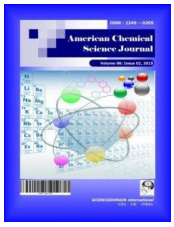

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Cytotoxic and Genotoxic Impacts of Pharmaceutical Effluent from KP Pharmaceutical Industry, Nigeria

A. C. Udebuani[1], A. O. Ofoma[1], O. Otitoju[2*], P. N. Abara[3], T. N. Ezejiofor[1] and M. C. Chukwuma[4]

[1]Department of Biotechnology, Federal University of Technology, PMB 1526, Owerri, Nigeria.
[2]Department of Biochemistry, Federal University Wukari, PMB 1020, Wukari Taraba State, Nigeria.
[3]Department of Biology, Federal University of Technology PMB 1526, Owerri, Nigeria.
[4]Department of Soil Science and Technology, Federal University of Technology, PMB 1526, Owerri, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author ACU designed the study, while author MCC performed the statistical analysis. Author TNE wrote the protocol. Authors ACU and OO wrote the first draft of the manuscript. Author AOO managed the analyses of the study. Author OO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The potential genotoxicity and cytotoxicity of water effluent from a pharmaceutical industry in Ogidi, Anambra State, Nigeria was evaluated using *Allium cepa Linn* assay. This is an alternate first-tier assay to experiments on animals for preliminary toxicity screening in accordance with the council directive 86/609/EEC art-23 that encourages research on alternative techniques to animals' use. The cytotoxic effects were evaluated on the basis of strong growth retardation in high concentrations of the effluent that resulted in root growth inhibition. Data collected were statistically analyzed using ANOVA. Results obtained showed that root growth inhibition was 5.33 to 1.33 while decrease in mitotic index from 68.4 to 52.8 which were statistically significant ($p<0.05$). Genotoxicity based on chromosomal aberrations induced in the onion root tips include sticky chromosomes, bridges, laggard, vagrant, polar deviation and polyploidy. Concentrations of heavy

**Corresponding author: E-mail: otitojuo@fuwukari.edu.ng;*

metals Zn, Cd, Pb, Cr, Ni and Cu, in the contaminated samples were 0.010, 0.006, 0.003, 0.300, 0.018 and 0.020 g/kg respectively. These concentrations far exceeded the EPA recommended limit. Chromium concentration in the effluent was found to be the highest and may be responsible for the observed genotoxic effects on the onion root tip cells. This study therefore, establishes the potential cytotoxic and genotoxic effects of this pharmaceutical waste on the environment.

Keywords: Cytotoxicity; genotoxicity; pharmaceuticals; wastewater; heavy metals; pollution.

1. INTRODUCTION

Contamination of our immediate environment by some emerging pollutants constitutes a major hazard to human health. Pharmaceuticals, commonly referred to as products used by individuals for personal health improvement, cosmetic reasons and to enhance growth and health of livestock, are classified as part of these emerging pollutants [1,2,3]. Despite the numerous benefits of pharmaceutical products, there is a growing concern over the environmental occurrence and effects on both terrestrial and aquatic ecosystem. A wide range of pharmaceutical products are massively produced annually both from industrial settings and in different localities. Many cosmetic products are produced at our back yards and their wastes are discharged within our immediate surroundings. This has made the industrial sector to be one of the major contributors of waste [4,5]. However, Valcarcel et al. [6] reported the various pharmaceutical active compounds (PhACs) found in different environmental compartments. These wastes obviously, are not being generated uniformly due to the varieties of pharmaceuticals produced during any given process. Similarly, low levels (nanogram per liter to micrograms per litre range) have been found in surface waters (rivers, lakes and seas), groundwater, sediments and tap water [6,7,8]. Pharmaceutical active compounds are not always degraded during conventional water treatment process; they are eventually discharged into surface water, which serves as a source of drinking water to some communities and perhaps for other domestic uses [9]. In Nigeria, the scenario is more complicated as pharmaceutical substances are discharged into the environment untreated [10,11]. Additional routes through which pharmaceutical compounds enter into the environment include removal of administered dose from the body. Michael and Christian [12] noted that up to more than 95% of administered dose are eliminated. Many are discharged, as non-metabolized form from urine and feces. These wastes are carried away by wastewater and are discharged into the sewage treated plant [9,13].

