

Evaluation of genotoxicity of ethanolic extracts of *Platostoma palustre* by micronucleus assay

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Abstract

Platostoma palustre (Pp) jelly is a traditional food. Pp has been used as folk medicine and is effective against heat-shock, hypertension and diabetes. Therefore, the aim of this study was to evaluate the ethanolic extracts of Pp' genotoxicity. The ethanolic extracts of Pp by using 40% ethanol for extraction. Evaluation of genotoxicity of ethanolic extracts of Pp by micronucleus assay was performed *in vivo*. During the *in vivo* genotoxicity-evaluated experiment, the experimental animal's clinical behavior, body weight (BW), food consumption, and the percentage of RET/RBCs (reticulocytes/red blood cells) and MN-RET/RETs (micronucleated reticulocytes/reticulocytes) after the treatments of Pp ethanolic extracts were evaluated. Both sexes Institute of Cancer Research (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 48 h after the last treatment, K2-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 animal's clinical behaviors were normal in all groups. The BW and food consumption were no significant difference between all groups. RET/RBCs (‰) in male or female ICR mice in the negative control group, the normal control group, the high dose of Pp ethanolic extract group, the middle dose of Pp ethanolic extract group, and the low dose of Pp ethanolic extract group were respectively $7.8 \pm 0.8 / 8.6 \pm 0.8$, $23.2 \pm 1.5 / 22.1 \pm 1.3$, $22.8 \pm 1.6 / 22.1 \pm 1.7$, $23.2 \pm 1.5 / 22.6 \pm 1.0$ and $22.2 \pm 1.9 / 23.9 \pm 1.9$; MN-RET/RETs (‰) in male or female ICR mice in the negative control group, the normal control group, the high dose of Pp ethanolic extract group, the middle dose of Pp ethanolic extract group, and the low dose of Pp ethanolic extract group were $2.0 \pm 0.0 / 2.0 \pm 0.0$, $43.2 \pm 10.6 / 39.6 \pm 10.9$, $1.8 \pm 0.4 / 1.6 \pm 0.5$, $1.6 \pm 0.5 / 1.4 \pm 0.5$, and $1.8 \pm 0.4 / 1.6 \pm 0.5$, respectively. Both RET/RBCs (‰) and MN-RET/RETs (‰) in male or female ICR mice in the negative control group were significantly difference than the other groups ($p < 0.001$). Taken all results together, Pp ethanolic extracts were without genotoxicity. Therefore, Pp ethanolic extracts were safety.

Keywords: Ethanolic extraction; Genotoxicity; *In vivo*; Micronucleus assay; Reticulocytes; *Platostoma palustre* extracts

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1. Introduction

In recent years, factors such as changes in people's health concepts, changes in living habits, emphasis on dietary intake, the promotion of alternative medical concepts, and advances in life science and technology have promoted the vigorous development of today's health food industry. Due to the gradual aging of the world and the rising market demand in Association of Southeast Asian Nations (ASEAN) countries, it is estimated that the functional food market is promising. *Platostoma palustre* (*Pp*) is an annual plant that is mainly distributed in tropical and subtropical regions, including Taiwan, Indonesia, Vietnam, southern China, and Burma [1-6]. The related food with *Pp* as tea, herbal jelly, and sweet soup with herbal jelly are popular during the summer. Additionally, the heated herbal jelly with *Pp* is admired by many Taiwanese in winter. *Pp* has been used as folk medicine. *Pp* has been verified that possessed many functional compounds such as polysaccharides (gum) with a unique aroma and texture, sterol compounds, stigmasterol, α -sitosterol, tripterene compounds, oleanolic acid, volatile compounds (caryophyllene oxide, α -caryophyllene, eugenol, benzene acetaldehyde, and 2,3-butanedione etc), essential oil (n-hexadecanoic acid, linoleic acid, and linolenic acid), volatile oil (chavibetol, n-hexadecanoic acid, and α -cadinol), and ursolic acid [7-10]. Therefore, these functional compounds of *Platostoma palustre* have indicated that many biological effects are effective against and attenuating the metabolic syndrome, heat-shock, hypertension, diabetes, liver disease, muscle and/or joint pains, hyperglycemia, inflammation, oxidant activity, free radical scavenging effects, acute and chronic hepatitis, and cancer growth [11-13]. However, whether the safety of *Pp* should be evaluated, especially genotoxicity.

