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Research Paper

An Exploratory Study of Mammary and Cardiovascular Alterations in Female Long-Evans Rats Chronically Exposed to Brown HT

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ABSTRACT

Background: Chocolate Brown HT (CBHT), a synthetic food color, is widely used in food and beverages but has raised health concerns due to prolonged consumption and its potential to advance diseases, such as cancer. This exploratory study aimed to evaluate mammary and cardiovascular alterations associated with CBHT exposure in female Long-Evans rats.

Methods: CBHT was characterized by gas chromatography-mass spectrometry, and twenty virgin female Long Evans rats were divided into five groups (n = 4 each). The negative control received the standard diet, while the positive control received 7,12-Dimethylbenz(a)anthracene. Brown HT was administered orally at doses of 200, 400, or 600 mg/kg for 50 weeks. Biochemical assays and histopathological examination of mammary and cardiac tissue were used to assess toxicological effects.

Results: Body weight initially increased, then decreased, especially in the F400 and F600 groups. Exposure to Brown HT was associated with a dose-dependent increase in triglycerides, total cholesterol, low-density lipoprotein, Creatine kinase-MB, creatinine, uric acid, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alpha-fetoprotein, and cancer antigen 15-3. High-density lipoprotein decreased compared with the negative control ($P < 0.05$). Mammary tissue showed changes in acinar architecture, cellular atypia, and fibrotic tissue. Heart tissue changes included edema, inflammation, angiogenesis, and hypertrophy, with more evident changes at higher doses.

Conclusion: Exposure to low levels of Brown HT over time led to dose-mediated changes in cardiovascular parameters and mammary tissue in female Long Evans rats. These findings suggest that Brown HT may affect the breasts and the cardiovascular system; however, more research with a larger sample size is required to confirm these results.

Keywords: Alpha-fetoprotein, Cancer antigen 15-3, Chocolate brown HT (CBHT), Creatine kinase-MB, Fibrotic stroma

Introduction

Breast cancer appears as one of the most prevalent cancers affecting women globally. The World Health Organization and the International Agency for Research on Cancer reported that in 2020, there were around 2.3 million new cases of breast cancer worldwide, representing nearly 11.7% of all new cancer cases [1,2]. Cancer is one of the leading causes of death in women because it often deteriorates metabolic disorders, cardiovascular diseases, diabetes, hypertension, renal failure, and neurological disorders. The complexity of breast cancer is influenced by a multitude of factors, particularly those related to the environment and lifestyle [3]. Recent research suggests that synthetic colors or artificial dyes in food, mainly chemical compounds classified as azo dyes, may pose health hazards, including cancer, when ingested over extended periods. Azo dyes are extensively used in food, medicines, cosmetics, and the textile sector, prompting public health concerns [4,5].

Known for its chocolate brown color, Brown HT is an azo dye that is frequently used in a variety of food products, including cakes, candies, baked goods, and ice cream, which are liked by both children and women [6]. Although they can have adverse effects, synthetic food dyes enhance the appearance and accessibility of food. Consuming this dye over a prolonged period may be very harmful to health. It may result in hyperactivity, allergic reactions, and, in certain situations, an increased risk of cancer [7]. Although Brown HT has been extensively studied, many aspects of its toxicity and effects on the human body remain unknown. Though it is frequently found in food in many countries, further study is required to completely comprehend its harmful effects and long-term implications for human health.

When azo dyes enter the body, they are broken down by liver enzymes, particularly CYP1A2. This enzyme breaks down the azo link (-N=N-) in the dyes, turning them into harmful aromatic amines that can be highly reactive and harmful for health [8,9]. Reactive oxygen species (ROS) are extremely reactive compounds that these aromatic amines can produce. ROS cause oxidative stress, which harms DNA, proteins, and lipids. This damage can lead to DNA changes and lipid peroxidation, which can affect cell function and raise the risk of many chronic diseases, including cancer [10,11]. Azo dyes can promote tumor growth by blocking genes that stop tumors from growing, such as p53, and activating inflammatory pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells. Azo dyes are also destructive for the heart because they cause oxidative stress, change the endothelium of blood vessels, raise inflammation, accelerate atherosclerosis, and change how lipids are broken down. These metabolites increase the possibility of hypertension, dyslipidemia, and thrombosis. They disrupt the body's hormonal balance, potentially leading to hypertension and increasing the risk of cardiovascular disease, stroke, and other cardiovascular diseases [12,13].

This study aimed to identify possible toxicological impacts of the widespread food, cosmetic, and textile dye Brown HT using biochemical, histopathological, and chemical characterization approaches. The direct mutagenic activities of Brown HT have been reported to be limited under experimental conditions; however, under certain conditions, the dye might be metabolized to products that could interact with biological molecules. Janet Echelman's previous work has indicated that some azo dye metabolites could play a role in cellular responses, DNA damage, and oxidative stress reactions, but further study of the mechanisms and health effects of Brown HT would be helpful [14,15]. Hence, this study gives a preliminary idea of the possible biological effects from long-term exposure to Brown HT.

Materials and Methods

Chemicals and Reagents

In this study, 801 Chocolate Brown, an azo dye used as a food-coloring agent, was obtained from Choto Raypara, Gazaria, Munshiganj, Bangladesh. The carcinogenic compound 7,12-Dimethylbenz(a)anthracene (DMBA) was sourced from Sigma-Aldrich (St. Louis, MO) for experimental induction. Uric acid reagents were purchased from Human GmbH (Wiesbaden, Germany) for biochemical investigations, while creatinine reagents were obtained from DiaSys Diagnostic Systems GmbH (Holzheim, Germany).

Preparation of Brown HT Food Color for Gas Chromatography-Mass Spectrometry (GC-MS) Process

For the gas chromatography-mass spectrometry (GC-MS) analysis of Brown HT food color, 1.0 g of the food color sample was initially conditioned with 17 mL of citrate buffer solution (0.06 M, pH 6.0) and sonicated (ultrasonic bath) for 15 minutes. The sample was heated in a tightly closed reaction vessel at 70°C for 30 minutes. Subsequently, 3.0 mL of sodium dithionite (200 mg/mL) was added to the reaction mixture, and the temperature was maintained at 70°C for an additional 30 minutes with continuous stirring at 400 rpm. After the reaction, the solution was rapidly cooled by transferring it to a refrigerator and then filtered under vacuum using a sintered glass Buchner funnel into a collecting flask. For the extraction process, a binary solvent mixture consisting of 100 µL 1,2-dichloroethane and 100 µL chloroform was combined with 2.0 mL ethanol and injected into 8.0 mL of the sample solution. The cloudy solution formed was vortexed for 15 seconds and centrifuged at 6000 rpm for 2 minutes to facilitate rapid phase separation. Approximately 60 µL of the sedimented organic phase was carefully transferred into conical-shaped glass insert vials for further analysis. A 1.0 µL aliquot of the extracted solution was then injected into the GC-MS system for analysis [16].

