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Evaluation of the 90-Day Administration of *Cyperus rotundus* Tubers on Nrf2, SOD, and MDA Profiles by Flow Cytometry

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase (SOD), and malondialdehyde (MDA) are components involved in the antioxidant system and the body's response to oxidative stress. The bioactive compounds of *Cyperus rotundus* tuber (CRT) have pharmacological effects as antioxidants and anti-inflammatory. This study aimed to evaluate the impact of CRT administration for 90 days on the profiles of Nrf2, SOD, and MDA. 24 male Wistar rats were used in this study and divided into 4 treatment groups: Control, dose 1 (300 mg/kg BW), dose 2 (600 mg/kg BW), and dose 3 (900 mg/kg BW). Oral administration of CRT was carried out for 90 days. On day 91, liver isolation was performed to determine the profiles of Nrf2, SOD, and MDA using Flow Cytometry. Statistical analysis using One-Way ANOVA with $P<0.05$ was conducted using SPSS. The administration of CRT to Wistar rats for 90 days did not induce toxic or harmful effects. The Nrf2 and MDA profiles did not show excessive activation with CRT administration. The SOD profile increased with D1 administration compared to the control and other doses. The administration of CRT for 90 days has the potential to be used as a natural alternative to address diseases related to oxidative stress.

1. Introduction

Oxidative stress is a condition caused by an imbalance between the production of free radicals and the antioxidant system (Adwas *et al*. 2019). These highly reactive free radicals can damage crucial molecules such as proteins, lipids, and DNA (Martemucci *et al*. 2022). While the body naturally produces free radicals as part of normal metabolic processes, an increase in production or a decrease in antioxidant capacity can lead to oxidative stress. Oxidative stress has been identified as a major contributor to various degenerative diseases such as cancer, diabetes, and cardiovascular diseases (García-Sánchez *et al*. 2020).

Several factors that can lead to oxidative stress involve external exposures such as radiation, air pollution, exposure to heavy metals, and toxic chemicals (Zheng *et al*. 2020; Jimenez *et al*. 2022). Additionally, internal factors such as inflammation, infections, and abnormal metabolism can also contribute to oxidative stress (Wang *et al*. 2020; Vona *et al*. 2021). Under normal circumstances, the human body possesses an efficient antioxidant system to neutralize free radicals and prevent damage caused by oxidative stress (Picos-Salas *et al*. 2022).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a protein that is a key regulator in the cellular response to oxidative stress. Nrf2 plays a

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role in activating antioxidant defense and cellular detoxification genes. When oxidative stress occurs within a cell, Nrf2 activates and translocates to the cell nucleus, interacting with regulatory elements on the promoters of antioxidant and detoxification genes. The activation of Nrf2 has been a research subject in health and therapeutic development. Some studies suggest that enhancing Nrf2 activity may provide protection against degenerative diseases and chronic inflammation (Cuadrado *et al*. 2019; Karunatilleke *et al*. 2021).

Several genes regulated by Nrf2 include those encoding antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase (Picos-Salas *et al*. 2022). Superoxide Dismutase (SOD) is an enzyme that plays a crucial role in protecting cells from oxidative stress. The primary function of SOD is to catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. It is critical in cellular antioxidant defense because other enzymes, such as catalase, can further break down the generated hydrogen peroxide. SOD is an essential component of the antioxidant defense system. Some studies suggest that supplementing SOD or strategies to enhance SOD activity may have potential health benefits by reducing oxidative stress (Rosa *et al*. 2021).

Indicators of oxidative stress and cellular damage can be observed through an increase in Malondialdehyde (MDA) (Huang *et al*. 2019; Mohideen *et al*. 2021). Lipid oxidation occurs when fat molecules react with free radicals, producing various oxidative products, including MDA (Huang *et al*. 2019). The reduction of MDA can reflect the effectiveness of antioxidants in protecting cells from damage caused by free radicals.

