

Taro (*Colocasia Esculenta* (L.) Schott) Extract didn't Increase Superoxide Dismutase (SOD) but Decrease C-Reactive Protein (CRP) in Male Wistar Rat (*Rattus Norvegicus*) Diabetes Mellitus Model

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Abstract

Backgrounds: In hyperglycemia induced by streptozotocin, free radicals and oxidative stress conditions occur. This also reduced SOD enzymes while increasing CRP. This study aimed to analyze effect of flavonoid-rich taro leaves ethanol extract on SOD levels and serum CRP levels in rats diabetes mellitus model induced by streptozotocin. **Methods:** Experimental study with a pretest-posttest control group design 26 healthy rats were randomly divided into 2 groups. The control group was given 2 ml aquadest and treatment group was given 400mg/kgBW ethanol extract of taro leaves in 2 ml aquadest. Before and after treatment, blood serum was collected for SOD and CRP analysis using the ELISA technique. **Results:** SOD level in control group before and after given placebo were reduce insignificantly (from 5.37 ± 0.69 ng/ml to 5.26 ± 1.50 ng/ml ; $p = 0.830$). Furthermore, CRP level in control group before and after given placebo were not significantly different (from 3.39 ± 0.24 ng/ml to 3.56 ± 0.43 ng/ml ; $p = 0.308$). SOD level in treatment group before and after given taro extract were increase but not significant (from 5.33 ± 1.14 ng/ml to 6.20 ± 2.64 ng/mL; $p = 0.239$). However, CRP level in control group after given placebo decrease significantly (from 3.56 ± 0.43 ng/mL to 2.93 ± 0.47 nmol/mL ; $p = 0.009$). **Conclusion:** ethanol extract of taro leaves did not increase SOD levels but decrease CRP levels in rats model diabetes mellitus induced by streptozotocin. Further studies are needed to confirm extract of taro leaves as external antioxidant.

Keywords: CRP, Diabetes mellitus, SOD, Streptozotocin, Taro leaves

1. Introduction

Diabetes mellitus is a chronic hyperglycemic condition that causes autoimmune, metabolic and hereditary problems (Egan and Dinneen, 2019). Insulin action and pancreatic beta cell dysfunction are two factors that induce diabetes mellitus. The breakdown of the function between insulin action and insulin production, resulting in hyperglycemia, is the initial factor that causes diabetes mellitus. The existence of pancreatic beta cell dysfunction is the second factor (Noormohammadi *et al.*, 2022). When insulin production is diminished, the body is unable to perform its physiological duty of maintaining glucose levels. Insulin resistance causes a rise in glucose in the liver as well as a reduction in glucose absorption in the muscle, liver, and adipose tissue. If this pattern persists, diabetes mellitus develops (Galicia-Garcia *et al.*, 2020). Hyperglycemia induces the development of reactive oxygen species on pancreatic cells in diabetes mellitus. In the body, an imbalance of reactive oxygen species with antioxidants causes oxidative stress, insulin resistance, decreased insulin secretion, DNA, RNA, protein, and lipid damage, endothelial damage in both large and small blood vessels, increased expression of proinflammatory cytokines, growth factors,

procoagulant factors, and decreased NO release (Bigagli and Lodovici, 2019; Hector Eloy, Tamez Perez, 2015). CRP levels will increase in response to an increase in proinflammatory cytokines (Wang *et al.*, 2013). Furthermore, in order to combat oxidative stress, the body produces antioxidants, one of which is SOD, which may absorb reactive oxygen species (Nimse and Pal, 2015).

Taro (*Colocasia esculenta*) is a nutrient-dense food with a high fiber content. The taro plant is well-known not just for its tubers, but also for its leaves and stems, which provide a variety of health advantages. Taro leaves, which are known for their broad leaves and distinctiveness, as well as being waterproof, are also frequently used in the community (Rashmi *et al.*, 2018). Taro leaves are used to cure a variety of ailments, including wounds, diarrhea, and high blood pressure medicine. Taro leaves are high in beneficial active substances such as anthocyanins, anthraquinones, sterols, vitamins A and C, and flavonoids (Bisala *et al.*, 2019; Habibah and Astika, 2020; Rubiono *et al.*, 2020).

