RESEARCH ARTICLE

Safety Concerns of *Tectona grandis* L.f. Leaf Extract as a Natural Food Colorant: Evidence of Irreversible Organ Pathology in Subchronic Toxicity Study

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Abstract

BACKGROUND: *Tectona grandis* Lf (TG) leaves are traditionally used in Indonesia for natural dyeing, and are gaining popularity as food colorants globally. However, their safety profile remains unclear. Acute toxicity studies reported no fatalities at doses up to 5000 mg/kg BW, histological analyses revealed inflammation and necrosis in the stomach, raising concerns regarding the long-term safety of TG leaf extract. Therefore, this study was performed to evaluate subchronic toxicity of TG leaf extract in both males and females Wistar rats.

METHODS: TG leaf extracts were obtained by water extraction and extract powder was suspended in sodium carboxymethyl cellulose (Na-CMC). Male and female rats were administered TG leaf extract at doses of 0, 10, 20, and 40 mg/kg BW for 28 days, with a 14-days recovery phase in the satellite groups (as controls). Hematology profiles and biochemistry were analyzed using hematology analyzer and spectrophotometry. Histology analysis was performed to investigate TG effects on the organs.

RESULTS: Hematological analysis revealed reversible reductions in hemoglobin, erythrocyte, and hematocrit levels, along with irreversible decreases in leukocyte and thrombocyte. While TG leaf extract did not significantly affect serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), or creatinine levels, bilirubin levels increased, but remained within normal ranges. However, histopathological findings at 40 mg/kg BW revealed congestion and inflammation in the digestive organs, as well as neutrophil infiltration and congestion in metabolism-related organs, the lungs, liver, kidneys, and lymph nodes. These pathological changes persisted throughout the recovery period.

CONCLUSION: TG leaf extract raises safety concerns, particularly at a dose of 40 mg/kg BW, as it induces irreversible organ pathology despite reversible changes in blood parameters.

KEYWORDS: Indonesian *Tectona grandis* Lf, subchronic, toxicity, natural, food, colorants

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Introduction

Food additives are not only utilized by the food industry but are also widespread in traditional foods in Indonesia. These additives include food colorants, preservatives, antioxidants, emulsifiers, thickeners, flavors, and sweeteners. Among these, food colorants are most used. Food colorants are chemical substances that are added to foods to enhance their visual appeal. They are generally categorized as artificial or natural agents. Common artificial colorants include tartrazine (E102), sunset yellow (E110), ponceau 4R (E124), Allura red (E129), and quinoline yellow (E104). (1) Although these artificial colorants have been tested

and approved for use, their long-term consumption raises concerns about potential health risks. Studies have indicated that prolonged use of artificial food colorants may promote carcinogenicity and hypersensitivity reactions. In particular, the proven food-dyeing agent Red 3 has been identified to induce cancer in animals. Other four USA-certified food dyeing agents including Blue 1, Red 40, Yellow 5, and Yellow 6 have raised concern for safety due to promoting hypersensitivity effects. With the growing global awareness of potential health risk associated with artificial colorants there is an increasing demand for natural alternatives in both food and non-food industry.(1,2)

Consequently, the use of natural food colorants has increased significantly in both urban and traditional settings. A popular natural colorant is the leaf of *Tectona grandis* L. f. (TG), which is rich in bioactive compounds such as anthocyanins and tannins and is used to produce yellow, orange, or red hues in food, demonstrating its potential as a sustainable and renewable natural dye.(3) However, the assumption that natural agents, such as Indonesian TG leaves, are always safe and healthy remains unproven.

Acute toxicity studies of Indonesian TG extracts have shown insignificant clinical symptoms in rats. However, histological analysis has raised concerns about the safety of Indonesian TG extract in the stomach, causing edema and inflammation at doses of 2000 and 5000 mg/kg BW. In general, acute toxicity tests of TG leaf extract have been conducted in category 5, indicating low acute toxicity. However, under particular circumstances, it may pose a hazard to vulnerable populations.(3,4) Although long-term toxicity assays of Indonesian TG leaves have not been reported, concerns have been raised owing to its acute toxicity. Therefore, it is suggested that Indonesian TG leaf extract may affect some organ histology profiles over a longer testing period.(3,5)

As concluded from our previous research on the acute toxicity effects of Indonesian TG leaf extract in rats, there were insignificant acute effects.(3) However, the subchronic toxicity of Indonesian TG leaf extracts remains unknown. Therefore, this study was performed to investigate the subchronic toxicity of TG leaf extracts in rats. Utilizing the modified guideline from the Organization for Economic Co-operation and Development (OECD) 407, this study evaluates the clinical presentation and potential toxicity effects of TG leaf extract on Wistar rats following 28 days of oral exposure.(6) The evaluated parameters included clinical manifestations, body weight variations, and patterns of food and water intake. Additionally, a comprehensive analysis of blood parameters, including hemoglobin, erythrocyte count, hematocrit, leukocyte count, thrombocyte count, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), creatinine, and bilirubin levels, was conducted. Urine characteristics such as color, pH, presence of precipitation, and blood clots were also examined. Furthermore, histopathological analysis of vital organs, including the stomach, small intestine, lungs, kidneys, liver, and lymph nodes, was performed.

Methods

Study Design

This study was constructed as an experimental investigation, by following the guideline of OECD 407, which outlines procedures for a 28 days repeated dose oral toxicity study in rodents with minor modifications.(6,7) The primary focus of this study was on evaluating the toxic effects of TG leaf extract. The main parameters included urine and blood biochemistry, as well as histological profiles were also investigated. Briefly, animals were treated with various concentrations of TG leaf extract for 28 days. The control group and the highest dose of 40 mg/mL BW were treated for another 14 days as satellite groups (Figure 1).

TG Leaf Extract Suspension

The aqueous extract of the TG leaves was sourced from the Laboratory of Chemical Separation Processed at the Faculty of Chemical Engineering, Universitas Gadjah Mada, Yogyakarta. The extract, delivered in powder form, was comprised of 20% pure extract and 80% malt dextrin. TG leaf powder has low solubility in water. Thus, sodium carboxymethyl cellulose (Na-CMC) was selected as the suspension agent. TG leaf extract was suspended in 0.5% Na-CMC at various concentrations (10, 20, and 40 mg/kg) before administration.

