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Evaluation of Genotoxic and Cytotoxic Activities of *Eremomastax speciosa* (Hochst.) Cufod. (Acanthaceae) Leaf Extract

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Abstract

There is a growing interest in the therapeutic potentials of natural products and the mounting need to ensure their safety and efficacy. *Eremomastax speciosa* (Hochst.) Cufod. (Acanthaceae) is one of such medicinal plants used traditionally to treat various diseases in Nigeria. The leaf extract was investigated for genotoxic and cytotoxic effects using *Allium cepa* test. The effect of the *Eremomastax speciosa* leaf extract on the root meristem cells of *Allium cepa* bulb was investigated using onion bulbs exposed to varying concentrations of the extract (2.5 mg/mL, 5 mg/mL, and 10 mg/mL) for macroscopic and microscopic analysis. Tap water was used as a negative control and Methotrexate (0.1 mg/ml) was used as a positive control. There was statistically significant ($p< 0.05$) inhibition of root growth depending on concentration by the extract when compared with the negative control group. All the tested concentrations of extract were observed to have cytotoxic effects on cell division in *A. cepa.* The extract-induced chromosomal aberrations and micronuclei (MNC) formations in *A. cepa* root tip cells were significant ($p<0.05$) when compared with control group. The extract treatment further induced cell death, ghost cells, cells membrane damage, and binucleated cells. These results suggest that the leaf extract of *Eremomastax speciosa* possess cytotoxic and genotoxic effects on *A. cepa* root meristem cells.

Keywords: *Eremomastax speciosa*; cytotoxic; genotoxic; Allium cepa; meristem; apoptosis.

INTRODUCTION

Eremomastax speciosa (Hochst.) Cufod (Acanthaceae) is a medicinal plant widely found in West Tropical Africa (Burkill,1985; Neuwinger,2000) especially in Nigeria and Cameroon where it is used locally as blood tonic and for the treatment of various diseases such as anaemia, diarrhea, jaundice, fever, female infertility, menstrual cramps, dysentery, urinary tract infection, haemorrhoids and gastric ulcers (Burkill,1994; Bassey and Effiong, 2011; Oben et al., 2006; Siwe et al., 2021). It is popularly known in Niger Delta of Nigeria (Ibibio) as Edem ididot ("golden seal" or "African blood tonic") and in Cameroon as pang nyemshe (red on one side) (Erhabor *et al.*,2013). The leaves are also used ethnomedically to enhance fertility and arrest postpartum bleeding in Nigeria and Cameroon (Telefo *et al*., 2011; Mboso *et al.,* 2013). The plant has been reported to posses aphrodisiac (Erhabor *et al.,* 2013; Nchegang et al., 2016), antianaemic (Okokon et al., 2007; Mboso et al., 2014), antiulcer (Tan et al., 1996; Amang *et al.*, 2014a;b; Amang et al., 2017a;b). and antioxidant (Amang et al., 2014; Sagnia et al., 2014). Phytochemical screening of the leaf extract of *E. speciosa* revealed the presence of tannins, alkaloids, resins, flavonoids, anthocyanins, phenols, quinones, oils, sterols, triterpenoids, glycosides and proteins (Amang *et al* ,2014). Chemical constituents identified in the leaves include; hydroxyandrographolide and stigmasterol glucoside,(Z)-4-coumaric Acid, 4-O-β-D-apiofuranosyl-(1''→2')-O-β-D-glucopyranoside,5-methoxy-4,4′-di-O-methyl-secolariciresinol-9′-monoacetate. Other major fatty acid esters were ethyl-9,12,15-octadecatrienoate (11.51%), 9,12-octadecadienoic acid ethyl ester (8.05%),i-propyl 9,12-octadecenadienoate (5.87%), (Z,Z,Z)-9,12,15-octadecatrienoic acid methyl ester(4.27%), methyl hexadecanoate (4.21%), ethyl octadecanoate (2.62%) and (Z,Z)-9,12-octadecadienoic acid methyl ester (1.83%). (Mboso *et al.*, 2020). Despite its wide use in traditional medicine in the treatment of various diseases, there is paucity of scientific information on the safety profile of this plant extract. Thus, we investigated the genotoxic and cytotoxic activities of ethanol leaf extract of *Eremomastax speciosa* on the root meristem cells of *Alium cepa* bulb.

