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Elevation of MDA Levels and Change in Histopathological of Balb/c Mice's Liver on Acute Test of Fortification of Ethanolic Roselle Flower (*Hibiscus sabdariffa L.*) Extract in Yogurt

Aldila Noviatri[1*], Ani Setianingrum[2], Novita Efrianti Tulak[3]

[1] Department of Veterinary Pharmacology, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia.
[2] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia.
[3] Student, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia.

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Corresponding Author:
Aldila Noviatri
noviatria25@ub.ac.id

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Abstract: The bioactive peptides in yogurt and flavonoids in roselle flower extract have antioxidant activity that benefits the body's health. Roselle flower extract supplementation at a 5000 mg/kg dose is known not to cause death in rats. However, the toxic dose and safety of consuming fortification of roselle and yogurt have not been studied. This study aimed to determine the effect of fortification of roselle flower extract and yogurt on liver histopathology and MDA levels in the liver of the mice. Twenty male Balb/c mice (*Mus musculus*) were divided into four groups: healthy and untreated (control) and groups of treatment that were given fortification of ethanolic roselle flower extract and yogurt. Each treatment group was given a single dose (1000, 1400, and 7000 mg/kg) of the extract orally and observed for any clinical signs, change of habit, and death for 14 days, and they were euthanized the day after. MDA levels were measured using the Thiobarbituric Acid-Reactive Substance (TBARS) method. Hematoxylin-Eosin staining was used for histopathology and analyzed with the Knodell scoring method. The MDA levels were analyzed using one-way Analysis of Variance (ANOVA) and post-test (α=0.05). The liver degeneration and focal necrosis were analyzed statistically using the Kruskal Wallis and Mann Whitney post-test (α=0.05). The 1400 and 7000 mg/kg dosage elevated the liver MDA levels ($p<0.05$). Degeneration and focal necrosis scores in the liver due to fortification administration were escalated at all doses compared to the control group ($p<0.05$).

Keywords: Liver; MDA; Mice; Roselle calyx; Yogurt

Introduction

Cow's milk has a very beneficial content for the body. Casein in milk is a bioactive peptide precursor with antioxidant effects (Pereira, 2014). Milk can be processed into various products, such as yogurt, a dairy product fermented using lactic acid bacteria such as Lactobacillus bulgaricus and Streptococcus thermophilus (Meilanie et al., 2018). Yogurt positively impacts the body, balancing the normal intestinal flora by inhibiting pathogenic bacteria if consumed regularly (Jannah et al., 2014). Yogurt can also be consumed by people who are lactose intolerant (Hwang et al., 2023). Yogurt has bioactive peptides and probiotics that are very beneficial to the body.

Indonesia has roselle plants (Hibiscus sabdariffa L.) that are often processed into various healthy products because of flavonoid and ascorbic acid contents (Oktaviani et al., 2018). According to Cid-Ortega et al. (2015), adding roselle extract to yogurt can also improve the yogurt quality. Consumer preference for consuming fortified roselle flower extract and yogurt increases with increasing roselle concentration due to its more attractive color presentation based on the hedonic quality test (Noviatri et al., 2020). Adding roselle extract to yogurt will make the antioxidant content of roselle more stable and increase antioxidant activity because the

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antioxidant content in roselle is stable at pH 2-5, and yogurt has a pH of 4.4-4.5.

Bioactive peptides and flavonoids are antioxidants that can prevent oxidative stress (Nurkhasanah et al., 2020). Excessive doses of roselle extract can cause oxidative stress (Procházková et al., 2011). Oxidative stress conditions occur due to excess amounts of ROS in the liver. Oxidative stress can affect lipid peroxidation reactions that will cause damage to liver cells. Malondialdehyde (MDA) is the final compound produced from lipid peroxidation (Zainuri et al., 2012). Histopathological examination of the hepatic lobule can be observed with degeneration parameters and focal necrosis in the liver (Knodell et al., 1981).

Despite its health benefits, toxicity testing must be conducted to estimate the damage caused by a compound to biological material since adding roselle in yogurt increases the antioxidant compound. This study aimed to determine the effect of fortification of roselle flower extract and yogurt on liver histopathology and MDA levels in the liver of the mice.

Method

Roselle Flower Extraction

The procedure for making roselle flower extract with 96% ethanol solvent uses the modified Moeksin et al. (2009) method. Roselle-dried flowers were mashed into simplicia with a blender. 100 g of the simplicia were soaked with 96% ethanol as much as 1 liter. Roselle flower flowers were soaked for three days and filtered with filter paper. The filtrate was evaporated with a rotary evaporator until the solvent separated.

