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CELLULAR AND MOLECULAR BIOLOGY

# Hepatotoxicity and Mutagenicity assessment during chronic *in vivo* exposure to aqueous extracts from *Peperomia pellucida*

YU-WEN HUANG, ARIANNE JAN TUOZO & ROGER S. TAN

**Abstract:** Studies on herbal medicine have exposed some toxic effects on humans. *Peperomia pellucida* (L.) HBK *(P. pellucida)* is one of the herbal medicines recommended as an alternative to synthetic medicine for diseases. Studies exist on the pharmacological activities of *P. pellucida* extracts, but studies on the potential hepatotoxic and mutagenic effects of subchronic administration of *P. pellucida* aqueous extracts, which is very important knowledge when we venture into alternative medicine, are lacking. In this study, two concentrations (60 mg/kg and 30 mg/kg) of *P. pellucida* aqueous extracts — decoction and freeze-dried extracts —were administered *in vivo* to *BALB/c* mice for nine (9) weeks. Significant differences were observed between the 60 mg/kg freeze-dried extract and the control in terms of mice weight and micronucleus frequency at 7-8 weeks of treatment. Also, no significant differences were found between groups in serum transaminases levels. Generally, there is no sufficient evidence to show that subchronic exposure to *P. pellucida* aqueous extracts is hepatotoxic though 60 mg/kg concentration may be mutagenic. This study suggests that although the herbal medicine is safe for prolonged consumption, users are advised to take precautions and moderations of its use due to the possibility of potential mutagenic effects.

**Key words**: *Peperomia pellucida* (L.) HBK, Hepatotoxicity, Mutagenicity, AST, ALT.

## INTRODUCTION

Herbal medicines have been used for the treatment of various ailments around the world since ancient times. Herbal medicines are thought to be eco-friendly, readily available, and milder in effect compared to synthetically derived medications (Van den Berg et al. 2011, Morilla et al. 2014, Dubey et al. 2014). Various studies have proven the capability of herbal medicines to alleviate certain diseases such as diabetes, bone-related damage, and immune and liver disorders (Van den Berg et al. 2011, Abe & Ohtani 2013). The Department of Health (DOH) in the Philippines enacted the Republic Act of 8423 in 1997 to promote herbal medicine (Philippines Department of Health 1997); however, these natural sources are not necessarily safe. In a study of 30 botanical compounds, it was shown that 18 of those had both genotoxic and carcinogenic characteristics. The 18 compounds included estragole, methyl eugenol, safrole, and β-asarone, which can be found in basil, nutmeg, cinnamon, and other herbal plants (Dubey et al. 2014). *Peperomia pellucida* (L.) HBK *(P. pellucida)* is one of the herbal medicines recommended by the Department of Health (DOH) in the Philippines as an alternative to synthetic medicine and is consumed as a decoction or as tea. *P. pellucida* extracts were found to exhibit analgesic (Arrigoni-Blank et al. 2004), anti-inflammatory (Arrigoni-Blank et al. 2004), antipyretic (Khan et al. 2008a), antioxidant

(Oloyede et al. 2016), antihyperuricemic (Kartika et al. 2016), antihyperglycemic (Hamzah et al. 2012), gastroprotective (Roslida & Noor 2009), antiosteoporotic (Ngueguim et al. 2017), depressant (Khan et al. 2008b), immunostimulatory (Lee et al. 2016), antimicrobial and free-radical scavenging (Zubair et al. 2015, Oloyede et al. 2016, Okoh et al. 2017), and cytotoxic (Narayana et al. 2018) activities. It was also found to accelerate bone fracture healing due to the presence of calcium, phosphorus, magnesium, sodium, and potassium (Florence et al. 2017). Despite the number of studies regarding the pharmacological activities of *P. pellucida* extracts, there have been no studies regarding the hepatotoxicity and mutagenicity of subchronic administration of *P. pellucida* leaf aqueous extracts, which we believe is a very crucial knowledge in alternative medicine as people are consuming this plant regularly for a longer period. In this study, hepatotoxicity and mutagenicity of subchronic administration of *P. pellucida* leaf aqueous extracts were studied to give users, of this medicinal plant, insights into its effects, during prolonged exposure, and advised them to take precautions in consuming the medicinal plant extract.