MATERIALS AND METHODS

Plants collection

The fresh leaves of *Eremomastax speciosa* were collected in November, 2024 at Farmland in Uyo, Uyo LGA, Akwa Ibom State, Nigeria. The leaves were identified and authenticated as *Eremomastax speciosa* by a taxonomist in the Department of Botany and Ecological studies, University of Uyo, Uyo. Nigeria. Herbarium specimen was deposited at the Faculty of Pharmacy Herbarium, University of Uyo, Nigeria.

Extraction

The plant parts (leaves) were washed and air- dried on laboratory table for 2 weeks. The dried leaves were pulverized using a pestle and mortar. The powdered leaf was macerated in 70% ethanol for 72 hours. The liquid ethanol extract obtained by filtration was evaporated to dryness in a water bath at 60˚C. The yield of the extract was stored in a refrigerator at -4˚C until it was used for the experiment reported in this study.

Allium cepa test.

This was carried out according to the method of Grant (1994) and Ikechukwu et al., (2024). Small onions bulbs, *A. cepa*, were procured from Elele market, Elele, Ikwere LGA, Rivers State, Nigeria. The bulbs were processed for the study by scarifying the bulbs and bottom base without destroying the root primordia using a small sharp knife. Distilled water (200 mL) was used to dissolve each of the extract (20 g) which were thereafter diluted to different concentrations of each extract 2.5 mg/mL, 5 mg/mL and 10 mg/mL respectively from the stock solution. Test concentrations of the leaf extract at 2.5 mg/mL, 5 mg/mL, and 10 mg/mL concentrations were prepared in 50 mL beakers and arranged in a series of 5 per test concentration. One *A. cepa* bulb was placed on top of each beaker, with the root primordia downward toward the liquid. Tap water was used as negative control and Methotrexate (0.1 mg/mL) was used as positive control. After 24 hours, the test samples were changed in the controls and all test concentrations and photographs of the growing *A. cepa* roots were taken. This continued for 72 hours, after which the roots were counted per beaker in all the tested concentrations and mean root number was calculated. Similarly, the roots' lengths were measured using a metre rule and the mean root length was calculated. These were also done for the control groups. Several root tips were cut at a length of 10 mm from the bulbs at 8:30 am, and respectively fixed in 3:1 (v/v) ethanol: glacial acetic acid and 1N HCL before putting them in sample bottles and storing in a refrigerator until use.

Microscopy

The root tips were each placed in a test tube with 1N HCL and heated at 50ºC for 6 minutes in order to fix and macerated them. Thereafter, the root tips were placed on microscopic slides on a blank background with a forcep and were cut off at terminal tips. Two drops of 2% (w/v) orcein stain was added and mixed with the rootlets properly by knocking and stirring with a stirring spatula.

Then a cover slip was placed at 45º to avoid air bubbles. After that, the cells were squashed by placing a filter paper on the cover slip and pressed lightly with a thumb. The cover slip was sealed with a clear finger nail polish and each slide was examined using a Light Microscope at a magnification of x40. Microphotographs were taken to show chromosomal aberrations. The mitotic index and frequency of chromosomal aberration were calculated based on the number of aberrant cells per total cells counted at each concentration of the test extract (Bakare *et al.,* 2000; Magnus *et al.,* 2024). The mitotic inhibition was determined using the following formula:

$$Mitotic\ index = \frac{Number\ of\ dividing\ cells}{Total\ number\ of\ cells} \times 100$$

The following indices were considered for evaluation of cytotoxicity and genotoxicity: (i) the mitotic index (MI was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage and (ii) chromatin aberrations (stickiness, bridges, breaks and polar deviation) were used as endpoints for assessment of cytogenetic effects and micronuclei (MNC) were scored in interphase cells per 500 cells.

Statistical Analysis

Data obtained from this work were analysed statistically using one –way ANOVA followed by Tukey-Kramer multiple comparison test using Instat Graphpad software, (San Diego, USA). Differences between means were considered significant at 5% level of significance ie $p \leq 0.05$.

