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*Original Article*

# Effect of *Nigella sativa* L. extract on improving memory function through reducing GSK3β activation and Aβ42/40 ratio

[Efecto del extracto de *Nigella sativa* L. en la mejora de la función de la memoria mediante la reducción de la activación de GSK3β y la relación Aβ42/40]

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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 GSK3β activation and Aβ42/40 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 $AlCl_3$ 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 β (Aβ), neurofibrillary tangles (NFT), glycogen synthase kinase 3β (GSK3β), and Aβ42/40 ratio.

*Results*: There were significant differences in the memory test (p = 0.000), the expression Aβ40 (p = 0.000), Aβ42 (p = 0.000), NFT (p = 0.000) and GSK3β levels (p = 0.000). The lowest expression of Aβ40 in the NS100 group, Aβ42 in the NS400, NFT in the CEE group, and GSK3β levels in the NS100 group. Administration of NS increased the plasma ratio of Aβ42/40 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 Aβ, NFT, GSK3β in ovariectomized Wistar rats supplemented with $AlCl_3$.

*Keywords*: Alzheimer's disease; amyloid-beta; glycogen synthase kinase 3β; 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 GSK3β y la relación Aβ42/40 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 $AlCl_3$, 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 β amiloide (Aβ), ovillos neurofibrilares (NFT), glucógeno sintasa cinasa 3β (GSK3β) y la relación Aβ42/40.

*Resultados*: Hubo diferencias significativas en la prueba de memoria (p = 0,000), la expresión Aβ40 (p = 0,000), Aβ42 (valor p = 0,000), NFT (p = 0,000) y los niveles de GSK3β (p = 0,000). La expresión más baja de Aβ40 en el grupo NS100, Aβ42 en el NS400, NFT en el grupo CEE y niveles de GSK3β en el grupo NS100. La administración de NS aumentó la proporción plasmática de Aβ42/40 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 Aβ, NFT, GSK3β en ratas Wistar ovariectomizadas suplementadas con $AlCl_3$.

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

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## INTRODUCTION

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 (Aβ) 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 Aβ 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 Aβ42/40 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-3β (GSK-3β) 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-3β 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 Aβ aggregation (Norsharina et al., 2013) and defend hippocampal and cortical neuronal cultures of mouse embryos against Aβ-induced toxicity and neurotoxicity (Alhebshi et al., 2019).

## MATERIAL AND METHODS

### 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 $AlCl_3.6H_2O$ 70 mg/kg intraperitoneal (i.p)/day, (3) ovariectomy group (OVX), (4) $AlCl_3.6H_2O$ + OVX, (5) $AlCl_3.6H_2O$ + OVX + conjugated equine estrogen (CEE) 0.018-0.0036 mg/day, and the next 3 groups with induction $AlCl_3.6H_2O$ + 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 darkroom 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 SPPS 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 $AlCl_3.6H_2O$ + OVX + CEE) coincides with group 6-8 (K6, K7, K8) (treat with $AlCl_3.6H_2O$ + OVX+ NS) 100, 200 and 400), while group 4 (K4) (treat with $AlCl_3.6H_2O$ + 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 $AlCl_3.6H_2O$) 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.

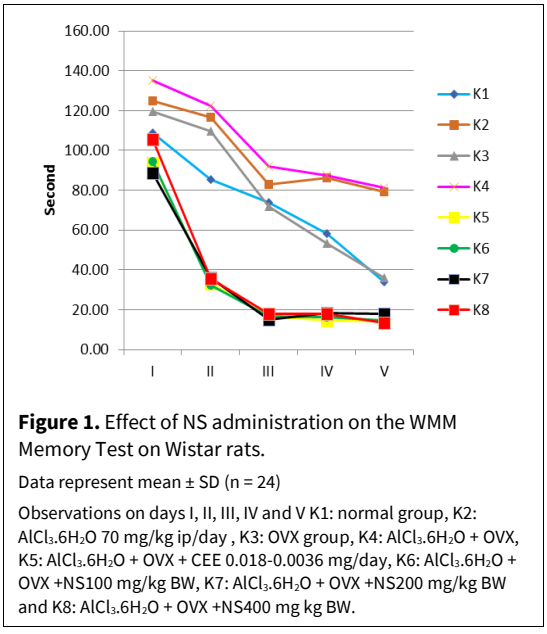


**Figure 1.** Effect of NS administration on the WMM Memory Test on Wistar rats.

Data represent mean ± SD (n = 24)

