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Therapeutic effects of four molecular-weight fractions of Kurozu against dextran sulfate sodium-induced experimental colitis

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Background/aims: *Kurozu has been reported to ameliorate colitis in mice and to have an anti-oxidative effect. However, the active components and mechanism of action remain unknown. Here, as a first step to identify the active components, we chromatographically fractionated Kurozu and investigated the anti-colitis activity of the fractions, focusing on anti-nitration activity.* ***Methods:*** *Kurozu was divided into 4 molecular-weight fractions (fraction I, >4,000 daltons; II, 2,000~4,000 daltons; III, 800~2,000 daltons; IV, <800 daltons). Forty C57black6 mice were divided into 5 groups as follows: the control group received standard CE-2 diet, and Groups I~IV received CE-2 diet containing Kurozu fractions I~IV, respectively. Dextran sulfate sodium was administered to the mice for 12 days to induce colitis. Body weight and bloody stool frequency were monitored as indices of severity of colitis after administration of dextran sulfate sodium, and at 12 days, all mice were sacrificed for examination of colonic pathology and nitrotyrosine production in the colon tissues.* ***Results:*** *Colitis was markedly ameliorated in Group III, followed by Group II, while Group IV showed little difference from the control. The colonic nitrotyrosine level in Group III was significantly reduced compared with the control.* ***Conclusions:*** *The major protective components in Kurozu appear to have molecular weights in the range of 800~4,000 daltons, and their action appears to be related, at least in part, to anti-oxidative and anti-nitration effects.*

Key words: Kurozu, dextran sulfate sodium-induced colitis, nitrotyrosine, peroxynitrite, oxidative stress

Dekstran sülfat sodyum ile oluşturulan deneysel kolitte dört farklı moleküler ağırlıkta Kurozu fraksiyonunun teropötik etkisi

Amaç: *Kurozu'nun farelerde koliti düzelttiği ve anti-oksidan etkileri olduğu rapor edilmiştir. Ancak, içeriğindeki aktif madde ve etki mekanizması bilinmemektedir. Burada, aktif içeriğinin tespit edilmesinde ilk basamak olarak, Kurozu'nun kromatografik olarak ayrıştırılması ile elde edilen kimyasalların kolite karşı olan etkileri incelenmiş ve özellikle nitrasyona karşı olan aktivitenin üzerinde durulmuştur.* ***Yöntem:*** *Kurozu molekül ağırlığına göre dörde ayrıştırılmıştır (Fraksiyon I: >4000 dalton; II: 2000-4000 dalton; III: 800-2000 dalton; IV:<800 dalton). Kırk adet C57black6 faresi; standard CE-5 diyetini alan kontrol grubu ve Kurozu fraksiyonlarını içeren CE-5 diyeti alan dört grup olmak üzere (sırasıyla grup I-IV) beşe bölünmüştür. Kolit gelişmesi için farelere 12 gün boyunca dekstran sodyum sülfat uygulanmıştır. Farelerin ağırlıkları ve kanlı ishal sıklığı indüklenen kolitin şiddetinin tespit edilmesi amacı ile takip edilmiş; 12. günde tüm fareler sakrifiye edilmiş ve patolojik inceleme ve nitrotirozin düzeyinin araştırılması amacı ile kolon dokusu örnekleri alınmıştır.* ***Bulgular:*** *Sırasıyla, grup III ve II'de kolitin belirgin olarak önlendiği, ancak grup IV ile kontrol grubu arasında çok hafif bir fark olduğu gözlemlenmiştir. Kolon dokusunda nitrotirozin seviyesi grup III'de kontrole göre anlamlı olarak düşük bulunmuştur.* ***Sonuç:*** *Kurozu'nun bileşenlerinden koruyucu olanlarının moleküler ağırlıkları 800-4000 dalton arasındadır ve bu etkisi; en azından kısmen, anti-oksidatif ve anti-nitrasyon etkinliğine bağlıdır.*

Anahtar kelimeler: Kurozu, dekstran sülfata bağlı kolit, nitrotirozin, peroksinitrit, oksidatif stres