Preparation of Mother Culture of Yogurt

The procedure for making yogurt using a modified Suliasih et al. (2019) method. 50 mL of fresh milk is incubated at 45°C for 30 minutes. Yogurt starter (Yógourmet Yogurt Starter, LYO-SAN. INC 500 Aéroparc, C. P. 598, Lachute, QC. Canada, J8H 4G4) weighed 0.25 g and mixed in milk until homogen and then incubated at 45°C for 6-8 hours until the pH of the mother culture is 4.4-4.5. After completion, the mother culture is stored in the refrigerator.

Yogurt Making

The procedure for making yogurt using the method of Suliasih et al. (2019). 50 mL of fresh milk was mixed with 1.5 mL of activated mother culture and incubated at 45°C for 6-8 hours until the pH was 4.4-4.5 and stored in the refrigerator.

Fortification of Roselle Flower Extract and Yogurt

Fortification was prepared by mixing 12 mL of yogurt and 3 mL of roselle extract (20%; V/V). Furthermore, it homogenized with a hand mixer. The fortification was stored in the refrigerator.

Acute Test Study

Balb/c male mice (Mus musculus) aged 6-8 weeks were separated into four groups: a control (A) and three groups of treatment 1000 (B), 1400 (C), and 7000 (D) mg/kg BW roselle extract fortification yogurt. Before the supplementation, all mice fasted overnight and had free access to water. The fortified yogurt was given orally in a single dose. The mice were fed with standard feeding and ad libitum drinking water. Clinical signs, habit change, and death were observed throughout 14 days. At the end of the trial (15th day), mice were euthanized. The liver was collected for MDA and histopathological examination. This study has been approved by the ethical clearance committee of Universitas Brawijaya No.

MDA Level Measurement

TBARS method was used for the measurement of MDA. 0.1 g of liver was crushed with mortar on ice, added with 1 mL of TCA, and centrifuged at 100,000 rpm for 10 minutes. Solution of 400 µL of the supernatant mixed with 400 µL of sterile TBA, 1 mL of aquades, and 200 µL of HCL 1N were homogenized and centrifuged at 500 rpm for 10 min. The solution was heated in a 95°C water-bath container for 15 minutes, then put in the refrigerator at 8°C for 15 minutes, and then centrifuged at 10,000 rpm for 10 minutes. The solution's absorbance value was measured using a spectrophotometer with a 512 nm wavelength (Himah et al., 2018).

Histopathology Analysis

The tissue was kept in a 10% neutral buffered formaldehyde solution for histological investigation. The liver sample was dehydrated with repeated ethanol solutions and embedded in paraffin. A microtome-cut micrometer segment was stained with hematoxylin and eosin (H&E), examined under a light microscope, and observed for pathological changes in degeneration and focal liver necrosis. Histopathology scoring method based on Knodell et al. (1981) based on the form of the lesion: (0) no degeneration; (1) Degeneration of acidophile bodies, ballooning degeneration and necrotic foci 1/3 lobule (mild); (2) degeneration of acidophile bodies, ballooning degeneration, and necrotic foci 1/3-2/3 lobules (medium); (3) Degeneration there are acidophile bodies, ballooning degeneration and necrotic foci >2/3 lobules (severe).

Statistical Analysis

The MDA data were evaluated using a one-way ANOVA and the Tukey test. Kruskal-Wallis and Mann-Whitney nonparametric tests (=5%) were used to

analyze degeneration and focal necrotic statistically. P<0.05 was regarded as statistically significant.

Result and Discussion

The score of degeneration and focal necrosis in the control group was the lowest (0.12). Meanwhile, there was an escalation in the average score in the fortification treatment group. Sequentially, the average scores in the treatment group (1000, 1400, and 7000 mg/kg of roselle flower extract fortified in yogurt) were 1.52, 1.8, and 2.25. It means that adding roselle flower extract into yogurt increased the focal degeneration and necrosis starting at a dose of 1000 mg/kg and significantly different from the control group (p<0.05). The 7000 mg/kg dose of the extract showed the highest score. There was no difference between the 1000 and 1400 mg/kg doses as in 1400 and 7000 mg/kg (p>0.05). The histopathology of mice's liver can be seen in Figure 1. The scoring results showed an increase in the average score of degeneration and focal necrosis of mice's liver, which can be seen in Table 1.

