



Evaluation of the bio-toxicity and cytogenetic effects of methanolic leaf extract of *Heliotropium indicum* L.

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ABSTRACT

Heliotropium indicum L. has been shown to be very important in traditional healing medicine. However, many reports have indicated that the toxicity is not unconnected with pharmaceutical properties. This study therefore investigated the cytotoxic, genotoxic and biotoxic activities of methanolic leaf extract of *H. indicum* using Brine Shrimp lethality assay. The results revealed that the leaf extract of *H. indicum* showed lethality against the Brine Shrimps nauplii. The highest mortality was recorded at a concentration of 1000 µg/ml. The LC₅₀ value of the Brine Shrimps mortality of the extract was recorded to be 461.04±10.02 µg/ml. In addition, the extract inhibited mitotic division in *A. cepa* root meristematic cells. The mitotic index was reduced from 59.99% in the control to 2.2% at 1000 µg/ml of the leaf extract. Chromosomal aberrations were observed at different concentrations. These include sticky chromosomes at metaphase and anaphase, chromosome breakage, degenerated and disoriented chromosomes at metaphase plate. It can be concluded from this present study that methanolic leaf extract of *H. indicum* was cytotoxic and genotoxic to Brine Shrimps and *A. cepa* cells respectively. Hence, caution should be exercised with respect to the consumption of *H. indicum* leaf in any form.

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Keywords: Mitotic index, chromosome aberrations, Brine Shrimps Lethality assay, *Allium cepa* assay, leaf extract.

INTRODUCTION

Heliotropium indicum popularly known as “Ogbe-ori akuko” (Cock’s comb) in Yoruba land, Nigeria belongs to the family, Boraginaceae (Dash and Abdullah, 2013). About 250-275 species have been documented in the genus and *H. indicum* has its centre of origin in Asian continent (Dash and Abdullah, 2013; Roy, 2015; Khurm et al., 2016). It has a wide distribution worldwide with the

exception of dry regions. *Heliotropium indicum* has pale violet flowers which are regular, sessile and are arranged in two rows. It flowers throughout the year (Dash and Abdullah, 2013). In response to biotic and abiotic stresses, phytochemicals are produced naturally in plants to protect themselves (Tembe Fokunang et al., 2019). Phytochemicals such as saponin, tannis, alkaloids, helotrine, flavonoids, phenols and

steroids have been isolated from *H. indicum*. Flavonoids and tannins were reported to be responsible for anti-oxidant property reported in this plant (Akinlolu et al., 2008; Roy, 2015; Santhosha et al., 2015).

Heliotropium indicum has been reported to have multiple traditional uses (Rashed et al., 2018) such as in the treatment of arthritis, rheumatism, eye infections, diarrhea, dysentery and malaria. It also has wound healing properties (Dash and Murthy, 2011; Yeo et al., 2011). It is a common ingredient in decoction for the treatment of fever in children among Yoruba people in Nigeria. In addition, it has been acknowledged to have anti-tumor, anti-microbial, anti-tussive, anti-inflammatory, anti-nociceptive, anti-fertility, anti-tuberculosis, anti-anaphylactic activities. It also possesses histogastro protective properties (Rahman et al., 2001; Akinlolu et al., 2008; Sivajothi et al., 2015; Villa et al., 2016; Ghosh et al., 2018). However, *Heliotropium* species have been reported to be toxic (Dash and Abdullah, 2013) despite their importance in traditional healing medicine. Many reports have indicated that the toxicity is connected with pharmaceutical properties (Bayala et al., 2019). Furthermore, *H. indicum* has been shown to have toxic side effects (Sathosha et al., 2015). The pyrrolizidine alkaloids isolated in *H. indicum* were proved to be hepatotoxic. This alkaloid was also reported to have been isolated in *H. curassavium* which was said to be toxic too and the toxicity was attributed to pyrrolidizing alkaloid in the plant (Reza et al., 2018). Moreover, *H. strigosum* which has been used in the treatment of various infections is cytotoxic and phytotoxic (Khurm et al., 2016). However, the toxicity of *H. indicum* has not been fully studied (Dash and Abdullah, 2015; Ghosh et al., 2018).

