Journal of Medicinal Plants Research Vol. 6(35), pp. 4915-4921,12 September, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.883
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Stephanostema stenocarpum (Apocynaceae) extract is a potential remedy for bacterial infections in domestic animals

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Accepted 22 December, 2010

***Stephanostema* is a monotypic genus whose sole specie *Stephanostema stenocarpum* is endemic to Tanzania. The liquid from boiled roots of this plant is being used by the local communities as a remedy of stomach-ache related ailments. The aim of the study was to evaluate antimicrobial activity of *S. stenocarpum* extracts against some common bacterial and fungal pathogens in domesticated animals and examine whether the crude extracts found active have cytotoxic effect or not. The standard growth inhibition technique was used for *in vitro* antimicrobial activity assay and the brine shrimp (BST) test was used to evaluate cytotoxicity of the extracts. Ethanolic extracts exhibited bacterial inhibitory growth activity against animal bacteria pathogens: *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum and Escherichia coli*, but not active against the fungal strains tested. The study also showed that the extracts have no cytotoxicity effects. These results provide baseline information on the medicinal potential of *S. stenocarpum* as a new potential source of veterinary medicines.**

Key words: *Stephanostema stenocarpum*, phytotherapy, antibacterial, antifungal, cytotoxicity, veterinary.

INTRODUCTION

Apocynaceae is a family mostly of tropical plants with about 1700 species found in the region (Wills, 1973; Cronquist, 1981; Brummitt, 1992). The family Apocynaceae has been exploited for medicinal purposes and many species have been used medically in varieties of ways. For example, *Carissa edulis* has been used for indigestion, lower abdominal pain in pregnant women, chest pain, gastric ulcers and in the treatment of "msati" (Chaga) a plague-like disease, cough remedy, anthelminthic in man and cattle, and as remedy for other venereal diseases (Watt and Breyer-Brandwijk, 1962).

Stephanostema is a monotypic genus in the family Apocynaceae, whose sole specie is *Stephanostema stenocarpum* (Sennblad and Bremer, 2002). According to botanical reports available to date, the plant is only found in Tanzania, from the remaining part of "Sachsenwald" which date from the German periods in Dar es salaam (Barink, 1983), this area is now known as Ukonga (Gongolamboto) cemetery. The plant is also well established in areas like Pugu Mwakanga and Pugu Kajiungeni where the soil is sandy. The plant is one of the endangered plant species (Barink, 1983; Mabberley, 1987; Walter and Gillett, 1997). Traditionally, the liquid from boiled roots of this plant is being used by the Matumbi people and other neighbouring communities as a remedy of stomach-ache related ailments, and the plant is known by the Matumbi people as "Mzigizigi". Despite being in families of plants with several others having medicinal potential, the antimicrobial activities of extracts from *S. stenocarpum* had not been known before this study.

The aim of this study was to evaluate the antimicrobial activity of *S. stenocarpum* extracts against pathogenic bacteria and fungus of veterinary importance and examine whether or not the extracts found active have cytotoxic effects. The extracts from root barks, stembarks and leaves

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of *S. stenocarpum* exhibited *in vitro* antimicrobial activity against some pathogenic microorganisms of veterinary importance such as *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum and Escherichia coli*. There was difference in strength of antimicrobial activity of crude extracts from different parts of the same plant, with the rootbarks exhibiting the highest activity followed by the stembark and leaves. The plant extracts were not toxic against shrimp larvae, implying that the plant extracts have no cytotoxicity. The findings of this study will increase the list of plants known to possess antimicrobials against some common animal pathogenic microorganisms. Also, the revelation that *S. stenocarpum* possesses *in vitro* growth inhibitory activity against microorganisms causing diseases to animals provides baseline information of their medicinal values for future investigation in search for new sources of medicines. The increased awareness of the medicinal value of *S. stenocarpum* will contribute to its sustainable exploitation and hence help towards conservation of its natural habitats which once instituted on a national scale will have indirect but important economic benefit of stabilizing our natural forests, woodlands and grasslands. Also, it will encourage the establishment of medicinal plant farms and botanical gardens for conservation and subsequent utilization.