Animals and Experimental Design

Adhering to the principles outlined in the "Guide for the Care and Use of Laboratory Animals" [17], a total of twenty healthy virgin female Long-Evans rats, aged between 7 to 8 weeks and weighing 100-105 grams each, were obtained from ICDDR, B. Prior to experimentation, the animals were adjusted for seven days under standard laboratory conditions, including a temperature of 25°C ± 2°C, relative humidity of 55 ± 10%, and a 12-hour light/dark cycle.

Following the 7-day acclimatization period, the rats were allocated to five experimental groups (n = 4 per group) by simple randomization using a lottery method.

Group 1: Control rats fed normal food (NC)

Group 2: Considered as a positive control, fed normal food with 3 mg/kg body weight of DMBA (7,12-Dimethylbenz[a]anthracene) (PC)

Group 3: Considered as the experimental group; feed normal food with 200 mg/kg body weight of Brown HT (F200)

Group 4: Considered as experimental group; fed normal food with 400 mg/kg body weight of Brown HT (F400)

Group 5: Considered as experimental group; fed normal food with 600 mg/kg body weight of Brown HT (F600)

Biochemical and Bio-cancer Marker Index

Blood was collected from each animal 50 weeks after the initiation of the experiment using the cardiac puncture method. The collected blood samples were allowed to clot at room temperature and subsequently centrifuged at 3,000 rpm for 15 minutes [7]. The alpha-fetoprotein

(AFP) and cancer antigen 15-3 (CA 15-3) levels in serum were measured using the HITACHI Cobas c 411 analyzer from Roche Diagnostics Ltd., a fully automated clinical chemistry analyzer. This serum was subsequently used for various biochemical tests, such as cardiac, hepatic, and renal biomarkers.

Histological Examination

Mammary tumor and heart tissue were fixed in 10% neutral buffered formalin solution to protect the cellular structure. All tissues were then cleaned with xylene and cut into small pieces for embedding in molten paraffin, and dehydrated using alcohol in a conventional dehydration series. Molten paraffin-embedded tissues were cut into 5 µm-thick sections using a microtome. Hematoxylin and eosin were used for staining cells and clear visualization [18]. The histological characteristics of the mammary tumors were examined using a confocal microscope.

Ethical Approval

All animal experiments were performed in accordance with the ARRIVE guidelines and received approval from the Ethical Review Committee of Islamic University, Bangladesh (Approval Ref. No: FBS/ERC/IU-2024/05).

Statistical Analysis

Statistics were elaborated with GraphPad Prism v.10.1.2 software. Before applying parametric tests, the data distribution and normality were tested using the Shapiro–Wilk test. Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test, and the findings of the Brown HT-treated groups were compared to the control groups, which are normally distributed data. Data are presented as means and standard deviation (SD), and the statistical significance level was set as $p < 0.05$.

Results

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Mass spectra and retention times play a crucial role in identifying compounds in samples (Figure 1). Mass spectra, with their unique mass-to-charge ratios, provide a unique fingerprint for each compound, enabling accurate identification. Retention times, on the other hand, indicate the distinct retention behavior of each compound on the chromatographic column. The peak area or peak height in the mass spectrum is directly related to the amount of each compound, allowing for quantitative analysis. The GC-MS analysis of Brown HT food color identified several compounds with different retention times and mass spectra patterns. These compounds include 1,2 Benzenedicarboxylic Acid, 2-Cyclohexyl-isoxazolidin-5-ol, 9 Decen-1-ol, Bicycloocta, 8-Methoxy-2,2,4-trimethyl-1,2,3,4 tetrahydroquinoline, Tetradecanamide, 4,7-Methano-2H-inden-2-one, octahydro-, cis-1,3-Cyclohexanedicarbonitrile, 4-n-Butylthiane, S, S dioxide, and 2,5-Methanofuro[3,2-b] pyridine, octahydro- (Table 1). The NIST database was used to further verify these compounds' identification based on their m/z fragmentation patterns. The unique peaks in the mass spectrum indicate the fragment ions formed when the mass spectrometer ionizes and breaks down the sample.

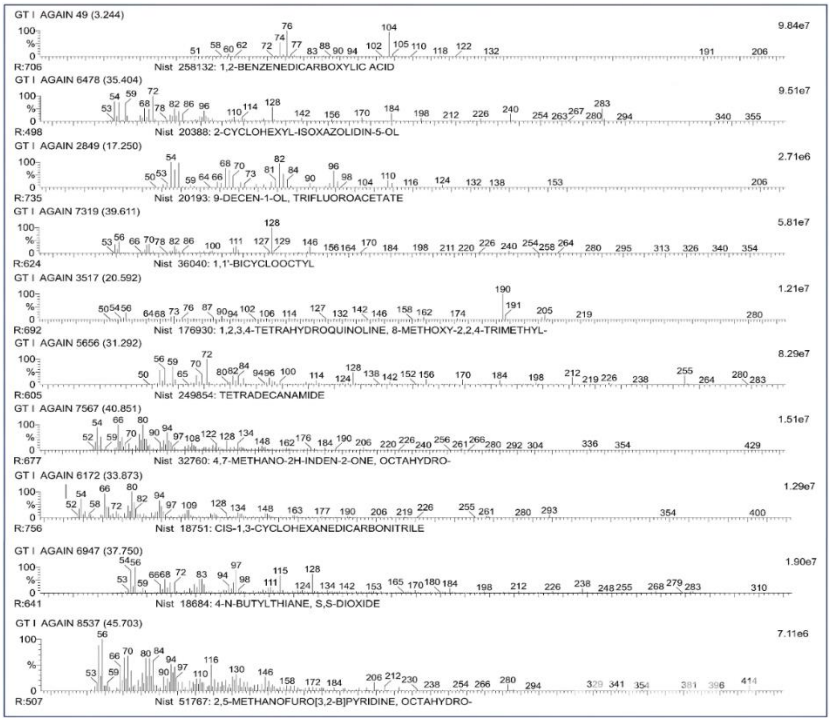

Figure 1. ***Gas*** Chromatography-Mass Spectrometry (GC-MS) Mass Spectra of Several Compounds Detected in Brown HT Food Color, with Corresponding Retention Times (RT) and Molecular Characteristics
The compounds were identified and confirmed by cross-referencing with the NIST Database to ensure accurate chemical profiling.

