Rosmarinus officinalis and Centella asiatica Affect Interleukin-6 Zebrafish Larvae Induced High Glucose

*Fajar Dwi Prastiwi¹, Sutini Lamadi¹, Husnul Khotimah², I Wayan Arsana Wiyasa³, Tri Yudani M. Raras²

¹Master Program of Midwifery, Faculty of Medicine, Brawijaya University, Indonesia. ²Department of Biomedical, Faculty of Medicine, University of Brawijaya, Indonesia. ³Department of Obstetrics and Gynecology, Faculty of Medicine, University of Brawijaya, Indonesia. *Email: Fajarprastiwi96@gmail.com

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Abstract: Gestational Diabetes Mellitus (GDM) is diabetes that occurs during pregnancy. GDM is characterized by hyperglycemia. Hyperglycemia causes an inflammatory response characterized by the excretion of pro-inflammatory cytokines, one of which is IL-6. Centella asiatica and Rosmarinus officinalis are known as anti-diabetic and anti-inflammatory. This study aims to study the effect of the combination of nanoemulsion Centella asiatica and Rosmarinus officinalis on the expression of Interleukin-6 in Zebrafish larvae induced by 3% glucose. Hyperglycemia is known from increased levels of Phosphoenolpyruvate carboxykinase in Zebrafish larvae. Phosphoenolpyruvate carboxykinase and Interleukin-6 expression were measured by conventional PCR method. The combination of Rosmarinus officinalis nanoemulsion and Centella asiatica was given with three different doses, namely at a concentration of 2.5, 5 and 10 µg/mL. The statistical analysis used was One Way ANOVA to measure Interleukin-6 expression levels. The results of this study prove that the combination of Rosmarinus officinalis and Centella asiatica nanoemulsions reduces the expression of Interleukin-6. The dose closest to the negative control was at a concentration of 5 µg/mL, although the final result was statistically significant (p<0.05). In conclusion, combining nanoemulsion Centella asiatica and Rosmarinus officinalis with the right dose reduced levels of Interleukin 6 in zebrafish larvae induced by high glucose.

Keywords: Centella; hyperglycemia; interleukin-6; Rosmarinus.

INTRODUCTION

Gestational Diabetes Mellitus (GDM) has experienced an increase in prevalence globally, especially in developing countries, reaching 3.8% to 21%. (McIntyre et al., 2019) obtained data from WHO that the Southeast Asian region has the second-largest prevalence of Gestational Diabetes Mellitus in the world. While data on the prevalence of GDM complications in Indonesia is 2 out of 5 diabetic women are in the reproductive age range. In 2015 there were 16.2% of births with signs of hyperglycemia during pregnancy; 85.1% were due to Diabetes Mellitus (Negara KS, 2015).

According to the American Diabetic Association (ADA), GDM is a condition of glucose intolerance due to insulin retention due to metabolic changes detected during pregnancy from the second to third trimester (without being detected before). Insulin resistance occurs because of the need for higher and more stable blood glucose levels for the growth and development of the baby (Negara KS, 2015). The predisposing factor for GDM is a combination of environmental, lifestyle, and genetic factors.

Corresponding Author: Fajar Dwi Prastiwi
Master Program of Midwifery, Faculty of Medicine, Universitas Brawijaya, Indonesia
Email: Fajarprastiwi96@gmail.com
Women with GDM have a risk of complications for both the mother and the fetus/child, both now and in the future (Kementerian Kesehatan Republik Indonesia, 2017). The incidence of pregnant women with GDM is also directly proportional to the incidence of DM in children and adolescents (Blotsky et al., 2019). Increased glucose and insulin levels in the mother's blood circulation will impact the fetus's metabolism, affecting its growth and development (Sweeting & Ross, 2020).