Animal Treatment and Grouping

The study utilized male and female Wistar rats aged 8 weeks, weighing between 100 and 200 g, that were obtained from the Unit Pengembangan Hewan Percobaan (UPPH-LPPT), Universitas Gadjah Mada. The animal ethics protocol was approved by UPPH-LPPT (No. KEC/LPPT/53/12). Rats were acclimatized for a minimum of 14 days at the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada, under standard conditions of room temperature (25°C) and normal humidity, and provided with adequate food and water. The rats were divided into four groups: a control group and three

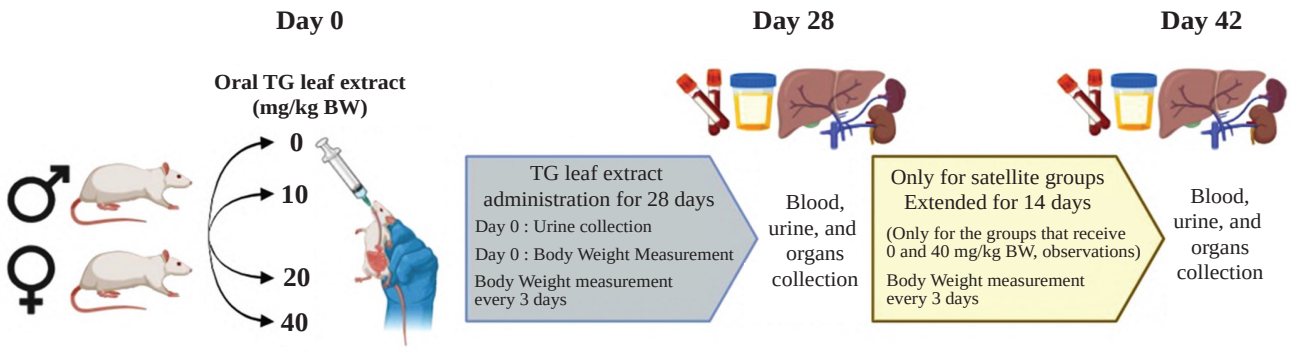

Figure 1. Subchronic toxicity study design. The study design was primarily based on OECD 407 guidelines. Male and female Wistar rats were utilized. The rats were divided into a control group and three treatment groups with different dose as shown. Before treatment started, data at day-0 were collected as stated in grey arrow box. Each rat received treatment for 28 days prior to main data collection, covering urine, blood, and organs collection. Additionally, the control group and the highest dose group (40 mg/kg BW) were observed for an additional 14 days as mentioned in yellow arrow box, forming the satellite groups before data were collected at day-42.

treatment groups of TG leaf extract at doses of 10, 20, and 40 mg/kg BW. Male and female rats were separated and grouped as previously described. To ensure consistency and control variability, the study minimized differences in age, sex (differentiating both male and female rats), strain, body weight, and the number of animals. The TG leaf extract treatment schedule was carefully set to maintain uniformity throughout the study.

This study was carried out within the 28 days of treatment period for all groups. The observation period was extended to 14 days for only the control and 40 mg/kg BW satellite groups. Rats were administered TG leaf extract daily within a specified time frame from 07.00 to 08.00 AM. Throughout the treatment period, body weight was monitored every 3 days and used for the dose adjustment. On day-28, all groups of both males and females were euthanized for data collection. Half of the rats from both males and females of the control and the highest tested dose of 40 mg/kg BW were kept for an additional 14 days observation period without exposure to TG leaf extract. This extension period allowed for monitoring of their growth and behavior under normal conditions before the rats were euthanized on day 42 of the study.

Examination and Observation of Clinical Symptoms

During the 28-day treatment period, clinical symptoms were observed, and this monitoring continued for an additional 14 days post-treatment in the satellite groups. Clinical or toxic symptoms were recorded daily for one hour following exposure. Additionally, the body weight of the rats was measured every three days, and food and water intake were documented every seven days. Observations adhered to the criteria specified in Supplementary 1.

Examination of Blood and Urine Parameters

Blood and urine parameters were evaluated at three intervals: before treatment (day-0), after 28 days of treatment (day-28), and 14 days after the end of observation (day-42). Rats were anesthetized with a low dose of isoflurane prior to blood collection via the retro-orbital route using a heparin-coated glass capillary tube.

Whole blood samples were collected in anticoagulant EDTA-coated tubes and aspirated for hematology analysis using Hematology Analyzer Sysmex KX-21 (Sysmex, Kobe, Japan). Hematological data included hemoglobin, erythrocytes, hematocrit, leukocytes, and platelets. For blood chemistry analysis, samples were centrifuged at 2000 rpm for 10 minutes to separate plasma from blood cells. SGPT and SGOT levels were analyzed using a colorimetric AST/ALT assay kit (Cat No. MBS169585; MyBiosource, San Diego, CA, USA) following the manufacturer's protocol. SGPT and SGOT activity were measured at 340 nm using a UV-Vis Spectrophotometry (Shimadzu, Kyoto, Japan). Creatinine concentration was determined by following Jaffe method, which involves the reaction between creatinine and picric acid in an alkaline medium and was measured at 520-530 nm against blank using the UV-Vis spectrophotometry. Bilirubin concentration was assessed using the Diazo method, where sulfanilic acid solution mixed with sodium nitrite was added to plasma and incubated for 10 minutes in room temperature. Total bilirubin levels were measured at 540 nm using UV-Vis spectrophotometry.

Additionally, along with blood sample collection, the urine samples were also collected from each subjects. The urine parameters analyzed including the color, pH, presence of sediment, and blood spots, which were evaluated through the macroscopic observation.

Organs Isolation and Histology Preparation

Prior to organs isolation mice were anesthetized with the isoflurane and immediately abdomen and thorax were dissected using the sterile instruments. Rats were perfused with saline, ensuring the organs clean from blood as in detailed available in previous publication.(8) Organs including stomach, small intestine, liver, kidney, lymph, and lung were carefully excised and immediately rinsed in normal saline before fixated in 10% formalin for 48 hours. Tissues were then prepared for histology preparation, started with tissue dehydration in a graded ethanol followed by clearing in xylene, continued with embedding in paraffin wax. Tissues were then sectioned into 4 µm thick slices using a microtome (Cat. No. YD-315; Yidi, Zhejiang, China). Sections were floated in warm water (40-45°C) and mounted onto glass slide. Dried slides were then deparaffinized and rehydrated through graded ethanol series. Sections were stained with hematoxylin and eosin (HE) for general histological analysis. Stained sections were dehydrated, cleared in xylene, and mounted with coverslip using a synthetic resin. Slides image were captured using Olympus CX23 binocular microscope (Olympus, Tokyo, Japan).

Results

TG Leaf Extract Did Not Affect Clinical Symptoms

In this study, clinical symptoms were observed throughout the research period, which included 28 days of treatment and 14 days post-treatment, to assess the potential for delayed effects or reversibility after cessation of administration. The substance was administered daily at the same time to ensure consistent levels in the rats. Intensive observations were carried out for one hour each day immediately following administration. Investigation of clinical symptoms indicated that throughout the 28 days of treatment and 14 days post-treatment periods, no toxic symptoms were observed in any of the groups. Clinical symptom parameters are provided in Supplementary 1.

