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Brine Shrimp Cytotoxicity and Anti-Mitotic Activity of Aqueous Root-Bark extract of *Securidaca longepedunculata* (Polygalaceae)

Ridwan A. Lawal[1]*, Sodiq K. Lawal[2], Omolola S. Odesanmi[1], Olufemi Isiaq[3], Ikeoluwapo O. Adefisan[4], Ibrahim A. Badmus[1], Jimoh O. Yusuf[1], Osaretin A.T. Ebuehi[1]

[1]*Department of Biochemistry, College of Medicine, University of Lagos, P.M.B 12003, Lagos, Nigeria.*
[2]*Department of Human Anatomy, St Francis University College of Health and Allied Sciences, Ifakara, Tanzania.*
[3]*Department of Computing, Southampton Solent University, United Kingdom.*
[4]*Department of Biological Sciences, College of Basic & Applied Sciences, Mountain Top University, Ogun State, Nigeria.*

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ABSTRACT

Securidaca longepedunculata is a savannah shrub found growing in South-West Nigeria and some other areas in Africa. It has been reported to be used in the treatment of over a hundred ailments. Its root bark is included in several anti-cancer decoctions in Nigeria. Despite its reported use as an anti-cancer plant, there is a dearth of information on the anti-cancer potential of *Securidaca longepedunculata* in Nigeria. The aim of this research is to determine the cytotoxic and anti-mitotic activity of aqueous root bark extract of *Securidaca longepedunculata*. *Securidaca longepedunculata* aqueous root-bark extract was prepared and used for the study. Cytotoxic activity of *S. longepedunculata* extract was determined by the brine shrimp toxicity assay and *Allium cepa* assay was used to assess the anti-mitotic activity. Brine shrimp toxicity assay showed an LC_{50} of 25.1 µg/mL and the *Allium cepa* assay revealed that 100 mg/mL extract caused a reduction in mitotic index (MI) which was comparable to that of the standard drug, methotrexate. *Securidaca longepedunculata* has potential as a cytotoxic agent.

***Keywords*:** *Allium cepa*, Brine Shrimp, *Securidaca longepedunculata*, cytotoxic, cancer.

Introduction

Cancer is an abnormal growth of cells in the body and can be treated by correcting the imbalance observed in the regulation of cell growth.[1] It remains one of the leading causes of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008.[2] Over 22.4 million people are reportedly suffering from cancer and approximately 10.1 million new cases are reported annually.[3] Apart from surgery and radiography, chemotherapeutic agents are useful in the treatment of cancer with associated toxicity.[4] Hence, there is always a continuous search for novel natural anticancer agents that will decipher the difference between cancerous and normal cells.[5] The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care.[6] The National Cancer Institute was reported to have collected about 35,000 plant samples from 20 countries and screened about 114, 000 extracts for anticancer activity.[7] The search for anticancer agents from plants led to the discovery and development of the vinca alkaloids (vincristine and vinblastine), the cytotoxic podophyllotoxins[5] as well as taxol (a cytotoxic diterpene alkaloid) from the Pacific yew tree, *Taxus brevifolia* (Newman et al., 2000). In 1997, Cragg *et al.*[9] reported that 60% of the 92 anticancer drugs available were of natural origin. Anticancer drugs exert their chemotherapeutic effect by blocking cell cycle progression and triggering apoptotic cell death.[10] Mitosis is an important phase of cell division cycle.[11] The *Allium cepa* assay method has been used severally to demonstrate the anti-mitotic activity of plant extracts and drugs.[12] Brine shrimp lethality assay is a useful tool to test extracts and drug for their toxicity.[13]