Hyperglycemic conditions trigger the process of gluconeogenesis, which will increase glucose levels in the liver. Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme catalyzing gluconeogenesis in the liver and kidneys, so that PEPCK can be a marker of hyperglycemic conditions (Khotimah, Alita, et al., 2021). The essence of the pathophysiology of GDM is the occurrence of insulin resistance (Plows et al., 2018). Insulin signalling is related to insulin receptor phosphorylation or insulin receptor substrate (IRS)-1, even though the number of cell surface receptors is stable. Pro-inflammatory cytokines excreted by adipose (fat) tissue are also present to play a role in the advancement of insulin resistance mechanisms. Pro-inflammatory cytokines, such as TNF-α, IL-1-β, and IL-6, play a role in inhibiting insulin signalling by inhibiting IRS-1 through serine phosphorylation (Filardi et al., 2020). High IL-6 levels during pregnancy, especially in the second trimester, cause insulin resistance by inhibiting insulin signalling in multiple pathways (Amirian et al., 2020). Inhibition of insulin signalling by inhibiting insulin receptor performance (IRS-1) due to the role of proinflammatory cytokines (IL-6) is the main factor in the emergence of GDM because it inhibits glucose uptake by cells by inhibiting the performance of glucose transporter proteins (GLUT4) (Sharma et al., 2022).

Zebrafish (Danio rerio) aged 0 – 72 hour post fertilization (hpf) is analogous to a fetus in the womb. Zebrafish have up to 70% homologous genes with vertebrates (humans). Research has proven that Zebrafish meet the requirements to be used as a model of diabetes mellitus. Another advantage is that Zebrafish are easy to breed to produce a large number of individuals in a short time (Khotimah, Alita et al., 2021).

Management of prevention and early management of diabetes is still limited. It is known that RO contains phenolic acids, and CA is known to contain pentacyclic triterpenoid saponins (asiaticoside, madecassoside, sapogenin) with strong therapeutic functions and is known as antihyperglycemic, antioxidant, and anti-inflammatory (Garcia et al., 2018) and (Teh et al., 2020).

Gonçalves et al. (2022) studied in vivo state that the Rosmarinus officinalis can be anti-inflammatory in mice and rats by suppressing IL-6 levels as a pro-inflammatory cytokine. Those studies used an experimental animal model of mice and rats as mammal vertebrates, while this study used an experimental animal model of zebrafish as Pisces vertebrates. Research by Kusumastuti et al. (2019) stated that Centella could increase glucose consumption in a coculture of 3T3-L1 adipocytes by inhibiting IL-6 excretion. In vitro, technique is used in those studies, while this study used in vivo technique. Finally, the novelty of this research is the application of 2 natural ingredients, which are combined and presented in the nanoemulsions. Based on its potential, this research will study the effect of the RO and CA nanoemulsion combination on zebrafish (Danio rerio) embryos with 3% high glucose induction on Interleukin-6 expression.

MATERIALS AND METHODS
Zebrafish Euthanasia
The approval for this study was obtained from the Ethics Commission of Medical Faculty, Brawijaya University No.50/EC/KEPK/03/2023. Zebrafish embryos
aged 0 dpf obtained from fertilization of adult wild-type Zebrafish that have been developed and maintained in an aquarium at the Reproductive Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Brawijaya. The research samples were Zebrafish embryos aged two hpf (hour post-fertilization) to 6 dpf (day post-fertilization) with a total sample of 30 samples per well which were repeated four times. Sampling was done by random allocation. This research group consisted of 5 groups, so the total number of samples was 600 Zebrafish embryos. The inclusion criteria for this study sample were Zebrafish embryos aged 0-2 hpf with a clear, transparent chorion, round shape, and no white fibres or fungus when viewed under an optimal microscope (Khotimah, Alita et al., 2021). In contrast, the exclusion criteria for this research sample were non-viable embryos characterized by bleaching (white chorion, not clear), embryos that were not fertilized, and fungus (white fibres). The composition for the embryonic medium is 0.25-gram CaCl powder, 0.15-gram KCl, 5 gram NaCl, 0.815 gram MgSO4, and 500 ml distilled water (Khotimah, Alita et al., 2021).

**Preparation of a Combination of *Rosmarinus officinalis* (RO) and *Centella asiatica* (CA) Nanoemulsions**

Preparation combination of nanoemulsion *Centella asiatica* and *Rosmarinus officinalis* uses 5 grams of *Centella asiatica* extract and 5 grams of *Rosmarinus officinalis* extract. Take 5 grams of *Centella asiatica* extract and 5 grams of *Rosmarinus officinalis* extract. Mix all the oil and surfactant ingredients (PEG 400 (40%), Span 80 (11.56%), PEG 40 (32.07%), soybean oil (16.37%)) as much as 100 mL using an overhead stirrer at 40 °C for 15 minutes, at 1000 rpm. Once homogeneous, add 5 grams of *Centella asiatica* and 5 grams of *Rosmarinus officinalis* extract, then stir using an overhead stirrer at 40°C for 15 minutes while always keeping the temperature not to exceed 60°C, with a speed of 1000 rpm until homogeneous. Put the *Centella asiatica* and *Rosmarinus officinalis* nanoemulsion combination into a closed bottle and store it in the refrigerator at 4°C.

