Acute and Sub-Acute Toxicity Studies of Hawm Nil Brown Rice Kefir Powder

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Abstract

The present study was designed to determine acute and sub-acute toxicities of Hawm Nil brown rice kefir powder (HNKP) in male Wistar rats. In this acute toxicity study, HNKP at doses of 1,000, 2,000 and 4,000 mg/kg were once administered to the rats orally. Symptoms of toxicity and mortality were observed within 24 h and over a further period for 14 days. The results showed that all the doses of HNKP did not produce mortality or symptoms of toxicity. HNKP at a dose of 1,000 mg/kg produced the best body weight gain, food intake and FCR. Moreover, blood biochemistry including TP, Alb, Glob, BS, BUN, CREA, UA, TB, AST, ALT, and ALP, cholesterol, hematological values including WBC, RBC, Hb, Hct, MCH, MCHC, MCV, and Plt, and relative organ weight (ROW) of the rats received all the doses of HNKP did not differ from those in controls. In a sub-acute toxicity study, HNKP at the doses of 500, 1,000 and 2,000 mg/kg were given orally to the rats every 2 days for 14 days. Again, the result showed that all the doses did not produce mortality or symptom of toxicity. HNKP at a dose of 500 mg/kg produced the best body weight gain, food intake and FCR. Furthermore, the rats that received HNKP at this dose had blood biochemistry, cholesterol, hematological values, and ROW close to those in controls. However, the rats received high doses of HNKP (1,000 and 2,000 mg/kg) and long term application altered Alb, Glob, BS, BUN, UA, AST, and ALP levels. These results indicate that long term and high dose application of HNKP can affect renal and hepatic functions (p<0.05). In addition, TG and HDL of the rats that received HNKP were significantly (p<0.05) less than those in controls. Interestingly, the rats that received HNKP had fewer neutrophils while lymphocytes were significantly higher than that in controls (p<0.05). These findings indicate that Hawm Nil brown rice kefir powder had no acute and sub-acute toxicities. However, long term application at high doses (1,000 and 2,000 mg/kg) of HNKP may cause hepatic and renal dysfunctions. Its activity on decreasing neutrophils and increasing lymphocytes resulted in increased globulin leads to improve immunomodulatory activity.

Keywords: Hawm Nil rice, rice kefir, acute toxicity, sub-acute toxicity, kefir powder

Introduction

Kefir is a fermented milk product. It contains lactic acid bacteria, yeasts and acetic acid bacteria that produce jelly-like grain. Kefir grain is white or lightly yellow, gelatinous irregular masses and sized between 0.3-3.5 cm. diameter[1-2]. Both bacteria and yeasts are surrounded by a water-soluble branched glucogalactan called kefiran[1]. Kefir has been reported to possess antibacterial[3-7], antifungal[7], antitumor[8], antioxidant[5, 9-11], anti-allergic[12], antineoplastic and pro-digestive[13-14], antidiabetic[15-16], and immunomodulatory activities[17-18]. Moreover, it is important to anti-inflammatory activity on the liver[19], lung[12, 16, 20] and colon[21]. Kefir can modulate the intestinal mucosa immune response. It induced the helper T cell type 2 response by increasing the number of immunoglobulin A, interleukins type 4, 6 and 10 cells, and induced simultaneously the production of pro-inflammatory cytokines (IFN γ and TNFα) but without tissue damage[17-18]. It can also improve lactose digestion and tolerance[22]. Fermented milk from kefir has high antioxidant activity and reduces the accu-

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mulation of reactive oxygen species (ROS) including superoxide ($O2^{-}$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO\cdot$)[23]. In addition, kefir has high nutritional value as a source of proteins and calcium. It has a long tradition of being regarded as good for health in many countries[24]. Kefir can be considered as a probiotic resource, as it produces good activities for health.

Recently, the γ-aminobutyric acid (GABA), α-tocopherol, γ-tocopherol and total phenolics compounds (TPC) it is reported, has been found in Hawm Nil rice [25-26]. Moreover, total phenolic content and total anthocyanin content are found in Hawm Nil rice (Black) more than in white and red colored rice[27-28]. In addition, it exerts high levels of antioxidant activity[28]. Kefir from rice milk has higher antioxidant activity than cow's milk[5]. The antioxidants were higher in Kefir produced from plants as a result of the phenolic compounds presence in the plants[29].