The Brine Shrimp lethality assay (BSLA) represents a rapid, inexpensive and simple bioassay for testing bioactivity of a plant extracts, which often correlates with cytotoxic and anti-tumor properties (Stephanie et al., 2018). This test is an indication of cytotoxicity, anticancer, antiviral, pesticidal, antimicrobial and other different pharmacological activities (Stephanie et al.,

2018). Although, BSLA is neither adequate in determining the mechanism of action of the bioactive substances in plants nor specific for antitumor activity; it however provides a preliminary screening that can be supported by a more specific bioassay, once the active compounds have been isolated (Ogbole et al., 2017). Plants that are found to be toxic to Brine Shrimp are likely to be a good candidate for anti-cancer research (Ogbole et al., 2017).

The effects of toxic substances can also be revealed at the level of chromosome through manifestation of chromosomal aberrations. Many plant systems such as *Allium cepa* have been widely used to evaluate DNA damages and it have been known to have a good correlation with other test systems (Celik, 2012). This study aimed at determining the cytotoxic and genotoxic effects of *H. indicum* leaf extract on Brine Shrimps and *Allium cepa* cells in order to predict its effect on human system.

MATERIALS AND METHODS

Plant collection

Fresh leaves of *H. indicum* were collected at Irebami Area, Fajuyi, Ile-Ife, Osun State Nigeria (N7° 30'12.9186" E 4°33'42.3652") during the dry season. The plant was identified and authenticated at the IFE Herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria. A voucher specimen has been deposited in IFE Herbarium with Voucher number, IFE-17807 (Figure 1).

Preparation of plant extract

The leaves of *H. indicum* were air dried for three weeks, ground into powdered form using a laboratory blender and then extracted with methanol (1:5 w/v) which was intermittently stirred for a period of 48 hours. The extract was then filtered using Whatman filter paper. The filtrate was concentrated in a rotary evaporator (Buchi RII, Switzerland) at 40 °C.

Bioassay effect of extract on Brine Shrimps

The procedure for Brine Shrimp lethality assay (BSLA) described by Jahangir

et al. (2019) was modified to investigate the cytotoxic property of the extract. Brine Shrimps (*Artemia salina*) were hatched using Brine Shrimp eggs in a conical shaped hatching chamber (1L), filled with sterile artificial seawater under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of Brine solution (seawater). In each experiment, different volume of extract was added to 4.5 ml of Brine solution to give different concentration (200, 400, 600, 800 and 1000 µg/ml) and maintained at room temperature for 24 h under the light after which the number of surviving larvae were counted. Experiments were conducted along with control (vehicle treated) of the test substances and potassium dichromate at different concentrations (20, 40, 60, 80, and 100 µg/ml) was used as the positive control. Three replicates were prepared per dose.

Cytotoxic and genotoxic effects of extract on *Allium cepa* meristematic root cells

Allium cepa assay was used to investigate the antimutagenic activity of the leaf extract as previously described (Gadano et al., 2002; Solange and Haywood, 2012). Dried commercially obtained *A. cepa* bulbs were sprouted in distilled water for 48 h. Sprouted

bulbs were then transferred to varying concentrations (200, 400, 600, 800, 1000 µg/ml) of methanolic leaf extract of *H. indicum* for 24 h. Sprouts in distilled water represented the untreated control. After 24 h, the roots were harvested and slides were prepared according to the squash technique used by Azeez et al. (2019). The slides were viewed under a light microscope (Olympus, model CKX31). The number of cells in each stage of mitotic division was counted and twenty-five fields were observed. Mitotic index was expressed as follows:

$$\text{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}}$$

The chromosome aberration frequency was expressed as percentage which was calculated as follows:

$$\text{Chromosome aberration frequency} = \frac{\text{Number of cells with chromosome aberration}}{\text{Total number of cells}} \times 100$$

Statistical analysis

GraphPad Prism version 5.0 and Probit software were used for statistical analysis. Data were expressed as mean with standard error in Brine Shrimp assay. Duncan's multiple range test was employed to determine the significant difference among the mitotic parameters for different concentrations in *A. cepa* assay.



Figure 1: Habit of *Heliotropium Indicum*.