**Administration of High Glucose Exposure and Combination of RO and CA Nanoemulsion**

Each well contains 30 Zebrafish embryos. The division of the treatment groups was as follows: 1) Negative controls were only given 5 mL of embryonic medium, 2) Positive controls were given 5 mL of embryonic media that had been exposed to 3% glucose, 3) Treatment group 1 (P1) was given 5 mL of liquid containing 3% glucose, a combination of 2.5 μg/mL RO and CA nanoemulsion and embryo media, 4) Treatment group 2 (P2) was given 5 mL of liquid containing 3% glucose, a combination of 5 μg/mL RO and CA nanoemulsion and embryo media, 5) Treatment group 3 (P3) was given 5 mL of liquid containing 3% glucose, combination of 10 μg/mL RO and CA nanoemulsion and embryo media. The RO and CA nanoemulsion combination treatment was given starting from 2 hpf, 24 hpf, up to 72 hpf in embryo media which will be replaced every 24 hours.

**Expression Measurement**

Expression of beta-actin, PEPCK and IL-6 will be measured in this study. Beta-actin as an internal control. PEPCK as an indicator of hyperglycemia. IL-6 as an indicator of inflammation. Expression results are expressed in numbers of density values. Observations were carried out using conventional PCR methods. After the zebrafish larvae are harvested at 3 dpf (day post-fertilization), they are frozen. Then Zebrafish larvae were extracted to extract pure RNA. Purified RNA was processed to obtain Zebrafish cDNA. This zebrafish 3 dpf cDNA will be sampled. After obtaining ready-to-use samples, proceed with the conventional PCR method with the
stages of pre-denaturation 95 0 C (3 minutes), denaturation 95 0 C (30 seconds), annealing 62.3 0 C (30 seconds), extension 72 0 C (1 minute) and 72 0 C (5 minutes) for 40 cycles. After conventional PCR for approximately 3-3.5 hours, the result was observed using the 5% agarose gel electrophoresis method. This PCR used the primer on Interleukin-6 (forward 5'-ATGCCATCCGCTCAGAAACAG-3'; reverse 5'-CCAAGGAGACTCTTTACGTCCA-3')

**Statistical Analysis**

Statistical analysis using IBM Statistical Package for the Social Sciences (SPSS) ver. 26. Changes in the expression levels of PEPCK and Interleukin-6 mRNA were analyzed using one-way ANOVA. The statistical probability significance level is P<0.05.

**RESULTS AND DISCUSSION**

**PEPCK expression**

Based on the research results, it appears that the expression of PEPCK in the positive control group (glucose-induced 3%) increased compared to the negative control. PEPCK expression will decrease by combining RO and CA nanoemulsions at P1, P2, and P3. The results of the observations will be presented in Figure 1.

![PEPCK expression](image1)

Figure 1. Results Impact of Nanoemulsion RO and CA on PEPCK Expression In Zebrafish Larvae Exposed to 3% Glucose

**Expression of Interleukin-6**

Based on the research results, it appears that the expression of Interleukin-6 in the positive control group (glucose-induced 3%) increased compared to the negative control. Furthermore, the expression of Interleukin-6 in various ways by administering a combination of RO and CA nanoemulsion at P1 (2.5 μg/mL), P2 (5 μg/mL), and P3 (10 μg/mL). From negative control (K-) to positive control (K+) indicates an increase in IL-6. IL-6 increased from k positive to P1 and P3. The analysis test gives a significant
statement of p=0.005 (Table 1). The results of the observations will be presented in the following figure 3.