Securidaca longepedunculata has in recent times been reported to be used to cure a variety of ailments.[14] *Securidaca longepedunculata* has been described as the Mother of all plants in Northern Nigeria and the root bark extract is reported to possess nephrotoxic and hepatotoxic activity.[15] The aqueous root bark extract of the plant has also been shown to have significant pro-oxidant activity.[16] The anti-cancer activity of *Securidaca longepedunculata* root bark extract has been reported.[17] *Securidaca longepedunculata* has been shown to possess cytotoxic materials such as phenol, 2, 4-bis (1, 1-dimethylethyl) and bis (2-ethylhexyl) phthalate.[18] A study by Dibwe *et al* had earlier reported the constituents of *S. longepedunculata* to include four novel highly oxygenated xanthones, named muchimangins A–D.[19] *Securidaca longepedunculata* also displayed potent preferential cytotoxicity when used on human pancreatic cancer PANC-1 cell line in a nutrient-deprived medium.[20] However, the mechanism of anti-cancer activity of *Securidaca longepedunculata* root bark extract has not been elucidated nor reported. This study aims to determine the brine shrimp cytotoxicity and the anti-mitotic activity of aqueous root bark extract of *Securidaca longepedunculata*.

Materials and Methods

Materials

Methotrexate (Biochem), Onion bulbs, universal bottles, blade, filter paper, microscope slides, coverslips, distilled water, Orcein stain, glacial acetic acid. All other chemicals used were of analytical grade available locally.

*Corresponding author. E mail: alawal@unilag.edu.ng
Tel: +234-8056036852

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Plant collection

Securidaca longepedunculata root barks were collected from Osogbo, South-West, Nigeria in January 2010. Plant material was identified and authenticated by Mr. Odewo. A voucher specimen was deposited in the University Herbarium, University of Lagos, Lagos, Nigeria with voucher number: LUH 3593.

Preparation of aqueous extract of Securidaca longepedunculata

The plant materials were shade dried for 3 days and pulverized into powder. Aqueous extract of the coarsely powdered material was obtained by macerating 1 kg of root bark in 1 L of distilled water for 72 h. The macerate was filtered and the filtrate was concentrated using a Rotary Evaporator and further concentrated to constant weight *in vacuo* using a lyotrap. The percentage yield was calculated using the formula;

$$\% \text{ Yield} = \frac{\text{Extract Weight}}{\text{Plant Weight}} \times 100$$

Brine Shrimp Toxicity Assay

Brine shrimps (*Artemia salina*) eggs were hatched in sea water. After 24 h incubation at room temperature, the nauplii were attracted to one side of the vessel with a light source. Brine shrimps exposed to various concentrations (1000, 100, 10 and 1 µg/mL) of *Securidaca longepedunculata* root bark aqueous extract were used for the assay. A stock solution of 1000 µg/mL was prepared. Subsequent concentrations of 100, 10 and 1 µg/mL were obtained from the stock solution through serial dilution. Ten nauplii were drawn through a glass capillary and placed in beakers containing various concentrations of the extract. A negative control experiment was set up with 10 mL of seawater and 10 nauplii while the positive control was set up using 10 mL of fluorouracil and 10 nauplii at concentrations of 1, 10, 100 and 1000 µg/mL. The experiments were maintained at room temperature for 24 h under light and the surviving nauplii were counted and the mortalities were recorded.[21] The LC_{50} (Lethal concentration that kills 50% of organisms) was calculated using the Finney Probit Analysis method.[22]

Allium cepa Assay

The method of Saboo *et al.*[12] was used for this assay. Briefly, 10g/100 mL stock solutions of *Securidaca longepedunculata* aqueous root bark extract was prepared. Different concentrations of 10, 20, 50 and 100 mg/mL of the extract were prepared separately from the stock solutions. A concentration of 125 µg/mL methotrexate solution was also prepared, serving as positive control while distilled water was used as negative control. *Allium cepa* bulbs initially sprouted in water were then treated with the various concentrations of *Securidaca longepedunculata* root bark aqueous extract, methotrexate and the distilled water. After 24 h of treatment, root tips were cut and fixed before squashing on microscopic slides. The tissues were stained with Orcein and the slides were viewed under the light microscope at a magnification of ×40. The total number of cells in Prophase, Metaphase, Anaphase, Telophase and Interphase were counted.

The total number of dividing cells = number of cells observed in prophase + metaphase + anaphase + telophase.
The mitotic index was calculated using the formula;

Mitotic Index = (Number of dividing cells/ Total number of cells x 100).[23]

Statistical analysis

Results were expressed as Mean ± Standard Error of Mean. SPSS version 17.0 software was used for the analysis of results.