RESULTS

Physicochemical Characterization.

The effects of *Eremomastax speciosa* leaf extract on levels of the physicochemical parameters (root number and root length) are presented in Table 1. This result shows that all tested concentrations of *Eremomastax speciosa* leaf extract caused significant inhibition of roots' growth in comparison to negative and positive control groups. The root number and root length were found to be decreased as the leaf extract concentration increases. The mean root lengths in negative and positive control (methotrexate) groups were 4.82±0.12 and 0.10±0.01 cm respectively. However, mean root lengths in 2.5 and 10 mg/mL treatment groups were observed to have decreased significantly relative to negative control; 2.20±0.02 and 0.26±0.02 cm respectively for

Eremomastax speciosa (Table 1). Mean root lengths in treatment groups decreased according to concentration, significantly ($p<0.05$) relative to negative control. The root morphology and appearance were normal in the negative control group, but root tips treated with 2.5 mg/mL of *Eremomastax speciosa* leaf extract, appeared slightly yellow and at 5 and 10 mg/mL of *Eremomastax speciosa* leaf extract, the roots tips were observed to appear brownish. (Table 1).

Cytogenetic Analysis.

Table 2 shows the effects of *Eremomastax speciosa* leaf extract on cytogenetic parameters of *Alium cepa* roots. Cytogenetic analysis performed showed that the leaf extract caused concentration-dependent and significant ($p<0.05$) decreases in the mitotic index relative to that of negative control group. The leaf extract of *Eremomastax speciosa* at 2.5 mg/ml and 10 mg/mL had mitotic index of 35.20±2.89 and 16.00 ±1.44 as compared to 70.80±3.22 recorded in the negative control group (Table 2).

Cytogenetic alterations caused by the extract are shown in Table 3. Chromosome and cytological alterations were observed in negative control, methotrexate and *Eremomastax speciosa* leaf extract-treated groups as depicted in Table 3. Analysis of chromosome aberrations observed in the study showed that fragmented chromosomes, laggard, bridge and polar deviation were observed in the different concentrations' treatments especially in the highest concentration (Table 3) (Figure 1(A, C, D and F). This was significant ($p<0.05$) when compared to negative control group. Chromosome bridges and laggard, and cell wall damage were observed to be in high concentrations (5 and 10 mg/mL) of the leaf extract (Table 3; figure 1A, C, D and F). Binucleated cells, apoptotic cells and dead cells were also observed (Figures 1(B, E and F) in the extract-treated groups. It was generally observed that these abnormalities were concentration dependent. The total aberrant cells (aberrant cells include bridge, laggard and stickiness) were statistically significant ($p<0.05$) when compared with the negative control and concentration dependent (Table 3). However, the methotrexate-treated group (positive control) had the highest value of aberrant cells (Table 3). Genotoxic activities of the extract were further demonstrated by the presence of micronuclei in the root tip meristem cells of *A. cepa* which were prominent in groups treated with methotrexate and 2.5 mg/mL of *Eremomastax speciosa* which were statistically significant ($p< .05$) compared to negative control, (Figure 1(A)).

Table 1. Cytotoxicity of *Eremomastax speciosa* leaf extract on growing roots of Onion (*Allium cepa*).

Treatment group	Concentration of extract (mg/mL)	Average root Number ± S.D	Average root length (cm)± S.D
Negative control	Tap water	26.40±3.82	4.82±0.12
Methotrexate	0.1	2.10±0.02[a]	0.10±0.01[a]
Eremomastax speciosa	2.5	24.50±3.96[a]	2.20±0.08[a]
	5.0	10.25±3.42[a]	1.10±0.04[a]
	10.0	6.25±0.47[a]	0.26±0.02[a]

Values are expressed as mean ±SEM (n=5). Significant at $p<0.05$ when compared to negative control

Table 2. Dividing and total cells counted under microscopic observations and mitotic values in control and treatment concentrations.