Administration TG Leaf Extract Resulted in Insignificant Body Weight, Food-Water Consumption, and Organs Gross Pathology

Body weight was measured every three days, and the average weight change for each group was calculated to determine the average daily weight gain. To address the initial disparities in body weight among rats, the average daily weight gain was utilized to facilitate a clearer observation of weight changes over time. The average daily weight gain was then quantified to achieve 3 days of body weight change in rats (Figures 2A and 2B). This method can be used to monitor the impact of the administered substance on body weight. Although fluctuations appeared, statistical analysis indicated that there was no difference between the groups in body weight changes (Figures 2A and 2B).

The body weight fluctuation correlated to daily food and liquid intake; thus, these data were examined (Supplementary 2). Data were collected by weighing food and measuring water consumption every seven days, with the aim of investigating the relationship between weight gain and intake. Statistical analysis indicated that there was no significant difference between the treatment groups and the control group in terms of food and water consumption (Supplementary 2). This data aligns with the body weight fluctuation, which indicated no significant effect of TG leaf extract exposure on rats (Figures 2A and 2B).

In addition, organ weight and appearance corresponded to pathological signs. Therefore, the gross pathology of the organs, including the liver, lung, stomach, kidney, lymph, and the small intestine were observed. Treatment with the TG leaf extract did not affect organ appearance (data not shown). The weight of the organs may change under some pathological conditions; thus, organ weight was also measured. As expected according to the organs presentation, organs weight did not show any significant difference when compared to their control (Figures 2C and 2D).

Effect of TG Leaf Extract on Hematological Parameters

To investigate the effect of TG leaf extract on blood parameters, the hematological profiles, including hemoglobin, erythrocytes, hematocrit, platelet levels, leukocytes, and thrombocyte levels, were evaluated. The results indicated that the average normal baseline levels of hemoglobin, erythrocytes, and hematocrit across all experimental groups in both male and female were evenly distributed (Figures 3A–3F). Statistically, there were no significant differences in the baseline levels of these parameters between groups. Notably, compared to their baseline, exposure to TG leaf extract across all doses led to a significant reduction in these parameters in both male and female rats, as indicated by the red line suppression (Figures 3A–3F). However, these values remained within the lower limit of the normal range. Further analysis of the extended 14 days satellite group, which included the control and 40 mg/kg BW treatments, revealed a return of these parameters to baseline levels similar to their baseline levels, as indicated by the green line (Figures 3A–3F).

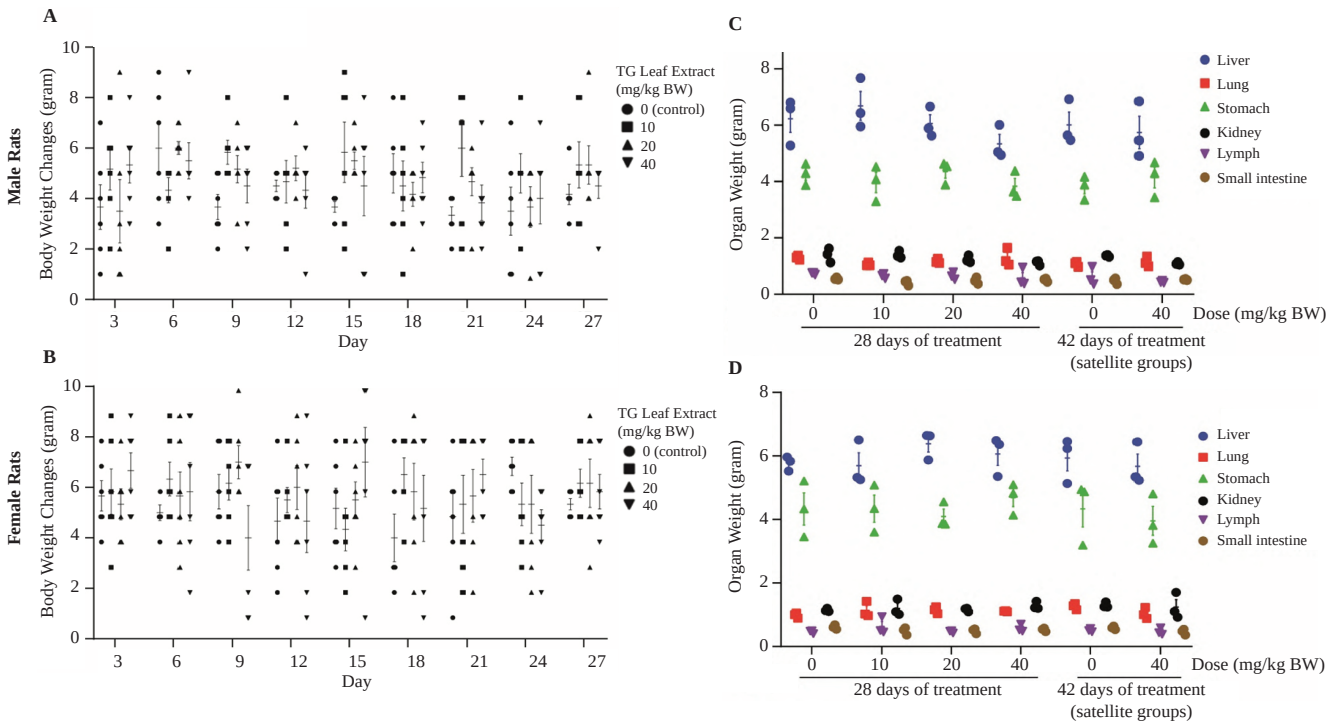

Figure 2. Effect of TG leaf extract on body and isolated organs weighty. A and B: Rat's body weight changes were recorded every 3 days and plotted individually in graphs of day of data collection (X bar) versus body weight changes in gram (Y bar). Data were collected from all groups, including control and three treatment doses (10, 20, and 40 mg/kg bw), for both male (A) and female (B) rats. Each group is indicated by specific symbols as depicted in the legend. The graphs present the mean body weight changes± SEM. C and D: Organs weight graphs were presented with horizontal bar of doses and days of data collection, while vertical bar presenting the weight of organs in gram. Organs were harvested at day-28 and day-42 for satellite from both male (C) and female (D) group. Organs were then weighed from at 3 rats each sex group indicated with specific symbol and color in figure legends. Sample sizes ranging from 3 to 6 rats per group.

To examine the potential effects of the TG extract on rat immunity and platelet levels, leukocytes and thrombocytes were measured. The quantification of these parameters indicated that the baseline levels were within the normal range for all groups of both males and females (Figures 3G–3J). Administration of TG leaf extract at all doses to Wistar rats over a 28 days period led to a downregulation in leukocyte concentration compared to that in the control group and their baseline levels, as indicated by the red dashed line (Figures 3G and 3H). Notably, a 14 days recovery period for the satellite group resulted in the normalization of leukocyte levels in female rats, but not in male rats (Figure 3G). In contrast, treatment with TG extract at all doses resulted in the suppression of thrombocyte levels compared to baseline measurements. Despite this suppression, thrombocyte levels remained within the normal range. Remarkably, a 14 days recovery period was sufficient to return thrombocyte levels to baseline values (Figures 3I and 3J).

