

Effects of Kefir Fermentation on Antioxidation Activities (*in vitro*) and Antioxidative Stress (*in vivo*) of Three Thai Rice Milk Varieties Prepared by Ultrasonication Technique

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ABSTRACT

Aims: The effects of kefir fermentation were investigated on antioxidation activities (*in vitro*) and antioxidative stress (*in vivo*) for different Thai rice; Hawm Nil rice, Red Hawm rice and Khao Dawk Mali 105 rice. **Methodology:** Antioxidant activity (*in vitro*) was investigated using ferric reducing antioxidant power and 2, 2'-diphenyl-1-picrylhydrazyl assays. In addition, antioxidative stress (*in vivo*) was performed using colitis rat models to study nitric oxide (NO), lipid peroxidation (LPO) and superoxide dismutase (SOD) compared with rats treated with prednisolone and cow's milk kefir. **Results:** Antioxidant activity of rice kefir powder from both assays had higher antioxidant activity than cow's milk kefir powder. NO levels of colitis rats received Hawm Nil rice kefir powder (HNKP) was reduced when compared to phosphate buffered saline (PBS) group. Moreover, colitis rats received HNKP did not differ in NO levels from colitis rats that received prednisolone and non-colitis rats. The result of LPO product malondialdehyde (MDA) indicated that colitis rats treated with HNKP had reduced TBARS compared to PBS group, and did not differ in TBARS levels from rats that received prednisolone and non-colitis rats. Surprisingly, increase in SOD activity was observed in colitis rats that received HNKP compared to PBS, with similar results of increased SOD in rats that received prednisolone and cow's milk kefir powder. **Conclusion:** Hawm Nil rice kefir may offer a protective effect for antioxidative stress resulting from chemical induction; it has potential as a supplementary food with high antioxidant activity and is regarded as safe for consumer health.

Key words: Antioxidant, Antioxidative stress, Lactic acid, Rice kefir, Thai rice

INTRODUCTION

Kefir is an acidic, fermented milk beverage that originated thousands of years ago in the Caucasus Mountains.¹ Popularity and availability of kefir are increasing globally due to the well-known health benefits and longevity related to daily consumption.²⁻¹⁰ Kefir beverage is commonly manufactured by fermenting milk with kefir grains. This process supports a complex microbial symbiotic mixture of lactic acid bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*) and yeasts (e.g., *Kluyveromyces* and *Saccharomyces*).¹¹

The main products of kefir fermentation are lactic acid, ethanol and carbon dioxide which confer the beverage with viscosity, acidity and low alcohol content. Minor components include diacetyl, acetaldehyde, ethyl and amino acids which contribute to the flavor.^{12,13} demonstrated kefirs as potential antioxidants; interacting with a wide range of species that are directly responsible for oxidative damage.² stated that kefir could be made from any type of milk: cow, goat, sheep, coconut, soy and rice; however, cow is commonly used.¹⁴ Reported that cereal grains, especially rice, contain special phenolic acids (such as ferulic,

p-coumaric and diferulic) that are not present in significant quantities in fruit and vegetables,¹⁵ found that brown rice milk kefir powders had higher α -tocopherol, γ -Aminobutyric acid (GABA) and phenolic contents than cow's milk kefir powder.

The antioxidant activity of plant phenolic is primarily due to their redox properties which allow them to act as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers.^{16,17} showed that rice milk kefir had high antioxidant activity compared to butylated hydroxyanisole (BHA). Moreover,¹⁵ reported rice milk kefir powder as having higher antioxidant activity than that cow's milk kefir. Many authors have examined the antioxidant activity of rice milk *in vitro* but few have considered the antioxidative stress of rice milk kefir *in vivo*. Therefore, this study investigated the unique properties of rice milk kefir and analyzed the effects of different varieties of Thai rice fermentation with kefir grains on the content of antioxidant activity *in vitro* and antioxidative stress *in vivo*.

