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Ethanolic extract of *Salacca zalacca* peel reduce IL-1β and apoptosis in high glucose induced zebrafish embryo

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Abstract

Gestational diabetes is a glucose intolerance level first identified during pregnancy. This condition is caused by hormone secretion that hinders insulin action from the placenta which caused insulin resistance that will cause hyperglycemia. This condition will increase oxidative stress, and in consequence increasing inflammation and cell death which can hinder fetal growth and development. *Salacca zalacca* peel is a food waste that contains antioxidants and anti-inflammatory properties. This research aimed to study the effect of SZ peel ethanolic extract in the expression of IL-1β and cell death marker (BAX and Apaf-1) in glucose 3% induced zebrafish embryo. Glucose metabolism is measured by the level of *Phosphoenolpyruvate carboxykinase* (PEPCK) at the age of 3 dpf. The expression of PEPCK, IL-1β, Bax, and Apaf-1 is measured using reverse transcriptase PCR (RT-PCR) method. *Salacca zalacca* peel extract is given in the concentration of 0,1; 0,2; and 0,4 mg/mL. The result of this research proves that PEPCK increases in the group which was given glucose 3%. *Salacca zalacca* peel extract significantly decreases the expression of IL-1β and Bax in the concentration of 0,4 mg/mL, and also able to decrease Apaf-1 expression with the result that most closely resemble the negative control in the concentration of 0,4 mg/mL. In conclusion, *Salacca zalacca* peel extract protects from gestational diabetes condition through the decrease of IL-1β, Bax, and Apaf-1.

Keywords: Glucose; *Salacca zalacca*; PEPCK; IL1-β; BAX; Apaf-1

1. Introduction

The population of gestational diabetes mellitus patients within the few decades had shifted, in which the trend of the age onset occurred in female between the age of 18 – 29 years old [1]. Gestational diabetes is predicted to be occur in 14% of pregnancies, or in 1 in every 7 pregnancies globally [2].

Gestational diabetes mellitus is the level of glucose intolerance that initially started, or successfully identified, during pregnancy [3]. This condition is enabled because placenta produces hormones like Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) and Placental protein-14 (PP14) which could hinder insulin action, causing insulin resistance [4] and in consequence, causing hyperglycemia [5]. Hyperglycemic condition causes elevation of glucose level in the liver duet to gluconeogenesis process. Phosphoenolpyruvate carboxykinase (PEPCK) is a rate-controlling enzyme for gluconeogenesis in the liver and kidney, so, PEPCK can be used as a marker for hyperglycemic condition [6].

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The increase of oxygen component in the body will increase free-radical oxygen (O^-) production which will oxidize glucose [7]. This will activate pathways that can elevate the production of Reactive Oxygen Species (ROS). Excessive ROS will activate the production mechanism for advanced glycation end products (AGE) and its receptors, and the interaction in cell surface will trigger inflammatory response [9]. Interleukin-1β (IL-1β) cytokine will be produced through the mitochondrial pathway, and subsequently, IL-1β and transcription factor NF-ƙB will trigger and enhance the activities of pro-apoptosis {BAX (Bcl-2 Associated X) and Apaf-1 (Apoptotic protease activating factor 1)} [10]. Increased excessive apoptosis will disrupt fetal organogenesis process [11].

Zebrafish embryo (*Danio rerio*) aged 0 – 72 hours post fertilization (hpf) is equal to the fetal inside the womb [12]. Zebrafish has gene homologue with human for up to 70%, and early studies had proven that zebrafish can be used as a diabetes mellitus model animal [13]. Besides that, zebrafish could produce plentiful eggs, up to 200 eggs, that can survive without food for a couple of days. After hatching, drugs that are being tested could diffuse through the skin, mouth, and gills of the fish embryo, and that is an advantage that became a reason for the use of zebrafish for the study of embryotoxicity and the teratogenic effects of drugs or other toxic substances [14].