A toxicity study of medicines or plant products should be carried out to see whether they are safe for human application. The present study was therefore designed to determine acute and sub-acute toxicity of Hawm Nil brown rice kefir powder in the rats.

Materials and methods

1. Hawm Nil brown rice, fermentation and kefir powder preparation

1.1 Hawm Nil brown rice: Hawm Nil brown rice harvested during the year 2013-2014 from Selaphum, Roi Et province, Thailand was used in this study. The rice was dried, weighed, soaked in distilled water (1:5, W:V) at 25°C for 2 h and thoroughly ground by using a blender and filtrated to obtain rice milk. The rice milk was pasteurized at 70°C for 15 min and then directly cooled at 4°C.

1.2 Hawm Nil brown rice fermentation: A 0.2 g freeze-dried Kefir grain was inoculated into 250 mL flask with 200 mL of Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth and incubated under anaerobic conditions; the flask was put into a 5L anaerobic jar. After that the sample jars were kept at 30°C for 24 h, and then centrifugation (1000×g, 15 min at 4°C) to obtain the cells. The cells were washed and re-suspended in sterile saline solution (0.85% NaCl) and then diluted with sterile 0.85% NaCl (1:10; V:V). Subculture, kefir starter was inoculated into fresh milk (20:200; V:V) and incubated under aerobic conditions at 30°C for 48 h to obtain activated kefir grain. Then activate kefir grain were cultured and fermented by inoculating into Hawm Nil brown rice milk adding with 2.5% sucrose (100:1,000, V:V) and incubated under anaerobic conditions at 30°C for 24 h to get the final pH of about 4.8-4.9.

1.3 Hawm Nil brown rice kefir powder (HNKP) preparation: the Hawm Nil brown rice milk kefir was freeze-dried and powdered. The kefir powder was kept at -20°C until used.

2. Animals

Forty-eight male Wistar rats weighing 280-300 g were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were kept in an animal laboratory and acclimated for 7 days in environmental conditions (22-25°C, 50%-55% humidity and under a 12-hour light/dark cycle). The rats were fed with a standard diet (Perfect Companion Group Co., Ltd.) and water *ad libitum*. All experimental protocols were maintained in accordance with the Guidelines of Committee Care and Use of Laboratory Animal Research, National Research Council of Thailand and advice of the Institutional Animal Care and Use Committee, Mahasarakham University, Thailand.

3. Acute toxicity study

Rats were weighed and divided randomly into four groups with 6 rats in each; group 1; rats received phosphate buffered saline (PBS) (control group), group 2, 3 and 4; rats received HNKP 1,000, 2,000 and 4,000 mg/kg respectively[30]. The doses of HNKP were once administered to the rats orally. Symptom of Toxicity (seizures, vomiting, diarrhea, and nausea) and rat mortality were observed within 24 h and over a further period for 14 days. Body weight and food intake were recorded daily. On day 14, the rats were fasted overnight, weighed and sacrificed by overdoses of chloroform. Blood sample was collected from the rat heart to determine blood biochemistry and hematological values. Visceral organs including liver, lung, heart, kidney and spleen were removed and weighed.

4. Sub-acute toxicity study

The rats were randomly divided into four groups with 6 rats in each; group 1; rats received PBS (control group), group 2, 3 and 4; rats received HNKP 500, 1,000 and 2,000 mg/kg respectively[31]. HNKP was given orally to the rats every 2 days for 14 days. Symptoms of toxicity were observed within 14 days. Body weight and food intake were recorded daily. At the end of experiments, the rats were fasted, weighed and then euthanasia by overdose of chloroform. Blood samples were collected from the rat hearts to determine blood biochemistry values and hematological values. Visceral organs including liver, lung, heart, kidney and spleen were removed and weighed.