![IL-6 and β-actin](image)

Figure 3. Results of the Effect of Giving a Combination of RO and CA Nanoemulsion on IL-6 Expression In Zebrafish Embryos Larvae to 3% Glucose

![Graph of Interleukin-6 Expression in Zebrafish Larvae Exposed to 3% Glucose](image)

Figure 4. Graph of Interleukin-6 Expression in Zebrafish Larvae Exposed to 3% Glucose

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-: (Embryonic medium)</td>
<td>0.75±0.65</td>
<td></td>
</tr>
<tr>
<td>K+: (Embryonic medium + Glucose 3%)</td>
<td>0.13±0.23</td>
<td></td>
</tr>
<tr>
<td>P1: (Embryonic medium + Glucose3% +Nanoemulsion RO CA 2.5μg/mL)</td>
<td>1.06±0.03</td>
<td>0.005*</td>
</tr>
<tr>
<td>P2: (Embryonic medium + Glucose 3% + Nanoemulsion RO CA 5 μg/mL)</td>
<td>1.14±1.12</td>
<td></td>
</tr>
<tr>
<td>P3: (Embryonic medium + Glucose 3% + Nanoemulsion RO CA 10 μg/mL)</td>
<td>0.70±0.24</td>
<td></td>
</tr>
</tbody>
</table>

*Significance (p< 0.05) with One Way ANOVA test.

Decreased Expression of PEPCK Due to High Glucose-Induced (3%)

PEPCK expression in the negative control group of Zebrafish larvae models (without 3% glucose exposure and RO CA nanoemulsion combination) showed an average value of 206700 ± 45000. It increased in the positive control group (with 3% glucose exposure) with an average value of 803190 ± 574440. The results of this study
are the same as those of a study by (Khotimah, Prima, et al., 2021), which showed an increase in PEPCK expression in hyperglycemic conditions. The use of PEPCK for analysis of hyperglycemia conditions was because Zebrafish larvae aged 3 dpf did not have enough blood for analysis, so they used enzyme levels related to glucose activity. PEPCK can increase in hyperglycemic conditions because more ROS will be produced to increase the activation of oxidative stress pathways, which results in insulin resistance. Hepatic insulin resistance activates p38 MAP kinase, which causes phosphorylation of ATP 2. This phosphorylation activates the PEPCK promoter. Through another mechanism, with hyperglycemic conditions, the PI3k/Akt/mTOR insulin transduction cascade pathway will be inhibited so that PEPCK and glucose 6-phosphatase (G6Pase) will increase (Khotimah, Alita, et al., 2021).

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme catalyzer for gluconeogenesis in the liver. It is known that the liver is the organ responsible for maintaining homeostasis of blood glucose levels through the mechanism of gluconeogenesis. Increased endogenous glucose production, especially in the liver, is the main indicator of fasting hyperglycemia in type 2 and gestational diabetes mellitus. Therefore, reducing liver glucose levels is an indicator in treating diabetes mellitus and gestational diabetes. Hyperglycemia conditions increase hepatic gluconeogenesis, whereas, in normoglycemic conditions, insulin suppresses the work of PEPCK expression and hyperglycemia, which triggers insulin resistance (Khotimah, Alita et al., 2021). To compensate for blood glucose, the PEPCK enzyme is a catalyst for converting oxaloacetate to phosphorus-pyruvate at the start of gluconeogenesis (Althurwi et al., 2022). Increased PEPCK expression will induce gluconeogenesis and suppress insulin through the phosphoinositide 3-kinase (PI3K/Akt) mechanism (Liu et al., 2015).

PEPCK decreased in groups P1, P2, and P3. The group sequentially was the group with glucose treatment 3%+ 2.5 μg/mL RO CA nanoemulsion combination (P1), 3%+ 5 μg/mL RO CA nanoemulsion combination (P2), 3%+ 10 μg RO CA nanoemulsion combination/mL (P3). This means that PEPCK expression decreased in hyperglycemic conditions exposed to the RO CA nanoemulsion combination. The best decrease was in the P2 group because it was closest to the control group, namely 173985+/-36585 au, and the use of medium doses. This is in line with research by (Feng et al., 2021) that saponins contained in CA at medium doses will optimally work to suppress PEPCK with a mechanism of increasing insulin signal transduction through the IRS-1/PI3K/AKT pathway. RO is known to contain phenolic acid compounds. Phenolics will inhibit nitric oxide (NO) expression and transcription factor activity by NF-kB, so insulin excretion increases in the PI3k/Akt pathway. Increased insulin excretion will suppress PEPCK (Khotimah, Alita, et al., 2021).

