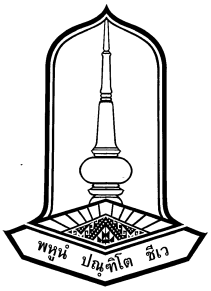

BIOCHEMICAL COMPONENTS, BIOACTIVITIES AND TOXICITIES OF DIFFERENT BROWN RICE MILK KEFIR POWDERS

SUPAPORN CHUNCHOM

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University

February 2016

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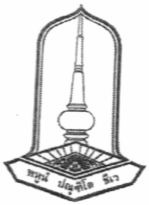

The examining committee has unanimously approved this dissertation, submitted by Miss Supaporn Chunchom, as a partial fulfillment of the requirements for the degree for Doctor of Philosophy in Biology at Mahasarakham University.

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Supaporn Chunchom

TITLE Biochemical components, bioactivities and toxicities of different brown rice milk kefir powders

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ABSTRACT

Kefir is a fermented milk product comprising several lactic acid bacteria, acetic acid bacteria and yeasts. Kefir from rice milk has been reportedly possessed an antioxidant activity higher than that from cow milk. Study on biochemical components and bioactivities of rice kefir are limited, and to see whether rice kefir is safe and probably provides a new and good biochemical resource for human health utilizations. The present study was therefore designed to determine the biochemical components, antioxidant and anti-inflammatory activities, and toxicity of kefir powder from Khao Dawk Mali 105 (KDMLKP), Red Hawm (RHKP) and Hawm Nil (HNKP) brown rice. The results showed that rice milk kefir powder significantly ($p \leq 0.05$) provided gamma amino butyric acid (GABA), α-tocopherol and total phenolic content (TPC) higher than cow milk kefir powder and the highest amount were in HNKP.

Ferric reducing antioxidant power (FRAP) assay and 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay showed that rice kefir powder significantly ($p \leq 0.05$) exhibited antioxidant activity higher than cow milk kefir powder and the highest potent was in HNKP.

Antioxidative stress in rat ulcerative colitis induced by using trinitrobenzene sulphonic (TNBS) acid revealed that the nitric oxide (NO) and thiobarbituric acid reactive substances (TBARS) levels in serum of colitis rats received PBS (control) were significantly ($p \leq 0.05$) increased when compared to those in the non-colitis rats. However, NO and TBARS levels in the colitis rats treated with HNKP were significantly ($p \leq 0.05$) decreased, but superoxide dismutase (SOD) activity was increased when compare to that in colitis rats received PBS (controls).

Anti-inflammatory effect on rat colitis, tumor necrosis factor-α (TNFα) level in the rat colitis treated with HNKP did not differ from that treated with prednisolone (agent) and cow kefir powder. Nevertheless, TNFα in serum of the rat colitis treated with HNKP also significantly ($p \leq 0.05$) reduced when compared to that in controls.

Acute toxicity using a single administration of various doses of kefir powder (1000, 2000 and 4000 mg/kg) showed that HNKP had no acute toxicity as it did not produce any signs or symptoms of acute toxicity. Moreover, the rat death and alteration of blood biochemistry were not observed. However, KDMLKP and RHKP at a dose of 4,000 mg/kg exerted the adverse effects on enzymes relating the hepatic and renal functions, ALP and AST. Sub-acute toxicity by an administration at the same doses of kefir powder to the rats every 2 days for 14 days revealed that KDMLKP, RHKP and HNKP exhibited non sub-acute toxicity when the dose less than 500 mg/kg was administered. Nevertheless, repeat administration of the kefir powder at and above 1,000 mg/kg could affect hepatic and renal functions. Its activity on significantly ($p \leq 0.05$) decreasing neutrophils and increasing lymphocytes resulted in increasing globulin probably leading to improve immunomodulatory activity. Sub-chronic toxicity study revealed that repeat administration of KDMLKP, RHKP and HNKP did not produce sub-chronic toxicity when a dose of 150 mg/kg was administered for 90 days. However, RHKP significantly ($p \leq 0.05$) increased HDL more than other kefirs.

These findings indicate that Hawm Nil rice kefir powder exerts potent anti-inflammatory activity and is safe and probably a new and good biochemical resource for human health utilizations.

ชื่อเรื่อง องค์ประกอบทางชีวเคมี ฤทธิ์ทางชีวภาพ และความเป็นพิษของผงคีเฟอร์จากนมข้าวกล้องต่างชนิดกัน
ผู้วิจัย นางสาวสุภาพร ชื่นชม
ปริญญา ปรัชญาดุษฎีบัณฑิต **สาขาวิชา** ชีววิทยา
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บทคัดย่อ

คีเฟอร์ เป็นผลิตภัณฑ์ที่ได้จากการหมักนม ประกอบด้วยจุลินทรีย์หลายชนิด ได้แก่ แลคติกแอซิดแบคทีเรีย อะซิติกแอซิดแบคทีเรีย และยีตส์ มีรายงานวิจัยว่า คีเฟอร์ที่ได้จากการหมักในนมข้าวขาวดอกมะลิ 105 มีฤทธิ์ต้านอนุมูลอิสระสูงกว่าคีเฟอร์ที่ได้จากการหมักในนมวัว เพื่อให้ทราบข้อมูลเกี่ยวกับปริมาณสารออกฤทธิ์ที่สำคัญของผงคีเฟอร์ เป็นแนวทางในการนำผงคีเฟอร์ไปพัฒนาเป็นผลิตภัณฑ์ใหม่สำหรับมนุษย์ และเพื่อให้ทราบข้อมูลด้านความปลอดภัยของผงคีเฟอร์ที่ได้จากการหมักในนมข้าว งานวิจัยครั้งนี้จึงมีวัตถุประสงค์ เพื่อศึกษาองค์ประกอบทางชีวเคมี ฤทธิ์ทางชีวภาพ และพิษวิทยาของผงคีเฟอร์ที่ได้จากการหมักในนมข้าวของข้าวกล้องข้าวขาวดอกมะลิ 105 (KDMLKP) ข้าวหอมแดง (RHKP) และข้าวหอมนิล (HNKP) ในหนูทดลอง ผลจากการวิเคราะห์ปริมาณองค์ประกอบทางชีวเคมี ได้แก่ ปริมาณ gamma amino butyric acid (GABA) ปริมาณ α-tocopherol และปริมาณ total phenolic content (TPC) พบว่า ปริมาณสารชีวเคมีดังกล่าวของผงคีเฟอร์จากนมข้าวสูงกว่าจากนมวัว และยังพบว่า HNKP มีปริมาณสารสูงที่สุด การวิเคราะห์ฤทธิ์ต้านอนุมูลอิสระด้วยวิธี ferric reducing antioxidant power (FRAP) assay และ 2, 2´-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay พบว่า ผงคีเฟอร์จากนมข้าวมีฤทธิ์ต้านอนุมูลสูงกว่าจากนมวัว โดยHNKP มีฤทธิ์ต้านอนุมูลอิสระสูงที่สุด การศึกษาสภาวะเครียดออกซิเดชันในหนูที่มีภาวะลำไส้ใหญ่อักเสบที่เกิดจากการชักนำโดยใช้ trinitrobenzenesulphonic (TNBS) พบว่า ระดับของ nitric oxide (NO) และ thiobarbituric acid reactive substances (TBARS) ในซีรั่มของหนูที่มีภาวะลำไส้ใหญ่อักเสบที่ได้รับ phosphate buffered saline; PBS (กลุ่มควบคุม) สูงกว่ากลุ่มที่ไม่มีภาวะลำไส้ใหญ่อักเสบ แต่อย่างไรก็ตาม หนูที่มีภาวะลำไส้ใหญ่อักเสบที่ได้รับ HNKP มีระดับของ NO และ TBARS ลดลง ($p \leq 0.05$) แต่มีกิจกรรมของเอนไซม์ superoxide dismutase activities (SOD) สูงขึ้น ($p \leq 0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนการศึกษาฤทธิ์ต้านการอักเสบ พบว่า หนูที่มีภาวะลำไส้ใหญ่อักเสบที่ได้รับ HNKP มีระดับของ tumor necrosis factor-α (TNF_α) ในเซรั่มไม่แตกต่างจากหนูที่มีภาวะลำไส้ใหญ่อักเสบที่ได้รับยา prednisolone และผงคีเฟอร์จากนมวัว แต่หนูทั้ง 3 กลุ่ม มีระดับ TNF_α ลดลงอย่างมีนัยสำคัญ ($p \leq 0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม สำหรับการศึกษาพิษเฉียบพลัน พบว่า HNKP ไม่ก่อให้เกิดความเป็นพิษต่อตับและไต ในขณะที่ KDMLKP ขนาด 4,000 mg/kg มีความเป็นพิษต่อตับ และ RHKP ขนาด 4,000 mg/kg มีความเป็นพิษต่อตับและไต ขณะที่การศึกษาพิษกึ่งเฉียบพลัน พบว่า KDMLKP, RHKP และ HNKP ขนาดน้อยกว่าหรือเท่ากับ 500 mg/kg ไม่ก่อให้เกิดความเป็นพิษต่อตับ

และไต แต่ KDMLKP, RHKP และ HNKP ขนาดตั้งแต่ 1,000 mg/kg ขึ้นไปมีพิษต่อการทำงานของตับและไต และจากการศึกษาพิษกึ่งเรื้อรัง พบว่า KDMLKP, RHKP และ HNKP ขนาด 150 mg/kg ไม่ก่อให้เกิดความเป็นพิษต่อตับและไต และยังพบว่า RHKP ทำให้ระดับ HDL สูงขึ้น เมื่อเปรียบเทียบกับกลุ่มอื่นๆ อย่างไรก็ตาม ผงคีเฟอร์จากนมข้าวทั้ง 3 ชนิดนี้ มีผลต่อการลดลงของนิวโตรฟิลส์ และการเพิ่มขึ้นของลิมโฟไซต์ ส่งผลให้ปริมาณโกลบูลินในซีรั่มเพิ่มขึ้น ซึ่งอาจนำไปสู่การกระตุ้นภูมิคุ้มกันให้ทำงานได้ดีขึ้น

จากผลการศึกษาทั้งหมดชี้ให้เห็นว่าผงคีเฟอร์จากนมข้าวกล้องหอมนิลมีความปลอดภัย มีฤทธิ์ต้านอนุมูลอิสระ และต้านการอักเสบสูง ดังนั้น ผงคีเฟอร์จากนมข้าวกล้องหอมนิลจึงมีคุณสมบัติที่เหมาะสมในการนำมาพัฒนาเป็นอาหารเสริมสุขภาพ และผลิตภัณฑ์อื่นๆ สำหรับมนุษย์

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CHAPTER 1

INTRODUCTION

1.1 Background

Kefir is a fermented milk product. It consists of lactic acid bacteria (LAB), acetic acid bacteria and yeasts that produce jelly-like grains. Kefir grains are white or lightly yellow color, gelatinous irregular masses and size between 0.3-3.5 cm. in diameter (Micheli et al., 1999; Witthuhn et al., 2005). Both bacteria and yeasts are surrounded by a water-soluble branched glucogalactan called kefiran (Micheli et al., 1999). Kefir from cow's milk has been reported to possess antibacterial (Bakken, 2009; Carasi et al., 2012; Deeseenthum and Pejovic, 2010; Kolakowski and Ozimkiewicz, 2012; Rodrigues et al., 2005), antifungial (Rodrigues et al., 2005), antitumor (Moreno de LeBlanc et al., 2007), antioxidant (Deeseenthum and Pejovic, 2010; Kesenkaş et al., 2011; Liu et al., 2005a and 2005b), anti-allergic (Lee et al., 2007), antineoplastic and pro-digestive (Saloff-Coaste, 1996; Urdaneta et al., 2007), antidiabetic (Hadisaputro et al., 2012; Punaro et al., 2014), and immunomodulatory activities (Vinderola et al., 2005; Vinderola et al., 2006). Moreover, it is important to anti-inflammatory activity on liver (Meydani and Ha, 2000), lung (Kwon et al., 2008; Punaro et al., 2014; Lee et al., 2007) and colon (Bolla et al., 2013). Wherewith, 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 (IFNγ and TNFα) without tissue damage (Vinderola et al., 2005 and 2006). It can also improve lactose digestion and tolerance (Hertzler and Clancy, 2003). In addition, kefir has high nutritional value as a source of proteins and calcium. Kefir has a long tradition of being regarded as good for health in many countries (Farnworth, 1999). It can be considered as a probiotic resource, because it has well activities on health.

Inflammatory bowel disease (IBD) refers to Crohn's disease (CD) and ulcerative colitis (UC). IBD is a group of chronic inflammatory disorders of the

gastrointestinal tract. This disease is caused by lesions occurring within the gastrointestinal tract and may be associated with extra-intestinal manifestation. The progression of the disease is chronic, with exacerbations switch on calm. The UC only found in the mucosa of colon, while CD found in many part and transmural of the gastrointestinal tract (Papadakis and Targan, 1999). At present, the pathogenesis of IBD is still not clear. But, the evidence that would be caused by the deformity immune response to the bacterial flora (Chassaing and Darfeuille-Michaud, 2011; Shih et al., 2008; Strober and Fuss, 2011), with there are some environmental factors (Molodecky and Kaplan, 2010). IBD have more affected with American and European (Loftus, 2004). In the present report, the incidence of the above disease is rising steadily in the Eastern (Manatsathit, 2012). The CD patients in Western countries are more than UC patients. In contrast, Thailand has UC patients more than CD patients, as many Eastern countries (Molodecky et al., 2012). Both diseases are characterized by mucosal inflammation, diarrhea, hematochezia, abdominal pain, fever, anorexia, weight loss, pale and extra-intestinal manifestation (Manatsathit, 2012). In Thailand, the current guidelines for the treatment of IBD are still very limited. Because the medicines such as 5-aminosalicylic acid (5-ASA) and infliximab are the very expensive and most people cannot afford the treatment. Moreover, some medicine such as prednisolone may be a major risk to the side effect (Kishore et al., 2004). Many researchers try to find plants or plant products with high antioxidant and anti-inflammatory effects that can prevent UC.

Fermented kefir milk has high antioxidant activity and reduces the accumulation of reactive oxygen species (ROS) including superoxide (O_2.-), hydrogen peroxide (H_2O_2) and nitric oxide (NO·) (Kaizu et al., 1993). Moreover, kefir from rice milk showed higher antioxidant than cow milk (Deeseenthum and Pejovic, 2010). McCue and Shetty (2005) suggested that kefir from plants exhibited high antioxidant effect as a result from phenolic compounds in the plants.

Kefir differs from other fermented milks in its starter, which exists in the form of grains (Simova et al., 2002). The grains are the product of fermentation by a mixed group of microflora confined to kefiran in matrix. The kefiran is a slimy polysaccharide matrix as the bioactivity of milk kefir (Marshall and Cole, 1985).

Rice (*Oryza sativa,* L.) is one of the most important food and agricultural commodities (ranked by value) in Thailand. Thailand is the 6th rice producing (FAOSTAT, 2014) and 3rd rice exporting country in the world (USDA, 2013). Several compounds with pharmacological activity have been previously isolated from brown rice. It has been reportedly possessed γ-aminobutyric acid (GABA), α-tocopherol, γ-tocopherol and total phenolics compounds (TPC) (Moongngarm and Saetung, 2010; Moongngarm et al., 2012). Moreover, rice and rice product comprise antioxidant activity (Deeseenthum and Pejovic, 2010; Selamassakul et al., 2013) and also anti-inflammatory activity (Hadisaputro et al., 2012; Kwon et al., 2008; Nui et al., 2013; Shalini et al., 2012).

Studies on chemical constituents as well as the effects of Thai rice kefir are still limited. There are only one report about the antioxidant activity of Thai rice kefir milk (Deeseenthum and Pejovic, 2010) and only one report about the antioxidant activity of fermented Khao Dawk Mali 105 brown rice by LAB (Selamassakul et al., 2013). However, scientific reports describing the pharmacological activity of Thai rice kefir powder has not yet been reported. To see whether different activities or properties of kefir powder that depend on the microflora grown on the process of kefir fermentation. The present study was therefore aimed to investigate the biochemical components, antioxidant activity and toxicity of rice kefir powder from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice. And also, its anti-inflammatory activity in the experimental model of trinitrobenzenesulphonic (TNBS) acid-induced rat ulcerative colitis was examined.

1.2 Objectives

The present study was designed to determine and compare the biochemical components, bioactivities and toxicities of rice kefir powder from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice.

1.3 Scope of Research

Material used ; Brown rice kefir powder prepared from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice.

1.3.1 Study on an effect of kefir powder in vitro

1.3.1.1 The amount of biochemical components including GABA and alpha-tocopherol (α-tocopherol) content were determined by using high performance liquid chromatography (HPLC) method. Total phenolic content (TPC) was determined by using an UV-Visible spectrophotometer.

1.3.1.2 Antioxidant activity including 2, 2′-diphenyl-1-picrylhydrazyl free radical scavenging assay was determined by using a microplate reader spectrophotometer and Ferric reducing/antioxidant power assay was determined by using an UV-Visible spectrophotometer.

1.3.2 Study on effect of kefir powder in vivo

1.3.2.1 Antioxidative stress activity

a) Colitis induction was performed by using TNBS.

b) Anti-oxidative stress activities including

(1) Nitric oxide (NO) estimation was determined by using Griess reaction commercial kit

(2) Superoxide dismutase (SOD) activity was determined by using SOD assay kit-WST

(3) Lipid peroxidation (LPO) estimation was determined by using thiobarbituric acid reactive substances (TBARS) commercial kit.

1.3.2.2 Anti-inflammatory activity

a) Blood chemistry and hematological values was investigated using automatic detection.

b) TNFα was detected using anti-TNFα by commercial kits.

c) Colonic histology was investigated using paraffin embedding H&E method.

1.3.2.3 Toxicity studies were examined.

a) Acute toxicity

b) Sub-acute toxicity for 14 days

c) Sub-chronic toxicity for 90 days

1.4 Research definition

1.4.1 Rice kefir powder is a kefir powder prepared from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice.

1.4.2 Biochemical components are chemical compounds found in rice kefir powder including GABA, alpha-tocopherol and total phenolic content.

1.4.3 Bioactivities are activities from rice kefir powder occurred in Wistar rats including antioxidant, anti-oxidative stress and anti-inflammatory activity.

1.4.4 Toxicity studies are the studies on the alterations of biology and physiology in experimental rats resulting from rice kefir powder including acute toxicity, sub-acute toxicity and sub-chronic toxicity.

CHAPTER 2

LITERATURE REVIEW

2.1 Kefir

2.1.1 Biological characteristic of kefir

Kefir is a fermented milk product. It consists of LAB, acetic acid bacteria and yeasts that produce jelly-like grains. Kefir grains are white or lightly yellow color, gelatinous irregular masses and size between 0.3-3.5 cm. in diameter (Micheli et al., 1999; Witthuhn et al., 2005). Both bacteria and yeasts are surrounded by a water-soluble branched glucogalactan called kefiran (Micheli et al., 1999). The relative composition and total microorganism number of kefir grains are variable. This depends on the origin of grains, the method and substrates used in the kefir fermented process (Londero et al., 2012). The microflora of kefir grains are shown in tables 2.1 and 2.2.

2.1.2 Activities of kefir

2.1.2.1 Antibacterial and anti-mycerial activities

The researches have been a growing interest in kefir grains for antibacterial and anti-mycerial activities. Rodrigues et al. (2005) reported that both kefir grains and kefiran were effective to inhibit *Staphylococcus aureus, S. salivarius, S. pyogenes, Pseudomonas aeruginosa, Candida albicans, Salmonella typhimurium*, *Listerica monocytogenes* and *Escherichia coli*. According to Deeseenthum and Pejovic (2010) found that both rice milk kefir and pasteurized cow milk kefir were effective to inhibit *Staphylococcus aureus, Bacillus subtilis, E. coli* and *P. fluorescens*. Thereafter, Kolakowski and Ozimkiewicz (2012) showed that the kefir grains grown in pasteurized *E.coli* contaminated milk. The kefir grains were separated from fermented milk and washed with water shown to be not contaminated with *E.coli*. In contrast, *E.coli* population increased in cow milk incubated without kefir grains. Moreover, in the case of *Clostridium difficile*-associated diarrhea patients were treated with daily intake of kefir products. As a result, all patients successfully resolved *C. difficile* infection and did not have diarrhea after completion of treatment. In addition, S-layer protein from

aggregating *Lactobacillus kefir* strains could antagonize the cytopathic effects of toxins from *C. difficile on* eukaryotic cells. S-layer proteins showed a higher inhibitory ability of *C. difficile* (Carasi et al., 2012). According to Bolla et al. (2013) who demonstrated the protective effect of a microorganism isolated mixture from kefir including *Lactobacillus plantarum, Lactobacillus kefir, Lc. lactis, Kluyveromyces marxianus*, and *Saccharomyces cerevisiae* in hamster infection by *C. difficile*. An only 1 of 7 hamsters had diarrhea and no mortality, while control group had 6 of 7 hamsters showed diarrhea and 5 of 7 died.

Table 2.1 Bacterial microbiota isolates in different batches of kefir grains

Actual nomenclature	**Obsolete nomenclature**	**Cited in**
Lactobacillus acidophilus	-	Angulo et al., 1993; Nalbantoglu et al., 2014
Lactobacillus casei subsp. casei	*Lactobacillus casei*	
Lactobacillus paracasei subsp. *paracasei*	-	Simova et al., 2002; Simova et al., 2006
Lactobacillus delbrueckii subsp. *bulgaricus*	*Lactobacillus casei* subsp. *pseudoplantarum*	
Lactobacillus brevis	-	Simova et al., 2002; Simova et al., 2006; Nalbantoglu et al., 2014
Lactobacillus helveticus	-	
Lactobacillus bulgaricus	-	Simova et al., 2006
Lactobacillus fermentum	-	Witthuhn et al., 2005
Lactobacillus plantrarum	-	
Lactobacillus kefiri	-	Garrote et al., 2001; Garrote et al., 2004; Nalbantoglu et al., 2014
Lactobacillus kefiranofaciens subsp. *kefiranofaciens*	*Lactobacillus kefiranofaciens*	Yokoi et al., 1990; Nalbantoglu et al., 2014

Table 2.1 (Continued)

Actual nomenclature	Obsolete nomenclature	Cited in
Lactobacillus kefiranofaciens subsp. *kefirgranum*	*Lactobacillus kefirgranum*	Takizawa et al., 1994; Nalbantoglu et al., 2014
Lactobacillus parakefiri	-	
Lactobacillus delbrueckii subsp. *lactis*	*Lactobacillus lactis*	Kwon et al., 2003; Simova et al., 2006
Lactobacillus hordei	-	Gulitz et al., 2011
Lactobacillus nagelii	-	
Lactobacillus sunkii	-	Nalbantoglu et al., 2014
Lactobacillus johnsonii	-	
Lactobacillus crispatus	-	
Lactobacillus otakiensis	-	
Lactobacillus kalixensis	-	
Lactobacillus rapi	-	
Lactobacillus diolivorans	-	
Lactobacillus buchneri	-	
Lactobacillus papabuchneri	-	
Lactobacillus parafarraginis	-	
Other bacteria		
Acetobacter aceti	-	Angulo et al., 1993
Enterococcus durans	-	Yuksekdag et al.,2004; Simova et al., 2002; Simova et al., 2006
Lactococcus lactis subsp. *cremoris*	*Lactococcus cremoris; Streptococcus cremoris*	
Streptococcus thermophiles	-	
Lactococcus lactis subsp. *lactis*	*Lactococcus lactis; Streptococcus lactis*	Garrote et al., 2001; Witthuhn et al., 2005; Yuksekdag et al., 2004

Table 2.1 (Continued)

Actual nomenclature	Obsolete nomenclature	Cited in
Leuconostoc. mesenteroides subsp. *cremoris*	-	Witthuhn et al., 2005
Leuconostoc. delbrueckii subsp. *delbrueckii*	-	
Pediococcus lolii	-	Nalbantoglu et al., 2014
Tetragenococcus halophilus	-	

(t) : teleomorph

(a): anamorph

- : No data

Table 2.2 Fungal microbiota isolates in different batches of kefir grains

Actual nomenclature	Obsolete nomenclature	Cited in
Candida friedrichii	-	Angulo et al., 1993
Candida humilis	-	Kumura et al., 2004
Candida inconspicua	-	Simova et al., 2002
Candida maris	-	
Cryptococcus humicolus	-	Witthuhn et al., 2005
Debaryomyces hansenii (t)/ *Candida famata* (a)	-	Kumura et al., 2004; Loretan et al., 2003
Debaryomyces (Schwanniomyces) occidentalis	-	Kumura et al., 2004
Dekkera anomata (t)/ *Brettanomyces anomalus* (a)	-	Angulo et al., 1993; Loretan et al., 2003; Pintado et al., 1996; Wyder et al., 1997
Galactomyces geotrichum (t)/ *Geotrichum candidum* (a)	-	Pintado et al., 1996; Witthuhn et al., 2005
Issatchenkia orientalis (t)/ *Candida krusei* (a)	-	Witthuhn et al., 2005
Kazachstania unispora	*Saccharomyces unisporus*	Loretan et al., 2003; Marguina et al., 2002; Wyder et al., 1997; Puerari et al., 2012
Kluyveromyces marxianus (t)/ *Candida kefyr* (a)	*Kluyveromyces marxianus* var. *marxianus*	Kwon et al., 2003; Marguina et al., 2002; Witthuhn et al., 2005; Wyder et al., 1997

Table 2.2 (Continued)

Actual nomenclature	Obsolete nomenclature	Cited in
Kluveromyces lactis var. lactis	*Kluyveromyces lactis;* *Kluyveromyces marxianus* var. *lactis*	Angulo et al., 1993; Marguina et al., 2002; Simova et al., 2002
Kluyveromyces lodderae	-	Kumura et al., 2004
Pichia fermentans (t)/ *Candida fermentaria* (a)	*Candida lambica*	Rohm et al., 1992; Witthuhn et al., 2005
Saccharomyces exiguus	*Torulopsis holmii; Candida holmii*	Angulo et al., 1993; Iwasawa et al., 1982; Marguina et al., 2002
Saccharomyces cerevisiae	-	Angulo et al., 1993; Marguina et al., 2002
Saccharomyces pastorianus	*Saccharomyces carlsbergensis*	Koroleva, 1988
Sacchaomyces turicensis sp. nov	-	Wyder et al., 1999
Torulaspora delbrueckii (t)	*Saccharomyces delbrueckii;* *Candida colliculosa*	Wyder et al., 1997
Yarrowia lipolytica (t)/ *Candida lipolytica* (a)	-	Kumura et al., 2004
Zygotorulaspora florentina	-	Gulitz et al., 2011
Zygosaccharomyces rouxii	-	Loretan et al., 2003; Witthuhn et al., 2005

(t) : teleomorph

(a): anamorph

- : No data

2.1.2.2 Antitumor activity

Moreno de LeBlanc et al. (2007) injected mice with breast tumor cells there administered kefir and a kefir cell-free fraction (KF) for 2 or 7 days. The administration of both products every 2 days increased the number of IgA cells and delayed tumor growth. The mice received KF every 2 days administration found increases in the number of apoptotic cells ($p \leq 0.05$), while decreases in the number of Bcl-2 cells and alters in the CD4+ and CD8+ cells balancing in the mammary organs.

2.1.2.3 Antioxidant activity

Kefirs demonstrated significantly ($p \leq 0.05$) greater scavenging effects and superoxide radicals, an inhibition effect peroxidation, and more substantial reducing power, while reduced glutathione peroxidase (GSH-Px) activity when compared with

cow milks. Kefirs possess antioxidant activity, consequently suggesting that kefir is a potential candidate for the useful natural antioxidant supplements for the human diet (Kesenkaş et al., 2011; Liu et al., 2005a and 2005b). Moreover, Deeseenthum and Pejovic (2010) also suggested that the antioxidant activity of rice milk kefir and cow's milk kefir. The methods from DPPH radical all scavenging activity of extracts, lipid peroxidation assay and hydroxyl radical scavenging activity showed the rice milk kefir was significant greater of antioxidant activity ($p \leq 0.05$) than cow milk kefir.

2.1.2.4 Anti-allergic activity

The mice asthma models administered by intra-gastric injection with kefir (50 mg/kg) showed significantly decreased ($p \leq 0.05$) in the total inflammatory cell count and the eosinophil count in bronchoalveolar lavage fluid (BALF). The type 2 helper T cell (Th2) cytokines were reduced to normal levels as well. In addition, kefir also inhibited eosinophilia in lung tissue and mucus hypersecretion by goblet cells in the mice airway (Lee et al., 2007).

2.1.2.5 Antineoplastic and pro-digestive activities

Kefir is considered to be probiotic and involved the host through its effects in the gastrointestinal tract. Kefir has affect to enzymes and proteins present in the intestine. Urdaneta et al. (2007) reported that the weight of the organs examined no significant differences in control and kefir supplemented group. An intestinal enzymatic analysis was carried out. The results showed an increase of this activity in addition to the uptake of D-galactose by brush border membrane vesicles, while glycaemia was significantly lower in the kefir group. Likewise, Zoumpopoulou et al. (2008) showed that *Lactobacillus fermentum* was against five streptococci including the two pathogenic strains of *Streptococcus oralis* and *S. pneumoniae. Streptococcus macedonicus* was against the majority of the strains tested. *Lactobacillus fermentum* also should elevated levels of the anti-inflammatory IL-10.

2.1.2.6 Antidiabetic activity

Punaro et al. (2014) treated diabetic rats with kefir by dairy gavage at a dose of 1.8 mL/day for 8 weeks. The rats treated with kefir showed a significant ($p \leq 0.05$) improvement of proteinuria, nitric oxide (NO), urea, creatinine, thiobarbituric acid reactive substances (TBARS), and C-reactive protein (CRP) in blood and urine

samples when compared with diabetic rats and control rats. In addition, the Western blot analysis showed the expression of inducible NO synthase (iNOS) on the diabetic rats treated with kefir were significantly lower ($p \leq 0.05$). The rats treated with kefir also presented a significant reduction ($p \leq 0.05$) of glycogen accumulation within the renal tubules. Hadisaputro et al. (2012) reported that the hyperglycemia rats induced by streptozotocin received kefir 3.6 cc/day could decrease of glucose in blood sample.

2.1.2.7 Anti-inflammatory activity

Many investigators have studied the therapeutic and preventive anti-inflammatory effects of kefir and kefiran products on animal models. Kwon et al. (2008) states that kefiran significantly inhibited ($p \leq 0.05$) the release of inflammatory cells into BALF and lung tissue of the hyperglycemia rats. Histological studies demonstrate that kefiran substantially inhibited eosinophilia in lung tissue by hematoxylin and eosin staining protocol. Lee et al. (2007) reported that kefir significantly suppressed in the total inflammatory cell count and eosinophilia in lung tissue of mouse asthma model. Hadisaputro et al. (2012) showed that kefir supplementation 3.6 cc/day on diabetic rats could significantly decrease ($p \leq 0.05$) pro-inflammatory cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα)). Additionally, anti-inflammatory (interleukin-10) also showed significant increase ($p \leq 0.05$) when compared with the control rats. Bolla et al. (2013) found that the colon of hamsters in histological section had decreased oedema and inflammatory infiltrates with neutrophils and crypt abscesses in hamster group fed with mixture kefir-isolated microorganisms.

