Chronic Toxic Effects of Chocolate Brown HT Dye on Hepatorenal Functions *In Vivo*

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Abstract

Background: Synthetic colors are prevalent in modern food processing, with Chocolate Brown HT (E155) being widely used to meet the demand for chocolate colors. This study examined the long-term health effects of E155 on both male and female subjects. Methods: Six treatment groups received E155 in low to high dosages (200, 400, and 600 mg/kg body weight) for 40 weeks, while control groups consumed a normal diet. After the feeding period, biochemical and histological evaluations were conducted alongside regular physical observations. Results: The female high-dose (FHD) group exhibited the most significant decrease in body weight. Body Mass Index (BMI) dropped notably in females at moderate (FMD) and high doses (FHD). Serum levels of cholesterol, LDL, and triglycerides increased dose-dependently, with males being more susceptible. Elevated SGPT and SGOT levels indicated liver function impairment due to E155. Both genders showed centrilobular necrosis and fibrosis at high doses, with immune cell invasion even at low doses. Serum creatinine levels, especially in males, were significantly elevated. Females experienced severe injuries, including arteriolar hyalinosis at low doses and IgA nephropathy in the FHD group. Conclusion: The findings underscore serious public health concerns regarding the long-term intake of E155, which can cause significant hepatic and renal damage, particularly in females. This highlights the need for regulatory review and potential restrictions on the use of E155 in food products.

Keywords: Brown HT, Lipid Profile, Centrilobular Necrosis, Hyalinosis, Nephropathy

Significance | This study showed the gender-specific toxicological impacts of Chocolate Brown HT as safer food colorant practices and regulatory guidelines.

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Introduction

The visual appeal of food and drink is attributed to their color. To enhance the visual appeal of food and beverages, one can incorporate more vibrant colors. Therefore, the food business develops chemicals that have the ability to transform the visual presentation of a recipe into a vibrant and seemingly limitless array of colors(Saxena & Sharma, 2015). Most natural or artificial food colors that have undergone conventional toxicity studies have been found to be hazardous. The ingestion of some food colors can lead to liver damage. These compounds have the ability to undergo a transformation within our bodies, resulting in the production of extremely reactive free radicals that cause damage to our liver cells. Given that hepatocyte injury can be inherently detrimental (Escobar et al., 1996). The prevalence of artificial colors in food, either alone or in combination with natural colors, has significantly increased. Approximately 800,000 kg of synthetic food colors are manufactured and utilized annually on a global scale. The colors employed can be classified into two main groups: dyes that are

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chemically inactive and do not have any impact on living organisms, and dyes that can lead to detrimental health consequences. Approximately 10-15% of the dyes manufactured in the food business exhibit a propensity to migrate into the environment instead of remaining confined to the dyed food(Moutaouakkil et al., 2003). Prior to being included into the human food supply, food additives undergo a very stringent testing process compared to other types of chemicals. Food additives undergo routine toxicological testing to evaluate their acute, sub-acute, and chronic toxicity. However, we need to observe the outcome and functionality of the product once it is released and being utilized. Obtaining information about the long-term safety of utilizing these compounds and the likelihood of individual reactions to them is challenging to locate. Individual responses can vary due to factors such as the dosage of the chemical they were exposed to, their age, gender, health condition, and genetic composition. Additionally, the duration of exposure to chemicals and additives in their diet at low concentrations can also influence their response(Elbanna et al., 2017). Recently, there has been an increased concern about the use of natural components as a substitute for artificial chemicals in order to mitigate the adverse consequences associated with the latter. The adverse effect of dye on people is related to its degradation process, which generates potentially cancer-causing aromatic amines. These amines have the ability to build up in the body and have been demonstrated to cause the formation of bladder, breast, and other forms of cancer. Azo dyes can undergo conversion into aromatic amines by bacterial activity in the intestines or via specific enzymes present in the liver or stomach(Chequer et al., 2011). Undoubtedly, azo dyes will undergo complete conversion into aromatic amines through reduction. Subsequently, these substances can function as substrates for NADPH-dependent enzymes that are classified as cytochrome P450 monooxygenases. Subsequently, these enzymes will catalyze the oxidation of aromatic amines to N-hydroxy derivatives by reacting with molecular oxygen (O2)(Demirkol et al., 2012). This type of metabolic conversion takes place in various mammalian species, including humans(Chequer et al., 2011). The undesirable consequences of azo dyes, like as mutations, cancer, and toxicity, can arise from either the dye itself or the disintegration of the azo bond between the two benzene rings(Chequer et al., 2011). Prolonged exposure to azo dyes might lead to various health complications. These can vary from relatively basic illnesses like anemia, to significantly more severe ailments such as liver, brain, kidney, and spleen disorders, or the formation of tumors and malignancies. They can also result in detrimental outcomes for the offspring of individuals who have utilized them, including congenital abnormalities, ocular issues that may lead to vision loss, and even impaired physical development(Sayed et al., 2012).

