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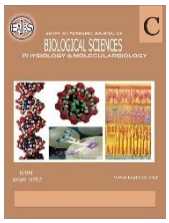

In vivo Evaluation of the Thawing Effect on the Safety of Frozen Carbonated Beverage from a Leading Global Brand

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ABSTRACT

Background : Hot climate in Sudan, increases the consumption of soft drinks. Freezing and de-freezing of Coca-Cola may affect its safety.**Objectives :**To determine the effects of thawing of frozen Coca-Cola on its consistency and safety .**Methods:** Samples were randomly purchased from local groceries, placed in the fridge and allowed to freeze, then removed and allowed to liquefy. Apparent physical changes in the beverage due to thawing were reported. Procedure was repeated until a highly concentrated extract was obtained.Thirty-six rats were randomly divided into 6 groups. Controls were treated orally with distilled water or Coca-Cola. Test groups were treated oral single daily doses of 100, 300, 600 or 5000 mg/kg body weight of the thawed soft drink extract. Body weight changes, food consumption, water intake, and relative organ weight were measured. Liver enzymes, creatinine and urea estimates at day 15, retro-orbital or cardiac puncture blood, were determined. All results were analyzed statistically and compared to controls .**Results:**Liver weights were significantly decreased especially at higher doses of the treatment. High albumin levels and significantly high urea level with the limit doses were determined in the test groups. Hemoglobin concentrations, RBCs, WBCs and PCV counts were significantly decreased at higher doses of the treatment .**Conclusions and recommendations:**De-frozen Coca-Cola thaws into a bilayer liquid. The extract of this liquid decreases liver mass, elevates serum albumin and uric acid levels, and generates a global decrease of blood cells. Storage and consumption of these beverages and adherence to manufacturer instructions is recommended.

INTRODUCTION

Coca-Cola is a carbonated soft drink sold in stores, restaurants, and vending machines throughout the world (Anonymous, 2012). It often is referred to simply as Coke (Anonymous A., 2024).

The primary ingredients of Coca-Cola drink include either high fructose corn syrup or sucrose derived from cane sugar, caramel color, caffeine, phosphoric acid, coca extract, lime extract, vanilla, and glycerin (D'Amato *et al.*, 2011). With the advent of modern life, the consumption of all kinds of drinks increases, but the consumption of Coca-Cola shows a much more marked increase than any other drink (Tsimihodimos *et al.*, 2009).

During the previous years, important concerns have been raised about the effects of Coca-Cola on human health. In addition to the possible detrimental effects of chronic Coca-Cola consumption, enamel softening (Jensdottir *et al.*, 2006), bone demineralization (Tucker *et al.*, 2006), development of metabolic syndrome, diabetes mellitus (Dhingra *et al.*, 2007), hypokalemia (Tsimihodimos *et al.*, 2009), and delay in alveolar bone healing (Teófilo *et al.*, 2010) were reported. The so called `Coca-Cola Incident` describes the outbreak of health complaints that occurred in Belgium, in June 1999, among schoolchildren and members of the public in relation to the consumption of Coca-Cola (Nemery *et al.*, 2002).

On the other hand, it has been reported that Coca-Cola Zero is a useful alternative solvent for polyethylene glycol for bowel preparation and results in better quality cleansing prior to colonoscopy (Seow-En and Seow-Choen, 2015). Other investigators have reported the treatment of gastric phytobezoars with Coca-Cola given via oral route (Gökhan *et al.*, 2012).Sudan's climate ranges from hot and dry in the north to humid and tropical in the equatorial south (Anonymous-B, 2024). Electric current must be supplied to different districts in rations due to shortage of production. Because of this hot climate and electric supply instability, deep freezers have widely replaced fridges. Natives have the tradition of freezing foodstuff, including soft drinks, that thereafter is left at room temperature to liquefy prior to consumption.The observation that a thawed Coca-Cola forms a biphasic liquid signals concern regarding the effects of temperature changes on the stability and consequently the safety of the soft drink.Reports of freeze-thaw instability of foodstuff include white sauces (Arocas *et al.*, 2009), vegetables, fruits, meat and dairy products (Rastogi *et al.*, 2007), potatoes (María *et al.*, 2010), apple juice (Rafał et al., 2016), and orange juices (Fei *et al.*, 2001).

Utilizing protocol E425 guidelines, described by The Organization for Economic Co-operation and Development (Anonymous, 2022), the current study aimed to determine whether the instability of Coca-Cola generated by repeated freezing/ de-freezing affects its safety to the consumers.

