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Research Article

ASSESSMENT OF BIOLOGICAL SAFETY OF FERMENTED *PHYLLANTHUS EMBLICA* FRUIT JUICE

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ABSTRACT

Objective: The present study evaluated the subchronic toxicity of *Lactobacillus* mediated fermented *Phyllanthus emblica* fruit juice (FPJ) using a rat as a model system.

Methods: FPJ was prepared, and estimated the changes in pH by pH meter, and microbial load by a plating method. Rats were fed with different dose of FPJ for 60 days. The changes in the body mass were noted. The blood and organs of the experimental rats were collected, after 60 days of intervention. Then, they were analyzed for the selected hematological and biochemical parameters by following standard hospital protocols.

Results: The pH of FPJ after 30 days of fermentation was 3.16. FPJ was rich in probiotic *Lactobacillus* spp. (7.23 Log CFU per mL) without contamination. The supplementation of FPJ was not significantly affected the body weight of the experimental animals, except the female rats in posteffective dose (PED) group showed significant changes (20.83±8.49 g) compared to control (40±17.22 g). The internal organs of the rat were not affected by the FPJ supplementation. The changes observed in blood urea nitrogen, creatinine, cholesterol, triglyceride, aspartate aminotransferase; alanine aminotransferase, and alkaline phosphatase level of experimental rats, both male and female, were not significantly differed from the respective controls. The average of lymphocytes level was significantly increased in continuous dosing group of males and females. Interestingly, the increase in red blood cell and hemoglobin (HGB) were statistically significant for ED group and PED in both sexes, except for females with no effect on HGB content.

Conclusion: The prepared FPJ was enriched with probiotic *Lactobacillus* spp. The supplementation of FPJ (up to 9 mL/kg/day) for 60 days was not significantly influenced the body weight, internal organs, biochemical and hematological parameters of experimental rats (both male and female). The results revealed that FHJ is suitable for the human consumption.

Keywords: *Phyllanthus emblica*, Lactic acid bacteria, Fermented plant juice, Subchronic, Toxicity.

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INTRODUCTION

Phyllanthus emblica, also known as *Emblica officinalis*, Indian gooseberry, emblic, belongs to Phyllanthaceae family. *P. emblica* is one of the commonly used medicinal herbs in traditional Indian medicine. *P. emblica* is rich in phytonutrients, proteins, fibers, fat, vitamins (especially Vitamin-C), minerals, and trace elements (calcium, chromium, copper, iron, magnesium, nicotinic acid, phosphorus, potassium, and zinc) [1]. Almost all the part of *P. emblica* plant (fruit, root,and leaves) was reported for health benefits and pharmacological importance such as anti-inflammation, antihyperglycemia, anti-hyperlipidemia, anti-cancer, anti-mutagenic, anti-pyretic, anti-microbial, anti-diarrheal, anti-hyperthyroidism, anti-venom cardioprotective, neuroprotective, etc., [2-20]. *P. emblica* is consumed as raw fruit, fruit juice, fermented fruit juice, pickles, and powder forms. *P. emblica* fruit pickling is most common practice in southern India, which has been consumed by the people every day along with regular diet. Southeast Asians widely use the fermented *P. emblica* fruit juice (FPJ). The fermentation of plant materials with desired microbial starter culture improved the nutritional value and bioactive compounds [21-23].

Fermentation is an ancient method of preparing, improving and preserving the food. Asian peoples extensively use the fermented foods, and they believe that fermented foods are a cure for several diseases [24]. The microbes isolated from naturally fermented foods are reported for glutaminase, and glutamate decarboxylase, and γ-aminobutyric acid-producing ability [25,26]. The fermented plant juices are reported for several pharmacological applications. For example, the supplementation of lactic acid bacteria (LAB) fermented mushroom juice help to diminish the diabetic consequences [27]. *Lactobacillus paracasei* HII01 mediated fermented *P. emblica* exhibited high polyphenolic compounds, and enhanced free-radical scavenging activity compared to control [21].