The aesthetic appeal of food products greatly impacts the views of individuals during their purchase, particularly youngsters who prefer to prioritize vividly colored foods such as chocolate cake, cookies, ice cream, and candy, among others. Among all the colors used in food, the chocolate color stands out for its exceptional versatility and extensive application in the bakery and food processing industry. Therefore, we have selected chocolate brown HT dye for our investigation. Chocolate Brown HT, also referred to as Brown HT, is a synthetic bis-azo dye that is extensively utilized as a food coloring agent. This dye is primarily used to enhance the color of a wide variety of food items, including chocolate cakes, brown bread, biscuits, baked goods, snacks, soft drinks, ice cream, puddings and sauces, cheese desserts (including flavored milk products), yoghurts, jams, fruit products, dessert mixes, canned meat, sugar confectionery, flour confectionery(Bawazir, 2012; EFSA Panel on Food Additives and Nutrient Sources (ANS), 2010; Hong et al., 2014; Shokrollahi & Ahmadi, 2017). The purpose of this study is to thoroughly examine the effects of long-term Brown HT exposure on gender-specific reactions. To do this, we used comprehensive serum biochemical assays to analyze key markers of liver and kidney function as well as perform a thorough evaluation of liver and kidney tissue histopathology. By examining these markers in males and females, we hope to gain a much more nuanced and detailed understanding of the specific hepatorenal damage that exposure to long-term Brown HT can cause in either gender. The findings of this investigation could generate a major breakthrough in comprehending the physiological and toxicological repercussions of Brown HT.

2. Material and methods

2.1.1 Study Area

The study was carried out in the Department of Applied Nutrition and Food Technology, Islamic University, Bangladesh. The biochemical analysis was conducted at Doctor's Lab, Kushtia. The histological examinations of the selected organs were carried out in the anatomical laboratory, National Medical College, Dhaka Bangladesh.

2.1.2 Chemicals and reagents

Chocolate Brown E155 (ECHO Food Color and Aroma LTD), Uric Acid Liquicolor (Human GmbH, Germany), Creatinine Liquicolor (Diasys, Germany), SGOT (Chronolab Ag, Switzerland), SGPT (Tulip Group, India), Direct Bilirubin (Randox, UK), Cholesterol Liquicolor (Biocon diagnostic, Germany), Triglyceride Liquicolor (Biocon diagnostic, Germany), LDL Cholesterol Liquicolor (Diasys, Germany).

2.1.3 Animal grouping and experimental design

All experimental protocols were approved by the Animal Ethics Committee of the University of Islamic University (Reference NO: FBS/ERC/IU-2021/05) under the guidelines “Guide for the care and

use of laboratory animals"(National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals., 2011). Both male and female Long-evens rats (8–9 weeks) were obtained from the icddr,b. Rats were individually housed in a temperature-controlled (23±1℃), 12-hour light/dark conditions with 12g of cack and water. Rats were randomly distributed into eight groups, each with 5 rats. Male and female Long-evens rats that were not fed Brown HT are included in control group and named as MC and FC respectively. Male and female groups feeding low dosage of brown HT (200 mg/kg BW/Day) were named as MLD and FLD respectively. Moderate dosage (400 mg/kg BW/Day) were fed to male and female groups named as MMD and FMD respectively. While high dosage (600 mg/kg BW/Day) were given to male and female treatment groups termed MHD and FHD respectively. The experiment was carried out for a period of 40 week.

3.1.4 Body Mass Index (BMI) and Lee's Index

The body weight of the rats was determined using a digital scale. The rats were weighed the day before each investigation, then twice a week, and finally on the last day of the experiment. The length from the nose to the anus was measured in centimeters, and the weight was measured in grams at the end of the trials. These measurements were used to calculate the Body Mass Index (BMI) in gm/cm^2.(NASCIMENTO et al., 2008)

3.1.5 Blood sampling

Blood samples was collected after 9 months and the end of the experiment from the direct cardiac puncture of each rat. The blood sample was allowed to coagulate at room temperature and centrifuge at 3000 rpm for 10 min for separation of serum to determine the biochemical tests(Eraslan et al., 2007).

3.1.6 Biochemical analysis

The enzymatic colorimetric methods with commercial kits were used to measure the concentrations of serum cholesterol, triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), serum glutamate pyruvate transaminase (SGPT), and serum glutamic-oxaloacetic transaminase (SGOT). These measurements were conducted using an automatic analyzer (Labomed, Inc BAS-100TS, Los Angeles, CA, USA).

3.1.7 Histopathological examinations

Liver and kidney tissue specimens were separated and small pieces from them were taken, fixed in neutral buffered formalin 10%, dehydrated, cleared, and paraffin ionized for paraffin blocks and 5-µm sections were obtained, mounted on glass slides, and stained with hematoxylin and eosin (H&E)(Ahmed et al., 2020) and examined under confocal microscope (Nikon Ti2).

3.1.5 Statistical analysis

The analysis of the data from the control and Chocolate Brown HT exposed groups was performed using ANOVA, followed by post-hoc Tuckey HSD using SPSS version 21 for windows. A Student's t-test was conducted for matched values of males and females. A significance level of $p<0.05$ was employed to ascertain statistical significance.