By examining the complex interactions among biomolecules such as Nrf2, SOD, and MDA, it was expected that valuable contributions could be made to the field of health research and provide a basis for the development of more effective natural-based antioxidant therapies. Natural-based antioxidant therapies can be explored by harnessing the properties of plants known for their antioxidant effects. One such plant known for these effects is the *Cyperus rotundus* tuber (CRT). *Cyperus rotundus* is a type of plant more commonly recognized as a weed. However, in recent decades, *Cyperus rotundus* has garnered attention due to its potential antioxidant activity (Boonyuenyong *et al*. 2023). Bioactive compounds in the *Cyperus rotundus*, such as polyphenols and terpenoids, may protect cells from oxidative damage (Ngoc and Minh 2021). Therefore, this research is conducted to evaluate the effects of CRT administration on factors related to oxidative stress, such as Nrf2, SOD, and MDA. This study provides a fundamental understanding of the interaction between CRT and the antioxidant and detoxification systems. It provides insights into potential clinical applications in addressing human oxidative stress-related diseases.

2. Materials and Methods

2.1. Animal

A total of 24 male Wistar rats (*Rattus norvegicus*) with 150-200 grams were used in this study. Wistar rats were obtained in good health condition from UD Mandiri, Bangil, Malang, Indonesia, under the supervision of the Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. The rats were housed in the animal room located in the Faculty of Medicine, University of Muhammadiyah Malang, Indonesia, under controlled conditions with standard temperature and pressure. The rats were maintained at a room temperature of 22-24°C, with a 12-hour light/dark cycle. Each rat was placed in a standard box and provided access to food and water. Paddy husks were used as bedding material and were cleaned regularly every two days for the period of treatment. The animals were acclimatized to laboratory conditions for 7 days before the experiments. All the procedures of this study were approved by the Ethics Commission Healthy Research University of Muhammadiyah Malang with registration number No.E.5.a/212/KEPKUMM/VII/2023.

2.2. Plant Materials and Aqueous Extraction

The CRT for this research was obtained from the same source as the previous study (Agustini *et al*. 2023). This study focuses on using *Cyperus rotundus*, specifically its tuber part. The extraction process refers to the research conducted by Lestari *et al*. (2022).

2.3. Experimental Design

A total of 24 male rats were divided into four treatment groups, each consisting of 6 rats. The four groups include a control group (without CRT administration) and three different dosage groups. Dose 1 is 300 mg/kg BW, dose 2 is 600 mg/kg BW, and dose 3 is 900 mg/kg BW. The administration of CRT aqueous extract was conducted orally daily for a treatment period of 90 days.

2.4. Organ Isolation

On the 91st day, all rats were euthanized to observe the effects of CRT administration on the parameters of Nrf2, SOD, and MDA. The organ taken for analysis was the liver. The livers from each rat were placed in separate containers according to the treatment labels. The organs were washed using PBS to remove blood debris and maintain cell viability. Subsequently, the organs were transferred to a petri dish containing 3-5 ml of PBS and gently crushed until homogenized. The resulting homogenate was carefully pipetted and placed in a 15 ml propylene tube. Ensure that the homogenate taken is only liquid and does not include any debris or organ fragments. The homogenate was centrifuged at 2500 rpm for 5 minutes at 10°C. The centrifugation resulted in a supernatant and a pellet. The supernatant was discarded, and the pellet was resuspended with an additional 1 ml of PBS. The resuspended solution was then divided into 1.5 ml microtubes containing 50 microliters. The cells were stored in an icebox at 4°C for subsequent antibody staining.

