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Original Research Article

Neuroprotective Activity and Antioxidant Effect of *Salacca zalacca* Peel Ethanol Extract on High Glucose Induced Zebrafish (*Danio rerio*) Embryo

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ABSTRACT

Hyperglycemia in gestational diabetes (GDM) could lead to oxidative stress that result in reduction of superoxide dismutase (SOD) level and sirtuin (SIRT)-1 expression. Reduction of SOD and SIRT-1 inhibit CAMP response element-binding protein (CREB), a regulator of brain derived neurotrophic factor (BDNF) transcription, and reduces BDNF expression. Decreased BDNF expression in newborns could lead to autism spectrum disorder (ASD) and retinopathy of prematurity (ROP) which can interfere with memory and vision function during growth and development. *Salacca zalacca* (SZ) peel contains antioxidant neuroprotectant substances and this study was aimed to prove the effect of *Salacca zalacca* peel extract on the development of embryos induced by high glucose. The study used zebrafish embryos induced by 3% of glucose as a model of gestational diabetes. *Salacca zalacca* peel extract was given at concentrations of 0.1, 0.2, and 0.4 mg/mL. The expression of SIRT-1, BDNF, and SOD of zebrafish embryos was evaluated by reverse transcriptase PCR (RT-PCR). The results showed a significant increase in the expression of SIRT-1, BDNF, and SOD at the concentration of 0.4 mg/mL. It was concluded that *Salacca zalacca* peel extract has a neuroprotectant and antioxidant effects.

Keywords: BDNF, Glucose, Salacca zalacca, SIRT-1, SOD.

Introduction

Diabetes mellitus is a group of metabolic diseases with hyperglycemia characteristics that occur due to defects in insulin secretion, insulin action, or both. Diabetes mellitus (DM) can be categorized based on its etiology, namely type 1 DM, type 2 DM, gestational diabetes mellitus (GDM), and other types of DM. The International Diabetes Federation stated that 1 in 6 pregnancies (16.8%) is affected by diabetes, of which 86.4% are GDM.[1] Gestational diabetes is diabetes that occurs during pregnancy, where glucose intolerance is first found during pregnancy, usually in the second and third trimesters. Gestational Diabetes Mellitus is widely associated with increased perinatal complications.[2] Hyperglycemia in GDM will induce oxidative stress through several mechanisms, including increased production of reactive oxygen species (ROS) in mitochondria, polyol pathway and hexosamine pathway, protein kinase C pathway, activation of the pathway for the formation of advanced glycation end-products (AGE), and changes in biomarkers of antioxidant protection.[3]

Increased oxidative stress will trigger a decrease in sirtuin (SIRT)-1 expression.[4] SIRT-1 is the most studied SIRT member due to its very crucial role in the human body since it has a function as a regulator of cellular functions such as metabolism of glucose, lipids, mitochondrial biogenesis, inflammation, autophagy, and circadian rhythms, and various other things such as defense against stress, apoptosis. and chromatin silencing.[5] Decreased SIRT-1 expression will cause a decrease in CAMP response element-binding protein (CREB) which binds to the brain-derived neurotrophic factor (BDNF) promoter so that BDNF expression will also decrease. In addition, modulation of BDNF by SIRT-1 can also take place through deacetylation of methyl-CpG binding protein 2 (MeCP2). If the deacetylation process decreases due to decreased SIRT-1 expression, then the BDNF transcription process will decrease as well.[6] The decrease in SIRT-1 expression will also decrease the transcriptional activity of the peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) which can inhibit the formation of endogenous antioxidants in mitochondria such as Superoxide Dismutase (SOD).[5]

Brain derived neurotrophic factor or BDNF is a neurotrophin that plays a role in synapse development, synaptic plasticity, and cognitive function. BDNF plays a role in the physiological function of the central nervous system and the development of cortex maturation and synaptic plasticity so that BDNF has a role in learning and memory processes. In addition, BDNF is also found abundantly in the retina. A decrease in BDNF expression in newborns could lead to autism spectrum disorder (ASD) and retinopathy of prematurity (ROP) that could impair vision.[7] The decrease in BDNF expression will reduce the production of SOD as an antioxidant and nitric oxide (NO). Nitric oxide has the capability to increase the production of cyclic guanosine monophosphate (cGMP) and the expression of protein kinase G (PKG-1) so that it will increase the expression of the antioxidant thioredoxin and antiapoptotic protein (Bcl-2).[8] In short, a decrease in BDNF will lead to an increase in the level of apoptosis. Increased apoptosis, if it occurs during the process of fetal organogenesis, will disrupt the development of brain organs in the endoderm layer, putting the fetus at a risk for cognitive dysfunction in children.[9] Superoxide dismutase (SOD), which is an endogenous antioxidant, can reduce ROS production by converting superoxide anions ($O_2^{\bullet-}$) into hydrogen

