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Research Article
Investigating the Angiogenic Modulating Properties of *Spirostachys africana* in MCF-7 Breast Cancer Cell Line

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Abstract

Background and Objectives: Angiogenesis is the process of forming new capillary vessels from existing ones. This process is implicated in tumour growth, metastasis and wound healing. During tumour metastasis, angiogenesis is amplified as new capillaries are needed for cancer to spread. However, when this process is inhibited, wound healing is impaired. Cyclooxygenase-2 (COX-2), nitric oxide synthase (NOS) and lipoxygenase (LOX) are some of the enzymes that are highly expressed during angiogenesis and therefore provide a useful way to detect angiogenesis. Some medicinal plants have shown to possess angiogenetic-modulating properties, which has led to the development of anti-angiogenic drugs for the treatment of cancer. *Spirostachys africana* sond has been used historically to treat open wounds, ulcers and cancer. Nevertheless, its mechanism of action is still unclear. The aim of this study was to determine mechanisms of anticancer and wound healing activities of *S. africana* by evaluating its effects on the proliferation of MCF-7 cells and on the activities of COX-2, LOX and NOS. **Materials and Methods:** The dried plant materials were extracted with water and sequentially with organic solvents in their order of increasing polarity. Extracts were screened for cell growth inhibitory activity against breast cancer MCF-7 cells and for selectivity against normal breast MCF-10A cells. Extracts that showed growth inhibitory activity with IC_{50} values <10 μg mL^{-1} were further evaluated for effects on COX-2, LOX and NOS enzymatic activity. **Results:** The non-polar extracts of all plant parts had anti-proliferation activity with IC_{50} of 10 μg mL^{-1} or lower. All the leaf extracts showed selectivity for MCF-7 breast cancer. The selected extracts also induced NOS activity and inhibited LOX and COX-2 activity in a concentration dependent manner. **Conclusion:** It was concluded that the selected extracts may suppress angiogenesis by inhibiting COX-2 and LOX and induce apoptosis by increasing NOS activity. *Spirostachys africana* was found to contain tannins, glycosides, saponins and alkaloids which could be responsible for the biological activity observed.

Key words: Angiogenesis, *Spirostachys africana*, cyclooxygenase-2, lipoxygenase, nitric oxide synthase, MCF-7 breast cancer cells

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Angiogenesis is the processes of forming new capillary vessels from existing ones. It is a normal biological process that occurs during female reproductive cycle and wound healing[1]. Studies have shown that abnormal angiogenesis is related to tumour growth and tumour progression to a metastatic phenotype[2]. For metastasis to occur, new vessels are required to supply the tumour with nutrients and oxygen[1]. Angiogenesis also involves growth factors, cytokines and enzymes such as cyclooxygenases (COX), nitric oxide synthase (NOS) and lipoxygenases (LOX).

The COX are enzymes that catalyse the oxidation of a wide variety of xenobiotics, including prostaglandins[3]. Cyclooxygenase-2 (COX-2) is inducible and functions in inflammation and tumour angiogenesis[4,5]. The LOX are a group of enzymes with a non-heme iron atom that catalyse the oxidation of polyunsaturated fatty acids like arachidonic acid[6]. The expression of 12-LOX in prostate cancer was found to induce tumour angiogenesis[7]. More 5-LOX transcripts were found in brain tumours than in normal bovine brain and 5(S)-hydrooxyeicosa-6E,8C,11Z,14Z-tetraenoic acid (5-HETE), a product of 5-LOX, stimulated the growth of breast cancer cells, whereas a 5-LOX selective inhibitor reduced cell growth[8,9]. NOS catalyse the formation of (NO) from L-arginine. Cianchi *et al.*[10] found elevated levels of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) in colorectal cancer and a high activity of iNOS in metastatic tumours than in non-metastatic ones. Thus, COX-2, LOX and NOS have been selected as the focus of this study due to their significant role in angiogenesis and their involvement in tumour initiation and growth.

