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Original Article

Effect of *Centella asiatica* (L.) Urb. extracts through expression of SIRT1 and Per2 on zebrafish (*Danio rerio*) larvae insomnia model

[Efecto de los extractos de *Centella asiatica* (L.) Urb. a través de la expresión de SIRT1 y Per2 en el modelo de insomnio de larvas de pez cebra (*Danio rerio*)]

Zamroni Afif[1,2], Nurvia Andriani[2], Rodhiyan Rakhmatiar[2], Shahdevi Kurniawan[2], Holipah[3], Husnul Khotimah[4*], Nurdiana[4], Irawan Satriotomo[5]

[1]Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[2]Department of Neurology Faculty of Medicine, Universitas Brawijaya, Saiful Anwar General Hospital, Malang, Indonesia.
[3]Department of Public Health, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[4]Departement of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[5]Indonesian Neuroscience Institute, Malang, Indonesia.
*E-mail: husnul_farmako.fk@ub.ac.id

Abstract

Context: Insomnia is difficulty falling asleep or maintaining sleep or sleeping conditions that cannot adequately restore the body and mental state. However, insomnia therapy has not been satisfying for improving the condition. Several alternative therapy options for treating insomnia include *Centella asiatica* (CA). Many pathways lead to sleep regulation, including circadian rhythms and cellular reactions. Further research is necessary to treat insomnia through circadian rhythms by administering CA with observations on zebrafish models of insomnia.

Aims: To evaluate the influence of CA extract administration on deacetylase sirtuin-1 (SIRT1) and period circadian regulator-2 (Per2) expression in zebrafish (*Danio rerio*) larvae in an insomnia model.

Methods: A laboratory experimental study with a randomized control group post-test only was used, with a sample of *D. rerio* larvae insomnia model that was given treatment with CA ethanolic extract (2.5, 5, and 10 µg/mL). The *D. rerio* larvae were prepared for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to evaluate SIRT1 and Per2 levels.

Results: This study found a significant effect of CA ethanolic extract on the expression of SIRT1 (p=0.001), with CA extract 10 µg/mL elevating SIRT1 expression in the group of *D. rerio* larvae with the insomnia model. There was no significant effect of CA extract on Per2 expression in the group of *D. rerio* larvae with the insomnia model (p=0.051).

Conclusions: Administration of CA extract at a concentration of 10 µg/mL can elevate the expression of SIRT1 and have no significant effect on Per2 expression in *D. rerio* larvae with insomnia model.

Keywords: *Centella asiatica*; circadian rhythm; deacetylase sirtuin-1; insomnia; period circadian regulator-2; zebrafish.

Resumen

Contexto: El insomnio es la dificultad para conciliar o mantener el sueño o las condiciones de sueño que no pueden restaurar adecuadamente el cuerpo y el estado mental. Sin embargo, la terapia del insomnio no ha sido satisfactoria para mejorar la condición. Varias opciones de terapia alternativa para tratar el insomnio incluyen *Centella asiatica* (CA). Muchas vías conducen a la regulación del sueño, incluidos los ritmos circadianos y las reacciones celulares. Es necesario seguir investigando para tratar el insomnio a través de los ritmos circadianos mediante la administración de CA con observaciones en modelos de insomnio con pez cebra.

Objetivos: Evaluar la influencia de la administración de extracto de CA en la expresión de la deacetilasa sirtuina-1 (SIRT1) y del regulador circadiano del periodo-2 (Per2) en larvas de pez cebra (*Danio rerio*) en un modelo de insomnio.

Métodos: Se utilizó un estudio experimental de laboratorio con un grupo de control aleatorizado post-test únicamente, con una muestra de larvas de *D. rerio* modelo de insomnio a las que se administró tratamiento con extracto etanólico de CA (2,5; 5 y 10 µg/mL). Las larvas de *D. rerio* se prepararon para la reacción en cadena de la polimerasa de transcripción inversa-cuantitativa (RT-qPCR) para evaluar los niveles de SIRT1 y Per2.

Resultados: Este estudio encontró un efecto significativo del extracto etanólico de CA sobre la expresión de SIRT1 (p=0,001), con el extracto de CA 10 µg/mL elevando la expresión de SIRT1 en el grupo de larvas de *D. rerio* con el modelo de insomnio. No hubo un efecto significativo del extracto de CA sobre la expresión de Per2 en el grupo de larvas de *D. rerio* con el modelo de insomnio (p=0,051).

Conclusiones: La administración de extracto de CA a una concentración de 10 µg/mL puede elevar la expresión de SIRT1 y no tener un efecto significativo sobre la expresión de Per2 en larvas de *D. rerio* con modelo de insomnio.