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INTRODUCTION

In Japan, various fermentative products, including *Kurozu, Natto* and *Koji,* are consumed as health food products. *Kurozu* is a traditional Japanese black vinegar, produced from unpolished rice by prolonged (more than one year) fermentation in earthenware jars. The supernatant is *Kurozu,* while the sediment is called *Kurozu moromimatsu.*

Kurozu has been reported to have an anti-inflammatory effect as well as anti-cancer activity against *in vitro*-cultured colon cancer cells (1) and in an animal model of colon cancer (2). However, little is known about the protective effects of *Kurozu* against inflammatory bowel disease (IBD), such as ulcerative colitis (UC), in humans or animal models. We previously reported the protective effects of *Kurozu* against dextran sulfate sodium (DSS)-induced colitis in mice as a part of our study on dietary factors effective against UC (3). We found that acetic acid, which is the main component of *Kurozu* (3), showed no anti-colitis activity, and the active components of Kurozu remained unidentified.

UC is an obstinate IBD. UC patients are generally treated with prednisolone or immunosuppressive drugs, but aggressive treatments are potentially toxic. Moreover, severe UC may require long-term abstinence from food intake. Effective nutritional therapies for UC patients have not yet been established, in contrast with the case of Crohn's disease. Therefore, it would be useful to identify dietary factors that might ameliorate colitis.

Multiple factors, including oxidative stress and nitration stress, are involved in the pathogenesis of UC and influence its severity (4). In particular, enhanced release of reactive oxygen species (ROS), such as superoxide, hydroxyl radical, and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), such as peroxynitrite generated via nitric oxide (NO), plays an important role in both clinical UC and DSS-induced colitis (5).

Superoxide has a protective action, for example against microorganisms, but excessive superoxide generation results in tissue damage. Moreover, superoxide reacts rapidly with endogenous NO to generate peroxynitrite, which is strongly cytotoxic (6). Nitrotyrosine is a good index of the generation of peroxynitrite and is known to be formed via at least two pathways. One is nitration of protein tyrosine residues by peroxynitrite generated by superoxide and NO. The other is reaction of myeloperoxidase (MPO) and nitrite (7). Nitrotyrosine is widely used as a marker of oxidative or nitration stress.

Kurozu has been reported to have potent anti-oxidative stress activity (8). Our previous study revealed an anti-colitis effect of *Kurozu* (3), and oxidative and nitration stress are known to be involved in the pathogenesis and severity of UC (4). Therefore, we chromatographically fractionated *Kurozu* and investigated the protective effects of four molecular-weight fractions against DSS-induced colitis in mice, focusing on anti-oxidative and anti-nitration stress activity.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Experimentation Committee, Tokai University, School of Medicine, Japan.

Column Chromatography

Freeze-dried *Kurozu* (Sakamoto Kurozu Inc., Kagoshima, Japan) was thawed and applied to a Bio-Gel P4 (Bio-Rad, Hercules, CA, USA) poly-acrylamide column. The column was eluted with super-pure water, with monitoring by absorbance measurement at 280 nm. The eluates were divided into 4 fractions based on molecular weight, as follows: I, >4,000 daltons (Da); II, 2,000~4,000 Da; III, 800~2,000 Da; IV, <800 Da.

Experimental Animal Model

Forty C57black6 female mice (6 weeks of age) were supplied by CLEA Japan Inc. (Tokyo, Japan) and bred under specific-pathogen-free conditions. These mice were randomized into 5 dietary groups (each group: n=8). The control group received standard CE-2 diet (CLEA Japan Inc.) and Groups I~ IV received CE-2 diet containing 3.2% *Kurozu* fractions I~ IV, respectively. The special CE-2 diets containing fractions of *Kurozu* were supplied by Sakamoto *Kurozu* Inc. CE-2 is a standard rodent diet, and includes soybean or white fish meal as source of protein, soybean oil or germ as source of lipids, rice bran or alfalfa as a source of carbohydrate, vegetable fiber, several vitamins, and minerals. The *Kurozu* content in these diets was selected based on volumes typically ingested by humans, adjusted for body weight. These diets were started a week before the initial administration of DSS (Sigma-Aldrich, St. Louis, MO, USA). In all groups, a 3.5% solution of DSS in water was given orally for 12 days to C57black6 mice to prepare the DSS-induced colitis animal model.

