



Effectiveness of *Rosmarinus officinalis* and *Centella asiatica* Nanoemulsions Against Caspase 3 Gestational Diabetes Mellitus Expression

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Abstract: Gestational diabetes mellitus (GDM) is closely related to oxidative stress conditions in insulin resistance conditions that increase Reactive Oxygen Species (ROS) against the body's defense antioxidant mechanism. Chronic complications due to hyperglycemia in patients with GDM increase BAX / BCL2 levels which then activates the change of procaspase 3 to caspase 3, an activator of apoptosis. This study aims to determine the effect of the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsions on caspase 3 expressions in zebrafish models of gestational diabetes mellitus, with a posttest-only controlled group design. The samples were divided into five groups, namely K- (EM), K+(EM+3% Glucose), P1, P2 and P3 (3% Glucose + Combination of *Rosmarinus officinalis* and *Centella asiatica* 2.5µg/ml, 5µg/ml and 10µg/ml), which will be tested for PEPCK and Caspase expression at the age of 3dpf using Real Time-PCR. The results of the correlation test of the group with caspase 3 obtained ($p = 0.045$) negative direction. These results show that the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion decreased Caspase 3 expression in zebrafish models of gestational diabetes mellitus. The combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion has the potential to reduce blood glucose levels and reduce the risk of apoptosis in gestational diabetes mellitus patients.

Keywords: Caspase 3; *Centella asiatica*; gestational diabetes mellitus; nanoemulsion; *Rosmarinus officinalis*.

INTRODUCTION

The compensatory mechanism against pregnancy causes pancreatic β cells to secrete more insulin to maintain the euglycemic condition. The occurrence of insulin secretion insufficiency from pancreatic β cells that cannot adapt to maintain maternal blood glucose homeostasis during pregnancy causes gestational diabetes mellitus (Choudhury & Devi Rajeswari, 2021). The prevalence of diabetics in Indonesia ranks sixth globally, with 10.3 million patients (Perkeni, 2021). Gestational diabetes mellitus (GDM) is closely related to oxidative stress conditions that occur in insulin resistance conditions that induce an increase in Reactive Oxygen Species (ROS) against the body's defense antioxidant mechanism (Rueangdetnarong et al., 2018). Increased ROS can directly impair cellular antioxidant protection (Newsholme et al., 2016).

Chronic complications due to hyperglycemia in patients with GDM produce oxidative stress that can induce cell damage by various pathways, namely by

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increasing BAX/BCL2 levels which then activates the change of procaspase 3 to caspase 3, which is an activator of apoptosis (Rasouljan et al., 2019). Signal apoptosis in GDM occurs intracellularly, disrupting mitochondria, thus releasing cytochrome C as an electron carrier in mitochondrial oxidation phosphorylation from the intermembrane. Cytochrome C binds to a cytoplasmic protein called Apaf-1, activating an initiator of caspase-9 in the cytoplasm, thus activating downstream procaspase 3 to caspase 3 (Sari, 2018).

Hyperglycemia causes gluconeogenesis which stimulates the liver and kidneys, increasing the secretion of the PEPCK enzyme to compensate for homeostatic mechanisms. In very small organisms, such as zebrafish larvae that do not yet have enough blood to analyze, it can be assessed by elevated levels of PEPCK. Zebrafish (*Danio rerio*) is suitable as a model for diabetes research because langerhans pancreas zebrafish contain β cells to produce insulin which are surrounded by α cells to produce glucagon, just like humans (Seth et al., 2013). The process of fertilization of zebrafish occurs extrauterine. Age 2 hpf (hours post fertilization) in zebrafish is the stage of embryogenesis. Making an environment with high glucose conditions in zebrafish with exposure to glucose at the embryogenesis stage causes hyperglycemia and represents gestational diabetes mellitus conditions (Singh et al., 2019).

The drugs available today for diabetes therapy have various side effects if used over a long period. In addition, treatment using insulin and chemical antidiabetic drugs also requires relatively expensive costs (Rosmiati & Fernando, 2018). Improper handling of hyperglycemia during pregnancy can have an impact on the condition of the mother and fetal development, including a high risk of type 2 diabetes.

Rosmarinus officinalis contains carnosic acid polyphenols and rosmarinic acid, which have been shown to have antidiabetic effects that can inhibit lipid peroxidation, otherwise improving insulin sensitivity (Nieto et al., 2018). The content of triterpenoids in *Centella asiatica* plants is known to reduce oxidative stress by improving the enzymatic endogenous antioxidant system (Sun et al., 2021). The combination of *Rosmarinus officinalis* and *Centella asiatica* extracts is expected to increase the desired therapeutic effect through a synergistic mechanism (Rita, 2020). To increase the efficacy of plant extract-based antidiabetic drugs, a nanoemulsion method is used that may improve the effectiveness of plant extract products and reduce the number of needs and side effects (Manocha et al., 2022). Nanoemulsions are proven to be a good alternative option for improving drug efficacy and absorption, including increased antidiabetic effects (Naseema et al., 2021). Previous studies have revealed the benefits of herbal therapy for treating type 2 diabetes, but research on its use in GDM is still limited (Keshavarzi & Golsheh, 2019).

