Pinostrobin alleviates chronic restraint stress-induced cognitive impairment by modulating oxidative stress and the function of astrocytes in the hippocampus of rats

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Abstract. Chronic stress has been recognized to induce the alterations of neuronal and glial cells in the hippocampus, and is thus implicated in cognitive dysfunction. There is increasing evidence to indicate that natural compounds capable of exerting neuroprotective and antioxidant activities, may function as potential therapeutic agents for cognitive impairment. The present study examined the neuroprotective effects of pinostrobin from *Boesenbergia rotunda* (L.) against chronic restraint stress (CRS)-induced cognitive impairment associated with the alterations of oxidative stress, neuronal density and glial fibrillary acidic protein (GFAP) of astrocytes in the hippocampus. For this purpose, male Wistar rats were administered once daily with pinostrobin (20 and 40 mg/kg, *per os*) prior to exposure to CRS (6 h/day) for 21 days. The cognitive behaviors, the concentration of malondialdehyde, and the activities of superoxide dismutase and catalase were determined. Histologically, the alterations in astrocytic GFAP and excitatory amino acid transporter 2 (EAAT2) in the hippocampus were examined. The results revealed that pinostrobin potentially attenuated cognitive impairment in the Y-maze and in novel object recognition tests, with a reduction in oxidative stress. Furthermore, pinostrobin effectively increased neuronal density, as well as the immunoreactivities of GFAP and EAAT2 in the hippocampus. Taken together, these findings indicate that treatment with pinostrobin alleviates chronic stress-induced cognitive impairment by exerting antioxidant effects, reducing neuronal cell damage, and improving the function of astrocytic GFAP and EAAT2. Thus, pinostrobin may have potential for use as a neuroprotective agent to protect against chronic stress-induced brain dysfunction and cognitive deficits.

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Introduction

Chronic stress affects brain function and induces long-term alterations in various neural systems related to anxiety, depression, cognition and insomnia (1-3). Prolonged exposure to stress has been found to play a crucial factor in the progression of dementia and in the development of neurological disorders, including Alzheimer's disease (AD) and major depressive disorder (4-6). Animal models of chronic restraint stress (CRS) are commonly used to induce learning and memory deficits, and neuronal damage to the hippocampus (7,8). The hippocampus is one of the brain regions which is most vulnerable to stress-induced damage, due to the high expression of mineralocorticoid receptors and glucocorticoid receptors (GR) (9,10). Previous studies have demonstrated that exposure to stress induces alterations in hippocampal neurogenesis, synaptic plasticity and neuronal cell survival (11-13). Additionally, chronic stress also provokes dendritic atrophy and causes spatial memory impairment (14,15). Furthermore, exposure to chronic stress can interrupt the balance of oxidants and antioxidants, leading to the overproduction of free radicals which can in turn induce brain damage (16). Oxidative stress is considered a crucial factor involved in the pathogenesis of neurodegenerative diseases, including AD, Parkinson's disease (PD), depression and memory impairment (17,18). High levels of oxidative stress can induce memory impairment mediated by changes in hippocampal synaptic plasticity (19). In addition, there is also evidence to indicate that antioxidants can ameliorate oxidative stress-induced neuronal damage (20). Therefore, the use of antioxidant agents may represent a successful strategy for the prevention and treatment of cognitive dysfunction.

Herbal medicinal substances exerting neuroprotective effects have been considered to be pharmacological agents

for the treatment of several neurodegenerative diseases. Pinostrobin, a natural bioflavonoid compound isolated from the rhizomes of *Boesenbergia rotunda* (L.) or Krachai in Thai, has been revealed to exhibit numerous biological properties, including antioxidant, anti-inflammatory and neuroprotective activities (21-23). Pinostrobin has been shown to ameliorate β-amyloid-induced neurotoxicity in PC12 cells by suppressing reactive oxygen species (ROS) and calcium overload (23). In addition, pinostrobin has been reported to reduce the loss of dopaminergic neurons by suppressing oxidative stress in a model of PD (24). However, to date, at least to the best of our knowledge, there are no available data on the protective effects of pinostrobin in an animal model of CRS-induced cognitive deficits. Therefore, the present study aimed to examine the neuroprotective potential and possible mechanisms of action of pinostrobin in chronic stress-induced brain damage and cognitive dysfunction in rats.

Materials and methods

Animals. A total of 28 adult male Wistar rats (weighing 200-220 g, at the commencement of the experiment) were obtained from the Nomura Siam International (Bangkok, Thailand). The animals were allowed a week for acclimatization prior to the commencement of the experiments. All experimental procedures were conducted during the dark phase of the light cycle with standard chow and water available *ad libitum* in a room with a constant temperature (21±1˚C) and humidity (35-60%). All experiments were approved by the Ethics Committee of the Laboratory Animal Research Center, University of Phayao, Thailand (approval no. 640104006).

Preparation of pinostrobin. Fresh rhizomes of *B. rotunda* were collected from Phayao, Thailand. The voucher specimen (no. S. Sedlak 19-1) was authenticated by the Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham, Thailand. The ethanolic crude extract (251 g) was resuspended in methanol to yield a pale yellow methanol-insoluble solid (50 g). The obtained solid was further subjected to an open column chromatography using silica gel (Arch. No. 7734, pore size 60 Å, particle size 70-230 mesh, Merck) as an adsorbent. The column was eluted with the mixture of 40-80% dichloromethane-hexane under gradient separation. The similar pattern fractions were combined based on thin-layer chromatography analysis and recrystallized with methanol to yield pure pinostrobin, while the purity was found to be >98% based on HPLC analysis. Structural elucidation was performed using 13C- and 1H-NMR spectroscopy (Bruker Avance DRX500 Spectrometer) and the NMR spectra were compared with those in the published literature (25) as follows: 1H NMR (500 MHz, acetone-d6): 2.80 (dd, J=17.2, 3.0 Hz; 1H, H-3a), 3.06 (dd, J=17.2, 13.0 Hz; 1H, H-3b), 3.79 (s, 3H; -OCH3), 5.39 (dd, J=13.0, 3.0 Hz; 1H, H-2), 6.06 (m, 2H, H-6, H-8), 7.41 (m, 5H; H-2', H-3', H-3', H-5', H-6'). 13C NMR (150 MHz, acetone-d6): 43.1 (C-3), 55.7 (C-7-OCH3), 79.2 (C-2), 94.3 (C-8), 95.1 (C-6), 103.1 (C-10), 126.1 (C-2', C-3', C-5', C-6'), 128.9 (C-4'), 138.4 (C-1'), 162.8 (C-5), 164.1 (C-9), 167.9 (C-7), 195.8 (C-4). (NMR data were recorded on January 13, 2021, Central Science Laboratory, Faculty of Science, Chiang Mai University, Thailand). The chemical structure of pinostrobin is illustrated in Fig. 1.