Though *P. emblica* is used in several traditional medicines, the reports on fermented LAB mediated FPJ is inadequate. The supplementation of 0.5 g/kg of *P. emblica* leaves aqueous extract (PLAE), and 10 g/kg of an ethanolic extract of emblica fruit (EEEF) were not shown any toxicity in mice. Whereas, intraperitoneal injection of PLAE and EEEF exhibited LD50 of 0.288–0.415, and 4.8 g/kg body weight of mice, respectively [28,29].

There was no subchronic toxicity study on LAB-mediated FPJ. The present study was executed to evaluate the subchronic toxicity of *L. paracasei* HII01 mediated fermented *P. emblica* using rodent model system.

METHODS

Preparation of FPJ

The inoculum preparation and fermentation process were performed as detailed in a previous study [21]. Briefly, the crushed *P. emblica* fruits, sterile water, and cane sugar were mixed in the ratio of 3:10: 1, and sterilized. Then, 10% of *L. paracasei* HII01 was inoculated, and fermented for 30 days.

pH, and microbial load

The pH of FPJ was kinetically measured using pH meter (Inola, pH level 2, Weilheim) [21]. The microbial load of FPJ was determined by spread plate method using specific media as prescribed earlier [30].

Animals, intervention, and sample collection

The Sprague Dawley rats of 150–180 g of weight, obtained from National Laboratory Animal Center of Mahidol University, Thailand, were casually divided into groups. The test intervention was 1.2 mL/kg/day of FPJ (effective dose [ED]), 9.00 mL/kg/day of FPJ (high dose [HD]) for 60 days, and 1.2 mL/kg/day of FPJ (post-ED [PED]) for 53 days along with standard animal feed. The control animals were fed with laboratory food and drinking water for 60 days. After 60 days of study, blood and internal organs of the experimental rats were collected for inspection. The experiments were ethically approved by the Ethical Committee of Faculty of Medicine, Chiang Mai University (CMU) (Approved protocol no: 1/2552 dated 23 June 2009).

Measurement of body mass and assessment of hematological and biochemical parameters

The difference in weight of experimental rats was recorded using laboratory weighing machine. The weight of internal organs (brain, liver, spleen, eyes, heart, lung, kidneys, stomach, and adrenal gland) was measured. The change in the weight was calculated by the following formula.

Deviations in weight = Final weight - Initial weight.

The biochemical and hematological parameters (hemoglobin [HGB], hematocrits, white blood cell (WBC) count, polymorphonuclear cell, lymphocyte, platelets, red blood cell (RBC) count, level of aminotransaminase, alanine aminotransaminase, alkaline phosphatase [ALP]) were measured at MT InterMed (Hosptial) Growth Diags Co., Ltd., Ching Mai, Thailand, as per the standard procedures.

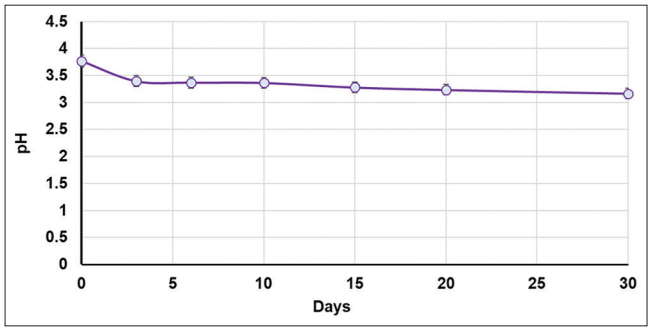

Fig. 1: The changes in the pH of fermented *Phyllanthus emblica* fruit juice

Statistical analysis

The experiments were completed in triplicate. The values were signified as a mean ± standard deviation. Duncan's new multiple range tests determined the significant differences, at the 95% confidential level ($p<0.05$) by SPSS v.17 (Chicago, SPSS Inc, U.S.A).

RESULTS AND DISCUSSION

The fermentation of *P. emblica* fruit juice (FPJ) was carried out. The pH of FPJ was gradually reduced during fermentation. The pH of initial day of fermentation was 3.76, while after 30 days of fermentation the pH was 3.16 (Fig. 1).

