ORIGINAL ARTICLE

Effects of Sauropus Androgynus Extract on Cognitive Function Improvement and Neuron Enhancement in Trimethyltin Chloride-Induced Rats

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ABSTRACT

INTRODUCTION: This study examines the effects of Sauropus androgynus on cognitive function, Bax expression, and neuronal number in the hippocampus and prefrontal cortex of rats induced by Trimethyltin chloride (TMT). TMT is a compound utilized to induce neurotoxicity. **MATERIALS AND METHODS:** This is an experimental study. Twenty-five male Rattus norvegicus. were divided into five groups: Group 1, no treatment (G1); Group 2, TMT-induced (G2); and Groups 3, 4, and 5, TMT-induced with ethanol extract of S. androgynus at doses of 75, 150, and 300 mg/kg body weight, respectively (G3, G4, and G5). The TMT is at a dose of 6 mg/kg body weight. The cognitive function of rats was measured by using the Y-maze spontaneous alternation test. Brain tissues of killed rats were collected for histological preparations using toluidine blue staining and immunohistochemistry with Bax antibody. Microscopic observations were performed to count the number of neurons and Bax expression. **RESULTS:** The percentage of alternation in the S. androgynus extract groups (G3, G4, G5) was higher than in the TMT group (G2). The number of neurons in the CA1 area of the hippocampus, dentate gyrus, and prefrontal cortex in the three groups (G3, G4, and G5) was higher than in group G2. The expression of Bax in groups G4 and G5 was lower than that in group G2. Different doses of 75, 150, and 300 mg/kg body weight did not provide significant differences in the percentage of alternation and the number of neurons. **CONCLUSION:** The S. androgynus extract could improve memory function, inhibit Bax expression, and increase the number of neurons in the CA1 area of the hippocampus, dentate gyrus, and prefrontal cortex in rats induced with neurotoxicity using TMT.

Keywords:
Sauropus androgynus, Trimethyltin, memory, hippocampus, prefrontal cortex.

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INTRODUCTION

Sauropus androgynus is a tropical plant known for its edible leaves and medicinal properties in Indonesia. It contains various phytochemical compounds, including vitamin C, which enhance its antioxidant capabilities.[1,2] S. androgynus is rich in bioactive substances such as sterols, saponins, alkaloids, terpenoids, phenols, tannins, flavonoids, and catechol. Traditionally, it has been used to boost lactation, treat diabetes and diabetic ulcers, and manage obesity.[2,3,4] Case reports have documented successful recoveries from heart stroke induced by ischaemic stroke using S. androgynus-based traditional therapies without serious complications.[5]

This study investigates the effects of S. androgynus on cognitive function, neuron counts in the hippocampus and prefrontal cortex, and Bax expression in rats induced with neurotoxicity using trimethyltin (TMT). TMT is a toxic compound used to induce neurodegeneration, causing extensive damage across various brain regions, including the hippocampus, olfactory area, cerebellum, pons, mammillary nucleus, and others.[6,7] Rats treated with intraperitoneal TMT exhibited impaired learning and memory, increased aggressive behavior, and hyperactivity.[8] TMT administration reduces acetylcholine levels, impacting cognitive function, similarly seen in Alzheimer's

disease.[9] TMT alters biomarkers linked to memory formation, including cAMP response element-binding protein (CREB), protein kinase C (PKC), neuronal nuclear protein (NeuN), nerve growth factor (NGF), and ionized calcium-binding adaptor molecule 1 (IBA1) within the hippocampus.[10]

TMT can help impair memory function and decrease the number of neurons in the hippocampus's CA1, CA3, and dentate gyrus areas.[11,12] TMT can cause pathological changes in the dentate gyrus and lead to an increase in necrotic neurons. Administering TMT at a dose of 6 mg/kg body weight to rats can result in brain damage characterised by necrosis and cell death in the hippocampal region, accompanied by heightened activity of glial cells. TMT-induced neurotoxicity in the brain is linked to mitochondrial dysfunction, affecting Na+/K+ ATPase function in cell membranes, disrupting adenosine triphosphate (ATP) synthesis, and triggering neuroinflammation. This inflammation is marked by significant activation of glial cells and the production of proinflammatory cytokines, contributing to neurodegenerative damage.[13,14]

