

**ASSESSMENT OF SUBCHRONIC TOXICITY OF FERMENTED *HOUTTUYNIA CORDATA* THUNB. USING RODENT MODEL SYSTEM****CHAIYAVAT CHAIYASUT<sup>1\*</sup>, BHAGAVATHI SUNDARAM SIVAMARUTHI<sup>1</sup>, YODSAWEE DUANGJITCHAROEN<sup>2</sup>, PERIYANAINA KESIKA<sup>1</sup>, SASITHORN SIRILUN<sup>1</sup>, KHONTAROS CHAIYASUT<sup>3,4</sup>, SARTJIN PEERAJAN<sup>4</sup>**

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**ABSTRACT**

**Objective:** The present study was intended to evaluate the subchronic toxicity of *Lactobacillus*-mediated fermented *Houttuynia cordata* juice (FHJ) using rodent model system.

**Methods:** FHJ was prepared and the microbial load, lactic acid content, and pH was estimated. Rats were fed with different doses of FHJ for 60 days. The body mass changes were measured during FHJ supplementation. After the treatment period, blood and organs of the experimental rats were collected. The samples were subjected to hematological and biochemical analysis by following standard hospital protocols.

**Results:** The pH of FHJ after 30 days of fermentation was 3.63. The lactic acid content of FHJ was gradually increased and reached 19.70 mg per mL after 30 days of the fermentation process. *Lactobacillus* load was high in FHJ after 30 days and no *Bacillus* spp. and yeast were detected in FHJ at any point of fermentation. There were no significant changes in body weight of male and female experimental rats supplemented with FHJ, irrespective of dose. There were no significant treatment-related pathological changes found in any organ of the experimental rats at all tested dose levels when compared with organs in control animals. There were no significant changes observed in red blood cells (RBCs), white blood cells (WBCs), hemoglobin, hematocrits, lymphocyte, and platelets level of male rats of all groups. Whereas, significant ( $p < 0.01$ ) changes were observed in the RBC ( $1.02 \pm 0.26 \times 10^6/\text{mm}^3$ ) of female rats in effective dose (ED) group compared to control. Similarly, significant ( $p < 0.01$ ) changes were detected in the WBC level of female rats in high dose ( $-7.53 \pm 0.03 \times 10^3/\text{mm}^3$ ), and post-ED group ( $-8.86 \pm 0.75 \times 10^3/\text{mm}^3$ ) compared to control. There were no alterations in tested biochemical parameters of experimental rats.

**Conclusion:** The FHJ was rich in probiotic *Lactobacillus*. The supplementation of FHJ (9 mL/kg/day) for 60 days did not significantly affect the body mass, internal organs, hematological, and biochemical parameters of rats. The results suggested that FHJ is qualified for the human consumption.

**Keywords:** *Houttuynia cordata* Thunb., Lactic acid bacteria, Fermented plant juice, Subchronic, Toxicity.

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**INTRODUCTION**

*Houttuynia cordata* Thunb., known as Chinese lizard tail, heartleaf, chameleon plant, fish wort, and Khow Tong in Thai, is one of the known perennial herbs for the pharmacological benefits, comes under the family of Tutupalli and Chaubal [1,2]. Two chemotypes of *H. cordata* species are habited in Asian countries. The plant is native to Northeast India, Japan, Korea, southern China, Thailand, and Burma regions, and is frequently used by the local tribes for the therapeutic purposes and as a dietary vegetable [3]. *H. cordata* is rich in organic acids (linoleic, palmitic, and aspartic acids), volatile compounds ( $\alpha$ -pinene, myrcene, d-limonene, decanoyl acetaldehyde, lauric aldehyde, and methyl nonyl ketone), flavonoids (hyperin, reyoutrin, quercetin, isoquercitrin, afzelin, and rutin), essential amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine), water-soluble polysaccharides, Vitamin-C, minerals, and trace elements (K, Fe, Zn, Cu, and Mn) [4].