Experimental procedure. All animals were randomly divided into four experimental groups (7 rats per group) as follows: i) The control group, no stress; ii) the vehicle + CRS group; iii) the pinostrobin 20 mg/kg + CRS goup; and iv) the pinostrobin 40 mg/kg + CRS group. The rats were exposed to restraint stress using a wire mesh restraint secured with butterfly clips that closely fit the body of the rats, as described in a previous study (26). The restraint stress procedure was repeated once daily for 6 h per day (10.00 am to 16.00 pm) for 21 consecutive days. During the restraint sessions, the unstressed control animals were handled for 2 min. The animals were administered 1% carboxymethylcellulose (CMC) used as the vehicle or pinostrobin (at doses of 20 or 40 mg/kg) via oral gavage daily at 30 min prior to stress exposure for 21 days. The learning and memory performance of all animals were assessed by using a Y-maze and novel object recognition (NOR) tests (as described below) on day 0 (prior to beginning the experiment) for baseline data and on the last day of the experiment (day 21) for the therapeutic effects of pinostrobin. At 24 h after the last behavioral test, all animals were sacrificed by transcardial perfusion with 0.1 M PBS for the evaluation of biochemical and immunohistochemical parameters. The doses of pinstrobin were selected based on a previous study (27).

Tissue processing and immunohistochemistry. All animals were deeply anesthetized via an intraperitoneal injection of thiopental sodium (70 mg/kg body weight) and perfused with ice-cold 0.1 M PBS. The brains (n=28) were immediately removed and cut at the midline, dividing the brain into two hemispheres. The hippocampus from the left hemisphere was separated and stored at -80˚C for biochemical determination. The right hemisphere was fixed with ice-cold 4% paraformaldehyde and cryopreserved in 12.5% sucrose for immunohistochemistry. The brains were sectioned into 30-μm-thick sections using a cryostat microtome (AST500, Amos Scientific Pty Ltd.) and stored in an anti-freeze solution (4˚C). The coronal sections were rinsed and incubated in 3% H_2O_2 and followed by 3% normal horse serum (cat. no. A9647, Sigma-Aldrich; Merck KGaA) for non-specific blocking. Subsequently, sections were incubated with the primary antibodies, mouse anti-glial fibrillary acidic protein (GFAP; 1:500, cat. no. MAB5628, MilliporeSigma), or rabbit anti-excitatory amino acid transporter 2 (EAAT2; (1:200, cat. no. ab41621, Abcam) at 4˚C overnight. The sections were washed in 0.1 M PBS for 30 min and incubated for 2 h at room temperature with biotinylated donkey anti-mouse secondary antibody (1:500, cat. no. 715-065-150, Jackson ImmunoResearch Europe, Ltd.) or anti-rabbit (1:500, cat. no. 711-065-152, Jackson ImmunoResearch Europe, Ltd.). The sections were rinsed in 0.1 M PBS followed by 1 h of incubation in 0.1% extravidin peroxidase (1:1,000, cat. no. E2886, Sigma-Aldrich; Merck KGaA) at room temperature, and then rinsed again. Immunolabeling was developed using a nickel-enhanced 3,3'-diaminobenzidine (DAB) reaction (cat. no. D12384, Sigma-Aldrich; Merck KGaA). Finally, the sections were washed in 0.1 M PBS and, then mounted on positive charged

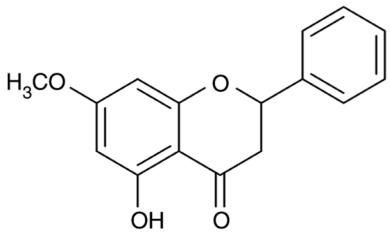

Figure 1. Chemical structure of pinostrobin.

slides, dehydrated using graded alcohols, cleared in xylene, and cover-slipped using mounting medium (cat. no. 107961, Sigma-Aldrich; Merck KGaA). For Nissl staining, the sections were stained with 0.1% cresyl violet (cat. no. 1.05235.0025, Sigma-Aldrich; Merck KGaA) for 8 min at 60˚C, dehydrated with ethanol, cleared in xylene, and coverslipped using mounting medium.

Determination of the malondialdehyde (MDA) level. The level of MDA was evaluated as an indicator of lipid peroxidation. A total of seven hippocampi from each group were homogenized in 0.1 M PBS (pH 7.4), centrifuged at 9,279 x g at 4˚C for 15 min, and the supernatant was subjected to the thiobarbituric acid reaction according to the protocol previously described by Nakmareong *et al* (28) with minor modifications. Briefly, the mixture consisted of 75 μl sample or standard (1,1,3,3-tetraethoxypropane) (cat. no. 108383, Sigma-Aldrich; Merck KGaA), 10% TCA (cat. no. 100807, Merck KGaA), 5 mM EDTA (cat. no. AR1240, RCI Labscan Ltd.), 8% SDS (cat. no. S/5200/53, Thermo Fisher Scientific, Inc.) and 0.5 μg/ml BHT (cat. no. 02381, LOBA Chemie Pvt. Ltd.) was incubated at room temperature for 10 min. The mixture was then supplemented with 250 μl 0.6% TBA (cat. no. 108180, Merck KGaA), and boiled in a water bath for 30 min. After cooling, the mixture was centrifuged at 10,000 x g for 5 min at 4˚C. The absorbance was measured at 532 nm by microplate reader (Synergy H1, BioTek Instruments, Inc.). The results are as expressed as μmol/mg protein.

