GENOTOXICITY AND CYTOTOXICITY ACTIVITIES OF STEMBARK EXTRACT OF *Mammea africana*

Abstract

Mammea africana, a medicinal plant, used in ethnomedicine for the treatment of malaria, diabetes, poisoning and inflammatory diseases was investigated for cytotoxic and genotoxic effects on the root meristem cells of *Allium cepa*. Onion bulbs were exposed to 2.5 mg/mL, 5mg/mL, and 10 mg/mL concentrations of the stembark 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 effect 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 *Mammea africana* root extract possesses cytotoxic and genotoxic effects on *A. cepa* which is as a results of its phytochemical constituents.

Keywords: *Mammea africana*, genotoxicity, cytotoxicity, *Allium cepa*

1.INTRODUCTION

Mammea africana Sabine (Guttiferae) (syn. *Ochrocarpus africana* Oliv.) (*M. africana*) is a large forest tree of 50 to 100 feet high with bark often yellow with pale scales and resinous yellow sap [1]. The plant is widely distributed in tropical Africa. The stembark of the plant is traditionally used by the Ibibios, of Niger Delta region of Nigeria, in the treatment of malaria related fever, diabetes, microbial infections and mental disorders. The stembark is also traditionally used to treat stomach pains, rheumatism pains, scabies, cough and hypertension [2,3]. The stembark extract has been reported to possess cytotoxic activity, *in vitro* [4,5]. Ouahouo *et al.,* [6] reported cytotoxic coumarins with anti-microbial activity against *Staphylococcus aureus* from the plant stembark. The stembark has been reported to have anti-plasmodial [7], cardioprotective [8], anti-diabetic , hypolipidaemic [9,10],

vasorelaxant [11](Dongmo *et al.,*2007), anti-hypertensive [12], anti inflammatory, analgesic [13], antioxidant [14], anti-diarrheal, anti-ulcer [15], immunomodulatory, anti-lesihmanial [5], depressant and anti-convulsant [16] as well as nephroprotective [17] and hepatoprotective [18] activities. The stembark has been reported to have 5,-7-dihydroxy-8-(12- methyl-butryl) – 4 –N -pentylcoumarins and mesuxanthone B [19-21], 4-phenyl and 4- alkylcoumarins [22]. Alkaloids have been reported to be absent in the entire plant parts [23]. We report in this study the genotoxic and cytotoxic activities of the stembark extract.

2. MATERIALS AND METHODS

2.1 Plants collection

The plant material *Mammea africana* (stembark) were collected in Anwa forest in Uruan area, Akwa Ibom State, Nigeria in January 2022. The plant was identified and authenticated by a taxonomist of Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria.

2.2 Extraction

The stembarks were washed and shade-dried for two weeks. The dried plants' materials were further chopped into small pieces and reduced to powder using electric grinder. The powdered material (1.5 kg) was macerated for 72 h in 50% ethanol. This was thereafter filtered and the liquid filtrate was concentrated and evaporated to dryness in *vacuo* 40°C using a rotary evaporator (BuchiLab, Switzerland). The extract was stored in a refrigerator at -4°C, until used for the proposed experiments.

2.3 *Allium cepa* test.

Small bulbs of the common onion*, A. cepa*, were procured from Itam market in Uyo, Akwa Ibom State 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. 20 g of the plant extract was dissolved in 200 mL of distilled water. From this stock solution, different concentrations of the extract; 2.5 mg/mL, 5 mg/mL and 10 mg/mL were prepared.. Test concentrations of the plants' extract (2.5 mg/mL, 5 mg/mL, and 10 mg/mL) prepared in 50 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 (0.1 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. 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 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. 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.

2.4 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 slight 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 scored at each concentration of each sample [24].The mitotic inhibition was determined using the following formula:

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

%Aberrant cells =$\frac{Number\ \ of\ Aberrant\ \ cells}{Total\ \ number\ \ of\ cells}$ x100

%root growth of control = $\frac{Overall\ \ mean\ \ root\ \ lengt\ h\ of\ \ test\ solution}{Overall\ \ mean\ \ root\ \ lengt\ h\ of\ \ control}$ x 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.

2.5 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 $p \leq 0.05$.

3. RESULTS

3.1 Physicochemical Characterization.

The effect of *Mammea africana* stembark extracts on the physicochemical parameters (root number and root length) are presented in Table 1. This result shows that all tested concentrations of *M. africana* stembark extract caused significant inhibition in the growth of roots in comparison to negative control and positive control. The inhibition of root number and root length was greater with increasing concentrations of the two extracts. The average root length 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 groups were decreased significantly compared to that of the negative control; 0.39±0.19 cm for *M. africana* (Table 1). Average root lengths in treatment groups were decreased depending on concentration, significantly ($p<0.05$) when compared to negative control. The root morphology was nearly normal in the negative control treatment, but at 2.5mg/mL of *M. africana* stembark, the roots appeared slightly yellow and at 5 and 10 mg/mL of *M. africana* stembark extract, the roots appeared brownish (Table 1).