2.1.2.8 Immunomodulatory activity

Innate immunity that protects against pathogens in the tissues and circulation is the first line of defense in the mammalian immune reaction. Vinderola et al. (2006) found that the liquid supernatant fraction of kefir had an immunomodulating capacity of solids including bacteria by studying the cytokines produced of peritoneal macrophages and the adherent cells from Peyer's patches on mice. The numbers of cytokine (IL-1a, IFNγ, TNFα, IL-6 and IL-10) producing cells was determined on peritoneal macrophages and adherent cells from Peyer's patches. Both kefir fractions induced IL-1a, IL-6 and IL-10 on peritoneal macrophages. All cytokines studied on

adherent cells from Peyer's patches were enhanced after feeding, except for IFNγ after liquid supernatant administration. The liquid supernatant fraction had a significantly higher percentage ($p \leq 0.05$) of induced of IL-10 cells on adherent cells from Peyer's patches than the one induced by solids including bacteria fraction. Vinderola et al. (2005) reported that pasteurized kefir was able to modulate the intestinal mucosa immune response in mice. Kefir induced a typical Th_2 response by increasing the number of IgA+, IL-4+, IL-6+ and IL-10+ cells.

2.2 Rice

2.2.1 Characteristics of rice

Rice (*Oryza sativa,* L.) is a widely used name for more than 84 annual varieties in Thailand (BRRD, 2014) and is one of the most important crops in the world in addition to wheat and corn. Rice is cultivated in over 100 countries around the world and is a staple food for about half of the world population. It is one of the first most important food and agricultural commodities in Thailand in the year 2012. Moreover, Thailand is the fifth highest rice producing country in the world (Figures 2.1 and 2.2; FAOSTAT, 2014) and top three highest the export volume of rice (Table 2.3; USDA, 2014). Rice accounts for over 22% of global energy in take. While the production and consumption of rice are concentrated in Asia, which contains about 92% of the world's total production, rice is also an important crop in specific regions of North and South America, Africa and Europe (Ohtsubo et al., 2004). Ideally, alternatives that diversify the application of rice in human nutrition and improve its nutritional value should be investigated.

Table 2.3 Export volume of Thailand rice

The export volume of rice (Million tons)			
Countries	**2553/54**	**2554/55**	**2555/56**
India	4.6	10.3	10.0
Vietnam	7.0	7.7	7.4
Thailand	10.6	6.9	7.0
United	3.2	3.3	3.4
Pakistan	3.4	3.4	3.0
Cambodia	0.9	0.8	1.0
Uruguay	0.8	1.1	0.9
Egypt	0.3	0.6	0.9
Other	5.3	5.1	4.8
Total	**36.2**	**39.1**	**38.3**

(World Grain Situation and Outlook, USDA, 2014)

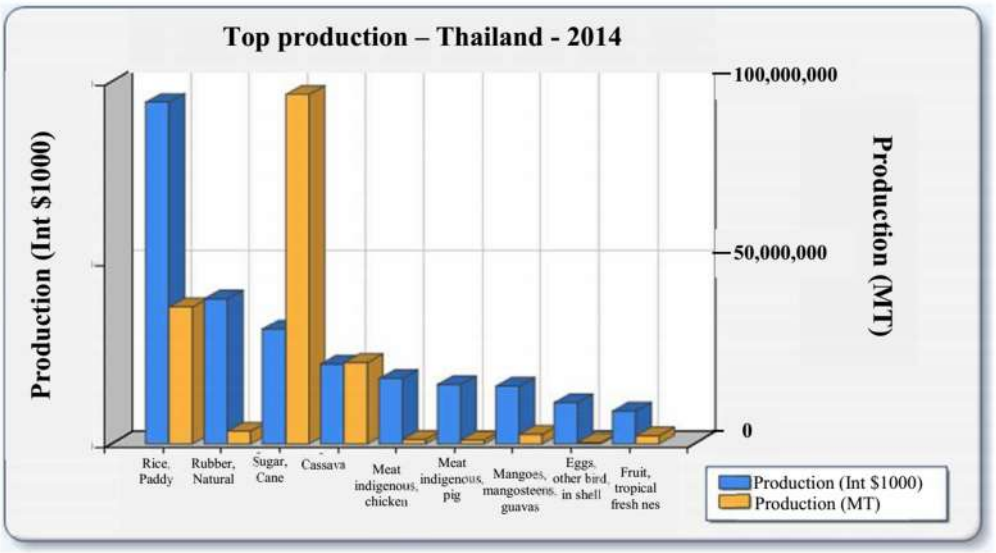


Figure 2.1 The ranked by value of 9th most important food and agricultural commodities in a given country for the year 2014 (FAOSTAT, 2014)

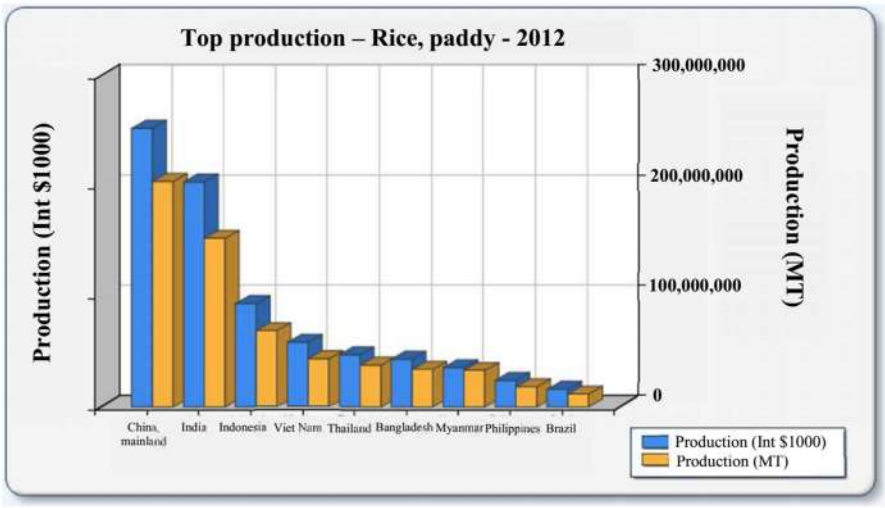


Figure 2.2 The 9th highest producing countries of rice for the year 2012 (USDA, 2014)

2.2.2 Varieties of rice

2.2.2.1 Khao Dawk Mali 105 rice

Variety: Khao Dawk Mali 105 rice (KDML 105)

History: Khao Dawk Mali 105 rice has a long history of rice cultivation and development.

Figures 2.3 shows morphological characters ofKhao Dawk Mali 105, a non-glutinous rice variety. Its seed dormancy is about 8 weeks. The average yield is 12,875 kilograms per hectare (Chaitep, 2010). One thousand grain weigh is about 27.9 gram. The paddy is about 10.4 mm long, 2.6 mm wide and 2.0 mm thick. The brown rice shape is slender with a 7.4 mm long, 2.1 mm wide and 1.7 mm thick.

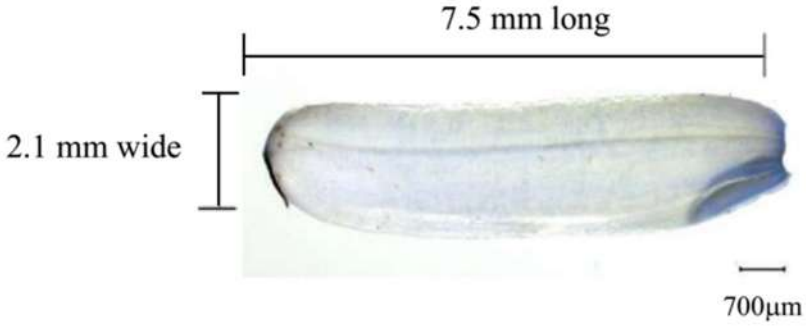


Figure 2.3 Morphological characters of Khao Dawk Mali 105 brown rice grain

2.2.2.2 Red Hawm rice

Variety: Red Hawm rice (KDML105R-PSL-E-14)

History: Red Hawm rice is a natural mutant derived from the Khao Dawk Mali 105 rice.

Figures 2.4 shows morphological characters of KDML105R-PSL-E-14, a non-glutinous rice variety. Its seed is about 9.6 mm long, 3.9 mm wide and 2.3 mm thick. The brown rice shape is slender, 7.5 mm long, 2.1 mm wide and 1.7 mm thick. The seed dormancy is about 8 weeks. The average yield is 16,075 kilogram per hectare (BRRD, 2014).

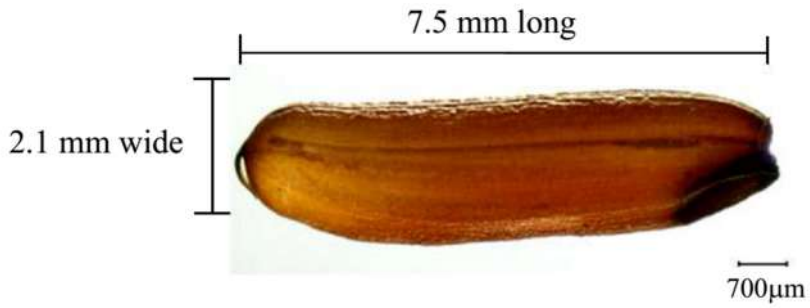


Figure 2.4 Morphological characters of Red Hawm brown rice grain

2.2.2.3 Hawm Nil rice

Variety: Hawm Nil rice (PSL00288-4-21-5R)

History: Hawm Nil rice is a natural mutant derived from the short plant form of Chinese glutinous rice.

Figures 2.5 shows morphological characters of Hawm Nil rice, a non-glutinous rice variety. It is low sensitive to photoperiod and can be harvested 3 times per year. The brown rice grains are slender less than Khao Dawk Mali 105 rice and Red Hawm rice, purple, soft, sticky and aromatic. The mature plant with researches is 60-75 centimeters height. The culm is erect with color morph between green-purple of leaves and purple color of leaf sheath, flower and husk. The brown rice grain is 6.5 mm long, 2.3 mm wide and 1.7 mm thick (BRRD, 2014).

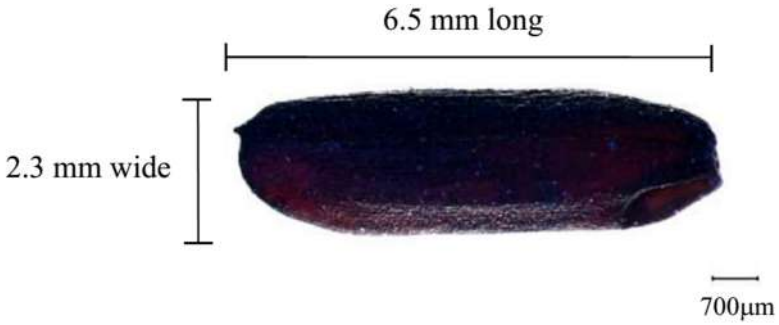


Figure 2.5 Morphological characters of Hawm nil brown rice grain

2.2.3 Chemical composition, phytochemical contents and antioxidant activity of Thai brown rice

Brown rice (BR) is composed of external thin layers (bran) that enclose the embryo and endosperm. The nutritional components in BR mainly exit in the germ and bran layers which are mostly removed as a consequence of milling or polishing (Monks et al., 2013). Moongngarm and Saetung (2010) found that the most significant changes, in γ-aminobutyric acid, glycine, lysine and leucine, were observed in the germinated rough rice and the germinated rice extracted powder. In addition, Moongngarm et al. (2012) also showed that the rice germ fraction was high in protein, lipid, and fiber, whilst rice bran layer fraction was a good source of carbohydrate and ash. In the phytochemical compositions and antioxidant activity study, rice germ contained the highest amount of α-tocopherol, γ-tocopherol, and indicated the strongest antioxidant activity. The rice bran layer showed the highest level of γ-oryzanol in black waxy rice. Moreover, Suwannalert and Rattanachitthawat, (2011) reported that the strains of Leum Phua, Klam, Hawm Nil and Black Rose showed high levels of phenolic content: 1.36±0.03, 0.78±0.02, 0.61±0.01 and 0.57±0.02 mg gallic acid/g sample, respectively and the unpolished Thai rice strain of Leum Phua showed the highest antioxidant activity. It was also highest in anthocyanin pigment. Interestingly, phytophenolic chromatogram and anthocyanin pigment levels showed a strong correlation.

2.2.4 Activity of fermented Thai brown rice

Deeseenthum and Pejovic (2010) investigated the antioxidant activity of 24 and 48 h old of rice kefir milk and cow kefir milk. Antioxidant activity of rice kefir milk was measured using three different methods: DPPH free radical scavenging activity assay, lipid peroxidation assay and hydroxyl radical scavenging activity assay. Rice kefir milk displayed significantly greater antioxidant activity than cow kefir milk. In addition, Selamassakul et al. (2013) studied the brown rice (Khao Dawk Mali 105) was fermented with Lactococcus lactis for 0, 3, 5, 7, 14, and 21 days to produce fermented brown rice protein hydrolysate (f-RPH). Degree of hydrolysis, total phenolics and antioxidant activity of the f-RPH increased when fermentation time was increased. Antioxidant activity with respect to DPPH and ABTS radicals of f-RPHs was two- or three-fold greater than the brown rice (0 day). The molecular weight of f-RPHs was less than 20 kDa when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Manosroi et al., (2011) also assayed for the bioactive compounds and the biological activities on white plain, purple plain, brown plain, white glutinous and purple glutinous rice were fermented with a mixed culture of yeasts and molds. The fermented purple plain sap samples at day 6^{th} of the fermentation period showed high Antioxidative activity, the highest tyrosinase inhibition and MMP-2 inhibition activities with low cytotoxicity to normal human skin fibroblast by SRB assay in comparing to other rice samples.

2.3 Free radicals

2.3.1 Free radical defined

A free radical is an atom, molecule, or ion that has unpaired valence electrons or an open electron shell, and therefore may be seen as having one or more unbalanced incomplete covalent bonds. It has been known for years that the reactive oxygen species (ROS) including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$) easily to found in vascular and cardiac tissues (Griendling and Ushio-Fukai, 1997). ROS have been shown produce and be regulated every cell type in the vascular wall of small mammal (Suzuki and Ford, 1999). Xanthine oxidase has subsequently

been established that smooth muscle cells and fibroblasts account for the majority of $O_2^{\cdot -}$ produced in the normal vessel wall (Rajagopalan et al., 1996).

2.3.2 Free radicals sources

As a consequence of aerobic metabolism, small amounts of reactive oxygen species, including superoxide radicals, hydroxyl radicals, hydrogen peroxide and peroxide radicals and its related radicals, are constantly generated within certain cells of certain organisms. The accumulation of peroxidases in the human body has been reported to be associated with disorders such as cancer, atherosclerosis, hypertension, and arthritis (Ham et al., 2003). To avoid cellular damage by these peroxidants, most biological systems have developed inherent antioxidant systems, for example, superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and uric acid, in order to protect from damage caused by the peroxidants. Such systems, however, are not totally effective and do not prevent damage universally (Simic,1988); hence, there is an increasing interest world-wide in finding natural food-based antioxidants that are able to protect the human body from attack by free radicals and thus retard the progress of many chronic diseases, as well as retarding the lipid oxidative rancidity in foods (Pryor, 1991).

2.3.3 Nitric oxide and intestinal inflammation

The several investigations showed that the inhibition of NO causes to the features of intestinal inflammation, because the delivery of exogenous NO reduces the subsequent of acute inflammation. The NO synthesis inhibition has been found to increase acute damage of the intestinal mucosal from immune-mediated stress (Kubes, 1993). Administration of exogenous NO protects the mucosa against the aforementioned models, and this protective effect may be exerted at different levels, including maintenance of blood flow, inhibition of platelet and leucocyte adhesion and⁄or aggregation within the vasculature, down-regulation of mast cell reactivity, and modulation of oxidative stress and stabilization of I-κB, resulting in the inhibition of nuclear factor-κB (NF-κB) translocation (Peng et al., 1995). In addition, NO can reduce superoxide and induced damage either by inhibiting NADPH oxidase and superoxide release from neutrophils, or by scavenging neutrophil-derived superoxide. Accordingly,

NO donors have been found to double the plasma antioxidant capacity of animals subjected to reperfusion-induced mucosal injury.

2.3.4 Effect of antioxidants on human health

Free radicals as reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems, exposure to different pathological states and physiochemical conditions. A balancing of antioxidants and free radicals is necessary for appropriate physiological functions. If free radicals possessed the body's ability for regulate them, a condition known as oxidative stress. Free radicals have an effect to lipids, proteins and DNA altering and stimulus a number of human diseases. Thus application of antioxidants from external source may assist in handling this oxidative stress. Many synthetic antioxidants such as butylated hydroxyl toluene and butylated hydroxyl anisole have recently been reported to be dangerous for human health. Therefore, the investigation on natural compounds for effective, non-toxic and high antioxidative activity has been intensified in recent years (Lobo et al., 2010).

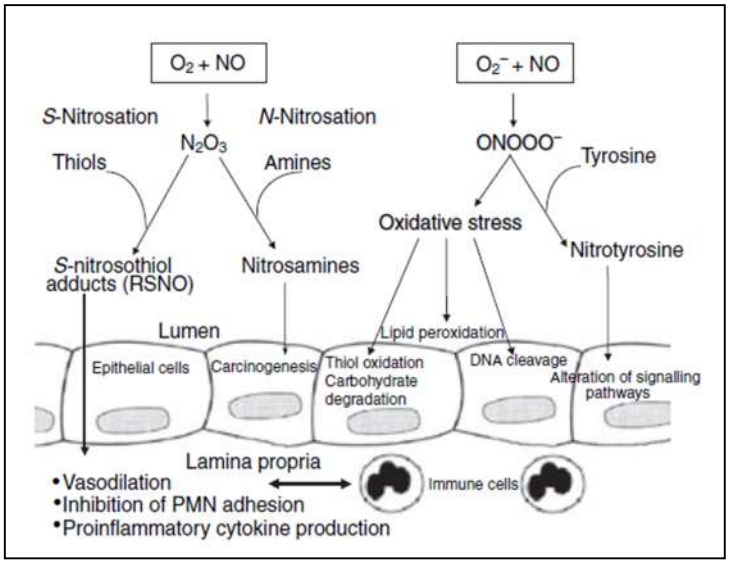


Figure 2.6 The role of nitric oxide in gut immunology (Kolios et al., 2004)

2.4 Intestinal bowel disease (IBD)

2.4.1 The classical of IBD

Ulcerative colitis (UC) and Crohn's disease (CD) are the two most common forms of IBD in humans. Important features of untreated UC in biopsy specimens include diffuse involvement of the colorectum without skip lesions, lack of submucosal involvement, lack of granulomas (except those related to mucin or foreign bodies), and lack of terminal ileum involvement (with the exception of a minor degree of inflammation associated with backwash ileitis). In approximately 5% of inflammatory bowel disease cases, a definite diagnosis of ulcerative colitis or Crohn's disease cannot be established, in which case the term "indeterminate" colitis is used. Most cases of indeterminate colitis are related to fulminant colitis, a condition in which the classic features of UC or CD may be obscured by severe ulceration with early superficial fissuring ulceration, transmural lymphoid aggregates, and relative rectal sparing. Approximately 20% of patients with indeterminate colitis develop severe pouch complications, which is intermediate in frequency between UC (8–10%) and CD (30–40%)(Odze, 2003).

2.4.2 Anti-inflammatory mechanism

The following account is based on the etiology of inflammatory bowel disease (IBD) remains debated but seems to result mainly from an aberrant immune response in genetically predisposed subjects. IBD studies are mainly focused on mechanisms involving immune cells but other cell types such as epithelial or endothelial cells also play a key role in the inflammatory process (Deban et al., 2008). In fact, microvascular endothelial cells (EC) regulate the migration of leukocytes from the intravascular compartment into the inflammatory tissue. Migration of leucocytes is a key process in inflammation that involves adhesion molecules such as ICAM-1 and VCAM-1. Primary cultures of human intestinal microvascular endothelial cells (HIMEC) have been developed to evaluate the role of EC in IBD (Deban et al., 2008; Haraldsen, et al., 1996). Under inflammatory conditions, mimicked in vitro by pro-inflammatory stimuli, adhesion molecules such as ICAM-1 and VCAM-1 are up-

regulated in HIMEC (Haraldsen, et al., 1996). Thus, HIMEC may be a cellular target for a specialized pharmacological or nutritional modulation. The key role of angiogenesis during IBD has been recently emphasized: increased vascularisation in IBD mucosa, up-regulation of angiogenic factors and angiogenic activity shown by IBD mucosal extracts-induced HIMEC migration (Deban et al., 2008). In chronic inflammatory disorders, angiogenesis appears deleterious by promoting tissue damage. Inhibition of angiogenesis may contribute to decrease immune cells recruitment and production of pro-inflammatory mediators (Ibrahim et al., 2012). Before birth, the gastrointestinal tract (GIT) has matured to digest human milk and to tolerate the invasion by billions of bacteria. The GIT produces mucins and defensins and has normal proliferation, restitution and cellular homeostasis. Not surprisingly, GIT inflammatory reactions are common in newborn infants, particularly incompromised infants such as those born preterm and/or growth-restricted. The most severe GIT inflammatory condition of very preterm infants is necrotizing enterocolitis (NEC), which has a high mortality (10–50%; Neu and Walker, 2011). Causative factors of NEC in preterm newborns include reduced peristalsis, impaired epithelial barrier function and GIT immaturity characterized by impaired enterocyte restitution (Figure 2.7). Other factors include a leaky mucosal barrier, dys-colonization, bacterial translocation, reduced mesenteric perfusion and excessive milk feeding.

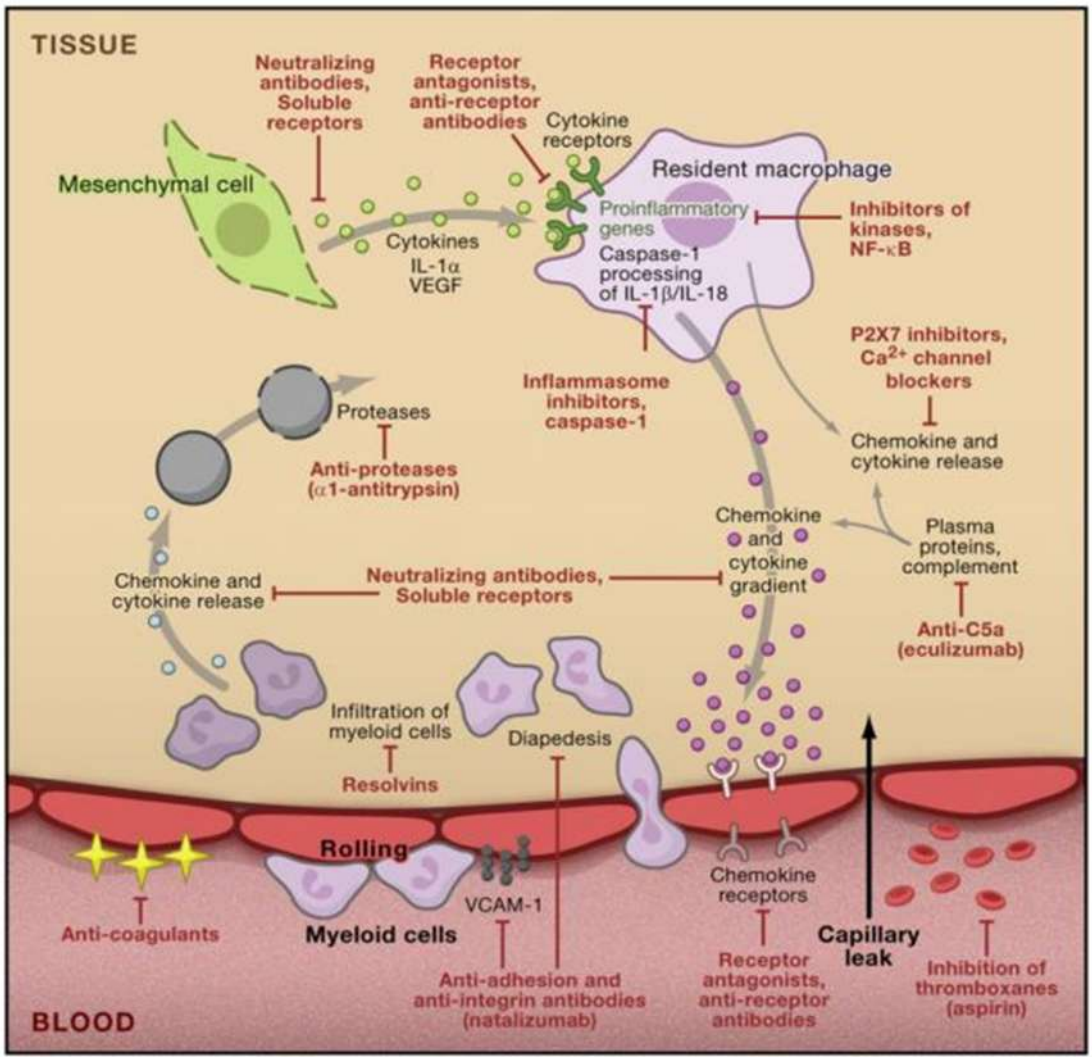


Figure 2.7 Inflammation and points of inhibition by anti-inflammatory agents (Dinarello, 2010)

CHAPTER 3

METHODOLOGY

3.1 Experimental animals

Male Wistar rats (weighing 180–200 g) were purchased from National Laboratory Animal Center, Mahidol University, Thailand. The rats were kept in an animal laboratory and acclimated for 7 days in environmental conditions (23±2°C and 50-55% relative humidity under a 12-h light/dark cycle). The rats were fed on 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 (ID : 0008/2557).

3.2 Kefir powder preparation

3.2.1 Brown rice samples

Khao Dawk Mali 105 brown rice, Red Hawm brown rice and Hawm Nil brown rice harvested during the year 2013–2014 from Selaphum, Roi Et Province, Thailand was used. The rice was dried, weighed, and soaked in distilled water (1:5, w:v) at 25°C for 2 h, and then thoroughly ground using a blender and filtrated to obtain rice milk. The rice milk was pasteurized at 70°C for 15 min and then immediately cooled at 4°C.

3.2.2 Kefir starter

A 0.2 g freeze-dried kefir grain from the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand was inoculated into a 250 ml flask with 200 ml of Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth and incubated under anaerobic conditions; the flask of kefir was put into a 5 L anaerobic jar. The sample jars were kept at 30°C for 24 h, and then centrifuged (1000 rpm, 15 min at 4°C) to obtain the cells. The cells were washed and re-suspended in sterile saline

solution (0.85% NaCl) and diluted with sterile 0.85% NaCl (1:10, v:v). Kefir was sub-cultured by inoculating kefir starter into fresh milk (20:200, v:v, Khao Dawk Mali 105 brown rice milk, Red Hawm brown rice milk, Hawm Nil brown rice milk, and cow's milk), and incubated under anaerobic conditions at 30°C for 48 h to obtain activated kefir grain. The activate kefir grain were sub-cultured by inoculating into fresh milk with 2.5% sucrose (100:1,000, v:v). Then the kefir was incubated under anaerobic conditions at 30°C for 24 h resulting in the final pH of about 4.8–4.9 of milk kefir.

3.2.3 Kefir powder

All milk kefir pH 4.8–4.9 were freeze-dried using SJIA-10N freeze dryer (Shanghai Beiyi Bioequip Information Co., Ltd., China.) at –55°C. The freeze-dried kefir was powdered to obtain kefir powder with a mortar and pestle under aseptic condition. The kefir powder including Khao Dawk Mali 105 (KDMLKP), Red Hawm (RHKP), Hawm Nil (HNKP) and cow's milk (CMKP) were packed into bottles and the caps were tightened and wrapped with foil. The kefir bottles were kept at –20°C until required for use.

3.3 Effect of kefir powder in vitro

3.3.1. Biochemical component determinations

3.3.1.1Gamma amino butyric acid (GABA) content

The content of GABA was determined using high performance liquid chromatography as described previously (Torino et al., 2013). Briefly, 0.5 g of sample was suspended in 12 ml distilled water. The suspension was stirred at 4°C for 16 h. Independent extractions were performed for each replicate. Samples were centrifuged at 15,000 rpm at 10°C for 20 min. The supernatant was vacuum-dried and dissolved in 500 µl of distilled water. A volume of 50 µl of kefir powder from Khao Dawk Mali 105 brown rice, Red Hawm brown rice, Hawm Nil brown rice, and cow's milk extracts were added to 10 µl of an internal standard solution containing 1.2 mg/ml allyl-L-glycine and 20 µl of 20% (v/v) triethylamine in 50% methanol (v/v). Mixtures were derived by adding 30 µl of phenyl iso-thiocyanate. Subsequently, samples were vacuum-dried, reconstituted in 500 µL of 0.1 M ammonium acetate pH 6.5 (mobile phase A), and

centrifuged at 13,000 rpm at 10°C for 5 min. Supernatants were filtered through a 0.22 µm nylon filter.

HPLC analyses were performed with an Alliance Separation Module 2695 (Waters, Milford, USA), equipped with a photodiode array detector 2996 (Waters). Samples (20 µl) were injected onto a C-18 Alltima (250 x 4.6 mm i.d., 5 µm particle size) column equipped with a guard column, both thermo stated at 40°C. The chromatograms were developed at a flow rate of 0.7 ml/min by eluting the sample in mobile phase A (0.1 M ammonium acetate pH 6.5), and mobile phase B (0.1 M ammonium acetate, acetonitrile, methanol, 44/46/10, v/v/v, pH 6.5) as follows: isocratic flow 100% A for 15 min, gradient flow from 100% A to 100% B for 27 min, isocratic flow 100% B for 8 min and finally equilibrated with 100% A for 5 min. Data acquisition and integration was performed using Empower II software (Waters). GABA was identified by retention time and spiking the sample with a standard solution. GABA content was quantified by using an external GABA standard calibration curve with a linear range over 0–240 µg/ml. Analyses were carried out in duplicate. The results were expressed in mg GABA/100 g of sample on dry matter basis (d.m.).