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peroxidase (H_2O_2) and molecular oxygen (O_2) so that superoxide anions become harmless.[10]

Zebrafish (*Danio rerio*) embryos aged 72 hpf (hours post fertilization) are equivalent to fetuses in the womb.[11] Zebrafish have 70% genes homology with humans and previous studies have proven that zebrafish can be used as a model of diabetes mellitus.[12] In addition, zebrafish have similarities with humans in terms of the dopaminergic system, endocrine, and exocrine functions. In addition, zebrafish produce plenty of eggs, up to 200 eggs, and can live without eating for several days. These advantages are the reason why zebrafish are suitable for embryotoxicity studies.[13]

Agroindustry is one of Indonesia's biggest sectors. Data published by Indonesia's Ministry of Environment and Forestry stated that the agroindustrial sector produce 23,8% of waste in 2019. *Salacca zalacca* peel is one of the agro-industrial waste that currently cannot be processed. The peel itself turns out to contain high levels of bioactive compounds in the form of ferulic acid, proline, flavonoids, and tannins which are antioxidants.[14] In addition, the peel also contains asaticoside and salidroside which function as neuroprotective in the central nervous system.[15,16]

Based on its potential as an antioxidant and neuroprotectant, the study investigated the effect of ethanol extract of *Salacca zalacca* peel on zebrafish (*Danio rerio*) embryos induced by high glucose on the expression of SIRT-1, BDNF, and SOD.

Materials and Methods

Sample

Wild type adult zebrafish, which were identified by their color patterns (a succession of 4 or 5 blue horizontal stripes alternating with 4 golden-silvered ones, giving the striking "zebra" pattern from which the species derives its vernacular name) and certified at the Hydrology Laboratory of the Faculty of Fisheries and Marine Sciences, Brawijaya University.[17] All fish were kept in a 60L aquarium at the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University with a dark cycle of 10 hours and a light cycle of 14 hours. The ratio of male: female is 2:1. Embryonic medium consist of 0.25 gr CaCl; KCl 0.15 g; NaCl 5 g; and 0.815 g of $MgSO_4$ dissolved in 500 mL of aquadest with a concentration of 10x.

Feeding was done every 8 hours. Embryos were collected 30 minutes after the light cycle and transferred to embryonic media in less than 2 hpf. Healthy embryos were cleaned and placed on 6 well plates with 30 fertile eggs in each well.

Salacca zalacca Peel Extraction

The ethanol extract of the peel of *Salacca zalacca* was obtained from the waste of the *Salacca zalacca* chips industry in Arjosari, Malang City. Extraction was made by the maceration method, 10 mg Salacca zalacca peel extracts were dissolved in 10 ml water and 98% ethanol. The metabolites of *Salacca zalacca* peel were analyzed by gas chromatography-mass spectrometry (GC-MS). This study used *Salacca zalacca* peel extract with the concentrations of 0.1 mg/ml, 0.2 mg/ml, and 0.4 mg/ml that was based on a previous study done by Khotimah, 2021.[18]

High Glucose Exposure and Salacca zalacca Peel Ethanol Extract

A total of 30 embryos were used for each treatment group,[19] namely: 1) Negative control, given 5mL of the embryonic medium, 2) Positive control, given exposure to embryonic medium and 3% glucose in the embryonic medium, 3) 4) and 5) was given 3% glucose treatment and given *Salacca zalacca* peel extract 0.1, 0.2 and 0.4 mg/ml respectively. Each exposure was done in triplicate, so for the whole experiment need total 450 embryos.[20] Exposure to glucose and *Salacca zalacca* peel extract was given from the age of 2-72 hpf by changing the medium every 24 hours.[21]

SIRT-1, BDNF dan SOD Expression Measurement with Reverse Transcriptase PCR (RT-PCR)

