Enhancement of superoxide dismutase gene expression in mice following the administration of cinnamon bark extract

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Abstract. Proliferation of free radicals in the body, particularly in the form of reactive oxygen species (ROS), induces oxidative stress. One can enhance the antioxidant levels in the body by ingesting antioxidant substances. The cinnamon bark (*Cinnamomum zeylanicum*) is rich in flavonoids, cinnamic acid derivatives, coumarins, and polyfunctional organic acids. These compounds can serve as significant antioxidants in neutralizing radicals. The objective of this study was to investigate the impact of cinnamon extract on the expression of the superoxidase dismutase (SOD) gene in hepatocytes and spermatozoa of male mice. The treatments included: C: no administration of cinnamon extract, T1: administration of cinnamon extract at a concentration of 75 mg/kgBW, T2: administration of cinnamon extract at a concentration of 150 mg/kgBW. The amount of SOD cDNA after cinnamon extract administration was quantified using quantitative real-time PCR with β-Actin as internal control. This study shows that cinnamon extract (Cinnamomum zeylanicum) had a statistically significant effect ($p < 0.05$) on the upregulation of the superoxidase dismutase (SOD) gene expression in sperm (3.44) as well as liver cells (3,73) of male mice. The optimal dosage is 150 mg/kg body weight.

1 Introduction

The generation of free radicals is an unavoidable result of aerobic metabolism that can lead to oxidative stress when reactive oxygen species (ROS) are overproduced. Some of ROS like hydrogen peroxide and superoxide anions could disrupt cellular homeostasis by actively damage the cell components like nucleic acid, protein, carbohydrate and lipid. Inequality between the antioxidant availability and the generation of reactive oxygen species (ROS) results in oxidative stress, which plays a significant role in the development of numerous degenerative conditions, including neurodegenerative disorders, cardiovascular diseases, and cancer [1, 2].

Endogenous antioxidant enzymes, like superoxide dismutase (SOD), are essential for mitigating ROS by catalyzing the conversion of superoxide radicals into oxygen and

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hydrogen peroxide, which is subsequently reduced to water by catalase or glutathione peroxidase [3, 4]. However, under conditions of excessive oxidative stress, endogenous antioxidants may be insufficient to counteract the elevated ROS levels, necessitating the supplementation of exogenous antioxidants.

Cinnamomum zeylanicum has garnered attention for its rich content of bioactive compounds, including flavonoids, cinnamic acid derivatives, and polyphenolic compounds, which exhibit potent antioxidant properties [5-7]. Numerous studies have shown that cinnamon extracts can boost the activity of antioxidant enzymes in vivo, such as SOD, suggesting its potential therapeutic value in mitigating oxidative stress. However, the molecular mechanisms underlying the upregulation of SOD gene expression by cinnamon extract remain poorly understood.

This study aims to elucidate the effects of *Cinnamomum zeylanicum* crude extract on the expression of the SOD gene in male mice (*Mus musculus* L.), with a focus on liver and sperm cells. By employing quantitative real-time PCR (rt-PCR), this research seeks to provide insights into the transcriptional regulation of antioxidant defences in response to cinnamon extract supplementation, thereby contributing to the broader understanding of its role in oxidative stress management.

2 Materials and methods

2.1 Animals preparation

This research employed 40 healthy male mice (*Mus musculus* L.). The mice were sourced from the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang. They were divided into three groups (C, T1, T2), with each group housed in a plastic cage containing ten mice. The cages were maintained in an environment with adequate ventilation, humidity, and lighting. Food in the form of pellets and water was provided *ad libitum*. These procedures are under approval of the Research Ethics Committee of Medical Faculty Andalas University Padang with the description of ethical approval number 168/UN.16.2/KEP-FK/2000.

2.2 Preparation of cinnamon bark extract and experimental animal induction

Cinnamon bark extract was prepared from dried cinnamon bark powder. The powder was made by grinding the bark using a mortar and sieving it through a mesh. The treatment group 1 (T1) received a dosage of 75 mg/kg/day [8], and treatment group 2 (T2) received 150 mg/kg/day [5]. The treatment was administered for 4 weeks. All the active compounds in cinnamon bark for antioxidants are standardized by the reference dosage [8].

