ORIGINAL ARTICLE

Effect of Tualang Honey-Mediated Silver Nanoparticles on TNF-α level, Caspase-3 Activity and Hippocampal Morphology in Kainic Acid-Induced Neurodegeneration in Male Rats

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ABSTRACT

INTRODUCTION: Despite being common disorder, the curative treatment for degenerative diseases are not yet available. Although Tualang honey (TH) has been reported to protect against neurodegeneration, but the effect of TH-mediated silver nanoparticles (THSN) on neurodegeneration is poorly understood. Thus, we conducted this study aimed to determine the effects of THSN on the levels of tumour necrosis factor alpha (TNF-α), caspase-3 activity, and hippocampal morphology in Kainic Acid (KA) induced neurodegeneration in rats. **MATERIALS AND METHODS:** A total of 72 Male Sprague Dawley rats were randomized into six groups which were the control, THSN 10mg, THSN 50 mg, KA only, KA+THSN 10 mg, and KA+THSN 50 mg groups. Each group was pre-treated orally with either distilled water or THSN (10 mg/kg or 50 mg/kg), according to their respective group. Following the last pre-treatment, each rat was injected with KA (15 mg/kg) or saline. After 24 h and 5 days of KA induction, all rats were sacrificed, and the hippocampus from each rat was harvested. Cresyl Violet and Fluoro Jade C staining were carried out to examine the number of viable cells and degenerating neurons. TNF-α level and caspase-3 activity in the hippocampus were measured using commercially available ELISA kits. **RESULTS:** Rats with KA-induced neurodegeneration demonstrated a significant increase ($p<0.05$) of TNF-α level and caspase-3 activity with a lower number of viable cells and increased number of degenerating neurons in the hippocampus. The pre-treatments of THSN groups improved these changes by lowering the TNF-α level and caspase-3 activity and decreasing the number of degenerating neurons. **CONCLUSION:** THSN could have potential neuroprotective effects in ameliorating TNF-α level, caspase-3 activity, and hippocampal damage in KA-induced male rats.

Keywords
Tualang Honey, Silver Nanoparticles, TNF-α, Caspase-3, Rats' Hippocampus

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INTRODUCTION

Neurodegenerative diseases are associated with of neurodegenerative disorders involves excitotoxicity, in glutamatergic dysfunction[1] involving the production of which neuronal cells are injured and die as a result of free radicals, release of pro-inflammatory mediators and overstimulation of neurotransmitters, such as glutamate.[3] programmed cell death resulting in progressive The structural and functional changes in the brain affected neurodegeneration of the brain tissue.[2] The pathogenesis by neurodegenerative disorders are characterised by

cognitive and emotional dysregulation[4], and associated with reduced adult neurogenesis in the hippocampus.[5] The neurodegenerative process may be triggered by genetic factors related to intrinsic susceptibility, ageing, and environmental factors.[6] Previously, several studies used kainic acid (KA) as a chemical neurotoxicant in animal experiments to investigate the mechanisms involved in excitotoxicity and neurodegeneration by inducing pathological processes such as neuroinflammation and apoptosis.[7-9]

Previous findings suggested that TNF-α that derived from KA-activated microglia can increase the excitotoxicity of hippocampal neurons and induce neuronal apoptosis *in vitro* and *in vivo*.[10,11] Systemic administration of KA has been shown to cause neuronal degeneration primarily in the hippocampus hilus, CA1, and CA3 regions in animals.[12-14] The distribution and selective sensitivity of the KA receptors in the brain are related to KA-induced selective vulnerability in hippocampal neurons.[15] Therefore, this study used KA-induced neurodegeneration in rats as a model to explore the pathogenesis of excitotoxicity in neurodegenerative disorders.

