

Original Research Article

Effect of Polyherbal X administration on malondialdehyde levels, superoxide dismutase activity, and hippocampal histopathology in rat models of ischemic stroke

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Abstract

Purpose: To assess the effect of polyherbal X containing *Moringa oleifera* L., *Phyllanthus niruri* L., and *Nigella sativa* L. on superoxide dismutase (SOD) activity, malondialdehyde (MDA) levels, and hippocampal histopathology in rat models of ischemic stroke.

Methods: This study examined seven groups of male Wistar rats, namely normal control, negative control, positive control, and four treatment groups with graded doses of polyherbal mixture, viz. 50, 100, 200, and 400 mg/kg body weight (BW). These rats were subject to ischemic stroke induction using a single intraperitoneal administration of trimethyltin chloride (0.5 mg/kg BW). Polyherbal X was administered orally to rat models of ischemic stroke in treatment groups once daily for 2 weeks. After that, blood samples were analyzed for their malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity, and hippocampal histopathology was determined.

Results: Rat models in the group treated with 400 mg/kg BW of polyherbal X had the lowest MDA level (1.68 nmol/mL) and the highest SOD activity (190.33 U/mL). The effective dose of polyherbal X was 200 mg/kg BW, which resulted in MDA level of 2.25 nmol/mL and SOD activity of 184.44 U/mL. Furthermore, histopathological analysis revealed that polyherbal X at 200 and 400 mg/kg BW significantly reduced the number of damaged neurons ($p < 0.05$), with results comparable to those of positive control.

Conclusion: Polyherbal X significantly affects MDA levels and SOD activity, ultimately reducing neuronal damage in rat models of ischemic stroke.

Keywords: *Moringa oleifera* L, *Phyllanthus niruri* L, *Nigella sativa* L, Ischemic stroke, Malondialdehyde, Superoxide dismutase

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INTRODUCTION

The growing number of young adults suffering from ischemic stroke highlights the special need

for targeted preventive measures for this age group. While the number of stroke survivors has nearly doubled to 33 million between 1990 and 2010, epidemiological projections indicate that

this number will escalate to reach 77 million by 2030 [1]. Meanwhile, therapeutic efficacy in treatment of ischemic stroke remains very limited, thus emphasizing the necessity of protecting against this disease.

Cerebral infarction, which is associated with thrombosis in the arteries or blockage of blood vessels by emboli, is the most prevalent form of stroke observed in clinical cases. Increased free radical production has been shown to contribute to brain ischemia and reperfusion [2]. Ischemia induces oxidative stress and lipid peroxidation reactions, both leading to secondary damage to nerve tissue [3]. Flavonoids, which exhibit antioxidant properties, are believed to be able to reduce oxidative stress and mitigate neuronal injury in ischemic stroke. Herbal remedies or related products possess neuroprotective properties and have the ability to dilate cerebral blood vessels, enhance brain circulation, inhibit platelet aggregation, protect against brain ischemia and reperfusion injury, and increase the tolerance of ischemic tissues to hypoxia [4]. Herbal medicines have multi-constituent, exhibit multi-effects at multiple targets, and have drawn the attention of numerous scientists due to the synergistic effects of their bioactive ingredients, especially in treating stroke [5,6]. Studies related to stroke treatment usually use animals such as Wistar rats to study the pathophysiological aspects of each type of stroke before being applied to stroke prevention and therapy in humans [7].

Moringa oleifera L. of the family Moringaceae is cultivated extensively in Asia, Polynesia, and the West Indies. Moringa leaves are rich in polyphenols, vitamin C, β -carotene, and vitamin E, which possess antioxidant properties [8]. The potential therapeutic benefits of *Moringa oleifera* L., including its anticancer, antidiabetic, anti-atherosclerosis, antifertility, antidepressant, and pain-relieving properties, have been proven in numerous investigations. According to recent studies, moringa leaf extracts also exhibit antioxidant properties, which may prevent oxidative damage [9].

Phyllanthus niruri L., or *meniran* of the family Euphorbiaceae, has various health benefits. Two endogenous antioxidants, namely catalase (CAT) and superoxide dismutase (SOD), are more active in the liver, kidney, cardiac tissues, and brain of rat models given methanol extract of *Phyllanthus niruri* L. In addition, bioagents found in *Phyllanthus niruri* L. prevent lipid peroxidation and superoxide overproduction brought on by chronic hyperglycemia, thereby alleviating abnormalities in lipoprotein metabolism, the ratio

of cholesterol to phospholipids, damage to biomembranes, and ROS-related lipid peroxidation [10,11].

