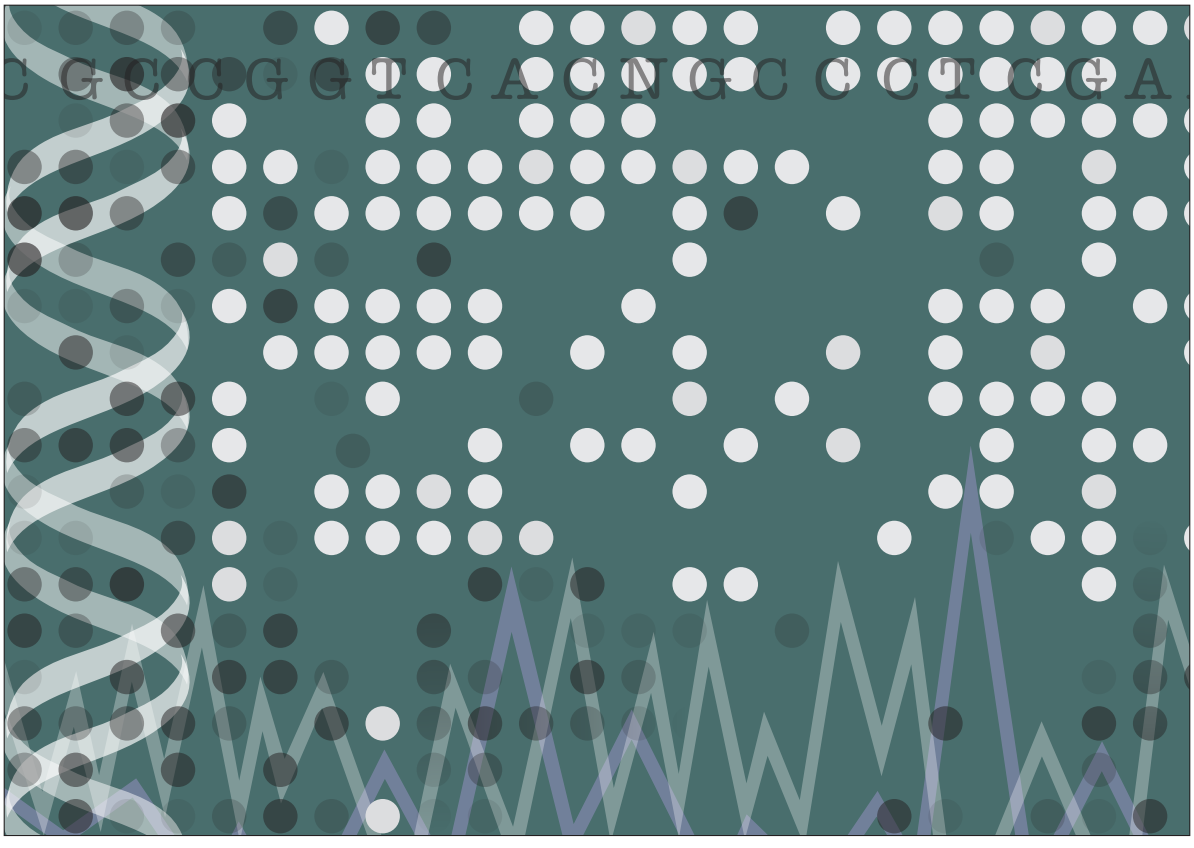

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Cytotoxic and Genotoxic Effects of Cassava Effluents using the *Allium cepa* Assay

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ABSTRACT

Agricultural wastes generated from pilled cassava tubers contribute significantly to pollution of the environment. In this study, we investigated the potential genotoxic effects of the effluents obtained from the processing of cassava tubers into three popular Nigerian cassava meals; *garri*, *lafun* and *akpu* using the modified *Allium cepa* assay. A series of 10 onion bulbs were cultivated in 0.001, 0.01, 0.1, 1.0 and 10% concentrations (effluents, v/v) of each of the test samples (*garri*, *lafun* and *akpu*). At 48 h, root tips from the treated bulbs were processed for cytological studies by the acetocarmine or orcein-orcein squash technique. At 72 h, their cytotoxic effects on the onion root tips showed strong growth retardation in high concentrations of all the effluents with EC_{50} values of 1.5, 2.5 and 3.5% for *garri*, *lafun* and *akpu* effluents, respectively while total phytotoxic effects was induced by the undiluted effluents. The physico-chemical properties of the effluents revealed the presence of significant amounts of cyanide and heavy metals. Root length inhibition, breakages and malformations were characterized by the presence of crochet hooks and c-tumors at low effluent concentrations (10^{-1}, 10^{-2} and 10^{-3}%). There was a rapid decrease in mitotic index with increasing effluent concentration. Effluents-induced chromosome aberrations in the root tip cells were statistically significant ($p<0.05$). The present findings indicate that the substances contained in the cassava effluents may be toxic to living organisms and may pollute the environment.