Research is needed to study the synergism of active compounds by administering a combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsions in reducing caspase 3 expression as an activator of apoptosis triggered by gestational diabetes mellitus. This study aims to analyze the effect of the combination of *Rosmarinus officinalis* and *Centella Asiatica* on caspase 3 expression in a zebrafish (*Danio rerio*) model of gestational diabetes mellitus.

MATERIALS AND METHODS

Research Protocol

This type of research is a true experimental laboratory with a posttest-only controlled group design research design. The procedure in this study has received ethical approval from the Faculty of Medicine, Universitas Brawijaya, with Ethical

Approval Letter No.50/EC/KEPK/03/2023. This research was conducted at the Pharmacology Laboratory, Biomedical Central Laboratory, and Bioscience Laboratory of Universitas Brawijaya.

The preparation of extracts of *R. officinalis* and *C. asiatica* starts with simplicial *R. officinalis* and *C. asiatica* are crushed using a blender. Simplicia *R. officinalis* and *C. asiatica* were obtained from the Herbal Materia Medica Batu laboratory, Indonesia. The refined Simplicia results were then taken as much as 300 grams each, which were placed in a separate container and mixed with 3 liters of 96% ethanol, stirred for ± 60 minutes with an overhead stirrer at 500 rpm, and left to settle for 24 hours. The top layer of the *R. officinalis* and *C. asiatica* powder bath is removed and filtered using a Buncher funnel, then evaporated using a rotary evaporator and heated at 80°C temperature. The ethanol is allowed to separate by stirring. The ethanol is allowed to separate with the active substance already present in the flask tube and waited until the ethanol flow stops dripping on the holding flask, which takes $\pm 1.5-2$ hours. The extraction results are then placed in a closed container and stored in a refrigerator at 4°C. The next step is to prepare the nanoemulsion combination, starting with taking 5 grams of *Rosmarinus officinalis* extract and 5 grams of *Centella asiatica* extract in separate containers, mixed with surfactants PEG 400 (40%), Span 80 (11.56%), PEG 40 (32.07%) with 100 ml of soybean oil (16.37%) using an overhead stirrer speed of 1000 rpm at 40°C for 15 minutes. Once homogenized, 5 grams of *R. officinalis* and 5 grams of *C. asiatica* are added to the emulsion, stirred using an overhead stirrer at a speed of 1000 rpm for 15 minutes at 40°C until homogenized, transferred to a sealed bottle and stored in a refrigerator at a temperature of 4°C.

The study sample was zebrafish embryos aged 2 hpf (hour post-fertilization) to 3 dpf (day post-fertilization). The number of samples per well amounted to 30 embryos; 4 repetitions were taken using random sampling. The total sample was 600 zebrafish embryos that met the inclusion criteria; Transparent chorion without white fibers was ascertained under an optimal microscope. The sample is divided into five groups, consisting of; Negative control (K-) given embryonic medium (EM) 5 ml, Positive control (K+) given EM 5 ml and 3% Glucose, Treatment 1 (P1) given EM 5 ml and 3% Glucose and combination of *R. officinalis* and *C. asiatica* 2.5 µg/ml, Treatment 2 (P2) given EM 5 ml and 3% Glucose and combination of *R. officinalis* and *C. asiatica* 5 µg/ml and Treatment 3 (P3) given EM 5 ml and 3% Glucose and combination of *R. officinalis* and *C. asiatica* 10 µg/ml. Zebrafish embryos aged 2 hpf to 72 hpf were given medium embryonic and 3% glucose replaced every 24 hours.

RNA Extraction

Each treatment group was then taken as many as 40 zebrafish embryos at the age of 72 hpf. The sample is transferred to eppendorf, and centrifuged at a speed of 14000rpm, remove the liquid that is still present in the eppendorf was removed properly, add 400 µl wash buffer in each eppendorf, homogenized with micropipette, insert 4µl 2-mercaptoethanol in each eppendorf vortex sample and transfer the sample into the column filter to get pellets and continue centrifugation at 1000rpm for 30 seconds, transfer the supernatant in a new tube, then add 400 µl of 70% ethanol into each tube, and transfer in spin column, centrifuge at a speed of 14,000-16,000 rpm for 1 minute. Take the pellet formed, add W1 as much as 400µl, centrifuge at a speed of 14,000 rpm for 30 seconds, remove the remaining liquid before adding wash buffer that has been mixed with ethanol 600µl /tube, centrifuge again at a speed of 14,000 rpm for 30 seconds, remove the remaining liquid (this step is repeated two times). Dry the spin column by centrifuge for 3 minutes, transfer the spin column in eppendorf, add 50µl rinase free water, let stand for 2 minutes, and centrifuge again at 14.00 rpm

for 1 minute. Measure the purity level of isolation by entering 2µl of RNA isolation results in nanodrop spectrophotometry with purity levels used 1.8 to 2.0.

