

Potential anticancer activity of Acetone extracts of *Toona ciliata*, *Seriphium plumosum* and *Schkuhria pinnata* on HeLa cervical cancer cells

Mxolisi Justice Ndlovu¹, Victor Patrick Bagla¹, Matlou Phenius Mokgotho¹,
Marema Ephraim Makgatho², Thabe Moss Matsebatlela¹

1. Department of Biochemistry, Microbiology and Biotechnology Faculty of Sciences and Agriculture.
2. Department of Pathology and Medical Sciences, Faculty of Health Sciences, University of Limpopo, South Africa.

Author emails:

MJ. Ndlovu: mxolisi.ndlovu@ul.ac.za; VP. Bagla: victor.bagla@ul.ac.za; MP. Mokgotho: matlou.mokgotho@ul.ac.za; ME. Makgatho: ephraim.makgatho@ul.ac.za; TM Matsebatlela: thabe.matsebatlela@ul.ac.za

Abstract

Background: Cervical cancer is common in women in less developed regions of the world. The plant biomolecules can be employed for synergistic activity with chemo- and radiotherapy. This combinations might result in reduced toxicity and increased efficacy of the treatment regimen.

Objectives: The anti-HeLa cells activity of the acetone extracts of *S. plumosum*, *T. ciliata* and *S. pinnata* was assessed using different parameters.

Methods: Secondary metabolite detection and antioxidant activity quantification were determined using the DPPH and ferric iron reducing assays. HeLa cell growth inhibition and mechanistics were assessed by employing MTT and Annexin-V fous assays.

Results: Observations revealed the presence of phenolic, flavonoids, tannins steroids and coumarins in all the plants extracts. High amount of total phenolic and flavonoid content were detected in *S. plumosum* and *T. ciliata*. *S. plumosum* extract had the best DPPH scavenging activity and ferric reducing powers.

Conclusion: Observable concentration dependent cell proliferation inhibition by test materials was exhibited. The leaf extracts from *T. ciliata*, *S. plumosum* and *S. pinnata* contain compounds of various polarities with free-radical, antioxidant and anti-cancerous activities that may play a beneficial role in treatment.

Keywords: Medicinal plants, anticancer activity, antioxidant activity.

DOI: <https://dx.doi.org/10.4314/ahs.v21i2.23>

Cite as: Ndlovu MJ, Bagla VP, Mokgotho MP, Makgatho ME, Matsebatlela TM. Potential anticancer activity of Acetone extracts of *Toona ciliata*, *Seriphium plumosum* and *Schkuhria pinnata* on HeLa cervical cancer cells. *Afri Health Sci.* 2021;21(2). 663-672. <https://dx.doi.org/10.4314/ahs.v21i2.23>

Introduction

The prevalence of cervical cancer has reached alarming global levels where in 2010 it was reported to be 4-fold higher prevalence of cervical cancer in low to middle-income countries¹. Countries ranked low in the Human Development Index (HDI), the disease is ranked as the second most common type of cancer

and second highest cause of cancer-related mortality amongst women after breast cancer. In Africa, cervical cancer is the most diagnosed and the leading cause of cancer-related death among². The disease affects one in 42 women and available reports from Statistics South Africa (2014) estimates that 16.84 million women over the age of 15 are at risk of the disease. At present, more than 8 South African women die from the disease every day³. This has prompted the observance in the month of September of each year as the cervical cancer awareness month in South Africa, to highlight the impact of the disease. Although vaccines are available for the prevention of the disease and can be administrated to girls between 9 and 13 years, they are unaffordable³.

Corresponding author:

MJ Ndlovu,
Department of Biochemistry,
Microbiology and Biotechnology Faculty
of Sciences and Agriculture.
Email: mxolisi.ndlovu@ul.ac.za

Immunization has been considered to unlikely generate significant therapeutic effects for establishing antibody-mediated neutralization⁴. Therapeutic agents with fewer side effects can serve to bridge this gap. The plants under investigation were selected based on their traditional indication in the treatment of patients exhibiting symptoms of cervical cancer by traditional healers in Limpopo Province of South Africa. *T. cilliata* is reported to be used mostly on chronic dysentery, ulcer and blood complaints as a cardiogenic, antihelminthic and as an expectorant⁵. *S. pinnata* is used mainly for blood cleansing, malaria and urinary tract infections⁶, while *S. plumosum* is used mainly for heart problems, epilepsy and postural realignment⁷. The present study focused on the anti-proliferative effects of acetone extracts of selected medicinal plants on proliferation of a HeLa cancer cell line, antioxidant activity, secondary metabolites, phytochemical constituencies and apoptotic effects.

Materials and Methods

Plant collection and verification of leaves.

S. plumosum and *S. pinnata* were collected from Mankweng and *T. cilliata* from Tzaneen in Limpopo province, South Africa. Plants were identified by the Larry Leach Herbarium, University of Limpopo and vouchered as *S. plumosum* (UNIN 121065), *T. cilliata* (UNIN 12331) and *S. pinnata* (UNIN 121066).