Palabras Clave: *Centella asiática*; deacetilasa sirtuin-1; insomnio; pez cebra; regulador circadiano del periodo-2; ritmo circadiano.

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AUTHOR INFO
ORCID: 0000-0002-2736-0941 (ZA)
0009-0007-8091-6834 (NA)
0009-0005-5353-5134 (RR)
0000-0002-9351-7505 (SNK)
0000-0002-6143-8239 (H)
0000-0002-2374-4358 (HK)
0000-0002-7953-1124 (N)
0000-0003-0519-9509 (IS)

INTRODUCTION

Insomnia is the disintegration of molecules that regulate the rhythm of waking and sleeping in the brain. It is critical to distinguish between normal cyclical and dysregulated rhythms that cause circadian rhythm oscillators to be disrupted. Circadian rhythm deviations can lead to poor rhythms and disruption of cellular core oscillator components. Circadian rhythms are regulated by the core circadian locomotor output cycles kaput (CLOCK) gene, which includes three per genes (Per1, Per2, and Per3), CLOCK, ARNTL (also called BMAL1), and two homologous cryptochrome genes (CRY1 and CRY2). These genes control a proportion of the genome. Approximately 10% of all expressed genes are thought to be regulated by clock genes (Qiu et al., 2016). In controlling circadian rhythms, deacetylase sirtuin-1 (SIRT1) is an accessory component of cellular circadian oscillators. It can increase the amplitude of circadian rhythms, influencing melatonin through circadian rhythms (Hardeland, 2021) and period circadian regulator-2 (Per2), a CLOCK gene that regulates circadian rhythms. Per2 and SIRT1 have a reciprocal relationship as SIRT1 binds to CLOCK-BMAL1 circadian and promotes deacetylation and degradation of Per2 (Asher et al., 2008).

Currently, pharmacological therapy for insomnia has several therapeutic targets, one of which is benzodiazepines. It works by increasing the activity of the neurotransmitter gamma-aminobutyric acid (GABA) through modulation of the type A GABA receptor complex. However, these drugs have side effects in long-term use (more than four weeks), including dependence, discontinuation syndrome, psychomotor retardation, learning difficulties, memory impairment and potential for cognitive impairment, delirium, accidents/falls, abuse, and a death potential for patients with chronic pulmonary insufficiency or sleep apnea. Consequently, the clinical use of off-label drugs and new drugs that do not target the GABAergic system is increasing (Atkin et al., 2018). Adverse long-term effects of insomnia therapy require alternative therapies from natural ingredients that have minimal side effects. One of which is by developing this study, whose effect of *Centella asiatica* (L.) Urb. (family *Apiaceae*) (CA) on zebrafish models of insomnia, with observations primarily through SIRT1 and Per2 in improving sleep activity in zebrafish with models of insomnia that have not been discussed in previous studies. The research on the effect of CA extract on the expression of SIRT1 and Per2 in zebrafish larvae insomnia model used the previous preliminary research by Afif et al. (2022), which observed zebrafish exposed to light to induce an insomnia model.

The light treatment used in the preliminary study refers to the research method of Pinheiro-da-Silva et al. (2017), included the negative control group (exposure to 12 hours light:dark), the positive control group with 24 hours light, 16 hours light 8 hours dark flash (2 minutes light:2 minutes dark), and 16 hours light 8 hours dark flash (4 minutes light:1 minute dark). This preliminary study obtained the 24-hour light group as an insomnia induction model based on observations of determining the insomnia model using light treatment induction of the light and dark phases on days 5, 6, and 7. The research obtained prolonged latency to first or sleep latency in the larval model zebrafish exposed to light for 24 hours. Continuous exposure to light can cause this condition, which aligns with the theory that zebrafish have endogenously controlled circadian rhythm behaviors that light can influence. Light regulation of sleep-wake cycles is related to the regulation of melatonin and the hypocretin/orexin (Hcrtr) system (Afif et al., 2022).

CA acts as a neuroprotector against various neurological disorders. The potency of this species is attributed to its antioxidant, anti-inflammatory, anxiolytic, and anti-stress properties (Gohil et al., 2010). CA administration in rats with sleep deprivation for 72 hours significantly improved locomotor activity, anti-anxiety effects, lowered cortisol levels, and improved neuronal inflammation and apoptosis response (Chanana and Kumar, 2017). The asiatic acid (AA) contained in CA can affect the expression of SIRT1 through peroxisome proliferator-activated receptor-γ-coactivator-1 α (PGC-1α) in cells. From one study, it was stated that cells preincubated with AA showed upregulation of SIRT1 and PGC-1α compared to those without AA so that SIRT1 activated NAD^+ deacetylated PGC-1α, which binds to the accessory rhythm component of retinoic acid receptor-related orphan receptor α (RORα). RORα responds to signaling to promote BMAL1 and CLOCK. BMAL1 and CLOCK are activated by rhythm-controlling genes PPARα. PPARα can directly activate the expression of REV-ERBα and Per2. BMAL1 and CLOCK activate the expression of the Per2 gene. When Per2 protein is accumulated maximally, it forms a complex with the BMAL1-CLOCK heterodimer, which causes suppression of Per2 transcription. SIRT1 binds CLOCK-BMAL1 and promotes deacetylation and degradation of Per2 (Borrás et al., 2021).

