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In vivo Genotoxicity Evaluation of *Hemerocallis fulva* Leaf Powder by Micronucleus Assay

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Abstract

In recent years, various factors such as shifts in health consciousness, changes in lifestyle habits, increased focus on dietary intake, the promotion of alternative medical concepts, and advancements in life sciences and technology have propelled the robust growth of the health food industry. An increasing number of reports suggest that "functional foods," containing beneficial functional components, may enhance short-term well-being and are increasingly regarded as healthful. Building upon these findings, this study aims to enhance the production capacity of high-value agricultural products. *Hemerocallis fulva* has demonstrated multiple functional properties in various studies. Its extracts possess significant antioxidant and anti-inflammatory activities, which may help mitigate oxidative stress and inflammation-related conditions. Additionally, evidence suggests potential hepatoprotective, neuroprotective, and anti-fatigue effects, possibly through the modulation of biochemical pathways and enhancement of cellular resilience. Traditional use and experimental findings also indicate its sedative and anxiolytic potential. These findings support the potential application of *H. fulva* as a functional ingredient in health-promoting formulations. However, for quality assurance, toxicity assessment of functional foods is imperative. There is a growing necessity to employ *in vivo* genotoxicity assays to evaluate the carcinogenic potential of these products. In previous study, we have successfully established *in vivo* genotoxicity evaluation platform via acridine orange induction. In this study, administration of Cyclophosphamide monohydrate (CPP) or *H. fulva* leaf powder in ICR mice showed no adverse effects on body weight or clinical signs. All animals survived throughout the study. Reticulocyte and micronucleated reticulocyte analyses at 48 and 72 hours post-administration indicated that the negative control group exhibited significantly decreased reticulocyte ratios and increased micronucleus frequencies compared to the normal control group ($p < 0.05$). In contrast, all *H. fulva* leaf powder dose groups showed no significant differences from the normal control group ($p > 0.05$), suggesting that *H. fulva* leaf powder did not induce hematological toxicity or genotoxicity under the test conditions. Taken all results together, *H. fulva* leaf powder were without genotoxicity. Therefore, *H. fulva* leaf powder were safety.

Keywords: Genotoxicity; *Hemerocallis fulva* leaf powder; ICR mice; *In vivo*; Micronucleus assay; Reticulocytes

1. Introduction

In recent years, the global health food industry has experienced rapid growth, driven by a confluence of factors including heightened health awareness, evolving lifestyle and dietary habits, increasing interest in natural and preventive health strategies, and the advancement of life sciences and biotechnology. Particularly in the Asia-Pacific region, and notably

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within the Association of Southeast Asian Nations (ASEAN), market demand for functional foods has surged in parallel with an aging population and rising disposable incomes. As consumers increasingly seek out products that offer not just basic nutrition but also added health benefits, the development of functional ingredients derived from natural sources has become a focal point for research and commercial innovation [1-6].

Taiwan, with its robust foundation in agricultural science, has demonstrated strong research output and international recognition. Agricultural science publications from Taiwan are cited at a higher rate compared to other disciplines, underscoring the nation's research strength and the effectiveness of its scientific endeavors [7-13]. More recently, there has been a growing emphasis on transforming upstream scientific achievements into tangible, value-added products through industrialization strategies. Government policy and public interest have converged to support the commercialization of research results, presenting a timely opportunity for Taiwan's agricultural and biotech sectors to showcase their innovative capabilities and contribute to the global functional food market.

Despite the substantial technological capacity in research and development, a critical bottleneck remains in the translation of laboratory findings into safe, effective, and market-ready products. To address this gap, it is essential to establish standardized, accurate, and rapid preclinical screening systems. In particular, animal models remain indispensable for evaluating the safety and efficacy of functional foods prior to market introduction. A reliable in vivo genotoxicity assessment platform-such as the mammalian erythrocyte micronucleus assay-is increasingly recognized as a necessary tool to screen for potential mutagenic or carcinogenic risks of newly developed health food ingredients [14-19].

Functional foods, defined as foods that provide health benefits beyond basic nutrition due to the presence of bioactive compounds, are gaining attention not only for their role in long-term disease prevention but also for their potential to improve short-term physiological performance and overall well-being. However, the safety of these bioactive compounds must be carefully evaluated through validated toxicological approaches to ensure consumer protection [20-23].

