



Unveiling the Safety of *Cassia alata* Leaf Extract: A Subchronic Oral Toxicity in Rats

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Abstract | *Cassia alata* has been employed as a traditional herbal medicine for several disorders, demonstrating anticancer, antiallergic, antioxidant, anti-inflammatory, antifungal, and antidiabetic properties. The main aim of this study was to evaluate the subchronic oral toxicity of the extract obtained from *Cassia alata* leaves (CALE). Male and female rats were orally administered four different dosages of CALE (0, 100, 300, and 900 mg/kg/day) for a duration of 28 days. Hematological and biochemical data, organ weights, histological findings, clinical symptoms, mortality rates, food and fluid intake, and fluctuations in body weight were rigorously observed. The CALE data reveal no statistically significant changes in mortality is 0%, hematological, biochemical and organ weights parameters are within normal range, food and water consumption is normal, body weight increases with age and no clinical symptoms appear. The histopathological analysis indicated no changes in the kidneys or liver. We conclude that no observed adverse effects of rats for both sexes after 28 days of oral CALE treatment at ≤ 900 mg/kg/day. CALE has the potential to develop into herbal medicine.

Keywords | *Cassia alata*, Herb medicine, OECD guidelines, Phytochemical analysis, Safety assessment, Subchronic toxicity

Received | November 17, 2024; **Accepted** | February 03, 2025; **Published** | March 18, 2025

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Citation | Fadholly A, Sa'diah S, Pristihadi DN, Harlina E, Ramadhan MK, Budiharjo YAK, Adinugroho CR, Rahmah AA, Azizah NF (2025). Unveiling the safety of *Cassia alata* leaf extract: A subchronic oral toxicity in rats. Adv. Anim. Vet. Sci. 13(4): 817-823.

DOI | <https://dx.doi.org/10.17582/journal.aavs/2025/13.4.817.823>

ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331



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INTRODUCTION

The use of herbal medicine has risen in various countries in recent decades, despite its established traditional effectiveness, it still encounters challenges regarding

inadequate quality control and a lack of scientific evidence pertaining to its efficacy and safety (Wang *et al.*, 2023; An-riyanto *et al.*, 2024). *Cassia alata* is a herbaceous medicinal plant that has demonstrated potential benefits according to research findings. In Indonesia, *Cassia alata* is referred to as

Ketepeng Cina. Prior research has shown that the root of *Cassia alata* is utilized in the management of rheumatism (Fatmawati *et al.*, 2020). The seeds and leaves serve as potent fungicides, therapeutic agents for dermatitis, and laxatives for alleviating constipation. Fresh leaves, when pulverized with water, garlic, red chalk, and balm, can effectively remedy skin affected by ringworm or fungus through direct application to the affected region (Chew *et al.*, 2022; Yon *et al.*, 2023). It is said to alleviate stomach discomfort during pregnancy, migraines, and paralysis (Colin *et al.*, 2024).

Cassia alata has various kinds of phytochemicals that affect its biological functions. *Cassia alata* functions as an antibacterial agent by causing permanent damage to bacterial cell membranes, resulting in the loss of cytoplasmic contents, ion leakage, and depletion of energy sources like glucose and ATP. *Cassia alata* functions as an antifungal agent by blocking the enzyme lanosterol 14- α demethylase. *Cassia alata* functions as an anti-inflammatory drug by diminishing TNF- α and IL-8 levels through the reduction of reactive oxygen species (ROS). *Cassia alata* functions as an antioxidant by neutralizing harmful free radicals and chelating metal ions to prevent the generation of deleterious radicals that threaten vital biomolecules (Toh *et al.*, 2023; Collin *et al.*, 2024). The leaves of *Cassia alata* are particularly rich in flavonoids, such as kaempferol and its derivatives, isoflavones, and rutin. Anthraquinones, including emodin and rhein, as well as terpenes, glycosides, alkaloids, coumarins, saponins, and other chemicals are also present (Turista *et al.*, 2023; Keng *et al.*, 2024).