CA extract concentrations (2.5, 5, and 10 µg/mL) used in this study have been referred to by Khotimah et al. (2015). Meanwhile, the concentration of the ethanol extract of CA has been widely used in other research, for example, the study of Parkinson's models, stunting, and also research to determine the neuroprotective effect on CA with experimental animal models of zebrafish larvae (Khotimah et al., 2015). This concentration has been proven optimal to show the pharmacological effect of CA extract. This study aimed to prove that the administration of CA ethanolic extract influences SIRT1 and Per2 expression in the zebrafish (*Danio rerio*) larvae insomnia model.

MATERIAL AND METHODS

Research design

The Faculty of Medicine at the University of Brawijaya's Health Research Ethics Committee has approved this project. Ethics Permit No: 237/EC/KEPK/11/2022. The research design used in this study was a true laboratory experiment with a randomized control group post-test approach. Zebrafish larvae were divided into 5 groups: control negative group (exposed to 12 hours of light and 12 hours of darkness); control positive group or insomnia group (24 hours light exposure); insomnia group with 2.5 µg/mL of CA (CA 2.5 µg/mL); insomnia group with 5 µg/mL of CA (CA 5 µg/mL), and insomnia group with 10 µg/mL of CA (CA 10 µg/mL).

Maintenance of zebrafish embryos

Wild-type zebrafish at age 0–7 days post fertilization (dpf) was used in this study, obtained from the Reproductive Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Brawijaya. Embryos were maintained at 28 ± 10°C. Maintenance of zebrafish embryos with dark:light condition for 12:12 hours and feeding plankton is given 3 times a day, conductivity 350-600 S and salinity 0-0.6 ppt (parts per thousand) zebrafish larvae were taken on the 7 dpf and placed in an ice pack. After the zebrafish larvae were unconscious (showing no spontaneous movements), the bottles were tightly closed and stored at -80°C and tissue analysis was performed. RNA was isolated using the MEGAshortscript T7 kit (Ambion. Inc., Austin, Texas).

Induction of insomnia

The light exposure used in this study to cause insomnia conditions in zebrafish larvae refers to the research method of Pinheiro-da-Silva et al. (2017). with modification of light exposure as follows: negative control group with light exposure 12 hours light and 12 hours dark (normal group), and positive control group includes light treatment 24 hours light (24 hours) (Pinheiro-da-Silva et al., 2017). Light is given at 200 lux from 0 dpf until the age of 7 dpf. Modifications were made to the design of lamps and tools used during the study (Afif et al., 2022).

Phytochemicals of CA

Scientific studies have revealed that CA contains more than 70 phytochemicals. CA is rich in triterpenes, flavonoids, essential oils, alkaloids, and amino acids. CA has diverse and complex components of chemically active compounds in which the main groups include terpenes (monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes), phenolic compounds (flavonoids, tannins), alkaloids, carbohydrates, vitamins, minerals and amino acids (Sabaragamuwa et al., 2018).

Geographical location of CA specimen collection

C. asiatica specimen collection was obtained and identified by Laboratory Materia Medica Batu with coordinate points -7.867086,112.519319.

Drying CA extract process

The stems and leaves that were above the ground were washed thoroughly and then cut into pieces of 1-2 cm to speed up the drying process. Next, put the oven at 40°C to dry (free of water content).

Extraction CA process

CA was mashed with a blender until smooth. The dry sample weighed 100 g and was put into a 1-liter Erlenmeyer glass. Then, it was soaked with 98% ethanol to a volume of 900 mL. Shake until thoroughly mixed (± 30 minutes). Let stand one night until settled. The top layer of ethanol (solvent) mixture with the active substance has been mixed (can be filtered using filter paper). The soaking process was carried out thrice, followed by evaporation. The solution from the extraction process was put into a 1-liter evaporation flask. The evaporating flask was installed on the evaporator with the water bath set to 70°C.

Exposure to CA extract

The administration of CA ethanol extract with concentrations of 2.5, 5, and 10 µg/mL (Khotimah et al., 2015) was sequentially introduced into the treatment group with 24 hours of light exposure group. CA extract was induced once a day from the third day to the seventh day of dpf (day post-fertilization).