MATERIALS AND METHODS

Plant material

S. stenocarpum rootbarks, stembarks and leaves were collected from Pugu Majohe, a village 20 km South west of Dar es Salaam city. The plant materials were air-dried in a well-ventilated room (25 to 28°C). The dried materials were ground into powder. Ethanol extraction was done by 3× soaking in ethanol (1:3 ratios) with magnetic steering at room temperature for 8 h, and collecting the liquid part (the extract). The 3 extracts were pooled together and filtered by vacuum filtration and the filtrate was concentrated by using rotary evaporator at 40°C. The known concentration of the crude extract was tested for *in vitro* activity against microbial pathogens isolated from sick domesticated animals.

Isolation and culturing of the test microorganisms

The diagnosis of microbial infections in sick domestic animals, specimen taking, isolation and identification of the microorganisms were done in the laboratory of the Department of Veterinary Microbiology and Parasitology of Sokoine University of Agriculture. Bacteria *S. agalactiae, S. aureus, S. gallinarum and E. coli,* and fungi *Microsporum* sp. and *Trichophyton* sp. isolates were used. The media used were as follows: *S. gallinarum* and *E. coli* each onto MacConkey agar (MA) Petri-dishes, *S. aureus* and *S. agalactiae* each streaked onto sterile nutrient agar (NA) and blood agar (BA) petri-dishes. The culture media were prepared according to manufacture's specifications in slunts or culture plates. The plates were incubated, and observed for growth and purity after the 24 h at 37°C. The observation was recorded, and the positive result plates were stored at 4°C. For the fungi, agar plate was inoculated with *Microsporum* sp., or *Trichophyton* sp. The inoculated media were kept at room temperature (25 to 28°C) and observed for growth of fungi from a period of five days to two weeks. The observation was recorded, and the positive result plates were stored at 4°C. Sub-culturing of pure strains was done at UDSM microbiology laboratory monthly, with the aim to maintain the strains.

Antimicrobial activity assay

The antimicrobial activity of the extracts was tested by agar well method (AWM) as previously described by Rojas et al. (2006) and Moshi et al. (2006), and was ascertained using disc method (DM) by Aszalos (1986) with minor modifications in both methods.

Preparations of inocula

Pure cultures of *S. aureus, S. gallinarum* and *E. coli* were grown on sterile NA slant, *S. agalactiae* was maintained on BA slant at 37°C for 24 h, and SDA slants were used to grow the dermatophytic fungi *Microsporum* sp. and *Trichophyton* sp at room temperature for a week. A loopful from the aforementioned pure culture slant was inoculated into 100 ml of sterile broth medium in 200-volumetric flask, which was then incubated at 37°C, for 24 h for the bacteria or for 120 h for the dermatophytic fungi in a shaking incubator. The broth culture was diluted to make an inoculum of approximately 10^5 C.F.U/ml. Determination of the number of cells in the broth cultures was done by the viable cell count method according to Grigorova and Norris (1990).

Preparation of assay plates for antimicrobial assay

For the AWM 0.2 ml of the estimated 10^5 C.F.U/ml inoculum was introduced into sterile 15 ml Muller Hinton Agar (bacteria) or SDA (fungi) in a petridish (90 mm effective inner diameter) under sterile conditions in a laminar flow and was mixed well. The inoculate agar plates were allowed to solidify and wells of 7 mm diameter with depth of 3.7 mm were made into the inoculated agar medium in the petridishes by using a sterile cork borer. For the DM, a drop from the 10^5 C.F.U/ml inoculum was introduced and spread over a sterile solidified agar in a Petri dish.

Antimicrobial assay

For the AWM, labelled wells in the culture plate were filled with 1 mg/ml of the plant extract dissolved in 10% solution of ethanol in water, or with 10% solution of ethanol in water as a control. The treated inoculated plates were kept at 4°C for 6 h to allow for diffusion of the active agents before the microbes were incubated at 37°C for 24 h for bacteria and at room temperature for 120 h for the dermatophytic fungi (Aszalos 1986). For the DM, 7 mm filter paper discs were dipped into 1 mg/ml of the plant extract dissolved in 10% solution of ethanol in water (or into 10 % solution of ethanol in water as a control) for 5 min, and the discs were air dried for 5 min. The dried discs were placed onto the inoculated agar plates. One plate could accommodate 4 discs of different antimicrobial agents. The plates were kept at 4°C for 6 h to allow for diffusion of the active agents before the microbes were allowed at their respective favourable conditions.

