



# Effect of *Nigella sativa* L. extract on improving memory function through reducing GSK3 $\beta$ activation and A $\beta$ 42/40 ratio

[Efecto del extracto de *Nigella sativa* L. en la mejora de la función de la memoria mediante la reducción de la activación de GSK3 $\beta$  y la relación A $\beta$ 42/40]

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## Abstract

**Context:** Alzheimer's disease (AD) is one of the biggest causes of public health problems today, especially in older, where women have a greater risk of developing AD than men. However, the current AD therapy has not been satisfactory. *Nigella sativa* (NS) has bioactive compounds of flavonoid compounds and estrogenic effects that could be alternative medicine.

**Aims:** To evaluate an extract of *N. sativa* on improving memory function through reducing GSK3 $\beta$  activation and A $\beta$ 42/40 ratio in a model of AD in rats.

**Methods:** This research was an experimental study with the post-test-only group design using 24 Wistar rats divided into eight groups. Conjugated equine estrogen (CEE) 0.018-0.0036 mg/day and NS 100, 200, and 400 mg/kg BW were administered orally two weeks before intraperitoneal AlCl<sub>3</sub> induction, then continued for up to 8 weeks. In the last five days of the study, the Morris water maze memory test was performed to analyze the expression of amyloid  $\beta$  (A $\beta$ ), neurofibrillary tangles (NFT), glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), and A $\beta$ 42/40 ratio.

**Results:** There were significant differences in the memory test ( $p = 0.000$ ), the expression A $\beta$ 40 ( $p = 0.000$ ), A $\beta$ 42 ( $p = 0.000$ ), NFT ( $p = 0.000$ ) and GSK3 $\beta$  levels ( $p = 0.000$ ). The lowest expression of A $\beta$ 40 in the NS100 group, A $\beta$ 42 in the NS400, NFT in the CEE group, and GSK3 $\beta$  levels in the NS100 group. Administration of NS increased the plasma ratio of A $\beta$ 42/40 with the highest mean in the CEE group ( $p = 0.001$ ).

**Conclusions:** The administration of NS extract affected improving memory function by decreasing the expression of A $\beta$ , NFT, GSK3 $\beta$  in ovariectomized Wistar rats supplemented with AlCl<sub>3</sub>.

**Keywords:** Alzheimer's disease; amyloid-beta; glycogen synthase kinase 3 $\beta$ ; neurofibrillary tangles; *Nigella sativa*.

## Resumen

**Contexto:** La enfermedad de Alzheimer (EA) es una de las mayores causas de problemas de salud pública en la actualidad, especialmente en las personas mayores, donde las mujeres tienen un mayor riesgo de desarrollar EA que los hombres. Sin embargo, la terapia actual contra la EA no ha sido satisfactoria. *Nigella sativa* (NS) tiene compuestos bioactivos de compuestos flavonoides y efectos estrogénicos que podrían ser una medicina alternativa.

**Objetivos:** Evaluar un extracto de *N. sativa* sobre la mejora de la función de la memoria mediante la reducción de la activación de GSK3 $\beta$  y la relación A $\beta$ 42/40 en un modelo de EA en ratas.

**Métodos:** Se realizó un estudio experimental con el diseño utilizando 24 ratas Wistar divididas en ocho grupos. Se administraron por vía oral estrógeno equino conjugado (CEE) 0,018-0,0036 mg/día y NS 100, 200 y 400 mg/kg de peso corporal dos semanas antes de la inducción intraperitoneal de AlCl<sub>3</sub>, y luego continuaron hasta 8 semanas. En los últimos cinco días del estudio, se realizó la prueba de memoria del laberinto de agua de Morris para analizar la expresión de  $\beta$  amiloide (A $\beta$ ), ovillos neurofibrilares (NFT), glucógeno sintasa cinasa 3 $\beta$  (GSK3 $\beta$ ) y la relación A $\beta$ 42/40.

**Resultados:** Hubo diferencias significativas en la prueba de memoria ( $p = 0,000$ ), la expresión A $\beta$ 40 ( $p = 0,000$ ), A $\beta$ 42 (valor  $p = 0,000$ ), NFT ( $p = 0,000$ ) y los niveles de GSK3 $\beta$  ( $p = 0,000$ ). La expresión más baja de A $\beta$ 40 en el grupo NS100, A $\beta$ 42 en el NS400, NFT en el grupo CEE y niveles de GSK3 $\beta$  en el grupo NS100. La administración de NS aumentó la proporción plasmática de A $\beta$ 42/40 con la media más alta en el grupo de CEE ( $p = 0,001$ ).