Results and Discussion

The brine shrimp lethality assay has been used severally as a preliminary tool in toxicity testing of plant extracts.[13,24] The result of the toxicity evaluation of the plant extracts by brine shrimp lethality assay is as shown in Table 1. There was a dose-dependent increase in mortality of brine shrimps exposed to various concentrations of aqueous root bark extract of *S. longepedunculata*. The exposure of shrimps to sea water only did not cause any nauplii death. However, exposure of nauplii in each group to concentrations of 1, 10, 100 and 1000 µg/mL *S. longepedunculata* caused a percentage death of 0, 50, 70 and 100%, respectively (Table 1). LC_{50} is the concentration of a substance that is lethal to 50% of the organisms exposed to it in a toxicity test. The LC_{50} value of *Securidaca longepedunculata* aqueous root bark extract was 25.1 µg/mL using the Finney probit analysis method. 5-Fluorouracil (5-FU) is one of the most frequently used antitumor agents for the treatment of solid tumours.[25] According to Saif *et al.*,[26] 5-fluorouracil-related toxicity has become a serious and common issue for many cancer patients. Hence, 5-FU was used as a positive control in this research. The LC_{50} value for fluorouracil on brine shrimp was 363 µg/mL. In toxicity evaluation of plant extracts by brine shrimp toxicity assay, the LC_{50} values lower than 1000 µg/mL are considered bioactive.[27] The LC_{50} value of *Securidaca longepedunculata* aqueous root bark extract is quite lower compared to the report of Ayo *et al.*[24] The methanol extract of *Khaya senegalensis* seeds showed a lower mitotic index than the petroleum ether extract. This indicates that polar extracts showed better mito-depressive activity than non-polar extracts. Hence the aqueous extract of *Securidaca longepedunculata* root bark may be considered biologically active with potential cytotoxic activity.

The cytotoxic effect of *S. longepedunculata* aqueous root bark extract on root tip cells of *A. cepa* is as shown in Table 2. The observation of cells in interphase (non-dividing phase) and cell division is used as an indicator of an adequate proliferation of the cell which can be measured through the *Allium cepa* assay. *Allium cepa* is commonly used for toxicity test (*Allium cepa* assay) in most laboratories.[28] Exposure to *Securidaca longepedunculata* extract inhibited the mitotic index in a concentration-dependent manner when compared to the mitotic index of 14.92% in control. The mitotic index decreased significantly ($P < 0.05$) at 50 and 100 mg/mL. The mitotic indices were 0.5 and 0.0% respectively at 50 and 100 mg/mL compared to mitotic indices at 10 and 20 mg/mL which were 2% and 0.8%, respectively. This indicates that *Securidaca longepedunculata* exerted a more anti-mitotic effect at 100 mg/mL. The mitotic indices in treated cells were lower when compared to the distilled water (negative control) which was 14.92. However, no dividing cell was observed on exposure of *A. cepa* root tip cells to 125 mg/mL methotrexate (positive control). Hence, the mitotic index for the methotrexate-treated *A. cepa* cells was 0.0%. The decline in the mitotic index suggests that *Securidaca longepedunculata* aqueous root bark extract interferes with the cell cycle. Figure 1 shows the features of *A. cepa* cells exposed to water. A-cells in anaphase (sister chromatids move to opposite poles), EM–cells in early metaphase (microtubules attached to kinetochore), M–cells in metaphase (chromosomes align in the equator of the cell), P–cells in prophase (chromatin becomes visible i.e chromatin condensation). Figure 2 shows the features of *A. cepa* cells exposed to 10 mg/mL of *Securidaca longepedunculata* aqueous root bark extract. T-cells in telophase (nuclear membranes re-form around each set of chromatids, the nucleoli also reappear), I–cells in early interphase (resting stage and preparation for cell division), P–cells in prophase (chromatin condensation), A–cells in anaphase (sister chromatids move to opposite poles). Figure 3 shows the features of *A. cepa* cells exposed to 100 mg/mL *Securidaca longepedunculata* aqueous root bark extract and methotrexate.