Nigella sativa L., or black cumin of the family Ranunculaceae, is native to Mediterranean and East Asian countries. Previous studies have demonstrated multifarious therapeutic potential of *Nigella sativa* L. extract, encompassing its immunomodulatory, antitumor, and antidiabetic properties. It also exhibits hypoglycemic effects with its ability to increase serum insulin and reduce lipid levels, serum cholesterol, and triglycerides. Furthermore, it has been observed that *Nigella sativa* L. extract provides renoprotective effects, inhibits hepatic necrosis, and enhances T3 serum concentration, which in turn positively influences the nervous system [12,13].

Polyherbal X contains *Moringa oleifera* L., *Phyllanthus niruri* L., and *Nigella sativa* L., which have tremendous potential to treat ischemic stroke. However, there is scant scientific proof of the properties of these plants. Therefore, this study was to determine whether polyherbal X (a mixture of the aforementioned plant species) has therapeutic potential for stroke treatment and whether its administration effectively lessens the effects of oxidative stress and brain cell damage in animal models of ischemic stroke.

EXPERIMENTAL

Materials

Polyherbal X, containing 300 mg moringa (*Moringa oleifera* L.) leaf extract, 100 mg *meniran* (*Phyllanthus niruri* L.), and 100 mg black cumin (*Nigella sativa* L.), was obtained from the 500 mg Onoake® capsule produced by Nucleus Farma (PT. Natura Nuswantara Nirmala) following Good Manufacturing Practices for Traditional Medicine (*Cara Pembuatan Obat Tradisional yang Baik*/CPOTB). This study also used piracetam 500 mg/5 mL syrup; distilled water; standard MDA solution from 1,1,3,3-tetraethoxypropane (TEP); 20 % trichloroacetic acid (TCA) and 0.67 % thiobarbituric acid (TBA) for MDA measurement; sodium carbonate buffer solution (pH 10.2) and epinephrine solution (0.01 M) for SOD measurement; Sampling materials: ketamine (0.01 mg/kg BW) and xylazine (0.02 mg/kg BW); physiological saline solution (0.9 % NaCl); and Phosphate Buffered Saline (PBS).

Ethical approval

Ethical approval was granted by the Animal Ethics Committee at the Faculty of Veterinary

Medicine and Biomedical Sciences, IPB University (approval no. 016/KEH/SKE/VII/2022). Animals were handled according to guidelines described in the care and use of laboratory animals [14].

Animals

Animal models used in this study were 3 – 3.5-month-old male *Rattus norvegicus* Wistar rats weighing between 200 and 250 g. The rats were divided into 3 control groups and 4 treatment groups with different doses of polyherbal X. Federer's formula (Eq 1) was employed to calculate the number of experimental animals per group.

$$(n-1)(t-1) \geq 15 \dots\dots\dots (1)$$

where: t = number of groups; n = number of experimental animals

If t = 7, then the number of experimental animals (n) is:

$$(n-1)(7-1) \geq 15 \rightarrow 6(n-1)(1) 15 \rightarrow n(1) 4$$

These computations indicate that four rats per group is the minimum number of animals needed for this study. Thus, the total number of animals needed is 28 rats.

Sample preparation

A 1 % stock solution was obtained by dissolving 1 g of test material (contents of polyherbal X capsule) into 100 mL of distilled water and stirring it until homogeneous. Trimethyltin chloride (TMT) Solution (0.5 mg/kg BW) was prepared by dissolving TMT in 0.9 % NaCl.

Rat doses and treatment

Male Wistar rats in each group were given different treatments for 14 days as follows:

Rat models (normal control) were given distilled water and regular food orally. Rat models (negative control) were induced by intraperitoneal administration of trimethyltin chloride intraperitoneally and given distilled water and regular food orally. Rat models (positive control) were given 5 mL of piracetam syrup (100 mg/mL) orally after TMT administration. Tested treatment with 50 mg/kg BW of polyherbal X was administered orally to rat models after TMT administration. Tested treatment with 100 mg/kg BW of polyherbal X was administered orally to rat models after exposure to TMT administration.

Treatment with 200 mg/kg BW of polyherbal X was administered orally to rat models after exposure to TMT administration.

