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Cytotoxicity and Genotoxicity of Three Major Commercially Available Energy Drinks in Nigeria Using the Allium Cepa Assay

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Abstract

Many studies have been conducted for the evaluation of the toxicity of various complex mixtures, however, there is limited research on the cytogenotoxic effect of energy drinks despite its worldwide consumption. This study aimed at investigating the possible cytotoxicity through root growth inhibition and genetic damage through induced chromosomal aberrations in Allium cepa by three major energy drinks in Nigeria. Onion bulbs were grown at 1.57, 3.13 and 6.25 % concentrations of each of the three energy drinks with 10 ppm lead nitrate and tap water as the positive and negative controls, respectively. At 48 and 72 hours, respectively, genotoxic and root growth inhibition analyses were carried out. Concentration-dependent inhibition of root growth and cell division was observed in the exposed onions which were significant ($p < 0.05$) in comparison with the negative control. Additionally, significant chromosomal abnormalities like spindle disturbances, sticky chromosomes, lagging chromosomes, fragmented chromosomes and binucleated cells were observed in the exposed onion roots. Onions grown at concentrations above the maximum concentration used in this study did not grow at all. These findings suggest that energy drinks contain compounds able to induce cytotoxicity and genotoxicity in somatic cells. This is significant for public health in nations where people rely on energy drinks for energy boost for their day-to-day activities.

Keywords: *Allium Cepa Assay, Cytotoxicity, DNA Damage, Energy Drinks, Genotoxicity.*

INTRODUCTION

Humans need extra energy surge for extra work, however there are many additional energy sources besides food and water. For best health, especially in youth, choosing the right supplements, timing of intake, and supplementation are crucial. Energy drinks are non-alcoholic beverages that are marketed as providing an extra boost of energy for daily tasks. These are carbonated drinks that are high in sugar and caffeine, along with mixes of unusual herbal extracts, B vitamins, and amino acids, designed to provide short-term energy boosts and mental clarity (Alford, 2001). Energy drinks provide individuals aged 18 to 55 with energizing benefits as compared to a placebo. These effects peaked 30 to 60 minutes after consumption and lasted for at least 90 minutes (Smit *et al.,* 2004).

Although energy drinks first became prominent in Europe and Asia in the 1960s, the more recent trend of aggressively promoting energy drinks with high caffeine content was spurred when Red Bull was launched in 1987 in Austria and 1997 in the United States. The industry for energy drinks has expanded rapidly since its start, and in 2006, about 500 new brands were introduced globally (Johnson, 2006). There are several reasons why there is increase in the consumption of energy drinks in recent years, a major reason is the aggressiveness of the beverage companies in the promotion and marketing of this type of drinks by primarily targeting adolescents and young adults (Wolk et al., 2012). The promotion and marketing most times focus on the association of energy drinks with high-energy activities and extreme sports, as well as their stimulating and energizing effects. Secondly, there is a belief that energy drinks are capable of improving physical and cognitive performance. The main goal of many energy drink consumers is to improve their concentration and focus, and boost their energy levels, however, while the consumers might enjoy these short-term benefits, the potential long-term health effects have not been well studied (Gutierrez-Hellin and Varillas-Delgado, 2021). Finally, increased consumption of energy drinks has been linked with their increasing availability in retail outlets such as the petrol stations and grocery stores. The promotion of energy drinks as portable and convenient source of stimulation and energy has made them popular option for people with busy lifestyles or those who regularly travel. However, easy access to energy drinks also implies the possibility of their excess consumption thereby increasing the risk of their detrimental effects.

Stimulants such as L-carnitine, taurine, and guarana present in energy drinks can increase energy, attention, alertness, as well as increase breathing, heart rate, and blood pressure (Seifert et al., 2011), and have been shown to exert cytotoxic effects on human neuronal cells and other cell types (Wolk et al., 2012). Energy drinks have been linked to adverse health effects, particularly in children, teenagers, and young

adults such as impaired driving, harm to adolescent users, increased risk-taking behaviors, and other negative side effects (Mateo-Fernández et al., 2021). The amount and dosage of caffeine in different energy drink brands varies significantly. Although single doses of energy drinks often do not contain enough caffeine to cause serious side effects, the usage of these drinks has been linked to at least one death (Reissig *et al*., 2009). There is a lot of worry about caffeine toxicity and poisoning when using energy drinks. In young children, caffeine dosages of 78 mg/kg have been shown to have significant negative effects. In healthy adults, the lethal dose of caffeine has been reported to be 5–10 g, however, in people with underlying medical issues, such as cardiac or seizure disorders, this amount may be lower (Babu *et al*., 2008).