5. Relative organ weight and feed conversion ratio

The relative organ weight (ROW) of each animal was calculated using the following equation;

$$\mathrm{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat (g)}} x100 \qquad (1)$$

The feed conversion ratio (FCR) of each animal was calculated as follow;

$$\mathrm{FCR} = \frac{\text{Food intake (g)}}{\text{Body weight gain (g)}} \qquad (2)$$

6. Determination of blood biochemistry and hematological values

Blood samples were put into heparinized and non-heparinized tubes. Blood was centrifuged at 1500 g for 10 min to separate serum. The serum from the non-heparinized blood was assayed for biochemistry including total protein (TP), blood sugar (BS), blood urea nitrogen (BUN), creatinine (Crea), uric acid (UA), cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), albumin (Alb), globulin (Glob), total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

Heparinized blood was used for hematological analysis. Hematological analysis included red blood cell (RBC) count, white blood cell (WBC) count, hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (Plt), neutrophils (Neu), and lymphocytes (Lym).

7. Statistical analysis

The results were presented as mean±SEM and analyzed using one-way ANOVA. The differences among means were detected by using the Duncan's Multiple Range Test and values of p£0.05 were consi dered statistically significant.

Results and Discussion

1. Acute toxicity

1.1 Symptoms of toxicity, body weight gain, food intake and FCR

All the doses of HNKP did not produce any symptom of toxicity and mortality of the rats during 14 days. HNKP at a dose of 1,000 mg/kg produced the best body weight gain, food intake and FCR (Fig.1A–1C). Moreover, the rats received HNKP at a dose of 1,000 mg/kg had hepatic and renal functions (Table 1), lipid profile (CHO, TG, HDL and LDL) (Table 2), hematological values (Table 3), and ROW (data not shown) close to those in controls. However, the rats received all the doses of HNKP had neutrophils less than while lymphocytes were higher than that in controls (p<0.05) (Table 3).

These findings indicate that HNKP has no acute toxicity on rats. Its activity on decreasing neutrophils and increasing lymphocytes resulting in increased globulin leads to improve immunomodulatory activity[17-18].

1.2 Blood biochemistry and ROW

Since, AST, ALT and ALP enzymes are involved in hepatic function and TB, TP, Alb, Glob, BS, BUN, CREA, and UA are involved in renal function[32]. The results from acute toxicity study revealed that TB, TP, Alb, Glob, BS; BUN, CREA, UA, AST, ALT, and ALP enzymes in the rats received all the doses of HNKP did not differ from those in controls (Table 1), suggesting that HNKP had no effect on hepatic and renal functions.

Hyperlipidemia is well known as one of the major risk factors for atherosclerosis which leads to coronary artery disease (CAD)[33]. The total cholesterol

was increased in the rats received HNKP at the doses of 2,000 and 4,000 mg/kg. However, TG, HDL and LDL were not altered in the rats treated with all the doses of HNKP compared to those in controls. These data indicate that HNKP has an effect on total cholesterol but not on TG, HDL and LDL (Table 2). The ROW in the rats treated with HNKP did not differ from that in controls.

1.3 Hematological values

(Table 3), WBC, RBC, Hb, Hct, MCV, MCH, MCHC, and Plt in the rats received HNKP did not differ from those in controls. Interestingly, the rats that received all the doses of HNKP had significantly less neutrophils while lymphocytes were significantly higher than that in controls (p<0.05), consistent with globulin increased. Since the total WBC count did not change, this result suggests that HNKP acts in opposite way in the differentiation of hematopoietic cells by suppressing neutrophils and stimulating lymphocytes. According to previous reports, kefir induced the helper T-lymphocytes type 2 proliferations by increasing the number of immunoglobulin A (IgA), interleukins type 4, 6 and 10 cells[17-18], in agreement with the increase of lymphocyte and globulin in this study.

Table 1 Blood biochemistry in rats treated with HNKP and PBS from acute toxicity study (mean±SEM).

Blood biochemistry	PBS	HNKP (mg/kg)		
		1,000	2,000	4,000
BS (mg/dl)	182.33±23.55	176.67±38.09	214.83±21.09	235.33±25.04
BUN (mg/dl)	20.17±0.48	19.75±0.37	19.63±0.33	20.62±0.51
CREA (mg/dl)	0.91±0.03	0.88±0.02	0.85±0.02	0.87±0.02
UA (mg/dl)	3.91±0.55	3.72±0.31	3.65±0.31	4.12±0.07
TP (g/dl)	5.65±0.11	5.47±0.17	5.47±0.12	5.63±0.02
Alb (g/dl)	3.47±0.04	3.45±0.08	3.48±0.07	3.47±0.03
Glob (g/dl)	2.20± 0.06	2.25±0.06	2.18±0.05	2.17±0.04
TB (mg/dl)	0.11±0.01	0.09±0.01	0.09±0.00	0.09±0.00
AST (U/L)	147.67±3.95	143.83±4.80	152.83±1.08	143.50±2.79
ALT (U/L)	39.33±1.17	38.17±2.21	35.00±0.68	37.50±0.96
ALP (U/L)	124.33±3.06	118.67±3.01	123.00±2.31	126.00±5.39

