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Neuroprotective effect of water lily (*Nymphaea pubescens* Willd) seed ethanolic extract against trimethyltin-induced cognitive impairment and neurodegeneration in mice

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ABSTRACT

Introduction: Cognitive impairments are profound outcomes of neurodegenerative disease, a global health issue. Water lily (*Nymphaea pubescens*, Nymphaeaceae) extracts have been reported to counteract oxidative stress. However, their protective effects against neurodegenerative disease remain to be fully investigated. The current study aimed to determine the neuroprotective effect of water lily seed ethanolic extract on trimethyltin (TMT)-induced cognitive impairment and neurodegeneration in a mouse model.

Methods: A single dose of TMT (0.6 mg/kg BW) was intraperitoneally injected to young adult male mice followed by daily oral treatments with different doses of water lily seed extract (0, 100, 200, and 400 mg/kg BW) for 28 days. Thereafter, cognitive behaviors were assessed, malondialdehyde (MDA) levels and catalase (CAT) activities were determined, followed by histopathological examination of the brain.

Results: The results revealed that, compared to the non-treated group, the water lily extract at doses of 100–400 mg/kg BW was effective in counteracting the decline in memory and spatial cognition of TMT-induced impairment ($P<0.05$). Moreover, the extract, particularly at doses of 200 and 400 mg/kg BW, substantially lowered the MDA level while elevating the CAT activity level ($P<0.05$). Water lily seed extract also significantly reduced TMT-induced pyramidal cell degeneration in the hippocampus and cerebral cortex ($P<0.05$).

Conclusion: Our findings demonstrated that ethanolic extract from water lily seeds could effectively reduce TMT-induced cognitive impairment and MDA levels and enhance CAT activity thereby precluding neurodegeneration in the hippocampus and cerebral cortex. Thus, water lily seed extract is a potent candidate for a natural anti-neurodegenerative supplement.

Implication for health policy/practice/research/medical education:
This study revealed the beneficial effects of water lily seed extract as a potent neuroprotector. Thus, it could be formulated as an affordable natural supplement to counteract neurodegenerative diseases including Alzheimer's disease.

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Introduction

Neurodegenerative disease is a set of pathological alterations that leads to neuronal damage, ultimately causing the progressive loss of normal motor and cognitive functions. Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases worldwide affecting millions of people (1). Various toxic chemicals such as lead and mercury in soil and water can have neurotoxic effects. Trimethyltin (TMT) is a by-product of the production of lead compounds that has wide applications in agriculture and industry, such as PVC heat stabilization (2). Animals exposed to TMT experienced behavioral changes (hyperactivity and aggression), cognitive impairment (memory loss and learning disorders), and spontaneous seizures (3). Rats injected with a single dose of 8 mg/kg body weight (BW) of TMT showed an increase in rat plasma and brain malondialdehyde (MDA) and decreased activity of endogenous antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and, glutathione (GSH)

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(4). Moreover, TMT induces damage in hippocampus pyramidal cells and dentate neurons that can occur progressively two to three days after intoxication. Such damage is rooted in severe stimulation of microglia and astroglia in the hippocampus. Experimentally, TMT is a reliable inducer of neurodegenerative disease (5).

Water lily (*Nymphaea pubescens* Willd, Nymphaeaceae) is one of the most prominent and widely used traditional medicinal herbs. It has been used to treat various diseases, including diabetes, diarrhea, dysentery, hemorrhages, indigestion, eye illnesses, jaundice, cancer, and inflammation (6). It is rich in bioactive compounds, including alkaloids, flavonoids, steroids, glycosides, tannins, terpenoids, and saponins (7). A previous study has reported that water lily leaf contains various phenolic compounds that have antidiabetic and hypolipidemic effects (8). The flower also contains flavonoids, phenolics, and saponins, which have been shown to exert antioxidant and anti-inflammatory effects in mice treated with CCl_4 (9). Another water lily species, *N. lotus*, is reported to enhance learning and memory capabilities in mice (10). In addition, the seeds of other water lily species such as *Nelumbo nucifera* have been used as supplements to improve brain function (11). Taken together, the findings suggest that water lily is a potent medicinal herb to counteract diseases, including cognitive disorders. However, to date, there is no report demonstrating the neuroprotective effects of water lily seeds against neurodegeneration and subsequent cognitive impairments. Therefore, the present study aimed to investigate the medicinal benefits of water lily seed extract (NPE; *Nymphaea pubescens* seed extract) in a mouse model.