3.2 Cytogenetic Analysis.

Table 2 shows the effect of *Mammea africana* stembark on cytogenetic parameters of *Allium cepa* roots. Cytogenetic analysis performed showed that the stembark extract caused concentration-dependent and significant ($p<0.05$) decreases in the mitotic index when compared to that of negative control. The extract of *M. africana* at 10 mg/mL had mitotic indices of 5.60±1.02 as compared to 57.60±12.34 recorded in the negative control group (Table 2).

Table 1: Cytotoxicity of *Mammea africana* stembark 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]
Mammea africana	2.5	16.0±5.10 [a]	2.0±0.44 [a]
	5.0	13.0±6.20 [a]	1.27±0.23 [a]
	10.0	12.0±5.14 [a]	0.39±0.19 [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	288	57.60±12.34
Methotrexate	0.1	500	15	3.00±0.68[a]
Mammea africana	2.5	500	146	29.20±5.40[a]
	5.0	500	86	17.20±4.66[a]
	10.0	500	28	5.60±1.02[a]

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

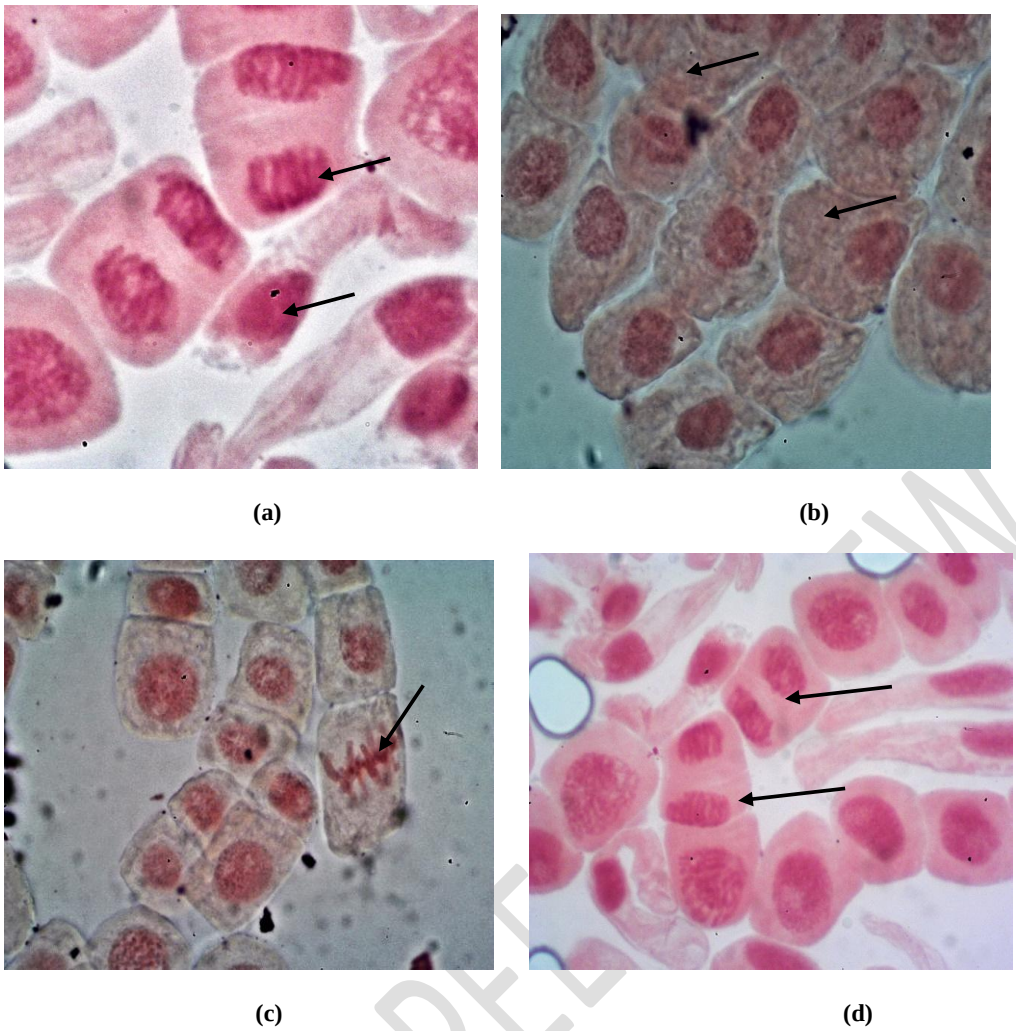
Cytogenetic alterations caused by the extract are shown in Table 3. Chromosome and cytological alterations were observed in negative control, methotrexate, and *Mammea africana* stembark extract-treated groups as depicted in Table 3. An analysis of chromosome aberrations observed showed that most of the fragments detected in the different treatments were of chromosome type especially in the highest concentration of *M. africana* stembark (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 (Figures 1(b) and 1(c)) in the extract-treated groups but were more frequent in the group treated with the highest concentration of *Mammea africana* (10 mg/mL). Sticky metaphase were also observed in the extract- treated groups. It was generally observed that these abnormalities increased with increasing concentrations of the 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 highest concentration exerting the highest effects and higher frequencies of aberrations. However, the highest value of aberrant cells was observed in methotrexate-treated group (positive control)(Table 3). Genotoxic activity of the extract wasfurther 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 *Mammea africana* had high numbers of cells with micronuclei in the test compared to negative control, which were statistically significant ($p< .05$) (Figure 3(a)). The increase occurred in the positive control. In *Allium* test, the strong toxic effect of the extract was observed, supported by great occurrence of sticky metaphases, leading to cellular death (mitotic index decrease). In addition, cells with membrane damage (Figure 1(d)), binucleated cells (Figure 1(e)), and nucleus damage (Figures 1(g) and 1(h)) were found in various frequencies. Also, apoptotic cells (Figure 1(f)) were detected in the group treated with the extract.