Effect of TG Leaf Extract on Liver and Kidney Function

In order to evaluate the effect of TG leaves extract on liver and kidney functions, the SGPT and SGOT were investigated. The baseline levels of SGPT and SGOT were within the normal concentration range, as marked by the gray area across all groups, regardless of sex (Figures 4A–4D). Following a 28 days treatment with TG leaf extract, no significant changes in the levels of SGPT and SGOT were observed in any of the groups. As anticipated, the 14 days recovery period for the satellite groups showed comparable results to the control group and the 40 mg/kg BW treatment group (Figures 4A–4D).

As the data showed an insignificant effect of liver and kidney toxicity parameters, the alternative indicators, such as creatinine and bilirubin were further examined. Quantitative analysis of plasma creatinine and bilirubin levels across all groups indicated that the concentrations remained within the normal range as indicated in grey area (Figures 4E–4H). Treatment with TG leaf extract for 28 days did not significantly alter creatinine levels across any dose or group (Figures 4E and 4F). Notably, TG leaf extract at doses of 10 and 40 mg/kg BW led to increased bilirubin concentrations in male rats compared to the control and baseline levels indicated by the red dashed line (Figures 4G and 4H). In contrast, female rats exhibited elevated

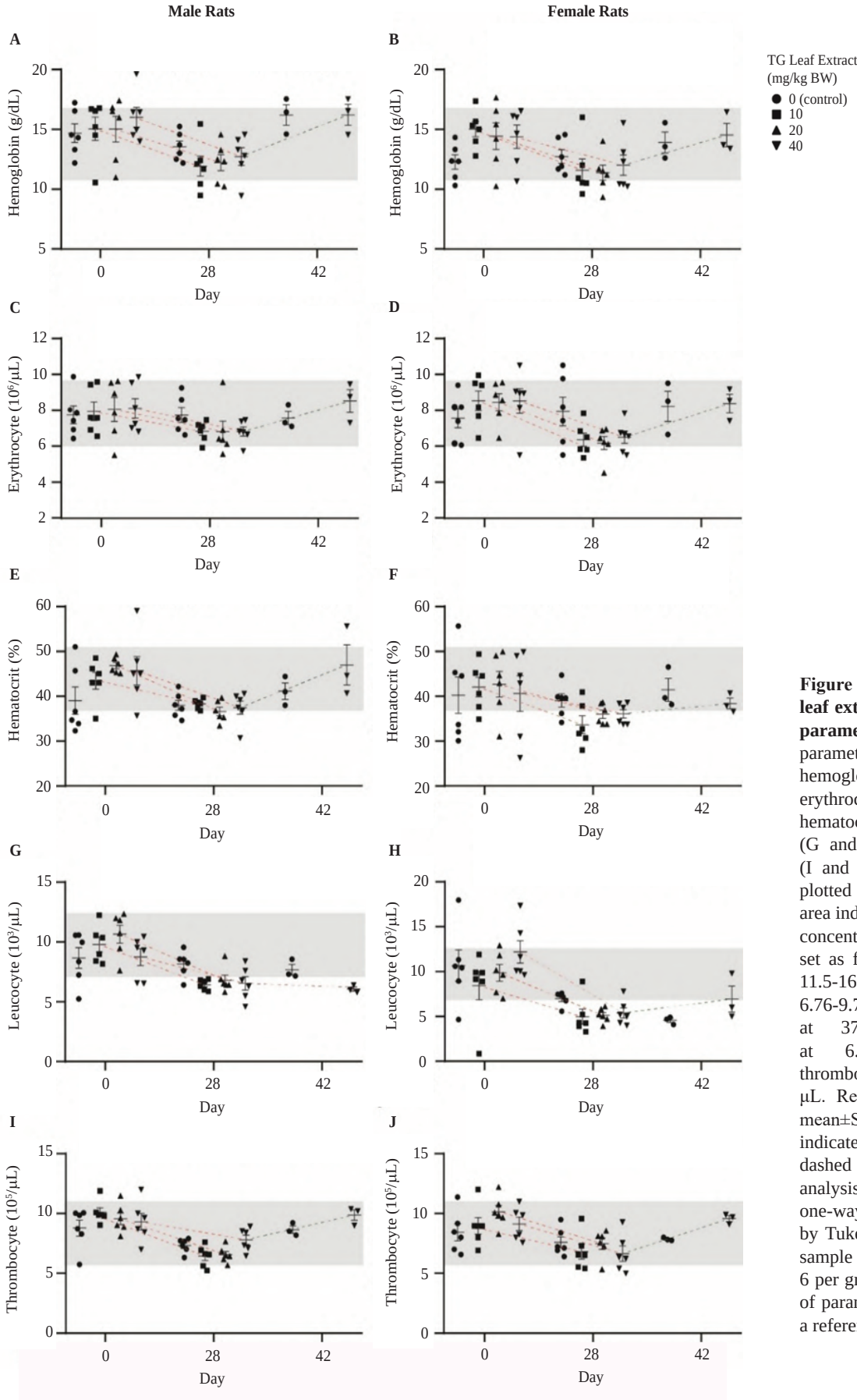

Figure 3. The effect of TG leaf extract on hematological parameters. Hematological parameters analyzed including hemoglobin (A and B), erythrocytes (C and D), and hematocrit (E and F), leucocyte (G and H) and, thrombocyte (I and J) were analyzed and plotted individually. The grey area indicates the normal range concentration as a reference and set as follows: hemoglobin at 11.5-16.1 g/dL, erythrocytes at 6.76-9.75×10^6/µL, hematocrit at 37.2-50.6%, leukocyte at 6.6-12.6×10^3/µL, and thrombocyte at 510-1100×10^3/µL. Results are presented as mean±SEM, with significance indicated by red and green dashed lines. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with sample sizes ranging from 3 to 6 per group. The normal range of parameter values was set as a reference (21).