Exogenous antioxidants are present in a variety of foods and natural products, including Tualang honey (TH). Studies have shown that TH contains many phytochemical components, such as flavonoids and phenolic acids, which display a variety of biological activities, including anti-apoptosis[6], anti-inflammatory[16], and neuroprotective effects.[17] The role of TH in neuroprotective activity against excitotoxicity in KA-induced rats' brain model has been reported in recent studies.[6,18] Moreover, TH has also been explored in nanotechnology fields and was found to be a good reducing and stabilising agent and, importantly, to function as a precursor in nanoparticles byproducts.[19-21] Nowadays, nanoparticles are one of the treatment options for several neurological disorders like Alzheimer's disease, Parkinson disease, and vascular dementia.[22] In addition, nanoparticles synthesized using antioxidants are becoming potential implications as a treatment and prevention of neurodegenerative illnesses.[23]

Various strategies have been explored to enhance honey's absorption, stability, and bioavailability, including the development of TH-mediated silver nanoparticles (THSN).[19-20] Nanotechnologies are extensively used to deliver neurotrophic agents to the brain, increasing the bioavailability of compounds and treatment for diseases affecting the brain tissue.[24,25] The nanoparticles increase the bioavailability and bioactivity of compounds by reducing the size of the particles[26], surface modification[27], attaching or entrapping the phytomedicine with different nanomaterials.[28] The rapid uptake of nanoparticles by cells is the main reason for their enhanced antioxidant capacity inside the brain cells.[29] THSN was shown to possess high antioxidant activity and ferric/reducing antioxidant power, with an average size of 22 nm, which most likely improves its bioavailability in the body.[20]

Our studies have recently reported that THSN was shown to ameliorate seizures, locomotor activity, memory function, and hippocampal oxidative damage in KA-induced rats.[30,31] However, the effects of THSN on neuroinflammation and apoptosis in an *in vivo* rat model of KA-induced neurodegeneration were poorly understood. Thus, the purpose of the current study was to investigate the neuroprotective effects of THSN in an *in vivo* rat model of KA-induced neurodegeneration, focusing on the levels of tumour necrosis factor alpha (TNF-α) and caspase-3 activity, as well as morphological changes in the hippocampus following KA administration.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (200 to 250 g) were acquired from the Animal Research and Service Centre (ARASC) at the Universiti Sains Malaysia (USM) Health Campus. The animals were acclimatised at a temperature of 25±2° C with a light-dark cycle of 12:12 hours for seven days with free access to food and water. All procedures were carried out in accordance with the guidelines approved by USM Animal Ethics Committee [USM/IACUC/2018/(111)(904)].

Preparation of THSN

TH was purchased from the Federal Agricultural Marketing Authority (FAMA), Kelantan, Malaysia. THSN was prepared via the green synthesis method.[20] TH was diluted with distilled water before being heated in the oven (10-15 min, at 60°C). The honey solution was then adjusted to pH 8.0 before being added to the silver nitrate (0.1 M) solution. After that, the mixture was vigourously stirred. Lastly, the solution was dried overnight at 60°C. The nanoparticles were obtained in powder form and dissolved in 0.5 ml of distilled water before each use.

Experimental Groups

A total of 72 male rats were randomly divided into two major groups (24 h and 5 days post-KA induction), and each group contained six subgroups (n=6) as the following:

Group (1): Control	Group (4): KA only
Group (2): THSN 10 mg	Group (5): KA + THSN 10 mg
Group (3): THSN 50	Group (6): KA + THSN 50 mg

Each group was pre-treated orally with distilled water or THSN (10mg/kg or 50mg/kg), according to their respective group, five times at 12 h of intervals. The THSN dosages used in this study were based on previous research.[32,33] A recent study found that a daily dose of 10mg/kg silver nanoparticles (low dose) of *Azadirachta indica* extract may be safer for rats.[34] Therefore, the current study utilised THSN at 10mg/kg (10mg equivalent of TH/kg body weight) (low dose) and 50mg/kg (50mg equivalent of TH/kg body weight) (high dose) to compare their effects on KA induction in rats. After the last pre-treatments, the animals were injected subcutaneously with KA (15mg/kg) or saline.