Examination of real-time quantitative chain reaction (RT-qPCR)

BDNF was measured on samples taken from the tissue of zebrafish larvae by the qRT-PCR method. Primers used in this study for SIRT1: forward primer CAA GGA AAT CTA CCC CGG ACA GT reverse primer CAG TGT GTC GAT ATT CTG CGT GT. Per2 was measured on samples taken from the tissue of zebrafish larvae by the qRT-PCR method. The primers

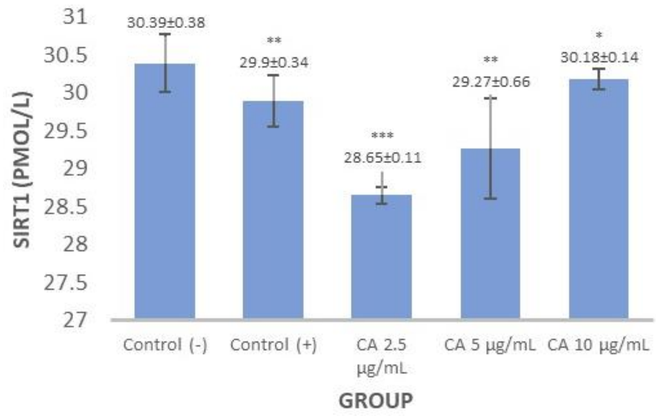

Figure 1. SIRT1 in zebrafish larvae model of insomnia after exposure to CA extract.

Data are expressed as mean ± SD (n = 15).*p<0.05; **p<0.01; ***p<0.001. One-Way ANOVA difference test followed by Tukey's post-hoc test.

Table 1. Quantitative data normality test and homogeneity test results for SIRT1 expression in zebrafish larvae insomnia model.

Variable	Group	p-value normality test	Description	P-value homogeneity test	Description
SIRT1	Control (-)	0.402	Normal		
	Control (+)	0.205	Normal		
	CA 2.5 µg/mL	0.899	Normal	0.064	Homogeneous
	CA 5 µg/mL	0.586	Normal		
	CA 10 µg/mL	0.411	Normal		

used in this study were for Per2: Forward primer (5′–3′) ACGAGGACAAGCCAGAGGAACG, reverse primer (5′-3′) GCACTGGCTGGTGATGGAGA (Ren et al., 2017).

Statistical analysis

The research data were analyzed using IBM SPSS Statistics 27 software and expressed in terms of mean ± standard deviation (mean ± SD). The normality test used the Shapiro-Wilk Test of normality, and the homogeneity test used the Homogeneity of Variance test; then, if the data was normally distributed and homogeneous, it was continued with the One-Way ANOVA difference test. Significant difference data with p<0.05 was followed by Tukey's post-hoc test.

RESULTS

SIRT1 examination results with RT-qPCR

The results of the SIRT1 examination using the RT-qPCR method in the form of mean SIRT1 in the zebrafish larvae model of insomnia after exposure to CA extract are presented in Fig. 1.

The diagram shows that the group exposed to CA extract exhibited an increased SIRT1 expression in insomnia model zebrafish larvae along with an increase in concentrations of CA extract, with the most optimum concentration of 10 µg/mL compared to others. The lowest of increased SIRT1 expression was at a concentration of 2.5 µg/mL compared to others. In the negative control, i.e., in normal zebrafish larvae (light treatment 12 hours light: 12 hours dark), the average SIRT1 expression was close to the mean SIRT1 expression in zebrafish larvae with insomnia model given exposure to CA extract 10 µg/mL, which showed that the higher the CA extract concentration, the more it can increase SIRT1 expression in insomnia model zebrafish larvae. In the positive control, the zebrafish larvae that were exposed to light for 24 hours, the mean expression results showed higher results than the insomnia model zebrafish larvae that were exposed to the lowest concentration of CA extract, 2.5 µg/mL.

The results of quantitative SIRT1 expression data were tested for normality and homogeneity. Normality testing was carried out using the Shapiro-Wilk test. The normality test is fulfilled if p>0.05. Using SPSS software, the normality test results are obtained as in Table 1.

From Tables 1 and 2, it can be seen that the SIRT1 variable data in each group was normally distributed. Therefore, the following variant homogeneity test was performed with the Levene test. The homogeneity test results were fulfilled with p>0.05. Statistical analysis results showed that the SIRT1 expression variable

Table 2. Average SIRT1 expression in insomnia model zebrafish larvae.