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METHODOLOGY

Rice materials and kefir samples

Thai colored rice varieties namely Khao Dawk Mali 105 rice (KDML 105; white rice), Red Hawm rice (KDML105R-PSL-E-14; red rice) and Hawm Nil rice (PSL00288-4-21-5R; dark purple or black rice) were used. The rice materials were obtained from Selaphum Farmer Group, Roi Et Province, Thailand (2014 harvest season), who used grain from Roi-et Rice Seed Center, Rice Seed Division, Rice Department, Thailand. Rice material and kefir sample preparations followed¹⁸ with minor modifications. Each 250 g of rice was left in 500 mL of water for 24 h before ultra-sonication using a Vibra-Cell Ultrasonicator (20 KHz) with tip diameter (25 mm), intensity (low), volume (500-1,000 mL, amplitude (60%) and time (5 min). The rice was blended, and then filtered with a cotton sheet and pasteurized at 75°C for 15 min. The pasteurized rice milks were immediately cooled and stored in dark plastic bags at a cool temperature of 4°C until required for use. The rice and cow milks prepared earlier were used to produce kefir. All the milk samples were maintained at 25°C for 24 h with 10% (v/v) of kefir starter culture. Following this, the milk kefirs were blended, filtered with a cotton sheet and pasteurized at 75°C for 15 min. All experiments were conducted in triplicate. Rice milk cultures were inoculated at 3% w/v and the kefir grains were incubated at 25°C with fermentation at pH 4.2. Samples were freeze-dried and analyzed.

Kefir powder

All milk kefirs at pH 4.2 were freeze-dried using a SJIA-10N Freeze Dryer (Shanghai Beiyi Bioequip Information Co., Ltd., China) at -55°C. The freeze-dried kefirs from the Khao Dawk Mali 105 rice, Red Hawm rice, Hawm Nil rice and cow milks were powdered with a mortar and pestle under aseptic condition and packed into bottles; the caps were tightened, wrapped with foil and the bottles were kept at -20°C until required for use.

Antioxidant activity *in vitro*

2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging assay

2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging assay determination followed the method of¹⁹ with some modifications. Briefly, 100 µL of DPPH solution was added to 50 µL of each kefir sample. Methanol was used as the control, mixed well and incubated for 30 min in the dark at room temperature. Absorbance of each sample was measured at 517 nm using a micro plate reader. Percentage of inhibition was calculated using the following equation:

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

The standard curve of DPPH was prepared as 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg/mL, absorbance was measured at 517 nm using a micro plate reader and the graph was plotted. The DPPH radical scavenging activity was expressed as the IC₅₀ value; this represented the amount of antioxidant in the kefir solution.

Necessary to reduce the initial DPPH concentration by 50%. The IC₅₀ value was determined from the standard curve of percent scavenging plotted against the rice kefir powder solution concentration. All experiments were performed in triplicate.

Ferric reducing antioxidant activity (FRAP) assay

A FRAP assay was performed following the method of Benzie and Strain (1999)²⁰ with slight modifications. FRAP reagent was prepared by adding 0.0270 g of ferric chloride to 5 mL of distilled water and mixing. Then, 300 mM of acetate buffer was prepared by adding 2.4609 g of sodium acetate in water with pH adjusted to 3.6. Next, 40 mM of HCl was

prepared in the ratio 1:1 with water and 0.66 mL was pipetted and added with 99.44 mL water. Then, 10 mM of 2,4,6-Tripyridyl-s-triazine (TPTZ) solution was prepared by adding 2,4,6-Tripyridyl-s-triazine 0.0156 g in 5 mL of 40 mM HCl, 300 mM of acetate buffer, 10 mM TPTZ, and 20 mM of iron (III) chloride solution. The prepared FRAP reagent was used as follows: 20 µL of each kefir sample was added to 1.50 µL of FRAP reagent. The mixture was stirred thoroughly and incubated in the dark at room temperature for 30 min.