Plant extracts preparation. The leaves of the plants were dried in the dark at 25°C for two weeks and grinded into fine powder (1g) which was exhaustively extracted using 10 ml of acetone⁸. The supernatants were filtered into pre-weighed glass vials. The quantity of plant materials extracted was determined and stored in air-tight glass vials in the dark until use. The dry plant extracts were reconstituted in dimethylsulphoxide (DMSO) (Sigma Aldrich, SA) for cell-based assays.

Phytochemical profiling of plant material.

Extracts of the selected plants were reconstituted in acetone (1 mg/ml) and spotted on aluminium backed thin layer chromatography plates (TLC) (Macherey-NEGEL, GMBH & CO.KG) for analysis. Each extract (10 µl) was loaded onto a TLC plate and developed in three different mobile phase viz: Chloroform: Ethyl acetate: Formic acid [CEF] 10:8:2 (v/v/v) (intermediate polarity/acidic), Ethyl acetate: Methanol: Water [EMW] 10:1, 35:1 (v/v/v) (polar/neutral) and Toluene: Ethanol: Ammonium hydroxide [TEA] 18:2:0.2 (v/v/v) (non-polar/basic). The separated compounds were vis-

ualized under ultraviolet light at wavelengths of 254 and 360nm. The plates were sprayed with vanillin-sulphuric acid (0.1 g vanillin (sigma): 28 ml methanol) and heated at 110°C to visualize coloured bands on the plates.

Qualitative antioxidant analysis using DPPH.

Thin layer chromatographic plates were prepared as described for in the previous section and sprayed with 0.2% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol solution to detect the presence of compounds with antioxidant activity. A positive result was represented by the presence of yellow bands on a purple background and scanned using a scanner (CanoScan Lide 700F)⁹.

Quantitative antioxidant activity assay (DPPH assay)

The plant extracts were tested for scavenging activity using quantitative methods as described by¹⁰. Thin layer chromatography (TLC) plates were used to separate compounds of the extracts with antioxidant activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) indicator, was sprayed on the plates to assist in viewing the antioxidant compounds. The quantitative method was performed using micro-titer plates, where 100 µl of each plant extract was serially diluted with water and was tested for antioxidant activity at various concentrations. DPPH (100 µl) was added into the wells after dilution. Ascorbic acid (vitamin C) was used as positive control and absorbance was read at 540 nm using M8500 UV-Visible spectrophotometer made in China.

Qualitative screening of various phytochemical constituencies from the selected medicinal plants

Phenols. The presence of phenols was indicated by a blueish and greenish colour change after additional of test material and distilled water¹¹.

Tannins. After the addition of the plant extract and distilled water as well as ferric chloride, the presence of a greenish-black colour was indicative of tannins¹².

Flavonoids. Flavonoids tested positive due a yellow colour after adding the test material, ammonia solution and concentrated H₂SO₄¹³.

Steroids. The Salkowski's test was used¹⁴.

Coumarins. A total volume of 2 ml of the extract was mixed with 3 ml of NaOH (10%), a yellow coloration was observed to draw an inference, indicating the presence of coumarins¹⁵.

Determination of total phenolic content.

The total phenolics in extracts was determined spectrophotometrically using Folin-Ciocalteu's phenol reagent. Garlic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalence (GAE). Concentration of 0.01, 0.02, 0.03 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentration of 0.1 and 1mg/ml of plant extracts were also prepared in methanol and 0.5ml of each sample were introduced into the test tubes and mixed with 2.5 ml of a 10- fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at 25°C before the absorbance was read at 760 nm using (Beckman Coulter DU® 730 and Life Science UV-Visible spectrophotometer). All determinations were performed in triplicate. Folin-Ciocalteu reagent being using sensitive to reducing compounds including polyphenols produce a blue color upon reaction which was measured spectrophotometric¹⁶. (Beckman Coulter DU® 730 and Life Science UV-Visible spectrophotometers was used) South Africa.

Determination of total flavonoids. The presence of flavonoids in the plant extracts was determined using aluminium chloride colorimetric assay as described by¹⁷. Volume of 1 ml of plant extract was mixed with 4 ml of distilled water and subsequently with 0.30 ml of a NaNO₂ solution (10%). After 5 minutes, 0.30 ml solution AlCl₃ (10%) was added followed by 2 ml of 1% NaOH solution to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was determined at 510 nm versus the blank using T60 UV- Visible spectrophotometer (Roche, S.A). Standard curve of quercetin was prepared (0-12 mg/ml) and the results were expressed as quercetin equivalents (mg quercetin/gm dried extract).

Cell culture. An immortalised human cell line (HeLa) was used in this study (ATCC® CCL-2™, Rockville, USA). The cells were cultured and maintained in RPMI media (Lonza, BioWhittaker®), supplemented with 10% foetal bovine serum (Hyclone, Thermo Scientific) at 37°C, in an atmosphere of 5% CO₂ in a humidified incubator (Heracell 150i CO₂ incubator, Thermo Scientific)

Determination of the effects of plant extracts on HeLa cell proliferation using the MTT assay.