2.5. Antibody Staining and Flowcytometry

The antibodies used in this study are Nrf2 (Rabbit anti-rat-Nrf2 Polyclonal Antibody, PE-Cy7 Conjugated/ catalog bs-1074R-PE-Cy7/bioss), SOD (Rabbit anti-rat-SOD1 Polyclonal antibody, Cy5.5 Conjugated/ catalog: bs-10216R-Cy5.5/bioss), dan MDA (FITC Anti-Malondialdehyde antibody (ab27615)/abcam). A total of 50 microliters of cells inside the microtube were initially mixed with 50 microliters of cytofix (fixation beffer, cat: 420801/ biolegend), resuspended, and incubated in an icebox at 4°C for 20 minutes. After incubation, 300-400 microliters of washperm (Intracellular Staining Permeabilization Wash Buffer (10×), cat: 421002/ biolegend) were added, resuspended, and incubated for an additional 5-10 minutes. Subsequently, centrifugation was performed at 2500 rpm, 5 minutes, 10°C. The supernatant was removed, and the pellet was mixed with the appropriate antibody, 50 microliters each, and incubated for 20 minutes in the icebox at 4°C. This step was carried out for all added of antibodies. Following the antibody addition, 400-500 microliters of PBS were added, resuspended, and transferred to a cuvette. The cuvette containing cells treated with antibodies in the previous steps was then ready to flow cytometry for subsequent readings on the FCM BD FACSCalibur™ machine (BD Biosciences, San Jose, CA). The results were analyzed using BD CellQuest Pro™ software (BD Biosciences, San Jose, CA).

2.6. Statistical Analysis

The flow cytometry analysis results were tabulated in Excel and subsequently analyzed using SPSS version 16 for Windows. A completely Randomized Design (CRD) using one-way ANOVA with a significance level of $p< 0.05$ was used in this study. If the data were normally distributed and homogenous, further analysis was conducted using Tukey's post hoc test to determine the significance between treatment groups.

3. Results

3.1. Effect of CRT Administration for 90 Days on Nrf2

The analysis of Nrf2 has been conducted on Wistar rat models that were administered CRT for 90 days. The analysis results indicate that the administration of CRT with three doses does not exhibit a significant difference ($p<0.05$) compared to the control group without CRT administration. However, the graph (Figure 1) shows that the administration of CRT doses 1 and 2 increases the percentage of Nrf2 compared to the control, although not statistically significant. The rate of Nrf2 in the control group is 7.36%, while the treatments with doses 1 and 2 are 9.46% and 10.9%, respectively. It is assumed that CRT administration can potentially increase the percentage of Nrf2 profiles. Based on these results, CRT is known to have antioxidant effects because the high percentage of Nrf2 indicates Nrf2 activation, which has a positive effect, namely, protecting cells against oxidative stress.

3.2. Effect of CRT Administration for 90 Days on SOD

The SOD profile in this study was analyzed using flow cytometry and subsequently subjected to statistical analysis to assess the impact of CRT administration on healthy rats. The results of the analysis indicate that the administration of CRT at dose 1 significantly influences an increase in the SOD profile compared to the control. In the control group, the SOD profile shows a percentage of 14.56% (Figure 2). Administration of dose 1 results in a significant increase ($P<0.05$) in the SOD profile (22.12%) compared to the control group. This result suggests that CRT administration enhances the SOD profile, and the increased SOD is known to have a positive impact on the effective performance of