MATERIALS AND METHODS

The Coca-Cola used in the experiment was purchased from different stores and supermarkets in different localities of Khartoum during February 2021. Bottles were placed in a fridge until they were frozen. They were then removed and allowed to liquify at room temperature. Thawing generated a biphasic solution with a relatively clear small supernatant layer compared to the dark lower part of the solution. The upper layer was then removed, and the lower layer was returned into the fridge and the procedure was repeated many times until a highly concentrated extract was obtained, (Fig. 1).

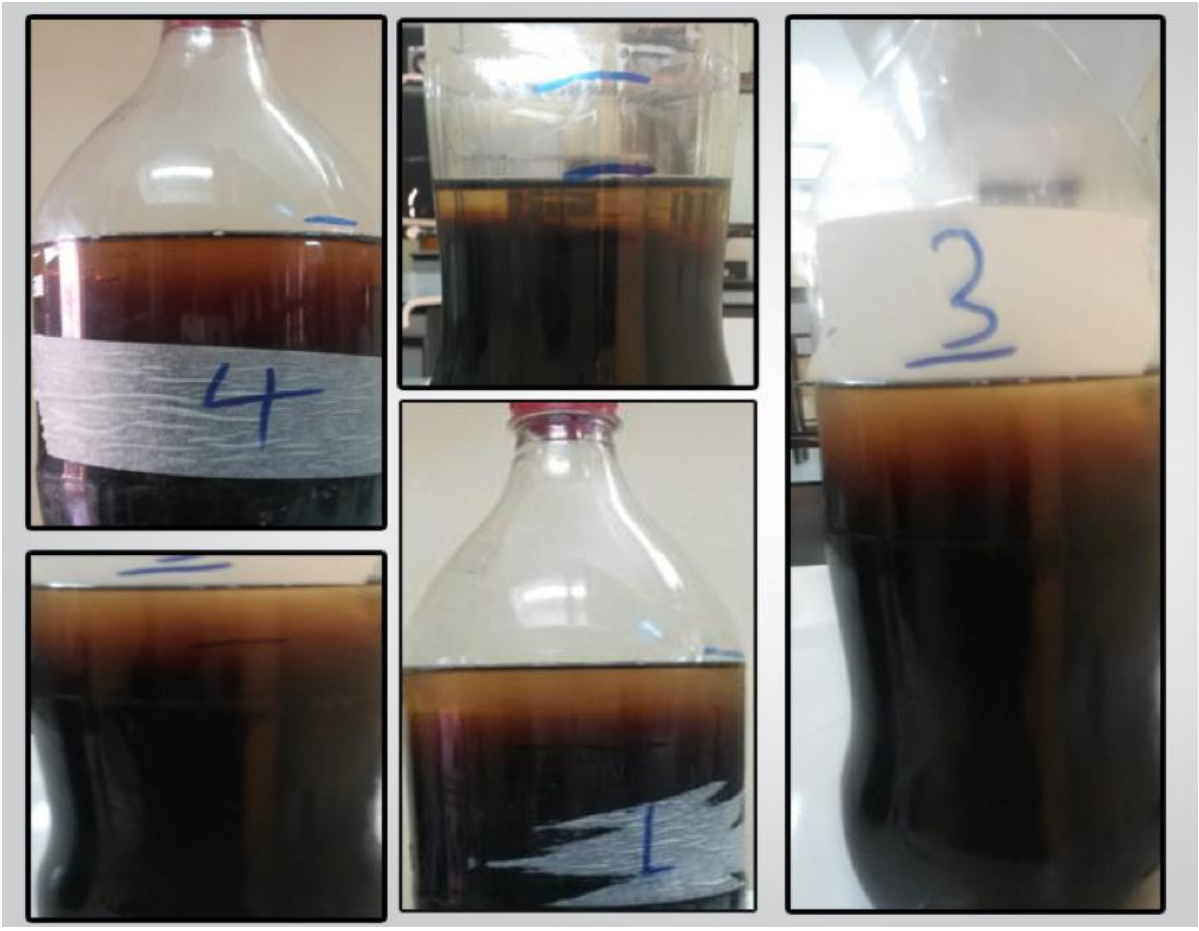

Fig. 1: Formation of biphasic layer due to thawing of frozen Coca-Cola

A total of 36 young adult Albino rats of both sexes, weighing 60-110 g, at 8-12 weeks of age, were used in the experiment. Rats were obtained from the Sudan National Centre for Research, Khartoum Sudan, and kept in the Faculty of Pharmacy, International University of Africa. They were housed at room temperature of (25 ± 1) ºC with 12 hours light/12 hours dark cycle. Food and water were provided daily. The animals were handled according to guidelines. Ethical approval had been obtained from the Ethical Committee of the International University of Africa, Faculty of Pharmacy Department of Pharmacology Approval Certificate number (IUA/I.A.E.C./Exp. Tox. Ph. 013/02). Before the experiment, rats were fasted overnight for 14-16 hours. Group C1 received oral untreated Coca-Cola throughout the study period. Group C2 received oral distilled water throughout the study period. These groups served as positive and negative controls respectively. Groups 1, 2, and 3 were administered oral single daily doses of the test Coca-Cola extract at 100, 300 or 600 mg/kg body weight, respectively. Group 4 was administered the limit dose of 5000 mg/kg body weight. The study was conducted according to the