The use of wastewater for agricultural purpose has become a common and widespread practice in many countries particularly those facing increase water scarcity [14]. A number of health risks such as cancer, neuro-degeneration and disruption of the endocrine system have been associated with consumption and exposure to pharmaceutical active compounds.

Heavy metals and pharmaceuticals such as endocrine disrupting compounds are considered great threat to living organisms [15]. Heavy metals such as Lead, Mercury, Cadmium, Nickel, Chromium and other toxic organic chemicals or phenolic compounds discharged from pharmaceutical industries are known to affect the surface and ground waters [16]. Due to mutagenic and carcinogenic properties of heavy metals, much attention has been paid to them since they have direct exposures to humans and other organisms. Heavy metals are natural components of the earth crust. These metals enter into living organisms through food or proximity to emission sources. They tend to bio-accumulate and are stored faster than excreted [17,18]. Industrial exposure accounts for a common route of contact in adults and ingestion for children [11,19,20]. There is therefore need to have a better understanding of the toxicological effects of these pollutants in the environment. Therefore, the aim of this study was to evaluate the potential and cytotoxic effects of pharmaceutical effluent on *Allium cepa* root tips.

2. MATERIALS AND METHODS

Pharmaceutical effluent samples were collected from Kingsize Pharmaceuticals (KP) Nigeria Limited in Ogidi, Idemili North Local Government Area of Anambra State in South-eastern Nigeria. Ogidi falls within the lowland areas Southeastern Nigeria [21]. The climate falls under type AW in the Kopper-Geiger classification of wet and dry climate and zone B of the Nigeria's

eco-climatological zone [21]. The area is a rain forest zone with geographical co-ordinates of longitude 6° 9^1 N and latitude 6° 52^1 E. it has an estimated population of 70,000.

2.1 Collection of Pharmaceutical Effluent

Five (5) liters of effluent waste water was collected two times in the month of July and August from KP effluent discharge point. The effluent was stored at 4°C until analyzed for physicochemical properties and was used for the assay.

2.2 *Allium cepa* Assay

Pharmaceutical wastewater samples were aseptically collected from the industrial discharge site. *Allium cepa* bulbs were exposed to graded concentrations of the pharmaceutical wastewater (0.1, 1.0, 10.0 and 20.0%). Onion (*A. cepa)* roots exposed to distilled water served as the control. Minimal statistical guidelines for conducting early seedling growth tests were used in the analysis of measured root length. The EC50 is the effective concentration where root growth amounts to 50% from the plot of percentage root growth of control against the effluent percentage concentrations. After 48 h, one root tip was removed from each bulb, fixed in ethanol: Glacial acetic acid (3:1, v/v) for 24 hours and hydrolyzed with a solution of 1N HCl at 65°C for 3 mins. Two root tips were squashed on each slide and processed for cytological studies by the conventional aceto-carmine technique. Microscopic observation at 100X was done with Nikon Microscope (Model YS 2-H fitted with Nikon Cool Pix 990 Digital Camera – 3.34 megapixels). And 1000X magnifications were used for microscopic studies. After taking some necessary steps, the slides were examined under the microscope to determine the genotoxic effect in terms of mitotic index (MI) and chromosomal aberrations. The data were expressed in terms of mitotic index, number and percentage of cytological aberrations for each sample respectively [22].

2.3 Macroscopic Parameters

After 72 hours growth in the test solution, we measured the root length and noted related parameters as the shape of the roots, number, color [22].

