Preventive Effect of Antioxidant From Purple Rosella Yoghurt on Duodenum and Colon Histopathology of *Rattus norvegicus* Exposed to Rhodamine B And Saccharin

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Abstract

Rodhamine B and Saccharin are often misused by food producers as food additive, which, if their use is not monitored, can produce free radicals that cause oxidative stress conditions in the body, leading to dangerous damage to the body. The addition of purple rosella in yoghurt can increase the functional value of the yoghurt, namely increasing the antioxidant content. The purpose of this study was to determine the benefits of giving purple rosella yoghurt in preventing damage to the duodenum and colon of (*Rattus norvegicus)* caused by Rodhamine B and Saccharin. This study used Completely Randomized Design (CRD) with 28 male *R. novergicus*, *Wistar* strain in aged 8-12 weeks. Group K was not given any treatment; R was given Rodhamine B, S was given Saccharin, KRS was given Rodhamine B and Saccharin, YR was given Rodhamine B and yoghurt, YS was given Saccharin and yogurt, YRS was given Rodhamine B, Saccharin and yogurt. The dose of Rodhamine B was 22.5 $mg.kg^{-1}$, Saccharin was 157.77 $mg.kg^{-1}$, and the volume of yoghurt was 1 $mL.head^{-1}$. They were administered orally with a gastric tube for 14 days. The concentration of purple rosella extract added to yoghurt was 15% (v/v). Parameters were observed in histopathology of the duodenum and colon with the scoring method. Data analysis was done with the Kruskal-Wallis test followed by the Mann-Whitney test. The KRS group showed the heaviest damage, namely necrosis, villi erosion, and inflammatory cell infiltration of >75% of the total visual field in the duodenum and colon. The YR, YS, and YRS groups showed significant changes compared to the R, S, and KRS groups, namely necrosis, villous erosion, and inflammatory cell infiltration, which appeared to be 25-50% of the total visual field. However, this did not match the condition of group K, which appeared to be <25% of the total visual field. The provision of purple rosella yoghurt can prevent 50% damage to the duodenum and colon of *Rattus novergicus* due to Rodhamine B and Saccharin.

Keywords: colon, duodenum, Rodhamine B, Saccharin, yoghurt.

INTRODUCTION

In food industries these days, more and more food producers are using food additives in food and beverage processing for various purposes. If food additives are not used in accordance with the dosage in the long term, it will cause harm to health and may cause diseases. The dosage of food additives needs to be better monitored. Even though it has been banned, synthetic dyes such as Rodhamine B and artificial sweeteners such as Saccharin are still sold in the market. Rodhamine B is often misused to color sausages, corned beef, and sauces. Rodhamine B is typically used in the textile industry, paper industry, and fabric dyes [1,2]. The impact of repeatedly consuming Rodhamine B in large quantities can lead to body precipitation, causing digestive tract irritation [3]. Apart from artificial coloring, artificial sweeteners such as Saccharin are often used because they taste 400 times sweeter than sucrose. Saccharin is usually found in products that use sweeteners, such as ice cream. Excessive consumption of Saccharin can cause side effects, such as diarrhea, allergies, hypertension, and bladder cancer [4].

Rodhamine B and Saccharin are xenobiotic substances that can produce free radicals as side products. Free radicals will induce the formation of Reactive Oxygen Species (ROS) and oxidative stress. Free radicals that are formed will be distributed throughout the body, including the duodenum and colon [2]. The best way to protect the body from oxidative stress exposure is to strengthen or consume foods that contain antioxidants. Along with technological developments, there has been an increase in the popularity of foods like yoghurt [5].

