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Full Length Research Paper

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

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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 (CCl_4), chloroform (CC), aqueous methanol (AM) and butanol (BT) fractions were determined by bioautography. Each fraction was loaded onto thin layer chromatography (TLC) plates and eluted using 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 were 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 CCl_4 extract was active against six out of the nine microbial strains used and was particularly active against *Staphylococcus aureus*, *Enterococcus faecalis*, *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans* and *Microsporum canis* with minimum inhibitory concentration (MIC) of 0.04, 0.04, 0.1, 0.1, 0.06 and 0.03 mg/ml, respectively. *M. canis* was the most sensitive organism with the lowest average MIC of 0.16 mg/ml. Qualitative antioxidation using diphenylpicryl- hydrazyl (DPPH) and qualitative assay using both 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate (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 was 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 EC_{50} value of 0.62 ± 0.03 µg/ml. The lower the EC_{50} 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 overall shows that the water fraction contains more antioxidants. The presence of antimicrobial and antioxidant activities in the fractions of *L. alata* suggests that this plant may be a source of pharmacologically active substances with beneficial actions towards disease control and treatment in animals and humans.

Key words: Medicinal plant, antibacterial, antifungal, serial microdilution, minimum inhibitory concentration, antioxidants.

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 medicinal activeity. 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; O'Neill and Lewis, 1993; Buwa and Staden, 2006).

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.

L. 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) have been isolated from the leaves of *L. alata* (Drewes, et al., 1998). The aim of the present study is 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.

MATERIALS AND METHODS

Plant collection and processing

L. 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.

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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 five litres of acetone (technical grade-Merck) in a macerating bottle. The bottle was shaken for 1 h in Labotec model 20.2, shaking machine at low speed. Lower speed extraction method was employed for about 24 h in other 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).

Microorganisms and medium

Fungal organisms used were *A. fumigatus*, *Candida albicans*, *C. neoformans*, *Microsporum 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.

Minimum inhibitory concentration (MIC) determination

The MIC was determined using a serial microdilution assay (Eloff, 1998). 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. 40 µl of INT was added to each of the microtitre wells. Thereafter, the plates were incubated at 37°C for 1 h. 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 h periods for bacteria and 24 and 48 h for fungi. The MIC is defined as the lowest concentration of fraction that inhibits bacterial growth as indicated by INT formazan formation.

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 bio-safety 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 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.

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 quantified 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 min. 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:

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

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 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.

Reactive oxygen species (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 h 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 dichlorofluorescin diacetate (DCFH-DA) as an indicator for intracellular ROS production, for 30 min and washed with phosphate buffered saline (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.

RESULTS AND DISCUSSION

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 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.

Bioautography

The bioautographic results against *S. aureus* and *A. fumigatus* are shown in Figure 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.