TP = total serum protein; Alb = albumin; Glob = globulin; BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid; TB= total bilirubin; AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

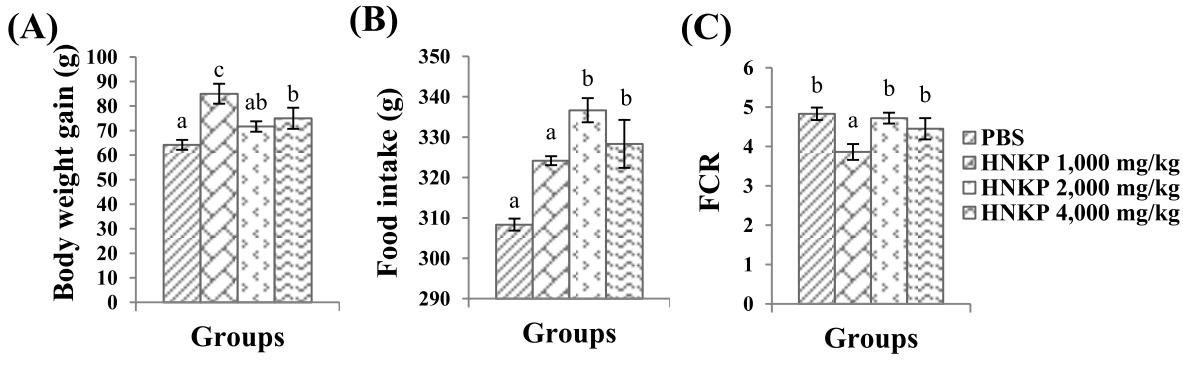

Figure 1 Body weight gain (A), food intake (B) and FCR (C) in rats treated with HNKP and PBS from acute toxicity study at the end experiment (mean±SEM). Mean values with different letters are significantly different, Duncan's test at p< 0.05.

Table 2 Cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) in rats treated with HNKP and PBS from acute toxicity study (mean±SEM).

Lipid profiles	PBS	HNKP (mg/kg)		
		1,000	2,000	4,000
CHO (mg/dl)	53.67±1.28[a]	56.50±1.91[ab]	58.67±0.88[b]	58.67±1.63[b]
TG (mg/dl)	130.00±2.62	127.33±4.14	123.50±3.64	123.17±4.73
HDL (mg/dl)	16.48±0.27	16.57±0.55	17.10±0.57	16.62±0.29
LDL (mg/dl)	32.50±2.11	32.00±0.68	32.17±0.98	35.33±0.88

Mean values within each row with different superscripts are significantly different, Duncan's test at $p<0.05$.

Table 3 Hematological values in rats treated with HNKP and PBS from acute toxicity study (mean±SEM).

Hematological values	PBS	HNKP (mg/kg)		
		1,000	2,000	4,000
WBC (10^3 cell/mm^3)	5.93±0.16	5.55±0.23	5.93±0.22	5.40±0.27
RBC (10^6 cell/mm^3)	8.87±0.13	8.76±0.21	9.00±0.12	8.99±0.05
Hb (g/dl)	17.07±0.40	16.30±0.21	16.35±0.32	16.28±0.28
Hct (%)	53.83±0.87	52.67±1.31	51.83±1.14	51.50±0.22
MCV (fl)	58.33±0.88	59.33±0.21	57.67±1.56	59.67±0.42
MCH (pg)	19.60±0.08	19.53±0.10	19.65±0.26	19.95±0.08
MCHC (g/dl)	32.33±0.34	32.55±0.21	33.07±0.46	33.13±0.18
Plt (10^3 cell/mm^3)	943.17±27.15	904.50±13.18	883.00±23.23	924.83±17.63
Neu (%)	8.50±0.22[c]	6.17±0.17[b]	2.50±0.43[a]	2.33±0.33[a]
Lym (%)	91.00±1.21[a]	94.00±0.26[b]	97.00±1.21[c]	97.83±0.31[c]

Mean values within each row with different superscripts are significantly different, Duncan's test at $p< 0.05$. WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

Table 4 Blood biochemistry in rat treated with HNKP and PBS from sub-acute toxicity (mean±SEM).