So far, the preventive mechanism for gestational diabetes is still limited. *Salacca zalacca* peel contains bioactive substances that has antidiabetic effect and antioxidants such as ferulic acid, proline, flavonoid, and tannin [15].

According to the potential, this research will study the effect of *Salacca zalacca* peel ethanolic extract on zebrafish (*Danio rerio*) embryo which will be induced with high glucose to the expression of IL-1β, BAX, and Apaf-1.

2. Methods

2.1. Zebrafish Husbandry

Adult wild-strain zebrafish with male to female ratio of 2:1 will be kept in a 60L aquarium in the Pharmacology Laboratory, Faculty of Medicine Universitas Brawijaya which is identified and certified by Hydrology Laboratory, Faculty of Marine and Fisheries, Universitas Brawijaya. The feeding cycle is every 8 hours. Dark cycle is every 10 hours, and bright cycle every 14 hours. Eggs aged 0 -2 hpf will be moved to a medium. Every well will be filled with 30 fertile eggs. Infertile eggs (eggs with white dot in the middle of the egg, murky, and or not round in morphology) will be separated from fertile eggs [16].

The composition of the embryonic medium used with 10 times concentration is CaCl 0,25 gr, KCl 0,15 gr, NaCl 5 gr, and $MgSO_4$ 0,815 gr, that are diluted into 500 mL of aquadest [16].

2.2. *Salacca zalacca* Peel Extraction

Salacca zalacca Gartner Voss ethanol peel extract is obtained from *Salacca zalacca* chips industry waste in Arjosari, Malang City, East Java, Indonesia. Extraction is made using 98% ethanol as a solvent using maceration method. This study used the concentration of *Salacca zalacca* of 0,1 mg/mL, 0,2 mg/mL, and 0,4 mg/mL.

2.3. High Glucose Exposure and *Salacca zalacca* Peel Ethanolic Extract

30 embryos are used for every treatment group as follows: 1) Negative control is given only 5 mL of embryo medium, 2) Positive control is given glucose 3% exposure and embryo medium, 3) Treatment group 1 is given glucose 3%, 0,1 mg/mL *Salacca zalacca* peel extract, and embryo medium. 4) Treatment group 2 is given glucose 3%, 0,2 mg/mL *Salacca zalacca* peel extract, and embryo medium, 5) Treatment group 3 is given glucose 3%, 0,1 mg/mL *Salacca zalacca* peel extract, and embryo medium. Exposure is given starting from age 2 hpf, 24 hpf, up to 72 hpf. *Salacca zalacca* peel extract and glucose 3% are given in 2–72 hpf in embryonic medium which will be replace in every 24 hours.

2.4. Expression Measurement

Zebrafish embryos were frozen and for the isolation of RNA and Reverse Transcription PCR, total RNAs were extracted using TRIzol reagent (Sigma; St. Louis, MO, USA). Isolated RNA was treated with DNase (DNA-free kit, Ambion, Austin, TX, USA). The integrity of RNA were checked using agarose gel electrophoresis. Extracted RNA was reverse-transcribed in a final volume of each polymerase chain reaction/PCR tube consisted of 200 ng template, 1 μL primer, 25 μL MyTaq HS Red Mix, and 50 μL water. We did 40 cycles in which each cycle of reverse transcriptase (RT-PCR) consisted of initial denaturation (95°C for 1 minute), denaturation (95°C for 15 seconds), annealing according to temperature of optimization for 15 seconds, and extension (72°C for 10 seconds). The PCR products were visualized by ethidium bromide staining under UV light followed by electrophoresis on a 2% agarose gel.

List of primers used are displayed on table 1.