No.	Treatment	SIRT1 expression	p-value
1	Control (-)	30.39 ± 0.38	0.001
2	Control (+)	29.9 ± 0.34	
3	CA 2.5 µg/mL	28.65 ± 0.11	
4	CA 5 µg/mL	29.27 ± 0.66	
5	CA 10 µg/mL	30.18 ± 0.14	

Data are expressed as mean ± SD (n = 15).

Table 3. Tukey post-hoc test results effect of treatment on SIRT1 expression.

Treatment		p-value	Description
	Control (+)	0.549	Not significantly different
	CA 2.5 µg/mL	0.002**	Significantly different
	CA 5 µg/mL	0.031*	Significantly different
Control (-)	CA 10 µg/mL	0.956	Not significantly different
	CA 2.5 µg/mL	0.017**	Significantly different
	CA 5 µg/mL	0.316	Not significantly different
Control (+)	CA 10 µg/mL	0.897	Not significantly different
	CA 5 µg/mL	0.349	Not significantly different
CA 2.5 µg/mL	CA 10 µg/mL	0.005***	Significantly different
CA 5 µg/mL	CA 10 µg/mL	0.089	Not significantly different

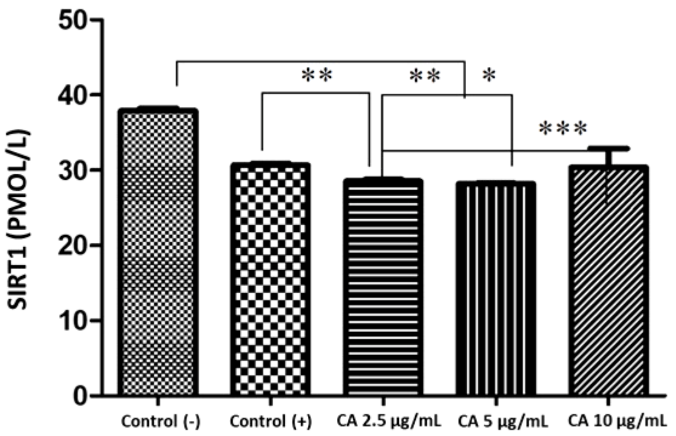

Figure 2. Tukey post-hoc test results effect of treatment on SIRT1 expression.

Data are expressed as mean ± SD (n = 15).*p<0.05; **p<0.01; ***p<0.001. One-Way ANOVA followed by Tukey's post-hoc test.

data was homogeneous. Based on the results of the normality and homogeneity tests of the data, it can be concluded that the statistical testing process was carried out using a parametric statistical approach using the One-way ANOVA test because the data was normally distributed and homogeneous.

Based on the analysis results using the One-way ANOVA test, a p<0.001 was obtained. Thus, this test obtained a significant effect of exposure to CA on SIRT1 expression in zebrafish larvae with the insomnia model. In addition, a posthoc Tukey test was carried out on the expression of SIRT in zebrafish larvae with the insomnia model to find out the differences in each treatment (Table 3, Fig. 2).

In Tukey's posthoc test, the group exposed to CA at 2.5 µg/mL concentrations and 5 µg/mL significantly differed from the negative control group. In comparison, exposure to CA at 10 µg/mL concentration was not significantly different. It indicates that the values were close to normal.

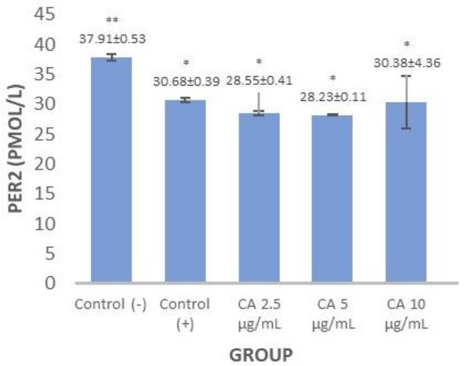

Figure 3. Mean Per2 expression in zebrafish larvae model of insomnia after exposure to CA extract.

Data are expressed as mean ± SD (n = 15).*p<0.05; **p<0.01; ***p<0.001. One-Way ANOVA difference test followed by Tukey's post-hoc test.

Table 4. Quantitative data normality test results for Per2 Expression in zebrafish larvae model of insomnia.

Variable	Group	p-value normality test	Description	P-value homogeneity test	Description
Per2	Control (-)	0.205	Normal		
	Control (+)	0.105	Normal		
	CA 2.5 µg/mL	0.6	Normal	0.001	Inhomogeneous
	CA 5 µg/mL	0.11	Normal		
	CA 10 µg/mL	0.025	Abnormal		

Table 5. Mean Per2 expression in zebrafish larvae insomnia model.