**Conclusiones:** La administración de extracto de NS afectó la mejora de la función de la memoria al disminuir la expresión de A $\beta$ , NFT, GSK3 $\beta$  en ratas Wistar ovariectomizadas suplementadas con AlCl<sub>3</sub>.

**Palabras Clave:** enfermedad de Alzheimer; beta amiloide; glucógeno sintasa cinasa 3 $\beta$ ; ovillos neurofibrilares; *Nigella sativa*.

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## INTRODUCTION

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Alzheimer's Disease (AD) is defined as dementia accompanied by a decrease in memory function, thinking, language, and learning capacity. AD's characteristics are the accumulation of amyloid-beta ( $A\beta$ ) and the formation of neurofibrillary tangles (NFT) due to the hyperphosphorylation in the brain (Wakawa et al., 2018). Prevalence in the world is around 35 million people and is predicted to double in 2030 and triple in 2050 (Martin et al., 2013).

The etiology of AD is still unclear and is related to genetic and environmental factors, including exposure to aluminum (Al) as a neurotoxin (Rather et al., 2018). AD's characteristics could be determined by measuring  $A\beta$  levels in cerebrospinal fluid (CSF) and neuroimaging using positron emission tomography (PET). Invasive examination, high price, and limited PET tools make this examination challenging to do for many people (Fandos et al., 2017). The plasma  $A\beta_{42}/40$  ratio was an alternative biomarker, although there were some contradictions from several research results (Fandos et al., 2017).

One of the most frequently used hypotheses in AD is the amyloid cascade. Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) plays a role in the amyloid cascade and is involved in the production of amyloid-beta, NFT formation, and neuronal degeneration. Increased activity of GSK-3 $\beta$  plays a role in cognitive changes in the early stages and neurodegeneration in advanced AD (Barage and Sonowane, 2015).

Women tend to experience AD more than men because many women live longer after menopause. Administration of estradiol (E2) as hormone replacement therapy (HRT) has manifested to reduce the risk of AD. Research by Gleason et al. (2015) proved no cognitive changes in women with early menopause who were supplemented with HRT. The Women's Health Initiative Memory Study (WHIMS) published that HRT is not recommended for AD therapy in women 65 or older. HRT is recommended at perimenopause or shortly after menopause (Wakawa et al., 2018). The timing of estrogen exposure as HRT is essential for AD therapy and related to the critical window hypothesis (Merlo et al., 2017).

*N. sativa* has high estrogen activity through its flavonoid and phenolic components (Yessuf, 2015). Several other studies have demonstrated that it has an estrogenic effect (Parhizkar et al., 2016). The application of thymoquinone (active compounds of NS) in AD research include *in vitro* tests on neuronal cultures with the results that thymoquinone can intercept  $A\beta$  aggregation (Norsharina et al., 2013) and defend hip-

pocampal and cortical neuronal cultures of mouse embryos against  $A\beta$ -induced toxicity and neurotoxicity (Alhebshi et al., 2019).

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## MATERIAL AND METHODS

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### Experimental animals

This research was conducted at the animal house, the Biomedical Laboratory of the Faculty of Medicine, Brawijaya University of Malang, and the Central Laboratory of Life Sciences (LSIH), Brawijaya University Malang, Malang, Indonesia.

The experimental animals used were 24 *Rattus norvegicus* strain Wistar aged 8-10 months with 200-300 grams. Experimental animals were randomly grouped into 8 groups: (1) negative control, (2) induction  $AlCl_3 \cdot 6H_2O$  70 mg/kg intraperitoneal (i.p)/day, (3) ovariectomy group (OVX), (4)  $AlCl_3 \cdot 6H_2O$  + OVX, (5)  $AlCl_3 \cdot 6H_2O$  + OVX + conjugated equine estrogen (CEE) 0.018-0.0036 mg/day, and the next 3 groups with induction  $AlCl_3 \cdot 6H_2O$  + OVX + *N. sativa* extract (NS) 100, 200 and 400 mg/kg body weight.

CEE administration in group 5 and NS at various doses in groups 6-8 were given two weeks first. In continued for another 6 weeks,  $AlCl_3$  was administered to groups 2 and 4, and concurrently with CEE or NS administration in groups 5-8.

Experimental animals were placed in individual cages. They were provided by drinking water *ad libitum* and standard feed. The cages were placed at room temperature, adjusted humidity, and normal circulation with light and dark periods of 12 hours each. In the last five days of the study, the Morris Water Maze (MWM) memory test was conducted, then the Wistar rats were sacrificed. The research was conducted after obtaining approval from the Research Ethics Commission of Brawijaya University with the reference number 043-KEP-UB-2020.