Zebrafish embryos were frozen. Total RNAs of the zebrafish embryo were extracted using TRIzol reagent (Sigma; St. Louis, MO, USA). Isolated RNA was then treated with DNase (DNA-free kit, Ambion, Austin, TX, USA). To check the integrity of RNA, agarose gel electrophoresis was used. Following the manufacturer's protocol (QuantiTect SYBR Green PCR Kit (1000) Qiagen), extracted RNA was reverse-transcribed in a polymerase chain reaction/PCR tube composed of 200 ng template, 1 µL primer, 25 µL MyTaq HS Red Mix, and 50 µL water then followed by 40 cycles of PCR. The reverse transcriptase PCR (RT-PCR) protocol of each primer used was listed in Table 2. To visualize the PCR products, the samples were stained by ethidium bromide under UV light and followed by electrophoresis on a 2% agarose gel. Visualization of the band was done by Gel Doc and the quantification of the absorbances was done by ImageLab. The list of primers used is shown in Table 1.[22–24]

Statistical Analysis

The statistical analysis was carried out with a one-way analysis of variance (ANOVA) followed by a posthoc Tukey HSD test using SPSS software 22.0 (SPSS, USA). Statistically significant differences were defined by a p-value < 0.05.

Table 1: List of Primers Used [22–24]

Forward Primer	
SIRT-1	5′- ACAGTTCCAGCCATCTCCATGTCA -3′
BDNF	5'-ATAGTAACGAACAGGATGG-3'
SOD	5′- GGC CAA CCG ATA GTG TTA GA-3′
β-Actin	5'-CGAGCAGGAGATGGGAACC-3'
Reverse Primer	
SIRT-1	5'- CTGGTTGAAATAGCCTTGATGAC -3'
BDNF	5'- GCTCAGTCATGGGAGTCC -3'
SOD	5′- CCA GCG TTG CCA GTT TTT AG -3′
β-Actin	5'-CAACGGAAACGCTCATTGC-3'

Table 2: Reverse Transcriptase Protocol of Each Primer

Steps	**Marker**		
	Sirt-1	**BDNF**	**SOD**
Initiation(time/temperature)	3'/95^{o}C	4'/95^{o}C	3'/95^{o}C
Denaturation(time/temperature)	15''/95^{o}C	30''/95^{o}C	15''/95^{o}C
Annealing(time/temperature)	30''/50,3^{o}C	30'/55^{o}C	30''/50,3^{o}C
Extention(time/temperature)	30''/72^{o}C	75'/72^{o}C	30''/72^{o}C
Cycles	39 cycles	39 cycles	39 cycles

Results and Discussion

In the previous study, exposure to glucose at the concentration of 3% increased the expression of PEPCK which is a marker of hyperglycemic conditions.[18] This condition will increase oxidative stress which will trigger a decrease in sirtuin (SIRT)-1 expression.[4,25,26]

In Figure 1, it can be inferred that 3% glucose decreased SIRT-1 expression, and administration of 0.4 mg/mL *Salacca zalacca* peel extract was able to significantly increase SIRT-1 expression. Sirtuins are regulators of metabolic disease development processes.[27] In mammals there are 7 types of sirtuins ranging from sirtuin 1 to 7. Sirtuin-1 (SIRT-1) is a key in regulating endothelial progenitor cell (EPCs) dysfunction and will increase deacetylation of peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1a) which will increase the expression of endogenous antioxidants such as Superoxide Dismutase (SOD). SIRT-1 also has the effect of decreasing the nuclear factor-κB p65 subunit (NFkb p65) which induces the formation of inflammatory mediators such as tumor

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necrosis factor-α (TNF- α), and chemoattractant protein-1 (MCP-1).[28] Increased expression of SIRT-1 EPCs reduces the rate of endothelial dysfunction induced by a high-fat and glucose diet.[29] In conditions of insulin resistance and diabetes, the amount of ROS will increase through 4 pathways, namely an increase in the hexosamine flux pathway, an increase in the polyol pathway, activation of protein kinase C (PKC), and the formation of advanced glycation end-products (AGEs).[3] Increased reactive oxygen species (ROS) induce oxidative stress that could lead to reduced NAD+ levels and this will inhibit SIRT-1 activity.[30]

Table 2 and Figure 2 display the result of *gas chromatography-mass spectrometry* (GC-MS) of *Salacca zalacca* peel. *Salacca zalacca* peel contains protein [D-(+)-Proline, Leucylproline, and L-Phenylalanine), ferulic acid, phenolic (Phenol, Tyrosol, and Caffeic Acid Phenethyl Ester), flavonoid (Tetramethoxyflavanone, Genistein, Hesperidin, and Keampferol), and glycoside (Salidroside dan Asiaticoside). The results show that *Salacca zalacca* peel contains a high value of phenolic acid and flavonoid that encompass antioxidant properties and could act as an inhibitor of *α*-glucosidase.[31] The content of flavonoids and polyphenols also has the ability to suppress ROS formation by inhibiting enzymes or binding the free radical element's, ROS scavenging, as well as enhancement of endogenous antioxidant effects.[32] Based on the results, it can be inferred that treatment with *Salacca zalacca* extract was able to increase the expression of SIRT-1 in zebrafish embryos induced by high glucose (Figure 1).