Flavonoids are active chemicals found in plants that are a component of polyphenols. Anti-viral, anti-inflammatory, cardioprotection, anti-cancer, anti-aging, antidiabetic, and antioxidant flavonoid effects have also been discovered. Flavonoids, as antioxidants, can protect cells against the effects of reactive oxygen species (ROS). Flavonoids' capacity to act as an anti-diabetic has also been extensively researched (Al-Khayri *et al.*, 2022; Bisala *et al.*, 2019; Noviardi *et al.*, 2020). Various plants have been studied and searched for flavonoid content in order to employ them as a medicine companion or supplement. Another research of flavonoids in diabetic rats found that they operate as gene regulators, glucose homeostasis inhibitors, cell apoptosis inhibitors, insulin secretion activators, and cellular replication activators (Brodowska, 2017). Flavonoids are antioxidants that fight free radicals both directly (by giving hydrogen ions) and indirectly (by enhancing gene expression, namely Nrf2). This flavonoid action reduces free radical toxicity while enhancing gene activity, resulting in increased GLUT4 translocation via the PI3K/AKT and AMPK pathways (Simanjuntak, 2021).

The purposes of this study were to evaluate the antioxidant property of taro leaf ethanol extract, as measured by SOD and CRP levels in streptozotocin – induced diabetes mellitus rats.

2. Methods

This study used a randomized pretest – posttest controlled group design. This study was conducted in Laboratorium Biomedik Terpadu Faculty of Medicine Udayana University Bali, Indonesia.

Experimental Animal

Twenty six healthy male Wistar (*Rattus norvegicus*) rats (180 – 200 g) were taken from animal unit of Medical Faculty, Udayana University. The adaption period was done in for one week with 10 gram standard food mixture and water were administered *ad libitum*. The samples were administered with a 45 mg/kgBW streptozotocin solution intraperitoneally, and blood sugar levels were determined to be greater than 230 mg/dl. The samples then divided into 2 groups of intervention randomly (n = 13). Pre-test SOD and CRP levels were determined using blood serum drawn through the medial canthus of the orbital sinus following anesthesia with ketamine 20 mg/kgBW and xylazine 5 mg/kgBW. The control group were given 2 ml aquadest as a placebo, whereas the treatment group were given 400 mg/kgBW in 2 ml of aquadest, force - feeding using a probe once a day. After 2 weeks after treatment, the rats were anesthetized. The rats were sedated once more after 14 days, and the levels of SOD and CRP (post – test) were measured through the medial canthus of the orbital sinus.

Reagen Preparation

Standard solution prepared before SOD and CRP determination in Table 1 and Figure 1.

Table 1.
Standard solution prepared before SOD and CRP

Dose	Standard	Description
12 ng/ml	Standard No. 5	120 μ l Original Standard + 120 μ l Standard Diluent
6 ng/ml	Standard No. 4	120 μ l Standard No.5 + 120 μ l Standard Diluent
3 ng/ml	Standard No.3	120 μ l Standard No.4 + 120 μ l Standard Diluent
1.5 ng/ml	Standard No.2	120 μ l Standard No.3 + 120 μ l Standard Diluent
0.75 ng/ml	Standard No.1	120 μ l Standard No.2 + 120 μ l Standard Diluent

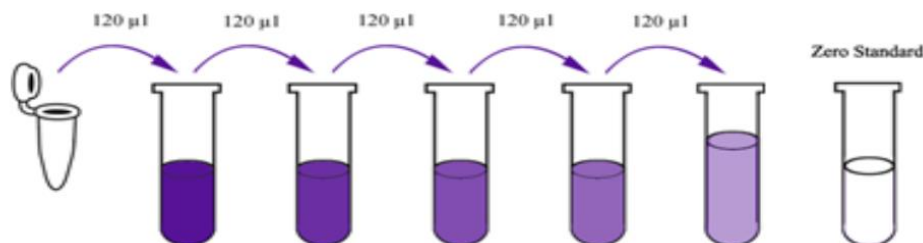


Figure 1
Standart concentration

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
24 ng/ml	12 ng/ml	6 ng/ml	3 ng/ml	1.5 ng/ml	0.75 ng/ml