Absorbance was then measured at 595 nm using a micro plate reader. The standard curve ($R_2 = 0.9995$) for FRAP was plotted and prepared as 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/mL. A calibration curve was drawn with concentration of FeSO₄·7H₂O on the X-axis and optical density (OD) on the Y-axis. Values obtained were expressed in µg/ml of ferrous equivalent Fe (II) per µg of each kefir sample.

Anti-oxidative stress activity studies

Experimental design

The Hawm Nil rice kefir power is the highest antioxidant activity, biochemical components values such as gamma amino butyric acid (GABA) content, alpha-tocopherol (α-tocopherol) content and total phenolic content. Moreover, this rice kefir power also had not toxicity in the rat model.¹⁵ So that, the experimental design for antioxidative stress activity in this studied chose only Hawm Nil rice kefir power from three rice kefir powder. In addition, the method was followed the originally method described by Deeseenthum *et al.*¹⁸ Each 6 rats were randomly divided into 7 groups; (1): non-colitis rats received phosphate buffered saline (PBS), (2): non-colitis rats received Hawm Nil brown rice kefir powder (150 mg/kg dissolved in PBS), (3): non-colitis rats received cow's milk kefir powder (150 mg/kg dissolved in PBS), (4): colitis rats received PBS, (5): colitis rats received Hawm Nil brown rice kefir powder (150 mg/kg dissolved in PBS), (6): colitis rats received cow's milk kefir powder (150 mg/kg dissolved in PBS), and (7): colitis rats received prednisolone (5 mg/kg).

Rat colitis groups were induced on day 4 by 2,4,6-trinitrobenzene sulfuric acid (TNBS) while groups treated with Hawm Nil brown rice kefir power, cow's milk kefir powder or prednisolone were left for 10 days.

Colitis induction

Rats were colitis induced on day 4 and thereafter. Colitis induction followed the method originally described by Scarminio *et al.*²¹ After fasting overnight, the rats were anesthetized with halothane. Under anesthesia, they were given 10 mg of TNBS dissolved in 0.25 mL of 50% (v/v) ethanol by means of a 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.

Serum sample collection

At the end of the experiment, rats were fasted for 24 h, weighed and then euthanized with 50 mL of chloroform. Blood samples were placed in heparinized and non-heparinized tubes and centrifuged at 1,500 g for 10 min to separate serum.

Nitric oxide measurement

Serum samples were treated with Centricon 10 (7,500 rpm, 4°C, 1 h) to remove hemoglobin and proteins. The nitric acid (NO) content was assessed by the Griess reaction method using 23479 Nitrate/Nitrite Assay Colorimetric Kit (Sigma-Aldrich, Inc., USA), that is a commercial kit for NO assay. Briefly, preparation of the nitrite calibration curve was performed by adding sodium nitrite (NaNO₂) standard solution and buffer solution to each well. Plot the concentration of NaNO₂ solution

on the X-axis and the absorbance value on the Y-axis to prepare the calibration curve. Plot the concentration of NaNO_3 solution on the X-axis and the absorbance value on the Y-axis to prepare the calibration curve. Determine the concentration of nitrite in the sample solution from the calibration curve. Determine the concentration of nitrate + nitrite in the sample solution using the calibration curve. Then, nitrate concentration can be obtained by the following equation:

$$[\text{Nitrate}] = [\text{Nitrate} + \text{Nitrite}] - [\text{Nitrite}]$$

Lipid peroxidation estimation

The lipid peroxide (LPO) product malondialdehyde (MDA) was estimated using a Lipid Peroxidation (MDA) Assay Kit of thiobarbituric acid reactive substances (TBARS) in serum (Sigma-Aldrich, Inc., USA), that is a commercial kit for Lipid Peroxidation (MDA) Assay. The LPO products were expressed in terms of nmole MDA/ μL . Concentration of MDA can be obtained by the following equation:

$$(\text{Sa/Sv}) \times 4 \times \text{D} = \text{C}$$

Where,

Sa is the amount of MDA in unknown sample (nmole) from the standard curve

Sv is the sample volume (μL) or amount (μg) added into the wells

C is the concentration of MDA in the sample

D is the dilution factor

4 is the correction factor using 200 μL of 800 μL reaction

Superoxide dismutase (SOD) activity

The SOD activity was estimated using a SOD Assay commercial Kit-WST (19160 SOD determination kit, Sigma-Aldrich, Inc., USA). Read the absorbance at 450 nm using a micro plate reader. Calculate the SOD activity (inhibition rate %) using the following equation:

$$\text{SOD activity} = \left\{ \frac{(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})}{(A_{\text{blank1}} - A_{\text{blank3}})} \right\} \times 100$$

Statistical analysis

The experiment used a randomized block split-plot design. Plot effect was rice type and the sub-plot was fermentation variable when comparing fermentation effect. Complete block design was used to compare the antioxidant activities of different rice milks and rice kefir samples. All experiments were performed in triplicate. Experimental data were analyzed for divergence using Duncan's multiple range test and SPSS (SPSS 19.0, SPSS Inc. statistical program). Significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

DPPH free radical scavenging and FRAP values

Antioxidant activity of Red Hawm rice kefir powder showed the highest % inhibition of DPPH and was significantly different ($p \leq 0.5$) from the other kefirs (85.79 \pm 0.34). Cow's milk kefir powder showed the lowest antioxidant activity from DPPH free radical scavenging with FRAP assays at 77.59 \pm 0.24 and 2.672 \pm 0.115, respectively (Table 1).

Anti-oxidative stress in rat models

Nitric oxide

The NO level in the serum was higher in colitis rats that received PBS (control) compared to non-colitis rats (Figure 1, $p \leq 0.05$). However, colitis rats that received Hawm Nil kefir powder (HNKP) gave reduced

NO levels compared to negative controls ($p \leq 0.05$). Moreover, colitis rats that received HNKP did not show reduced NO levels compared to rats that received prednisolone and non-colitis rats (Figure 1).

Nitric oxide is a potent, endogenous vasodilator that modulates renal function and plays a key role in endothelial dysfunction.²² Colitis rats that received PBS (control) had higher NO levels in serum than non-colitis rats. However, colitis rats treated with HNKP showed reduced NO levels compared to the controls. These findings indicated that rice kefir powder may reduce NO excretion in colitis rats compared to controls. Other authors investigated the role of probiotic bacteria in the generation of local NO in the intestinal lumen by nitrate reduction or acid dependent mechanisms. This may be counteracted through rapid NO consumption by other strains or diffused into the surrounding tissues,²³ and explain some of the health promoting effects of this kefir by reducing NO levels in rat models.

Lipid peroxidation

Colitis rats that received PBS showed increased TBARS in serum (20.78 \pm 0.58, $p \leq 0.05$). However, colitis rats treated with HNKP gave reduced TBARS compared to controls (10.10 \pm 1.06 vs 20.78 \pm 0.58, $p \leq 0.05$). Moreover, colitis rats that received HNKP showed similar TBARS levels to rats that received prednisolone and non-colitis rats (Figure 2).

Table 1: Antioxidant activity in cow and pigmented rice's milk fermented with kefir grain at pH 4.2.

| Treatments | (%) Inhibition of DPPH | FRAP values |
|--------------------------------------|---------------------------------|--------------------------------|
| Cow Milk kefir powder | 77.59 \pm 0.24 ^a | 2.672 \pm 0.115 ^b |
| Khaw Dawk Mali 105 rice kefir powder | 82.51 \pm 0.12 ^{a,b} | 2.725 \pm 0.107 ^a |
| Red Hawm Rice kefir powder | 85.79 \pm 0.34 ^b | 2.874 \pm 0.197 ^a |
| Hawm Nil Rice kefir powder | 78.14 \pm 0.16 ^a | 2.876 \pm 0.145 ^a |