Hemerocallis fulva, commonly known as daylily, is a perennial herbaceous plant that has long been cultivated and consumed throughout East Asia, including in China, Korea, and Taiwan. Traditionally, its flower buds and leaves have been used both as culinary ingredients and in herbal medicine for their nutritional and therapeutic properties. Historical texts and ethnobotanical records highlight its calming and sedative effects, with preparations such as soups and teas commonly used to reduce anxiety and improve sleep quality. In recent years, phytochemical investigations have identified a wide array of functional constituents in *H. fulva*, including flavonoids, phenolic acids, and polysaccharides, which possess antioxidant, anti-inflammatory, hepatoprotective, neuroprotective, and anti-fatigue activities. These multifunctional properties make *H. fulva* a promising candidate for development into a functional food ingredient that aligns with current health trends and consumer preferences [24].

Given the increasing interest in *H. fulva* as a functional botanical, it is crucial to evaluate its safety profile using rigorous scientific methods. The present study employs the mammalian erythrocyte micronucleus assay to detect chromosomal damage in erythroblasts, serving as a biomarker for genotoxic potential. This sensitive and widely accepted *in vivo* assay [25] enables the quantification of micronuclei formation in peripheral blood reticulocytes, thereby providing essential data to assess the mutagenic risk of *H. fulva* leaf powder. The findings will contribute to the overall toxicological evaluation and help support the safe use of this traditional plant in the functional food industry.

2. Materials and Methods

2.1. Chemicals and Reagents

Cyclophosphamide monohydrate (CPP; Sigma-Aldrich, Cat. No. C0768), acridine orange solution (Sigma-Aldrich, Cat. No. 65-61-2), reverse osmosis water, phosphate-buffered saline (PBS; Sigma-Aldrich, Cat. No.P3813), saline (Taiwan Biotech Co., LTD, Cat. No. 100-120-1101), and Zoletil 50 (Virbac, Carros, France) were used in this study [25].

2.2. Experimental Animals and Experimental Design

Adult male ICR mice [6 weeks old; 25 mice; body weight (BW) between 26-27g)] with specific pathogen-free conditions were used for this study, were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). These ICR mice were fed with standard laboratory diet (No. 5001, LabDiet®; PMI Nutrition International, St. Louis, MO, USA) and distilled water ad libitum during the experimental period. The environment was maintained room temperature (24-27°C) and 60%-70% humidity with a photoperiod of 12-hr light/12-hr dark cycle. The study will begin after a week acclimation. The

Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC-112089 approved by the IACUC ethics committee. The 25 ICR male mice were randomly divided respectively into as the normal control group (n = 5), the negative control group (n = 5), *H. fulva* leaf powder-low dose (n = 5), *H. fulva* leaf powder-median dose (n = 5), and *H. fulva* leaf powder-high dose (n = 5). The CPP-induced mice' genotoxicity via intraperitoneal injection were performed in the negative control group and three *H. fulva* leaf powder groups. In the normal control group, the mice were treated with reverse osmosis water via gavage. The clinical behaviors, BW, food consumption, and blood smear exanimation were monitored and performed during the experiment.

2.3. Collection of Peripheral Blood from ICR Mice

The ICR mice were anesthetized with Zoletil 50 and the blood collection site was cleaned with 70% alcohol. The blood was stored in anticoagulant tubes containing K_2-EDTA for use in the subsequent experiments.

2.4. Preparation of Blood Smear and Acridine Orange Staining with Fluorescence Microscopic Examination

After 48 and 72 hours post-administration of the test substance, blood samples were collected via performing submandibular blood collection from the restrained mice, and 5 μL of blood was obtained to prepare blood smears. Blood was placed on pre-dyed slides with 0.1% acridine orange solution, gently covered with a cover slip, and spread evenly to a single layer thickness of blood cells. After incubating at room temperature and avoiding light for 3 hours, the number of reticulocytes and micronucleated reticulocytes in the blood were observed under a fluorescence microscope. The 1,000 reticulocytes were observed in each ICR mouse, the number of micronuclei was recorded, and the proportion of reticulocytes in total red blood cells was calculated.

2.5. Statistical Analysis

The data of BW, reticulocyte percentage, and reticulocyte micronucleus incidence rate for each group of mice were expressed as mean and standard deviation (SD). Mice' BW were analyzed using one-way ANOVA followed by Duncan's multiple range test in SPSS statistical software. Reticulocyte percentage and micronucleated reticulocyte incidence rate were analyzed using SPSS software to calculate the median and Mann-Whitney U test. The p value < 0.05 indicates significant differences between groups.

