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Assessment of the Safety of a Fermented Multi-fruit Beverage

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Fruits have been widely considered as the "default health foods" as they contain numerous vitamins and minerals needed to sustain human health. To fully enhance their nutritive and flavor features while prolonging their shelf lives, fermentation strategies have been utilized to prepare healthy yet readily consumable fruit products. However, albeit their innumerable health-promoting effects, a lot of people are still concerned if consuming live bacteria and their metabolites are safe for humans. In this study, the safety of a fermented multi-fruit beverage using five different fruits (kiwis, guavas, papayas, pineapples, and grapes) was investigated. Safety assessment experiments performed on the multi-fruit beverage showed that it is non-mutagenic to five strains of *Salmonella typhimurium*, non-cytotoxic, and is unable to induce chromosome aberrations in Chinese Hamster Ovary K1 (CHO-K1) cells, as well as non-toxic in male ICR mice compared to the water-fed control group after 28 d of feeding. Therefore, these results collectively indicated that the fermented multi-fruit beverage is safe for human consumption.

Keywords: fermentation, fruits, safety assessment

INTRODUCTION

A diet rich in fruits and vegetables has been universally recommended due to their ability to promote health and well-being owing to their abundant concentration of vitamins, minerals, dietary fiber, and phytochemicals. These substances can function as antioxidants, phytoestrogens, and anti-inflammatory agents (Slavin and Lloyd 2012). In fact, a lot of epidemiological researches and ecological studies have already demonstrated that frequent fruit and vegetable consumption has been associated with the decreased risk for cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, and age-related diseases (Lampe 1999; Temple 2000; Willett 1994, 1995). However, the main problem with fruits is that they are easily perishable due to their high water and nutrient content that it is difficult to meet the global demand for fruit supply because of the increasing population (Swain *et al*. 2014). Lactic acid fermentation plays a very significant role in solving this problem by preserving fresh fruits while also enhancing their nutritive values, improving their sensory and flavor features, as well as reducing their toxicity (Swain *et al*. 2014). Furthermore, since most consumers tend to prefer foods that are ready to eat or ready to drink (Endrizzi *et al*. 2009), fermentation is a great practice in preparing healthy yet readily consumable fruit products.

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Fermented fruit products are great sources of probiotics that have a lot of impact on human health. They are able to enhance the bioavailability of the nutrients present in the food (Parvez *et al*. 2006) but more importantly, they also exhibit excellent immunomodulatory functions (Bermudez-Brito *et al*. 2012). However, despite having a lot of nutritional advantages, a lot of people are still concerned regarding the safety of consuming fermented products as they contain live bacteria and their metabolites.

In fact, a lot of fermented food products are considered to be safe for consumption without the need for reheating (Motarjemi and Nout 1996). Commonly used probiotic strains such as *Lactobacillus* spp*., Bifidobacterium* spp*.,* and *Streptococcus* spp. also has been given a Generally Recognized as Safe status by the US Food and Drug Administration, and also has a long history for safe use in the fermented food manufacturing industry (Gawai and Prajapati 2017). Furthermore, due to the competitive activity and metabolite production of these starter microorganisms, fermented food products are considered to be less likely to be a breeding ground for pathogens that can cause food contamination or intoxication compare to fresh foods (Nout 1994). Although there have been some testimonies of clinical-pathological conditions of bacteremia and endocarditis that have been associated with the consumption of lactic acid bacteria (LAB) fermented products (Lara-Villoslada *et al*. 2007), it is likely that these infections are opportunistic that occur in immune-compromised individuals (ICMR-DBT 2011). These reports of LAB-associated infections, even though rare and not likely, still prompted a concern for the use of LAB fermentation in food manufacturing (Gawai and Prajapati 2017). Therefore, safety evaluation and risk assessments of fermented food products are still considered as a very important task that must be done before it can be deemed safe for human consumption. In this study, the safety of a fermented multi-fruit beverage was investigated.

MATERIALS AND METHODS

Preparation of the Fermented Multi-fruit Beverage

All the microorganisms used for the fermentation process were purchased from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). As described by Xu (2013), the fermented multi-fruit beverage was prepared as follows. Kiwis, guavas, papayas, pineapples, and grapes were obtained from a local market in Taipei, Taiwan, washed with water, manually peeled, then sliced. Afterward, each fruit was blended and filtered to remove the larger pulp fragments. To prepare the fruit mixture, the blended kiwi, papaya, pineapple, guava, and grape juices were mixed in a ratio of 5:15:70:5:5 (w/w), inoculated with 5% (w/v) of *Saccharomyces cerevisiae* BCRC 21447, then incubated at 30 ˚C for 5–7 d or until the mixture has reached a pH of 3.5. Then, the fermentation is continued by further inoculating the fruit pulp with 5% (w/v) of *Lactobacillus acidophilus* BCRC 10695, 1.0% (w/v) of *Pediococcus dextrinicus* BCRC 12842, 1.0% (w/v) of *Lactobacillus plantarum* BCRC 10069, and 2.5% (w/v) of *Acetobacter pasteurianus* BCRC 14145. The mixture was further incubated at 30 ˚C for about 14 d or until the mixture reaches a pH of 3.0. Afterward, it was subjected to vacuum filtration using a 3-µm pore size filter paper and incubated at 30 ˚C for 8 wk. Lastly, the final fermented fruit pulp was vacuum filtered (0.45-µm pore size) and stored at 4 ˚C until use for *in vitro* and *in vivo* experiments.