Determination of superoxide dismutase (SOD) activity. SOD activity was determined using a colorimetric assay kit (cat. no. S19160, Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. The results were presented as the inhibition rate (%).

Determination of catalase (CAT) activity. Catalase activity was measured based on the enzyme degradation of H_2O_2 according to previously published study with some modifications (29). Briefly, 20 μl of sample were mixed with 100 μl of 6 mM H_2O_2 for initiating the enzymatic reactions followed by incubation at 37˚C for 1 min. The reaction was then terminated with 100 μl of 32.4 mM ammonium molybdate and the absorbance at 405 nm was measured using a microplate reader (Synergy H1, BioTek Instruments, Inc.). The results are presented as U/mg protein.

Cell count analysis and thresholding. To determine the density of neurons, hippocampal images were captured at x40 magnification using a bright-field microscope (Nikon Corporation). Images of subregions of the hippocampus (at x40 magnification), including CA1, CA2 and CA3 were subjected to exhaustive manual counts using NIS Elements imaging software version 5 (Nikon Corporation). For thresholding function, the immunoreactivity of the astrocytes within the hippocampus was determined using the thresholding function of Image J software (Version 1.53, National Institutes of Health). Image thresholding is the frequently used technique to quantitatively determine the alterations in immunolabelled material as previously described (30). The data are presented as the percentage of threshold material.

Y-maze test. The Y-maze test was used to measure working memory in animal by recording a spontaneous alternation (31). The Y-maze consisted of three arms (40 cm long x 33 cm high x 15 cm wide, separated by an angle of 120˚, Laboratory Animal Research Center, University of Phayao, Thailand). All animals were individually placed at one of three enclosed arms for free exploration for total of 8 min. A spontaneous alternation was defined as any combination of three sequential entries in which the animal entered all three arms (e.g., ABC, CAB, or BCA, but not ABB). The percentage of spontaneous alternation was used as an index of working memory and calculated according to the following equation: The spontaneous alternation (%)=[(number of alternations)/(total arm entries-2)] x100 (32). After each session, the maze was cleaned with 70% ethanol to avoid odors.

Novel object recognition (NOR) test. The NOR test was performed in a black open field box (45x65x45 cm, Laboratory Animal Research Center, University of Phayao, Thailand) to determine the recognition memory. The test composed of three sessions (habituation phase, training phase and test phase) using a previously described method with minor modifications (33). At the end of the treatment period, the animals were allowed to explore the empty open field for 5 min during the habituation phase. During the training phase, two identical objects (A1 and A2) were placed in two corners of the open field. The rats were placed in the middle of the open field and allowed to freely explore these two identical objects for 5 min and then the animals were returned to home cage. After 4 h of post-training phase, a new object (B) was placed and the animals were left to investigate the two objects for 5 min. The exploration time identifying as pointing the nose to the object at a distance ≤2 cm was manually recorded using a stopwatch. A recognition index (RI) was calculated using the following formula: [TB/(TA + TB) x100], where TA and TB are the time spent exploring familiar object A and novel object B, respectively (34).

Statistical analysis. All data were analyzed using GraphPad Prism 9 (GraphPad Software, Inc.). Data are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of pinostrobin against oxidative stress in the hippocampus. The antioxidant activities of pinostrobin

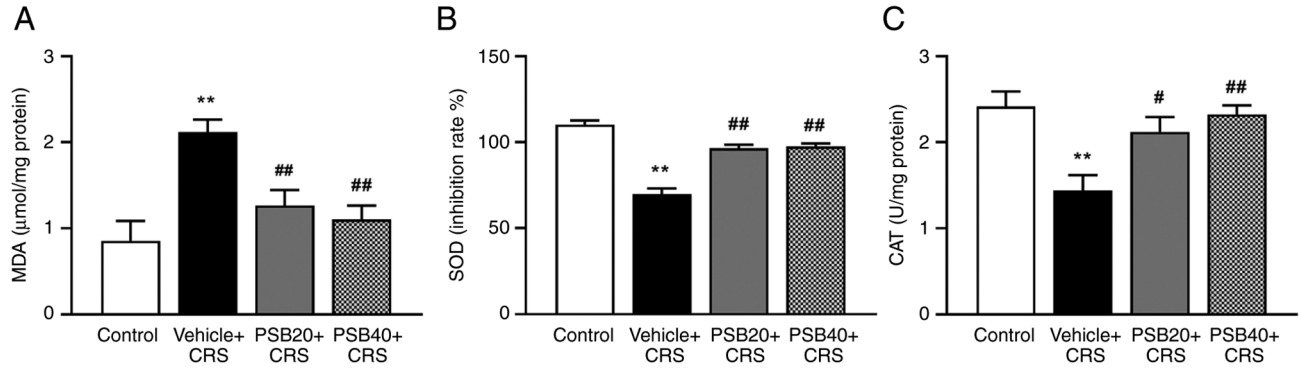

Figure 2. Effects of pinostrobin on oxidative stress markers in the hippocampus. Graphs demonstrate the (A) MDA level, (B) SOD activity, and (C) CAT activity. Data are expressed as the mean ± SEM. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the vehicle-CRS group. CRS, chronic restraint stress; PSB, pinostrobin; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase.

