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Off-label use of tramadol combined with *Lacasera*® soft drink: A sub-acute toxicological study using Wistar rats to evaluate the effects of combination on human basic haematological parameters and weight changes

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Abstract

Tramadol is a synthetic opioid that is used to treat moderate to severe pain in both acute and chronic conditions. Tramadol is predominantly used off-label for premature ejaculation and euphoria among male youth in Nigeria, especially when dissolved in *Lacasera*® soft drink, which gives it a pleasant taste. Research has not yet examined the effects of this combination on hematological parameters, despite the fact that this type of abuse is common among young people and poses a public health risk. A 28-day subacute study was conducted with Wistar rats weighing 160-180g. The rats were separated into 8 groups of 6 each. The control groups: 1 and 2 were treated with *Lacasera*® and deionized water respectively. Groups 3, 4 and 5 received 35mg/kg per day, 70.7mg/kg per day and 106mg/kg per day of Tramadol dissolved in deionized water respectively. Groups 6, 7 and 8 received similar doses of Tramadol dissolved in *Lacasera*®. Blood samples were analyzed using auto-analyzer and standard methods. The data was analyzed using a statistical tool software (SPSS version 27). All results were presented as mean ± SD. Values were considered significant at $p<0.05$. Hematological analysis of groups treated with *Lacasera*®-Tramadol combination shows dose-dependent significant decreases ($p<0.05$) in PCVs, RBCs, and Hbs. WBCs and platelets increased in groups that received *Lacasera*®-Tramadol combination. The study concludes that regular or infrequent users of *Lacasera*® without Tramadol are unlikely to experience serious hematological complications. However, chronic Tramadol-*Lacasera*® users will experience increased toxicity over time, leading to hemolytic anemia.

Keywords: Anemia; Premature Ejaculation; *Lacasera*®; Tramadol; Off-label

1. Introduction

The abuse of Tramadol, a synthetic prescription opioid analgesic, is well around the world acknowledged as a problem of global health [1]. Some Tramadol off-label uses, such as euphoria and delayed ejaculation, may be responsible for its abuse [2], particularly among adult male users. Unfortunately, Tramadol trafficking and abuse continue to gain prominence on a global scale[1]. Tramadol has been designated as a restricted substance in a number of different countries due to an increase overdoses and fatalities caused by tramadol over the past ten years [3].

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In 2019, global seizures of Tramadol trafficked illegally increased from less than 10 kg in 2010 to a record high of 125 tons in 2017, according to the United Nations Office on Drugs and Crime (UNODC)[4], [5].In various sub-regions of the world, it has turned into an opioid of significant public health concern, especially across many areas of Asia and Africa [5]. Studies in Egypt reported that Tramadol is used at a high incidence of 8.8 to 12.3% among high school and university students. [6]. Tramadol addiction and misuse is also becoming a public health concern in many developed countries[7].

Despite the fact that the link between Tramadol and sexual function appears to be questionable, using Tramadol off-label may help men who have premature ejaculation [8],[9].This could be one of the primary reasons for misuse of Tramadol by young people in Nigeria. To mask the bitter taste of Tramadol, most addicts dissolve it in *Lacasera*® soft drink [10], though some use other liquid media. Notwithstanding the positive outcome of several study on the administration of Tramadol to treat premature ejaculation (PE), it is not medically indicated for PE. In Nigeria and Ghana, off-label use of Tramadol for purposes other than pain relief has been linked to three major causes: psychological, favorable effects for physical or manual labor, and economic factors based on availability and affordability[7].

Generally, in Nigeria, *Lacasera*® soft drink is popularly consumed among other soft drinks because of its flavor. *Lacasera*® soft drink contains malic acid, citric acid, caramel, acidulants , carbonated water ,sodium benzoate, sucralose and apple juice concentrate. A popular acidulant used as ingredient in such soft drink is phosphoric acid due to it's strong effect on acidity and flavor of the carbonated soft drinks [11]. Utilization of phosphoric acid is controversial due to its association with harmful health effects [12]. Phosphoric acid has a significant effect on pH and is widely used to give a particular taste profile to cola style beverages [13]. Also, according to study done by Bamise et al., [14], the pH of each of the well-known soft drinks used for the study in Nigeria had a notable erosive potential among *Lacasera* was found to be very acidic.

There is significant worry about preservatives and sweeteners in general, and some compounds may be harmful to health if ingested in large quantities[15]. It is not known if these chemicals might interact with Tramadol resulting in synergistic or additive effects since *Lacasera*® soft drink is highly acidic. Moreover, it is not known if there is a strong relationship between hematological abnormalities and consumers of this combination based on local clinical reports. This knowledge gap prompted a subacute toxicity study on the effects of Tramadol dissolved in *Lacasera*® soft drink on hematological parameters in Wistar rats.

2. Materials and methods

2.1. Animals

Healthy male adult Albino Wistar rats weighing 160–180g were employed in the studies**.** The rats were domesticated at the Animal House facility of the Nnamdi Azikiwe University's School of Pharmaceutical Sciences in Awka, Nigeria. **Tramadol:** Tramadol used in the study was obtained from a licensed retail Community Pharmacy in Port-Harcourt, Nigeria. Tramadol capsules B.P 50mg (B.G Tragesic® N-1339, Stallion Laboratory PVT. Limited, India) with NAFDAC approval number (A4-2740) was used. **Rodent Feed:** The rats were fed with commercial standard rodent feed (Vital Feeds®, Nigeria Limited). ***Lacasera*® Soft Drink:** A 50 ml *Lacasera*® *soft* drink was purchased from a supermarket (*Lacasera*®; Nigeria) with NAFDAC approved number (01-4278) was used.

2.2. Animal Handling

The Wistar rats were procured and maintained in the same manner as previously described by [16]. Animals were between 8 and 12 weeks old at the start of the study, and their weights were within 20% of the mean initial weight according to Organization for Economic Co-operation and Development (OECD)[17] recommendation. They were kept in their cages for about two weeks before dosing to allow time for adjustment and adapting to the laboratory environment. The animals were carefully chosen to ensure that they were available in the appropriate sizes and ages for the duration of the study. The temperature in the animal housing was controlled at 22°C (±3°C), as suggested by the National Research Council(NRC) and the relative humidity was 50-60%[18] . Artificial lighting was used, with 12 hours of light followed by 12 hours of darkness. They were fed with commercial rat feed ad libitum and provided enough de-ionized water. The animals were cared for in accordance with the National Research Council's Guideline for Laboratory Care of Animal and Use.