Tested treatment with 400 mg/kg BW of polyherbal X was administered orally to rat models after exposure to TMT administration.

Sampling

Following the end of treatment, all animals were euthanized by injecting 0.01 mg/kg BW of ketamine and 0.02 mg/kg BW of xylazine. A necropsy was performed to obtain brain tissue samples. The collected organs were subsequently rinsed with a 0.9% NaCl solution to remove any residual blood. After that, the organs were dried with filter paper and weighed using analytical scales. Rat brain organs were destroyed using a mortar & pestle and dissolved in PBS. After centrifuging the brain homogenate at 3000 rpm for 10 minutes, the supernatant was pipetted into a test tube [15].

Data retrieval

Thiobarbituric acid reactive substances (TBARs) test was employed to determine MDA levels of rat models. For this measurement, 200 μ L of the sample (supernatant from the centrifuged homogenate) was added to 2 mL of 0.67 % TBA and 1 mL of 20 % TCA. Mixture was then homogenized and heated in a water bath for 10 minutes. A UV-VIS spectrophotometer operating at a wavelength of 532 nm was used to measure the absorbance. The obtained values (nmol/mL) were utilized to calculate MDA levels [15]. Meanwhile, SOD activity was measured using Misra and Fridovich method. In this process, 100 μ L of the sample (supernatant from the centrifuged homogenate) was combined in a test tube with 2800 μ L of sodium carbonate buffer (pH 10.2) and 100 μ L of epinephrine solution (0.01 M). A similar procedure was followed for the blank using distilled water. After that, the mixture was transferred into a cuvette, and absorbance was recorded at wavelength of 480 nm for the first, second, third, and fourth minutes. The same absorbance readings were taken for the blanks at the corresponding time intervals [10].

SOD activity was calculated using Eqs 2 and 3.

$$\text{Resistance (\%)} = \{(A-B/A)\}100 \dots\dots\dots (2)$$

$$\text{SOD activity (U/mL)} = \{(\text{Resistance (\%)} / 50\%) \cdot 1 \text{ unit} / 10 \mu\text{L}\}100 \dots\dots\dots (3)$$

where A = average absorbance difference per minute without sample (blank); B = average absorbance difference per minute with sample; 50 % = unit of SOD activity; defined as the amount of SOD needed to prevent epinephrine oxidation by 50 % (SOD₅₀)

Rats' hippocampal incisions were prepared for histopathology analysis using Hematoxylin and Eosin (H&E) staining technique. Hippocampus was observed through a field of view (FOV) observation. For one preparation, five FOV observations were made, each observing the number of normal pyramidal cells and those with damage (pyknosis, karyorrhexis, and karyolysis). From these observations, the percentage of damaged cells in each FOV and the average percentage of cell damage in each treatment were calculated.

Data analysis

IBM SPSS version 20 was utilized to statistically analyze MDA levels and SOD activity data from each treatment group, with Kolmogorov-Smirnov test (one-sample) performed for the normality test. Normally distributed data ($p > 0.05$) were examined using parametric One-Way ANOVA; the post-hoc LSD follow-up test was carried out in the event of a significant difference ($p < 0.05$). Conversely, the non-parametric Kruskal-Wallis test was used to evaluate abnormally distributed data, and Mann-Whitney test was carried out if the data were significantly different ($p < 0.05$).

The value is considered significant ($p < 0.05$) at the 95 % confidence interval.

RESULTS

Effects of polyherbal X on malondialdehyde (MDA) levels in rat models of ischemic stroke

This study performed the TBARS test method to measure MDA levels in this study. It involved reacting 0.67 % thiobarbituric acid (TBA) and 20 % trichloroacetic acid (TCA) with one molecule of MDA in acidic conditions to form the pink-colored complex compound MDA-TBA. Furthermore, the absorption of this compound was measured with a UV-VIS spectrophotometer set at a wavelength of 532 nm. Figure 1 shows the results of MDA levels in each group of rat models [15].

Effects of polyherbal X administration on SOD activity in rat models of ischemic stroke

As one of the enzymatic antioxidants, SOD has a catalytic function in neutralizing free radicals into H₂O₂ and O₂. The quantity of product generated per unit time is used to calculate the enzyme's activity. Measurement is based on the reaction of the product with a compound that produces a certain color. SOD activity was measured using Misra and Fridovich method. This procedure is predicated on SOD's capacity to prevent epinephrine autooxidation to adrenochrome, which gives substances a brown hue.