Table 1 List of Primers Used

Forward Primer	
PEPCK	5'- GAGAATTCTCACACACACACACGTGAGCAGTA -3'
IL-1β	5'-ATGCTCATGGCGAACGTC-3'
BAX	5'- GAGCTGCACTTCTCAACAACT -3'
Apaf-1	5'-TTCTACAGTAAACGCCCACC-3'
B-Actin	5'-CGAGCAGGAGATGGGAACC-3'
Reverse Primer	
PEPCK	5'- GTAAAAGCTTTCCGCCATAACATCTCCAGCAGAA -3'
IL-1β	5'-TGGTTTTAGTGTAAGACGGCACT-3'
BAX	5'- CTGGTTGAAATAGCCTTGATGAC -3'
Apaf-1	5'-TATCTAGTATTTCCCCATATTCC-3'
B-Actin	5'-CAACGGAAACGCTCATTGC-3'

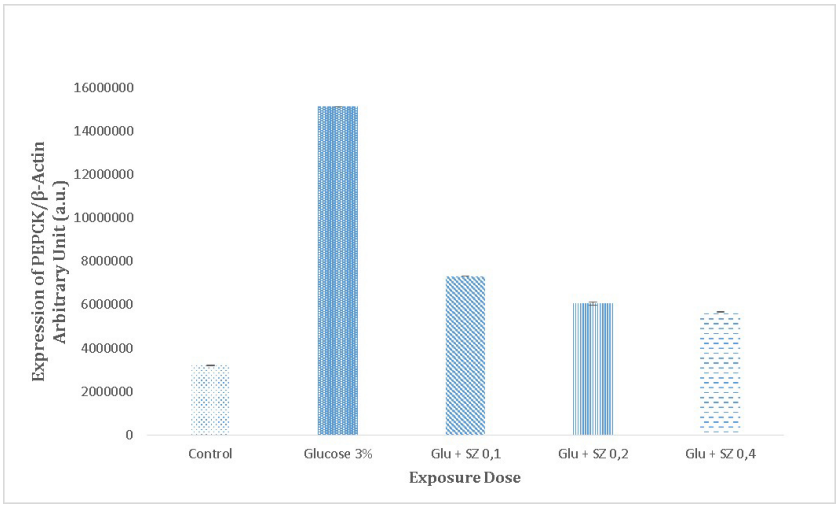
2.5. Statistical Analysis

Data are analyzed using IBM Statistical Package for the Social Sciences (SPSS) software ver. 22. Changes in the mRNA expression level of PEPCK, IL-1β, BAX, dan Apaf-1 are analyzed using one-way ANOVA. The level of statistical probability significancy is $P<0.05$.

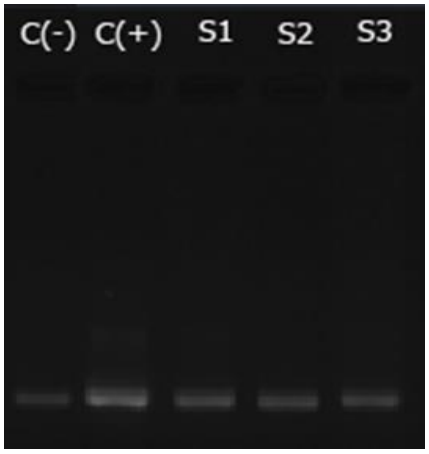
3. Results

3.1. PEPCK Expression

According to the result obtained, PEPCK expression is higher in groups that are exposed with glucose 3%, compared to control group. PEPCK expression data in each group is displayed on figure 1.



(a)