The toxic effects of TMT in the brain involve elevated calcium levels, excitotoxicity, mitochondrial dysfunction, oxidative stress, and neuroinflammation processes. Increased oxidative stress produces reactive oxygen species (ROS), which can damage mitochondria by causing swelling, reducing mitochondrial membrane potential, and activating pathways that initiate apoptosis. Elevated ROS levels affect apoptosis-related proteins like Bcl-2, BAX, and caspase-3.[15,16] TMT administration could lead to increased expression of caspase 3, oxidative stress, elevated calcium levels, and mitochondrial damage, especially in the CA1 area of the hippocampus, resulting in a decrease in the number of neurons.[17] This compound can induce cytotoxicity, evidenced by a lower ratio of phosphorylated Akt (p-Akt)/Akt compared to controls. Akt commonly promotes cell survival by phosphorylating proteins such as glycogen synthase kinase-3 (GSK-3), BAD, and caspase 9. TMT can also upregulate BAX expression, leading to increased mitochondrial permeability, release of cytochrome c, and activation of the caspase cascade, ultimately leading to apoptosis.[9] Moreover, TMT can enhance microglial activation, increasing proinflammatory cytokines such as Tumor Necrosis Factor alpha (TNFα), interleukin-1β (IL-1β), and Interleukin (IL-6). It also elevates mRNA levels of pro-inflammatory microglial markers like TNFα, IL-1β, IL-6, and nitric-oxide synthase-2 (NOS2). Additionally, TMT may reduce cholinergic immunoreactivity (ChAT, AChE), brain-derived neurotrophic factor (BDNF), and cAMP-response element-binding protein (CREB) in the hippocampus, affecting choline acetyltransferase (ChAT) and acetylcholinesterase (AChE).[8]

MATERIALS AND METHODS

This experimental study was conducted using a post-test-only group design. It was approved by the Ethics Committee of the Faculty of Medicine, Universitas Islam Indonesia. The experimental animals used consisted of 25 male rats of Rattus norvegicus (Wistar strain), aged 2-3 months. They were divided into five groups: Group 1, no treatment (G1); Group 2, TMT-induced (G2); and Groups 3, 4, and 5, induced with TMT and administered with ethanol extract of SA at doses of 75, 150, and 300 mg/kg body weight, respectively (G3, G4, G5).

Ethanol Extract of Sauropus androgynus

S. androgynus leaf simplicia was obtained from local farmers in Tegalyasan Farm, Tegalarum Village, Sempu District, Banyuwangi Regency, East Java Province, Indonesia. The leaves of S. androgynus were extracted using a maceration technique. The leaves were dried under sunlight until thoroughly dehydrated and completely dry. Next, the dried leaves were made into powder. Subsequently, the powder was mixed with 96% ethanol in a ratio of 1:10. The mixture was soaked for 72 hours and stirred every 24 hours. The mixture was filtered using filter paper. Next, a rotatory vacuum evaporator and a water bath evaporator were utilized to concentrate the filtrate at approximately 60 degrees Celsius until the extract concentration was 100%.[18]

Intervention of Ethanol Extract of S. androgynus and Induction of TMT

The rats were housed in cages measuring 40 cm x 45 cm x 15 cm, with one rat per cage. The cages were maintained

at room temperature between 24-26 degrees Celsius and with a 12-hour light-dark cycle. The light cycle began at 6 am, while the dark cycle started at 6 pm. Food and water were provided ad libitum. TMT (Sigma-Aldrich) was administered via intraperitoneal injection at a dose of 6 mg/kg body weight on the first day of the study. Rats in groups G1 and G2 were injected with normal saline. Meanwhile, groups 3, 4, and 5 (G3, G4, and G5) were treated with the ethanol extract of S. androgynus leaves using a probe daily for 28 days.