Evaluation of Manifestations in Mice

Changes in body weight and bloody stool frequency were monitored every 2 days for 12 days after the start of DSS administration. Body weight after DSS administration is given as % of basal body weight before DSS administration, taken as 100%. On day 12, all mice were sacrificed and the middle colon was resected.

Histological Examination

Macroscopic findings of the resected colon were examined. For microscopic examination, resected colonic tissues from all mice were stained with hematoxylin-eosin (H-E). Moreover, histological findings were evaluated. Colon histology in all groups was scored according to a previous report (9).

For mucosal damage: normal, 0; 3–10 intraepithelial cells (IEL)/high-power field (HPF) and focal damage, 1; >10 IEL/HPF and rare crypt abscesses, 2; and >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, 3.

For submucosal damage: normal or widely scattered leukocytes, 0; focal aggregates of leukocytes, 1; diffuse leukocyte infiltration with expansion of submucosa, 2; and diffuse leukocyte infiltration, 3.

For muscularis damage: normal or widely scattered leukocytes, 0; widely scattered leukocyte aggregates between muscle layers, 1; leukocyte infiltration with focal effacement of the muscularis, 2; and extensive leukocyte infiltration with transmural effacement of the muscularis, 3.

Nitrotyrosine Assay

Nitrotyrosine levels of resected colon tissues were compared between Group III and the control group at 12 days after the start of DSS administration because group III showed the greatest attenuation of body weight reduction and blood stool frequency among the *Kurozu*-treated groups. Levels of nitrotyrosine in colon tissues were measured using a commercial ELISA kit (NWLSS, Funakoshi Co., Osaka, Japan).

Statistical Analysis

Differences in body weight reduction and histological scores among the 5 groups were statistically analyzed by means of one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Differences in bloody stool frequency were statistically analyzed using contingency tables. The levels of nitrotyrosine in the colonic tissues in 2 groups were compared using the unpaired t-test. The criterion of significance was $p<0.05$. Values of body weight (%) of mice, histological scores and nitrotyrosine levels of the resected colon are given as mean and standard deviation (SD). Bloody stool frequency in mice after DSS administration is given as percent (number of affected mice divided by total number of mice, x 100).

RESULTS

Group III, followed by Groups II and I, showed significant attenuation of body weight reduction compared with the controls and Group IV in the 4-12 days after the start of DSS administration. However, there was no significant difference between Group IV and the controls at any time point. At 10-12 days after the start of DSS administration, Group III showed significant attenuation of body weight reduction compared with all other groups, while Group II showed significant attenuation versus the control and Groups I and IV, and Group I did so versus the controls and Group IV. There was no significant difference between the control group and Group IV throughout days 2-12 (Table 1).

Table 1. Changes in body weight after administration of DSS

Days†	2	4	6	8	10	12
Control Group	93.6±3.3	89.1±3.6	86.9±4.4	82.5±7.2	70.1±7.5	66.6±8.5
Kurozu I Group	94.0±1.6	93.3[a]±4.9	93.0[a]±5.8	89.2[a]±10.6	78.1[b]±10.1	71.4[b]±6.9
Kurozu II Group	94.7±2.3	94.3[a]±3.4	93.8[a]±5.2	91.5[a]±6.2	87.1[c]±4.5	83.0[c]±8.4
Kurozu III Group	96.0±3.1	94.8[a]±4.7	94.1[a]±4.8	92.9[a]±3.2	91.5[d]±5.1	88.9[d]±8.3
Kurozu IV Group	93.5±3.9	88.7±4.8	87.1±6.2	82.1±5.2	68.4±4.8	65.5±8.1

[a]$p<0.01$ versus the control and IV groups. [b]$p<0.01$ versus the control, II, III and IV groups.

[c]$p<0.01$ versus the control, I, III and IV groups. [d]$p<0.01$ versus the control, I, II and IV groups.

There was no significant difference between the control group and group IV throughout 2-12 days.

†Days after administration of DSS

Body weight after DSS administration is given as % of basal body weight before DSS administration, taken as 100%.