2.3. Animal grouping, selection, and experiment design

The rats used in the study were randomly selected, and the sample size was calculated using Charan and Kantharia equation [19]. In the 28-day study, 48 male Wistar rats were employed in the sub-acute study, with 16% attrition. The animals' body weights were measured at the start of the study (initial weights), then after 7, 14, 21, and 28 day respectively. The rats were separated into eight groups of six rats each (n=6 each group). According to their body weights, Group 1 received 5ml/kg of deionized water. Control group (2) received 5ml of *Lacasera*® soft drink per kilogram of body weight. Groups 3, 4, and 5 received standard (35 mg/kg per day) and supra-therapeutic (70,7mg/kg per day and 106mg/kg per day) graded dosages of Tramadol dissolved in deionized water (i.e. Tramadol + water). Groups 6, 7, and 8 were administered standard dose (35 mg/kg per day) and supra-therapeutic (70.7 mg/kg per day and 106mg/kg per day) graded dosages of Tramadol dissolved in *Lacasera*® soft drink (i.e. Tramadol + *Lacasera*®).

2.4. Dosage preparation and administration

Prior to administration, fresh daily dosages were made. The rats in the control groups were administered 5ml/kg of fluid (either de-ionized water or *Lacasera*®). The total daily doses of test solution were given in split doses of 0hr, 6hrs, 12hrs, and 18hrs. Dosing lasted 28 days, with daily and weekly weights of the animals taken. To prevent injury, the solutions were administered orally through an appropriate cannula to the rats. The rats received graded test doses of oral Tramadol in divided dosages of 5.71 mg/kg per day, 11.4 mg/kg per day, and 17.1 mg/kg per day in each group. According to WHO [4], the maximum daily dose required for Tramadol administered in divided doses in humans is 5.71 mg/kg (400 mg/70kg) . In order to attain similar therapeutic effects expected in a 70 kg man per day in rats, a conversion formula by Nair and Jacob[20] was used to calculate the required equivalent doses of 35 mg/kg/ day,70.7 mg/kg /day and 106 mg/kg per day respectively. The 35 mg/kg per day of Tramadol administered to the rats (in divided doses) is the equivalent maximum dose (5.71 mg/kg per day) administered in human. The supra-therapeutic doses (70.7 mg/kg per day and 106 mg/kg per day) were also administered to mimic uncontrolled supra-therapeutic doses used by human chronic addicts. The doses for the animals in groups 3, 4, and 5 were dissolved in deionized water. Similar doses of Tramadol dissolved in *Lacasera*® soft drink were prepared for animals in groups 6, 7, and 8 respectively.

2.5. Acute Toxicity Test (LD_{50})

The modified Lorke method [21] was used in the determination of oral acute toxicity of Tramadol. Two LD_{50} s of Tramadol were determined using water and *Lacasera*® soft drink as solvent media respectively. A total of 13 male Albino Wistar rats were used in determination of each of the LD_{50} s.

In phase I, Wistar rats were divided into three groups (n = 3). Solution of Tramadol (i.e. dissolved in deionized water) was orally administered to each of the animal using cannula in the dosages of 10, 100 and 1000 mg/kg per group . The rats were housed in the same environment and monitored for toxicity symptoms for six hours after administering Tramadol. They were scored for mortality and general behavior after 24 hours. Dosing was move to phase II when mortality at 1000 mg/kg was recorded.

In phase II, four groups of rats (n = 1) were treated with Tramadol solutions containing 200, 300, 400, and 500 mg/kg, respectively. Tramadol's LD_{50} was calculated by taking the geometric mean of the highest non-toxic dose (a) and the least fatal dose (b), [$LD_{50} = \sqrt{(ab)}$][22],[23].Same procedure were followed in the determination of LD_{50} of Tramadol using *Lacasera*® soft drink.

2.6. Determination of pH of Graded Doses of Tramadol Dissolved in 50cl(500ml) in De-ionized Water and *Lacasera*® Soft Drink

The pH of the two solutions was determined using a digital pH meter after calibration and standardization in a manner similar to that described by Chowdbury et al., [24] at 28.6 ^{o}C . The graded Tramadol doses (400 mg, 800 mg & 1200 mg) were dissolved in 50cl (500 ml) of Tramadol and deionized water respectively. A 40ml of both Tramadol solutions were measured using calibrated glass cylinders into two groups of beakers respectively. The beakers were labelled according to their contents (Tramadol dissolved in deionized water and Tramadol dissolved in *Lacasera*® soft drink respectively). The pH of each solution of graded dose of Tramadol were determined by dipping the electrode into the solution for about 60secs. After measuring each solution three times, average pH values were calculated. Before a new dose is measured, the electrode was washed with distilled water. The same process was used to determine the pH of more doses.

2.7. Hematological Analysis

The analysis was carried out using Sysmex automated hematology analyzer (Sysmex Kx-21N, United States). The mode of operation is such that the total blood count is measured using an automated analyzer in either whole blood or pre-diluted mode. The sample probe draws whole blood into the sample rotor valve, which measures 6 ul of blood and delivers it, together with 1.994 ml of diluent, to the WBC transducer chamber. A 1:500 dilution sample is done by adding 1.0 ml of WBC/HGB lyse at the same time. When the solution is left to react in this state for 10 seconds, red blood cells (RBC) are hemolyzed, platelets decrease, and the membrane of white blood cells (WBC) remains intact. Simultaneously, haemoglobin (Hb) is converted into red meth-haemoglobin. In the WBC transducer chamber, roughly 1.0ml of the diluted/hemolyzed material is transferred to the Hb flow cell. The hole is used to aspirate 500ul of material from the WBC transducer. The DC detection method counts blood cell pulses as they pass through the aperture. The material in the Hb flow cell is subjected to a 555nm wavelength light emitting diode beam (LED). This sample's absorbance is used to calculate its concentration. Before the addition of the sample, the diluent's absorbance alone, which produced Hb, was measured (haemoglobin value).

The blood samples were mixed in a blood mixer for at least 10 minutes prior to analysis. A self-check was performed after the analyzer was turned on. When it said "Ready," the device was ready for use . The probe was used to inject test and control samples into the device. The results displayed on the machine's screen after around five minutes. The machine was shut off in accordance with the standard operating procedure (SOP) for turning off the unit after recording the results.