The Measurement of Cognitive Function

The cognitive function of the rats was assessed using the Y-maze spontaneous alternation test. The spontaneous alternation referred to the number of rats entering the arms of the Y-maze within 8 minutes, with the condition that all four legs enter the arms of the Y-maze. Next, the researchers calculated the percentage of alternation by using a formula: Alternation% = Number of Alternations/(Total number of arm entries-2) x 100.19

In this study, the Y-maze test was conducted on the 28th day. It measured 50 cm x 10 cm x 20 cm and was placed in a closed room. Before assessment, the rats were acclimatized to the experimental room for 30 minutes. Then, the experiment was conducted on the Y-maze using the spontaneous alternation method to assess their spatial working memory. The researchers recorded and placed the Wistar rats at label A, facing towards the center, allowing them to explore the sides of the Y-maze for 8 minutes. After 8 minutes, the rats were returned to their respective cages. The researchers calculated the percentage of alternation by using the formula: Alternation % = Number of Alternations / (Total number of arm entries – 2) x 100.[18]

Necropsy and Histological Preparation

On the 29th day, the rats were anesthetized with Tiletamine + Zolazepam (Zoletil™) via intramuscular injection, followed by transcardial perfusion. Transcardial perfusion was done by inserting sodium chloride fluid through the heart to flush and cleanse the blood from the tissues. The brain tissue was then taken and fixed for 24 hours using a 10% neutral buffered formalin solution. The brain tissue was embedded in paraffin blocks and sectioned using a microtome with a thickness of 3 μm. The brain tissue sections were stained with Toluidine Blue (TB) and immunohistochemical staining with Bax antibody.

Observation of histopathological specimens

Observation of histopathological specimens to count the number of neurons and Bax expression was conducted using a light microscope connected to a camera, with a magnification of 400x across the entire field of view. The observations were performed in all CA1 hippocampus, dentate gyrus, and prefrontal cortex areas. The number of neurons was counted by observing the entire CA 1 area of the hippocampus, dentate gyrus, and prefrontal cortex. Next, the average number of neurons from that area was calculated for each research subject (rat).

Data Analysis

A One-Way ANOVA statistical analysis was performed to determine the difference in the alternation percentage and mean number of neurons.

RESULTS

Figures 1, 2, and 3 illustrate these findings of the study, including the percentage of alternation, counts of CA1 neurons in the hippocampus, dentate gyrus, and prefrontal cortex, and BAX expression.

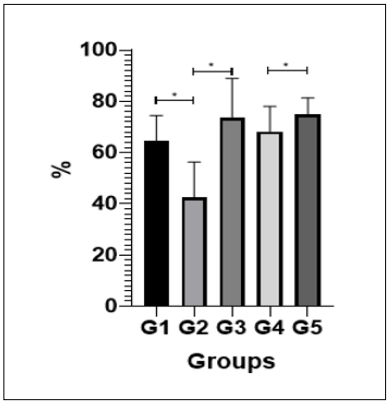

Figure 1. Alternation percentage G1: Group without TMT induction. G2: Group with TMT induction. G3, G4, and G5: Groups with TMT induction and administration of ethanol extract of SA leaves at doses of 75, 150, and 300 mg/kg body weight per day, respectively.

*significant difference between G1 and G2 (indicate as p=0.046), G2 and G3 (indicate as p=003), G2 and G4(indicate as p= 0.016), G2 and G5(indicate as p=0.002)

The difference in S. androgynus doses of 75, 150, and 300 mg/mg/kg body weight did not show significant differences in the Alternation percentage. No significant difference between G3 and G4 (indicated as p=0.944), G3 and G5 (indicated as p=1.000), and G4 and G5 (indicated as p=0.887).

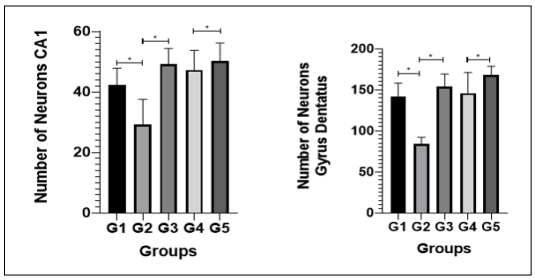

Figure 2. Number of hippocampal neuron and dentate gyrus neuron. G1: Group without TMT induction. G2: Group with TMT induction. G3, G4, and G5: Groups with TMT induction and administration of ethanol extract of SA leaves at doses of 75, 150, and 300 mg/kg body weight per day, respectively.
*significant difference in the number of hippocampal neurons between G1 and G2 (indicate as p=0.039), G2 and G3 (indicate as p=001), G2 and G4 (indicate as p= 0.002), G2 and G5 (indicate as p=0.000).
*significant difference in the number of dentate gyrus neurons between G1 and G2 (indicate as p=0.000), G2 and G3 (indicate as p=000), G2 and G4(indicate as p=0.000), G2 and G5 (indicate as p=0.000).