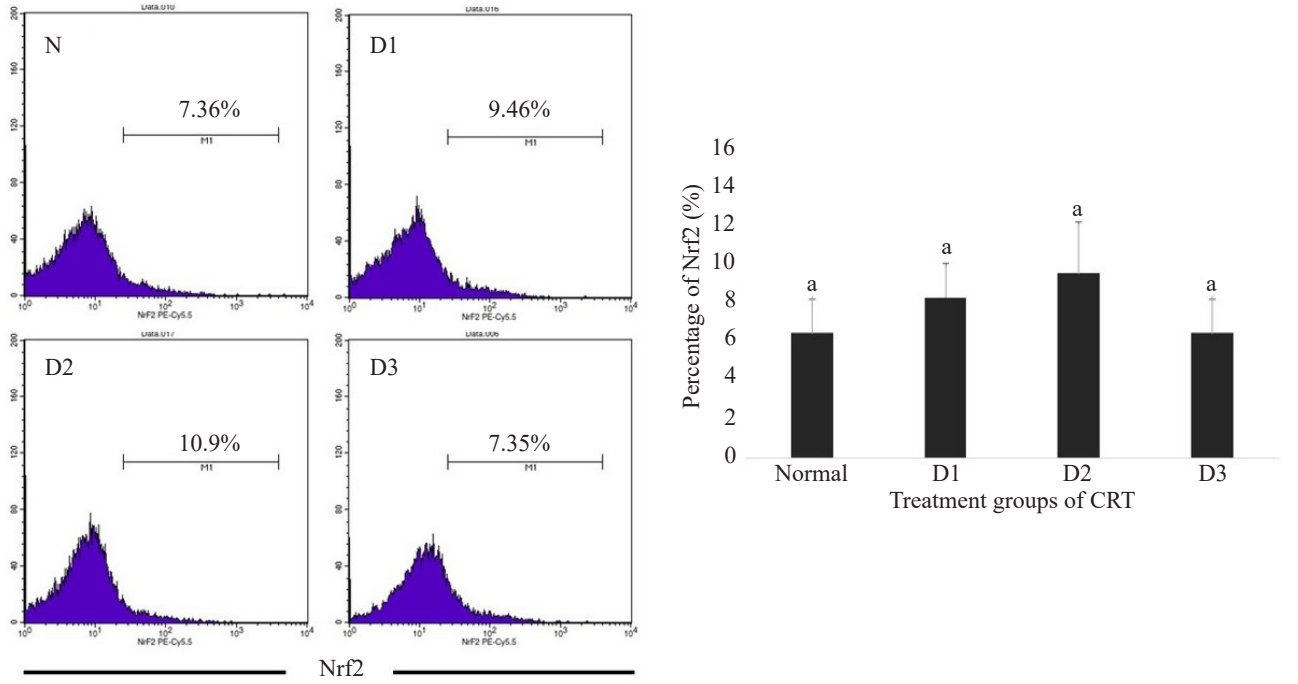

Figure 1. Evaluation of Nrf2 post 90 days CRT treatment. N= normal (healthy rat, without treatment of CRT), D1= CRT treatment dose 1 (300 mg/kg BB) for 90 days, D2= CRT treatment dose 2 (600 mg/kg BB) for 90 days. D3= CRT treatment dose 3 (900 mg/kg BB) for 90 days. The data were obtained from the mean of ± SD in each treatment with a p-value ≤ 0.05. Different subsets indicate significant differences based on the Tukey test