### Memory test

MWM memory test was performed to examine spatial memory in Wistar rats using a circular pool with a diameter of 150 cm, a height of 35 cm, and filled with water (height 21 cm) with a water temperature of 24-26°C. In addition, a hidden platform made of transparent glass with a diameter of 10 cm and a height of 20 cm was placed upside down in the middle of the pool.

For three consecutive days before the start of the study, the Wistar rats were accustomed to the pool for 30 seconds without a hidden platform. In the final five days of the study, Wistar rats were evaluated to

be released in the pool 4 times per day from 4 different points, and the time to reach the hidden platform was recorded.

### Nigella sativa extract

*Nigella sativa* powder was purchased and identification in Materia Medica, Batu, East Java, Indonesia. The powder was extracted with ethanol solvent by maceration. Fifty grams of NS powder was added with 150 mL of 96% ethanol and then homogenized. The solution was stored in a refrigerator at 4°C overnight. The NS extract in ethanol was then concentrated in a rotary evaporator at 35°C.

Furthermore, the extract was evaporated to obtain solid crystals. The drying product was 1000 mg then dissolved with 1 mL DMSO. The ethanol extract was then diluted to obtain a dilution fraction of 100 mg/mL (Farah, 2005).

### Immunofluorescence (IF)

Experimental animals were sacrificed under anesthesia. The brain was slowly removed. Half of the brain was put in 10% formalin buffer for IF. The other was stored in a clean container in cold storage at -20°C (for ELISA examination) (Schneider Gasser et al., 2006).

Brain organs were cut coronally, 7 mm from the bregma, then performed a 6 µm cryostat sectioning of brains and attached to a poly-L-lysine slide. The obtained slides were processed according to the guidelines of the existing reagent kit. The primary antibodies used were abeta40 antibody (Novusbio, NBP1-44047), Aβ42 (Bioss, BSM-0107M), and NFT (Thermo Fisher, MN1020). Finally, slides were dried, mounted with Entellan (Merck), and then observed in the dark-room using an Olympus IX71 binocular microscope (Fahmi et al., 2020).

### ELISA

To determine the levels of Aβ42, Aβ40 in plasma and GSK3β in brain tissue, a quantitative sandwich immunoassay technique was applied according to the protocol from the kit. Abeta40 ELISA kit, catalog number MBS765853, sensitivity <46,875 pg/mL, with detectable results ranging from 78.125–5000 pg/mL. Abeta1-42 ELISA kit, catalog number MBS7265579 sensitivity 1pg/mL. GSK3β ELISA kit (ab205710), with 5 g/mL sensitivity.

### Statistical analysis

The observations were recapitulated in a descriptive table followed by normality and homogeneity tests. The difference significance of memory tests in groups was tested using Multivariate Analysis of

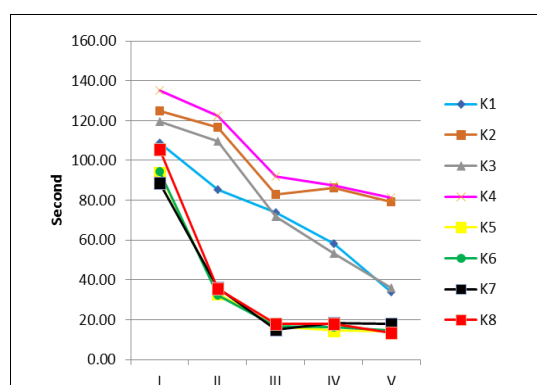
Variance (MANOVA) while other variables were tested using ANOVA. If the MANOVA and ANOVA tests were significant, post hoc Bonferroni tests were continued. Data were analyzed using SPSS 24. The results are presented as mean ± SD. P<0.05 was considered statistically significant.

## RESULTS

### Memory test

Fig. 1 showed that the time to reach the hidden platform was lowered in group 5 (K5) (treat with AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + CEE) coincides with group 6-8 (K6, K7, K8) (treat with AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX+ NS) 100, 200 and 400), while group 4 (K4) (treat with AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX ) demonstrates the most prolonged time compared to all groups.

Based on the MANOVA test F<0.05), values were less than 0.05. From the *post hoc* Bonferroni test, it was found that group 2 (K2) (treated with AlCl<sub>3</sub>.6H<sub>2</sub>O) was not significantly different from group 4 on all days. Group 3 (K3) (treat with OVX) was not significantly different from group 1 (K1) on all days. Group 5 was significantly different from groups 1-4 on days 2-5, and no different from groups 6-8 on days 1, 3-5.