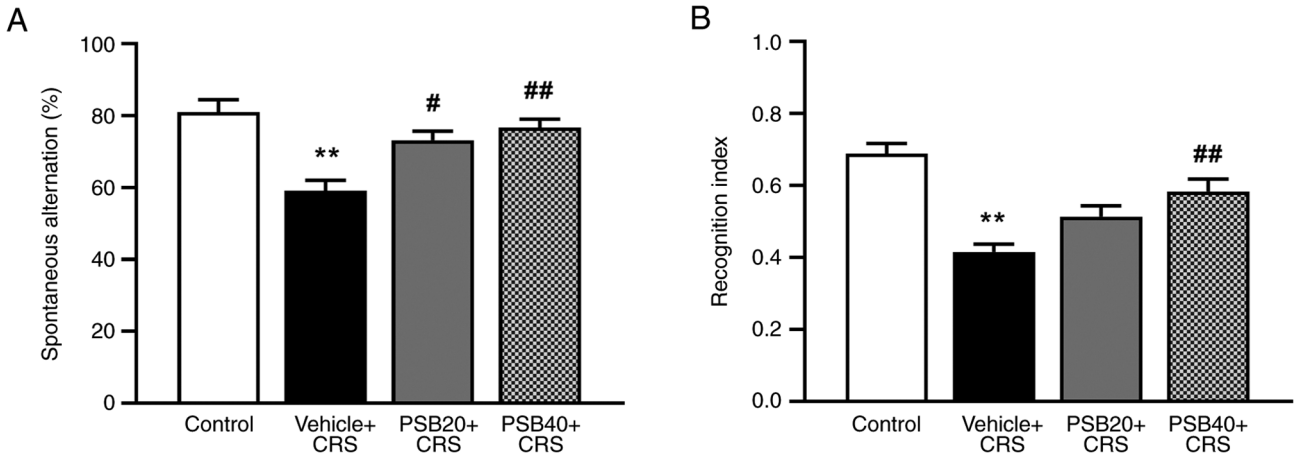

Figure 3. Effects of pinostrobin on learning and memory performance. Graphs demonstrate the percentage of spontaneous alternation using (A) the Y-maze test, and (B) the recognition index using the novel object recognition test. Data are expressed as the mean ± SEM. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the vehicle-CRS group. CRS, chronic restraint stress; PSB, pinostrobin.

were determined by measuring the levels of MDA, which is an indicator of lipid peroxidation, and the activities of some of the main scavenging enzymes, such as SOD and CAT in the hippocampus (Fig. 2). The results demonstrated that the amount of hippocampal MDA was markedly enhanced, whereas the activities of SOD and CAT were significantly decreased in the rats subjected to CRS (P<0.01, Fig. 2). Notably, treatment with pinostrobin (20 and 40 mg/kg) significantly decreased the level of MDA in the hippocampus compared to the vehicle-chronic stress group (P<0.01, Fig. 2A). In addition, treatment with pinostrobin at a low dose (20 mg/kg) markedly attenuated the chronic stress-induced reduction in the levels of antioxidant enzymes in the hippocampus compared with vehicle chronic stress-treated group (SOD, P<0.01, Fig. 2B; CAT, P<0.05, Fig. 2C). Furthermore, treatment with pinostrobin at a dose of 40 mg/kg also significantly increased the activities of SOD and CAT (SOD, P<0.01, Fig. 2B; CAT, P<0.01, Fig. 2C).

Effects of pinostrobin on CRS-induced memory impairment of rats in the Y-maze and NOR tests. The Y-maze and NOR tests were used to determine whether pinostrobin could reverse CRS-induced cognitive impairment (Fig. 3). Prior to the commencement of the experiment (day 0), the cognitive function of the rats was determined using Y-maze and NOR tests for baseline data. It was found that there were no significant differences between the groups (data not shown). Additionally, behavioral assessment revealed that 21 consecutive days of exposure to restraint stress affected the working memory of the animals by decreasing the percentage of spontaneous alternation in the Y-maze compared to the control animals (P<0.01). However, this impairment was considerably restored following treatment with pinostrobin (20 mg/kg, P<0.05; 40 mg/kg, P<0.01; Fig. 3A). After the Y-maze test, the animals were immediately examined for recognition memory using the NOR test. In rats exposed to CRS, the administration of pinostrobin at a dose of 40 mg/kg significantly improved cognitive function by enhancing the recognition index compared to vehicle-treated group (P<0.01, Fig. 3B).

Effects of pinostrobin on neuronal damage in the hippocampus. Chronic stress is considered an important risk factor that can induce neuronal damage (7). Therefore, in the present study, the neuronal density in the hippocampus was assessed for the possible involvement of neuroprotective mechanism. The effects of pinostrobin on neuronal damage

