Original Research Article

Genotoxic and Cytotoxic Activities of *Hippocrateaafricana*Loes. Ex. Engl. Celastraceae Root Extract

Abstract

Hippocrateaafricana, a medicinal plant, used in ethnomedicine for the treatment of antimalarial, diabetes, poisoning and inflammatory diseases was investigated for cytotoxic and genotoxic effects on the root meristem cells of *Allium cepa*. The extract and fractions was also screened for total flavonoid and phenol content using standard methods. Onion bulbs were exposed to 2.5 mg/ml, 5mg/ml, and 10 mg/ml concentrations of the leaf extract for macroscopic and microscopic analysis. Tap water was used as a negative control and Methotrexate (0.1 mg/ml) 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 the 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 *Hippocrateaafricana*root extract possess cytotoxic and genotoxic effects on *A. cepa* which is as a results of its phytochemical constituents.

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Keywords: *Hippocrateaafricana*; genotoxicity, cytotoxicity, *Aliumcepa*

INTRODUCTION

Hippocrateaafricana (Willd.) Loes. ex Engl. (Celastraceae) syn. *Loeseneriellaafricana* (Willd.) N.Halléis a green forest perennial climber widely distributed in tropical Africa **[1]**. It is commonly known as African paddle-pod and 'Ebaenangenang' by the Ibibios of Nigeria. The plant root has been variously used traditionally by the Ibibios of the Niger Delta region of Nigeria to treat ailments such as fever, convulsion, malaria, body pains, diabetes and diarrhea **[2]**. The plant's root is also use for its antidote or antipoison potential to treat liver diseases such as jaundice and hepatitis**[3,4,5]**.Previous reports showed that the root extract possess antimalarial **[2,6]**, antioedema and antinociceptive**[7]**, antidiabetic and hypolipidemic**[8,9]**, antidiarrhoeal and antiulcer **[10]**, hepatoprotective**[11]**, antileishmanial, cytotoxicity and cellular antioxidant **[12]**, antibacterial, anticonvulsant and depressant**[13]**, in vivo alpha amylase and alpha glucosidase inhibitory **[6]**, *in vitro* antioxidant **[6,9]**activities . Earlier studies had reported the presence of spirohexane-1-carboxylic acid, ethyl ester, 3-methoxy-2-methylphenol,2,3-benzofurandione,6-hydroxy-4-(p-hydroxybenzyl), $^{\delta}$-3-Carene and α-terpineol in ethyl acetate fraction**[14]**and the presence of monoterpenes (thujene, limonene, linalool, α-phellandrene, α-terpineol and sabinene) and sesquiterpenes (dehydromevalonic lactone), in the n-hexane fraction of the root extract **[11]**. Also, two xanthones; 1,3,6,7-tetrahydroxyxanthone and 1,3,6-trihydroxy-7-

methoxyxanthone, have been isolated from the root of *H. africana***[15]**. In this study, genotoxic and cytotoxic potentials of the root extract of *H africana*on *Aliumcepa* test are reported.

MATERIALS AND METHODS

Preparation of extract

The plant parts (roots) were washed and air- dried on laboratory table for 2 weeks. The dried materials were pulverized using a pestle and mortar. The powdered material was macerated in 50% ethanol for 72 hours. The liquid ethanol extract obtained by filtration was evaporated to dryness in a rotary evaporator 40˚C. The extract was stored in a refrigerator at 4˚C until used for experiment reported in this study.

***Allium cepa*test.**

Small bulbs of the common onion, *A. cepa*, were procured from Jos, Northern region of Nigeria. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia using a small sharp knife and collected in a jar of water. The plant extract (20 g) was dissolved in 200 mL of distilled water. Different concentrations of 2.5 mg/mL, 5 mg/mL and 10 mg/mL respectively were prepared from the stock solution. Test concentration of the plants' extract at 2.5 mg/mL, 5 mg/mL, and 10 mg/mL concentrations prepared in 15 mL beakers were arranged in a series of 5 per test concentration and filled up for each 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 (0.1mg/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. cep*a roots were captured. This continued for 72 hours, after which the roots were counted per beaker in all the tested concentrations and mean root number mean root length was calculated. These were also done for the control. 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.