The Food and Drug Administration (FDA) reopened its investigation into energy drinks, a caffeine-containing product, in 2012 due to safety concerns. Numerous varieties of these products, caffeinated drinks in particular, have been connected to unanticipated deaths in otherwise healthy individuals, prompting calls for increased monitoring and potential regulation (USFDA, 2012). The FDA deemed drinks with both alcohol and caffeine to be dangerous in 2010 due to the caffeine's ability to overshadow some important sensory cues required by individuals in making informed decision about their intoxication level (USFDA, 2010). Despite the increased demand for energy drinks and surge in the production of this type of drink worldwide, limited information exist on the cytotoxicity and the possible genetic danger of consuming it, hence, the need for this study. Therefore this study investigated the possible cytotoxicity through root growth inhibition and genetic damage through induced chromosomal aberrations in *Allium cepa* by three major energy drinks commercially available in Nigeria.

MATERIALS AND METHODS

Collection of Sample

Three major commercially available energy drinks labeled sample A, B and C for ethical reasons were obtained from Akure, Ondo State, Nigeria. They came in plastic containers and can of 400 mL, 440 mL, and 500 mL, respectively, and were kept at room temperature throughout the period of the study. Table 1 contains information of the constituents of each of the energy drink as given by the producers.

Table 1. Constituents of the energy drink used in this study as given by the producers.

Energy Drink Samples	Constituents as stated by the producers
A	Preservatives (Sodium Benzoate E 211 and Potassium Sorbate E 202), Vitamin B12, Ginseng Extract, Vitamin B6, Carbon dioxide, Flavouring (Taurine, Inositol, Caffeine, Acidity Regulator Sodium Citrates E 331, and Niacin), citric acid E 330, Sugar, Colours (Sunset Yellow FCF E110 and Tartrazine E 102), and water.
B	Carbonated Water, Sugar, Caffeine (0.03%), Maltodextrin, Citric Acid E330, Sodium Citrates E331 (Acidity Regulator), Taurine (0.1%), Preservatives (Sodium Benzoate E211 and Potassium Sorbate E202), Non-Nutritive Sweetener (Sucralose E955), Salt, Nicotinamide (83), Colourant (Tartrazine E102), Inositol (0.001%), Flavourings, and Vitamin B6.
C	Cyanocobalamin, Caffeine, Maltodextrin, Citric Acid, Riboflavin, Taurine, Pyridoxine Hydrochloride, Sodium Citrate, Sucralose, Panax Ginseng Root Extract, L-Tartrate, Sorbic Acid, Benzoic Acid, Niacinamide, Sodium Chloride, Glycine Max Glucuronolactone, Inositol, Guarana Seed Extract, L-Carnitine, Color Added, Natural Flavors, Glucose, Sucrose, and carbonated water.

Biological Sample

Healthy onions (*Allium cepa*) with no obvious infection were purchased at Shasha Market, Akure, Ondo State, Nigeria. Twice the number of onions needed for the experiment was purchased in order to cover for loss due to mould infestation, decay/rottening, theft, or rodent attack. The onions were transported to the premise of Federal University of Technology, Akure and were sun dried for two weeks in a well-aerated environment.

Allium Cepa Assay

After carefully removing the primordial scales, the onion bulbs were washed with tap water for cleaning and to prevent the drying of the primordial roots, and air-dried. Then, the onion bulbs were chosen to fit on top of each beaker. Three concentrations each: 1.57, 3.13 and 6.25 % (v/v energy drink/tap water) of the three energy drinks, 10ppm lead nitrate (positive control) and tap water (negative control) were added to their corresponding beakers. The two concentrations of the energy drinks were purposefully chosen after initial study that showed no root growth in concentrations above 6.25% of the three energy drinks. Ensuring that the concentrations were only touching the onion bulb's base, the onion bulbs were placed on each beaker containing the different concentrations and put inside a dark cabinet. Each set up had ten replicates. After 24 hours, the onion bulbs were examined to gauge growth and determine whether topping with corresponding samples was necessary.