2.3 Termination, sample collection, and storage

One day after the final treatment, mice were decapitated through the cervical dislocation method. The target organs (testis and liver) were immediately removed and stored at -80°C for total RNA isolation.

2.4 Total RNA isolation and RNA quality and quantity measurements

The total RNA isolation procedure followed the Zymo Research kit manual. The quality and quantity of total RNA were consecutively measured by nanodrop (Implen N50) at wavelengths of λ260/λ280 and λ260.

2.5 Primer design

A pair of primers was designed to amplify mice SOD cDNA (NM-011434.2) using Geneious Prime software and the Pick Primer tool. The amplification product was 240 bp.

2.6 cDNA synthesis and amplification of SOD and β-Actin

cDNA of SOD and β-Actin was synthesized following the Bioline Sensifast cDNA synthesis kit manual (Cat. No BIO-65053). β-Actin used as an internal standard. The composition of the RT-PCR reaction and the amplification cycle profile are detailed in Tables 1 and 2.

Table 1. Real-time-PCR reactions for SOD and β-Actin cDNA amplification

Reaction Compound	Initial Conc.	End Conc.	Volume (µl)
Nuclease-free water	-	-	2.4
Forward primers	10 µM	0.3 µM	0.3
Reverse primers	10 µM	0.3 µM	0.3
SensiFAST™ SYBR NO-ROX Kit	2X	1X	5
cDNA samples			2
Total volume			10

Table 2. Temperatures, duration, and cycles of RT-PCR for cDNA Amplifications

Steps	Temperatures (ºC)	Duration	Cycles
Pre-denaturation	95ºC	2 min	1 x
Denaturation	95ºC	5 second	40 x
Annealing-Extension	59.4ºC	15 second	40 x
Melting curve	65-95ºC	5 second	1 x
Temporary stored	4 ºC	∞	-

2.7 Data Analysis

A one-way analysis of variance (ANOVA) and the Tukey HSD test were used to analyze the data.

3 Results and Discussions

3.1 RNA quality and quantity

Total RNA was isolated from both liver and sperm cells. The qualities and quantities of extracted total RNA were in a good range for the next steps (Table 3).

Table 3. The quality and quantity of total RNA extracted from liver and sperm cells

Cell Sources	Mean of RNA Concentration (ng/ul)	Mean of RNA Purity 260/280	Range of Acceptable Purity
Sperm	233.47	2.00	1.8 – 2.0
Liver	275.5	2.00	

3.2 Candidate Primers for SOD Gene Expression Analysis

A pair of primers was designed to amplify the SOD cDNA after the reverse transcription process of RNA. The forward primer was located at nucleotide 252-273, and the reverse primer at nucleotide 491-471 (Table 4). The primer melting temperatures (Tm) range from 58.99-60.34ºC with 62% GC content. Forward primer is 22 nucleotides in length, while the reverse primer is 21 nucleotides. Specificity tests showed that the primers were 100% identical to the target (SOD cDNA) (Table 5). Based on BLAST tests, the selected primers were in the appropriate criteria.

Table 4. A Pair of primer designed to amplify SOD cDNA using *real-time* PCR

cDNA	Primers Sequences	Melting Temperature (ºC)	% GC	Length (bases)	Start position (nucle.)	Product Size (bp)
SOD	F: 5'-GGACAATACACAAGGCTGTACC-3'	58,99	50,00	22	252	240
	R: 5'-TTCCACCTTTGCCCAAGTCA-3'	60,34	47,62	21	491	

3.3 SOD and housekeeping gene expressions

The real-time PCR analysis showed that the primers specifically amplified the cDNA of SOD in sperm and liver cells, as indicated by the single peak curve in each cycle (Figures 1A). The amplification curves (Figures 1B) showed that the samples crossed the threshold line between cycles 15 to 30 for sperm cells and cycles 20-30 for liver cells. The β-Actin used as housekeeping gene exhibited low variation in Cq values (graphic not shown), indicating stable expression across samples [9].