Spirostachys africana sond is a member of the family *Euphorbiaceae.* The plant is indigenous to the southern and central regions of Africa[11]. Historically, the plant has been used to treat both cancer and acute wounds. In South Africa, its stem and bark are used to treat stomach pains, stomach ulcers, coughs and eye complaints[12-16]. Additionally, in Tanzania, its stem barks and roots are used to treat cervical, colon, breast and liver cancer[17]. There is no scientific information on the anti-cancer and wound healing ability of this plant. Hence, this study aimed to investigate the effects of *S. africana* on the process of angiogenesis, which is implicated in both cancer and wound healing. This study determined the effects of *S. africana* on the growth of MCF-7 breast cancer cell line and its angiogenesis modulating properties, by evaluating its inhibitory effects on COX-2, LOX and NOS. The MCF-7 cell line was selected for the study because it has the characteristics of a tumor and expresses the selected enzymes in high levels[18,19].

MATERIALS AND METHODS

Plant collection and extraction: This study was conducted at the Central University of Technology and the University of the Free State, in Bloemfontein, South Africa, from April, 2017 until September, 2018. The plants were collected from Pretoria and Kruger National Park in Gauteng and Limpopo Provinces of South Africa during the summer season. The plants were authenticated by a botanist in the Department of Botany at University of the Free State. The plants were washed with tap water upon arrival at the laboratory and dried at room temperature. The dried plant material was ground, weighed and then extracted with water or organic solvents (hexane, DCM, ethyl acetate and methanol) in their order of increasing polarity. The plant-solvent mixtures were left to shake for 48 h before filtering. The filtrates were concentrated by freeze drying (water extract) and rotatory evaporation (organic extracts). The percentage yields were calculated.

Phytochemical analysis: The powdered plant material was subjected to different methods to determine the presence of phytosterols, pentose, tannins, glycosides, triterpenoids, anthraquinones, saponins, flavonoids and alkaloids[20].

Cell culturing: The MCF-7 cells were grown in DMEM media supplemented with 10% Fetal Bovine Serum (FBS) and 0.6% streptomycin. The culture was maintained at 37°C in humidified atmosphere and concentrated with CO_2 (5%). Following the methods described by Huang *et al.*[20], the cells were sub-cultured once they reached ~80% confluency. Subsequently, the cell viability was determined by trypan blue staining and automated cell counter was used to obtain a cell concentration of 1×10^5 cells mL^{-1}, which was used in all experiments. Thereafter, the cells were plated in 96-well plate and incubated for 24 h to adhere. After the 24 h incubation period, the media was aspirated and cells were treated with 100 µL of 100, 10 and 1 µg mL^{-1} dilutions of the crude extracts (in triplicate). Aliquots of 100 µL of media were added to make a final volume of 200 µL. The plates were further incubated for 48 h. Cell proliferation was measured by MTT assay following incubation[21] and absorbance was read at 540 nm wavelength.

Selectivity was assessed on non-cancerous normal MCF-10A breast cells. Cells were grown in EMEM media supplemented with epidermal growth factors and 10% FBS.

Nitric oxide synthase activity: The Griess reagent method was used to measure the amount of NO produced by the cells after exposure to the plant extracts[22]. The assay relies on a diazotization reaction to detect nitrite (NO^{-}_{2}), which is a stable form of NO. The NO^{-}_{2} reacts with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. In this assay, cells (1×10^{5} cells mL^{-1}) were incubated with different concentrations of the extracts for 48 h. The cell culture supernatant (100 µL) was obtained from the culture and mixed with sulfanilamide (100 µL). The mixture was incubated for 10 min at room temperature. The NED solution (100 µL) was then added. The experiment was incubated for 10 min. Thereafter, the absorbance was measured at 550 nm. The nitrite (NO^{-}_{2}) concentration was calculated from the NO^{-}_{2} standard curve. Cells without extract were used as a negative control.

LOX activity assay: Lipoxygenase activity was determined using the lipoxygenase activity assay kit obtained from BioVision Life Science in South Africa. In this assay, LOX converts its substrate into an intermediate that reacts with the probe and in doing so, it generates a fluorescent product that is measured with a spectrophotometer. The cells (4×10^{5} cells mL^{-1}) were homogenised with 100 µL ice-cold LOX buffer and kept on ice for 10 min, followed by centrifugation at 10 000×g for 15 min. The cell lysate (10 µL) was then mixed with 10 µL plant extract at different concentrations (100, 10 and 1 µg mL^{-1}), 20 µl LOX substrate, 2 µL LOX probe and 78 µL of LOX buffer. The mixture was immediately placed on the fluorescence plate reader (PHERAstar FS, BMG) and the fluorescence was measured at 30 sec interval for 30 min, at an excitation wavelength of 500 nm and emission wavelength of 536 nm. A standard curve was generated from the LOX substrate and pure LOX enzyme supplied. The LOX inhibitor was used as a positive control. The experiment was performed in duplicates and repeated twice.