Materials and Methods

Collection of plant material and authentication

Water lily seeds were procured from a local farmer in Amuntai (Banjarmasin, Borneo, Indonesia). The species identity of the plant was authenticated by a certified botanist at Herbarium ANDA, Andalas University. A specimen deposited there with herbarium number 235/K-ID/ANDA/V/2021.

Preparation of extract

The extraction was performed as per procedures previously described elsewhere (7). Briefly, 1000 g of water lily seeds were ground into be powder before being macerated in 2000 mL of 70% ethanol (Merck, Darmstadt, Germany). Thereafter, the sample was filtered and the filtrate was concentrated using a vacuum rotary evaporator (BUCHI Rotavapor RII, Switzerland) under reduced pressure at 40°C. The extract (NPE; *Nymphaea pubescens* seed extract) was kept in a dark sealed container until use.

Animal model and experimental design

Twenty-five young male mice (DDY strain, 4 weeks old, 18 ± 4 g of BW) were purchased from the Veterinary Monitoring and Investigation Center, Baso, Bukittinggi, West Sumatra, Indonesia. Prior to the experiment, all mice were allowed to be accustomed to an animal room for a week. The mice were kept in a 12/12 hours light/dark cycle, fed a chow diet (Rat Bio, PT Citra Ina Feedmill, Jakarta) and tap water *ad libitum*. All procedures involving the animals were approved by the Research Ethic Committee Faculty of Medicine, Andalas University (528/UN.16.2/KEP-FK-2021). After acclimatization, mice were randomly assigned into five groups (n = 5 per group) as follows: Group 1: CMC-Na 0.5% (NC, negative control), Group 2: TMT, Group 3: TMT + NPE 100 mg/kg BW (TMT + NPE 100), Group 4: TMT + NPE 200 mg/kg BW (TMT + NPE 200), and Group 5: TMT + NPE 400 mg/kg BW (TMT + NPE 400). The CMC and TMT were injected intraperitoneally (i.p). The dose of TMT (0.6 mg/kg BW; Trimethyltin chloride, Sigma-Aldrich, Inc., St. Louis, MO) was determined based on a previous study (12). The oral gavages of NPE were started three days after TMT injection and performed continuously once a day at 09.00 AM for 28 days. The NPE doses were chosen based on the previous study (10).

Cognitive behavioral tests

At the end of treatment, the cognitive behavioral tests were deployed. A Morris water maze test to assess the spatial cognitive performance was conducted as per protocol previously described elsewhere (13). The escape latency, average swimming speed, and total distance traveled were calculated using ANY-maze software (Stoelting Co., USA). Moreover, the Hebb-Williams maze test to examine memory intelligence was carried out by following the procedure as per protocol previously described (11) by recording the time to complete the maze. All tests were carried out in the afternoon (03:00 PM).

Measurement of malondialdehyde (MDA) level

Two days after cognitive behavioral tests, animals were sacrificed and the brain samples were collected. The levels of MDA in the brain tissue homogenates were determined by thiobarbituric acid method as previously described (14).

Measurement of CAT activity level

The levels of CAT activity in the brain tissue were measured using a CAT assay kit (#K773-100, BioVision). The procedures for the measurements of CAT were as per the protocol described in the kit instructions (15).

Histopathological examination of the brain

Immediately upon scarification, mice brain samples were preserved in a 10% of formalin solution. Afterward, the samples were subjected to tissue processing by following the standard protocols as described previously (16). The samples were stained with hematoxylin-eosin. The examinations of histopathological alterations in

the brain tissues, particularly in the hippocampal and cerebral cortex regions, were performed using a light microscope (Olympus BX51, Tokyo Japan). The numbers of degenerated pyramidal cells in the hippocampus and cerebral cortex were counted using ImageJ software (ImageJ 1.49v software, USA).