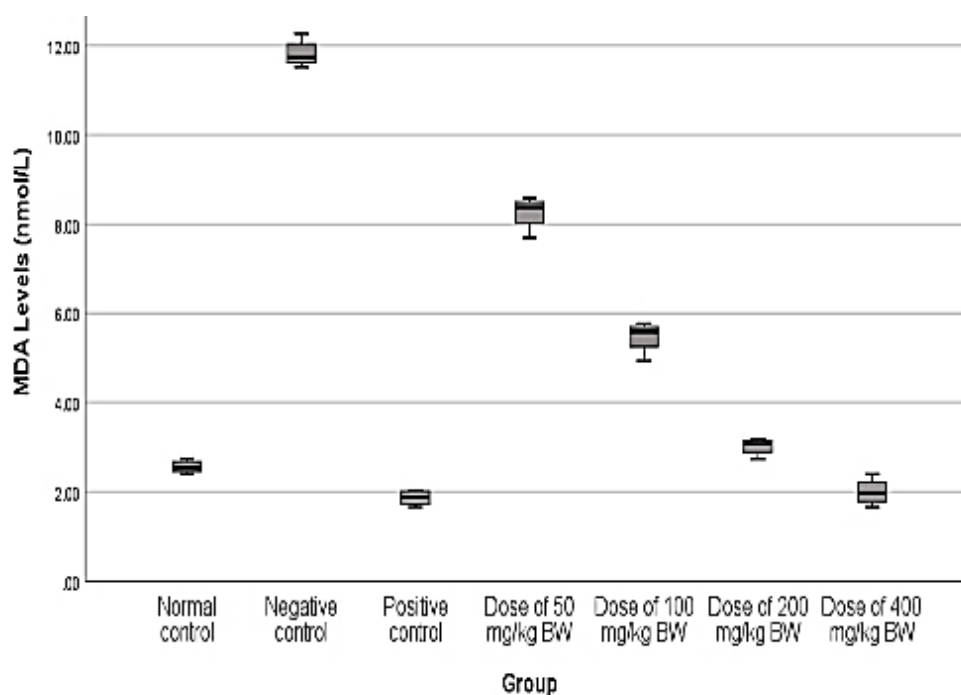


Figure 1: Results of MDA levels (nmol/mL)

The greater the SOD content of the sample, the greater the inhibition and the lesser the color intensity. Using a spectrophotometer, the brown color is detected at a wavelength of 480 nm [10]. The results of SOD activity in each group of rat models are presented in Figure 2.

As shown in Figure 2, the negative control group had the lowest average SOD activity compared to the other groups. In this study, trimethyltin chloride (TMT) was administered to the

experimental animals (rat models) for 7 days. This may increase free radical production, leading to more damage to neurons. The elevated levels of free radicals result in the use of endogenous antioxidants [16]. The rat models in the negative control group used much more endogenous antioxidants than those in the other groups since they did not receive exogenous antioxidants. An adrenochrome assay was used in this study as it is a sensitive and simple method to measure the SOD activity.