Cyclooxygenase-2 activity assay: Inhibition of COX-2 in MCF-7 cells was determined using COX activity kit from BioVision Life Science in South Africa. The cells (4×10^{5} cells mL^{-1}) were homogenised with 100 µL ice-cold LOX buffer and kept on ice for 10 min, followed by centrifugation at 10 000×g for 15 min. In each well, cell lysate (10 µL) was mixed with 10 µL plant extract at different concentrations, viz: (100, 10 and 1 µg mL^{-1}), 2 µL COX probe, 4 µL diluted COX cofactor and 74 µL COX assay buffer. The mixture was immediately placed on the fluorescence plate reader and the fluorescence was measured at 30 sec interval for 30 min, at an excitation wavelength of 536 nm and emission wavelength of 587 nm. A standard curve was generated using the supplied standard. The COX-2 inhibitor (supplied) was used as a positive control. The experiment was performed in duplicates and repeated twice.

Statistical analysis: The values are presented as the mean±standard deviation (SD) for 2 experiments performed independently.

RESULTS

The yields of the extraction process are summarized in Table 1. Methanol extracts had the highest yield for both leaves and barks while the lowest yield was observed in DCM stem extract.

Phytochemical analysis: Phytochemical composition of the 3 parts of *S. africana* plant viz: Leaves, stems and barks was analysed for the presence of various secondary metabolites. Tannins, glycosides, saponins and alkaloids were present in all plant parts. All the phytochemicals tested were detected in the bark except flavonoids, which were absent in all plant parts (Table 2).

Cell growth inhibition studies: Figure 1 illustrates cell growth inhibition of MCF-7 cells following exposure to *S. africana* extracts. The bark had 4 extracts: Hexane (TB1), dichloromethane (TB2), methanol (TB3) and ethyl acetate (TB4). From the 4 extracts, the dichloromethane extract (TB2) had the highest anti-proliferative activity, with concentrations of 10 and 1 µg mL^{-1} inhibiting more than 50% of the culture (IC_{50}). All the leaf extracts showed considerable activity with IC_{50} ranging from 1-10 µg mL^{-1}. For the stem extracts, only the DCM extract (TS2) indicated anti-proliferation activity at concentrations of <10 µg mL^{-1}.

Table 1: Percentage yields obtained from extraction of *S. africana* plant material with various solvents

Plant parts	Hexane (%)	DCM (%)	MeOH (%)	Ethyl acetate (%)
Leaves	0.5	0.70	21.1	0.2
Barks	5.9	5.10	39.9	5.9
Stems	1.9	0.02	ND	ND

ND: Not done

Table 2: Phytochemical screening results of *S. africana* plant parts

Phytochemicals	Stem	Leaf	Bark
Phytosterols	+	-	+
Pentose	-	-	+
Tannins	+	+	+
Glycosides	+	+	+
Triterpenoids	+	-	+
Anthraquinones	-	-	+
Saponins	+	+	+
Flavonoids	-	-	-
Alkaloids	+	+	+