*H. cordata* plant has been used to treat hyperglycemia, dysentery, cholera, and acute renal failure and to purify the circulating system [5,6]. The shoots of *H. cordata* are used as antidote and antivenom [2,7]. Several scientific reports revealed that *H. cordata* has a vast range of pharmacological effects such as anticancer (against leukemia),

antileukemic, antioxidant, adjuvanticity, antimutagenic, anti-inflammation, antibacterial, and antiviral (anti-HSV-1, anti-influenza, and anti-HIV-1) [4,8-13].

Fermented plant juices are famous among the people of Asian countries; especially, Thai people believe that consumption of traditionally prepared fermented plant juices has the supremacy to cure most of the illness [14]. The fermented foods are rich in bioactive microbes, which offer the health benefits to the consumer and improved the quality of the food [15]. The preparation of fermented plant-based food using specific starter culture improved the nutritional and pharmaceutical values of the food [16-18]. The fermented plant juice with bioactive principles can be an alternative food supplement with regular medication to control the metabolic disorders like diabetes [19].

Although *H. cordata* is used in several folk medicines to treat several diseases, the reports on fermented *H. cordata* juice (FHJ) are very limited. Kwon and Ha [20] reported that *Bacillus* spp. (isolated from the naturally fermented *H. cordata*) based fermentation increased the flavonoid content of the fermented *H. cordata*. The fermented *H. cordata* exhibited high protective effects against lipopolysaccharide (LPS)-induced inflammation with no cytotoxicity in RAW264.7 cells. Banjerdpongchai and Kongtawelert [21] stated that ethanolic extract of fermented *H. cordata* was strong toxic to human leukemic Molt-4 and HL-60 cells.

The subchronic toxicity studies are needed before any clinical trials. Thus, the present study was aimed to assess the subchronic toxicity of *Lactobacillus*-mediated fermented *H. cordata* using rodent model system.

## METHODS

### Preparation of fermented *H. cordata* thunb juice

*H. cordata*, water, and cane sugar were mixed in the ratio of 3:10:1 and subjected to pasteurization. After sterilization and cooling of the medium, 10% of *Lactobacillus paracasei* HII03 was inoculated, and fermented for 30 days.

### Physical observation of FHJ

The changes in the color, odor, and consistency of FHJ during and after fermentation were monitored by organoleptic techniques [22-24].

### Determination of pH, lactic acid content, ethanol content, and microbial load

The experiments were performed as described in the previous studies. The pH of FHJ was kinetically assessed using pH meter (Inola, pH level 2, Weilheim) [16]. The lactic acid content of FHJ was determined by high-performance liquid chromatography [25]. The gas chromatography method was employed to assess the ethanol content of FHJ [26]. The microbial load of FHJ was determined by spread plate method using specific media [27].

### Animals, intervention, and sample collection

Sprague Dawley rats (150–180 g of weight) were purchased from National Laboratory Animal Center, Mahidol University, Thailand, and randomly divided into different groups as follows:

1. Control group: Standard laboratory foods (Commercial food no. C.P. 082, Perfect Companion Group Co., Ltd., Bangkok, Thailand) and water.
2. Low-dose group (effective dose [ED]): Standard laboratory foods and 1.2 mL/kg/day of FKJ.
3. High-dose group (HD): Standard laboratory foods and 9.0 mL/kg/day of FKJ.
4. Post-ED group (PED): Standard laboratory foods and 1.2 mL/kg/day of FKJ for 53 days (supplementation was stopped before 1 week of animal scarification and testing). The experimental rats were supplemented with test intervention for 60 days. After the intervention period, blood and internal organs were collected for examination. The experiments were ethically approved by the ethical committee of Faculty of Medicine, Chiang Mai University (Approved protocol no: 1/2552 dated 23 June 2009).