In positive results, clear zones of inhibition appeared around the wells (AWM) and around the discs (DM), the diameters (mm) of zones of inhibition including the area covered by the discs were measured using a transparent measuring ruler and the averages for each extract (n = 10) were recorded. The mean values ± SD for each test were determined and used to draw bar charts (Figure 1), and the differences between the means were analyzed by using a Single

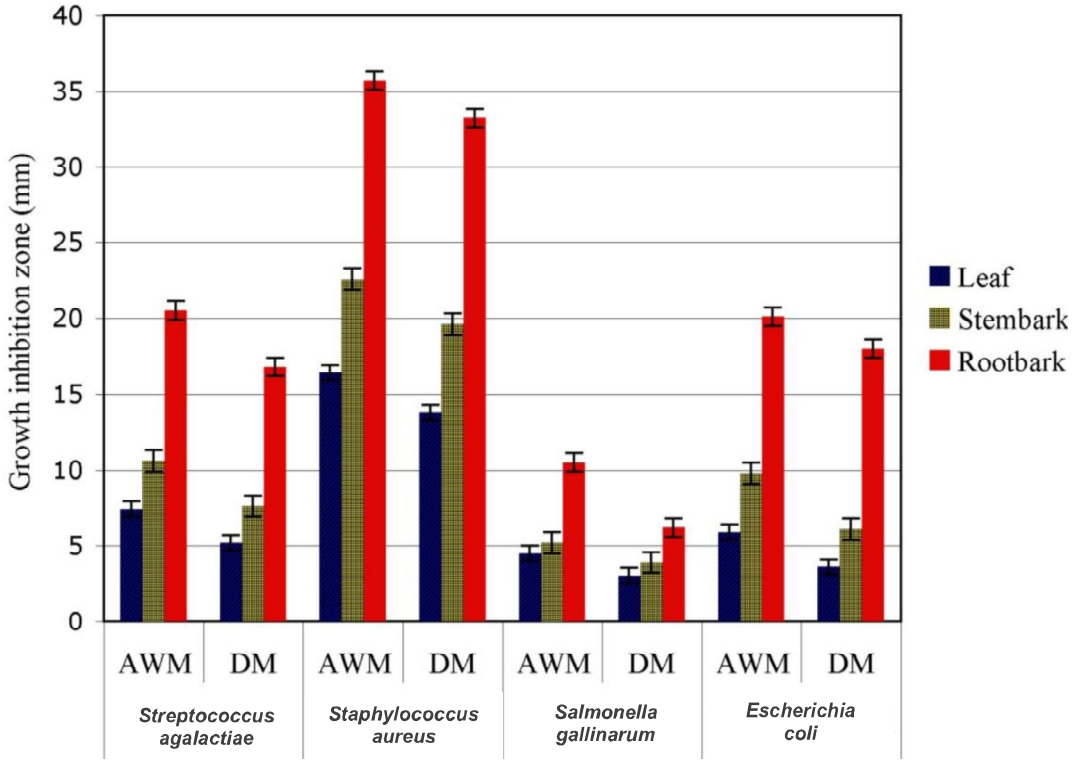


Figure 1. Growth inhibition zones (in mm ± SD) of animal pathogens *Staphylococcus aureus, Salmonella gallinarum, Escherichia coli* treated with ethanolic extracts from leaves, stembarks and rootbarks of *Stephanostema stenocarpum* as determined by using Agar Well Method (AWM) and Disk Methods (DM), n = 10.

Factor Analysis of Variance (ANOVA) test as described by Zar (1996) and exemplified in Table 1.

Determination of MIC and MBC of the active crude extracts

MIC (minimum inhibition concentration) and MBC (minimum bactericidal concentration) of active plant extracts were determined by broth (or tube) dilution method according to Garrod (1985). The MIC was determined only on the microbes (the bacteria) which had earlier shown sensitivity to the plant extract, and the MIC values were tabulated and expressed as µg/ml. Determination of MBC involved analysing the viability of the bacterial cells by subculturing 10 µl of the solution from each of the test tubes, showing no growth on Muller Hinton agar (Difco) plates and incubated at 37°C for 24 h. The resulting bacterial colonies on the plates were compared to that of the untreated control. Then the MBC values were recorded in µg/ml. Number of colonies similar to that from the control tube before incubation, indicated bacteriostatic action. When there was no growth in the culture plate then it indicated complete killing (complete bactericidal action).