2.8. Statistical Analysis

The data were analyzed using the Statistical analytical Package software (SPSS, version 27). The results were presented as the mean ± standard deviation (SD). The means of the hematological parameters were compared between the control and experimental groups at each dose level using the independent t-test and one-way analysis of variance (ANOVA). At $p< 0.05$, the values were deemed statistically significant**.**

3. Results and discussion

3.1. Acute Toxicity Study

Acute systemic toxicity investigates the unfavorable consequences of exposing experimental animals to a single or repeated doses of a test substance through a known route (oral, cutaneous, or inhalation) within 24 hours. [25]. Modified Lorke's[22] method was used to determine the **LD_{50}** of Tramadol solutions (i.e. Tramadol dissolved in deionized water and Tramadol dissolved in *Lacasera*® soft drink). A comparison of the LD_{50} s in tables 1 and 2 indicates that toxicity in the Tramadol-*Lacasera* treated group develops rapidly. The difference in the doses that resulted in death between the two solutions suggests that the Tramadol-*Lacasera* (245 mg/kg) combination is more lethal than the Tramadol-deionized water (346 mg/kg) combination.

Table 1 LD_{50} of Tramadol Dissolved in Deionized Water by Modified Lorke's Method

Phases	Dose(mg/kg)	Onset of Toxicity(hour)	Mortality	Remarks
Phase I	10	0	0/3	No evident signs of intoxication.
	100	0	0/3	No evident signs of intoxication.
	1000	1.25	3/3	Death
Phase II	200	0	0/1	No evident signs of intoxication.
	300	0	0/1	No evident signs of intoxication.
	400	1.53	1/1	Death
	500	1:46	1/1	Death

No. of rats (n=13); LD50 = $\sqrt{(300X400)}$=346 mg/kg, total no. of death recorded=5

Table 2 LD_{50} of Tramadol Dissolved in *Lacasera*® Soft Drink by Modified Lorke's Method

Phases	Dose(mg/kg)	Onset of Toxicity(hour)	Mortality	Remarks
Phase I	10	0	0/3	No evident signs of intoxication.
	100	0	0/3	No evident signs of intoxication.
	1000	0.51	3/3	Death
Phase II	200	0	0/1	No evident signs of intoxication.
	300	1.46	1/1	Death
	400	1.32	1/1	Death
	500	1.28	1/1	Death

No. of rats (n =13); LD50 = $\sqrt{(200X300)}$=245 mg/kg; Total no. of death recorded=6

The oral LD_{50} of Tramadol dissolved in deionized water determined in this study was consistent with the LD_{50} s (300–350 mg/kg) determined by Grond & Sablotzki[26] and Matthiesen *et al.,*[27]. In contrast, the obtained LD_{50} values for Tramadol dissolved in *Lacasera*® soft drink were lower than those reported by Grond & Sablotzki [28] and Matthiesen *et al.,*[27].The LD_{50} values for the two solutions (245 mg/kg; 346 mg/kg) were compared using Loomis & Hayes[29] toxicity classification (50-500 mg/kg) as a guide.

Because the animals (in table 2) died at a lower dose (300mg/kg) and for a shorter period in phase II than Tramadol solution (in table 1) may be due to pharmacokinetic or pharmacodynamics factors. A variety of factors can impede or facilitate absorption. The presence and density of membrane transporters, as well as the solubility of lipids and the pH of the medium, all have a greater effect on the absorption rate[30].Additionally, high concentration of the drug promotes absorption[31] . If pH is to be considered as a factor in the Tramadol-*Lacasera*® solution's rapid onset of action in rats, both the stomach and the solution's pH should be considered.

The stomach is an acidic environment with a pH ranging from 1.5 to 3.5.[31]. Tramadol hydrochloride is a highly acidic compound. However, Smyj *et al* .[32] demonstrated that Tramadol's pH can be adjusted from weakly acidic (3.5) to weakly basic (7.5) using phosphate buffer . According to the International Union of Basic and Clinical Pharmacology (IUPHAR)[33] , when drugs classified as weak acids are exposed to an acidic environment, the drugs will absorb a proton and become unionized. :H+ + A—> HA. This increases the rate of absorption in the stomach. Racemic tramadol is extensively absorbed in the gut when taken orally, with a bioavailability of roughly 75% for 100 mg tramadol [34].

Although the stomach is acidic, its thick mucus layer and tiny surface area make it unsuitable for drug absorption, even for mild acids [33]. Rather than being an organ of absorption, the stomach serves as a "storage" organ. In contrast, the abundance of numerous villi and microvilli in the small intestines results in a huge surface area that is available for absorption. As a result, acidic medications have a higher likelihood of being absorbed in the proximal duodenum's acidic areas, whereas basic medications are more likely to be absorbed in the distal ileum's more alkaline parts [33]. *Lacasera*® soft drink contains acidulants, which may have contributed to the Tramadol-*Lacasera*® solution's pH being further lowered[35]). As a result, the Tramadol-*Lacasera*® solution was absorbed more readily, increasing its systemic bioavailability. As a result, the onset of action and toxicity are accelerated.

3.2. Effect of Lacasera®-Tramadol Combination on pH

As shown in table 3, when *Lacasera* soft drink was used to dissolve Tramadol, the pH of the medium became strongly acidic, in contrast to the pH of the same dose of Tramadol dissolved in the same amount of portable water, which became alkaline. Moreover, it was observed that the pH of the *Lacasera*-Tramadol combination decreased (becoming more acidic) as Tramadol doses increased. Although Tramadol is an acidic drug, manufacturers always adjust the pH of the drug using phosphate buffer to alkaline status as demonstrated by Smyj et al., [32]. This alkalinity makes it a better alternative in treatment of pains in ulcer patients whose case might get worse when NSAIDs are taken. The pH of Tramadol dissolved in *Lacasera*® obtained in Table 3 was changed from alkaline (7.35,7.41 & 7.46) to acidic (1.36, 1.38 & 1.40) state . The low pH values obtained in our study support the findings of a study on pH of popular soft drinks in Nigeria conducted by Bamise *et al*[36] , who concluded that the study's selection of commonly consumed soft beverages in Nigeria had high erosive potential, and that *Lacasera®'s* pH was confirmed to be extremely acidic. Furthermore , Chowdhury *et al*., [24]state that the addition of citric acid lowers the pH of the formulated diets, owing to the unknown concentration of citric acid and other organic acids in *Lacasera*® soft drink.