Table 1. Identification of Compounds in Brown HT Food Color via Gas Chromatography-Mass Spectrometry (GC-MS) Analysis with Cross-Matching to NIST Database

Compound Name	Retention Time	Area %	Molecular Formula	Molecular Weight
1,2-benzenedicarboxylic acid	3.244	0.871624	$C_8H_6O_4$	166.1308
2-Cyclohexyl-isoxazolidin-5-ol	35.404	10.6114	$C_9H_{17}O_2N$	171.2368
9-Decen-1-ol	17.250	0.211305	$C_{12}H_{19}O_2F_3$	252.2733
Bicyclooctyl	39.611	1.444209	$C_{16}H_{30}$	222.4094
8-Methoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline	20.592	0.21861	$C_{13}H_{19}ON$	205.2961
Tetradecanamide	31.292	8.700335	$C_{14}H_{29}ON$	227.3862
4,7-Methano-2H-inden-2-one, octahydro-	40.851	2.384119	$C_{10}H_{14}O$	150.2176
cis-1,3-Cyclohexanedicarbonitrile	33.873	0.58553	$C_8H_{10}N_2$	134.1784
4-n-Butylthiane, S,S-dioxide	37.750	0.058762	$C_9H_{18}O_2S$	190.303
2,5-Methanofuro[3,2-b] pyridine, octahydro-	45.703	1.624167	$C_8H_{13}ON$	139.1949

Body Weight Change

The NC group showed a consistent and linear trend of body weight gain, starting from 100.67 g in Week 1 and increasing to 294.10 g by Week 50. This growth reflects normal physiological development under controlled conditions (Figure 2). In contrast, the PC group exhibited a different pattern, with body weight gradually increasing to 203.79 g by Week 15, but then declining steadily to 165.91 g by Week 50. The F200, F400, and F600 dose-treated groups exhibited a notable trend of rapid weight gain during the first 15 weeks, reaching weights of 280.43 g for F200, 285.43 g for F400, and 283.89 g for F600. However, from Week 20 to Week 50, all three groups exhibited an apparent decline in weight, indicating that the initial doses contributed to rapid weight gain. However, the animals were unable to maintain this growth in the long term in the presence of mammary tumors.

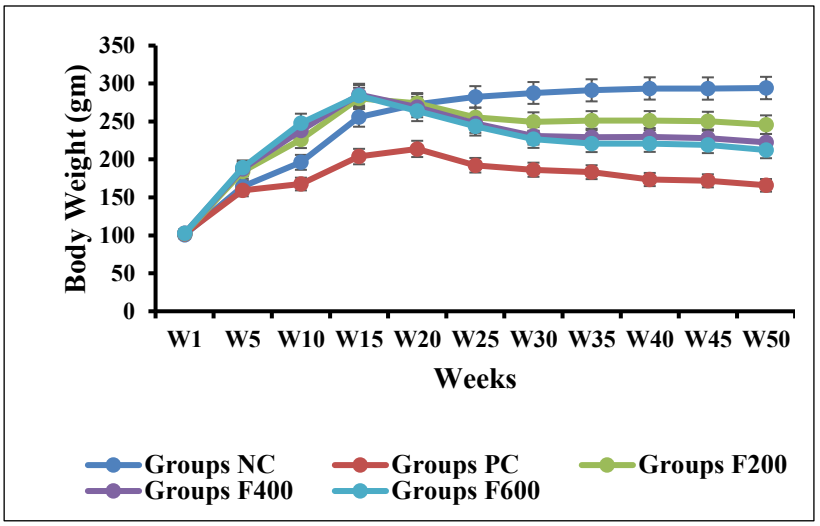

Figure 2. Body Weight Change as Mean ± SD (n = 4) Obtained from One-Way Analysis of Variance (ANOVA) Followed by Tucky Honestly Significant Difference (HSD)
The different experimental groups included NC, PC, and various treatment groups 200, 400, and 600 mg/kg body of Brown HT food color (F200, F400, and F600), over a 50-week observation period.

Biochemical Interpretation of Lipid Profile Parameters

The lipid profile analysis revealed significant group-wise variations in cholesterol levels, reflecting the dose-dependent toxicological effects of the tested compounds (Figure 3). The NC group had a normal cholesterol level of 68.57 ± 2.47 mg/dL. In contrast, the PC group exhibited a substantial increase to 124.97 ± 4.32 mg/dL, a value higher than the control and significant ($P < 0.05$). A gradual rise in cholesterol was observed across the dose-dependent groups F200, F400, and F600, indicating potential dose-related metabolic disturbances. High-Density Lipoprotein (HDL), commonly referred to as "good cholesterol," was highest in the NC group (38.20 ± 1.88 mg/dL). However, in the PC group, HDL levels declined drastically to 16.37 ± 0.45 mg/dL. The treatment groups exhibited a consistent pattern of decreasing HDL levels, with no statistically significant difference between PC, F400, and F600. Low-density lipoprotein (LDL), known as "bad cholesterol," remained within safe limits in the NC group (16.07 ± 1.38 mg/dL), whereas the PC group reached 36.95 ± 0.36 mg/dL. The groups receiving the dose also had higher LDL levels over time. Triglyceride levels in the NC group were normal, but the PC group showed an apparent increase (99.10 ± 1.82 mg/dL) with no significant difference between F600.

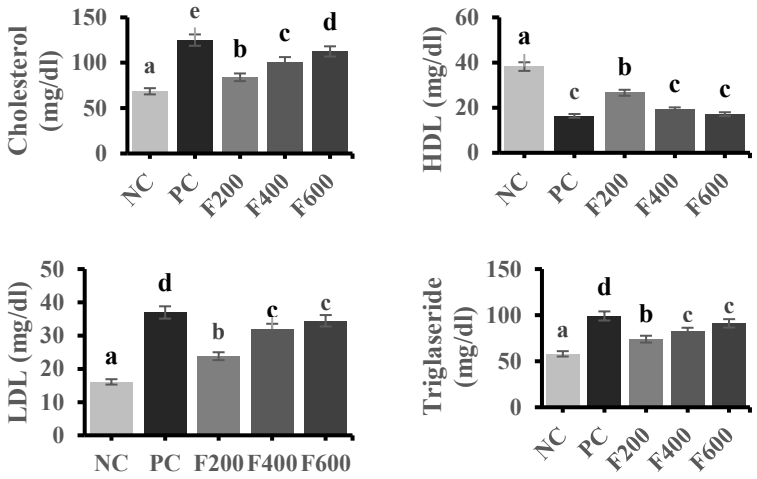

Figure 3. Each Value as Mean ± SD (n = 4) Obtained from One-Way Analysis of Variance (ANOVA) Followed by Tucky Honestly Significant Difference (HSD)

Values in the same bar followed by a different letter (a-e) are significantly different ($P < 0.05$). NC, PC (feed 3 mg/kg B.W of DMBA), F200- feed 200 mg/kg B.W of Brown HT, F400- feed 400 mg/kg B.W of Brown HT, and F600- feed 600 mg/kg B.W of Brown HT.

