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Antioxidant Activity and Cardioprotective Potential of a Nanoemulsion Mix of *Rosmarinus officinalis* and *Centella asiatica* in Gestational Diabetes Mellitus Zebrafish Larvae Model

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ABSTRACT

Hyperglycemia affects approximately 16.7% of pregnancies worldwide, with gestational diabetes mellitus (GDM) accounting for nearly 84% of these cases. GDM, characterized by glucose intolerance during pregnancy, presents significant health risks. *Rosmarinus officinalis* (RO) and *Centella asiatica* (CA) are known for their antidiabetic and antioxidant properties, including the ability to enhance insulin secretion and inhibit phosphoenolpyruvate carboxykinase (PEPCK) expression in the gluconeogenesis pathway. This study aims to assess the impact of a nanoemulsion of RO and CA combination (RO-CA) on heart rate, superoxide dismutase (SOD), nuclear factor erythroid 2-related factor 2a (Nrf2a), sirtuin 1 (SIRT-1), and tyrosine hydroxylase (TH) expression in zebrafish larvae model of GDM. GDM was induced by exposure of zebrafish embryo to a 3% glucose solution in an embryonic medium. The GDM phenotype was confirmed by elevated PEPCK expression as a hyperglycemia marker. GDM zebrafish was administered RO-CA nanoemulsion at concentrations of 2.5, 5, and 10 µg/mL.from 2 hpf to 72 hpf. Heart rate was monitored using stereoscopic imaging connected to a camera, while expression levels of PEPCK, Nrf2a, SOD, SIRT-1, and TH were quantified using reverse transcriptase polymerase chain reaction (RT-PCR). Results revealed a significant decrease in PEPCK expression in the treatment groups compared to the glucose untreated group. Notably, the nanoemulsion maintained heart rate frequency and upregulated Nrf2a, SOD, SIRT-1, and TH expression, particularly at a concentration of 2.5 µg/mL. Overall, these findings suggest that the RO-CA nanoemulsion exhibits enhanced antioxidant activity and holds promise as a potential cardioprotective agent in GDM.

Keywords: Gestational diabetes mellitus, Phosphoenolpyruvate carboxykinase, Nuclear factor erythroid 2-related factor 2a, Superoxide dismutase, Sirtuin 1, Tyrosine hydroxylase

Introduction

Gestational diabetes mellitus (GDM), a disorder characterized by glucose intolerance, is first detected during pregnancy and is typically diagnosed through routine screening conducted between the 24th and 28th weeks of gestation.[1,2] Hyperglycemia affects approximately 16.7% of pregnancies worldwide, with GDM accounting for nearly 84% of these cases.[3,4]

Physiologically, during normal pregnancy, alterations in glucose regulation occur to support fetal nutrient uptake, leading to a reduction in insulin sensitivity by approximately 56%.[5] Pancreatic cells respond to this change by augmenting insulin production.

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However, if the pancreatic cell response is inadequate in considering insulin resistance, hyperglycemia can induce an elevation in reactive oxygen species (ROS).[6,7]

Oxidative stress ensues from an imbalance between pro-oxidants and antioxidants, often initiated by increased ROS production.[8] This oxidative environment can have a deleterious effect on the developing heart, leading to defects in both structure and function. Hypertrophic cardiomyopathy emerges as the predominant cardiac complication in 40% of diabetic pregnancies, concurrently elevating the mean fetal heart rate.[9-11] When nuclear factor erythroid 2–related factor 2 (Nrf2) activation is impaired, hyperglycemia suppresses cellular antioxidants and diminishes superoxide dismutase (SOD) activity.[12,13] The resultant oxidative stress, characteristic of hyperglycemia in GDM, exerts significant effects on the expression of sirtuin-1 (SIRT-1) and tyrosine hydroxylase (TH).[14-16] Notably, TH and SIRT-1 play pivotal roles in maintaining the integrity and functionality of the cardiovascular system.[17,18] The current approach to preventing GDM primarily revolves around managing maternal lifestyle, including diet and physical activity.[19] However, this strategy has proven insufficient in effectively preventing GDM and mitigating fetal complications associated with the condition.[20] Consequently,