The various benefits of the chemical components of the *Cassia alata* plant enhance its traditional application. To preserve this history, it can be enhanced by creating a profile of the substance composition and safety assessment data of the *Cassia alata* plant. Sub-chronic toxicity evaluations can be performed to validate cumulative effect data from plants and dosages that may elicit toxic, carcinogenic, teratogenic, and mutagenic effects in animals or humans. The choice of these tests provides corroborative evidence for the safety of test preparations utilizing experimental animals (Jităreanu *et al.*, 2023). A restricted number have undergone clinical testing, leading to persistent consumer skepticism about the efficacy and safety of the *Cassia alata* plant. These investigations will ascertain the harmful effects of *Cassia alata* on biological systems and provide standard dose-response data within the effective dosage range for prolonged usage durations (Gatt *et al.*, 2024).

MATERIALS AND METHODS

PLANT MATERIAL

The leaves of *Cassia alata* were gathered and verified at the Tropical Biopharmaca Research Centre, IPB University, Indonesia.

EXTRACT PREPARATION

One kilogram of *Cassia alata* leaf samples undergoes a drying process in an oven at 40°C for two days. Samples were ground using a blender, and the desiccated simplicia were weighed. Simplicia is produced by a unique maceration method employing a solvent consisting of 70% ethanol, with a simplicia to solvent ratio of 1:10. When the ethanol concentration is above 70%, the extraction rate of target components is diminished marginally, likely due to increased diffusion resistance from protein denaturation at elevated ethanol levels. Following this, *Cassia alata* extract was subjected to phytochemical screening (Chua *et al.*, 2019).

ANIMALS

The subchronic toxicity test was performed following the protocol specified in the Organization of Economic Co-operation and Development (OECD) guideline 408 for chemical assessment (OECD, 2008). Rats of both sexes, aged 6–8 weeks (200–250g), were randomly assigned to six groups: one control group, three treatment groups, and two satellite groups ($n = 60$; 30 males and 30 females). Each group was divided into 5 males and 5 females. Rats received oral administration of single doses of 0, 100, 300, and 900 mg/kg of *Cassia alata* leaf extract daily for a duration of 28 days. Two additional groups were also formed as a satellite group (0 and 900 mg/kg BB) to observe the reversal sign of any toxicity throughout a duration of 14 days.

CLINICAL OBSERVATIONS AND MORTALITY

All animals were observed daily, and clinical signs and mortality, if observed, were recorded throughout a duration of 28 days. Body weight, as well as food and drink intake, was evaluated weekly. After a week of acclimatization, rats were observed bi-daily (before and after treatment) for typical symptoms and mortality. Food and water consumption was recorded once before treatment commenced and approximately weekly afterward by evaluating the discrepancy between the quantities provided and the amounts remained the following day. The body weight of each rat group was measured every three days consistently. Clinical manifestations including changes in the skin, hair, eyes, mucous membranes, secretions, excretions, autonomic processes (including lacrimation, piloerection, pupillary response, and respiratory patterns), disturbances in gait, behavior, and the presence of seizures. At the end of the fourth week, an evaluation of sensory responses to stimuli (auditory, visual, proprioceptive, and motor, including grip strength) was performed (Sutrisni *et al.*, 2019).

HEMATOLOGY AND SERUM BIOCHEMISTRY

Blood samples collected from all animals were analyzed to determine the red blood cell count, hemoglobin concentration, hematocrit level, platelet count, white blood cell count (WBC), differential WBC count, and reticulocyte count. All indicators were evaluated using a Mindray Animal Care

BC-5000 Vet (China). Serum biochemistry parameters were assessed, including blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine (CREA) using a Mindray BA-88a (China) (Aydemir *et al.*, 2022).

POST MORTEM AND HISTOPATHOLOGICAL EXAMINATIONS

Following blood collection, the organs (liver, spleen, kidneys, heart, lungs) were removed, weighed, and analyzed morphologically. The overall weight was assessed, and the relative organ weight (as a percentage of body weight) was calculated. For histological analysis, the organ samples were immersed in a 10% neutral buffered formalin solution and incubated for 24 hours at 4°C, thereafter subjected to a tissue processor overnight, followed by embedding in paraffin using embedding molds and stored at 4°C. All specimens were sliced and stained with hematoxylin and eosin (Wehrle *et al.*, 2024).