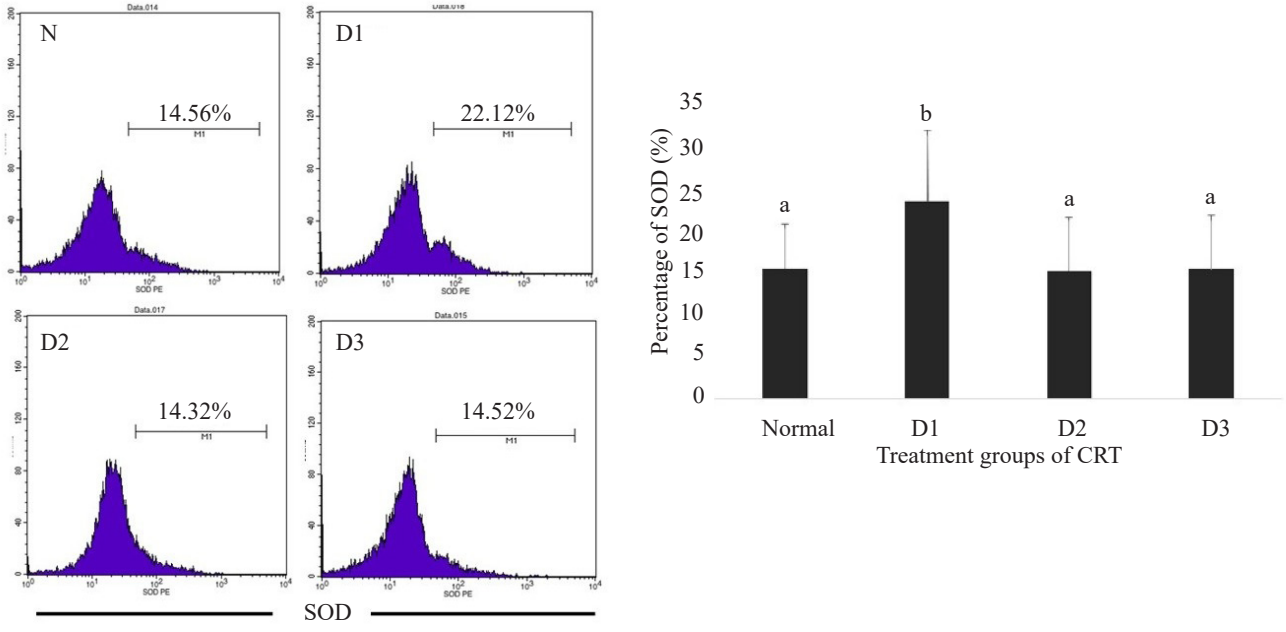

Figure 2. CRT administration for 90 days to SOD profile. N= normal (healthy rat, without treatment of CRT), D1= CRT treatment dose 1 (300 mg/kg BB) for 90 days, D2= CRT treatment dose 2 (600 mg/kg BB) for 90 days. D3= CRT treatment dose 3 (900 mg/kg BB) for 90 days. The data were obtained from the mean of ± SD in each treatment with a p-value≤0.05. Different subsets indicate significant differences based on the Tukey test

endogenous antioxidants in protecting cells from oxidative stress damage. Administration of CRT doses 2 and 3 showed no significant difference compared to the normal group. These results imply that CRT administration is assumed to maintain the health condition of rats during the treatment period.

3.3. Effect of CRT Administration for 90 Days on MDA

This study evaluates the impact of CRT administration on the Wistar rats model for 90 days to the MDA profile. CRT administration with three different doses does not show a significant increase in MDA compared to the control (Figure 3). The escalating doses, doses 1, 2, and 3 at 300 mg/kg BW, 600 mg/kg BW, and 900 mg/kg BW, respectively, do not result in a significant MDA increase compared to the control. This result suggests that even the highest dose of CRT, at 900 mg/kg BW, does not induce cell damage, leading to oxidative stress and the production of MDA. In the control group, the percentage of the MDA profile is 9.99%. Although smaller than the dose one percentage of 13.23%, this difference is insignificant. Doses 2 and 3, with percentages of 9.59% and 10.32%, respectively, also do not significantly differ from the control.

4. Discussion

The administration of CRT to the Wistar rats model indicates that, even at the highest dose in this study, 900 mg/kg BW, there is no evidence of toxicity based on the parameters of Nrf2, SOD, and MDA. This study shows that the administration of CRT at doses 1, 2, and 3 for 90 days does not affect Nrf2 activity compared to the control group. Wardaya *et al*. (2024) found that Nrf2 is activated in response to oxidative stress, which can be caused by ROS generation; hence, its upregulation indicated oxidative stress-induced activation. According to Calabrese and Kozumbo (2021), Nrf2 activation occurs when cells defend themselves against oxidative stress, and Nrf2 plays a key role in cellular responses to oxidative stress (Ngo and Duennwald 2022). Therefore, in this study, it can be assumed that CRT administration does not induce oxidative stress in cells, preventing the activation of Nrf2.