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there has been a growing interest in exploring the potential of plant-derived bioactive compounds for GDM prevention. Scientific evidence suggests that phytochemicals possess anti-diabetic properties, with compounds found in *Rosmarinus officinalis* (RO) and *Centella asiatica* (CA) showing promise in this regard.[21,22] These compounds have been shown to enhance insulin secretion, reduce expression of key enzymes involved in gluconeogenesis, and modulate glucose uptake pathways, including the AMP-activated protein kinase (AMPK) pathway.[23,24] Additionally, both plants exhibit antioxidant properties attributed to their rich content of polyphenols, flavonoids, and terpenes, which effectively counteract ROS production associated with diabetes.[25,26]

Nanoemulsions represent a promising drug delivery system for enhancing the efficacy and bioavailability of anti-diabetic herbal formulations.[27] This technique enables the production of nanoparticles with sizes smaller than one micron, thereby facilitating improved absorption of bioactive herbal compounds.[28] Moreover, the synergistic interactions observed in herbal combination formulations amplify their bioactivity, surpassing that of individual herbal constituents.[29,30] Such synergy operates through both pharmacokinetic and pharmacodynamic mechanisms, enhancing absorption, distribution, metabolism, and elimination of active compounds while simultaneously targeting multiple pathways with complementary therapeutic effects.[30] Mixtures of compounds, for example polyphenols and flavonoids, as sources of antioxidants, have better antioxidant activity than single compounds in suppressing insulin resistance and oxidative stress.[31,32]

The zebrafish (*Danio rerio*) emerges as a valuable model for investigating metabolic diseases like diabetes due to its genetic similarity to humans, sharing approximately 70% orthologous genes, as well as possessing analogous metabolic processes in major organs.[33,34] Additionally, zebrafish offer practical advantages such as high fecundity and rapid embryonic development, facilitating efficient experimental protocols.[35]

This study aims to assess the impact of RO-CA nanoemulsion combination on heart rate and the expression of key biomarkers associated with oxidative stress and metabolic regulation, including SOD, Nrf2a, SIRT-1, and TH, using zebrafish larvae model of GDM. Through elucidating the effects of this novel formulation on multiple targets of GDM pathophysiology, the present study seeks to contribute to the development of effective preventive and therapeutic strategies for this prevalent pregnancy-related disorder.

Materials and Methods

Experimental design

Zebrafish embryos aged 0 - 2 hours post-fertilization (hpf) were used for the study. One hundred and twenty (120) 120 embryos were divided into four groups of 30 embryos each, and were distributed into 6-well plates. The groups were delineated as follows:

Control group (C): Received 5 mL of embryonic medium per well.

Glucose group (G): Administered 5 mL of embryonic medium per well and exposed to a 3% glucose solution.

Treatment groups: Received 5 mL of embryonic medium per well, exposure to a 3% glucose solution, and a combination of RO-CA nanoemulsion. The concentrations of nanoemulsion used were 2.5 µg/mL (T1), 5 µg/mL (T2), and 10 µg/mL (T3).

Exposure to glucose and the RO-CA nanoemulsion commenced at 2 hpf and continued until 72 hpf, covering the zebrafish larval stage.[36]

Embryonic medium

A 10-fold stock solution of 0.25 g CaCl, 0.15 g KCl, 5 g NaCl, and 0.815 g $MgSO_4$ was made by dissolving in distilled water, and made up to 500 mL.[13] A 10-fold dilution of the stock solution was made by mixing one part of the stock solution with nine parts of distilled water.[37]

Zebrafish model of GDM

The zebrafish model of GDM was induced by exposure of zebrafish embryo (2 hpf to 72 hpf) to a 3% glucose solution in an embryonic medium.[36] Zebrafish embryos were incubated in a high-glucose environment, this offers a straightforward means of elevating both blood and tissue glucose levels effectively.[38] This gestational model aligns with the developmental equivalence of zebrafish embryos aged 0 - 72 hpf to the fetal stage *in utero*.[36] This temporal adaptation underscores the suitability of zebrafish as a model organism for studying gestational conditions.Validation of the GDM model was achieved through the observed upregulation of phosphoenolpyruvate carboxykinase (PEPCK) expression, serving as a reliable marker of hyperglycemia. PEPCK's sensitivity to glucose levels renders it a valuable indicator for assessing glucose dysregulation or homeostasis, particularly in microscopic species like zebrafish larvae where direct blood glucose analysis is impracticable. The observed increase in PEPCK expression in zebrafish larvae further corroborates the fidelity of this experimental model in mimicking hyperglycemic conditions.[36,39]