2.4 Laboratory Studies

2.4.1 Analysis of pharmaceutical effluent

The following physicochemical parameters of the pharmaceutical effluent were studied: Dissolved oxygen, biochemical oxygen demand, pH, total dissolved solids, total hardness, turbidity, color and selected heavy metals (these are Ni, Cd, Zn, Cu, Cr and Pb). The dissolved oxygen determination method was used in accordance with standard methods for the determination method of water and wastewater [23]. Same standard procedure was used for the determination of biochemical oxygen demand. A total dissolved solid was determined following the modified procedure of Osaigbovo et al. [24] using electrical conductivity (EC) meter. A calibrated nephelometer whose unit is Nephelometric turbidity Units (NTU) was used to measure the turbidity of the pharmaceutical effluent (EPA Environmental Monitoring Systems Laboratory [5]. The total hardness of the pharmaceutical effluent was determined using the Langelier Saturation Index [25]. To determine the pH of the pharmaceutical effluent, 80 mls of the effluent was poured into a 100 ml glass beaker. Then, the electrode of the pH meter was inserted into the effluent in the beaker and the pH reading was taken. The following heavy metal: Zn, Cd, Pb, Cr Ni and Cu were determined. Samples were analyzed on graphite atomic absorption.

Spectrophotometer (Perkin Elmer model 2380). The atomic absorption spectrophotometer was calibrated for each element using standard solution of known concentration before sample injection [26].

2.4.2 Data presentation and statistical analysis

The results of the root inhibition and chromosome aberrations were presented as means and standard deviation for 5 onion bulbs per concentration. One-way ANOVA was used for testing significance. Statistical significant differences between the control and the different concentrations of the effluent were determined using Turkey post-hoc test at $p<0.05$ degree of freedom. All statistical analysis was carried out using SPSS version 16.0 statistical package.

3. RESULTS

3.1 Root Growth Inhibition

The result of the macroscopic parameter (number of leaves and root length) used in testing for the general toxicity of *Allium cepa* exposed to pharmaceutical effluent are presented in Table 1. The estimated EC_{50} (the concentration of chemical producing 50% of total effect) was 5.6, the effluent concentration at 0-1.0% did not inhibit the growth of roots, however inhibition in root growth was observed at 20% effluent concentration. Generally, strong growth retardation or inhibition (Plate 1) was observed in onion roots growing in high concentration of the pharmaceutical effluent and the effects were less severe at low concentration. It was also observed that the number of root did not reduce at 0-0.1 effluent concentrations. However, the number of roots reduced by 63% at 20% effluent concentration. At 10-20% effluent concentration, different malformations were observed at the root tips of *Allium cepa*. The observed malformations include twists, crotchet, hooks (root tips bent upwards resembling a hook) and swelling of root tips. Signs of wilting appeared on very few of the exposed sample at 20% effluent concentration (Plate 1).

3.2 Microscopic Effects

The microscopic effects of *Allium cepa* exposed to different percentage effluent concentrations are summarized in Table 2. Generally exposure to pharmaceutical effluent concentration lowered significantly the mitotic activity of *Allium cepa* compared to the control. Mitotic index was higher in the control (untreated sample) compared to the exposed. Significant reduction in mitotic index was not observed in effluent concentration at 0 -1.0, however the lowest mitotic index value of 61.8 and 52.8 were recorded for 10 and 20% effluent concentration respectively.

Plate 1. Growth response of *Allium cepa* L. roots exposed to different concentrations of pharmaceutical waste

Table 1. Root length and overall number of *Allium cepa* after cultivation in different concentrations of pharmaceutical effluents

Concentration (%)	Overall number of roots	Mean root length (cm)	RG (%) of control
0.0	100	5.33±0.74	100
0.1	100	3.83±1.09	71.86
1.0	100	3.06±1.20	57.79
10.0	89	2.35±0.80	44.28
20.0	63	1.38± 0.80	26.08

The results obtained also showed significant increase in mitotic inhibitions in relation to increase concentration of pharmaceutical effluent (Table 2) but a decline in mitotic index and increase in mitotic inhibition was generally observed.