## MATERIALS AND METHODS

### Materials

Methanol and Giemsa stain were from Sigma. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) kits were secured from BIOBASE. Unless otherwise noted, solvents and other reagents were purchased from Aldrich Chemical Co.

### Plant sample extraction

Plant sample of *Peperomia pellucida* (L.) HBK (*P. pellucida*) was obtained from Claveria, Misamis Oriental, Philippines (8°36'36"N 124°53'41"E). The plant specimen's scientific name was correctly identified and certified using the available materials from the Institute of Biology, Jose Vera Santos Memorial Herbarium (PUH), College of Science, University of the Philippines, Diliman, Quezon City in the Philippines, by a Botanist Dr. Edwino S. Fernando. The whole plant (except roots) was used in this study. The plants were washed with distilled water and roots were removed. Then the samples were oven-dried at 60 °C for four (4) hours, after which they were ground to a uniform powder. Sample preparation was done every three days. The samples were covered, labeled, and stored at 4 °C. Two types of extract preparations were done — decoction and freeze-dried. For freeze-dried preparations, 24.06 g of dried leaves were cut into small pieces, placed in an Erlenmeyer flask with 420 mL of distilled water, and was heated overnight at 50 °C. The mixture was filtered, and the supernatant was lyophilized. After freeze-drying, a 1.28 g of dark green extract was obtained, giving a percent yield of 5.33%. For the decoction preparations, approximately 25 g of dried leaves were heated at 100 °C, in an Erlenmeyer flask with 420 mL of distilled water, for 20 minutes. The extract was then dried in a lyophilizer. The crude extract afforded was 2.1538 g giving an 8.62% yield. For each preparation, two concentrations were tested, which were 30 mg leaves/ kg body weight and 60 mg leaves/ kg body weight.

### Animals and Ethics

The study was conducted in strict accordance with the recommendations for animal care and handling according to the guidelines set by the National Ethical Guidelines for Health Research of the Philippine National Health Research System, abiding RA number 8485-Animal Welfare Act of 1998 and its implementing rules and regulations (DA Administrative Order number 40 series of 1998 and the Code of Practice for

the Care and Use of Laboratory Animals in the Philippines, 2nd edition, 2002 developed by the Philippine Association for Laboratory Animal Science (PALAS)) and the Code of Research Ethics set by the University Research Council of De La Salle University-Manila. In this study, Thirty (30) *BALB/c* mice used were from the Philippines Food and Drug Administration (FDA). Animals were individually placed in mice cages, allowed access to food and water *ad libitum*, and maintained in a 12-hour light/dark cycle at room temperature. The experiments were performed after mice were acclimatized for one (1) week. Mice were treated with the extracts through oral gavage daily for nine weeks. Seven (7) mice were treated with 30 mg/kg decoction (Group DD), five (5) mice were treated with 60 mg/kg decoction (Group DC), seven (7) mice were treated with 30 mg/kg of freeze-dried extract (Group FD), five (5) mice were treated with 60 mg/kg of freeze-dried extract (Group FC), and six (6) mice served as the control group treated only with distilled water (Group C). After the treatment period, mice were sacrificed via cervical dislocation. Blood was obtained through cardiac puncture and the serum was then separated and analyzed.

### Micronucleus test

The method was according to Hayashi et al. with modifications and performed every two weeks to monitor the mutagenic effect of the extracts. Briefly, blood samples were collected by making a small incision on the foreskin of the mouse tail and a drop of blood was smeared on pre-cleaned and coded microscope slides. The blood smears were fixed with methanol, air-dried, and stained with Giemsa stain for 15 minutes. The micronucleated polychromatic erythrocytes (MPCEs)were then scored under a compound microscope with a magnification of 1200x using a 5.0 megapixel CMOS sensor SVS-C1042997 microscope camera (SHIVISION, China). Three (3) slides per mouse were prepared and at least 1000 cells were scored per slide.