Evaluation of Cardiac Function

The analysis of Creatine Kinase-MB (CK-MB), a crucial biomarker for cardiac function, indicated significant differences between the groups. The NC group had a CK-MB activity level of 46.36 ± 0.84 U/L, while the PC group had a CK-MB level of 127.43 ± 0.75 U/L, which is almost three times higher than that of the control group. The CK-MB value of group F200 was 83.15 ± 1.5 U/L, which was significantly higher than the NC group. The CK-MB levels in the F400 group increased further, reaching 108.90 ± 1.23 U/L, representing a larger difference from the baseline (Figure 4). Finally, the F600 group had a CK-MB level of 126.77 ± 1.43 U/L, which was almost the same as that of the PC group and not statistically significantly different between the PC and F600 groups.

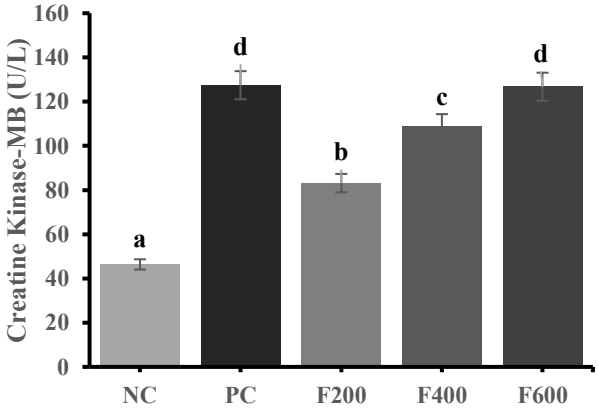

Figure 4. Quantitative Evaluation of Cardiac Function Biomarker Levels in Female Long-Evans Rats, Based on Serum Creatine Kinase-MB (CK-MB) Concentrations across Control and Treatment Groups

Values are expressed as mean ± SD. Bars bearing different superscript letters (a–d) indicate statistically significant differences among groups ($P < 0.05$), as determined by one-way ANOVA followed by a post hoc multiple comparison test.

Comparative Profile of Hepatic and Renal Function Markers

The study analyzed creatinine levels in different groups, with the NC group showing the lowest value at 0.63 ± 0.04 mg/dL. The PC group showed a significant ($P < 0.05$) increase, indicating potential renal stress. The F200 and F400 groups showed moderately elevated levels, but in the F600 group, the creatinine level increased to 1.00 ± 0.06 mg/dL (Table 2). Serum glutamic oxaloacetic transaminase (SGOT) levels were lowest in the NC group, nearly doubled in the PC group, and showed a dose-dependent rise in the F200, F400, and F600 groups, with no significant difference between the F600 groups. Serum glutamic pyruvic transaminase (SGPT) levels were lowest in the NC group, but rose sharply in the PC group to 65.55 ± 1.67 IU/L. Uric acid levels were lowest in the NC group, but increased significantly in the PC and F600 groups. Bilirubin levels were lowest in the NC group, but increased not only in the PC group but also in the treatment groups. Bilirubin levels showed a gradual dose-dependent rise in the F200, F400, and F600 groups. In conclusion, creatinine, SGOT, alanine aminotransferase, and bilirubin levels showed no statistically significant difference between PC and F600.

Evaluation of Tumor-Specific Biomarkers

The serum AFP and CA 15-3 levels among the experimental groups differed significantly ($P < 0.05$) (Table 3). The NC group had the lowest AFP and CA 15-3 levels, while the PC group had the highest levels. There was a tendency for both biomarkers to increase with dose in both the Brown HT-treated groups but were lower than those observed in the PC group. AFP is not a marker exclusively of mammary gland tumors, however, and

neither AFP nor CA 15-3 can be used on its own to confirm malignancy. As such, these elevations were interpreted as a biochemical chnge only, not specifically a carcinogenic or mammary cancer effect.

Table 2. In Vivo Evaluation of Key Hepatic and Renal Biomarkers to Determine Functional Alterations in Response to Brown HT Exposure

Biochemical Parameter	Groups				
	NC	PC	F200	F400	F600
Creatinine (mg/dl)	0.63±0.04^{a}	1.10±0.05^{d}	0.78±0.02^{b}	0.92±0.06^{c}	1.00±0.06^{cd}
SGOT (IU/L)	44.10±2.23^{a}	80.27±1.03^{d}	55.15±2.51^{b}	67.95±3.15^{c}	80.02±3.62^{d}
SGPT (IU/L)	22.87±3.81^{a}	65.55±1.67^{d}	45.77±2.07^{b}	57.52±1.54^{c}	63.30±2.02^{d}
UA (mmol/L)	42.88±0.92^{a}	84.31±2.68^{e}	52.98±0.30^{b}	62.53±1.56^{c}	70.26±1.23^{d}
Bilirubin (mg/dl)	0.27±0.01^{a}	0.39±0.01^{c}	0.32±0.01^{b}	0.36±0.02^{c}	0.38±0.01^{c}

Each value in the table is represented as mean ± SD (n = 4) obtained from One-way ANOVA followed by Tucky HSD. Values in the same row followed by a different letter (a-e) are significantly different ($P < 0.05$). NC- Control, PC- Positive Control (feed 3 mg/kg B.W of DMBA), F200- feed 200 mg/kg B.W of Brown HT, F400- feed 400 mg/kg B.W of Brown HT, and F600- feed 600 mg/kg B.W of Brown HT. Abbreviations: HSD, honestly significant difference; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; UA, uric acid.