In this experiment, two time points were selected, which were 24 h and 5 days. The rats were divided into two time periods of sacrifice as we expected that the different durations might have different effects on alteration of neuroinflammation, apoptosis and morphology in the hippocampus tissue. Following KA administration, an acute phase, lasting 24 to 48 h, corresponded to the damaging effect of neuronal apoptosis which occurred at early stage of excitotoxic insult.[7] Besides that, previous study showed a persistent hippocampal neuronal death in rodents at 1 to 5 days after KA treatment.[35] Therefore, the current study duration was extended to 5 days after KA injection to evaluate the potential protective effects at a later stage after KA injection.

Preparation of the Brain Homogenate

After 24 h and 5 days of KA induction, the animals were anesthetized with an overdose of sodium pentobarbital by intraperitoneal injection. The hippocampus from each animal was extracted. First, the isolated hippocampus was weighed and homogenized (10% w/v) in ice-cold 0.1 M phosphate-buffered saline (pH 7.4). Then, the homogenates were centrifuged (10,000 x g) for 10 min, and the supernatants were stored at -80°C until assayed.

Assay Procedures for Biochemical Analysis

TNF-α and caspase-3 activity were measured using commercially available kits (Qayee, Wuhan) according to the manufacturer's instructions. A double antibody enzyme-linked immunosorbent one-step process was used to determine the level of each parameter in the sample. The samples were analyzed at 450 nm using a microplate reader. The OD values were calculated according to the standard concentration using the standard curve linear regression equation.

Histological Analysis

Preparation of Brain Tissue

After all animals were anesthetised with an overdose of intraperitoneal injection of sodium pentobarbital, cardiac perfusion was performed. Then, the brain was removed and placed in the fixative solution, 4% PFA in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for overnight fixation. At post-fixation, the tissue samples were processed overnight in the automated tissue processing centre. The processed samples were embedded and blocked in paraffin for sectioning. The tissue blocks were serially sectioned (5 μm thickness) in the coronal plane using a rotary microtome. In each rat, three hippocampus sections were randomly taken from the anteroposterior Bregma -2.28 mm to Bregma -3.96 mm.

Cresyl Violet and Fluoro Jade C (FJC) Staining

Cresyl violet staining was performed to estimate the neuronal loss by identifying the positively bright purple, finely granular stained cells. Cresyl violet-positive cells were counted and defined as those with normal morphology and exhibiting round nuclei stained with granular-purple cresyl violet. Neuronal cells (pyknotic) that demonstrated shrunken nuclei or neuronal cells with no nucleoli or unclear bodies with surrounding empty spaces were excluded. Meanwhile, FJC staining was performed to further evaluate neuronal degeneration in the rats' hippocampus. FJC-positive cells were counted and defined as bright green fluorescence. In contrast, normal neurons that appeared darker than the background with lightly stained nucleoli or unstained nuclei were not counted. The sample sections were viewed and imaged using an Olympus BX41-32PO2 microscope (Olympus Corporation, Japan).

Six non-overlapping hippocampal areas were identified and photographed at 400X magnification for each slide. The number of cells was counted by taking the mean of six fields of CA1, CA2, and CA3 from each section. These regions were chosen as the function of CA1 pyramidal neurons provide a major output of the hippocampus proper.[36] Meanwhile, CA2 and CA3 pyramidal neurons will receive input from all the sources and this information will be segregated.[37] These regions are essential in the hippocampal function, like learning, long-term memory, spatial memory, and mood.[38] Therefore, these areas are important to consider in relation to neurodegenerative diseases.

Statistically Analysis

The IBM Statistical Package for Social Sciences (SPSS) software (Version 26, Chicago, USA) was used to analyze the results. Data with normal distribution and equal variance were analyzed using a parametric test; one-way analysis of variance (ANOVA) and followed by Tukey's post hoc test for multiple pairwise comparisons. The mean ± standard error of the mean (SEM) was used to express all values. The differences were considered statistically significant at $p<0.05$.