3. Results

3.1. Normality of Mice' Clinical Behavior in Two Groups

In this study, the clinical behavior observation indexes of mice in each group were normal during the experiments. During the experiments, the mice in each group had smooth hair, normal hair color, and the normal activity. Moreover, all mice were survival until the end of the experiments. The survival rate was 100% (25/25) (Table 1).

Table 1 Mice' clinical observation daily in each group during the experiment

Group	Dose (mg/kg BW)	Daily Clinical Observation			
		D0	D2	D3	D4
Normal control	-	N	N	N	N
Negative control	50	N	N	N	N
L	250	N	N	N	N
M	500	N	N	N	N
H	1,000	N	N	N	N

N: Normal. Normal control group was administered reverse osmosis water via gavage; Negative control group was intraperitoneally injected with 50 mg/kg BW cyclophosphamide monohydrate (CPP). "L" represents the low-dose group (250 mg/kg BW) of *H. fulva* leaf powder; "M" represents the medium-dose group (500 mg/kg BW) of *H. fulva* leaf powder. "H" represents the high-dose group (1,000 mg/kg BW) of *H. fulva* leaf powder. Each group was 5 ICR mice (n = 5/group).

3.2. Change of Mice' BW in Two Groups

The BW of mice was detected on day 1 and day 4. During the experiment, the mice' BW continued to rise and there was no statistically significant difference in BW between 5 groups ($p > 0.05$) (Figure 1).

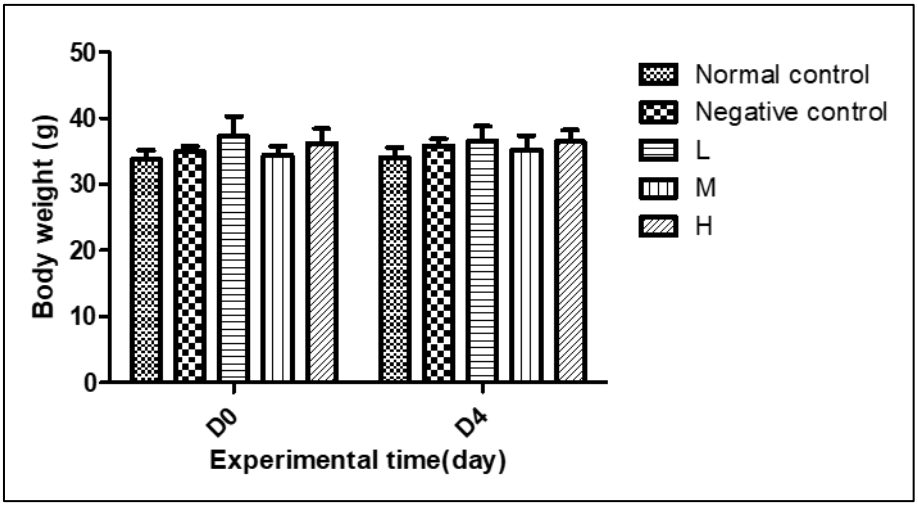

Figure 1 Change of body weight before and after the treatments of three doses of *H. fulva* leaf powder. Normal control group was administered reverse osmosis water via gavage; Negative control group was intraperitoneally injected with 50 mg/kg BW cyclophosphamide monohydrate (CPP). "L" represents the low-dose group (250 mg/kg BW) of *H. fulva* leaf powder; "M" represents the medium-dose group (500 mg/kg BW) of *H. fulva* leaf powder. "H" represents the high-dose group (1,000 mg/kg BW) of *H. fulva* leaf powder. All data are expressed as mean ± SD. Each group was 5 ICR mice (n = 5/group)

3.3. No Significant Change of Mouse's Food Consumption in All Groups

In this study, the mouse's food consumption in each group were monitored during the experiments. The food consumption of mice was recorded daily after the experiment. During the experiments, there were no significant difference between five groups on the food consumption (data not shown).