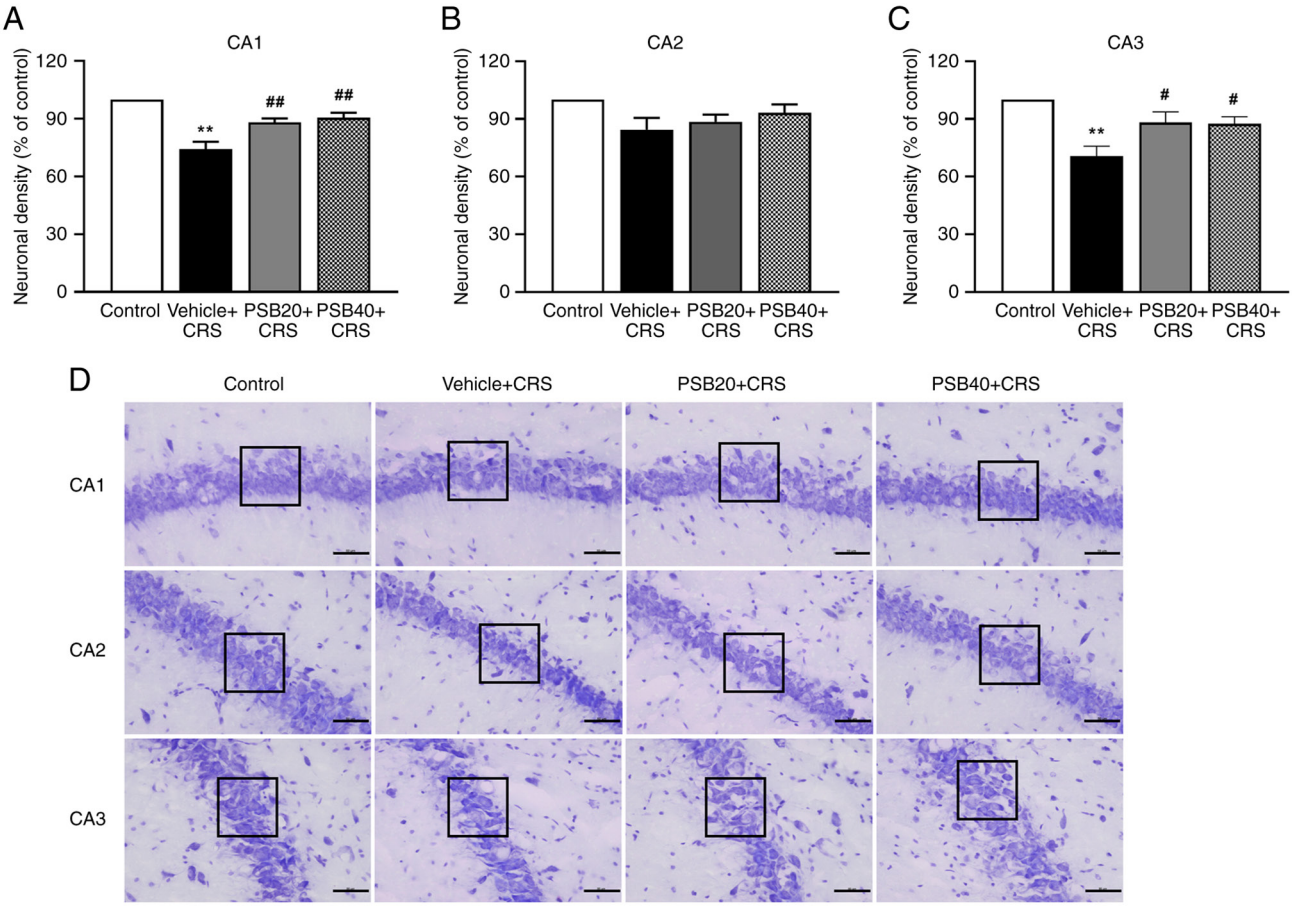

Figure 4. Effects of pinostrobin on neuronal damage in the hippocampus. (A-C) Graphs demonstrate neuronal density in the hippocampal CA1, CA2 and CA3 regions. (D) Representative images of Nissl staining of the hippocampal CA1, CA2 and CA3 regions at x40 magnification. Black squares illustrate the changes in neuronal density. Scale bar, 50 μm. Data are expressed as the mean ± SEM. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the vehicle-CRS group. CRS, chronic restraint stress; PSB, pinostrobin.

were investigated by detecting Nissl staining in the hippocampus (Fig. 4). The animals exposed to CRS exhibited a significant reduction in neuronal density in the hippocampal CA1 and CA3 regions. As shown in Fig. 4D, the neurons in the hippocampal CA1 and CA3 regions of the CRS group were evidently damaged, and missing. However, the administration with pinostrobin at doses of 20 and 40 mg/kg markedly improved the density of surviving cells in the CA1 and CA3 regions of the hippocampus (CA1, P<0.01 at 20 and 40 mg/kg, Fig. 4A; CA3, P<0.05 at 20 and 40 mg/kg, Fig. 4C). However, no significant difference was found in the neuronal density in the hippocampal CA2 region.

Effects of pinostrobin on the alteration of GFAP immunoreactivity in the hippocampus. To examine whether CRS affects astrocytes, the alteration of GFAP-immunoreactive astrocytes in the hippocampus was further evaluated using immunohistochemistry and threshold analysis. As shown by the results illustrated in Fig. 5, the rats exposed to CRS exhibited a significant reduction in GFAP immunostaining in all regions of the hippocampus compared to the controls (P<0.01). Notably, it was found that the astrocytes of the rats exposed to CRS exhibited small cellular bodies with less and thinner processes, characterized as atrophy (Fig. 5A). However, treatment of these rats with pinostrobin at 20 and 40 mg/kg markedly ameliorated the CRS-induced downregulation of GFAP in the hippocampus compared with the vehicle-treated group (P<0.01, Fig. 5B-D).

Effects of pinostrobin on the alteration of EAAT2 immunolabeling in the hippocampus. To determine the mechanisms through which pinostrobin altered the level of glutamate transporter expressed in astrocytes, the immunoreactivity of EAAT2 was further investigated using immunohistochemical analysis. As illustrated in Fig. 6, EAAT2 immunoreactivity was markedly decreased in the rats exposed to CRS, whereas treatment with pinostrobin at a dose of 20 mg/kg upregulated the expression of EAAT2 in the CA1 and CA3 regions of the hippocampus (CA1, P<0.01; CA3, P<0.05; Fig. 6A and C). Notably, a high dose of treatment (40 mg/kg) enhanced the expression of EAAT2 in all subregions of the hippocampus (P<0.01).

Discussion

The present study investigated the neuroprotective effects of pinostrobin on cognitive performance in rats exposed to CRS. The results suggested that the administration of pinostrobin ameliorated chronic stress-induced cognitive impairment by exerting antioxidant effects, reducing neuronal cell damage,