The rate of chromosomal aberrations increased as the percentage effluent concentration increases (Table 3). The highest value of percentage aberration was obtained at 20% (21.1%) effluent concentration and the least percentage aberrant cells were obtained at control (0). Different kinds of chromosomal aberration were found in cells of *Allium cepa*. The highest values of all the chromosomal aberration were found in highest effluent concentration (20% concentration of pharmaceutical effluent).

The observed aberrations in Table 3 were stick chromosome at metaphase stage. It was observed that the centromeres were not in the rolls. Stickiness of the chromosome was often observed in this study. While in the control samples, the short and stick chromosomes aligned themselves on the metaphase plate and the chromosomes were clearly arranged in rows (Fig. 1).

Anaphase stage observed in this study showed poor separation of the chromosome in the exposed samples. Vagrants, bridged and fragmented chromosome were also observed in anaphase stage (Fig. 1) at different concentration of pharmaceutical effluents, while in the unexposed samples, the centromere were observed to break normally, releasing the sister chromatids as individual chromosome.

Telophase stage of *Allium cepa* exposed to different concentrations of pharmaceutical effluent showed a multipolar and disoriented chromosome moving slowly to opposite poles while in the unexposed samples (Fig. 1), the chromosome separation was complete and reaching the opposite ends of the poles.

3.3 Physicochemical Parameter of Pharmaceutical Effluent

The physicochemical analysis results (Tables 4 and 5) showed that the pharmaceutical effluent had turbidity index of 51.5 with unpleasant odor compared to the control, which was odorless and colorless. The pH of the effluent was 5.0 compared to the control, which was 7.0, reduction in pH is an indication of slight acidity of the effluent water. Biochemical oxygen demand (5.0 mg/L) was lower than the control. Result obtained also showed that lead, zinc, copper, nickel and cadmium were lower in the pharmaceutical effluent water compared to the control. However chromium was higher in the pharmaceutical effluent water than in the control.

Table 2. Cytological effects of pharmaceutical effluents on *Allium cepa*

Effluent conc. (%)	No of dividing cells	Mitotic index	Mitotic inhibition	Percentage aberrant cells	Mean aberrant cells
0	342	68.4±5.22	0.0	1.5	1±0
0.1	336	67.2±11.23	1.80	5.10	34±1.95
1.0	318	63.6±7.13	7.02	10.38	7±2.35
10.0	509	61.8±4.0	9.62	17.15	10±1.58
20.0	784	52.8±10.81	17.0	21.13	12±1.58

500 cells per concentration of the effluent and the control

Table 3. Mitotic index and chromosome aberrations for the various concentration of pharmaceutical effluent

Effluent conc. (%)	0	0.1	1.0	10	20
Mitotic index	68.4	67.2	63.6	61.8	56.8
Bridge	-	7	63.6	14	13
Stickiness	-	5	10	13	14
Laggard	-	2	12	7	6
Vagrant	-	3	4	9	8
Polar deviation	-	-	4	6	10
Disoriented cells	-	-	3	4	8
Polyploidy	-	-	1	-	1
Total aberrant cells	5	17	33	53	60

Table 4. Physical characteristics of the pharmaceutical effluent

Parameter	Pharmaceutical effluent	Control	FEPA (1991)
Appearance	Turbid	Clear	6-9
Odor	Unpleasant	Odorless	-
Color	Orange	Colorless	-
Turbidity	51.5±2.0 NTU	90±10 NTU	-

Table 5. Chemical characteristics of the pharmaceutical effluent

Parameter	Pharmaceutical effluent	Control	FEPA (1991)
pH	5.0±1.0	7.0±0.04	6-9
DO	3.5±1.0 mg/L	5.2±1.3	2000 mg/L
BOD	1.8 ±1.0 mg/L	5±1.0	50 mg/L
TDS	361±100 mg/L	0.03±0.01	-
Hardness	50±1.5 mg/L	22.0±2.20	-