Purple rosella extract can be added to improve the quality of the yoghurt. Purple rosella (*Hibiscus sabdariffa* L.) contains anthocyanins with high antioxidant activity. Anthocyanin can act as an antioxidant to maintain and protect the

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body from free radical effects [6]. Anthocyanins can capture and counteract free radicals (scavengers) by donating one hydrogen atom of the phenolic hydroxyl groups when reacting with free radicals. Anthocyanins have a conjugated double bond structure capable of destroying and counteracting free radicals, as well as preying on various types of reactive oxygen-derived free radicals, such as hydroxyl (OH), peroxyl (ROO), and single oxygen (O2) group [7]. Therefore, this research was conducted to determine the benefits of giving yoghurt with the addition of 15% purple rosella, as a source of antioxidants, in preventing damage that occurs to the duodenum and colon of *Rattus norvegicus* using the toxicity model of Rodhamine B and Saccharin.

MATERIAL AND METHOD

Experimental Research Design and Sample Preparation

This research used a Completely Randomized Design (CRD) with a sample of 28 male *Rattus norvegicus*, Wistar strain, aged 8 to 12 weeks, body weight 150-200 g, obtained from Iwan Farm Pakisaji, Malang City. The research used experimental animals, which have received an Ethical Clearance from the Ethics Commission of Universitas Brawijaya (No. 079-KEP UB-2022). Animals were acclimated for seven days to adapt to the condition and environment through animal cages of the Experimental Animal Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya. BR-1 feeding and drinking water were given ad libitum. Each treatment group, consisting of four individuals of *Rattus norvegicus*, has been shown in Table 1.

Table 1. Group treatment of *Rattus norvegicus*

Groups	Rodhamine B ($mg.kg^{-1}BW^{-1}$)	Saccharin ($mg.kg^{-1}BW^{-1}$)	purple rosella yoghurt ($rat.day^{-1}$)
K	-	-	-
R	22.5	-	-
S	-	157.77	-
KRS	22.5	157.77	-
YR	22.5	-	1 mL
YS	-	157.77	1 mL
YRS	22.5	157.77	1 mL

The administration of Rodhamine B, Saccharin, and purple rosella yoghurt to *Rattus norvegicus* was carried out using the gavage method, namely orally using a gastric probe for 14 days. Purple rosella yoghurt was administered four hours before Rodhamine B and Saccharin administration. Rodhamine B and Saccharin were obtained from Duta Wijaya Labware, Malang City.

Purple rosella yoghurt is made at the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya. The purple roselle (*Hibiscus sabdariffa* L.) used in making yoghurt is obtained from local plantations in Kediri City. Dried purple rosella flower petals were ground with a blender to a powder, sifted through a 60 mesh sieve, dissolved in distilled water in a ratio of 1 : 5 (g/v), pasteurized using the Low-Temperature Long Time (LTLT) technique at 63℃ for 30 minutes, filtered with filter paper to separate liquid and sediment, the liquid that has been obtained can be stored in a refrigerator at 4℃ [5].

The first stage of making purple rosella yoghurt is making a yoghurt starter; as much as 50 mL of milk is pasteurized using the High-Temperature Short Time (HTST) technique at 72℃ for 15 seconds. Milk is obtained from KUD Batu Jeding Kulon, Batu City. Pasteurized milk is added with freeze-dried Yogourmet® starter powder, which contains *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus* as much as 0.5% (g/v), homogenized using a stir bar, incubated at 45℃ incubator until pH reaches 4.4 - 4.5 (± 4 hours incubation). The second step is to make yoghurt, 150 mL of milk is pasteurized using the HTST technique at 72℃ for 15 seconds, added with 3% (v/v) yoghurt starter, homogenized using a stir bar, and incubated at 45℃ until the pH reaches 4.5 - 5 (± 2-3 hours of incubation) [8]. The third step is to make purple rosella yoghurt. As much as 15% (v/v) of purple rosella liquid was added to 100 mL of yoghurt and homogenized using a blender. Purple rosella yoghurt can be stored in the refrigerator at 4℃ [5].