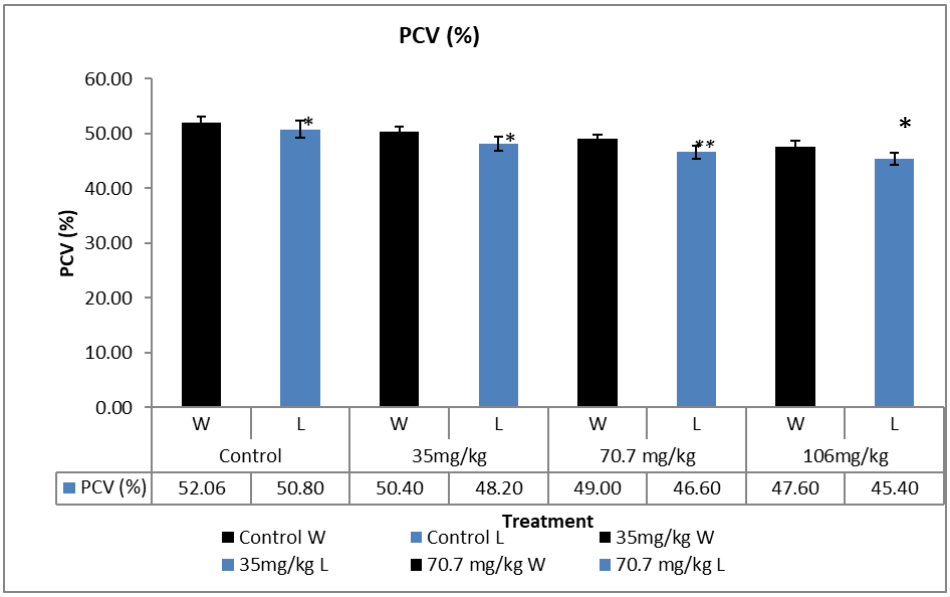
Table 3 pH of Graded Doses of Tramadol Dissolved in 50cl (500 ml) of De-ionized Water and *Lacasera*® Soft Drink at 400 mg, 800 mg and 1200 mg

	Dose(mg)	Volume of Solution (ml)	pH Readings			Average pH	Temp. (°C)
			1st	2nd	3rd		
	400	500 ml	7.35	7.36	7.36	7.35	29.0°C
T+W	800	500 ml	7.41	7.39	7.42	7.41	29.0°C
	1200	500 ml	7.47	7.46	7.45	7.46	29.0°C
	400	500 ml	1.39	1.40	1.40	1.40	29.0°C
T+*L*	800	500 ml	1.38	1.37	1.38	1.38	29.0°C
	1200	500 ml	1.35	1.37	1.36	1.36	29.0°C

Key: T + W=*Tramadol dissolved in de-ionized water;* T+ L=*Tramadol dissolved in Lacasera® soft drink;* pH of *Lacasera*® drink =1.46

Although the body, through the kidneys and lungs has compensatory mechanisms for the body's abnormal pH; the integrity of the stomach or mucosa could be affected by increased acidity. Soft drinks, which, in addition to causing gastric distension and stomach ulcers linked to dyspepsia, contain CO_2 gas and increase acid production. [37] .According to Armstrong [38] ,a pH of 4 has been established as the point at which refluxed stomach contents cause esophageal injury. Our results show pH lower than 4 suggesting high acidic condition of the mucosa which resulted into slight ulceration. As a result, persistent ingestion of Tramadol mixed in the extremely acidic *Lacasera*® soft drink may have other effects in the body than stomach ulceration and tooth erosion.

3.3. Effects of Tramadol and *Lacasera*® Soft Drink Basic Hematological Parameters



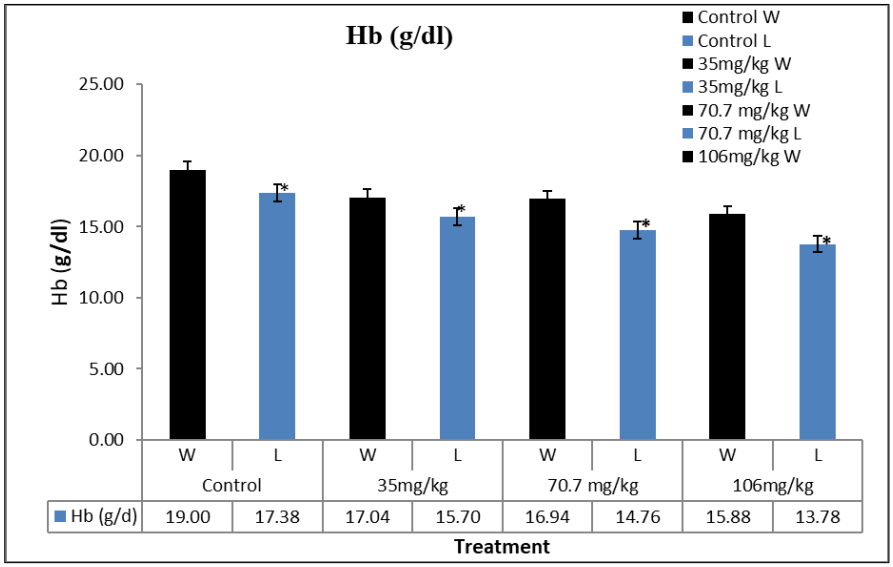
Values are presented as mean ± Standard Deviation, n =5. nsp > 0.05: Not significantly different statistically from W (water group). *p<0.05: Statistically significantly different from W (water group). **: Significant at 0.01.

Figure 1 Effects of graded doses of Tramadol-deionized water versus Tramadol-*Lacasera*® on the PCV of Wistar rats

Packed cell volume, also known as hematocrit, is the volume of erythrocytes/red blood cells in whole blood, or the percentage of blood volume occupied by RBC[39].In our study, we observed that as the doses of Tramadol increases in each of the groups ,there is corresponding slight decreases in PCVs recorded as seen in figure 1. A dose-dependent reduction that is statistically significant ($p<0.05$) was observed in the Tramadol-*Lacasera*® treated group when the means of PCVs at each dose levels were compared to those in the Tramadol-deionized group. According to Wilson [40], anemia is defined by a reduction in hematocrit or PCV. It is a manifestation of an underlying pathology [41].Anemia can

be caused by the loss of erythrocytes , the destruction of erythrocytes (hemolysis), or insufficient erythrocyte synthesis by the bone marrow [42].This implies that combination of Tramadol and *Lacasera*® might have played a part in destruction of erythrocytes leading to decreased PCV recorded. Our study is consistent with Aldiwan et al., [43]but indicates significant effect of *Lacasera*® on PCV of male rats used.