Legend; DO= Dissolved Oxygen; BOD=Biochemical Oxygen demand; TDS= Total Dissolved Solid

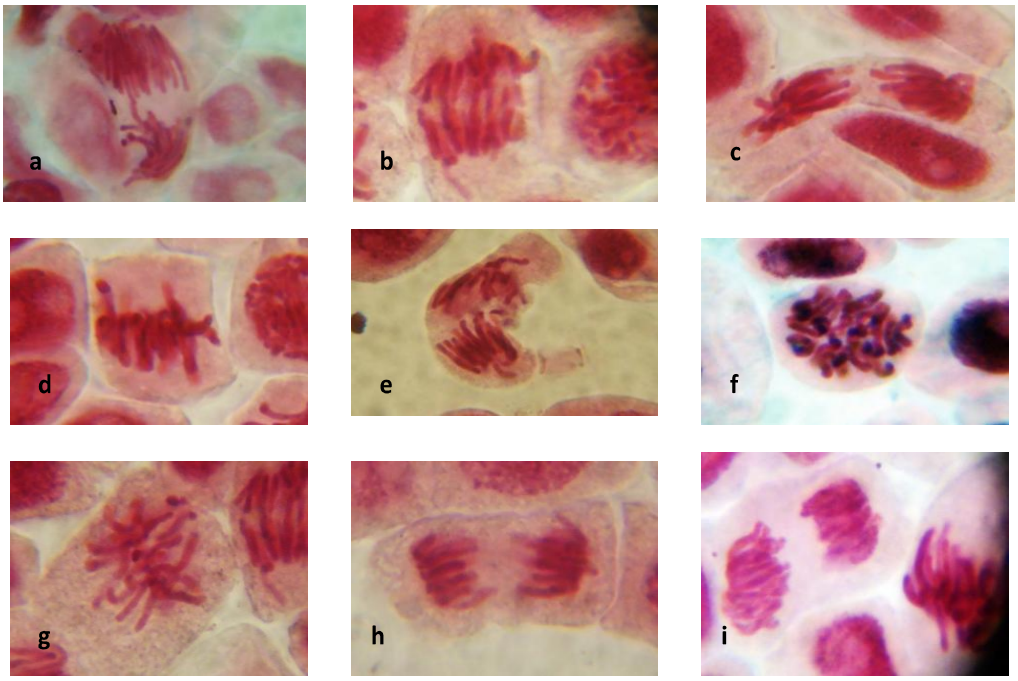

Fig. 1. Some common chromosomal aberration induced by pharmaceutical waste; (a) laggards, (b) anaphase bridged, (c) multipolar bridge, (d) sticky metaphase, (e) vagrant anaphase, (f) C-mitosis, (g) Normal mitotic divisions observed in Allium cepa that is unexposed to pharmaceutical waste effluent, (h) anaphase, (i) telophase {x1000}

Table 6. Concentrations of heavy metals (g/kg) in pharmaceutical effluent water and the control

Heavy metals	Pharmaceutical effluent	Control	FEPA
Nickel	0.010 ± 0.01	0.02±0.001	-
Cadmium	0.006±0.001	0.010±0.01	0.05
Zinc	0.020±0.01	0.100±0.01	0.30
Copper	0.02±0.001	0.10±0.001	0.01
Chromium	0.30±0.1	0.010±0.01	-
Lead	0.003±0.001	0.010±0.01	-

Heavy metal concentration in the effluent shows that Ni, Cd, Zn and Pb were generally low compared with the control and FEPA standard. However, chromium concentration was the highest (0.30 g/kg).