Determination of SOD Level

Superoxide dismutase was extracted from blood plasma samples and analyzed using Rat Superoxide Dismutase ELISA method, Cat. No E0168Ra, BT – LAB kit. The test was performed in room temperature. Add 50 μ l standard to standard well, 40 μ l sample to sample well, 10 μ l antiSOD antibody to sample well, 50 μ l streptavidin HRP to sample well and standard well, mixed then incubated for 60 minutes at 37°C. After the sealer was removed, rinsed 5 times, then wet with 300 μ l wash buffer for 30 seconds to 1 minute. Suck all tubs and wash 5 times with washing buffer, tapping dishes against paper towels or other absorbent material to drain the plate. Add 50 μ l of substrate solution A to each well, followed by 50 μ l of substrate solution B. Plates were incubated for 10 minutes at 37 °C in the dark before being coated with fresh sealer. The blue color turned to yellow as soon as 50 μ l of Stop Solution was added to each tube. Within 10 minutes of administering the stopping solution, read a microplate using a microplate reader set to 450 nm with computer – based curve – fitting software.

Determination of CRP Level

C – Reactive Protein was extracted from blood plasma samples and analyzed using Rat C – Reactive Protein ELISA method , Cat.No E0053Ra, BT – LAB kit. The test was performed in room temperature. Add 50 μ l standard to standard well, 40 μ l sample to sample well, 10 μ l antiCRP antibody to sample well, 50 μ l streptavidin HRP to sample well and standard well, mixed then incubated for 60 minutes at 37°C. After the

sealer was removed, rinsed 5 times, then wet with 300 μ l wash buffer for 30 seconds to 1 minute. Suck all tubs and wash 5 times with washing buffer, tapping dishes against paper towels or other absorbent material to drain the plate. Add 50 μ l of substrate solution A to each tube, followed by 50 μ l of substrate solution B. Plates were incubated for 10 minutes at 37 °C in the dark before being coated with fresh sealer.

The blue color that turned to yellow as soon as 50 μ l of Stop Solution was added to each tube. Within 10 minutes of administering the stopping solution, read a microplate using a microplate reader set to 450 nm with computer – based curve – fitting software.

Data Analysis

The Statistical Package for Social Science (SPSS) software version 26 for Windows was used to conduct the statistical analysis. All data were tested its normality using Saphiro –Wilk. The comparasion data using parametric test (Independent T test and paired T test) for the normal distribution data ($p > 0.005$) and non parametric test (Wilcoxon and Mann Whitney) for abnormal distribution data ($p < 0.005$)

3. Results

The research included 26 rats, however one died in the therapy group. The control group included 13 rats, but the treatment group had 12 rats which could be analyzed. The results of the data analysis were in table 2.

Table 2
Characteristics of Data

Variable	Control Group		Treatment Group	
	Pretest	Post-test	Pretest	Post-test
SOD level (ng/ml)				
Mean $\pm$ SD	5.37 $\pm$ 0.69	5.26 $\pm$ 1.50	5.33 $\pm$ 1.14	6.20 $\pm$ 2.64
median	5.46	5.31	5.44	6.06
(Min-Max)	(3.63 – 6.30)	(1.72 – 6.92)	(3.07 – 7.01)	(1.33 – 11.54)
CRP level (ng/ml)				
Mean $\pm$ SD	3.39 $\pm$ 0.24	3.56 $\pm$ 0.43	3.21 $\pm$ 0.37	2.93 $\pm$ 0.47
median	3.34	3.72	3.25	3.06
(Min – Max)	(2.92 – 3.72)	(2.92 – 4.06)	(2.51 – 3.94)	(1.70 – 3.45)

Distribution of Data with SOD and CRP Variables between Groups

SD = Standard Deviation; Min = Minimum Value; Max = Maximum Value

Comparability analysis aimed to compare the data levels of SOD and CRP. Data on SOD and CRP levels were data with a numerical scale and had homogeneous data variants and generally distributed. This study was conducted in 2 groups in pairs. Because the data was normally distributed, the comparibility analysis used the parametric test. Paired t test for compared pretest and posttest data, independent t test used for control and treatment group. Comparability analysis is presented in table 3.