cDNA synthesis

Reacted gDNA by mixing 264 4x DNA master mix with 5.4 gDNA, adding diacetylated water with a total volume of 10µl. Denaturation of the sample at 65°C for 5 minutes, then place on ice. Add 20µl of gDNA reaction and 50µl of nuclease water free, then incubate at 98°C for 5 min. Add 20µl 5x RT Mix II, then centrifuge, incubate at 37°C for 15 minutes, at 50°C for 5 minutes, then at 98°C for 5 minutes, and store the sample at 4°C-20°C. The expression of PEPCK and caspase 3 was then examined using real-time PCR. PEPCK expression was measured to determine the homeostatic level that occurs as an effect of 3% glucose administration to create hyperglycemic conditions in zebrafish models of gestational diabetes mellitus. Caspase 3 will be investigated for potential apoptosis as a manifestation of hyperglycemia characterized by increased expression of caspase 3.

The real-time PCR method is based on the following: The sample was placed on ice, the primer was diluted to 100 pmol from a 100g preparation with nuclease-free water, and 0.4µl of reverse and forward primer stocks were taken into the PCR tube together. The sample was run together with the housekeeping gene, beta-actin, in the following sequence: forward: 5'-CGA GCT GTC TTC CCA TCC A-3', reverse: 5'-TCA CCA ACG TAG CGT CTT TCT G-3', PEPCK in the following order: Forward: 5'-GAG AAT TCT CAC ACA CGT GAG CAG T-3', reverse: 5'-GTA AAA GCT TTC CGC CAT AAC ATC TCC AGC AGA A-3', and caspase 3 primer with the sequence forward: 5'-TGC TCG AGG ATG CCA AGC CTC AAT CCC ATG CC-3', reverse: 5'-TTC TCG AGT TAA GGA GTG AAG TAC ATC TCT TTG GT-3'. Then add 2x sensitive cyber 5µl, samples 0.5µl and 3.7µl water nuclease-free to give a total of 10µl, then close the PCR tube, input into the Biorad CFX96 machine and run the PCR with the program: Initial denaturation: 95°C for 2 minutes, denaturation: 95°C for 5 seconds, annealing: 60°C for 30 seconds, extension: 72°C for 2 minutes, with cycle: 40.

Statistical Data Analysis

The results of PCR quantification were analyzed using SPSS 25.0 with a 95% confidence level, starting with the prerequisite tests, namely the Shapiro-Wilk normality test and Levene's homogeneity test. If the data are normal and homogeneous, the parametric one-way ANOVA test will be performed between each group (K-, K+, P1, P2 and P3) to determine the effect of the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsions on PEPCK expression and caspase 3 expressions in the zebrafish (*Danio rerio*) gestational diabetes mellitus model, followed by posthoc LSD (Least Significant Difference) analysis to find out which groups have meaningful differences and test correlations using Pearson.

RESULTS AND DISCUSSION

The results of PCR quantification are fold change values which are then carried out parametric prerequisite tests using Shapiro Wilk for data normality tests and Levens't test for homogeneity tests (Table 1), showing that the data obtained are normally distributed and homogeneous with p values > 0.05 so that they can be continued with One Way Anova parametric tests.

The highest PEPCK expression was in the positive control group, or those given only medium embryonic and 3% glucose exposure of 3.05 ± 0.27 , and the lowest PEPCK expression was in the P2 treatment group given medium embryonic, 3% glucose exposure and a combination of *R.officinalis* & *C.asiatica* 5µg/ml nanoemulsion of 0.54 ± 0.49 (Table 2). PEPCK expression of the P1 treatment group

given a combination of *R. officinalis* nanoemulsion and *C. asiatica* concentration of 2.5 µg/ml, an increase of 1.63 fold from the housekeeping gene with deviation standard 0.6. In the P2 treatment group with a combination of *R. officinalis* nanoemulsion and *C. asiatica* concentration of 5 µg/ml, PEPCK expression increased 0.54 fold compared to the housekeeping gene and deviation standard 0.49. In the P3 treatment group, given the combined effect of *R. officinalis* and *C. asiatica* nanoemulsions, a concentration of 10 µg/ml increased PEPCK expression 1.06 fold from the housekeeping gene and 0.33 deviation standard. PEPCK expression was significantly decreased by the combined administration of *R. officinalis* and *C. asiatica* nanoemulsions compared to positive controls (Figure 1).