Treatment group	Concentration of extract (mg/mL)	Total Number of cells	Dividing cells	M.I (%)± S.E
Negative control	Tap water	500	354	70.80±3.22
Methotrexate	0.1	500	14	2.80±0.10[a]
Eremomastax	2.5	500	176	35.20±2.89[a]
speciosa	5.0	500	80	16.00±1.44[a]
	10.0	500	23	4.60±1.26[a]

Values are expressed as mean ±SEM (n=5). Significant at $p<0.05$ when compared to negative control.

Table-3. Chromosomal and mitotic aberrations in the root meristematic cells of *Allium cepa* after treatment of extract of *Eremomastax speciosa.*

Treatment group	Concentration of extract (mg/mL)	Chromosome breaks (%)±S.E	Stickiness (%)±S.E	Polar deviation (%)±S.E	Aberrant cells (%)±S.E	MNC (%)± S.E
Negative control	Tap water	-	0.28±0.02	0.15±0.01	2.03±0.13	-
Methotrexate	0.10	3.23±1.38[a]	34.12±2.38[a]	15.23±2.34[a]	51.12±3.42[a]	3.12±0.56[a]
Eremomastax	2.5	-	20.33±0.55[a]	-	26.28±4.19[a]	1.02±0.12[a]
speciosa	5.0	0.24±0.08[a]	26.86±5.48[a]	-	30.64±2.28[a]	0.66±0.05[a]
	10.0	1.55±0.74[a]	30.34±6.49[a]	-	44.69±6.92[a]	0.88±0.02[a]

Values are expressed as mean ±SEM (n=5). Significant at $p<0.05$ when compared to negative control.

DISCUSSION

In this study, toxic effects of *Eremomastax speciosa* leaf extract were assessed by evaluating the growth and morphology of root. The root growth was inhibited by various concentrations of the extract as observed in this study and these were statistically significant relative to control group. Furthermore, the extract was observed to caused light yellow, light brown and brownish colouration of the roots. Cyto- and genotoxicity were determined by assessing cytological parameters such as the mitotic index and number of chromosome abnormalities, including chromosome breaks, stickiness, and polar deviations. The mitotic index (MI) of *A. cepa* meristematic cells treated with methotrexate (2.80%) was significantly decreased when compared to control. Significant inhibition in the onion roots treated with the *Eremomastax speciosa* leaf extract (35.20%, 16.00% and 4.60% compared to the negative control) was observed (Table 2). The inhibition of root growth was found to increase with decreased of Mitotic Index. The reduction of mitotic index below 22% in comparison to negative control can have lethal impact on the organism (Antonsie-Wiez, 1990), while a decrease below 50% usually has sublethal effects (Panda and Sahu, 1985) and is called cytotoxic limit value (Sharma, 1983). Mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics (Rojas *et al.,* 1993). Reduction in the mitotic activity could be due to inhibition of DNA synthesis or a blocking in the G2 phase of the cell cycle, preventing the cell from entering mitosis (Sudhakar *et al.*, 2001). Mitodepressive effects of some herbal extracts, including the ability to block the synthesis of DNA and nucleus proteins, were reported earlier (Mercykutty and Stephen,1980; Schulze and Kirschner, 1986). Several other herbal extracts have been reported to inhibit mitosis (Johnny *et al.*, 2023; Okokon *et al.*,2023; Ikechukwu *et al*.,2024). The decreased mitotic index in *A. cepa* roots treated with *Eremomastax speciosa* leaf extract is probably due to either disturbances in the cell cycle or chromatin dysfunction induced by extracts-DNA interactions. The results herein suggest that the tested extract concentrations have inhibitory, mito-depressive effects on root growth and cell division of *A. cepa* and it can prevent DNA synthesis and the reduction in number of the dividing cells in roots produced by the cytotoxic effects of compounds found in the extract. The observation of sticky metaphase supports the hypothesis of the toxic effect of the extract. Metaphases with sticky chromosome, loses their normal appearance, and they are seen with a sticky "surface," causing chromosome agglomeration (Babich *et al.,* 1997). Stickiness has been attributed to the effect of pollutants and chemical compounds on the physical-chemical properties of DNA, protein or both, on the formation of complexes with phosphate groups in DNA, on DNA condensation or on formation of inter- and intra chromatid cross links (G''om''urgen, 2005; T¨urkoglu, 2007). Chromosomal aberrations (CA) are changes in chromosome structure resulting from a break or exchange of chromosomal material. Most of the CA observed in cells are lethal, but there are many related aberrations that are viable and that can cause genetic effects, either somatic or inherited (Swierenga *et al.,* 1991). The presence of chromosome fragments is an indication of chromosome breaks, and can be a consequence of anaphase/telophase bridges (Sharma and Sen, 2002). Fragment was not observed in this study in all the extract concentrations- treated groups. The extract was found to interfere with the cell cycle, and affect chromatin organization or DNA replication. Frequencies of total chromosome aberrations increased significantly following exposure to the extract which indicates clastogenic activity (Table 3). The extract significantly induced the formation of MNC in *A. cepa* root cells at 2.5–10 mg/mL concentrations. Frequencies of MNC were found to increase in the groups treated with 2.5 mg/mL of the leaf extract. However, MNC frequency decreased in *A. cepa* roots treated at the highest concentration of the extract (10 mg/mL), due to high cytotoxicity. The frequency of cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals. Micronuclei (MN) often results from the acentric fragments or lagging chromosomes that fail to incorporate into the daughter nuclei during telophase of the mitotic cells and can cause cellular death due to the deletion of primary genes (Albertini *et al.*, 2000; Krishna and Hayashi, 2000). Previous studies have suggested MNC-induced effect of various plant extracts such as *Lavandula stoechas* and *Ecballium elaterium* (As *et al.*, 2007; As *et al.*, 2009), *Azadirachta indica* (Soliman, 2001) *Psychotria* species (Akinboro and Bakare, 2007).