3.4. Change of RETs/1,000 RBCs and Mn-RETs/1,000 RETs Percentages during the Experiment

Mice' peripheral anticoagulant blood were collected at 48th and 72th hours-experiment, respectively. Blood samples were prepared for blood smears and processed with acridine orange staining. After blood sample processing, they were examined by using fluorescence microscope. The percentages of RETs/1,000 RBCs and Mn-RETs/1,000 RETs were evaluated. Results of reticulocyte analysis at 48 hours after administration of CPP or *H. fulva* leaf powder in ICR mice showed that the median reticulocyte ratio was 45‰ in the normal control group and 18.0‰ in the negative control group. The reticulocyte ratio in the negative control group was significantly lower than that in the normal control group ($p < 0.05$). The median reticulocyte ratios in the low-, medium-, and high-dose *H. fulva* leaf powder groups were 45‰, 43‰, and 52‰, respectively, with no significant differences compared to the normal control group ($p > 0.05$) (Figure 2A); Results of reticulocyte analysis at 72 hours after administration of CPP or *H. fulva* leaf powder in ICR mice showed that the median reticulocyte ratio was 45‰ in the normal control group and 20‰ in the negative control group. The ratio in the negative control group was significantly lower than that in the normal control group ($p < 0.05$). The median reticulocyte ratios in the low-, medium-, and high-dose *H. fulva* leaf powder groups were 48‰, 50‰, and 43‰, respectively, with no significant differences compared to the normal control group ($p > 0.05$) (Figure 2B).

Results of micronucleated reticulocyte analysis at 48 hours after administration of CPP or *H. fulva* leaf powder in ICR mice showed that the median micronucleated reticulocyte frequency was 1‰ in the normal control group and 12‰ in the negative control group. The frequency in the negative control group was significantly higher than that in the normal control group ($p < 0.05$). The median micronucleated reticulocyte frequencies in the low-, medium-, and high-dose *H. fulva* leaf powder groups were 2‰, 2‰, and 1‰, respectively, with no significant differences compared to the normal control group ($p > 0.05$) (Figure 2C); Results of micronucleated reticulocyte analysis at 72 hours after administration of CPP or *H. fulva* leaf powder in ICR mice showed that the median micronucleated reticulocyte frequency was 1‰ in the normal control group and 10‰ in the negative control group. The frequency in the negative control group was significantly higher than that in the normal control group ($p < 0.05$). The median micronucleated reticulocyte

frequencies in the low-, medium-, and high-dose *H. fulva* leaf powder groups were 1‰, 1‰, and 2‰, respectively, with no significant differences compared to the normal control group ($p > 0.05$) (Figure 2D).