Preparation of RO-CA nanoemulsion

Rosmarinus officinalis and *Centella asiatica* were identified and authenticated based on the criteria of UPT Materia Medica Batu, Malang, East Java, Indonesia. The powdered plant leaves were extracted by maceration in ethanol (96%). The combined extracts of *Rosmarinus officinalis* and *Centella asiatica* (RO-CA) in a 1:1 ration was formulated into nanoemulsion according to the method described by Moukette *et al*. (2017).[32] The extract was converted into a nanoemulsion using the self nanoemulsifying drug delivery system (SNEDDS) technique, with PEG 400 (40%), Span 80 (11.56%), PEG 40 (32.07%), and soybean oil (16.37%) as the main ingredients. The size of the nanoparticles (< 100 nanometers) was confirmed by examining the zeta potential (Zeta Sizer Ver. 7.01) and particle size analysis using the particle size analyzer (Malvern v2.2).

Measurement of heart rate and marker expressions

The heart rate of zebrafish larvae (age 3-6 dpf) was measured on a stereotypical microscope (Olympus SZ61) with 40x magnification connected to a Panasonic Lumix GF8 camera, and a recording device. The heart rate was recorded for 15 seconds, using a tally counter, the reading was multiplied by 4 to measure the number of heartbeats per minute.[40] The PEPCK, Nrf2a, SOD, SIRT-1, TH, and Actb2 expressions were measured by reverse transcriptase polymerase chain reaction (RT-PCR). Zebrafish larvae (40 per group) aged 3 days post fertilization (dpf) were euthanized by rapid cooling, and then frozen in a -80^{o}C freezer. The total RNA of the zebrafish larvae was extracted according to the protocol of the Total RNA Mini Kit (Geneaid, catalog number RT100, New Taipei City, Taiwan). Isolated RNA was transcribed into a cDNA template following the protocol of the ReverTra Ace qPCR RT Mastermix with gDNA Remover kit (TOYOBO catalog number FSQ-301, Osaka, Japan).[41] RT-PCR was done using the protocol of GoTaq Green Master Mix (Promega, Madison, WI, USA) and run in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA).[42] The list of primers and RT-PCR programs used is shown in Table 1 and Table 2, respectively. PCR products were analyzed using 2% agarose gel electrophoresis and stained with ethidium bromide.[13]

Statistical Analysis

IBM SPSS software (Version 25) was used for the statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) with a p-value of less than 0.05 used to establish the significant difference between means.

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Results and Discussion

Effect of RO-CA nanoemulsion on PEPCK expression in zebrafish larvae model of GDM

Phosphoenolpyruvate carboxykinase (PEPCK) is a crucial enzyme in gluconeogenesis that maintains glucose homeostasis.[43] PEPCK can be used as a marker to determine changes in glucose metabolism caused by hyperglycemic conditions.[44] This proposition was supported by the findings from the present study, which showed that the zebrafish exposed to 3% glucose had noticeably higher levels of PEPCK expression than the control (Figure 1).

Table 1: List of primers' sequences used

Parameters	Forward Primer	Reverse Primer
PEPCK	5'-GAGAATTCTCACACAC ACACACGTGAGCAGTA-3'	5'-GTAAAAGCTTTCCGCCA TAACATCTCCAGCAGAA-3'
Nrf2a	5'-GAGCGGGAGAAATCA CACAGAATG-3'	5'-CAGGAGCTGCATGCACT CATCG-3'
SOD	5'-GGCCAACCGATAGTG TTAGA -3'	5'-CCAGCGTTGCCAGTT TTTAG -3'
SIRT-1	5'- ACAGTTCCAGCC ATCTCCATGTCA -3'	5'- AAGACCCGTGG CACTGAATGATCT -3'
TH	5'- CCCCACCTGG AGTATTTTGTG -3'	5'- ATCACGGGCG GACAGTAGACC-3'
Actb2	5'-CGA GCT GTC TTC CCA TCC A-3'	5'-TCA CCA ACG TAG CGT CTT TCT G-3'