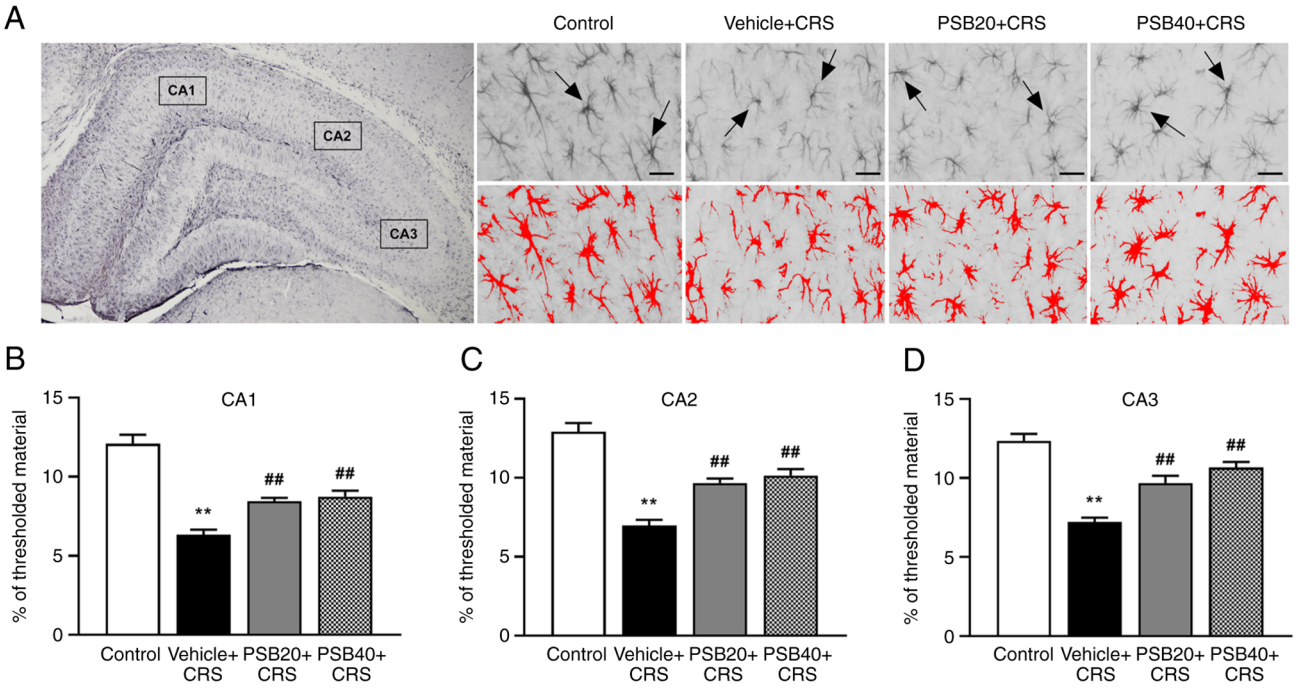

Figure 5. Effects of pinostrobin on the alteration of GFAP-labeled astrocytes in the hippocampus. (A) Immunohistochemical staining for GFAP in the hippocampus and threshold images of GFAP in CA1. (B-D) Graphs demonstrate the GFAP expression in subregions of the hippocampus. Black arrows illustrate morphological changes of astrocytes. Scale bar, 100 μm. Data are expressed as the mean ± SEM. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the vehicle-CRS group. CRS, chronic restraint stress; PSB, pinostrobin; GFAP, glial fibrillary acidic protein.