Observations on days I, II, III, IV and V K1: normal group, K2: $AlCl_3.6H_2O$ 70 mg/kg ip/day , K3: OVX group, K4: $AlCl_3.6H_2O$ + OVX, K5: $AlCl_3.6H_2O$ + OVX + CEE 0.018-0.0036 mg/day, K6: $AlCl_3.6H_2O$ + OVX +NS100 mg/kg BW, K7: $AlCl_3.6H_2O$ + OVX +NS200 mg/kg BW and K8: $AlCl_3.6H_2O$ + 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.

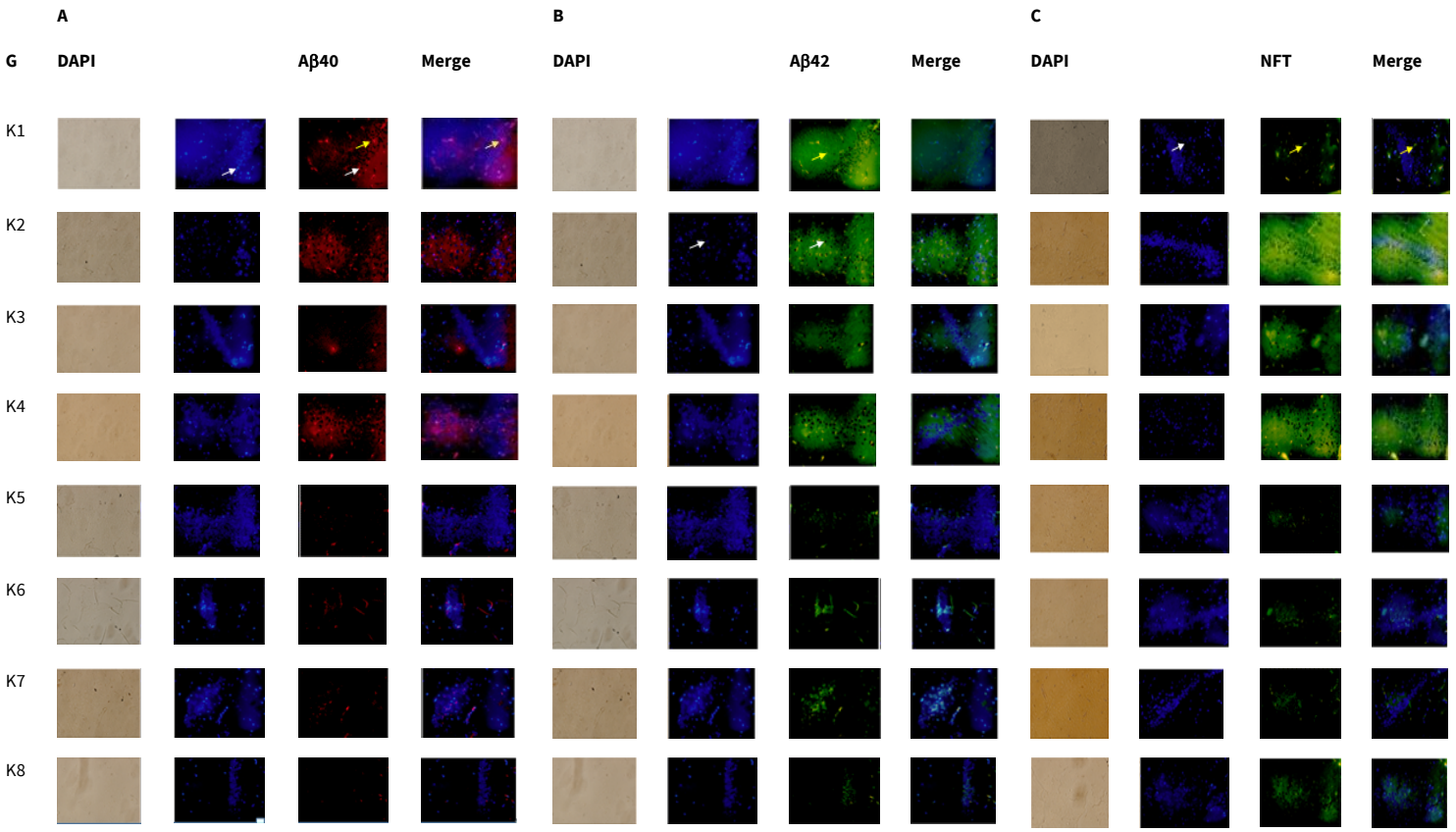


**Figure 2.** Overview of Aβ40, Aβ42 and NFT expression in the hippocampus of Wistar rats examined by the IF method.

White arrow: pyramidal cells. Yellow arrows **(A)** Aβ40; **(B)** Aβ42; and **(C)** NFT. Magnification 40×. G: Group. K1: normal group, K2: $AlCl_3.6H_2O$ 70 mg/kg ip/day , K3: OVX group, K4: $AlCl_3.6H_2O$ + OVX, K5: $AlCl_3.6H_2O$ + OVX + CEE 0.018-0.0036 mg/day, K6: $AlCl_3.6H_2O$ + OVX +NS100 mg/kg BW, K7: $AlCl_3.6H_2O$ + OVX +NS200 mg/kg BW and K8: $AlCl_3.6H_2O$ + OVX +NS400 mg kg BW.

**Table 1.** Effects of NS administration on the expression of Aβ40, Aβ42, and NFT in the hippocampus of Wistar rats.

| Group | Integrated density (pixel$^2$) | | | | | |