Table 2: Reverse Transcriptase PCR Protocol

Steps	Marker					
	PEPCK	Nrf2a	SOD	SIRT-1	TH	Actb2
Initiation	94^0C/3'	95^0C/3,5'	95^0C/3'	95^0C/3'	95^0C/3,'	94^0C/3'
Denaturation	94^0C/1'	95^0C/30"	95^0C/15"	95^0C/15"	95^0C/30"	94^0C/1'
Annealing	58^0C/1'	$57,6^0$C/30"	$50,3^0$C/30"	63^0C/30"	$53,7^0$C/30"	58^0C/45"
Extention	72^0C/1'	72^0C/1'	72^0C/30"	72^0C/30"	72^0C/1'	72^0C/30"
Cycles	35 cycles	35 cycles	40 cycles	39 cycles	35 cycles	35 cycles

There was a significant reduction in PEPCK expression following the administration of RO-CA nanoemulsions. Rosmarinic acid, a polyphenolic compound found in *Rosmarinus officinalis*, has been reported to downregulate PEPCK expression within the gluconeogenesis pathway, while also modulating glucose uptake via the AMPK pathway.[23,45] Additionally, flavonoids such as kaempferol and triterpenoids including madecassoside, asiaticoside, madecassic acid, and asiatic acid present in *Centella asiatica* have demonstrated the ability to decrease PEPCK enzyme levels involved in gluconeogenesis.[46,47]

In the present study, even the lowest concentration (2.5 µg/mL) of the nanoemulsion elicited a reduction in PEPCK expression comparable to that of the control group. Notably, the combination of these two herbal components is known to exhibit synergistic effects. Indeed, formulations combining multiple herbs often exhibit heightened bioactivity compared to individual herbal preparations, owing to synergistic interactions.[29,30] Moreover, nanoemulsions have demonstrated efficacy as a viable alternative for augmenting drug potency and absorption, while also amplifying anti-diabetic effect.[27]

Antioxidant activity of RO-CA nanoemulsion in zebrafish larvae model of GDM

Elevated glucose levels in GDM resulted in the accumulation of reactive oxygen species (ROS), and a corresponding decline in the antioxidant defense system, resulting in oxidative stress.[48] Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that plays a crucial role in mediating the oxidative stress response, is responsible for coordinating the transcription of many antioxidant genes and subsequently modulating cellular antioxidant defense systems.[49] Notably, Nrf2a, a homolog of mammalian Nrf2 found in zebrafish triggers the transcriptional activation of enzymatic antioxidants, controls a significant portion of cytoprotective gene expression during oxidative stress.[50,51] Superoxide dismutase (SOD) is a prominent enzymatic antioxidant that acts as a main cellular antioxidant and scavenger of ROS.[52] As shown in Figures 2 and 3, it is evident that the administration of RO-CA nanoemulsion significantly augmented the expression of Nrf2a and SOD, whereas exposure to 3% glucose resulted in diminished expression of both genes. These findings align with previous research which indicated that GDM impairs Nrf2 activation and reduces SOD expression in both maternal and placental tissues.[53] Furthermore, *Centella asiatica*, enriched with triterpenoids, has been shown to enhance Nrf2 and SOD activation.[54]

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Moreover, the polyphenolic compounds present in *Rosmarinus officinalis* contain unsaturated β-carbonyl groups, serving as electrophiles that potentially facilitate Nrf2 activation, thereby facilitating the synthesis of endogenous antioxidants.[55] There was an increase in the Nrf2a expression in the group administered RO-CA nanoemulsion at a concentration of 2.5 µg/mL (T1). Interestingly, SOD expression demonstrated a concentration-dependent increase; even at the lowest concentration (2.5 µg/mL), the RO-CA nanoemulsion elicited a significant enhancement in SOD expression. Thus, it can be inferred that the administration of RO-CA nanoemulsion at a minimal concentration (2.5 µg/mL) augmented antioxidant activity by upregulating the expression of both Nrf2a and SOD. This underscores the synergistic interaction between herbal combination and nanoemulsion formulation, resulting in increased herbal efficacy and effective dose reduction.[30,56]

a)

b)