According to the research and investigation, Taiwan's agricultural science papers are cited more than other fields, showing Taiwan's agricultural science and technology has R&D energy that cannot be ignored. Recently, the industrialization of scientific and technological achievements has attracted the attention of the government and the public. At the same time, the demonstration of this global trend is also an opportunity for Taiwan's agricultural science and technology to once again demonstrate the miracle. Looking at the industrialization development of agricultural science and technology in Taiwan today, a considerable number of resources and technologies have been accumulated in upstream R&D, but how to quickly transform technologies and resources into commodities through the industrialization development platform, and then form settlements and industries, is a topic worthy of attention. Therefore, it is an urgent need to establish a stable, accurate and rapid animal test evaluation system to evaluate the safety of healthy foods and functional foods as a pre-market screening tool [14-19].

An increasing number of reports are shown that "functional foods" are containing some functional component benefits may be observed to enhance short-term well-being and increasingly considered healthful. Based on these findings, the study was aimed to upgrade the production capacity of high value-added agricultural products. For the quality assurance, toxicity assessment for the functional foods is necessary. There is an emerging requirement to apply *in vivo* genotoxicity assays to evaluate the carcinogenic potential of these functional foods. Based on these requirements, we suggested that the mammalian erythrocyte micronucleus assay is used for the detection of damage induced by the test substance to the chromosomes of erythroblasts [20-23]. In this study, we tried to institute a sensitive and stable method for quantifying the formation of micronuclei in erythrocytes *in vivo*. The objective of this study was to evaluate the safety of *Pp* ethanolic extracts via micronucleus assay *in vivo*. The resulting data will enable us to evaluate the potential toxicity assessment of agricultural functional products in the future.

2. Material and methods

2.1. Chemicals and Reagents

Mitomycin C (Cayman, CAS 50-07-7, Item No.11435), Giemsa staining solution kit (Baso corporation, WGO-020), phosphate-buffered saline (PBS; Sigma-Aldrich, Cat. No. P3813), saline (Taiwan Biotech Co., LTD, Cat. No. 100-120-1101), Zoletil 50 (Virbac, Carros, France), and DPX mountant (Sigma-Aldrich, Cat. No. 44581).

2.2. Source of Dried *Platostoma palustre*

The dried *Pp* were ordered from the traditional markets in Miaoli, Taiwan.

2.3. 40% Ethanolic Extraction

10-15 g samples of *Pp* powder were soaked for 24 h at room temperature with 200 mL of 40% ethanol, followed by filtration through Whatman #1 filter paper. The filtrates were then evaporated in vacuo to dryness and weighed to determine the yields.

2.4. Preparation of Mitomycin C

Weighing 0.75 mg of mitomycin C and add it to 3 mL of saline and mix it to a concentration of 250 µg/mL mitomycin C. To draw mitomycin C solution [2 mg/kg body weight (BW)] with a 1 mL syringe for the subsequent intraperitoneal injection (the injection volume is adjusted according to the BW of mice).

2.5. Experimental Animals and Experimental Design

Adult male and female ICR mice [6 weeks old; 50 mice; BW between 26-27 g] 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-110045 approved by the IACUC ethics committee. The 25 ICR male mice and 25 ICR female mice were divided respectively into as the normal control group (n = 10; 5 male and 5 female mice), the negative control group (n = 10; 5 male and 5 female mice), the high dose (1,000 mg/kg BW) of *Pp* ethanolic extract group (n = 10; 5 male and 5 female mice), the middle dose (500 mg/kg BW) of *Pp* ethanolic extract group (n = 10; 5 male and 5 female mice), and the low dose (250 mg/kg BW) of *Pp* ethanolic extract group (n = 10; 5 male and 5 female mice). The mitomycin C-induced genotoxicity were performed in the negative control group. The clinical behaviors, BW, food consumption, and blood smear examination were monitored and performed during the experiment.