### Measurement of body mass and Assessment of hematological and biochemical parameters

The changes in body mass of the experimental rats were measured using digital weighing balance. The difference in the weight was calculated as per the following formula:

Changes in body mass = Final weight - The initial weight of the experimental rat.

The weight of brain, eyes, heart, lung, liver, spleen, stomach, kidneys, and adrenal gland of the rat was measured. The hematological (hemoglobin, hematocrits, white blood cells (WBCs) count, lymphocyte, platelets, and red blood cells [RBCs] count), biochemical parameters for liver and kidney function (aminotransaminase, alanine aminotransaminase, alkaline phosphatase), and lipid profile (triglyceride and cholesterol) of the experimental rats were determined at MT InterMed (Hospital) Growth Diags. Co., Ltd., Chiang Mai, Thailand, as per the standard procedures.

### Statistical analysis

The experiments were completed in triplicate. The values were signified as a mean  $\pm$  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 FHJ was prepared as detailed. The pH of FHJ was measured periodically and found that the pH was gradually reduced after 30 days of fermentation. The pH of FHJ after 30 days of fermentation was 3.63 (Fig. 1a). The lactic acid content of FHJ was gradually increased and reached 19.70 mg per mL after 30 days of the fermentation process (Fig. 1b).

The microbial load in FHJ was estimated kinetically at different time points such as 3, 6, 10, 15, 20, and 30 days of fermentation. The total bacterial count (8.8–6.6 Log CFU/mL) and *Lactobacillus* spp. (8.64–6.4 Log CFU/mL) were gradually decreased over the period of fermentation. The reduction in bacterial load is possibly due to the high acidic condition and nutrient depletion in fermenting medium. At the end of the fermentation process, after 30 days, *Lactobacillus* spp. concentration was high (6.4 Log CFU/mL) in FHJ with respect to the total microbial load. *Bacillus* spp. and yeast were not found in FHJ at any point of fermentation, which suggested that FHJ was microbiologically safe (Fig. 2).

The changes in body mass of experimental rat were measured and tabulated (Table 1). The body mass increases in ED, HD, and PED group male rats were 175 $\pm$ 24.32, 187 $\pm$ 28.57, and 176 $\pm$ 30.68 g, respectively, while control rat was 187.50 $\pm$ 35.83 g. The body mass increases in ED, HD, and PED group female rats were 68.57 $\pm$ 14.43, 62.14 $\pm$ 17.99, and 63.57 $\pm$ 13.97 g, respectively, while control rat was 75.83 $\pm$ 18 g. There were no significant changes in body weight of male and female experimental rats supplemented with FHJ, irrespective of dose (ED, HD, and PED) (Table 1).

All the experimental rats were dissected after the study period to check macroscopic morphology of the organs. The organs brain, eyes, heart, lung, liver, spleen, stomach, kidneys, and adrenal gland were collected to determine the organ weight and physical changes. The changes in the organ weight, derived from the control animal, were reported (Table 2). There were no significant treatment-related pathological changes found in any organ of the experimental rats at all tested dose levels when compared with organs in control animals (Table 2).

The hematological changes in the experimental rats were determined. The differences, compared to representative control, were represented (Table 3). There were no significant changes observed in RBC, WBC, hemoglobin, hematocrits, lymphocyte, and platelets level of male rats