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

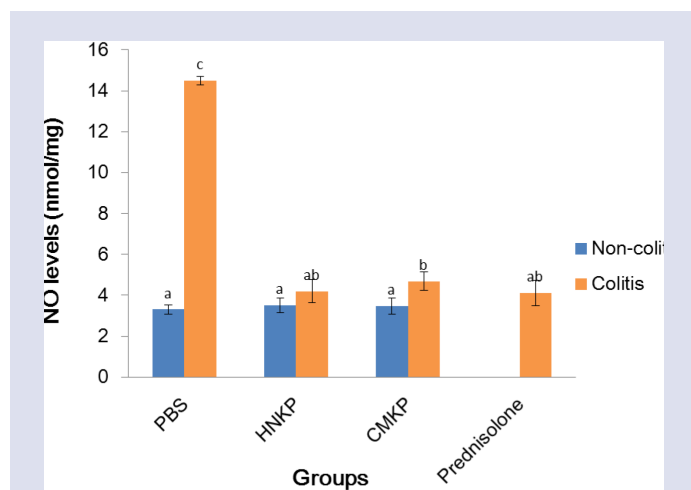


Figure 1: Nitric oxide (NO) levels in the treated rat colitis compared to those in controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate buffered saline, HNKP = Hawm Nil Rice kefir powder, CMKP = Cow Milk kefir powder.

Lipid peroxidation is the degradation of lipids that occurs because of oxidative damage and a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with end products such as malondialdehyde (MDA). Lipid peroxidation may contribute to the pathology of many diseases including atherosclerosis, diabetes and Alzheimer's. Here, a significant increase of LPO (TBARS) was recorded in colitis rats, indicating that peroxidative injury involved the reduction of antioxidant defense mechanisms and development of colitis complications.²⁴ Colitis rats treated with RKP showed significantly decreased LPO. Bioactive peptides released during fermentation e.g., α -tocopherol, γ -amino butyric acid and total phenolic contents, by proteolytic lactic acid bacteria can scavenge reactive oxygen species (ROS) and inhibit LPO, consistent the reported by Pihlanto.²⁵

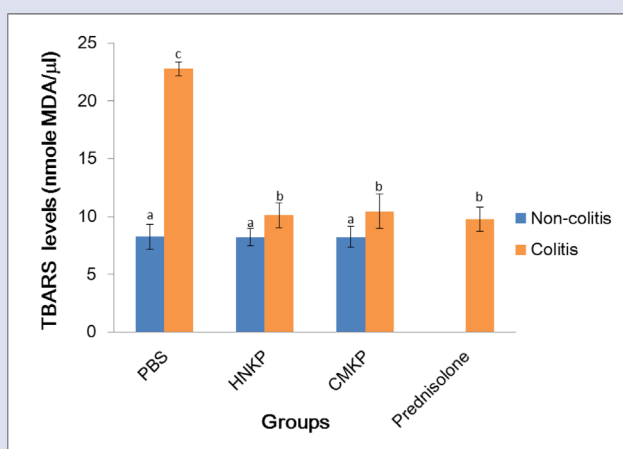


Figure 2: Thiobarbituric acid reactive substances (TBARS) levels in the treated rat colitis compared with controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate buffered saline, HNKP = Hawm Nil Rice kefir powder, CMKP = Cow Milk kefir powder.

