

## Biological Activities of Compounds Isolated from *Loxostylis alata* (Anacardiaceae) Leaf Extract

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### Abstract

In a random screening of antimicrobial activity of tree leaves, *Loxostylis alata* (Spreng.) f. ex Reichb had shown activity against the pathogenic fungi; *Cryptococcus neoformans*. This stimulated further interest to investigate its antimicrobial activity. Extracts and compounds isolated from leaves of *Loxostylis alata* by bioassay-guided fractionation were evaluated for their *in vitro* antimicrobial, anti-inflammatory (Cyclooxygenase-1 and -2) activities and 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 a serial dilution microplate assay. 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 isolates of *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporium canis* and *Candida albicans*. A bioassay guided fractionation of the crude extract yielded two antimicrobial compounds namely, Lupeol 1 and  $\beta$ -sitosterol 2. In addition  $\beta$ -sitosterol exhibited selective inhibition of COX-1 (IC<sub>50</sub> = 55.3  $\pm$  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

### Introduction

About 60% of the world's population relies almost entirely on herbal remedies to treat different ailments [1]. Plant derived drugs have for ages been regarded as an essential source of therapeutically effective medicines and still remain important with about 25% of the drugs prescribed worldwide being herbal formulations [1]. 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 [1]. In 1997, the world market for phytomedicine products was estimated at US\$10 billion [2]. Recent development of natural products into chemotherapeutic armamentarium include the antimalarial drug artemisinin and the anticancer agents taxol, docetaxel and camptothecin. Therefore, the use of natural products is one of the most successful strategies for the discovery of new medicines [3]. Where natural compounds failed to show sufficient activity or were too toxic, they may serve as lead compounds, allowing the design and rational planning of new drugs that could be more effective [4].

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 [5].

*Loxostylis alata* is a member of the family Anacardiaceae [6]. The bark and leaves of *Loxostylis alata* are used in South African traditional medicine during childbirth to relieve pain [7] and also to stimulate the immune system [8] but no indication of its use in combating microbial infections could be found. Ginkcol (3-(8Z-pentadecenyl) phenol) and ginkgolic acid (6-(8Z-pentadecenyl) salicylic acid) were previously isolated from the leaves of *L. alata* [9]. To date no studies have been carried out on the plant to determine its pharmacologically active constituents.

In a screening study of seven South African plant species active against *Cryptococcus neoformans*, *Loxostylis alata* had the highest

activity [10]. The primary objective of this study was to isolate the antimicrobial compound(s) from the crude extracts of *Loxostylis alata* based on bioautography assays. The isolated compounds were investigated further for their anti-inflammatory (cyclooxygenase-1 and -2), mutagenic (*Salmonella*/microsome assay) and cytotoxic (MTT assay) effects in order to find a safer, efficacious and cheap chemical compounds that could be used to treat microbial infections and other related diseases.

The plant extract had activity against experimental infection with *Aspergillus fumigatus* in chickens [11]. It will be worthwhile to identify and isolate compound(s) that are responsible for the antifungal effect of the plant. Therefore, the study was aim at identifying and isolating the biologically active compounds in the crude extract of *L. alata*.

### Materials and methods

#### Plant collection

Leaves of *Loxostylis alata* (Spreng.) f. ex Reichb were collected at the 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)

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was deposited at the Schweikert Herbarium of the Department of Plant Science, University of Pretoria, South Africa.

### 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  $\times$  3). The solvent of the combined extracts was removed *in vacuo*.

The acetone extract (70 g) was subjected to solvent-solvent fractionation using carbon tetrachloride, hexane, chloroform, aqueous methanol, butanol and water [12]. The carbon tetrachloride ( $\text{CCl}_4$ ) fraction had the highest antimicrobial activity and were therefore, chosen for further isolation of active compounds. Column chromatography (37 $\times$ 5 cm, silica gel 60) of the  $\text{CCl}_4$  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 successive steps. This was followed by an ethyl acetate: methanol gradient where ethyl acetate was reduced to 0% by the addition of 10% methanol in successive increments. Thirteen fractions were collected and each tested for activity against *S. aureus* using the bioautographic method [13]. Based on the bioautography profile, fractions containing active compounds with the same  $R_f$  value (F3-F8, F9-F13) were combined and referred to as F2-1 and F2-2 respectively. Fractions F2-1 (3 g) and F2-2 (2 g) were further fractionated by column chromatography eluted isocratically with hexane: ethylacetate (7:3) to give lupeol as a white amorphous powder and  $\beta$ -sitosterol as white-yellowish amorphous powder respectively.