RESULTS

Level of TNF-α Level in Rats' Hippocampus

The level of hippocampal TNF-α was found to be significantly different ($p < 0.05$) between the groups at 24 h and 5 days post-KA induction (Figure 1). The post-hoc Tukey test demonstrated that the group pre-treated with 10mg/kg of THSN had significantly lower ($p<0.05$) TNF-α levels at both 24 h and 5 days post-KA induction when compared to KA only group. Meanwhile, pre-treatment with 50mg/kg of THSN reduced TNF-α level significantly ($p<0.05$) after 5 days but not at 24 h post-KA induction.

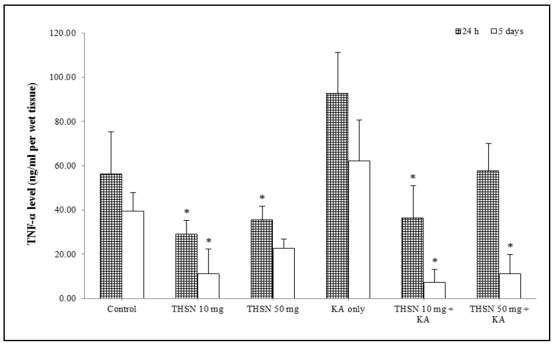

Figure 1. Level of TNF-α in the hippocampus of KA-induced rats. $p<0.05$ versus KA-only group. The results were expressed as mean ± SEM.

Caspase-3 Activity in Rats' Hippocampus

In the present study, the hippocampal caspase-3 level showed a significant difference ($p<0.01$) between groups at 24 h and 5 days post-KA induction (Figure 2). The post-hoc Tukey test revealed that, as compared to the KA-only group, pre-treatment with a low dose of THSN

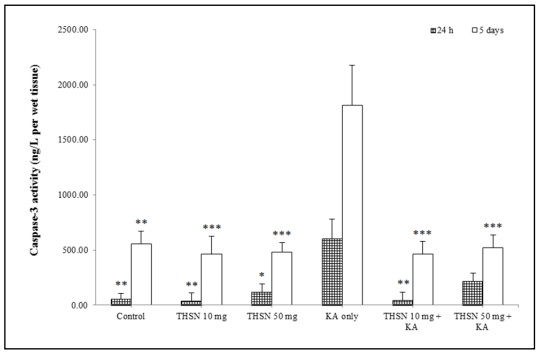

Figure 2. Level of caspase-3 in the hippocampus of KA-induced rats. *** $p < 0.001$ versus KA-only group; ** $p < 0.01$ versus KA-only group; * $p < 0.05$ versus KA-only group. The results were expressed as mean ± SEM.

(10mg/kg) remarkably decreased ($p<0.01$) caspase-3 activity in both time frames, while pre-treatment with THSN (50mg/kg) significantly reduced ($p<0.001$) the caspase-3 level after 5 days, but not at 24 h post-KA induction.

The Number of Cresyl Violet-Positive Cells in the Rats' Hippocampus

The number of cresyl violet-positive cells was shown to have significant differences ($p < 0.05$) between the groups in the CA1, CA2, and CA3 hippocampal regions, at 24 h post-KA induction. The post-hoc Tukey test demonstrated a significant decrease ($p<0.05$) in the KA-only group compared to other groups. Meanwhile, pre-treatment with THSN significantly improved ($p<0.05$) the number of cresyl violet-positive cells in the CA2 and CA3 regions at 24 h post-KA induction (Table I).

At 5 days of post-KA induction, the number of cresyl violet-positive cells in the CA1 and CA2 hippocampal regions revealed a significant difference ($p<0.05$) between the groups. The results revealed that as compared to the KA-only group, the pre-treatment with both doses of THSN significantly improved ($p<0.05$) the morphology of the CA2 region at both time frames. In contrast, in the CA3 region, the number of cresyl violet-positive cells was significantly increased ($p<0.05$) by the pre-treatment with THSN after 24 h, but not at 5 days of post-KA induction (Figure 3).