### Blood biochemical assays

The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum was estimated using standard kits from BioBase following the manufacturer's protocol recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

### Statistical analysis

PHStat4 was the program used for statistical analysis. Outliers were removed after analysis with the Q test at a level of significance of 0.05. The data were then analyzed through one-way ANOVA with a subsequent posthoc Tukey test if significant differences were found in ANOVA. The level of significance used was also 0.05.

## RESULTS AND DISCUSSION

### Mice weights

As shown in Figure 1, no significant differences were observed between groups DD, DC, and FD concerning the weights of mice from the control group (C). However, a significant decrease in weights was observed in group FC compared to the control group, indicating that, among the treated groups, mice in group FC were significantly affected by the extract. The increased number of components, emphasized by its higher concentration, of the 60 mg/kg freeze-dried extract may have contributed to the decrease in mice's appetite. Botanical medicines are usually lyophilized due to the assumption of preserving their components (Abascal et al. 2005). Abascal and his colleagues showed that extraction through freeze-drying is a good method for the preservation of condensed tannins with large molecular weight (Abascal et

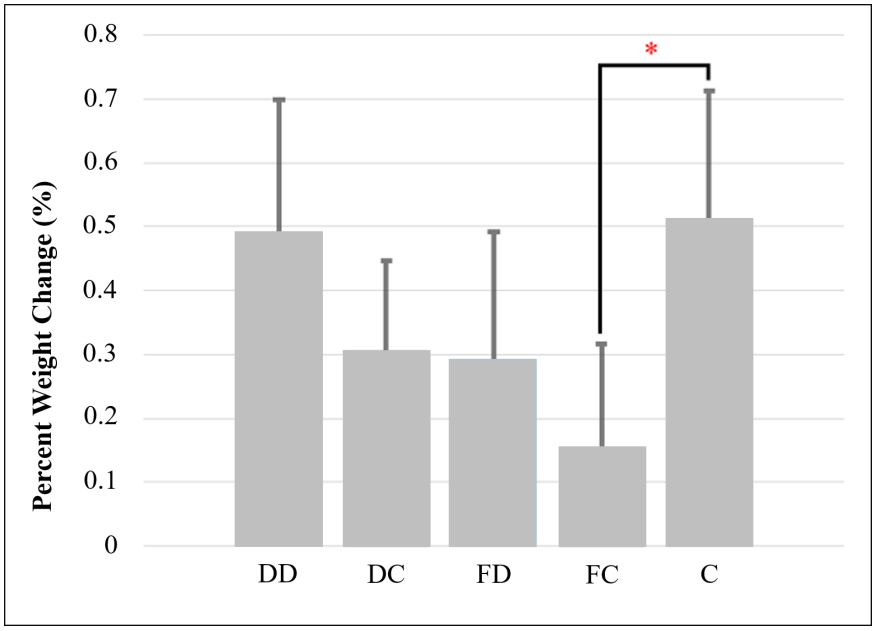


**Figure 1. Average daily weight change for the whole treatment period (9 weeks). DD = group treated with 30 mg/kg decoction; DC = group treated with 60 mg/kg decoction; FD = group treated with 30 mg/kg freeze-dried extract; FC = group treated with 60 mg/kg freeze dried extract; C = control group treated with only distilled water. Significant differences ($p<0.05$) between groups are indicated by asterisks.**

al. 2005); thus, the freeze-dried extract has more components preserved through lyophilization. Tannins, a type of polyphenol, were shown to be present in *P. pellucida* plants of different locations when subjected to phytochemical screening (Majumder 2011, Awe et al. 2013, Waty et al. 2017). In a study, tannins in the diet were shown to result in weight loss and high feces-to-food ratios (Freeland et al. 1985). The greater amount of tannins in the 60 mg/kg freeze-dried extract, compared to 30 mg/kg, could potentially lead to a decreased appetite that led to less weight gain for the FC group.