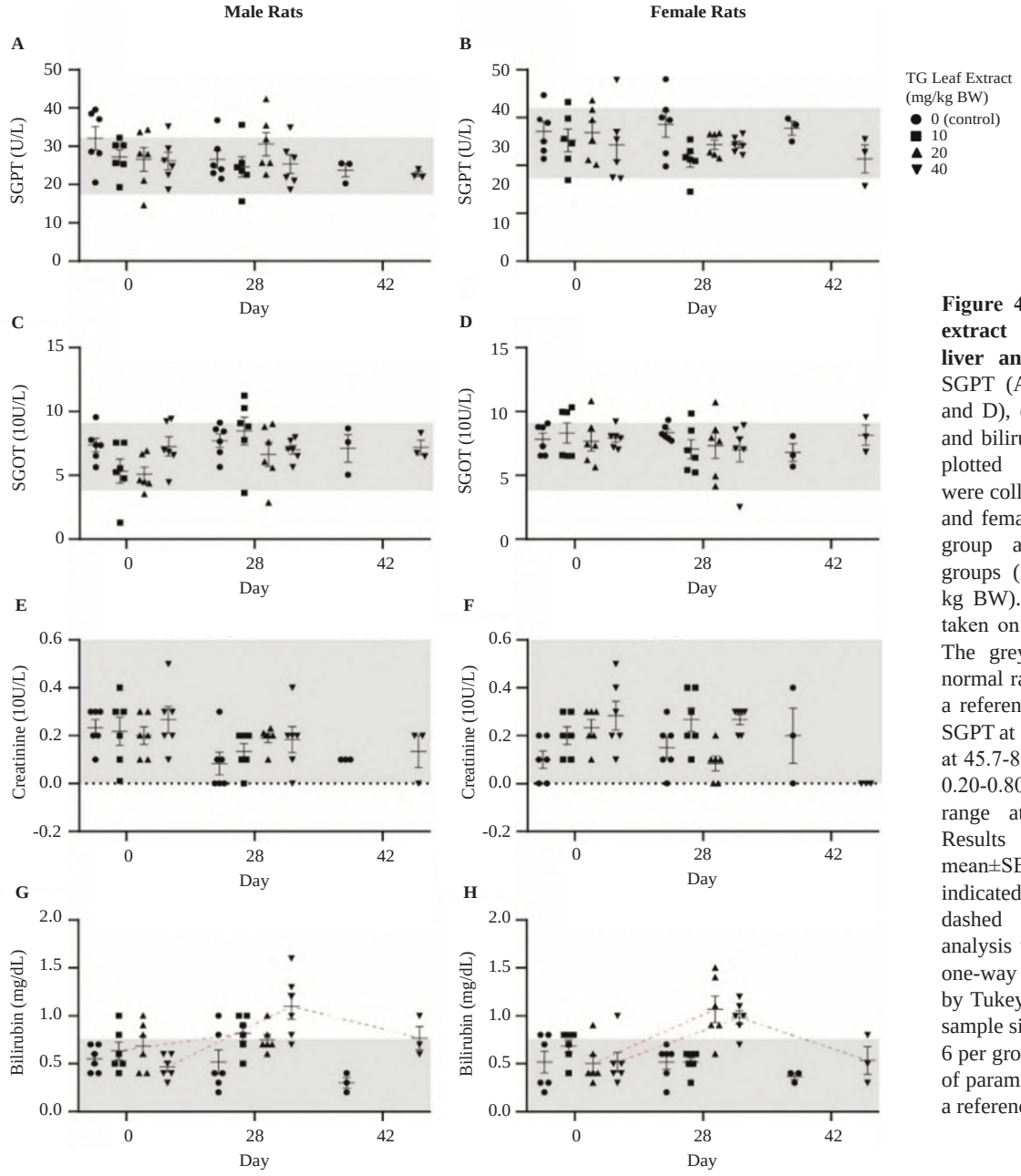

Figure 4. Effect of TG leaf extract on biomarkers of liver and kidney function. SGPT (A and B), SGOT (C and D), creatinine (E and F), and bilirubin (G and H) were plotted individually. Data were collected from both male and female rats in the control group and three treatment groups (10, 20, and 40 mg/kg BW). Measurements were taken on day-0, -28, and -42. The grey area indicates the normal range concentration as a reference an set as follows: SGPT at 17.5-30.2 U/L, SGOT at 45.7-80.8 U/L, creatinine at 0.20-0.80 mg/dL, and bilirubin range at 0.00-0.55 mg/dL. Results are presented as mean±SEM, with significance indicated by red and green dashed lines. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with sample sizes ranging from 3 to 6 per group. The normal range of parameter values was set as a reference (21).

bilirubin levels at doses of 20 and 40 mg/kg BW relative to control and baseline values, as indicated by the red dashed line (Figure 4H). Remarkably, bilirubin levels in both male and female rats returned to baseline values following a 14-day recovery period, as observed in the satellite group and indicated by the green dashed line (Figures 4G and 4H).

Effect of TG Leaf Extract on Urine Parameters

Macroscopic analysis of urine parameters evaluated in this study including color, pH, and sedimentation or blood clots. The urine pH value recorded in this study was 9, indicating that the urine was alkaline. For sediment examination, a direct observation method was employed to detect turbidity in rat urine. The presence of visible sediment could suggest severe damage to the metabolic or excretory organs of rats, particularly the kidneys. In this study, no turbidity was detected in rat urine. Furthermore, no blood clots were identified in any of the groups.

Effect of the TG Leaf Extract on Organs Histology

Since gross pathological analysis did not reveal significant changes in organ appearance or weight, further investigation using histological analysis was deemed necessary. Oral exposure to TG leaf extract may affect digestive organs, including the stomach and small intestine. Unlike the gross pathological analysis, histological assessment demonstrated that exposure to TG leaf extract at a dose of 40 mg/kg BW caused congestion and inflammation in both male and

female rats. Furthermore, extending the recovery period to 14 days did not alleviate pathological conditions (Figure 5 and Supplementary 3). The small intestine was examined, in addition to the stomach. Histological analysis of the small intestine revealed that exposure to the same dose of the TG leaf extract (40 mg/kg BW) induced inflammation and neutrophil infiltration in both male and female rats. This pathological condition appeared to be irreversible, as indicated by the small intestine histological analysis of the satellite group, which still exhibited inflammation and neutrophil infiltration (Figure 5 and Supplementary 3).

Further, we examined the organs associated with metabolism and detoxification, including the lungs, liver, kidneys, and lymph nodes. Histological analysis of the lungs revealed that the TG leaf extract at a dose of 40 mg/kg BW induced neutrophil infiltration. Additionally, the extract