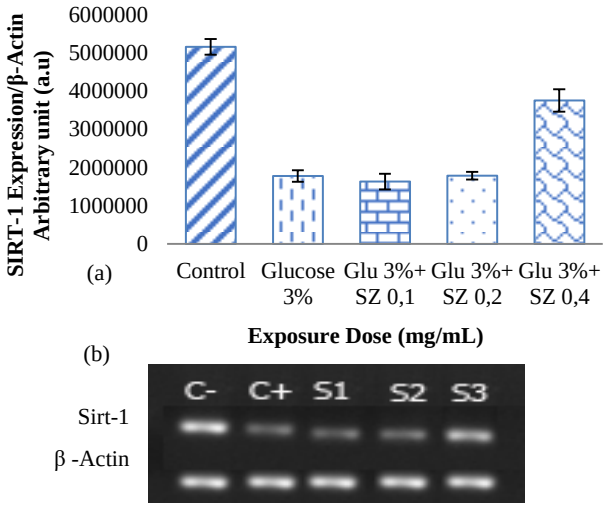

Figure 1: SIRT-1 expression graph (a) SIRT expression in 3 dpf (n=90 embryos in each group) show significant difference at the dose of 0,4 mg/mL ($p < 0,001$) compared to other groups. (b) SIRT electrophoresis.

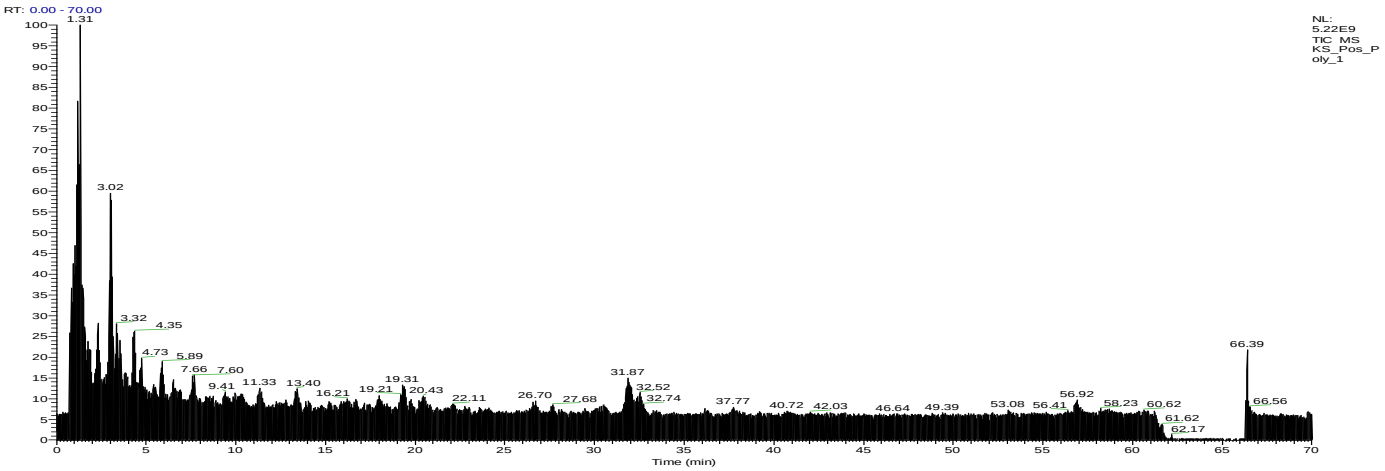

Figure 2: Chromatogram graphic from *gas chromatography-mass spectrometry* of *Salacca zalacca* peel