+: Present, -: Absent

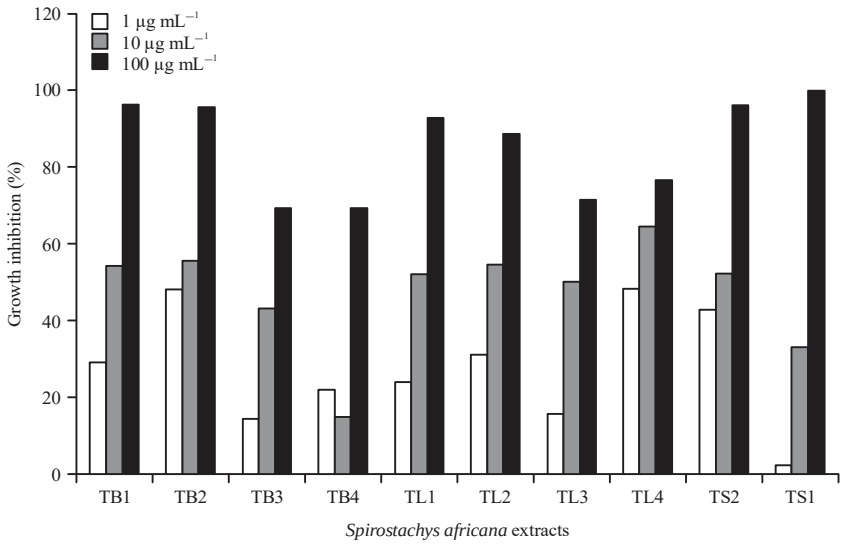

Fig. 1: Cell growth inhibitory effects of *S. africana* extracts on MCF-7 cells
TB: Bark extract, TL: Leaf extract, TS: Stem extract, 1: Hexane, 2: DCM, 3: Methanol, 4: Ethyl acetate

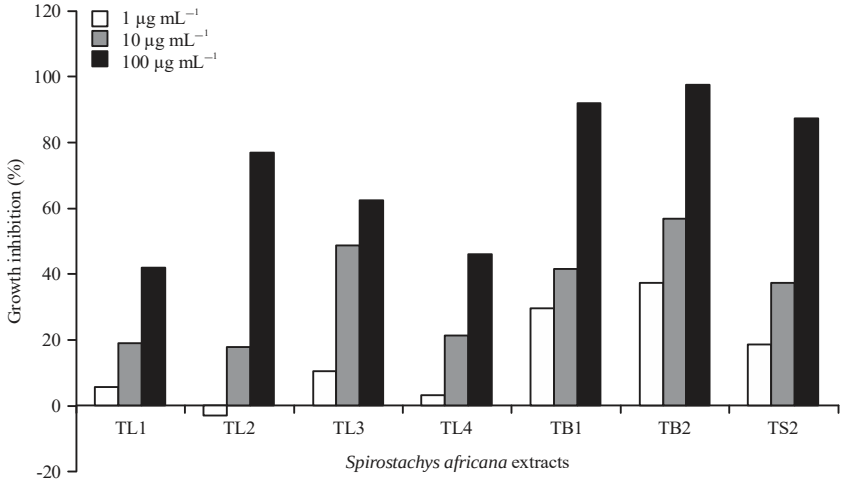

Fig. 2: Inhibitory effects of *S. africana* bark, stem and leaf extracts on normal human breast MCF-10A cell growth
TL: Leaf extract, TB: Bark extract, TS: Stem extract, 1: Hexane, 2: DCM, 3: Methanol, 4: Ethyl acetate

The hexane and DCM extracts of the bark (TB1 and TB2), all the leaf extracts (TL1-TL4) and the DCM extract of the stem (TS2), achieved IC_{50} at a concentration of 10 µg mL^{-1} and were selected for further studies.

Selectivity: Selectivity of the selected extracts was studied by determining their anti-proliferative activity against the normal human breast MCF-10A cells. All the leaf extracts showed low anti-proliferation activity against MCF-10A cells as compared to activity against MCF-7 cells (Fig. 2).

Generally, extracts that exhibited IC_{50} of <10 µg mL^{-1} against MCF-7 showed reduced inhibition (20% inhibition and less) at the same concentration against the normal breast cell line. The leaf extracts indicated minimal activity (<10% growth inhibition) against the normal breast cells at a concentration of 1 µg mL^{-1}. The hexane leaf extract (TL1), which had the highest cytotoxic activity against the breast cancer cells, showed the least activity on MCF-10A cells, suggesting some selectivity for MCF-7 cells.