MTT Cytotoxicity Assay

The fermented multi-fruit beverage was tested at five two-fold dilutions of 5, 2.5, 1.25, 0.6, and 0.3 µL/mL. Complete F-12K growth medium was used as the assay negative control, while 0.1M HCl (Sigma-Aldrich, St. Louis, MO, USA) was used as the assay positive control. CHO-K1 cells were first seeded into 24-well plates at a seeding density of 5 x 10^4 cells/well in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA), then incubated at 37 °C in the presence of 5% CO_2. After 24 h, the growth medium was then replaced with a new medium supplemented with the different concentrations of fermented multi-fruit beverage and control samples. Each sample was tested in triplicate. After 20 h more incubation at 37 °C in a 5% CO_2 incubator, the supernatant was aspirated, and 0.5 mL of 0.5 mg/mL of MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The reaction was allowed to proceed for 2 h at 37 °C in a 5% CO_2 incubator. After 2 h, the supernatant was aspirated and 200 uL of DMSO was added to each well and then mixed uniformly. One hundred (100) uL of the reaction mixture was pipetted into a 96 well ELISA plate, and the absorbance was measured at 570 nm. Cell viability was calculated as the percentage of the absorbance of the fermented multi-fruit beverage treated group relative to that of the negative control group.

In Vitro Mammalian Chromosome Aberration Test

The fermented multi-fruit beverage was tested at five different two-fold dilutions: 5, 2.5, 1.25, 0.6, and 0.3 µL/mL. Sterile distilled water was used as the assay negative control. For the test with S9 metabolization, the positive control used was 10 µg/mL of cyclophosphamide (CPP) (Sigma-Aldrich, St. Louis, MO, USA). For the test without S9 activation, the control used was 0.07 µg/mL of mitomycin C (mitC) (Sigma-Aldrich, St. Louis, MO, USA).

CHO-K1 cells were first cultured at a density of $1x10^6$ cells into a 6-cm cell culture dish for a total of 21 dishes, then incubated for 24 h at 37 °C in a 5% CO_2 incubator. After 24 h, the cultures were divided into three groups, where each group has seven dishes. The cells of the first group were treated with the test substances (five doses of fermented multi-fruit beverage, CPP, and mitC) for 3 h in the presence of the S9 metabolic activation mixture, after which the supernatant was then aspirated and replaced with fresh complete F-12K culture medium. The second group was treated with the same test substances as that of the first group and incubated for 3 h but without the addition of S9. After 3 h, the supernatant was also aspirated and replaced with a new culture medium. The third group was also treated with the various test substances without the addition of S9; however, the incubation period was extended to 20 h. After 20 h of test substance treatments, the medium of the cells of all the three groups was changed to one containing 5 μg/mL of Colcemid (Sigma-Aldrich, St. Louis, MO, USA) and then incubated for an additional of 2 h to induce the cells to arrest in the metaphase stage. After 2 h, the cells were then transferred in a centrifuge tube and then centrifuged at 1,000 rpm for 5 min. The pellet was gently resuspended with 3 mL of a warm hypotonic 0.56% KCl solution and incubated at 37 °C for 20 min. After centrifugation, 4 mL of chilled fixative solution (methanol: glacial acetic acid = 3:1) was used to gently resuspend the pellet, and the mixture was allowed to sit at room temperature for 30 min. This procedure was repeated twice. After the last resuspension in a fresh fixative solution, the mitotic cells were then prepared as chromosome slides by dropping the cell suspension onto a glass slide surface. Three slides were prepared for each treatment. After drying, the slides were stained with 5% Giemsa and viewed under a light microscope using oil immersion lenses. A total of 100 metaphase cells were analyzed per treatment group for chromosomal aberrations such as gaps, breaks, dicentrics, and rings.

Bacterial Reverse Mutation Test (Ames Test)