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Microscopy

The root tips were each placed in a test tube with 1N HCL and heated at 60ºC 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 slight with a thumb. The cover slip was sealed with a clear finger nail polish as suggested by Grant (1982) and each slide was examined using a Light Microscope at a magnification of x40. Microphotographs were taken to show chromosomal aberrations with the aid of an MD500 Amscope. The mitotic index and frequency of chromosomal aberration were calculated based on the number of aberrant cells per total cells scored at each concentration of each sample **[35]**.The mitotic inhibition was determined using the following formula:

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

$$\%\text{Aberrant cells} = \frac{Number\ of\ Aberrant\ cells}{Total\ number\ of\ cells} \times 100$$

$$\%\text{root growth of control} = \frac{Overall\ mean\ root\ length\ of\ test\ solution}{Overall\ mean\ root\ length\ of\ control} \times 100$$

The following parameters were used for determination 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 (i) chromatin aberrations (stickiness, bridges, breaks and polar deviation) were used as endpoints for determination 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 InstatGraphpad software, (San Diego, USA). Differences between means were considered significant at 5% level of significance ie $p \leq 0.05$.

RESULTS

Physicochemical Characterization

The effect of *Hippocrateaafricana*rootextract on different physicochemical parameters (root number and root length) of *Allium cepa* root tips are presented in Table 1. These results show that all tested concentrations of *H. africana*root extract (2.5 -10.0 mg/mL) caused significant ($p<0.05$) inhibition roots growth relative to negative control. The inhibition of root number and root length was greater with increasing concentrations of the two extracts. The average root lengths in negative and positive control (methotrexate) groups were 4.16±1.26 and 0.25±0.22 cm respectively. However, average root lengths in 10 mg/mL treatment group decreased significantly compared to that of the negative control; 1.51±0.41cm for *H. africana* (Table 1). Average root lengths in treatment groups decreased depending on concentration, significantly ($p<0.05$) when compared to negative control. The root morphology was nearly normal in the negative control group, but at *H. africana*(2.5 mg/mL)- treated group, the roots appeared slightly brownish and at 5 and 10 mg/mL of *H. africana*root extract, the roots appeared brownish (Table 1).

Table 1: Cytotoxicity of *Hippocrateaafricana* root 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	34.10±4.62	4.66±1.26
Methotrexate	0.1	8.16±3.28[a]	0.25±0.22 [a]
HippocrateaAfricana	2.5	19.4±2.87[a]	3.46±0.75 [a]

5.0	13.8±2.78 [a]	2.59±0.92 [a]
10.0	25.60±4.27[a]	1.51±0.41 [a]

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

Cytogenetic Analysis.

Table 2 shows the effects of *Hippocrateaafricana*rootextract on cytogenetic parameters of *Allium cepa* roots. Cytogenetic analysis performed showed that the extract caused concentration-dependent and significant ($p<0.05$) decreases in the mitotic index when compared to that of negative control. *H.africana*root extract(10 mg/mL) had mitotic index of 18.0 ±1.02 as compared to 57.60±12.34 recorded in the negative control group (Table 2).

Comment [MF3]: put table 2 here below this section

Cytogenetic alterations caused by the extract are shown in Table 3. Chromosome and cytological alterations were observed in negative control, methotrexate, and *Hippocrateaafricana*root extract-treated groups as depicted in Table 3. An analysis of chromosome aberrations observed showed that most of the fragments detected in the different treatments were of chromosome type especially in the highest concentration of *H. africana*root (Table 3) (Figure 1(a)). The observation of chromosome breaks showed the clastogenic effect of extract. This was significant ($p<0.05$) when compared to negative control group. Sticky metaphase and polar deviations (wrong directions of chromosome movement) were also observed (Figure 1(b))in the extract-treated groups but were more frequent in the group treated with the highest concentration of *Hippocrateaafricana* (10 mg/mL). It was generally observed that these abnormalities increased with increasing concentrations of the root extract. A concentration-dependent and statistically significant ($p<0.05$) increase in total aberrant cells (aberrant cells include chromosome breaks, stickiness and polar deviation) as compared with the negative control (Table 3) was observed with the extract and the highest effects were recorded in the highest concentration (10 mg/mL) treated group showing higher frequencies of aberrations(Table 3). Genotoxicactivity of the extract was further demonstrated by the induction of micronuclei in the root tip meristem cells of *A. cepa*. Micronucleus formation in 500 cells per slide (‰MNC value) was not concentration-dependent as the groups treated with methotrexate and 2.5 mg/mL of *Hippocrateaafricana* had higher number of cells with micronuclei in the test compared to negative control, which were statistically significant ($p<.05$). The increase also occurred in the positive control. In *Allium* test, a strong toxic effect of the extract was observed and supported by great occurrence of sticky metaphases, leading to cellular death (mitotic index decrease). In addition, cells with membrane damage, ghost cells and nucleus damage (Figure 1(c and d))were found in various frequencies.