HeLa cells were seeded in 96 well tissue culture plates at a density of 2 x 10⁴ cells/ml and treated with various concentrations (50 – 1000µg/ml) of extract for 24 hours. After 24 hours of incubation, MTT solution (2.5 mg/ml) was added to each well (20 µl). The cells were incubated for a further 4 hours at 37°C, and the supernatant was removed. The pellet was then dissolved by addition of 100 µl of DMSO. The antiproliferative activity of extracts was determined following the conversion of tetrazolium by the enzyme dehydrogenase to purple formazan¹⁹ which directly represent the number of viable cells. The absorbance was measured at 560 nm using a Glomax microtiter plate (Promega, U.S.A). Actinomycin D was used as positive control while untreated cells were used as negative control. Percent cell viability was calculated using the formula: Percentage viability = (A_{490nm} of sample) x 100/ (A_{490nm} of control)

Determination of apoptotic activity effects of the selected plant extracts on HeLa cells.

The cells were seeded over a cover slip and put inside a six well plate for 24 hours. The cells were then treated and incubated for 24 hours in a CO₂ incubator. The slips were washed followed by addition of paraformaldehyde and incubated for 30 minutes. After incubation it was followed by a washing step and addition of Annexin V FITC solutions for 30 minutes. This was followed by viewing under fluorescent of X60 magnification with Nikon Ti- E inverted microscope (Nikon Japan) using different excitations.

Results

Extraction yield, TLC profile and qualitative antioxidant activity of acetone leaf extracts. The plant leaf materials (10 g) exhaustively extracted using 100 ml of acetone yielded 0,0432g for *S. plumosum*, *T. ciliata* (0,1786g) and *S. pinnata* (0,0295g). A representative fingerprint profile of the three acetone extracts in the eluent system (CEF) that best separated compounds with antioxidant activity is presented (figure 1). Antioxidant activity profile show *S. plumosum* to contain more compounds with antioxidant activity as compared to the other two plant extracts (figure 1B).

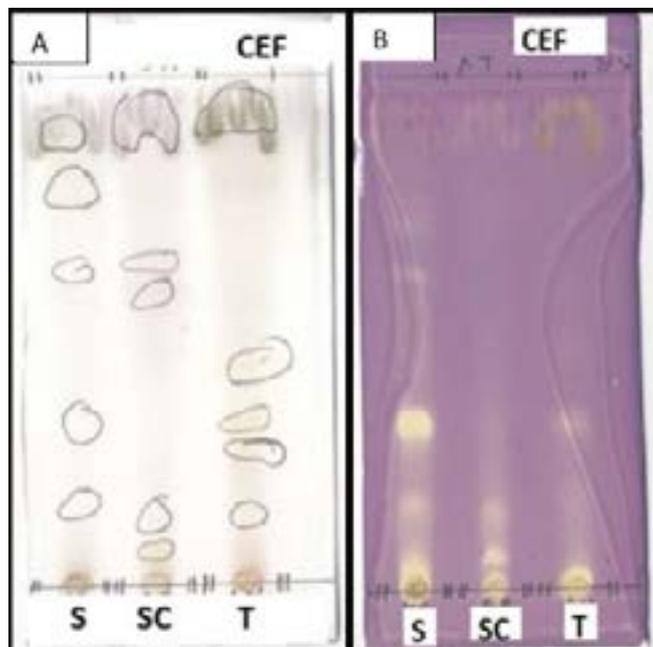


Figure 1: Thin layer chromatograms of acetone leaf extracts obtained from *S. plumosum* (S), *S. pinnata* (SC) and *T. cilliata* (T) developed in CEF as mobile phases. The chromatograms were sprayed with vanillin/H₂SO₄ (A) and DPPH for detection of antioxidant compounds (B).

Evaluating the presence of secondary metabolites in the acetone extracts of the selected medicinal plants

Table 1: Types of secondary metabolites detected in the acetone leaf extracts of *S. plumosum*, *S. pinnata* and *T. cilliata* using chemical methods.

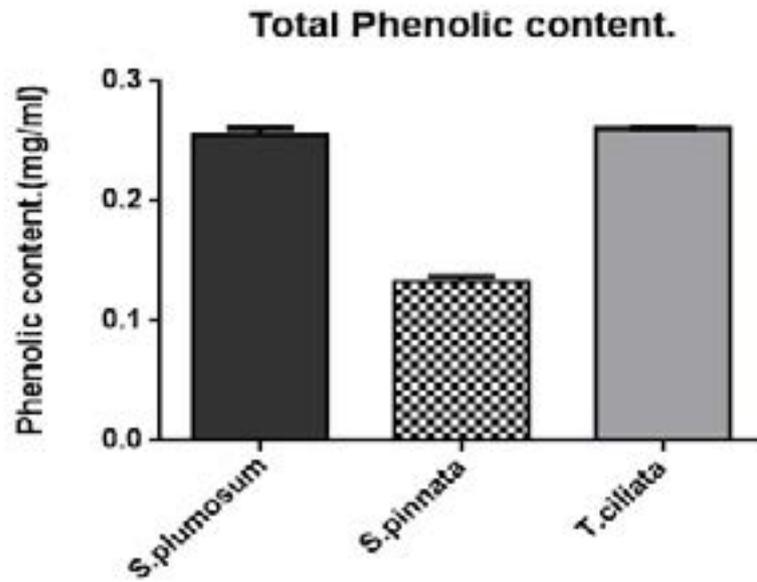
Secondary metabolites	Type of extract		
	S	SC	T
Phenolics	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Coumarins	+	+	+
Steroids	+	+	+