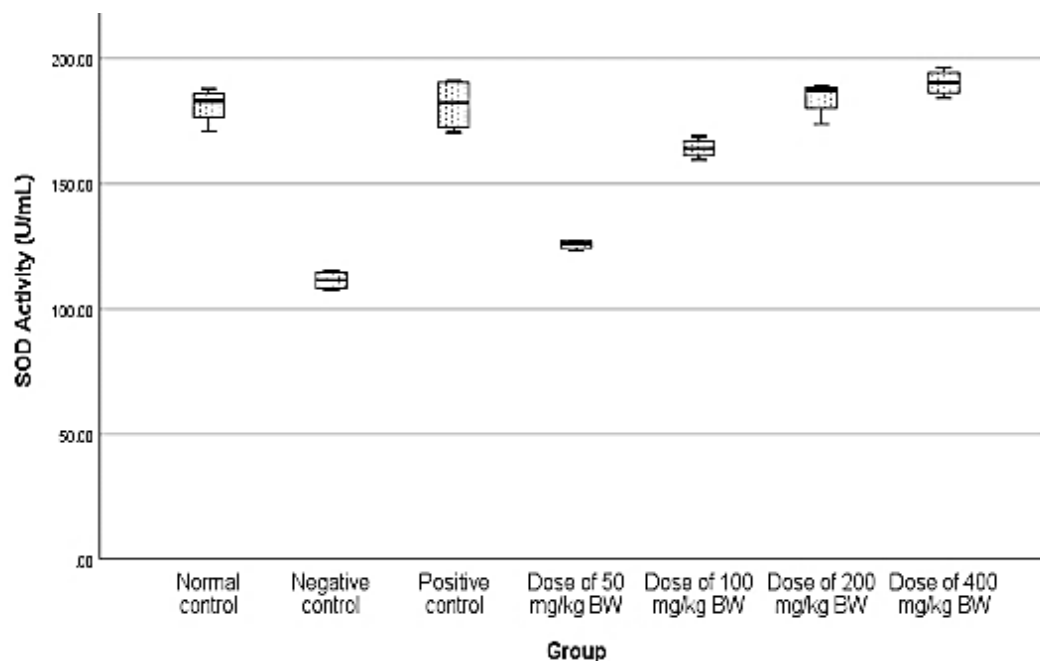


Figure 2: Results of SOD activity (U/mL)

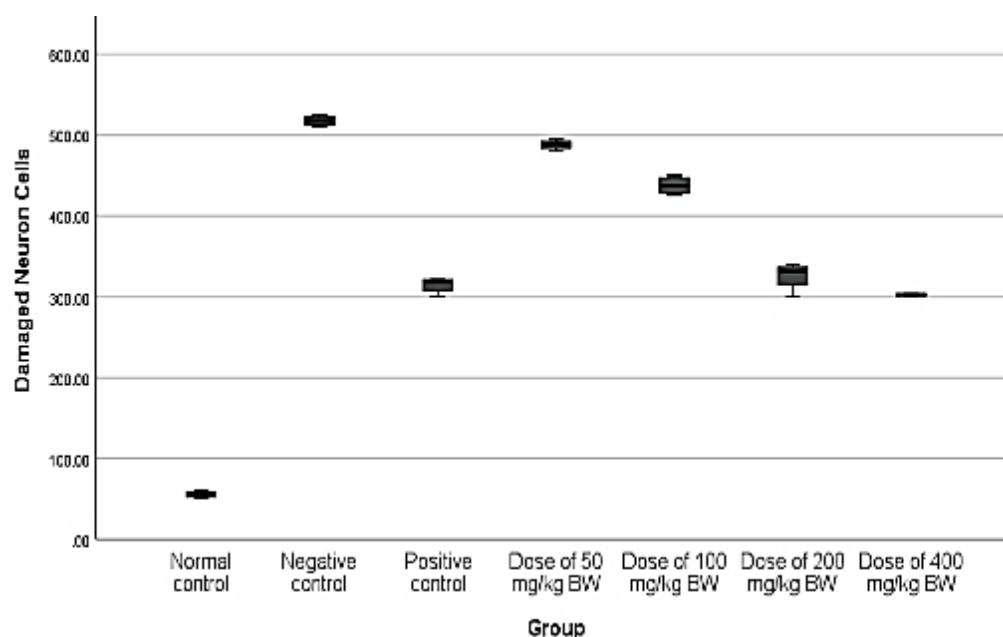


Figure 3: Damaged neurons in each group

Effects of polyherbal X administration on hippocampal histopathology

The number of damaged neurons in the negative control group differs significantly from that of the positive control group (Figure 3). This indicates a statistically significant difference between the positive control and treatment groups receiving polyherbal X at doses of 50 mg/kg BW and 100 mg/kg BW. Conversely, no significant difference is found between the positive control and the groups treated with polyherbal X at doses of 200 mg/kg BW and 400 mg/kg BW.

Table 1: Data processing of damaged neurons

Test	Method	P-value	α -value
Normality	Kolmogorov-Smirnov	0.856	0.05
Homogeneity	Levene	0.063	0.05

Statistical analyses were carried out to determine the validity of parametric testing for the data of damaged neurons (Table 1). First, a normality test was done using the Kolmogorov-Smirnov method, obtaining a significance (p -value = 0.856). This exceeds the threshold value of 0.05, signifying that the data are normally distributed. After that, the homogeneity of variance was assessed using Levene's test, achieving a significance value of 0.063 ($p > 0.05$). This confirms that the data exhibit homogeneous variance among groups. With the fulfillment of

both assumptions for parametric analysis, a one-way ANOVA was performed to evaluate differences between treatment groups, yielding a significance value of 0.000 ($p < 0.05$). This indicates the statistically significant differences in the number of damaged neurons across the groups.