Key words: Chromosomal aberration, processed cassava effluents, mitotic index, cyanide, *Allium cepa*

INTRODUCTION

The Food and Agricultural Organisation of the United Nations has estimated cassava production in Nigeria to be approximately thirty-four million metric tones (FAO, 2004). Cassava has a 'cyanogenic potential' (meaning that, though not there, cyanide (hydrocyanic acid-HCN) is produced through enzymatic processes when the plant cells are bruised, grated or bitten) and thus needs to be processed so as to increase shelf life and to remove cyanide (Lancaster *et al*., 1982; Nweke and Bokanga, 1994; Osiru *et al*., 1995). Health effects due to cyanide exposure arise only from insufficient processing of the bitter varieties (Wheatley *et al*., 1984; Padmaja, 1995) and one of the most common practices employed in the processing is fermentation. About 75% of the

harvested roots in Africa are processed to fermented products (Westby, 1991), leading to improvements in shelf life, taste and flavour. The three most common fermented cassava products in Nigeria are *garri*, *fufu* and *lafun* (Oyewole and Odunfa, 1988; Westby and Twiddy, 1992; Bokanga, 2001). While *garri* involves fermentation of grated roots, *fufu* and *lafun* on the other hand involves fermentation of soaked roots (Okafor *et al*., 1998).

Cassava processing is generally considered to contribute significantly to environmental pollution and environmental nuisance (Ubalua, 2007) because the effluents produced during and after processing are usually discharged indiscriminately into the environment, particularly on farmland (Ogboghodo *et al*., 2001, 2006). Indiscriminate discharge of untreated or partially treated wastewaters directly or indirectly into aquatic bodies may render water resources unwholesome and hazardous to man and other living systems (Bakare *et al*., 2003, 2009; Fawole *et al*., 2008; Kumar, 2008).

We are not aware of any report on the genotoxicity of effluents obtained from cassava processing using the *Allium cepa* assay. Such studies would be of importance in determining the genetic basis of root damage and growth inhibition in exposed plant communities, as well as provide baseline data that are vital for the formulation of guidelines for pollution control with regard to discharge of cassava wastewaters into the environment. In this study, we investigated the cytotoxic and genotoxic effects of effluents from cassava processing mills using the modified *Allium cepa* assay.

MATERIALS AND METHODS

Test material: The biological materials were equal-sized onion (*Allium cepa* -2n = 16) bulbs of the purple variety (average size 15-22 mm diameter) purchased locally in Benin City, Edo State in Nigeria (latitudes 6° 06'N, 6° 30' N and longitudes 5° 30' E, 5° 45' E). They were sun-dried for 6 weeks before use. This study was conducted from 2006 to 2008.

Test effluent: Fresh effluents from the processing of cassava into *garri*, *lafun* and *akpu* were obtained from small-scale cassava processing mills in Uselu Quarters, Benin City (6°15' N and 5°25' E). The effluents were scooped from ten different positions into 10 L plastic containers from moulds in the mills in order to form a homogenous mixture, where grated cassava packed into sacs are kept before putting them into the manual pressing machines. The effluents obtained from the processing of the cassava roots were collected at three different times (morning, afternoon and evening) and stored at 4°C until analysed for physico-chemical properties and used for the assay.