No	Treatment	Mean ± SD	p-value
1	Control (-)	37.91 ± 0.53	
2	Control (+)	30.68 ± 0.39	
3	CA 2.5 µg/mL	28.55 ± 0.41	0.051
4	CA 5 µg/mL	28.23 ± 0.11	
5	CA 10 µg/mL	30.38 ± 4.36	

This shows that exposure to CA extract with a concentration of 10 µg/mL was the most optimum in increasing SIRT1 expression in zebrafish larvae with the insomnia model.

Per2 examination results with RT-qPCR

The results of the Per2 expression examination using the RT-qPCR method to obtain data on average Per2 expression in zebrafish larvae model insomnia after exposure to CA extract are presented in the following Fig. 3.

Based on the figure above, it can be seen that the group exposed to 10 µg/mL CA extract had a higher average Per2 expression value compared to other concentrations. However, the highest Per2 expression average value was in the negative control. The lowest mean value of Per2 expression was in the group exposed to CA extract of 5 µg/mL.

From the quantitative data of Per2 expression, normality, and homogeneity tests were conducted. Normality testing was performed using the Shapiro-Wilk test. The assumption of normality if p>0.05. Using the help of SPSS software, the normality test results are showed in Table 4.

Table 5 shows that the Per2 variable data in each group is normally distributed, except for the 10 µg/mL group. Furthermore, the variance homogeneity test was carried out using the Levene test. The assumption of homogeneity of variance is fulfilled if p>0.05. The results of statistical analysis show that the Per2 variable data is inhomogeneous. Based on the results of the normality and homogeneity tests of the data, it can be concluded that the statistical testing process was carried out using a non-parametric statistical approach using

Table 6. Tukey post-hoc test results effect of treatment on expression of Per2.

Treatment		p-value	Description
CA 2.5 µg/mL	Control (+)	0.248	Not significantly different
	Control (-)	0.248	Not significantly different
CA 5 µg/mL	CA 10 µg/mL	1.000	Not significantly different
	CA 2.5 µg/mL	1.000	Not significantly different
	Control (+)	0.248	Not significantly different
	Control (-)	0.248	Not significantly different
CA 10 µg/mL	CA 2.5 µg/mL	1.000	Not significantly different
	Control (+)	1.000	Not significantly different
	Control (-)	0.248	Not significantly different

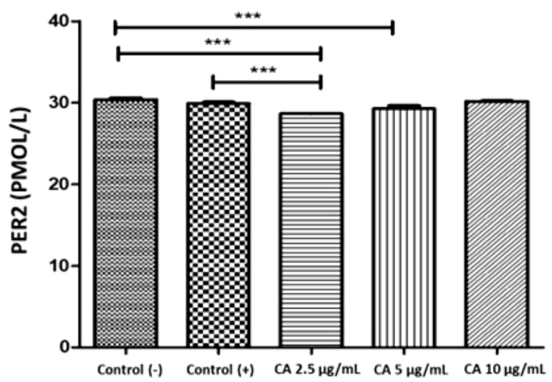

Figure 4. Tukey post-hoc test results effect of treatment on expression of Per2.
Data are expressed as mean ± SD (n = 15).*p<0.05; **p<0.01; ***p<0.001. One-Way ANOVA followed by Tukey's post-hoc test.

Kruskall Wallis because the data was not normally distributed and was inhomogeneous (Table 6).

Based on the Kruskall Wallis analysis results, a p-value was 0.051 ($p>0.05$). Therefore, it can be concluded that there is no significant effect of the concentration of CA extract on the expression of Per2 in insomnia model zebrafish. Thus, this test shows that there was no significant effect of concentration of CA extract on Per2 expression, or there was no significant difference in Per2 levels due to treatment (Fig. 4).

DISCUSSION

Insomnia is the result of disruption of the endogenous circadian system. For example, many physiological markers of the circadian phase exhibit sleep-wake inhibiting patterns. There is also evidence that individuals with insomnia have hypersensitivity to nocturnal melatonin suppression due to bright light. They also have decreased oscillator sensitivity to alignment and long circadian cycles. Furthermore, the duration and timing of environmental light and dark exposure may be essential in expressing the insomnia phenotype. Insomnia has also been reported in cases of mild head trauma and has a genetic basis. Some cases of this syndrome can be hereditary due to autosomal dominance. Other evidence supporting the genetic basis of insomnia is research reports of polymorphisms in circadian genes such as hPer3, arylakylamin N-acetyltransferase, human leukocyte antigen, and Jam, which are associated with diurnal preferences and insomnia (Riemann et al., 2010).