Table 1. Data Normality and Homogeneity Test Results

No	Variable	P-Value	
		Shapiro Wilk	Levene's test
1.	PEPCK Expression	0,214	0,396
2.	Caspase 3 Expression	0,658	0,156
	Explanation	Normally	Homogeneous

The secretion of placental lactogen and prolactin hormones in pregnancy causes β pancreas cells to experience hyperplasia, consequently elevating the need for insulin. Gluconeogenesis in diabetic conditions triggers an increase in the enzyme PEPCK in the kidneys and liver to maintain homeostatic balance. Zebrafish as a model of gestational diabetes mellitus in this study, prepared using the 3% glucose immersion method from 2 hpf to 72 hpf, showed an elevate in PEPCK expression in the positive control group of 3.05 ± 0.27 of the housekeeping gene (beta-actin) and the positive control experienced an escalate in the multiple values of the negative control. This is in line with research conducted by Khotimah et al. (2021), which provided 3% glucose exposure in zebrafish embryos aged 2hpf to age 3dpf and obtained an increase in PEPCK expression. In addition, Singh et al. (2019) also create an environment with high glucose conditions through exposure to glucose at the embryogenesis stage, resulting in hyperglycemia while representing gestational diabetes mellitus conditions.

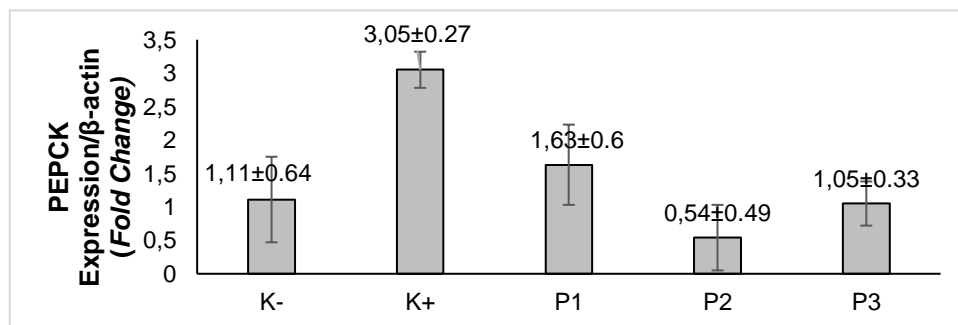


Figure 1. PEPCK Expression in Zebrafish Models of Gestational Diabetes Mellitus.

K-: negative control, K+: positive control, P1: treatment 1 with a combination of *R. officinalis* and *C. asiatica* 2.5µg/ml nanoemulsions, P2: treatment 2 with a combination of *R. officinalis* and *C. asiatica* 5µg/ml nanoemulsions, P3: treatment 3 with a combination of *R. officinalis* and *C. asiatica* 10µg/ml nanoemulsions.

One Way Anova parametric test of PEPCK expression (Table 1) showed that the combination of *R. officinalis* and *C. asiatica* nanoemulsions in the P1, P2, and P3 treatment groups could significantly reduce PEPCK expression with a p-value of 0.001 (<0.05), followed by a Post Hoc test, it can be seen that positive controls have significant differences with negative controls and treatment groups. Meanwhile, between treatment groups P1, P2 and P3 did not show significant differences.

Table 2. PEPCK Expression of Zebrafish Model of Gestational Diabetes Mellitus

Treatment	Mean±SD One Way Anova	p- value
K- : (Embryonic Medium)	1,11±0,64 ^a	
K+ : (Embryonic medium + Glucose 3%)	3,05±0.27 ^b	
P1 : (Embryonic medium + Glucose 3% + Combination nanoemulsion <i>R.officinalis</i> and <i>C.asiatica</i> 2.5µg/ml)	1,63±0.60 ^a	0,001
P2 : (Embryonic medium + Glucose 3% + Combination nanoemulsion <i>R.officinalis</i> and <i>C.asiatica</i> 5µg/ml)	0,54±0.49 ^{ac}	
P3 : (Embryonic medium + Glucose 3% + Combination of nanoemulsions <i>R.officinalis</i> and <i>C.asiatica</i> 10µg/ml)	1,05±0.33 ^a	

If the mean±SD contains letters that indicate a significant difference (p<0.05), and if there are the same letters, it means there is no significant difference (p>0.05)

The carnosol content of *R. officinalis* extracts downregulated gluconeogenesis through the cyclic response of Adenosine Monophosphate (cAMP) to the PEPCK promoter. In addition, carnosol compounds in *R. officinalis* can also suppress cAMP-mediated gene expression so that gluconeogenesis decreases (Bao et al., 2020) in line with research conducted by Tu et al. (2013), which showed that *R. officinalis* could have a significant effect with metformin therapy. *R. officinalis* affects glucose metabolism, namely gluconeogenesis and glycogen levels and induces glycolysis in cells. The content of rosmarinic acid, carnosol acid can inhibit pancreas lipase.

The antidiabetic effect resulting from *C. asiatica* plant extract is also known to be comparable to the use of metformin drugs. *Centella asiatica* provides a protective effect against oxidative stress through increased synthesis and antioxidant activity, so it significantly provides a better alternative therapy for people with diabetes mellitus (Masola et al., 2018). *Centella asiatica* has a high molecular weight of active triterpenoids, so the absorption is low and difficult to pass through cell membrane lipids. *C.asiatica* nanoemulsions can improve their effectiveness and absorption (Jusril et al., 2022).