4. Result

4.1 Physical Parameters

4.1.1 Body weight

Figure 1 represents the body weight of male and female rats over a span of 40 weeks, starting from the 1st week. It is evident that the weight of male control rats (Group MC) did not exhibit a significant increase after the 20th week ($p>0.05$). There was no significant difference in the weight of female control rats after the 30th week, indicating stability. The data clearly indicates that the body weight of male rats fed with brown HT tended to decline after the 20th week. By the 40th week, all the groups that were given brown HT experienced a significant loss in weight, which was even lower than their weight at the 20th week. The weight of control has increased by 195.92% for males and 161.05% for females from the 1st week to the 40th week. The percentage increase in the male treatment groups reduced by 145.92%, 109.31%, and 94.06% for doses of 200, 400, and 600 mg/kg body weight, respectively. In the female treatment group, the rate of body weight rise is reduced by 141.09%, 101.13%, and 73.62% at doses of 200, 400, and 600 mg/kg body weight, respectively.

4.1.2 Body Mass Index

The long-term ingestion of chocolate brown HT had a significant impact on the body mass index (BMI) of both male and female rats, when compared to the control groups (Table 1). The One-way ANOVA analysis demonstrated a statistically significant influence of the period of time on the alterations in BMI. In case of males, the rats who were given a diet of 200 mg/kg and 600 mg/kg body weight experienced a drop in BMI from the 1st to the 40th week, with reductions of 5.88% and 4.08% respectively. Surprisingly, the group that consumed a diet of 400 mg/kg body weight showed an increase in BMI of 3.85%. In contrast, female treatment groups that were given chocolate brown HT at doses of 200, 400, and 600 mg/kg BW experienced a reduction in BMI of 10.42%, 29.41%, and 30.77% respectively, demonstrating the dose-dependent effect. It clearly shows (Table 1) that nearly all the groups experienced a significant decrease in BMI at the 10th week. Subsequently, there was a gradual increase in BMI until the 20th to 30th week, after which it began to reduce again due to the influence of the brown HT color.

4.2 Biochemical Observation

Table 2 demonstrates the impact of varying concentrations of chocolate brown HT color on male and female rats. A t-test was conducted to examine the impact of gender on the manifestation of the detrimental effects of this dye. A one-way analysis of variance

(ANOVA) was conducted to examine the differences between the control and treatment groups.

4.2.1 Effect of chocolate brown HT on lipid profile

Varying concentrations of the chocolate brown HT color had a significant impact on the lipid profile of male and female rats, as seen in Figure 2. Following a 40-week period of feeding, the serum cholesterol level in both male and female rats showed a substantial increase when compared to the control group. The rise in low and moderate dosage group in both genders exhibited statistical significance. However, there were no significant differences between moderate and high dosage group. The results also indicated a statistically significant difference ($p<0.05$) in serum cholesterol and LDL levels between male and female treatment groups (Table 2). The levels of serum LDL and triglyceride increased dramatically in direct proportion to the dosage, surpassing those of the control group although showing no difference between male and females. Conversely, an increase in dosage was associated with a decrease in blood HDL level.

4.2.2 Effect of chocolate brown HT on liver function

Extended intake of chocolate brown HT also exhibited an adverse effect on liver function (Figure 3). The levels of Serum glutamic oxaloacetic transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) have shown a substantial increase in both genders while consuming varying dosages of chocolate brown HT, as compared to the control groups. Serum levels of SGOT and SGPT significantly ($p<0.05$) increased as the dose increased. There was no statistically significant difference in SGPT and SGOT levels between the male and female treatment groups, however a variance was observed in bilirubin levels. The bilirubin level exhibited a positive correlation with the dosage; however, the rate of increase did not reach statistical significance. The male rats who ingested a dosage of 600 mg/kg of body weight dye had the most elevated levels of bilirubin, SGOT, and SGPT.

4.2.3 Effect of chocolate brown HT on renal function

Elevated levels of creatinine were observed in both male and female rats undergoing treatment, in comparison to the control groups (Figure 4). Furthermore, the creatinine levels rose proportionally with the dosage administered. Male subjects exhibited a significantly elevated creatinine level in comparison to female counterparts (Table 2). The treatment groups exhibited an elevation in serum uric acid levels, which progressively rose with higher doses in both males and females. Although there was no significant difference in uric acid levels among the female groups, there were significant differences in uric acid levels across the male treatment groups in comparison to the control group.

4.3 Histopathological Analysis

4.3.1 Liver

Our research has shown that male and female rats may exhibit distinct responses to liver damage caused by Brown HT. These distinct reactions seem to be influenced by the specific alterations in liver tissue composition. Both male and female rats exhibited indications of hepatic injury and the early phases of fibrosis, even when administered a minimal dose equivalent to 200 mg/kg body weight of Brown HT. Consequently, ongoing modifications in the livers of male rats were characterized by the invasion of immune cells and the activation of the cell death mechanism called apoptosis, while the alterations observed in female rat livers mostly impacted the blood vessels in the liver. As the liver damage progressed, the concentration of the Brown HT increased. Male rats exhibited ballooning degeneration and necrosis when administered a dosage of 400 mg/kg body weight. In contrast, female rats had hepatocytes (liver cells) that had undergone atrophy and were in the process of necrosis. The presence of ballooning degeneration in male rats suggests the occurrence of significant cellular damage and swelling in the body, mostly caused by the disruption of cellular equilibrium. Both males and females experienced significant liver damage when exposed to the highest dose of 600 mg/kg, regardless of their body weight. Male rats exhibited centrilobular necrosis and fibrosis, while female rats demonstrated similar centrilobular necrosis and fibrosis patterns to those reported in males. Centrilobular necrosis refers to liver damage mostly localized in the areas of the liver lobules rather than other regions. This form of necrosis typically occurs when the liver is exposed to potentially hazardous substances, particularly in the core regions of the lobes.