Keynote: (+) presence; (-) absence; *S. plumosum* (S), *S. pinnata* (SC), *T. cilliata* (T).

Determination of Phenolic contents of the acetone extracts of the selected medicinal plants

The total phenolics in extracts were determined (figure

2) using Garlic acid as a standard and expressed as mg/ml gallic equivalence (GAE).



Determination of total flavonoids contents of the acetone extracts of the selected medicinal plants
 The flavonoid content of extracts was measured by the aluminum chloride colorimetric assay (figure 3).

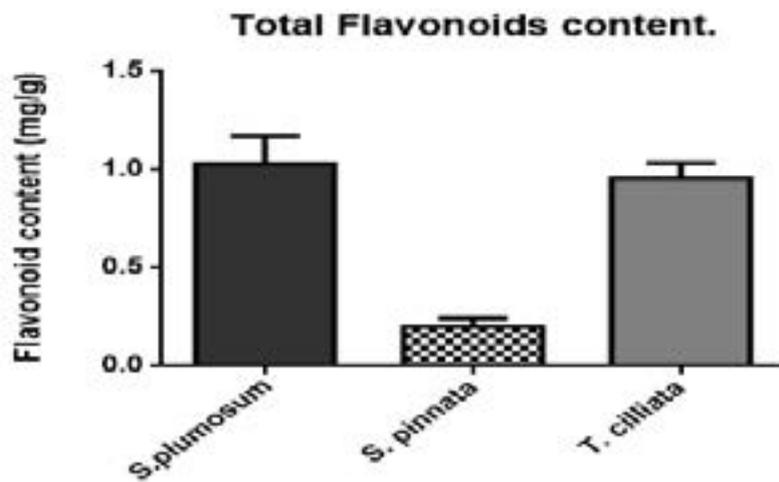


Figure 3: Total flavonoids content

Cell proliferation determination of the acetone leaf extracts of the selected medicinal plants

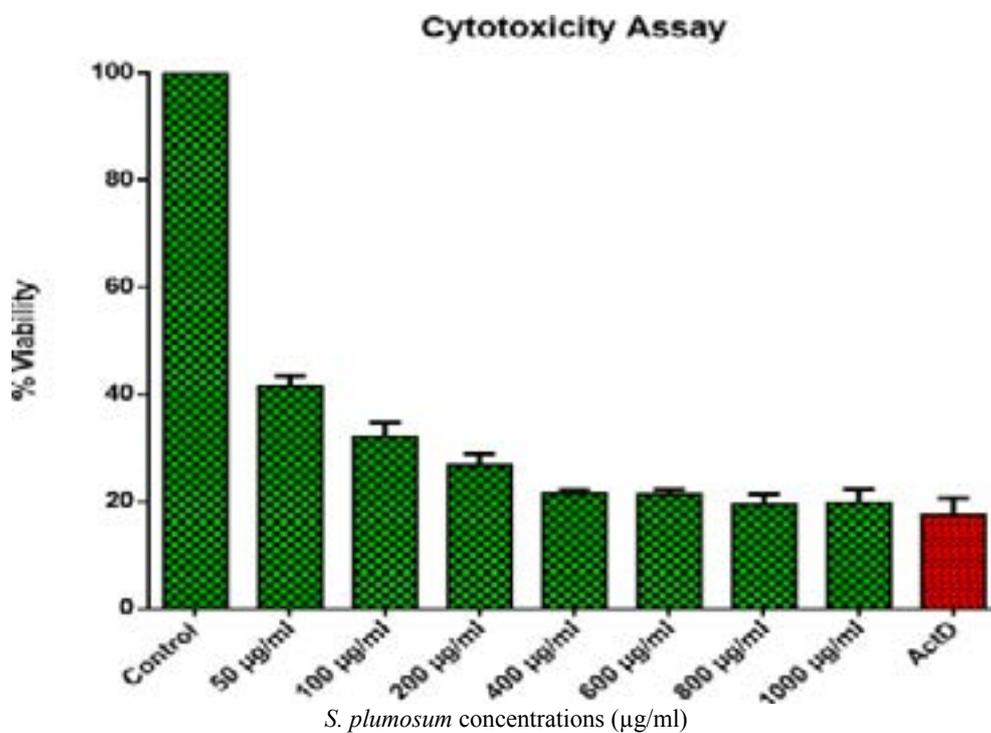


Figure 4: Cytotoxicity effects of *S. plumosum* acetone leaf extract. HeLa cells at a density of 1×10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 µg/ml - 1000 µg/ml. The results are presented as \pm SEM from triplicate of three independent experiments. *ActD stands for Actinomycin D.