The essential content of *R. officinalis* is also known to have sensitive properties to light, temperature, and oxidative reactions that can cause loss of quality and pharmacological properties so that *R. officinalis* nanoemulsions thermodynamically form small droplets, which can prevent the evaporation of components and increase the solubility, absorption, and bioavailability of herbal compounds used (Martin-Piñero et al., 2019). The combination of *R. officinalis* and *C. asiatica* nanoemulsions can trigger an increase in the encapsulation capacity of hydrophilic and lipophilic molecules in one particle so that it has the potential to protect molecular compounds expected from herbal extracts on an ongoing basis (Ding et al., 2017). This is also like research conducted by Rita (2020), which states that combining *C. asiatica* and *R. officinalis* can synergistically increase cell proliferation and migration in herbal therapy.

The highest mean expression of caspase 3 (1.14 ± 0.07) was obtained in the positive control group or only embryonic medium and 3% glucose, and the lowest in the P3 treatment group (0.70 ± 0.18). However, there was no significant difference in the average expression of caspase 3 from each treatment group, as indicated by a significance value of 0.281 (Table 5.4). The multiples of caspase 3 expression in the positive control group raised 1.14 fold from the housekeeping gene (beta-actin), and positive control is higher than negative control. In the P1 treatment group, which received a combination of *R. officinalis* and *C. asiatica* nanoemulsion at a concentration of 2.5 µg/ml, caspase 3 expression promoted 0.95 fold from the housekeeping gene with 0.22 deviation standard and was lower than the positive control. In the P2 treatment group, with a combination of *R. officinalis* and *C. asiatica* nanoemulsion at a concentration of 5µg/ml, caspase 3 expression from the housekeeping gene marked up 0.85 fold and 0.41 deviation standard. In contrast, caspase 3 expression from the positive control decreased. In the P3 treatment group, which received a combination of *R. officinalis* and *C. asiatica* nanoemulsion at a concentration of 10µg/ml, caspase-3 expression elevated by 0.71 fold from the housekeeping gene with 0.18 deviation standard, conversely, the caspase-3 expression descend from the positive control (Figure 2).

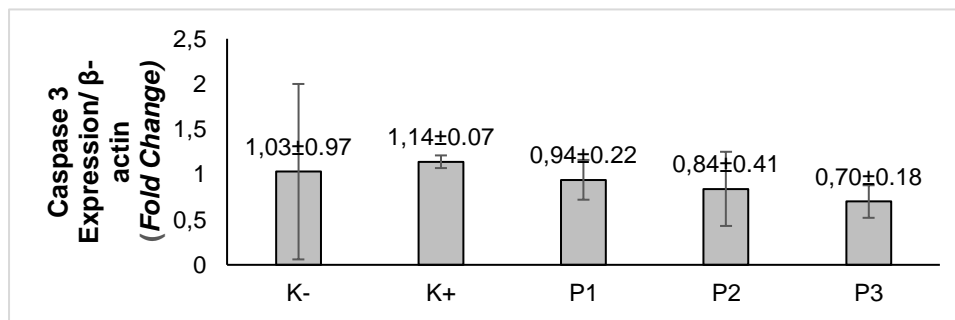


Figure 2. Caspase 3 Expression in Zebrafish Model of Gestational Diabetes Mellitus
 K-: negative control, K+: positive control, P1: treatment 1 with a combination of *R. officinalis* and *C. asiatica* 2.5µg/ml nanoemulsions, P2: treatment 2 with a combination of *R. officinalis* and *C. asiatica* 5µg/ml nanoemulsions, P3: treatment 3 with a combination of *R. officinalis* and *C. asiatica* 10µg/ml nanoemulsions.

Table 3. Caspase Expression 3 Zebrafish Model of Gestational Diabetes Mellitus

Treatment	Mean ± SD	p-value
K- : (Embryonic Medium)	1.00±0.97 ^a	0,281
K+ : (Embryonic medium + Glucose 3%)	1,14±0.07 ^{ab}	
P1 : (Embryonic medium + Glucose 3% + Combination nanoemulsion <i>R.officinalis</i> and <i>C.asiatica</i> 2.5µg/ml)	0,94±0.22 ^a	
P2 : (Embryonic medium + Glucose 3% + Combination nanoemulsion <i>R.officinalis</i> and <i>C.asiatica</i> 5µg/ml)	0,84±0.41 ^a	
P3 : (Embryonic medium + Glucose 3% + Combination of nanoemulsions <i>R.officinalis</i> and <i>C.asiatica</i> 10µg/ml)	0,70±0.18 ^{ab}	

If the mean±SD contains letters that indicate a significant difference ($p < 0.05$), and if there are the same letters, it means there is no significant difference ($p > 0.05$).

The results of the Anova parametric test (table 3) showed that combining *R. officinalis* and *C. asiatica* nanoemulsions in the P1, P2 and P3 groups could decrease caspase 3 expression with increasing nanoemulsions doses. However, there was no statistically significant difference, with a p-value of 0.281 (>0.05).