**Figure 1.** Effect of NS administration on the WMM Memory Test on Wistar rats.

Data represent mean ± SD (n = 24)

Observations on days I, II, III, IV and V K1: normal group, K2: AlCl<sub>3</sub>.6H<sub>2</sub>O 70 mg/kg ip/day, K3: OVX group, K4: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX, K5: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + CEE 0.018-0.0036 mg/day, K6: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS100 mg/kg BW, K7: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS200 mg/kg BW and K8: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS400 mg/kg BW.

### Xpressions A, 40, 42 and NFT

This study examined the Wistar rat brains that focused on the CA1 area located in the hippocampus. The calculation of the expression area of Aβ40, Aβ42, and NFT in the hippocampus was carried out using the ImageJ application. The lowest expression of Aβ40 was found in group 6, Aβ42 in group 8, and NFT in group 5. The mean expression in groups 5-8 was sig-

nificantly different from groups 2-4 (see Table 1 and Fig. 2).

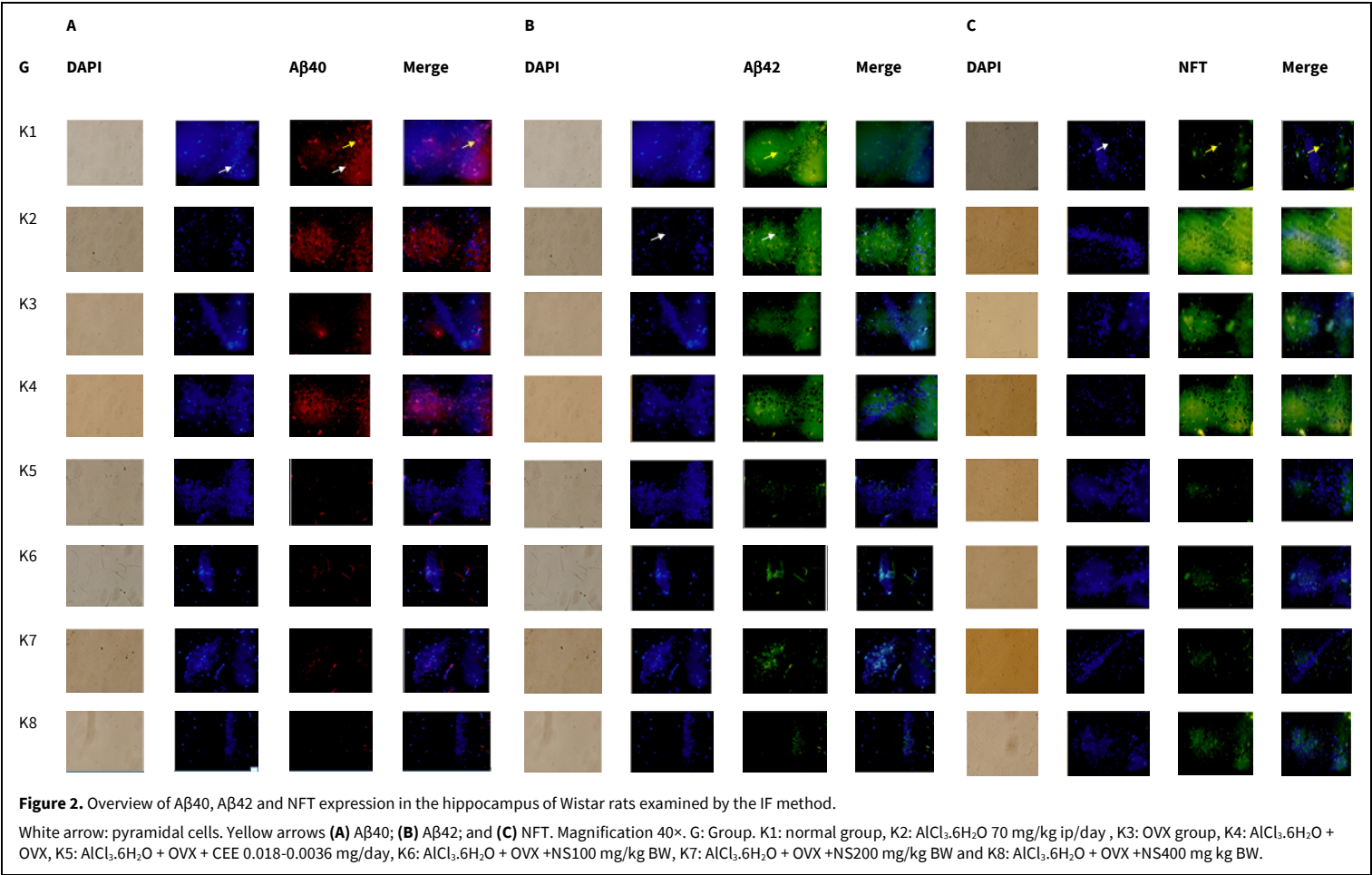
Ratio Aβ42/Aβ40 in plasma

The highest plasma concentrations of Aβ42 and Aβ40 were found in group 4. Otherwise, the lowest levels of plasma Aβ40 were found in group 5 and plasma Aβ42 in group 6. The highest Aβ42/Aβ40 ratio was found in group 5. The lowest was in group 4, followed by groups 1 and 3. Although the ratio Aβ42/Aβ40 tended to decrease in groups 6-8, this ratio was higher than groups 1-4. From the *post hoc*

Bonferroni test, there were significant differences between group 5 and groups 1-4 and 8. There was no significant difference between groups 6 and 7 with all groups (see Table 2).

GSK3 activation

From this study, the lowest concentration of GSK3β was obtained by group 6, followed by groups 7 and 8, while the highest concentration was in groups 4 and 2 (Table 3). There were no significant differences, only between K2 and K4 and K5 with K7 and 8.





**Table 1.** Effects of NS administration on the expression of A $\beta$ 40, A $\beta$ 42, and NFT in the hippocampus of Wistar rats.

Group	Integrated density (pixel <sup>2</sup> )					