Data Collection and Analysis

Rattus norvegicus was euthanized using the cervical os dislocation technique on the 15th day of the study treatment to take samples of the duodenum and colon. The duodenum and colon were washed using 0.9% NaCl to remove residual blood and put into an organ pot containing 10% BNF as an organ fixation fluid. Histopathological preparations were made for Hematoxylin Eosin (HE) staining. Histopathological preparations of the duodenum and colon were observed under a light microscope with magnification of 100x and 400x. Images were taken using the Optilab digital microscope camera and the Optilab Viewer application. The histopathological changes were

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scored in 5 visual fields based on the scoring criteria shown in Table 2.

Table 2. Scoring Criteria of Duodenum and Colon [9] with modification

Damage Percentage	Score
Lesion <25% total field of view	1
Lesion 25-50% total field of view	2
Lesion 50-75% total field of view	3
Lesion >75% total field of view	4

The data obtained was analyzed using the Kruskal-Wallis Test, followed by the Mann-Whitney Test. The analysis was assisted by the application of the Statistical Product and Service Solution (SPSS) program for Windows Version 26.

RESULT AND DISCUSSION

Histopathology of *R. norvegicus* Duodenum

The observed duodenum histopathology was the presence or absence of lesions, including necrosis, villous erosion, and inflammatory cell infiltration. No lesions were detected in the duodenum of group K. Meanwhile, groups R, S, and KRS showed lesions, i.e. necrosis, villous erosion, and a quite heavy infiltration of inflammatory cells. In the YR and YRS groups, lesions were not as severe as those in the R, S, and KRS groups. Even close to the condition of group K (Fig. 1).