Table I: Number of cresyl violet-positive cells in rats' hippocampal regions at 24 h and five day of post-KA induction.

Groups	Number of cresyl violet-positive cells (0.01 mm²)					
	CA1		CA2		CA3	
	24 h	5 days	24 h	5 days	24 h	5 days
Control	64.65 ± 5.30	57.55 ± 3.283.28	74.71 ± 3.40[b]	41.39 ± 2.58[b]	41.39 ± 2.58[a]	41.59 ± 1.17
THSN 10 mg	69.45 ± 4.14[c]	69.95 ± 4.29[c]	79.74 ± 5.28[a]	43.67 ± 2.5[b]	43.67 ± 2.50[a]	43.72 ± 2.04
THSN 50 mg	62.69 ± 5.73	58.16 ± 3.22	74.91 ± 3.95[b]	38.77 ± 3.52[b]	38.77 ± 3.52[b]	39.36 ± 1.54
KA-only	49.02 ± 3.29	55.93 ± 2.99	47.89 ± 3.41	15.14 ± 2.66	15.14 ± 2.66	26.76 ± 5.50
KA + THSN 10 mg	65.55 ± 3.92	62.86 ± 2.73	72.69 ± 4.48[b]	38.98 ± 5.99[c]	38.98 ± 5.99[b]	28.14 ± 4.88
KA + THSN 50 mg	65.00 ± 4.25	66.68 ± 2.25	71.86 ± 5.89[b]	40.66 ± 3.51[c]	40.66 ± 3.51[c]	30.34 ± 6.66

The results were expressed as mean ± SEM. a $p<0.001$ versus KA-only group; b $p<0.01$ versus KA-only group; c $p<0.05$ versus KA-only group.

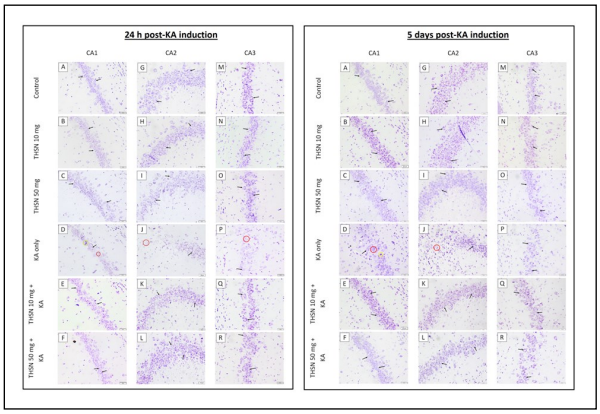

Figure 3. Morphology of CA1, CA2, and CA3 hippocampal regions among all groups after 24 h and 5 days of post-KA induction. (A – R) The arrows indicate the cells of interest; (D, J, and P) the neurons in the red circle show reduced staining intensity of the cresyl violet; (D) the appearance of pyknotic neuron in the yellow circle represents an injured neuron (Cresyl violet staining × 400, scale bar: 50 µm).

The Number of FJC-Positive Cells in the Rats' Hippocampus

In the present study, the post-hoc Tukey test demonstrated that the higher number of FJC-positive cells in the CA2 and CA3 hippocampal regions in KA-only group was significantly reduced ($p<0.05$) by both doses of THSN pre-treatments at 24 h post-KA induction (Table II). The hippocampal neuronal degeneration upon KA exposure was recognized by the appearance of shrunken or smaller-sized in their cell bodies compared to normal neurons (Figure 4).