Statistical analysis

The data were statistically analyzed by one-way ANOVA followed by Duncan's new multiple range test using SPSS version 25 and $P < 0.05$ was considered statistically significant.

Results

Effects of *Nymphaea pubescens* seed extract on cognitive behaviors

In cognitive behavioral tests performed at the end of treatment TMT-exposed mice exhibited significant cognitive deficits compared to the control (NC) mice (Figure 1). The Morris water maze test demonstrated that, during early trials, the escape latency time of the TMT group was significantly higher than the control group. However, the escape latency time of the TMT+NPE400 group was significantly reduced compared to the TMT group (Figure 1A). The TMT group exhibited learning impairments with a longer latency to find the platform, while the NPE treatments (100, 200, and 400 mg/kg) shortened the latency (Figure 1B). In the test, the TMT group showed distinct memory deficits and thus spent a longer time, traveled a greater total distance, and rarely crossed the platform (Figure 1C). The swimming speed did not differ between the groups indicating that the locomotor activity of mice was not affected by TMT and was thus independent of the ability to find the platform in the test (Figure 1D). In particular, the TMT+NPE 100, 200, 400 mg/kg BW groups spent a similar amount of time in the W zone as the NC group. Furthermore, TMT groups exhibited improvements over the TMT-induced abnormal travel trajectories (Figure 1E). NPE treatment significantly alleviated the cognitive deficits of TMT-induced mice. The Hebb–Williams maze test (Figure 2) also demonstrated the cognitive impairment in the TMT group showing the longest average maze completion time of all the groups. Mice in NPE-treated groups demonstrated significantly shorter times in completing the maze task compared to the TMT group. Furthermore,

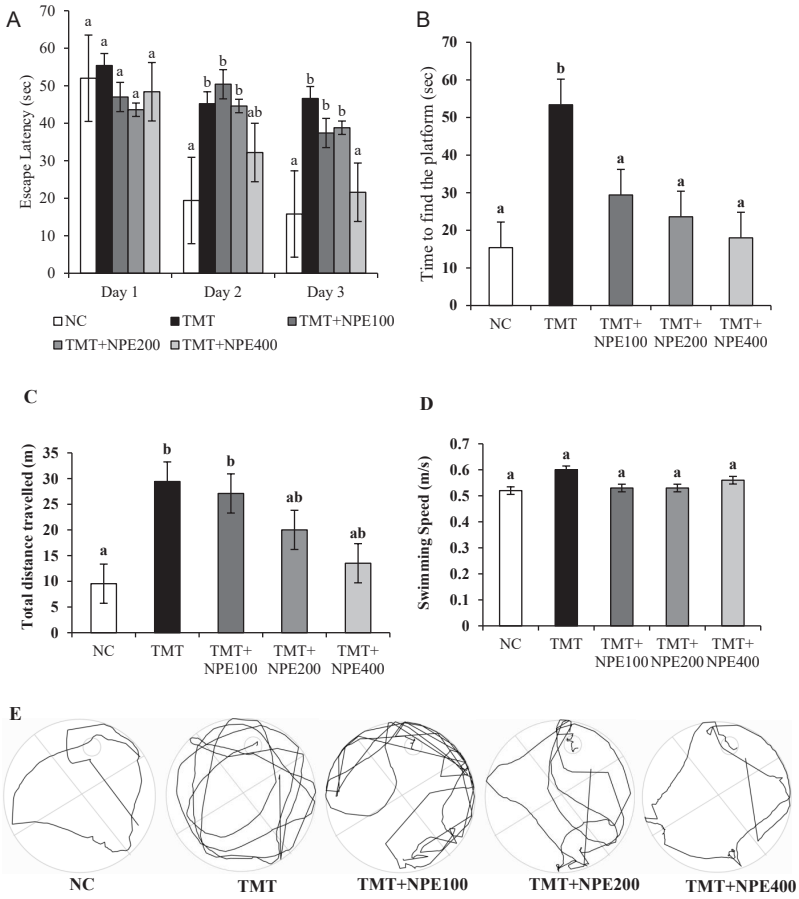

Figure 1. Spatial cognitive performances of mice assessed by Morris Water Maze (MWM). A) Escape latency; B) Time to find the platform; C) Total distance travelled; D) Swimming speed; E) Representative track plot of the MWM. NC (negative control), TMT (injected with 0.6 mg/kg trimethyltin), NPE 100, 200, 400 (orally gavaged with 100, 200, and 400 mg/kg seed extract of water lily). Different characters above the bars reveal statistical differences among groups ($P < 0.05$).