	A $\beta$ 40 (mean $\pm$ SD)	p value	A $\beta$ 42 (mean $\pm$ SD)	p value	NFT (mean $\pm$ SD)	p value
K1	428.54 $\pm$ 63.61		416.58 $\pm$ 205.71		378.55 $\pm$ 176.26	
K2	1045.66 $\pm$ 95.74		1848.52 $\pm$ 590.05		1735.79 $\pm$ 438.78	
K3	721.35 $\pm$ 170.58		1877.34 $\pm$ 566.283		995.48 $\pm$ 593.93	
K4	1126.09 $\pm$ 74.78	0.000*	2055.28 $\pm$ 886.64	0.000*	2008.51 $\pm$ 495.73	0.000*
K5	317.53 $\pm$ 86.32		502.81 $\pm$ 24.67		157.20 $\pm$ 53.13	
K6	178.73 $\pm$ 100.48		310.66 $\pm$ 193.73		259.71 $\pm$ 83.52	
K7	292.05 $\pm$ 66.48		318.95 $\pm$ 100.51		171.81 $\pm$ 59.52	
K8	231.73 $\pm$ 115.16		247.03 $\pm$ 82.63		579.84 $\pm$ 305.97	

\*p $\leq$ 0.05 statistically significant (n = 24). K1: normal group, K2: AlCl<sub>3</sub>.6H<sub>2</sub>O 70 mg/kg ip/day, K3: OVX group, K4: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX, K5: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + CEE 0.018-0.0036 mg/day, K6: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS100 mg/kg BW, K7: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS200 mg/kg BW and K8: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS400 mg/kg BW.

**Table 2.** Effects of NS administration on concentrations of A $\beta$ 42, A $\beta$ 40 and the ratio of A $\beta$ 42/A $\beta$ 40 in Wistar rats.

Group	A $\beta$ 40 (pg/mL)	A $\beta$ 42 (pg/mL)	Ratio A $\beta$ 42/A $\beta$ 40 (mean $\pm$ SD)	p value
K1	172.50	925.02	5.50 $\pm$ 1.09	
K2	165.00	911.98	5.91 $\pm$ 1.91	
K3	180.00	924.70	5.74 $\pm$ 2.47	
K4	389.49	1339.42	4.51 $\pm$ 4.64	0.001*
K5	55.00	884.79	16.94 $\pm$ 1.37	
K6	81.67	819.79	10.22 $\pm$ 2.96	
K7	95.83	822.60	9.33 $\pm$ 3.26	
K8	112.50	861.46	8.31 $\pm$ 1.36	

\*p $\leq$ 0.05. K1: normal group (n = 24). K2: AlCl<sub>3</sub>.6H<sub>2</sub>O 70 mg/kg ip/day, K3: OVX group, K4: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX, K5: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + CEE 0.018-0.0036 mg/day, K6: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS100 mg/kg BW, K7: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS200 mg/kg BW and K8: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS400 mg/kg BW.