Meanwhile, there was a significant difference ($p<0.05$) between the groups in the hippocampal CA2 and CA3 regions at 5 days of post-KA induction. The post-hoc Tukey test presented that, as compared to the KA only group, both doses of THSN pre-treatment significantly improved ($p<0.05$) the morphology of the CA2 region at both time frames, whereas the morphological alteration in the CA3 region could be seen at 24 h, but not at 5 days of post-KA induction. Therefore, THSN pre-treatments might exhibit protective effects on KA-induced neuronal degeneration in the hippocampus after 24 h.

Table II: Number of FJC-positive cells in rats' hippocampal regions at 24 h and five days of post-KA induction.

Groups	Number of FJC-positive cells (0.01 mm²)					
	CA1		CA2		CA3	
	24 h	5 days	24 h	5 days	24 h	5 days
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[c]
THSN 10 mg	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[a]	0.00 ± 0.00[b]	0.00 ± 0.00[a]	0.00 ± 0.00[c]
THSN 50 mg	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[a]	0.00 ± 0.00[b]	0.00 ± 0.00[a]	0.00 ± 0.00[c]
KA-only	4.06 ± 3.35	1.76 ± 1.75	14.67 ± 2.07	13.16 ± 5.74	23.20 ± 2.88	18.19 ± 4.75
KA + THSN 10 mg	0.24 ± 0.15	0.64 ± 0.39	1.81 ± 1.06[a]	1.46 ± 1.46[b]	6.31 ± 2.74[a]	12.01 ± 6.69
KA + THSN 50 mg	0.40 ± 0.35	0.11 ± 0.04	4.31 ± 2.78[a]	3.01 ± 1.37[c]	7.69 ± 3.62[a]	12.43 ± 6.18

The results were expressed as mean ± SEM. a $p<0.001$ versus KA-only group; b $p<0.01$ versus KA-only group; c $p<0.05$ versus KA-only group.

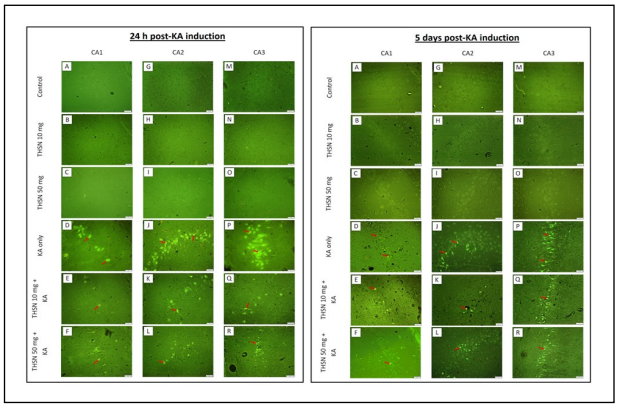

Figure 4. Morphology of CA1, CA2 and CA3 hippocampal regions among all of the groups after 24 h and 5 days of post-KA induction. The red arrows indicate the cell damages (FJC staining × 400, scale bar: 50 µm).

DISCUSSION

Our study found that hippocampal TNF-a level was remarkably increased in KA-only group at 24 h and 5 days of post-KA induction. The dysregulation of inflammatory factors like TNF-α, TLR-4, and NF-ϰb is thought to play a role in KA -induced excitotoxicity. Previous study reported that the high TNF-α level in rats' hippocampus can still be detected at 24 h, 3, and 21 days after KA induction.[39,40] Apart from that, previous finding suggests that the proinflammatory cytokine TNF-α derived from KA-activated microglia can promote neuronal apoptosis.[10] Apoptosis is the final stage of neurotoxicity and can be evaluated by measuring caspase- 3 activity. In the present study, a significant increase in caspase-3 activity was observed in KA- only group in both time frames, indicating a high rate of neuronal apoptosis in the hippocampus. These results closely resemble the previous findings that reported a significant increase in hippocampal caspase-3 level at 24 h after KA administration in rats.[41]