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

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]
Mammea africana	2.5	3.45±0.42 [a]	6.02±1.15 [a]	4.84±1.38 [a]	30.03±2.28 [a]	2.24±0.94 [a]
	5.0	5.83±1.38 [a]	12.83±2.58 [a]	8.20±2.13 [a]	36.34±5.21 [a]	0.13±0.01 [a]
	10.0	8.21±1.37 [a]	20.19±2.58 [a]	6.12±1.56 [a]	42.18±4.32 [a]	1.01±0.12 [a]

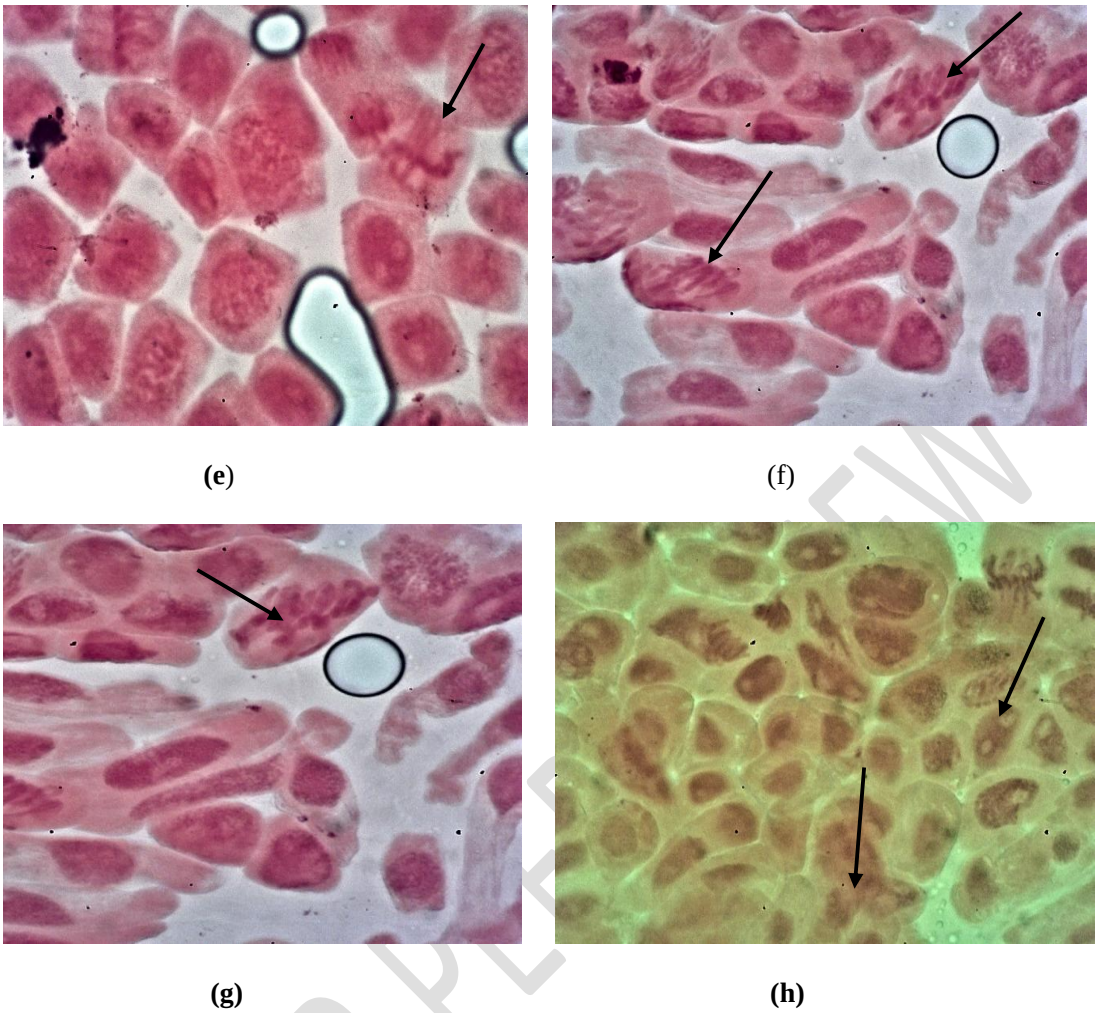
Values are expressed as mean ±SEM (n=5). Significant at $p<0.05$ when compared to negative control