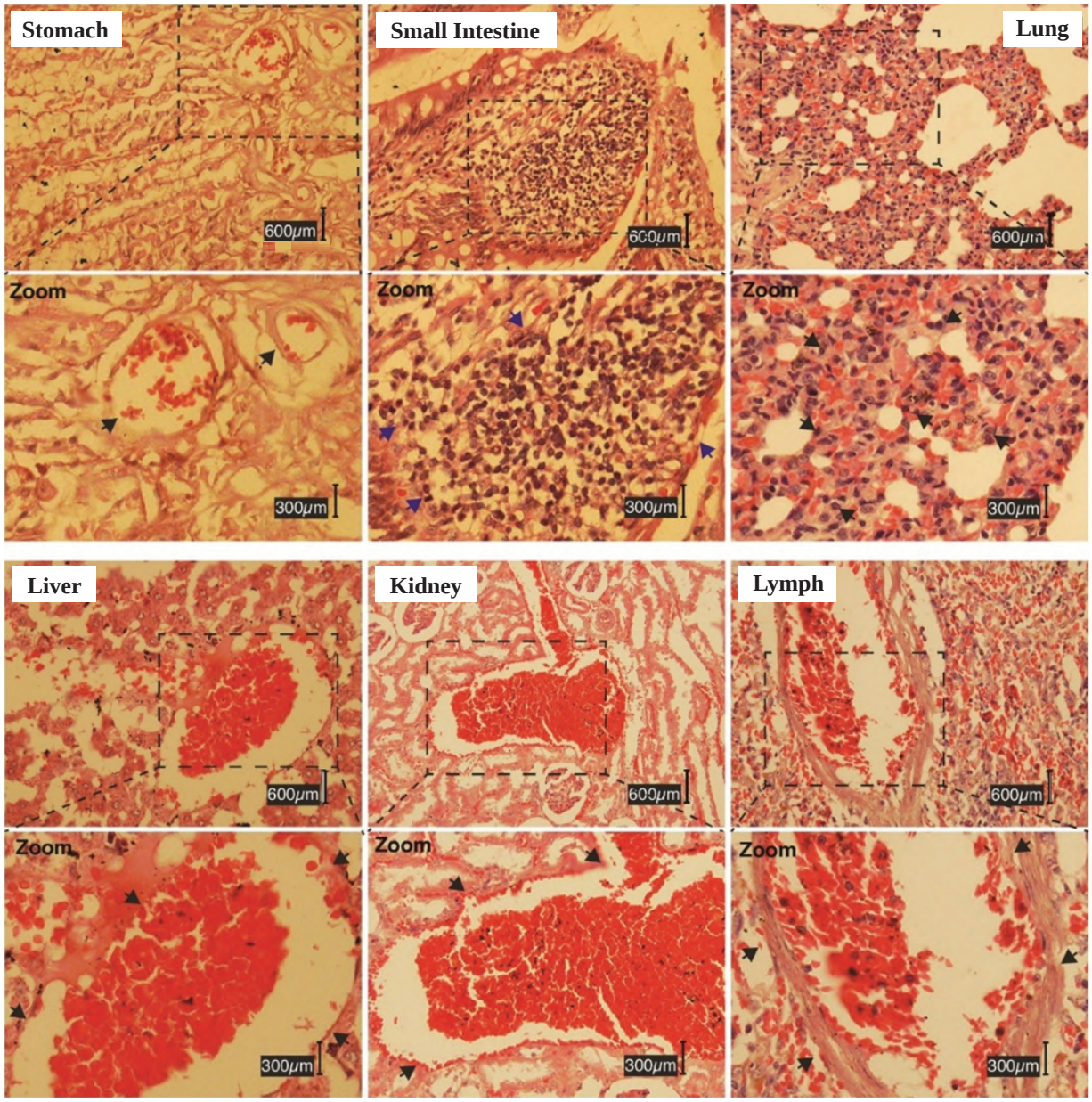

Figure 5. Effect of TG leaf extract on organs histology. Histological profile of digestive organs covering stomach and small intestine. Treatment with TG leaf extract at a dose of 40 mg/kg bw induced congestion and inflammation in the stomach and small intestine of both male and female rats, as observed in the treatment and satellite groups. Representative histological images of the stomach, highlighting congestion and inflammation indicated by black arrows. While representative histological images of the small intestine, with inflammation and neutrophil infiltration marked by blue arrows. Effect of TG leaf extract on metabolism and detoxification organs were assessed through lung, liver, kidney and lymph. Exposure of TG leaf extract in both male and female rats induce lung neutrophils infiltration and congestion of liver, kidney and lymph at the dose of 40 mg/kg BW including satellite group. Images are presented in original and a magnified view.

promoted congestion of the liver, kidneys, and lymph nodes. These pathological conditions were observed in both the male and female groups, indicating an irreversible state, as the satellite group continued to exhibit these conditions (Figure 5 and Supplementary 3).

Discussion

This study aimed to identify the potential subchronic toxicity of the Indonesian TG leaf extract in rats by following the modified OECD 407 guidelines (Figure 1). The study covered several key observations and examinations, including the assessment of body weight, monitoring of food and water intake, hematological analysis, biochemical blood analysis, urinalysis, and gross pathology and histopathological analysis.(6)

Body weight, along with food and water consumption, are critical indicators of overall health condition and substance tolerability.(9,10) The lack of significant differences between the treated and control groups suggests that the TG leaf extract does not adversely affect appetite, hydration, or growth (Figures 2A–2B and Supplementary 1). These findings are consistent with previous studies that involved exposure to either water or methanolic TG leaf extract.(3,4,11) Furthermore, although different serial doses were used, exposure to the Cameroonian TG leaf extract at the doses higher than 90 mg/kg BW induced higher level of total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL); however, it did not affect the body weight elevation.(5) In contrast, the reduction in total cholesterol led to decreased body weight in rats after exposure to β-carotene in a subchronic toxicity assay.(12) Thus, it is suggested that TG leaf extract exposure probably reduced specific lipoprotein levels, which had a minor impact on total cholesterol levels and eventually inadequate affect on body weight (Figure 2).

Further, hemoglobin, erythrocytes and hematocrit were assessed, as xenobiotics are known to influence their production, function, and lifespan, often resulting in decreased levels.(13) TG leaf extract did not induce abnormal hemoglobin concentrations compared to the control. However, when compared to their baseline levels, TG leaf extract treatment resulted in a significant reduction in hemoglobin levels, as well as erythrocyte and hematocrit levels. Remarkably, in the satellite groups, in which rats underwent a 14 days recovery period, these parameters returned to baseline levels (Figures 3A–3F). This reversibility suggests that the suppressive effects of TG leaf extract are temporary and may not result in long-term hematological alterations. Similarly, oral administration of Cameroonian TG leaf extract at higher doses, 30–810 mg/kg BW resulted in no significant alterations of hemoglobin levels in rats.(5)

Exposure to substances typically elicits an immune response. Therefore, the measurement of leukocytes is essential for toxicity assessment. Leukocytes play a crucial role in combating infections and other foreign pathogens in the body.(14) Indeed, our data showed a decrease in leukocyte levels to the lower limit of normal after 28 days of treatment with TG leaf extract compared to their baseline levels on day 0 (Figures 3G and 3H). In the male group, extending the recovery period for an additional 14 days did not restore leukocyte levels to their baseline levels, indicating an irreversible effect of the TG leaf extract on leukocytes (Figure 3G). The methanolic extract of TG seeds, rich in alkaloids, tannins, and flavonoids, likely exerts immunosuppressive effects by targeting hematopoietic stem cells and leukocyte precursors through mechanisms involving DNA synthesis inhibition, oxidative stress, and nutrient deprivation.(11,15,16) On the other hand, thrombocyte levels were suppressed following exposure to TG leaf extract when compared to their baseline levels at day-28. Unlike leukocytes, thrombocyte levels recovered after a 14 days recovery period in the satellite group, indicating a reversible effect of the TG leaf extract on thrombocyte levels (Figures 3I and 3J).