**Table 3.** Effects of NS administration on GSK-3 $\beta$  concentration in the brain of Wistar rats.

Group	Concentration GSK 3 $\beta$ ( $\mu$ g/mL)	
	(mean $\pm$ SD)	p value
K1	374.14 $\pm$ 9.28	
K2	545.47 $\pm$ 11.96	
K3	264.06 $\pm$ 10.15	
K4	553.29 $\pm$ 17.01	0.000*
K5	217.91 $\pm$ 13.12	
K6	163.17 $\pm$ 7.24	
K7	197.91 $\pm$ 5.02	
K8	199.96 $\pm$ 2.69	

\*p $\leq$ 0.05. K1: normal group (n = 24). K2: AlCl<sub>3</sub>.6H<sub>2</sub>O 70 mg/kg ip/day, K3: OVX group, K4: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX, K5: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + CEE 0.018-0.0036 mg/day, K6: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS100 mg/kg BW, K7: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS200 mg/kg BW and K8: AlCl<sub>3</sub>.6H<sub>2</sub>O + OVX + NS400 mg/kg BW.

## DISCUSSION

### Memory test

This MWM memory test is quite simple to perform on numerous experimental animal models of AD and involves the function of the hippocampus -the area first affected by AD (Petraseket al., 2016). Hypoestrogen conditions accompanied by  $\text{AlCl}_3$  induction in this study demonstrated worse memory and cognitive than other groups. There was some research evidence that NS has beneficial effects on memory and learning. These studies demonstrate that long-term and short-term administration of NS oil was effective on working memory, especially short-term memory in Wistar rats tested using the radial arm maze test (Safitri and Andriana, 2017).

*N. sativa* oil can repair damage to spatial and non-spatial working memory in rats tested using the T-maze alternation task and object recognition test (Sahak et al., 2013). In addition, long-term administration of NS would improve memory and learning processes in rats by increasing serotonin (5-HT) levels in the brain (Rhandawa and Alenazi, 2016) and decreasing 5-HT degradation (Perveen, 2014). A trial of 20 volunteers women aged 55-57 years old supplemented with 500 mg capsules containing NS two times a day for nine weeks found improvements in memory, attention, and cognitive function (Bin Sayeed et al., 2013).

Topcagic et al. (2017) reported that quercitrin, quercetin, and kaempferol were the main phenolic constituents of NS. Administration of 40 mg/kg quercetin orally for 16 weeks was known to improve memory and learning ability, reduce senile plaque, reduce mitochondrial dysfunction as indicated by increased mitochondrial membrane potential, ATP levels, decreased ROS production, increased AMP-activated protein kinase (AMPK) activity in the APP<sup>swe</sup>/PS1<sup>dE9</sup> transgenic mice AD model (Wang et al., 2014).

Quercetin was involved in the molecular pathway of BDNF signaling by activating BDNF-TrKB binding in cell membranes. The binding between TrKB and mBDNF will activate (1) ERK1/2 pathway, which will activate Bcl-2 (2) PI3K/Akt pathway, which will activate mTOR (3) PLC $\gamma$ /IP3 pathway (phospholipase C gamma/inositol triphosphate), which will regulate homeostasis calcium. The activation of the ERK1/2 and PI3K/Akt pathways will help maintain cell viability, synaptic plasticity and prevent apoptosis (Numakawa and Odaka, 2021).

### Expressions A $\beta$ 40, A $\beta$ 42 and NFT

A $\beta$  peptides or their aggregates were cleared from

the brain through several pathways, one of which was transcytosis through the blood-brain barrier (BBB) through receptor intermediaries. In AD conditions, BBB dysfunction occurred, resulting in increased BBB permeability, microbleeds, decreased expression of tight junctions, and accumulation of blood cell products in perivascular, and degeneration of pericytic and endothelial cells (Sweeney et al., 2018).

No studies in AD experimental animals have analyzed the possibility that E2 administration could improve BBB function. Studies in other disease models have shown that E2 administration could protect BBB function (Xiao et al., 2018).

Studies in ovariectomized rats or via aromatase inhibition demonstrated increased degeneration of hippocampal neurons, increased A $\beta$  in AD animal models, increased loss of dopaminergic neurons. In contrast, administration of E2 reduced neuronal damage in brain injury rat models, cerebral ischemia, and decreased the incidence of seizures. The neuroprotective effect of E2 was also detected in Arko mice and those supplemented with aromatase inhibitors (Overk et al., 2012). Alhebshi et al. (2019) reported that thymoquinone (TQ) as the main component of NS could prevent the formation of A $\beta$ , decrease A $\beta$  neurotoxicity in hippocampal and cortical neurons. Administration of TQ prevented neurotoxicity due to A $\beta$ 1-42 and inhibited oxidative stress and mitochondrial potential membrane depolarization (Samarghndian et al., 2018).