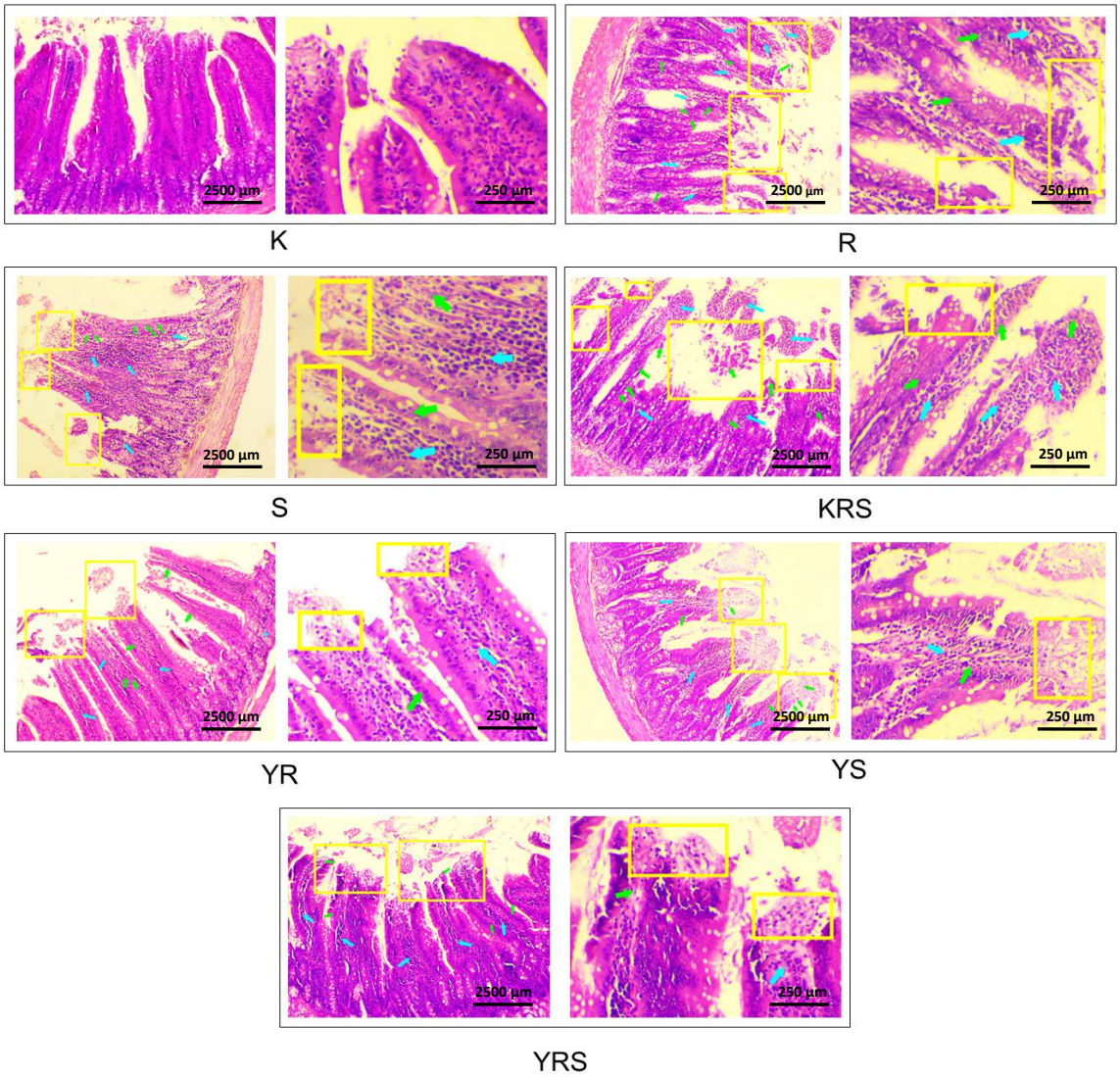

Figure 1. Histopathology of *Rattus norvegicus* Duodenum in All Treatment Groups Using Hematoxylin Eosin (HE) Staining. Magnification 100x (left), 400x (right)
Necrosis
Villi erosion
inflammatory cell infiltration

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The statistical test results showed significant differences between the R, S, KRS groups and the K, YR, YS, and YRS groups. The K group was not significantly different from the YR, YS, and YRS groups (Table 3). The necrosis in the YR (1.75 ± 0.58) group was better than in the R group (2.75 ± 0.50); necrosis in the YS group (1.50 ± 0.58) was better than in the S group (2.75 ± 0.50); and necrosis in the YRS group (1.50 ± 0.58) than KRS group (3.50 ± 0.58). Villous erosion in the YR group (1.50 ± 0.50) was better than R group (3.25 ± 0.50); villous erosion in the YS group (1.50 ± 0.58) was better than in the S group (2.75 ± 0.50); and villious erosion in YRS group (1.50 ± 0.58) better than in KRS group (3.50 ± 0.58). Inflammatory cell infiltration in YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); inflammatory cell infiltration in YS group (1.50 ± 0.58) better than S group (3.00 ± 0.82); and inflammatory cell infiltration in YRS group (1.75 ± 0.50) better than in KRS group (3.75 ± 0.50). This shows that giving purple rosella yoghurt prevent 50% damage to the duodenum due to exposure to free radicals caused by Rodhamine B and Saccharin.

Table 3. Duodenum Histopathological Scoring Average

Groups	Lesion Average ± SD		
	Necrosis	Villi Erosion	Inflammatory Cell Infiltration
K	1.25 ± 0.50[a]	1.25 ± 0.50[a]	1.25 ± 0.50[a]
R	2.75 ± 0.50[b]	3.25 ± 0.50[b]	3.25 ± 0.50[b]
S	2.75 ± 0.50[b]	2.75 ± 0.50[b]	3,00 ± 0.82[b]
KRS	3.50 ± 0.58[b]	3.50 ± 0.58[b]	3.75 ± 0.50[b]
YR	1.75 ± 0.58[a]	1.50 ± 0.50[a]	1.50 ± 0.58[a]
YS	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.50 ± 0.58[a]
YRS	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.75 ± 0.50[a]

Notes: Results are shown as mean values ± SD with different letter superscripts showed a significant difference ($p<0.05$)

K = no treatment
R = Rodhamine B 22.5 mg.kg^{-1}BW^{-1}
S = Saccharin 157.77 mg.kg^{-1}BW^{-1}
KRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1}
YR = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YS = Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}

The duodenum as a part of the small intestines, a place for digestion and absorption of food, which is susceptible to damage caused by oxidative stress. One of the changes caused by free radicals due to exposure to Rodhamine B and Saccharin is a change in the properties of cell membranes and cytoplasmic membranes of cell organelles, such as lysosomes and mitochondria. The most critical component in the cell membrane contains Polyunsaturated Fatty Acids (PUFA), which are very susceptible to free radical attack and cause the cell membrane to become brittle [10]. Free radicals will damage the structure of the cell membrane. It allows free radicals to enter the cytoplasm and damage the cell nucleus, which causes necrosis, as in the treatment group [11].