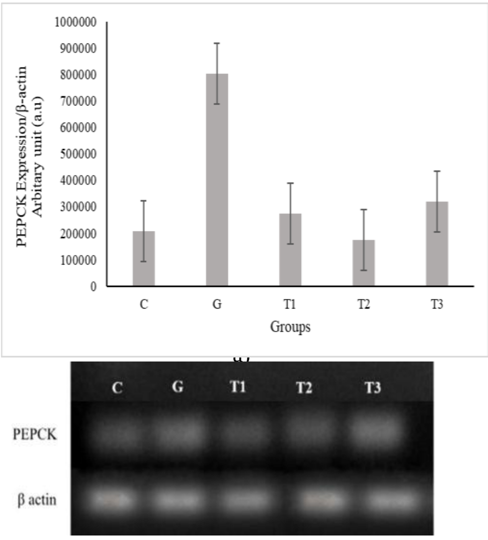

Figure 1: a) PEPCK expression graph of zebrafish at 3 dpf. **b)** PEPCK electrophoresis. (Glu: glucose; RO-CA: the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion. C: control; G: glucose 3%; T1: glu+RO-CA 2,5 µg/mL; T2: glu+RO-CA 5 µg/mL; T3: glu+RO-CA 10 µg/mL.

a)

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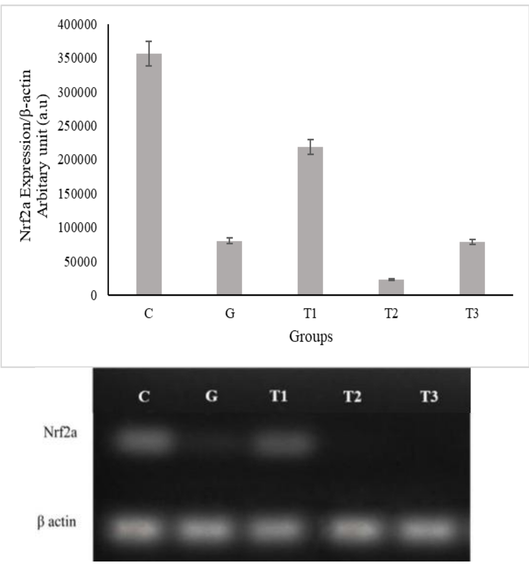

Figure 2: a) Nrf2a expression graph in 3 dpf zebrafish larvae. Nrf2a expression increased significantly ($p = 0.002$) in the treatment 1 group (2.5 µg/mL) compared to the glucose group. **b)** Nrf2a electrophoresis. (Glu: glucose; RO-CA: the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion. C: control; G: glucose 3%; T1: glu+RO-CA 2.5 µg/mL; T2: glu+RO-CA 5 µg/mL; T3: glu+RO-CA 10 µg/mL.

Cardioprotective effect of RO-CA nanoemulsion in zebrafish larval model of GDM

Hyperglycemia in GDM initiates a cascade of events characterized by heightened ROS production, thereby inducing oxidative stress, a phenomenon well-documented in the literature.[6,7] This oxidative stress exerts a notable impact on the fetal cardiovascular system, primarily mediated through the intricate interplay of key regulatory elements such as SIRT-1 and tyrosine hydroxylase (TH), as elucidated in prior studies.[14,16,57] The present investigation, as depicted in Figures 4 and 5, reveals a discernible reduction in both SIRT-1 and TH expression

a)

b)

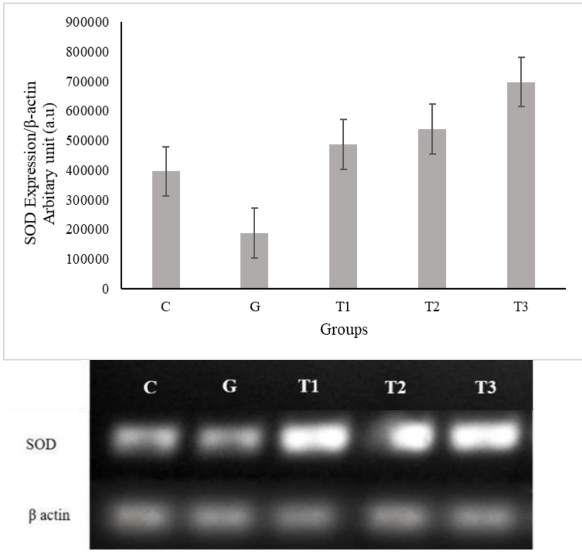

Figure 3: a) SOD mean expression graph in 3 dpf zebrafish larvae. Compared to the glucose group, SOD expression increased dramatically ($p < 0.05$) in all treatment groups. **b)** SOD electrophoresis. (Glu: glucose; RO-CA: the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion. C: control; G: glucose 3%; T1: glu+RO-CA 2.5 µg/mL; T2: glu+RO-CA 5 µg/mL; T3: glu+RO-CA 10 µg/mL.