Evaluation of Chromosomal Aberration and Mitotic Inhibition

Two onions with good growth were harvested after 48

hours to check for chromosomal aberration per group. The root tips of the two onion bulbs within 0.5-1.0cm were cut into a beaker containing 1:3 of glacial acetic acid/ethanol to fix them. The beakers containing the root tips were then refrigerated at 4°C for 24 hours. After being fixed for 24 hours, the fixative was decanted and 1N HCl was added to the root tips. The roots were then hydrolysed at 60°C for 5 minutes before been washed 3-4 times with distilled water, squashed on labelled slides and stained for 10 minutes with acetocarmine (1%). Filter paper was used to remove excess stain on the slides before cover slips were carefully lowered on slides excluding air bubbles, and then sealed with nail polish. In each concentration, six slides were prepared and 1000 cells were counted in each slide to investigate the number and frequency of all the cell cycle phases. The total mitotic cells in the controls (negative and positive) and each concentration of the samples were counted, and the mitotic index (MI) calculated thus:

$$Mitotic\ Index = \frac{Number\ of\ Dividing\ cells}{Total\ number\ of\ cells\ counted}$$

Fifty dividing cells per concentration was further analysed for chromosome aberrations and the calculation of the percentage aberrations in relation to the total mitotic cells was recorded (Alabi et al., 2022).

Assessment of Root Growth Inhibition

After 72 hours of planting the onion bulbs, length of each root from the remaining eight onions grown on the various concentrations, including the positive and the control, were harvested, measured, and recorded. Also, examination of the effect of the energy drinks on root morphology was carried out.

Statistical Analysis

The data obtained were all analyzed with SPSS 20.0 package and expressed as mean ± standard error of mean of the different concentrations. The difference between each concentration and the negative control was analyzed at the probability level of 0.05.

RESULTS

Evaluation of Chromosomal Aberration and Mitotic Inhibition

The effects of the different energy drinks on cell division in *A. cepa* are shown in Table 2. The different concentrations of energy drinks altered cell division in exposed onion cells. The total dividing cells in the negative control was 995 with a MI of 16.58%. The MI significantly ($p<0.05$) decreased as concentration increased from 1.57 to 6.25% in all the samples tested as against the negative control. Values recorded for the MI of the different concentrations of the three energy drinks ranged from 3.48-4.45% with sample B having the lowest MI.

Table 2. Mitotic division and the mean±SE of chromosome aberrations in *Allium cepa* grown on three different energy drinks.

Concentrations	Total cells counted	Total dividing cells	Percentage Mitotic index	Total aberrant cells	Frequency of aberrant cells
Negative	6000	995	16.58	05	0.50
A 1.57%	6000	267	4.45*	29	10.86*
3.13%	6000	230*	3.83*	35	15.22*
6.25%	6000	220*	3.66*	42	19.09*
B 1.57%	6000	229*	3.83*	37	16.16*
3.13%	6000	213*	3.55*	46	21.60*
6.25%	6000	209*	3.48*	58	27.75*
C 1.57%	6000	230*	3.83*	32	13.91*
3.13%	6000	224*	3.73*	39	17.41*
6.25%	6000	212*	3.53*	46	21.70*
Positive	6000	406*	6.77*	115	28.32*

Values with * are statistically ($p \leq 0.05$) different as against the negative control.

The total number of chromosome aberrations induced by the samples and their frequencies of occurrence are shown in Table 2. The negative control had a total of 5 aberrations with a percentage frequency of 0.50. All the concentrations of the three energy drinks induced significantly ($p<0.05$) increased chromosome aberrations in the exposed onion bulbs compared to the negative control group. Frequencies of the various aberrations was from 10.86% in A1.57% to 27.75% in B6.25%. Generally, the highest concentration induced the highest chromosome aberration. The energy drinks induced chromosomal abnormalities like spindle disturbances, sticky chromosomes, lagging chromosomes, fragmented chromosome, and binucleated cells (Figure 1).