Table 3
SOD Level Comparability Analysis

Group	Pretest (ng/ml $\pm$ SD)	Posttest (ng/ml $\pm$ SD)	P*
Control Group	5.37 $\pm$ 0.69	5.26 $\pm$ 1.50	0.830
Treatment Group	5.33 $\pm$ 1.14	6.20 $\pm$ 2.64	0.239
P**	0.916	0.296	

P* = Paired t test; P** = Independent t test; SD = Standard Deviation

In the results of the compatibility analysis of SOD levels, the significance value was not significant in the unpaired group with independent t test ($p > 0.05$). So it can be concluded that the levels of SOD in the control and treatment groups were not much different, namely $p = 0.296$ in the pretest and $p = 0.296$ in the posttest. In the paired group after paired t test test, the significance value was also not significant where $p > 0.05$, namely $p = 0.830$ in the control group and $p = 0.239$ in the treatment group. Thus, it can be concluded that before and after being given placebo and ethanol extract in the control and treatment groups, SOD levels did not increase or decrease significantly.

Table 4
CRP Level Comparability Analysis

Group	Pretest (ng/ml $\pm$ SD)	Posttest (ng/ml $\pm$ SD)	P*
Control Group	3.39 $\pm$ 0.24	3.56 $\pm$ 0.43	0.308
Treatment Group	3.21 $\pm$ 0.37	2.93 $\pm$ 0.47	0.182
P**	0.112	0.009	

P* = Wilcoxon test ; P** = Mann – Whitney test ; SD = Standard Deviation

In the comparability analysis of CRP levels (Table 4), in the unpaired group with the Mann Whitney test, the significance value in pretest was not significant ($p = 0.112$), but in the posttest group was significant ($p = 0.009$). In the paired group with Wilcoxon test, the significance value in the control group was not significant ($p = 0.308$) and in the treatment group was not significant too ($p = 0.182$). So it can be concluded that the levels of CRP before and after being given a placebo and ethanol extract in the control and treatment groups, CRP levels was decrease significantly but the levels of CRP before and after given taro extract in treatment group was not decrease significantly.

4. Discussion

Effect of Streptozotocin induction on SOD and CRP levels

Streptozotocin is a diabetogenic agent that causes an increase in the production of ROS and RNS free radicals. Streptozotocin that enters cells through GLUT2 causes DNA alkylation, spontaneous NO release and superoxide through the mitochondrial respiratory chain (Goud *et al.*, 2015). In this study, the control and treatment groups were induced by streptozotocin 45 mg/kgBW, to make the mice hyperglycemic (> 230 mg/dl). According to Afanas'ev (2010), hyperglycemia here increases ROS (superoxide) through NADPH oxidase and mitochondria. The increase in ROS causes a decrease in the expression of the SOD enzyme (Omoruyi *et al.*, 2020). In this study, the pretest SOD level certainly increased. SOD levels in the control group were 5.37 ng/ml, the treatment group was 5.33 ng/ml, and were not significantly different.

Hyperglycemia increases the expression of TNF- α and IL-6 in cells, resulting in an increase in plasma TNF- α and IL-6. Increased levels of TNF- α and IL-6 will increase CRP levels (Sun *et al.*, 2014). In this study, pretest CRP levels also certainly increased. CRP levels in the control group were 3.35 ng/ml, the treatment group was 3.21 ng/ml and did not differ significantly.

The control group's posttest SOD level was 5.26 ng/ml a decrease but not significant from 5.37 ng/ml. This demonstrates that, while the process of superoxide ROS generation is still ongoing, the SOD activity to overcome ROS is also still ongoing. Posttest CRP levels in the control group increased but not significant from 3.39 ng/ml to 3.56 ng/ml. From these results, it is concluded that the inflammatory process in diabetic rats was still ongoing.