a)

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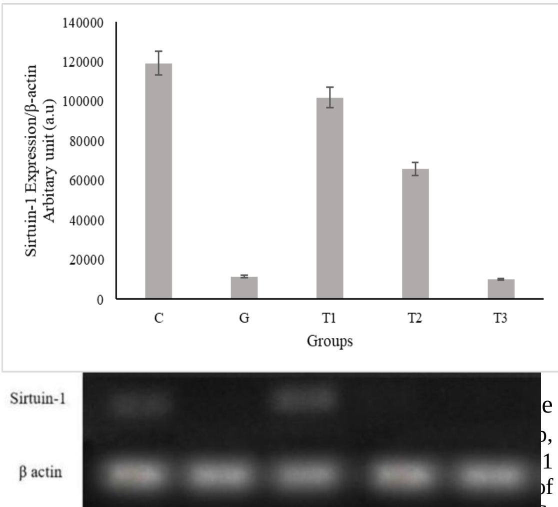

Figur e 4: a) S ... e treatmen ... p, SIRT-1 e ... 1 electropho ... of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion. C: control; G: glucose 3%; T1: glu+RO-CA 2.5 µg/mL; T2: glu+RO-CA 5 µg/mL; T3: glu+RO-CA 10 µg/mL.

consequent to exposure to elevated glucose levels. This decline in SIRT-1 expression or activity assumes significance in the context of fetal cardiovascular health, given its established role in cardiovascular regulation.[58] Furthermore, the intricate modulation of TH expression by SIRT-1 underscores the multifaceted nature of their interaction,

wherein aberrations in either component can disrupt the delicate balance necessary for embryonic myocardial cell differentiation and the maintenance of physiological cardiac rhythm.[17,18] Notably, our findings corroborate existing literature suggesting a negative correlation between glucose levels and TH expression, with implications for heart rate variability, thereby reinforcing the clinical relevance of this study.[59]
Figure 6 provides an additional support to these observations, illustrating a significant increase in heart rate in zebrafish larvae exposed to elevated glucose concentrations. Moreover, the mechanism behind this phenomenon can be attributed to oxidative stress-mediated S-nitrosylation, leading to calcium dysregulation and subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This cascade, involving a serine-threonine kinase, is pivotal in modulating the excitability of cardiac cell membranes, culminating in an elevation of heart rate - a phenomenon in line with the present experimental observations.[60] In summary, the present study underscores the intricate interplay between hyperglycemia, oxidative stress, and fetal cardiovascular health, shedding light on the mechanistic pathways involving SIRT-1, TH, and CaMKII. These insights do not only augment our understanding of the pathophysiology of GDM-associated fetal cardiovascular complications, but also offer potential avenues for therapeutic intervention and prenatal care strategies.