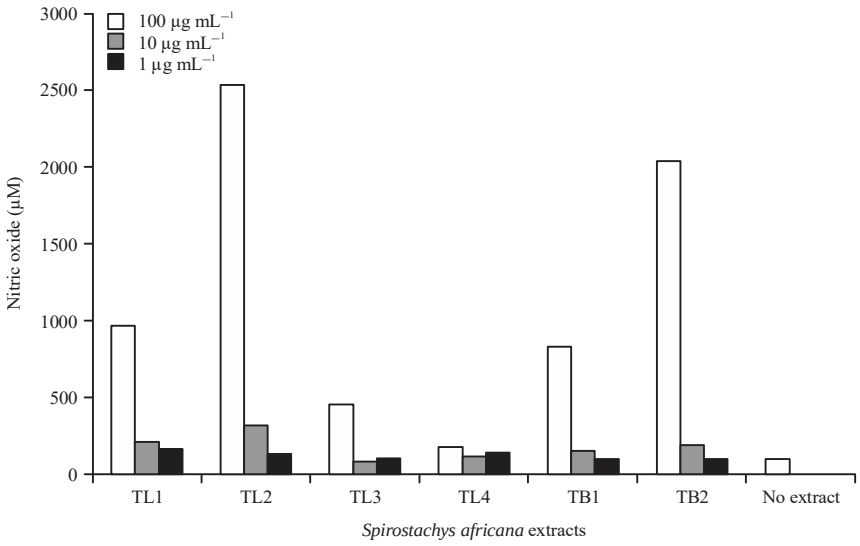

Fig. 3: Effects of *S. africana* extracts on NOS

TB: Bark extract, TL: Leaf extract, 1: Hexane, 2: DCM, 3: Methanol, 4: Ethyl acetate

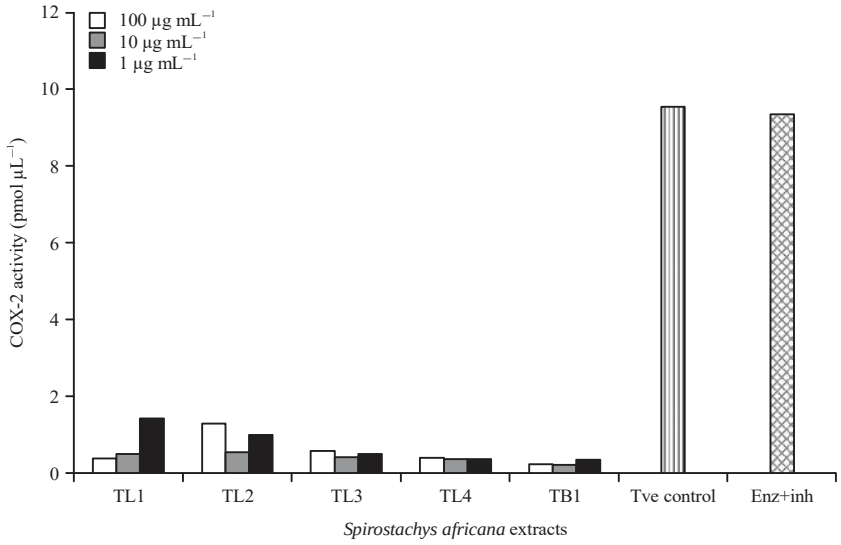

Fig. 4: Inhibition of activity of COX-2 by *S. africana* extracts

TB: Bark extract, TL: Leaf extract, 1: Hexane, 2: DCM, 3: Methanol, 4: Ethyl acetate, Tve control: Positive control (activity of pure enzyme supplied), Enz+inh: Pure enzyme with celecoxib (COX-2 inhibitor)

NOS inhibition studies: The effects of plant extracts on the activity of NOS are depicted in Fig. 3. The results demonstrate that the levels of NO produced by the cells following exposure to the extracts increased with increasing concentration of the extracts.

The hexane and DCM extracts of the both the leaf and bark (TL1, TL2, TB1 and TB2), which had exhibited the highest cell growth inhibition against MCF-7 cells, induced high levels of NO (over 1000 and 2000 µM, respectively) as compared to cells that were not exposed to the extracts. This implies a strong association between the high levels of NO and cell death.

COX-2 inhibition studies: The results depicted in Fig. 4 shows that COX-2 was inhibited by all selected *S. africana* extracts in a concentration dependent manner. The hexane extract of the bark-(TB1) exhibited the highest inhibition of enzyme activity (~0.26 pmol μL^{-1} at 100 µg mL^{-1}), as compared to celecoxib, a known inhibitor of COX-2, which had activity of 9.3 pmol μL^{-1}.