This may be due to abnormal conditions of the cells induced by treatments with these substances (*Securidaca longepedunculata* and methotrexate) that could best be described as mitotic inhibitors. The results of this study indicate that methotrexate inhibits mitosis to a higher degree than *Securidaca longepedunculata*. Mitotic inhibitors are also useful in genetics, where they stop cell division at a stage where chromosomes can be examined.[29] The reduction of the mitotic index may be due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing of the rate of cell cycle progression through mitosis.[30] Smaka-Kincl *et al.*[31] reported that the decrease in mitotic index was the result of cytotoxic effect.

Table 1: Cytotoxic effect of *Securidaca longepedunculata* on Brine shrimps.

Dose (µg/mL)	Log_{10} Dose	Dead	Mortality (%)	Corrected Mortality (%)	Probit
0	0	0	0	0	0.00
1	0	0	0	3	3.12
10	1	5	50	50	5.00
100	2	7	70	70	5.52
1000	3	10	100	98	7.05

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Table 2: Anti-mitotic activity of aqueous extract of *Securidaca longepedunculata*.

Treatment (µg/mL)	Prophase cells	Metaphase cells	Anaphase Cells	Telophase cells	Total Dividing Cells	Total number of cells	Mitotic Index
Control	6.00 ± 0.71	5.25 ± 0.63	4.50 ± 1.32	3.75 ± 0.85	19.50 ± 3.09	177.00 ± 54.21	14.92 ± 5.09
125 MET	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	238.25 ± 71.75	0.00 ± 0.00*
10	0.25 ± 0.25*	2.00 ± 0.71*	1.00 ± 0.00*	1.50 ± 0.65*	4.75 ± 1.03*	274.00 ± 83.53	2.08 ± 0.69*
20	0.00 ± 0.00*	1.50 ± 0.50*	0.25 ± 0.25*	0.00 ± 0.00*	1.75 ± 0.48*	238.00 ± 64.01	0.82 ± 0.17*
50	0.25 ± 0.25*	0.50 ± 0.50*	0.00 ± 0.00*	0.00 ± 0.00*	0.75 ± 0.48*	115.50 ± 26.80	0.51 ± 0.35*
100	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	149.00 ± 83.37	0.00 ± 0.00*

*Results are expressed as Mean ± standard error of mean number of cells. Values carrying different superscripts are significantly different from control.

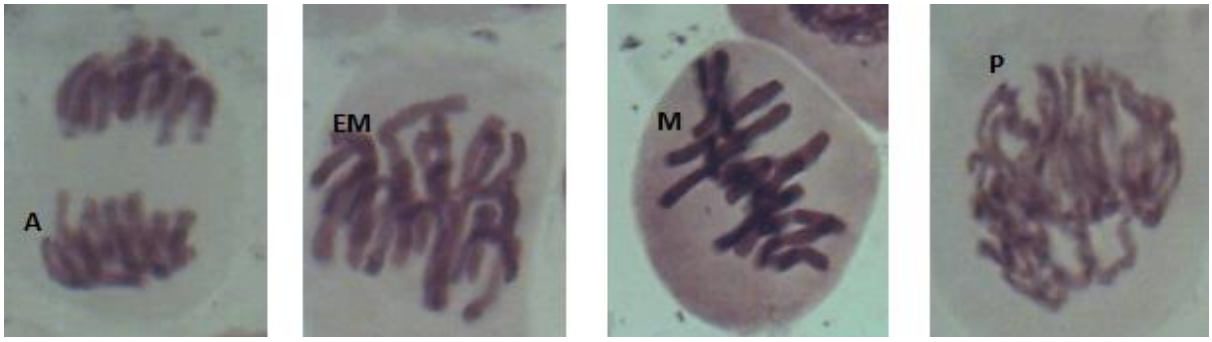

Figure 1: Mitotic phases in *Allium cepa* sprouted in water (x 400).