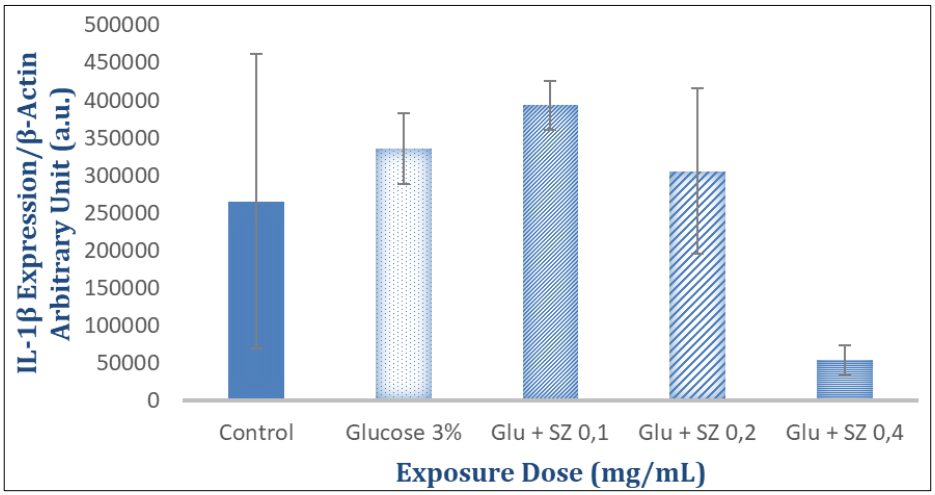


(b)

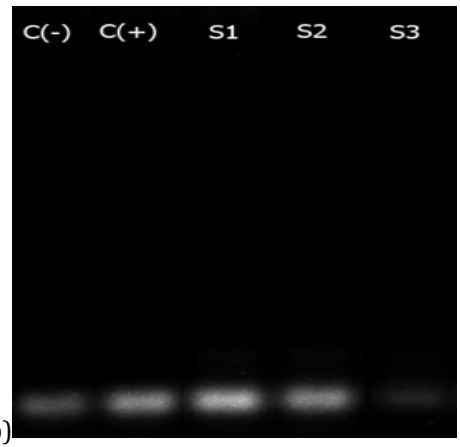
Figure 1 PEPCK expression graph (a) PEPCK expression on 3 dpf (n=90 embryos of each group) (b) PEPCK electrophoresis (n=90 embryos of each group)

3.2. IL-1β Expression

This study observed a change in the expression of IL-1β in zebrafish embryo (Figure 2). Significant change is observed on the dosage of 0.4 mg/mL.



(a)



(b)

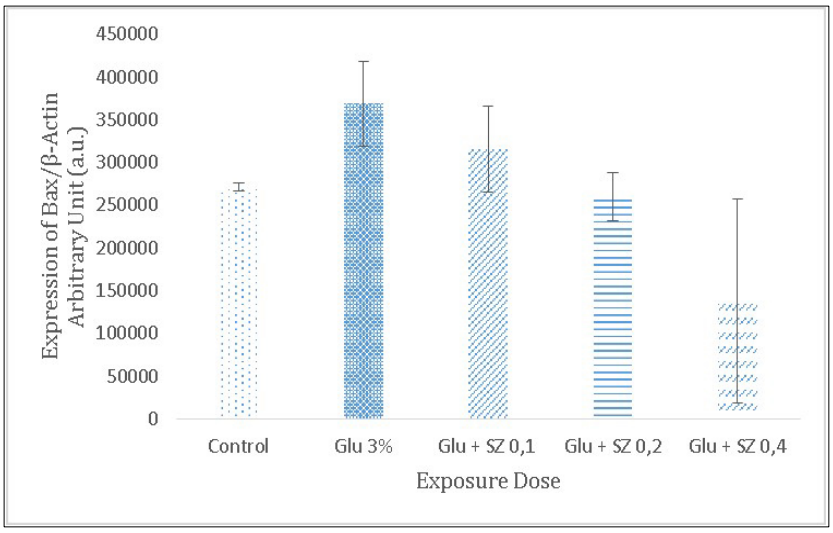
Figure 2 Il-1β expression graph (a) IL-1β expression graph on 3 dpf. IL-1β graph shows significant change in the dosage of 0,4 mg/mL ($p = 0.048$) compared to other groups, with exception of the control group; *$p<0,05$ compared to the

control group (b) IL-1β electrophoresis. (Glu: glucose; SZ: *Salacca zalacca* peel ethanolic extract; K(-): control; C(+): glucose 3%; S1: glu+SZ 0,1; S2: glu+SZ 0,2; S3: glu+SZ 0,4)

3.3. BAX Expression

This study observed a change in the expression of BAX in zebrafish embryo (Figure 3). Significant change is observed on the dosage of 0.4 mg/mL.