After being induced with ischemic stroke, Wistar rats were given polyherbal extract in various doses for 14 days. Figure 4 presents microscopic visualization of neurons after extract administration.

DISCUSSION

Data revealed that the average MDA levels increased in negative control group. This is because trimethyltin chloride-induced brain cell damage occurs in the rat models. Furthermore, trimethyltin chloride administration produces free radicals that result in oxidative stress, thus triggering an increase in lipid peroxidation processes [16]. Malondialdehyde (MDA) is a byproduct of lipid peroxidation that exist in either free or complex form within tissues in the body [17].

In comparison to other groups, results revealed a modest reduction in MDA levels in the positive control group, signifying that the administration of piracetam prevents the process of lipid peroxidation.

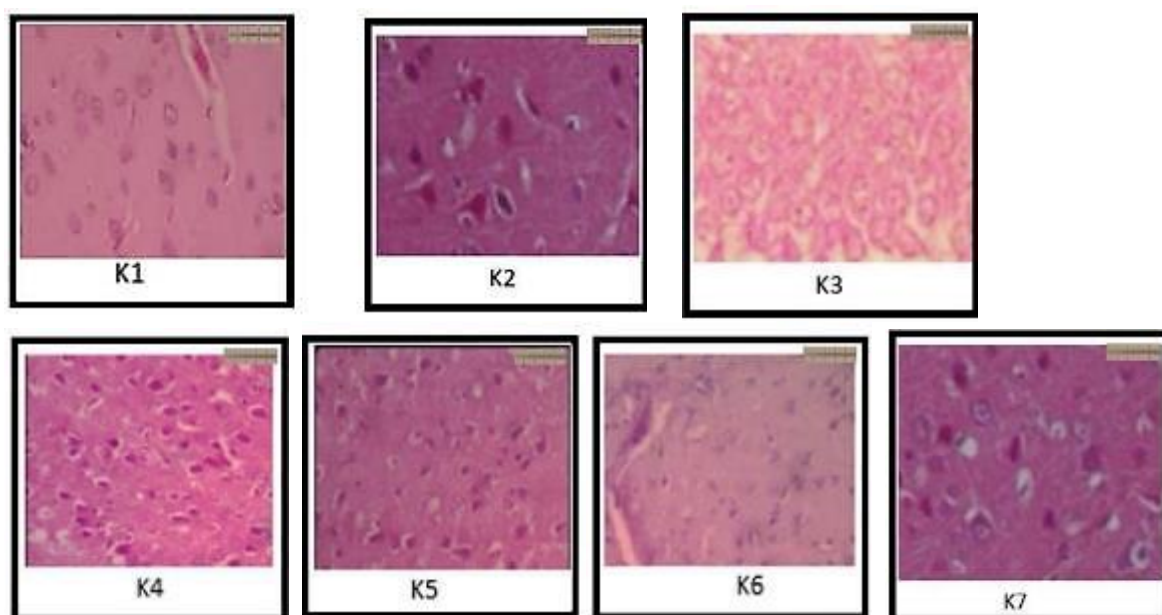


Figure 4: Microscopic visualization of neurons after extract administration. Group I = normal control; Group II = negative control; Group III = positive control; Group IV = treatment dose of 50 mg/kg BW; Group V = treatment dose of 100 mg/kg BW; Group VI = treatment dose of 200 mg/kg BW; and Group VII = treatment dose of 400 mg/kg BW

Piracetam belongs to the category of neuroprotector drugs, which are medications designed to lessen the likelihood of cell damage. Piracetam contributes to cell membrane repair and blood flow enhancement in the brain. Blood aids in the removal of cellular waste and supplies the oxygen and glucose needed for cell metabolism. Increased oxygen and nutrition delivered to neurons by improved blood flow enhance focus, memory, and cognitive function [9]. Administration of trimethyltin chloride causes damaged neurons to recover.

As for treatment groups, the rat models administered 400 mg/kg BW of polyherbal X had an average MDA level lower than those given other doses of polyherbal X. This indicates that polyherbal X has a greater effect on lowering MDA levels at larger doses.

