isolated from plant material.

In the qualitative assay, the aqueous methanol, butanol and water fractions showed antioxidant compounds with R_f values ranging from 0.1-0.6 (Figure 6.3). The aqueous methanol fraction had three major antioxidative compounds but not one of the had the same R_f value as the antifungal compounds found by bioautography. This is not surprising because antioxidant compounds are usually polar in nature and in the experience of the Phytomedicine Programme antimicrobial compounds are usually relatively non-polar. These results also indicate the potential of using a crude extract rather than the separated fractions because the crude would contain both antimicrobial and anti oxidant activities.

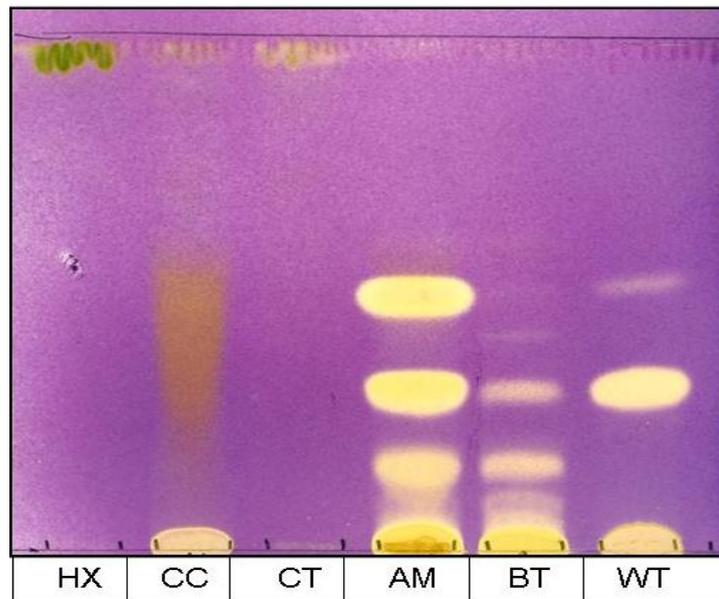


Figure 6.3. Chromatogram of hexane (HX), chloroform (CC), carbon tetrachloride (CT), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *Loxostylis alata* separated with CEF mobile phase and sprayed with 0.2% DPPH. Antioxidant compounds are indicated by yellow areas over purplish background.

6.3.6. Determining qualitative antioxidant activity

Qualitative antioxidant assays had a good correlation with the qualitative assay, with the aqueous methanol, butanol and water fractions having significant ($p \leq 0.05$) antioxidant activity in the DPPH assay (Table 6.2). The aqueous methanol, butanol and water fractions had respective EC_{50} values of 1.82 ± 0.03 , 1.05 ± 0.06 and 0.62 ± 0.03 . The chloroform, carbon tetrachloride and hexane fractions had EC_{50} values of 2.76 ± 0.18 , 3.42 ± 0.09 and $12.42 \pm 0.13 \mu\text{g/ml}$, respectively. The lower the EC_{50} value of an extract, 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 is indicative of good antioxidant activity, as it is higher than that of the reference compound. Polyphenols, although not the only compounds, are the major plant secondary metabolites with antioxidant activity. The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties (Galato et al., 2001), which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Perhaps similar compounds are present in the aqueous methanol, butanol and water fractions of *L. alata*, which could be responsible for their antioxidant action.

Table 6.2. Fraction yield and antioxidant activity of fractions of *L. alata*.

Fraction	Fraction yield (%)	Antioxidant values		
		TEAC	DPPH (EC ₅₀ ± SEM, µg/ml)	DPPH ascorbic acid equivalent*
Chloroform	1.30	0.45	2.76 ± 0.18 ^b	0.34
Carbon tetrachloride	25.61	0.12	3.42 ± 0.09 ^b	0.27
Hexane	3.33	0.56	12.42 ± 0.13 ^c	0.075
Butanol	38.64	2.21	1.05 ± 0.06 ^a	0.90
Aqueous methanol	3.57	1.55	1.82 ± 0.03 ^a	0.52
Water	11.92	2.97	0.62 ± 0.03 ^a	1.52
Trolox	-	1.0	-	-
L-ascorbic acid	-	-	0.94 ± 0.11 ^a	1.0

Means within the same column and with different superscript letters differ significantly ($p \leq 0.05$)

* calculated by dividing ascorbic value with value for fraction

6.3.7. Reactive oxygen species inhibition by fractions

Diclofenac inhibits the transport of malate and glutamate into mitochondria via the malate–aspartate shuttle, the most important mitochondrial metabolite transport system in the kidney, liver, and heart (Ng et al., 2006). Another plausible mechanism explaining its toxic effect is its ability to cause production of ROS (Naidoo and Swan, 2009). ROS production by Vero monkey kidney cells was greatly reduced by incorporating the fractions of *L. alata* (Figure 6.4). The greatest reduction of ROS production was also achieved by the water fraction. Reduction of ROS production by the fractions, however, suggests that they can either reduce diclofenac-induced ROS production or neutralize ROS when produced. Since diclofenac also interfered with uric acid (an important antioxidant) transport in the kidneys (Naidoo and Swan, 2009). It will be reasonable to assume also that the fractions have an important role in preventing the interference of uric acid transport in the kidneys, hence another important mechanism of their antioxidant action.