Damaged cells will release chemical compounds to signal inflammatory cells by the circulatory system to the damaged site. This condition is called inflammatory cell infiltration. Villi erosion and necrosis can trigger an increase in inflammatory cells that move from vessels to the damaged site [12,13]. Damage in the toxification process occurs due to the reactions between toxic substances and molecules in the body. The appearance of inflammatory cells is a form of the body's defense against foreign antigens. Inflammatory cells provide rapid and strong body defense against pathogens [10].

The human body needs antioxidants as the main compounds of free radicals scavengers to reduce the adverse effects of free radicals. Antioxidants release an electron to free radicals, inhibiting free radical activity [6]. The anthocyanin in purple rosella yoghurt has a conjugated double bond structure that destroy and ward off free radicals, as well as prey on various types of free radicals derived from reactive oxygen, such as hydroxyl (OH), peroxyl (ROO), and single oxygen (O_2) [14,15]. Therefore, the treatment groups that was given purple rosella yoghurt had a mild damaging effect on the duodenum. It has antioxidant compounds that can capture free radicals [16]. However, in this study, damage prevention was not optimal, because the concentration of purple rosella extract added to the yoghurt was not adequate.

Histopathology of *R. norvegicus* Colon

Observed colon histopathology is the lesions, including mucosal epithelial erosion, loss of goblet cells, and inflammatory cell infiltration. There were no lesions detected in the colon of group K. Group R, S, and KRS showed lesions, namely erosion of the mucosal epithelium, loss of goblet cells, and infiltration of inflammatory cells, which is quite heavy. In the YR, YS, and YRS groups, lesions were not as severe as those in the R, S, and KRS groups. Even almost close to the conditions of group K (Fig. 2).