### Antimicrobial activity

**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*, *Microsporium 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.

### Bioautography

The antibacterial and antifungal bioautographic assays were carried out according to the method described by Begue and Kline [13] with slight modification for fungi by Masoko et al. [14]. Briefly, Thin Layer Chromatography (TLC) plates were loaded with 100  $\mu$ g of each fraction or 10  $\mu$ g 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 cold 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 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 [11]. 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.

### 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 [14,15]. In brief, two-fold serial dilutions of the samples were prepared in wells of 96-well microtitre plates. Bacterial or fungal culture (100 $\mu$ l 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-Iodo Nitro Tetrazolium 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 positive controls, respectively with the solvents as negative controls. The samples were tested in triplicate and the assays were repeated twice to confirm results.

### 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). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of  $0.5 \times 10^5$  cells into each well of a 96-well microtitre plate. After overnight incubation at 37°C in a 5% CO<sub>2</sub> 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  $\mu$ g/ml). The method described by Mosmann [16] 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 shown by Shai et al. [17]

Selectivity index (SI) = Lethal concentration 50 (LC50)/Minimum inhibitory concentration (MIC)

This ratio gives the relative safety of each fraction.

### 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. One hundred microliters of bacterial stock was incubated in 20 ml of Oxoid Nutrient 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 [18]. 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.

### Anti-inflammatory assay

Inhibition of prostaglandin biosynthesis by the plant extract and isolated compounds was investigated using both the COX-1 and COX-2 assays [19,20]. 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 [<sup>14</sup>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 2 N 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 [<sup>14</sup>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<sub>50</sub> was calculated from at least 5 concentrations. Results are presented as mean ± SEM. of two experiments carried out in duplicate.

## Results and Discussion

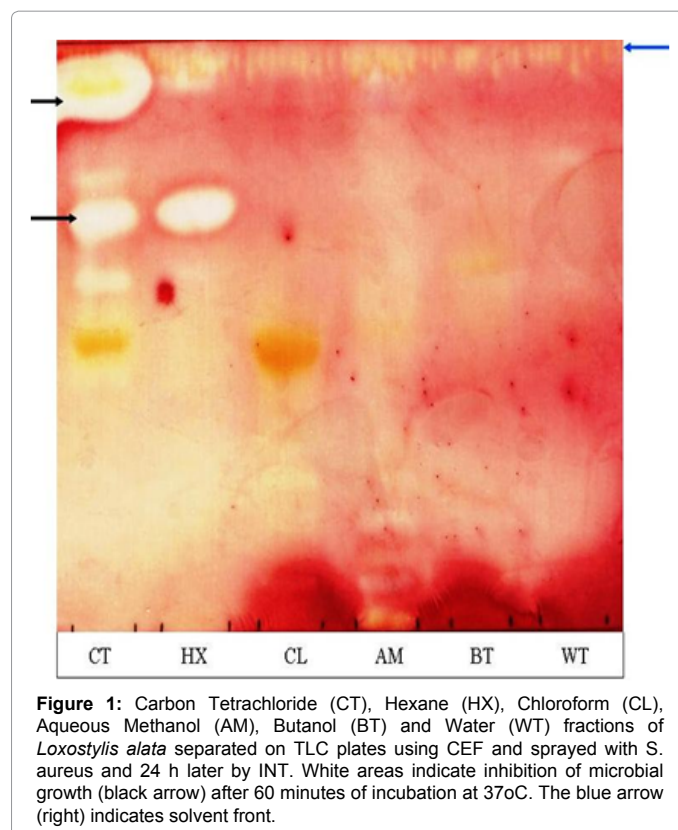
### Biological activity of the extract

In a preliminary random screening of antimicrobial activity of acetone extracts of tree leaves, *Loxostylis alata* had promising activity against *Cryptococcus neoformans*. In this study, an investigation on the antifungal effect 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 1. Total Activity (TA) is calculated by dividing the quantity extracted by each solvent 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 [21]. A higher value of total activity indicates increased usefulness and economic value of the plant species and is of benefit in enabling rural use of extracts of the species. The CC<sub>14</sub> 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<sub>4</sub> fraction can be diluted in 3201.79 ml of the solvent used and still inhibit the growth of *A. fumigatus*. Similarly, the CC<sub>14</sub> fraction had a greater area of inhibition against all the tested pathogens. Hexane and aqueous methanol fractions had a low area of inhibition, while