2.6. 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 K₂-EDTA for use in the subsequent experiments. ICR mice in three doses of *Pp* ethanolic extract groups were respectively administered three doses of *Pp* ethanolic extracts (the interval between each time must exceed 24 hours), and peripheral blood in all groups must be collected at 48th hours after the last administration of *Pp* ethanolic extracts.

2.7. Preparation of Blood Smear and Giemsa Staining with Light Microscopic Examination

Blood smear on the glass slides were prepared by a senior researcher. The air-dried blood smears were stained with 1 mL of Giemsa stain for 1-3 minutes, then add 2 mL of PBS (pH 7.2) or distilled water for 2-6 minutes. The slides were washed with the running water, air-dried and mounted by DPX mountant, and then placed under a light microscope (1,000× magnification) to observe and count the number of cells. At least, 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.

2.8. Statistical Analysis

The data were expressed as mean ± SD. All comparisons were made by one-way ANOVA and all significant differences are reported at ****p* < 0.001.

3. Results

3.1. Change of Mouse's BW in All Groups

The ethanolic extracts of *Pp* by using 40% ethanol for extraction were provide in the oral administration in the three *Pp* ethanolic extract 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% (50/50) (data not shown). The BW of mice was detected on day 0, day 7, day 8, day 9, and day 10. The day 0 was mice entering the animal room. The day 7 to day 9 were genotoxicity induction. The day 9 to day 10 were three doses of *Pp* ethanolic extract administration. During the experiments, the mice's BW continued to rise and there was no statistically significant difference in BW between all groups (Figure 1).

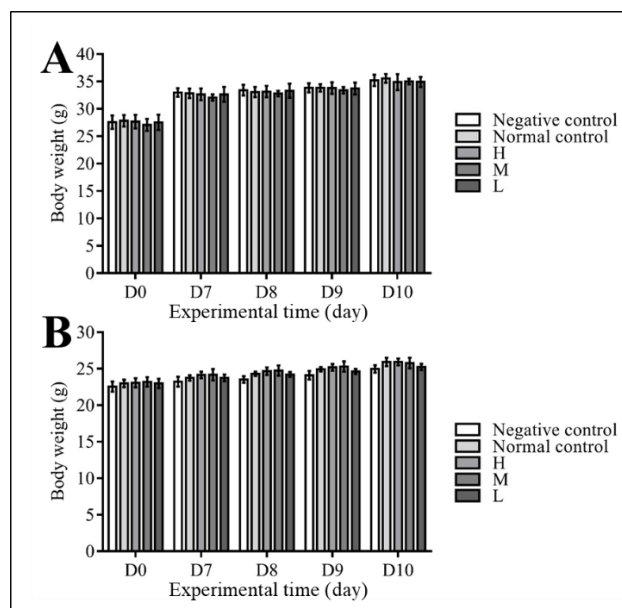


Figure 1 Change of body weight before and after the treatments of three doses of *Platostoma palustre* ethanolic extracts. (A) Male ICR mice. (B) Female ICR mice. All data are expressed as mean $\pm$ SD. 'H' is high dose (1,000 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'M' is middle dose (500 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'L' is low dose (250 mg/kg BW) of *Platostoma palustre* ethanolic extract group