|---|---|---|---|---|---|---|
| | Aβ40 (mean ± SD) | p value | Aβ42 (mean ± SD) | p value | NFT (mean ±SD) | p value |
| K1 | 428.54 ± 63.61 | | 416.58 ± 205.71 | | 378.55 ± 176.26 | |
| K2 | 1045.66 ± 95.74 | | 1848.52 ± 590.05 | | 1735.79 ± 438.78 | |
| K3 | 721.35 ± 170.58 | | 1877.34 ± 566.283 | | 995.48 ± 593.93 | |
| K4 | 1126.09 ± 74.78 | 0.000* | 2055.28 ± 886.64 | 0.000* | 2008.51 ± 495.73 | 0.000* |
| K5 | 317.53 ± 86.32 | | 502.81 ± 24.67 | | 157.20 ± 53.13 | |
| K6 | 178.73 ± 100.48 | | 310.66 ± 193.73 | | 259.71 ± 83.52 | |
| K7 | 292.05 ± 66.48 | | 318.95 ± 100.51 | | 171.81 ± 59.52 | |
| K8 | 231.73 ± 115.16 | | 247.03 ± 82.63 | | 579.84 ± 305.97 | |

*p≤0.05 statistically significant (n = 24). K1: normal group, K2: $AlCl_3.6H_2O$ 70 mg/kg ip/day , K3: OVX group, K4: $AlCl_3.6H_2O$ + OVX, K5: $AlCl_3.6H_2O$ + OVX + CEE 0.018-0.0036 mg/day, K6: $AlCl_3.6H_2O$ + OVX +NS100 mg/kg BW, K7: $AlCl_3.6H_2O$ + OVX +NS200 mg/kg BW and K8: $AlCl_3.6H_2O$ + OVX +NS400 mg kg BW.