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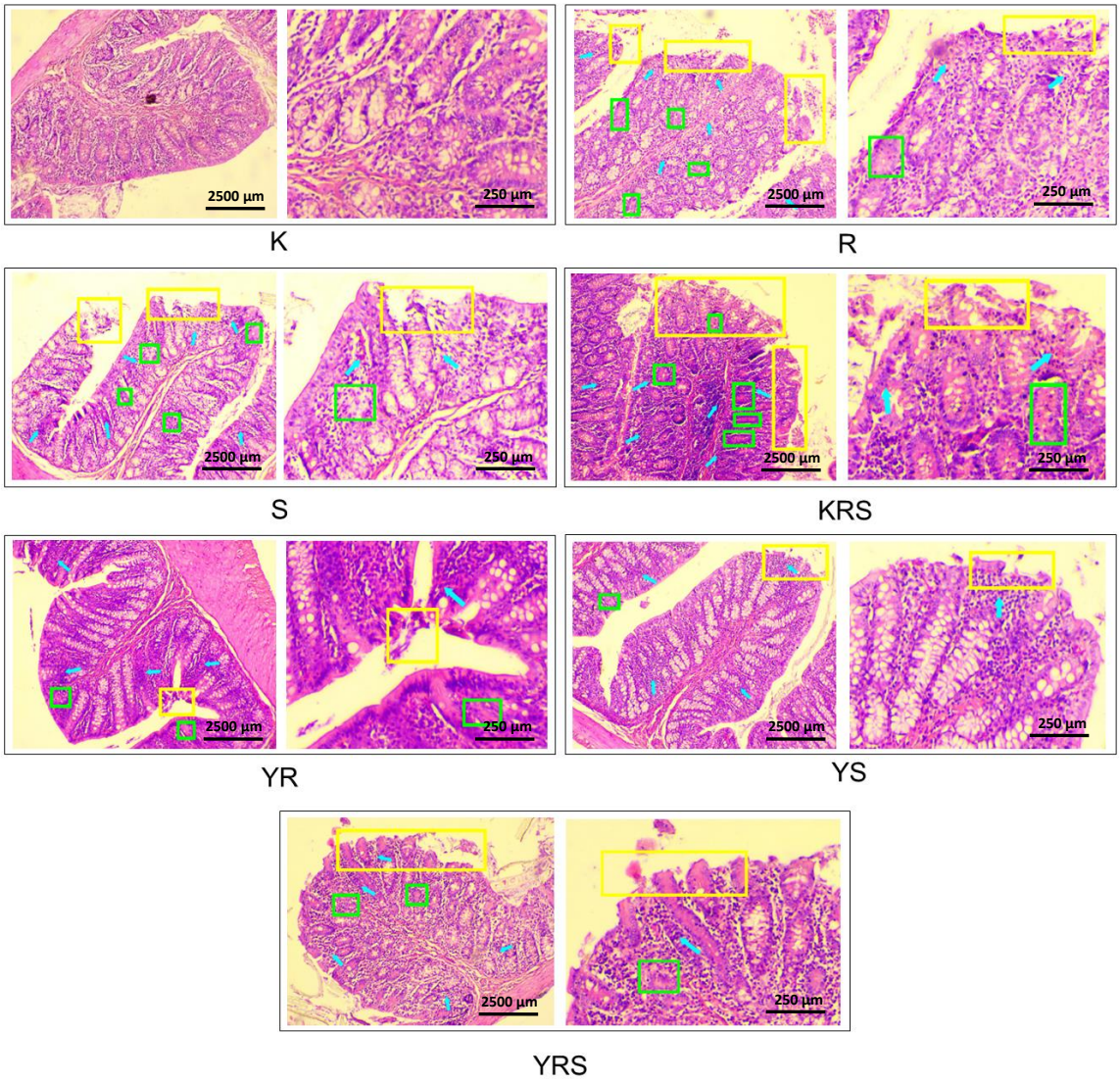

Figure 2. Histopathology of *Rattus norvegicus* Colon in All Treatment Groups Using Hematoxylin Eosin (HE) Staining. Magnification 100x (left), 400x (right) colon and superscripted.
lost of Goblet cells
Mucosal epithelial erosion
inflammatory cell infiltration

The statistical test results showed significant differences between the R, S, KRS groups and the K, YR, YS, and YRS groups (Table 4). The K group was not significantly different from the YR, YS, and YRS groups. Table 4 showed that erosion of the mucosal epithelium in the YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); erosion of the mucosal epithelium in YS group (1.25 ± 0.50) better than in S group (2.75 ± 0.50); and erosion of the mucosal epithelium in YRS group (1.75 ± 0.50) better than in KRS group (3.50 ± 0.58). The loss of goblet cells in YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); the loss of goblet cells in YS group (1.50 ± 0.58) better than in S group (2.75 ± 0.50); and the loss of goblet cells in YRS group (1.75 ± 0.50) better than in KRS group (3.50 ± 0.58). Inflammatory cell infiltration in YR group (1.75 ± 0.50) better than in R group (3.25 ± 0.50); inflammatory cell infiltration in YS group (1.50 ± 0.58) better than in S group (2.75 ± 0.50); and inflammatory cell infiltration in YRS group (1.75 ± 0.50) better than in KRS group (3.75 ± 0.50). This generally shows that giving purple rosella yoghurt is able to prevent damage to the colon due to exposure to free radicals caused by Rodhamine B and Saccharin.