The microbial content of FPJ was increased during fermentation. After thirty days of fermentation, total bacterial count, and *Lactobacillus* spp. count was found as 7.39, and 7.23 Log CFU per mL, respectively. At 3–6 days of fermentation high concentration of *Lactobacillus* spp. (8.33–8.63 Log CFU per mL) was observed in FPJ. Then, the number of *Lactobacillus* spp. was reduced steadily, possibly due to the depletion of nutrients. Yeast and *Bacillus* spp. were not found in FPJ at any point of fermentation, which suggested that FPJ was microbiologically safe (Fig. 2).

The average body weight of ED, HD, and PED group male rats was 104.29±31.47, 98.57±28.24, and 115.83±20.66 g, respectively, while control rat was 123.33±30.33 g after 60 days of the experimental period. The average body weight of ED, HD, and PED group female rats was 33.57±13.14, 32.86±12.2, and 20.83±8.49 g, respectively while control rat was 40.00±17.22 g after 60 days of the experimental period. There were no significant changes in body weight of male rats supplemented with FPJ, irrespective of dose (ED, HD, and PED). Whereas, female rats in PED group showed significant changes (20.83±8.49 g) compared to control (40±17.22 g) (Table 1).

Animals were euthanized after the intervention of FPJ for 60 days. The organs (brain, eyes, heart, lung, liver, spleen, stomach, kidneys, and adrenal gland) were collected for macroscopic examination and weighed. There were no significant treatment-related pathological changes observed in any organ of the experimental rats at all tested dose levels when compared with organs in control animals (Table 2). The changes observed in blood urea nitrogen, creatinine, cholesterol, triglyceride, aspartate aminotransferase; alanine aminotransferase, and ALP level of experimental rats, both male and female, were not significantly differed from the respective controls (Table 3).

The tested hematological parameters were significantly varied from control (Table 3). The average of WBC, hematocrits, and platelets level was not altered significantly in male rats of all groups. The average WBC, HGB, hematocrits levels were not changed in female rats of ED group. The average of lymphocytes level was significantly increased

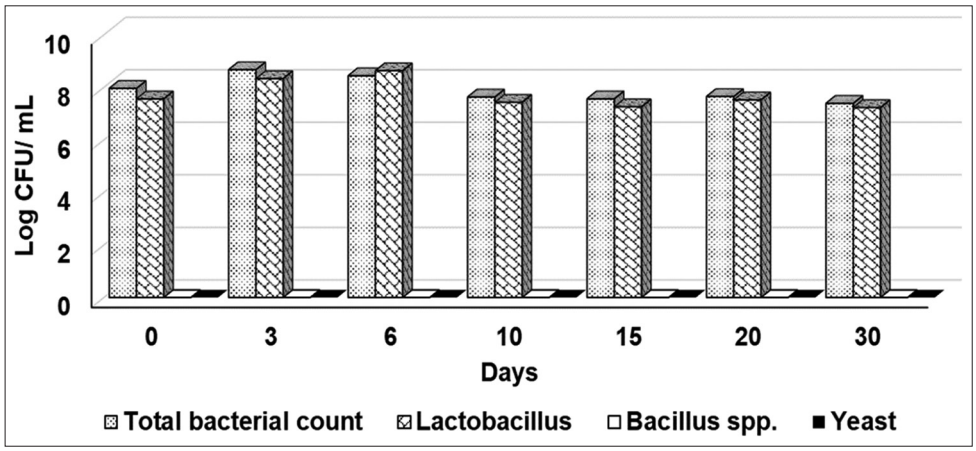

Fig. 2: Microbial load in *Phyllanthus emblica* fruit juice during fermentation

Table 1: The difference in body mass of test animals during the experimental period. The values were derived from the baseline values

Day	Body weight (g)							
	Male				Female			
	Control	ED*	HD**	PED***	Control	ED*	HD**	PED***
5	15.00±11.69	12.14±4.15	16.43±7.18	10.00±2.94	6.67±3.29	8.57±0.29	6.43±0.32	5.00±1.36
15	40.00±17.51	24.29±8.56	25.00±6.59	38.33±13.93	12.50±3.20	6.07±2.29	9.51±3.57	8.61±0.00
30	88.33±20.74	55.00±18.7	60.00±27.06	78.33±15.94	29.17±12.81	15.00±9.57	16.43±4.27$^{\#}$	13.33±7.58$^{\#\#}$
60	123.33±30.33	104.29±31.47	98.57±28.24	115.83±20.66	40.00±17.22	33.57±13.14	32.86±12.2	20.83±8.49$^{\#\#}$

