Cytotoxic Effects and Genotoxic Screening of Pharmaceutical Effluents using Onion Bulbs (*Allium cepa L.*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SEO and AEO managed the analyses of the study. Author OAA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Pharmaceutical effluents like other pharmaceutical effluents are toxic waste water generated from drug industries which when discharged directly into the environment without proper handling and treatments may cause deleterious effects on human health and environment.
Aim: This research is aimed at investigating the potential cytotoxic and genotoxic effect of effluents released by three different pharmaceutical industries in Sango Industrial Area of Ogun State in Nigeria using the USEPA recommended *Allium cepa* test.
Methodology: Each of the effluents were prepared into different concentrations using distilled water (1%, 2%, 3%, 4%, 5%, 10%, 25%, 50% and 100% respectively) and their cytotoxicity on the root length of a series of onion bulbs was observed after three days of exposure. Cytological studies on the root tips of onion bulbs using five concentrations of each effluent were also

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examined by Aceto-orcein squash technique. Mitotic index which is the ratio of the number of cells dividing to number of cells counted was observed to decrease with increasing concentration of all the effluents compared to the control. Chromosome aberrations induced at different concentrations were observed under microscopes.

Result: Significant results were observed in the Root length Evaluation and Chromosomal Aberration Evaluation tests respectively after three days. Effluent C seems to be the most toxic on the root length of the onion bulbs as it reduced from 1.2cm to as low as 0.30cm, followed by effluent B and A with 0.60cm and 0.70cm respectively. But Effluent A has the most % of aberrant cells with 5.32% and Effluent C with 3.03%. Most of the aberrations induced were vagrants, bridges and sticky chromosomes.

Discussion and Conclusion: The results from this study showed that effluents from these pharmaceutical industries have toxic chemicals and that *Allium cepa* test in relation to this study has proved to be an effective tool that may be employed by environmental toxicologists in monitoring industrial effluents before they are discharged into the environment.

1. INTRODUCTION

The Increase in demand of pharmaceuticals in Nigeria as a result of its growing disease burden has led to consequential pharma boom in Nigeria [1] and therefore, increase in the amount of effluents generated. A larger number of these industries which are involved in manufacturing of Pharmaceutical and Personal Care Products (PPCP) suffer from inadequate effluent treatment due to the presence of recalcitrant substances. The wastewater is mainly generated through the washing activities of equipments. Though the wastewater discharged is small in volume, it highly pollutes because of presence of substantial amount of organic pollutants [2]. Level of wastewater pollution varies from industry to industry depending on the type of process and the size of industry [3].

Most pharmaceutical effluents are known to contain different concentration of organic compounds (such as pharmaceuticals) and total solids as well as heavy metals [4,5]. Even though much attention has been paid on heavy metals due to their mutagenic and carcinogenic effects when exposed directly to humans and other organisms [6], Pharmaceuticals are also known to exhibit some health effects over time [7].

This research is aimed at studying the cytotoxic effects and genotoxic screening of pharmaceutical effluents collected from three pharmaceutical industries in the industrial area of Sango Ota in Ogun State Nigeria. An *Allium*

cepa test looks like a potential tool for this investigation.

Onions bulbs (*Allium cepa*) has largely been used for culinary purposes, but apart from its nutritional significance, the common onion is one of the most outstanding higher plant recommended by United States Environmental Protection Agency (USEPA) and the American Society for Testing and Materials (ASTM) in 1982 and 1994 respectively [8-10]. They are useful as an excellent and alternative first–tier indicator for safety evaluation of cytogenetic and mutagenic effects of drinking water and environmental pollutants as their root length inhibition and chromosome aberration bioassay are sensitive, cost effective and valid indicator of toxicity test for the routine monitoring of water pollution due to the important activating enzymes the root tip cells possesses [11-14]. They have shown good correlation with other test systems involving genotoxicity [15].

2. EXPERIMENTAL

2.1 Collection of Pharmaceutical Effluents

Effluents from three Pharmaceutical industries denoted as "A, B and C" located in Sango Industrial Area of Ogun State-Nigeria were collected from their discharged points into the environment during the period of high production. Effluents from these industries are released into the adjacent water bodies such as rivers and canals.