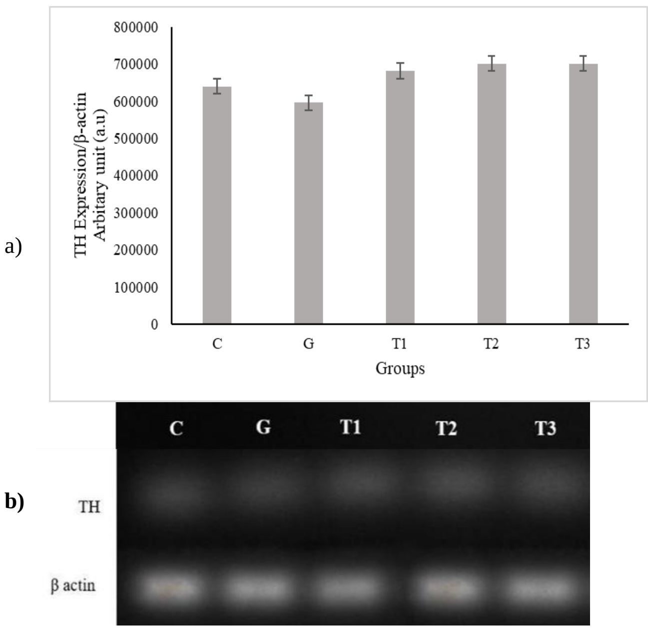

Figure 5: a) TH expression graph of zebrafish at 3 dpf. When TH expression was compared to the glucose group, it increased significantly ($p < 0.05$) in all treatment groups. **b)** TH electrophoresis. (Glu: glucose; RO-CA: the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion; C: control; G: glucose 3%; T1: glu+RO-CA 2.5 μg/mL; T2: glu+RO-CA 5 μg/mL; T3: glu+RO-CA 10 μg/mL.

Furthermore, in the present investigation, there was a notable increase in the expression of SIRT-1 in the experimental group that received RO-CA nanoemulsion at a concentration of 2.5 μg/mL (T1). Additionally, these findings indicated a dose-dependent increase in TH expression. Specifically, the administration of RO-CA nanoemulsions at the lowest concentration (2.5 μg/mL) led to a significant increase in TH expression. Furthermore, the results demonstrated that the combined therapy with RO-CA effectively preserved heart rate, closely approximating that of the control group.
The observed effects can be attributed to a synergistic interaction facilitated by the combination of RO-CA nanoemulsions. Constituents such as rosmarinic acid and carnosol derived from *Rosmarinus officinalis*, along with madecassoside and asiaticoside from *Centella asiatica*, have been implicated in enhancing both TH and SIRT-1 expression.[61-63] It is important to note that herbal formulations employing the nanoemulsion drug delivery system offer several advantages, including enhanced bioavailability, improved absorption, and reduced dosage requirements to mitigate potential toxicity.[64] administration of RO-CA nanoemulsion at the lowest concentration (T1) exerts cardioprotective effects by augmenting the expression of both SIRT-1 and TH, thereby contributing to the maintenance of heart rate. This underscores the potential of nanoemulsion-based herbal formulations as promising therapeutic strategies for maintaining cardiovascular health.

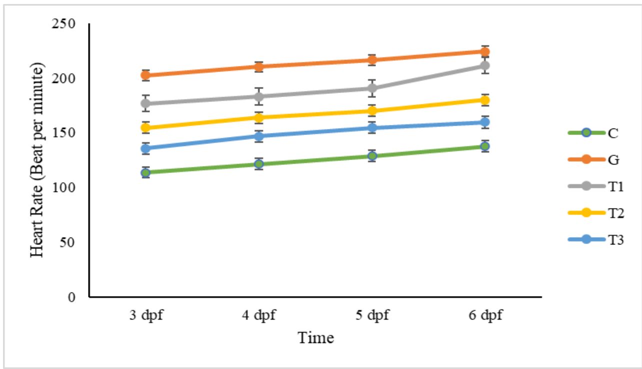

Figure 6: Mean zebrafish larvae heart rate. Heart rate decreased significantly in all treatment groups compared to the glucose group ($p < 0.05$). (Glu: glucose; RO-CA: the combination of *Rosmarinus officinalis* and *Centella asiatica* nanoemulsion; C: control; G: glucose 3%; T1: glu+RO-CA 2.5 μg/mL; T2: glu+RO-CA 5 μg/mL; T3: glu+RO-CA 10 μg/mL.

Conclusion

The combination of RO-CA nanoemulsions has demonstrated the capacity to attenuate PEPCK expression and mitigate hyperglycemia in zebrafish larvae subjected to elevated glucose levels from 2 to 72 hours post-fertilization, a time analogous to the gestational period in humans. Moreover, at a concentration of 2.5 μg/mL, this nanoemulsion blend sustains heart rate frequency while concurrently upregulating the expression of key antioxidative and protective genes, including Nrf2a, SOD, SIRT-1, and TH. These findings signify a notable enhancement in antioxidant activity induced by the RO-CA nanoemulsion, suggesting its potential utility as a cardioprotective agent in gestational diabetes mellitus (GDM). However, further research is imperative to validate these observations and elucidate the prospective clinical applications of this nanoemulsion in managing GDM-related complications.

Conflict of interest

The authors declare no conflict of interest.

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Authors' Declaration

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

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