*ED: 1.2 ml/kg/day, **HD: 9 ml/kg/day, ***PED: Post-effective dose (intervention has been stopped before 7 days of final assessments). $^{\#}$Significant difference ($p<0.05$) between control and test-group; $^{\#\#}$significant difference ($p<0.01$) between control and test group. ED: Effective dose, HD: High dose

Table 2: Changes in the organ weight after oral supplementation of fermented *P. emblica* juice. The values were derived from the control values (the difference between control value and experimental value, after the experimental period), and were represented as a mean±standard deviation

Organs	Weight (g)					
	Male			Female		
	1.2 ml/kg/day (ED)	9 ml/kg/day (HD)	PED	1.2 ml/kg/day (ED)	9 ml/kg/day (HD)	PED
Brain	−0.06±0.11	−0.02±0.05	−0.26±0.43	0.01±0.02	0.02±0.10	−0.21±0.57
Eyes	−0.03±0.05	−0.021±0.04	0.42±0.79	−0.02±0.07	0±0.02	0.05±0.2
Heart	−0.02±0.39	0.129±0.41	0.17±0.55	−0.2±0.30	−0.22±0.24	−0.31±0.44
Lung	0.29±0.98	−0.2648±0.43	0.08±0.82	−0.19±0.43	−0.16±0.56	−0.11±0.79
Liver	−0.41±2.15	−1.55±1.3	−1.09±2.38	−5.03±0.69	−4.78±0.62	−5.88±1.77
Spleen	−0.09±0.09	−0.11±0.05	−0.11±0.12	−0.04±0.05	−0.12±0.22	−0.14±0.19
Stomach	−0.22±±0.95	0.17±0.28	0.04±0.13	0.14±0.12	0.11±±0.11	−0.06±0.55
Kidneys	−0.04±0.27	−0.13±0.23	−0.33±0.42	0.11±0.11	0.17±0.15	−0.09±0.62
Adrenal Grand	−0.01±0.02	−0.02±0.02	−0.02±0.02	0.02±0.01	0.02±0.01	0±0.03

P. emblica: Phyllanthus emblica, ED: Effective dose, HD: High dose, PED: Post-effective dose

Table 3: Effect of supplementation of fermented *P. emblica* juice on the hematological and biochemical parameters in hamster after 60 days of treatment. The values were derived from the control values (the difference between control value and experimental value, after the experimental period) and were represented as a mean±standard deviation.

Parameters	Male			Female		
	ED	HD	PED	ED	HD	PED
Hematological parameters						
RBC (10^6/mm^3)	−3.9±0.18**	−0.26±0.19	−0.52±0.19**	−0.21±0.08*	−0.22±0.07	−0.23±0.12*
WBC (10^3/mm^3)	0.34±0.26	0.16±0.20	0.04±0.25	0.24±0.19	0.13±0.20	0.46±0.25
HGB (g/dL)	−1.08±0.61*	−0.83±0.58	−1.12±0.33*	−0.64±0.50	−0.16±0.58	−0.92±0.92
Hematocrits (mL%)	−3.29±1.80	−2.57±1.81	−3.33±1.03	2.50±1.47	3.42±1.26	2.17±2.74
Lymphocyte (mL%)	−7.90±3.1*	−10.33±8.04*	−6.16±2.64	−11.5±2.83***	−9.64±3.53**	−10.00±4.37**
Platelets (10^3/mm^3)	15.84±11.13	15.84±11.13	15.50±19.00	−31.21±16.33*	−16.93±13.95	−11.33±26.63
Biochemical parameters						
B.U.N. (mg/dL)	2.84±7.10	6.00±4.71	12.34±8.61	7.33±6.44	−4.86±2.95	2.00±4.09
Creatinine (mg/dL)	0.35±0.63	−0.14±1.24	−0.02±1.36	0.63±0.94	−0.05±1.42	0.48±1.03
Cholesterol (mg/dL)	−20.00±16.34	−6.5±44.23	6.17±76.79	0.5±39.69	5.19±65.99	20.17±89.36
TG (mg/dL)	−4.67±7.87	1.16±11.34	4.83±10.64	18.83±9.81	15.90±9.57	13.83±9.33
AST (IU/L)	−11.17±25.37	−24.17±29.46	−12.57±30.02	0.2±32.33	−0.71±26.37	7.6±24.96
ALT (IU/L)	−0.33±5.00	3.50±6.77	−5.33±6.71	2.33±4.37	−0.46±5.12	3.83±4.58
ALP (IU/L)	3.08±9.06	10.58±2.94	11.08±3.71	7.77±8.73	9.74±4.71*	11.43±2.04*