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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	288	57.60±12.34

Methotrexate	0.1	500	15	3.00±0.68[a]
Hippocrateaafricana	2.5	500	140	28.0±3.29[a]
	5.0	500	132	26.40±5.23[a]
	10.0	500	90	18.0±1.02[a]

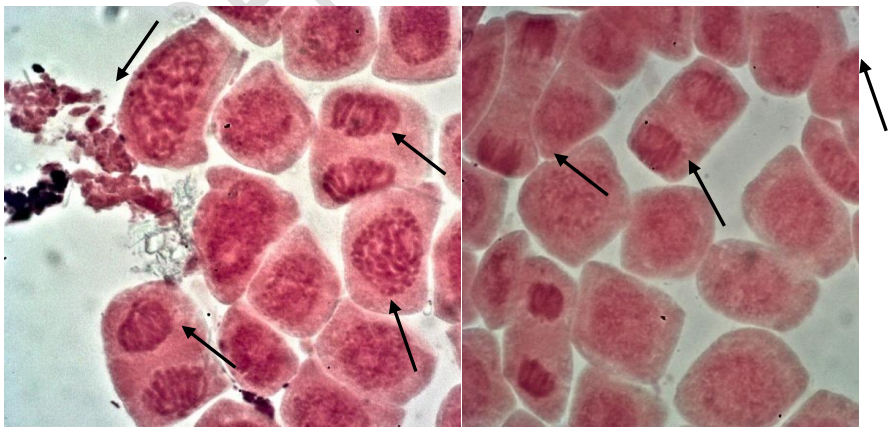
Values are expressed as mean±SEM (n=5). Significant at $p<0.05$ when compared to negative control.

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

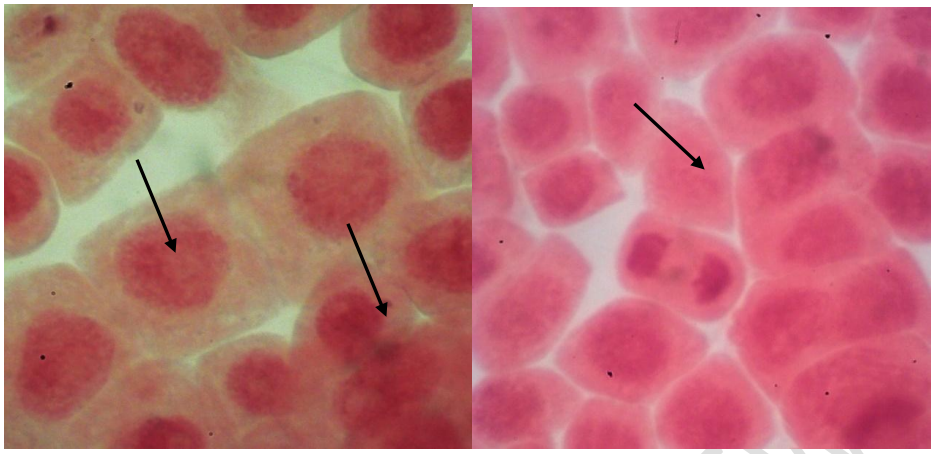
Treatment group	**Concentration of extract (mg/mL)**	**Chromosome breaks(%) ±S.E**	**Stickiness (%)±S.E**	**Polar deviation (%)±S.E**	**Aberrant cells (%)±S.E**	**MNC (%)± S.E**
Negative control	Tap water	-	0.11±0.08	0.31±0.04	1.05±0.56	-
Methotrexate	0.10	2.34±1.23[a]	21.34±5.38[a]	10.55±2.28[a]	45.13±4.22[a]	2.28±0.86[a]
HippocrateaAfricana	2.5	2.46±0.57[a]	24.68±3.14[a]	0.36±0.10[a]	34.33±6.64[a]	4.12±1.29[a]
	5.0	5.04±1.68[a]	63.28±7.45[a]	6.73±1.35[a]	68.93±7.46[a]	1.13±0.11[a]
	10.0	9.34±0.91[a]	72.64±8.65[a]	4.32±1.86[a]	83.34±9.10[a]	2.11±0.55[a]

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

Light Microscopy



(a) (b)



(c) **(d)**

Figure 1: Photomicrography showing the mitotic and chromosomal aberrations after the *Hippocrateaafricana* extract treatments in *Allium cepa* root tip meristem cells visualized with light microscopy at magnification X40. **(a)** visible fragments and polar deviation **(b)** polar deviations and stickyness at metaphase **(c)** micronucleus **(d)** ghost cells.