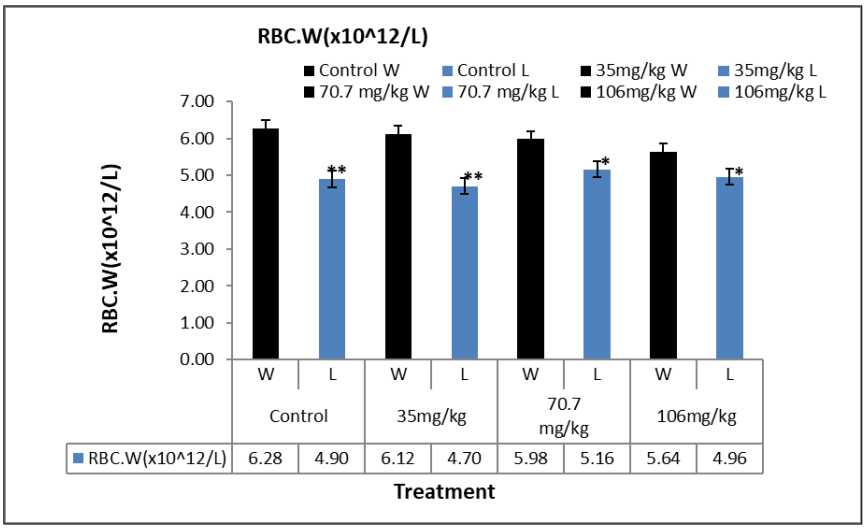
In red blood cells, haemoglobin, which is made up of four protein chains, transports oxygen from the blood to organs and tissues [44]. One of its most essential functions include supplying the tissues with oxygen from the lungs[45], primarily to enable oxidative phosphorylation in the mitochondria. As carbaminohaemoglobin, it aids in the transfer of carbon dioxide from tissues to the lungs[46]. In our study, both treatment groups experienced a dose-dependent decrease in Hb. This is to be expected, as Tramadol is known to have a depressing effect on the respiratory system, causing a reduction in total CO_2 sensitivity[3], which Hb plays a part in .However, Tramadol-induced Hb reduction has not been reported at normal therapeutic doses. However, a mean ±SD comparison of Tramadol-*Lacasera*® versus Tramadol-deionized water shows a significant ($p<0.05$) decrease in Hb values of the Tramadol-*Lacasera*® treated group at normal therapeutic doses used in rats. The decrease in Hb increases at the doses increases: 35 mg/kg per day, 70.7 mg/kg per day and 106 mg/kg per day respectively.



Values are presented as mean ± Standard Deviation, n =5. nsp>0.05: Not statistically significantly different from W (water group).

Figure 2 Effects of graded doses of Tramadol-deionized water versus Tramadol-*Lacasera*® on the Hb of Wistar rats

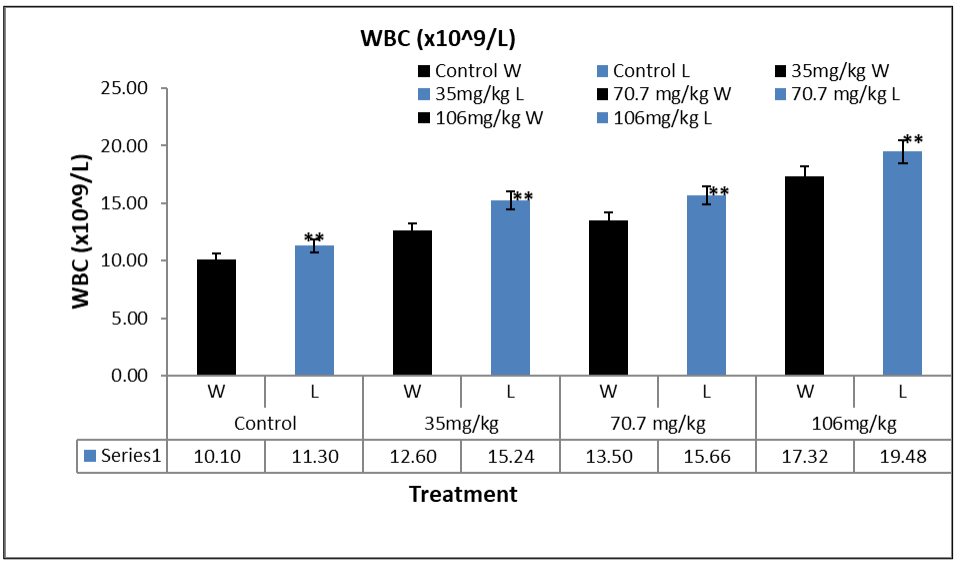
Tramadol and *Lacasera*® had different effects on Wistar rats' RBCs, as seen in figure 3 (Tramadol-deionized water vs. Tramadol-*Lacasera*®). Our study shows that Tramadol had no statistically significant effects on RBCs in either group (within), particularly in the control and normal therapeutic dose groups. But comparison of mean ±SD values of RBCs recorded in control groups and normal doses of Tramadol-deionized water and Tramadol-*Lacasera*® groups shows a significant decrease in RBCs even at $p<0.01$. Generally, such reduction in RBCs is an indication of anemia. According to Chaparro & Suchdev, [47]anemia is a condition in which a person's Hb concentration and/or RBC count are below normal and insufficient to meet their physiological needs of the body. Our study is consistent with Aldiwan et al.,[48] but indicates significant effect of *Lacasera*® on RBCs of male rats used.



Values are presented as mean ± Standard Deviation, n =5. nsp>0.05: Not statistically significantly different from W (water group).