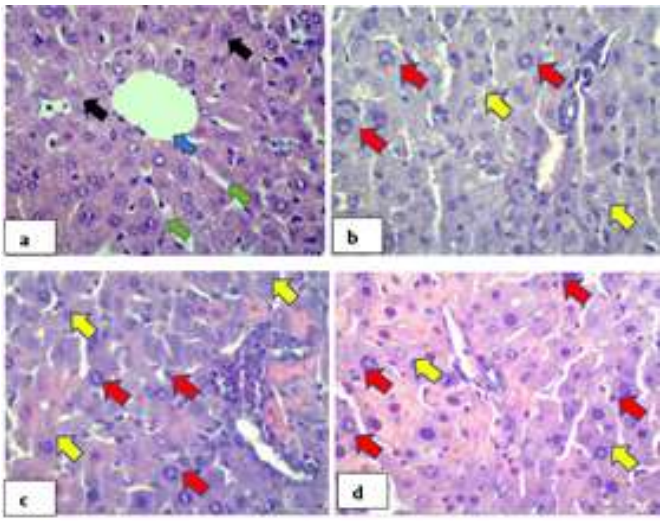

Figure 1. Histopathology features of mice's liver, 400x magnification. Description: (a) control; (b) yogurt+roselle flower extract dose 1000mg/kgBB; (c) Yogurt+roselle flower extract dose 1400mg/kgBB; and (d) yogurt+roselle flower extract dose 7000mg/kgBB; central vein (blue arrow), hepatocytes (black arrow), sinusoids green arrow, degeneration (yellow arrow) and necrosis (red arrow).

High flavonoid concentrations can influence the increase in average scoring in mice's liver. High concentrations of flavonoids reduce Fe3+ into Fe2+ and produce highly toxic hydroxyl radicals. Reactive Oxidative Species (ROS) from Fenton reactions can affect lipid peroxidation. During the lipid peroxidation process, ROS can remove electrons contained in lipids in cell membranes. ROS imbalance in the body can cause oxidative stress (Su et al., 2019). Oxidative stress conditions can cause impaired mitochondrial function in cells and increased cell membrane permeability (Berawi et al., 2017). Disrupted mitochondria can lead to decreased ATP production, so the energy required for sodium ions inside the cell to exit is insufficient. The accumulation of sodium in cells can cause disruptions in cell permeability, and fluid outside the cell will enter the cell (Lieberman et al., 2018). If there is no healing response in cells, hydropic degeneration conditions will continue and can cause necrosis. Focal necrosis is necrosis in groups of hepatocyte cells in lobules describing lobular changes in liver damage (Krishna, 2017).

Table 1. Mean of degeneration and focal necrosis scoring

Treatment	Mean of Degeneration and Focal Necrosis Score
Control (A)	0.12[a]
Yogurt+Roselle Flower Extract dose 1000 mg/kg BW (B)	1.52[b]
Yogurt+Roselle Flower Extract dose 1400 mg/kg BW(C)	1.8[bc]
Yogurt+Roselle Flower Extract dose 7000 mg/kg BW (D)	2.25[bc]

Description: Different superscript notations show a significant difference (p<0.05).

Lipids are an essential component in maintaining cell structure. Lipids are the main target of free radicals. Conditions of oxidative stress can cause an increase in Malondialdehyde (MDA) in the body. MDA is an aldehyde compound produced by lipid peroxidation (Yekti et al., 2018). MDA levels in mice livers can be seen in Table 2.

Table 2. Mean of Liver's MDA Levels

Treatment	Mean of MDA ± SD (ng/mL)
Control (A)	335.02±8.81[a]
Yogurt + Roselle Flower Extract dose 1000 mg/kg BW (B)	355.78±9.56 [ab]
Yogurt + Roselle Flower Extract dose 1400 mg/kg BW(C)	362.17±18.8[b]
Yogurt + Roselle Flower Extract dose 7000 mg/kg BW (D)	368.83±18.3[b]

Description: Different superscript notations show a significant difference (p<0.05).

The average MDA levels in the control group that did not receive treatment amounted to 335.02±8.81 ng/mL. The average MDA levels in the treatment group sequentially (1000, 1400, and 7000 mg/kg of roselle flower extract fortified in yogurt) were 355.78±9.56, 362.17±18.8, and 368.83±18.3 ng/mL. There was no significant difference in MDA levels between the 1000 mg/kg dose and the control (p>0.05). A significant increase in MDA levels compared to the control group

($p<0.05$) was shown in the treatment group at 1400 and 7000 mg/kg of roselle flower extract fortified in yogurt. MDA levels between groups of treatment (1000, 1400, and 7000 mg/kg of roselle flower extract fortified in yogurt) showed that there was no noticeable difference statistically ($p>0.05$).