Organization for Economic Co-operation and Development (OECD) 425 guidelines for acute oral toxicity. Animals were randomly divided into six groups consisting of 6 rats each. Treatment followed the above-mentioned procedures for 14 days. The animals were closely observed for the first 4 hours after each dosing to examine any toxic signs. Body weight on day 0, 7 and 14 of the experiment were recorded. Food consumption and water intake were measured and recorded daily. On day 14, the animals were subjected to overnight fasting, anaesthetized and blood samples were collected via retro orbital and cardiac puncture on day 15. Blood samples for CBC were collected in E.D.T.A tubes using non-heparinized capillary tubes. All serum

samples were sent to Soba Teaching Hospital Laboratory within the same day and analyzed using automatic analyzing machine. Several serum biochemical parameters including the liver enzymes aspartate transaminase (AST), alkaline phosphatase (ALP), and alanine transaminase (ALT); and the renal function parameters creatinine and urea were determined. All the animals were dissected, hearts, livers, lungs, kidneys, and spleens were collected and weighed to determine their relative weight and were examined for possible gross abnormalities. All results were analyzed using SPSS 20 and Sigma-plot 11.0 programs and expressed as mean ± standard error of the mean. The two-tailed unpaired T-test was also used to compare the findings. The level of significances was set at $p< 0.05$ compared to the control groups.

RESULTS

All the animals survived the experiment with zero mortality. Observations revealed that rats have behaved normally during the experiment period. Be that as it may, eyes changes, including color and increased pupil size (Experimental exophthalmos), and hyperactivity associated with dose administration, were observed especially at dose 5000 mg/kg. These results are shown in Figure 2.