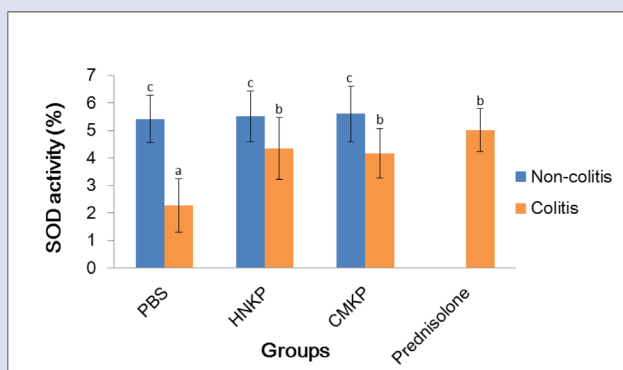


Figure 3: Superoxide dismutase activities (SOD) in the treated rat colitis compared with controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate buffered saline, HNKP = Hawm Nil Rice kefir powder, CMKP = Cow Milk kefir powder.

Superoxide dismutase (SOD)

Increase in SOD activity was observed in colitis rats that received HNKP compared to colitis rats that received PBS (controls). This indicated that the antioxidant defense system was functional in colitis rats that received HNKP; similar findings of increased SOD were seen in rats that received prednisolone and cow's milk kefir powder (Figure 3).

Moreover, increase in SOD activity was observed in colitis rats treated with HNKP compared to colitis rats that received PBS (controls). SOD is the primary enzymatic antioxidant defense system in the cell and catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen as one of the most important anti-oxidative enzymes. Several direct and indirect methods have been developed to determine SOD activity. A common, convenient and easy indirect method uses nitro blue tetrazolium (NBT). However, there are several disadvantages to the NBT method such as poor water solubility of the formazan dye and interaction with the reduced form of xanthine oxidase. The SOD Assay Kit-WST is very convenient and utilizes Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye on reduction with a superoxide anion.²⁶ The IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Antioxidant defense systems were functional in colitis rats that received HNKP, with similar findings of increased SOD in rats subjected to prednisolone and CMKP.

CONCLUSION

Thai rice kefir powder included high antioxidant activity. Hawm Nil rice kefir powders had the highest antioxidant activity, followed by Red Hawm rice kefir powder and Khao Dawk Mali 105 rice kefir powder, respectively. Moreover, the Hawm Nil rice kefir powder also may offer protection against chemically induced antioxidative stress such as nitric oxide, lipid peroxidation, while stimulate superoxide dismutase. Thus, this rice milk kefir has potential as a supplementary food with high antioxidant activity and it is regarded as safe for consumer health.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ABBREVIATIONS

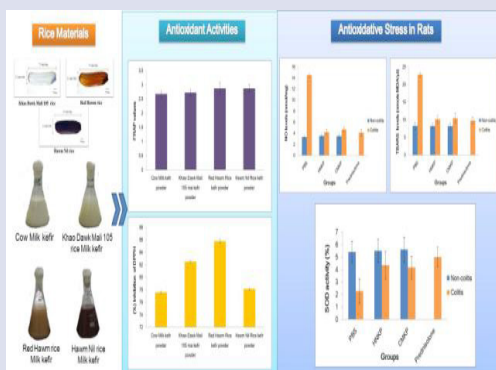
PBS: Phosphate buffered saline; **HNKP:** Hawm Nil Rice kefir powder; **CMKP:** Cow Milk kefir powder; **TNBS:** 2, 4, 6-trinitrobenzene sulfonic acid; **FRAP:** Ferric reducing antioxidant power; **DPPH:** 2, 2'-diphenyl-1-picrylhydrazyl; **NO:** Nitric oxide; **LPO:** Lipid peroxidation; **SOD:** Superoxide dismutase; **MDA:** Malondialdehyde; **TBARS:** Thiobarbituric acid reactive substances.

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GRAPHICAL ABSTRACT



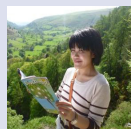
SUMMARY

- The rice kefir powder had high antioxidant activity.
- Hawm Nil rice kefir powder (HNKP) can reduce NO levels in the colitis rats.
- HNKP can reduce TBARS levels in the colitis rats.
- Surprisingly, this rice kefir takes SOD activity increased in colitis rats.
- The NO, TBARS and SOD levels of colitis rats that received HNKP had no differ from the prednisolone, which is a current medicine.

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