Figure 3 Effects of graded doses of Tramadol-deionized water versus Tramadol-*Lacasera*® on the RBC of Wistar rats

In figure 4, our study recorded dose-dependent increase in WBCs in either (within) of the groups. When the WBCs in both groups are compared, the mean ± SD of the Tramadol-deionized water treated group is statistically significantly ($p<0.05$) lower than the WBCs in the *Lacasera*®–Tramadol treated group. Although, many hematological disorders can be affected by drug toxicities that are caused by a number of mechanisms [49], effects of Tramadol-induced elevated WBCs at normal therapeutic dose has not been reported. According to Mank and Brown [50] ,the increase in WBCs could be the result of physical stress and internal organ inflammation which was observed in the histopathological analysis of kidney and liver tissues. According to several studies, tramadol has no effect on immune cell activity. It has been demonstrated that it increases NK activity, lymphocyte proliferation, and IL-2 production.[51].The increased WBC levels recorded in our study could be due to Tramadol-*Lacasera*®-induced necrosis seen in histological examinations of the stomachs of the rats used in the study. The body launches a chemical signaling cascade in reaction to injury, which encourages responses targeted at repairing afflicted tissues.[52]. These signals stimulate leukocyte chemotaxis, or the movement of white blood cells, from the general circulation to the area of injury [52].

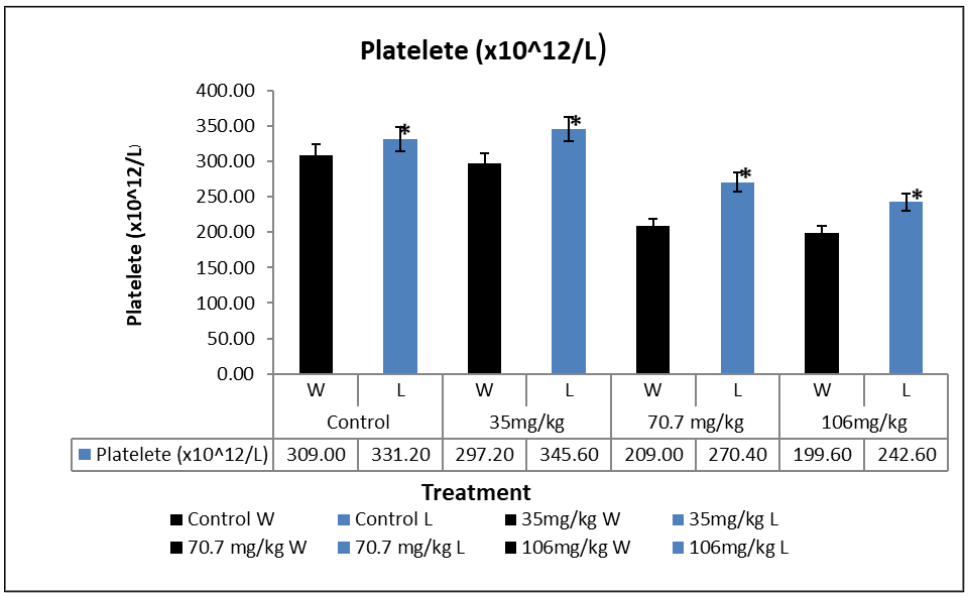


Values are presented as mean ± Standard Deviation, n =5. nsp>0.05: Not statistically significantly different from W (water group). *p<0.05: Statistically significantly different from W (water group). **: Significant at 0.01.

Figure 4 Effects of graded doses of Tramadol-deionized water versus Tramadol-*Lacasera*® on the WBC of Wistar rats

Also, in comparing the mean platelet counts of rats administered graded dosages of Tramadol in both groups, the Tramadol-*Lacasera*® treated group shows statistically significant higher platelet counts ($p <0.05$) than the Tramadol-deionized water treated group. The dose-dependent increase in platelets counts could be as a result of secondary

thrombocytosis caused by injured tissues , liver or kidney[53] as was observed in the histological examination of these organs . According to Williams [54] and Rokkam and Kotagiri [53], such increase could be due to inflammation or hemolytic anemia. The study partially agreed with the hematological studies done by Owoade et al.,[55], which used graded Tramadol doses of 10 mg/kg, 50 mg/kg, and 100 mg/kg administered over a 28-day period. Comparing the Tramadol-*Lacasera*® treated group to the Tramadol-deionized group shows a dose-dependent decrease in PCV, Hb, and RBC .Similarly, all of the hematological parameters recorded in the study agreed completely with the study conducted by El-shafey [56] .Normally, decreases in such blood parameters would indicate anemia caused by hemolysis[57];[58] Based on the findings in the *Lacasera*®-Tramadol treated group, the combination could worsen or lead to severe anemia if used for a considerable amount of time. The significant increase in WBC observed was consistent with previous research by Aldiwan *et al.,* [43], which indicated possible immunological stimulation. According to Mintzer et al.,[49], almost the full spectrum of hematological abnormalities can be brought on by drugs that impact white cells, red cells, platelets, and the coagulation system. Moreover, the findings were consistent with those of Mohammed et al.[59] , who indicated that Tramadol caused platelet aggregation..



Values are presented as mean ± Standard Deviation, n =5. nsp>0.05: Not statistically different from W, although not by much (water group).
*p<0.05: Statistically different from W in a significant way (water group).

Figure 5 Effects of graded doses of Tramadol-deionized water versus Tramadol-*Lacasera*® on the Platelet of Wistar rats

3.4. Weight Changes in Rats Administered Tramadol-*Lacasera*® Versus Tramadol-Water Combination Within Four Weeks of Study

Figure 6a shows mean differences in weights of Wistar rats administered 35 mg/kg, 70.7mgkg and 106 mg/kg of Tramadol dissolved in deionized water for 28 days (4 weeks). Figure 6b shows mean differences in weights of animals administered 35 mg/kg, 70.7mgkg and 106 mg/kg of Tramadol dissolved in *Lacasera*® soft drink for 28 days (4 weeks).A combined plot of data obtained in figures 6a and 6b was used in plotting figure 6c. In comparison of figures 6a and 6b, the weekly mean weight changes of rats in the control (L) group treated with *Lacasera*® soft drink increased more progressively than those in the tramadol-deionized water control group (w). *Lacasera*® , like other carbonated beverages, contains acidulants such as citric, malic, and phosphoric acid, as documented by Kregiel ,[12]. Since *Lacasera*® was always administered after feeding the rats, the presence of these acidulants increases the stomach's acidity, which aids in the breakdown of food for faster digestion[60]. This could have accelerated the digestion and absorption of the feed consumed by the rats, given the ease with which acidic xenobiotics are absorbed in low pH environments such as the stomach [61].