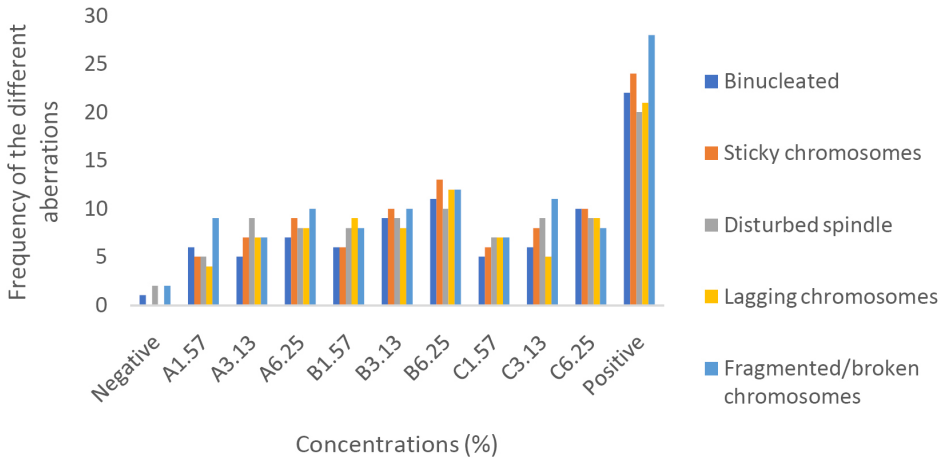

Figure 1. The frequency of the different chromosome aberrations observed in *Allium cepa* root tips grown on three energy drinks at different concentrations.

Analysis of Root Growth Inhibition

Onions grown on the negative control had good growth. Short, scanty and twisting growths were observed in the root tips of onions grown on the energy drinks at the different concentrations (Figure 2). Figure 3 shows the percentage inhibition of the root growth of onions from different concentrations of the three energy drinks as against the negative control. Compared to the negative control, the inhibition of the root growth observed in all the concentrations was significantly ($p<0.05$) higher with sample B inducing the highest root growth inhibition and sample C the least. The inhibition of the root growth in the onions was concentration dependent, with the highest concentration inducing the highest root growth inhibitions in the three energy drinks.

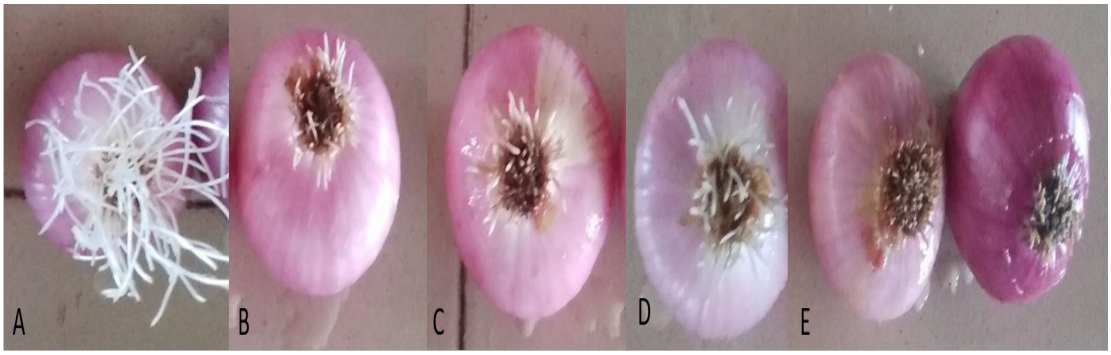

Figure 2. Representative macroscopic effects observed in *Allium cepa* grown on three energy drinks. (**A**) Control onion with normal root growth, (**B**) roots were scanty and short; and rottenness at the basal plate in sample A, (**C**) roots were scanty and short in sample B, (**D**) short and scanty roots in sample C, (**E**) No root growth and rottenness in onions exposed to concentrations higher than 6.25% in the three energy drinks.