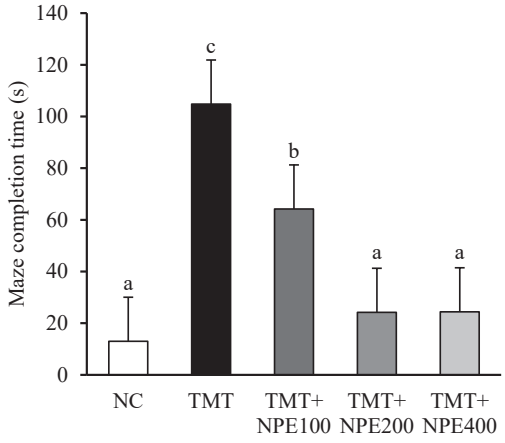

Figure 2. Cognitive behavioral performances of mice assessed by Hebb-William maze. NC (negative control), TMT (injected with 0.6 mg/kg trimethyltin), NPE 100, 200, 400 (orally gagged with 100, 200, and 400 mg/kg seed extract of water lily). Different characters above the bars reveal statistical differences among groups ($P < 0.05$).

there was a dose-dependent effect of NPE, in which the groups treated with higher doses (200 and 400 mg/kg BW) exhibited significantly faster completion time of the maze than the lower dose (100 mg/kg BW). Moreover, the durations in maze completion in higher-dose of NPE-treated groups were statistically comparable to those of the control group.

Effects of *Nymphaea pubescens* seed extract on MDA level and CAT activity

As shown in Figure 3A, MDA levels in the brain tissue were significantly increased in the TMT group compared with the NC group. However, NPE treatment resulted in a significant decrease in MDA levels compared with the TMT group. Furthermore, as presented in Figure 3B, CAT activity in the TMT group significantly decreased in the brain tissue compared with the NC group. The treatments with 100 mg/kg and 200 mg/kg NPE tended to increase CAT activity, while a significant elevation was achieved at a higher dose of NPE (400 mg/kg BW).

Effects of *Nymphaea pubescens* seed extract on brain histopathology

Histological observations in the brain were focused on pyramidal cells in the hippocampus and cerebral cortex. As depicted in Figure 4, the pyramidal cells in the CA1 region of the hippocampus were markedly reduced in TMT mice (Figure 4C), while they were sustained in the NPE-treated groups (Figures 4D-F). The proportion of pyramidal dead cells in the hippocampus was the highest in the TMT group as compared with other groups, including those treated with NPE (Figure 4G). However, the proportion of dead cells also remained higher in NPE-treated groups (doses of 100-400 g/kg BW) as compared to the control group. Similar patterns were also observed in the cerebral cortex (Figure 5). The TMT group exhibited substantial elevation of the pyramidal dead cells as compared with other groups (Figures 5C and 5G), while in the NPE-treated groups, cell death was reduced in a dose-dependent manner (Figure 5D-G). Statistically, the proportion of pyramidal dead cells in the cerebral cortex of NPE-treated groups remained incomparable to that of the control group.

Discussion

The present study demonstrated the neuroprotective effects of water lily seed ethanolic extract (NPE) against TMT-induced cognitive impairments and neurodegeneration in mice. Oral administration of NPE at doses of 100, 200, and 400 mg/kg BW effectively prevented learning impairments, oxidative stress, and neurodegeneration in memory-related brain areas such as the cerebral cortex and hippocampus.