**Table 2.** Effects of NS administration on concentrations of Aβ42, Aβ40 and the ratio of Aβ42/Aβ40 in Wistar rats.

| Group | Aβ40 (pg/mL) | Aβ42 (pg/mL) | Ratio Aβ42/Aβ40 (mean ± SD) | p value |
|---|---|---|---|---|
| K1 | 172.50 | 925.02 | 5.50 ± 1.09 | |
| K2 | 165.00 | 911.98 | 5.91 ± 1.91 | |
| K3 | 180.00 | 924.70 | 5.74 ± 2.47 | |
| K4 | 389.49 | 1339.42 | 4.51 ± 4.64 | 0.001* |
| K5 | 55.00 | 884.79 | 16.94 ± 1.37 | |
| K6 | 81.67 | 819.79 | 10.22 ± 2.96 | |
| K7 | 95.83 | 822.60 | 9.33 ± 3.26 | |
| K8 | 112.50 | 861.46 | 8.31 ± 1.36 | |

*p<0.05. K1: normal group (n = 24). K2: $AlCl_3.6H_2O$ 70 mg/kg ip/day , K3: OVX group, K4: $AlCl_3.6H_2O$ + OVX, K5: $AlCl_3.6H_2O$ + OVX + CEE 0.018-0.0036 mg/day, K6: $AlCl_3.6H_2O$ + OVX +NS100 mg/kg BW, K7: $AlCl_3.6H_2O$ + OVX +NS200 mg/kg BW and K8: $AlCl_3.6H_2O$ + OVX +NS400 mg kg BW.

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

| Group | Concentration GSK 3β (µg/mL) (mean ± SD) | p value |
|---|---|---|
| K1 | 374.14 ± 9.28 | |
| K2 | 545.47 ± 11.96 | |
| K3 | 264.06 ± 10.15 | |
| K4 | 553.29 ± 17.01 | 0.000* |
| K5 | 217.91 ± 13.12 | |
| K6 | 163.17 ± 7.24 | |
| K7 | 197.91 ± 5.02 | |
| K8 | 199.96 ± 2.69 | |

*p<0.05. K1: normal group (n = 24). K2: $AlCl_3.6H_2O$ 70 mg/kg ip/day , K3: OVX group, K4: $AlCl_3.6H_2O$ + OVX, K5: $AlCl_3.6H_2O$ + OVX + CEE 0.018-0.0036 mg/day, K6: $AlCl_3.6H_2O$ + OVX +NS100 mg/kg BW, K7: $AlCl_3.6H_2O$ + OVX +NS200 mg/kg BW and K8: $AlCl_3.6H_2O$ + 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 $AlCl_3$ 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 APPswe/PS1dE9 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) PLCγ/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 Aβ40, Aβ42 and NFT

Aβ 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 Aβ 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 Aβ, decrease Aβ neurotoxicity in hippocampal and cortical neurons. Administration of TQ prevented neurotoxicity due to Aβ1-42 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 GSK3β 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 GSK3β 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 okaidic 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 nigeglanine 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, Aβ1-40, Aβ1-42, 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 Aβ42/Aβ40 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, Aβ peptides could be found in plasma membranes, blood, and CSF. In general, the concentration of Aβ40 in body fluids was higher than that of Aβ42. 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 Aβ1-40 and Aβ1-42 in plasma were 10-20% lower than in CSF, so a susceptible test was needed to detect the concentrations of Aβ1-40 and Aβ1-42 in plasma accurately.

Several investigators have reported that low plasma Aβ42/Aβ40 ratio was associated with a higher risk of dementia and more significant cognitive decline. Low plasma Aβ42/Aβ40 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 Aβ 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 Aβ42/Aβ40 in plasma and AD (Lovheimet et al., 2017). There were numerous Aβs in the brain, but the predominant ones were Aβ1-42, pyroglutamate-modified Aβ3-42, and Aβ4-42, but only Aβ1-42 secreted by cells could be detected. Aβ1-40 was frequently found in the brains of AD patients as well, but Aβ1-40 was more reflective of Aβ 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 Aβ 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 Aβ levels were influenced by inflammation and renal function. AIBL also reported that absolute levels of Aβ40 or Aβ42 were not associated with AD or Aβ pathology in the neocortex (Rembach et al., 2014).

These contradictory conditions reflected the complexity of plasma Aβ measurements. Several studies have shown that the plasma ratio of Aβ42/Aβ40 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).

**GSK3β activation**

Over-activation of GSK3β 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 GSK3β 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 α and β, and the level of GSK3β in the cerebral cortex.