Antimicrobial killing rate determination

Antimicrobial killing rate was determined by evaluating the bactericidal effects of the most active *S. stenocarpum* root extract on the most sensitive test bacteria *S. aureus*. To each of 4 sterile 50 ml capacity conical flasks 20 ml of nutrient broth maintained at 37°C, and 1 ml of the inoculum with cell concentration necessary to bring the final cell concentration of about 10^7 cells/ml were added. The flasks were left to stand for 15 min to allow the bacterial cells to adapt to the new culture conditions. Then 50 µl was taken from each of the 4 culture flasks for viable cell count and this was taken as at time t = 0. Then, root extract solution of known concentration was added so as to make the final concentration at MBC level. 50 µl volumes were then taken at 5 min intervals for 30 min and immediately maintained at 4 to 5°C in a refrigerator so as to arrest further growth. The samples were later analyzed for cell population by Viable Cell Count method as explained in earlier sections. The rate of killing of bacteria by the root extract was estimated as a slope of the graph plotting the *ln* of the number of bacteria killed to the y-axis against time (min) to the x-axis.

Cytotoxicity test

The presence and levels of toxicity of the active plant crude extracts were determined by the Brine Shrimp Test (BST) method described by Meyer et al. (1982), by which a crude extract is considered to be cytotoxic if it kills at concentrations less or equal to 240 µg/ml.

RESULTS

Figure 1 shows that the ethanolic crude extracts from leaves, stembarks and rootbarks of *S. stenocarpum*

Table 1. Analysis of the difference in antimicrobial activity (in mm) among crude extracts from rootbarks (Column 1), stembarks (Column 2) and leaves (Column 3) of *Stephanostema stenocarpum* against *Staphylococcus aureus*.

Anova: Single Factor				
Summary				
Groups	**Count**	**Sum**	**Average**	**Variance**
Column 1	10	164	16.4	0.711111111
Column 2	10	226	22.6	1.6
Column 3	10	357	35.7	1.122222222

ANOVA						
Source of variance	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	1941.8	2	970.9	848.3592233	4.27676E-25	3.354131195
Within groups	30.9	27	1.144444444			
Total	1972.7	29				

The null hypothesis is rejected; therefore there was significant difference among the three plant parts ($p << 0.0005$).

Table 2. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of *S. stenocarpum* ethanolic extracts against bacterial pathogens of veterinary importance. NO = no killing.

Plant part	*Staphylococcus aureus*		*Salmonella gallinarum*		*Escherichia coli*	
	MIC (µg/ml)	**MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**
Leaf	31.25	250	4000	NO	4000	NO
Stembark	31.25	250	4000	NO	250	NO
Rootbark	31.25	125	4000	NO	125	500

exhibited growth inhibitory activity *in vitro* against domestic animal pathogens *S. agalactiae*. *S. aureus*, *E. coli* and *S. gallinarum*. However, none of the extracts did show inhibitory growth activity against the test fungal strains *Microsporum* sp. and *Trichophyton* sp. The mean zones of the bacterial growth inhibition ranged from 3.0 to 35.7 mm, with the highest activity observed from the rootbark extract against *S. aureus*. The growth inhibition zones were relatively bigger for the agar well method (AWM) than for the disc method (DM).

There was significant difference in strengths of antimicrobial activity among the extracts from different parts of a plant, namely rootbark, stembark and leaf ($p < 0.0005$) against *S. aureus* (Table 1), *S. agalactiae*, *E. coli* and *S. gallinarum* strains of veterinary importance. The rootbark extract exhibited the strongest activity and the leaf extract exhibited the least activity. In Table 2, again *S. aureus* is shown to be the most sensitive to the extracts, and the rootbarks extract exhibited the highest activity. The minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) of the rootbark extract against *S. aureus* were found to be 31.25 and 125 µg/ml, respectively. The MIC values were very high for Gram negative bacteria (125 to 4000 µg/ml) as compared to *S. aureus*. The extracts inhibited the growth but no killing of *S. gallinarum* cells. The rootbarks extract killed the *E. coli* at least at 500 µg/ml. Figure 2 shows the specific killing rate of *S. aureus* by the rootbark extract as 10% cells per min. The time in which 50% of the cells died estimated as ln 2/specific death rate (0.693/0.1) was 6.9 min.