STATISTICAL ANALYSIS

All experimental data were documented in Microsoft Excel and statistically analyzed using SPSS 19.0. The initial test conducted was assessing normality and homogeneity of variance, subsequently followed by one-way analysis of variance (ANOVA) and the Tukey HSD post hoc test. The paired t-test was utilized to compare data gathered before and after the trial. P values under 0.05 were considered significant (Bemidinezhad *et al.*, 2023).

RESULTS AND DISCUSSION

Animal toxicity studies are commonly employed to assess potential health concerns to humans arising from the intrinsic harmful effects of chemical constituents in plant extracts (Patrick-Iwuanyanwu *et al.*, 2015). Subchronic studies assess the adverse effects of repeated or prolonged exposure to plant extracts or chemicals over a segment of the typical lifespan of experimental animals, such as rodents. Furthermore, subchronic assessment can aid in determining appropriate dosage regimens for extended studies (Wresdiyati *et al.*, 2023).

Phytochemical analysis is a crucial phase in exploring the potential of medicinal plant resources (Dubale *et al.*, 2023). Moreover, variables like soil composition, temperature, humidity, climate, and altitude influence the results of phytochemical studies. The analytical data may yield insights into several types of secondary metabolites, such as triterpenoids, alkaloids, flavonoids, and steroids (Brahmi *et al.*, 2022). The leaves of *Cassia alata* exhibit a good presence of flavonoids, saponins, and quinones (Table 1). This pertains to numerous studies utilizing *Cassia alata* leaves for various therapeutic applications. Flavonoids and saponins are extensively utilized for their antioxidant and anti-in-

flammatory properties, while quinones provide antifungal, antimalarial, and laxative functions. This content indicates that the appropriate dosage of *Cassia alata* leaves will yield significant advantages (Sun and Shahrajabian, 2023).

Table 1: Phytochemical screening results of *Cassia alata* leaves extract.

Parameters	Results
Flavonoids	Positive
Alkaloids	Negative
Tannin	Negative
Saponin	Positive
Quinone	Positive
Steroid	Negative
Terpenoids	Negative

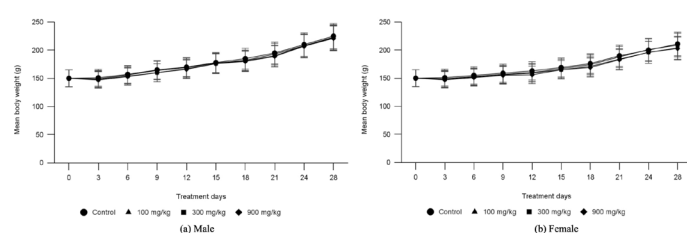


Figure 1: Mean body weight changes in male (a) and female (b) rats treated with CALE at dose levels of 0 (control), 100, 300, and 900 mg/kg/day for 28 days.

Clinical symptoms were observed to assess the delayed toxicity of the formulation in rats (Parasuraman, 2011). The assessment of clinical symptoms in test animals during therapy revealed no abnormalities in skin changes, fur condition, eye health, mucous membranes, secretions, excretions, autonomic function, locomotion, behavior, seizures, or sensory responses. Both male and female rats have diverse behaviors. Furthermore, no fatalities were recorded after 28 days, indicating that CALE preparation is not toxicologically relevant in any treatment group relative to the control groups. The qualitative assessment of daily food and beverage consumption revealed no significant changes in intake during the research. The results indicate no reduction in appetite (data not provided). Related with the body weight measurements of the treatment group during 28 days, the data showed no significant difference compared to the control group. All groups exhibited an increase in body weight corresponding to the number of days administered. Body weight measurements are essential for determining the maximal dosage that experimental animals can endure and serve as a sensitive indicator of their overall health (Asto *et al.*, 2017). The weight increase in experimental animals after CALE treatment can be ascribed to the nutritional constituents of CALE and the lack of disruption to the digestive tract and nutrient absorption. The outcomes of the CALE delivered to rats are depicted in Figure 1, illustrating the average body weight of each group.