Effect of Taro Leaf Extract on SOD and CRP Levels.

Taro leaf ethanol extract contains rich antioxidants, one of which is flavonoids. Flavonoids are excellent reducing compounds because they may capture free radicals (ROS/Reactive Oxygen Species or RNS/Reactive Nitrogen Species) via electron transfer and limit peroxidation processes (Bisala *et al.*, 2019). Flavonoids increase SOD by activating nuclear factor erythroid 2 related factor 2 (Nrf2), which activates extracellular signal regulated protein kinase (ERK), c-jun n-terminal kinase (JNK), and p38, which is responsible for phosphorylation of nuclear factor erythroid 2 related factor 2 (Nrf2) in the cytoplasm, which then moves to the nucleus and binds to antioxidant responsive element (Fadli *et al.*, 2015)

The SOD level of the pretest treatment group was 5.33 ng/ml, while the posttest was 6.20 ng/ml, with no significant increase. The insignificant difference might be due to a lack of time (14 days). This study took the time reference from the study of Nugroho (2016) in rats with diabetes mellitus who were also induced with streptozotocin 65 mg/kgBW and given cherry leaf extract with IC₅₀ 25.74 g/ml and flavonoid levels 93.21 mgEQ/g extract could increase SOD levels significantly for 14 days. The difference in the higher IC₅₀ content (126.4216 mg/L) means it has a lower antioxidant capacity in this study did not give a significant difference in results. Meanwhile, based on the results of this study, Similar to the study conducted by Andrestian *et al* (2019), in diabetic rats (streptozotocin 40 mg/kgBW) given torbangun leaf extract with an IC₅₀ of 306.28 ppm can increase SOD levels but not significantly for 14 days. Similar to the study conducted by Luandayanti (2013), in diabetic rats induced with streptozotocin 100 mg/kgBW and given mahogany seed methanol extract (IC₅₀ 117.73 mg/L) there was an increase in SOD levels but not significant. The use of 14 days was decided based on a preliminary study that in 14 days, SOD levels increased, but their significance was not tested. Similar to the study conducted by Luandayanti (2013), in diabetic rats induced with streptozotocin 100 mg/kgBW and given mahogany seed methanol extract (IC₅₀ 117.73 mg/L) there was an increase in SOD levels but not significant. The use of 14 days was decided based on a preliminary study that in 14 days, SOD levels increased, but their significance was not tested. Similar to the study conducted by Luandayanti (2013), in diabetic rats induced with streptozotocin 100 mg/kgBW and given mahogany seed methanol extract (IC₅₀ 117.73 mg/L) there was an increase in SOD levels but not significant. The use of 14 days was decided based on a preliminary study that in 14 days, SOD levels increased, but their significance was not tested.

An insignificant increase in SOD levels for 14 days, may also be due to the ongoing production of superoxide free radicals by streptozotocin (Fachri *et al.*, 2019; Ngestiningsih *et al.*, 2019; Tang *et al.*, 2018). Streptozotocin enters cells via the GLUT2 transporter and spontaneously forms NO free radicals which produce peroxynitrite. Streptozotocin that has entered the cell causes DNA damage through DNA alkylation and peroxynitrite. The DNA damage causes xanthine to produce superoxide free radicals through xanthine oxidase (Goud *et al.*, 2015). SOD works to convert superoxide into hydrogen peroxide. Superoxide produced due to DNA damage by streptozotocin is still running, so that within 14 days the increase in SOD has not been seen to be significant.

In addition, a dose of 400 mg/kgBW 1 time a day can also cause an insignificant increase in SOD. A dose of 400 mg/kg BW based on studies by Akter et al (2013), Tendean et al (2017) and Sudhakar et al (2017) can lower blood sugar, but these studies did not look at its antioxidant activity. In the preliminary study, the use of a dose of 400 mg/kgBW used the research reference above and it could indeed increase SOD levels but the significance was not seen. Research on the half-life of taro leaf doses needs to be carried out in order to obtain the optimal daily dose to significantly increase SOD.