3.3.1.2 Alpha-tocopherol content

The α-tocopherol analysis was minor modified by the reversed phase high performance liquid chromatography (RP-HPLC) method (Moongngarm et al., 2012). The Shimadzu HPLC system (model L-6200A) equipped with a Photo diode array detector and a computer system was applied. Detection was operated at 292, simultaneously. The spectra from 250 to 600 nm were recorded for all peaks. The samples were injected through a guard-column and separated on C-18 column (4.60x150mm, 4 µm,). Gradient elution at ambient temperature was used, mobile phase A was methanol, mobile phase B was water, and mobile phase C was butanol. The gradient was as follows: 0–12 min 92%A, 4%B and 4% C: 12–25 min linear gradient from 4%B to 3%B and 4%C to 5%C with flow rate of 1.5 ml/min and injection volume of 20 µl. The tocopherol was detected at 292 nm. Chromatograms were recorded, and peak areas were used to calculate the content of α-tocopherol compared with those of standards.

3.3.1.3 Total phenolic content

The amount of total phenolic content (TPC) in the solution of kefir powder was determined using the Folin-Ciocalteu reagent according to the method for the modified procedure of Bonli et al., (2004) using gallic acid as a standard. Fifty microliters of each kefir solution (0.1 g/ml) were mixed with 3 mL of 10% Folin-Ciocalteu reagent (diluted 10 fold with distilled water). The mixture solution was allowed to stand at room temperature for 15 min. After that 1.5 mL of 10% (w/v) sodium carbonate solution was added to the mixture and then left at room temperature for 15 min. The absorbance of all samples was measured at 750 nm using an UV-Visible spectrophotometer. This experiment was carried out in triplicate and average values were calculated. The total phenolic content was analyzed against gallic acid calibration standard curve and expressed as milligrams of gallic acid equivalents (mg GAE) per grams of dry weight (g of DW).

3.3.2 Antioxidant activities

3.3.2.1 DPPH free radical scavenging assay

Free radical scavenging activity of aqueous extract were determined using a stable 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) following a modified method of Chan et al. (2007). A total of 1.0 ml of kefir solution was added to 2.0 mL of 0.1 mM DPPH solution. The mixture solution was incubated at room temperature in a dark condition for 30 min. Absorbance of all samples were measured at 517 nm using a microplate reader. The percentage of inhibition was calculated using the following equation;

$$\text{Inhibition (\%)} = [(A_{517control} - A_{517sample}) / A_{517control}] \times 100$$

BHA dissolved in methanol was analyzed as control. DPPH radical scavenging activity was expressed as IC_{50} value, which represented the amount of antioxidant in the aqueous extract necessary to reduce the initial DPPH concentration by 50%. The experiments were performed in triplicate.

3.3.2.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted using a modified method described by Benzie and Strain (1996). The 100 µL of each kefir solution (0.1g/ml) was mixed with 3 ml of FRAP solution (300 mM acetate buffer (pH 3.6): 10 mM tripyridyltriazine solution: 20 mM ferric chloride solution (in 40 mM HCl) of 10: 1: 1 by volume) and 300 µl of deionized water, then incubated at 37˚C for 4 min. The absorbance was then measured at 593 nm using an UV-Visible spectrophotometer. The results were expressed as millimolar ferrous sulfate per grams of dry weight (mM Fe (II) g^{-1} of DW).

3.4 Effect of kefir powder in rat models

3.4.1 Rat colitis studies

3.4.1.1 Experimental design

The rats were randomly divided into 7 groups with 6 rats in each;

(1) Group 1: non-colitis rats received phosphate buffered saline (PBS)

(2) Group 2: non-colitis rats received Hawm Nil brown rice kefir powder (150 mg/kg dissolved in PBS)

(3) Group 3: non-colitis rats received cow's kefir powder (150 mg/kg dissolved in PBS)

(4) Group 4: colitis rats received PBS

(5) Group 5: colitis rats received best brown rice kefir powder (150 mg/kg dissolved in PBS)

(6) Group 6: colitis rats received cow's kefir powder (150 mg/kg dissolved in PBS)

(7) Group 7: colitis rats received prednisolone (5 mg/kg)

Rat colitis groups were induced on day 4 by TNBS, those groups treated with Hawm Nil brown rice kefir power, cow's milk kefir powder or prednisolone were left for 10 days.

3.4.1.2 Colitis induction

The rats were colitis induced on day 4 and thereafter. Colitis induction followed the method originally described by Scarminio et al. (2012). After fasting overnight, the rats were anesthetized with halothane. Under anesthesia, they were given 10 mg of trinitrobenzenesulfonic acid (TNBS) dissolved in 0.25 ml of 50% (v/v) ethanol by means of Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthesia. Rats from the non-colitis group received 0.25 ml of saline.

3.4.1.3 Anti-oxidative stress activity studies

a) Nitric oxide measurement

Serum or plasma samples were treated with Centricon 10 (7,500 rpm, 4°C, 1 h) to remove hemoglobin and proteins. Figure 3.1, the NO content was assessed by the Griess reaction method using 23479 Nitrate/nitrite Assay Kit Colorimetric kit (Sigma–Aldrich, Inc., USA).

Preparation of nitrite calibration curve

(1) Add $NaNO_2$ standard solution and buffer solution to each well as follows.

(2) Add 20 µl of buffer solution to each well (total volume is 100 µl/well).

(3) Add 50 µl of Griess Reagent A to each well, and mix.

(4) After 5 min, add 50 µl of Griess Reagent B to each well, and mix.

(5) Incubate for 10 min at room temperature, and measure the absorbance of each well at 540 nm with a microplate reader.

(6) Subtract the absorbance of the blank solution (well A) from the absorbance of each well.

(7) Plot the concentration of $NaNO_2$ solution on X-axis and the absorbance value on Y-axis to prepare the calibration curve.

Preparation of nitrate + nitrite calibration curve

(1) Add NaNO3 standard solution and buffer solution to each well as follows.

(2) Add 10 µl of the Nitrate Reductase solution and 10 µl of the Enzyme Co–factors solution to each well.

(3) Incubate the plate at room temperature (25°C) for 2 h.

(4) Add 50 µl of Griess Reagent A to each well, and mix. After 5 min, add 50 µl of Griess Reagent B to each well, and mix.

(5) Incubate for 10 min at room temperature, and measure the absorbance at 540 nm with a microplate reader.

(6) Subtract the absorbance of the blank solution (well E) from the absorbance of each well.

(7) Plot the concentration of $NaNO_3$ solution on X-axis and the absorbance value on Y-axis to prepare the calibration curve.

Determination of nitrite concentration in sample solution

(1) Add 80 µl of a sample solution to one well.

(2) Add 20 µl of buffer solution to each well.

(3) Add 50 µl of Griess Reagent A to each well, and mix.

(4) After 5 min., add 50 µl of Griess Reagent B to each well, and mix.

(5) Incubate for 10 min at room temperature, and measure the absorbance at 540 nm with a microplate reader.

(6) Subtract the absorbance of the blank solution (well A) from the absorbance of each well.

(7) Determine the concentration of nitrite in the sample solution from the calibration curve.

Determination of Nitrate + Nitrite Concentration in Sample Solution

(1) Add 80 µl of a sample solution to one well.

(2) Add 10 µl of the Nitrate Reductase solution and 10 µl of the enzyme co-factors solution to each well.

(3) Incubate the plate at 25°C for 2 h.

(4) Add 50 µl of Griess Reagent A to each well, and mix.

(5) After 5 min, add 50 µl of Griess Reagent B to each well, and mix.

(6) Incubate for 10 min at room temperature, and measure the absorbance at 540 nm with a microplate reader.

(7) Subtract the absorbance of the blank solution (well E) from the absorbance of each well.

(8) Determine the concentration of nitrate + nitrite in the sample solution using the calibration curve.

Determination of nitrate concentration in sample solution

Nitrate concentration can be obtained by the following equation.

[Nitrate] = [Nitrate + Nitrite] – [Nitrite]

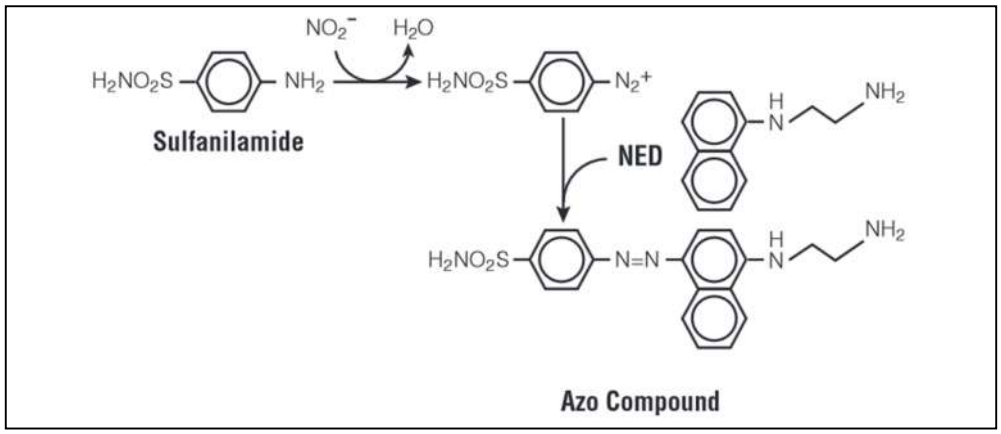


Figure 3.1 Coloring reaction scheme of NO^{2-} detection

b) Lipid peroxidation estimation

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde (MDA). Lipid peroxidation may contribute to the pathology of many diseases including atherosclerosis, diabetes, and Alzheimer's.

The LPO product malondialdehyde (MDA) was estimated by using Lipid Peroxidation (MDA) Assay Kit of thiobarbituric acid reactive substances (TBARS) in serum (Sigma–Aldrich, Inc., USA).

Preparation instructions

Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles. Use ultrapure water for the preparation of all reagents. Allow all components to come to room temperature before starting.

TBA solution – Reconstitute a bottle with 7.5 mL Glacial Acetic Acid, then adjust the final volume to 25 ml with water. Sonication can be used to assist dissolution if necessary. Store at room temperature and use within 1 week of preparation.

MDA standards for colorimetric detection

Dilute 10 µl of the 4.17 M MDA standard solution with 407 µl of water to prepare a 0.1 M MDA Standard Solution. Further dilute 20 µl of the 0.1 M MDA Standard Solution with 980 µl of water to prepare a 2 mM MDA Standard. Add 0, 2, 4, 6, 8, and 10 µl of the 2 mM MDA standard solution into separate microcentrifuge tubes, generating 0 (blank), 4, 8, 12, 16, and 20 nmole standards. Add water to each tube to bring the volume to 200 µl.

Sample preparation

Serum or Plasma samples (10 µl) were gently mixed with 500 µl of 42 mM H_2SO_4 in a microcentrifuge tube. Add 125 µl of phosphotungstic acid solution and mix by vortexing. Incubate at room temperature for 5 min and then centrifuge the samples at 13,000 rpm for 3 min. In a separate tube, add 2 µl of BHT (100x) to 100 µl of water. Re-suspend the pellet on ice with the water/BHT solution. Adjust the volume to 200 µl with water.

Assay reaction

(1) To form the MDA-TBA adduct, add 600 ml of the TBA solution into each vial containing standard and sample. Incubate at 95°C for 60 min. Cool to room temperature in an ice bath for 10 min. Pipette 200 ml from each reaction mixture into a 96 well plate for analysis.

(2) For colorimetric assays, measure the absorbance at 532 nm (A_{532}). The LPO products were expressed in terms of nmole MDA/µl.

Concentration of MDA

$$(S_a/S_v) \times 4 \times D = C$$

S_a = Amount of MDA in unknown sample (nmole) from standard curve

S_v = Sample volume (µl) or amount (µg) added into the wells

C = Concentration of MDA in sample

D = Dilution factor

4 = Correction factor for using 200 µl of 800 µl reaction

c) Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O_2.-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. To determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase. The SOD activity was estimated by using SOD Assay Kit–WST (19160 SOD determination kit, Sigma–Aldrich, Inc., USA).

SOD Assay Kit–WST allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST–1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in Figure 3.2 Therefore, the IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method.

Preparation of Working Solutions

(1) WST working solution: Dilute 1 ml of WST solution with 19 ml of buffer solution.

(2) Enzyme working solution: Centrifuge the enzyme solution tube for 5 sec. Mix by pipetting, and dilute 15 µl of enzyme solution with 2.5 ml of dilution buffer.

(3) SOD solution (for assay monitoring, if necessary): Dilute SOD with dilution buffer to prepare SOD standard solution as follows: 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, and 0.001 U/ml.

Concentrated protein assay

Refer to Table 3.1 for the amount of solutions in each well. If you are using a SOD standard, set up wells for it in the same manner as the sample.

(1) Add 20 µl of sample solution to each sample and blank 2 well, and add 20 µl of ddH_2O (double distilled water) to each blank 1 and blank 3 well.

(2) Add 200 µl of WST working solution to each well, and mix.

(3) Add 20 µl of Dilution Buffer to each blank 2 and blank 3 well.

(4) Add 20 µl of enzyme working solution to each sample and blank 1 well, and then mix thoroughly.

(5) Incubate the plate at 37°C for 20 min.

(6) Read the absorbance at 450 nm using a microplate reader.

(7) Calculate the SOD activity (inhibition rate %) using the following equation:

$$\text{SOD activity} = \{[(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})]/(A_{blank1} - A_{blank3})\} \times 100$$

Table 3.1 Amount of each solution for sample, blank 1, 2 and 3

	Sample	**Blank 1**	**Blank 2**	**Blank 3**
Sample solution	20 µl		20 µl	
ddH_2O		20 µl		20µl
WST working solution	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20µl		
Dilution buffer			20 µl	20 µl

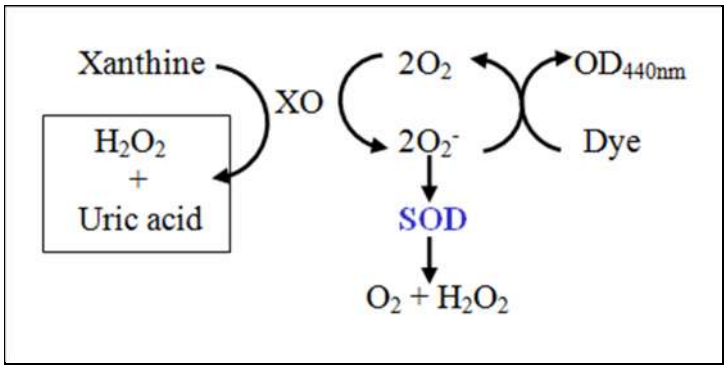


Figure 3.2 Principle of the SOD assay kit

3.4.1.4 Anti-inflammatory activity studies

a) Blood chemistry and hematological values

At the end of experiment, the rats fasted for 24 h, weighed and then euthanized with 50 mL of chloroform. 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 (Stanbio LiquiColor®) 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 analyses including 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) were performed (Stanbio LiquiColor®).

b) Tumor necrosis factor-α determination

Tumor necrosis factor-α (TNFα) is secreted by macrophages, monocytes, neutrophils, T cells, NK cells following their stimulation by bacterial lipopolysaccharides. TNFα shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Within hours after injection

TNFα leads to the destruction of small blood vessels within malignant tumors. TNFα also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes, and also modulates the expression of many other proteins.

TNFα determination was performing by using the Rat TNFα ELSA (Enzyme-Linked Immunosorbent Assay; Sigma-Aldrich, Inc., USA) kit. The kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of rat TNFα in cell lysate and tissue lysate. This assay employs an antibody specific for rat TNFα coated on a 96–well plate. Standards and samples are pipetted into the wells and TNFα present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-rat TNFα antibody is added. After washing away unbound biotinylated antibody, HRP–conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNFα bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

c) Colonic histology

The colon tissue was fixed in 10% buffer formaldehyde for 24 h. Cross sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non-colitis group. Full-thickness sections of 5 μm were obtained at different levels and stained with hematoxylin and eosin. The histologic was damage evaluated by one observer who was blinded to the experimental groups, according to the criteria described previously by Stucchi et al. (2000). Results were means of three histological slices for each animal, for each feeding period.

3.4.2 Toxicity studies

3.4.2.1 Acute toxicity

Acute toxicity study was performed according to Organization for Economic Cooperation and Development (OECD) guideline 423 adoption (2001). Rats were weighed and divided randomly into four groups with 8 rats in each; group 1; rats received phosphate buffered saline (PBS) (control group), group 2, 3, and 4; rats received KDML105KP 1,000, 2,000, and 4,000 mg/kg respectively, group 5, 6 and 7; rats received RHKP 1,000, 2,000, and 4,000 mg/kg respectively, and group 8, 9, and 10; rats received HNKP 1,000, 2,000, and 4,000 mg/kg respectively. The kefir powder was administered to the rats orally. Symptoms 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 euthanized by overdoses of chloroform. Blood samples were collected from the rat heart for the determination of blood biochemistry, and hematological values by using commercial kits (Stanbio LiquiColor®). Visceral organs including liver, lung, heart, kidney, and spleen were removed and weighed to calculate of the relative organ weight (ROW).

3.4.2.2 Sub-acute toxicity

Sub-acute toxicity study was conducted according to Organization for OECD guideline 407 (2001). The rats were randomly divided into four groups with 8 rats in each; group 1; rats received PBS (control group), group 2, 3 and 4; rats received KDML105KP 500, 1000, and 2000 mg/kg respectively, group 5, 6 and 7; rats received RHKP 500, 1000, and 2000 mg/kg respectively, and group 8, 9, and 10; rats received HNKP 500, 1000, and 2000 mg/kg respectively. The kefir powder was given orally to the rats every 2 days for 14 days. Symptoms of toxicity and mortal rats 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 euthanized by overdose of chloroform. Blood samples were collected from the rat hearts for the determination of blood biochemistry values, and hematological values by using commercial kits (Stanbio LiquiColor®). Visceral organs including liver, lung, heart, kidney, and spleen were removed and weighed to calculate the ROW.

3.4.2.3 Sub-chronic toxicity

Sub-chronic toxicity study was conducted according to the guidelines for sub-chronic toxicity studies for natural and traditional Chinese medicine. The rats were randomly divided into four groups with 8 rats in each; group 1; rats received PBS (control group), group 2; rats received 150 mg/kg of Khao Dawk Mali 105 brown rice kefir powder, group 3; rats received 150 mg/kg of Red Hawm brown rice kefir powder, and 4; rats received 150 mg/kg of Hawm Nil brown rice kefir powder respectively. The kefir powder was given orally to the rats every day for 90 days. Symptoms of toxicity and mortality of the rats were observed within 90 days. Body weight and food intake were recorded daily. At the end of experiments, the rats were fasted, weighed and then euthanized by overdose of chloroform. Blood samples were collected from the rat hearts

for the determination of blood biochemistry values, and hematological values by using commercial kits (Stanbio LiquiColor®). Visceral organs including liver, lung, heart, kidney, and spleen were removed and weighed to calculate the ROW.

3.5 Statistical analyses

The data 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 $p \leq 0.05$ were considered statistically significant.

CHAPTER 4

RESULTS

4.1 Biochemical components

4.1.1 Gamma amino butyric acid (GABA) content

GABA content performed by using HPLC revealed that GABA content in HNKP (10.87±0.01 mg/100 g) was significantly higher than that in RHKP (7.43±0.02 mg/100 g). However, it was not found in CMKP and KDML105KP (Table 4.1).

Table 4.1 The GABA values (mg GABA/100 g DW) of CMKP, KDMLKP, RHKP and HNKP.

Treatments	**GABA (mg/100 g DW)**
CMKP	Not detected
KDMLKP	Not detected
RHKP	7.43±0.02[b]
HNKP	10.87±0.01[a]

Mean values within column with different superscripts ([a and b]) are significantly different, *t-test* at p≤0.05.

4.1.2 Alpha-tocopherol (α-tocopherol) content

Figure 4.1 shows α-tocopherol content performed by using HPLC. HNKP and KDMLKP produced α-tocopherol higher than CMKP. The highest amount of α-tocopherol content (2.01±0.01 mg/100 g) was found in HNKP. The α-tocopherol content in KDMLKP was 1.61±0.02 mg/100 g, while the lowest α-tocopherol content (0.63±0.01 mg/100 g) was in RHKP.

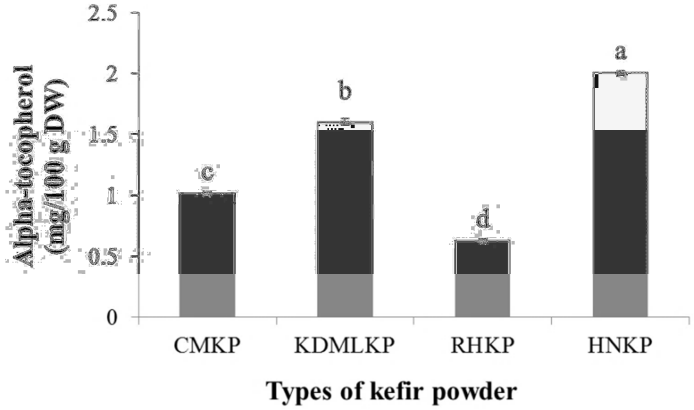


Figure 4.1 Alpha-tocopherol content (mg/100 g DW) of CMKP, KDMLKP, RHKP, and HNKP. Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

4.1.3 Total phenolic content (TPC)

TPC performed by using UV-Vis spectrophotometer revealed that HNKP exerted the highest TPC (29.88±0.81 mg GAE/g DW). Moreover, TPC in HNKP, KDMLKP (9.33±1.16 mg GAE/g DW) and RHKP (8.13±0.20 mg GAE/g DW) were significantly ($p \leq 0.05$) higher than that in CMKP (1.57±0.28 mg GAE/g DW) (Figure 4.2).

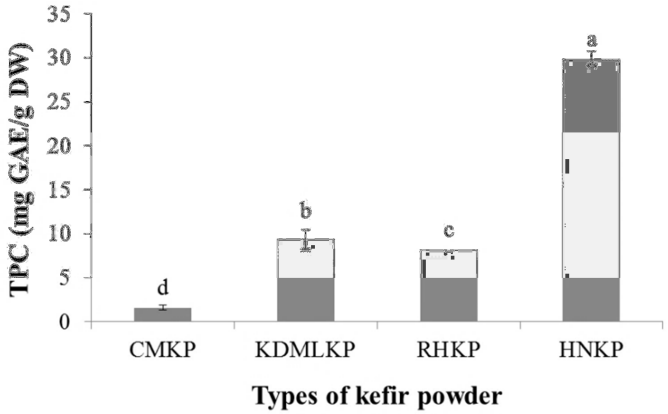


Figure 4.2 TPC (mg GAE/gDW) of CMKP, KDMLKP, RHKP and HNKP. Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

4.2 Antioxidant activity

4.2.1 DPPH radical scavenging assay

Antioxidant activity using DPPH assay revealed that RKP exibited antioxidant activity higher than CMKP (154.85± 9.87 mg/mL). The HNKP exerted the highest antioxidant activity with the lowest IC_{50} of 15.66±2.84 mg/mL. KDMLKP produced antioxidant activity with IC_{50} of 37.14±1.15 mg/mL. RHKP produced the lowest antioxidant activity with IC_{50} of 66.30±3.11 mg/mL (Table 4.2).

4.2.2 Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity using FRAP assay also revealed RKP exibited antioxidant activity higher than CMKP (2.40±0.22 mM $FeSO_4$/g). The rice kefir powder, HNKP exerted the highest FRAB value with 43.44±0.85 mM $FeSO_4$/g. RHKP produced FRAB value with 17.26±0.31 mM $FeSO_4$/g, while KDMLKP produced the lowest FRAB value with 14.02±0.25 mM $FeSO_4$/g (Table 4.2).

Table 4.2 Antioxidant activity of CMKP, KDMLKP, RHKP and HNKP.

Groups	**Antioxidant activity**	
	IC_{50} of DPPH radical scavenging activity (mg/ml)	**FRAB values (mM $FeSO_4$/g DW)**
Ascorbic acid	0.006±0.001[a]	-
CMKP	154.853±9.872[e]	9.929±0.612[d]
KDMLKP	37.142±1.151[c]	47.570±.633[c]
RHKP	66.300±3.106[d]	51.627±.568[b]
HNKP	15.657±2.841[b]	119.850±1.554[a]

Mean values within column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at $p \leq 0.05$.

4.3 Antioxidative stress in rats colitis

4.3.1 Nitric oxide (NO)

The NO level in the serum was higher in the colitis rats that received PBS (control) when compared to non-colitis rats (Table 4.16, $p \leq 0.05$). However, the colitis rats that received HNKP produced NO reduced levels when compared to negative controls ($p \leq 0.05$). Moreover, the colitis rats that received HNKP did not differ in NO level from the rats that received prednisolone, and non-colitis rats (Table 4.3).

Table 4.3 Nitric oxide (NO) levels in the treated rat colitis compared to those in controls.

Groups	**NO levels (nmol/mg)**
Non-colitis	
PBS	3.30 ± 0.24^{a}
HNKP	3.50 ± 0.36^{a}
CMKP	3.47 ± 0.41^{a}
Colitis	
PBS	14.50 ± 0.20^{c}
HNKP	4.20 ± 0.58^{ab}
CMKP	4.69 ± 0.44^{b}
Prednisolone	4.11 ± 0.62^{ab}

Mean values within each column with different superscripts $^{(a, b, c, \ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$.

4.3.2 Lipid peroxidation estimation (LPO)

The colitis rats that received PBS showed increased TBARS in serum (20.78 ± 0.58, $p \leq 0.05$). However, the colitis rats treated with HNKP produced reduced TBARS when compared to controls (10.10 ± 1.06 vs 20.78 ± 0.58, $p \leq 0.05$). Moreover, the colitis rats that received HNKP did not differ in TBARS levels from the rats that received prednisolone, and non-colitis rats (Table 4.4).

Table 4.4 TBARS levels in the treated rat colitis compared with controls.

Groups	TBARS levels (nmole MDA/μl)
Non-colitis	
PBS	8.25±1.06[a]
HNKP	8.23±0.75[a]
CMKP	8.24±0.88[a]
Colitis	
PBS	22.78±0.58[c]
HNKP	10.10±1.06[ab]
CMKP	10.45±1.49[b]
Prednisolone	9.78±1.05[ab]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at $p \leq 0.05$.

4.3.3 Superoxide dismutase activities (SOD)

An increase in SOD activity was observed in the colitis rats that received HNKP when compared to colitis rats that received PBS (controls). This indicated that the antioxidant defense system was functional in the colitis rats that received HNKP, similar findings of increased SOD were seen in the rats that received prednisolone, and cow's milk kefir powder (Table 4.5).

Table 4.5 Superoxide dismutase activities (SOD) in the treated rat colitis compared with controls.

Groups	SOD activity (%)
Non-colitis	
PBS	5.42±0.86[c]
HNKP	5.51±0.92[c]
CMKP	5.60±1.02[c]

Table 4.5 (Continued)

Groups	SOD activity (%)
Colitis	
PBS	2.27±0.97[a]
HNKP	4.35±1.13[b]
CMKP	4.18±0.90[b]
Prednisolone	5.01±0.78[b]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at $p \leq 0.05$.

4.4 Anti-inflammatory effect in rats colitis

4.4.1 Blood biochemistry

The colitis rats that received PBS (controls) significantly ($p \leq 0.05$) increased BS, BUN, UA, and TP when compared to the non-colitis rats (Table 4.6). Moreover, the colitis rats treated with rice kefir powder produced a reverse of BS, BUN, UA, and TP to normal levels, similar with prednisolone and cow's kefir powder. In addition, the colitis rats that received PBS also significantly ($p \leq 0.05$) increased ALT and ALP, while decreased AST when compared to the non-colitis rats (Table 4.7). However, the colitis rats treated with rice kefir powder exerted a reverse of ALT, ALP, and AST to normal levels.

The colitis rats treated with kefir powder and control did not differ in lipid profiles (Table 4.8).

Table 4.6 Blood biochemistry; BS, BUN, CREA, UA, TP, Alb, and Glob in the treated rat colitis compared to those in controls.

Groups	BS (mg/dl)	BUN (mg/dl)	CREA (mg/dl)	UA (mg/dl)	TP (g/dl)	Alb (g/dl)	Glob (g/dl)
Non-colitis							
PBS	268.70±2.96[a]	21.01±0.96[a]	0.95±0.04[a]	2.80±0.44[a]	6.05±0.10[a]	2.90±0.08[a]	2.61±0.40[a]
HNKP	255.61±1.90[a]	20.06±1.01[a]	0.96±0.06[a]	2.75±0.73[a]	6.03±0.11[a]	2.98±0.10[a]	2.65±0.55[a]
CMKP	266.50±1.08[a]	21.05±0.86[a]	0.94±0.74[a]	2.81±0.72[a]	6.05±0.14[a]	2.80±0.22[a]	2.64±0.62[a]
Colitis							
PBS	298.25±3.54[b]	25.90±0.67[b]	0.87±0.08[a]	3.15±0.40[b]	6.37±1.22[b]	2.92±0.14[a]	2.70±0.11[a]
HNKP	265.40±2.06[a]	21.01±0.97[a]	0.95±0.02[a]	2.70±0.90[a]	6.09±0.15[a]	2.88±0.87[a]	2.62±0.21[a]
CMKP	277.67±1.34[a]	21.12±1.63[a]	0.95±0.00[a]	2.67±0.81[a]	6.00±0.17[a]	2.97±0.13[a]	2.62±0.33[a]
Prednisolone	267.50±0.98[a]	21.15±1.60[a]	0.94±0.07[a]	2.80±0.70[a]	6.04±0.11[a]	2.85±0.21[a]	2.61±0.67[a]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at $p \leq 0.05$. BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid TP = total serum protein; Alb = albumin; Glob = globulin.

Table 4.7 Blood biochemistry; AST, ALT, and ALP in the treated rat colitis compared to those in controls.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
Non-colitis			
PBS	128.60±6.57[c]	47.67±1.53[ab]	94.67±6.60[a]
HNKP	124.50±8.63[c]	46.67±1.57[a]	93.82±8.44[a]
CMKP	126.44±5.55[c]	45.33±0.98[a]	93.90±7.64[a]
Colitis			
PBS	68.47±7.33[a]	78.67±1.44[c]	136.65±5.33[b]
HNKP	115.10±6.67[bc]	56.67±1.43[b]	93.67±8.24[a]
CMKP	116.50±7.58[bc]	55.48±1.21[b]	94.20±7.41[a]
Prednisolone	117.10±5.80[bc]	56.89±1.60[b]	93.85±8.55[a]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at $p \leq 0.05$. AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

Table 4.8 Cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) in the treated rat colitis compared to those in controls.

Groups	Lipid profiles (mg/dl)			
	CHO	TG	HDL	LDL
Non-colitis				
PBS	52.12±2.33	148.15±4.89	17.53±0.67	28.11±1.76
HNKP	51.67±1.37	146.22±5.40	18.12±0.45	27.15±1.42
CMKP	51.88±2.11	147.18±5.88	17.69±0.92	28.02±1.54
Colitis				
PBS	51.94±0.98	151.01±1.46	17.00±0.25	27.51±0.98
HNKP	50.17±1.56	146.17±3.67	17.77±0.60	27.76±1.70
CMKP	51.18±2.41	147.37±5.83	17.43±0.68	28.17±1.77
Prednisolone	51.19±2.52	148.10±2.91	17.73±0.55	27.32±1.98

4.4.2 Hematological values

The colitis rats produced increased WBC and Neu while RBC, Hb, and Hct decreased when compared with the colitis rats treated with kefir powder and prednisolone. In addition, the colitis rats treated with kefir powder did not differ in hematological values from non-colitis rats (Table 4.9).