2.2 Procedure for Root Length Evaluation

Onion bulbs (*Allium cepa*, L, 2n=16) of the purple variety with average size (15-22mm diameter) were bought locally in Lagos State, Nigeria. They were dried for about six weeks and the dried roots present at the base of the onion bulbs were carefully shaved off with a new razor blade in order to expose the fresh meristematic tissues. The bulbs were then placed in freshly prepared distilled water to prevent the drying up of the primodial cells.

To account for a number of bulbs in the population that would show poor or slow growth, three replicate bulbs were used for each test samples (i.e. pharmaceutical effluents from each industry). The bulbs were taken out from distilled water and placed on blotting paper to remove excess water.

To evaluate the root length or growth inhibition, the bulbs were exposed directly in to control (tap water) and then in to 1, 2, 3, 4, 5, 10, 25, 50 and $100\%^v/v$ (effluent/tap water) of each test sample. Five onion bulbs were used for each concentration of the test samples and the control. The base of each onion bulb was suspended on the effluents inside a 50ml bottle in the dark for three days (72hr). The test effluents were changed daily and the three bulbs showing best growth were selected for measurement. Their root length (in cm) was measured with a calibrated metre rule for three consecutive days and the mean root length of the three bulbs for each test sample concentration was determined statistically and recorded.

2.3 Procedure for Chromosomal Aberration Evaluation

Three onion bulbs were suspended in 1, 2, 3, 4 and $5\%^v/v$ concentration of each of the effluents and the control (distilled water). At the end of 72hr, root tips from these bulbs were cut and fixed in acetic acid alcohol. Acetic acid alcohol is used here as the fixative and preservative for the specimen. For each root concentration, a clean plain glass slide was taken using a pair of forceps and the root tip was placed on the glass slide. A razor blade was used in cutting its tip 5mm and the remaining was discarded.

A drop of 1N HCL was used to dehydrate and soften its tissue for maceration for 2 minutes. The excess 1N HCL was removed neatly with filter paper. A dissecting needle was then used to macerate the root tips after which a drop of Acetic Orcein stain was placed on the tissue to stain for 15-20 minutes.

After staining the tissue, the specimen on the slide was gently covered with a cover slip, allowing the stain to spread evenly over the square parts of the cover slip to eliminate air bubble. The slide with the specimen was then placed in between two folds of the filter paper and using the blunt end of the biro, gentle tapping and pressure was applied around the square area of the cover slip for evenly squashing of the specimen. Finally, the square edges of the cover slip of the squashed slide was sealed with white transparent nail hardener as suggested by Grant [11] to prevent drying out of the preparation by the heat of the microscope [16].

Three slides were prepared for each concentration and control. The slides were viewed under the microscope at × 1000 magnification to observe mitotic stages and chromosomal aberrations for photomicrographs.

The mitotic index (MI) was calculated as the ratio of number of dividing cells to number of observed cells [8]. The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of each effluent.

2.4 Statistical Analysis

The means, with 95% confidence limits and the standard errors for results of the root inhibition and chromosome aberrations at each concentration of the effluents were calculated. Data were expressed as Mean ± Standard Error of Mean (SEM). Differences between the control and different concentrations of the effluents were analyzed using independent-test. Values of $p \leq 0.05$ were considered to be significant. All statistical analyses were carried out using SPSS[®] 16.0 statistical packages.

3. RESULTS AND DISCUSSION

The mean root length of *Allium cepa* (onion bulbs) grown in three pharmaceutical industrial effluents are presented in Table 1. In general,

most of the onion bulbs exposed to different concentrations of the effluents showed growth retardation when compared to the control (Tap water). Low growth rate was observed in onion bulbs exposed to high concentrations of the effluents and vice versa.