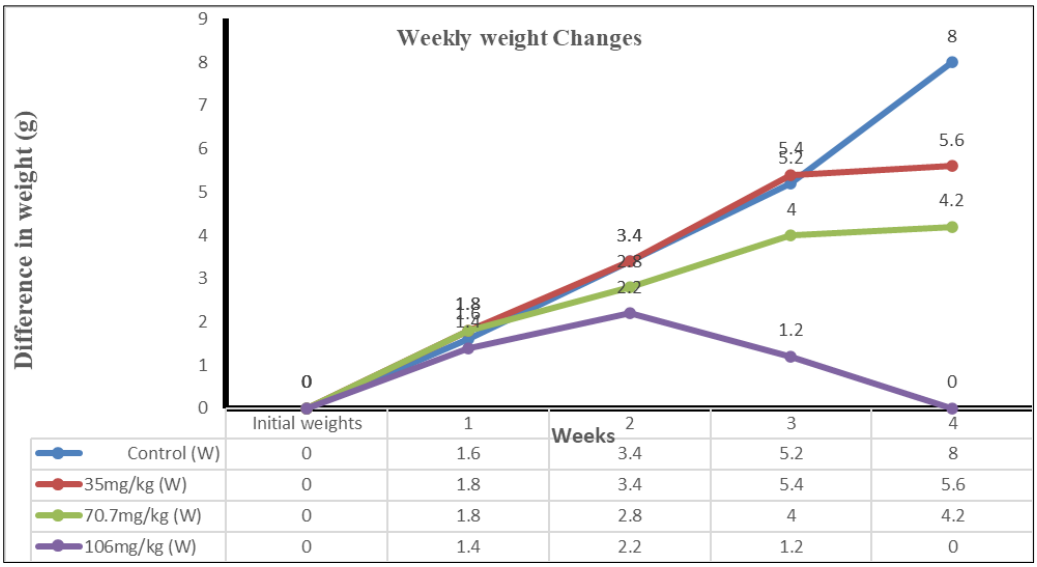


Figure 6(a) Mean differences in weights of Wistar rats administered 35 mg/kg, 70.7 mg/kg and 106 mg/kg of Tramadol dissolved in deionized water for 28 days (4 weeks)

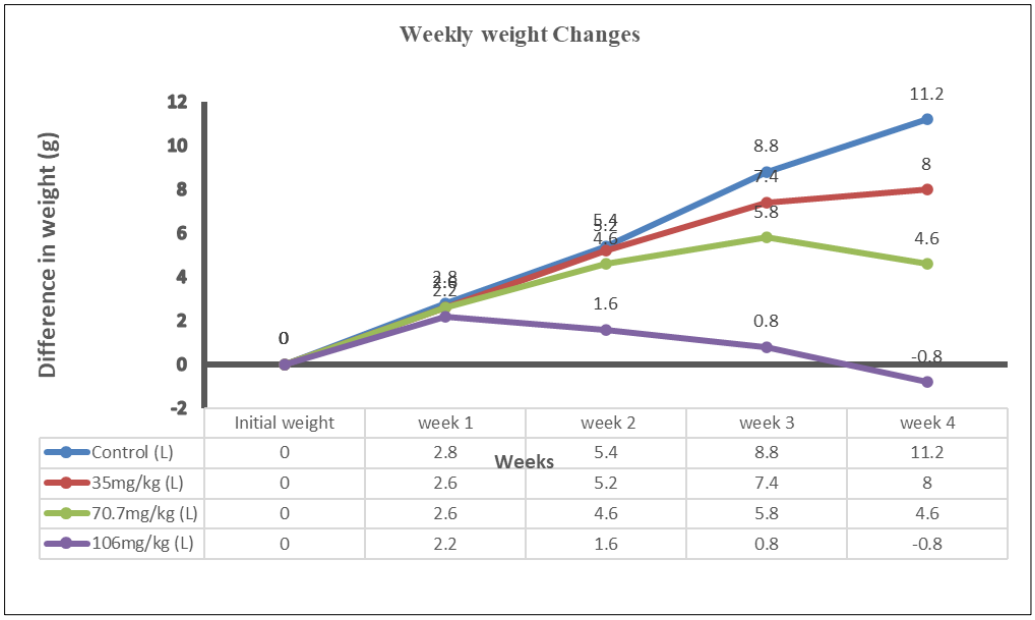
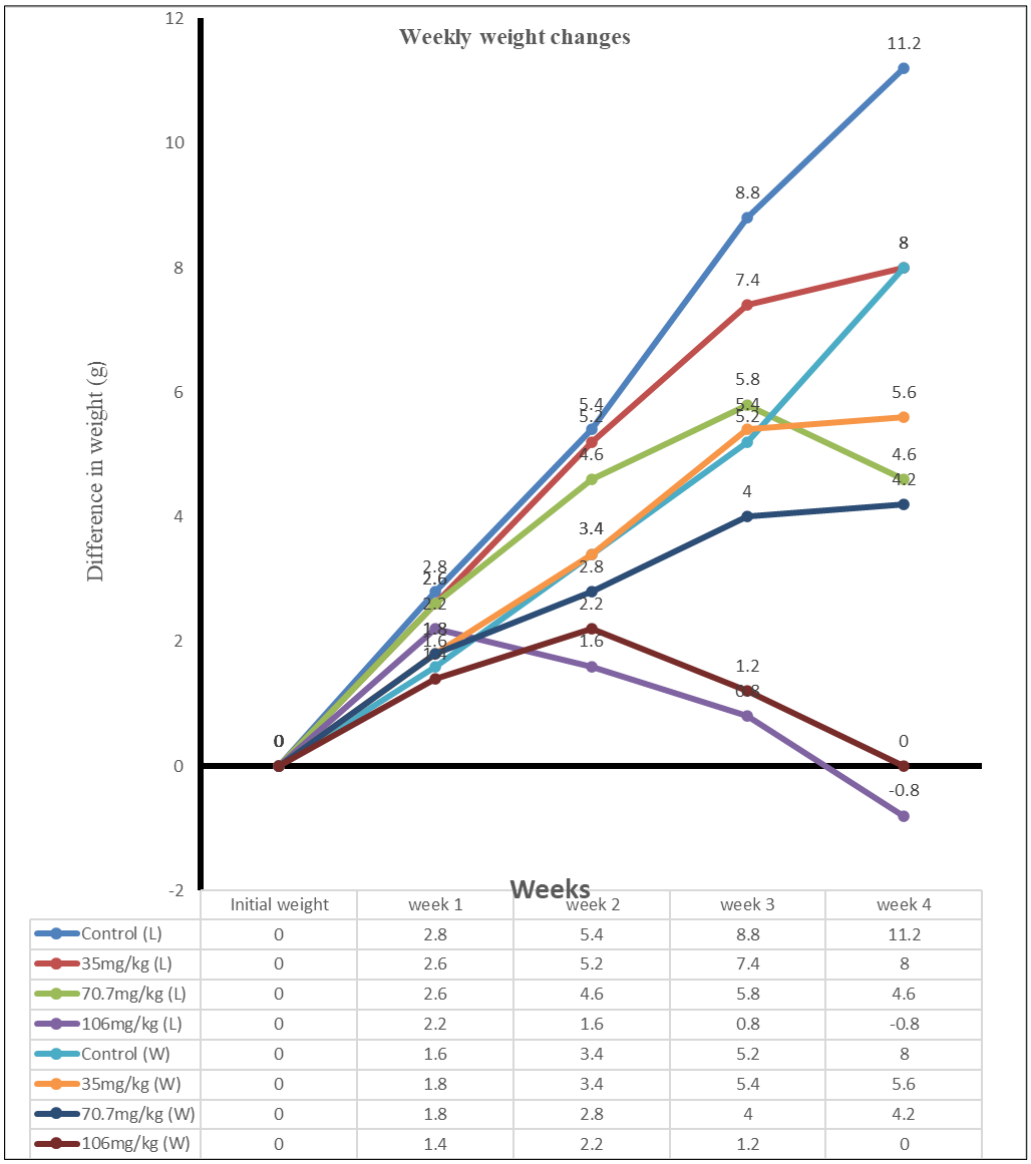