(a)



(b)

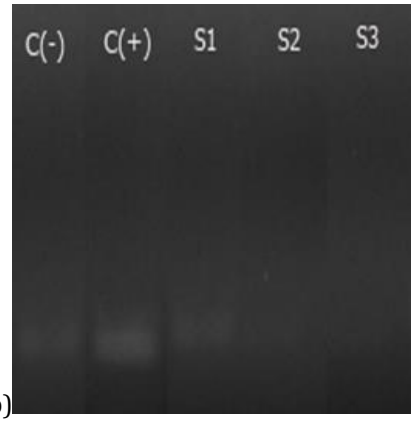
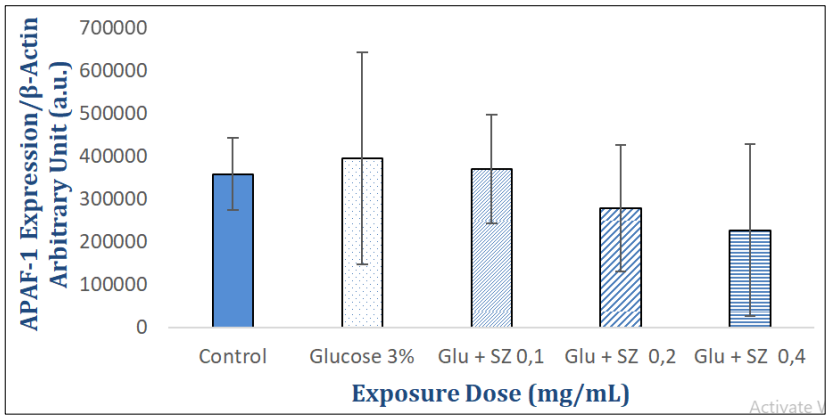


Figure 3 BAX expression graph (a) BAX expression graph on 3 dpf. BAX expression showed significant change in the dosage of 0,2 mg/mL ($p = 0.038$) and 0,4 mg/mL ($p = 0.001$) compared to other groups, with exception of the control group; *$p<0,05$ compared to the control group (b) BAX electrophoresis. (Glu: glucose; SZ: *Salacca zalacca* peel ethanolic extract; C(-): control; C(+): glucose 3%; S1: glu+SZ 0,1; S2: glu+SZ 0,2; S3: glu+SZ 0,4)

3.4. Apaf-1 Expression

This study observed a change in the expression of Apaf-1 in zebrafish embryo (Figure 4). There is no significant change in all dosages.

(a)



(b)

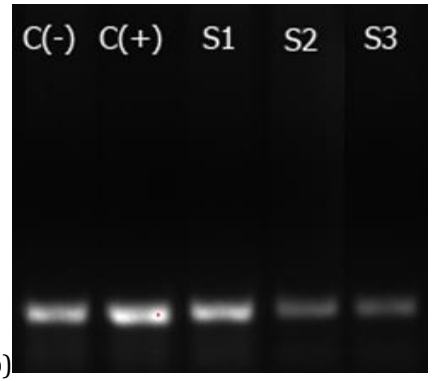


Figure 4 Apaf-1 expression graph (a) Apaf-1 expression graph on 3 dpf. Apaf-1 expression didn't show any significant change in all groups (b) Apaf-1 electrophoresis. (Glu: glucose; SZ: *Salacca zalacca* peel ethanolic extract; C(-): control; C(+): glucose 3%; S1: glu+SZ 0,1; S2: glu+SZ 0,2; S3: glu+SZ 0,4)