Table 3. Serum AFP and CA 15-3 Concentrations in Female Long-Evans Rats

Cancer Biomarker	Groups				
	NC	PC	F200	F400	F600
AFP (ng/mL)	2.06 ± 0.12^{a}	50.41 ± 1.26^{e}	24.12 ± 1.33^{b}	36.94 ± 0.95^{c}	47.85 ± 1.00^{d}
CA 15-3 (U/mL)	67.33 ± 1.42^{a}	143.58 ± 2.22^{e}	82.42 ± 166^{b}	86.47 ± 0.73^{c}	96.35 ± 1.19^{d}

Serum alpha-fetoprotein and cancer antigen 15-3 concentrations in the experimental groups. Values are presented as mean ± SD (n = 4 per group). Values with different superscript letters differ significantly at $P < 0.05$.
Abbreviations: AFP, alpha-fetoprotein; CA 15-3, cancer antigen 15-3; NC, control; PC, positive control.

Histology of Mammary Gland Analysis

The large mammary tumors were found in an experimental rat and surgically removed, indicating significant growth. The tumor's morphology showed a smooth, shiny surface with some discoloration, suggesting internal changes (Figure 5). Histopathological examination of mammary tumor morphology in virgin female Long Evans rats following exposure to Brown HT revealed several notable histological changes. In the positive control group (A), rats were administered DMBA, which caused several notable histological changes. These changes included loss of standard acinar architecture, dense fibrotic stroma, cellular atypia, and hyperplastic glands with variably sized glands and papillary infoldings. In the treatment group F200 (B), similar histopathological alterations were observed, indicating that Brown HT has a tumor-promoting effect, albeit less severe than in the positive control (Figure 6). In rats treated with 400 mg/kg Brown HT (C), further histological changes were noted, including Corpora amylacea, spherical, hyaline bodies within the tissue linked to the accumulation of secretions or metabolic by-products in tumor tissue. Cellular atypia, characterized by irregular cell structure that reinforces potential malignancy, and a dense fibrotic stroma indicating an ongoing scarring process in response to tumor development, were also observed. In the group exposed to continued 600 mg/kg Brown HT (D), additional alterations were observed, including loss of standard acinar architecture, increased number of acinar units, cellular atypia, and dense fibrotic stroma, further demonstrating the body's ongoing response to tumor development.

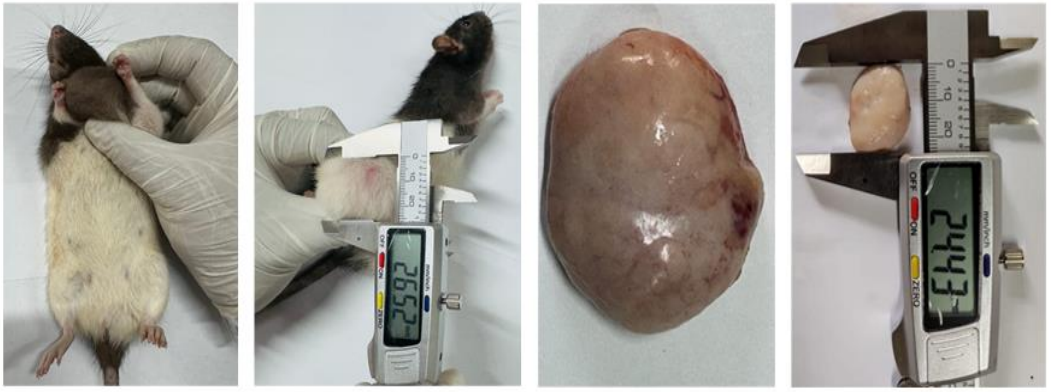

Figure 5. An Experimental Rat with a Mammary Tumor, the Tumor After Surgical Excision, and the Measurements of Both the Rat and the Tumor Taken with a Digital Caliper
Tumors were not found in the control group, were distinctly observed in the positive control group, and showed variability in size and presence within the treatment group.

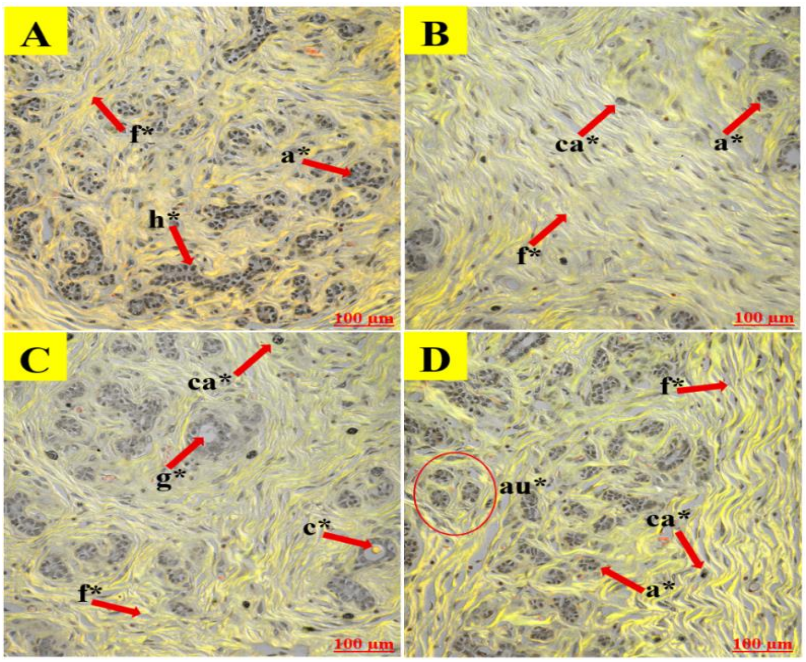

Figure 6. Advanced Histopathological Investigation of Mammary Tumor Morphology in Virgin Female Long-Evans Rats Following Exposure to Brown HT Tissue sections were imaged using a confocal microscope (Nikon Ti2-E) at 40× magnification, with a 100 µm scale bar for reference: (A) Positive control animals administered 7,12-dimethylbenz[a]anthracene (DMBA) at 3 mg/kg body weight; and treatment groups receiving Brown HT at doses of (B) 200 mg/kg, (C) 400 mg/kg, and (D) 600 mg/kg body weight, respectively. a* (Loss of normal Acinar architecture), au* (Increased number of Acinar Units), c* (Corpora Amylacea), ca* (Cellular Atypia), f* (Dense Fibrotic Stroma), g* (Glands lined by double layered Epithelium), h* (Hyperplastic glands variably sized glands with papillary infoldings).