Centella asiatica (CA) can improve the regulation of SIRT1 gene expression

From the results of this study, the mean expression of SIRT1 in the insomnia model of zebrafish larvae in the exposure group CA was found. It can be explained that the expression level of the SIRT1 gene increased with increasing concentrations of CA extract as evidenced by the results of Tukey's posthoc test, i.e., the exposure group of CA with concentration 2.5 µg/mL and 5 µg/mL significantly different from the negative control group. In comparison, exposure to a CA concentration of 10 µg/mL did not differ significantly, indicating that the values were close to normal. This shows that exposure to CA extract with a concentration of 10 µg/mL is the most optimum in increasing SIRT1 expression in zebrafish larvae with the insomnia model.

This study found that SIRT1 gene expression increased with increasing concentrations of CA extract. In theory, the SIRT1 cascade through the active substance of CA extract, and the triterpene content, asiatic acid (AA), can increase SIRT1 gene expression. AA content in CA can influence SIRT1 expression through PGC-1α in cells; preincubation of SIRT1 with AA shows upregulation of SIRT1 and PGC-1α compared to those without AA (Xu et al., 2013). SIRT1 interacts with the core oscillator, which increases the amplitude of circadian oscillators in the center and peripherally. SIRT1 expression in the central circadian rhythm through the expression of CLOCK (circadian locomotor output cycles kaput), which depends on the NAD+ sirtuin substrate which oscillates with the expression of the NAD enzyme, nicotinamide phosphoribosyltransferase (NAMPT) through the promoter of the BMAL1 core oscillator component (brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1), which binds CLOCK. NAD+-activated SIRT1 deacetylates PGC-1α, which binds to the accessory clock component RORα (retinoic acid receptor-related orphan receptor α) (Mendelsohn and Larrick, 2017).

Based on other studies stated that the circadian system is a complex multioscillator machine that is useful in avoiding many diseases and disorders. The circadian amplitude decreases with age in central and peripheral oscillators, so increased amplitude through SIRT1 is of high value for healthy aging supported mitochondrial function by SIRT1, which can contribute to the avoidance of excessive oxidative stress. Sirtuins from various studies have relevance to mitochondrial function, which is useful in therapeutic aspects related to its role in metabolic homeostasis (Yamazaki et al., 2002).

In addition, SIRT1 is also known to be involved in mitochondrial proliferation, especially in central nervous system cells. Neuron cells subjected to oxidative stress, excitotoxic, or other cell stress, mitophagy can cause a reduction in mitochondria in the peripheral parts of the cell, leading to decreased connectivity leading to loss of function, as in neurodegenerative diseases such as Alzheimer's disease and occurs to some degree in normal aging. Thus, mitochondrial proliferation by SIRT1 may maintain functions, including regulation of PGC-1α and PPARγ factors, as both influence circadian rhythms through antioxidant effects associated with traumatic or ischemic brain injury and damage by mitochondrial toxins, such as doxorubicin. In ischemic and traumatic brain injury, the neuroprotective function of SIRT1 is anti-inflammatory and antiapoptotic. Research publications mention that neuroprotective effects can be mediated by SIRT1 (Hernández-Jiménez et al., 2013).

In the positive control, the zebrafish larvae that were exposed to light for 24 hours, the mean expression results showed higher results than the insomnia model zebrafish larvae that were exposed to the lowest concentration of CA extract, 2.5 μg/mL. Theoretically, SIRT1 decreases in insomnia conditions, but various regulatory conditions can influence the increase and decrease of SIRT1. SIRT1 is an accessory component of a cellular circadian oscillator that is part of a circadian system whose complexity, aside from the main clock cycle activity of the hypothalamus, is the suprachiasmatic nucleus (SCN). However, it also comprises a peripheral oscillator in every tissue and countless nucleated cells. The role of SIRT1 in the oscillation process provides a systemic role of sirtuins throughout the body, influencing a large number of functions controlled by circadian rhythms, ranging from ups and downs of regulation of genes and their respective products to regulation of metabolism, endocrine, excitatory, immunological, and many other physiological functions. This is a crucial aspect of circadian gene expression concerning epigenetic modulation by the interaction of core components and accessory oscillators, chromatin remodeling, and modulation of noncoding RNA. Therefore, the increase in the positive control can affect the ups and downs of the regulation of genes and their respective products to regulate the metabolism, endocrine, excitatory, immunological, and many physiological functions of the zebrafish larvae (Mendelsohn and Larrick, 2017).

Kazimi and Cahill (199) found that environmental conditions affect the circadian rhythm of zebrafish larvae through the influence of light. In this study, it was stated that the optimum temperature that best influences circadian rhythms, one of which is an increase in melatonin, is a temperature of 28.58°C. These environmental conditions may be affecting SIRT1 expression in positive controls (Kazimi and Cahill, 1999).