Mutagenic activity of the fermented multi-fruit beverage was evaluated using the *Salmonella* microsome assay or Ames test. The tester strains used were TA98, TA100, TA102, TA1535, and TA1537. These strains were revived from frozen cultures by first streaking them on master plates for 48 h at 37 °C and a loopful of the bacteria was cultured in Oxoid No. 2 nutrient broth (Oxoid Ltd, Cheshire, England) for 16 h at 37 °C before performing the Ames test proper. The S9 fraction (MOLTOX, NC, USA), prepared from livers of Sprague-Dawley rats treated with polychlorinated biphenyl mixture Aroclor 1254, was freshly prepared before each test. The complete metabolic activation mixture consisted of 4% S9 fraction, 1% 0.4 M $MgCl_2$, 1% 1.65 M KCl, 0.5% 1 M D-glucose-6-phosphate disodium and 4% 0.1 M NADP, 50% 0.2 M phosphate buffer, and 39.5% sterile distilled water. For the Ames test, the fermented multi-fruit beverage was tested at five different concentrations diluted in sterile water: 0.3, 0.6, 1.3, 2.5, and 5 mL/plate. Water or DMSO was used as the assay negative control, depending upon the solvent that was used for the positive control. In the test without the presence of the S9 mixture, the positive controls used were 2-nitrofluorene (2-NF) (1 μg/plate) for the TA98 strain, sodium azide (AzNa) (1 μg/plate) for TA100 and TA1535, MitC (0.5 μg/plate) for TA102, and 9-aminoacridine (9-AA) (1 μg/plate) for TA1537. In the test containing the S9 mixture, benzo(a)pyrene (BAP) (1 μg/plate) was used for TA98, and 2-aminoanthracene (2-AA) (4 μg/plate) was used for TA100, TA102, TA1535, and TA1537. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In a sterile test tube, 0.1 mL of the fermented multi-fruit beverage or the various positive and negative control samples were mixed with 0.1 mL of a 16-h culture of the *S. typhimurium* tester strain (activated or not with 0.5 mL of the S9 metabolic activation mixture), and then incubated for 30 mins at 37 °C. Next, 2 mL of soft agar containing 0.5 M of histidine/biotin was then added. The mixture was then vortexed uniformly and poured on glucose minimal agar plates. After the top agar has solidified, the plates were inverted and then incubated at a constant temperature incubator at 37 °C. After 48 h of incubation, the number of His+ revertant colonies were manually counted for each plate. All samples were analyzed in triplicate. The sample was considered mutagenic if its mean number of His+ revertant colonies is significantly higher than the mean number of spontaneous revertants in the negative control plates.

28-day Repeated Dose Oral Toxicity Study

Male ICR mice were purchased from BioLASCO (Taipei City, Taiwan) at 7 wk of age and allowed to acclimatize for one week before the oral gavage began. They were kept on a 12-h light/dark cycle in the Institute of Biotechnology Animal Room in the Institute of Biotechnology at National Taiwan University. The animal room was maintained at 25–29 °C and relative humidity between 50–70%. Mice were randomly housed in cages and consumed Mouse Diet 20 LabDiet 5058 (LabDiet, PMI Nutrition International Inc., Brenwood, MO) and sterilized tap water *ad libitum*. After 1 wk, mice were fed by oral gavage with the fermented multi-fruit beverage for 28 consecutive days. Mice body weights were measured every week. Protocols used in this animal study were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (NTU105-EL-00101) and was also performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the IACUC of National Taiwan University.

For the experiment, mice were fed with three different doses of the fermented multi-fruit beverage: low dose at 3 mL/kg/d, medium-dose at 7.5 mL/kg/d, and high dose at 15 mL/kg/d through a stomach tube at a feeding volume of 10 mL per kg of body weight. For the control group, mice were orally fed with an equal volume of sterile distilled water. Animals were then sacrificed after 28 d of oral gavage.

A 0.25-mL aliquot of the whole blood sample was taken from each mouse on the day of sacrifice with anesthesia and placed in a BD Microtainer® tube with dipotassium EDTA (BD Biosciences, San Jose, CA, USA). The blood was then analyzed for the following parameters: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular value (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and lymphocyte count (LYMPH). The remaining whole blood sample was placed in a BD Microtainer® tube with serum separator additive BD Microgard™ closure (BD Biosciences, San Jose, CA, USA) and then centrifuged at 3000 x *g* for 15 min at 4 °C to separate the serum. The collected serum was then analyzed for the following biochemical parameters: glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), cholesterol (CHOL), triglycerides, calcium, phosphorus, sodium, potassium, chloride. The organs of the animals – including the heart, liver, spleen, lungs, kidneys, adrenal glands, testis, and accessory organs – were harvested, weighed, and placed in 10% formalin fixative for 1 wk. The organs of the control and high groups were then sectioned and then sent to the National Institute of Veterinary Medicine, National Chung Hsing University for a histopathological examination.

Statistical Analysis

The data were analyzed using GraphPad Prism 7 by analysis of variance (ANOVA) with Dunnett's multiple comparison test or Bonferroni's multiple comparison test compared with the control group. The results were presented as mean ± standard deviation (SD).

RESULTS

In assessing the *in vitro* cytotoxic effects of the fermented multi-fruit beverage, MTT assay results showed that there is a significant difference in the percentage of viable CHO cells between the fermented multi-fruit beverage treated groups and the positive control HCl-treated group (Figure 1). On the other hand, Figure 2 shows the number of cells with chromosomal aberrations per 100 metaphase cells counted for the fermented multi-fruit beverage, positive control, and sterile water treated cultures. Three types of culture system were used: cells that were treated with the test chemical for 3 h with S9 metabolic activation (Figure 2A), cells that were treated for 3 h without S9

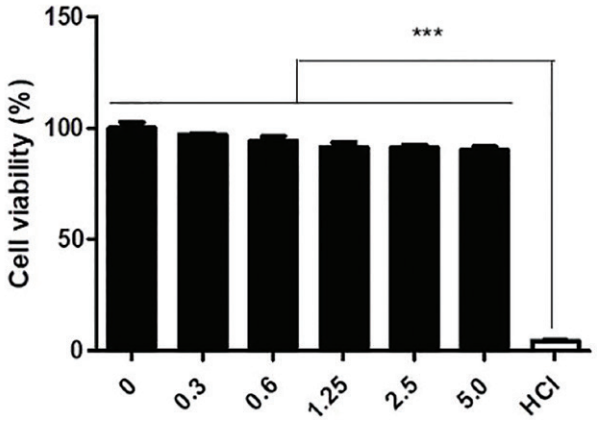

Figure 1. Effect of fermented multi-fruit beverage on the viability of CHO cells as determined by the MTT assay. Values are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test with positive control (0.01 M HCl). ***$p < 0.001$.