Histology of Heart Tissue Analysis

Many changes were observed in hearts after exposure to Brown HT under histological examination, including cardiac edema, cardiac inflammation and cardiac hypertrophy. In the NC-control group (A), normal heart tissue was observed; in contrast, in the PC group (B), marked changes were observed, including cardiac edema, cardiac inflammation and cardiac hypertrophy (Figure 7). In the treatment group (C), the changes were weaker than in the control group; however, the effects on cardiac edema and Angiogenesis were significantly pronounced. The increase in changes of severity was detected in the treatment group (D), and in the group dosed at 600 mg/kg of body weight, additional severe changes were noted. These results highlight the importance of understanding the histological alterations in heart tissue and the impact of exposure to Brown HT on cardiac health.

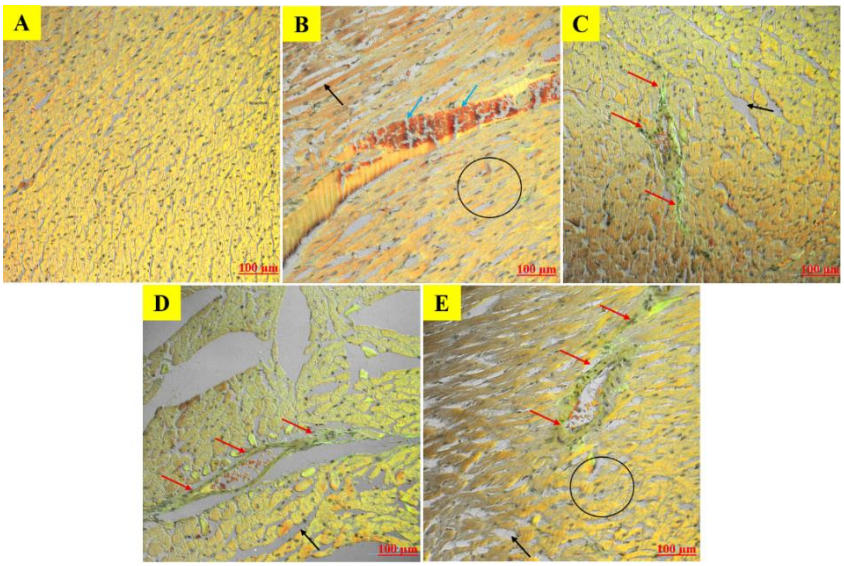

Figure 7. Advanced Histopathological Investigation of Heart Tissue Morphology in Virgin Female Long-Evans Rats Following Exposure to Brown HT Tissue sections were imaged using a confocal microscope (Nikon Ti2-E) at 40× magnification, with a 100 µm scale bar for reference: (A) NC- Control, (B) Positive control animals administered 7,12-dimethylbenz[a]anthracene (DMBA) at 3 mg/kg body weight; and treatment groups receiving Brown HT at doses of (C) 200 mg/kg, (D) 400 mg/kg, and (E) 600 mg/kg body weight, respectively. Black arrow (Cardiac Edema), Read arrow (Angiogenesis), Blue arrow (Cardiac Inflammation), Black circle (Cardiac Hypertrophy).

Discussion

Some azo dyes are metabolized to form aromatic amines, which have been shown in previous toxicological studies to have genotoxic or carcinogenic properties. However, these effects are compound-specific and should remain unexplained, as noted by Brown HT [4,12]. Various effects of some synthetic food dyes on endocrine, metabolic, and cardiovascular functions have also been suggested. Still, there has not yet been much direct evidence linking Brown HT intake to breast cancer or to cardiovascular disease in humans. The results found should therefore be thought of as initial observations at high experimental doses and not as human disease risk [13,19].

Some unexpected compounds, including 1,2-benzenedicarboxylic acid, 4-n-Butylthiane, S, S-dioxide, and 8-methoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline, were discovered by the GC-MS assessment on Brown HT food color. These are typical industrial chemicals used to increase the flexibility of plastics [20,21]. Concerns have been raised regarding the potential health hazards of these substances. Similar chemicals, some of which are hazardous to health, have been discovered in assessments of food colorants in previous studies by Liu et al. (2016) and Khodot et al. (2022). These preliminary GC–MS findings provide a basis for further targeted analysis to confirm their identity and toxicological significance [22,23].

In the study, Brown HT treated groups showed a biphasic pattern of body weight change. Initially, body weight increases rapidly during the first 15 weeks; this is possibly due to metabolic stimulation or increased appetite. However, a consistent body weight decline was observed from week 20 to week 50, which was directly associated with the development of mammary tumors. This pattern aligns with cancer cachexia, a physiological condition where tumors disrupt the body's metabolic balance, leading to nutrient depletion, muscle wasting, and systemic weight loss [24]. Reza et al. (2019) and Smith et al. (2025) investigated the occurrence of metabolic imbalance and tumor-induced protein degradation during the initial increase in body weight, which afterward significantly decreased [25,26]. Overall, the biphasic body-weight pattern may reflect a progressive physiological response to prolonged Brown HT exposure, although the underlying metabolic basis requires further investigation.

Brown HT has shown dose-dependent effects on cardiovascular and metabolic health. The treatment groups exhibited substantial changes in their lipid profiles and cardiac enzyme levels, which could suggest that the exposure was toxic. The total cholesterol and LDL levels of the animals in the treatment groups were higher, despite the significant ($P < 0.05$) decrease in HDL levels. These findings indicate that Brown HT can interfere with hepatic lipid homeostasis and cause dyslipidemia, which inflammatory and oxidative stress pathways could mediate. Similar outcomes were also obtained by John et al. (2022) and Sensoy et al. (2025), who found that prolonged exposure to food dyes of synthetic origin altered lipid metabolism and reduced the activity of liver enzymes in experimental animal models [13,19]. CK MB was very high in both PC and high-dose groups, such that F600 was very high, showing a possibility of the treatment damaging the heart. Overall, the changes in lipid profiles and CK-MB levels were consistent with dose-associated metabolic and cardiac alterations following Brown HT exposure, with the most pronounced changes observed at the highest dose.

During the investigation, higher levels of creatinine, SGOT, SGPT, uric acid, and bilirubin were noted, implying a possible nephrotoxic and hepatotoxic effect. The results obtained are in line with the findings of Islam et al. (2024), which have previously shown that exposure to synthetic food color in animal models can lead to liver enzyme elevation, oxidative stress, and histopathological lesions in hepatic and renal tissues [26,27]. The hypothesis of liver insufficiency and metabolic disorder is supported by the continued rise of bilirubin and uric acid, possibly associated with protein metabolism disorders and inability to discharge bile [28]. These findings suggest that prolonged Brown HT exposure may be associated with adverse biological effects in rats. However, the present study did not evaluate short-term safety, and the findings should not be directly extrapolated to typical human dietary exposure.