Table 2: Relative organ weights of rats treated orally with CALE (percentage of body weight) for 28 days.

Parameters	Male				Female			
	Control	100 mg/kg	300 mg/kg	900 mg/kg	Control	100 mg/kg	300 mg/kg	900 mg/kg
Liver	2.73 ±0.08	2.73 ±0.07	2.78 ±0.02	2.77 ±0.01	2.75 ±0.03	2.72 ±0.08	2.76±0.06	2.77±0.01
Kidneys	0.65±0.12	0.65±0.22	0.66±0.15	0.66±0.04	0.65±0.02	0.66±0.03	0.7±0.03	0.75±0.07*
Heart	0.28±0.05	0.27±0.04	0.27±0.01	0.27±0.05	0.32±0.02	0.31±0.07	0.31±0.04	0.33±0.09
Spleen	0.16±0.08	0.15±0.03	0.15±0.02	0.16±0.07	0.18±0.08	0.18±0.03	0.18±0.08	0.19±0.06
Lungs	0.3±0.05	0.3±0.02	0.3±0.02	0.31±0.04	0.44±0.04	0.42±0.05	0.42±0.03	0.45±0.05

*significant differences from control group (p < 0.05).

Relative organ weights may be assessed using macroscopic evaluation, serum biochemical analysis, and microscopic histopathology in toxicity studies to examine the liver and kidneys (Nouioura *et al.*, 2023). Evaluating organ weight is essential in toxicity studies as it aids in forecasting toxicity, acute injury, physiological disruptions, and enzyme induction. The study's findings indicate that the vital organs heart, liver, spleen, kidneys, and lungs were not impacted by the treatment and exhibited no clinical signs of toxicity; the only statistically significant weight increase was observed in the relative kidneys of female rats administered CALE (Table 2). No discernible clinical indications were noted, and these changes remained within the usual parameters. These adverse effects may manifest as alterations in biomolecule concentrations, including enzymes and metabolic byproducts, alongside fluctuations in organ function (Heindel *et al.*, 2017). No treatment-related histopathological abnormalities were identified in either sex after 28 days of CALE and histological analysis. Neither the liver nor the kidney exhibited any abnormalities, including degeneration and necrosis of liver zonation, proximal tubules, and mesangial cells (Figure 2). Thus, these fluctuations in relative kidney weight were not considered aberrations attributable to CALE. The absence of any decrease in body and relative organ weights of the treated animals across all supplied doses indicates that the extract is harmless to the evaluated organs.

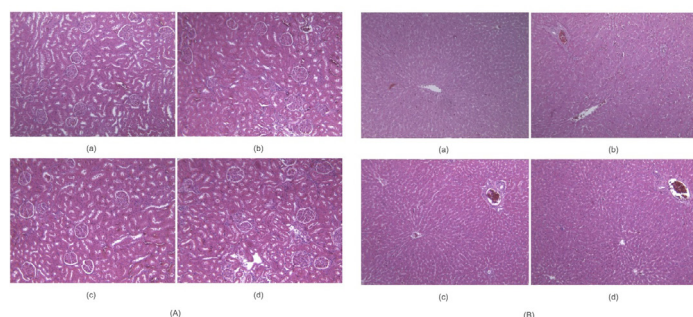


Figure 2: Representative images of histopathologic changes in kidneys of male rats (A) and liver of female rats (B) in each dose group for 28 days of CALE administration. (a): control; (b): 100 mg/kg/day; (c): 300 mg/kg/day; (d): 900 mg/kg/day.