3.2. Change of Mouse's Food Consumption in All Groups

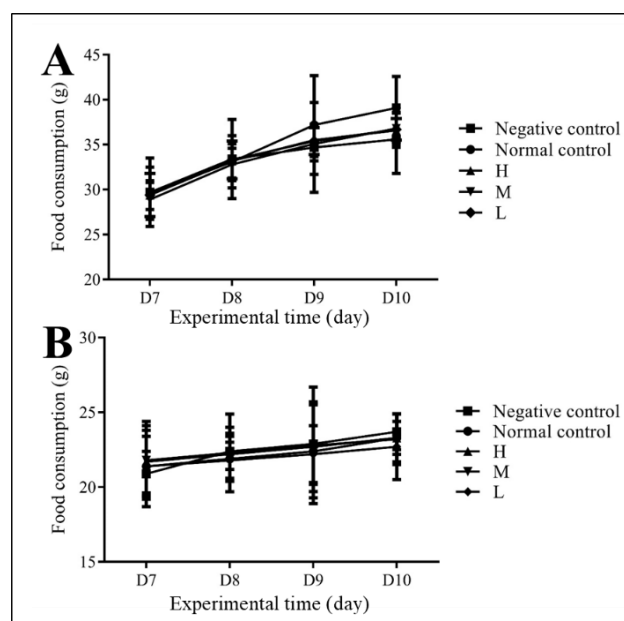


Figure 2 Change of food consumption after the treatments of three doses of *Platostoma palustre* ethanolic extracts. (A) Male ICR mice. (B) Female ICR mice. All data are expressed as mean $\pm$ SD. 'H' is high dose (1,000 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'M' is middle dose (500 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'L' is low dose (250 mg/kg BW) of *Platostoma palustre* ethanolic extract group

In this study, the mouse's food consumption in each group were monitored during the experiments. The food consumption of mice was detected on day 0, day 7, day 8, day 9, and day 10. The day 0 was mice entering the animal room? The day 7 to day 9 were genotoxicity induction. The day 9 to day 10 were three doses of *Pp* ethanolic extract administration. During the experiments, three doses of *Pp* ethanolic extracts did not cause a decrease in appetite and abrupt decrease in food intake (Figure 2).

3.3. Change of RET/RBCs and MN-RET/RETs Percentages in All Groups

Mice' peripheral anticoagulant blood were collected at 48 hours after the third administration, respectively. Blood samples were prepared for blood smears and processed with Giemsa staining. After blood sample processing, they were examined by using light microscope. The percentages of RET/RBCs and MN-RET/RETs were evaluated. The results showed that the male and female mice in the negative control group were induced genotoxicity. The percentage of RET/RBCs in the negative control group was significantly decrease than the other groups ($p < 0.001$) (Figure 3A-B). Except for the negative control group, there were no significant difference for the comparison of the other groups ($p > 0.05$) (Figure 3A-B). RET/RBCs (%) in male or female ICR mice in the negative control group, the normal control group, the high dose of *Pp* ethanolic extract group, the middle dose of *Pp* ethanolic extract group, and the low dose of *Pp* ethanolic extract group were respectively 7.8 ± 0.8 / 8.6 ± 0.8 , 23.2 ± 1.5 / 22.1 ± 1.3 , 22.8 ± 1.6 / 22.1 ± 1.7 , 23.2 ± 1.5 / 22.6 ± 1.0 , and 22.2 ± 1.9 / 23.9 ± 1.9 (Figure 3A-B). The percentage of MN-RET/RETs in the negative control group was significantly increase than the other groups ($p < 0.001$) (Figure 3C-D). Except for the negative control group, there were no significant difference for the comparison of the other groups ($p > 0.05$) (Figure 3C-D). MN-RET/RETs (%) in male or female ICR mice in the negative control group, the normal control group, the high dose of *Pp* ethanolic extract group, the middle dose of *Pp* ethanolic extract group, and the low dose of *Pp* ethanolic extract group were 2.0 ± 0.0 / 2.0 ± 0.0 , 43.2 ± 10.6 / 39.6 ± 10.9 , 1.8 ± 0.4 / 1.6 ± 0.5 , 1.6 ± 0.5 / 1.4 ± 0.5 , and 1.8 ± 0.4 / 1.6 ± 0.5 , respectively (Figure 3C-D). Taken all results together, *Pp* ethanolic extracts were without genotoxicity. Therefore, *Pp* ethanolic extracts were safety.

