International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 5 (4), pp. 001-006, April, 2016. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

A novel pentacyclic triterpene glycoside from a resin of *Commiphora glandulosa* from Botswana

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Accepted 13 January, 2016

A novel ursane-type pentacyclic triterpene glycoside, 1β,2β,3β-trihydoxy-urs-12-ene-23-oic-rhamnoside was isolated from the resin of *Commiphora glandulosa* Schinz by bioassay guided fractionation. Crude aqueous and chloroform extracts of the resin as well as the isolated compound exhibited good *in vitro* antibacterial activity against gram positive bacteria (*Bacillus subtilis*: NCTC 10073), (*Clostridium perfringens*: NCTC 8237), (*Staphylococcus aureus*: NCIMB 9518) and also drug resistant strains (*S.aureus*:XU212-tetracycline resistant strain), (*S. aureus* SA1199B-norfloxacin resistant strain). There was no observed antimicrobial activity against gram negative bacteria (*Escherichia coli*: NCTC 9002), (*Pseudomonas aeruginosa*: NCIMB 10421*), (Klebsiella aerogenes*: NCTC 5055); and fungal strains (*Candida albicans*: NCPF 3179, *Aspergillus fumigatus*: NCPF 7097), *Trichophyton tonsurans*: NCPF 995). The structure of the isolated compound was elucidated on the basis of spectral data using ^{13}C-NMR, ^{1}H-NMR and mass spectroscopy.

Key words: *Commiphora glandulosa* resins, antibacterial activity, novel pentacyclic triterpene, Botswana medicinal plants, antibacterial activity.

INTRODUCTION

Medicinal plants have long been the subject of human curiosity and need. Plant derived products are present in most pharmaceutical preparations that are currently recommended by medical practitioners and they still form an important part of the health-care system in the world (Phillipson and Anderson, 1989). There are several reports in the literature regarding the antimicrobial activity of plant crude extracts and the bioassay guided fractionation to yield active principles (Rabe and van Staden, 2000; Polombo and Semple, 2001; Portillo et al., 2001).

There is an urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there is an alarming increase in the incidences of new and re-emerging infectious diseases (Rojas et al., 2003). There is also need for development of new antibacterial substances against multi-resistant gram positive bacteria.

Commiphora glandulosa Schinz (Burseraceae) resins are extensively used by rural dwellers in Botswana to treat stomach aches and wound infections. *C. glandulosa* is a spiny shrub or small tree and can reach up to 8 m in height. The bark is yellowish green or greyish green flaking in small yellowish papery pieces. It produces gummy brownish resinous exudate from the stem. Resins from numerous species of *Commiphora* are used as antiseptics (Tyler et al., 1988), anti-inflammatory (Fourie

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Abbreviations: MIC, Minimum inhibitory concentration; **MBC,** minimum bactericidal concentration; **TLC,** thin layer chromatography; **NMR,** nuclear magnetic resonance; **HSQC,** heteronuclear single quantum correlation; **HMBC,** heteronulear multiple bond correlation.

and Synckers, 1989) particularly in India, Arabian countries and Africa. Resins provide trees with a versatile set of defences against an ever increasing number of organisms attacking them (Langenheim, 1969). Thomas et al. (1961; 1964), found that the acid component of *C. glandulosa* Schinz resin consisted of small amounts of the monohydroxy acids Commic acid A and B, together with two dihydroxy acids Commic acids C and D and a trihydroxy acid Commic acid E. The acids were obtained by hydrolysis of their corresponding esters (Figure 1). The antibacterial and anti-inflammatory activity of the acids was not investigated.

Resins are therefore, a potential source of biologically active therapeutic tools. This study reports antibacterial activity of crude chloroform, aqueous extracts and 1β, 2β,3β-trihydoxy-urs-12-ene-23-oic-rhamnoside isolated from the resin chloroform extract of *C. glandulosa* Schinz.

MATERIALS AND METHODS

Selection and collection of plant material

The plant was selected on the basis of its use in traditional medicine. The traditional healers provided information relating to the use of the plant, mode of preparation of part of the plant used, method of administration and the ailments for which the plant is prescribed. A questionnaire was used to collect the ethnological data.