To assess the overall impact of TG leaf extract on key metabolic organs, such as the liver and kidneys, this study evaluated SGPT, SGOT, creatinine, and bilirubin levels. Surprisingly, among the biomarkers of organ damage measured, most showed an insignificant effect, but bilirubin was an exception (Figure 4). TG leaf extract at doses of 20 and 40 mg/kg BW resulted in increased bilirubin levels in both male and female rats. Interestingly, these effects declined after 14 days of recovery in the satellite groups (Figures 4G and 4H). The observed increase in bilirubin levels suggests that TG leaf extract may transiently affect bilirubin metabolism or hepatic function. Bilirubin is a key biomarker of liver function, and elevated levels often indicate altered hepatic processes, such as impaired bilirubin conjugation, hepatocellular injury, or disruption of bile secretion pathways.(17) The reversible nature of this effect, as shown by the decline in bilirubin levels after a 14 days recovery period in the satellite groups, suggests that the TG leaf extract does not cause permanent damage to liver function (Figures 4G and 4H). This could be attributed to the liver's regenerative capacity and its ability to restore

normal metabolic functions after the cessation of exposure to the extract.(18) The reversible increase in bilirubin levels observed in this study aligns with findings from other plant-based toxicity studies, where transient effects on hepatic biomarkers were noted without long-term adverse effects.(19) Possible explanations include the presence of specific phytochemicals in TG leaf extract, such as tannins or alkaloids, which may interact with hepatic enzymes involved in bilirubin metabolism.(20) Additionally, the potential influence of sex-specific differences in metabolism and detoxification pathways should be explored to fully understand the effects of the extract.

Urine analysis in this study showed no significant changes in macroscopic observations. The urine pH was consistently 9 across all groups, indicating alkalinity. Although this value exceeds the normal range of 7.3 to 8.5, it remains acceptable considering the 0.5 deviation of the pH paper used.(21-23) Uniform pH values across all groups, including controls, indicated that TG leaf extract did not induce alkalosis in rats.

Analysis of digestive organ histology revealed that the treatment with 40 mg/kg BW TG leaf extract induced congestion and inflammation in the stomach and small intestine of both male and female rats. These pathological changes were characterized by signs of vascular congestion and neutrophil infiltration (Figure 5). Congestion and inflammation in the stomach could be linked to the direct interaction of these phytochemicals present in the TG leaf extract with the gastric mucosa.(20,24) Prolonged exposure or higher doses of these compounds could lead to oxidative stress or trigger inflammatory pathways, resulting in neutrophil infiltration and tissue damage.(25) In small intestine (Figure 5), the observed neutrophil infiltration suggests activation of the immune response, potentially due to irritation or damage to the intestinal epithelium caused by TG leaf extract.(20) This finding aligns with previous studies on other plant extracts that reported similar inflammatory responses in the gastrointestinal tract following exposure to high doses of phytochemicals.(26) Further, this result is also consistent with the study on the acute toxicity of TG leaf extract which showed that it promotes inflammation of stomach and small intestine.(3) Several studies have reported that bioactive compounds, such as tannins and alkaloids, which are known to be present in TG leaves, can exert both protective and irritative effects depending on their concentration and duration of exposure.(24,27)

Additionally, we investigated the histological patterns of the metabolism and detoxification organs including the lungs, liver, kidneys, and lymph nodes, in both male and female rats (Figure 5). Histological analysis revealed that exposure to TG leaf extract at a dose of 40 mg/kg BW induced pathological changes. Specifically, neutrophil infiltration was observed in the lungs, whereas congestion was evident in the liver, kidneys, and lymph nodes (Figure 5). These findings suggest that higher doses of TG leaf extract may cause localized inflammation and impair normal physiological functions in these organs. The neutrophil infiltration in the lungs indicates an inflammatory response, possibly triggered by bioactive compounds in TG leaf extract, such as alkaloids and tannins, which have been associated with pro-inflammatory effects at higher concentrations.(15,20) Lung inflammation could also result from systemic exposure to plant-derived compounds, which may activate immune pathways and lead to pulmonary infiltration of neutrophils.(16,24,25)

Congestion of the liver, kidneys, and lymph nodes suggest vascular disturbances, potentially due to increased permeability or impaired microcirculation caused by the extract. Congestion of the liver and kidneys, key organs in metabolism and detoxification, could indicate a temporary disruption in their functional capacity, although no direct signs of organ damage (*e.g.*, elevated SGPT, SGOT, or creatinine levels) were observed (Figure 4). This aligns with previous studies showing that plant-based bioactive compounds may influence vascular integrity and organ perfusion without necessarily causing irreversible damage. (20,26) Moreover this result is supported by the previous study of TG leaf extract on acute toxicity which showed the hydropic generation of liver.(3)

The presence of inflammation and neutrophil infiltration in the digestive and metabolism-detoxification organs suggests that pathological changes induced by TG leaf extract may involve a combination of immune activation, oxidative stress, and endothelial dysfunction. Previous studies on plant-based extracts with similar phytochemical profiles have reported comparable adverse effects at high doses, supporting the dose-dependent toxicity of TG leaf extract. Particularly, the observed vascular congestion is likely a result of local vasodilation mediated by inflammatory mediators such as prostaglandins, histamine, and cytokines released in response to irritation or injury. (20,25) Neutrophil infiltration into the affected tissues further supports the hypothesis of an acute inflammatory response, as neutrophils are typically recruited to sites of tissue damage or infection through the activation of chemotactic factors, including interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α).(28) In this study, these pathological changes were also observed in the satellite

groups, indicating that these effects persist even after a recovery period (Figure 5). However, further investigation is needed to determine whether longer recovery periods can completely reverse these effects.

Taken together, this study highlights the importance of evaluating the duration of adverse effects following exposure to plant extracts, and determining whether the observed effects are reversible or progressive over time. TG leaf extract pro-inflammatory and congestive effects at higher doses emphasize the need for careful dose optimization and long-term safety assessments. Although the study adhered to modified OECD 407 guidelines, which include both sexes to assess potential sex-specific effects and involve the analysis of hematological, biochemical, and histological parameters, this study is not without limitations. The minimal number of animals and relatively short duration of exposure, including the recovery period, may not fully capture the effects of prolonged or chronic use. Future studies should consider longer observation periods and additional endpoints, such as oxidative stress markers and molecular mechanisms underlying the observed effects. Moreover, while this study focused on oral administration, other routes of exposure relevant to cosmetic and textile applications should be explored.

Conclusion

Besides the potential of TG leaf extract as a promising natural food dyeing agent, its subchronic toxicity at higher doses raises concerns. While the reversible effects on hematological and biochemical parameters suggest low systemic toxicity, persistent histopathological changes at 40 mg/kg BW underscore the need for dose optimization and further safety evaluations.