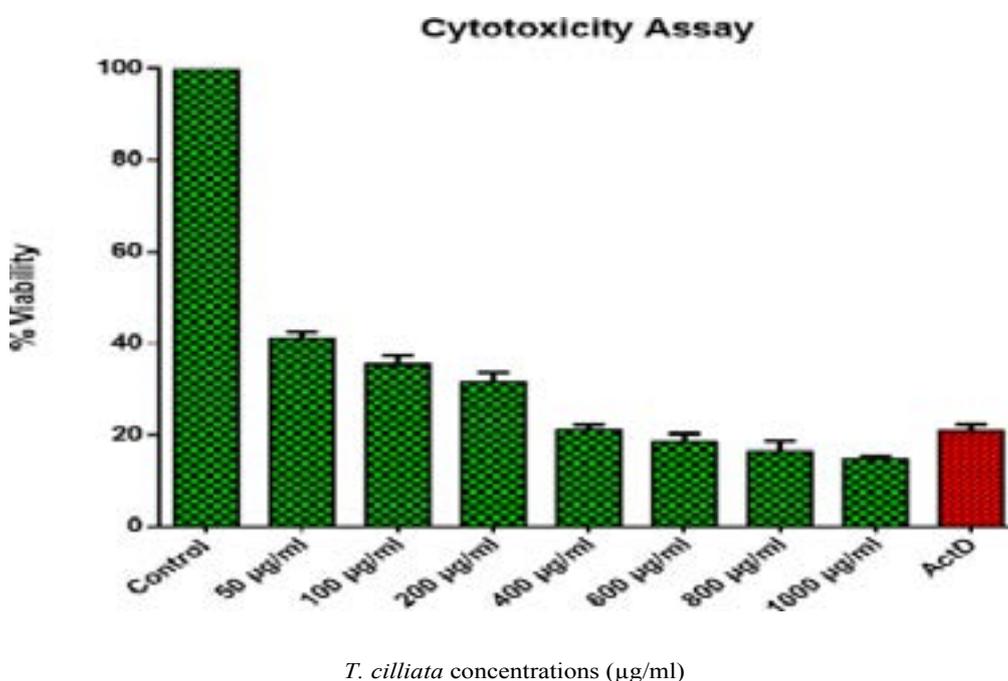


Figure 5: Cytotoxicity effects of *T. cilliata* acetone leaf extract. HeLa cells at a density of 1×10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 µg/ml - 1000 µg/ml. The results are presented as \pm SEM from triplicate of three independent experiments. *ActD stands for Actinomycin D.

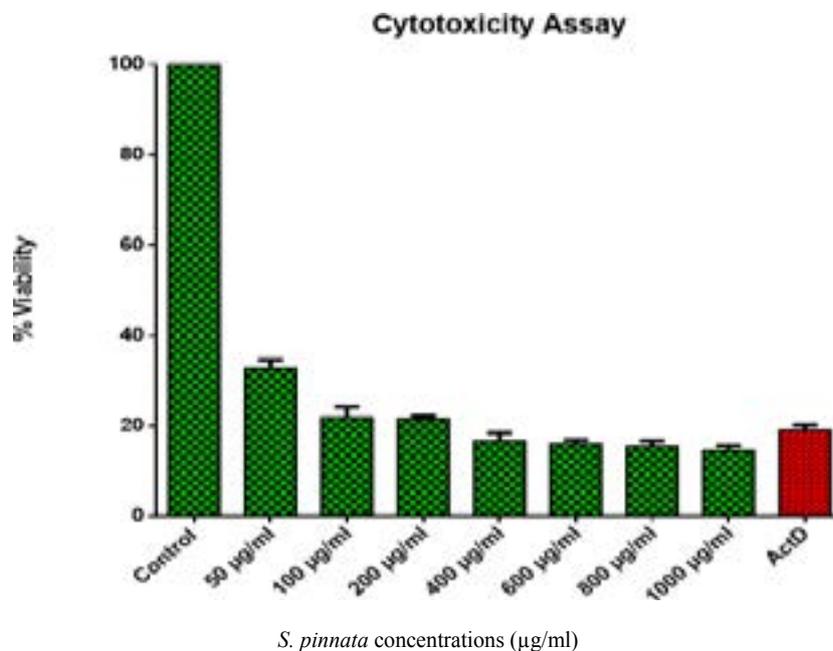


Figure 6: Cytotoxicity effects of *S. pinnata* acetone leaf extract. HeLa cells at a density of 1×10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 µg/ml - 1000 µg/ml. The results are presented as \pm SEM from triplicate of three independent experiments. *ActD stands for Actinomycin D.