The plant was selected and the samples collected under the guidance of traditional healers. The plant material was collected in June (winter season). The traditional healers harvests the resins during the dry season, because, during the rainy season, the plants produce less resin or any resin left to ooze out of incisions would be washed off by rain. The resinous exudate was harvested by means of making several incisions at different regions of the plant's stem. A cream-white resinous material was left to ooze out of the incision for a week. The solid cream-white crystalline resinous material that resulted was harvested and packaged into black polythene bags.

Plant material and extract preparation

The resins of *C. glandulosa* stem bark were extracted in 2.5 L of either chloroform or water using Soxhlet apparatus for 36 h. The crude chloroform extract was concentrated to dryness by means of a rota vapour and the aqueous extract was freeze dried. The resulting crude extracts were then tested for antibacterial and antifungal activity. The plant was authenticated by comparison with Herbarium sample at the Botswana National Herbarium and Gallery, Gaborone, Botswana, where voucher specimen (DMT26) has been deposited.

Microorganisms

Escherichia coli (NCTC 9002); *Pseudomonas aeruginosa* (NCIMB 10421); *Klebsiella aerogenes* (NCTC 5055); *Bacillus subtilis* (NCTC 10073) ; *Clostridium perfringens* (NCTC 8237); *Staphylococcus aureus* (NCIMB 9518); *S. aureus* (XU212-tetracycline resistant strain); *S. aureus* (SA1199B-norfloxacin resistant strain); *Candida albicans* (NCPF 3179); *Trichophyton tonsurans* (NCPF 995); *Aspergillus fumigatus* (NCPF 7097).

Antimicrobial assays

Analysis by microdilution assay

A 96-well microplate was used for the quantitative determination of the Minimum inhibitory concentration of the test sample. This technique provides a valuable tool in assessing the susceptibility of a given microorganism to a given antimicrobial agent. The technique is also widely used to search for substances with the ability to potentiate the activity of clinical antibiotics (antibiotic modulation).

A 96-well plate microdilution method can be used for minimum inhibitory minimum bacteriostatic or bactericidal concentrations.

MIC determination

2 mg crude extract was soaked in 160 l of sterile DMSO (dimethylsulfoxide) and left at room temperature overnight. Then the solutions were made up to 2000 l with sterile distilled water. Two fold serial dilutions (ranging from 1000 to 7.8 g/ml) of the extracts were prepared as shown in Table 1. Then 100 l of the test concentrations of the extract was introduced into wells. 80 l of sterile double strength nutrient broth was introduced into all the wells. Then 20 l of 10^4 CFU/ml bacteria were introduced into all wells. Tetracycline HCl was used as a control drug (against bacteria) at concentrations ranging from 100 to 0.78 g/ml. Miconazole was used a control drug for fungi. The plate was incubated at 36ºC overnight. After 24 h the plate was observed for the presence of clear wells (activity) and the MIC values were recorded. Triphenyl tetrazolium chloride (TTC) was used to detect growth of microbes in wells.

MBC determination

Minimum bactericidal concentrations were determined by removing 5 l of samples from the clear wells that is, wells that showed no bacterial growth, onto plates containing sterile growth medium without plant extract. The plates were incubated overnight at 37ºC and observed for presence or absence of growth. The MBC was then recorded as the concentration at which no microbial growth recurred (Table 2).

Antimicrobial analysis by bioautography TLC

The antimicrobial activities of the crude chloroform extract and that of the purified compound were further assessed using direct bioautography on TLC plates (Figure 3) [Merck Si-gel 60 F_{254nm}, 025 mm thick] according to the method of Slusarenko et al. (1989).

Preparation of the TLC plate

Isolation of active components from resin of C. glandulosa

10 – 100 l of extract from a stock solution (1 mg/ml) was spotted onto a TLC plate (20cm × 20 cm). The plates were prepared in duplicate (plates 1 and 2) and both plates were developed in a tank saturated with a solvent system, petroleum spirit, ethyl -acetate, methanol (5:4:1) for 25 min. The plates were then removed from the tank, air dried and observed under ultra-violet 254 and 365 nm. Some compounds show quenching at 254 nm while others show fluorescent quenching at 365 nm. Plate 1 was sprayed with anisaldehyde- sulphuric acid spray reagent and thereafter heated on a hot plate at 110°C for 5 min to visualize separated spots (Figure 3). Plate 2, was dried overnight by being left in the laminar flow