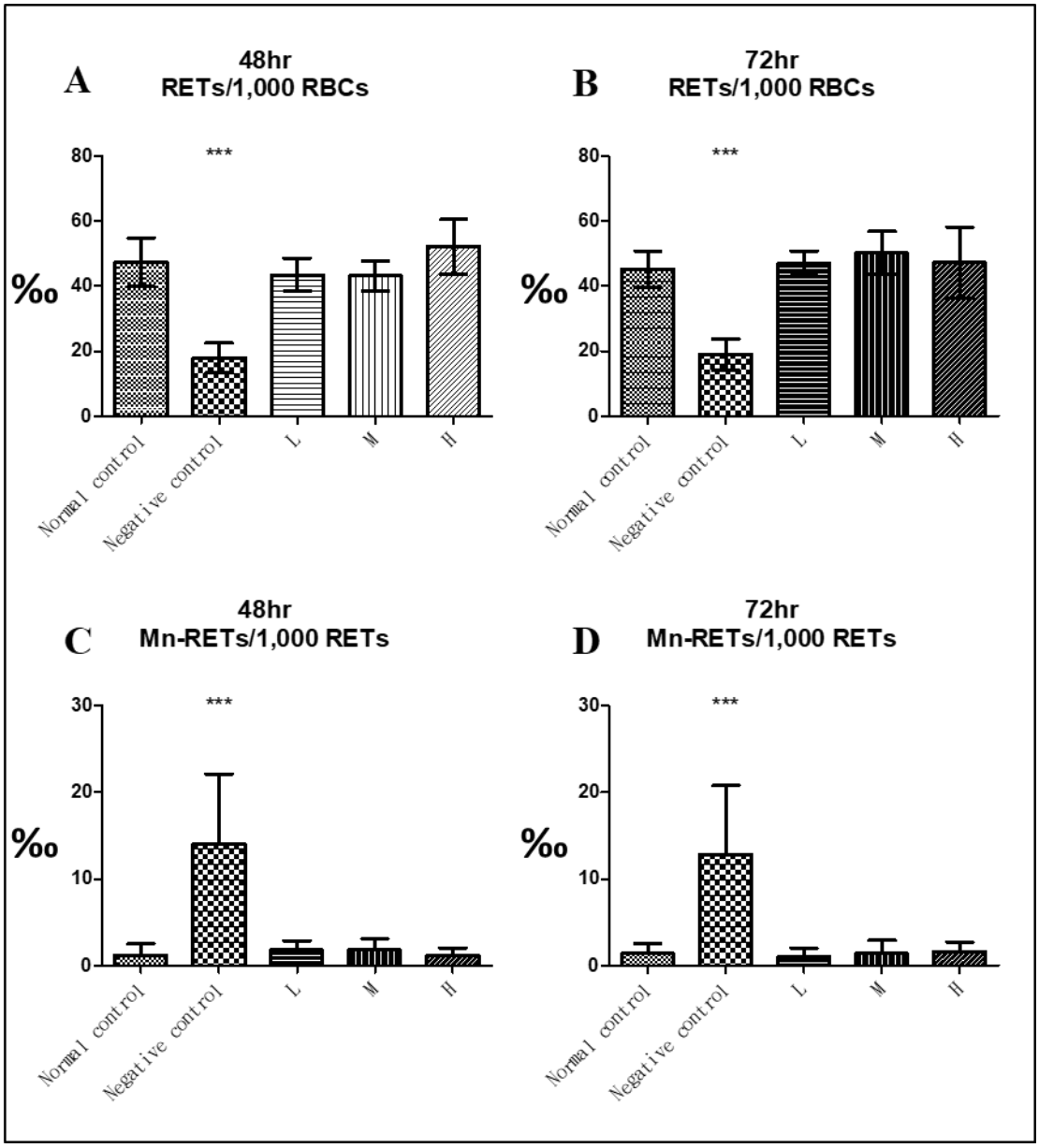

Figure 2 Change of RET/RBCs and MN-RET/RETs in each group at 48th hour- and 72th hour-experiment. (A) RETs/1,000 RBCs (‰) at 48th hours-experiment. (B) RETs/1,000 RBCs (‰) at 72th hours-experiment. (C) Mn-RETs/1,000 RETs (‰) at 48th hours-experiment. (D) Mn-RETs/1,000 RETs (‰) at 72th hours-experiment. Normal control group was administered reverse osmosis water via gavage; Negative control group was intraperitoneally injected with 50 mg/kg BW cyclophosphamide monohydrate (CPP). "L" represents the low-dose group (250 mg/kg BW) of *H. fulva* leaf powder; "M" represents the medium-dose group (500 mg/kg BW) of *H. fulva* leaf powder. "H" represents the high-dose group (1,000 mg/kg BW) of *H. fulva* leaf powder. All data are expressed as mean ± SD. 5 ICR mice in each group was (n = 5/group). All significant differences compared to negative control group were reported at $^{***}p < 0.001$

4. Discussion

In order to evaluate the safety of health food, Taiwan government has formulated a method for evaluating the safety of health food [30]. Among health food safety assessment methods, genotoxicity test methods can be divided into *in vivo* and *in vitro* tests, the purpose of which is to detect the genetic damage and extent directly or indirectly caused by the test substances, including the microbial gene mutation assays, the *in vitro* genotoxicity analysis of mammalian cells, and

the genotoxicity analysis of animals *in vivo* [26-28]. *In vivo* genotoxicity assays in animals generally were used chromosomal damage assays of rodent hematopoietic cells, including bone marrow cell micronucleus assays, chromosomal abnormalities assays or peripheral blood micronucleus assays. Due to the high sensitivity of the micronucleus assays, this method is the most commonly used method for testing toxic drug-induced chromosomal aberrations *in vivo* [29-30].