Cells in the colonic mucosa are more susceptible to damage caused by the induction of oxygen radicals and through direct contact with

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toxic substances. The colon is the organ that exposed longest to toxic substances in the feces and is easily damaged due to potent oxidizing agents [17]. The loss of PUFA causes damage to the structure of the cell membrane, thereby affecting the permeability and function of the cell membrane. It is known that PUFA is most sensitive to free radicals and will form lipid peroxide chain reactions, causing cell membranes to lose integrity and rupture. Cell damage can occur if the damage continues to affect the lysosomal membrane, causing hydrolytic enzymes to be released and damage other organelles [18].

Table 4. Colon Histopathological Scoring Average

Groups	Lesion Average ± SD		
	Mucosal Epithelial Erosion	Loss of Goblet Cells	Inflammatory Cell Infiltration
K	1.25 ± 0.50[a]	1.25 ± 0.50[a]	1.25 ± 0.50[a]
R	3.25 ± 0.50[b]	3.25 ± 0.50[b]	3.25 ± 0.50[b]
S	2.75 ± 0.50[b]	2.75 ± 0.50[b]	2.75 ± 0.50[b]
KRS	3.50 ± 0.58[b]	3.50 ± 0.58[b]	3.75 ± 0.50[b]
YR	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.75 ± 0.50[a]
YS	1.25 ± 0.50[a]	1.50 ± 0.58[a]	1.50 ± 0.58[a]
YRS	1.75 ± 0.50[a]	1.75 ± 0.50[a]	1.75 ± 0.50[a]

Notes: Results are shown as mean values ± SD with different letter superscripts showed a significant difference ($p<0.05$)

K = no treatment
R = Rodhamine B 22.5 mg.kg^{-1}BW^{-1}
S = Saccharin 157.77 mg.kg^{-1}BW^{-1}
KRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1}
YR = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YS = Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}

The increase of free radicals caused by exposure to Rodhamine B and Saccharin can cause damage to epithelial cells and interfere with the absorption of nutrients. Lipid peroxidation is the process of converting unsaturated fatty acids into free radicals through the absorption of hydrogen. Erosion of the mucosal epithelium is one of the damages in the colon, in which the colon loses some of the epithelial cells in the mucosal lining [19]. Mucosal erosion causes an increase in epithelial permeability so that the normal flora damages the mucosa and causes an inflammatory response to occur. The inflammatory response can increase the amount of proinflammatory cytokines. An increase in the number of proinflammatory cytokines in areas experiencing inflammation is directly proportional to the rise in ROS, which will cause damage to colonic tissue [20].

Erosion of the colonic mucosal epithelium results in the loss of goblet cells. Goblet cells in the colon function as a barrier to the colonic mucosa by secreting mucin compounds. The barrier function of goblet cells prevents foreign antigens and pathogens from entering the mucosal tissue; therefore, colonic homeostasis can be maintained [21]. Goblet cells and secreted mucin prevent pathogens, which will cause inflammation from entering the mucosal tissue. If inflammation occurs, it indicates that the pathogen has succeeded in entering the mucosal tissue by destroying goblet cells [22].

The antioxidant content in purple rosella can suppress ROS by inhibiting the activity of the superoxide dismutase enzyme so that it can protect cells in the body from free radical attacks [23,24]. The anthocyanin compounds contained in purple rosella yoghurt are able to prevent damage to the *Rattus norvegicus* colon. Anthocyanin compounds will release a hydrogen atom (H+) to ROS, causing ROS to become stable compounds and unable to take atoms from cells, thus stopping the process of cell damage. This situation decreases oxidative stress in the tissue [9,25]. Therefore, reduced oxidative stress in the tissues will prevent lipid peroxidation [16], so the treatment group given purple rosella yoghurt can suppress damage to the duodenum and colon more than the treatment group without given purple rosella yoghurt, but not optimal.

CONCLUSION

Giving purple rosella yoghurt can prevent 50% damage to the duodenum and colon of *Rattus novergicus* due to exposure to free radicals from Rodhamine B and Saccharin. It is recommended that further research be carried out by increasing the concentration of purple rosella extract added to yoghurt by more than 15% (v/v).

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