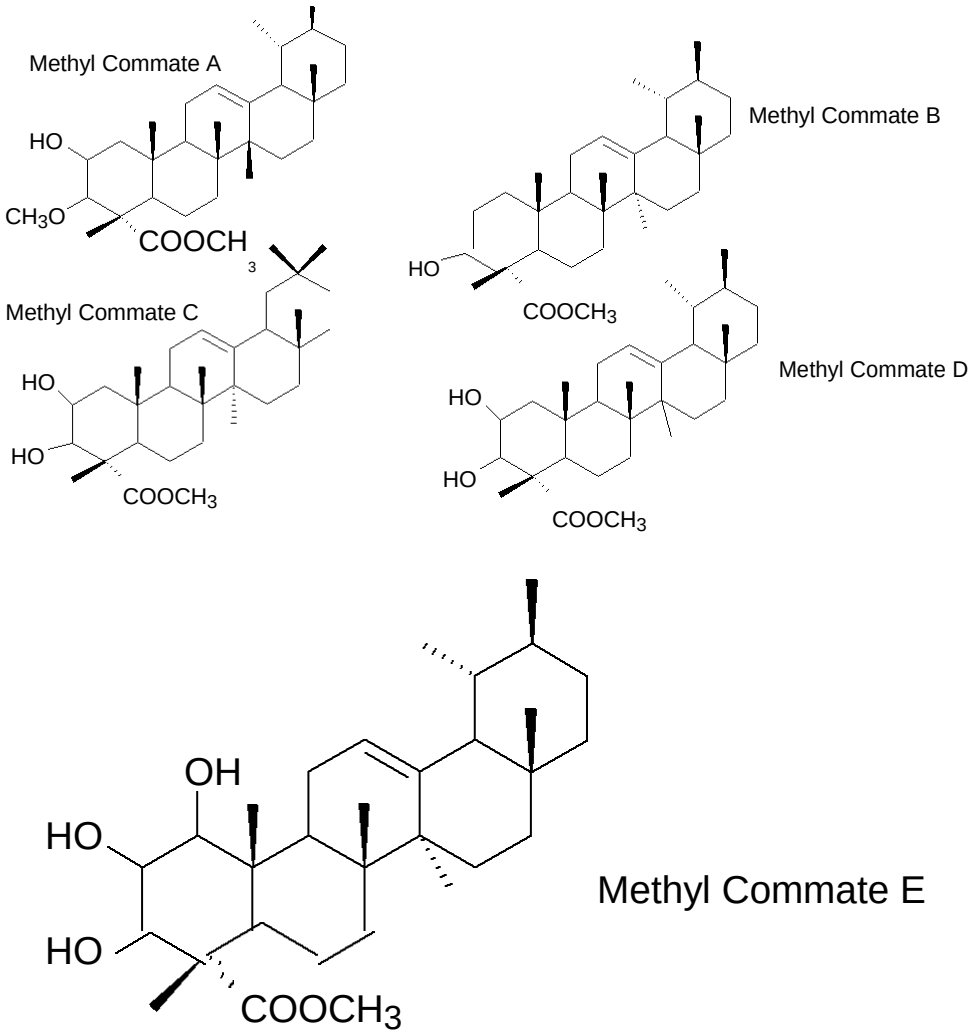

Figure 1. Previously isolated compounds from *C. glandulosa* Schinz resin.

extraction hood. (Drying by heat should be avoided at all costs as some compounds are heat labile). Then a *B. subtilis* seeded molten agar was applied/poured evenly on the TLC plate 2 (Figure 3). The plate was covered and incubated at 37°C overnight. The TLC (plate 2) was then sprayed with 1%TTC (as described in plate 1 above) and incubated for further 30 min. Areas where separated spots had activity showed clear zones indicating no growth (inhibition), while those which turned pink / red background indicated the growth of bacteria.