4.3.2 Kidney

This study focuses on significant gender-specific differences in the response to exposure to Brown HT. The histopathological abnormalities observed in both male and female rats were influenced by the dosage administered. However, the nature and severity of these changes varied between the male and female. At the maximum dosage, only the female subjects exhibited the presence of IgA nephropathy, a condition that was not evident in males. This suggests that different immune responses may be involved in the process of coloration in bodies, and that hormones may contribute to the likelihood of experiencing this coloration. Male and female rats both exhibit degenerative alterations that may be easily detected using normal histopathology techniques and compared with the control group. Beneficial changes involve the reduction of specific types of inflammation, the contraction of glomeruli, and diverse alterations in the blood vessels of the kidneys that appear to be actively promoting the development of a healthier and well-maintained kidney. The detrimental alterations encompass further indications of fibrosis and hyalinosis. When doing toxicological research, it is crucial to consider the possibility of varying responses between males and females to a specific dosage. This necessitates carefully examining every dosage for the circumstances in which it is administered and the specific location on which it has an effect. Significant disparities in the nature or

magnitude of effects should be regarded with suspicion since they suggest the existence of potential, if not definite, individual susceptibilities. It is crucial to examine the subsequent outcomes that occur when these variables are altered in any manner. Renal impairment has been observed in both male and female rats when administered a dosage of 200 mg/kg body weight. The injury manifests as a withered glomerulus, inflammation in the interstitial region, and irregular renal tubules. Arteriolar hyalinosis was exclusively observed in female rats. Administration of a dose of 400 mg/kg body weight resulted in considerably more significant alterations. Both male and female rats exhibited inflammation and the buildup of hyaline material between the renal tubules. Cellular apoptosis was minimal and predominantly observed in female rats, whereas male rats experienced a substantial reduction in body mass. Moreover, at a dosage of 600 mg/kg, it induced a visible microscopic change in the morphology of the arterial walls in both male and female rats. The arterial wall exhibited a conspicuous increase in thickness, accompanied by bleeding within the wall. This bleeding serves as a potential conduit for the formation of blockages, specifically atherosclerosis. In addition, the male volunteers exhibited vasoconstriction, while the female group showed a correlation with IgA nephropathy, suggesting an enhanced immune response and kidney dysfunction.

5. Discussion

The worldwide use of synthetic dyes, particularly azo dyes, in food has significantly risen. There are several underlying factors contributing to this phenomenon, including the synthetic dye's ability to retain its color for an extended period of time while producing vibrant and bright colors that attract consumers. While relevant authorities regulate the use of artificial dye, there has been a noticeable increase in malpractices such as the use of unauthorized dyes and exceeding the permissible limits of dye. In Bangladesh, a country characterized by underdevelopment and a huge population, the occurrence of food adulteration is widespread, although the regulation of this issue remains inadequate(AK, 2018; Hoque, 2023). Our study aimed to examine the impact of chocolate brown HT, a frequently applied dye by food manufacturers in Bangladesh, on male and female rats in order to identify gender-specific variations in its effects. Previous research has identified a correlation between azo dyes and body weight(Aboel-Zahab et al., 1997; Shaker et al., 1989; Takeda et al., 1992; Zralý et al., 2006). Our investigation found that the chocolate brown HT had a noteworthy effect on male and female rats. The results indicate that continuous consumption of chocolate brown HT hue resulted in notable weight reduction, especially in female rats, aligning with prior studies(Al-Shinnawy & Elkattan, 2013; Hassan & Salman, 2016) The fluctuations in body weight resulted in proportional modifications in the BMI of male and female rats. This could be attributed to a possible decrease in appetite and impaired nutrient absorption. Body weight loss and decline in BMI is regarded as a dependable and sensitive sign of toxicity(Arefin et al., 2017; Ezeuko Vitalis et al., 2007). Therefore, the reduction in body weight and BMI seen in this study may serve as an indicator of the negative impact of the dye. This observation contradicted the results of previous investigations, which indicated that body weight tended to increase after consuming azo dyes(Gautam et al., 2010; Sharma et al., 2005). Abnormal changes in lipid profile were observed in both males and females suggesting the presence of toxicity and the detrimental effects of the chocolate brown HT color. The findings are in line with previous study (ABDEL-RAHIM et al., 2019) which showed that triglyceride level increased as per the dose. Another study also noted a significant increase in the levels of serum triglyceride in rats that were administered synthetic color(Himri et al., 2011). There appears to be a disparity in the effect of chocolate brown HT on lipid profiles between males and females. Excessive consumption of Brown HT has been discovered to have a detrimental impact on the liver, elevating levels of short-term (SGPT) and long-term (SGOT) liver enzymes as well as bilirubin to a hazardous range. This discovery is consistent with prior inquiries(ABDEL-RAHIM et al., 2019; Hassan & Salman, 2016). Elevated values of SGOT and SGPT indicate liver injury. The reason for conducting these tests is to detect the presence of intracellular enzymes in the bloodstream, which indicates that liver cells have been damaged or destroyed during the process of dying. (Abd-El-Rahim et al., 1987; Roosdiana et al., 2019). Research indicates that the presence of Hypercoloration (HT) dye in chocolate brown is linked to kidney injury. The HT dyes are hair colorants that are semi-permanent and contain p-phenylenediamine, which is a well-known nephrotoxin. Extended exposure may result in severe health complications such as renal failure. Creatinine is the most dependable measure of diminished kidney function, and it can be easily obtained in a clinical laboratory environment(Wei et al., 2012). Elevated levels of creatinine may be associated with illnesses such as acute infectious meningitis. There is a hypothesis that levels of creatinine rise due to glomerular hyper-perfusion.(Lautrette et al., 2012; Praga, 2005). Renal insufficiency can lead to elevated amounts of uric acid(Crawley et al., 2022). Higher plasma levels of uric acid can be attributed to either reduced excretion, increased production, or a combination of both(de Oliveira & Burini, 2012).