The role of E and NS in this study can be seen from the low concentration of GSK3β 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 GSK3β, 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 Aβ1-42 via a micro-osmotic pump to the hippocampus. This study found that TQ reduced Aβ expression, tau phosphorylation, and BACE-1 protein. However, administration of TQ had no therapeutic effect on AKT/GSK3β nor the MAPK signaling pathway (Elibol et al., 2020).

Quercetin could inhibit GSK3β activity and tau hyperphosphorylation by modulating the PI3K/Akt/GSK3β 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 (Suganthy et al., 2016).

## CONCLUSION

Administration of *Nigella sativa* extracts at a dose of 100 mg/kg body weight affected memory improvement, increased Aβ42/40 ratio, decreased GSK3β activity, expression of Aβ40, Aβ42, 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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## REFERENCES

Alhebshi AH, Gotoh M, Suzuki I (2019) Neuroprotective efficacy of thymoquinone against amyloid beta-induced neurotoxicity in human induced pluripotent stem cell-derived cholinergic neurons. Bioch Biophys Rep 3(17): 122–126.

Andriana K, Nurdiana, Barlianto W, Arsana IWW, Rahayu M (2021) *In silico* prediction of potential compounds of *Nigella sativa* as aromatase agonists and ability to cross the blood-brain barrier for treatment of Alzheimer's disease. Indian J Forensic Med Toxicol 15(2): 267–275.

Azcoitia I, George E, Luiz M, Garcia-Segura LM (2019) Molecular mechanisms and cellular events involved in the neuroprotective actions of estradiol. Analysis of sex differences. Front Neuroendocrinol 55: 100787.

Banu J, Bhattacharya A, Rahman M, O'Shea M, Fernandes G (2006) Effects of conjugated linoleic acid and exercise on bone mass in young male Balb/c mice. Lipids Health Dis 23: 5–7.

Barage SH, Sonawane KD (2015) Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. Neuropeptides 52: 1–18.

Bin Sayeed MS, Asaduzzaman M, Morshed H, Hossain MM, Fahim KM, Rahman MR (2013) The effect of *Nigella sativa* Linn. seed on memory, attention and cognition in healthy human volunteers. J Ethnopharmacol 148: 780–786.

Chouraki V, Beiser A, Younkin L, Preis SR, Weinstein G, Hansson O, Skoog I, Lambert JC, Au R, Launer L, Wolf PA, Younkin S, Seshadri S (2015) Plasma amyloid-beta and risk of Alzheimer's disease in the Framingham Heart Study. Alzheimers Dement 11: 249–257.

Elibol B, Beker M, Terzioglu-Usak S, Dalli T, Kilic U (2020) Thymoquinone administration ameliorates Alzheimer's disease-like phenotype by promoting cell survival in the hippocampus of amyloid beta1–42 infused rat model. Phytomed 79: 153324.

Fahmi AF, Tri AE, Rachmi E (2020) Modul Dry Workshop Teknik dan Analisis Citra Digital Immunofluorescence. Fakultas Kedoktran, Universitas Brawijaya Malang, Indonesia.

Fandos N, Pérez-Grijalba V, Pesini P, Olmos S, Bossa M, Villemagne VL (2017) Plasma amyloid β 42/40 ratios as biomarkers for amyloid β cerebral deposition in cognitively normal individuals. Alzheimers Dement 8: 179–187.

Farah IO (2005) Assessment of cellular responses to oxidative stress using MCF-7 breast cancer cells, black seed (*N. sativa* L.) extracts and $H_2O_2$. Int J Environ Res Public Health 2(3): 411–419.

Gleason CE, Dowling NM, Wharton W, Manson JE, Miller VM, Atwood CS (2015) Effects of hormone therapy on cognition and mood in recently postmenopausal women: Findings from the randomized, controlled KEEPS-cognitive and affective study. PLoS Med 12: e1001833

Grigoryan GA (2014) The role of glycogen synthase kinase 3 in the mechanisms of learning and memory. Neurosci Behav Physio 44(9): 1051–1058.