The primary function of tau protein was to bind and stabilize microtubules by copolymerizing with tubulin. Under dephosphorylated conditions, tau protein is more efficient in microtubule formation. Tau phosphorylation is a dynamic process that depends on the interaction of numerous kinases and phosphatases. The kinases involved in the abnormal phosphorylation of tau protein *in vivo* were GSK3 $\beta$  and CDK-5, while the phosphatases involved in the dephosphorylation of tau were protein phosphatase 1 (PP1), PP2A, PP2B, and PP5. In AD, PP2A activity was reduced by 50%, causing tau hyperphosphorylation, memory deficits, and triggering increased GSK3 $\beta$  activity (Martin et al., 2013).

Low levels of E and P due to the aging process increased tau phosphorylation in the rat hippocampus. *In vitro* studies by administering E2 to neuroblastoma cells and rat cortical neurons showed tau dephosphorylation as measured using the Tau-1 antibody. In another study, administration of E2 for 24 hours decreased okadaic acid-induced tau hyperphosphorylation in neuroblastoma cells. The impact of E administration on tau phosphorylation was thought to be via the Akt pathway (Azcoitia et al., 2019).

Quercetin and kaempferol are two types of flavonoids in NS. Flavonoids were proved to cross the BBB. The potential of NS and its metabolites to cross the BBB was predicted in an *in silico* test. From this analysis, ten metabolites were obtained with an analysis number >0.95, with an average analysis value of 0.91. The highest value (0.9890) was found in nige-glamine compounds (Andriana et al., 2021). Thymoquinone and thymohydroquinone (THQ) exist in a glycosidic form that binds to aglycones that allow them to cross the BBB (Jukic et al., 2007). Administration of quercetin to triple transgenic AD mice was reported to significantly reduce extracellular beta amyloidosis, astrogliosis, tauopathy, and reduced microgliosis in the amygdala and hippocampus. Quercetin also significantly decreased PHF, A $\beta$ 1-40, A $\beta$ 1-42, and BACE-1 mediating APP breakdown (Zaplatic et al., 2019). The effects of phytoestrogens on NS were not yet fully understood. There may be two mechanisms, through dependent ER and independent ER. Many studies have demonstrated that phytoestrogens are bound to the ER and showed significant E effects in humans, animals, and cell cultures (Liu et al., 2004; Banu et al., 2006; Hu et al., 2007; Suzuki et al., 2008).

### Ratio A $\beta$ 42/A $\beta$ 40 in plasma

Identification of cerebral amyloidosis using amyloid-beta levels in CSF and PET has a high diagnostic and prognostic rate and could be used to demonstrate early pathological signs of AD. Blood-based biomarkers were expected to be a solution to the global threat of the AD epidemic (Vergallo et al., 2019).

Under physiological conditions, A $\beta$  peptides could be found in plasma membranes, blood, and CSF. In general, the concentration of A $\beta$ 40 in body fluids was higher than that of A $\beta$ 42. Many studies have shown that A42 was more toxic and was directly related to AD pathology (Qiu et al., 2015). Liu et al. (2020) found that the levels of A $\beta$ 1-40 and A $\beta$ 1-42 in plasma were 10-20% lower than in CSF, so a susceptible test was needed to detect the concentrations of A $\beta$ 1-40 and A $\beta$ 1-42 in plasma accurately.

Several investigators have reported that low plasma A $\beta$ 42/A $\beta$ 40 ratio was associated with a higher risk of dementia and more significant cognitive decline. Low plasma A $\beta$ 42/A $\beta$ 40 ratio was associated with a higher risk of dementia and more significant cognitive decline (Chouraki et al., 2015). Detection of pathological changes and accumulation of A $\beta$  in the brain was associated with increased uptake (18F) of Flortaucipirtau- a PET marker for AD (Risacher et al., 2019). Other investigators reported no association between the ratio of A $\beta$ 42/A $\beta$ 40 in plasma and AD (Lovheimet et al., 2017). There were numerous A $\beta$ s in the brain, but the predominant ones were A $\beta$ 1-42,

pyroglutamate-modified A $\beta$ 3-42, and A $\beta$ 4-42, but only A $\beta$ 1-42 secreted by cells could be detected. A $\beta$ 1-40 was frequently found in the brains of AD patients as well, but A $\beta$ 1-40 was more reflective of A $\beta$  deposits in blood vessel walls (Vergallo et al., 2019). Factors that caused the absence of a relationship between plasma amyloid and CSF were amyloid circulating in peripheral blood produced by platelets. Platelets could express and process APP. APP could be degraded by circulating enzymes or metabolized in the liver (Vergallo et al., 2019).