RESULTS AND DISCUSSION

Microdilution assay

Bioautography guided TLC of C. glandulosa chloroform crude extract

All fungal strains and gram negative bacteria were not sensitive to the crude extracts and compound 1. Tetracycline was bacteriostatic. Values with superscript "c„ represent bactericidal effect. *B.s*; *B. subtilis*, *S.a(w)*; wildtype *S.aureus, C.p; C.perfringens,* XU212 *and* SA1199B are multiple drug resistant strains of *S. aureus.*

Spectral characteristics compound 1 isolated from C. glandulosa resin

The structure of compound 1 was elucidated with the help of ^{13}C, ^{1}H, DEPT 135, HSQC and HMBC NMR, and Mass spectroscopy. 1β,2β,3β-trihydroxy-urs-12-ene-23-oic rhamnoside ($C_{36}H_{59}O_9$). Melting point = 169 - 172°C. The peak at 1291.8 is consistent with $[2M^+ + Na]$ = (2 × 634) + 23 = 1291 m/z (100%) at 657.4 is consistent with $[M^+ + Na]$ = [634 + 23] = 657.

Bioassay guided fractionation of the chloroform extract of the resin of *C. glandulosa* led to the isolation and characterisation of a new ursane-type triterpene glycolside,

1β, 2β, 3β–trihydroxy-urs-12-ene-23-oic-rhamnoside (Figure 4). The structure of the compound was determined by spectroscopic techniques, among which

Table 1. Two fold serial dilutions for the test extracts.

	Wells							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Initial prepared concentration (µg/ml)	1000	500	250	125	62.5	31.2	15	7.8
Effective concentration in wells (µg/ml)	500	250	125	62.5	31.2	15	7.8	3.9

Tetracycline; (100 - 0.78 µg/ml) was used as positive control.

Table 2. MIC and MBC values of extracts of *C. glandulosa* on different bacteria.

	MIC (µg/ml)					MBC (µg/ml)				
	B.s	***S.a(w)***	***C.p***	**XU 212**	**SA 1199B**	***B.s***	***S.a(w)***	***C.p***	**XU 212**	**SA 1199B**
Tetracycline	*6.25*	*6.25*	*6.25*	>100	6.25	>100	*25*	*25*	>100	>100
Water extract	15.6	15.6	15.6	125	62.5	250	125^{c}	31.3^{c}	$>10^{3}$	$>10^{3}$
Chloroform extract	15.6	62.5	7.8	125	62.5	125^{c}	125^{c}	7.8^{c}	$>10^{3}$	$>10^{3}$
Compound 1	15.6	31.3	7.8	125	15.6	>500	31.3^{c}	7.8^{c}	>100	>100

All fungal strains and gram negative bacteria were not sensitive to the crude extracts and compound 1. Tetracycline was bacteriostatic. Values with superscript "c" represent bactericidal effect. B.s; B. subtilis, S.a(w); wildtype *S.aureus*, C.p; *C.perfringens*, XU212 and SA1199B are multiple drug resistant strains of *S. aureus*.

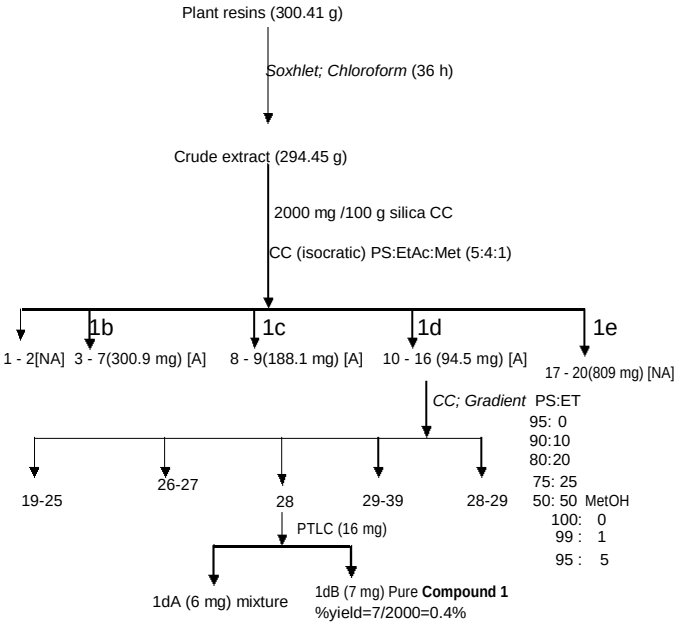

Figure 2. Scheme of isolation of active principles from resin of *C. glandulosa* extract. PS, petroleum spirit; ET, ethylacetate; MetOH, methanol; CC, Column chromatography; TLC, preparative TLC; [A], active (inhibition of microbial growth); [NA], not active (lack of inhibition of microbial growth).