Male rats administered with 200 mg/kg of Brown HT exhibited cell infiltration, early fibrosis, continuous infiltration, and death of cell nuclei. This newly acquired evidence indicates that exposure to the Brown HT elicits an inflammatory reaction in the body. This occurs prior to and predicts the development of fibrosis. Fibrosis first indicates the beginning of scarring in the liver, while ongoing infiltration is indicative of chronic liver inflammation(Elbanna et al., 2017). Similar alterations were observed in the tissue of female

Table 1. Effects of long-term consumption of chocolate brown HT on body mass index (BMI)

Week	Body Mass Index of Male Rats (BMI)			
	Group MC	Group MLD	Group MMD	Group MHD
1st	0.46±0.04[a]	0.51±0.02[a]	0.52±0.02[a]	0.49±0.01[a]
10th	0.38±0.03[b]	0.38±0.01[b]	0.47±0.01[b]	0.44±0.01[b]
20th	0.51±0.04[ac]	0.48±0.01[ac]	0.58±0.02[cd]	0.54±0.03[c]
30th	0.53±0.02[c]	0.56±0.04[d]	0.55±0.01[dc]	0.49±0.01[a]
40th	0.54±0.02[c]	0.48±0.01[c]	0.54±0.00[ad]	0.47±0.02[a]
Week	Body Mass Index of Female Rats (BMI)			
	Group FC	Group FLD	Group FMD	Group FHD
1st	0.45±0.02[a]	0.48±0.01[a]	0.51±0.04[a]	0.52±0.01[a]
10th	0.31±0.02[b]	0.35±0.02[b]	0.44±0.01[b]	0.47±0.02[b]
20th	0.37±0.01[c]	0.44±0.02[c]	0.39±0.02[c]	0.39±0.02[c]
30th	0.41±0.02[d]	0.50±0.02[a]	0.41±0.02[bc]	0.42±0.02[cd]
40th	0.40±0.02[cd]	0.43±0.03[c]	0.36±0.01[c]	0.36±0.01[c]

Each value in the table is represented as mean ± SD (n = 4). Values in the same column followed by a different letter ([a-e]) are significantly different ($P < 0.05$).

Table 2. Effects of different concentration of brown HT color on biochemical parameters of male and female rats.

Biochemical parameters	Group Control		Group Low-Dose		Group Medium-Dose		Group High-Dose	
	Male (Mean±SD)	Female (Mean±SD)	Male (Mean±SD)	Female (Mean±SD)	Male (Mean±SD)	Female (Mean±SD)	Male (Mean±SD)	Female (Mean±SD)
S. Cholesterol(mg/dl)	68.30±0.84*	64.73±1.92	84.73±3.46**	77.30±1.66	88.50±1.13***	81.65±1.67	90.75±1.21**	84.53±1.64
S. HDL(mg/dl)	36.88±1.46	37.55±0.58	23.75±1.45	24.85±1.11	22.60±0.84	23.75±0.80	19.18±0.77	19.20±0.77
S. LDL(mg/dl)	11.68±0.75	13.25±1.31	15.24±1.96	17.03±0.71	29.05±2.36**	18.70±0.29	35.85±2.61***	23.20±2.04
S. Triglyceride(mg/dl)	54.35±4.32	51.85±3.56	67.93±1.40	65.65±3.09	71.94±8.54	69.03±3.25	80.04±5.45	79.86±0.83
S. Creatinine(mg/dl)	0.68±0.01**	0.60±0.04	0.78±0.01**	0.71±0.02	0.85±0.02*	0.81±0.01	0.97±0.02**	0.90±0.01
Uric Acid (mg/dL)	2.83±0.24	2.48±0.24	2.90±0.24	2.73±0.26	3.18±0.07*	2.93±0.13	3.43±0.11	2.94±0.53
Bilirubin (mg/dl)	0.34±0.04	0.30±0.05	0.35±0.03*	0.30±0.02	0.37±0.03*	0.32±0.03	0.39±0.02	0.37±0.01
SGOT (IU/L)	44.45±1.26**	41.20±1.19	51.85±1.54	48.85±3.09	68.78±2.01	66.28±5.18	79.05±0.96	76.55±4.35
SGPT(ALT) (IU/L)	25.85±2.91	23.33±3.46	33.13±4.43	32.45±4.86	47.35±2.62	45.40±3.26	52.13±1.45	50.60±8.30

N.B.: Each value in the table is represented as mean ± SD (n = 4). Asterisks (*) represent significant differences between male and female (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$).