Table 3: Identified Metabolites on *Salacca zalacca* Ethanol Extract

n	*Retention Time*	*Area (%)*	*Probability (%)*	*Tentatives Metabolites*
1	0.885	401,146	74.7	D-(+)-Proline
2	9.429	157,300	99	(E)-Ferulic acid
3	12.197	339,777	80.2	Tyrosol
4	9.091	904,189	70	Tetramethoxyflavanone
5	2.996	12,503,471	88.3	Phenol
6	12.802	377,249	77	Salidroside
7	27.665	585,409	81.4	Asiaticoside
8	32.537	1,046,116	65.9	Genistein
9	1.92	1,034,401	78.6	Leucylproline
10	17.999	948,569	93.9	Hesperidin
11	1.551	224,404	95.4	L-Phenylalanine
12	12.767	456,666	90.4	Kaempferol
13	20.421	311,154.72	91	Caffeic acid phenethyl ester

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Based on Figure 3, it can be inferred that *Salacca zalacca* peel extracts can increase BDNF expression in embryos exposed to 3% glucose. The highest average of BDNF expression was found in 3% glucose + 0.4 mg/mL *Salacca zalacca* peel extract. Increased oxidative stress lead to a decrease in SIRT-1 expression. SIRT-1 deficiency conditions will upregulate miR-134 expression, inhibit the deacetylation process of methyl-CpG binding protein 2 (MeCP2) and inhibit the activation of Transcription Co-activator-1 (TORC1) to bind to CREB and activate the BDNF transcription pathway.[33,34] In summary, the decreased expression of SIRT-1 due to hyperglycemic conditions causes a decrease in the expression of BDNF.

Brain-derived neurotrophic factor (BDNF) is very important for neuronal growth, survival, and differentiation of neuron cells in the central nervous system. In the brain, BDNF synthesis occurs in the hippocampus and the cortex and affects neuronal activity, synaptic plasticity, and also influences apoptosis control. BDNF is the first neurotrophin that mediates the resistance and uptake of dopamine to dopaminergic neurons in the embryo's midbrain in the substantia nigra, so that in the early stages of development, BDNF is used as an important protein in ASD. In addition to the brain, BDNF is also abundant in the eyes. BDNF is produced by glial cells in the retina so that a decrease in BDNF can cause retinal function disorders in the eye.[27]

Salaca zalacca peel extract, besides containing phytoconstituents as listed earlier in Table 2 and Figure 2, also contains asiaticoside and salidroside which can work as neuroprotectants through the mechanism of decreasing levels of NR2B receptors, causing the opening time of NMDA receptors to shorten so that calcium influx into neurons decreased as a result.[15,16] Flavonoid also acts as a competitive inhibitor of ubiquinone that will inhibit the attachment of complex 1 electron transport chain and result in the reduction of H_2O_2 production.[35] Reduction of H_2O_2 production leads to decreasing oxidative stress and increase of BDNF expression. *Salacca zalacca* with the concentration of 0,4 mg/ml shows the highest expression of BDNF. This overexpression of BDNF has a beneficial effect on the neuron due to the protective effect against the degenerative process.[36]

Based on Figure 4, administration of 3% glucose did not cause a significant decrease in SOD levels in zebrafish embryos. On the other hand, administration of *Salacca zalacca* extracts was able to significantly increase the SOD of zebrafish embryos at a concentration of 0.4 mg/mL.

Hyperglycemia in gestational diabetes increases oxidative stress due to excessive production of reactive oxygen species (ROS) and in consequence, will reduce endogenous antioxidant excretion.[14] Decreased SIRT-1 expression will also reduce SOD through the transcriptional activity of peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) which can inhibit the formation of endogenous antioxidants in mitochondria such as Superoxide Dismutase (SOD).[5] Besides SIRT-1, BDNF also affects SOD expression. BDNF bind to the TrkB receptor and in return TrkB will cause the activity of tyrosine kinase which will activate PI3K. PI3K phosphorylates phosphatidylinositol from cell membranes produces phosphatidylinositol-3,4,5-phosphate (PIP3) from phosphatidylinositol-4,5-phosphate (PIP2), which then along with protein kinases such as Akt will travel to the plasma membrane. Activation of Akt will cross-talk to the mitogen-activated protein kinase (Erk) and causes activation and translocation of NF-κB.[37] NF-κB will upregulate SOD expression as an antioxidant and suppress apoptosis by binding to the Bcl-2 promoter which is an anti-apoptotic protein. Binding with Bcl-2 will inactivate caspase 3, an enzyme that initiates the process of apoptosis.[38] In that aspect, the increase in BDNF can increase the expression of SOD. *Salacca zalacca* peel extract, which can increase the expression of SIRT-1 and BDNF, also has the effect of increasing SOD. Flavonoids and polyphenols in *Salacca zalacca* peel can suppress the formation of ROS through inhibition of enzymes or binding of free radical elements, ROS scavenging, and increased expression of endogenous antioxidants.[39] Superoxide dismutase (SOD), which is an endogenous antioxidant, can reduce ROS production by converting superoxide anions ($O_2^{\bullet}$) into hydrogen peroxidase (H_2O_2) and molecular oxygen (O_2) so that superoxide anions become harmless.