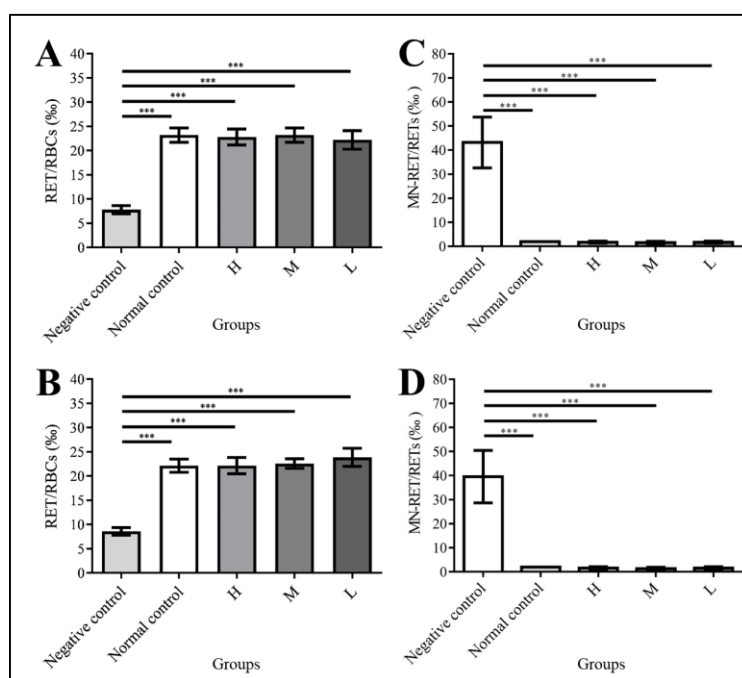


Figure 3 Change of RET/RBCs and MN-RET/RETs after the treatments of three doses of *Platostoma palustre* ethanolic extracts. (A) RET/RBCs (%) in male ICR mice. (B) RET/RBCs (%) in female ICR mice. (C) MN-RET/RETs (%) in male ICR mice. (D) MN-RET/RETs (%) in female ICR mice. 'H' is high dose (1,000 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'M' is middle dose (500 mg/kg BW) of *Platostoma palustre* ethanolic extract group. 'L' is low dose (250 mg/kg BW) of *Platostoma palustre* ethanolic extract group. All data are expressed as mean $\pm$ SD. 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 [28]. 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* [24-26]. *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* [27].

Previously, the effect of different ethanolic concentrations on antioxidant properties and cytoprotective activities of *Pp* has been verified. The antioxidant activities of *Mesona procumbens* ethanolic extracts which displayed variable antioxidant levels. The 60% *Mesona procumbens* ethanolic extracts exhibited higher antioxidant activities that possessed a protective capability for the biological membrane system to prevent and treat oxidative stress-related disorders [11-13]. According to our previous data, the bio-functional components and concentrations of *Pp* ethanolic extracts via 90% ethanol extraction were chlorogenic acid (0.30 mg/mg *Pp* ethanolic extracts), caffeic acid (0.70 mg/mg *Pp* ethanolic extracts), rutin (0.80 mg/mg *Pp* ethanolic extracts), astragaloside IV (7.50 mg/mg *Pp* ethanolic extracts), and rosmarinic acid (15.90 mg/mg *Pp* ethanolic extracts). Among of these bio-functional components of *Pp* ethanolic extracts via 90% ethanol extraction, the contents of astragaloside IV and rosmarinic acid were higher than others. Many literatures have been demonstrated that astragal side IV and rosmarinic acid possessed anti-tumor abilities. In this study, *Pp* ethanolic extracts via 40% ethanol extraction were used. Therefore, we want to understand the functional effects of *Pp* ethanolic extracts and their component concentrations.

5. Conclusion

The purpose of this study was to induce genotoxicity in ICR mice with mitomycin C and evaluate whether *Pp* ethanolic extracts were safe. Taken these results together, we successfully established the phenomenon of micronucleus in peripheral blood cells induced by mitomycin C in ICR mice. After detecting via this genotoxicity mouse platform, we have demonstrated that *Pp* ethanolic extracts were no genotoxicity. In the future, we hope this genotoxicity mouse platform will be provided to detect the genotoxicity in the test samples.

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-110045 approved by the IACUC ethics committee.

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