All the *S. stenocarpum* extracts were found to be devoid of cytotoxicity; the rootbarks extract killed less, only 30% of the shrimp (*A. salina*) larvae, at 240 µg/ml, which is supposed to be the IC_{50}. The leaves and stembarks extract had no effect at all on the larvae (Table 3).

DISCUSSION AND CONCLUSION

It is well known that plants contain antimicrobial substances and many plant extracts derived compounds are used as leads in the developments of antibiotics (El-Said et al., 1971; Lewis, 1980; Zaria et al., 1975; Ibekwe et al., 2001; Akujobi et al., 2006). The results of the present study agree essentially with the reports of these previous researchers. The result shows that ethanolic extracts from *S. stenocarpum* exhibit antimicrobial activity

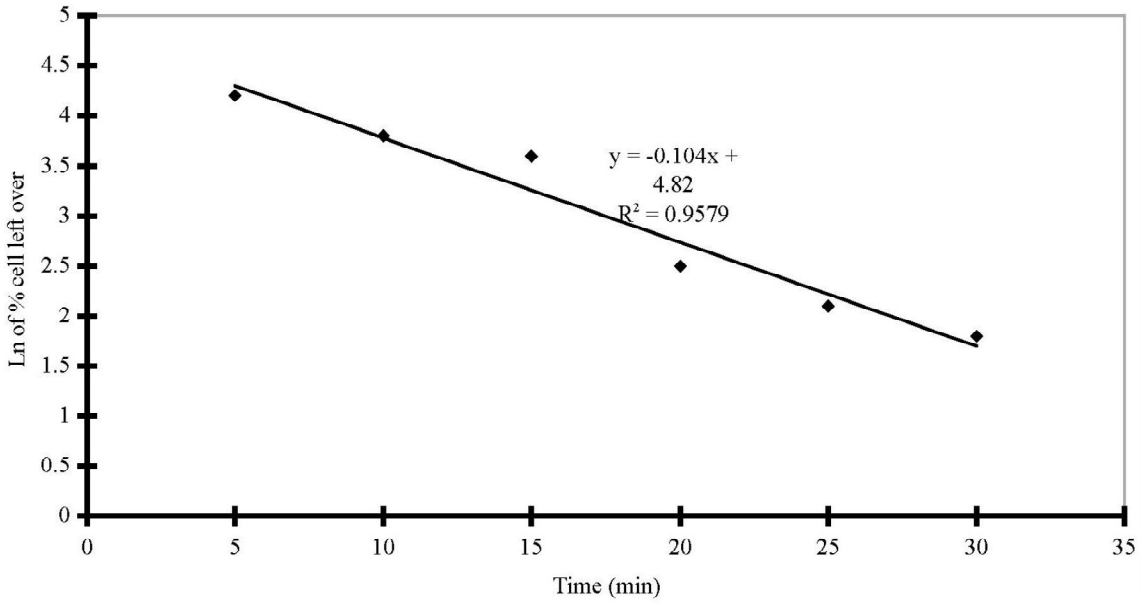


Figure 2. Ln of the percentage viable cell number of *S.s aureus* cells left over in time (min) in broth culture treated with 125 µg/ml (MBC) of *S. stenocarpum* rootbarks extract. The slope gives the specific death rate of 10% cells min^{-1}.

Table 3. Brine shrimp test for the *S. stenocarpum* extracts cytotoxicity test. IC_{50} means the concentration at which 50% of the shrimp larvae dye, and NT abbreviates "non toxic".

Plant part	% Deaths of *Artemia salina* larvae at 24 h for different concentrations						
	8 (µg/ml)	24 (µg/ml)	40 (µg/ml)	80 (µg/ml)	120 (µg/ml)	240 (µg/ml)	IC_{50}
Leaves	0	0	0	0	0	0	NT
Stembarks	0	0	0	0	0	0	NT
Rootbarks	0	0	0	0	10	30	NT

against the animal disease causing agents *S. agalactiae and S. aureus* (Gram positive bacteria) and *S. gallinarum* and *E. coli* (Gram negative bacteria) (Figure 1). This is not surprising since ethanol is generally able to dissolve multivariable types of compounds; polar and non-polar, simple and complex chemical structures (Cowan, 1999). The relative amount of phytochemical substances from plant extraction depends on the solubility of the phytochemical in the solvent used for extraction (Olowosulu and Ibrahim, 2006). This is the first time *S. stenocarpum* has been reported to exhibit antibacterial activity against such bacterial strains of veterinary importance.