RESULTS

Cytotoxic effect of methanolic leaf extract of *Heliotropium indicum*

The methanolic leaf extract of *Heliotropium indicum* showed varying level of lethality against the Brine Shrimp nauplii (Tables 1 and 2). The degree of lethality was observed to be directly proportional to the concentration of the extract (Figure 2). Highest and lowest levels of mortality were observed at highest and lowest concentrations of the leaf extract respectively. The LC_{50} values of the Brine Shrimp obtained through regression plot analysis for the extract and the positive control (potassium dichromate) were $461.04 \pm 10.02 \mu\text{g/ml}$ and $49.65 \pm 0.25 \mu\text{g/ml}$ respectively (Figure 2).

Cytotoxic and genotoxic effect of methanolic leaf extract of *H. indicum*

The methanolic leaf extract of *Heliotropium indicum* inhibited mitotic division in actively dividing *A. cepa* meristematic cells (Table 3). The result showed decreasing cell activities with increase

in concentrations of the extract. The mitotic index was reduced from 59.99% in the control to 2.2% at the highest concentration of 1000 $\mu\text{g/ml}$ investigated. The mitotic index in the control was significantly higher than the mitotic indices recorded in all the treatments. There was no significant difference between mitotic indices as well as percentage of cell abnormality recorded in 200 and 400 $\mu\text{g/ml}$. Similarly, 800 and 1000 $\mu\text{g/ml}$ did not show any significant difference in their mitotic indices and percentage cell deformity (Table 3). Deformation and elongation of non-dividing cells as well as chromosome aberrations started with 200 $\mu\text{g/ml}$ treatment. It should be noted that larger percentage of the cells were already degenerated at 1000 $\mu\text{g/ml}$. There was no single normal cell at this concentration. Chromosome aberrations observed include sticky chromosomes at metaphase and anaphase, chromosome breakage at anaphase, degenerated chromosomes and disoriented chromosomes at metaphase plate (Figures 3 and 4).

Table 1: Brine Shrimp lethality data at different concentrations of the *Heliotropium indicum* methanolic extract.

Dose($\mu\text{g/mL}$)	No of Shrimps Exposed	Number Responding	$LD_{50}(\mu\text{g/mL})$
200	30	9	461.04 ± 10.02
400	30	11	
600	30	21	
800	30	24	
1000	30	27	

Table 2: Brine Shrimp lethality data at different concentrations of the Potassium dichromate.

Dose($\mu\text{g/mL}$)	No of Shrimps Exposed	Number Responding	$LD_{50}(\mu\text{g/mL})$
20	30	8	49.65 ± 0.25
40	30	9	
60	30	19	
80	30	25	
100	30	29	