Determination of apoptotic effects of the selected plant extracts.

Annexin-V and PI apoptosis detection assay was used

to test for the ability of the acetone leaf extracts of *S. plumosum*, *T. ciliata* and *S. pinnata* to induce apoptosis (figure 7, 8 and 9).

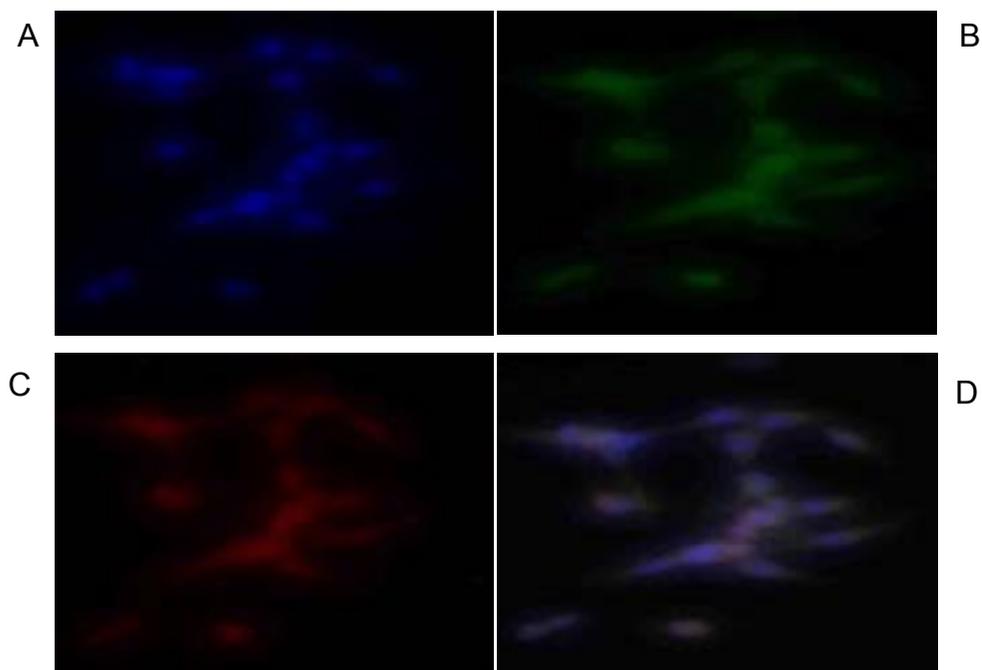


Figure 7: Apoptotic effects

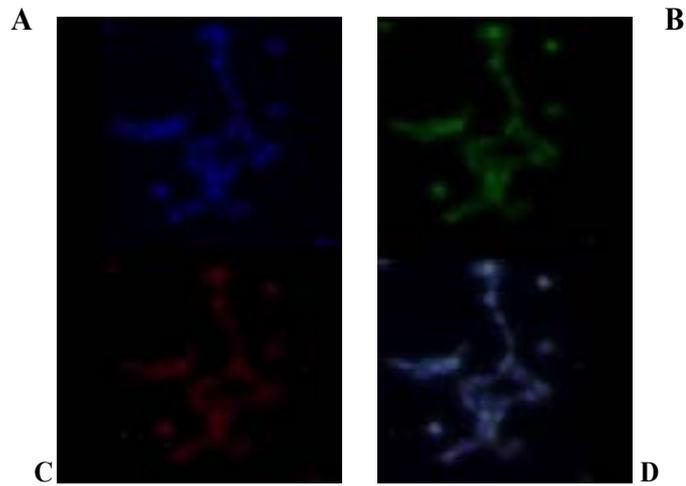


Figure 8: Apoptotic effects of *T. ciliata* acetone leaf extract. Cells were cultured at a density of 6×10^6 cells/ml, on coverslip in micropetri-dishes. (A) represents a slide incubated with 4',6-diamidino-2-2 phenylindole (DAPI), (B) represents a slide incubated after adding Annexin V Fluos and shows the flipping of phosphatidylserine, (C) represents the disintegrated nucleus after the movement of annexin V into the cell and (D) represents the slide after overlaying with all the colours.