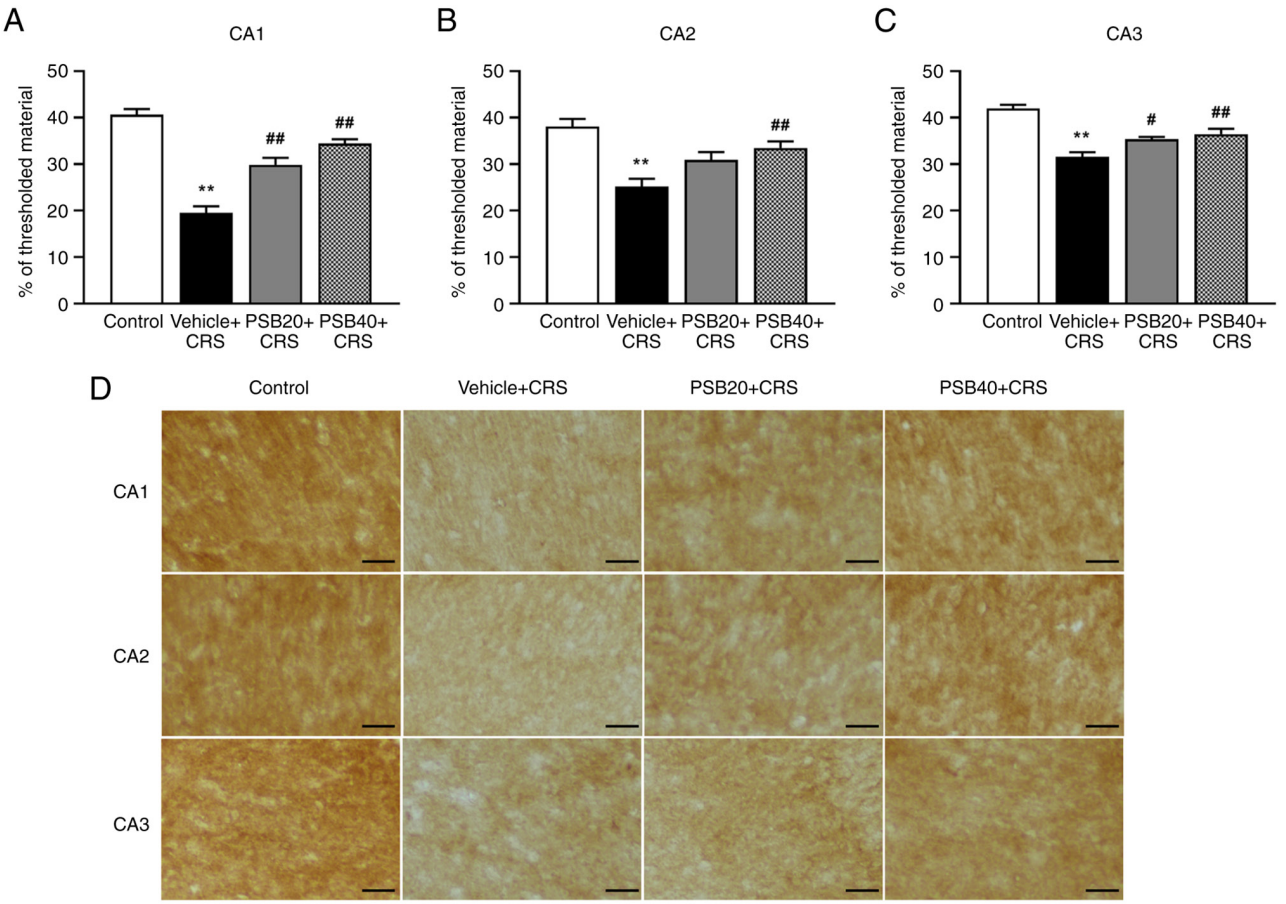

Figure 6. Effects of pinostrobin on the alteration of astroglial EAAT2 immunoreactivity in the hippocampus. Graphs demonstrate EAAT2 expression in the hippocampal (A) CA1, (B) CA2, and (C) CA3 subregions. (D) Representative images of EAAT2 immunoreactivity in the hippocampal CA1, CA2 and CA3 subregions. Scale bar, 100 μm. Data are expressed as the mean ± SEM. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the vehicle-CRS group. CRS, chronic restraint stress; PSB, pinostrobin; EAAT2, excitatory amino acid transporter 2.

and enhancing the expression of astrocytic GFAP and EAAT2 in the hippocampus. Previous studies have revealed that chronic stress is an important regulatory factor for the development of cognitive dysfunction (35-37). As the main structure of the brain associated with cognition and mood, the hippocampus is extremely susceptible to exposure to chronic stress. The hippocampus contains high quantities of GRs. High glucocorticoid levels trigger ROS production, inducing oxidative damage in the hippocampus, resulting in cognitive impairment. Additionally, chronic stress promotes the structural and functional alterations of the hippocampus (38,39), leading to disruptions in cognitive function. The enhanced production of ROS and reduced levels of antioxidants have been implicated in the pathogenesis of neurodegenerative diseases, such as depression and cognitive disorders (40). Exposure to chronic stress can alter the balance of oxidants and antioxidants, leading to a large generation of free radicals, thus inhibiting the antioxidant efficacy. The elevated amounts of lipid peroxidation and diminished antioxidant levels lead to the incidence of oxidative damage in the hippocampus of rats subjected to chronic stress. The findings of the present study demonstrated that chronic stress significantly enhanced MDA levels, accompanied by decreased levels of the major antioxidant enzymes, SOD and CAT, in the hippocampus; these findings are consistent with those of previous studies (41,42).

According to previous findings, the supplementation of natural compounds can ameliorate learning and memory deficits involved in the CRS-induced production of oxidative stress (37,43). Pinostrobin exhibits a several of pharmacological activities, such as antioxidant, anti-inflammatory and neuroprotective properties (21-23). Pinostrobin has been previously reported to exert antioxidant effects on ethanol-induced ulcers in rats by reducing the MDA and nitric oxide levels (27). In addition, pinostrobin has been found to inhibit the production of inflammatory cytokines, such as TNF-α and IL-1β in both *in vitro* and *in vivo* experiments, indicating anti-inflammatory activity (22). Pre-treatment with pinostrobin has been reported to exert neuroprotective effects against β-amyloid-induced neurotoxicity by suppressing oxidative damage and neuronal apoptosis (23). Moreover, pinostrobin has been shown to reduce the loss of dopaminergic neurons in a model of PD by exerting antioxidant and neuroprotective effects by decreasing lipid peroxidation and enhancing the levels of antioxidant enzymes (24). Therefore, in the present study, the antioxidant and neuroprotective potential of pinostrobin was determined. The results demonstrated that chronic treatment with pinostrobin at doses of 20 and 40 mg/kg significantly reduced the level of hippocampal lipid peroxidation induced by CRS. In addition, treatment with pinostrobin significantly reversed the CRS-induced decrease in hippocampal SOD and CAT activities. This may be one of the possible underlying mechanisms for the neuroprotective potential of pinostrobin in the cognitive impairment of rats with CRS. However, the present study determined oxidative stress markers only in the hippocampal homogenate, and not by using histological analysis. This is a limitation of the present study. Thus, further study are required to examine the alterations in oxidative stress using histological methods.

Moreover, the ameliorative effects of pinostrobin on cognitive deficits were determined using the Y-maze test. Working memory is one of the short-term memories that can be decreased in Alzheimer's disease. The Y-maze test was extensively used to evaluate the function of hippocampal spatial working memory by counting the number of arm entries and calculating the percentage of spontaneous alternation (31). The animals typically prefer to explore a new arm of a maze instead of going to the previously visited arm, which is related to the spatial working memory. Similar to a previous study, the present study demonstrated that repeated stress for 21 days impaired working memory by decreasing the percentage of spontaneous alternations in the Y-maze compared to the control group not exposed to stress (44). However, treatment with pinostrobin significantly enhanced the percentage of spontaneous alternations, indicating its ability in ameliorating cognitive impairment. Moreover, the effects of pinstrobin on the recognition memory were examined by conducting the novel object recognition test. The results demonstrated that the administration of pinostrobin at a dose of 40 mg/kg expressively attenuated the impairment of recognition memory by increasing the recognition index following prolonged exposure to stress. These findings demonstrated that pinostrobin improved cognitive performance in both the Y-maze and NOR tests. There are numerous behavioral tests to determine cognitive function in rodent models, such as the Morris water maze (MWM), NOR test, Y-maze, T-maze and passive avoidance task. The previous study by Xu *et al* (45) used the MWM as a test of spatial memory in a model of chronic stress. Additionally, some studies have used two behavioral tests, including the NOR and Y-maze tests (46), or MWM and NOR test (47). Therefore, the use of the NOR and Y-maze tests was considered adequate to evaluate cognitive deficits in the present study. However, further research is required using several cognitive tests to provide more comprehensive data associated with hippocampal functions.