The hematological and biochemical studies were conducted to assess potential changes in hepatic and renal functions affected by the CALE. Assessing liver and kidney function is essential for evaluating the toxicity of pharmaceuticals and botanical extracts, as both organs are crucial for an organism's survival. The liver governs bodily homeostasis, encompassing metabolism, biotransformation, protein synthesis, bile acid production, and energy storage. Alterations in the activity and concentrations of tumor marker enzymes, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in hepatic tissue, may indicate hepatotoxicity (Thakur *et al.*, 2024). Elevated levels of ALT, AST, and alkaline phosphatase indicate liver impairment or hepatotoxicity. The minor variations in ALT, AST, and alkaline phosphatase levels in both male and female rats across all dosages indicate that subchronic CALE treatment does not impact hepatocyte function in the rats. Renal function was evaluated using simultaneous measures of blood urea nitrogen (BUN) and creatinine (CREA), with normal readings indicating a diminished probability of renal problems. Renal cells regulate metabolic waste in the circulation to maintain homeostasis (Imenez-Silva and Mohebbi, 2022). Elevated BUN and CREA levels in the blood signify impaired renal function (Sujino *et al.*, 2019). The ALT, AST, BUN, and CREA levels demonstrated in normal range, indicating both normal renal and kidney function (Table 3). Histological analyses indicate no significant pathological alterations in the morphology and architecture of the rat's liver and kidneys.

Hematological parameters are assessed to elucidate physiology and assist in disease diagnosis in animals. This methodology is pertinent to risk assessment, as alterations in the hematological system have greater predictive significance for toxicity (Pessini *et al.*, 2020; Wiono *et al.*, 2023). A haemogram was conducted for all CALE-treated and control groups, revealing no significant effects. The negligible effect of the extract on total red blood cells, mean corpuscular hemoglobin, and platelets suggest that the CALE does not influence erythropoiesis, morphology, or osmotic fragility of red blood cells. Erythrocytes are the principal indicators of oxygen distribution inside the body. Leukocytes serve as the primary cellular defense against viral, bacterial, and parasitic

Table 3: Serum biochemical values of rats treated orally with CALE for 28 days.

Parameters	Male				Female			
	Control	100 mg/kg	300 mg/kg	900 mg/kg	Control	100 mg/kg	300 mg/kg	900 mg/kg
BUN (mg/dL)	14.8±1.54	14.6±0.89	14.5±0.11	13.7±0.93	18.4±0.43	16.9±1.56	17.1±0.11	17.4±0.82
CREA (mg/dL)	0.56±0.09	0.55±0.21	0.55±2.32	0.57±0.11	0.71±0.77	0.69±0.34	0.69±1.45	0.69±1.98
ALT (IU/L)	35.1±0.67	33.8±0.04	33.9±1.89	34.2±0.07	34.4±0.14	37.9±0.41	37.1±0.07	38.8±0.89
AST (IU/L)	124.9±1.33	119.4±0.06	116.1±1.87	121.4±0.4	116.8±0.23	123±01.45	121±0.48	118±1.33

Table 4: Hematological values of rats treated orally with CALE for 28 days.

Parameters	Male				Female			
	Control	100 mg/kg	300 mg/kg	900 mg/kg	Control	100 mg/kg	300 mg/kg	900 mg/kg
RBC (X10 ⁶ /μL)	9.56±0.12	9.11±0.46	9.67±1.78	9.38±0.34	8.23±0.13	8.44±0.88	8.43±1.34	8.57±0.21
HGB (g/dL)	16.57±1.66	16.2±0.52	16.6±0.57	16.3±1.22	16.0±0.5	16.11±1.45	16.2±0.87	16.4±0.44
HCT (%)	49.32±1.96	50.21±0.11	50.25±0.8	49.6±1.76	48.8±1.33	48.06±0.51	47.1±0.9	47.56±0.16
MCV (fL)	53.2±0.77	54.05±1.11	54.8±0.08	53.5±0.56	55.9±0.11	55.41±0.47	56.1±1.88	56.5±0.31
MCH (pg)	17.9±0.55	17.41±0.32	17.7±1.49	17.9±0.54	18.4±1.89	19.8±0.84	19.5±1.53	19.61±0.41
MCHC (g/dL)	32.5±1.14	32.8±0.44	32.9±1.77	33.1±1.89	33.7±0.19	33.6±1.31	33.8±1.99	34.2±0.76
PLT (x10 ³ /μL)	1197±61.1	1084±0.33	1048±1.1	1097±0.35	1065±0.8	1075±0.76	1056±1.3	1011±1.97
WBC (x10 ³ /μL)	13.32±2.89	11.89±0.55	11.94±1.9	10.6±0.51	8.11±0.17	8.56±0.09	8.14±0.96	7.88±0.11
Reticulocyte (%)	2.9±0.97	2.1±0.58	2.38±2.08	2.7±0.05	2.7±0.19	2.6±0.71	2.7±0.04	2.3±0.19
Neutrophils (%)	15.06±0.98	16.22±0.67	16.34±1.4	19.3±0.56	12.97±0.1	13.09±0.11	13.1±0.56	11.78±0.9
Lymphocytes (%)	79.6±1.95	76.9±0.54	77.4±1.62	75.45±0.1	82.9±0.66	81.76±0.11	81.1±1.86	82.9±0.15
Monocytes (%)	3.1±0.88	3.07±0.34	2.98±1.92	2.84±0.43	2.8±0.19	3.1±0.84	3.2±0.88	3.2±0.82
Eosinophils (%)	1.2±0.66	1.3±0.28	1.3±0.86	1.4±0.77	1.2±0.12	1.3±0.11	1.3±0.81	1.3±0.09
Basophils (%)	0.6±0.88	0.8±0.5	0.8±0.33	0.7±0.78	0.7±0.82	0.6±1.45	0.6±0.35	0.7±1.73