Blood biochemistry	PBS	HNKP (mg/kg)		
		500	1,000	2,000
BS (mg/dl)	207.67±11.25[c]	156.50±19.70[b]	128.67±9.30[b]	85.50±7.64[a]
BUN (mg/dl)	20.45±1.12[c]	19.27±0.71[bc]	17.53±0.55[ab]	16.47±0.27[a]
CREA (mg/dl)	0.82±0.03	0.90±0.07	0.78±0.03	0.83±0.03
UA (mg/dl)	2.65±0.34[b]	2.62±0.28[b]	1.65±0.25[a]	1.48±0.19[a]
TP (g/dl)	5.72±0.13	5.63±0.15	5.43±0.08	5.73±0.18
Alb (g/dl)	3.28±0.06[b]	2.72±0.05[a]	2.67±0.03[a]	2.67±0.05[a]
Glob (g/dl)	2.48±0.09[a]	2.73±0.05[b]	2.73±0.08[b]	3.02±0.06[c]
TB (mg/dl)	0.07±0.02[ab]	0.05±0.01[a]	0.05±0.01[a]	0.13±0.02[b]
AST (U/L)	104.00±1.53[a]	140.00±3.18[b]	151.67±5.16[c]	157.33±4.58[c]
ALT (U/L)	49.17±1.66	47.33±1.41	50.33±1.33	53.83±3.70
ALP (U/L)	132.83±4.66[b]	119.83±6.82[ab]	116.67±3.37[a]	112.67±3.25[a]

Mean values within each row with different superscripts are significantly different, Duncan's test at $p< 0.05$ (N=6). TP = total serum protein; Alb = albumin; Glob = globulin; BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid; TB= total bilirubin; AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

Table 5 CHO, TG, HDL and LDL in rats treated with HNKP and PBS from sub-acute toxicity study (mean±SEM).

Lipid profiles	PBS	HNKP (mg/kg)		
		500	1,000	2,000
CHO (mg/dl)	54.50±5.42	46.50±1.43	48.17±3.38	49.33±3.02
TG (mg/dl)	152.00±4.20[b]	109.00±4.56[a]	100.67±7.05[a]	94.33±4.62[a]
HDL (mg/dl)	22.55±1.04[b]	18.42±0.42[a]	16.93±0.84[a]	17.48±1.44[a]
LDL (mg/dl)	29.17±1.01	31.83±1.90	33.67±2.93	31.33±2.17

Mean values within each row with different superscripts are significantly different, Duncan's test at $p< 0.05$.

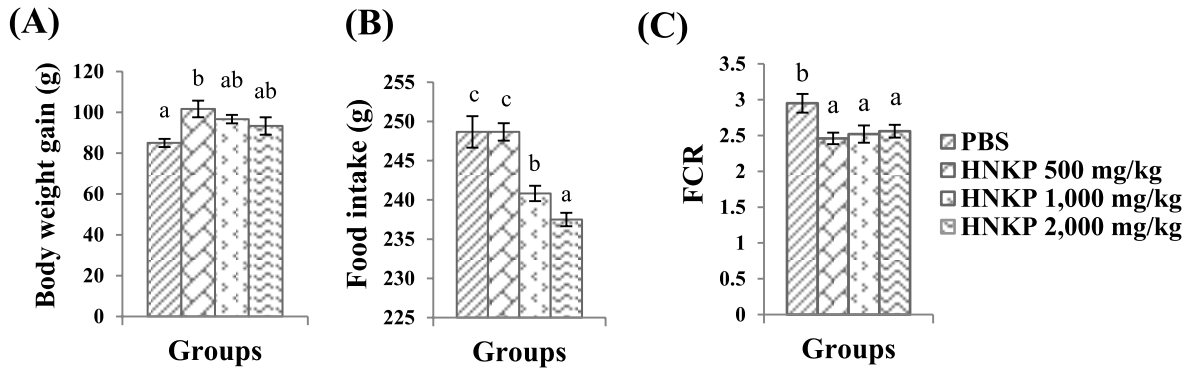

Figure 2 Body weight gain (A), food intake (B) and FCR (C) in rats treated with HNKP and PBS from sub-acute toxicity study at the end experiment (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p< 0.05$.