According to Lieberman et al. (2018), under normal conditions, the MDA body can still form because the formation of ROS still occurs. Singlet oxygen is formed in cellular respiration in mitochondria, which can form superoxide through electron transfer. Superoxide can be converted into other ROS when catalyzed with other enzymes, such as Mn-SOD. The accumulation of ROS formed will affect the lipid peroxidation process and produce MDA (Bansal et al., 2014).

The movement activity of mice can influence the insignificant MDA levels between the treatment groups in this study (Mushab et al., 2020). According to research conducted by Alyani et al. (2021), stress levels in experimental animals can affect the formation of free radicals that can affect the results of MDA. The amount of flavonoids absorbed can also influence insignificant levels of MDA reaching the liver, not as much as in the stomach and intestines. Flavonoids will be absorbed in the small intestine, and a small part will be absorbed in the large intestine with the help of microorganisms that will break down flavonoids so that they are more easily absorbed in the intestine (Kumar et al., 2013).

Increased levels of MDA in mice can be caused by flavonoids in roselle flower extract. High flavonoid concentrations can increase Fenton's reaction to produce ROS. In the Fenton reaction, flavonoids reduce with Fe3+ metal to Fe2 + or Cu2+ into Cu+ to produce hydroxyl radicals (Rahal et al., 2014). The accumulation of hydroxyl radical production can lead to lipid peroxidation. Hydroxyl radicals will bind to hydrogen, producing water and unstable fatty acids. Fatty acids will bind to oxygen molecules and produce unstable peroxyl fatty acids. This process will continue to repeat until the radical compound binds to antioxidants or other radical compounds to produce a more stable compound. Casein in yogurt can help stabilize free radicals by donating hydrogen atoms. Bioactive peptides will bind free radicals. Casein will prevent the formation of unstable free radicals to prevent oxidation stress (Padaga et al., 2018). In this condition, bioactive peptides in yogurt cannot ward off free radicals produced when a Fenton reaction occurs, causing an increase in MDA in mice. This study aligns with research conducted by Alyani et al. (2021) that administering roselle ethanol extract can increase MDA levels in rat liver.

Conclusion

The addition of roselle flower extract at doses of 1400 mg/kg BW and 7000 mg/kg BW fortified in yogurt can cause an increase in MDA levels in mice's liver ($p<0.05$). Degeneration and focal necrosis scores in the liver due to fortification administration were escalated at all doses (1000, 1400, 7000 mg/kg) compared to the control group ($p<0.05$).

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Author Contributions

Conceptualization, Aldila Noviatri and Ani Setianingrum; methodology, Aldila Noviatri and Ani Setianingrum; formal analysis, Novita Efrianti Tulak; data curation, Novita Efrianti Tulak; writing – original draft, Novita Efrianti Tulak; writing – review & editing, Aldila Noviatri; supervision, Aldila Noviatri; project administration, Aldila Noviatri; funding acquisition, Aldila Noviatri. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

References

Alyani, F. S., Yulianti, R., & Thadeus, M. S. (2021). The Effect of Roselle (Hibiscus sabdariffa) Extract on Malondialdehyde Level in Rat Liver. *Jurnal Gizi Dan Pangan*, *16*(1), 57–62. https://doi.org/10.25182/jgp.2021.16.1.57-62

Bansal, M., & Kaushal, N. (2014). *Oxidative Stress Mechanism*. Spinger.

Berawi, K. N., & Agverianti, T. (2017). Efek Aktivitas Fisik pada Proses Pembentukan Radikal Bebas sebagai Faktor Risiko Aterosklerosis. *Jurnal Majority*, *6*(2), 85–90. Retrieved from https://juke.kedokteran.unila.ac.id/index.php/majority/article/view/1019

Cid-Ortega, S., & Guerrero-Beltrán, J. A. (2015). Roselle calyces (Hibiscus sabdariffa), an alternative to the food and beverages industries: a review. *Journal of Food Science and Technology*, *52*(11), 6859–6869. https://doi.org/10.1007/s13197-015-1800-9

Hwang, E. S., & Kim, S. (2023). Quality Characteristics and Antioxidant Activity of Cookies Supplemented with Aronia Pomace Powder.