infections, as well as suppressing cellular growth (Pawlowska *et al.*, 2024). Moreover, no substantial alterations were detected in the neutrophils, lymphocytes, and monocytes following CALE administration. The conventional hematological profile of the CALE-treated groups further validated the non-toxic properties of CALE. The findings of the hematological assessment for both the control group and the group administered CALE are displayed in Table 4.

The toxicity of a chemical extract can impact essential organs involved in toxin processing, including the heart, lungs, spleen, liver, and kidneys (Bassan *et al.*, 2021). All organ systems can be susceptible to toxic exposure. Alterations in hazardous exposure encompass the location, quantity, dimensions, hue, morphology, and texture of organs, in addition to the serum biochemical parameters (Khan and Jia, 2023). The additional satellite groups were administered both zero and maximum dosages to assess reversibility, persistence, delayed onset, or delayed effects of the test preparation for systemic toxicity, as well as recovery within a 14-day post-treatment interval. Results indicated no reversibility or persistence of any toxic effects in the satellite group, thereby further confirming the nontoxic nature of CALE assessed in this study. Consequently, it is proposed

that the administration of *Cassia alata* leaf extract exhibits no detrimental effects in both male and female rats during a 28-day treatment period.

CONCLUSIONS AND RECOMMENDATIONS

Our results demonstrate that 28 days of uninterrupted oral administration of CALE did not produce any adverse effects in the assessment of clinical symptom parameters, body and organ weight, blood, blood chemistry, and histopathology of male and female rats at the maximal dosage of ≤ 900 mg/kg/day for both sexes. CALE has the potential to develop into a herbal medicine. Future studies may necessitate higher dosages to assess the potential adverse effects of CALE on test subjects and should include more species, with study potentially extending up to 90 days.

ACKNOWLEDGMENTS

This research received complete financing from Penelitian Dasar Dosen Muda 2024, reference number 23405/IT3/PT.01.03/P/B/2024, provided by IPB University, Indonesia.

The subchronic toxicity results on male and female rats can serve as foundational information for utilizing *Cassia alata* leaves extract in subsequent toxicity research.

AUTHOR'S CONTRIBUTIONS

Amaq Fadholly, Yumna Annisa Kurnia Budiharjo, Cyntia Rafi Adinugroho, Afifah Alawiyah Rahmah, Nahla Fifi Azizah, and Maharani Kartika Ramadhan contributed to data collection, data analysis, and the compilation of the original text. Siti Sa'diah, Diah Nugrahani Pristihadi, and Eva Harlina participated in the study design, manuscript review, and oversight. The authors have reviewed and sanctioned the final version of the text.

ETHICAL APPROVAL

All treatment procedures were conducted under the auspices of the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences (SVMBS), IPB University, Indonesia (Approval reference number: 244/KEH/SKE/IX/2024). All precautions were executed to mitigate animal pain throughout this experiment, adhering to the Animal Research: Reporting of In Vivo Experiments (ARRIVE guidelines).

CONFLICT OF INTEREST

The authors assert that they possess no conflicting interests.

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