Table 4.9 Hematological values in the treated colitis rat compared to those in controls.

Groups	WBC (10^3cell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
Non-colitis							
PBS	7.33±0.86^a	10.05±0.42^b	17.15±0.56^b	51.83±1.62^b	808.17±25.15^a	19.17±2.84^a	78.33±3.86^a
HNKP	7.35±0.77^a	9.67±0.25^b	16.70±0.52^b	51.00±1.68^b	866.50±31.20^a	18.75±2.24^a	77.25±1.65^a
CMKP	7.13±0.88^a	9.73±0.41^b	16.87±0.46^b	51.00±1.57^b	888.50±37.04^a	20.83±2.87^a	76.83±2.62^a
Colitis							
PBS	15.42±0.28^b	8.76±0.13^a	14.52±0.23^a	46.33±0.76^a	923.17±24.11^a	48.83±1.01^b	80.83±1.25^a
HNKP	7.34±0.56^a	10.04±0.40^b	17.18±0.57^b	51.80±1.02^b	817.11±25.15^a	18.57±2.64^a	77.63±1.46^a
CMKP	7.15±0.87^a	9.75±0.58^b	16.88±0.41^b	51.03±1.51^b	868.60±33.01^a	19.43±2.67^a	78.13±2.63^a
Prednisolone	6.95±0.77^a	9.87±0.28^b	16.76±0.50^b	51.68±1.62^b	855.50±34.00^a	17.95±2.21^a	77.15±1.05^a

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. WBC = white blood cells; RBC = red blood cells; Hb

= hemoglobin; Hct = hematocrit; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

4.4.3 Tumor necrosis factor-α (TNFα)

The TNFα level in serum of the colitis rats that received PBS (controls) was highest. However, the colitis rats treated with kefir powder produced reduced TNFα level when compared to those in controls (Table 4.10).

Table 4.10 Tumor necrosis factor-α (TNFα) levels in the treated colitis rat compared to those in controls.

Groups	**TNFα levels (ng/ml)**
Non-colitis	
PBS	0.23±0.02[a]
HNKP	0.22±0.01[a]
CMKP	0.23±0.01[a]
Colitis	
PBS	0.45±0.03[c]
HNKP	0.32±0.02[b]
CMKP	0.33±0.02[b]
Prednisolone	0.30±0.01[b]

Mean values within each column with different superscripts [(a, b, c,...)] are significantly different, Duncan's test at $p \leq 0.05$.

4.4.4 Histology of colon tissue

The colon tissues of rat colitis that received PBS showed high damage of cilia above villi (red arrow), the rat colitis treated with CMKP showed slight damage of cilia above villi (red arrow), while rat colitis treated with rice kefir powder (HNKP) and prednisolone did not produce damage when compare to those in controls (Figure 4.3).

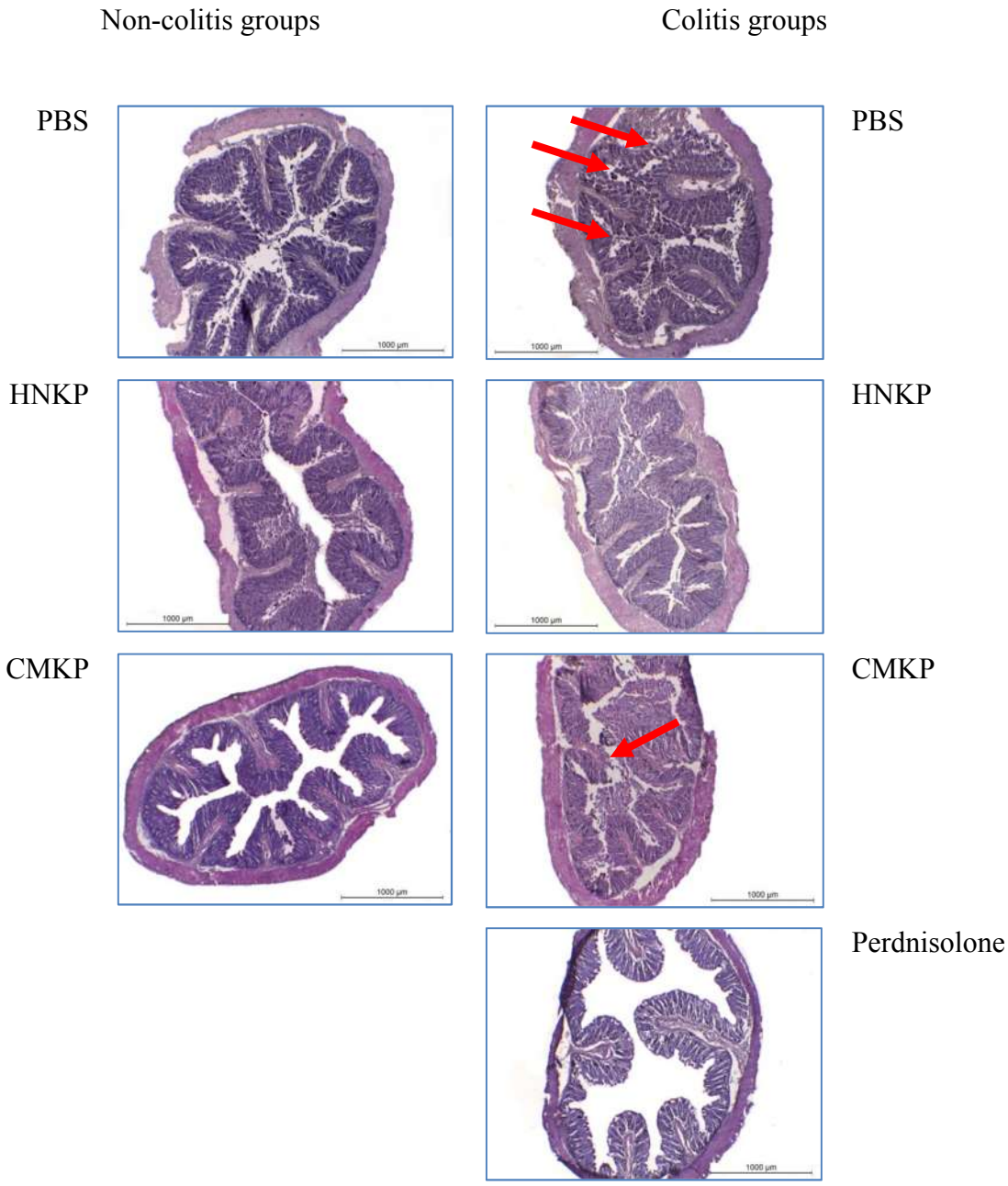


Figure 4.3 Colonic histological tissues of rat colitis and non-colitis at the end experiment.

4.5 Toxicity studies

4.5.1 Acute toxicity

4.5.1.1 Body weight gain, food intake, and feed conversion ratio

An increasing HNKP produced increase in body weight gain (Figure 4.4 C) while an increasing RHKP produced decrease in the body weight gain (Figure 4.4 B). However, RHKP did not alter the body weight gain (Figure 4.4 A) of the treated rats compared to that in controls. Food intake, increasing KDMLKP and RNKP also increased food intake (Figure 4.5 A and B). In contrast, increasing HNKP decreased food intake (Figure 4.5 C) in the treated rats compared to that in controls. FCR, increasing RHKP increased FCR (Figure 4.6 B). Nevertheless, KDMLKP and HNKP did not change FCR (Figure 4.6 A and C) in the treated rats compared to that in controls.

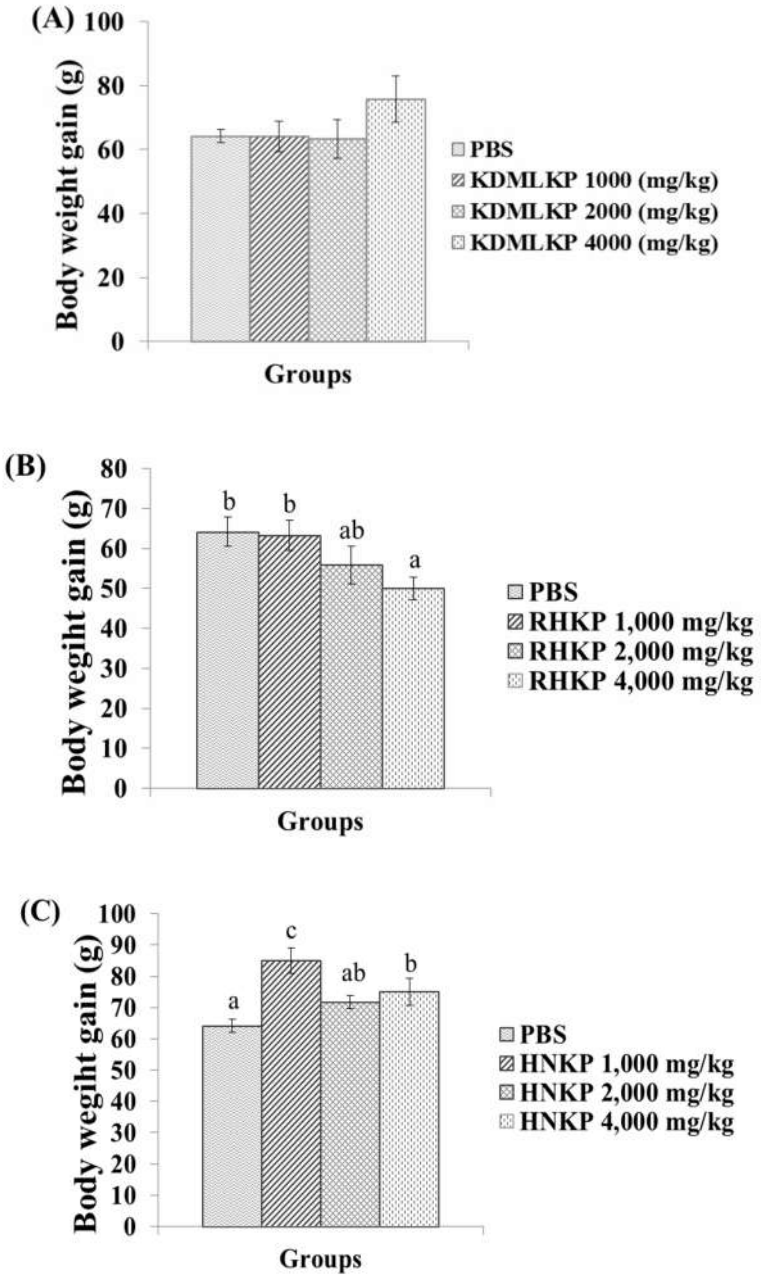


Figure 4.4 Body weight gain in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end experiment from acute toxicity (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

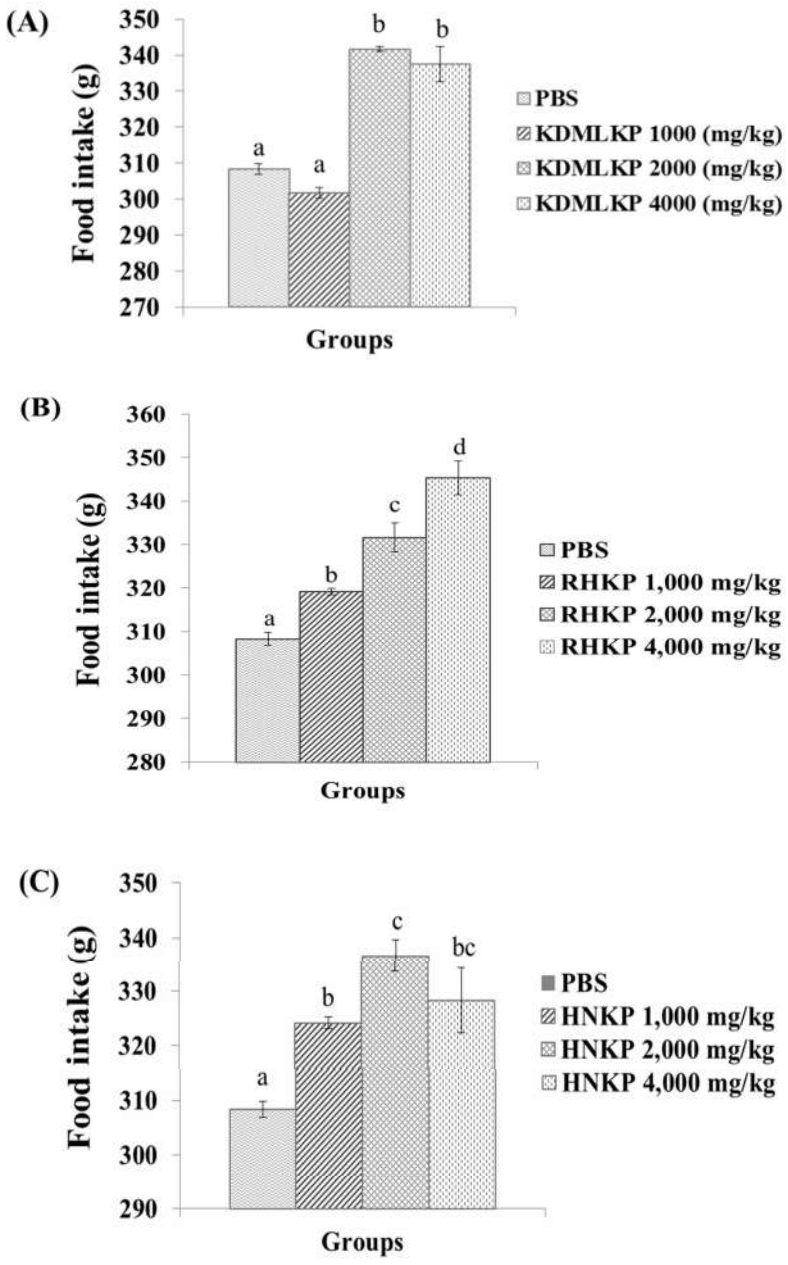


Figure 4.5 Food intake in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end experiment from acute toxicity (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

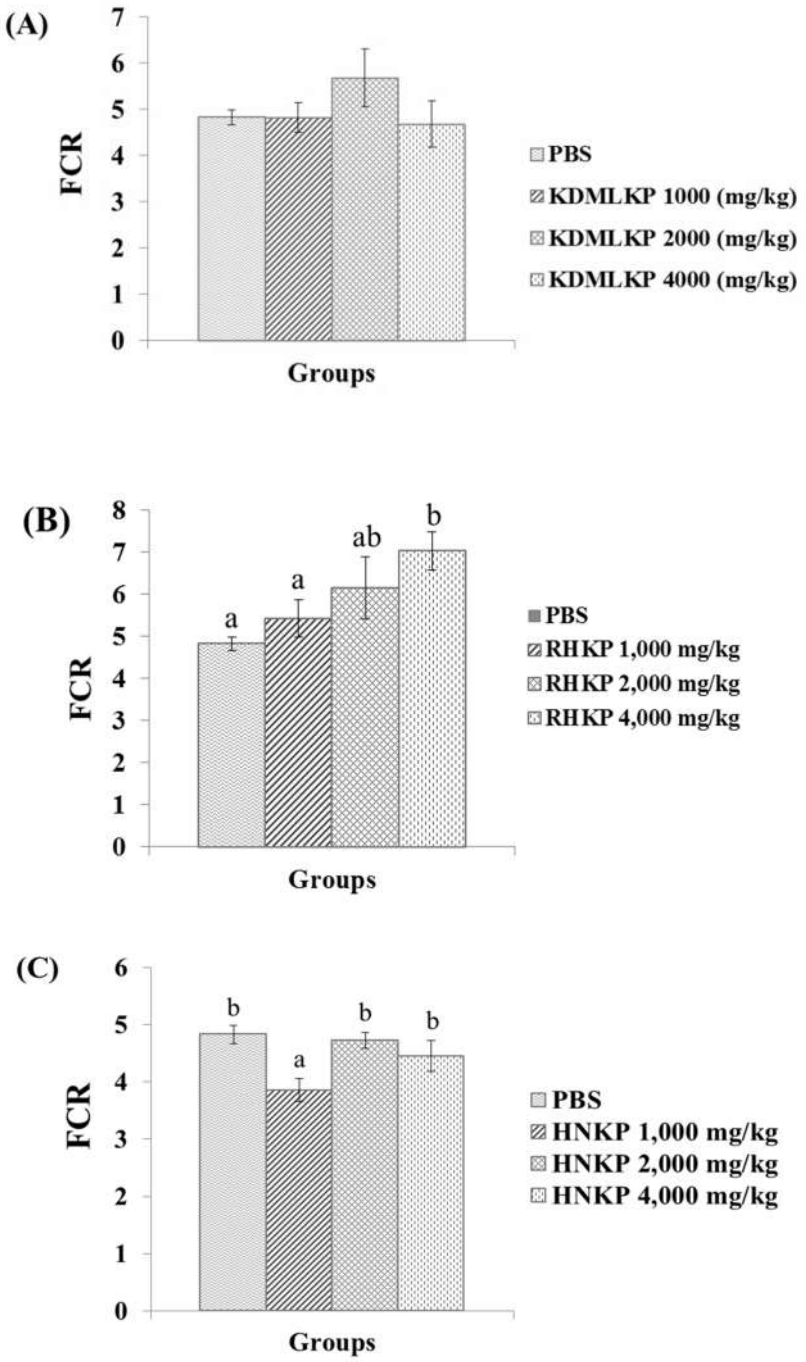


Figure 4.6 FCR in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end experiment from acute toxicity (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

4.5.1.2 Symptoms of toxicity, rat mortality, and relative organ weight

The rice kefir treated rats did not produce any symptoms of toxicity and mortality of the rats during 14 days (Data not shown). Moreover, the ROW in the rats treated with rice kefir powder did not differ from that in controls (Figure 4.7).

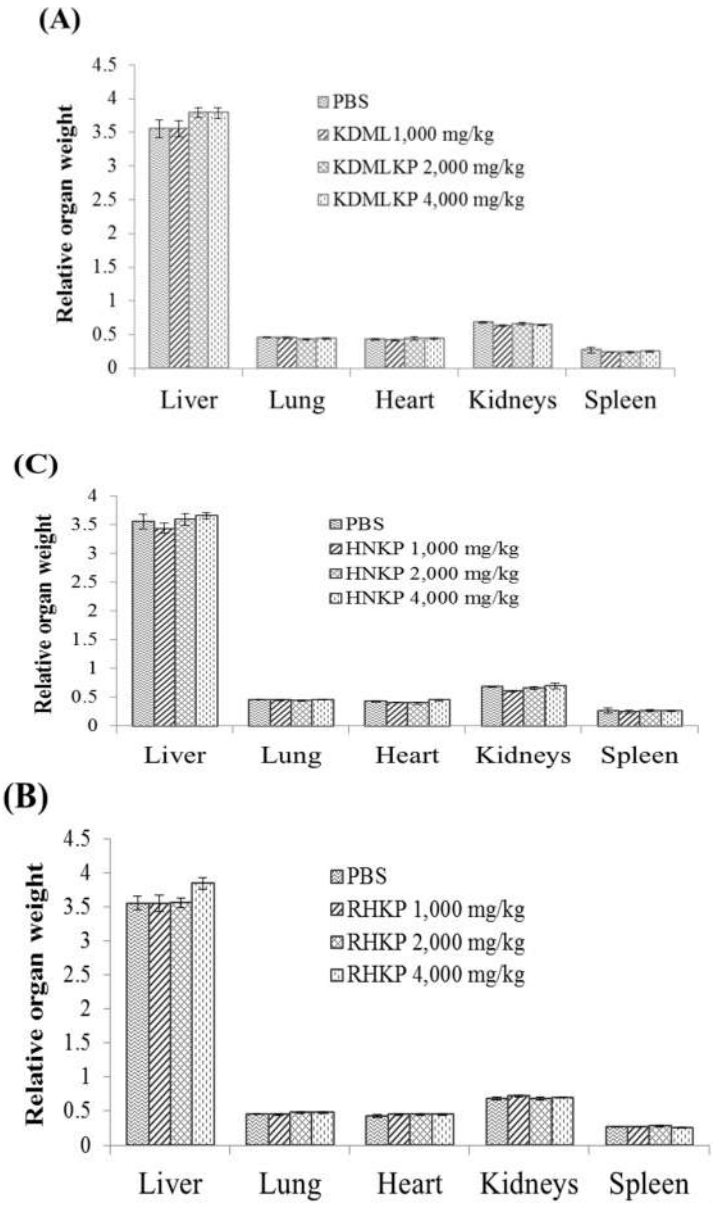


Figure 4.7 Relative organ weight in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to PBS at the end experiment from acute toxicity (mean±SEM).

4.5.1.3 Blood biochemistry

KDMLKP did not alter BS, BUN, CREA, and UA. However, increasing KDMLKP increased TP, Alb, and Glob ($p \leq 0.05$) in treated rats compared to those in controls. RHKP did not change BS, CREA, and Alb. Nevertheless, increasing RHKP increased BUN, TP, and Glob ($p \leq 0.05$) in treated rats compared to those in controls. On the other hand, reducing RHKP reduced UA in the treated rats. HNKP did not produce any alteration of BS, BUN, CREA, UA, TP, Alb, and Glob in the treated rats compared to those in controls (Table 4.11).

KDMLKP and HNKP did not alter AST, ALT, and ALP while increasing RHKP increased AST, and ALT ($p \leq 0.05$) in the treated rats compared to those in controls (Table 4.12).

These findings indicated that HNKP did not have an effect on hepatic and renal functions. In contrast, KDMLKP at a dose of 4,000 mg/kg had an effect on hepatic function, while this dose of RHKP had an effect both on hepatic and renal functions when compared with those in controls.

In addition, the rats treated with 4,000 mg/kg of KDMLKP, and RHKP significantly increased in CHO, TG, and HDL. However, the rats treated with HNKP did not differ from those in controls (Table 4.13).

Table 4.11 Blood biochemistry; BS, BUN, CREA, UA, TP, Alb, and Glob in the rats treated with PBS and kefir powder from acute toxicity study.

Groups	Blood biochemistry						
	BS (mg/dl)	**BUN (mg/dl)**	**CREA (mg/dl)**	**UA (mg/dl)**	**TP (g/dl)**	**Alb (g/dl)**	**Glob (g/dl)**
PBS	182.33±23.55^{ab}	20.17±0.48^{b}	0.91±0.03^{a}	3.61±0.$_{55ab}$	5.65±0.11^{ab}	3.47±0.04^{ab}	2.20± 0.06^{a}
KDMLKP							
1,000 mg/kg	189.50±11.84^{ab}	20.65±0.32^{b}	0.81±0.03^{a}	3.25±0.19^{ab}	5.85±0.06^{bc}	3.50±0.04^{ab}	2.40±0.06^{b}
2,000 mg/kg	200.50±6.99^{ab}	20.08±0.46^{ab}	0.82±0.02^{a}	3.50±0.20^{ab}	5.93±0.03^{bc}	3.52±0.03^{ab}	2.43±0.04^{bc}
4,000 mg/kg	213.83±7.98^{ab}	20.28±0.33^{b}	0.83±0.18^{a}	3.30±0.17^{ab}	6.00±0.18^{c}	4.03±0.59^{b}	2.45±0.04^{bc}
RHKP							
1,000 mg/kg	166.17±2.41^{a}	20.23±0.28^{b}	0.90±0.02^{a}	2.88±0.17^{a}	5.78±0.07^{bc}	3.37±0.07^{a}	2.35±0.07^{ab}
2,000 mg/kg	182.50±19.68^{ab}	19.87±0.42^{ab}	0.95±0.03^{a}	3.38±0.08^{ab}	5.87±0.02^{bc}	3.43±0.03^{ab}	2.50±0.08^{c}
4,000 mg/kg	202.17±11.50^{ab}	18.78±0.63^{a}	0.95±0.02^{a}	4.05±0.33^{b}	6.10±0.07^{c}	3.57±0.02^{ab}	2.53±0.06^{c}

Table 4.11 (Continued)

Groups	Blood biochemistry						
	BS (mg/dl)	BUN (mg/dl)	CREA (mg/dl)	UA (mg/dl)	TP (g/dl)	Alb (g/dl)	Glob (g/dl)
HNKP							
1,000 mg/kg	176.67±38.09^{ab}	19.75±0.37^{ab}	0.88±0.02^{a}	3.72±0.31^{ab}	5.47±0.17^{a}	3.45±0.08^{ab}	2.25±0.06^{ab}
2,000 mg/kg	214.83±21.09^{ab}	19.63±0.33^{ab}	0.85±0.02^{a}	3.65±0.31^{ab}	5.47±0.12^{a}	3.48±0.07^{ab}	2.18±0.05^{a}
4,000 mg/kg	235.33±25.04^{b}	20.62±0.51^{b}	0.87±0.02^{a}	4.12±0.07^{b}	5.63±0.02^{ab}	3.47±0.03^{ab}	2.17±0.04^{a}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid TP = total serum protein; Alb = albumin; Glob = globulin.

Table 4.12 Blood biochemistry; AST, ALT, and ALP in the rats treated with PBS and kefir powder from acute toxicity study.

Groups	Blood biochemistry (U/L)		
	AST	ALT	ALP
PBS	143.67±3.95^{ab}	39.33±1.17^{ab}	124.33±3.06^{ab}
KDMLKP			
1,000 mg/kg	136.33±5.29^{a}	42.33±1.69^{b}	127.33±2.24^{b}
2,000 mg/kg	148.33±5.98^{b}	40.67±1.23^{b}	127.50±3.27^{b}
4,000 mg/kg	144.50±3.95^{ab}	41.83±1.30^{b}	129.50±1.43^{bc}
RHKP			
1,000 mg/kg	142.50±8.61^{ab}	35.50±1.56^{a}	116.67±2.84^{a}
2,000 mg/kg	153.50±2.08^{bc}	42.33±3.12^{b}	132.67±3.68^{bc}
4,000 mg/kg	161.00±5.59^{c}	49.33±0.61^{c}	135.17±4.12^{c}
HNKP			
1,000 mg/kg	143.83±4.80^{ab}	38.17±2.21^{ab}	118.67±3.01^{a}
2,000 mg/kg	152.83±1.08^{bc}	35.00±0.68^{a}	123.00±2.31^{ab}
4,000 mg/kg	143.50±2.79^{ab}	37.50±0.96^{ab}	126.00±5.39^{b}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

Table 4.13 Cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) in rats treated with PBS and kefir powder from acute toxicity study.

Groups	Lipid profiles (mg/dl)			
	CHO	**TG**	**HDL**	**LDL**
PBS	53.67±1.28^{ab}	130.00±2.62^{ab}	16.48±0.27^{b}	32.50±2.11^{ab}
KDMLKP				
1,000 mg/kg	57.67±1.78^{bc}	128.17±4.61^{ab}	16.95±0.15^{bc}	36.33±2.11^{b}
2,000 mg/kg	58.67±1.56^{bc}	152.50±5.32^{bc}	17.10±0.13^{bc}	33.67±1.45^{ab}
4,000 mg/kg	59.67±2.17^{c}	154.50±4.62^{c}	19.85±0.91^{d}	31.50±1.80^{ab}
RHKP				
1,000 mg/kg	50.50±1.43^{a}	137.67±2.81^{b}	14.85±0.20^{a}	30.33±0.99^{a}
2,000 mg/kg	57.67±1.20^{bc}	142.33±3.76^{bc}	15.90±0.40^{ab}	33.67±1.36^{ab}
4,000 mg/kg	60.17±1.33^{c}	154.67±1.05^{c}	17.98±1.01^{c}	35.33±1.50^{b}
HNKP				
1,000 mg/kg	56.50±1.91^{bc}	127.33±4.14^{ab}	16.57±0.55^{bc}	32.00±0.68^{ab}
2,000 mg/kg	58.67±0.88^{bc}	123.50±3.64^{a}	16.62±0.57^{bc}	32.17±0.98^{ab}
4,000 mg/kg	58.67±1.63^{bc}	123.17±4.73^{a}	17.10±0.29^{bc}	35.33±0.88^{b}

Mean values within each column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$.

4.5.1.4 Hematological values

KDMLKP, RHKP, and HNKP did not alter WBC, Hb, Hct, and Plt in the treated rats compared to those in controls (Table 4.14) at a dose 4,000 mg/kg of RHKP. Nevertheless, the rats that received a dose 4,000 mg/kg of KDMLKP and RHKP showed RBC significantly reduced ($p \leq 0.05$) while the rats that received HNKP showed no significance difference compared to those in controls. Moreover, increasing KDMLKP, RHKP and HNKP decreased Neu ($p \leq 0.05$), and increased Lym ($p \leq 0.05$) compared to those in controls.

Table 4.14 Hematological values in the rats treated with PBS and kefir powder from acute toxicity study.

Groups	Hematological values						
	WBC (10^3 ell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
PBS	5.93±0.16^{ab}	8.87±0.13^{bc}	17.07±0.40^{ab}	53.83±0.87^{ab}	943.17±27.15^{b}	8.50±0.22^{d}	91.00±1.21^{a}
KDMLKP							
1,000 mg/kg	6.18±0.22^{ab}	8.88±0.12^{bc}	17.18±0.11^{b}	52.17±0.70^{ab}	830.00±53.83^{ab}	8.17±0.31^{d}	90.33±2.04^{a}
2,000 mg/kg	5.90±0.16^{ab}	8.29±0.34^{ab}	16.08±0.60^{ab}	51.17±1.49^{ab}	812.67±50.67^{ab}	5.67±0.67^{bc}	94.33±0.67^{bc}
4,000 mg/kg	6.38±0.17^{b}	8.22±0.22^{a}	16.45±0.27^{ab}	51.17±1.01^{ab}	851.33±46.61^{ab}	4.67±0.61^{b}	95.50±0.62^{b}
RHKP							
1,000 mg/kg	6.33±0.39^{b}	8.67±0.20^{b}	17.12±0.40^{b}	56.00±1.88^{b}	881.33±20.51^{b}	7.00±0.26^{cd}	91.33±0.42^{ab}
2,000 mg/kg	6.17±0.21^{ab}	8.33±0.10^{ab}	17.00±0.06^{ab}	52.00±0.45^{ab}	881.17±20.15^{b}	6.33±0.88^{c}	92.83±0.70^{ab}
4,000 mg/kg	5.93±0.35^{ab}	8.16±0.05^{a}	16.05±0.09^{ab}	51.83±0.48^{a}	743.50±74.12^{a}	3.67±0.56^{ab}	96.17±0.79^{bc}
HNKP							
1,000 mg/kg	5.55±0.23^{a}	8.76±0.21^{b}	16.30±0.21^{ab}	52.67±1.31^{ab}	904.50±13.18^{b}	6.17±0.17^{c}	94.00±0.26^{b}
2,000 mg/kg	5.93±0.22^{ab}	9.00±0.12^{c}	16.35±0.32^{ab}	51.83±1.14^{ab}	883.00±23.23^{b}	2.50±0.43^{a}	97.00±1.21^{c}
4,000 mg/kg	5.40±0.27^{a}	8.99±0.05^{c}	16.28±0.28^{ab}	51.50±0.22^{ab}	924.83±17.63^{b}	2.33±0.33^{a}	97.83±0.31^{c}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

4.5.2 Sub-acute toxicity

4.5.2.1 Body weight gain, food intake, and feed conversion ratio

Increasing KDMLKP significantly ($p \leq 0.05$) increased body weight gain, while increasing HNKP significantly ($p \leq 0.05$) decreased the body weight gain. However, all the doses of RHKP did not alter the body weight gain compared to those in controls (Figure 4.8). Food intake, increasing KDMLKP, and HNKP also significantly ($p \leq 0.05$) increased food intake. In contrast, increasing RHKP significantly ($p \leq 0.05$) decreased food intake in the treated rats compared to those in controls (Figure 4.9). Increasing RHKP significantly ($p \leq 0.05$) increased FCR. Nevertheless, KDMLKP and HNKP did not change FCR in the treated rats compared to those in controls (Figure 4.10).