Specifically, the mean root length of most onion bulbs grown in the three different pharmaceutical effluents were in decreasing order as concentration increases: For instance the % root length of control onion bulbs grown in stock sample (100% effluents) of A was 22.6

while those grown in effluents B and C were 19.3 and 9.7 respectively (i.e. mean root length: A>B>C). However, there are deviations at some concentrations which may be as a result of genetic variations among the onion bulbs used as vehicle for the experiment. At 95% confidence level (p value≤0.05), it was observed that the root growth inhibition was statistically significant for effluent A at 25%, effluent B at 10% and effluent C at 5% throughout the three days compared to the control i.e. the toxicity of these three effluents on the root growth of onion bulbs was A<B<C.

Table 1. The mean root length of *Allium cepa* cultivated in different concentrations of the effluents obtained from Pharmaceutical Industry A, B and C respectively for three days compared to the control

Concentration (%)	MRL ± S.E.M (cm) Day 1	MRL ± S.E.M (cm) Day 2	MRL ± S.E.M (cm) Day 3
Pharmaceutical effluent A			
0%(Control)	1.20±0.0	2.00±0.21	3.1±0.15
A1%	0.90±0.16	1.60±0.21	2.00±0.29*
A2%	0.80±0.16	1.30±0.14*	2.10±0.24*
A3%	1.30±0.08	1.70±0.15	2.20±0.15*
A4%	1.00±0.14	1.90±0.32	2.10±0.31*
A5%	0.80±0.06*	2.53±0.16	2.80±0.15*
A10%	0.90±0.10	1.70±0.10	2.00±0.10*
A25%	0.85±0.70*	1.10±0.10*	1.70±0.10*
A50%	0.70±0.20*	1.05±0.15*	1.10±0.15*
A100%	0.30±0.13*	0.70±0.23*	0.70±0.23*
Pharmaceutical Effluent B			
0%(Control)	1.20±0.0	2.00±0.21	3.1±0.15
B1%	1.00±0.23	1.60±0.30	1.93±0.23*
B2%	0.90±0.21	1.80±0.21	2.20±0.30
B3%	0.80±0.26	1.43±0.35	1.90±0.40*
B4%	0.90±0.13	1.63±0.37	2.00±0.43
B5%	0.90±0.22	0.60±0.52	1.90±0.66
B10%	0.60±0.05*	1.10±0.10*	1.70±0.10*
B25%	0.50±0.00*	0.90±0.10*	1.10±0.10*
B50%	0.60±0.05*	0.80±0.10*	0.90±0.10*
B100%	0.40±0.12*	0.60±0.10*	0.60±0.88*
Pharmaceutical Effluent C			
0%(Control)	1.20±0.0	2.00±0.21	3.1±0.15
C1%	0.90±0.20	1.80±0.34	2.80±0.35
C2%	0.80±0.15	1.40±0.35	2.10±0.57
C3%	0.80±0.11*	1.10±0.19*	1.90±0.47
C4%	0.90±0.19	2.50±0.52	2.70±0.44
C5%	0.50±0.08*	1.00±0.19*	1.63±0.32*
C10%	0.45±0.05	0.90±0.50*	1.40±0.10*
C25%	0.40±0.05*	0.70±0.50*	0.90±0.10*
C50%	0.30±0.00*	0.50±0.10*	0.80±0.50*
C100%	0.10±0.03*	0.30±0.17*	0.30±0.16*

P≤0.05 level of significance of root growth inhibition as compared with the controls, Values are expressed in Means Root length ± Standard Error Mean, MRL-Mean Root length, S.E.M (cm)-Standard Error Mean

The root tips of onion bulbs grown in the effluents when compared to the root tips of those grown in the control were characterized by malformations such as swollen roots, twists and crotchet roots (roots bent upwards resembling hooks). The induction of root malformation in *Allium cepa* has been shown to be useful indicator of toxicity [14,17,18].

Table 2 showed the mitotic index (MI) of the onion bulbs treated with the control (Tap water) and effluents collected from three pharmaceutical industries. There was decrease in the mitotic index with corresponding increase in the concentrations of all the effluents when compared to the mitotic index value of the control (9.40). Effluent C at 5% gave the lowest MI value of 4.04 while Effluents B and A gave MI values of 4.15 and 4.44 respectively at the same concentration. The decrease in the mitotic index with increasing concentrations of the effluents is a reflection of cytotoxicity [15,18]. Decline of mitotic index below 22% compared to the control can cause lethal effects on the organism [19].