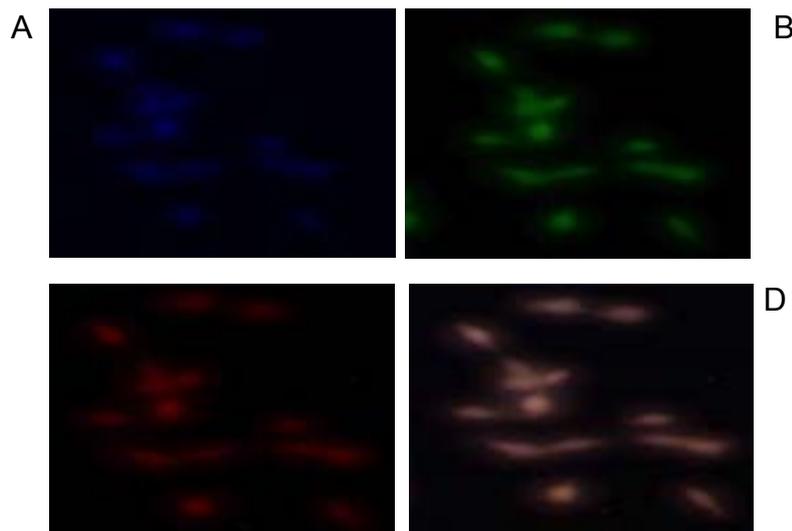


Figure 9: Apoptotic effects

Discussion

Cervical cancer is one of the detrimental and life-threatening female cancers with its early stages being cured with surgery and a combination of chemotherapy (cis-platinum) and radiation therapy. Thus, treatment that is effective, less toxic, affordable is desirable. Thus, the current study was therefore aimed at evaluating the extracts of the selected plants to induce apoptosis. Acetone was the choice in this study due to its ability to extract both polar and non-polar constituents.

Phytochemical analysis of the plant extracts revealed the presence of phenolics, flavonoids, tannins, steroids and coumarins in all the plants. Phenolic compounds are one of the largest and common groups of plant metabolites¹⁸. They have important biological properties such as anti-carcinogenic, anti-inflammatory, as well as the inhibition of the angiogenesis process¹⁹. All the plants tested positive for phenols which exhibit antioxidant and antiproliferative tumor effects whereas ethanolic extracts of *S. pinnata* have been reported to

exhibit anticancer activity against a human colon cancer cell line (Caco-2)²⁰. Flavonoids have been reported for playing an important role in preventing carcinogenesis through anti-proliferation, anti-oxidation and apoptosis activity in various cancer cell lines²¹, Tannic acid possible application versatility not only in cancer prophylaxis, as was initially thought, but also in adjuvant cancer therapy was also reported²². With steroids there was increasing research attention through investigating the novel therapeutic potential of steroidal cardiac glycosides in the past decade²³. Curcumin has also been reported to have anti-cancer activity in both non-small cell lung and small cell lung cancer²⁴. On the other hand, this study represents the first report of the anticancer activity of *T. cilliata* and *S. plumosum*. The detected phytochemical compounds contained in these plants have immersed bioactive activities and can be considered to be valuable reservoir of bioactive compounds of a high medicinal good.

These compounds, however, still need to be isolated and identified. The ferric reducing antioxidant power assay also showed an increase in percentage ferric iron reduction with increase in the concentration of the extracts and the positive control. This finding, furthermore, supported the antioxidant activities of the plant extracts. Toxicological studies were conducted on the plants to examine the toxicity profiles of these plant extract constituents to produce safe and less toxic new drugs.

Determination of apoptotic effects of the acetone leaf extracts was performed against cervical cancer HeLa cells. The method used in this study, is based on the degradation of the nucleus, condensation of the chromatin and flipping of the cell membrane²⁴. Following treatment of cells with 400 µg/ml of the different plant extracts, the cells were shown to have undergone apoptosis, this concentration was chosen because it was almost the intermediate of the highest concentration. The heavily stained nucleus in (A) represents the nuclei considered to have the abnormal phenotype. Sec. hoAlasrOdneed, u37c5eGdreferonbmriethrDerivfieg, uCrhearslo, tSte.svpillue,mVAo,s2u2m901 showed to have the highest concentration of the stain followed by *T. cilliata* and lastly *S. pinnata* which shows that it induced more cytotoxicity effects against the cell line.

Propidium iodide is a cell permeant fluorochrome that stains the DNA and is used to identify the mode of cell death and phase of apoptosis pictures (B). The Annexin V- FITC green fluorescence is used to identify the

mode of cell death through flipping of the membrane. The fluorescence intensity in (figure 7, 8 and 9) represent the phase and amount of cell undergoing apoptosis. The higher red fluorescence represents cell death through the disintegrations of the nucleus after the movement of annexin V into the cell, while (D) represents fluorescence after overlaying all the three images. After overlaying the images, cells displaying simultaneous reddish nuclear and a greenish membrane fluorescence, represent cells that have undergone apoptosis²⁰. Apoptosis is important since it does not promote continuous growth of cells that finally leads to cancer.

Conclusion

All leaf extracts were shown to contain various phytochemical constituents. *S. plumosum* showed more antioxidant activity as compared to the other extracts. All leaf extracts were shown to contain secondary metabolites such as Phenolics, flavonoids, tannins, steroids and coumarins. All the plant extracts were cytotoxic to HeLa cells. Studies will be ongoing to determine the effect of the extracts on the expression underlying mechanisms in cervical cancer progression with the aim of identifying therapeutic principles contained in the extracts for the treatment of cervical cancer.

Conflicts of interest

There is no conflict of interest.

Funding

Research reported in this publication was supported by Thuthuka funding awarded to Matlou P. Mokgotho, and the University of Limpopo.