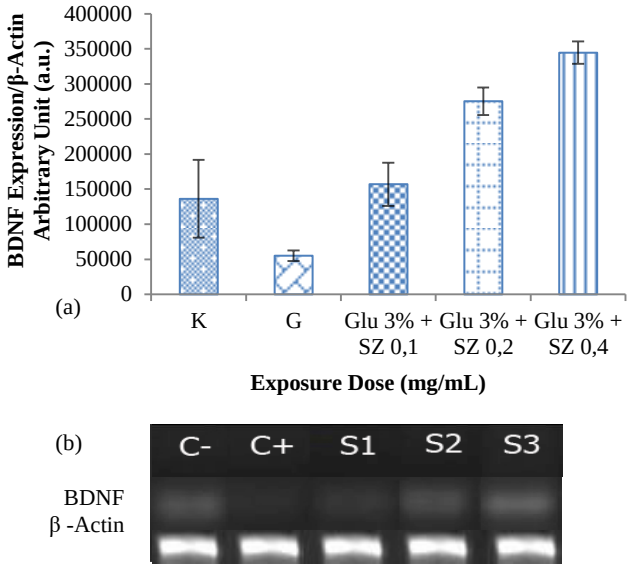

Figure 3: BDNF expression graph in 3 dpf. (a) BDNF expression show significant difference at the dose of 0,1 mg/mL ($p < 0,001$).
Significant difference can be seen at the dose of 0,2 mg/mL ($p < 0,001$) and 0,4 mg/mL ($p < 0,001$). (b) BDNF electrophoresis.

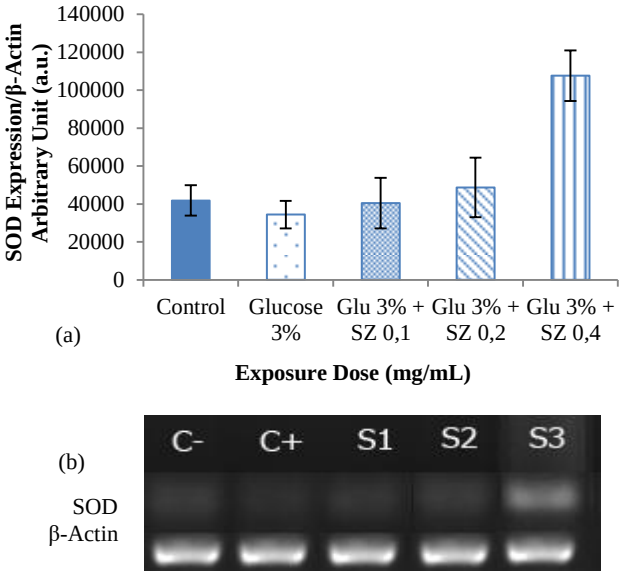

Figure 4: SOD expression graph in 3 dpf. (a) SOD expression did not show any significant difference at the dose of 0,1 mg/mL ($p = 0.972$) and 0,2 mg/mL ($p = 0.632$).
Significant difference can be seen at the dose of 0,4 mg/mL compared to other groups ($p < 0.001$). (b) SOD electrophoresis.

An increase in SOD can protect the organogenesis process that acts as the target of oxidative stress in various organs such as the cardiovascular, nerve, or respiratory system and also in the condition of inflammatory or ischemia.[10]

Conclusion

Salacca zalacca peel acts as an antioxidant and enhancer of endogenous antioxidants as well as potential neuroprotectivity properties due to its metabolites particularly flavonoids, polyphenols, asiaticoside, and salidroside. *Salacca zalacca* peels Ethanol extract was found to increase the expression of SOD, an endogenous antioxidant, and SIRT-1, a transcription factor of many endogenous

antioxidants including SOD. This study also found an increase in a neurotrophin called BDNF. These results mean that *Salacca zalacca* peel has an antioxidant effect and a potency to be developed as a neuroprotective agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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