Centella asiatica (CA) did not affect the regulation of Per2 gene expression

This study found that the average Per2 expression in insomnia model zebrafish larvae in the CA exposure group showed no significant effect of concentration of CA extracts on Per2 expression. This is evidenced by the analysis using Kruskall Wallis, where there was no significant effect of the concentration of CA extract on Per2 expression.

The content of AA in CA is an indirect pathway Per2 from the AA, and SIRT1 cascades through BMAL1 and CLOCK. BMAL1 and CLOCK activate the expression of the Per2 gene. When Per2 protein accumulates maximally, it will form a complex with the BMAL1-CLOCK heterodimer, which causes suppression of Per2 transcription. SIRT1 binds to

CLOCK-BMAL1 circadian and promotes deacetylation and degradation of Per2 (Xu et al., 2013).

Other research mentions that the regulation of wake and sleep rhythms in the brain can also be regulated by remodeling neuronal connectivity, either strengthening or weakening synaptic connections. The study mentioned that Per2 is a CLOCK gene that regulates circadian rhythms in entering light and dark information in vesicular glutamate transporter 1 (vGLUT1) in synaptic vesicles. Light can impact through Per2, so changes in light and dark, such as those experienced in jet lag and shift work, can affect behavior (Cox and Takahashi, 2019).

The mechanism of circadian rhythm generation relies on feedback loops that involve transcription factors positively and negatively. BMAL1 and CLOCK activate the expression of the Per and cryptochrome (Cry) genes when the Per and Cry proteins accumulate maximally, they form complexes with BMAL1-CLOCK heterodimers that cause suppression of their own gene transcription. Per2 and SIRT1 have a reciprocal relationship. It is said that SIRT1 binds CLOCK-BMAL1 circadianly and promotes deacetylation and degradation of Per2. The potential interaction of Per2 with other proteins is regulated by the accumulation of Per2 proteins during the circadian cycle (Asher et al., 2008).

Synchronization of circadian oscillators with environmental inputs and outputs of physiological circadian rhythm pathways requires complex transcriptional feedback loops. Per2 mediates the main output of the molecular oscillator. Direct interaction with nuclear receptors (NR) homo or heterodimers can affect promoters of the appropriate target genes and metabolic or physiological processes. Per2 activity can be modulated by input (e.g., light or food) to the circadian oscillator (Asher et al., 2008). Protein-protein interactions in circadian rhythms require coupling these proteins to circadian rhythm oscillators in nuclear receptor target genes. The high flexibility of this interaction allows Per2 to act as a coactivator or corepressor, depending on the balance of nuclear receptor activity. Several studies stated that the potential for Per2 interaction and its regulation with post-translational modification requires further experimentation. These interactions may form the basis of many of Per2's additional functions in metabolism and the brain. Other core circadian oscillators can interact with various other proteins and regulators. Per2 can directly interact with more transcriptional regulators (Di Rosa et al., 2015).

The interaction of Per2 with various proteins and circadian rhythm oscillator regulators supports the results of this study, which state that there is no significant effect of concentrations of CA extract on the expression of Per2 due to the influence of environmental factors (light, food) with nuclear receptors in cells related to various metabolisms outside the circadian rhythm including the dopaminergic system, inflammation, glucose metabolism, temperature, and fatty acid metabolism (Kim et al., 2019).

This study has a limitation in that the gene expression preparation sample uses the whole body or whole body of zebrafish larvae, so it is not specific to target organs or tissues such as SIRT1 and Per2 gene expression in the brain of zebrafish larvae. This research also does not explore specific active compounds from CA extract that improve sleep activity in insomnia events. Therefore, further research is needed to see the specific location of the SIRT1 and Per2 genes in the organs or brain tissue of zebrafish larvae in insomnia models, and it is necessary to measure levels of SIRT1 and Per2 and other genes involved in insomnia conditions. Further research is needed to determine which compounds from CA are the most effective for improving sleep activity in insomnia conditions.

CONCLUSION

Administration of *Centella asiatica extract* at a concentration of 10 µg/mL increased the expression of SIRT1 and had no effect on the expression of Per2 in zebrafish larvae of the insomnia model.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Afif Z	Andriani N	Rakhmatiar R	Holipah	Kurniawan S	Khotimah H	Nurdiana	Satriotomo I
Concepts or ideas	x	x	x	x	x	x	x	x
Design	x	x			x	x	x	x
Definition of intellectual content	x	x				x		
Literature search	x	x	x	x	x	x	x	x
Experimental studies	x	x				x	x	
Data acquisition	x	x						
Data analysis	x	x		x		x		
Statistical analysis	x	x		x	x	x		
Manuscript preparation	x	x	x	x				
Manuscript editing	x	x	x	x				
Manuscript review	x	x	x	x	x	x	x	x

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