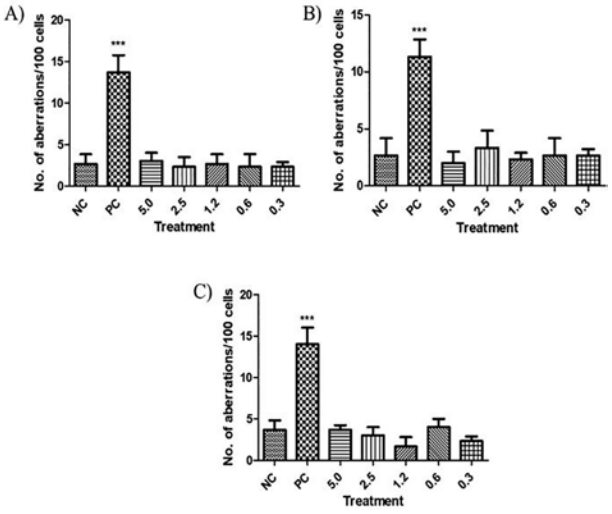

Figure 2. Effect of fermented multi-fruit beverage on inducing chromosomal aberrations (gaps, breaks, dicentrics, rings) in CHO-K1 cells. CHO-K1 cells were treated with the appropriate treatment for A) 3 h with S9 metabolic activation, B) 20 h without S9 metabolic activation, and C) 3 h without metabolic activation. Controls used were distilled water (NC), CPP (PC for cells without S9 activation), and mitC (PC for cells with S9). Cells were treated with fermented multi-fruit beverage at five different doses (µL/mL). Values are shown as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test with negative control. ***$p < 0.001$.

activation (Figure 2B), and cells that were treated for 20 h without metabolic activation (Figure 2C). It is clear that the number of chromosomal aberrations (gaps, breaks, dicentrics, rings) induced by different doses of the fermented multi-fruit beverage on CHO-K1 cells is not significantly different from the sterile water-treated negative control group.

To check whether the fermented multi-fruit beverage can induce any type of DNA mutations, a bacterial reverse mutation assay (Ames test) was performed by exposing the *Salmonella typhimurium* tester strains (Appendix I). Results indicate that the number of histidine-revertant colonies (CFU/plate) induced by the different doses of the fermented multi-fruit beverage was found to be not significantly different from the number of spontaneous revertants induced by the water control (Table 1).

Finally, a 28-day repeated dose oral toxicity study using male ICR mice was conducted to assess the *in vivo* toxic effects of the fermented multi-fruit beverage. Figure 3 shows the changes in the bodyweight of the control and fermented multi-fruit beverage treated mice after 28 d of feeding. Results showed that feeding with all three doses of the fermented multi-fruit beverage has resulted in a

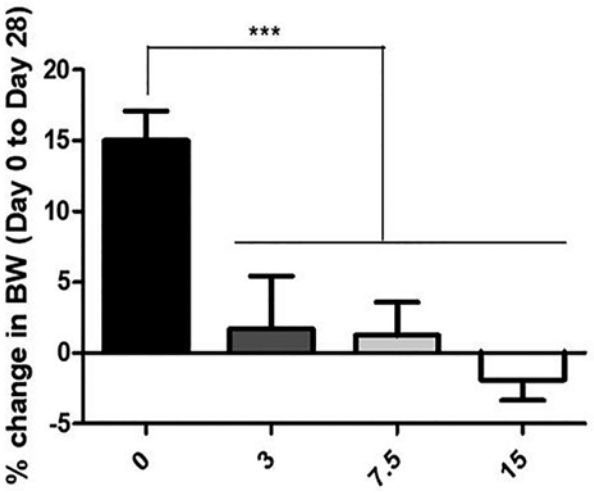

Figure 3. Bodyweight changes of control and fermented multi-fruit beverage treated mice after 28 d of feeding. Percentage change in body weight from Day 0 to Day 28 of the different groups of mice. Values are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test with the negative control 0 mL/kg/d group. ***$p < 0.001$.

Table 1. Number of revertants per plate for the *S. typhimurium* strains TA98, TA100, TA102, TA 1535, and TA1537 after treatment with various doses of fermented multi-fruit beverage, with (+S9) and without (−S9) metabolic activation.