Fraction	Fraction yield (%)	MIC (mg/ml)	Total activity (ml/fraction)	Cytotoxicity (LC50 ± SEM in mg/ml)	Selectivity index
Hexane	3.33	0.24	138.69	0.41 ± 0.003	
Carbon tetrachloride	25.61	0.08	3201.79	0.23 ± 0.001	1.71
Chloroform	1.86	0.94	19.76	0.67 ± 0.002	2.88
Aqueous methanol	3.50	1.88	18.62	NT	0.71
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

**Table 1:** Minimal inhibitory concentrations and safety evaluation of various fractions of *Loxostylis alata* against *Aspergillus fumigatus*.



**Figure 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 (black arrow) after 60 minutes of incubation at 37°C. The blue arrow (right) indicates solvent front.

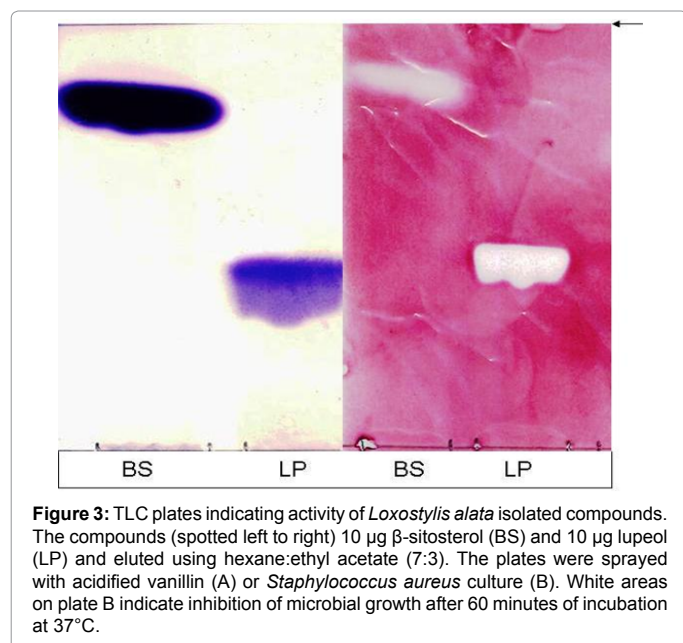
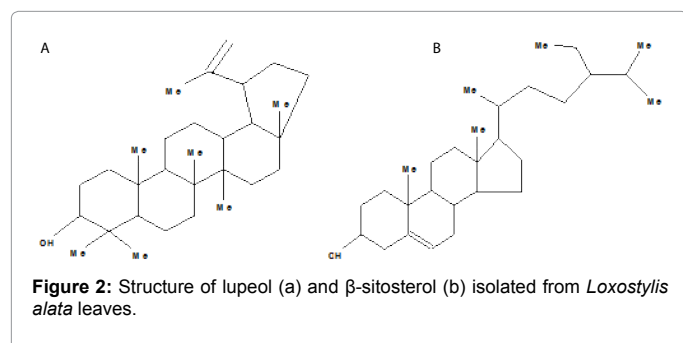
butanol and water fractions did not have any areas of inhibition when the TLC bioautographic method was used (Figure 1).

Bioactivity guided fractionation of the CC<sub>14</sub> fraction led to the isolation of two compounds 1 and 2. These compounds were identified, based on comparison of <sup>1</sup>H NMR, <sup>13</sup>C NMR and mass spectroscopic (MS) data with literature values, as lupeol and β-sitosterol, respectively.

The <sup>1</sup>H NMR and <sup>13</sup>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 [22-25]. Mass spectrum of the compound with M+426, and prominent signals at 218 and 207 confirmed that the compound is lupeol [24]. Analysis and interpretation of the spectroscopic data

obtained with previously reported data led to the proposed structure for the compound as lupeol (Figure 2a) with a molecular formula C<sub>30</sub>H<sub>50</sub>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 [26-28] led to the proposed structure of the compound to be β-sitosterol (Figure 2b). 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<sub>30</sub>H<sub>50</sub>O [26-28].