(a) **(b)**

(c) **(d)**

Figure 1: Photomicrography showing the mitotic and chromosomal aberrations after the *Mammea africana* extract treatments in *Allium cepa* root tip meristem cells visualized with light microscopy at magnification X40. (**a)** arrow indicates the presence of a micronucleus; **(b)** arrow indicates apoptotic bodies; **(c)** arrow chromosomal stickiness at metaphase; **(e)** arrow indicates chromosomal fragments.



(e) (f)

(g) **(h)**

Figure 1: Photomicrography showing the mitotic and chromosomal aberrations after the *Solanum anomalum* extract treatments in *Allium cepa* root tip meristem cells visualized with light microscopy at magnification X40. **(e)** arrow indicates polar deviations; **(f)** arrow indicates chromatids bridges and stickiness; **(g)** arrow indicates stickiness; **(h)** chromosomal breaks and damaged nucleus.

4. DISCUSSION

In this study, toxic effect of *Mammea africana* stembark was evaluated by analyzing root growth and root morphology. 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 slightly yellow, slightly brown and brownish colouration 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 *Mammea africana* stembark extract (29.20%, 17.20% and 5.60% 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 [25], while a decrease below 50% usually has sublethal effects [26] and is called cytotoxic limit value [27]. Mitotic index reflects the proportion of cells undergoing cell division whose inhibition could be interpreted as cellular death or a delay in the cell multiplication activities[28]. 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 [29]. Mitodepressive effects of some herbal extracts, including the ability to block the synthesis of DNA and nucleus proteins, were reported earlier [30,31].

Several other herbal extracts have been reported to inhibit mitosis [32-34]. The decreased mitotic index in *A. cepa* roots treated with *Mammea africana* 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 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 [35]. 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 [36,37]. 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 [38]. The presence of chromosome fragments is an indication of chromosome breaks, and can be a consequence of anaphase/telophase bridges [39]. Fragments were observed in this study especially in the *Mammea africana* stembark extract treated groups. The extract 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 extract which indicate clastogenic activity (Table 3). These were more frequent in the groups treated with the stembark of *Mammea africana.* The extract significantly induced the formation of MNC in *A. cepa* root cells at 2.5-10 mg/mL concentrations. Frequencies of MNC was found to increased in the groups treated with 2.5 mg/mL of the 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 [40,41]. Previous studies have suggested MNC-induced effect of various plant extracts such as *Lavandula stoechas* and *Ecballium elaterium* [33,42], *Azadirachta indica* [43], *Psychotria* species [34].

In this study, membrane damage cells were observed in groups treated with 5 mg/mL and 10 mg/mL of the extract especially (10 mg/mL) treated group. These results show that the extract over certain concentrations may cause cytotoxicity as they cause membrane damage. These results further support the cytotoxicity activity reported on *Mammea africana* stembark extract [5]. 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 [42]. Some ghost cells were observed in various frequencies in this study especially in *Mammea africana* stembark (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 extract 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 extract 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.

The results of this study show that stembark extract of *M. 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 extracts.

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

Conclusion

The results of this study show the stem bark extract of *Mammea africana,* exhibits cytotoxic and genotoxic effects on living cells as demonstrated by significant inhibition on the *Allium cepa* root lengths, root numbers, cellular mitosis and genetic processes. The results obtained from the *Allium cepa* test suggest that *Mammea africana* despite their potential benefits as a medicinal plant, can have toxic effects living organisms when used inappropriately.

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