*Significant difference ($p<0.05$) between control and test-group, **significant difference ($p<0.01$) between control and test group. ***Significant difference ($p<0.001$) between control and test-group. RBC: Red blood cells, WBC: White blood cells, HGB: Hemoglobin; PMNC: Polymorphonuclear cell; BUN: Blood urea nitrogen, TG: Triglyceride, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, ED: Effective dose, HD: High dose, PED: Post-effective dose, *P. emblica: Phyllanthus emblica*

in continuous dosing group of males and females. Interestingly, the increase in RBC and HGB were statistically significant for ED and PED in both sexes, except for females with no effect on HGB content. The results suggested that the supplementation of FPJ affected the hematological parameters. However, all the changes were in the normal range. Hence, supplementation of FPJ was not affect the tested parameters.

The phytochemical content and medicinal property of *P. emblica* has been reported with a possible molecular mechanism [1]. *P. emblica* fruit extract exhibited cytotoxicity against ovarian, liver and cervical cancer cells, and the toxic effect on ovarian cancer cells was attributed to the activation of autophagy [31-33]. The antiproliferative effect of emblica extracts on cancer cell lines was associated with the activation of apoptotic pathway, and both crude extract and purified compounds (such as quercetin, corilagin, pyrogallol, ellagic, chebulagic, and gallic acids) can inhibit the NF-κB activity [34,35].

The anti-inflammatory effect of hydroalcoholic extract of emblica fruit was reported using induced paw edema in rats. The emblica extract showed edema reduction in a dose-dependent manner, and results were similar to the commercial ant-inflammatory drug and indomethacin [36]. The methanolic extract of emblica fruits (400 mg/kg

of body weight) reduced the carrageenan-induced acute inflammation in rats up to 72.71% [37].

Guo and Wang [38] reported that aqueous extract of emblica fruit protects the human colon epithelial cells from mitotic abnormalities, and genetic variability by improving the function of spindle assembly checkpoint. The emblica fruit extract was reported for synergetic antimicrobial effect along with antibiotics against *Salmonella typhimurium* [19].

The changes in phytochemical content, antioxidant property, and other physical properties such as color, odor, taste, and gas formation in LAB LPFJ was reported previously [21]. The study proved that *L. paracasei* HII01 mediated fermentation process improved the quality of *P. emblica* fruit juice regarding nutritional value and antioxidant property. The authors also suggested that LPFJ can be used as a dietary supplement [21].

CONCLUSION

As we reported earlier, the prepared FPJ was rich in phytochemicals, antioxidants, and free from pathogenic microbes. The supplementation of different dose of FHJ does not affect the average body weight gain, internal organs' weight, and tested biochemical parameters significantly. However, hematological parameters were altered significantly on FHJ supplementation. The changes were within the normal range of respective parameters. Hence, the study suggested that the consumption of FPJ was not affected the rodent system in some disagreeable ways. The further detailed study is required to confirm the toxicity of FHJ.

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AUTHORS CONTRIBUTIONS

CC involved in the study design and finalization of the manuscript. BSS and PK contributed to data analysis, manuscript preparation, and critical revision of the manuscript. YD, SS, KC, and SP is responsible for wet laboratory experiments. All the authors agree with the content of the manuscript.

CONFLICT OF INTEREST

There is no conflict of interests.

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