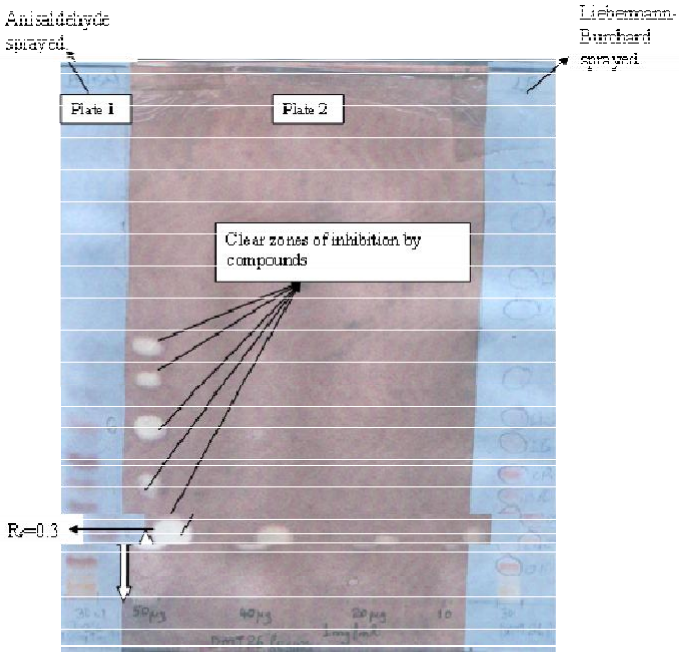

Figure 3. Bioautography guided TLC fractionation of resin chloroform extract of *C. glandulosa* Schinz against *B. subtilis.* 1 mg/ml crude extract, applied at 50, 40, 20 and 10 l. Solvent system (petroleum spirit: ethylacetate: methanol) (5:4:1).

^{13}C-NMR and mass spectroscopy (Table 3 and Figure 4) were useful. This compound has an unusual ester linkage with the sugar and this is what constitutes its novelty. The rhamnose residue was identified using proton and carbon NMR data and by comparison with published data (Ercil et al., 2004).

CONCLUSIONS

The chloroform and aqueous extracts were equally active against the antibiotic resistant strains SA1199B and XU212 with MIC values ranging from 62.5 – 125 µg/ml (Table 2). The isolated compound;

Table 3. ^{1}H AND ^{13}C-NMR FOR 1β,2β,3β –trihydroxy-urs-12-ENE-23-OIC rhamnoside(*D4*-methanol).

Experimental data for (1β,2β,3β –trihydroxy-urs-12-ene-23-oic-rhamnoside) compound 1			
Carbon number	**δC(ppm)**	**DEPT**	**δH(ppm) J(Hz)**
1	85.3	CH	3.16 (*d, J=9Hz*)
2	74.3	CH	3.38-3.42(*t, J=10Hz)*
3	77.6	CH	3.73 (*d, J=17Hz*)
4	55.6	C	-
5	49.8	CH	1.54-1.61(m)*
6	21.4	CH_2	1.72-1.75 (m)*
7	33.6	CH_2	1.56-1.59 (m)*
8	41.8	C	-
9	50.4	CH	1.88
10	43.9	C	-
11	27.8	CH_2	2.14; 2.64
12	126.8	CH	5.15 (br, s)
13	139.8	C	-
14	43.2	C	-
15	27.8	CH_2	1.86 + *
16	29.1	CH_2	2.03; 0.85
17	34.8	C	-
18	60.4	CH	1.34
19	41	CH	1.35
20	40.9	CH	1.44
21	32.3	CH	1.27; 1.39
22	42.6	CH_2	1.29; 1.42
23	175.8	C	-
24	12.8	CH_3	1.16 (*3H, s*)
25	14.1	CH_3	1.09 (*3H, s*)
26	17.8	CH_3	1.04 (*3H, s*)
27	24.1	CH_3	1.12 (*3H, s*)
28	29.3	CH_3	0.81 (*3H, s*)
29	21.8	CH_3	0.93 (*d, J = 6Hz*)
30	18	CH_3	0.83 (*d, J = 6Hz*)