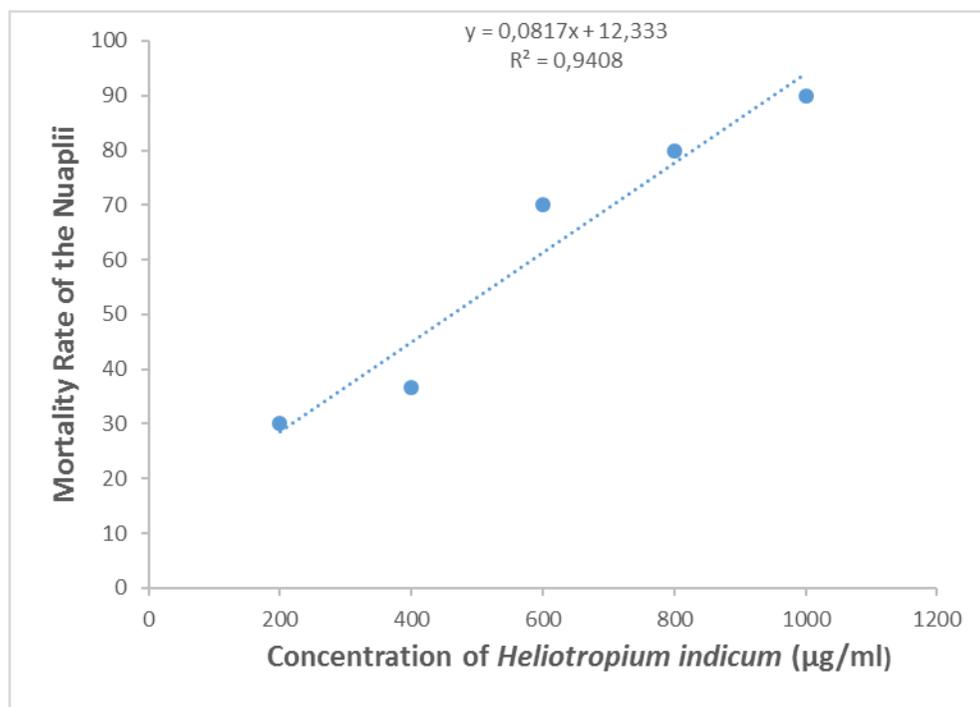


Figure 2: Average inhibition of Brine Shrimp growth at different concentrations of methanolic *H. indicum* leaf extract.

Table 3: The mitotic index, cell abnormality and chromosome aberration frequency in *Allium cepa* root tip cells after treatment with different concentrations of the *H. indicum* methanolic extract for 24 hours.

Concentration of <i>H. indicum</i> extract (µg/mL)	Mitotic Index (%)	Deformed /Elongated Non-dividing Cells (%)	Chromosome Aberration Frequency (%)
0	59.99 ^c	-	-
200	33.70 ^b	44.59 ^a	0.63 ^a
400	22.90 ^b	35.87 ^a	6.88 ^b
600	7.50 ^a	88.52 ^b	7.66 ^b
800	7.30 ^a	85.71 ^b	10.71 ^c
1000	2.20 ^a	90.9 ^b	9.01 ^c

The same letter showed that there was no significant difference ($P \leq 0.05$).

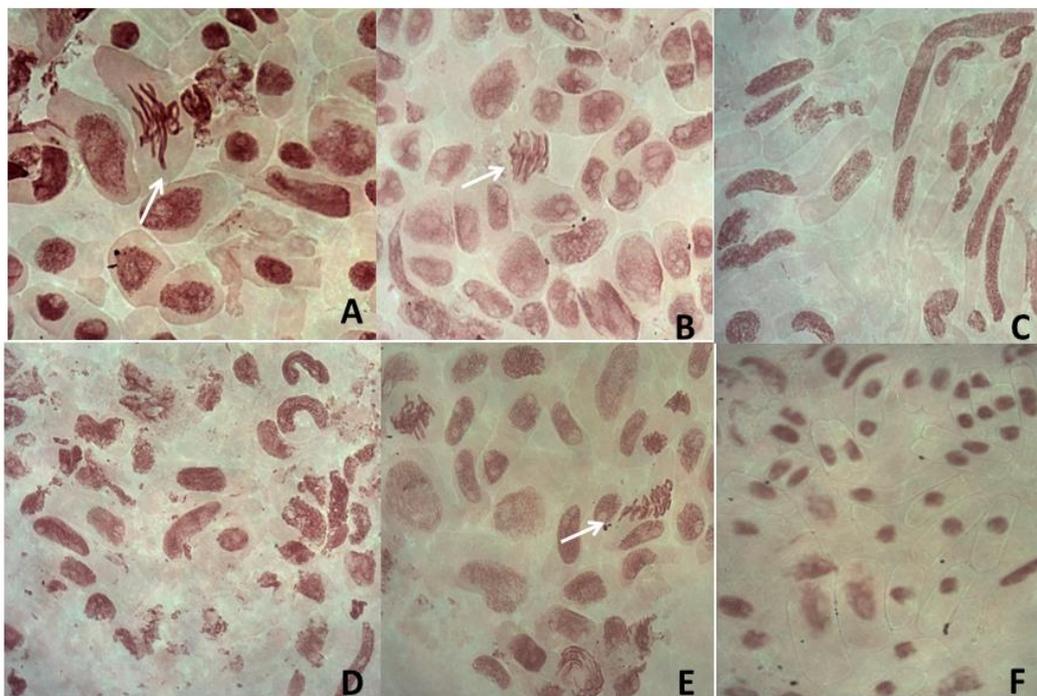


Figure 3: Cell divisions in *Allium cepa* root cells of untreated and those treated with *H. indicum* extract. **A:** Metaphase (arrowed) control; **B:** Metaphase (arrowed) at 200 µg/mL; **C:** Elongated cells at 200 µg/mL; **D:** Deformed cells at 200 µg/mL; **E:** Sticky metaphase (arrowed) at 400 µg/mL; **F:** Elongated cells at 400 µg/mL.