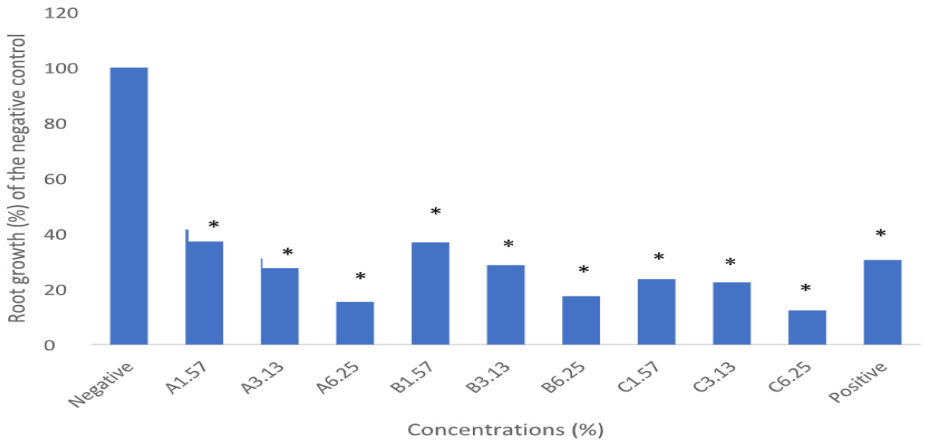

Figure 3. Percentage root growth inhibition by three energy drinks on the root growth of *Allium cepa.* *significant ($p \leq 0.05$) as against the negative.

DISCUSSION

The high rate of production of energy drinks has poised serious health concern due to its increased demand by hard labour workers who depend on the extra boost of energy from these beverages to do more work, and students who need to stay awake for a longer period of time for academic purposes. For this study, we grew onions in two different concentrations of three energy drinks to determine their cytogenotoxicity. The *A. cepa* assay utilized in this study is a popular plant assay used to investigate mutagens (Fiskesjo, 1997; Alabi et al., 2022). It is notably helpful in chromosomal integrity assessment, which allows toxins to be ranked according to their toxicity (Kumar *et al*., 2010). *Allium cepa* test has been used often for the evaluation of DNA damage like disturbance in the mitotic cycle and chromosome aberrations (Akdeniz and Özmen, 2011; Alabi et al., 2023) because of its reaction in the presence of several known mutagens and cycle duration (Evseeva et al., 2003). The outcome of this test could be an indication that certain mutagenic, genotoxic, and cytotoxic agents are present in the substance tested, representing direct or indirect danger for all organisms. Plant's cytological aberrations are very reliable monitoring system that can be used to detect chemicals with potential to pose genetic hazard. This test has been documented to have a good correlation with other *in vivo* mammalian tests (Leme and Marin-Morales, 2009, Alabi et al., 2022). It is observed in the present study that the consumption of energy drinks has cytogenotoxic effects, caused mitotic inhibition, root growth toxicity, and chromosomal aberrations in treated onions.

The observed decrease in MI in the exposed onion roots in this study showed that the three energy drinks are cytotoxic. According to Zulkipli *et al*. (2015), cells may not be able to complete the mitotic process if any stage is stopped. This would result in cell cycle arrest and ultimately cell death. These changes in cell division can affect the general health of the plants as well as hinder their development and generate irregular growth patterns (Bakare *et al.,* 2013). Mitotic index reliably detect agents capable of exerting cytotoxic effects (Kaymak and Goc-Rasgele, 2009; Radic et al., 2010) and Glińska et al. (2007) believed that when there is a decrease in the MI of onion roots after exposure to chemicals, there is a possibility of either chromatin dysfunction or disturbances in the cell cycle as a result of the interactions of DNA with the chemical. This buttresses the conclusion of Yuzbasioglu et al. (2003) that the complete halt of metabolic activities or pressure on DNA synthesis which prevent cells from entering mitosis is caused by decrease in MI (Metin and Bürün, 2010). The ability of these energy drinks on the onions could be a signpost for potential health risks to humans.