Hu Y, Hou TT, Xin HL, Zhang QY, Zheng HC, Rahman K, Qin LP (2007) Estrogen-like activity of volatile components from *Vitex rotundifolia* L. Indian J Med Res 126(1): 68–72.

Jukic M, Politeo O, Maksimovic M, Milos M, Milos M (2007) *In vitro* acetylcholinesterase inhibitory properties of

thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytother Res 21(3): 259–261.

Liu HL, Chiu MJ, Lin CH, Yang SY (2020) Stability of plasma amyloid-β 1–40, amyloid-β 1–42, and total tau protein over repeated freeze/thaw cycles. Dement Geriatr Cogn Disord Extra 10: 46–55.

Liu M, Xu X, Rang W, Li Y, Song Y (2004) Influence of ovariectomy and 17beta-estradiol treatment on insulin sensitivity, lipid metabolism and post-ischemic cardiac function. Int J Cardiol 97(3): 485–493.

Lovheim H, Elgh F, Johansson A, Zetterberg H, Blennow K, Hallmans G, Eriksson S (2017) Plasma concentrations of free amyloid-beta cannot predict the development of Alzheimer's disease. Alzheimers Dement 13: 778–782.

Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin M, Yardin C (2013) Tau protein phosphatases in Alzheimer's disease: The leading role of PP2A. Ageing Res Rev 12: 39–49.

Merlo S, Spampinato SF, Sortino MA (2017) Estrogen and Alzheimer's disease: Still an attractive topic despite disappointment from early clinical results. Eur J Pharmacol 817: 51–58.

Norsharina I, Maznah I, Iqbal S, Latiff LA (2013) Anti-aggregation effects of thymoquinone against Alzheimer's β-amyloid *in vitro*. Med Plants Res 7: 2280–2288.

Numakawa T, Odaka H (2021) Brain-derived neurotrophic factor signaling in the pathophysiology of Alzheimer's disease: Beneficial effects of flavonoids for neuroprotection. Int J Mol Sci 22(11): 5719.

Overk CR, Lu PY, Wang YT, Choi J, Shaw JW, Thatcher GR, Mufson EJ (2012) Effects of aromatase inhibition versus gonadectomy on hippocampal complex amyloid pathology in triple transgenic mice. Neurobiol Dis 45: 479–487.

Parhizkar S, Latiff LA, Parsa A (2016) Effect of *Nigella sativa* on reproductive system in experimental menopause rat model'. Afr J Pharm Pharmacol 6(1): 95–103.

Pérez-Grijalba V, Arbizu J, Romero J (2019) Plasma Aβ42/40 ratio alone or combined with FDG-PET can accurately predict amyloid-PET positivity: a cross-sectional analysis from the AB255 study. Alzheimers Res Ther 11: 96.

Perveen T (2014) Increased 5-HT levels following repeated administration of *Nigella sativa* L. (black seed) oil produce antidepressant effects in rats. Sci Pharm 82(1): 161–170.

Petrasek T, Skurlova M, Maleninska K, Vojtechova I, Kristofikova Z, Matuskova H, Stuchlik A (2016) A rat model of Alzheimer's disease based on Abeta42 and pro-oxidative substances exhibits cognitive deficit and alterations in glutamatergic and cholinergic neurotransmitter systems. Front Aging Neurosci 8: 83.

Qiu T, Liu Q, Chen Y-X, Zhao Y-F, Li Y-M (2015) Aβ42 and Aβ40: similarities and differences. J Pept Sci 21(7): 522–529.

Rather MA, Thenmozhi AJ, Manivasagam T, Bharathi MD, Essa M, Guillemin GJ (2018) Neuroprotective role of Asiatic acid in aluminium chloride induced rat model of Alzheimer ' s disease'. Front Biosci 1(10): 262–275.

Randhawa MA, Alenazi SA (2016) Neuropsychiatric effects of *Nigella sativa* (black seed) – A Review. Altern Integr Med 5: 209.

Rembach A, Faux NG, Watt AD, Pertile KK, Rumble RL, Trounson BO (2014) Changes in plasma amyloid beta in a longitudinal study of aging and Alzheimer's disease. Alzheimers Dement 10(1): 53–61.