SOD activity is based on its ability to inhibit the autooxidation of epinephrine. In an acidic state, an epinephrine solution will remain stable but oxidize spontaneously as the pH increases. Autooxidation occurs most rapidly at pH 10.2 and a temperature of 30 °C, accompanied by the formation of adrenochrome with a linear velocity [5].

Due to the administration of piracetam, mean SOD activity in Group III (positive control) was higher than that of the other control groups and treatment groups with 50 mg/kg BW and 100 mg/kg BW of polyherbal X. Through enhanced cholinergic neurotransmitter action, piracetam improves the effectiveness of the brain's telencephalon function which regulates human cognitive functions (memory, consciousness, learning, and others). In addition, piracetam stimulates oxidative glycolysis, increases oxygen consumption in the brain, influences cerebrovascular regulation, and has antithrombotic properties that reduce major neuronal lesions. Due to its remarkable qualities, piracetam is commonly used for the treatment of stroke, especially ischemic stroke. Piracetam affects brain activity by a multitude of different mechanisms, including the stimulation of neuron transmission in the brain, enhancement of brain metabolism, and improvement of microvasculature without vasodilation, thus maintaining SOD in the body [18].

Among all groups, Group VII (treatment with 400 mg/kg BW of polyherbal X) had the highest mean SOD activity, namely 190.33 U/mL. This shows that the body improves SOD activity more effectively when a larger dose of polyherbal X is administered. This is due to the flavonoid content

of polyherbal X, which functions to sustain SOD activity [19].

The average values of SOD activity in Group IV (treatment with 50 mg/kg BW of polyherbal X), Group V (treatment with 100 mg/kg BW of polyherbal X), and Group III (positive control) showed significant differences, namely 125.76 U/mL, 164.04 U/mL, and 181.52 U/mL, respectively. This suggests that piracetam increases SOD activity better than polyherbal X at doses of 50 mg/kg BW and 100 mg/kg BW.

The average SOD activity in Group VI (treatment with 200 mg/kg BW of polyherbal X) statistically showed no significant difference from that of the positive control (treatment with piracetam). This implies that the administration of polyherbal X at a dose of 200 mg/kg BW maintains SOD activity in the body as effectively as piracetam. SOD activity will increase when higher doses of polyherbal X are administered. Both polyherbal X (which contains flavonoids) and piracetam (which serves as an exogenous antioxidant and neuroprotective agent) maintain SOD activity in the body [19].

Reduced blood flow leads to a decrease in ATP production, resulting in the dysfunction of the Na⁺/K⁺ pump and subsequent neuronal membrane depolarization due to ionic imbalance. This depolarization triggers excessive glutamate release and a rise in intracellular Ca²⁺ levels, which in turn damages mitochondria and promotes axonal sprouting. Mitochondrial dysfunction increases the generation of reactive oxygen species (ROS) and initiates apoptotic pathways, ultimately leading to neuronal damage [20].

The number of damaged neurons decreases progressively with increasing doses of the polyherbal extract. This is attributed not only to the neuroprotective properties of quercetin found in *Moringa oleifera*—which aids in neuronal repair—but also to the presence of phyllanthin in *Phyllanthus niruri* as well as various other antioxidants contained in the polyherbal extract. Neuronal damage in this context is primarily mediated through the excitotoxicity pathway. Excitotoxic effects lead to cytotoxic edema due to water accumulation and increased expression of NMDA receptors and intracellular Ca²⁺, ultimately resulting in apoptosis and neuronal injury [21].

The result of damaged neuron counts in the positive control demonstrates the lowest level of neuronal injury compared to the other groups. This is due to the administration of piracetam, which reduces cellular damage through its

mechanism of action [22]. Meanwhile, among treatment groups, those treated with polyherbal X at doses of 200 mg/kg BW and 400 mg/kg BW also have lower neuronal damage.

CONCLUSION

Polyherbal X administration is shown to decrease MDA levels and increase SOD activity in rat models of ischemic stroke, with the optimal dose being 200 mg/kg BW. Polyherbal X at doses of 200 mg/kg BW and 400 mg/kg BW effectively reduced neuronal damage, showing effects comparable to positive control. However, further studies are needed to determine the toxicity and stability of polyherbal X. Future studies are also suggested to employ other methods of inducing ischemic stroke in animals to determine other effects of polyherbal X administration for the treatment of ischemic stroke.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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