Chromosome abnormalities observed in this report suggest that the energy drinks contain aneugenic and/or clastogenic compounds, which may exacerbate chromosomal damage in exposed plants. Chromosomal aberrations are modifications to the structure of the chromosomes which can result in exchange or a break of the chromosome. While the majority of chromosomal aberrations are fatal, many of their corresponding aberrations are still viable and can have hereditary or somatic consequences on a person's DNA (Swierenga *et al.*, 1991). One of the chromosomal aberrations observed is binucleated cell. In a culture, binucleated cells can arise due to incomplete cell division such as incomplete cytokinesis in karyokinesis or due to mitotic division of a pre-existing binucleated cell. Different types of daughter cells can arise from a binucleated cell's mitotic division. Binucleated cells can sometimes enter mitosis but remain binucleated after the division process fails. But, when the division process is successful (which it usually does), the results are either one binucleated and one mononucleated cell or two mononucleated cells (Rodilla, 1993). Stickiness of chromosomes is considered to be a common sign of adverse effects on the chromosomes which could likely lead to cell death (Fiskesjö, 1997; Metin and Bürün, 2008). It could also be an indication that the energy drinks altered the organization of the chromatin which could be related to an alteration to the balanced quantity of histones and/or other proteins controlling the correct structure of nuclear chromatin (Kurás 2004). Chromosome stickiness can also cause sticky bridges to be formed in anaphase and telophase, hence, preventing normal cytokinesis to take place. A pronounced stickiness of the chromatin matrix most times results in atypical metaphase and anaphases. Chromosome lagging can be considered to be an indication of loss of genetic material (Ford *et al.,* 1988; Alabi et al., 2020). Spindle disturbance occurs when there is a centromere abnormality that prevents spindle fibers from attaching to the chromosome and separating it to the cell's distal poles (Miko, 2008). All these are caused by aneugenic and/or clastogenic compounds present in the energy drinks.

The root growth inhibition data indicated that the energy drinks were cytotoxic resulting in reduced root growth in the exposed onions. The initial study (data not shown) tested the effect of the energy drinks on the onions at higher concentrations (100, 50, 25 and 12.5 %) than the one reported in this study but with no root growth. This showed that the energy drink are very cytotoxic at high concentration, hindering cell division and growth of onion roots. It is possible that the drinks' inhibitory effect on *A. cepa*'s root growth and cell proliferation is due to halting of metabolic activities thus preventing mitosis in the cells, DNA synthesis inhibition at the S phase, and the disruptions of the chromatin materials and/or the cell cycle (Bakare *et al.,* 2013). This may indicate how these drinks may affect genetic material in any biological system including humans.

The cytogenotoxicity of the energy drinks reported in this study is believed to be caused by some of the constituents. For example, the drinks contained caffeine which has been controversial when it comes to its role in inducing DNA damage. Ito et al. (2003) reported alterations to the DNA

repair system by caffeine present in soft drinks causing disease of different organs, especially cancer of the pancreas and cardiovascular anomalies. DNA damage in human cells caused by caffeine has also been documented by Timson (2012) in the study where cultured human lymphocytes were treated with caffeine leading to reduction in cell mitosis. The cytogenotoxicity of energy drinks in this study is similar to the results of George and George (2017) where Coca cola, Pepsi and Red Bull caused chromosomal aberrations in onions. Also, the study of Khalaf (2023) reported that energy drinks tested on epithelial cells exhibited cytotoxicity and caused damage to human DNA assessed by comet assay.

Allium cepa bulbs planted in the positive control had the highest growth followed by the ones planted in the negative control. This is consistent with the observation of Islam *et al.* (2008) where at high Pb concentrations, the rate of germination increased while simultaneously causing adverse effects. Indeed, the highest number of chromosome aberrations were found in the positive control in the present study.

CONCLUSION

In conclusion, energy drinks in this study were shown to be cytotoxic and genotoxic in *A. cepa* assay. The constituents of the drinks are believed to have caused these observed effects either individually or synergistically. The data reported in this study calls for further study using animal assays to further study the mechanism(s) of induced cytogenotoxicity in mammals with a focus on humans consuming them. This is important to prevent the possible genetic damage these type of drinks might have on exposed individuals.

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