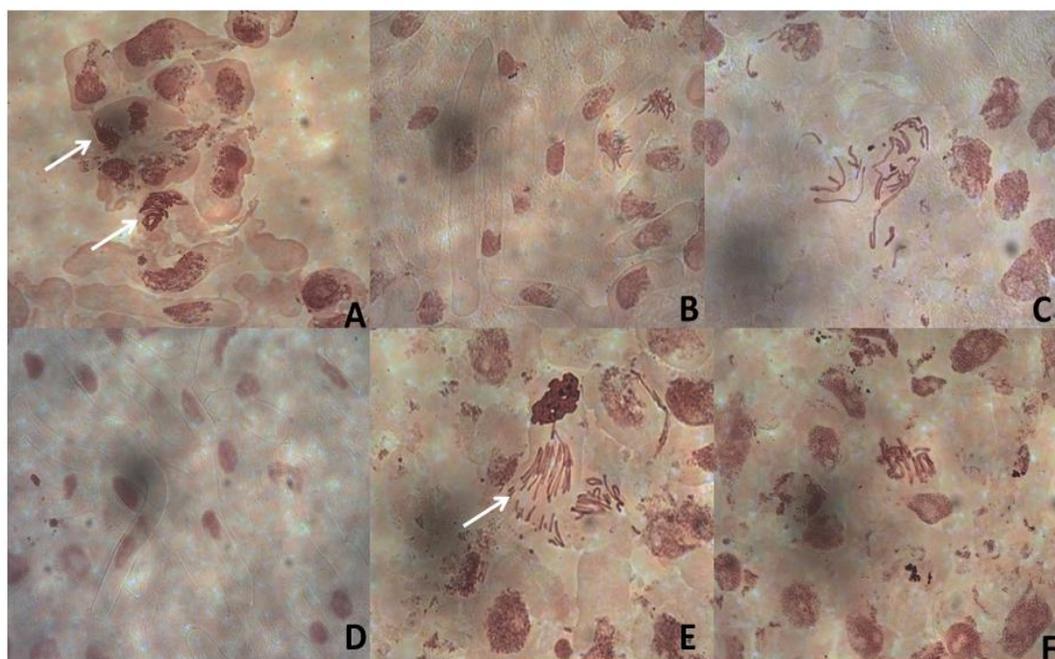


Figure 4: Cell divisions in the *Allium cepa* root cells treated with *H. indicum* extract. **A:** Sticky and degenerated chromosomes at anaphase and sticky metaphase (arrowed) at 600 µg/mL; **B:** Elongated cells at 600 µg/mL; **C:** Disoriented chromosomes at metaphase plate at 800 µg/mL; **D:** Elongated cells at 800 µg/mL; **E:** chromosome breakage at anaphase (arrowed) at 1000 µg/mL; **F:** sticky chromosomes at metaphase at 1000 µg/mL.

DISCUSSION

In this study, the cytotoxic activity of methanolic leaf extract of *H. indicum* showed LC₅₀ of 461.04±10.02 µg/ml which indicated cytotoxicity according to Meyer's toxicity index which stated that LD₅₀ below 1000 µg/ml indicates toxicity (Franklin et al., 2016). The cytotoxic potential of *H. indicum* recorded in this study is consistent with the previous studies in the field of antitumor drug discovery in different species of *Heliotropium* genus. Rahman et al. (2011) established that methanolic extract of the dried roots of *H. indicum* was strongly cytotoxic with a LC₅₀ of 47.86 µg/ml. Investigations on antimicrobial, cytotoxic, phytotoxic and antioxidant potentials of *H. strigosum* Willd established that dichloromethane extract of the whole plant of *H. strigosum* was cytotoxic with a LD₅₀ of 462 µg/ml (Muhammad et al., 2016). In addition, Ogbole et al. (2017) established that methanolic extract of aerial part of *H. indicum* from Nigeria possesses cytotoxic property with LC₅₀ of 391.30 ± 11.24 µg/ml. Cytotoxic property of *H. indicum* observed in BSLA in this present study might be due to various pyrrolizidine alkaloids that have been identified in the plant. The alkaloids reported in the plant include heliotrine, lasiocarpine, indicine, 12-acetyl indicine, indicinine, indicine-N-oxide, retronecine, trachelanthamide. The aerial parts contain echinatine, heleurine, lasiocarpine-N-oxide, supinine, heliotrine, indicine, indicine-N-oxide and lasiocarpine (Dash and Abdullah, 2013). Some of these active principles have been established to have several biological activities especially antitumor (Oluwatoyin et al., 2011).