Memory deficits can occur as a result of a TMT-induced increase in the enzyme acetylcholine esterase in mice. Acetylcholine esterase is responsible for the degradation of acetylcholine, which maintains learning and memory function (16). In the Morris water maze test and the Hebb–William maze test, mice receiving extract doses of 200 and 400 mg/kg BW were, on average, significantly faster to complete the maze than the control, suggesting that NPE

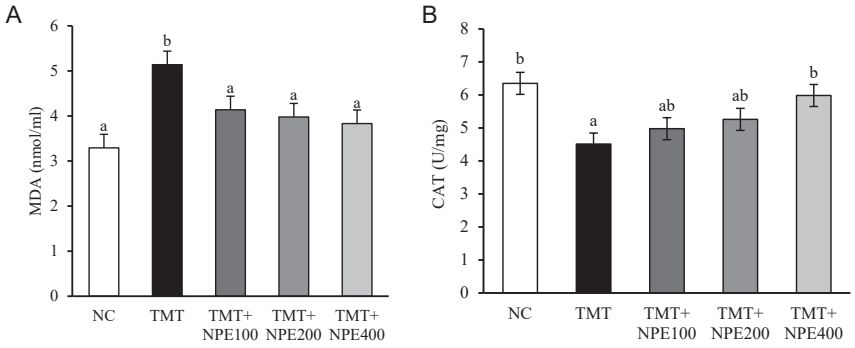

Figure 3. Effect of *Nymphaea pubescens* seed extract on oxidative stress and endogenous antioxidant levels in the brain. A) Level of malondialdehyde (MDA) in the brain tissue after 28 days of treatment, B) Catalase (CAT) activity in the brain tissue after 28 days of treatment. NC (negative control), TMT (injected with 0.6 mg/kg trimethyltin), NPE 100, 200, 400 (orally gavaged with 100, 200, and 400 mg/kg seed extract of water lily). Different characters above the bars reveal statistical differences among groups ($P < 0.05$).

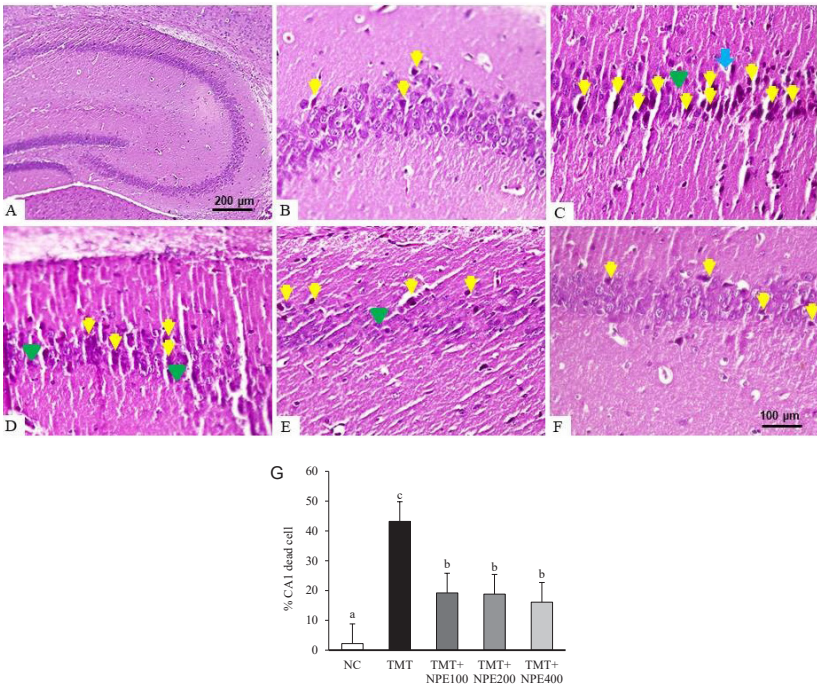

Figure 4. Effect of *Nymphaea pubescens* seed extract on the histopathological alteration of hippocampus. (A) Histology of hippocampus at a lower magnification; pyramidal cells of hyppocampus of mice in group (B) NC, (C) TMT, (D) TMT + NPE 100 mg/kg, (E) TMT + NPE 200 mg/kg, (F) TMT + NPE 400 mg/kg; (G) percentage of pyramidal death cells of hyppocampus. In B–F, yellow arrows indicate the pycnotic cells; green arrows indicate the eosinophilic cytoplasm, and blue arrows indicate the lysed cells. NC (negative control), TMT (injected with trimethyltin), NPE (orally gavaged with seed extract of water lily). Different characters above the bars reveal statistical differences among groups ($P < 0.05$).