4.5.2.2 Symptoms of toxicity, rat mortality, and relative organ weight

The results showed that all the doses of the rice kefir did not produce any symptoms of toxicity and mortality of the rats during 14 days (Data not shown).

Moreover, the ROW in the rats treated with rice kefir powder did not differ from that in controls (Figure 4.11).

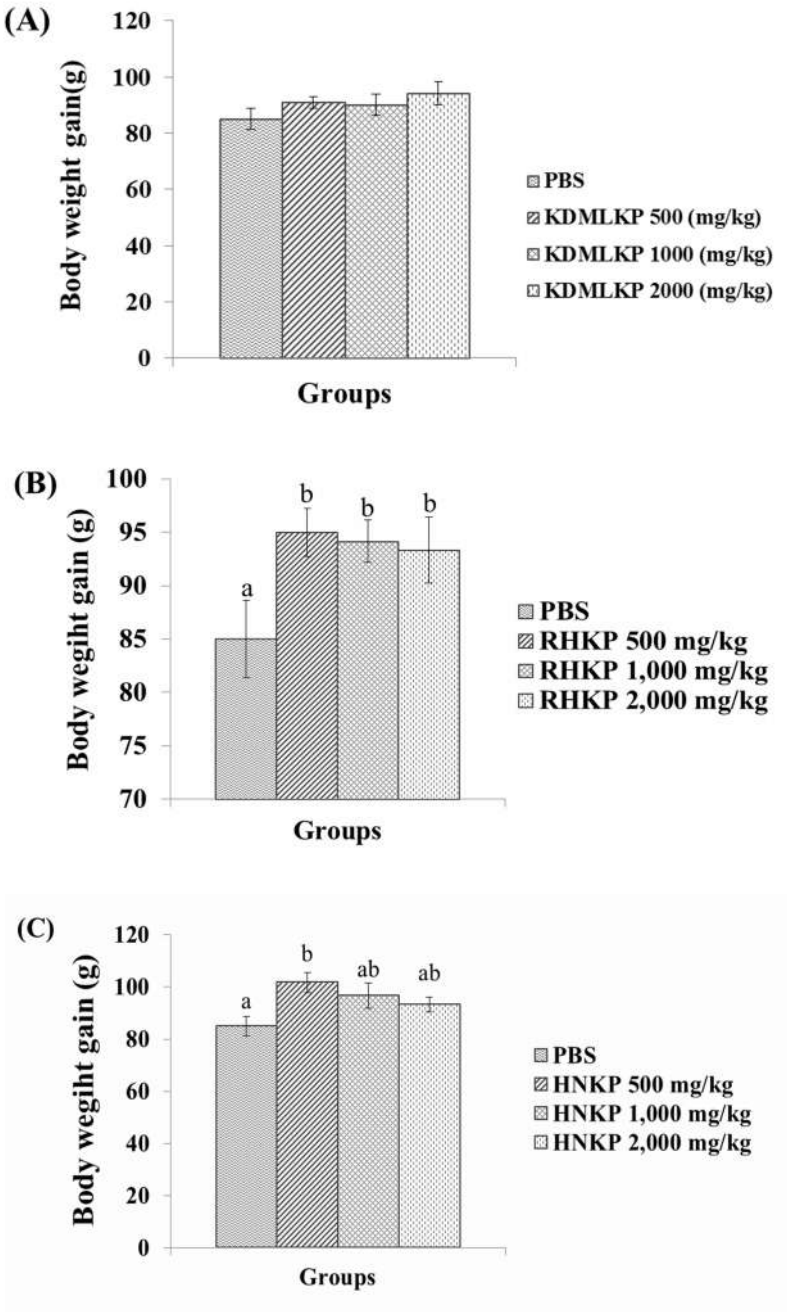


Figure 4.8 Body weight gain in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end of the experiment on sub-acute toxicity study (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

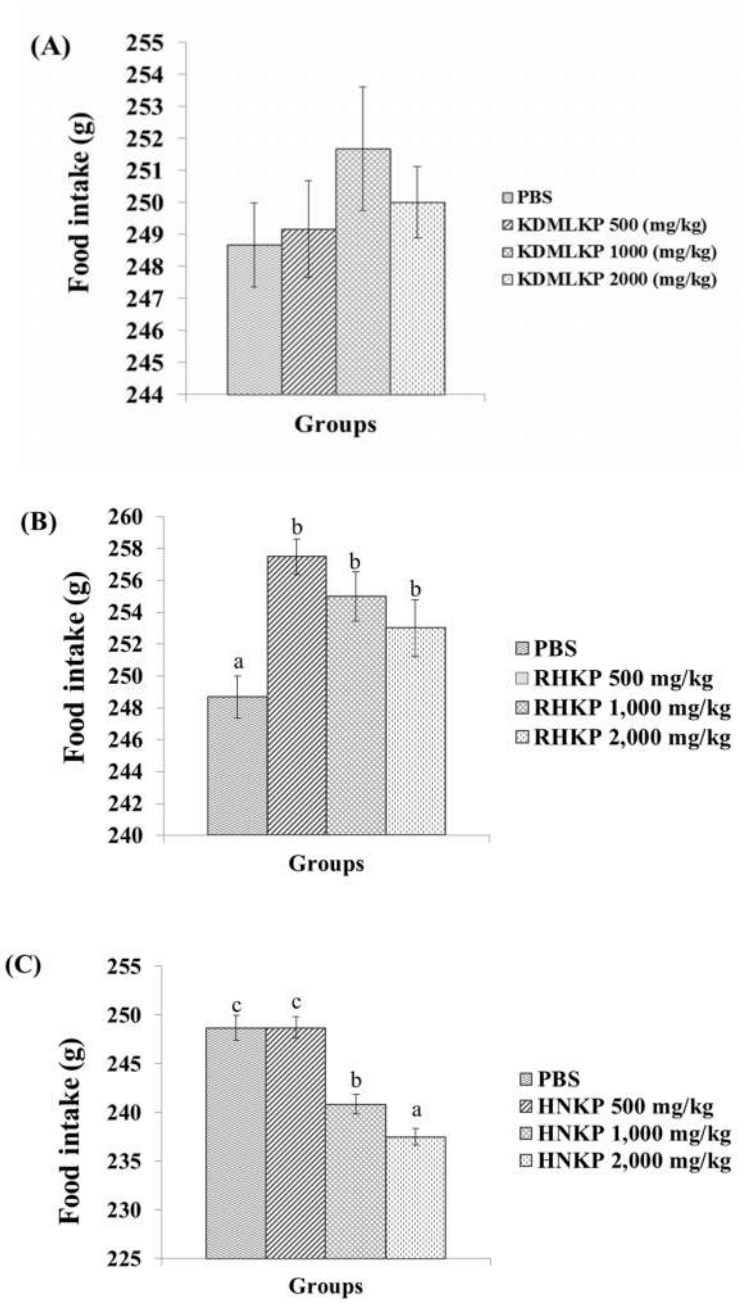


Figure 4.9 Food intake in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end of the experiment on sub-acute toxicity (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

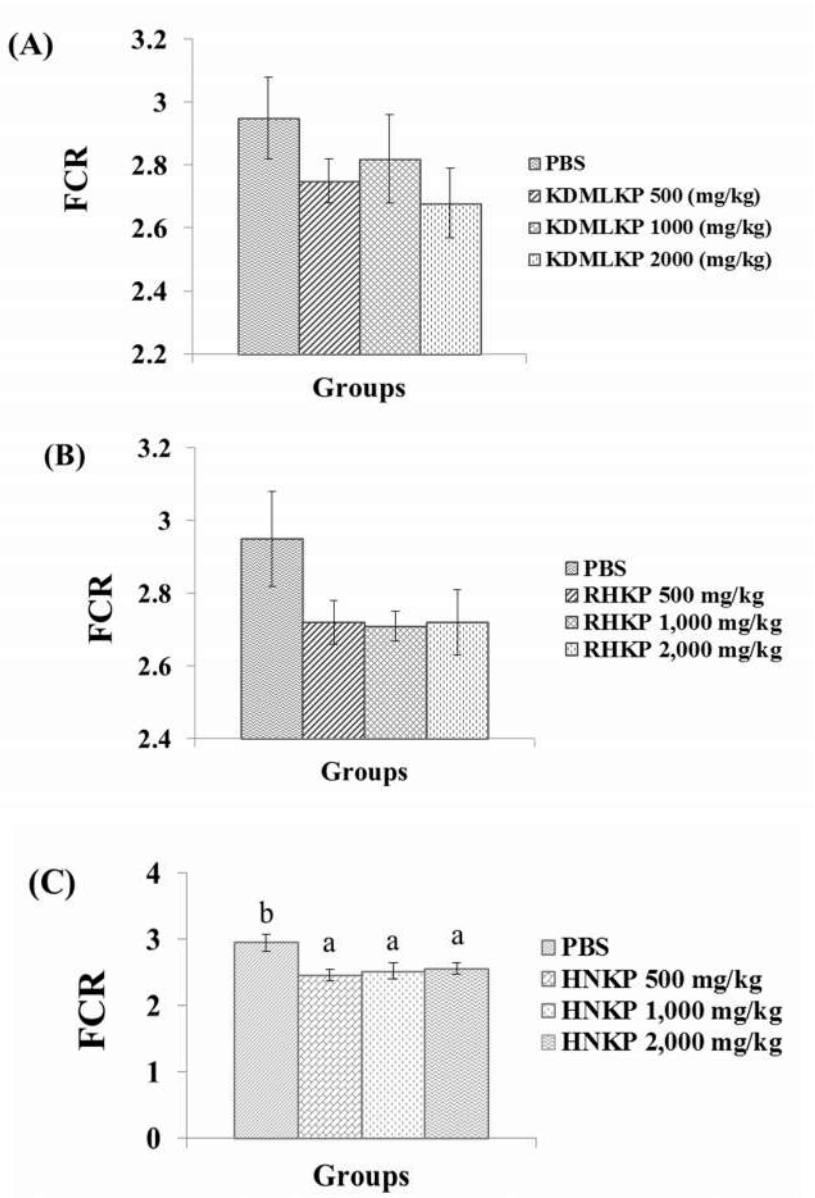


Figure 4.10 FCR in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to those in control (PBS) at the end of the experiment on sub-acute toxicity (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

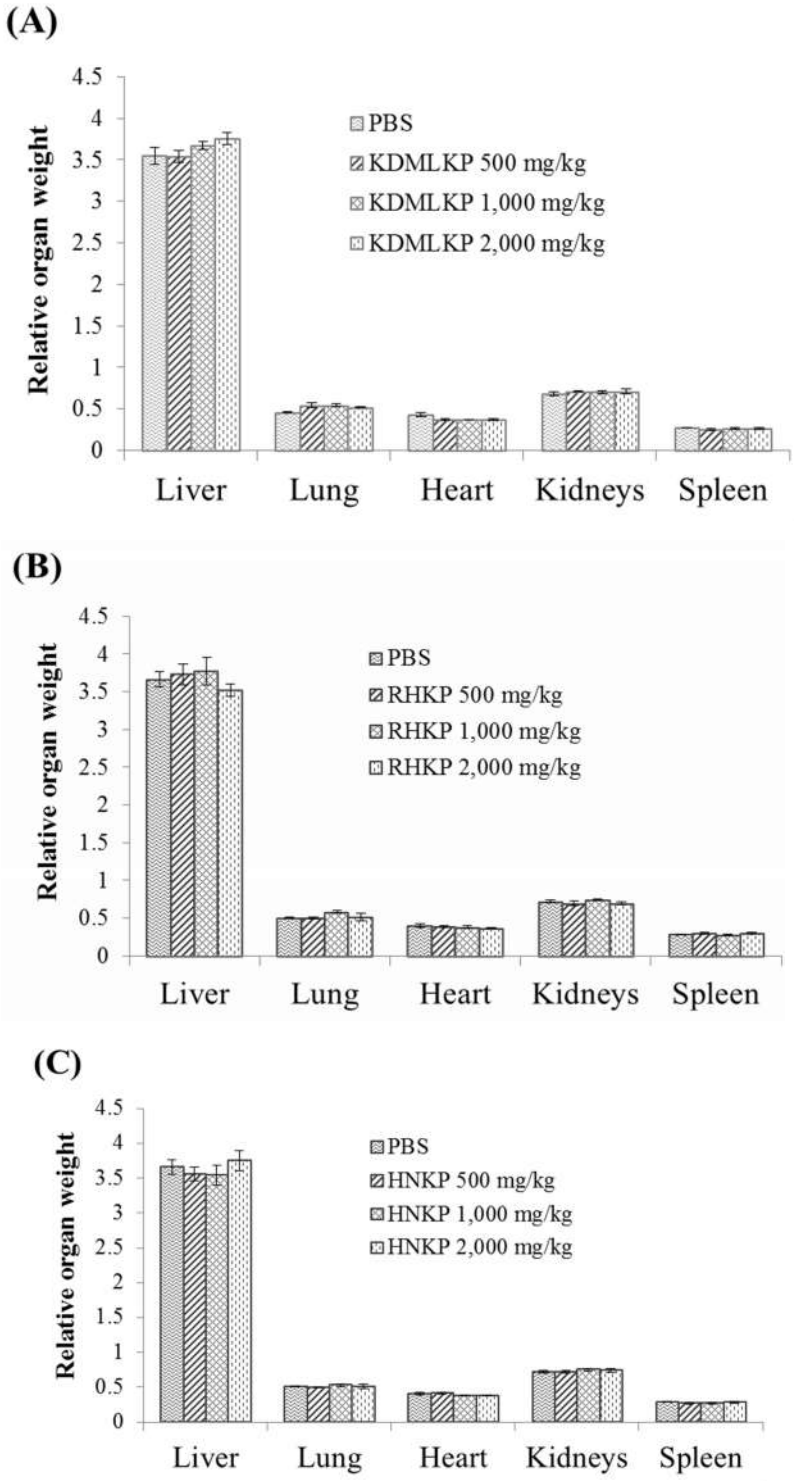


Figure 4.11 Relative organ weight in rats treated with KDMLKP (A), RHKP (B), and HNKP (C) compared to PBS at the end of the experiment on sub-acute toxicity (mean±SEM).

Table 4.15 Blood biochemistry; BS, BUN, CREA, UA, TP, Alb, and Glob in the rats treated with PBS and kefir powder from sub-acute toxicity study.

Groups	BS (mg/dl)	BUN (mg/dl)	CREA (mg/dl)	UA (mg/dl)	TP (g/dl)	Alb (g/dl)	Glob (g/dl)
PBS	195.83±7.25^{d}	20.45±1.12^{c}	0.82±0.03^{bc}	2.65±0.34^{b}	5.72±0.13^{ab}	3.28±0.06^{b}	2.48±0.09^{a}
KDML105KP							
500 mg/kg	176.83±7.78^{c}	21.10±1.03^{c}	0.87±0.02^{c}	2.72±0.36^{b}	5.92±0.10^{b}	3.25±0.08^{b}	2.60±0.06^{ab}
1,000 mg/kg	160.67±7.53^{bc}	20.12±0.91^{c}	0.80±0.02^{b}	2.28±0.14^{ab}	5.77±0.12^{ab}	3.22±0.08^{b}	2.72±0.17^{ab}
2,000 mg/kg	139.83±8.95^{b}	20.07±0.59^{c}	0.80±0.04^{b}	2.20±0.36^{ab}	5.70±0.14^{ab}	3.12±0.05^{b}	2.85±0.04^{bc}
RHKP							
500 mg/kg	190.50±9.35^{d}	19.22±0.70^{bc}	0.85±0.06^{b}	2.37±0.38^{ab}	5.60±0.14^{ab}	2.77±0.06^{a}	2.72±0.07^{ab}
1,000 mg/kg	144.17±14.15^{b}	19.22±0.66^{bc}	0.77±0.02^{ab}	2.02±0.28^{ab}	5.58±0.10^{ab}	2.75±0.07^{a}	2.80±0.06^{bc}
2,000 mg/kg	99.00±9.02^{a}	19.15±0.53^{bc}	0.73±0.03^{a}	1.53±0.17^{a}	5.52±0.12^{ab}	2.73±0.04^{a}	2.88±0.09^{bc}
HNKP							
500 mg/kg	156.50±19.70^{bc}	19.27±0.71^{bc}	0.90±0.07^{c}	2.62±0.28^{b}	5.63±0.15^{ab}	2.72±0.05^{a}	2.73±0.05^{ab}
1,000 mg/kg	128.67±9.30^{ab}	17.53±0.55^{ab}	0.78±0.03^{ab}	1.65±0.25^{a}	5.43±0.08^{a}	2.67±0.03^{a}	2.73±0.08^{ab}
2,000 mg/kg	85.50±7.64^{a}	16.47±0.27^{a}	0.83±0.03^{bc}	1.48±0.19^{a}	5.73±0.18^{ab}	2.67±0.05^{a}	3.02±0.06^{c}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid TP = total serum protein; Alb = albumin; Glob = globulin.

Table 4.16 Blood biochemistry; AST, ALT, and ALP in the rats treated with PBS and kefir powder from sub-acute toxicity study.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
PBS	137.00±1.53^{ab}	49.17±1.66^{a}	132.83±4.66^{ab}
KDMLKP			
500 mg/kg	114.67±5.13^{a}	51.33±2.58^{ab}	137.50±3.82^{b}
1,000 mg/kg	117.33±3.56^{a}	51.00±3.13^{ab}	133.33±3.05^{ab}
2,000 mg/kg	144.67±4.52^{bc}	50.50±1.38^{a}	131.00±3.47^{ab}
RHKP			
500 mg/kg	141.33±1.93^{b}	49.33±1.11^{a}	128.83±9.19^{ab}
1,000 mg/kg	152.83±6.36^{bc}	49.50±1.69^{a}	125.83±8.09^{ab}
2,000 mg/kg	164.83±3.50^{cd}	57.67±2.59^{b}	114.17±3.59^{a}

Table 4.16 (Continued)

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
HNKP			
500 mg/kg	140.00±3.18^{b}	47.33±1.41^{a}	119.83±6.82^{ab}
1,000 mg/kg	151.67±5.16^{bc}	50.33±1.33^{a}	116.67±3.37^{a}
2,000 mg/kg	157.33±4.58^{cd}	53.83±3.70^{ab}	112.67±3.25^{a}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

4.5.2.3 Blood biochemistry

The results of this study showed the rats that received KDMLKP, RNKP, and HNKP decreased BS, BUN, CREA, UA, and Alb, while Glob increased when compared to those in controls (Table 4.15). However, the rats that received all the doses of rice kefir powder did not differ in AST, ALT, and ALP when compared to those in controls, without a dose of 2,000 mg/kg of RHKP, and HNKP (Table 4.16).

These findings indicated that KDMLKP, RNKP, and HNKP may have an effect on renal function but not an effect on hepatic function. In contrast, RNKP and HNKP at a dose of 2,000 mg/kg had an effect on hepatic function.

Moreover, increasing KDMLKP decreased TG, while increasing RHKP and HNKP decreased CHO, TG, and HDL when compared to those in controls. However, all the doses of rice kefir powder did not have an effect on LDL level in the treated rats (Table 4.17).

Table 4.17 Cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) in rats treated with PBS and kefir powder from sub-acute toxicity study.

Groups	Lipid profiles (mg/dl)			
	CHO	TG	HDL	LDL
PBS	54.50±3.97^{bc}	148.83±4.67^{d}	20.21±0.66^{ab}	31.17±0.74^{a}
KDMLKP				
500 mg/kg	57.33±1.82^{c}	147.17±2.79^{d}	21.14±0.36^{b}	31.17±1.30^{a}
1,000 mg/kg	52.33±1.69^{b}	123.00±4.76^{c}	20.41±1.12^{ab}	34.04±1.94^{a}
2,000 mg/kg	50.67±1.47^{ab}	122.33±4.51^{c}	20.54±0.81^{ab}	34.79±2.12^{a}
RHKP				
500 mg/kg	48.67±0.94^{ab}	108.67±7.95^{bc}	19.22±0.87^{a}	29.42±2.14^{a}
1,000 mg/kg	45.83±1.38^{a}	106.17±9.95^{bc}	19.14±0.88^{a}	29.42±2.19^{a}
2,000 mg/kg	45.83±1.01^{a}	80.83±5.91^{a}	19.01±0.82^{a}	31.29±1.19^{a}
HNKP				
500 mg/kg	49.33±2.21^{ab}	109.00±3.34^{bc}	19.45±0.74^{ab}	31.17±1.46^{a}
1,000 mg/kg	48.17±2.47^{ab}	100.67±5.16^{bc}	18.34±1.10^{a}	32.54±2.27^{a}
2,000 mg/kg	46.50±1.05^{a}	94.33±3.38^{ab}	18.75±1.34^{a}	30.79±1.63^{a}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$.

Table 4.18 Hematological values in the rats treated with PBS and kefir powder from sub-acute toxicity study.

Groups	WBC (10^3cell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
PBS	6.42±0.28^{b}	8.76±0.13^{a}	17.52±0.23^{b}	53.33±0.76^{b}	923.17±24.11^{a}	8.83±1.01^{d}	90.83±1.25^{ab}
KDMLKP							
500 mg/kg	6.93±0.44^{bc}	8.46±0.20^{a}	16.78±0.32^{ab}	51.83±0.87^{ab}	882.83±4.82^{a}	8.00±0.58^{c}	89.83±1.22^{a}
1,000 mg/kg	6.95±1.10^{bc}	8.40±0.13^{a}	16.95±0.32^{ab}	51.50±0.76^{ab}	888.33±8.35^{a}	6.50±0.56^{bc}	93.17±0.79^{bc}
2,000 mg/kg	8.63±0.74^{d}	8.37±0.13^{a}	16.90±0.27^{ab}	51.83±0.87^{ab}	922.83±14.71^{a}	3.67±0.56^{a}	95.50±0.81^{c}
RHKP							
500 mg/kg	7.80±0.45^{bc}	8.28±0.12^{a}	16.83±0.28^{ab}	50.67±1.11^{ab}	837.00±68.15^{a}	8.00±1.37^{c}	90.67±1.47^{ab}
1,000 mg/kg	5.53±0.28^{ab}	8.24±0.09^{a}	16.63±0.24^{ab}	49.83±0.48^{ab}	890.33±29.69^{a}	7.00±1.79^{bc}	91.00±3.88^{ab}
2,000 mg/kg	5.13±0.28^{a}	8.19±0.19^{a}	16.30±0.28^{a}	48.50±0.92^{a}	947.50±31.95^{a}	5.00±1.12^{ab}	92.50±1.98^{b}

Table 4.18 (Continued)

Groups	WBC (10^3cell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
HNKP							
500 mg/kg	5.77±0.43^{ab}	8.38±0.14^{a}	16.70±0.35^{ab}	51.00±0.68^{ab}	887.33±24.78^{a}	7.33±1.54^{bc}	91.83±1.87^{ab}
1,000 mg/kg	6.50±0.26^{b}	8.63±0.41^{a}	16.88±0.44^{ab}	50.67±1.38^{ab}	893.67±42.25^{a}	5.83±0.94^{b}	95.17±0.87^{c}
2,000 mg/kg	5.60±0.40^{ab}	8.13±0.18^{a}	16.32±0.47^{a}	48.67±1.36^{a}	905.67±46.40^{a}	4.83±1.05^{ab}	96.33±0.71^{c}

Mean values within each column with different superscripts $^{(a, b, c,\ldots)}$ are significantly different, Duncan's test at $p \leq 0.05$. WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

4.5.2.4 Hematological values

KDMLKP, RHKP, and HNKP did not alter WBC, Hb, Hct, and Plt in the treated rats compared to those in controls at a dose 2,000 mg/kg. Nevertheless, the rats received at a dose 2,000 mg/kg of KDMLKP, and RHKP showed RBC significantly reduced ($p \leq 0.05$), while the rats that received HNKP showed no significantly difference compared to those in controls. In addition, at this dose of RHKP and HNKP Hb and Hct were significantly reduced ($p \leq 0.05$) compared to those in controls. Increasing KDMLKP, RHKP, and HNKP decreased Neu ($p \leq 0.05$) while they increased Lym ($p \leq 0.05$), compared to those in controls (Table 4.18).

4.5.3 Sub-chronic toxicity

4.5.3.1 Body weight gain, food intake, and feed conversion ratio

The treated rats with KDMLKP, RHKP, and HNKP did not alter in body weight gain and food intake (Figure 4.12 A and B) when compared to those in controls. Nevertheless, the treated rats with KDMLKP produced a decreasing FCR, while RHKP and HNKP did not change FCR (Figure 4.12 C) when compared to those in controls.

4.5.3.2 Symptoms of toxicity, rat mortality, and relative organ weight

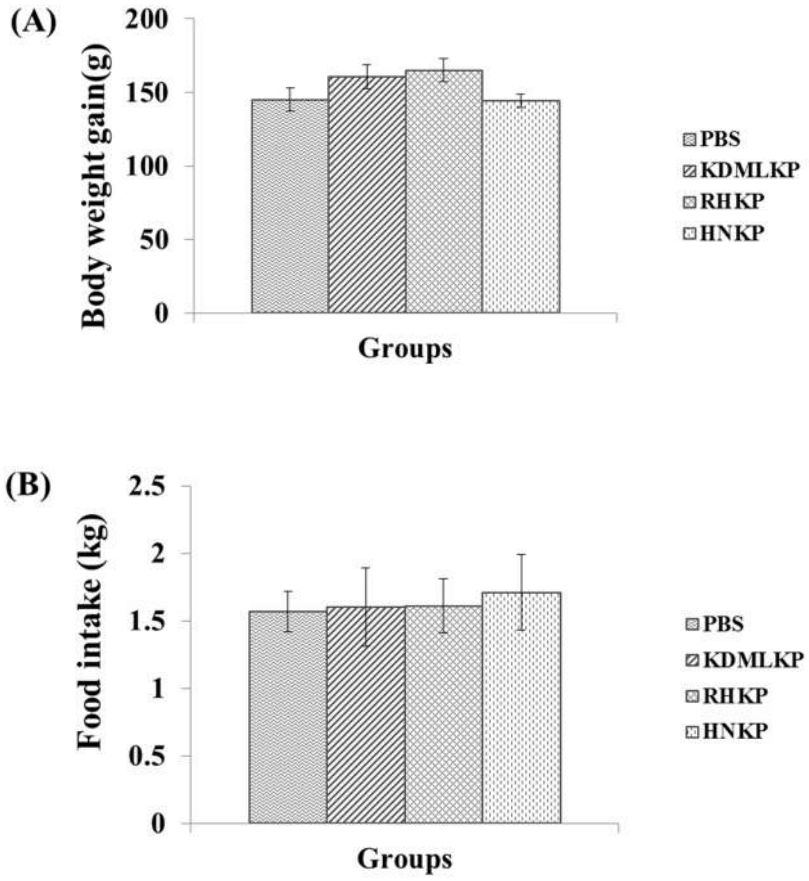
The rats that received kefir powder did not produce any symptoms of toxicity after 90 days. Moreover, the ROW in the rats treated with rice kefir powder did not differ from those in controls (Figure 4.13).

4.5.3.3 Blood biochemistry

Blood biochemistry including BS, BUN, CREA, UA, Alb, TP, Glob, AST, ALT, and ALP in the rats that received rice kefir powder did not alter from those in controls (Table 4.19 and 4.20).

These findings indicated that KDML105KP, RHKP, and HNKP had no sub-chronic toxicity as they had no effect on hepatic and renal functions.

In addition, the rats treated with KDMLKP and HNKP did not differ in CHO, TG, HDL, and LDL from those in controls. However, the rats treated with RHKP showed higher HDL than those in controls (Table 4.21).



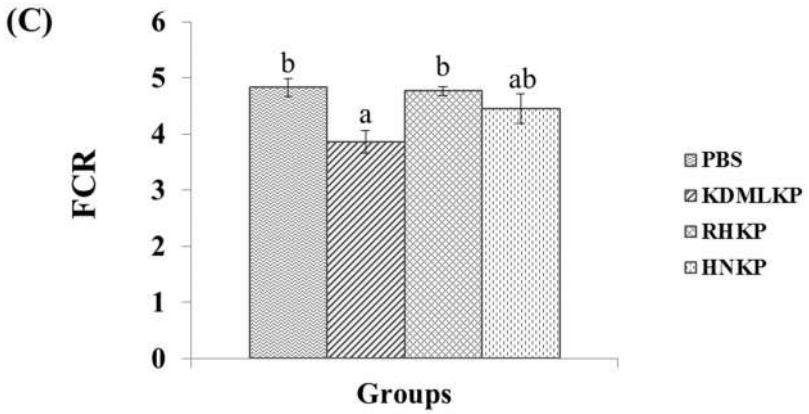


Figure 4.12 Body weight gain (A), food intake (B), and FCR (C) in rats treated with KDMLKP, RHKP, and HNKP compared to those in control (PBS) at the end of the experiment on sub-chronic toxicity study (90 days). Mean values with different letters are significantly different, Duncan's test at $p \leq 0.05$.