The Alzheimer's Disease Neuroimaging Initiative (ADNI) also reported that plasma A $\beta$  levels could not be used to distinguish AD patients from controls and amyloid-positive individuals from amyloid-negative individuals (Swaminathan et al., 2014). Australian Imaging Biomarkers and Lifestyle (AIBL) have reported that plasma A $\beta$  levels were influenced by inflammation and renal function. AIBL also reported that absolute levels of A $\beta$ 40 or A $\beta$ 42 were not associated with AD or A $\beta$  pathology in the neocortex (Rembach et al., 2014).

These contradictory conditions reflected the complexity of plasma A $\beta$  measurements. Several studies have shown that the plasma ratio of A $\beta$ 42/A $\beta$ 40 could be used as a screening tool to detect neuropathology in individuals at risk of AD (Risacher et al., 2019) and as an initial screening tool to monitor the management of AD patients. If a low ratio was obtained, it should be continued with CSF analysis or neuroimaging using PET (Pérez-Grijalba et al., 2019).

### GSK3 $\beta$ activation

Over-activation of GSK3 $\beta$  in AD causes long-term potentiation (LTP) in the hippocampus, associated with changes in synaptic transmission efficiency and dependent on NMDA receptors. Studies in transgenic mice have demonstrated that overexpression of GSK3 $\beta$  leads to neurodegeneration and tau phosphorylation in AD-associated phosphor epitope (Grigoryan, 2014). This study was in line with Xiao et al. (2017), which reported that hypoestrogenic conditions due to OVX increased mRNA levels from ER  $\alpha$  and  $\beta$ , and the level of GSK3 $\beta$  in the cerebral cortex.

The role of E and NS in this study can be seen from the low concentration of GSK3 $\beta$  in groups 5-8. There was no difference between the administration of CEE and NS at doses of 200 and 400 mg/kg BW. A possible mechanism of this action was the neuroprotective effect of E2 involving both genomic and non-genomic mechanisms directly or through interactions with other growth factor signal transduction cascades. Estrogen triggers PI3K/AKT, MAPK/ERK and inhibits Jun amino-terminal kinase. The effect of ER trig-

gering PI3K/AKT is the inhibition of GSK3 $\beta$ , causing phosphorylation of CREB and increasing BDNF expression. This results in the activation of several protective E2 signals, including up-regulation, anti-apoptosis, and down-regulation of pro-inflammatory and pro-apoptotic genes. The level of E2 as neuroprotective changes with age depending on IGF1 signaling. In another study, TQ was administered to rats induced with A $\beta$ 1-42 via a micro-osmotic pump to the hippocampus. This study found that TQ reduced A $\beta$  expression, tau phosphorylation, and BACE-1 protein. However, administration of TQ had no therapeutic effect on AKT/GSK3 $\beta$  nor the MAPK signaling pathway (Elibol et al., 2020).

Quercetin could inhibit GSK3 $\beta$  activity and tau hyperphosphorylation by modulating the PI3K/Akt/GSK3 $\beta$  pathway. Although quercetin exhibits various neuroprotective abilities *in vitro* and *in vivo*, its application in the pharmaceutical field is limited due to its low solubility, bioavailability, and stability (Suganthi et al., 2016).

## CONCLUSION

Administration of *Nigella sativa* extracts at a dose of 100 mg/kg body weight affected memory improvement, increased A $\beta$ 42/40 ratio, decreased GSK3 $\beta$  activity, expression of A $\beta$ 40, A $\beta$ 42, and NFT in the hippocampus. There was no difference in the effect of CEE and NS administration. Neuroprotective effects of NS administration may be possible through an estrogen-like effect, the mechanism of which remains to be studied further. It cannot be separated from the effect of each component and the effect of secondary metabolites together on memory improvement through anti-inflammatory, immunomodulatory, and antioxidant effects, which were not investigated in this study.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Andriana K	Nurdiana	Barlianto W	Wiyasa IWA	Rahayu M
Concepts or ideas	x		x	x	
Design	x	x			x
Definition of intellectual content		x	x		x
Literature search	x			x	
Experimental studies		x		x	x
Data acquisition	x	x			
Data analysis	x		x		
Statistical analysis	x		x	x	
Manuscript preparation	x				
Manuscript editing	x	x	x	x	x
Manuscript review	x	x	x	x	x

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