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Authors Contribution

AN and IPS were involved in the conceptualization of the study. CDH, DG, and DC performed the experiments. CDH, DG, DC, and IMM analyzed the collected data. CDH prepared the figures and draft the original manuscript. AN and IPS supervised the overall study. All authors agreed to the published version of the manuscript.

References

1. Ferreyra-Suarez D, Paredes-Vargas L, Jafari SM, García-Depraect O, Castro-Muñoz R. Extraction pathways and purification strategies towards carminic acid as natural-based food colorant: A comprehensive review. Adv Colloid Interface Sci. 2024; 323: 103052. doi: 10.1016/j.cis.2023.103052.
2. Center for Science in the Public Interest [Internet]. Synthetic Food Dyes: A Rainbow of Risks [cited 2024 Dec 31]. Available from: https://www.cspinet.org/cspi-news/synthetic-food-dyes-rainbow-risks.
3. Hamdin CD, Prasedya ES, Utami SW, Galanova D, Saputro DC, Nurrochmad A, *et al.* Acute toxicity of Indonesian natural food colorant Tectona grandis leaf extract in wistar rats. J Med Sci. 2019; 19(2): 69–74.
4. Hamdin CD, Cahyo D, Galanova D. Ketoksikan akut oral zat pewarna makanan daun jati (Tectona grandis L. f.) pada tikus wistar. Pro Food. 2017; 3(2): 240–6.
5. Kamsu GT, Djamen Chuisseu DP, Fodouop Chegaing SP, Laure Feudjio HB, Ndel Famen LC, Kodjio N, *et al.* Toxicological profile of the aqueous extract of Tectona grandis L.F. (Verbenaceae) leaves: A medicinal plant used in the treatment of typhoid fever in traditional Cameroonian medicine. J Toxicol. 2021; 2021: 6646771. doi: 10.1155/2021/6646771.
6. OECD [Internet]. Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption 018 [updated 2018 Sep 3; cited 2024 Dec 31]. Available from: https://www.oecd.org/en/publications/guidance-document-on-standardised-test-guidelines-for-evaluating-chemicals-for-endocrine-disruption-2nd-edition_9789264304741-en.html.
7. Sumiwi SA, Zuhrotun A, Hendriani R, Rizal M, Levita J, Megantara S. Subchronic toxicity of ethanol extract of Syzygium polyanthum (Wight) Walp. leaves on wistar rat. Indones Biomed J. 2019; 11(1): 30–5.
8. Hamdin CD, Wu ML, Chen CM, Ho YC, Jiang WC, Gung PY, *et al.* Dual-specificity phosphatase 6 deficiency attenuates arterial-injury-induced intimal hyperplasia in mice. Int J Mol Sci. 2023; 24(24): 17136. doi: 10.3390/ijms242417136.
9. Tcheutchoua YC, Bilanda DC, Mengue Ngadena YS, Djomeni Dzeufiet PD, Owona PE, Goufani RBA, *et al.* Acute and subchronic toxicity studies on the aqueous extract of the plant mixture (Bidens pilosa and Cymbopogon citratus aerial parts) in rat model. J Toxicol. 2022; 2022: 1998433. doi: 10.1155/2022/1998433.
10. López-García E, Banegas Banegas JR, Gutiérrez-Fisac JL, Pérez-Regadera AG, Gañán LD, Rodríguez-Artalejo F. Relation between body weight and health-related quality of life among the elderly in Spain. Int J Obes Relat Metab Disord. 2003; 27(6): 701–9.
11. Dokuparthi SK, Khan M, Anusha A, Bee M, Shaibaz Dr, Shahajeb S, *et al.* Acute oral toxicity study of Tectona grandis Linn. methanolic seed extract in albino mice. J Phytopharmacol. 2017; 6(3): 183–5.
12. Bohari B, Lestari FD, Rahmadi A. Body weight, cholesterol changes and sub-chronic toxicity of mice treated with an emulsion product rich in β-carotene. Mol Cell Biomed Sci. 2020; 4(1): 19–26.
13. Kawthalkar SM. Essentials of Haematology. New Delhi: Jaypee Brothers Medical Publishers; 2012.

14. Edo GI. Effects of paraquat dichloride on adult male wistar rat; An approach in the toxicity of body weights and hematological tissues. J Anal Pharm Res. 2022; 11(1): 1–7.
15. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: An overview. Scientific World Journal. 2013; 2013: 162750. doi: 10.1155/2013/162750.
16. Melo LFM de, Aquino-Martins VG de Q, Silva AP da, Oliveira Rocha HA, Scortecci KC. Biological and pharmacological aspects of tannins and potential biotechnological applications. Food Chem. 2023: 414: 135645. doi: 10.1016/j.foodchem.2023.135645.
17. Sedlak TW, Snyder SH. Bilirubin benefits: Cellular protection by a biliverdin reductase antioxidant cycle. Pediatrics. 2004; 113(6): 1776–82.
18. Michalopoulos GK. Liver regeneration. J Cell Physiol. 2007; 213(2): 286–300.
19. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, *et al.* Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol. 2000; 32(1): 56–67.
20. Pizzi A. Tannins medical / pharmacological and related applications: A critical review. Sustain Chem Pharm. 2021; 22: 100481. doi: 10.1016/j.scp.2021.100481.
21. Mitruka BM, Rawnsley HM. Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Normal Humans. New York: Masson Pub; 1981.
22. Berardi JM, Logan AC, Rao AV. Plant based dietary supplement increases urinary pH. J Int Soc Sports Nutr. 2008: 5: 20. doi: 10.1186/1550-2783-5-20.
23. Beddiar K, Fen-Chong T, Dupas A, Berthaud Y, Dangla P. Role of pH in electro-osmosis: Experimental study on NaCl–water saturated kaolinite. Transp Porous Med. 2005; 61(1): 93–107.
24. Anywar G, Kakudidi E, Byamukama R, Mukonzo J, Schubert A, Oryem-Origa H, *et al.* A review of the toxicity and phytochemistry of medicinal plant species used by herbalists in treating people living with HIV/AIDS in Uganda. Front Pharmacol. 2021; 12: 615147. doi: 10.3389/fphar.2021.615147.
25. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal. 2014; 20(7): 1126–67.
26. Li Y, Yuan F, He X, Fu S, Fan B. Anthocyanin extract from black rice attenuates chronic inflammation in DSS-induced colitis mouse model by modulating the gut microbiota. Open Chemistry. 2023; 2(1): 20220288. doi: 10.1515/chem-2022-0288.
27. Park JU, Kang JH, Rahman MAA, Hussain A, Cho JS, Lee YI. Gastroprotective effects of plants extracts on gastric mucosal injury in experimental sprague-dawley rats. Biomed Res Int. 2019; 2019: 8759708. doi: 10.1155/2019/8759708.
28. Özcan A, Boyman O. Mechanisms regulating neutrophil responses in immunity, allergy, and autoimmunity. Allergy. 2022; 77(12): 3567–83.