Table 5. BLAST identification of designed primers

cDNA	Accession Number	Primers	E-Value	Identify (%)
SOD	NM_011434.2	Forward Reverse	0,00	100

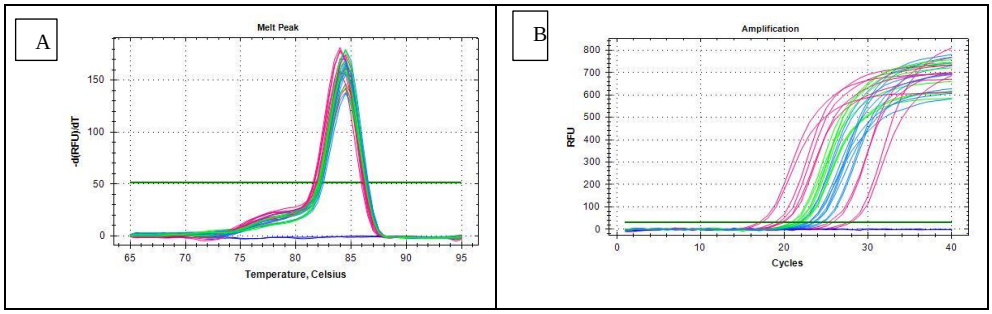

Fig. 1. Melt peak and amplification curve SOD cDNA of sperm samples; (A) Melt Peak (B) Amplification Curve. The similar condition was obtained from liver samples (figure not showed).

3.4 SOD gene expressions in mice sperm cells

The relative expression levels of the superoxide dismutase (SOD) gene in male mice sperm cells for each treatment group are presented in Figure 2. The mean relative expression of the SOD gene in sperm cells of the T2 group is 3.44 while 2.41 in T1 group and 1.18 in the control group. Statistical analysis revealed a significant difference in the cDNA quantity of the control and treatment groups.

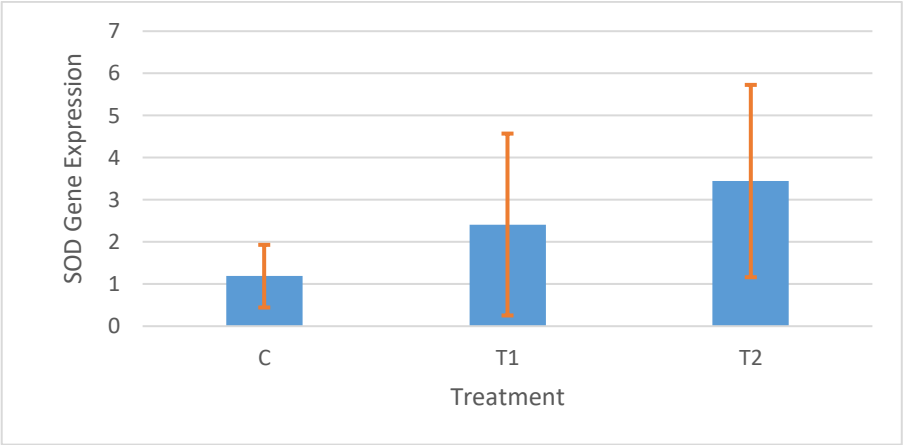

Fig. 2. Mean of relative quantity of SOD cDNA of mice sperm (*Mus musculus* L.) p-value = 0.039 (< 0.05). Significant differences in gene expression were observed between treatment and control groups.

3.5 SOD gene expressions in mice liver cells

The relative expressions of the SOD gene in mice liver cells is shown in Figure 3. The highest expression of the SOD gene was detected in the T2 group (3.73), and the lowest expression was found in the control group (1.15). Statistical analysis showed that there was a significant difference in the expression level of the SOD gene between the T2 dan control group. However, there was no significant difference between the T1 (2.24) and control group.