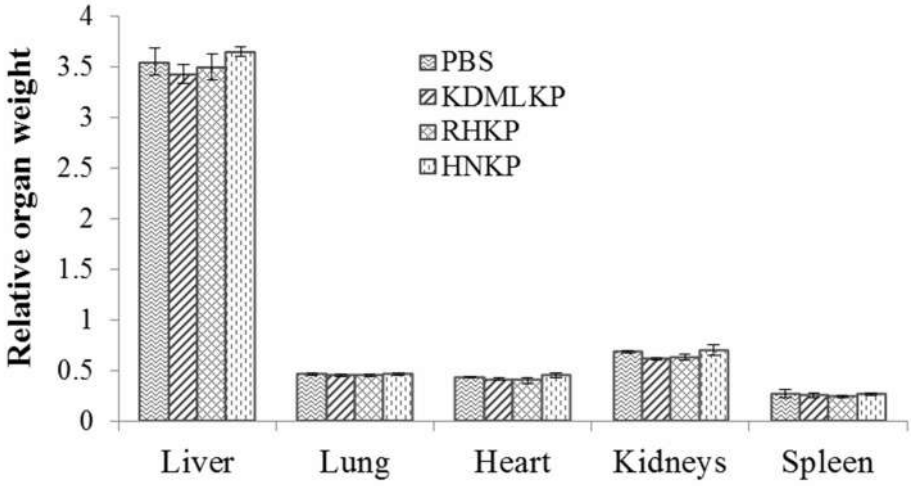


Figure 4.13 Relative organ weight in rats treated with PBS, KDMLKP, RHKP and HNKP at the end of the experiment on sub-chronic toxicity (mean±SEM).

Table 4.19 Blood biochemistry; BS, BUN, CREA, UA, TP, Alb, and Glob in the rats treated with PBS and kefir powder from sub-chronic toxicity study.

Groups	BS (mg/dl)	BUN (mg/dl)	CREA (mg/dl)	UA (mg/dl)	TP (g/dl)	Alb (g/dl)	Glob (g/dl)
PBS	259.50±4.99[a]	20.03±0.92[ab]	0.97±0.06[a]	2.82±0.25[a]	6.08±0.09[a]	2.97±0.07[a]	2.62±0.49[a]
KDMLKP (150 mg/kg)	135.00±8.57[a]	18.80±1.44[ab]	0.90±0.06[a]	1.88±0.31[a]	5.43±0.17[a]	2.67±0.14[a]	2.77±0.18[a]
RHKP (150 mg/kg)	193.25±5.84[a]	21.90±0.69[b]	0.97±0.08[a]	2.15±0.40[a]	5.37±1.22[a]	2.92±0.14[a]	2.70±0.18[a]
HNKP (150 mg/kg)	245.67±6.79[a]	18.38±0.44[a]	0.97±0.02[a]	2.73±0.50[a]	5.40±0.15[a]	2.48±0.20[a]	2.75±0.18[a]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at p≤0.05. BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid TP = total serum protein; Alb = albumin; Glob = globulin.

Table 4.20 Blood biochemistry; AST, ALT, and ALP in the rats treated with PBS and kefir powder from sub-chronic toxicity study.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
PBS	123.50±8.58[ab]	46.67±1.58[a]	93.67±8.64[a]
KDMLKP (150 mg/kg)	112.67±9.61[ab]	46.67±3.23[a]	86.00±6.98[a]
RHKP (150 mg/kg)	140.25±11.91[b]	47.50±4.09[a]	100.75±7.86[a]
HNKP (150 mg/kg)	108.00±9.61[a]	48.33±4.68[a]	82.50±5.95[a]

Mean values within each column with different superscripts [(a, b, c,…)] are significantly different, Duncan's test at p≤0.05. AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

Table 4.21 Cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) in rats treated with PBS and kefir powder from sub-chronic toxicity study.

Groups	**Lipid profiles (mg/dl)**			
	CHO	**TG**	**HDL**	**LDL**
PBS	51.17±2.31[a]	146.17±5.87[ab]	17.73±0.68[a]	28.17±1.70[a]
KDMLKP (150 mg/kg)	44.50±3.79[a]	162.33±9.26[ab]	16.88±0.88[a]	21.83±1.51[a]
RHKP (150 mg/kg)	43.50±4.55[a]	183.25±7.74[b]	22.52±0.95[b]	22.75±1.65[a]
HNKP (150 mg/kg)	43.00±3.44[a]	119.17±6.72[a]	19.02±1.84[ab]	22.17±2.84[a]

Mean values within each column with different superscripts [(a, b, c,...)] are significantly different, Duncan's test at $p \leq 0.05$.

4.5.3.4 Hematological values

KDMLKP, RHKP, and HNKP did not alter WBC, RBC, Hb, Hct, Plt, Neu, and Lym in the treated rats compared to those in controls (Table 4.22)

These findings indicated that rice kefir powder had no effect on hematological values when the rats received low doses (150 mg/kg) and long term administration.

Table 4.22 Hematological values in the rats treated with PBS and kefir powder from sub-chronic toxicity study.

Groups	WBC (10^3cell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
PBS	7.33±0.86	10.15±0.42	17.15±0.56	51.83±1.62	808.17±25.15	19.17±2.84	76.33±3.86
KDMLKP (150 mg/kg)	7.13±0.88	9.73±0.41	16.87±0.46	51.00±1.57	888.50±37.04	20.83±2.87	76.83±2.62
RHKP (150 mg/kg)	4.95±0.77	9.67±0.25	16.70±0.52	51.00±1.68	866.50±31.20	15.25±2.21	81.25±1.65
HNKP (150 mg/kg)	5.10±0.99	9.48±0.34	16.00±0.42	48.50±1.50	890.17±26.28	19.17±3.88	76.17±3.75

WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Biochemical components

The results of biochemical components showed that RKP had GABA, α-tocopherol and TPC higher than CMKP. The HNKP has the highest of biochemical components. These results may be due to high biochemical components presence in the brown rice (Moongngarm et al., 2012). GABA is a non-protein amino acid. It is known one of the main inhibitory neurotransmitters in the sympathetic nervous system and plays an important role in cardiovascular function (Gillis et al., 1980). In addition, TPC levels in all RKP were higher than that in non–fermented brown rice studied by Moongngarm et al. (2012).

5.2 Antioxidant activity in vitro

These results from Ferric reducing antioxidant power (FRAP) assay and 2, 2´-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay showed that RKP has high antioxidant activity than CMKP. Interestingly, the HNKP had the highest antioxidant activity. Our result supports rice kefir milk has antioxidant activity higher than cow kefir milk found by Deeseenthum and Pejovic, (2010).

Interestingly, the HNKP had the highest antioxidant activity. These results were also consistent with the results of the highest biochemical components in this kefir powder. Thus, these factors may high relative in kefir powder.

5.3 Antioxidative stress in vivo

NO is a potent, endogenous vasodilator that modulates renal function and plays a key role in endothelial dysfunction (Komers and Anderson, 2003). The rats colitis received PBS (control) has higher NO levels in serum than non-colitis rats. However, the colitis rats treated with HNKP produced NO levels reducing when compared to

those in controls. These findings indicate that rice kefir powder may reduce NO excretion in colitis rats compare those in controls. Another study showed the role of some probiotic bacteria in the generation of local NO in the intestinal lumen by nitrate reduction or acid dependent mechanisms, which may be counteracted by rapid NO consumption by other strains or diffused into the surrounding tissues (Sobko et al., 2006; Pautz et al., 2010). This may explain some of the health promoting effects of this kefir.

In the present study, there was a significant increase of LPO (TBARS) in colitis rats. This indicates that peroxidative injury involved in the reduction of antioxidant defense mechanisms and the development of colitis complications (Szkudelski, 2001). The colitis rats treated with RKP was significantly decreased LPO. It is known that the bioactive peptides released during fermentation by proteolytic lactic acid bacteria can scavenge the ROS and inhibit LPO (Pihlanto, 2006).

Moreover, an increase in SOD activity observed in the colitis rats treated with HNKP when compare to that in colitis rats received PBS (controls). SOD is the primary enzymatic antioxidant defense system in the cell. These indicated that antioxidant defense system was functional in the colitis rats received HNKP, similar findings of increased SOD in the rats received prednisolone and CMKP.

5.4 Anti-inflammatory effect in rats colitis

Blood biochemistry including BS, BUN, CREA, UA, TP, Alb, and Glob are involve in renal function and AST, ALT, and ALP enzymes are involved in hepatic function (Ramaiah, 2007). The colitis rats treated with RKP produced reverse of BS, BUN, UA and TP to normal levels similar with prednisolone and CMKP. In addition, the colitis rats received PBS significantly ($p \leq 0.05$) increased ALT and ALP while decreased AST when compared to those non-colitis. However, the colitis rats exerted reverse of ALT, ALP and AST to normal levels when treated with RKP.

The increased production of pro-inflammatory cytokines is thought to be a pivotal factor in the pathogenesis of UC. It is accepted that TNFα may be particularly important for inducing and sustaining intestinal inflammation in UC. It is known from many studies that TNFα is expressed in human gastrointestinal mucosa, and that the

expression is strongly enhanced in the inflammatory course of UC. Enhanced production of TNFα may induce some key enzymes of the inflammation cascade and neutrophils chemotaxis. TNFα can also induce more production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) (Geller et al., 1998).

Although, the TNFα level in serum of the colitis rats received PBS was highest. The colitis rats treated with kefir powder also produced TNFα level reducing when compared to those in controls.

These findings indicate that RKP may effect on protect tissues injury from chemical inducing.

5.5 Toxicity studies

Acute toxicity

HNKP has no acute toxicity but KDMLKP and RHKP at a dose of 4,000 mg/kg exerts acute toxicity as it produces adverse effect on hepatic function. In addition, RHKP at this dose also affects renal functions. Interestingly, the treated rats had neutrophils significant less than while lymphocytes were high levels, consistent with globulin increased. Since the total WBC count did not change, this result suggests that the kefir powder acts in opposite way in the differentiation of hematopoietic cells by suppressing neutrophils and stimulating lymphocytes. According to previous reports, the kefir induced the helper T-lymphocytes type 2 proliferations by increasing the number of immunoglobulin A (IgA) (Vinderola et al., 2005), in agreement with the increasing of lymphocyte and globulin in this study. In the other hand, the significant increasing of globulin may be results of B-lymphocytes were induced to IgA secreting cells.

Sub-acute toxicity

The kefir powder from brown rice, KDMLKP, RHKP and HNKP exerts non sub-acute toxicity when the dose less than or at a dose of 500 mg/kg is administrated every 2 days for 14 days. Repeat administration of the kefir powder at the doses of 1,000 and 2,000 mg/kg may affect hepatic and renal functions. Its activity on decreasing neutrophils and increasing lymphocytes resulting in increasing globulin is probably leading to improve immunomodulatory activity.

Sub-chronic toxicity

KDMLKP, RHKP and HNKP have no sub-chronic toxicity when the dose less than 150 mg/kg is administrated every day for 90 days. Moreover, RHKP produced higher HDL than other groups.

The overview, the Hawm Nil rice kefir powder exerts potent antioxidant and anti-inflammatory activities. Interestingly, it is safe and probably a new and good biochemical resource for human health utilizations.

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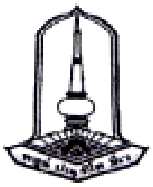
APPENDICES

APPENDIX A

Certificate



คณะกรรมการจริยธรรมการวิจัยในสัตว์ทดลอง มหาวิทยาลัยมหาสารคาม

ใบรับรองการอนุมัติ

เลขที่การรับรอง : 0008 / 2557

ชื่อโครงการวิจัย : องค์ประกองทางชีวเคมี ฤทธิ์ด้านอนุมูลอิสระ และฤทธิ์ด้านการอักเสบของผงคีเฟอร์นมข้าวต่อภาวะลำไส้ใหญ่อักเสบในหนู

ผู้วิจัย : นางสาวสุภาพร ขื่นชม

หน่วยงานที่รับผิดชอบ : คณะวิทยาศาสตร์

สถานที่ทำการวิจัย : จังหวัดมหาสารคาม

วันที่รับรอง : 17 ธันวาคม 2557 วันหมดอายุ : 18 ธันวาคม 2558

ข้อเสนอการวิจัยนี้ ได้รับการพิจารณาและให้ความเห็นชอบจากคณะกรรมการจริยธรรมการวิจัยในสัตว์ทดลอง มหาวิทยาลัยมหาสารคามแล้ว และอนุมัติในด้านจริยธรรมให้ดำเนินการศึกษาวิจัยเรื่องข้างต้นได้ บนพื้นฐานของโครงร่างงานวิจัยที่คณะกรรมการฯ ได้รับและพิจารณา หากมีการเปลี่ยนแปลงใดๆ ในโครงการวิจัย ผู้วิจัยจักต้องยื่นขอรับการพิจารณาใหม่

.....

(ศาสตราจารย์ปรีชา ประเทพา)

ประธานคณะกรรมการจริยธรรมการวิจัยในสัตว์ทดลอง

APPENDIX B

Solution preparation

1. Preparation of reagents for Free- radical scavenging activity (DPPH) assay

a. Preparation of 0.1 mM DPPH (MW= 394.33): A 0.1 mM DPPH was prepared by dissolving 0.0232 g of 85% DPPH in 500 mL and made up to volume with methanol in 500 mL volumetric flask.

b. Preparation of Stock standard 2 mg ml-1 BHA: Stock standard solution (2 mg mL-1) of BHA was prepared by dissolving 0.0521 g of 96% BHA and made up to volume with methanol in 25 mL volumetric flask.

2. Preparation of reagents for Ferric reducing antioxidant power (FRAP) assay

a. Preparation of 300 mM Sodium acetate buffer, pH 3.6: A 0.025 M sodium acetate buffer (pH 3.6) solution was prepared by dissolving 24.624 g of $CH_3COONa•3H_2O$ in 500 mL of deionized water. The pH value of 0.3 M of the solution was adjusted by using CH_3COOH and made up to volume with deionized water in a 1000 mL volumetric flask.

b. Preparation of 10 mM TPTZ (MW= 312.32): A 10 mM TPTZ solution was prepared by dissolving 0.0789 g of 99% TPTZ in 25 mL and made up to volume with 40 mM HCl in 25 mL volumetric flask.

c. Preparation of 20 mM Ferric chloride (MW= 162.21): A 20 mM ferric chloride solution was prepared by dissolving 0.1655 g of 98% $FeCl_3$ in 50 mL and made up to ending volume with deionized water in 50 mL volumetric flask.

d. Preparation of 40 mM Hydrochloric acid (MW= 36.441; 37%; d= 1.19): A 40 mM hydrochloric acid was prepared by dilute 3.30 mL of 37% HCl in 1000 mL and made up to volume with deionized water in 1000 mL volumetric flask.

e. Preparation of 10 mM Ferrous sulphate: Standard stock solution of 10 mM $FeSO_4$ was prepared by dissolving 0.0140 g of 99% $FeSO_4•7H_2O$ in 5 mL and made up to ending volume with methanol in 5 mL volumetric flask.

3. Preparation of reagents for total phenolic content

a. Preparation of 10% Folin-Ciocalteu reagent: A 10% Folin-Ciocalteu reagent was prepared by diluting 10 mL of Folin-Ciocalteu reagent in 900 mL of deionized water.

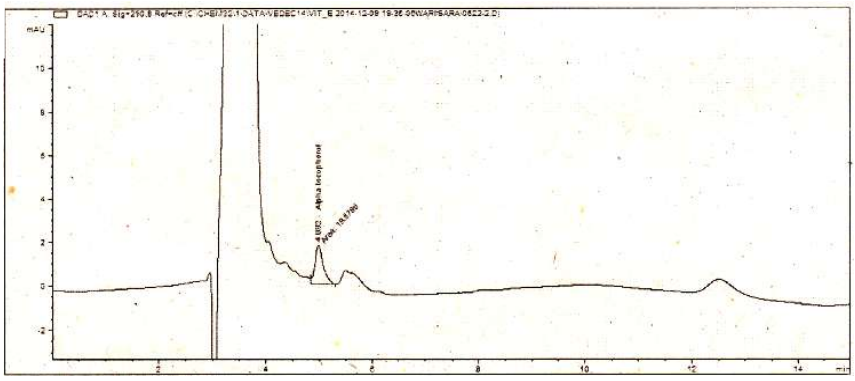
b. Preparation of 10% Sodium carbonate: A 10% sodium carbonate solution was prepared by dissolving 10.0502 g of 99.5% Na2CO3 in 100 mL of deionized water.

c.Preparation of standard Stock (2 mg mL-1) gallic acid: Standard stock solution (2 mg mL-1) of gallic acid was prepared by dissolving 0.051g of 98% gallic acid and made up to ending volume with deionized water in 25 mL volumetric flask.

APPENDIX C

HPLC output of α-tocopherol and GABA

(A)



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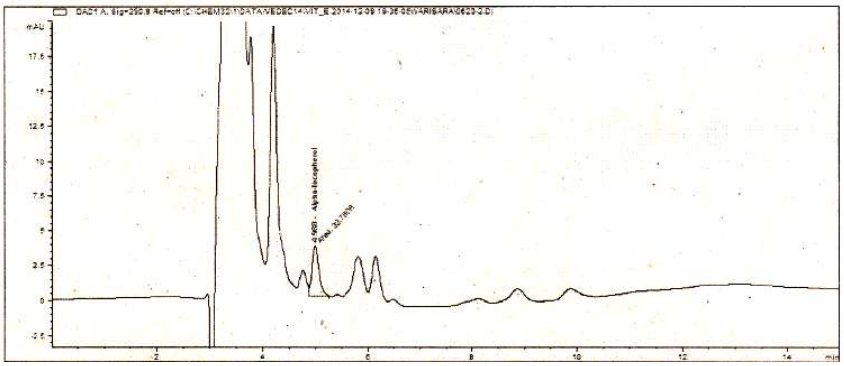

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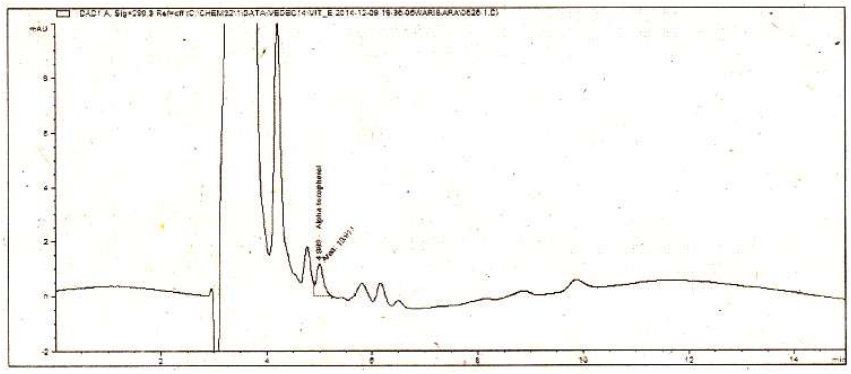


(D)

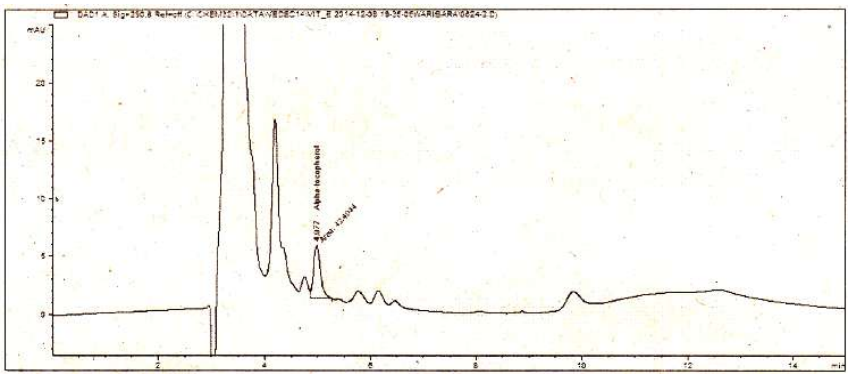
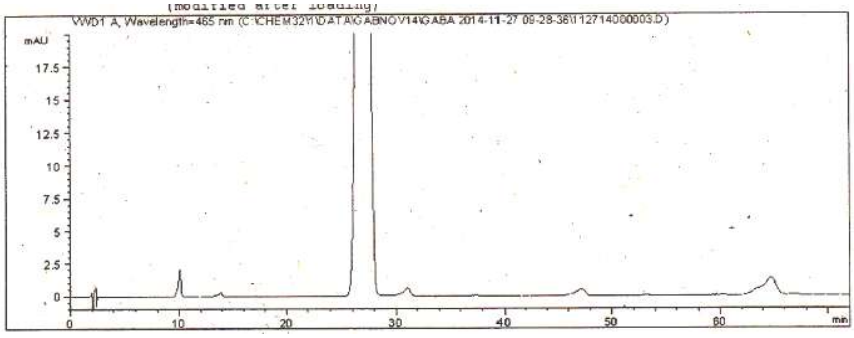


Figure AC1 HPLC output of α-tocopherol from kefir powder from Cow milk (A), KDML 105 rice (B), Red Hawm rice (C), and Hawm Nil rice (D).

(A)



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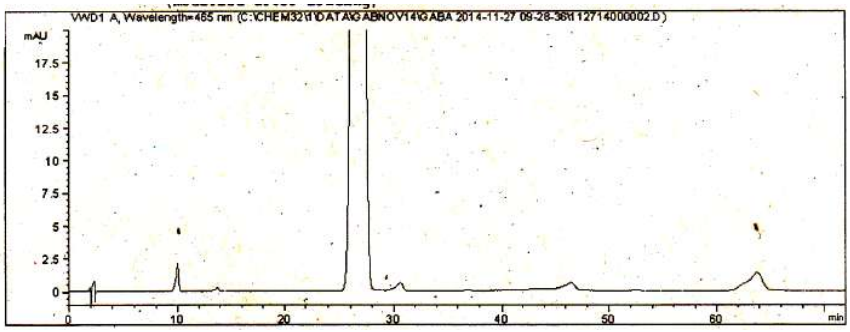


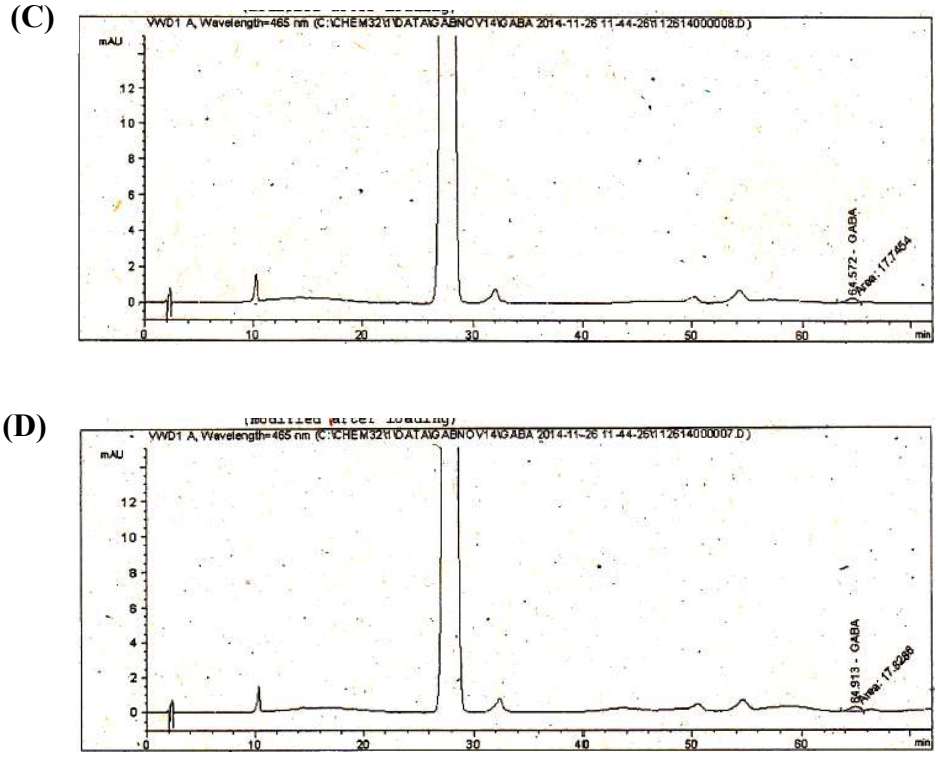
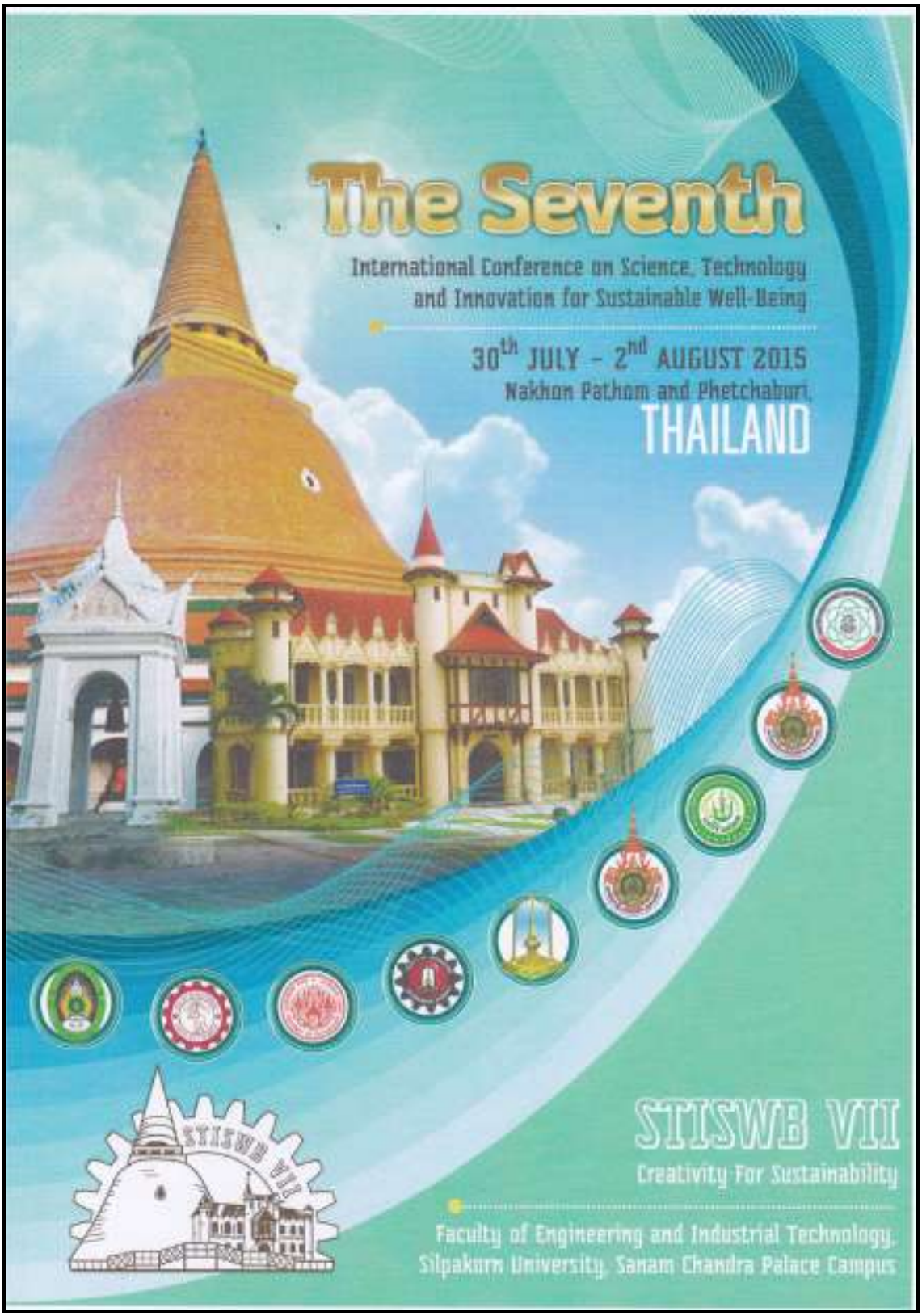


Figure AC2 HPLC output of GABA from kefir powder from Cow milk (A), KDML 105 rice (B), Red Hawm rice (C), and Hawm Nil rice (D).

APPENDIX D

Manuscripts



The 7th International Conference on Science, Technology and Innovation for Sustainable Well-Being (STISWB VII), 30 July - 2 August, 2015, Nakhon Pathom-Phetchaburi ,Thailand

MEE-036

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 acute toxicity study, HNKP at the 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 symptom 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 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 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 doses application of HNKP can affect renal and hepatic functions ($p<0.05$). In addition, TG and HDL of the rats received HNKP were significant ($p<0.05$) less than those in controls. Interestingly, the rats received HNKP had neutrophils significant less than while lymphocytes significant 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 resulting in increased globulin leads to improve immunomodulatory activity.

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

1. 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 color, gelatinous irregular masses and size 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 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 reduce the accumulation 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 on health.

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

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.

2. Materials and methods

2.1 Hawm Nil brown rice, fermentation and kefir powder preparation

2.1.1 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 grinded by using 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.

2.1.2 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.

2.1.3 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.

2.2 Animals

Forty-eight male Wistar rats weighing 280-300 g were purchased from National Laboratory Animal Center, Mahidol University, Thailand. The rats were kept in 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.

2.3 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.

2.4 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[131]. HNKP was given orally to the rats every 2 days for 14 days. Symptoms of toxicity and mortal rats 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.

2.5 Relative organ weight and feed conversion ratio

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

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

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

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

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 including 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) were performed.

2.7 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 $p \leq 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Acute toxicity

3.1.1 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].

3.1.2 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 involve 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.

3.1.3 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 received all the doses of HNKP had neutrophils significant less than while lymphocytes were significant 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, the 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 increasing of lymphocyte and globulin in this study. Other than, the significant increasing of globulin may be results of B-lymphocytes were induced to IgA secreting cells.

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.

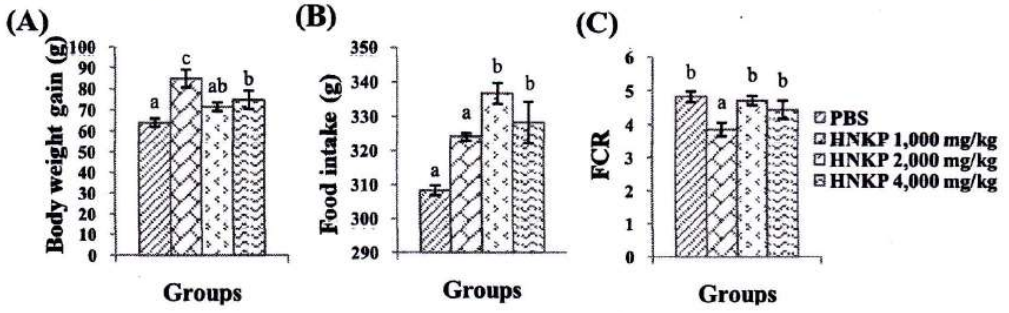

Fig. 1: Body weight gain (A), food intake (B) and FCR (C) in rats treated with HNKP and PBS from acute toxicity study at the end experiment (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p< 0.05$.

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 $p< 0.05$.