Figure 6(b) Mean differences in weights of animals administered 35 mg/kg, 70.7mgkg and 106 mg/kg of Tramadol dissolved in *Lacasera*® soft drink for 28 days (4 weeks)

Moreover, it is possible that *Lacasera*® soft drink activated the rats' reward system, resulting in hedonic hyperphagia[62], (Hoch et al ., 2014) in *Lacasera*® -treated rats, given the increased rate of feed consumption, which results in weight gain. However, an independent t-test comparison of the mean weights in the two control groups reveals a statistically significant increase ($p<0.01$) in weight gain in the *Lacasera*®-treated group and a moderate positive correlation ($r=0.68$) between rats administered *Lacasera*® soft drink and rats in the first control (W) group treated with deionized water [63],[64]. Moreover, a moderately positive correlation ($r=0.62$) was found between weight gained in the Tramadol-*Lacasera*® treated group and weight gained in the tramadol-deionized water treated group in an independent t-test comparing the mean weights of the two groups at a dose of 35 mg/kg.



	Initial weight	week 1	week 2	week 3	week 4
Control (L)	0	2.8	5.4	8.8	11.2
35mg/kg (L)	0	2.6	5.2	7.4	8
70.7mg/kg (L)	0	2.6	4.6	5.8	4.6
106mg/kg (L)	0	2.2	1.6	0.8	-0.8
Control (W)	0	1.6	3.4	5.2	8
35mg/kg (W)	0	1.8	3.4	5.4	5.6
70.7mg/kg (W)	0	1.8	2.8	4	4.2
106mg/kg (W)	0	1.4	2.2	1.2	0

Figure 6 (c) Combined plot of figure 8(a) and (b)

According to Gale and Zhang [65], at a dose of 70.7 mg/kg, an independent t-test comparison of the two groups' weekly mean weight changes reveals a p-value ($p<0.05$) that is not statistically significant .According to [63], the mean weights of the two groups had a weakly positive relationship ($r=0.38$). However, in figure 6c, the plot of mean weight differences versus weekly intervals demonstrates that rats treated with a 70.7 mg/kg Tramadol-*Lacasera*® combination gained weight rapidly from week 1 to week 3, after which the group's mean weight gradually decreased until week 4, when the study was terminated. The rise in mean weights during weeks 1-3 could be due to the *Lacasera*® soft drink activating the rat brain's reward system, resulting in hedonic hyperphagia[62] in Tramadol-*Lacasera*® treated rats. McCrickerd & Forde [66] acknowledge that sensory qualities of meals and beverages are active before, during, and after an eating event. Consequently, rapid weight gain may also be as a result of *Lacasera*® *'s* acidulants-facilitated digestion and absorption, as acidic xenobiotics are easily absorbed in low pH environments such as the stomach [61]. However, as the study progressed beyond week 3, toxicity may have occurred, resulting in the rats ingesting less feed and gradually losing weight. This is to be expected given that time-dependent xenobiotics Dawson et al., [67]may exhibit or produce observable harm over time.

Moreover, a comparison of the mean weights of rats receiving 106mg/kg in both groups reveals that they are not statistically significant ($p>0.44$), implying a weak positive correlation ($r=0.16$) between the mean weights obtained during the four-week study . Interestingly, in figure 6c, the Tramadol-*Lacasera*® treated group lost weight consistently after week 1, whereas the tramadol-deionized water treated group lost weight starting in week 2. The earlier onset of toxicity in the Tramadol-*Lacasera*® treated group is consistent with Kregiel [68]. At supra-therapeutic levels, this study

suggests that the Tramadol-*Lacasera* soft drink combination would likely cause more toxicity than the tramadol-deionized water combination.

4. Conclusion

It is logical to assume that the findings of this study would have similar effects on humans as they did on rats, given the physiological similarities that exist between the two species. Our findings imply that even at normal therapeutic doses, off-label chronic users of the Tramadol-*Lacasera*® combination for euphoria and premature ejaculation are most likely to develop haemolytic anaemia. However, neither irregular Tramadol users at normal doses nor irregular *Lacasera*® users are likely to suffer major health consequences. Our findings imply that when Tramadol and *Lacasera*® soft drink are combined, utilizing *Lacasera*® to mask Tramadol's bitter taste, haematological toxicity occurs even at conventional therapeutic doses of Tramadol. We anticipate that continued off-label use of this combination in humans may negatively impact the health of young individuals who abuse it. This, especially at supra-therapeutic doses, could eventually lead to a haematological disorder and weight loss, thereby posing a public health risk.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflicts of interest. The final manuscript was read and approved by all writers.

Statement of ethical approval

The animals used in the study were handled in accordance with the National Research Council's Guideline for Laboratory Care of Animal and Use**.** The study was extensively supervised and approved by Nnamdi Azikiwe University's Animal Research Ethics Committee (AREC), Awka. Reference approval number of this study is *NAU/AREC/2021/00008B.*

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