Our previous results have been published for establishing the genotoxicity evaluation platform via mitomycin C induction [30-32]. During the in vivo genotoxicity-evaluated experiment, the experimental animal's clinical behavior, BW, food consumption, and the percentage of RETs/RBCs (reticulocytes/red blood cells) and Mn-RETs/RETs (micronucleated reticulocytes/reticulocytes) were evaluated. Both sexes ICR mice were given three daily treatments by intraperitoneal injection of 2 mg/kg of mitomycin C (genotoxicity induction) or by oral route of 200 μL of PBS (normal control group). Until 48h after the last treatment, K_2-EDTA-anticoagulated peripheral blood specimens were collected. These blood samples were processed for the microscopy-based analysis using Giemsa stain and the percentage of reticulocytes and micronucleated reticulocytes was determined. The results were shown that the experimental mice' clinical behaviors were normal in all groups. The BW and food consumption were no significant difference between all groups. RETs/RBCs (‰) in male or female ICR mice in the negative control group and the normal control group were respectively 7.8 ± 0.8 / 8.6 ± 0.8 and 23.2 ± 1.5 / 22.1 ± 1.3; Mn-RETs/RETs (‰) in male or female ICR mice in the negative control group and the normal control group were 2.0 ± 0.0 / 2.0 ± 0.0 and 43.2 ± 10.6 / 39.6 ± 10.9, respectively. Both RETs/RBCs (‰) and Mn-RETs/RETs (‰) in male or female ICR mice in the negative control group were significantly difference than the normal control group ($p < 0.001$) [30-32]. In our previous study, we have successfully established *in vivo* genotoxicity evaluation platform via acridine orange induction. According to all results in this study, during the experiment, there were no statistically significant differences in BW between the negative control group and the normal control group ($p > 0.05$). Moreover, the clinical observations of the experimental mice showed that all mice survived until the end of the experiment, and no abnormal clinical symptoms were observed. The results of reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median reticulocyte percentage in the normal control group was 45‰, while in the negative control group it was 18.0‰. The reticulocyte percentage in the negative control group was significantly lower than that in the normal control group. The results of micronucleated reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median micronucleus incidence rate in the normal control group was 0‰, while in the negative control group it was 11‰. The micronucleus incidence rate in the negative control group was significantly higher than that in the normal control group. The results of reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median reticulocyte percentage in the normal control group was 48‰, while in the negative control group it was 20‰. The reticulocyte percentage in the negative control group was significantly lower than that in the normal control group. The results of micronucleated reticulocyte count in CPP-treated ICR mice at 72 hours showed that the median micronucleus incidence rate in the normal control group was 0‰, while in the negative control group it was 8‰. The micronucleus incidence rate in the negative control group was significantly higher than that in the normal control group. Comparison of mitomycin C-induced *in vivo* genotoxicity in ICR mice and acridine orange-induced *in vivo* genotoxicity in ICR mice revealed that the RETs/RBCs (‰) and Mn-RETs/RETs (‰) in the negative control group were all significantly different from those in the normal control group [25, 30-32].

In this study, at both 48 and 72 hours following administration of CPP or *H. fulva* leaf powder in ICR mice, reticulocyte and micronucleated reticulocyte analyses revealed no signs of hematotoxicity or genotoxicity in any dose group. Specifically, the median reticulocyte ratios at 48 hours were 45‰ in the normal control group and 18.0‰ in the negative control group, with the latter showing a significant decrease. In contrast, the low-, medium-, and high-dose *H. fulva* leaf powder groups showed ratios of 45‰, 43‰, and 52‰, respectively, with no significant differences compared to the normal control group. At 72 hours, a similar trend was observed that the normal and negative control groups had median ratios of 45‰ and 20‰, respectively, while the treatment groups showed values of 48‰, 50‰, and 43‰, again without significant differences. Micronucleated reticulocyte frequencies were also evaluated. At 48 hours, the normal control group showed a median frequency of 1‰, while the negative control group showed a significantly elevated frequency of 12‰. The treatment groups had frequencies of 2‰, 2‰, and 1‰ for the low-, medium-, and high-dose *H. fulva* leaf powder groups, respectively, with no significant differences from the normal control group. Similarly, at 72 hours, the normal and negative control groups showed median frequencies of 1‰ and 10‰, respectively, while the treatment groups reported 1‰, 1‰, and 2‰, without significant variation from the normal control. These results indicate that administration of *H. fulva* leaf powder does not induce hematotoxic or genotoxic effects in ICR mice under the tested conditions. All dose groups showed reticulocyte and micronucleated reticulocyte values comparable to the normal control, with no dose-dependent trends observed. These findings suggest that *H. fulva* leaf powder is non-mutagenic and safe for further development as a functional food ingredient.

5. Conclusion

This study was conducted in accordance with the "Safety Assessment Methods for Health Foods" issued by the Taiwan Food and Drug Administration (TFDA), Ministry of Health and Welfare, revised on December 8, 2020. A rodent peripheral blood micronucleus assay was performed to evaluate the genotoxicity of the test substance. Results showed that at 48 hours post-administration, there were no statistically significant differences in micronucleated reticulocyte frequencies among the low-, medium-, and high-dose groups compared to the normal control group, and no dose-dependent relationship was observed. Similarly, at 72 hours post-administration, no significant intergroup differences or dose-response relationships were found. Based on the results of this micronucleus assay in rodents, the test substance "*H. fulva* leaf powder" did not induce abnormal responses in ICR mice under the test conditions and is therefore considered non-mutagenic.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC 112089 approved by the IACUC ethics committee.

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