Discussion

In this study, toxic effect of *Hippocraeaafricana*rootextract was evaluated by analyzing root growth and root morphology of *Allium cepa*. Varying concentrations of the extract were observed to cause inhibition of root growth and these were statistically significant when compared to control group. In addition, the extract induced colouration of the root tips *Allium cepa* depending on the concentration. This colouration ranged from light brown to dark brown coloration of the roots. Cyto- and genotoxicity were estimated by observing 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 (3.00%) was significantly decreased when compared to control. Significant inhibition in the onion roots treated with the *Hippocrateaafricana*rootextract (28.0%, 26.40% and 18.0 % compared to the negative control) was observed (Table 2). The inhibition of root growth was found to be dependent on decrease of Mitotic Index. The decline of mitotic index below 22% in

comparison to negative control can have lethal impact on the organism **[16]**, while a decrease below 50% usually has sublethal effects**[17]** and is called cytotoxic limit value **[18]**. 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 **[19]**. 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 **[20]**. Mitodepressive effects of some herbal extracts, including the ability to block the synthesis of DNA and nucleus proteins, were reported earlier **[21,22]**. Several other herbal extracts have been reported to inhibit mitosis **[23,24,25]**. The decreased mitotic index in *A. cepa*roots treated with *Hippocrateaafricana* extract is probably due to either disturbances in the cell cycle or chromatin dysfunction induced by extracts-DNA interactions. The results of this study 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 extracts. The observation of sticky metaphase reinforces the hypothesis of the toxic effect of the extracts. Metaphases with sticky chromosome, loses their normal appearance, and they are seen with a sticky "surface," causing chromosome agglomeration **[26]**. 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 **[27,28]**. 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 **[29]**. The presence of chromosome fragments is an indication of chromosome breaks, and can be a consequence of anaphase/telophase bridges **[30]**. Fragments were observed in this study especially in the *Hippocrateaafricana*root extract treated groups. The extract used in this study was found to not only interfere with the cell cycle, but also affect chromatin organization or DNA replication, causing chromosome breaks. Frequencies of total chromosome aberrations increased significantly following exposure to the extracts which indicate 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 group treated with 2.5 mg/mL of *Hippocrateaafricana* root 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 **[31,32]**. Previous studies have suggested MNC-induced effect of various plant extracts such as *Lavandulastoechas*and *Ecballium elaterium***[33]**, *Azadirachtaindica***[34]***Psychotria* species **[25]**.

In this study, membrane damage cells were observed in groups treated with 5 mg/ml and 10 mg/ml of *Hippocrateaafricana* (10mg/mL).These results show that the extract over certain concentrations may cause cytotoxicity as which were prominent as membrane damage in this study. These results further support the cytotoxicity activities reported on *Hippocrateaafricana*root extract**[12]**. Multinucleated and binucleated cells have been 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 and the extracts the

process, 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 **[33]**. Some ghost cells were observed in various frequencies in this study especially in both extracts but mostly in *Hippocrateaafricana*root extract (10 mg/ml) treated groups (Figure 2). This could have resulted from the activities of the phytochemical constituents of the extracts leading to nucleus damage and prevention of cytoplasmic structures, thus resulting in ghost cells. In addition, the extracts also induced DNA damage and cell death and/or apoptosis in various frequencies in this study. In this study, high concentrations (5 mg/ml and 10 mg/ml) of the extracts were found to cause the induction of cell death and/or apoptosis. Cell death is a basic biological process of living organism. The cell death is induced by high concentrations of such as toxin, stress, heavy metals, chemicals and others.

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The results of this study show that the root extract of *H. africana* 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*(Figures 1(a), 1(b), 1(c), and 1(d)), suggesting cytotoxic and genotoxic activities of the extract.

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

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Conclusion

This study revealed that the root extract of *Hippocrataeaafricana* possesses cytotoxic and genotoxic effects, as seen in the effects elicited by all test concentrations of the plant extract on the root number, root length, and root morphology of the *Allium cepa* meristems after exposure. The degree of chromosomal aberrations (based on increasing extract concentration), the inhibition of cellular mitotic processes, and the general abnormalities observed in all root bulbs treated with the extract further indicate cytotoxic potentials of *Hippocrateaafricana* rootnot only in experimental plant tissues, but also quite possibly in animal systems as well.

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Comment [MF8]: the percent of recentness is 8.57 ,very poor and need update

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