*Mitotic index = $\dfrac{\text{Number of dividing cells}}{\text{Number of cells scored}} \times 100$

*Frequency (%) of aberration =

$\dfrac{\text{Number of aberrant cells}}{\text{Number of cells scored}} \times 100$

The summary of the microscopic analysis results of *Allium cepa* root tips grown in three pharmaceutical industries effluents are presented in Table 3. Chromosome aberrations were observed at all the tested concentrations of each effluent and most frequently chromosome aberrations observed were vagrants, bridges and sticky chromosomes with effluent a having the highest aberrations, followed by effluent B while effluent C had the least aberrations.

In *Allium cepa* test, there usually seems to be a relative decrease in root growth (cytotoxicity) and chromosomal deviations reduction (genotoxicity). Whenever chromosome aberrations occurred, there are almost always definite growth restrictions [8].

Fig. 1 represents the micrograph of the chromosome aberrations observed. Sticky chromosome is an indicator of poisoned chromosomes with sticky surface which

possibly bring about cell death [9]. The abundance of sticky chromosomes at metaphase (A_3) and anaphase (B_2) stages in this *Allium cepa* test indicate that these effluents contain toxic substances. This claim is supported by a previous research on the physicochemical analyses of effluents collected from this same industrial area which showed the presence of some poisonous toxicant heavy metals such as Nickel, Lead and Cadmium. The levels of Nickel (0.867ppm) and Cadmium (0.085ppm) were beyond the WHO permissible limits of 0.02ppm and 0.003ppm respectively [5]. Since industrial effluent A has the highest number of sticky chromosomes, it therefore suggests that it is the most toxic of all the three effluents. On the other hand, the industrial effluent C has the least sticky chromosomes which suggest that it is the least toxic. The inductions of bridges at anaphase were frequently observed and such anomaly is also an indication of mutagenic events in the cell [20].

Table 2. Cytological effects of different concentration of pharmaceutical effluents A, B and C respectively on the root tips of *Allium cepa* after three days compared to their respective controls

% Concentration	No of dividing cells	Mitotic index	% of Aberrant cells
Pharmaceutical effluent A			
0(control)	470	9.40	-
1	412	8.24	5.32
2	350	6.99	4.66
3	287	5.74	4.53
4	259	5.12	4.28
5	222	4.44	3.17
Pharmaceutical effluent B			
0(control)	470	9.40	-
1	363	7.28	5.12
2	326	6.51	4.82
3	298	5.97	4.48
4	252	4.15	4.73
5	208	6.85	4.15
Pharmaceutical effluent C			
0 (control)	470	9.40	-
1	342	6.85	4.38
2	284	5.68	3.98
3	270	5.41	4.50
4	301	6.03	5.08
5	203	4.04	3.03

5000 cells per concentration of the effluents and the control

Table 3. Chromosome aberrations induced in the root tips of *Allium cepa* cultivated in different concentration of pharmaceutical effluents A, B and C after three days compared to control