Our study demonstrated that the higher levels of TNF-α and caspase-3 were reduced by THSN pre-treatment after KA induction. A previous report found that silver nanoparticles can prevent protein denaturation, which is a common occurrence in inflammation and can interfere with the production of acute inflammatory mediators (such as histamines, serotonin, prostaglandins, and cyclooxygenase products) and counteract their action.[42] In a recent investigation, it was reported that the eco-friendly selenium nanoparticle produced using *M. oleifera* extract decreased melamine-induced nephrotoxicity in rats via reducing apoptosis.[43] Another finding also showed that green synthesis of silver nanoparticles was able to down-regulate the apoptotic pathways by reducing the caspase-3 level in rats' kidneys.[44] The reports cited above imply that green synthesized metallic nanoparticles can decrease inflammation more efficiently by inhibiting pro-inflammatory cytokines and preventing the apoptotic caspase-3 pathways.

Apart from that, the present study showed an increment in neuronal loss in hippocampus morphology, remarkably in CA1, CA2, and CA3 regions of KA-only group. However, the degenerated neuron in the CA1 region was detected at 24 h but not at day 5. This could be due to the cortical neuronal excitability that underwent an oscillating process after KA administration. Previous study showed that neuronal excitability increased rapidly in mice, decreased gradually at 8 h post-KA injection, and restored to a normal level one week later.[45] Concomitantly, kainate receptors were likewise found mostly in the CA2 hippocampal area with higher levels in low-excitability rats.[45] This observation can be explained by the existence of pyramidal neurons in different hippocampus regions, which demonstrated selective sensitivity to KA action, depending on the strain features of the animals' nervous system excitability. The present results showed that the morphology of the CA2 and CA3 regions were altered at both 24 h and 5 days after KA injection. This finding is consistent with previous reports demonstrating that CA2 and CA3 neurons were severely damaged due to KA-induced neurotoxicity.[46] Besides that, the CA3 region is the most sensitive and preferentially lesioned by KA since this region contains a high density of KA receptors.[47]

Our study result revealed that the morphological damage in the hippocampus after KA induction was attenuated by THSN. TH, a reducing agent used to synthesize the silver nanoparticles, contains various chemical compounds including acids, aldehydes, alcohol, ketones, terpenes, hydrocarbons, and furan derivatives as well as phytochemical compounds.[48] Meanwhile, THSN has been reported to contain alcohols, phenols, amides, carboxylate ions, and protein and exhibited excellent antioxidant activity.[20] The presence of antioxidant flavonoids and phenolic acids in TH could scavenge ROS and elevate enzymatic and non-enzymatic antioxidants

while decreases lipid peroxidation and inflammatory cytokine production; the combined effects contribute to the antioxidant and anti-inflammatory effects.[7,49] These compounds also reduce the degeneration of neurons and inhibit apoptosis via the downregulation of Bad, Bax, and cleaved caspase-3 expression levels and upregulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL.[50] The presence of flavonoids and phenolic acids in TH and THSN, as well as the synergistic interaction between these bioactive compounds may have contributed to THSN's protective effects on neuroinflammation and apoptosis, which in turn contributed to the reduction of neuronal degeneration in the hippocampus of KA-induced rats.

Overall, our study findings suggested that THSN exhibited neuroprotective activity against KA-induced neurodegeneration. Establishing nanoparticles with natural antioxidants can increase stability and biocompatibility while diminishing toxicity, besides preserving the desirable properties of the natural compound in THSN as reported in previous study.[20] However, the chemical composition and profiling of THSN were not explored in detail in order to find out the possible mechanisms behind their protective effects against KA-induced neurodegeneration.

CONCLUSIONS

THSN may have a protective effect on neurodegeneration by reducing the level of TNF-α and caspase-3 activity as well as improving the morphological alteration in the rats' hippocampus after KA administration. Thus, a further research is required to investigate the molecular level of the mechanism to provide better elucidation of the protective effects of THSN against KA-induced neurodegeneration in rats.

CONFLICT OF INTEREST

The authors declare no conflict of interests

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