Table 6 Hematological values in rats treated with HNKP and PBS from sub-acute toxicity study (mean±SEM).

Hematological values	PBS	HNKP (mg/kg)		
		500	1,000	2,000
WBC (10^3 cell/mm^3)	6.42±0.28	5.77±0.43	6.50±0.26	5.60±0.40
RBC (10^6 cell/mm^3)	8.76±0.13	8.38±0.14	8.63±0.41	8.13±0.18
Hb (g/dl)	17.52±0.23	16.70±0.35	16.88±0.44	16.32±0.47
Hct (%)	53.33±0.76	51.00±0.68	50.67±1.38	50.67±1.36
MCV (fl)	59.33±0.49	59.50±0.43	59.33±0.42	59.33±0.33
MCH (pg)	19.98±0.28	19.90±0.17	19.80±0.27	20.05±0.26
MCHC (g/dl)	33.72±0.35	33.43±0.28	33.37±0.47	33.75±0.36
Plt (10^3 cell/mm^3)	923.17±24.11	887.33±24.78	893.67±42.25	905.67±46.40
Neu (%)	8.83±1.01[b]	7.33±1.54[ab]	5.83±0.94[ab]	4.83±1.05[a]
Lym (%)	90.83±1.25[a]	91.83±1.87[ab]	95.17±0.87[bc]	96.33±0.71[c]

Mean values within each row with different superscripts are significantly different, Duncan's test at $p< 0.05$. WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

2. Sub-acute toxicity

2.1 Symptoms of toxicity, body weight gain, food intake and FCR

Again, the results showed that all the doses of HNKP did not produce any symptom of toxicity and mortality of the rats. HNKP at a dose of 500 mg/kg produced the best body weight gain, food intake and FCR (Figure 2A-2C). However, all the doses of HNKP had FCR better than that in controls (p<0.05). Kefir has been widely used in clinical practice to promote growth[13-14,24], and may be a source of nutritional compounds. Hawm Nil rice exerts high levels of antioxidant and phytochemical activities than white and red rice[25-28]. Thus, HNKP is suitable for further development as therapeutic agents for growth promotion.

2.2 Blood biochemistry and ROW

The results of this study showed the rats received HNKP at a dose of 500 mg/kg had hepatic and renal functions close to those in controls. Furthermore, the rats received high doses of HNKP (1,000 and 2,000 mg/kg) and long term were altered of Alb, Glob, BS, BUN, UA, AST, and ALP levels. These results indicate that long term and high doses application of HNKP can effect on renal and hepatic functions (p<0.05) (Table 4). The serum AST and ALP activities are widely used as sensitive markers of possible tissue damage, particularly liver toxicity[34]. Moreover, the triglycerides and HLD of the rats received kefir powder were significant less than that in controls (p<0.05). The decreasing of TG and HLD on rats received HNKP may be cause from hepatic function changing. However, the ROW in the rats treated with HNKP did not differ from that in controls (data not shown).

2.3 Hematological values

In line with the acute toxicity study, WBC, RBC, Hb, Hct, MCV, MCH, MCHC, and Plt in the rats that received HNKP did not differ from those in controls (Table 6). Nevertheless, the rats received all the doses of HNKP had neutrophils significant less than while lymphocytes were significant higher than that in controls (p<0.05). These results confirmed the non-toxicity of the application of Hawm Nil brown rice kefir powder at the doses less than 4,000 mg/kg.

Conclusions

HNKP has no acute and sub-acute toxicities when a dose less than 4,000 mg/kg is administered orally once, or a dose less than 500 mg/kg is administrated every 2 days for 14 days. In long term application, the powder at doses higher than 1,000 mg/kg may cause hepatic and renal functions as it produces Alb, BS, BUN, UA, and ALP levels decreasing while Glob and AST levels increase compared to those in controls. Furthermore, its activity on decreasing neutrophils and increasing lymphocytes resulting in increased globulin leads to improve immunomodulatory activity.

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