Test compound	Concentration	No. of revertant colonies (CFU/plate)[a]									
		TA98		TA100		TA102		TA1535		TA1537	
		–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
Fermented multi-fruit beverage[b]	5 µL/plate	7 ± 3	10 ± 1	62 ± 4	67 ± 9	129 ± 7	227 ± 18	10 ± 1	6 ± 2	4 ± 2	5 ± 2
	2.5 µL/plate	9 ± 5	11 ± 1	60 ± 3	64 ± 8	123 ± 6	137 ± 10	7 ± 4	9 ± 3	5 ± 4	3 ± 3
	1.25 µL/plate	7 ± 1	9 ± 2	52 ± 10	63 ± 3	113 ± 6	157 ± 36	9 ± 2	8 ± 3	5 ± 4	5 ± 4
	0.63 µL/plate	5 ± 1	8 ± 4	56 ± 12	52 ± 10	128 ± 8	136 ± 16	8 ± 2	10 ± 2	4 ± 2	4 ± 2
	0.31 µL/plate	6 ± 1	8 ± 4	43 ± 6	61 ± 8	133 ± 4	143 ± 27	5 ± 2	8 ± 1	3 ± 2	5 ± 4
Control (DMSO)[c]	NA	1 ± 1	10 ± 2	–	43 ± 6	–	185 ± 8	–	4 ± 2	11 ± 3	2 ± 1
Control (water)	NA	7 ± 1	7 ± 2	61 ± 1	61 ± 3	147 ± 8	171 ± 27	7 ± 4	11 ± 4	6 ± 1	5 ± 2
2-AA[d]	4 µg/plate	–	–	–	1627 ± 76*	–	571 ± 35*	–	168 ± 2*	–	187 ± 44*
BAP[d]	1 µg/plate	–	171 ± 3*	–	–	–	–	–	–	–	–
AzNa[d]	1 µg/plate	–	–	225 ± 6*	–	–	–	122 ± 22*	–	–	–
2-NF[d]	1 µg/plate	135 ± 8*	–	–	–	–	–	–	–	–	–
MitC[d]	0.5 µg/plate	–	–	–	–	949 ± 82*	–	–	–	–	–
9-AA[d]	1 µg/plate	–	–	–	–	–	–	–	–	73 ± 6*	–

*$p < 0.001$ (ANOVA with Bonferroni's multiple comparison test) versus control (water)
[a]Data are tabulated as mean ± SD of triplicates.
[b]Water was used as the solvent for the fermented multi-fruit beverage.
[c]DMSO controls are not present for all samples since the positive controls used [AzNa for TA100 (–S9) and TA1535 (–S9) and MitC for TA102 (–S9)] were dissolved in sterile distilled water.
[d]Positive controls: without S9: for strain TA98, 1 µg/plate of 2-NF was used; for strainsTA100 and TA1535, 1 µg/plate of AzNa was used; for strain TA102, 0.5 µg/plate of MitC was used; for strain TA1537, 1 µg/plate of 9-AA was used; with S9: for strains TA100, TA102, TA1535, and TA1537, 4 µg/plate of 2-AA was used; for strain TA98, 1 µg/plate of BAP was used.

significant decrease in the amount of weight gain from Day 0 to Day 28 of feeding. Moreover, the highest dose even resulted in a weight loss after 28 consecutive days of fermented multi-fruit beverage intake.

The hematologic profile of both the control and the fermented multi-fruit beverage treated mice are shown in Figure 4. Results showed that there is no significant difference between the control and the treated mice in terms of RBC parameters (Figure 4A), WBC parameters (Figure 4B), and platelet count (Figure 4C).

In evaluating the effect of the fermented multi-fruit beverage on liver and kidney function parameters, results showed that the GOT (Figure 5A), GPT (Figure 5B), and BUN (Figure 5C) levels of the fermented multi-fruit beverage treated mice were not significantly different from the control group; however, feeding with 3 mL/kg/d has resulted in a significant decrease in CRE levels (Figure 5D).

Figure 6 shows the serum CHOL, TG, and GLU levels of both the control and the fermented multi-fruit beverage treated mice. It was found that CHOL and GLU levels were not affected by fermented multi-fruit beverage intake. On the other hand, all three doses of the fermented multi-fruit beverage were found to be able to lower triglyceride levels, with the 3 mL/kg/d dose resulting in a significant decrease.

Lastly, histopathological analysis of the different tissue sections of the control and the high dose (15 mL/kg/d) treated mice showed that there were no significant changes noted in the adrenal gland, epididymis, heart, kidney, liver, spleen, and testes of both the control (Appendix III) and the high dose treated mice (Figure 7).

DISCUSSION

To assess the safety of the fermented multi-fruit beverage *in vitro*, MTT assay, Ames test, and chromosome aberration test were conducted. Results collectively indicate that the fermented multi-fruit beverage was found to have no cytotoxic effect (Figure 1) and no genotoxic effect (Figure 2) against mammalian cells, as well as no mutagenic potential as it did not induce significant DNA mutations in five of the *Salmonella typhimurium* tester strains (Table 1).

A complete investigation regarding the *in vivo* toxicity of a test chemical entails not only the determination of the test chemical's lethal toxicity; but, moreover, it should also attempt to evaluate the test chemical's sublethal and