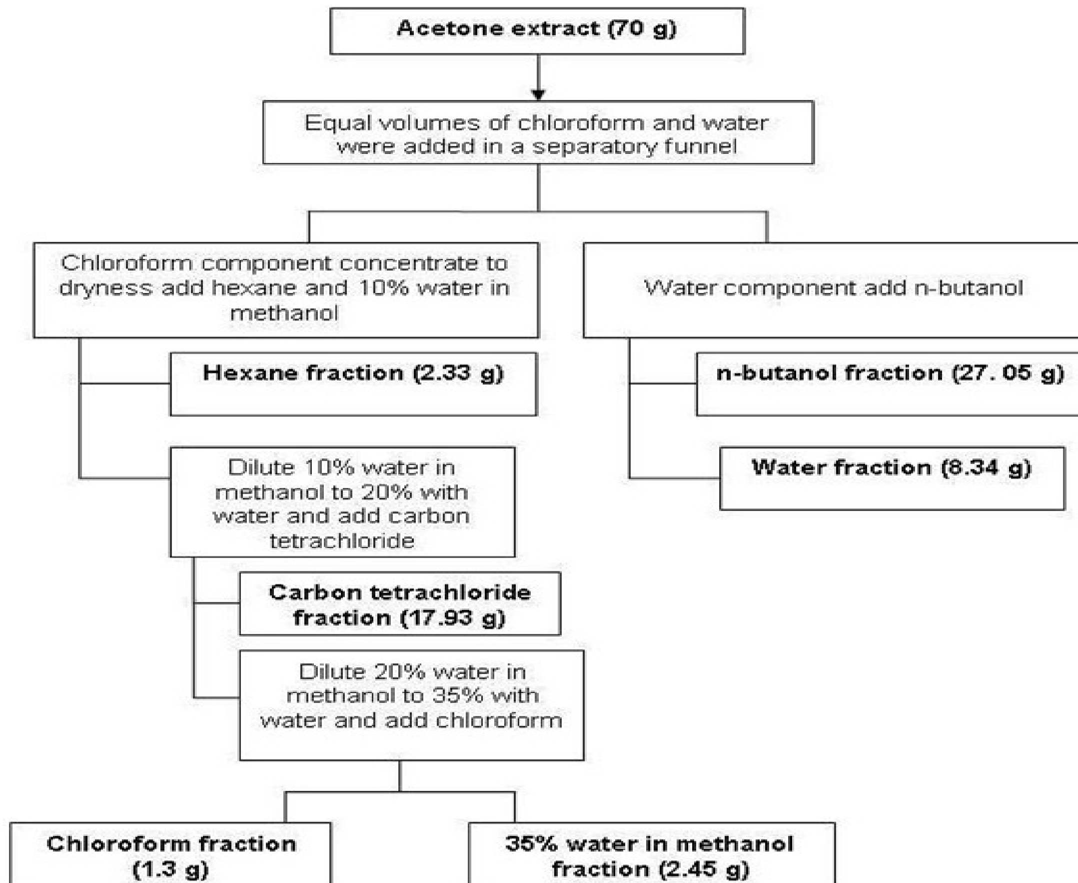

Figure 1. Stepwise procedure for the fractionation of *L. alata* acetone extract. Text in bold represents final collected fraction.

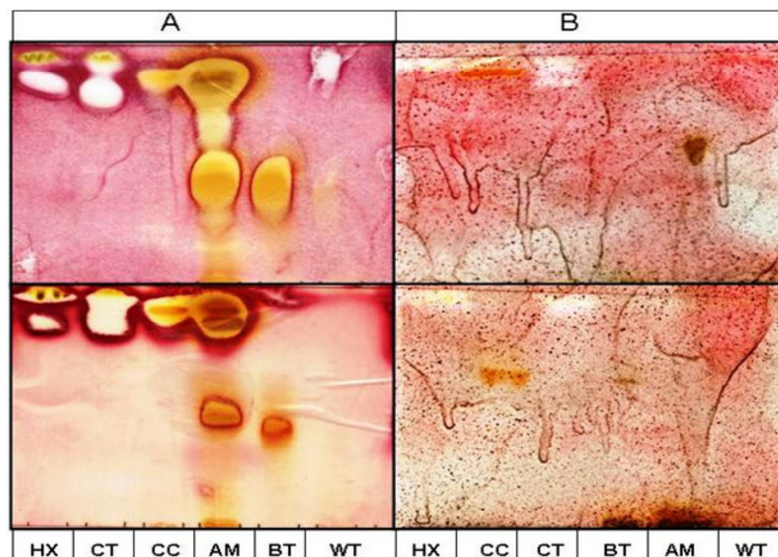

Figure 2. Hexane (HX), carbon tetrachloride (CT), chloroform (CC), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *L. alata* separated on TLC plates using CEF and EMW and sprayed with *S. aureus* (A) and *A. fumigatus* (B) and 24 h later by INT. White areas indicate inhibition of microbial growth after 60 min of incubation at 37°C.

Minimum inhibitory concentration of fractions

The MICs results are presented in Table 1. The chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4) fractions from *L. alata* leaf had interesting activity against both bacteria and fungi. The CCl_4 extract was active against six out of the nine 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 $CHCl_3$ 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 CCl_4 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 an average MIC of 1.63 mg/ml. Plant drugs prepared using water as solvent do not usually extract the more lipophilic compounds (Buwa and van 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.

Total activity and evidence for synergism

The total activity (TA) of the fractions was also calculated (Table 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 – 597667 ml. The CCl_4 fraction was the most active with TA value of 597 667 ml against *M. canis*. It therefore implies that if one gram of the CCL_4 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. Since the method used to determine the MIC was based on a two fold serial dilution, changes in the activity of approximately 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.

Qualitative analysis of antioxidant compounds in different fractions

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Antioxidants can help 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 were also determined. Antioxidants when present at low concentration 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

Table 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; NT = not tested; Amp. B= Amphotericin B; *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).

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 3). The aqueous methanol fraction had three major antioxidative compounds but not one of them 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 group, University of Pretoria, 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 antioxidant activities.

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 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 ± 0.13 µg/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.