## Micronucleus test

There were no significant differences in the frequency of micronucleated polychromatic erythrocytes (MPCEs) between the groups after the 2$^{nd}$, 4$^{th}$, and 6$^{th}$ weeks of treatment (Fig. 2). However, changes in the frequency of MPCEs were significantly observed between groups in the 8$^{th}$ week of treatment, between DC and C, FD and FC, and FC and C. The micronucleus test is a mutagenicity test indicative of both chromosomal aberrations and chromosomal loss that result in chromosomal abnormalities (Heddle et al. 1983). For most groups, the increasing micronucleus frequency indicated that the treatment induces micronuclei with daily administration. The differences among groups in the 8$^{th}$ week indicate that the frequency of micronuclei is dependent on the number of treatments (Tice et al. 1990); in this case, the group treated with the highest concentration, both decoction and freeze-dried, exhibited a higher micronucleus frequency. It is also worth noting that the increase in micronucleus frequency of the FC group happened almost simultaneously with its weight loss in the 7$^{th}$ week. Mice are good models for the micronucleus test because of their spleen's inability to remove micronucleated cells (Kasamoto et al. 2013). In the eighth week, it can be seen that the groups treated with 60 mg/kg extract were significantly different from the control, suggesting that the extracts of the highest concentration have a mutagenic effect, which could be linked to the reported increased amounts of phytoconstituents in the extracts (Majumder 2011, Awe et al. 2013, Waty et al. 2017). Though not