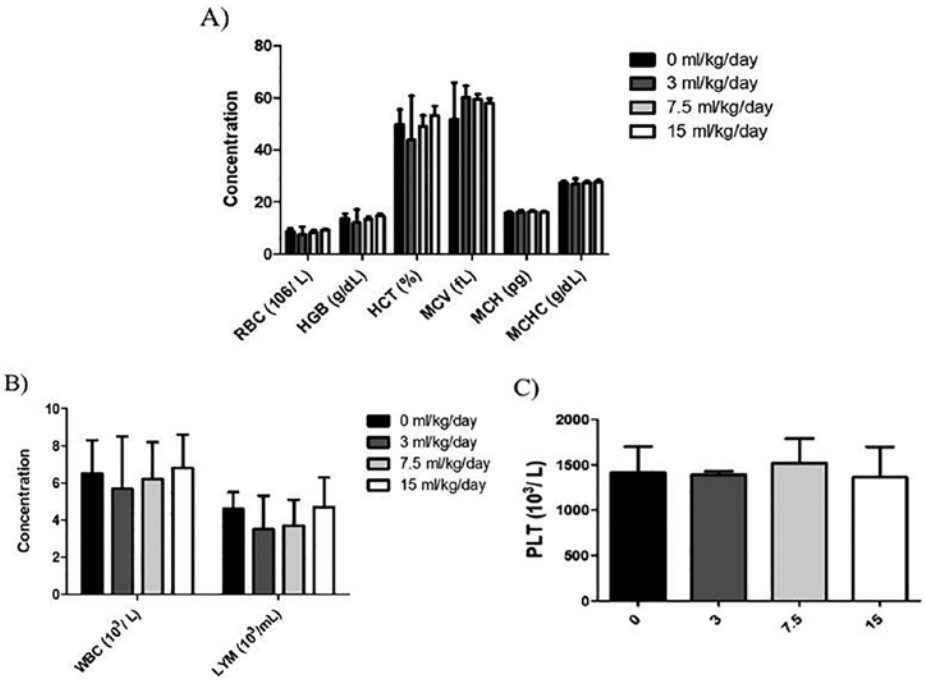

Figure 4. Hematologic profile of control and fermented multi-fruit beverage treated mice. A) RBC parameters in mouse whole blood: RBC – red blood cells; HGB – hemoglobin; HCT – hematocrit; MCV – mean corpuscular value; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration. B) WBC parameters in mouse whole blood: WBC – white blood cells; LYM – lymphocytes. C) Platelet (PLT) count in mouse whole blood. Values are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test with the negative control 0 mL/kg/d group.

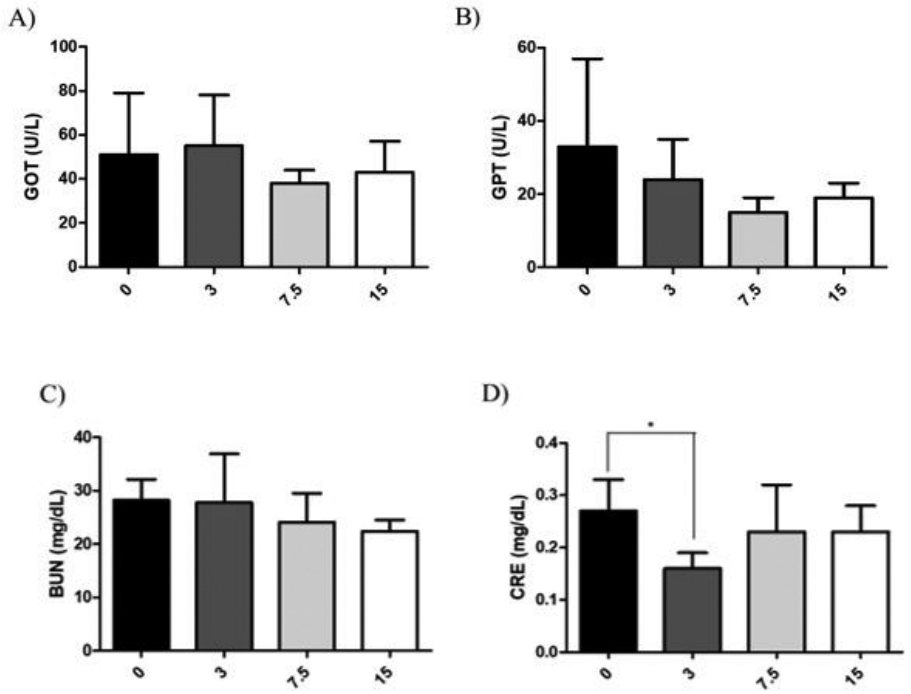

Figure 5. Liver and kidney function parameters of the control and fermented multi-fruit beverage treated mice. Liver function of mice was assessed by measuring A) GOT and B) GPT. Kidney function of mice was assessed by measuring C) BUN and D) CRE. Values are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test with the negative control 0 mL/kg/d group. *$p < 0.05$.

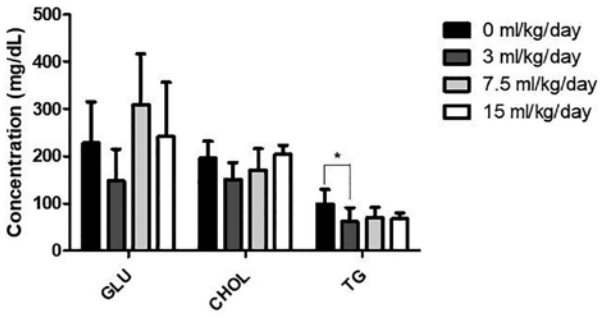

Figure 6. Blood chemistry profile of control and fermented multi-fruit beverage treated mice. Serum from whole mouse blood were collected and analyzed for GLU, CHOL, and TG levels. Values are shown as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test.