The difference in S. androgynus doses of 75, 150, and 300 mg/mg/kg body weight did not show significant differences in the number of hippocampal neurons. No significant difference between G3 and G4 (indicated as p=1.000), G3 and G5 (indicated as p=1.000), and G4 and G5 (indicated as p=1.000). The difference in S. androgynus doses of 75, 150, and 300 mg/mg/kg body weight did not show significant differences in the number of dentate gyrus neurons. No significant difference between G3 and G4 (indicated as p=1.000), G3 and G5 (indicated as p=1.000), and G4 and G5 (indicated as p=0.422).

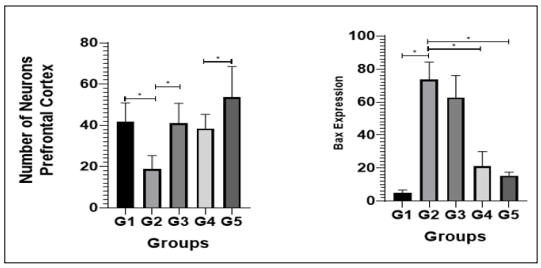

Figure 3. Number of Neuron in the prefrontal cortex and BAX expression. G1: Group without TMT induction. G2: Group with TMT induction. G3, G4, and G5: Groups with TMT induction and administration of ethanol extract of SA leaves at doses of 75, 150, and 300 mg/kg body weight per day, respectively.
*significant difference in the number of neurons in the prefrontal cortex between G1 and G2 (indicate as p=0.014), G2 and G3 (indicate as p=0.019), G2 and G4(indicate as p=0.049), G2 and G5(indicate as p=0.000).
*significant difference in BAX expression between G1 and G2 (indicate as p=0.000), G2 and G4 (indicate as p=0.000), G2 and G5(indicate as p=0.000).

The difference in S. androgynus doses of 75, 150, and 300 mg/mg/kg body weight did not show significant differences in the number of prefrontal cortex neurons. No significant difference between G3 and G4 (indicated as p=1.000), G3 and G5 (indicated as p=0.549), and G4 and G5 (indicated as p=0,236). S. androgynus doses of 150 and 300 mg/mg/kg body weight did not show significant differences in the Bax expression (indicated as p=1.000). S. androgynus doses of 75 mg/mg/kg body weight cannot reduce BAX expression. No significant differences in BAX expression between G2 and G3 (indicated as p=0.5999).

DISCUSSION AND CONCLUSION

Extract from S. androgynus enhances memory function The study observed higher alternation percentages in groups G3, G4, and G5 treated with S. androgynus extract compared to group G2 treated with trimethyltin alone. Significant differences in alternation percentage were noted between group G2 and groups G3 (indicate as p=003), G2 and G4(indicate as p= 0.016), G2 and G5 (indicate as p=0.002). These findings suggest that cognitive function was better in the trimethyltin-induced group treated with S. androgynus extract at doses of 75, 150, and 300 mg/kg body weight per day (G3, G4 and G5) compared to the group induced with trimethyltin only (G2). There was no significant difference between G3, G4, and G5. This shows that the difference in S. androgynus doses has no effect on improving cognitive function. A S. androgynus dose of 75 mg/kg body weight per day can improve cognitive function due to trimethyltin exposure.

A previous study supports these results, indicating that Sauropus androgynous leaf extract, administered at 150 mg/kg and 300 mg/kg, improves cognitive function in rat models of Alzheimer's disease by reducing β-amyloid plaques in the hippocampus.[18] The ethanol extract of S. androgynus leaves contains flavonoids and steroids, which may mitigate neuroinflammation, decrease β-amyloid production, and enhance insulin sensitivity, positively influencing memory.[1]