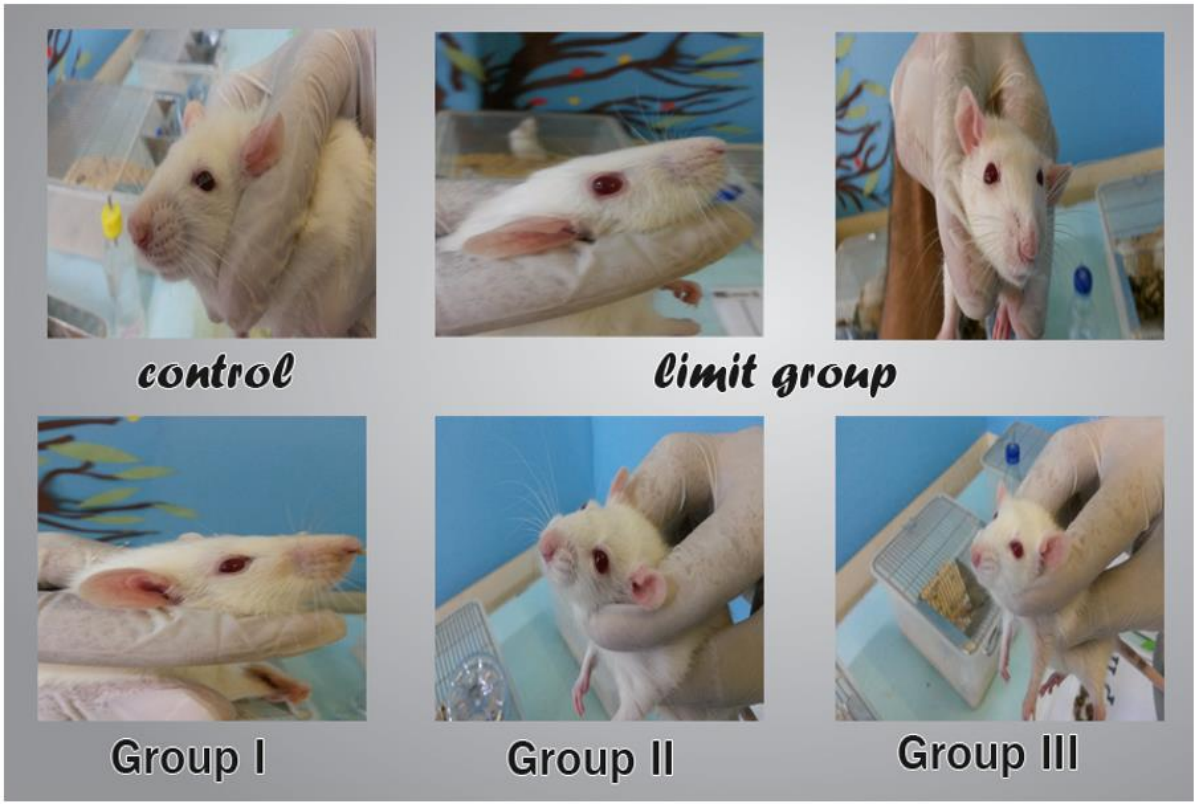

Fig. 2: Phenomenon of eyeball change (Proptosis) in white Albino rats treated with oral de-frozen Coca-cola extract

Gross necropsy has not shown any abnormality in organs morphology but has shown significant decrease in the weights of the hearts and livers cultivated from the rats of group 4. The group has been treated by the dose 5000 mg/kg. Decreases in heart weights were observed to treatments with 600 mg/kg and decreases in liver weights to treatments with 300 mg/kg compared to normal controls. These changes were not observed in animals from both positive and negative control groups. These results are shown in Table 1.

Table 1: Relative organ weights and lethality of white Albino rats treated with oral de-frozen Coca-cola extract.

Doses (mg/k)	Relative organ weight (g)					Lethality
	Heart	Liver	Kidneys	Lung	Spleen	
Control	0.48±0.07	4.33±0.21	0.71±0.05	0.75±0.05	0.26±0.03	0
5000	0.36±0.03*	2.88±0.29*	0.63±0.04	0.62±0.05	0.22±0.03	0
600	0.40±0.03*	3.81±0.29	0.68±0.04	0.75±0.11	0.23±0.02	0
300	0.43±0.02	3.60±0.24*	0.66±0.03	0.62±0.04	0.25±0.02	0
100	0.46±0.05	4.05±0.29	0.72±0.05	0.68±0.08	0.25±0.02	0

*Values are expressed in mean ± S.E.M; analyzed using T-test; *: P<0.05 significant different as compared to negative and positive control groups.*

A significant elevation (*P<0.001*) in urea level and (*P<0.005*) in albumin level were observed at dose 5000 mg/kg. Also, significant decreases in alanine transaminase (ALT) level were observed at the dose 600 mg/kg (*P<0.01*), and at the dose 100 mg/kg (*P<0.001*). These results are shown in Table 2.

Table 2: Results of liver and kidney functions tests of white Albino rats treated with oral de-frozen Coca-cola extract.

Doses (mg/kg)	Liver function tests					Kidneys function tests	
	ALP [u/L]	ALT [U/L]	AST [U/L]	T. protein [g/dl]	Albumin [g/dl]	Urea [mg/dl]	Creatinine [mg/dl]
Control	194.60 ±32.48	40.25 ±2.27	114.85 ±19.80	3.46 ±0.23	2.35 ±0.18	13 ±1.13	0.25 ±0.03
5000	130.10 ±18.59	30.06 ±10.89	115.67 ±44.47	3.93 ±0.22	2.94 ±0.11*	23.33 ±0.66***	0.25 ±0.02
600	167 ±31.48	24.31 ±2.83**	97.25 ±10.90	3.72 ±0.23	2.69 ±0.17	10.50 ±0.89	0.25 ±0.03
300	213.52 ±42.44	36.13 ±6.95	95.15 ±11.27	3.90 ±0.44	2.86 ±0.31	13.33 ±1.54	0.25 ±0.04
100	154.38 ±25.13	22.82 ±2.24***	80.22 ±5.82	3.05 ±0.22	2.17 ±0.19	14.33 ±1.22	0.25 ±0.02

*Values are expressed in mean ± S.E.M; analyzed using T-test; *: P<0.05, *: P<0.01, *: P<0.001 significant different as compared to negative and positive control groups.*

Results of hematological investigations of the test groups revealed that white blood cells (WBCs) counts, blood hemoglobin (HB) concentrations, red blood cells (RBCs) counts, and packed cells volumes (PCV), were all significantly low especially at high doses, compared to controls. These results are shown in Table 3.