Journal of the Korean Society of Food Science and Nutrition, *52*(9), 929–937. https://doi.org/10.3746/jkfn.2023.52.9.929

Jannah, A. M., Legowo, A. M., Pramono, Y. B., & Al-baarri, A. N. (2014). Total Bakteri Asam Laktat, pH, Keasaman, Citarasa dan Kesukaan Yogurt Drink dengan Penambahan Ekstrak Buah Belimbing. *C*, *3*(2), 7–11. Retrieved from https://jatp.ift.or.id/index.php/jatp/article/view/36

Knodell, R. G., Ishak, K. G., Black, W. C., Chen, T. S., Craig, R., Kaplowitz, N., Kiernan, T. W., & Wollman, J. (1981). Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology*, *1*(5), 431–435. https://doi.org/10.1002/hep.1840010511

Krishna, M. (2017). Patterns of necrosis in liver disease. *Clinical Liver Disease*, *10*(2), 53–56. https://doi.org/10.1002/cld.653

Kumar, S., & Pandey, A. K. (2013). Chemistry and Biological Activities of Flavonoids: An Overview. *The Scientific World Journal*, *2013*, 1–16. https://doi.org/10.1155/2013/162750

Lieberman, M., & Peet, A. (2018). *Marks' Basic Medical Biochemistry A Clinical Approach* (Fifth). Wolters Kluwer.

Meilanie, R. T., Arief, I. I., & Taufik, E. (2018). Karakteristik Yoghurt Probiotik dengan Penambahan Ekstrak Bunga Rosella (Hibiscus sabdariffa L) Selama Penyimpanan Suhu Dingin. *Jurnal Ilmu Produksi Dan Teknologi Hasil Peternakan*, *6*(1), 36–44. https://doi.org/10.29244/jipthp.6.1.36-44

Moeksin, R., & Ronald, S. (2009). Pengaruh kondisi, perlakuan dan berat sampel terhadap ekstraksi antosianin dari kelopak bunga rosella dengan pelarut akuades dan etanol. *Jurnal Teknik Kimia*, *16*(4), 11–18.

Mushab, M., Hairrudin, H., & Abrori, C. (2020). Perbandingan Peningkatan Kadar Malondialdehid (MDA) Serum setelah Olahraga Pagi dan Malam Hari pada Orang Tidak Terlatih. *Jurnal Kesehatan Andalas*, *9*(2), 211. https://doi.org/10.25077/jka.v9i2.1312

Noviatri, A., Setianingrum, A., & Haskito, A. E. P. (2020). Organoleptic properties evaluation of purple Hibiscus sabdariffa L (Roselle) calyx extract-fortified yogurt. *Journal of Physics: Conference Series*, *1430*(1), 12012. https://doi.org/10.1088/1742-6596/1430/1/012012

Nurkhasanah, N., & Amelia, A. (2020). The antioxidant activity and stability of yogurt fortified with rosella (Hibiscus sabdariffa Linn) calyx extract. *Pharmaciana*, *10*(3), 365. https://doi.org/10.12928/pharmaciana.v10i3.16690

Oktaviani, T., & Megantara, S. (2018). Review: Aktivitas Farmakologi Ekstrak Rosella. *Farmaka*, *16*(1), 345–351. https://doi.org/10.24198/jf.v16i1.17500.g8659

Padaga, M., Erika P. H, A., & Irawan, M. (2018). Efek Antioksidatif Kasein Yogurt Susu Kambing Terhadap Pencegahan Reprotoksik pada Hewan Model Rattus Norvegicus yang Dipapar 2, 3, 7, 8 Tetrachlorinedibenzo-P-Dioksin (TCDD). *Jurnal Ilmu Dan Teknologi Hasil Ternak*, *13*(2), 72–80. https://doi.org/10.21776/ub.jitek.2018.013.02.1

Pereira, P. C. (2014). Milk nutritional composition and its role in human health. *Nutrition*, *30*(6), 619–627. https://doi.org/10.1016/j.nut.2013.10.011

Procházková, D., Boušová, I., & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, *82*(4), 513–523. https://doi.org/10.1016/j.fitote.2011.01.018

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative Stress, Prooxidants, and Antioxidants: The Interplay. *BioMed Research International*, *2014*, 1–19. https://doi.org/10.1155/2014/761264

Su, L.-J., Zhang, J.-H., Gomez, H., Murugan, R., Hong, X., Xu, D., Jiang, F., & Peng, Z.-Y. (2019). Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. *Oxidative Medicine and Cellular Longevity*, *2019*, 1–13. https://doi.org/10.1155/2019/5080843

Suliasih, S., Legowo, A. M., & Tampoebolon, B. I. M. (2019). Aktivitas Antioksidan, BAL, Viskositas dan Nilai L*a*b* dalam Yogurt Drink Sinbiotik antara Bifidobacterium Longum dengan Buah Naga Merah (Hylocereus polyrhizus). *Jurnal Aplikasi Teknologi Pangan*, *7*(4), 151–156. https://doi.org/10.17728/jatp.3061