Table 2. Frequencies of bloody stool after administration of DSS

Days†	2	4	6	8	10	12
Control Group	9/10 (90%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Kurozu I Group	7/10 (70%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Kurozu II Group	0/10* (0%)	0/10*(0%)	0/10* (0%)	0/10* (0%)	1/10* (10%)	2/10*(20%)
Kurozu III Group	0/10* (0%)	0/10*(0%)	0/10* (0%)	0/10* (0%)	1/10* (10%)	1/10*(10%)
Kurozu IV Group	9/10 (90%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

*p<0.01 versus the control, Kurozu I and Kurozu IV Groups

†Days after administration of DSS

The bloody stool frequency in mice after DSS administration is given as % of the number of animals in each group.

Bloody stool was noted in all mice among the controls and Groups I and IV at 4-12 days after the start of DSS administration, but was not noted in Groups III and II at 2-8 days after the start of DSS administration. The bloody stool frequency at 12 days was only 10% in Kurozu Group III and 20% in *Kurozu* Group II (Table 2).

Macroscopic examination of resected colons revealed erosions and edematous changes in the control group and Group IV followed by Group I, while these changes were suppressed in Groups II and III. The H-E staining revealed epithelial abrasion, glandular destruction and inflammatory cell infiltration in mucosal and submucosal areas of the colon in the control group, as well as Groups I and IV. However, in Groups II and III, these findings were greatly alleviated compared with the control group (Figure 1a~e). Histological scores (9) were as follows: Control group, 5.88±0.33; Group I, 4.38±0.48; Group II, 0.88±0.33; Group III, 0.50±0.50; and Group IV, 5.63±0.48. In Groups II and III, the scores were significantly ($p<0.001$) reduced compared with the controls and Groups I and IV. Moreover, in Group I, the score was significantly ($p<0.001$) reduced compared with the controls and Group IV. There was no significant difference between Groups II and III, or between the controls and Group IV (Figure 2).