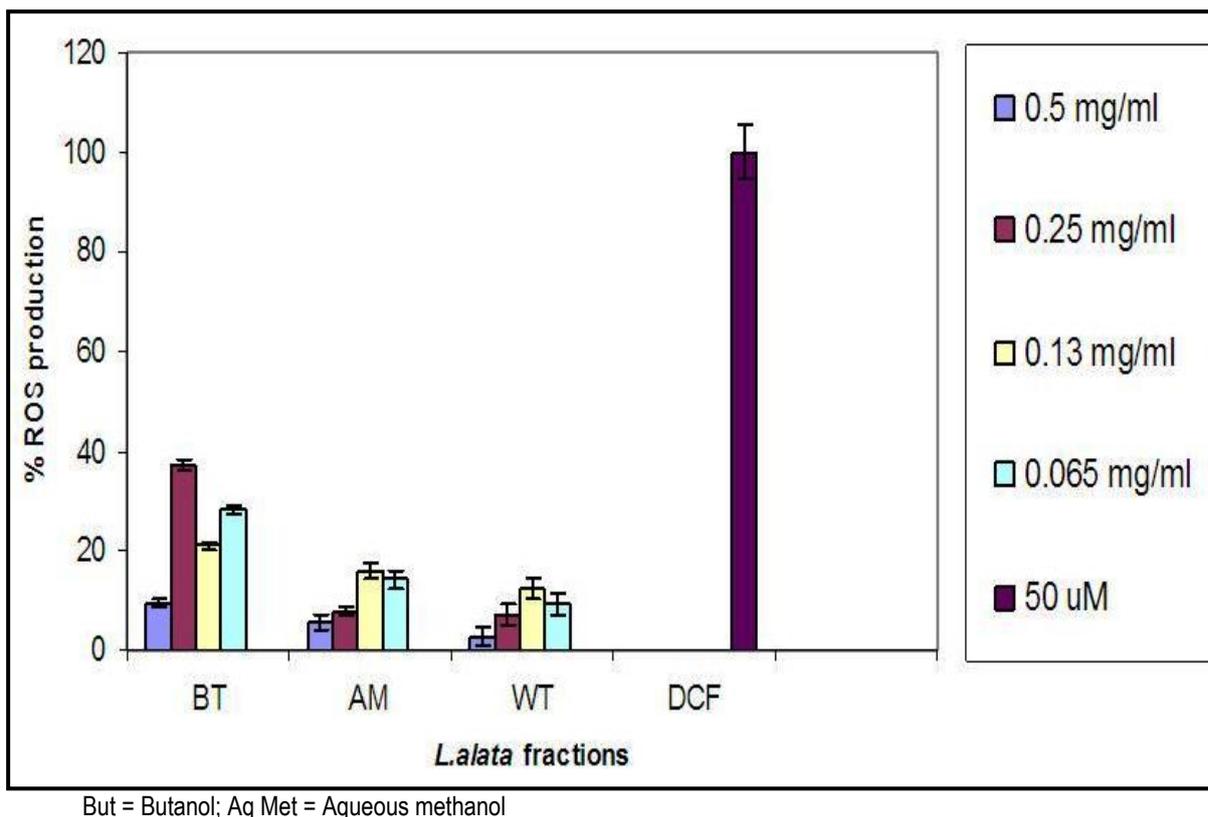


Figure 6.4. Inhibition of ROS production in Vero monkey kidney cell by *L. alata* fractions.

6.4. Conclusion

The presence of antimicrobial and antioxidant activities in the fractions of *Loxostylis alata* suggests that this plant may be a source of bioactive substances with multifaceted activity. The presence of antioxidant compounds in this plant may be responsible for its immunostimulant action, which is employed in traditional medicine (Pooley, 1993). Although the attempt to increase the antifungal activity by solvent-solvent fractionation activity did not yield good results, in terms of antifungal activity, it did facilitate efforts to isolate the antifungal compound from the crude extract.

Postscripts

The carbon tetrachloride fraction of *Loxostylis alata* had a higher activity than all the other fractions against *Aspergillus fumigatus* and other tested pathogens. The fraction was therefore chosen and subjected to column chromatography to isolate the active compound(s) present. Knowledge of the compound(s) that are active may assist in standardizing and formulating safer and effective dosages.