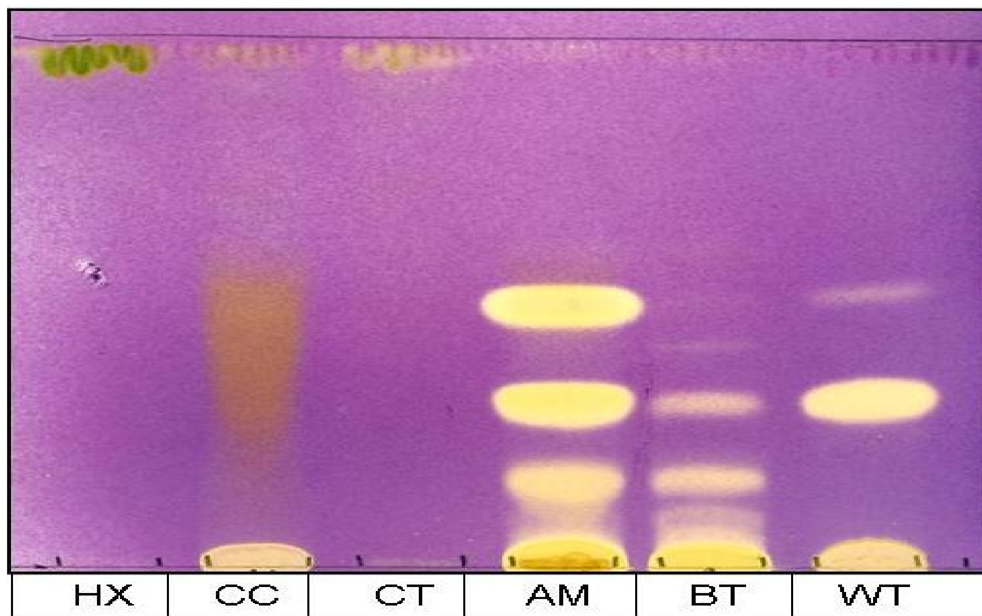

Figure 3. Chromatogram of hexane (HX), chloroform (CC), carbon tetrachloride (CT), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *Loxostylis alata* separated with CEF mobile phase and sprayed with 0.2% DPPH. Antioxidant compounds are indicated by yellow areas over purplish background.

Table 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.

Perhaps similar compounds are present in the aqueous methanol, butanol and water fractions of *L. alata*, which could be responsible for their antioxidant action.

Reactive oxygen species inhibition by fractions

Diclofenac inhibits the transport of malate and glutamate into the 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 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 mecha nism of their antioxidant action.

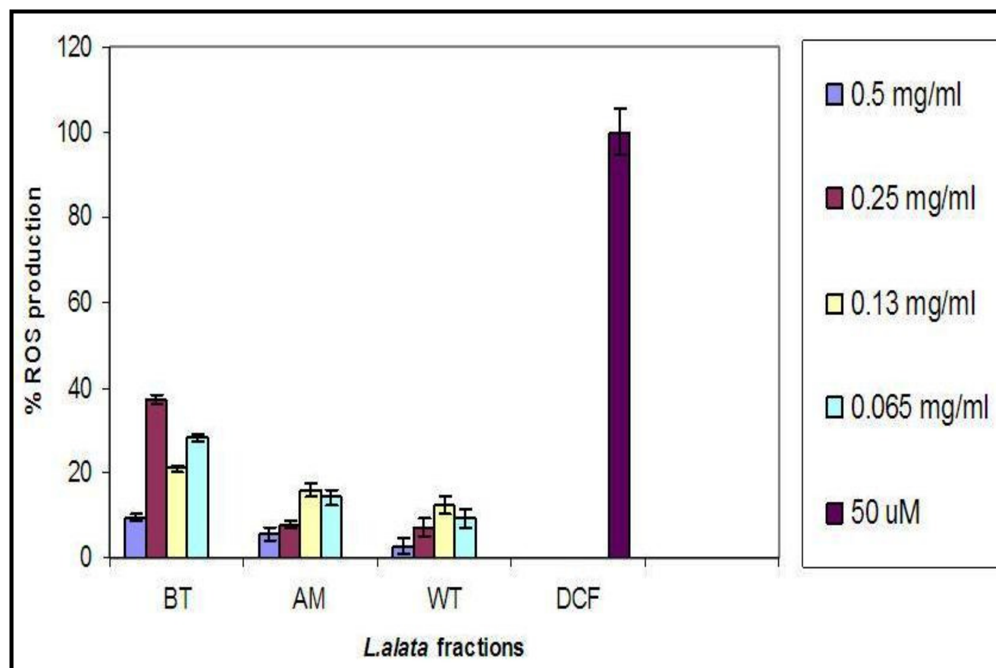

Figure 4. Inhibition of ROS production in Vero monkey kidney cell by *L. alata* fractions. BT = Butanol fraction; AM = Aqueous methanol fraction; WT = Water fraction; DCF = Diclofenac.

Conclusion

The presence of antimicrobial and antioxidant activities in the fractions of *L. 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, it facilitated efforts to isolate the antifungal compound from the crude extract.

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Abbreviations: CCl_4, Carbon tetrachloride; **CC,** chloroform; **AM,** aqueous methanol; **BT,** butanol; **TLC,** thin layer chromatography; **CEF,** chloroform/ethyl acetate/formic acid; **EMW,** ethyl acetate/methanol/water; **Rf,** relative front; **MIC,** minimum inhibitory concentration; **DPPH,** diphenylpicryl- hydrazyl; **ROS,** reactive oxygen species; **SD,** Sabouraud dextrose; **MH,** Mueller Hinton; **INT,** *p*-iodonitrotetrazolium violet; **MEM,** minimal essential medium; **PBS,** phosphate buffered saline; **TA,** total activity.

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