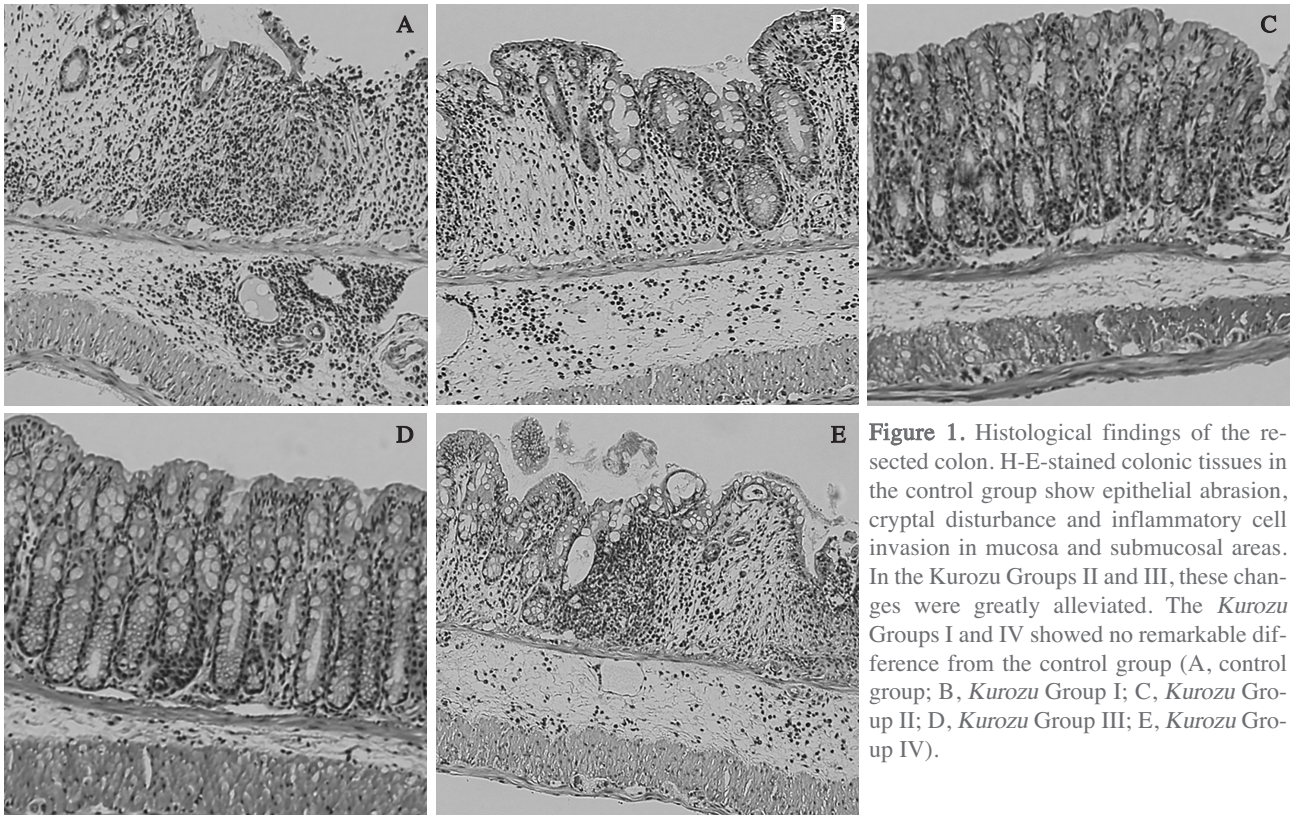

Figure 1. Histological findings of the resected colon. H-E-stained colonic tissues in the control group show epithelial abrasion, cryptal disturbance and inflammatory cell invasion in mucosa and submucosal areas. In the Kurozu Groups II and III, these changes were greatly alleviated. The *Kurozu* Groups I and IV showed no remarkable difference from the control group (A, control group; B, *Kurozu* Group I; C, *Kurozu* Group II; D, *Kurozu* Group III; E, *Kurozu* Group IV).

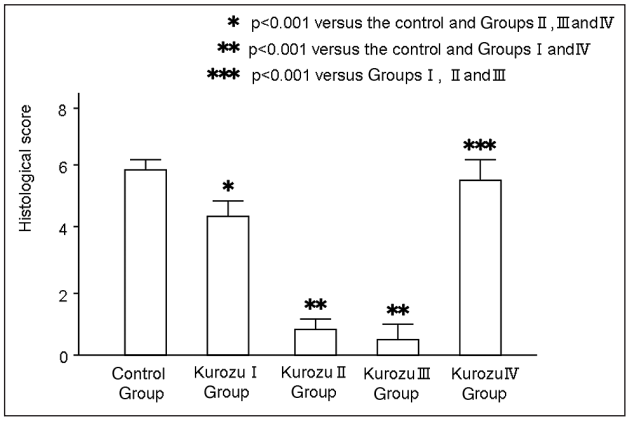

Figure 2. Comparison of histological scores of the colon among the 5 groups.The scores were significantly ($p<0.001$) reduced in Groups II and III compared with the controls and Groups I and IV. Moreover, the score was significantly ($p<0.001$) reduced in Group I compared with the controls and Group IV. There was no significant difference between Groups II and III or between the controls and Group IV.

Nitrotyrosine level in the resected colonic tissues, determined by ELISA, was significantly ($p<0.001$) reduced to 54.5±7.1 ng/g protein in Group III compared with 83.3±9.6 ng/g protein in the controls (Figure 3).

DISCUSSION

Our results indicate that the active components in *Kurozu* were present in the fractions corresponding to the molecular weight range of 800~4,000 Da (*Kurozu* fractions III and II). These fractions greatly ameliorated DSS-induced colitis, and fraction III suppressed generation of nitrotyrosine.

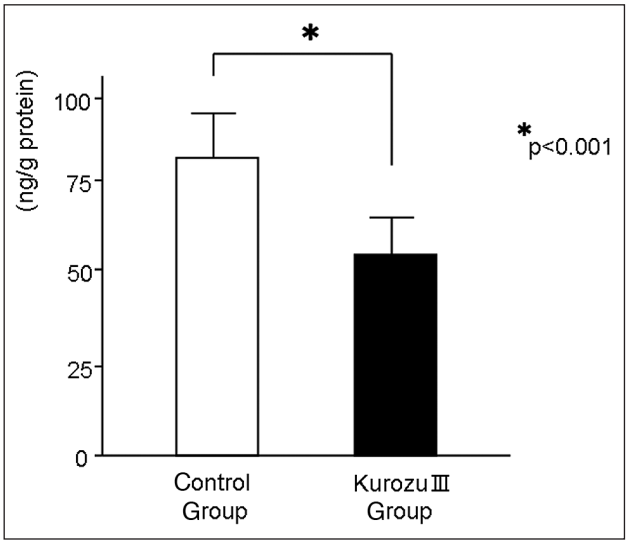

Figure 3. Nitrotyrosine levels in the resected colonic tissues. The *Kurozu* III treatment (54.5±7.1) reduced significantly nitrotyrosine levels in the resected colonic tissues in comparison to the control group (83.3±7.4).