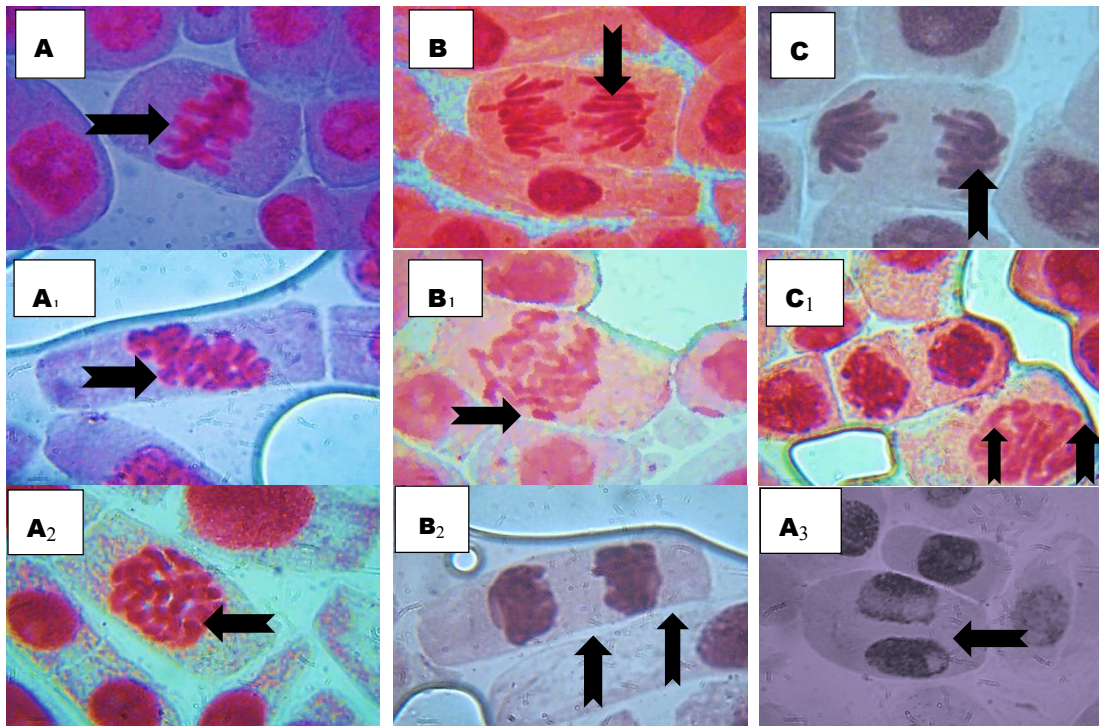| Pharmaceutical Effluent A							
Chromosome aberrations per 1000 cells							
Concentration	Stickiness	C-mitosis	Bridges	Vagrants	Binuclei	Mitotic index	% of aberrant cells
Control	0	0	0	0	0	9.40	-
A1%	13	0	16	24	0	8.24	5.32
A2%	20	0	12	15	0	6.99	4.66
A3%	18	0	9	18	0	5.74	4.53
A4%	15	3	12	12	0	5.12	4.28
A5%	9	0	6	16	0	4.44	3.17
Pharmaceutical Effluent B							
Control	0	0	0	0	0	9.40	-
B1%	16	0	13	21	0	7.28	5.12
B2%	11	6	14	17	0	6.51	4.82
B3%	15	0	12	18	0	5.97	4.48
B4%	16	6	13	13	0	5.04	4.73
B5%	16	0	0	16	0	4.15	4.15
Pharmaceutical effluent C							
Control	0	0	0	0	0	9.40	-
C1%	16	0	14	14	0	6.85	4.38
C2%	11	0	8	20	0	5.68	3.98
C3%	9	0	9	21	6	5.41	4.50
C4%	16	3	13	19	0	6.03	5.08
C5%	17	0	7	7	0	4.04	3.03

Fig. 1. Chromosome aberration observed in meristematiccells exposed to pharmaceutical effluents × 1000 magnification
(A) Normal metaphase; (A₁) Vagrant cells in metaphase; (A₂) C-mitosis or C-metaphase; (A₃) Binucleated cell in metaphase;
(B) Normal anaphase; (B₁) Anaphase with chromosome bridges; (B₂) Sticky anaphase
(C) Normal telophase; (C₁) Sticky telophase

4. CONCLUSION

The findings from this research is in agreement with earlier studies by Samuel et al, Olorunfemi et al and Olorunfemi and Ehwre who have previously worked on industrial effluents [15,18,21]. It pinpoints pharmaceutical effluents to have adverse cytogenetic effects which when exposed to human and other living organisms can lead to harmful effects on vital organs of the body and may extend to future offspring if not well managed. The discharge of pharmaceutical effluents without appropriate treatments can result in bioaccumulation of toxic substances in the environment, hence, it is strongly suggested, as recommended by Samuel et al, that the onion root growth should be integrated in the Whole Effluent Test (WET) programme by giving a particular EC_{50} that must be met by an industrial effluent before being allowed to be discharged into the environment [21]. *Allium cepa* test in relation to this study has proved to be an effective tool for monitoring the genotoxic effects

of pharmaceutical effluents before they are discharged into the environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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