The Role of RO and CA Nanoemulsion Combination against Interleukin-6 in Zebrafish Induced High Glucose (3%)

This study also examined the effect of a combination of RO and CA nanoemulsion on IL-6 expression in Zebrafish larvae aged 2 hpf-3 dpf larvae with high glucose exposure. The dose of RO and CA nanoemulsion combination was at concentrations of 2.5 μg/mL, 5 μg/mL, and 10 μg/mL. The examination was carried out using conventional PCR on 3 dpf larvae. IL-6 measurement was also started with a negative control group. The results showed that IL-6 expression increased in the 3% glucose induction group (positive control) (figure 4). That is, IL-6 increases in hyperglycemia conditions.

This study aligns with research from (Siddiqui et al., 2019), which confirmed a significant increase in IL-6 in pregnant women with GDM. In hyperglycemia, this
condition occurs because IL-6 acts as an insulin antagonist, so its presence inhibits insulin action, which is known as the cause of insulin resistance. As an inflammatory mediator, IL-6 plays a role in the mechanism of glucose intolerance. IL-6 works by inhibiting glucose transport by GLUT 4. In a pregnant condition, at least two conditions cause IL-6 to increase, namely fat mass and placenta (Amirian et al., 2020).

The study results showed that of the three treatment doses given, only P2 (5 μg/mL) doses could reduce IL-6 levels in the model. This is by research from Gonçalves et al. (2022) for RO and (Lee et al., 2020), which stated that RO and CA could suppress IL-6 expression as a pro-inflammatory cytokine. It is known that RO, through 1,8-Cineole from the monoterpenes and camphor groups, can suppress IL-6 excretion by adipose tissue. Meanwhile, CA, through the asiatic side content from the triterpenoid saponin group, can suppress the formation of IL-6 by adipose tissue. It is known that the RO CA nanoemulsion combination consists of 2 different types of plants, with each having a similar function. RO contains several active compounds, including 1,8-Cineole monoterpenes (Borges, Keita, et al., 2018), camphor (Borges, Lima, et al., 2018), and phenolic (Rahbardar & Hosseinzadeh, 2020). With these active compounds, RO plays a role in the therapeutic sphere as an anti-inflammatory, antimicrobial, antitumorigenic, antioxidant (Diass et al., 2021) and neuroprotective (Rahbardar & Hosseinzadeh, 2020). Whereas CA contains various active compounds, including tannins, potassium, sodium, magnesium, calcium, and iron (Pusat Kajian Hortikultura Tropika (IPB), 2018) and according to (Teh et al., 2020), the most dominant and prominent are pentacyclic triterpenoids (asiaticoside, madecassoside, and sapogenin). With these various active compounds, CA has broad therapeutic functions. Apart from being an anti-inflammatory, it is also known as an antioxidant, antibacterial, and anti-ageing.

IL-6 is known to play a role in chronic inflammatory processes; therefore, giving a dose that is too small will be prioritized for acute inflammatory pathways (Amirian et al., 2020). The high expression of IL-6 by administering a low dose of RO CA nanoemulsion combination allows RO to work in a role other than suppressing IL-6 with related compounds (Benincà et al., 2011). Enhancement of the dose at P3 (combination of 10 μg/mL RO CA nanoemulsion) will increase IL-6. This might happen because, at high doses and concentrations, RO is known to have a toxic effect (Diass et al., 2021). Giving RO in a nanoemulsion preparation with too high a concentration can disrupt existing regulations (Chamoun et al., 2021).

The limitation of this study is that the number of samples is only enough for two repetitions (Diplo). It is known that the more research repetitions, the variation of the data will be better. Another limitation of this study is that it does not compare with standard drug administration in the management of diabetes. A suggestion for future researchers is to add a variety of treatments to compare herbal treatments with standard GDM therapy drugs so that the comparison of significance in suppressing IL-6 is clearer.

CONCLUSION

The results showed that the potential benefit of combining Rosmarinus officinalis and Centella asiatica as a treatment for hyperglycemic conditions (diabetes mellitus) with a mechanism of mediating inflammation prevention is relevant for therapeutic purposes. The mechanism of action of Rosmarinus officinalis and Centella asiatica is by suppressing Interleukin-6 as a pro-inflammatory cytokine in Zebrafish Larvae. Inhibition of inflammatory reactions in conditions of hyperglycemia (diabetes mellitus) will increase insulin sensitivity.
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CONFLICT OF INTEREST

There is no conflict of interest in this research.

REFERENCES