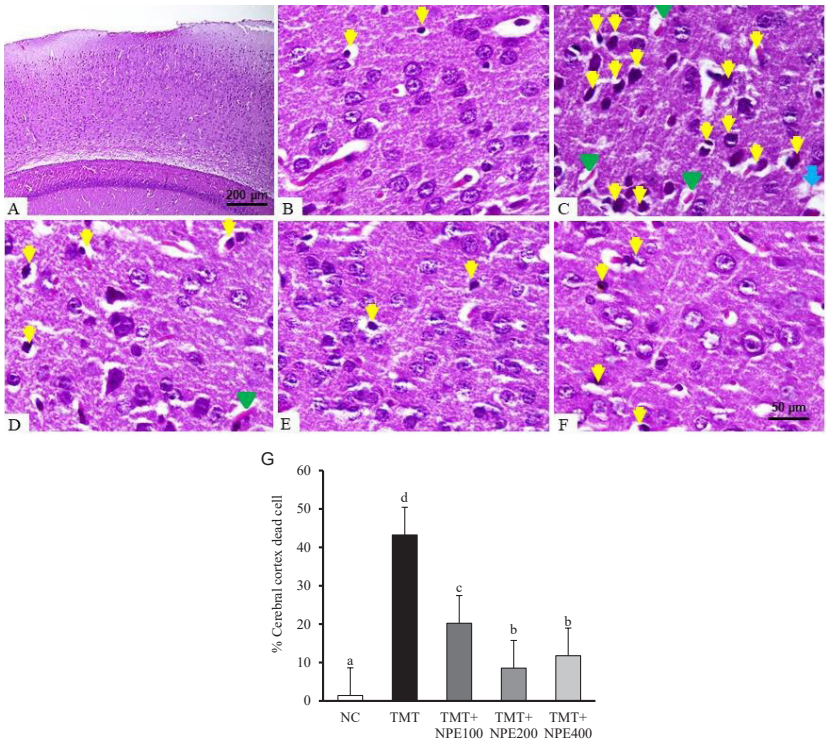

Figure 5. Effect of *Nymphaea pubescens* seed extract on the histopathological alteration of cerebral cortex. (A) Histology of cerebral cortex at a lower magnification; pyramidal cells of cerebral cortex of mice in group (B) NC, (C) TMT (0.6 mg/kg), (D) TMT + NPE 100 mg/kg, (E) TMT + NPE 200 mg/kg, (F) TMT + NPE 400 mg/kg. (G) percentage of pyramidal death cells of cerebral cortex. In B–F, yellow arrows indicate the pycnotic cells; green arrows indicate the eosinophilic cytoplasm and blue arrows indicate the lysed cells. NC (negative control), TMT (injected with trimethyltin), NPE (orally gavaged with seed extract of water lily). Different characters above the bars reveal statistical differences among groups ($P < 0.05$).

treatment not only suppressed the effects of stress but also increased learning ability and spatial working memory in mice. This result is consistent with previous findings that the administration of 2.8 mg/kg TMT caused substantial learning deficits in mice (17), while the administration of *N. lotus* leaf extract at the doses of 200 and 400 mg/kg BW could effectively counteract it. It was suggested that such protection is associated with the presence of antioxidants in the extract (18). Another study is also in line with our finding, showing that the protective effect of NPE correlated with the presence of flavonoid compounds that could diminish reactive oxygen species (ROS) production, thereby improving learning ability (19).