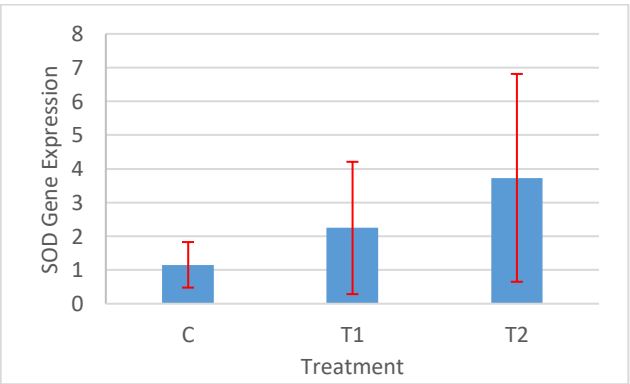

Figure 3. Mean of the relative quantity of SOD cDNA of Mice Liver cells. p-value = 0.040 (< 0.05). There were significant differences in gene expression between treatment and control groups.

3.6 Discussion

The findings of this study demonstrate significant upregulation of SOD gene expression in both liver and sperm cells of male mice following the administration of *Cinnamomum zeylanicum* crude extract. The highest level of SOD expression was observed in the group receiving 150 mg/kg/day body weight, indicating a dose-dependent response. This result aligns with previous research that highlighted the efficacy of cinnamon extract in enhancing antioxidant enzyme activity, thereby reinforcing the hypothesis that *Cinnamomum zeylanicum* possesses strong antioxidant properties [10, 11]. Moreover, this result aligned with Sandamali et al. [5], where administering 2 g/kg/day of *Cinnamomum zeylanicum* can reduce some sources of free radicals like lipid peroxidation and myeloperoxidase activity and increase glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase activity in rats injected by doxorubicin [5].

The increase in SOD expression can be linked to the high levels of flavonoids and polyphenolic compounds in cinnamon, which are known to activate the Nrf2 pathway (nuclear factor erythroid 2-related factor 2). Nrf2 is a transcription factor that regulates the production of antioxidant proteins, including SOD, by interacting with antioxidant response elements (ARE) in the promoter regions of the corresponding genes [12, 13]. The activation of Nrf2 by cinnamic acid derivatives and other bioactive compounds present in cinnamon could explain the upregulation of SOD observed in this study.

Moreover, the differential expression of SOD in liver and sperm cells suggests tissue-specific responses to oxidative stress. The liver, being a central organ in detoxification and metabolism, is constantly exposed to high levels of ROS, making the upregulation of SOD crucial for maintaining redox homeostasis [14]. In sperm cells, the increase in SOD expression may play a protective role against oxidative damage to DNA, which is critical for maintaining sperm integrity and preventing infertility [15].

The methodological approach of using rt-PCR to quantify SOD gene expression provided robust and sensitive detection of mRNA levels, allowing for precise quantification of the impacts of cinnamon extract on gene expression. The use of β-Actin as a housekeeping gene for normalization ensured the reliability of the results, as β-Actin is constitutively expressed and not influenced by external factors such as oxidative stress [16].

The implications of these findings are significant for the potential use of *Cinnamomum zeylanicum* as a dietary supplement to enhance endogenous antioxidant defences. Given the safety profile of natural antioxidants compared to synthetic ones, cinnamon extract could be a viable alternative for individuals seeking to mitigate oxidative stress-related conditions. Nonetheless, additional research is needed to investigate the long-term effects of cinnamon supplementation and its influence on humans since different species can give different physiological and cellular responses. This study provides compelling evidence that *Cinnamomum zeylanicum* extract can significantly enhance the expression of the SOD gene in mice key tissues, offering a promising strategy for combating oxidative stress. These findings add to the expanding body of literature supporting the therapeutic potential of natural antioxidants in disease prevention and management.

4 Conclusion

Cinnamomum zeylanicum crude extract increase SOD gene expressions in male mice sperm cells as well as liver cells. The most effective extract concentration is 150 mg/kg/day.

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