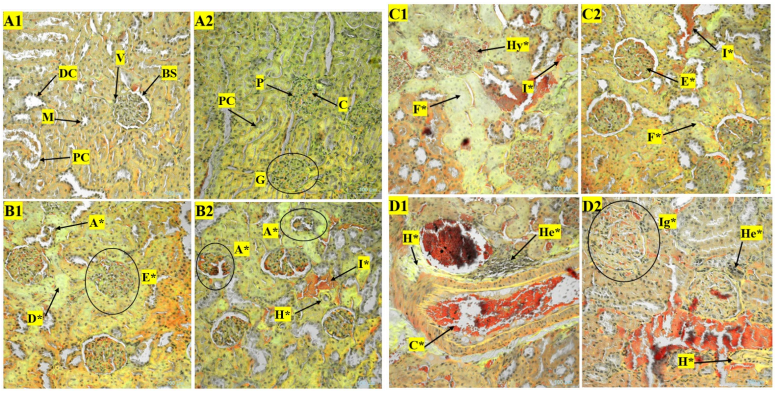

Figure 6 . Investigation of the kidney histological alterations caused by Brown HT (E-155) in rats. In the visualization of object x40 and 100µm scalebar (Mod: Ti2-E Nikon) a section of the liver of (A1) Control Male Rats, (A2) Control Female Rats, (B1) Dose 200 mg/kg body weight in Male, (B2) Dose 200 mg/kg body weight in Female, (C1) Dose 400 mg/kg body weight in Male, (C2) Dose 400 mg/kg body weight in Female, (D1) Dose 600 mg/kg body weight in Male, (D2) Dose 600 mg/kg body weight in Female. A*(atrophied glomerulus), BS (Bowman's space), C(Capillary), C*(congested blood vessel),), DC(Distal Convoluted Tubule), D* (degenerated epithelia of renal tubules), E*(Early crescent), F*(interstitial fibrosis), G (Glomerulus), H*(Arteriolar Hyalinosis), He*(hemorrhage), Hy*(hyalinosis), I*(Interstitial Inflammation), Ig*(IgA Nephropathy), M(Macula Densa), p(podocyte), PC(Proximal Convoluted

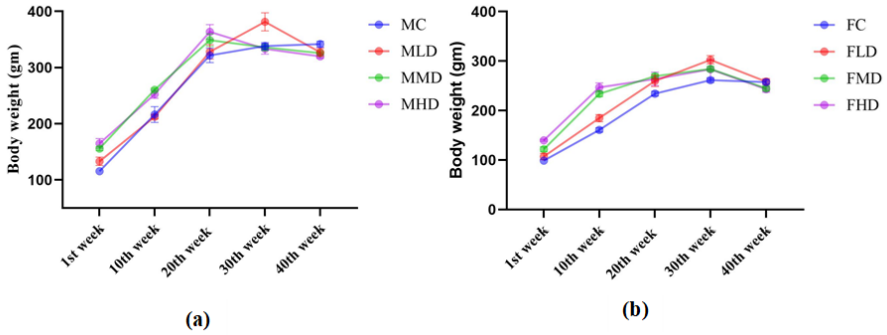

Figure 1. Effects of prolonged intake of chocolate brown HT on body weight of (a) Male and (b) Female groups

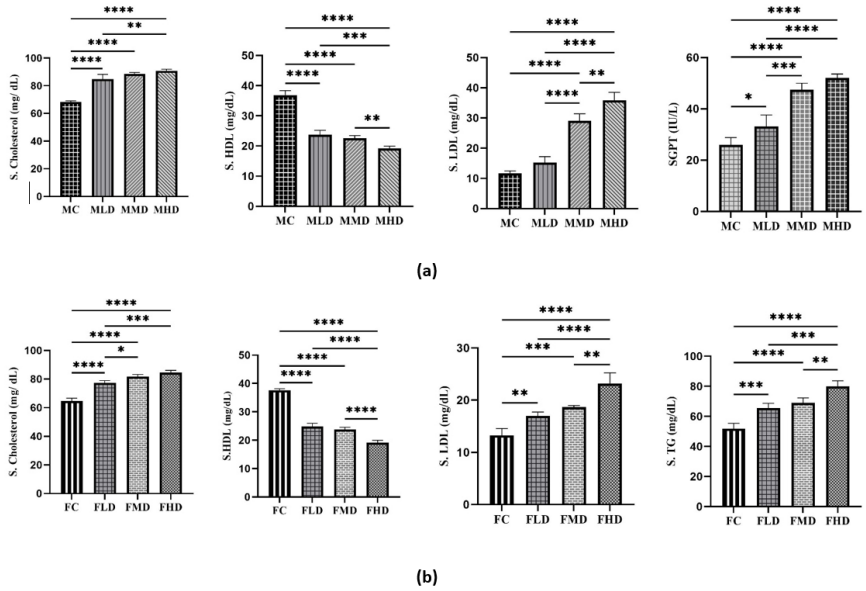

Figure 2. Effects of chocolate brown HT on lipid profile on (a) Male, (b) Female groups [Asterisks (*) represent significant differences between groups*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001]