4. Discussion

4.1. Increased PEPCK Expression in Zebrafish Embryos Exposed to High Glucose

In groups exposed with glucose 3%, we observed higher PEPCK expression compared to control group. The result we obtained is the same with the research by Shao, *et al.,* 2005 and Inui, *et al.,* 2016 which stated that PEPCK expression will rise in hyperglycemic condition [17-18]. This is possible because in hyperglycemic condition there will be an increase of ROS production which will activate oxidative stress pathway, causing insulin resistance. Hepatic insulin impairment will cause an activation of p38 MAP kinase which in turn will cause a phosphorylation of ATP 2 which activates PEPCK promoter. Besides that, hyperglycemic condition will inhibit the PI3k/Akt/mTOR pathway which is the insulin transduction cascade pathway, causing an elevation of PEPCK and glucose 6-phosphatase (G6Pase). The elevation of these two enzymes will cause a rise of hepatic glucose through the gluconeogenesis proses [19-21].

In zebrafish, organ development including the liver and pancreas, are complete on the age of 3 days. Because zebrafish embryo doesn't have enough blood for blood glucose analysis, PEPCK expression which will be transcript by glucagon and insulin is analyzed instead. Hence, PEPCK becomes a sensitive marker on glucose level [6]. *Salacca zalacca* peel extract which contains polyphenol will affect the regulation of pivotal pathway for carbohydrate metabolism and hepatic glucose homeostasis, including activation of glycogenesis and glycolysis pathway. Polyphenol component can increase catalytic activities of glucose phosphorylation. Phenolic component can inhibit the expression of nitric oxide (NO) and the activities of transcription factor NF-kB, which can elevate insulin secretion through PI3k/Akt pathway, causing the suppression of PEPCK and G6Pase [21,22].

4.2. *Salacca zalacca* Peel Reduces IL-1β and Apoptosis Markers Expression Due to High Glucose

According to the result of this study, administration of *Salacca zalacca* peel extract with the dosage of 0,4 mg/mL can significantly decrease the expression of IL-1β and BAX on zebrafish embryo aged 3 dpf. Apaf-1 expression also decreased on the administration of 0,4 mg/mL *Salacca zalacca* peel extract, but with unsignificant statistical value.

Salacca zalacca is an endemic fruit from Indonesia. The use of *Salacca zalacca* peel is aimed to utilize unused food waste from *Salacca zalacca* fruit processing. *Salacca zalacca* peel contains bioactive substances with antioxidant effect. Antioxidants contained in *Salacca zalacca* peel are flavonoid, polyphenol, ferulic acid, and high tannin [15]. Flavonoid and polyphenol work through the suppression of ROS production by inhibiting enzyme or binding to free radical elements, scavenging ROS, and elevating antioxidant effects [23]. Ferulic acid works by inhibiting the production of ROS and scavenging ROS [24]. Tannin works as a primary and secondary antioxidant which will donate hydrogen atom for free radical [25, 26]. This indicates that antioxidant contained in *Salacca zalacca* peel will inhibit oxidative stress which in turn will decrease inflammation process and apoptosis [15].