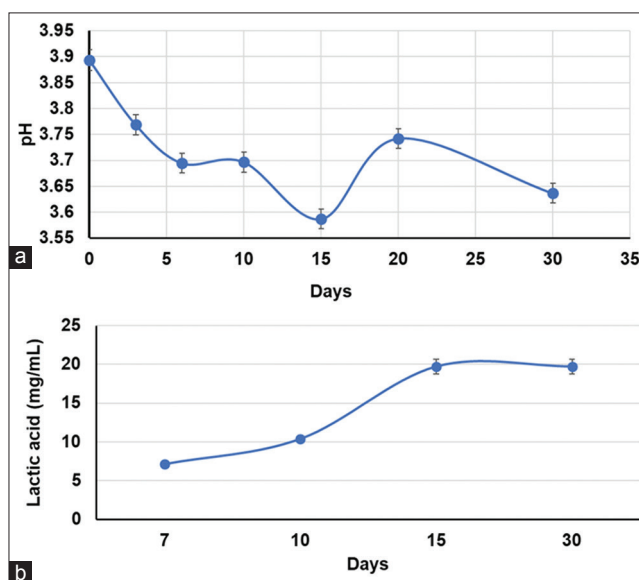


Fig. 1: The pH profile (a) and lactic acid content (b) of fermented *Houttuynia cordata* juice

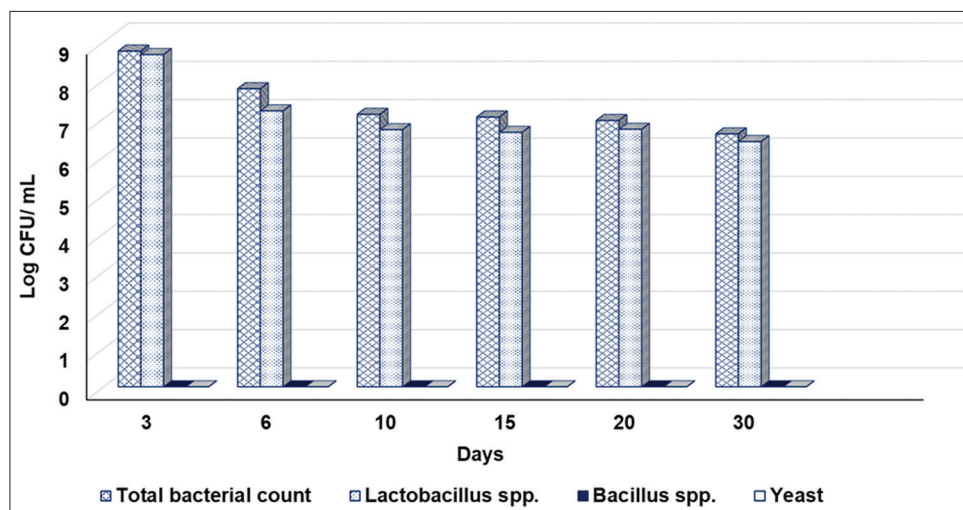


Fig. 2: The microbial load in fermented *Houttuynia cordata* juice during fermentation

Table 1: The increase in body mass of test animals during the experimental period compared to baseline value

Duration (day)	Body weight (g)							
	Male				Female			
	Control	ED*	HD**	PED***	Control	ED	HD	PED
7	44.17±16.36	43.57±15.99	37.14±12.25	42.85±21.93	14.17±3.32	19.28±9.75	9.32±5.0	11.42±3.80
30	128.33±29.43	116.42±22.30	128.57±27.60	129.28±26.27	58.33±13.66	51.42±9.06	35.00±14.55	40.00±10.17
60	187.50±35.83	175.00±24.32	187.87±28.57	176.42±30.68	75.83±18.00	68.57±14.43	62.14±17.99	63.57±13.97

\*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), ED: Effective dose, HD: High dose