4. DISCUSSION

Exposure to industrial waste may not pose overt toxicity effect in living organisms but its exposure may pose a lot of health risk such as immunosuppression, hormonal fluctuation, teratogenesis etc without causing any death. This is why it is necessary to develop rapid and alternative methods for evaluating their toxicological profile. The growth and development of root of *Allium cepa* was adversely affected by pharmaceutical effluent treatment. The observed effects of pharmaceutical effluents include; reduction in number of roots, stunted growth, with different root malformations (twist, crotchets, hooks swelling at the root tips and wilting). Similar results were obtained after treating *Allium cepa* roots cells with leachates from solid industrial waste [27,28] and they reported that induction of root malformation in the onion bulb is a useful sign of toxicity.

Plants exposed to different kinds of pollutants show depressed growth especially decrease in root elongation and enzyme activities [29,30]. Pharmaceutical effluent must have changed the medium conditions, which imposed stressful conditions on the plant. This study confirmed the fact that *Allium cepa* had induced such physiological stress. The root system of the plant is a complex and important part of the plant, providing the plant water and nutrient, it is also important to note that the depth of the rooting system can have big positive effect on the plant growth.

This study also showed the cytotoxic effect of different concentrations of pharmaceutical effluent on *Allium cepa* to include its effects on the mitotic cycle of the root tips of cell. Decrease in mitotic index and increase in mitotic inhibitions as the concentration of pharmaceutical effluents increased. This showed that the effluent affected the values obtained in mitotic index. Samuel et al. [31] reported that inhibition of mitotic activities is often used to trace cytotoxic substances.

The study also showed effluent induced chromosomal aberrations in the root tip cells of *Allium cepa*. This shows that pharmaceutical waste can cause severe problems to living organisms when not properly treated and disposed into any of the environmental compartment. Studies have reported chromosomal aberration in *Allium cepa* exposed to effluents and they concluded that the observation is an indication of environmental toxicity risk [32,33]. The negative impact of environmental toxicants is its involvement in specific reactions with certain chemical groups in many important sites of the tissues. Such interactions manifested as DNA strand breakage, chromosomal breakage, the chromosome will become fragmented in such a way that the integrity of chromosome is compromised. This study observed chromosomal aberration in all the different stages in mitotic cycle; such aberrations as sticky chromosome at metaphase, bridged and fragmented chromosome at anaphase stage, multipolar and disoriented chromosome, All manifestations of the chromosome suggest presence of cytotoxic and genotoxic substances in the pharmaceutical effluent indicating environmental toxicity risk [31,33]. The chromosomal manifestation can be used as biomarkers for detecting the present of mutagens such as those present in pharmaceutical effluent found in the environment [33].

Physicochemical parameters of the effluent showed the values obtained from the effluent to be beyond the permissible limit set by regulatory agencies [23]. The study showed the heavy metal content of the effluent to be higher in the control compared to the pharmaceutical effluent used in this study. The values of the metals assessed were within the permissible limit [23], however the values obtained for Chromium was higher in pharmaceutical effluent (0.30 g/kg) compared to control (0.10 g/kg). Chromium is considered one of the metals that induce chromosomal aberrations in most organisms [34]. There have been reports of concentration of heavy metals especially chromium in the root and leaves of spinach vegetables irrigated with untreated city effluent [15,35]. However, reports have shown that living cells exert unique mechanisms of repair inhibition due to the cross-linking with some metals [36]. Induction of DNA single strand breaks by inhibiting the activities of enzymes and by formation of radical species such as active oxygen [37]. Pharmaceutical effluents have been known to have toxic genotoxic mutagenic and carcinogenic effects on living things [38]. The interference of the effluent with the mitotic division and the interference of the effluent with the mitotic spindle proved the

toxic nature of the pharmaceutical effluent. These results are consistent with the earlier results of Akintowa et al. [39] who worked on the assessment of mutagenicity of some pharmaceutical effluents using *Allium cepa* as one of the test materials.

5. CONCLUSION

In this study, the *Allium cepa* roots consistently showed symptom of both genotoxicity and cytotoxicity as a result of influence of pharmaceutical effluent. Thus, this finding clearly elaborates the negative consequences of pharmaceutical effluents on both the terrestrial and possibly aquatic environment therefore through food chain human and animals are at risk too.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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