Analysis of effluents for physico-chemical parameters: The effluents were analyzed for a number of standard physico-chemical properties, including Chemical Oxygen Demand (COD), Total Dissolved Solids (TDS), alkalinity, Biochemical Oxygen Demand (BOD), chlorides, nitrates, ammonia and phosphates, according to methods described by APHA/AWWA/WEF (1998). Nine metals (including eight heavy metals) namely aluminum (Al), cadmium (Cd), copper (Cu), chromium (Cr), iron (Fe), mercury (Hg), zinc (Zn), nickel (Ni) and manganese (Mn) were analyzed in the effluents sample according to standard analytical methods (USEPA, 1996; APHA/AWWA/WEF, 1998). Briefly, 100 mL of the effluents were digested by heating with concentrated HNO_3 and the volume reduced to 3-5 mL. This volume was made up to 10 mL with 0.1 N HNO_3. Concentrations of the metals were estimated by using an Atomic Absorption Spectrophotometer.

***Allium cepa* assay:** The *Allium* test for macroscopic as well as microscopic evaluations was as described by Fiskesjö (1997) and Bakare and Wale-Adeyemo (2004). The outer scales of the onion bulbs and brownish bottom plate were removed, leaving the ring of root primordia intact. The peeled bulbs were put into fresh tap water during the cleaning procedure to protect the primordia from drying. Thereafter, the bulbs were exposed directly in 0, 0.001, 0.01, 0.1, 1.0, 10 and 20% concentrations (v/v, effluent/tap water), of each of the test sample (*garri*, *lafun* and *akpu* effluents). Twelve onion bulbs were set up in each series for each sample, out of which the best ten with good root growth were selected for analysis of root growth inhibition. Distilled water was used as negative control.The experiment was set up in the dark at 25±1°C for 72 h. Test liquids were changed daily. Photographs of test materials were taken with *Nikon* Digital Camera D80 (Nikon Corp., Japan) and special note was taken of change of colour of root tip and morphological changes. ASTM (1994) minimal statistical guidelines for conducting early seedling growth tests were used in the analysis of measured root length. The EC_{50} the effective concentration where root growth amounts to 50% of the controls, (100%) was interpolated for each test sample from the plot of root lengths against the log of effluent concentrations. After 48 h, one root tip was removed from each bulb, fixed in ethanol:glacial acetic acid (3:1, v/v) and hydrolysed with a solution of 1 N HCl at 65°C for 3 min. Two root tips were squashed on each slide and processed for cytological studies by the conventional acetocarmine technique. Microscopic observation at 100X was done with *Nikon* Microscope (Model YS 2-H fitted with Nikon CoolPix 990 Digital Camera -3.34 megapixels) and 1000x magnification was used for microscopic studies. The mitotic index was calculated as:

$$\frac{\text{No. of dividing cells}}{\text{Total No. of cells}} \times 100$$

Statistical analysis: The results of the root inhibition and chromosome aberrations are presented as Mean±SE for 10 onion bulbs per concentration and One-Way ANOVA was used for testing significance. Statistical significant differences between control and the different concentrations of the effluents were determined using Tukey post-hoc test. All statistical analyses were carried out using SPSS®14.0 statistical package.

RESULTS AND DISCUSSION

Physico-chemical characteristics of the cassava effluents: The physical and chemical characteristics of the effluents and tap water used in this study are shown in Table 1. Potassium and sodium were present in detectable amounts. The concentration of K was 98.50, 29.70 and 75.20 mg in *garri*, *akpu* and *lafun* effluents, respectively.

The concentration profile of Al, Mg, Ca, Mn, Cd, Cr, Hg, Cu, Pb, Fe and nitrate was not in any definite increasing/decreasing order, however, the concentrations of heavy metals were relatively high. For example in *garri* effluent, the concentration of Cd, Fe, Zn and Ni were 0.11, 3.90, 5.90 and 4.40 mg, respectively. The effluents were turbid with offensive odour and highly acidic with pH values of 3.96, 4.11 and 4.61 for *garri*, *akpu* and *lafun* effluents, respectively. The characteristics of the tap water showed that it was of good quality with a pH of 7.22 and considerable amount of calcium and magnesium without any toxic ions.