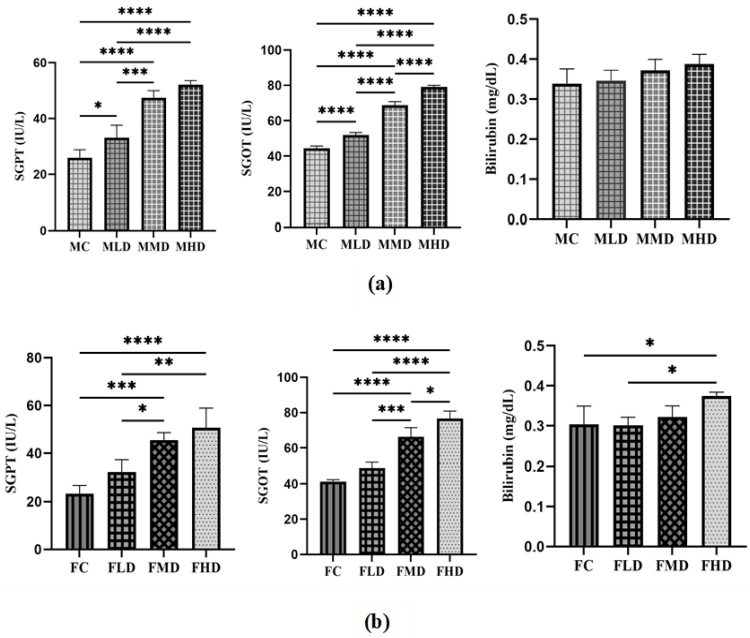

Figure 3. Effects of chocolate brown HT on liver function on (a) Male, (b) Female groups [Asterisks (*) represent significant differences

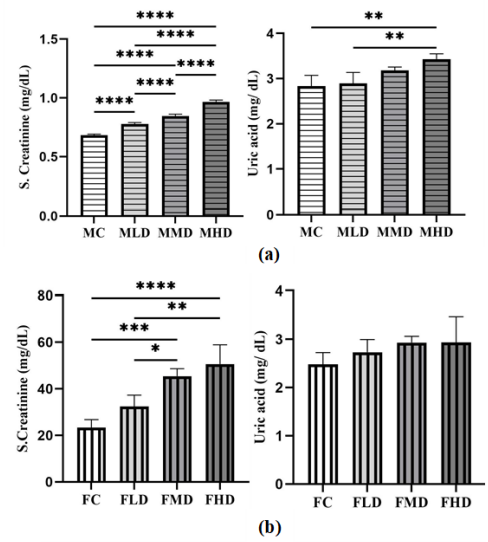

Figure 4. Effects of chocolate brown HT on renal function on (a) Male, (b) Female groups [Asterisks (*) represent significant differences between groups*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001]

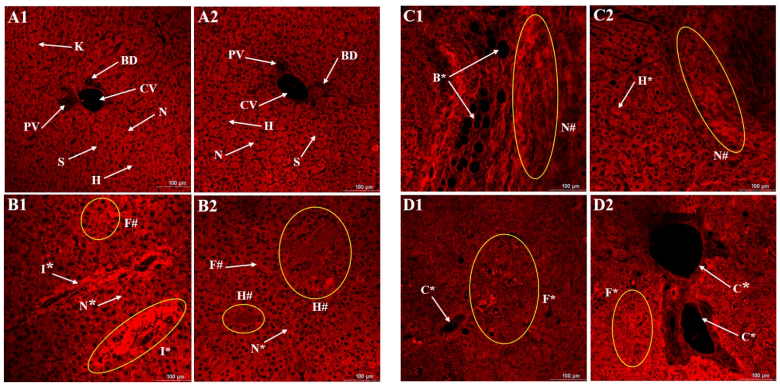

Figure 5. Investigation of the hepatic histological alterations caused by Brown HT (E-155) in rats. In the visualization of object x40 and 100μm scalebar (Mod: Ti2-E Nikon) a section of the liver of (A1) Control Male Rats, (A2) Control Female Rats, (B1) Dose 200 mg/kg body weight in Male, (B2) Dose 200 mg/kg body weight in Female, (C1) Dose 400 mg/kg body weight in Male, (C2) Dose 400 mg/kg body weight in Female, (D1) Dose 600 mg/kg body weight in Male, (D2) Dose 600 mg/kg body weight in Female. CV(Central vain), PV(Portal vain), BD(Bile duct), K(Kuffer cell), N(Neucleus), H(Hepatosite), S(Sinoside), B*(Ballooning degeneration), C*(Centrilobular necrosis), F*(Fibrosis), F#(*infiltration and early fibrosis*), H*(atrophied hepatocytes), H#(Hepatic hemorrhage), I*(Cronic *infiltration*), N*(apoptotic nuclei), N#(Necrosis).