Based on the Pearson correlation test between groups (Table 4) on PEPCK expression, p-value = 0.149 (>0.05), with a negative direction, it can be concluded that there is no significant correlation between the combination of *R. officinalis* and *C. asiatica* nanoemulsions 2.5µg / ml, 5µg / ml and 10µg / ml on the decrease in PEPCK expression in zebrafish models of gestational diabetes mellitus. In the correlation test between groups on caspase 3 expression, p-value = 0.045 (<0.05), with a negative direction, so it can be concluded that there is a significant correlation between the combination of *R.offiicinalis* nanoemulsion and *C.asiatica* 2.5µg/ml, 5µg/ml and 10µg/ml against the decline in caspase 3 expression in zebrafish gestational diabetes mellitus model.

Table 4. Correlation Test of PEPCK and Caspase 3 Expression of Zebrafish Model of Gestational Diabetes Mellitus

Correlation		Pearson Correlation Test	P-Value	Information
Group	PEPCK	-0,391	0,149	No correlation, negative direction
	Caspase 3	-0,523	0,045	The correlation is quite meaningful, negative direction

Hyperglycemia in patients with GDM causes chronic complications caused by oxidative stress, thus triggering cell damage by increasing BAX/BCI2 levels which can activate procaspase 3 to caspase 3 as an activator of programmed cell death (Rasoulia et al., 2019). Caspase is a proteolytic enzyme that has a role in the mechanism of apoptosis. The pathogenesis of diabetes indicates the involvement of caspase. Glucotoxins that occur due to hyperglycemia in diabetic conditions cause apoptosis and necrosis in β pancreas cells, inhibiting insulin secretion. Manifestations of cell dysfunction due to insulin resistance during pregnancy trigger a progressive risk of type 2 diabetes mellitus after pregnancy ends (Plows et al., 2018).

Caspase 3 is responsible for chromatin condensation and DNA fragmentation. The increase in caspase 3 fragment levels is a marker of stem cell differentiation that causes intrinsic and extrinsic apoptosis. Sun et al. (2021) stated that a correlation exists between increased caspase expression due to reduced cell mass β pancreas and mitochondrial dysregulation due to caspase 3 activation mediated by an increase in Reactive Oxygen Species.

Based on the results of the analysis of the multiple expression of caspase 3 as an activator of apoptosis in the treatment group (P1, P2, and P3), a combination of *R. officinalis* and *C. asiatica* nanoemulsions, a concentration of 2.5µg / ml is an effective dose, because it can down-regulated the expression of caspase 3 from positive controls, close to negative controls. Although in the P2 treatment group with a dose of 5µg/ml and the P3 treatment group with a dose of 10µg/ml, it was found that the expression of caspase 3 also decreased from the positive control, the impact was also seen in a significant decrease in PEPCK expression in the P2 and P3 treatment groups compared to the negative control group. Thus it can be assumed that the decrease in caspase 3 with concentrations of 5µg/ml and 10 µg/ml also decreased the expression

of PEPCK 2 times smaller than the negative control. This has an impact on increasing the production of pancreas glucagon, which is hypoglycemia, which is also an imbalance of mechanisms in the body. The body needs glucose as an energy source for the formation of genetic components such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), the main source of protein formation (Chen & Lu, 2011). In addition, erythrocytes also need glucose to produce bisphosphoglycerate to release oxygen to body tissues and ward off free radicals (Puji, 2022).

In the P1 treatment group, a combination of *R. officinalis* and *C. asiatica* nanoemulsions with a concentration of 2.5µg/ml was a lower dose preparation in the research group. The low dose in this study significantly reduced the expression of PEPCK, an enzyme that regulates gluconeogenesis. In addition, the expression of caspase 3 can also be suppressed by administering a combination of *R. officinalis* and *C. asiatica* nanoemulsions with a preparation of 2.5µg/ml. The advantage of herbal therapeutic nanoemulsion preparations is that they can increase bioavailability so that the therapeutic dose becomes smaller, as Baloch et al. (2019) showed in their research that low concentrations of nanoemulsion preparations could increase intracellular permeability by increasing lymphatic transport, reduce metabolism in the gut because it is easily absorbed, and thus achieve the expected therapeutic target.

Secondary metabolic compounds contained in *R. officinalis* extract, especially those containing flavonoids, alkaloids, tannins, and triterpenoids, are proven to have the ability to transfer hydrogen ions to eliminate reactive free radicals to be stable (Nabila et al., 2020). The flavonoid content in *C. asiatica* plants is proven to suppress the development of diabetes by neutralizing free radicals due to unbalanced blood glucose. *Centella Asiatica* also contains alkaloids that can inhibit glucose transport in the blood, stimulate glycogen synthesis and inhibit glucose synthesis. Alkaloids can also stimulate sympathetic nerves to secrete insulin through the process of regeneration of damaged pancreas β cells (Maulida et al., 2019). Increased ROS in hyperglycemia in gestational diabetes mellitus patients can be suppressed by giving *R. officinalis* and *C. asiatica*, which contain phenolic and triterpenoid main compounds that can bind free electrons so that they become non-reactive and stable (Nieto et al., 2018).