Rhamnose	**Experimental data for the rhamnose residue of compound 1**			**Published data by Ercil et al. (2004)**	
	δC (ppm)	DEPT	δH (ppm)J (Hz)	δC	δH J (Hz)
1´	95.5	CH	5.98 (*br, s*)	100.5	4.59 (*1H br, s*)
2´	71.1	CH	3.83 - 3.84(*m*)	70.8	3.2-3.9(*3H, m*)
3´	72.4	CH	3.68 - 3.71(*m*)	68.8	3.2-3.9 (*3H, m*)
4´	73.3	CH	3.46 (*t, J = 9.5Hz*)	74.3	4.69 (*t, J = 9.8 Hz*)
5´	72.7	CH	3.66 - 3.69 (*m*)	66.3	3.2-3.9 (*3H, m*)
6´	18	CH3	1.25 (*d, J = 6.5Hz*)	20.9	0.82 (*d, J = 6.2Hz*)

* proton signals were difficult to be seen from the spectrum and appeared as multiplets.

(1β,2β,3β –trihydroxy-urs-12-ene-23-oic-rhamnoside); exhibited good activity against the gram positive strains *C. perfringens* (7.8 µg/ml); *Bacillus subtilis* (15.6 µg/ml); and various strains of *S. aureus* (31.3 µg/ml). Both the tested crude extracts and the isolated compound exhibited a bactericidal effect against the various strains of *S. aureus* and *B. subtilis* at concentrations ranging between 7.8 and 125 µg/ml. The most sensitive strain was *C.perfringens* with MIC of 7.8 µg/ml. Neither crude extracts nor the isolated compound showed any antimicrobial activity against gram negative bacteria and fungi. Generally, inhibiting the growth of gram negative bacteria is more difficult than the gram positive bacteria. This is partly because the gram negative bacterial cells

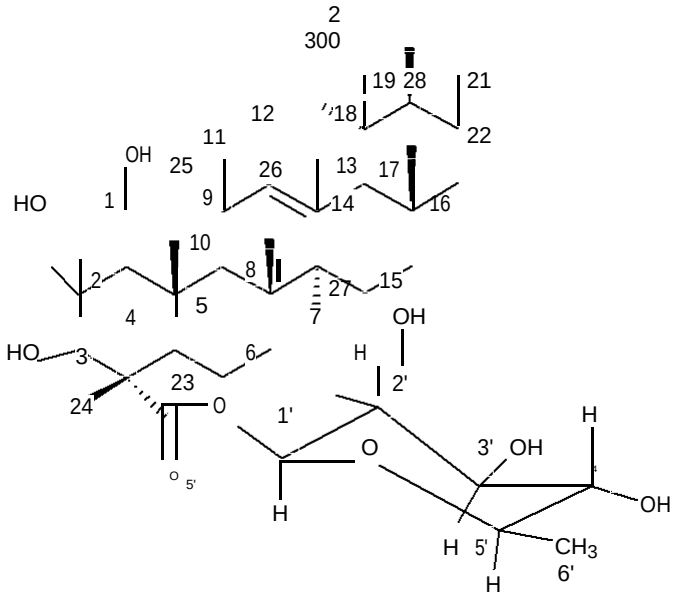

Figure 4. Structure of compound 1 isolated from *C. glandulosa* resin chloroform extract.

have multiple layers that afford protection by resisting the passage of chemicals such as antibiotics and salts (Alcamo, 2000). In addition, the Gram negative cells have periplasmic space which is heavily laden with antimicrobial deactivating enzymes.

As can be seen in Figure 3, there are other compounds present in the extract with antibacterial activity. It is strongly recommended that these compounds be isolated and characterised whilst these findings support the traditional use of *C. glandulosa* resins in controlling bacterial infections, it is strongly recommended that the extracts and the isolated compound be further tested in other models such as the anti-inflammation and cytotoxicity models because infections can yield inflammatory responses and can also promote tumour progression.

ACKNOWLEDEMENTS

The authors are grateful to the Botswana College of Agriculture for funding and traditional healers for providing the ethnological data.

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