Table 1: Physical and chemical characteristics of the cassava effluents assessed for genotoxicity

Parameters*±	*Garri*	*Lafun*	*Akpu*	Tap water	FEPA[a]	USEPA[b]
pH	3.96±0.08	4.11±0.06	4.61±0.08	7.22±0.04	6-9	6.5-8.5
Appearance	Turbid	Turbid	Turbid	Colourless	-	-
Odour	Unpleasant	Unpleasant	Unpleasant	Odourless	-	-
Hardness	75.00±3.45	55.00±2.98	47.00±4.22	22.00±2.20	-	0-75
BOD	6.20±1.70	4.80±1.04	3.40±0.92	1.80±0.40	50	-
COD	10.10±4.20	7.30±3.20	6.40±1.12	1.02±0.04	-	-
TDS	6.00±0.20	4.00±0.18	4.00±0.20	0.03±0.01	2000	500
Salinity	28.40±3.02	14.20±4.01	15.40±2.12	1.40±0.04	-	-
Total alkalinity	1.70±0.03	1.45±0.01	1.40±0.01	1.85±0.02	250	20
Total solids	5,600±120.50	4,630±98.80	4,780±68.34	20.03±5.35	-	-
Sulphates	78.00±8.20	55.00±10.40	57.00±9.56	25.00±7.44	20	250
Nitrates	97.00±4.60	40.00±6.40	68.00±6.80	0.53±0.02	-	10
Chlorides	516.30±44.20	149.10±32.70	113.60±24.76	13.60±4.40	-	250
Sodium	120.50±12.40	30.50±8.20	86.90±8.40	12.30±3.40	-	-
Calcium	94.30±6.30	55.90±4.20	81.10±5.60	62.50±7.20	-	-
Aluminium	71.50±4.60	69.00±4.50	80.00±6.00	70.00±5.00	-	-
Potassium	98.50±4.14	29.70±6.30	75.20±4.80	40.90±6.20	-	-
Magnesium	110.90±9.43	100.50±10.25	90.50±9.60	8.10±0.04	-	-
Lead	1.82±0.20	0.21±0.04	0.10±0.01	0.01±0.01	0.01	0.015
Iron	3.90±0.90	0.70±0.02	0.20±0.01	0.20±0.01	0.05	0.30
Copper	2.60±0.30	1.10±0.04	1.00±0.02	0.10±0.01	0.30	1.00
Manganese	2.10±0.04	1.10±0.02	1.00±0.02	0.01±0.01	0.05	0.05
Cadmium	0.11±0.02	0.04±0.02	0.04±0.01	0.01±0.01	0.05	0.05
Mercury	1.05±0.02	1.00±0.02	0.95±0.02	0.01±0.01	-	-
Chromium	0.14±0.02	0.09±0.02	0.06±0.01	0.01±0.01	-	-
Zinc	5.90±0.62	1.10±0.04	1.10±0.02	0.50±0.01	-	-
Silver	8.20±0.44	2.40±0.08	2.60±0.06	0.02±0.01	-	-
Nickel	4.40±0.78	4.00±0.04	3.60±0.04	0.02±0.01	-	-
Cyanide	685±14.50	346±20.80	159±18.20	0.00±0.00	-	-

Values are means of 3 replicates±SEM. *All values are in mg L^{-1} except pH with no unit. Cyanogenic potential is expressed as µg HCl mL^{-1} (ppm). COD: Chemical oxygen demand, BOD: Biochemical oxygen demand, TDS: Total dissolved solids, Federal Environmental Protection Agency (FEPA, 1991) Permissible limits for drinking water

Macroscopic effects: Table 2 shows root lengths of the *Allium* roots exposed to different effluents, this is a concentration dependent decrease in the root lengths of the various effluents. Effluent concentrations at 0, 0.001, 0.01, 0.1, 1 and 10% did not inhibit the growth of roots, however, 100% inhibition in root growth was observed at 20% effluent concentration. Strong growth retardation was observed in onion roots growing at high concentrations of all the effluents, the effects were less severe at low concentrations. For instance, mean root length of 3.20 cm was obtained for onion bulb grown in 10^{-3}% *garri* effluent while at 1% the root length was 0.28 cm. Similar trends were observed with *akpu* and *lafun* effluents. The root growth inhibition is concentration-dependent with EC_{50} values of 1.5, 2.5 and 3.5% for *garri*, *akpu* and *lafun* effluents, respectively. Restricted root growth was observed in all effluents however, there was a decreasing order of root growth inhibition in the order *garri>akpu>lafun* effluents.