The present study demonstrated that NPE ameliorates oxidative stress. This was represented by MDA reduction and a significant increase in the antioxidant enzyme CAT. An increase in MDA levels in the TMT-induced group suggests the involvement of cellular damage caused by free radicals. Indeed, TMT reduces the activity of several antioxidant enzymes in different parts of the brain, thus facilitating the spread of lipid peroxidation. Previous studies have reported that TMT treatment induces the production of MDA, and an elevation in the levels of the antioxidant marker GSH, SOD, and heme oxygenase-1 (HO-1) enzyme in mouse models (20). Among the antioxidant enzymes, CAT is crucial and directly eliminates hydrogen peroxide. This increase in CAT activity could be due to the presence of bioactive compounds present in NPE that could donate their proton to stabilize radicals formed during stress. A similar study has reported that the administration of 2.5 mg/kg BW TMT in mice caused an increase in MDA levels and a decrease in antioxidant enzymes (GSH, CAT, and SOD) (16). Another study showed that the administration of NPE was able to prevent the deficits in the number of pyramidal cells in the cornu ammonis 1 (CA1) region of the hippocampus and cerebral cortex of TMT-exposed mice. Previous studies have reported that the administration of TMT at a dose of 8 mg/kg BW caused widespread damage to the neurons in the hippocampal area, as demonstrated by significant decreases in the number of neurons in the stratum pyramidal of CA1, CA3, and dentate gyrus (DG) in a rat model of AD (21). The increase in reactive oxygen species associated with oxidative stress plays an important role in mediating TMT-inducing neurotoxic damage. TMT neurotoxicity induces oxidative stress, Ca^{2+} excess, apoptosis, and inflammation in TMT-sensitive hippocampal neurons, consequently leading to hippocampal neurodegeneration (2). TMT can lead to a cytotoxic response by triggering a series of molecular events and cellular pathways through the activation of various kinases, such as c-Jun N-terminal kinase (JNK), protein kinase C, and transcription factors, such as core factor kappa B (NF-κB) and stress protein (22). Thus, when taken together with previous findings, the present results indicate that NPE protects or rescues neurons, particularly in the hippocampal CA1 regions and cerebral cortex, from TMT-induced neurodegeneration.

The present results support our hypothesis that NPE is beneficial for treating cognitive dysfunctions caused by various insults related to AD, which lead to neuronal damage and/or degeneration. NPE contains various phytochemical compounds: alkaloids, tannins, saponins, glycosides, flavonoids, and steroids/triterpenoids. In addition, NPE is highly nutritional, containing 88.36% carbohydrate, 10.39% protein, 0.58% fat and 7.98% total dietary fiber (2). Previous studies succeeded in finding secondary metabolites of flavonoid species in *N. pubescens*, namely myricetin, arachidic acid, stigmasterol, urea, stearic acid, behenic acid, kaempferol, ergosterol, D-threitol, and oleic acid. Flavonoid compounds can inhibit apoptosis and other damage to neurons through neurotoxic species, but not directly. Flavonoids modulate several protein kinases and lipid kinase signaling cascades, such as PI3 kinase (PI3K)/Akt, tyrosine kinase, protein kinase C, and MAPK signaling pathways by inducing angiogenesis and new neuronal cell growth in the hippocampus (23,24). It has been reported that oleanolic acid present in NPE induces a reduction in intracellular ROS levels, activates GSH, and reduces MDA levels (25,26). Oleanolic acid effectively reduces the formation of ROS in the brain, decreases AR activity and expression, and decreases NF-kB activation. Oleanolic acid also causes anti-apoptotic effects by suppressing the production of Bax protein, caspase-3, and maintaining Bcl-2 expression (27).

Conclusion

In conclusion, our study demonstrated that NPE could effectively preclude spatial cognitive impairment and memory in TMT-exposed mice via a likely mechanism of reducing oxidative stress and enhancing endogenous antioxidant activity, thereby protecting neurons in the hippocampus and cerebral cortex. Hence, NPE has the potential to be developed as a neuroprotective drug or supplement against neurodegenerative diseases.

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Authors' contribution

PS, RR, DFB were involved in study design, experiments, and data analysis. DFB and PS wrote the manuscript with an interpretation of the results. All authors read and approved the final version of the manuscript for publication.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

All procedures involving the animals were approved by the Research Ethic Committee Faculty of Medicine, Andalas University (528/UN.16.2/KEP-FK-2022).

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