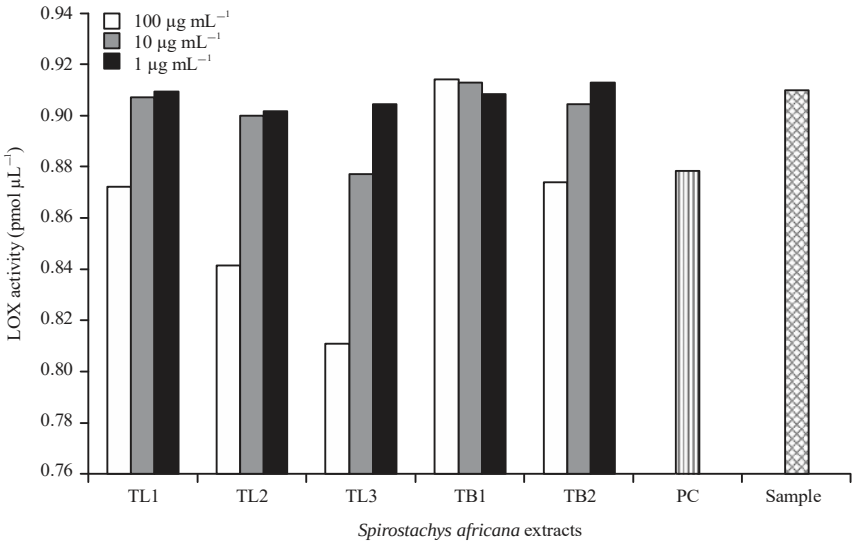

Fig. 5: Effects of selected extracts of *S. africana* on the activity of LOX enzyme

TB: Bark extract, TL: Leaf extract, PC: Positive control (cell lysate plus the supplied inhibitor), Sample: Cells without extract (negative control), 1: Hexane, 2: DCM, 3: Methanol

LOX inhibition studies: The LOX activity was determined by measuring the Relative Fluorescence Units (RFU) and deducing concentration from the LOX standard probe. The results are illustrated in Fig. 5. Extracts inhibited LOX activity in a concentration dependant manner with methanol leaf extract (TL3) demonstrating the highest inhibition (0.81 pmol μL^{-1}) at 100 µg mL^{-1}. The negative control (cells without the extract) had the highest activity of 0.91 pmol μL^{-1}.

DISCUSSION

The findings of this study confirm earlier research that many secondary metabolites such as tannins, glycosides, saponins and alkaloids are present in the S. *africana*. Previous studies reported that tannins have anticancer activity[23,24], while saponins, flavonoids and glycosides have anti-inflammatory and antioxidant activity[25-27], both of which are implicated in anticancer processes. In addition, the stem and the bark of *S. africana* ethanolic extracts were found to contain diterpenoids and triterpenoids[28]. Similarly, triterpenoids were detected in the same parts in the current study. It was also observed that all the non-polar extracts had the highest cell growth inhibitory activity. This suggests that the active phytochemicals are non-polar. The non-polar and semi-polar phytochemicals that could be present in the plant include alkaloids, phytosterols and triterpenoids[29].

The non-polar leaf and bark extracts which showed the highest inhibition of MCF-cells with IC_{50} values of 10 µg mL^{-1} and lower, also resulted in high production of NO. High levels of NO have been reported to promote apoptosis[30]. Results suggest that the non-polar leaf and bark extracts of *S. africana* induced the iNOS (inducible-nitric oxide synthase) to produce more NO, which promoted apoptosis.

The literature shows that LOX and COX-2 are highly expressed in tumour angiogenesis[31]. Inhibition of these enzymes inhibits tumour angiogenesis[32]. In this study, all leaf extracts and DCM bark extract of *S. africana* inhibited COX-2 activity more than the known COX-2 inhibitor. These extracts further inhibited the LOX enzyme. This implies that the extracts could inhibit the process of angiogenesis, which is associated with tumour initiation, growth and metastasis.

CONCLUSION

The *S. africana* leaves had few but essential phytochemicals that are known to have anti-inflammatory and anticancer activities. The leaf extracts selectively inhibited growth of breast cancer MCF-7 cells over normal breast cells and induced NO production. The extracts also inhibited COX-2 and LOX activities in a concentration dependent manner in MCF-7 cells. The results show selective inhibition of MCF-7 breast cancer cells, a correlation between cell death and stimulation of NOS and an inhibition of COX-2 and LOX enzymes.

The authors are not aware of similar studies that have been conducted on this plant.

SIGNIFICANCE STATEMENT

This study adds to the search for novel anti-cancer drugs from medicinal plants and contributes scientific knowledge on mechanisms exerted by medicinal plants on biological processes involved in cancer initiation and growth. *Spirostachys africana* exhibited anti-angiogenesis properties selectively on breast cancer cells. Active constituents could be isolated and further studied as potential anti-angiogenic chemotherapeutic lead compounds.

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