rats with a dosage of 200 mg/kg body weight. These modifications involved elevated levels of white blood cells actively migrating into the tissue. Additionally, there was evidence of initial fibrosis. The presence of bleeding in the liver indicates that the blood vessels have suffered damage, most likely due to the poisonous nature of the dye and its detrimental effects on the cells that line the blood vessels(Hussain et al., 2022). Male rats exhibited ballooning degeneration and necrosis when administered a dosage of 400 mg/kg of body weight. Ballooning degeneration occurs when cells undergo swelling, serving as a precursor to cellular demise. Cells undergo swelling due to the presence of a noxious chemical that they are unable to eliminate(Abd Elhalem et al., 2016). Administration of a dosage of 400 mg/kg resulted in hepatic impairment in female rats. The hepatocytes in their livers underwent atrophy, resulting in a reduction in size. Simultaneously, the liver underwent necrosis, resulting in cellular demise within the afflicted region. If the cells are shrinking and dying, it is evident that their vitality and function are damaged. Without a doubt, the cells being discussed are liver cells, more specifically known as hepatocytes(Alaguprathana & Poonkothai, 2021). Female rats with a specific dosage of Brown HT display hepatic tissue necrosis. The observed phenomenon in male rats at that dosage level is consistent with the toxicity to the liver that can occur at that concentration of Brown HT. Male and female rats subjected to the maximum tested dose (600 mg/kg) both showed centrilobular necrosis and fibrosis. Central necrosis inside liver lobules signifies severe hepatic injury, often caused by exposure to toxic substances(Elbanna et al., 2017).

Male rats with a dosage of 400 milligrams per kilogram of body weight of Brown HS exhibit evident indications of renal inflammation and enlargement. The pathological investigation shows significant renal fibrosis. This data indicates significant renal impairment. At this specific dosage, the Brown HT appears to pose a significant risk to kidney health. Fibrosis in the kidney's interstitium can be induced by detrimental conditions, potentially leading to adverse outcomes if left untreated(Ibrahim et al., 2020). Rats that were given a dosage of 400 mg/kg body weight had histological alterations characteristic of lungs with inflammation, fibrosis, and crescentic transformation. These data suggest that the asthma model is feasible. Within this quantity, crescent-shaped structures indicative of glomerulonephritis are observed in the kidneys of female rats. The renal damage that occurs due to this type of exposure is likely to follow an immune-mediated pathway(Barot & Bahadur, 2013). Observing the early creation of crescents may suggest a faster or more intense initiation of immune-mediated harm in female rats. Upon receiving a dosage of 200 mg/kg, the male rats exhibited reduced glomeruli size, early formation of crescents, and the disintegration of epithelial cells in the renal tubules. The presence of glomerular shrinkage and tubular degeneration observed here is consistent with chronic indications of renal damage that have the potential to result in renal failure if the exposure continues without intervention(Amin et al., 2010). Female rats with a dosage of 200 mg/kg of body weight exhibited contracted filters. Nevertheless, the female rats did not exhibit comparable levels of tubule damage in their kidneys, nor did they generate the same quantity of kidney cells that assume a characteristic crescent shape, as observed in the male rats. Both male and female rats exhibited centrilobular necrosis and fibrosis, with a maximal dose of 600 mg/kg. Hepatotoxicity usually occurs as a result of exposure to substances that are harmful to the liver, and it has substantial implications for liver function. When it is found in renal tissues, its existence suggests a harmful insult that has extensive and harmful consequences not just on the kidney but also on several other organs and systems of the body(Elbanna et al., 2017).

6. Conclusion

The results of this study show that both male and female rats experienced significant health effects after prolonged consumption of chocolate brown HT dye. Impaired liver and renal function were indicated by anomalies in lipid profiles, abnormal increases in serum creatinine, SGOT, and SGPT, and abnormal decreases in body weight. Liver histology also showed immune cell invasion, ballooning degeneration, atrophy, and necrosis as consequences. Symptoms such as arteriolar hyalinosis, a withered glomerulus, inflammation in the interstitial region, and IgA nephropathy were noted, which suggests an augmented immune response and renal failure. The female treatment groups showed a higher prevalence of these symptoms. These data indicate that the widespread use of E155, an azo dye, poses a significant public health risk. The unregulated application of this dye in food could potentially lead to widespread sickness among a large population, resulting in the loss of innocent lives and jeopardizing the national economy.

Author contributions

T.I., I.A.S., A.K.T., and I.S. conceptualized the study. Data curation and formal analysis were led by T.I., with support from N.C.M., S.A.R., and M.A.S. Project administration was managed by I.A.S., I.S., and A.K.T. T.I. and I.A.S. drafted the manuscript, with contributions from I.S. and N.C.M.

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Competing financial interests

The authors have no conflict of interest.

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