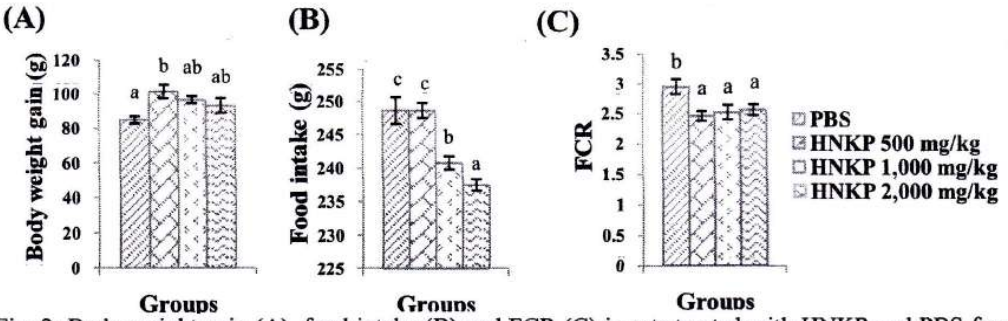

Fig. 2: Body weight gain (A), food intake (B) and FCR (C) in rats treated with HNKP and PBS from sub-acute toxicity study at the end experiment (mean±SEM). Mean values with different letters are significantly different, Duncan's test at $p< 0.05$.

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.

3.2 Sub-acute toxicity

3.2.1 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 (Fig.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.

3.2.2 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).

3.2.3 Hematological values

In line with the acute toxicity study, WBC, RBC, Hb, Hct, MCV, MCH, MCHC, and Plt in the rats 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.

4. Conclusions

HNKP has no acute and sub-acute toxicities when the dose less than 4,000 mg/kg is once oral administered or the dose less than 500 mg/kg is administrated every 2 days for 14 days. In long term application, the powder at the doses higher than 1,000 mg/kg my cause hepatic and renal functions as it produces Alb, BS, BUN, UA, and ALP levels decreasing while Glob and AST levels increasing 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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พิษเฉียบพลันและพิษกึ่งเฉียบพลันของผงคีเฟอร์จากน้ำนมข้าวกล้องขาวดอกมะลิ 105

Acute and Sub-acute Toxicities of Kefir Powder from KhaoDawk Mali 105 Brown Rice Milk

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บทคัดย่อ

การศึกษาพิษเฉียบพลันของผงคีเฟอร์จากน้ำนมข้าวกล้องขาวดอกมะลิ 105 (KDML105KP) โดยการป้อนKDML105KP ขนาด 1,000, 2,000 และ 4,000 mg/kg ครั้งเดียวจากนั้นสังเกตอาการของพิษภายใน 24 ชั่วโมง และสังเกตอาการต่อเนื่อง14 วัน การศึกษาพิษกึ่งเฉียบพลันโดยการป้อน KDML105KP ขนาด 500, 1,000 และ2,000 mg/kg ทุก 2 วัน เป็นเวลา 14 วัน ไม่พบการตายหรืออาการของพิษแต่พบว่าหนูที่ได้รับ KDML105KP ตั้งแต่1,000 mg/kg ขึ้นไป ติดต่อกันเป็นระยะเวลา 14 วัน มีค่า น้ำตาลในเลือด (BS) ลดลง แต่aspartate aminotransferase (AST)และ ไลโปโปรตีนความหนาแน่นต่ำ (LDL)เพิ่มขึ้น หนูที่ได้รับ KDML105KPตั้งแต่ 2,000 mg/kg ขึ้นไป มีโกลบูลิน(Glob)และ lymphocytesเพิ่มขึ้น แต่neutrophils ลดลง ในขณะที่หนูที่ได้รับ KDML105KP 4,000 mg/kg มี BS, Glob, คอเลสเตอรอล (CHO), ไตรกลีเซอไรด์ (TG) และไลโปโปรตีนชนิดความหนาแน่นสูง (HDL)เพิ่มขึ้น

ผลการศึกษาแสดงให้เห็นว่า ผงคีเฟอร์จากน้ำนมข้าวกล้องขาวดอกมะลิ 105 ปริมาณสูงมีผลต่อการทำงานของตับและไต และยังทำให้ปริมาณ neutrophils และ lymphocytes เปลี่ยนแปลงซึ่งส่งผลต่อ globulin และนำไปสู่การกระตุ้นภูมิคุ้มกัน

คำสำคัญ: ข้าวขาวดอกมะลิ 105 ผงคีเฟอร์ พิษเฉียบพลัน พิษกึ่งเฉียบพลัน

Abstract

The aim of this study was to determine acute and sub-acute toxicities of kefir powder from KhaoDawk Mali 105 brown rice milk (KDML105KP) in male Wistar rats. Acute toxicity study, KDML105KP at the doses of 1,000, 2,000 and 4,000 mg/kg were once administered to the rats orally. Symptoms of toxicity and mortality of the rats were observed within 24 h and over a further period for 14 days. Moreover, sub-acute toxicity study, KDML105KP at the doses of 500, 1,000 and 2,000 mg/kg were given orally to the rats every 2 days for 14 days. The results showed that all the doses of KDML105KP did not produce any symptom or mortality of the rats. The rats received KDML105KP at and above a dose of 1,000mg/kgfor 14 days produced blood sugar (BS) decreasing while aspartate aminotransferase (AST) and low density lipoprotein (LDL) increasing. In addition, the rats received KDML105KP at and above a dose of 2,000 mg/kg produced globulin (Glob) and lymphocytes increasing while neutrophils decreasing. However, the rats received KDML105KP at a doses 4,000 mg/kg produced BS,Glob, cholesterol (CHO), triglycerides (TG) and high density lipoprotein (HDL) increasing.

These results indicatedthat high dose application of kefir powder from KhaoDawk Mali 105 brown rice milk can affect renal and hepatic functions and also hematological values. Interestingly, its activity on decreasing neutrophils and increasing lymphocytes results in an increase globulin and leads to improve immunomodulatory activity.

Keywords: KhaoDawk Mali 105 (KDML 105), kefir powder, acute toxicity, sub-acute toxicity

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บทนำ

จากอดีตจนถึงปัจจุบันข้าวถือเป็นอาหารและสินค้าการเกษตรที่มีความสำคัญอันดับต้นๆ ของประเทศไทย ในปัจจุบันประเทศไทยมีความสามารถในการผลิตข้าว (*Oryza sativa*, L.) ได้มากเป็นอันดับ 5 ของโลก[1] ผลการวิจัยที่ผ่านมาพบว่าข้าวขาวดอกมะลิ 105 มีองค์ประกอบที่ส่งเสริมฤทธิ์ทางเภสัชวิทยาหลายชนิดสูงกว่าในข้าวขัดขาว ได้แก่ α-tocopherol, γ-aminobutyric acid (GABA) และ total phenolics compounds (TPC)[2-3]สารประกอบดังกล่าวมีรายงานฤทธิ์การยับยั้งและป้องกันอาการของโรคได้หลายชนิด เช่น ป้องกันภาวะ hypo adiponectinemiaในผู้ป่วยที่เป็นโรคเบาหวานชนิดที่ 2[4]ควบคุมการเผาผลาญไขมันในตับให้ดีขึ้นและลดความเสี่ยงต่อการเป็นโรคหัวใจและหลอดเลือดอุดตัน[5]ทั้งนี้เป็นผลอันเนื่องมาจาก α-tocopherol, GABA และ TPC มีคุณสมบัติในการต้านอนุมูลอิสระ[6]

นอกจากนี้ยังพบว่า คีเฟอร์ที่หมักในน้ำนมข้าวขาวดอกมะลิ 105 มีฤทธิ์ต้านอนุมูลอิสระสูงกว่าคีเฟอร์ที่หมักด้วยนมวัว[7]คีเฟอร์ หรือบัวหิมะ ถูกนำมาใช้ประโยชน์ในอุตสาหกรรมเครื่องดื่ม เช่น นมเปี้ยว และโยเกิร์ตมาเป็นเวลาช้านาน จากรายงานวิจัยพบว่าก้อนคีเฟอร์ที่ได้จากการหมักมีการสร้างและหลั่งสาร water-soluble branched glucogalactanหรือ kefiranออกมาละลายในน้ำนมที่ใช้หมัก[8] ซึ่งสาร kefiranนี้ มีฤทธิ์ทางเภสัชวิทยาหลายอย่างที่น่าสนใจ ได้แก่ ฤทธิ์ต้านเนื้องอก[9] ฤทธิ์ต้านอนุมูลอิสระ[10-11] ฤทธิ์ต้านเบาหวาน[12] ฤทธิ์ต้านการอักเสบ[13]และฤทธิ์กระตุ้นภูมิคุ้มกัน[14-15]ซึ่งอาหารที่มีฤทธิ์ต้านอนุมูลอิสระในระดับสูงจะส่งเสริมระบบภูมิคุ้มกันทำให้ผู้บริโภคมีภาวะเสี่ยงต่อการเกิดโรคน้อยลง[16]

การศึกษาฤทธิ์ทางเภสัชวิทยา และผลิตภัณฑ์จากสมุนไพรต้องคำนึงถึงความปลอดภัยต่อสุขภาพของผู้บริโภค ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาพิษเฉียบพลันและพิษกึ่งเฉียบพลันของผงคีเฟอร์จากน้ำนมข้าวกล้องขาวดอกมะลิ105 (KDML105KP) เพื่อใช้เป็นข้อมูลประกอบการตัดสินใจในการเลือกใช้คีเฟอร์ได้อย่างปลอดภัย และเป็นข้อมูลพื้นฐานในการศึกษาฤทธิ์ทางเภสัชวิทยาของ KDML105KP ต่อไป

วิธีการดำเนินงานวิจัย

การเตรียมข้าวกล้องขาวดอกมะลิ 105

ในการทดลองครั้งนี้ใช้ข้าวกล้องสายพันธุ์ขาวดอกมะลิ 105 ที่เก็บเกี่ยวในปี 2556-2557 จากกลุ่มเกษตรกรอำเภอเสลภูมิ จังหวัดร้อยเอ็ด นำข้าวที่ได้มาคัดเลือกสิ่งปลอมปนและอบให้แห้งที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 2 ชั่วโมง จากนั้นนำข้าวที่ได้มาชั่งน้ำหนักและแช่ข้าวในน้ำกลั่น (1:5, w:v) ที่อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 2 ชั่วโมง นำข้าวที่แช่น้ำจนอ่อนนุ่มมาปั่นรวมกับน้ำที่ใช้แช่ให้เป็นเนื้อเดียวกัน กรองเอากากออกเพื่อให้ได้น้ำนมข้าว นำน้ำนมข้าวใส่ในขวดรูปชมพูและนำไปพาสเจอร์ไรส์ที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 15 นาทีแล้วนำไปแช่ในตู้ 4 องศาเซลเซียสทันที เป็นเวลา 30 นาที

วิธีการหมักข้าว

เตรียมหัวเชื้อคีเฟอร์ในอาหารเหลว *Lactobacilli* de Man, Rogosa, and Sharpe (MRS) โดยใช้ผงคีเฟอร์DT 5001 ปริมาณ 0.2 กรัม เลี้ยงในขวดรูปชมพูขนาด 250 มิลลิลิตรที่มีอาหารเหลว MRS ปริมาณ 200 มิลลิลิตร จากนั้นทำการบ่มเชื้อในสภาวะไร้อากาศ (anaerobic condition) ที่อุณหภูมิ 30องศาเซลเซียส เป็นเวลา 24 ชั่วโมง นำอาหารเหลวที่เลี้ยงเชื้อไว้มาปั่นเหวี่ยงที่ 5000 g อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 10 นาที จากนั้นเทส่วนใสทิ้งเพื่อเก็บเซลล์ แล้วนำเซลล์ที่ได้มาล้างและละลายด้วยโซเดียมคลอไรด์0.85 เปอร์เซ็นต์ ปริมาณ 10 เปอร์เซ็นต์ของปริมาณเซลล์ที่เก็บได้เขย่าให้เข้ากันเทเซลล์ที่ละลายในโซเดียมคลอไรด์ 0.85 เปอร์เซ็นต์ลงในน้ำนมข้าวที่เตรียมไว้ในอัตราส่วน 10:100 (v:v)ทำการบ่มเชื้อในสภาวะไร้อากาศ ที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 48 ชั่วโมงได้เชื้อคีเฟอร์เริ่มต้น (Kefir starter) เพื่อใช้ในการหมักต่อไป ต่อจากนั้นทำการหมักอีกครั้งโดยใช้เชื้อคีเฟอร์เริ่มต้นที่ได้เลี้ยงในนมข้าวที่เติมน้ำตาลซูโครส 2.5 เปอร์เซ็นต์ในอัตราส่วน 100:1000 (v:v) แล้วบ่มเชื้อในสภาวะไร้อากาศที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 24ชั่วโมง หลังจากครบ 24 ชั่วโมง จะได้คีเฟอร์นมข้าวที่มีค่า pH ประมาณ 4.8-4.9

การเตรียมผงคีเฟอร์จากน้ำนมข้าวกล้องขาวดอกมะลิ 105 (KDML105KP)

นำคีเฟอร์นมข้าวที่ได้จากการหมักไปผ่านกระบวนการทำให้แห้งแบบแช่เยือกแข็ง (freeze-dried) แล้วนำมาบดให้เป็นผงละเอียด จากนั้นนำผงคีเฟอร์ที่ได้บรรจุในขวดสีชาที่มีฝาปิดสนิทและเก็บในตู้ควบคุมอุณหภูมิที่ -20 องศาเซลเซียสจนกว่าจะนำออกมาใช้ในการทดลอง

สัตว์ทดลอง

ในการทดลองครั้งนี้ใช้หนูขาวสายพันธุ์ Wistar เพศผู้น้ำหนักระหว่าง 280-300กรัม ที่ซื้อจากศูนย์สัตว์ทดลองแห่งชาติ มหาวิทยาลัยมหิดล ทำการเลี้ยงหนูทดลองก่อนทำการทดสอบเป็นเวลา 7 วัน เพื่อให้หนูได้ปรับสภาพเข้ากับสภาวะของห้องปฏิบัติการที่ใช้ในการเลี้ยงที่มีอุณหภูมิ 23±2องศาเซลเซียสความชื้นสัมพัทธ์ 50-55 เปอร์เซ็นต์ และให้แสงสว่าง 12 ชั่วโมงต่อวัน ทำการเลี้ยงสัตว์ทดลองในกรงสแตนเลสที่มี

ตะแกงปิดด้านบน ให้อาหารเม็ดสูตรมาตรฐาน (Perfect Companion Group Co., Ltd.)และน้ำแบบไม่จำกัด ซึ่งทุกขั้นตอนของการทดลองได้ปฏิบัติอยู่ภายใต้จริยธรรมการใช้สัตว์ทดลองแห่งชาติ และผ่านการอนุมัติการใช้สัตว์ทดลองจากกรรมการพิจารณาจริยธรรมการใช้สัตว์ทดลองมหาวิทยาลัยมหาสารคาม

การศึกษาพิษเฉียบพลัน

ทำการชั่งน้ำหนักหนูทดลองและสุ่มหนูทดลองออกเป็น 4 กลุ่ม กลุ่มละ 6 ตัว ดังนี้ กลุ่มที่ 1 คือ หนูที่ได้รับ phosphate buffered saline; PBS (กลุ่มควบคุม)กลุ่มที่ 2-4 คือหนูที่ได้รับ KDML105KP ขนาด 1,000, 2,000และ4,000mg/kgตามลำดับ ทำการป้อนสิ่งทดสอบครั้งเดียว จากนั้นสังเกตอาการความเป็นพิษ เช่น ชัก ท้องเสีย อาเจียน และ/หรือเดินเซ อย่างใกล้ชิดในชั่วโมงแรกและสังเกตอาการดังกล่าวทุกๆ 2 ชั่วโมง จนครบ 24 ชั่วโมง จากนั้นทำการสังเกตอาการต่อเนื่องจนครบ 14 วันระหว่างการทดลองทำการชั่งอาหารที่เหลือทุกวันและชั่งน้ำหนักของหนูทุกๆ 7 วัน เมื่อครบ 14 วัน ทำให้หนูอดอาหารเป็นเวลา 8-12 ชั่วโมงแล้วทำให้หนูตายอย่างสงบด้วยคลอโรฟอร์ม จากนั้นทำการผ่าตัดและเก็บเลือดจากหัวใจเพื่อใช้ในการวิเคราะห์ค่าชีวเคมีโลหิต และค่าโลหิตวิทยา จากนั้นตัดแยกเอาอวัยวะภายใน ได้แก่ ตับ ปอด หัวใจ ไต และม้าม ไปชั่งน้ำหนักเพื่อนำไปคำนวณหาน้ำหนักสัมพัทธ์ของอวัยวะ

การศึกษาพิษกึ่งเฉียบพลัน

ทำการชั่งน้ำหนักหนูทดลองและสุ่มหนูทดลองออกเป็น 4 กลุ่ม กลุ่มละ 6 ตัว ดังนี้ กลุ่มที่ 1 คือ หนูที่ได้รับ PBS (กลุ่มควบคุม) กลุ่มที่ 2-4 คือ หนูที่ได้รับ KDML105KP 500, 1,000และ2,000mg/kg ตามลำดับ ป้อนKDML105KPและPBSทุกๆ 2 วัน เป็นระยะเวลา 14 วัน ระหว่างการทดลองทำการชั่งอาหารที่เหลือทุกวันและชั่งน้ำหนักของหนูทุกๆ 7 วัน เมื่อครบ 14 วันทำให้หนูอดอาหารเป็นเวลา 8-12 ชั่วโมงแล้วทำให้หนูตายอย่างสงบด้วยคลอโรฟอร์ม จากนั้นทำการผ่าตัด และเก็บเลือดจากหัวใจเพื่อใช้ในการวิเคราะห์ค่าชีวเคมีโลหิต และค่าโลหิตวิทยา และชั่งน้ำหนักอวัยวะภายใน ได้แก่ ตับ ปอด หัวใจ ไต และม้าม เพื่อนำไปใช้ในการคำนวณหาน้ำหนักสัมพัทธ์ของอวัยวะ

น้ำหนักสัมพัทธ์ของอวัยวะและอัตราการแลกเนื้อ

การคำนวณหาน้ำหนักสัมพัทธ์ของอวัยวะ(relative organ weight: ROW)คำนวณหาน้ำหนักสัมพัทธ์ของอวัยวะของสัตว์ทดลองแต่ละตัว จากสมการ

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

การคำนวณหาอัตราการแลกเนื้อ (feed conversion ratio: FCR) คำนวณหาอัตราการแลกเนื้อของสัตว์ทดลองแต่ละตัว จากสมการ

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

การวิเคราะห์ค่าชีวเคมีโลหิตและค่าโลหิตวิทยา ทำการแยกเลือดตัวอย่างออกเป็น 2 หลอด ได้แก่ หลอดที่ 1 ไม่ได้เติมสารป้องกันการแข็งตัวของเลือด (non-heparinized) และหลอดที่ 2 เติมสารป้องกันการแข็งตัวของเลือด (heparinized) นำเลือดตัวอย่างในหลอดที่1 ไปปั่นเหวี่ยงที่ 1500 g เป็นเวลา10นาที เพื่อแยกเอาเซรั่ม นำเซรั่มที่ได้มาวิเคราะห์ค่าชีวเคมีโลหิตได้แก่ โปรตีนรวม (TP), น้ำตาลในเลือด (BS), ยูเรียไนโตรเจนในเลือด (BUN), ครีเอตินิน (Crea), กรดยูริค (UA), คอเลสเตอรอล (CHO), ไตรกลีเซอไรด์ (TG), ไลโปโปรตีนชนิดความหนาแน่นสูง (HDL), ไลโปโปรตีนความหนาแน่นต่ำ (LDL), อัลบูมิ (Alb), โกลบูลิน (Glob), บิลิรูบินรวม (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT) และ alkaline phosphatase (ALP) นำเลือดตัวอย่างที่เก็บในหลอดที่ 2 มาวิเคราะห์หาค่าโลหิตวิทยา โดยทำการวิเคราะห์ค่าต่างๆ ได้แก่ จำนวนเม็ดเลือดแดง (RBC), จำนวนเม็ดเลือดขาว (WBC), ฮีมาโทคริต (Hct), ฮีโมโกลบิน (Hgb), ปริมาตรของเม็ดเลือดแดงโดยเฉลี่ย(MCV), ปริมาณเฉลี่ยของฮีโมโกลบินในเม็ดเลือดแดง(MCH), ความเข้มข้นเฉลี่ยของฮีโมโกลบินในเม็ดเลือดแดง(MCHC), เกล็ดเลือด (Plt), เปอร์เซ็นต์ของเม็ดเลือดขาวชนิดนิวโทรฟิล (Neu) และเปอร์เซ็นต์ของเม็ดเลือดขาวชนิดลิมโฟซัยต์ (Lym)

การวิเคราะห์สถิติ

ผลที่ได้จากการศึกษาในครั้งนี้ นำเสนอในรูปแบบค่าเฉลี่ย±ค่าคลาดเคลื่อนมาตรฐานเฉลี่ย(mean±SEM) โดยวิเคราะห์ค่าความแปรปรวนของข้อมูลด้วย one-way ANOVA และวิเคราะห์ความ แตกต่างของค่าเฉลี่ยโดยใช้ Duncan's Multiple Range Test ที่ค่าความเชื่อมั่น 95 เปอร์เซ็นต์ ($p<0.05$)

ผลการทดลอง

พิษเฉียบพลัน

หนูที่ได้รับKDML105KP ทุกขนาดตลอดการทดลองไม่มีการตายหรือแสดงอาการความเป็นพิษ นอกจากนี้หนูที่ได้รับ KDML105KP ขนาด 1,000 mg/kg มีน้ำหนักตัวเพิ่มขึ้น การกินอาหาร และอัตราการแลกเนื้อไม่แตกต่างกันและไม่แตก

ต่างจากหนูกลุ่มควบคุม แต่อย่างไรก็ตามหนูที่ได้รับ KDML105KP ขนาด 2,000 และ 4,000 mg/kg มีการกินอาหารเพิ่มมากขึ้น (p<0.05) แต่กลับให้อัตราแลกเนื้อที่ไม่แตกต่างกันและไม่แตกต่างจากหนูกลุ่มควบคุม(Figure1A–1C)แต่อย่างไรก็ตามน้ำหนักสัมพัทธ์ของอวัยวะของหนูที่ได้รับ KDML105KP ไม่แตกต่างจากกลุ่มควบคุม (Table1)

จากการวิเคราะห์ค่าชีวเคมีโลหิตของหนูที่ได้รับ KDML105KP ขนาด 1,000, 2,000 และ 4,000 mg/kg (Table2) พบว่าหนูที่ได้รับ KDML105KP ขนาด 1,000 mg/kg มีค่าชีวเคมีโลหิตไม่แตกต่างกันและไม่แตกต่างจากกลุ่มหนูควบคุม ยกเว้นปริมาณ Glob ที่เพิ่มขึ้นแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ (p<0.05) ส่วนหนูที่ได้รับ KDML105KP ขนาด 2,000 mg/kg มีปริมาณ BS, Glob และ TG เพิ่มขึ้นแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ (p<0.05) และหนูที่ได้รับ KDML105KP ขนาด 4,000 mg/kg มีปริมาณ BS, Glob, CHO, TG และ HDL เพิ่มขึ้นอย่างมีนัยสำคัญ (p<0.05)

Table 3 แสดงให้เห็นว่า WBC, RBC, Hb, Hct, MCV, MCH, MCHC และ Pltในหนูที่ได้รับ KDML105KP ทุกขนาดไม่แตกต่างกันและไม่แตกต่างจากหนูกลุ่มควบคุม แต่อย่างไรก็ตามพบว่าปริมาณร้อยละของ Neuลดลง(p<0.05) ในขณะที่ Lymเพิ่มขึ้น (p<0.05) เมื่อเปรียบเทียบกับหนูกลุ่มควบคุม

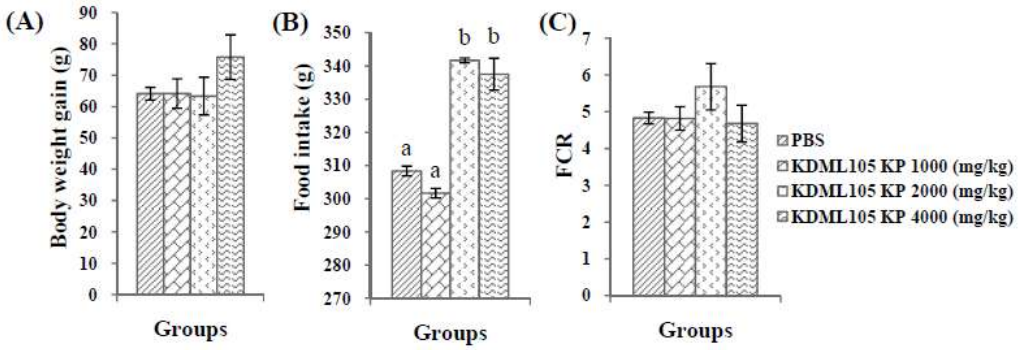

Figure 1 Body weight gain (A), food intake (B) and FCR (C) in rats treated with KDML105 KP and PBS from acute toxicity study at the end experiments (mean±SEM). Mean values with different letters are significantly different, Duncan's test at p<0.05.

Table 1 Relative organ weight in rats treated with KDML105KPand PBS from acute toxicity study at the end experiment (mean±SEM).

Visceral organs	PBS	KDML105KP (mg/kg)		
		1,000	2,000	4,000
Liver	3.55±0.13	3.55±0.12	3.79±0.07	3.79±0.08
Lung	0.46±0.01	0.45±0.01	0.43±0.01	0.44±0.01
Heart	0.43±0.01	0.42±0.01	0.44±0.02	0.44±0.01
Kidneys	0.68±0.01	0.63±0.01	0.66±0.02	0.64±0.01
Spleen	0.27±0.04	0.24±0.00	0.24±0.01	0.25±0.01

Table 2 Blood biochemistry in rats treated with KDML105KP and PBS from acute toxicity study (mean±SEM).

Blood biochemistry	PBS	KDML105KP (mg/kg)		
		1,000	2,000	4,000
BS (mg/dl)	174.00±6.19^{a}	189.50±11.84^{ab}	200.50±6.99^{b}	213.83±7.98^{b}
BUN (mg/dl)	20.17±0.48	20.65±0.32	20.08±0.46	20.28±0.33
CREA (mg/dl)	0.91±0.03	0.81±0.03	0.82±0.02	0.83±0.18
UA (mg/dl)	3.74±0.19	3.25±0.19	3.50±0.20	3.30±0.17
TP (g/dl)	5.65±0.11	5.85±0.06	5.93±0.03	6.00±0.18
Alb (g/dl)	3.47±0.04	3.50±0.04	3.52±0.03	4.03±0.59
Glob (g/dl)	2.20±0.06^{a}	2.40±0.06^{b}	2.43±0.042^{b}	2.45±0.043^{b}
TB (mg/dl)	0.11±0.01	0.13±0.01	0.14±0.01	0.14±0.01
AST (U/L)	147.67±3.95	136.33±5.29	148.33±5.98	144.50±3.95
ALP (U/L)	124.33±3.06	127.33±2.24	127.50±3.27	129.50±1.43
ALT (U/L)	39.33±1.17	42.33±1.69	40.67±1.23	41.83±1.30
CHO (mg/dl)	53.67±1.28^{a}	57.67±1.78^{ab}	58.67±1.56^{ab}	59.67±2.17^{b}
TG (mg/dl)	130.00±2.62^{a}	124.17±8.61^{a}	152.50±5.32^{b}	161.50±10.62^{b}
HDL (mg/dl)	16.48±0.27^{a}	17.10±0.15^{a}	16.95±0.13^{a}	19.85±0.91^{b}
LDL (mg/dl)	32.50±2.11	36.33±2.11	33.67±1.45	31.50±1.80

Mean values within each row with different superscripts are significantly different, Duncan's test at p< 0.05. PBS= phosphate buffered saline; BS = blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid; TP = total serum protein; Alb = albumin; Glob = globulin; TB= total bilirubin; AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase; CHO = Cholesterol; TG = triglycerides; HDL = high density lipoprotein; LDL = low density lipoprotein.

Table 3 Hematological values in rats treated with KDML105KP and PBS from acute toxicity study (mean±SEM).

Hematological values	PBS	KDML105 KP (mg/kg)		
		1,000	2,000	4,000
WBC (10^3 cell/mm^3)	5.93±0.16	6.18±00.22	5.90±0.16	6.38±0.17
RBC (10^6 cell/mm^3)	8.87±0.13	8.88±0.12	8.29±0.34	8.32±0.22
Hb (g/dl)	17.07±0.40	17.18±0.11	16.08±0.60	16.45±0.27
Hct (%)	53.83±0.87	52.17±0.70	51.17±1.49	51.17±1.01
MCV (fl)	58.33±0.88	59.50±0.22	59.67±1.02	59.67±0.42
MCH (pg)	19.60±0.08	19.50±0.19	19.73±0.05	19.70±0.32
MCHC (g/dl)	32.33±0.34	32.65±0.26	32.45±0.08	32.87±0.57
Plt (10^3 cell/mm^3)	943.17±27.15	830.00±53.83	812.67±50.67	851.33±46.61
Neu (%)	8.50±0.22^{b}	8.17±0.31^{b}	5.67±0.67^{a}	4.67±0.61^{a}
Lym (%)	91.00±1.21^{a}	90.33±2.04^{ab}	94.33±0.67^{bc}	95.50±0.62^{c}

Mean values within each row with different superscripts are significantly different, Duncan's test at p< 0.05. PBS= phosphate buffered saline; 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.

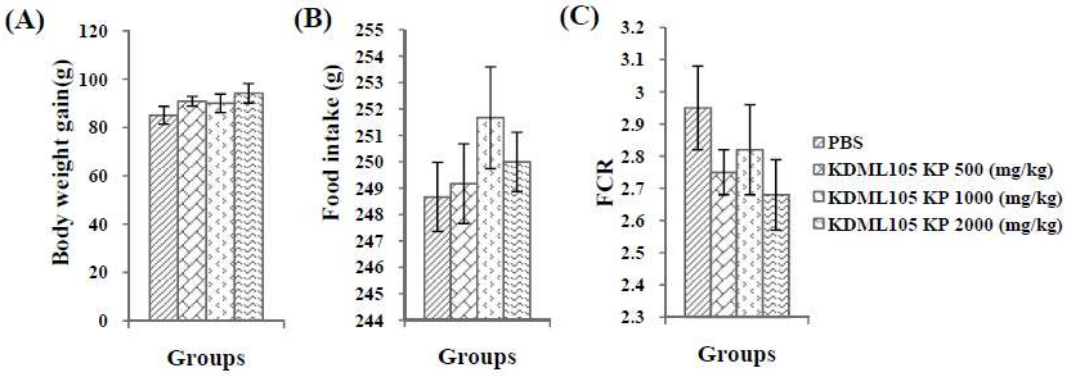

Figure 2 Body weight gain (A), food intake (B) and FCR (C) in rats treated with KDML105KP and PBS from sub-acute toxicity study at the end experiments (mean±SEM). Mean values were not significant different, Duncan's test at $p< 0.05$.