Pathohistological changes in mammals, as well as elevated serum levels of AFP and CA 15-3, were observed in all Brown HT-treated groups, especially F600. These changes included disruption of the normal acinar architecture, dense fibrotic stroma, cellular atypia, and glandular hyperplasia after chronic exposure to Brown HT. However, the data cannot be interpreted as evidence of tumor-

promoting activity or carcinogenicity, since AFP is not specific to the mammary gland and CA 15-3 does not always indicate malignancy. Similar changes in serum levels of these biomarkers and mammary tissue architecture were also reported after exposure to some food colorants [29–31]. However, such comparisons must be made with caution because dye combinations, dosage, exposure time, and experimental design differ.

Based on the results of a histopathological study of cardiac tissues by Brown HT, there was a dose-response of cardiotoxic effects. In the NC group, there was no interference with cardiac architecture, whereas in the PC group, there were intense changes, such as cardiac edema, inflammation, and hypertrophy. Both the F200 and F400 treatment groups showed abnormal cardiac changes, whereas the treatment group at the highest dose (F600), with severe pathological changes shows chronic cardiac stress as a result of prolonged exposure. The present results align with those of Israel Oyewole et al. (2016) and Sensoy et al. (2025), who have stated cardiotoxicity in the case of synthetic food dyes [19,32]. Taken together, cardiac histopathological alterations were more pronounced in the F600 group than in the lower-dose groups, indicating a dose-associated pattern of cardiac tissue response under the experimental conditions. However, without functional cardiac assessments and larger confirmatory studies, these changes cannot be interpreted as definitive evidence of cardiotoxicity.

While this study offers several insights into the component weights, limitations warrant discussion. The findings might not be generalizable because of the small sample size, which could not have yielded accurate statistics. Additionally, no specific study assessed potential mechanistic pathways, such as oxidative stress, inflammation, apoptosis, and DNA damage. Histopathological evaluation was not conducted anonymously, and AFP or CA 15-3 should be interpreted with caution, as they are not specific markers of mammary carcinogenesis. The compounds identified by GC–MS were not confirmed with authentic samples. Moreover, the relatively high doses used experimentally in this particular study may not reflect “real world” dietary exposure for humans. Thus, the findings are to be viewed as preliminary and should be further confirmed by larger, well-controlled studies that include mechanisms, validated chemical analysis, and exposure-relevant dose levels.

Conclusions

Females of the Long-Evans rat species exhibited mammary and cardiovascular changes, and higher doses of Brown HT caused greater changes, especially in the F600 group. These changes included mammary epithelial abnormalities, fibrotic changes in the stroma, lipid profile alterations, increased CK-MB levels, and histopathological changes in the heart. Positive results for serum AFP and serum CA 15-3 were also found, but they alone did not indicate a carcinogen or reveal direct causative links with mammary cancer. The small number of samples is a limitation of the study, and the results are preliminary. Future investigation is needed with more samples, proper statistical power, a systematic analysis of the tumors, a blinded histopathological disease evaluation, and a relevant mechanistic endpoint.

Conflict of Interests

There is no conflict of interest that may exist with relation to the presented study.

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Ethical Considerations

All experimental protocols were conducted in accordance with ethical guidelines and received approval from the Ethical Review Committee of Islamic University, Bangladesh (Approval Ref. No: FBS/ERC/IU-2021/05).

Authors Contributions

Tarique Muhammad Tawabul Islam: Investigation, Writing-original draft, Writing-review & editing, Methodology, Conceptualization. **Ivvala Anand Shaker:** Visualization, Methodology, Conceptualization, Writing-review & editing. **Md. Abul Kashem Tang:** Visualization, Methodology, Conceptualization, Writing-review & editing. **Nirmal Chandra Mahat:** Conceptualization, Data curation, Formal Analysis, Visualization, Writing-original draft, Writing-review & editing. **Mustakin Ahmed Shohel:** Conceptualization and Visualization. **Tahsin Bin**

Rabbani: Conceptualization and Visualization. **Bulbul Shaikat:** Conceptualization and Visualization.