### 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  $\beta$ -sitosterol (Figure 3). Lupeol showed the most pronounced zone of inhibition against *S. aureus*. Only lupeol was active when tested against the five fungal pathogens using bioautographic means (results not shown). Similarly, when MICs of the 2 compounds were determined, only lupeol had activity against the fungal pathogens. However,  $\beta$ -sitosterol showed some activity against *S. aureus* and *E. coli* (Table 2). In a similar study, lupeol had low activity against *Candida albicans* with MIC values of more than 250  $\mu$ g/ml but showed high activity against *Sporothrix schenckii* and *Microsporium canis* (MIC values of 12 and 16  $\mu$ g/ml,

respectively), this is in agreement with what Shai et al. [17], reported. Lupeol and  $\beta$ -sitosterol, previously isolated from the stem bark of *Buchholzia coriacea*, have been shown to have activity against some species of pathogenic bacteria and fungi [29]. Similarly,  $\beta$ -sitosterol was found to have good inhibitory activity against the fungi *Aspergillus niger*, *C. cladosporioides*, and *Phytophthora sp.* [30].

### Anti-inflammatory assay

The results from screening against cyclooxygenase enzymes -1 and -2 (COX-1 and -2) showed that the crude extract of *Loxostylis alata* and lupeol inhibited COX-1 in a concentration dependent manner (IC<sub>50</sub>=92.5  $\pm$  1.6 and 134.0  $\pm$  5.1  $\mu$ M, respectively). Indomethacin had an IC<sub>50</sub> of 3.30  $\pm$  0.006 and 122.5  $\mu$ M against COX-1 and COX-2, respectively. Neither the crude extract nor lupeol or 2 inhibited the activity of COX-2. Although lupeol was not active against COX-2, it did produce a dose-dependent inhibition of carrageenan induced paw oedema in rats [31]. 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 [32]. 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 believed to be behind the anti-inflammatory mechanism of action of NSAIDs [32]. However, prolonged use of both enzymes inhibitors may cause detrimental myocardial infarction (COX-2) [33], gastrointestinal damage and nephrotoxicity (COX-1) [32]. 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 [34]. Moreover, lupeol could form a base for the development of new semi-synthetic drugs for the management of thrombotic disorders.

### In vitro safety test

Compounds 1 and 2 were relatively non toxic with LC<sub>50</sub> of 76.66  $\pm$  4.13 and 136.60  $\pm$  7.20  $\mu$ g/ml, respectively compared to the reference compound berberine with LC<sub>50</sub> of 6.36  $\pm$  0.81  $\mu$ g/ml (Table 2).  $\beta$ -sitosterol which occurs as an sterol in many plants has activities potentially useful in improving human health such as anti-inflammatory, antipyretic, immunomodulating, and antineoplastic [35,36].

Results obtained from the mutagenicity test of the 2 compounds using *Salmonella* TA98 and TA100 strains are expressed as mean  $\pm$  SEM (Table 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) [18]. The two compounds were not mutagenic in the *Salmonella*/

Compound	MIC values against the tested pathogens ( $\mu$ g/ml)										Cytotoxicity (LC <sub>50</sub> $\pm$ SEM in $\mu$ g/ml)
	SA	EF	EC	PA	AF	CA	CN	MC	SS		
Lupeol	29	67	83	150	92	120	47	63	57	76.66 $\pm$ 4.1	
$\beta$ -sitosterol	90	>250	110	>250	>250	>250	>250	>250	>250	136.60 $\pm$ 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 $\pm$ 0.8	

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

**Table 2:** Minimal inhibitory concentrations and safety evaluation of compounds isolated from *Loxostylis alata*.

Treatment	Revertant/plate in <i>Salmonella typhimurium</i> strains					
	TA98 (µg/plate ± SEM)			TA100 (µg/plate ± SEM)		
	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
β-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 ± SEM

**Table 3:** 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.

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 [37].

## Conclusion

The compounds isolated from the leaf extract of *Loxostylis alata* had varying degrees of antimicrobial and cyclooxygenase inhibitory activities. However, detailed toxicity and efficacy studies of the extracts or isolated compounds in both laboratory and target animal species are required to justify its use in clinical practice. If these are effective, there is also need for investigating the mechanism underlying the antimicrobial actions of these compounds from *Loxostylis alata*.

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