In this research, high glucose condition will induce ROS accumulation through different metabolism pathways: polyol pathway, hexosamine pathway, and increase of AGEs production and activation of its receptors. The accumulation of ROS will induce mitochondrial dysfunction which will induce apoptosis and causing cell damage. Apoptosis that appeared due to the induction from oxidative stress mostly happened through MAPK pathway. Phosphorylation of JNK/p38-MAPK will activate BAX, induce initial hyperpolarization and oligomerization of BAX, which will trigger the appearance of Mitochondrial Outer Membrane Polarization (MOMP). MOMP allows BAX translocation and release of c cytochrome to cytosol, initiating the formation of apoptosome due to the binding of Apaf-1 to cytochrome that activates apoptosis cascade system. This can be observed on the increased expression of BAX and Apaf-1 in embryos with glucose 3% exposure. Earlier research studies showed that BAX has an important role in apoptosis process due to ROS, and increased BAX expression correlates with increased apoptosis rate [28-31]. The result of this study is linear to earlier studies which stated that increased Apaf-1 expression can increase apoptosis rate in zebrafish embryo through mitochondria-dependent pathway [32-34]. Besides that, in hyperglycemic condition, excessive ROS will activate the production mechanism of advanced glycation end products (AGE) and its receptors, and the interaction in cell surface can trigger inflammation response. Increased ROS can also become a factor that triggers inflammasome activation, and protein complex which regulates the maturation of proinflammatory cytokines such as IL-1β which will increase the level of IL-1β. This can be observed through the increased IL-1β expression in embryos with glucose 3% exposure compared to the control group [35,36]. This is linear to the result of the studies conducted by Iglesias in 2020, Bi in 2020, and Li in 2018, which stated that in hyperglycemic condition, there will be a rise in IL-1β proinflammatory cytokine due to increased oxidative stress condition. Hyperglycemia condition will elevate oxidative stress, and induce the increase of IL-1β proinflammatory cytokine through nucleotide-binding domain and leucine-rich repeat containing family pyrin (NLRP30 [37-39].

In our research, *Salacca zalacca* peel can decrease the expression of IL-1β due to high glucose exposure. *Salacca zalacca* peel can decrease IL-1β in embryo aged 3 dpf, with significant effect in the dosage of 0,4 μM/mL. Administration of *Salacca zalacca* peel as antioxidant is proven to inhibit the interaction of AGE-RAGE, thus preventing the excessive rise of proinflammatory cytokine in hyperglycemic condition. The excessive rise of IL-1β proinflammatory cytokine in maternal gestational diabetes can cause damage in the lungs, digestive system, and brain of the neonates [40].

We also observed that *Salacca zalacca* peel ethanolic extract can reduce apoptosis in zebrafish embryo. Figure 4 showed that the administration of *Salacca zalacca* peel extract in the dosage of 0,4 mg/mL can significantly reduce BAX expression in zebrafish embryo aged 3 dpf. This result is similar to the finding in another journal that observed anti-apoptosis effect of *Vitis vinifera* seed ethanolic extract on the liver of diabetic model mice induced with *Streptozotocin-Nicotinamide.* In that research, Giribabu, *et al.,* found that there is no further increase of mRNA BAX expression alongside the increased dose of *Vitis vinifera* seed ethanolic extract [41]. Antioxidant is known to work as anti-apoptosis by reducing ROS production and inhibiting MAPK pathways in order to prevent apoptosis mediated by BAX and Apaf-1 [42, 43]. The administration of *Centella asiatica* extract which contains antioxidant to stunting model zebrafish is also known to reduce BAX expression [44]. Preceding research showed that administration of mulberry fruit extracts which is known to have antioxidant activities can reduce Apaf-1 expression, hence slowing down the progression of diseases related to apoptosis [45]. This is linear to the result of our research. Figure 3 showed the reduced Apaf-1 expression on zebrafish embryos that were given *Salacca zalacca* peel extract [46,47]. Orzáez *et al*. showed that the administration of Apaf-1 inhibitor can reduce cytochrome-c release and apoptosome-mediated activation of procaspase 9 which in turn will prevent cell damage due to apoptosis [48]. Increased apoptosis in neonates born from mothers with gestational diabetes is known to be the cause for the malformation of brain, nervous system, heart, and eyes in neonates [47,49, 50].

5. Conclusion

Salacca zalacca peel ethanolic extract can protect gestational diabetes model zebrafish embryo through the reduction of IL1-β expression and the reduction of apoptosis markers (BAX and Apaf-1) expression.

Future Direction

The role of zebrafish as a nutrigenomic model was just being discovered. We hope that more studies will focus on the use of zebrafish as diabetic model in attempt to identify the effect of diabetes on developmental stage. We believe this information could be beneficial for human health.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

We declare that we have no conflict of interest.

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