References

1. World Health Organization. Breast cancer. [Link]
2. International Agency for Research on Cancer. IARC marks breast cancer awareness month 2024. [Link]
3. Cauchi J P, Camilleri L, Scerri C. Environmental and lifestyle risk factors of breast cancer in malta—a retrospective case-control study. EPMA J. 2016;**7**(1):20. [DOI: 10.1186/s13167-016-0069-z] [PMID: 27679672]
4. Chung KT. Azo Dyes and Human Health: A Review. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2016;**34**(4):233–261. [DOI: 10.1080/10590501.2017.1284570] [PMID: 27635691]
5. T M Tawabul Islam N C. M. I. A. S. Toxicological assessment of azo dye brown ht in vivo. J Angiother. 2024;**8**(8):1–8. [Link]
6. EFSA Panel on Food Additives and Nutrient Sources (ANS). Scientific opinion on the re-evaluation of brown ht (e 155) as a food additive. EFSA J. 2010;***8***(4). [DOI: 10.2903/j.efsa.2010.1536]
7. T M Tawabul, Islam, et al. Chronic toxic effects of chocolate brown ht dye on hepatorenal functions in vivo. J Angiother. 2024;**8**(7):1–11. [Link]
8. Mokkawes T. Visser SP. Melatonin activation by cytochrome p450 isozymes: how does cyp1a2 compare to cyp1a1? Int J Mol Sci. 2023;**24**(4):3651. [DOI: 10.3390/ijms24043651] [PMID: 36835057]
9. Kesharwani, SS, Nandekar PP, Pragyan P, Rathod V, Sangamwar AT. Characterization of differences in substrate specificity among cyp1a1, cyp1a2 and cyp1b1: an integrated approach employing molecular docking and molecular dynamics simulations. J Mol Recognit. 2016;**29**(8):370–390. [DOI: 10.1002/jmr.2537] [PMID: 26916064]
10. Istifli E S. Preliminary in silico studies of the interactions of certain genotoxic azo dyes with different double-stranded dna conformations. Colorants. 2022;**1**(2):236–255. [DOI: 10.3390/colorants1020015]
11. Ramamurthy K, PriyaPS, Murugan R, Arockiaraj J. Hues of risk: investigating genotoxicity and environmental impacts of azo textile dyes. Environ Sci Pollut Res Int. 2024;**31**(23):33190–33211. [DOI: 10.1007/s11356-024-33444-1] [PMID: 38676865]
12. Kumari R, Sunil D, Ningthoujam RS, Kumar NA. Azodyes as markers for tumor hypoxia imaging and therapy: an up-to-date review. Chem Biol Interact. 2019;**307**:91–104. [DOI: 10.1016/j.cbi.2019.04.034] [PMID: 3104791]
13. John, A, Yang H H, Muhammad S, Khan ZI, et al. Cross talk between synthetic food colors (azo dyes), oral flora, and cardiovascular disorders. Appl Sci. 2022;**12**(14):7084. [DOI: 10.3390/app12147084]
14. Shimada C, Kano K, Sasaki YF, Sato I, Tsudua S. Differential Colon DNA Damage Induced by Azo Food Additives between Rats and Mice. J Toxicol Sci. 2010;**35** (4):547–554. [DOI: 10.2131/jts.35.547] [PMID: 20686341]
15. Chequer FM, Lizier TM, de Felício R, Zanoni MV, Debonsi HM, Lopes NP, et al. The azo dye disperse red 13 and its oxidation and reduction products showed mutagenic potential. Toxicol in Vitro. 2015;**29**(7):1906–1915. [DOI: 10.1016/j.tiv.2015.08.001] [PMID: 26247324]
16. Balçık, Utku, et al. Liquid phase microextraction based sensitive analytical strategy for the determination of 22 hazardous aromatic amine products of azo dyes in wastewater and tap water samples by gc-ms system. Microchem J. 2020;**155**:104712. [Link]
17. Institute of Laboratory Animal Resources (US). Committee on care u. of l. animals. guide for the care and use of laboratory animals. us department of health and human services. National Institutes of Health.1986. [Link]
18. Rahman SS, Klamrak A, Mahat NC, Rahat RH, Nopkuesuk N, Kamruzzaman M, et al. Thyroid stimulatory activity of houttuynia cordata thunb. ethanolic extract in 6-propyl-Thiouracil-induced hypothyroid and stz induced diabetes rats: In Vivo and In Silico Studies. Nutrients. 2025;**17**(3):594. [DOI: 10.3390/nu17030594] [PMID: 39940455]
19. Şensoy, E. The potential histopathological effect of sunset yellow fcf on lungs and hearts of developing mice. British Food J. 2025. [DOI: 10.1108/BFJ-06-2024-0580]
20. Kumar A, Kaur S, Dhiman S, Singh PP, Bhatia G. Targeting Akt/NF-KB/P53 Pathway and Apoptosis Inducing Potential of 1,2-Benzenedicarboxylic Acid, Bis (2-Methyl Propyl) Ester Isolated from Onosma Bracteata Wall. against Human Osteosarcoma (MG-63) Cells. Molecules. 2022;**27**(11):3478. [DOI: 10.3390/molecules27113478] [PMID: 35684419]
21. Nekipelova TD, Kurkovskaya LN, Levina II., Shishkov VS, Kuzmin, VA. Studies of Photoinduced addition of water and alcohols to substituted dihydroquinolines. Russ Chem Bull. 2001;**50**(4):673–677. [Link]
22. Liu LL, Yu CX, Zhou W, Zhang QG, Liu SM, Shi Y, et al. Construction of Four Zn(II) Coordination Polymers Used as Catalysts for the Photodegradation of Organic Dyes in Water. Polymers (Basel*)*. 2016**;8**(1):3. [DOI: 10.3390/polym8010003] [PMID: 30979106]
23. Khodot EN, Golovina GV, Timokhina EN, Samigullina AI, Levina II, Kuzmin VA, et al. New azo dyes based on 8-methoxy-2,2,4-trimethyl-1,2-dihydroquinoline and n-substituted tetrazoles. Russ Chem Bull. 2022;**71**(10):2207-17. [Link]
24. Tisdale M J. Mechanisms of cancer cachexia. Physiol Rev. 2009;**89**(2):381-410. [DOI: 10.1152/physrev.00016.2008] [PMID: 19342610]
25. Reza MSA, Hasan MM, Kamruzzaman M, Hossain MI, Zubair MA, Bari L, et al. Hossain md k. study of a common azo food dye in mice model: toxicity reports and its relation to carcinogenicity. Food Sci Nutr. 2019;**7**(2):667-77. [DOI: 10.1002/fsn3.906] [PMID: 30847145]
26. Smith LA, Craven DM, Ho AN, Glenny EM, Rezeli ET, Carson MS, et al. Weight loss reverses the effects of aging and obesity on mammary tumor immunosuppression and progression. Cancer Prev Res. 2025. [DOI: 10.1158/1940-6207.CAPR-24-0514] [PMID: 40231562]
27. Islam TMT, Mahat NC, Shaker IA, Rahman SA, Kabir MH, Shohel MA, et al. Investigation of the relationship between brown ht dye exposure and mammary tumor development in female rats: an assessment of the potential risk of breast cancer. Cureus. 2024. [DOI: 10.7759/cureus.73351] [PMID: 39659309]
28. Josephy PD, Allen-Vercoe E. Reductive metabolism of azo dyes and drugs: toxicological implications. Food Chem Toxicology. 2023;**178**:113932. [DOI: 10.1016/j.fct.2023.113932] [PMID: 37451600]
29. Zingue S, Mindang ELN, Awounfack FC, Kalgonbe AY, Kada MM, Njamen D, et al. Oral administration of tartrazine (e102) accelerates the incidence and the development of 7,12-dimethylbenz(a) anthracene (dmba)-induced breast cancer in rats. BMC Complement Med Ther. 2021;**21**(1):303. [DOI: 10.1186/s12906-021-03490-0] [PMID: 34972512]
30. Liu Y, Han G, Gong J, Hua X, Zhu Q, Zhou S, et al. Intramolecular fluorescence resonance energy transfer strategy for accurate detection of afp-l3% and improved diagnosis of hepatocellular carcinoma. Spectrochim Acta A Mol Biomol Spectrosc. 2023;**300**:122950. [Link]
31. Alzarea AI, Zafar A, Alsaidan OA, Alanazi AS, Alzarea, SI, Alhassan HH,et al. Preventive effect of acemannan on dmba-induced mouse skin tumorigenesis by modulating inflammatory cytokines and apoptosis pathways: molecular docking and molecular dynamic simulation approaches. Int J Biol Macromol. 2025;**311**:143836. [DOI: 10.1016/j.ijbiomac.2025.143836] [PMID: 40318716]
32. Israel Oyewole, O. Assessment of cardiac and renal functions in wistar albino rats administered carmoisine and tartrazine. Adv Biochem. 2016;**4**(3):21. [Link]