Table 2: Changes in the organ weight after oral supplementation of FHJ

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	-0.07±0.16	0.06±0.08	-0.13±0.49	0.1±0.11	0.12±0.06	0.12±0.05
Eyes	0±0.05	-0.04±0.06	0.03±0.01	0.07±0.05	0.03±0.06	0.1±0.01
Heart	0.03±0.37	0.06±0.36	0.48±0.29	0.01±0.18	0.17±0.45	0.6±0.19
Lung	-0.37±0.22	-0.08±0.43	-0.29±0.14	-0.06±0.25	0.33±0.44	0.25±0.50
Liver	0.12±2.22	0.09±2.07	-1.31±1.01	1.1±0.07	0.7±0.88	0.6±0.01
Spleen	-0.003±0.13	0.017±0.14	-0.113±0.05	0.07±0.06	0.11±0.06	0.01±0.03
Stomach	-0.203±0.19	0.047±0.29	0.077±0.10	0.13±0.22	0.27±0.56	0.18±0.07
Kidneys	-0.22±0.42	0.12±0.45	-0.14±0.43	0.27±0.24	0.14±0.18	0.1±0.13
Adrenal gland	0.004±0.00	0.029±0.05	0.014±0.01	0.02±0.02	0±0.02	0.016±0.02

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. ED: Effective dose, HD: High dose, PED: Post-effective dose, FHJ: Fermented *Houttuynia cordata* juice

of all groups. Whereas, significant ( $p < 0.01$ ) changes were observed in the RBC ( $1.02 \pm 0.26 \times 10^6 / \text{mm}^3$ ) of female rats in ED group compared to control. Likewise, significant ( $p < 0.01$ ) changes were detected in the WBC level of female rats in HD ( $-7.53 \pm 0.03 \times 10^3 / \text{mm}^3$ ), and PED group ( $-8.86 \pm 0.75 \times 10^3 / \text{mm}^3$ ) compared to control (Table 3). There were no alterations in tested biochemical parameters (blood urea nitrogen, creatinine, cholesterol, triglyceride, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase) of experimental rats, both male and female, of all treatment groups (Table 3). The results suggested that the consumption of FHJ, even HD, did not affect the internal organ of the experimental rats.

Several reports are available on the pharmacological importance of *H. cordata* extracts. The ethyl acetate extract of *H. cordata* (EAEH) has been reported for antiviral property against dengue virus and mouse

hepatitis virus [28]. The EAEH exhibited hepatoprotective activity, inhibited the hydroxyl and alkyl free radicals *in vitro*, and prohibited the carbon tetrachloride-induced liver damage in mice [29], and the isolated alkaloids of *H. cordata* showed moderate hepatoprotective activity in d-galactosamine-induced WB-F344 cells, and anti-inflammatory activity [30,31].

The methanol extract of *H. cordata* induces apoptosis in human HepG2 hepatocellular carcinoma cells through activation of hypoxia-inducible factor-1A - Forkhead box O3 and MEF2A pathways [32]. The ethanolic extract of *H. cordata* persuades apoptosis in human leukemic Molt-4 cells through endoplasmic reticulum stress pathway, which was characterized by declined expression of Bcl-xl and improved levels of Smac/Diablo, Bax, and GRP78 proteins [33]. Kumar *et al.* [2] reported that ethanolic extract of *H. cordata* (EEH) displayed the antidiabetic activity

Table 3: Effect of supplementation of FHJ on the hematological and biochemical parameters in hamster after 60 days of treatment