This study was not preceded by a toxicity test on a combination preparation of *R. officinalis* and *C. asiatica* nanoemulsions, so it needs to be developed in further research to reduce bias due to exposure to treatment under hyperglycemic conditions and the level of apoptosis of the measured expression.

CONCLUSION

The combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsions significantly decreased caspase 3 expression in the zebrafish (*Danio rerio*) gestational diabetes mellitus model. Future studies need to examine the toxicity effect of the dosage of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsions used to reduce the bias that occurs to increase caspase 3 expression due to oxidative stress triggered by hyperglycemia.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Baloch, J., Sohail, M. F., Sarwar, H. S., Kiani, M. H., Khan, G. M., Jahan, S., Rafay, M., Chaudhry, M. T., Yasinzai, M., & Shahnaz, G. (2019). Self-nanoemulsifying drug delivery system (Snedds) for improved oral bioavailability of chlorpromazine: In vitro and in vivo evaluation. *Medicina (Lithuania)*, *55*(5), 1–13. <https://doi.org/10.3390/medicina55050210>
- Bao, T. Q., Li, Y., Qu, C., Zheng, Z. G., Yang, H., & Li, P. (2020). Antidiabetic effects and mechanisms of rosemary (*Rosmarinus officinalis* L.) and its phenolic components. *American Journal of Chinese Medicine*, *48*(6), 1353–1368. <https://doi.org/10.1142/S0192415X20500664>
- Chen, G. Q., & Lu, Y. (2011). Effector caspases and leukemia. *International Journal of Cell Biology*, *2011*. <https://doi.org/10.1155/2011/738301>
- Choudhury, A. A., & Devi Rajeswari, V. (2021). Gestational diabetes mellitus - A metabolic and reproductive disorder. *Biomedicine & Pharmacotherapy*, *143*, 112183. <https://doi.org/10.1016/J.BIOPHA.2021.112183>
- Ding, S., Anton, N., Akram, S., Er-Rafik, M., Anton, H., Klymchenko, A., Yu, W., Vandamme, T. F., & Serra, C. A. (2017). A new method for the formulation of double nanoemulsions. *Soft Matter*, *13*(8), 1660–1669. <https://doi.org/10.1039/c6sm02603f>
- Khotimah, H., Alita., Safira, N.A.P., Aninditha, D., Weningtyas, A., Wahyu P.E, Kalsum U., Rahayu, M., Handayani, D., & Nandar S.K. (2021). Ethanolic extract of *Salacca zalacca* peel reduce IL-1 β and apoptosis in high glucose induced zebrafish embryo. *GSC Biological and Pharmaceutical Sciences*, *16*(3), 024–033. <https://doi.org/10.30574/gscbps.2021.16.3.0213>
- Jusril, N. A., Abu Bakar, S. I., Khalil, K. A., Md Saad, W. M., Wen, N. K., & Adenan, M. I. (2022). Development and Optimization of Nanoemulsion from Ethanolic Extract of *Centella asiatica* (NanoSECA) Using D-Optimal Mixture Design to Improve Blood-Brain Barrier Permeability. *Evidence-based Complementary and Alternative Medicine*, *2022*. <https://doi.org/10.1155/2022/3483511>
- Keshavarzi, F., & Golsheh, S. (2019). IRS1- rs10498210 G/A and CCR5-59029 A/G polymorphisms in patients with type 2 diabetes in Kurdistan. *Molecular Genetics and Genomic Medicine*, *7*(5), 1–7. <https://doi.org/10.1002/mgg3.631>
- Manocha, S., Dhiman, S., Grewal, A. S., & Guarve, K. (2022). Nanotechnology: An approach to overcome bioavailability challenges of nutraceuticals. *Journal of Drug Delivery Science and Technology*, *72*, 103418. <https://doi.org/10.1016/J.JDDST.2022.103418>
- Martin-Piñero, M. J., Ramirez, P., Muñoz, J., & Alfaro, M. C. (2019). Development of rosemary essential oil nanoemulsions using a wheat biomass-derived surfactant. *Colloids and Surfaces B: Biointerfaces*, *173*, 486–492. <https://doi.org/10.1016/j.colsurfb.2018.10.024>
- Masola, B., Oguntibeju, O. O., & Oyenih, A. B. (2018). *Centella asiatica* ameliorates diabetes-induced stress in rat tissues via influences on antioxidants and