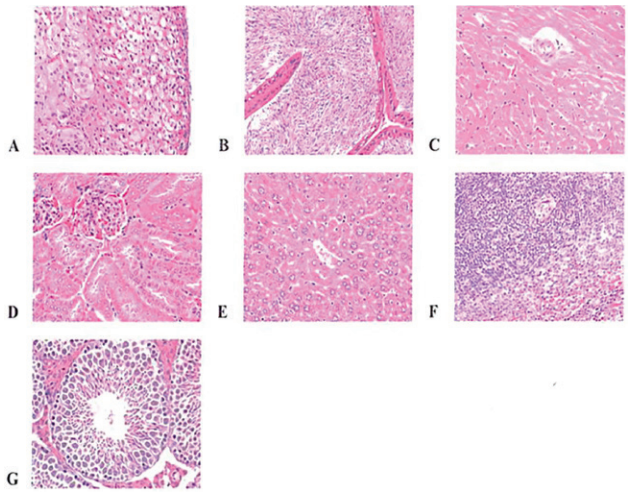

Figure 7. Histopathological examination of the different organs of the 15 mL fermented multi-fruit beverage/kg/day treated mice. No significant histopathological changes were noted in the A) adrenal gland, B) epididymis, C) heart, D) kidney, E) liver, F) spleen, and G) testes of the mice after 28 d of feeding with the fermented multi-fruit beverage (H&E stain, 400x).

target organ toxicity (Ballantyne *et al*. 2009). This can be accomplished by including bodyweight observations, hematology, and clinical chemistry profiling, as well as gross and microscopic histopathological analysis into the protocol (Ballantyne *et al*. 2009). Thus, in the 28-d repeated dosing study of the fermented multi-fruit beverage, we incorporated all of the aforementioned analyses into the experimental procedure in order to fully understand its safety and toxicological effects.

In terms of regulating body weight changes in mice, the fermented multi-fruit beverage was found to decrease the amount of weight gain after 28 d (Figure 3). To date, there has been no extensive study regarding the weight regulation effects of fermented mixed fruit products. However, there have been some studies regarding the bodyweight regulation effects of other fermented food products. For instance, fermented kimchi has been shown to be able to reduce body weight in obese patients, and that this is due to the dietary fiber, vitamins, calcium, capsaicin, and niacin contained in the kimchi (Kim *et al*. 2011). Fermented soybean paste has also been found to be able to reduce body weight and visceral fat in overweight adults, and the authors attributed its weight-reducing effects to genistein, an isoflavone that enhances the transcription of target genes involved in fatty acid beta-oxidation (Cha *et al*. 2012). Combining these studies altogether, the common denominator among these two food products is that aside from being fermented, they both contain dietary fiber. Dietary fiber has been extensively studied to be able to regulate body weight, energy intake, and subjective appetite (Howarth *et al*. 2001; Wanders *et al*. 2011). Moreover, when subjected to fermentation, fiber can ultimately result in short chain fatty acids that may enter the portal circulation, thus increasing satiety, as well as decreasing energy intake and fat storage (Pereira and Ludwig 2001). However, in this case of the fermented multi-fruit beverage, proximate analysis in our previous study showed that this beverage might not be a significant source of fiber as it only contains 0.5 g dietary fiber/100 mL (Sy *et al*. 2020). Moreover, the fiber consumed by the treated mice might not be significantly different from that consumed by the control mice as the mouse feed (Mouse Diet 20 LabDiet 5058) also contained dietary fiber. Instead, our analysis showed that the main component of this beverage were carbohydrates, mainly sugars (Sy *et al*. 2020). Therefore, we hypothesized that sugars may be the primary reason why the treated mice had a lesser weight gain compared to the control mice. To support this hypothesis, various studies have shown that carbohydrates and sugars can prevent weight gain (Stubbs *et al*. 2001), reduce hunger, and increase satiety (Anderson and Woodend 2003; Lavin *et al*. 2001). However, the mechanism as to exactly how carbohydrates or sugars may affect satiety is still unknown (Anderson and Woodend 2003; Stubbs *et al.* 2001).

In toxicological experiments, changes in organ weights are a well-accepted sensitive indicator of chemically induced changes to the target organs (Michael *et al*. 2007). Since the organ weights of the fermented multi-fruit beverage treated mice are not significantly different from the water-fed mice (Appendix II), this indicates that the fermented multi-fruit beverage has no observed toxic effect directed to these organs.

To further confirm the non-toxicity of the fermented multi-fruit beverage, hematologic parameters were also measured in mouse whole blood. For both humans and experimental animals, abnormalities observed in the number of certain blood cell types may be indicative of induced toxicity to hematopoietic organs such as the bone marrow, or to other organs affected by nutrition intake such as the kidney, which in turn also affects hematopoiesis (Marrs and Warren 2009). As seen in Figure 4, there is no significant difference in the number of RBC cell types, WBC cell types, and platelets between the fermented multi-fruit beverage treated mice and the water-fed mice. Moreover, all these values are within the published normal reference values for hematologic parameters in male ICR mice (Serfilippi *et al*. 2003). Therefore, the fermented multi-fruit beverage did not induce hematotoxicity to the male ICR mice.