The zones of inhibitions were bigger in the agar well method (AWM) than in disc method (DM), which is not surprising because the individual agar wells could accommodate higher amounts of extract than individual paper disc could accommodate. The sizes of the zones of inhibition varied from one crude extract to the other, this may be attributed to various factors including possible presence of different types and concentration of the active agents in different extracts (Platt, 1986). Since the present study was based on the treatment with crude extract, the identities and concentrations of the active compounds present in the extracts are not known and so the mode of actions too may not be clear at this stage. The antimicrobial property of extracts could be attributed to different substances interaction either by synergism, antagonism, additivity, or indifference in the same plant (Mtolera, 1991; Gutierrez et al., 2009). The MICs and MBCs against animal pathogen *S. aureus* were only 100 folds less than that of most common standard antibiotics against *S. aureus* ATCC 25923 (Barcia-Macay et al., 2006), and the rate of killing was comparable to the rate of killing by daptomycin in previous study (Vance-Bryan et al., 1992).

The antimicrobial properties exhibited by the *S. stephanostema* extracts may be associated with the presence of one or more of tannins, saponins, cardiac glycosides and alkaloids, the common molecules found in plant extracts. The antimicrobial activities of plant derived

compounds may be attributed to their ability to complex with extracellular and soluble proteins as well as their ability to complex with bacterial cell walls and disrupting microbial cells membranes (Tsuchiya et al., 1996). Also plant toxins may affect biochemistry, and lead to death of pathogenic microorganisms (Prost et al., 2005; Spoel et al., 2007). This is evidence that higher plants could be a potential source of antimicrobial agents. In addition, plants may carry non-naturally occurring antimicrobial chemical substances into their metabolism (Miller, 1973).

The identities and concentrations of the active components in the crude extracts are not known yet, this leads to difficulty to explain the differences in the sizes of the microbial growth inhibition zones among the plant extracts tested. The observed difference in the activity could be due to differences in molecular size or chemical nature of the active molecules, small molecules diffuses through the solidified medium more easily than large molecules (Hewitt, 1977; Pongtharangkul and Demirci, 2004).

The cause for the resistance to the plant extracts shown by fungal strains under the present study is still unknown. There is no existing information on the traditional use of the *S. stenocarpum* for skin fungal diseases; neither is there any record on the anti-*Microsporum* sp. or anti-*Trichophyton* sp. activity tests on this plant. It is possible, therefore, that the plant is lacking effective antifungal substances (Rippon, 1988; Carter, 1990; Güven et al., 2005).

The extracts had IC_{50} values lower than 240 µg/ml (Table 3) against shrimp (*A. salina*) larvae suggesting that the extract was not cytotoxic (Meyer et al., 1996). This means that the *S. stenocarpum* crude extracts are inhibitory to the growth of bacterial pathogens but not toxic to the eukaryotic host cells. This result gives baseline information on the medicinal values of this plant for further investigations in search of new sources of medicine (Ramachandran et al., 2011). This is important, especially, in the search for drugs.

The results of this study show that *S. stephanostema* has appreciable antimicrobial properties against bacterial infections in domestic animals thus suggesting the medicinal potential of this plant. This is good news for Tanzania because as the western medicines prices continue to shoot up beyond what local farmers can afford it could be studied further for use as alternative veterinary medicines in the country. *S. stenocarpum* is an endangered and nearly extinct species; studies on the possibilities of vegetative propagation and multiplication in botanical gardens would serve the plant from disappearance.

ACKNOWLEDGEMENTS

The material support from the Department of Molecular Biology and Biotechnology and the College of Natural and Applied Sciences of the University of Dar es salaam is highly appreciated. Thanks are also due to Dr. Henry Ndangalasi for accompanying the researchers to the fields, and helping with the identification of the plant.

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