Table 4 Relative organ weight in rats treated with KDML105KP and PBS from sub-acute toxicity study at the end experiment (mean±SEM).

Visceral organs	PBS	KDML105KP (mg/kg)		
		500	1,000	2,000
Liver	3.66±0.10	3.54±0.07	3.67±0.05	3.76±0.07
Lung	0.51±0.01	0.54±0.03	0.54±0.02	0.52±0.01
Heart	0.40±0.02	0.37±0.01	0.37±0.00	0.37±0.01
Kidneys	0.72±0.02	0.71±0.01	0.70±0.02	0.71±0.03
Spleen	0.29±0.00	0.25±0.01	0.26±0.01	0.26±0.01

Table 5 Blood biochemistry in rat treated with KDML105KP and PBS from sub-acute toxicity (mean±SEM).

Blood biochemistry	PBS	KDML105KP (mg/kg)		
		500	1,000	2,000
BS (mg/dl)	195.83±7.25^{c}	176.83±7.78^{bc}	160.67±7.53^{ab}	139.83±8.95^{a}
BUN (mg/dl)	20.45±1.12	21.10±1.03	20.12±0.91	20.07±0.59
CREA (mg/dl)	0.82±0.03	0.87±0.02	0.80±0.02	0.80±0.04
UA (mg/dl)	2.65±0.34	2.72±0.36	2.28±0.14	2.20±0.36
TP (g/dl)	5.72±0.13	5.92±0.10	5.77±0.12	5.70±0.14
Alb (g/dl)	3.28±0.06	3.25±0.08	3.22±0.08	3.12±0.05
Glob (g/dl)	2.48±0.09^{a}	2.60±0.06^{ab}	2.72±0.17^{ab}	2.85±0.04^{b}
TB (mg/dl)	0.07±0.02^{ab}	0.09±0.01^{b}	0.04±0.01^{ab}	0.04±0.00^{a}
AST (U/L)	104.00±1.53^{a}	114.67±5.13^{ab}	117.33±3.56^{b}	144.67±4.52^{c}
ALP (U/L)	132.83±4.66	137.50±3.82	133.33±3.05	131.00±3.47
ALT (U/L)	49.17±1.66	51.33±2.58	51.00±3.13	50.50±1.38
CHO (mg/dl)	54.50±5.42	57.33±2.48	52.33±2.30	50.67±2.01
TG (mg/dl)	135.33±7.54	147.17±3.81	123.00±6.51	122.33±6.16
HDL (mg/dl)	22.55±1.04	22.00±0.48	19.70±1.39	19.87±0.94
LDL (mg/dl)	29.17±1.01^{a}	31.83±1.68^{ab}	35.67±2.22^{b}	36.67±2.36^{b}

Mean values within each row with different superscripts are significantly different, Duncan's test at $p<0.05$.PBS= phosphate buffered saline;BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid; TP = total serum protein; Alb = albumin; Glob = globulin; TB= total bilirubin; AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase; CHO = Cholesterol; TG = triglycerides; HDL = high density lipoprotein; LDL = low density lipoprotein.

Table 6 Hematological values in rats treated with KDML105KP and PBS from sub-acute toxicity study (mean±SEM).

Hematological values	PBS	KDML105KP (mg/kg)		
		500	1,000	2,000
WBC (10^3 cell/mm^3)	6.42±0.28	6.93±0.44	6.95±1.10	8.63±0.74
RBC (10^6 cell/mm^3)	8.76±0.13	8.46±0.20	8.40±0.13	8.37±0.13
Hb (g/dl)	17.52±0.23	16.78±0.32	16.95±0.32	16.90±0.27
Hct (%)	53.33±0.76	51.83±0.87	51.50±0.76	51.83±0.87
MCV (fl)	59.33±0.49^{ab}	59.00±0.26^{a}	59.67±0.33^{ab}	60.50±0.43^{b}
MCH (pg)	19.98±0.28	19.85±0.31	19.97±0.17	20.60±0.17
MCHC (g/dl)	33.72±0.35	33.68±0.47	33.47±0.28	34.02±0.25
Plt (10^3 cell/mm^3)	923.17±24.11	882.83±4.82	888.33±8.35	922.83±14.71
Neu (%)	8.83±1.01^{bc}	8.00±0.58^{b}	6.50±0.56^{b}	3.67±0.56^{a}
Lym (%)	90.83±1.25^{ab}	89.83±1.22^{a}	93.17±0.79^{b}	95.50±0.81^{c}

Mean values within each row with different superscripts are significantly different, Duncan's test at $p<0.05$. PBS= phosphate buffered saline; 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.

พิษกึ่งเฉียบพลัน

หนูที่ได้รับ KDML105KP ทุกขนาดตลอดการทดลองไม่มีการตายหรืออาการความเป็นพิษเช่นเดียวกับการศึกษาพิษเฉียบพลัน และนอกจากนี้หนูที่ได้รับ KDML105KP มีน้ำหนักตัวเพิ่มขึ้น การกินอาหาร และอัตราการแลกเนื้อไม่แตกต่างกันและไม่แตกต่างจากหนูกลุ่มควบคุม(Figure 2A-2C)

แต่อย่างไรก็ตาม น้ำหนักสัมพัทธ์ของอวัยวะของหนูที่ได้รับ KDML105KP ไม่แตกต่างจากกลุ่มควบคุม (Table4)

จากการวิเคราะห์ค่าชีวเคมีโลหิตของหนูที่ได้รับ KDML105KP ขนาด 500, 1,000 และ 2,000 mg/kg เป็นระยะเวลา 14 วัน (Table5) พบว่าหนูที่ได้รับ KDML105KP ขนาด 500 mg/kg มีค่าชีวเคมีโลหิตไม่แตกต่างจากหนูกลุ่มควบคุม หนูที่ได้รับ KDML105KPขนาด 1,000 mg/kg มีค่า BS ลดลง ในขณะที่ค่า ASTและ LDL เพิ่มขึ้น (p<0.05) ส่วนหนูที่ได้รับ KDML105KPขนาด2,000 mg/kg มีค่า BS ลดต่ำลง ในขณะที่ค่า Glob, AST และ LDL เพิ่มสูงขึ้น (p<0.05) เมื่อเปรียบเทียบกับหนูกลุ่มควบคุม

จากการวิเคราะห์ค่าต่างๆ ได้แก่ WBC, RBC, Hb, Hct, MCV, MCH, MCHC และ Pltในหนูที่ได้รับ KDML105KP ไม่แตกต่างกันและไม่แตกต่างจากหนูกลุ่มควบคุม แต่อย่างไรก็ตามพบว่าปริมาณของเม็ดเลือดขาวชนิด neutrophils ในหนูที่ได้รับ KDML105KP ขนาด 2,000 mg/kg ลดลงอย่างมีนัยสำคัญ (p<0.05) ในขณะที่เม็ดเลือดขาวชนิดlymphocytes เพิ่มขึ้นอย่างมีนัยสำคัญ (p<0.05) เมื่อเปรียบเทียบกับกลุ่มควบคุม (Table 6)

สรุปผลและวิจารณ์ผลการทดลอง

เป็นที่ทราบกันโดยทั่วไปว่าคีเฟอร์มีฤทธิ์ทางเภสัชวิทยาหลายอย่างและยังเป็นแหล่งโภชนาการที่สำคัญอีกด้วย[9-16]มีรายงานวิจัยที่ระบุว่าข้าวกล้องขาวดอกมะลิ105 มีฤทธิ์ต้านอนุมูลอิสระและสารพฤษเคมีสูงกว่าในข้าวขัดขาว[2]ผลจากการวิจัยในครั้งนี้แสดงให้เห็นว่า KDML105KP ขนาด 1,000mg/kg และ 500 mg/kg ไม่ก่อให้เกิดพิษเฉียบพลันและพิษกึ่งเฉียบพลันตามลำดับ อีกทั้งไม่ทำให้หนูทดลองตาย KDML105KP ขนาดดังกล่าวจึงมีความเหมาะสมในการนำไปพัฒนาเป็นอาหารเสริมและยารักษาโรคได้ แต่อย่างไรก็ตามเนื่องจากการวิเคราะห์ชีวเคมีโลหิต พบว่าหนูที่ได้รับ KDML105KP ขนาด 2,000 และ 4,000 mg/kg ส่งผลต่อการเปลี่ยนแปลงBS และ Glob ในขณะที่หนูที่ได้รับ KDML105KP ขนาด 1,000 และ 2,000 mg/kg เป็นระยะเวลานานส่งผลต่อการเปลี่ยนแปลงBS, Glob และ AST ซึ่งระดับของ BS เป็นตัวชีวัดการทำงานของไต ขณะที่AST เป็นตัวชีวัดการทำงานของตับ[17-18] โดยระดับของ BS ในเลือดที่สูงขึ้นอาจเป็นผลจากการได้รับแป้งหรือน้ำตาลซึ่งเป็นองค์ประกอบหลักที่พบในข้าวในปริมาณสูงกกว่ากลุ่มควบคุมจึงอาจกล่าวได้ว่าการได้รับ KDML105KP ในปริมาณสูงเป็นระยะเวลานานมีผลต่อการทำงานของตับและไตจากการวิเคราะห์ไขมันในเลือดพบว่าหนูที่ได้รับ KDML105KP ขนาด 4,000mg/kg มีค่า CHO, TG และ HDL เพิ่มสูงขึ้น ซึ่งการเพิ่มขึ้นดังกล่าวมีความเสี่ยงต่อการเกิดภาวะหลอดเลือดตีบตัน (atherosclerosis) ที่นำไปสู่การเกิดโรคหลอดเลือดหัวใจ (coronary artery disease: CAD)[19]แต่อย่างไรก็ตามการได้รับ KDML105KP ขนาด 1,000 และ 2,000 mg/kg เป็นระยะเวลานานไม่ทำให้ระดับของ CHO, TG และ HDL เพิ่มขึ้น แต่มีผลต่อการเพิ่มขึ้นของ LDL ที่สำคัญคือ พบว่าระดับของเม็ดเลือดขาวชนิด neutrophils ลดลง ในขณะที่เม็ดเลือดขาวชนิดlymphocytes เพิ่มขึ้น และการเพิ่มขึ้นดังกล่าวยังสอดคล้องกับการเพิ่มขึ้นของ Glob ซึ่งผลจากการเพิ่มขึ้นดังกล่าวนำไปสู่การกระตุ้นภูมิคุ้มกัน (immunomodulatory activity)[14-15]

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบพระคุณภาควิชาเทคโนโลยีชีวภาพ คณะเทคโนโลยี มหาวิทยาลัยมหาสารคาม ที่ให้ความอนุเคราะห์ผงหัวเชื้อคีเฟอร์DT 5001 ที่ใช้ในการวิจัยครั้งนี้ และขอขอบคุณนิสิตภาควิชาชีววิทยาที่ทำงานวิจัยในสัตว์ทดลองที่ให้ความร่วมมือในการทำวิจัยในครั้งนี้

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Acute toxicityof brown rice kefir powder

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***Abstract*—Kefir is a fermented milk product comprising several lactic acid bacteria, acetic acid bacteria and yeasts. It has been reported to possess pharmacological and antioxidant activities. Recently, kefir from rice milk showed antioxidant activity higher than that from cow milk. To see whether rice and rice products such as rice kefir is safe for human applications. Therefore, the present study was designed to determine an acute toxicity of kefir powder from brown rice; KhaoDawk Mali 105 (KDMLKP), Red Hawm (RHKP) and Hawm Nil (HNKP). The study was carried out in male Wistar rats by once giving kefir powder at the doses of 1,000, 2,000 and 4,000 mg/kg to the rats orally. The results showed that the kefir powder of all the doses did not produce mortality and symptoms of toxicity. Moreover, the kefir powder did not alter relative organ weight (ROW) and feed conversion ratio (FCR) in the kefir powder treated rats compared to those in controls, but this was not a 4,000 mg/kg RHKP. Increasing KDMLKP significantly ($p \leq 0.05$) increased body weight gain and food intake, whilst increasing HNKP significantly ($p \leq 0.05$) decreased the body weight gain, but significantly ($p \leq 0.05$) increased food intake in the treated rats compared to that in controls. However, RHKP did not alter the body weight gain but increasing RHKP significantly ($p \leq 0.05$) decreased food intake in the treated rats compared to that in controls. KDMLKP and HNKP did not alter BS, BUN, CREA, UA, TP, Alb, AST, ALT, and ALP in the treated rats compared to those in controls. KDMLKP at a dose of 4,000 mg/kg significantly ($p \leq 0.05$) increased TP and Alb. Nevertheless, RHKP at a dose of 4,000 mg/kg significantly ($p \leq 0.05$) increased BUN, TP, AST, ALT, and ALP in the treated rats when compared to those in controls. KDMLKP, RHKP and HNKP did not alter WBC, Hb, Hct and Plt in the treated rats compared to those in controls but this was not at a dose of 4,000 mg/kg RHKP. Interestingly, the rats received 4,000 mg/kg KDMLKP and RHKP but not HNKP significantly ($p \leq 0.05$) reduced RBC compared to that in controls. Moreover, increasing KDMLKP, RHKP and HNKP significantly ($p \leq 0.05$) decreased Neu but increased Lym compared to those in controls.**

These findings indicate that the kefir powder from brown rice; KhaoDawk Mali 105 (KDMLKP), Red Hawm (RHKP) and Hawm Nil (HNKP) exhibit non acute toxicity with LD_{50} higher than 4,000 mg/kg. Kefir powder from this brown rice is probably a new good nutrition resource.

***Keywords*—KhaoDawk Mali 105, Red Hawm, Hawm Nil, rice kefir powder, acute toxicity**

I. Introduction

Rice (*Oryza sativa*, L.) is one of the most important economic plants in Thailand. It is an important nourishes nutrition resource. Phytochemicals such as protein, total free amino acids, α-tocopherol, γ-oryzanol, thiamine, niacin, and pyridoxine are higher in brown rice than in ordinary milled rice [1]. Several compounds such as γ-aminobutyric acid (GABA), α-tocopherol, γ-tocopherol and total phenolic compounds (TPC) in rice and rice products exert pharmacological and antioxidant activities [1-4].

Kefir is a fermented milk product comprising several lactic acid bacteria, acetic acid bacteria and yeasts [5]. It has been reportedly possessed anti-bacterial and anti-fungal [3, 6-7], anti-tumor [9], antioxidant [3, 9-10], anti-allergic [11], anti-diabetic [12-13], anti-inflammatory [11, 14-15], and immunomodulatory activities [16]. Kefir can modulate the intestinal mucosa immune response without tissue damage [16]. Fermented milk from kefir has high antioxidant activity [17]. Recently, kefir from rice milk showed antioxidant activity higher than that from cow milk [3]. The antioxidants in kefir produced from plants were higher as a result from the phenolic compounds presence in the plants [18]. As kefir produces good activities on health, therefore it can be considered as a probiotic resource.

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To see whether kefir produced from rice milk is safe for human applications, the present study was therefore, designed to determine acute toxicity of kefir powder from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice.

II. MATERIALS AND METHODS

A. *Brown rice, fermentation and kefir powder preparation*

Brown rice: Khao Dawk Mali 105, Red Hawm andHawm Nil 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 grinded by using 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.

Brown rice fermentation: A0.2 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 centrifuged (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 diluted with sterile 0.85% NaCl (1:10; V:V). Kefir was subcultured by inoculating kefir starter into fresh milk (20:200; V:V) and incubated under anaerobic conditions at 30°C for 48 h to obtain activated kefir grain. The activate kefir grain were cultured and fermented by inoculating into brown rice milk adding with 2.5% sucrose (100:1,000, V:V) and incubated under anaerobic conditions at 30°C for 24 h resulting in the final pH of about 4.8-4.9 of rice milk kefir.

Brown rice kefir powder preparation: Kefir milk from Khao Dawk Mali 105, Red Hawm and Hawm Nil brown rice were freeze-dried and powdered to obtain kefir powder from; Khao Dawk Mali 105 (KDMLKP), Red Hawm (RHKP) and Hawm Nil(HNKP). The kefir powder was kept at -20°C until be used.

B. *Animals*

Eightymale Wistar rats weighing 280-300 g were purchased from National Laboratory Animal Center, Mahidol University, Thailand. The rats were kept in animal laboratory and acclimated for 7 days in environmental conditions (23±2°C and 50-55% relative humidity 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 (ID:0008/2557).

C. *Acute toxicity study*

Acute toxicity study was performed according to Organization for Economic Cooperation and Development (OECD) guideline 423 adoption[19]. Rats were weighed and divided randomly into four groups with 8 rats in each; group 1; rats received phosphate buffered saline (PBS) (control group), group 2, 3 and 4; rats received KDML105KP 1,000, 2,000 and 4,000 mg/kg respectively, group 5, 6 and 7; rats received RHKP 1,000, 2,000 and 4,000 mg/kg respectively, and group8, 9 and 10; rats received HNKP 1,000, 2,000 and 4,000 mg/kg respectively. The kefir powder was 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 for the determination of blood biochemistry and hematological values by using comercial kits (Stanbio LiquiColor®). Visceral organs including liver, lung, heart, kidney and spleen were removed and weighed for a calculation of the relative organ weight (ROW).

Calculation of the relative organ weight and feed conversion ratio: the relative organ weight (ROW) of each animal was calculated using the following equation;

$$\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat (g)}} \times 100 \quad (1)$$

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

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

Determination of blood biochemistry and hematological values: blood samples were put into heparinized and non-heparinized tubes. Non-heparinzed blood was centrifuged at 1500 g for 10 min to separate serum. The serum was assayedfor biochemistry including total protein (TP), blood sugar (BS), blood urea nitrogen (BUN), creatinine (Crea), uric acid (UA), albumin (Alb), globulin (Glob), total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

Heparinized blood was used for hematological analysis including red blood cell (RBC), white blood cell (WBC), hematocrit (Hct), hemoglobin (Hb), platelets (Plt), neutrophils (Neu), and lymphocytes (Lym).

D. Statistical analysis

The data 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 $p \leq 0.05$ were considered statistically significant.

III. RESULTS AND DISCUSSION

A. Symptoms and mortality

The rice kefir treated rats did not produce any symptoms of toxicity and mortality of the rats during 14 days.

B. Body weight gain, food intake, FCR and ROW

Table I. An increase in KDMLKP produced an increase in body weight gain, while an increase in HNKP decreased the body weight gain. However, RHKP did not alter the body weight gain of the treated rats compared to that in controls. An increasing KDMLKP and HNKP also produced an increase in food intake. In contrast, increasing RHKP decreased food intake in the treated rats compared to that in controls. FCR, increasing RHKP increased FCR. Nevertheless, KDMLKP and HNKP did not change FCR in the treated rats compared to that in controls.

However, the ROW in the rats treated with HNKP did not differ from that in controls (data not shown).

C. Blood biochemistry

Blood biochemistry including BS, BUN, CREA, UA, TP, Alb, and Glob are involve in renal function and AST, ALT, and ALP enzymes are involved in hepatic function [20]. KDMLKP did not alter BS, BUN, CREA, and UA. However, increasing KDMLKP increased TP, Alb and Glob ($p \leq 0.05$) in treated rats compared to those in controls. RHKP did not change BS, CREA and Alb. Nevertheless, increasing RHKP increased BUN, TP and Glob ($p \leq 0.05$) in treated rats compared to those in controls. On the other hand, reducing RHKP reduced UA in the treated rats. HNKP did not produce any alteration of BS, BUN, CREA, UA, TP, Alb, and Glob in the treated rats compared to those in controls (Table II).

KDMLKP and HNKP did not alter AST, ALT and ALP while increasing RHKP increased AST and ALT ($p \leq 0.05$) in the treated rats compared to those in controls (Table III).

These findings indicate that HNKP did not effect on hepatic and renal functions because it did not change any blood biochemistry parameters (Table II and III). In contrast, KDMLKP at a dose of 4,000 mg/kg had effect on hepatic function as it had TP and Glob increasing (Table II). In addition, RHKP at this dose had affected both on hepatic and renal functions as it had BUN, UA, TP, Glob, AST, ALT, and ALP altering(Table II and III) when compared with those in controls.

D. Hematological values

KDML105KP, RHKP and HNKP did not alter WBC, Hb, Hct and Plt in the treated rats compared to those in controls (Table IV) but not a 4,000 mg/kg RHKP. Nevertheless, the rats received KDMLKP and RHKP at this dose had RBC significant reduced ($p \leq 0.05$) while the rats received HNKP had no significance different compared to those in controls. Moreover, increasing KDML105KP, RHKP and HNKP decreased Neu ($p \leq 0.05$) while they increased Lym ($p \leq 0.05$) compared to those in controls. Interestingly, the treated rats had neutrophils significant less than while lymphocytes were high levels, consistent with globulin increased. Since the total WBC count did not change, this result suggests that the kefir powder acts in opposite way in the differentiation of hematopoietic cells by suppressing neutrophils and stimulating lymphocytes.

According to previous reports, the kefir induced the helper T-lymphocytes type 2 proliferations by increasing the number of immunoglobulin A (IgA) [17], in agreement with the increasing of lymphocyte and globulin in this study. In the other hand, the significant increasing of globulin may be results of B-lymphocytes were induced to IgA secreting cells.

TABLE I. BODY WEIGHT GAIN, FOOD INTAKE AND FCR IN RATS TREATED WITH PBS AND KEFIR POWDER.

Groups	Body weight gain (g)	Food intake (g)	FCR
PBS	63.75±1.57^{ab}	308.33±1.26^{ab}	4.86±0.12^{ab}
KDML105KP			
1,000 mg/kg	62.50±3.89^{ab}	300.83±1.22^{a}	4.93±0.28^{b}
2,000 mg/kg	66.87±5.17^{ab}	341.25±0.61^{d}	5.37±0.51^{bc}
4,000 mg/kg	78.12±5.50^{cd}	334.79±3.96^{cd}	4.47±0.39^{ab}
RHKP			
1,000 mg/kg	75.00±5.51^{bc}	347.50±3.17^{d}	4.78±0.28^{ab}
2,000 mg/kg	61.87±4.11^{ab}	331.67±0.63^{c}	5.51±0.33^{bc}
4,000 mg/kg	58.12±5.08^{a}	321.04±2.74^{b}	5.87±0.57^{c}
HNKP			
1,000 mg/kg	85.62±3.33^{d}	323.54±0.91^{b}	3.82±0.16^{a}
2,000 mg/kg	77.50±3.66^{c}	325.00±4.88^{bc}	4.27±0.23^{ab}
4,000 mg/kg	71.87±1.62^{b}	338.33±2.44^{cd}	4.72±0.10^{ab}

Mean values within each column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$.

TABLE II. BLOOD BIOCHEMISTRY; BS, BUN, CREA, UA, TP, ALB, AND GLOB IN THE RATS TREATED WITH PBS AND KEFIR POWDER.

Groups	Blood biochemistry						
	BS (mg/dl)	BUN (mg/dl)	CREA (mg/dl)	UA (mg/dl)	TP (g/dl)	Alb (g/dl)	Glob (g/dl)
PBS	182.33±23.55^{ab}	20.17±0.48^{b}	0.91±0.03	3.91±0.55^{b}	5.65±0.11^{ab}	3.47±0.04^{ab}	2.20± 0.06^{a}
KDML105KP							
1,000 mg/kg	189.50±11.84^{ab}	20.65±0.32^{b}	0.81±0.03	3.25±0.19^{ab}	5.85±0.06^{bc}	3.50±0.04^{ab}	2.40±0.06^{b}
2,000 mg/kg	200.50±6.99^{ab}	20.08±0.46^{ab}	0.82±0.02	3.50±0.20^{ab}	5.93±0.03^{bc}	3.52±0.03^{ab}	2.43±0.04^{bc}
4,000 mg/kg	213.83±7.98^{ab}	20.28±0.33^{b}	0.83±0.18	3.30±0.17^{ab}	6.00±0.18^{c}	4.03±0.59^{b}	2.45±0.04^{bc}
RHKP							
1,000 mg/kg	166.17±2.41^{a}	20.23±0.28^{b}	0.90±0.02	2.88±0.17^{a}	5.78±0.07^{bc}	3.37±0.07^{a}	2.35±0.07^{ab}
2,000 mg/kg	182.50±19.68^{ab}	19.87±0.42^{ab}	0.95±0.03	3.38±0.08^{ab}	5.87±0.02^{bc}	3.43±0.03^{ab}	2.50±0.08^{c}
4,000 mg/kg	202.17±11.50^{ab}	18.78±0.63^{a}	0.95±0.02	4.05±0.33^{b}	6.10±0.07^{c}	3.57±0.02^{ab}	2.53±0.06^{c}
HNKP							
1,000 mg/kg	176.67±38.09^{ab}	19.75±0.37^{ab}	0.88±0.02	3.72±0.31^{ab}	5.47±0.17^{a}	3.45±0.08^{ab}	2.25±0.06^{ab}
2,000 mg/kg	214.83±21.09^{ab}	19.63±0.33^{ab}	0.85±0.02	3.65±0.31^{ab}	5.47±0.12^{a}	3.48±0.07^{ab}	2.18±0.05^{a}
4,000 mg/kg	235.33±25.04^{b}	20.62±0.51^{b}	0.87±0.02	4.12±0.07^{b}	5.63±0.02^{ab}	3.47±0.03^{ab}	2.17±0.04^{a}

Mean values within each column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$. BS= blood sugar; BUN = blood urea nitrogen; CREA = creatinine; UA= uric acid TP = total serum protein; Alb = albumin; Glob = globulin.

TABLE III. BLOOD BIOCHEMISTRY; AST, ALT AND ALP IN THE RATS TREATED WITH PBS AND KEFIR POWDER.

Groups	Blood biochemistry (U/L)		
	AST	ALT	ALP
PBS	143.67±3.^{95ab}	39.33±1.17^{ab}	124.33±3.06^{ab}
KDML105KP			
1,000 mg/kg	136.33±5.29^{a}	42.33±1.69^{b}	127.33±2.24^{b}
2,000 mg/kg	148.33±5.98^{b}	40.67±1.23^{b}	127.50±3.27^{b}
4,000 mg/kg	144.50±3.95^{ab}	41.83±1.30^{b}	129.50±1.43^{bc}
RHKP			
1,000 mg/kg	142.50±8.61^{ab}	35.50±1.56^{a}	116.67±2.84^{a}
2,000 mg/kg	153.50±2.08^{bc}	42.33±3.12^{b}	132.67±3.68^{bc}
4,000 mg/kg	161.00±5.59^{c}	49.33±0.61^{c}	135.17±4.12^{c}
HNKP			
1,000 mg/kg	143.83±4.80^{ab}	38.17±2.21^{ab}	118.67±3.01^{a}
2,000 mg/kg	152.83±1.08^{bc}	35.00±0.68^{a}	123.00±2.31^{ab}
4,000 mg/kg	143.50±2.79^{ab}	37.50±0.96^{ab}	126.00±5.39^{b}

Mean values within each r column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$. TB = total bilirubin; AST = serum aspartate aminotransferase; ALT = serum alanine aminotransferase; ALP = alkaline phosphatase.

TABLE IV. HEMATOLOGICAL VALUES IN THE RATS TREATED WITH PBS AND KEFIR POWDER.

Groups	Hematological values						
	WBC (10^3 cell/mm^3)	RBC (10^6cell/mm^3)	Hb (g/dl)	Hct (%)	Plt (10^3cell/mm^3)	Neu (%)	Lym (%)
PBS	5.93±0.16^{ab}	8.87±0.13^{bc}	17.07±0.40^{ab}	53.83±0.87^{ab}	943.17±27.15^{b}	8.50±0.22^{d}	91.00±1.21^{a}
KDML105KP							
1,000 mg/kg	6.18±0.22^{ab}	8.88±0.12^{bc}	17.18±0.11^{b}	52.17±0.70^{ab}	830.00±53.83^{ab}	8.17±0.31^{d}	90.33±2.04^{a}
2,000 mg/kg	5.90±0.16^{ab}	8.29±0.34^{ab}	16.08±0.60^{ab}	51.17±1.49^{ab}	812.67±50.67^{ab}	5.67±0.67^{bc}	94.33±0.67^{bc}
4,000 mg/kg	6.38±0.17^{b}	8.22±0.22^{a}	16.45±0.27^{ab}	51.17±1.01^{ab}	851.33±46.61^{ab}	4.67±0.61^{b}	95.50±0.62^{b}
RHKP							
1,000 mg/kg	6.33±0.39^{b}	8.67±0.20^{b}	17.12±0.40^{b}	56.00±1.88^{b}	881.33±20.51^{b}	7.00±0.26^{cd}	91.33±0.42^{ab}
2,000 mg/kg	6.17±0.21^{ab}	8.33±0.10^{ab}	17.00±0.06^{ab}	52.00±0.45^{ab}	881.17±20.15^{b}	6.33±0.88^{c}	92.83±0.70^{ab}
4,000 mg/kg	5.93±0.35^{ab}	8.16±0.05^{a}	16.05±0.09^{ab}	51.83±0.48^{a}	743.50±74.12^{a}	3.67±0.56^{ab}	96.17±0.79^{bc}
HNKP							
1,000 mg/kg	5.55±0.23^{a}	8.76±0.21^{b}	16.30±0.21^{ab}	52.67±1.31^{ab}	904.50±13.18^{b}	6.17±0.17^{c}	94.00±0.26^{b}
2,000 mg/kg	5.93±0.22^{ab}	9.00±0.12^{c}	16.35±0.32^{ab}	51.83±1.14^{ab}	883.00±23.23^{b}	2.50±0.43^{a}	97.00±1.21^{c}
4,000 mg/kg	5.40±0.27^{a}	8.99±0.05^{c}	16.28±0.28^{ab}	51.50±0.22^{ab}	924.83±17.63^{b}	2.33±0.33^{a}	97.83±0.31^{c}

Mean values within each column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$. WBC = white blood cells; RBC = red blood cells; Hb = hemoglobin; Hct = hematocrit; Plt = platelets; Neu = neutrophils; Lym = lymphocytes.

IV. CONCLUSIONS

HNKP has no acute toxicity but KDMLKP and RHKP at a dose of 4,000 mg/kg exerts acute toxicity as it produces adverse effect on hepatic function. In addition, RHKP at this dose also affects renal functions. Furthermore, its effects on neutrophils and lymphocytes lead to an improve immunomodulatory activity.

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Supaporn Chunchom, Sirirat Deeseenthum and Chusri Talubmook. Acute and sub-acute toxicities of kefir powder from Khao Dawk Mali 105 brown rice milk. Proceeding of the 11th Mahasarakham University Research Conference, 20-22 August 2015, 61-69. (Oral presentation)

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