Table 3: Results of hematological tests of white Albino rats treated with oral de-frozen Coca-cola extract*: P<0.05.

Hematological Test	Controls	Dose (g/kg)		
		100	300	600
Hb [g/dl]	13.0±0.1	12.3±0.2*	12.3±0.3	12.4±0.4
RBCs [*10^6/µL]	7.2±0.2	7.0±0.1	6.9±0.2*	7.3±0.2
WBCs [*10^3/µL]	7.6±1.4	7.1±0.6	4.7±0.7*	3.6±0.3*
Platelets [*10^3/µL]	770.7±50.6	853±58.8	894.2±38.3	788.8±46.1
PCV [%]	39.8±0.3	37.4±0.9	38.6±0.4	38.7±1.5

The level of water consumption has fluctuated in all groups. Food consumption has, likewise, been variable within a specific range. These results are shown in Figure 3.

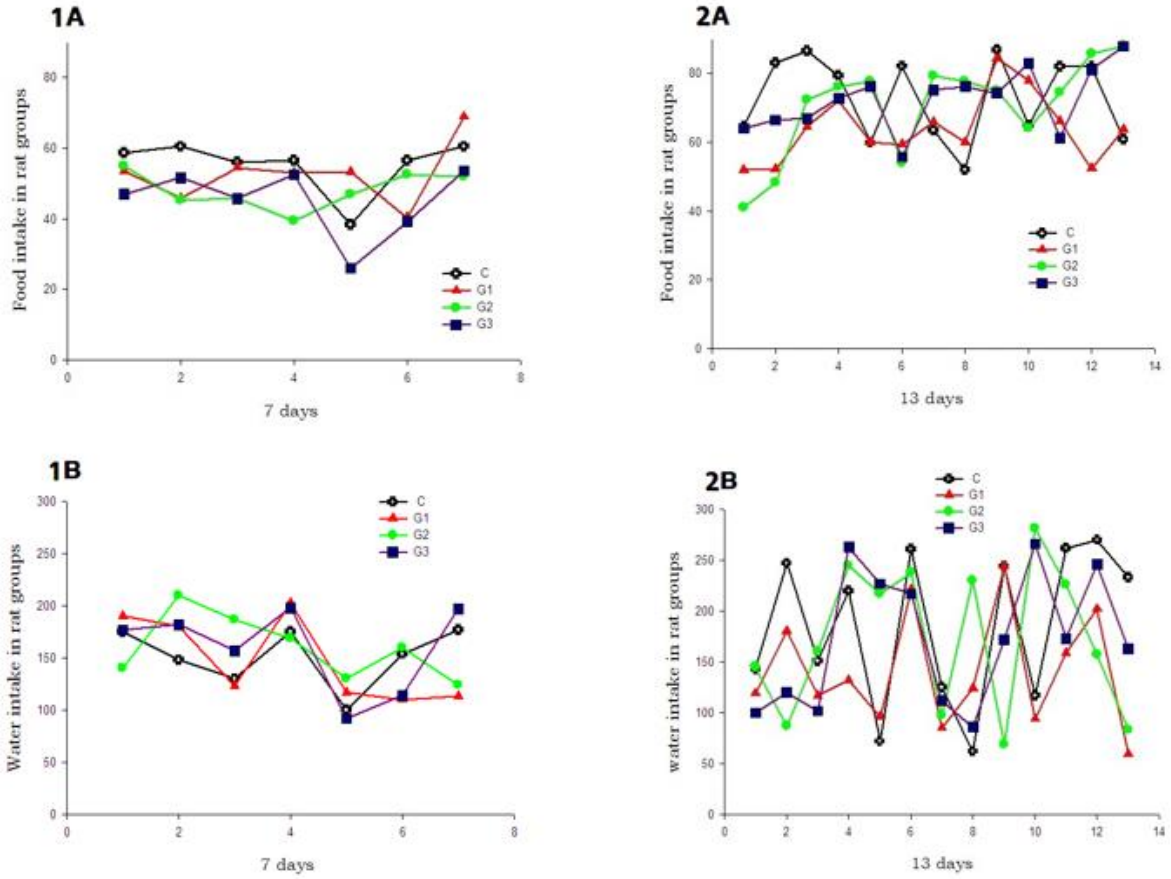

Fig. 3: Food consumption of white Albino rats, treated with oral de-frozen Coca-cola extract, in the adaptation period [1A] and during the experiment period [2A], and water consumption in the adaptation period [1B], and during the experiment period [2B].