- inflammatory cytokines. *Biomedicine and Pharmacotherapy*, 101(October 2017), 447–457. <https://doi.org/10.1016/j.biopha.2018.02.115>
- Maulida, U., Jofrisha, & Mauliza. (2019). Uji Aktivitas Antidiabetes Ekstrak Etanol Pada Tanaman Pegegan (*Centella asiatica* (L) Urban. *KATALIS Jurnal Pendidikan Kimia dan Ilmu Kimia*, 2(2), 1–8.
- Nabila, A., Puspitasari, C. E., & Erwinayanti, G. A. . S. (2020). Uji Aktivitas Antioksidan Ekstrak Etanol Daun Rosemary (*Rosmarinus officinalis* L.) dengan Metode DPPH dan FRAP serta Pengaplikasiannya sebagai Zat Aktif dalam Losion. *Jurnal Sains dan Kesehatan*, 3(1), 242–247.
- Naseema, A., Kovooru, L., Behera, A. K., Kumar, K. P. P., & Srivastava, P. (2021). A critical review of synthesis procedures, applications and future potential of nanoemulsions. *Advances in Colloid and Interface Science*, 287, 102318. <https://doi.org/10.1016/J.CIS.2020.102318>
- Newsholme, P., Cruzat, V. F., Keane, K. N., Carlessi, R., & De Bittencourt, P. I. H. (2016). Molecular mechanisms of ROS production and oxidative stress in diabetes. *Biochemical Journal*, 473(24), 4527–4550. <https://doi.org/10.1042/BCJ20160503C>
- Nieto, G., Ros, G., & Castillo, J. (2018). Antioxidant and Antimicrobial Properties of Rosemary (*Rosmarinus officinalis*, L.): A Review. *Medicines*, 5(3), 98. <https://doi.org/10.3390/medicines5030098>
- Perkeni, (Persatuan Endokrinologi Indonesia) (2021). *Pedoman Diagnosis Dan Penatalaksanaan Hiperglikemia Dalam Kehamilan 2021*. PB. Perkeni.
- Plows, J. F., Stanley, J. L., Baker, P. N., Reynolds, C. M., & Vickers, M. H. (2018). The pathophysiology of gestational diabetes mellitus. *International Journal of Molecular Sciences*, 19(11), 1–21. <https://doi.org/10.3390/ijms19113342>
- Puji, A. (2022). *Mengenal Glukosa : Manfaat dan Cara Tubuh Mengolahnya*. Accessed 10-08-2023. <https://hellosehat.com/nutrisi/fakta-gizi/manfaat-glukosa/>.
- Rasoulia, B., Hajializadeh, Z., Esmaeili-Mahani, S., Rashidipour, M., Fatemi, I., & Kaeidi, A. (2019). Neuroprotective and antinociceptive effects of rosemary (*Rosmarinus officinalis* L.) extract in rats with painful diabetic neuropathy. *Journal of Physiological Sciences*, 69(1), 57–64. <https://doi.org/10.1007/s12576-018-0620-x>
- Rita, C. I. (2020). *Pengaruh Kombinasi Transfersom Pegagan dan Nanoemulsi Rosemary Terhadap Ekspresi Transforming Growth Factor Beta dan Matrix Metalloproteinase-9 Pada Proses Penyembuhan Luka Insisi Tikus (*Rattus norvegicus*)*. Universitas Brawijaya.
- Rosmiati, K., & Fernando, A. (2018). Uji efektivitas antidiabetes ekstrak etanol daun ungu (*Graptophyllum pictum*) terhadap mencit putih jantan (*Mus musculus*). *Jurnal Sains dan Teknologi Laboratorium Medik*, 2(1), 8–13. <https://doi.org/10.52071/jstlm.v2i1.14>
- Rueangdetnarong, H., Sekararithi, R., Jaiwongkam, T., Kumfu, S., Chattapakorn, N., Tongsong, T., & Jatavan, P. (2018). Comparisons of the oxidative stress biomarkers levels in gestational diabetes mellitus (GDM) and non-GDM among Thai population: cohort study. *Endocrine Connections*, 7(5), 681–687. <https://doi.org/10.1530/EC-18-0093>
- Sari, L. M. (2018). Apoptosis: Mekanisme Molekuler Kematian Sel. *Cakradonya Dental Journal*, 10(2), 65–70. <https://doi.org/10.24815/cdj.v10i2.11701>
- Seth, A., Stemple, D. L., & Barroso, I. (2013). The emerging use of zebrafish to model metabolic disease. *DMM Disease Models and Mechanisms*, 6(5), 1080–1088. <https://doi.org/10.1242/dmm.011346>

- Singh, A., Castillo, H. A., Brown, J., Kaslin, J., Dwyer, K. M., & Gibert, Y. (2019). High glucose levels affect retinal patterning during zebrafish embryogenesis. *Scientific Reports*, 9(1), 1–13. <https://doi.org/10.1038/s41598-019-41009-3>
- Sun, J., Singh, P., Österlund, J., Orho-Melander, M., Melander, O., Engström, G., & Edsfeldt, A. (2021). Hyperglycaemia-associated Caspase-3 predicts diabetes and coronary artery disease events. *Journal of Internal Medicine*, 290(4), 855–865. <https://doi.org/10.1111/joim.13327>
- Tu, Z., Moss-Pierce, T., Ford, P., & Jiang, T. A. (2013). Rosemary (*Rosmarinus officinalis* L.) extract regulates glucose and lipid metabolism by activating AMPK and PPAR pathways in HepG2 cells. *Journal of Agricultural and Food Chemistry*, 61(11), 2803–2810. <https://doi.org/10.1021/jf400298c>