It is well known that oxidative stress and nitration stress are involved in the pathogenesis of UC and influence its severity (4). We found that nitrotyrosine formation in colonic tissues was reduced by administration of active fractions of *Kurozu*. Nitrotyrosine is produced via at least two pathways, i.e., reaction of nitrite and MPO, or reaction of superoxide and NO. Therefore, the active components of *Kurozu* may suppress either of these pathways, or both. This would cause a reduction of nitration stress, which in turn may be related to the amelioration of colitis.

Nitrite is derived from NO, and nitrite itself is not thought to cause tissue damage. Indeed, Ohtake et al. (10) reported that oral administration of nitrite ameliorated DSS-induced colitis. Moreover, to our knowledge, there is no report indicating that nitrite itself aggravates DSS-induced colitis.

Regarding the MPO pathway, MPO oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent (11). Since MPO is mainly released from neutrophils, active components of *Kurozu* may block MPO release from neutrophils. Therefore, we cannot rule out the possibility that the active components of *Kurozu* suppress the reaction of MPO and nitrite.

On the other hand, peroxynitrite seems more likely to play a key role in the induction of colitis in this study, because peroxynitrite exhibits cytotoxicity, and induces severe tissue damage (7). Suppression of superoxide and/or NO by active components of *Kurozu* may induce the suppression of peroxynitrite production, leading to a decrease of nitrotyrosine production. Superoxide itself is cytotoxic and is associated with tissue damage. Moreover, it is converted to H_2O_2, which in turn generates hydroxyl radical. Therefore, suppression of superoxide production would lead to decreased production of both hydroxyl radical and peroxynitrite production, thereby leading to amelioration of colitis. On the other hand, excessive NO production can aggravate inflammatory conditions. NO is mainly synthesized via inducible nitric oxide synthase (iNOS) under inflammatory conditions, and iNOS activity is increased in UC (12). Therefore, active components of *Kurozu* may suppress peroxynitrite formation by reducing superoxide and/or NO, although the effects of active components of Kurozu on iNOS activity and NO production remain to be examined.

Kurozu contains abundant components, including amino acids, oligopeptides, organic acids, minerals, vitamins, saccharides, carbohydrates, and lipids. However, acetic acid, which is the main component of *Kurozu,* was found to be ineffective against DSS-induced colitis in our previous study (3). Further, acetic acid was not present in the *Kurozu* fractions used in this study.

Several amino acids or their metabolic or degradative products are known to have anti-oxidative effects and preventive effects on inflammatory colitis in animals (13). However, the molecular weights of these compounds do not lie within the range of 800~4,000 Da. Similarly, organic metabolites generated by lactobacillus or *koji* bacillus, produced in the process of fermentation of *Kurozu,* improve IBD in UC patients (14), or an animal model (15), possibly by influencing the intestinal flora. However, these compounds are also mostly expected to have molecular weights below 800 Da.

On the other hand, oligosaccharides are also candidates because they have been reported to increase the growth of *Bifidobacterium* sp. and to ameliorate colitis (16). Moreover, oligosaccharides from agar suppress NO production in vitro (17), and a reduction of iNOS activity by oligosaccharides has a protective effect in a rat model of colitis (18). Therefore, oligosaccharides in *Kurozu* may suppress superoxide and/or NO generation, leading to reduced peroxynitrite formation.

In conclusion, our results support the idea that the anti-colitis activity of *Kurozu* involves anti-oxidative or anti-nitration effects and indicate that the active components of *Kurozu* are present in the 800~4,000 Da fractions. *Kurozu* might be a candidate for dietary therapy for UC, although further study will be needed to confirm this. We are planning to further fractionate the 800~4,000 Da fractions in Kurozu in order to identify the active compounds.

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