References

1. Bruni L, Diaz M, Castellsagué X, Ferrer E, Bosch FX, de Sanjosé S. (2010). Cervical human papillomavirus prevalence in 5 continents: Meta-analysis of 1 million women with normal cytological findings. *Journal of Infectious Diseases*. 202: 1789-1799.
2. Bray F, Ren JS, Masuyer E, Ferlay J. (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *International Journal of Cancer*. 132: 1133–1145.
3. Botha, M. H. and Dochez, C. (2012). Introducing human papillomavirus vaccines into the health system in South Africa. *Vaccine*. 30: 28 - 34.
4. Roden, B. S. R, Ling, M. and WU, C. T. (2004). Vaccination to Prevent and Treat Cervical Cancer. *Perspectives in Pathology*. 35: 971 - 982.
5. Katende, B., Birnie A. and Tengnas B. (1995). Use-

- ful trees and shrubs for Uganda. Identification, Propagation and Management for Agricultural and Pastoral Communities. Regional Soil Conservation Unit (RSCU), Swedish International Development Authority (SIDA). 1 - 710.
6. Muthaura, C. N., Rukunga, G. M., Chhabra, S. C., Omar, S. A., Guantai, A. N., Gathirwa, J. W., Tolo, F. M., Mwitari, P. G., Keter, L. K., Kirira, P. G. and Kimani, C. W. (2007). Antimalarial activity of some plants traditionally used in Meru district of Kenya. *Phytotherapy Research*. 21: 860-867.
 7. Rodrigo, G. C., Almanza, G. R., Akesson, B. and Duan, R. D. (2010). Antiproliferative activity of extracts of some Bolivian medicinal plants. *Journal of Medicinal Research*. 4: 2204 – 2210.
 8. Avenant, P. (2015). Production Scientist Report on the National bank bush (*Seriphium plumosum*) Survey. Agriculture. *Forestry and Fisheries*. 4-38.
 9. Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*. 60: 1 - 8.
 10. Deby, C. and Magotteaux, G. (1970). Relationship between essential fatty acids and tissue antioxidant levels in mice. *Comptes Rendus des Séances de la Société de Biologie et de ses Filiales*. 164: 2675.
 11. Katsube, T., Tabata, H., Ohta, Y., Yamaski, Y., Anuurad, E., Shiwaku, K. and Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *Journal of Agriculture Food Chemistry*. 5: 2391 - 2393.
 12. Harborne, J. B. (1973). Phytochemical Methods. *Scientific Research*. 49 - 188.
 13. Borokini, T. I. and Omotayo, F. O. (2012). Phytochemical and ethanobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research*. 6:1106-1118.
 14. Walboomers, J. M., Meijer, C. J. and Pathol, J. (1997). Do HPV-negative cervical carcinomas exist. 181: 253- 254.
 15. Savitree, M., Isara, P., Nittaya, S. L. and Worapan, S. (2004). Radical Scavenging Activity and Total Phenolic Content of Medicinal Plants Used in Primary Health Care. *Journal of Pharmaceutical Sciences*. 9: 32 - 35.
 16. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65: 55-63.
 17. Yadav, M., Chatterji, S., Gupta, S. K. and Watal, G. (2014). Preliminary phytochemical screening of six medicinal plants used in traditional medicine. *International Journal of Pharmacy and Pharmaceutical Sciences*. 6: 0975 - 1491.
 18. Singh, R., Singh, S. K. and Arora, S. (2007). Evaluation of antioxidant potential of ethyl acetate extract/fractions of *Acacia auriculiformis* A. *Cunn. Food and Chemical Toxicology* 45: 1216 – 1223.
 19. Han, X., Shen, T. and Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences*. 950 - 988.
 20. Rodrigo, G. C., Almanza, G. R., Akesson, B. and Duan, R. D. (2010). Antiproliferative activity of extracts of some Bolivian medicinal plants. *Journal of Medicinal Research*. 4: 2204 – 2210.
 21. Lea, M.A. (2015). Flavonol regulation in tumor cells. *Journal of Cellular Biochemistry*. 116: 1190–1194.
 22. Baer-Dubowska, W. Szaefer, H; Majchrzak-Celińska, A. and Krajka-Kuźniak V.(2020).Tannic Acid: Specific Form of Tannins in Cancer Chemoprevention and Therapy-Old and New Applications.*Current Pharmacology Reports*. 6:28 - 37.
 23. Chien-shan C., Jiaqiang W., Jie C., Kuei T. K., Jian T., Huifeng G., Lianyu C., Zhen C and Zhiqiang M. (2019). New therapeutic aspects of steroidal cardiac glycosides: the anticancer properties of Huachansu and its main active constituent Bufalin. . 19:92.
 24. Khan, N. and Mukhtar H. (2015). Dietary agents for prevention and treatment of lung cancer. *Cancer Letters*. 359: 155–164.
 25. Galluzzi, L. Zamzami, N., de La Motte Rouge, T., Lemaire, C., Brenner, C. and Kroemer, G. (2007) Methods for the assessment of mitochondrial membrane permeabilization in apoptosis. *Apoptosis*. 12: 803 - 13.