Exposure of *Allium* roots to the effluents at 0, 0.001 and 0.01% did not cause any change in colour of roots, however, at higher concentrations of 1 and 10%, roots were pale and at 20%, the roots were dark brown/black in color. The root malformations observed at these effluent

Table 2: Root length of *Allium cepa* after cultivation in different concentrations of cassava effluents

	Garri			*Akpu*			*Lafun*		
Conc. (%)	Mean root length±SE	RG (%) of control	95% CL	Mean root length±SE	RG (%) of control	95% CL	Mean root length±SE	RG (%) of control	95% CL
0	4.90±0.14	-	0.38	4.90±0.14	-	0.38	4.90±0.14	-	0.38
0.001	3.20±0.46	65.3	0.44	4.05±0.26	90.2	0.90	4.21±0.26	89.2	0.55
0.01	2.20±0.10	46.5	0.61	3.61±0.30	70.4	0.60	3.81±0.30	78.4	0.66
0.1	0.76±0.14[a]	46.4	0.60	1.23±0.14[a]	36.5	0.54	2.53±0.14[a]	68.4	0.54
1	0.28±0.04[a]	35.4	0.64	0.87±0.04[a]	64.4	0.65	1.09±0.04[a]	72.5	0.65
10	0.27±0.07[a]	18.4	0.42	0.76±0.07[a]	60.3	0.75	0.98±0.07[a]	81.2	0.48
20	Not measurable	-	-	Not measurable	-	-	Not measurable	-	-
EC_{50}		0.015			0.025			0.035	

RG (%) of control expressed as % root growth of the control. 95%CL: 95% confidence limit. $p<0.05$, level of significance of root growth inhibition compared with the untreated control. [a]p- values, (>0.05) level as compared to controls, NS: Non significant, Values are Mean±SEM

Table 3: Cytological effects of effluents of *garri, akpu* and *lafun* on cells of *Allium cepa*

	Garri			*Akpu*			*Lafun*		
*Conc. (%)	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells
0	565	14.13	-	565	14.13	-	565	14.13	-
0.001	261	6.53	5.4	347	8.68	6.9	347	8.68	6.9
0.01	252	6.30	3.6	293	7.33	5.8	293	7.33	5.8
0.1	190	4.75	2.6	276	6.90	6.2	276	6.90	6.2
1	142	3.55	9.9	205	5.13	11.7	205	5.13	11.7
10	71	1.78	8.5	198	4.95	9.1	198	4.95	9.1

*5000 cells per conc. of each effluent and the control

concentrations were twists, 'crotchet hooks' (root tips bent upwards resembling hooks) and c-tumors (abnormalities appearing as swellings of the root tips) (Fig. 1a, b).

Microscopic effects: The effect of the effluents on cell division and chromosome behaviour of *Allium cepa* are shown in Table 3. There was no chromosomal aberration in the control which had a Mitotic Index (MI) value of 14.13. Chromosomal aberrations were induced at all concentrations of the effluents and were statistically significant ($p<0.05$). With increasing concentration of the effluents however, there was concentration dependent decrease in the mitotic index, for instance, the MI at 1% effluent concentration was 3.55, 5.13 and 5.36 for *garri*, *akpu* and *lafun* effluents respectively compared to the negative control value of 14.13%, in all concentrations. The types of chromosomal aberrations induced by the effluents include multiple bridges and fragments, criss-cross at anaphase, polar deviations and disturbances of the mitotic spindle (Fig. 2a-f) at various concentrations.

The cytotoxic and genotoxic effects of *garri*, *akpu* and *lafun* effluents were evaluated using *Allium cepa* test. The severity of these effects was highest in *garri* followed by *akpu* and *lafun* in decreasing order. These observed effects may be due to the constituents of the effluents which were highest in *garri*. The characteristics of the constituents of the effluents reveal that they were

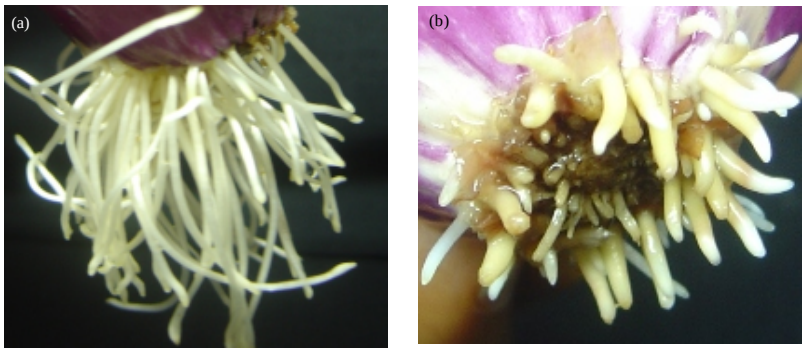

Fig. 1: (a, b) Macroscopic effects on Allium roots exposed to cassava effluents. (a) Crochet roots (1% *garri* effluent and (b) C-tumor roots (10% *garri* effluent)