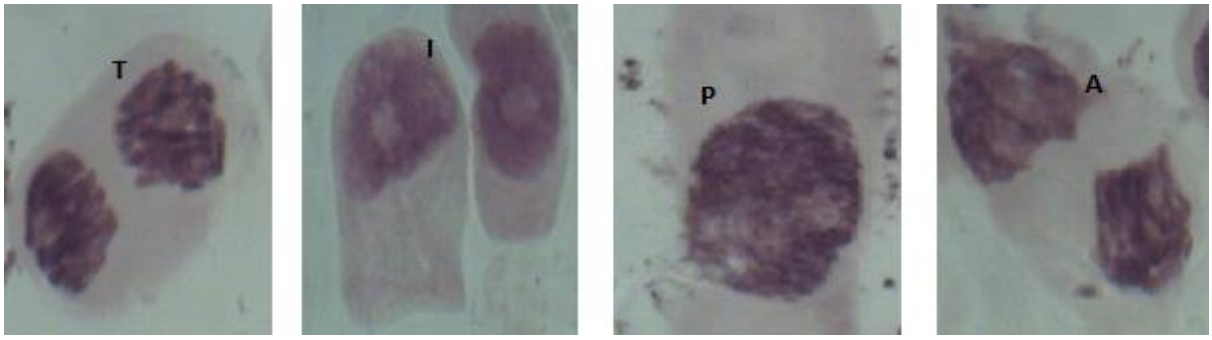

Figure 2: Mitotic phases observed in *Allium cepa* sprouted in 10mg/ml *S. longepedunculata* (x 400).

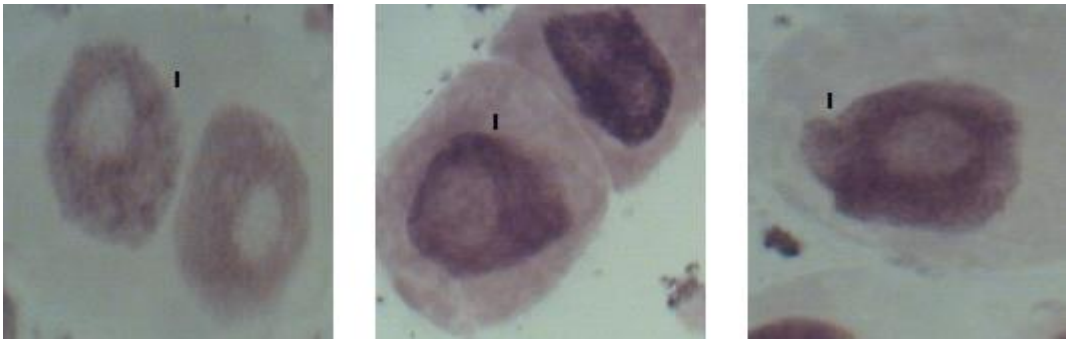

Figure 3: Mitotic phases observed in *Allium cepa* sprouted in 100 mg/ml *S. longepedunculata* (x 400).
I – cells in early interphase (resting stage and preparation for cell division)

The ability of the *Securidaca longepedunculata* extract to reduce mitotic index by inhibiting cell proliferation at the anaphase and metaphase stage is similar to the action of plant-derived anticancer drugs such as taxol, vinblastine and vincristine. Vinca alkaloids block the process of microtubule assembly whereas taxol stabilizes microtubule and promotes the formation of abnormal microtubule bundles.[32] The mito-depressive activity of *Securidaca longepedunculata* root bark extract on *A. cepa* root tip cells indicates the presence of cytotoxic substances in the extract while the observation of aberrant cells in the treated onion tip indicates genotoxic effects of the *Securidaca longepedunculata* aqueous root bark extract.[33] Water and ethanol extracts of *Pterospermum acerifolium* (10 mg/mL) leaves showed potent inhibition of dividing meristematic tissue cell cycle with mitotic indices of 12.14 and 14.24, respectively compared to the negative control of 52.73.[12] The mitotic indices reported in our study were lower compared to those observed for *Pterospermum acerifolium* at 10 mg/mL. This indicates the presence of larger quantities of anti-mitotic substances in *Securidaca longepedunculata* aqueous root bark extract. This study corroborates an earlier report by Lawal *et al.*[18] which identified several cytotoxic substances in *Securidaca longepedunculata* aqueous root bark extract. Hence, this study suggests that the mechanism of anti-cancer activity of *Securidaca longepedunculata* aqueous root bark extract may be through its ability to suppress mitosis in tumour cells.

Conclusion

Securidaca longepedunculata root bark extract has shown great potential as a potent cytotoxic agent and possible mitotic inhibitor.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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