There is accumulating scientific evidence to demonstrate that chronic stress can provoke hippocampal neurodegeneration, including neuronal apoptosis, decreased synaptic plasticity and the reduction of dendritic spine density, which results in learning and memory impairment (7,48). Therefore, the present study examined the protective effects of pinostrobin on neuronal damage by detecting Nissl staining in the hippocampus. The animals exposed to chronic stress exhibited noticeable neuronal damage in the CA1 and CA3 regions of the hippocampus, while CA2 did not exhibit a significant reduction in neuronal density. Chronic stress has been shown to induce morphological alterations in the hippocampus. Chronic stress causes the reduction of dendrites in the hippocampal CA3 and dentate gyrus (DG) neurons. The synaptic connection of the hippocampus involves the input from the entorhinal cortex to the CA3 and DG, with the feed-forward and feedback connections between these two subregions enhancing memory formation (49). In addition, alterations in CA3 associational axons can stimulate their neighbors, thereby magnifying the output of CA3. Consequently, the disruptions of the CA3 associational system causes the reduced activation of CA1 (50). As demonstrated in previous studies, exposure to chronic stress for 21 days promotes a substantial reduction of dendritic spine density in the hippocampal CA1 and CA3 regions (51,52). Additional studies have revealed that CRS leads to dendritic retraction in the CA3, which is an extremely vulnerable region

related to the alterations in *N*-methyl-d-aspartic acid receptors, and leads to neurotoxicity and neuronal death in the hippocampus (52,53). On the other hand, the hippocampal CA2 region has been reported to exhibit several features that distinguish it from the CA1 and CA3 regions, including a distinctive gene expression profile, inability to display long-term potentiation (LPT) and relative resistance to cell death (54). This may be the possible explanation as to why, in the present study, no significant difference was found in neuronal cell loss in the hippocampal CA2 region. Moreover, the structural and functional alterations in different hippocampal subregions may be due to the type, duration and intensity of stress stimuli (53). However, the results demonstrated that treatment with pinostrobin at doses of 20 and 40 mg/kg markedly enhanced the density of surviving cells in the hippocampal CA1 and CA3 regions, suggesting the occurrence of a neuroprotective effect in the chronically stressed rats.

To further elucidate the possible neuroprotective mechanisms of pinostrobin, the disruptions of astrocytes involved in CRS-induced memory deficits were determined. Previous studies have suggested that CRS-induced astrocyte dysfunction may be associated with learning and memory deficits (55,56). GFAP is a critical marker of intermediate filament protein in astrocytes. Astrocytes are the most common population of glial cells in the central nervous system, as they play a key role in neuronal homeostasis, supporting neurons and regulating synaptic transmission. In addition, astrocytes can remove excess glutamate out of the synaptic cleft through EAATs in neuronal protection against excitotoxicity. Previous studies have demonstrated that exposure to CRS markedly decreases the expression of GFAP in the prefrontal cortex and the hippocampus (26,57,58). Similarly, the present study revealed that exposure to chronic stress led to a significant reduction in the immunostaining of GFAP in the hippocampus compared to the controls. On the other hand, it was found that treatment with pinostrobin markedly and dose-dependently enhanced GFAP immunoreactivity in the hippocampal CA1, CA2 and CA3 regions following exposure to CRS. Apart from astrocytes, excitatory glutamate receptors, particularly EAAT2, which is the principal glutamate transporter expressed in astrocytes, plays a crucial role in the capacity of glutamate uptake and in preventing neuronal damage from glutamate neurotoxicity. A previous study suggested that the disruption of GFAP provokes a disruption in the clearance of glutamate over the synaptic cleft. Thus, it is possible that the reduction in GFAP expression induced by CRS may produce a decrease of EAAT2 in the hippocampus (59). Consistently, the findings of the present study revealed a reduction in EAAT2 immunoreactivity in the hippocampus of animals exposed to CRS. Of note, these changes were effectively restored by pinostrobin treatment. However, the present study did not measure the amount of glutamate and the synaptic plasticity in the hippocampus for the possible underlying mechanisms in neurotoxicity. Thus, further investigations are warranted in this regard.

The results demonstrated herein revealed the dose-dependent effects of pinostrobin in treating CRS-induced cognitive deficits. In a previous study on the toxicity of pinostrobin, it was reported that pinostrobin was non-toxic and was not mutagenic to male rats within the 1-100 mg/kg dose range (60). Therefore, the doses of pinostrobin (20 and 40 mg/kg) used in the present study are within the non-toxic dose range and as indicated by the results, these doses of pinostrobin significantly and dose-dependently improved cognitive impairments in rats exposed to CRS. Consistent with the findings of the present study, the study by Abdelwahab *et al* (27) demonstrated that pinostrobin at doses of 20 and 40 mg/kg significantly protected against ethanol-induced peptic ulcers in rats. In addition, another study demonstrated that pinostrobin at a dose of 40 mg/kg significantly inhibited the renal expression of cystic fibrosis transmembrane conductance regulator in rats with polycystic kidney disease (61). It is possible that treatment with higher concentrations of pinostrobin may exert therapeutic effects without toxicity. Thus, further studies use various doses (20, 40, and 80 mg/kg) of pinostrobin are required to confirm the dose-dependent effects of pinostrobin.

In conclusion, the present study indicated that treatment with pinostrobin significantly attenuated chronic stress-induced cognitive impairment by decreasing the levels of oxidative stress, reducing neuronal damage, and by enhancing the function of astrocytes and EAAT2 in the hippocampus of rats. Therefore, these findings suggest that pinostrobin may have potential medicinal value as a neuroprotective agent for the prevention and treatment of chronic stress-induced cognitive deficits and other cognitive disorders.

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Availability of data and materials

The database used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RK designed the study, performed all the experiments and wrote the manuscript. ST was involved in the study methodology and wrote the manuscript. SS was involved in the preparation of the extract. TP and JJ were involved in the data analysis and in editing the manuscript. All authors have read and approved the final manuscript. RK and ST confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of the Laboratory Animal Research Center, University of Phayao, Phayao, Thailand (approval no. 640104006).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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