Parameters	Male			Female		
	ED	HD	PED	ED	HD	PED
Hematological parameters						
RBC ( $10^6/\text{mm}^3$ )	-0.08±0.16	0.38±0.34	0.39±0.32	1.02±0.26**	0.47±0.33	0.84±0.21
WBC ( $10^3/\text{mm}^3$ )	0.42±0.43	4.57±0.95	-2.71±0.03	-2.86±0.04	-7.53±0.03*	-8.86±0.75*
HGB (g/dL)	-0.51±0.82	0.34±0.64	1.10±0.41	-0.51±0.88	-0.88±0.89	-0.75±0.70
Hematocrits (mL%)	-1.35±0.47	1.07±0.90	3.35±1.34	-1.80±0.60	-2.63±0.92	-2.30±0.07
Lymphocyte (mL%)	-1.35±4.14	0.92±4.42	0.64±3.07	1.70±4.84	1.20±4.19	2.53±2.87
Platelets ( $10^3/\text{mm}^3$ )	-0.25±4.97	2.76±5.28	-0.23±2.12	0.47±1.73	0.83±2.11	-1.14±1.90
Biochemical parameters						
BUN (mg/dL)	-1.67±1.55	-2.17±2.35	-3.84±2.31	0.96±3.73	-0.19±6.47	6.17±7.30
Creatinine (mg/dL)	-0.15±0.08	-0.14±0.04	-0.70±2.27	0.03±0.13	0.02±0.22	0.45±1.13
Cholesterol (mg/dL)	24.33 ± 11.76	29.83±9.54	-36.00±13.28	-9.64±20.96	-18.64±40.86	-15.83± 37.5
TG (mg/dL)	1.33±4.82	10.16±14.66	-2.00±14.15	-6.33±7.07	-6.04±10.48	4.53±16.71
AST (IU/L)	-38.67±24.43	22.67±49.11	41.29±23.45	-43.40±26.45	-31.60±122.27	20.71±23.45
ALT (IU/L)	-2.5±9.20	-5.47±11.56	-2.42±11.30	-6.59±8.16	4.84±9.07	-1.34±9.70
ALP (IU/L)	-7.34±9.50	-11.67±11.92	-4.83±16.70	10.67±12.58	8.38±25.40	4.34±6.31

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. RBCs: Red blood cells, WBCs: White blood cells, HGB: Hemoglobin, BUN: Blood urea nitrogen, TG: Triglyceride, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase. \* Significant difference ( $p<0.05$ ) between control and test group, \*\*Significant difference ( $p<0.01$ ) between control and test group

in streptozotocin-induced diabetic rats. The oral supplementation of 200–400 mg/kg body weight of EEH reduced the fasting glucose level, improved the insulin level, and normalized the biochemical parameters and the expression of glucose homeostatic enzyme-coding genes [2].

The ethanol extract of fermented *H. cordata* was reported for cytotoxicity effects in human leukemic HL-60 and Molt-4 cell. The apoptosis of cancer cell was achieved through activation and mitochondrial and oxidative stress pathway, and the study also suggested that fermented *H. cordata* was more toxic to human cancer cells than non-fermented *H. cordata* [21]. The fermented *H. cordata* protects the cells (RAW264.7 and RBL-2H3) from LPS-induced toxicity and suppresses the growth of HepG2 cells (human liver cancer cells) [20]. Senawong *et al.* [34] studied the anticancer activity of commercially available fermented *H. cordata* products (CFH) of Thailand. About seven phenolic compounds were detected in CFH, and some of the CFH exhibited dose-dependent-antiproliferative activity against HCT116, HT29, and HeLa cells.

An insufficient number of toxicity studies are there on *H. cordata* extracts and FHJ. The acute oral toxicity of EAEH was studied in C57BL/6 mice and found that 2000 mg/kg of EAEH supplementation did not cause any adverse behavioral and histopathological effects [28]. The ethanol extract of *H. cordata* leaves (EEHL) was studied for toxicity in F344/DuCrj rats. The supplementation of up to 5% of EEHL has not affected the hematological, ophthalmological parameters, and organs. The study reported that 1.5 and 0.5 % as dietary level of EEHL supplementation for male and female, respectively [35].

The results of the current study revealed that FHJ did not affected the behavioral character, body mass, internal organs, hematological, and biochemical parameters of experimental rats, and the consumption of FHJ was safe to rodent model system up to the concentration of 9 mL/Kg/day.

## CONCLUSION

The FHJ was rich in probiotic *Lactobacillus* strain (*L. paracasei* HII03) and acidic in nature. The supplementation of FHJ (9 mL/Kg/day) for 60 days did not significantly affect the body mass, internal organs, hematological, and biochemical parameters of rats. The results suggested that FHJ is eligible for the human consumption.

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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 are responsible for wet lab experiments. All the authors agree with the content of the manuscript.

## CONFLICTS OF INTEREST

There are no conflicts of interest.

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