DISCUSSION

A trend to consume de-frozen Coca-Cola, that has been liquefied under room temperature, affects the uniformity of the soft drink. This instability renders the safety of the soft drink questionable. Toxicological studies are the platform for hazard identification and safety assessment (Wallace, 2011). Acute exposure to the Coca-Cola extract at doses from 100 mg/kg to 5000 mg/kg has not been detrimental. This indicates that the oral LD50 value of the extract in rats was greater than 5000 mg/kg as has been estimated by the special statistical software [AOT425] with

three stopping criteria. This indicates the wide safety margin of the soft drink. Be that as it may, prolonged treatment with the extract may enhance the possibility of toxicity in rats. This is consistent with a previous work by Marcos *et al.* (2016) that has investigated the toxic/antitoxic, genotoxic/antigenotoxic, and chronic toxicity effects of Classic Coca-Cola and Caffeine- Free Coca-Cola *in vivo* using the *Drosophila* model, their cytotoxic activity using the HL-60 *in vitro* cancer model, and clastogenic DNA toxicity using inter-nucleosomal fragmentation and SCGE assays. The investigators reported a slight chemo-preventive effect of the two cola beverages against HL-60 leukaemia cells, a global genome hypo-methylation, but an overall demonstration of safety of this beverage in *in vivo* and *in vitro* models (Marcos *et al.*, 2016).

The present observations revealed that rats have behaved normally during the experiment period. Dose administration has induced changes in eye color, increased pupil size (experimental exophthalmos) and hyperactivity. These observations were particularly evident at dose 5000 mg/kg. Gross necropsy on the rats has not shown any abnormality in the surface of relative organ but has shown significant *(P<0.05)* decrease in heart and liver weight at dose 5000 mg/kg, decrease in heart weight at dose 600 mg/kg and decrease in liver weight at dose 300 mg/kg compared to control group. Albumin level was high, indicating a negative impact on liver function. These findings are consistent with a previous report that even a couple of cans Coca-Cola may raise the risk of liver damage (Valentine, 2016). ALT estimates were significantly low at the dose of 600 mg/kg (*P<0.01*); and at the dose of 100 mg/kg (*P<0.001*). These observations are partially consistent with a previous report that chronic drinking of Coca-Cola induced decreases in pancreatic mass, particularly pancreatic beta cells, of rats that have been ameliorated by exercise (Otero, 2016).

A significant elevation (*P<0.001*) in urea level was observed at dose 5000 mg/kg. This finding is consistent with a recent report that drinking two or more Coca-Cola bottles per day, whether artificially sweetened or not, is linked to a twofold risk of chronic kidney disease (Malik *et al.,* 2010). WBCs, HB, RBCs, and PCV counts were all significantly low especially at high doses. These findings are partially not consistent with a previous report that Coca-Cola is not cytotoxic to bone marrow cells (Düsman *et al.,* 2013). During the experiment, water consumption estimates of the rats from all the groups fluctuated. Food consumption estimates similarly fluctuated, within a specific range. An increase of the estimates of food consumption of the limit group, suggests an appetizer effect of Coca-Cola at higher doses.

Conclusions and Recommendations:

It can be concluded that the consumption of thawed Coca-Cola may be associated with serious side effects including albuminemia, uremia and pancytopenia. Be that as it may, one cannot extrapolate from these animal experiments a final effect pertaining to human subjects.Consumers and retail sellers should adhere to manufacturer instructions regarding the storage and consumption of these beverages. Subjecting these products to direct sunlight, freezing and thawing prior to consumption should be prohibited. Producers are urged to supervise limiting of the storage of their products to the fridges that they distribute to retailers. They are also urged to encourage research and public awareness regarding inappropriate storage of their products.

Declarations:

Ethical Approval: The study was approved by The Ethical Committee of the International University of Africa, Khartoum, Sudan, Faculty of Pharmacy, Department of Pharmacology. Approval Certificate number (IUA/I.A.E.C./Exp. Tox. Ph. 013/02).

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