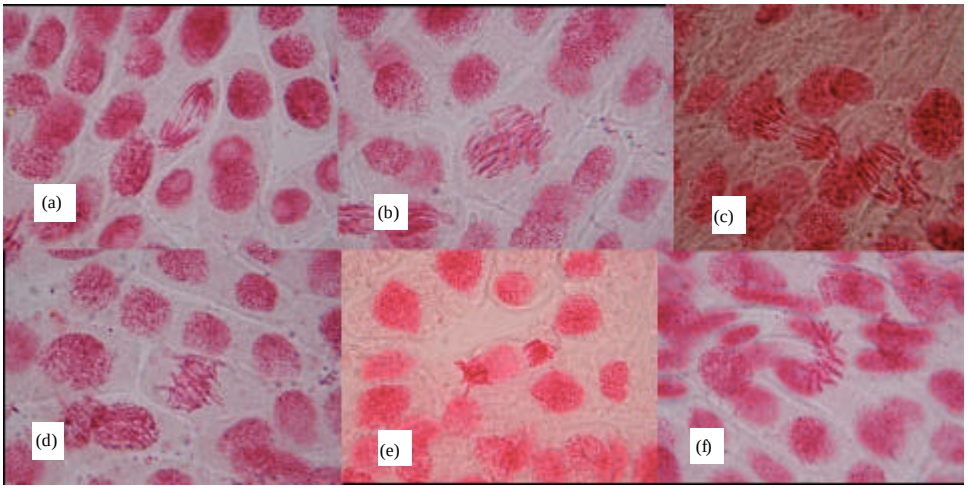

Fig. 2: (a-f) Chromosomal aberrations observed in root tip cells of *Allium cepa* exposed to cassava effluents. (a) Anaphase bridge, (b) multiple bridges, (c) anaphase bridge and fragments, (d) criss-cross at anaphase, (e) telophase with vagrant chromosome and (f) polar deviation

complex and the effluents were also highly acidic even at low concentrations. Compared to the allowable limits (FEPA, 1991; USEPA, 1988; UNESCO/WHO/UNEP, 1992) most of the parameters analyzed, especially the heavy metals were present in high concentrations. Ubalua (2007) stated that the claim that cassava wastewater can cause problems in some crops is based on anecdotal information. Macroscopic and microscopic results obtained from the *Allium* test in this study with the *garri*, *lafun* and *akpu* effluents clearly show that they are cytotoxic. The presence of detectable amounts of essential elements such as K and Ca necessary for plant growth in the effluents notwithstanding, this study provides factual evidence of inhibitory effects of cassava wastewaters on plants. The effluents induced root malformations which other workers (Fiskesjö, 1988; Odeigah *et al.*, 1997; Seetharaman *et al.*, 2004; Babatunde and Bakare, 2006; Bakare *et al.*, 2009) have shown to be useful signs of toxicity.

Ivanova *et al.* (2002) and Staykova *et al.* (2005) have established the genotoxic and mutagenic effects of open water contaminated with heavy metals and cyanide, further confirming the results

of the inhibitory effects of these effluents on seed germination and growth in earlier studies (Olorunfemi *et al.* 2007, 2008). The results from this study suggest that anomalies in cell division process and chromosome aberration induction in the *Allium cepa* root meristem could be as a result of heavy metals-cyanide interaction in the cassava waste waters. In a related study conducted to assess the haematological and histopathological effects of cassava effluent on adult female African catfish, *Clarias gariepinus*, the fish was found to show signs of gill and liver damage (Adeyemo, 2005). Similarly, histopathological examination of the kidney, gill and liver of the fingerlings of the Nile Tilapia, *Oreochromis niloticus* treated with cassava effluent indicated damage (Wade *et al.*, 2002). The genotoxic effects of the cassava effluents established in this study indicates that the effluents contain toxic substances which may constitute a risk to the environment and human health, more especially as the waste generated from cassava processing is not properly managed.

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