In this study, membrane damage cells were observed in all the treated groups. These results indicated the potential of the extract to exert cytotoxic effect over certain concentrations such as membrane damage. Multinucleated and binucleated cells were observed in extract-treated groups. This is due to the prevention of cytokinesis or cell plate formation. Microtubules have been implicated in cell plate formation by the extract, resulting in inhibition of cytokinesis. Ghost cell is a dead cell in which the outline remains visible, but whose nucleus and cytoplasmic structures are not stainable (As *et al.,* 2009). Some ghost cells were observed in various frequencies in this study especially in 10 mg/mL treated groups (Figure 2). This could have resulted from the activities of the phytochemical constituents of the extract leading to nucleus damage and prevention of cytoplasmic structures. In addition, the extract also induced DNA damage and cell death and/or apoptosis in various frequencies in this study. Cell death is a basic biological process of living organism. The cell death is induced by

high concentrations of toxin, stress, heavy metals, chemicals and others.

Chemical constituents identified in the leaves include; hydroxyandrographolide and stigmasterol glucoside,(Z)-4-coumaric Acid, 4-O-β-D-apiofuranosyl-(1''→2')-O-β-D-glucopyranoside,5-methoxy-4,4′-di-O-methyl-secolariciresinol-9′-monoacetate. Other major fatty acid esters were ethyl-9,12,15-octadecatrienoate (11.51%), 9,12-octadecadienoic acid ethyl ester (8.05%),i-propyl 9,12-octadecenadienoate (5.87%), (Z,Z,Z)-9,12,15-octadecatrienoic acid methyl ester(4.27%), methyl hexadecanoate (4.21%), ethyl octadecanoate (2.62%) and (Z,Z)-9,12-octadecadienoic acid methyl ester (1.83%). (Mboso *et al.*, 2020).

The phytochemical constituents of this extract may have been responsible for the observed effects in this study. The terpenes present in this extract may have contributed to the observed cytotoxic and genotoxic activities in this study

CONCLUSION

The results of this study show that the leaf extract of *Eremomastax speciosa* can induced cytogenetic alterations (cytoplasmic shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing, cytoskeleton alterations and appearance of apoptotic bodies) and cell death in root tips of *A. cepa*, suggesting cytotoxic and genotoxic activities of the extract.

Therefore, proper use of this plants in ethnomedicine is recommended and high doses should be avoided as it can cause cytotoxic and/or genotoxic effects.

Disclaimer (Artificial Intelligence): Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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