The activation of Nrf2 is the body's response to oxidative stress and is a crucial part of the cellular defense mechanism against damage caused by free radicals (Sadiq 2023). Nrf2 is a transcription factor that regulates genes involved in antioxidant defense and

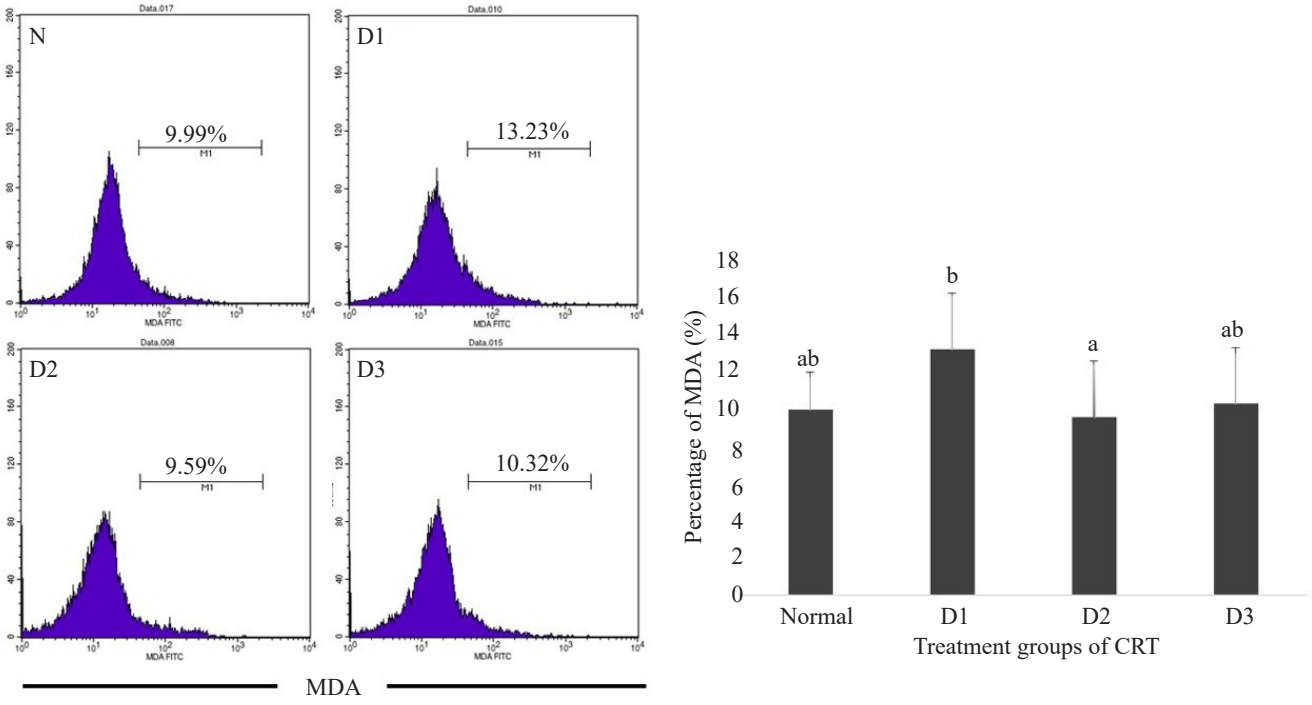

Figure 3. Effect of CRT administration for 90 days to reduce MDA profile. N= normal (healthy rat, without treatment of CRT), D1= CRT treatment dose 1 (300 mg/kg BB) for 90 days, D2= CRT treatment dose 2 (600 mg/kg BB) for 90 days. D3= CRT treatment dose 3 (900 mg/kg BB) for 90 days. The data were obtained from the mean of ± SD in each treatment with a p-value ≤ 0.05. Different subsets indicate significant differences based on the Tukey test