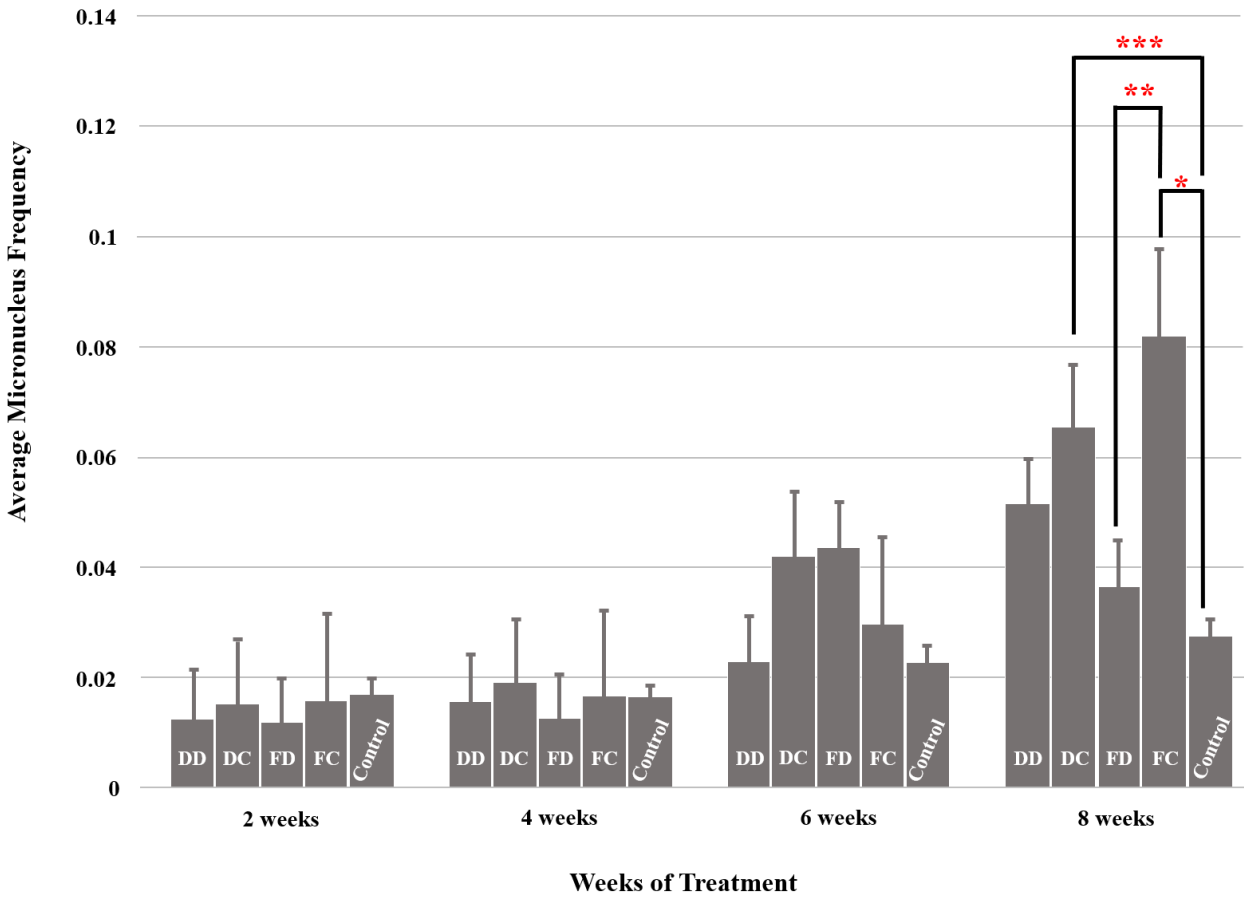


**Figure 2.** **Representative Average frequencies of micronucleated polychromatic erythrocytes (MPCEs) in weeks of treatment. DD = group treated with 30 mg/kg decoction; DC = group treated with 60 mg/kg decoction; FD = group treated with 30 mg/kg freeze-dried extract; FC = group treated with 60 mg/kg freeze-dried extract; C = control group treated with only distilled water. Significant differences ($p<0.05$) between groups are indicated by asterisks.**

all plant flavonoids are mutagenic, among the common naturally occurring mutagens are the plant flavonoids and their glycosides (National Research Council (US) Committee on Diet, Nutrition, and Cancer 1982). Some flavonoids, namely quercetin, kaempferol, luteolin, fisetin, chrysin, galangin, flavone, 3-hydroxyflavone, 5-hydroxyflavone, and 7-hydroxyflavone, were found to be mutagenic by Ames Test (Resende et al. 2012). Interestingly, a study on the effects of a flavonoid on oxidative DNA damage on human leukocytes *in vitro* showed ambiguous results. Oxidative DNA damage decreased at flavonoid concentrations lower than 50 µM, but the damage was aggravated at concentrations higher than 100 µM. High concentrations also showed a prooxidant effect (Wilms et al. 2008). The study conducted by Wei et al. in 2011 showed that phytol (37.88%) constitutes the major component of *P. pellucida* leaf extract followed by 2-Naphthalenol, decahydro (26.20%). Phytol was also found to be generally genotoxic (Islam et al. 2017). On the other hand, studies showed that 2-Naphthalenol caused DNA damage in human erythrocytes at as low as 100 µM concentration (Kapuci et al. 2014). The mutagenic activity observed in the concentrated extract (60 mg/kg) on the eighth week could be more likely

related to the higher phytol and 2-Naphthalenol presence in the crude extract compared to the 30 mg/kg extracts.

### Blood biochemical assays

Alanine aminotransferase (ALT) is an enzyme found at the highest levels in the liver and kidney tissues. It is involved in amino acid metabolism. Tissue destruction leads to the release of ALT into the circulating blood. Damaged liver due to a variety of diseases, such as hepatitis, mononucleosis, and cirrhosis is marked by elevated levels of serum ALT. High levels of ALT are not usually observed in other diseases making it a specific indicator of liver disease. As shown in Figure 3, the ALT levels of mice in different groups were comparable to that of the control, with no groups having higher ALT values than the control, indicating that the serum ALT levels of both the control and treated groups were normal. Although the ALT levels observed seemed to be normal, it is likely that the ALT activities increased sometime during the treatment period, but the detoxifying ability of the liver reversed this effect back to normal. This, however, could not be monitored since the ALT levels were only measured at the end of the treatment period. This means that the *P. pellucida* aqueous extracts did not incur any damage that could be detected by the serum transaminase levels. The aspartate aminotransferase (AST) activities, as shown in Figure 4, also showed no significant increase. These results, similar to ALT, indicated that the extracts did not incur liver damage in the mice.

## CONCLUSION

In conclusion, there is no sufficient evidence to show that subchronic exposure to *P. pellucida* aqueous extracts could cause hepatotoxicity, though the extracts have manifested to be mutagenic at high concentrations. There were no significant differences found between the control and the dilute (30 mg/kg) decoction group in terms of weight change, micronucleus, ALT levels, and AST levels. This study suggested that although the herbal medicine is safe for long-term consumption, users are advised to take precautions and moderations of its use due to the possibility of potential mutagenic effects when consumed in high concentration.