The decrease in mitotic index of *Allium cepa* root cells treated with various concentrations of *H. indicum* leaf observed in this present study is an indication of cell growth inhibition and cell death. It was observed that the mitotic index decreased with increasing concentrations of the leaf extract. It is also important to point out here that the

observations made in the study concerning the preponderance of elongated cells (Figure 3 and 4) which increased with increasing concentrations of the extract may indicate rapid cell differentiation. This phenomenon will of course reduce mitotic index since differentiated cells become specialized instead of going into replication; indicating reduction in meristematic cells. Moreover, decrease in mitotic index in the current study can also be attributed to the inhibition of DNA synthesis or the blocking of G2 phase in the cell cycle (Akinpelu et al., 2019). Mitotic index decrease of 12.5%, 12.16% and 3.67% at 600 µg/ml, 800 µg/ml and 1000 µg/ml respectively were recorded when compared with control in this present study. It has been documented that a decrease in mitotic index below 22% against control will have a lethal effect on the test organism (Iqbal et al., 2019). It could be noted from this present study that 600, 800 and 1000 µg/ml concentrations had similar effects on the *A. cepa* root tip cells except that less chromosome aberrations were recorded at 600 µg/ml. It can thus be inferred that the rate of cell replication decreased with increasing concentration of the active compounds in the leaf extract of *H. indicum* with 800 and 1000 µg/ml being the most lethal.

The chromosome breakage observed in this study might be as a result of formation of DNA-DNA and DNA-protein crosslinks (Iqbal et al., 2019). In addition, the disoriented chromosomes at metaphase noted here may be attributed to distorted segregation during cell division. The sticky chromosomes observed in this study suggest that *H. indicum* extract may be toxic. The stickiness has been documented to be connected to the excessive contraction and condensation of chromosomes as well as partial dissolution of nucleoproteins (Akinpelu et al., 2019). In addition, sticky chromosomes reveal toxic effect on chromatin, which has been documented to cause irreparable cell death. It was opined that this effect could be connected to the formation

of complexes of toxic agent with phosphate groups in DNA (Iqbal et al., 2019).

The reported genotoxic effects in the *Crassocephalum crepidioides* by Akinpelu et al. (2019) was linked to the pyrroizidine alkaloids that has been known to be hepatotoxic which has been isolated in the members of the genus. Similarly, the cytotoxic and genotoxic effects of *H. indicum* as shown from observations in this present study such as reduced mitotic index, sticky chromosomes and chromosome fragmentation may be attributed to pyrroizidine alkaloids which are present among the members of genus *Heliotropium*. The genotoxic and cytotoxic effects observed in this study may also be associated with the presence of multiple biological activities in *H. indicum*. In addition, the observed chromosome aberrations may be linked to the interactions of a great variety of chemical agents in this test plant extract with its DNA (Celik, 2012).

Conclusion

The cytotoxic and genotoxic effects of methanolic leaf extract of *H. indicum* were investigated. The extract was cytotoxic to Brine Shrimp nauplii and induced concentration-dependent reduction in the mitotic activities of *A. cepa* root meristematic cells. In addition, chromosomal aberrations observed in this study confirm the cytotoxic and genotoxic activities of *H. indicum*. In fact, it can be said that the leaf extract of *H. indicum* has lethal effect at higher concentrations. This present study therefore concluded that *H. indicum* leaf extract has both cytotoxic and genotoxic effects in the models investigated. From the observations in this study, caution should be exercised in consuming the leaf of *H. indicum*. Moreover, further work is recommended on this plant to isolate the active compounds responsible for its array of healing activities.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

SOA and OGA handled the *Allium cepa* assay aspect of the study while RAA and IJO carried out the Brine Shrimp assay. The manuscript was prepared and read by all the authors.

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