cellular detoxification (He *et al*. 2020; Bardallo *et al*. 2022). When cells undergo oxidative stress, for instance, due to exposure to free radicals or excessive oxidative reactions, Nrf2 is triggered. This activation prompts the translocation of Nrf2 into the cell nucleus, where it interacts with regulatory elements on the promoters of genes responsible for encoding antioxidant enzymes and detoxification proteins (Ngo and Duennwald 2022). Consequently, there is an augmentation in the production of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, along with other detoxification enzymes (Panieri *et al*. 2020; Awan *et al*. 2023). In essence, Nrf2 activation represents the body's strategy to counteract oxidative stress by amplifying the cell's ability to manage oxidative damage. Hence, a heightened level of Nrf2 activity can indicate that cells are actively endeavoring to shield themselves from the detrimental effects of oxidative stress.

Oxidative stress forms free radicals, which are produced by the normal metabolism of cells and should trigger the activation of Nrf2 (Ma 2013). However, the administration of CRT does not influence Nrf2 activation. This result could be attributed to bioactive compounds in *Cyperus rotundus* that function as antioxidants. Flavonoids are among the compounds found in CRT (Ngoc and Minh 2021). They can directly interact with free radicals, capturing unpaired electrons and stabilizing them. flavonoids assist in preventing chain reactions that may damage cells and molecules (Kaurinovic and Vastag 2019).

The evaluation of the effects of CRT administration on the SOD profile was also conducted in this study. The results indicate that only dose 1 significantly increased the SOD profile compared to doses 2 and 3. It is assumed that the higher level of SOD profile is attributed to the bioactive compounds in CRT capable of modulating SOD activity. Compounds in CRT may stimulate the activity of antioxidant enzymes, including SOD, which is a key enzyme in cellular defense against free radicals. The heightened SOD activity can contribute to protecting cells from oxidative damage (Younus 2018).

On the other hand, the administration of doses 2 and 3 does not significantly increase the SOD profile. It was suspected that CRT administration does not induce oxidative stress. Thus, it wasn't required for the activation of endogenous antioxidant enzymes such as SOD to counteract radicals formed due to oxidative stress. In this case, it can be inferred that CRT may work through multiple pathways. As observed in this study, a crude extract from CRT was used, and specific compound isolation was not performed. Therefore, the bioactive compounds in CRT can work simultaneously, resulting in various effects.

The previous study mentioned that the aqueous extract of CRT contains various bioactive compounds such as polyphenols, flavonoids, and tannins (Kilani-Jaziri *et al*. 2011; Badgujar and Bandivdekar 2015; Bezerra *et al*. 2022). CRT is also known to have pharmacological effects such as antioxidant (Boonyuenyong *et al*. 2023), anti-inflammatory (Bahi and Necib 2014), anti-cancer (Ngoc and Minh 2021), and antidiabetic properties (Dechakhamphu *et al*. 2023).

The administration of CRT for 90 days with three different dosages in this study did not significantly affect the MDA profile compared to the control. This result indicates that CRT can maintain the health condition of the Wistar rats model during the treatment period from oxidative stress activity. MDA is a lipid oxidation product often used to indicate oxidative stress in cells and tissues. Measuring MDA can provide insight into the level of oxidative damage in the cells (Cordiano *et al*. 2023). The MDA profile in this study did not show an increase during the 90-day administration of CRT. Therefore, CRT can be considered safe for prolonged use with appropriate doses. In addition to its antioxidant effects, CRT in this study is also suspected to have anti-inflammatory effects. This is suggested by the administration of CRT not leading to an increase in the MDA profile compared to the control, where excessive MDA production indicates the occurrence of inflammation (Xu *et al*. 2020).

In conclusion, the administration of CRT for 90 days does not induce toxic effects and has the potential to be used as an antioxidant and anti-inflammatory agent for treating diseases associated with oxidative stress.

Conflict of interest, No conflict of interest associated with this work.

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