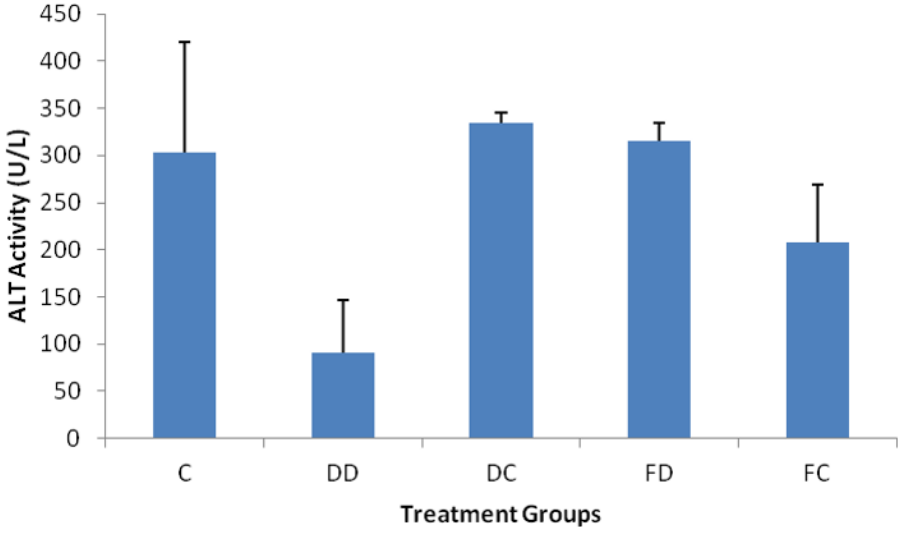


**Figure 3. Alanine aminotransferase activity of treated and control mice in Units per Liter. DD = group treated with 30 mg/kg decoction; DC = group treated with 60 mg/kg decoction; FD = group treated with 30 mg/kg freeze-dried extract; FC = group treated with 60 mg/kg freeze-dried extract; C = control group treated with only distilled water. The values presented are the averages of the different treatment groups. Error bar means the standard deviation of the trials.**

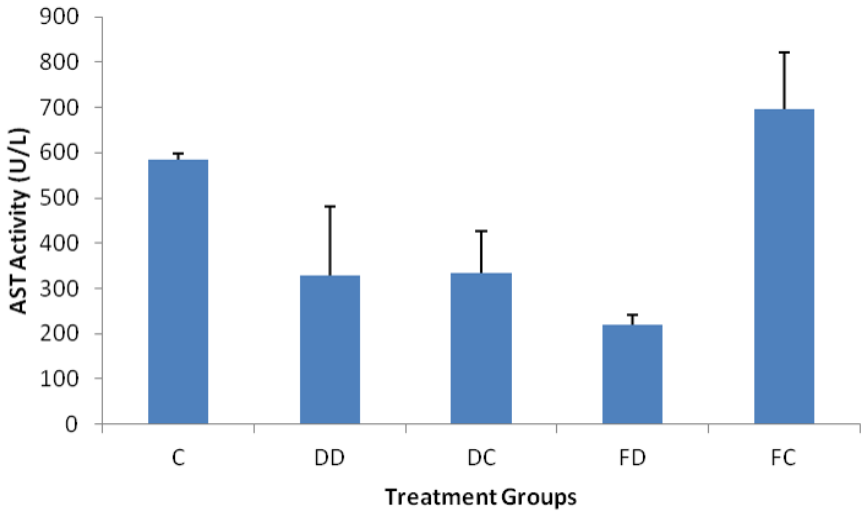


**Figure 4. Aspartate aminotransferase (AST) activity of treated and control mice in Units per Liter. DD = group treated with 30 mg/kg decoction; DC = group treated with 60 mg/kg decoction; FD = group treated with 30 mg/kg freeze-dried extract; FC = group treated with 60 mg/kg freeze-dried extract; C = control group treated with only distilled water. The values presented are the averages of the different treatment groups. Error bar means the standard deviation of the trials.**

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## Author contributions

Y-WH and AJT performed the experiments, generate data, and statistical analyses. RST planned and conceptualized the study, provides necessary reagents, finalized and check the correctness of data, and edited and finalized the paper. The authors declared no conflict of interest.