For the past 50 years, hepatotoxicity has been the leading single cause of drug withdrawals from the market; therefore, evaluating the test chemical's effect on liver function is indispensable in a safety assessment program (Gosselin *et al.* 2009). GOT and GPT are powerful indicators of hepatotoxicity and the occurrence of liver disease (Huang *et al*. 2006). GPT levels are normally elevated by any type of liver injury that primarily affects the hepatocytes, such as viral hepatitis, ischemic liver injury (shock liver), and toxin-induced liver damage (Gowda *et al*. 2009). On the other hand, elevated GOT levels are commonly associated with extensive tissue necrosis due to myocardial infarction, as well as with chronic liver diseases such as tissue necrosis and degeneration (Gowda *et al*. 2009). Based on Figure 5, it is noted that the GOT (Figure 5A) and GPT (Figure 5B) levels of the fermented multi-fruit beverage treated mice is not significantly different from the negative control group. All values were also within the normal reference range (Serfilippi *et al*. 2003). Thus, the fermented multi-fruit beverage was found to have no apparent hepatotoxic potential.

Because of the significant role of the kidney in maintaining a number of homeostatic functions in the body (Gosselin *et al.* 2009), safety assessment of chemicals also necessitates the measurement of kidney function parameters in order to ensure there is no drug-induced nephrotoxicity involved. In fact, in drug development, approximately 70% of compound termination resulted from the induction of urinary system toxicities, which is significantly higher than 55% for hepatobiliary injury (Olson *et al*. 2000). Nephrotoxicity is most readily evaluated by measuring changes in the glomerular filtration rate (GFR), and both BUN and CRE are standard indices of GFR due to the fact that these two proteins readily filtered across the glomerulus then ultimately excreted in the urine (Gosselin

et al. 2009). Elevated levels of BUN and CRE above the normal range is indicative of a decrease in GFR, thus forming the starting point for the investigation in drug-induced nephrotoxicity (Gosselin *et al*. 2009). From the results, it can be seen that there is no change in the BUN levels between the fermented multi-fruit beverage and the water treated mice (Figure 5C); however, there is a significant decrease in CRE levels in the low dose-treated mice (Figure 5D). Low creatinine levels are usually associated with the occurrence of hepatic disease (Takabatake 1988); however, since the GOT and GPT levels of the fermented multi-fruit beverage treated mice were normal, this decrease in CRE may not be a cause for concern. Additionally, all the BUN and CRE levels were within the published reference values (Serfilippi *et al*. 2003). Therefore, the fermented multi-fruit beverage was found to have no inherent nephrotoxicity.

In animal toxicity studies, molecules such as lipids (CHOL, TG) and carbohydrates (GLU) were also measured in the mice serum aside from enzymes and proteins. Results of this study indicate that there is no difference in the GLU and CHOL levels between the fermented multi-fruit beverage treated mice and the negative control; however, there is a decrease in TG levels in the fermented multi-fruit beverage mice, with the low dose group resulting to a significant decrease (Figure 6). Triglyceride levels are largely affected by how much fat the body stores in the cells. Therefore, the observed decrease in TG levels is probably due to the ability of the fermented multi-fruit beverage to decrease fat storage and control weight gain as previously mentioned. Since the weight loss observed in the fermented multi-fruit beverage treated mice was concluded to have no adverse effects based on the organ weights and daily routine observations of the mice during the 28-d feeding period, the decrease in TG may in fact be favorable as elevated levels of TG, or hypertriglyceridemia, is more commonly associated with cardiovascular diseases. In addition, increases and decreases in CHOL and TG occur relatively frequently in toxicity studies, where there are small changes that are indicative of minor alterations in the animals' lipid metabolism and, therefore, do not adversely affect its overall health status (Gosselin *et al.* 2009). Therefore, the fermented multi-fruit beverage – because of its ability to lower triglyceride levels – may in fact have the potential to reduce the risk of heart diseases.

Lastly, histopathological analysis of different tissue sections was performed to provide more conclusive evidence with regards to the safety of the fermented multi-fruit beverage. Histopathological analysis remains to be one of the most significant methods included in standard toxicological studies because of its ability to evaluate the interaction of the test chemical with complete organ systems rather than on single cells and organs (Greaves 2009). In fact, careful interpretation of other safety evaluation data such as organ weights, hematology, serum chemistry, and electrolyte levels all need to be correlated with histopathological findings in order to form sound conclusions regarding a test chemical's toxicity In this study, histopathological analysis of the high dose treated mice revealed that there were no significant findings in the different tissue sections sampled (Figure 7). Furthermore, these results also further confirmed that the decrease in CRE is not indicative of nephrotoxicity as there were no significant histopathological findings in the kidney tissue sections of the high dose treated mice. Therefore, the fermented multi-fruit beverage was found to be safe and has no apparent toxic effects in an experimental mice model.

CONCLUSION

The consumption of fermented food products has been demonstrated by numerous studies to have a lot of health-promoting effects; however, there are a limited number of scientific reports that have investigated the safety of fermented mixed fruit beverages. Our study concluded that the fermented multi-fruit beverage is safe for human consumption as it did not induce cytotoxicity and chromosomal mutations in cultured CHO-K1 cells, mutagenicity in five mutant strains of *Salmonella typhimurium*, and drug-induced toxicity in male ICR mice as evidenced by hematologic, biochemical, and histopathological analyses. However, its detailed mechanism in regulating calorie intake and weight gain still remains unclear and requires further research.

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STATEMENT ON CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

NOTES ON APPENDICES

The complete appendices section of the study is accessible at http://philjournsci.dost.gov.ph

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