As an anti-inflammatory, flavonoids work in several ways, which are still being studied, because each flavonoid has its own uniqueness. Quercetin and catechins as part of flavonoids work as anti-inflammatory by inhibiting c - Jun N terminal kinase and p 38 kinase, and inhibiting Nf - kB activity. Nf - kB works as a controller of proinflammatory cytokine expression, namely TNF α , IL -1 β and IL - 6. The inhibition of Nf - kB activity against proinflammatory cytokines causes hepatocytes to inhibit CRP production (Al-Khayri *et al.*, 2022).

Increased levels of CRP have a role as a biomarker of tissue damage due to the ongoing inflammatory process. The decrease in CRP levels is expected to prevent tissue damage due to free radicals (Giudice and Gangestad, 2018). CRP as a biomarker of inflammation is used as a parameter to see the course and complications of diabetes mellitus. Compared with other cytokine markers (IL -1, IL-6 and TNF -), CRP is easier to use because it is relatively stable in serum, easier to measure and the availability of quantifiers (Kanmani *et al.*, 2019).

In this study, CRP levels after treatment for 14 days, in the control group was 3.56 ng/ml, significantly higher than the treatment group, which was 2.93 ng/ml. Although when compared with the pretest data, there was an insignificant increase in the control group and an insignificant decrease in the treatment group. This study showed the same results in the study of Ajiboye et al (2021), CRP levels in diabetic rats (streptozotocin 35 mg/BW) given tropical chestnut (chestnut) leaf extract 300 mg/kgBW with IC₅₀ 114,659 g/ml and its flavonoid content 141.40 mg/gram extract can reduce CRP levels in 14 days.

The content of flavonoids contained in taro leaves can increase the expression of the Nrf2 gene to overcome free radicals⁸. In a meta-analysis study, Nrf2 activated by flavonoids directly binds to proinflammatory cytokine genes (IL-6 and IL-1) and inhibits their expression in M-1 type macrophages. In addition to activating Nrf2, flavonoids can also increase Sirtuin 1 (Sirt1) (Hošek *et al.*, 2011; Kolakul and Sripanidkulchai, 2017). Sirt1 prevents the release of proinflammatory mediators (IL-6, TNF- α and IL1) through binding to toll-like receptors 4. Prevention of increased proinflammatory mediators (IL-1, IL-6 and TNF- α) causes a decrease in CRP levels (Xie *et al.*, 2022).

Statistically the increase in SOD levels in this study was not significant, therefore further studies are needed to determine the clinical implications. From this increase, it may be possible to reduce clinical symptoms due to hyperglycemia. Significantly decreased CRP levels also need to be studied further to see the clinical significance of clinical symptoms that arise due to hyperglycemia.

Based on the results of phytochemical samples conducted at the Integrated Service Laboratory of the Faculty of Agricultural Technology, Udayana University, it was found that taro leaf extract contains flavonoids of 20817.55 mg/100g QE, phenol 1821.45 mg/100g GAE, tannin 2684.24 mg /100g TAE, antioxidant capacity 5926.36 mg/L GAEAC, and IC 50% 126.4216 mg/L.

There was no significant difference in SOD levels in this research, although there was a significant difference in CRP levels. Although SOD levels did not increase significantly, the trend seemed to increase. This increasing trend can be considered that the flavonoid content in taro leaf extract activates the Nrf2 gene and increases SOD levels. The significant difference due to the decrease in CRP levels indicates that the flavonoids in taro leaf extract can act as anti-inflammatory through the activation of the Nrf2 and Sirt1 genes, so that proinflammatory cytokines can be suppressed and CRP levels decreased significantly. Further studies needed to confirm the research time that taro leaf extract can increase SOD level significantly, the duration of administration, and an active substance, so taro leaf extract can use as an external antioxidant.

5. Conclusions

The ethanol extract of taro leaves (*Colocasia esculenta* (L.) Schott) did not increased SOD levels but decreased of CRP levels in white male rats (*Rattus norvegicus*) Wistar strain model diabetes mellitus

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Ethics In Research

Ethical permission was published by Faculty of Veterinary Medicine, Udayana University research ethics committee, Udayana University No. B/230/UN.14.2.9/2021,

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