Risacher SL, Fandos N, Romero J, Sherriff I, Pesini P, Saykin AJ, Apostolova LG (2019) Plasma amyloid beta levels are associated with cerebral amyloid and tau deposition. Alzheimer's Dement 11: 510–519.

Safitri F, Andriana K (2017) Black cummin extract (*Nigella sativa* Linn) on spatial memory performance in menopausal model of rat. Adv Health Sci Res 2: 254–263.

**S**ahak MKA, Mohamed AM, Hashim NH, Adli DSH (2013) *Nigella sativa* oil enhances the spatial working memory performance of rats on a radial arm maze. Evid Based Compl Altern Med 2013: 180598.

Samarghndian S, Farkhondeh T, Samini F (2018) A review on possible therapeutic effect of *Nigella sativa* and thymoquinone in neurodegenerative diseases. CNS Neurol Disord Drug Targets 17(6): 412–20.

Schneider Gasser EM, Straub CJ, Panzanelli P, Weinmann O, Sassoè-Pognetto M, Fritschy JM (2006) Immunofluorescence in brain sections: Simultaneous detection of presynaptic and postsynaptic proteins in identified neurons. Nat Protoc 1(4): 1887–1897.

Suganthy N, Devi KP, Nabavi SF, Braidy N, Nabavi SM (2016) Bioactive effects of quercetin in the central nervous system: Focusing on the mechanisms of actions. Biomed Pharmacother 84: 892–908.

Suzuki KM, Isohama Y, Maruyama H, Yamada Y, Narita Y, Ohta S, Araki Y, Miyata T, Mishima S (2008) Estrogenic activities of fatty acids and a sterol isolated from royal jelly. Evid Based Complement Alternat Med 5(3): 295–302.

Swaminathan S, Risacher SL, Yoder KK, West JD, Shen L, Kim S (2014) Association of plasma and cortical amyloid beta is modulated by APOE epsilon4 status. Alzheimers Dement 10(1): e9–e18.

Sweeney MD, Sagare AP, Zlokovic BV (2018) Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. Nat Rev Neurol 14: 133–150.

Topcagic A, Cavar Zeljkovic S, Karalija E, Galijasevic S, Sofic E (2017) Evaluation of phenolic profile, enzyme inhibitory and antimicrobial activities of *Nigella sativa* L. seed extracts. Bosn J Basic Med Sci 17(4): 286–294.

Vergallo A, Mégret L, Lista S, Cavedo E, Zetterberg, Blennow K (2019) Plasma amyloid β 40/42 ratio predicts cerebral amyloidosis in cognitively normal individuals at risk for Alzheimer's disease. Alzheimers Dement 15(6): 764–775.

Wakawa AI, Olufunke AS, Ibrahim A, Babagana KM (2018) Hormone replacement therapy and Alzheimers disease

in older women: A systematic review of literature. J Neurosci Behav Health 10(1): 1–8.

Wang DM, Li SQ, Wu WL, Zhu XY, Wang Y, Yuan HY (2014) Effects of long-term treatment with quercetin on cognition and mitochondrial function in a mouse model of Alzheimer's disease. Neurochem Res 39(8): 1533–1543.

Xiao H, Deng M, Yang B, Hu Z, Tang J (2018) Pretreatment with 17beta-estradiol attenuates cerebral ischemia-induced blood-brain barrier disruption in aged rats: involvement of antioxidant signaling. Neuroendocrinol 106: 20–29.

Xiao H, Deng M, Yang B, Tang J, Hu Z (2017) Role of glycogen synthase kinase 3 in ischemia-induced blood-brain barrier disruption in aged female rats. J Neurochem 142(2): 194–203.

Yessuf AM (2015) Phytochemical extraction and screening of bioactive compounds from black cumin (*Nigella sativa*) seeds extract. Am J Life Sci 3(5): 358–364.

Zaplatic E, Bule M, Shah SZA, Uddin MS, Niaz K (2019) Molecular mechanisms underlying protective role of quercetin in attenuating Alzheimer's disease. Life Sci 224(1): 109–119.

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|---|---|---|---|---|---|
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| 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 | | |
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