S. androgynus extract mitigates neuronal loss and reduces BAX expression.

The number of neurons in the hippocampus, dentate gyrus, and prefrontal cortex was higher in the groups treated with both trimethyltin and S. androgynus (G3, G4, and G5) compared to the group treated with trimethyltin only (G2). Significant differences in the number of CA1 hippocampal neurons between G1 and G2 (indicate as p=0.039), G2 and G3 (indicate as p=001), G2 and G4 (indicate as p=0.002), and G2 and G5 (indicate as p=0.000) were seen. Significant differences in the number of dentate gyrus neurons between G1 and G2 (indicate as p=0.000), G2 and G3 (indicate as p=000), G2 and G4 (indicate as p=0.000), G2 and G5 (indicate as p=0.000) were seen. Significant differences in the number of neurons in the prefrontal cortex between G1 and G2 (indicate as p=0.014), G2 and G3 (indicate as p=0.019), G2 and G4(indicate as p=0.049), and G2 and G5 (indicate as p=0.000) were seen. This study suggests that S. androgynus extract administration at doses of 75, 150, and 300 mg/kg body weight per day may prevent neuronal loss caused by TMT exposure. Additionally, BAX expression was significantly lower in groups G4 and G5 compared to group G2. Significant difference in BAX expression between G2 and G4 (indicated as p=0.000), G2 and G5 (indicated as p=0.000). This study implies that S. androgynus extract administration at doses of 150 and 300 mg/kg body weight per day inhibited pro-apoptotic protein BAX expression induced by TMT exposure.

No significant difference in the number of neurons in the hippocampus, dentate gyrus, and prefrontal cortex between G3, G4, and G5. This shows that the difference in S. androgynus doses does not inhibit neuronal loss. S. androgynus dose of 75 mg/kg body weight per day can inhibit neuronal loss due to trimethyltin exposure. No significant difference in the Bax expression between G4 and G5. S. androgynus doses of 150 and 300 mg/kg body weight per day can inhibit Bax expression due to trimethyltin exposure. S. androgynus dose of 75 mg/kg body weight per day cannot inhibit BAX expression due to trimethyltin exposure. Researchers found no side effects at higher doses (300 mg/ kg body weight per day). S. androgynus dose of 300 mg/kg body weight per day is safe to use.

According to phytochemical analysis, S. androgynus (SA) leaf extract contains alkaloids, flavonoids, saponins, polyphenols, quinone, monoterpene, and sesquiterpene.[20] Flavonoids identified include catechin, rutin, myricetin, quercetin, apigenin, and kaempferol.[21] Previous studies investigating the antioxidant properties of SA, using rats induced with sodium nitrite, demonstrated its ability to reduce malondialdehyde (MDA) levels, indicating antioxidant potential.[22] The antioxidants present in S. androgynus include vitamin C and flavonoids, particularly kaempferol and quercetin.1 Studies suggest that β-carotene, tocopherols, and ascorbic acid may also mitigate oxidative stress.[23]

Vitamin C plays a role in myelination during both the initial development of nerves and their recovery following injury.[24] In rat models of neurodegeneration, giving vitamin C (ascorbic acid) improved cognitive function and enhanced cellular proliferation, neuronal differentiation, and maturation. Vitamin C also prevented the decline in protein expression related to neuroplasticity in the hippocampus, including synaptophysin, phosphorylated Ca2+/calmodulin-dependent protein kinase II, and brain-derived neurotrophic factor (BDNF).[25] Additionally, it prevented the decline in Purkinje cell count and the decrease in osteopontin expression in the cerebellum caused by exposure to lead. Osteopontin is a protein crucial for axonal myelination and neuron development.[26]

Intake of S. androgynus may elevate vitamin E levels in the liver and boost coenzyme Q10 (CoQ10). CoQ10, also known as ubiquinol in its reduced form, is a potent lipophilic antioxidant that can regenerate other antioxidants such as tocopherol and ascorbate.[27] Vitamin E, known for its antioxidant, anti-inflammatory, and neuroprotective properties crucial for brain health, has been shown to benefit Alzheimer's therapy.[28] Vitamin E may help inhibit neurodegeneration in the brain by reducing the number and activity of microglia in cortical areas.[29] High levels of γ-tocopherol in the brain have been linked to presynaptic proteins in mid-frontal brain regions, suggesting that vitamin E may help maintain presynaptic protein levels.[30]

S. androgynus contains the flavonoid quercetin. The antioxidant effect of quercetin operates through several mechanisms, including increasing glutathione (GSH) synthesis, regulating antioxidant enzyme activity, modulating signal transduction pathways, and inhibiting reactive oxygen species (ROS) production.[31] Studies in rat models of hypoxia have demonstrated that quercetin administration increases levels of GSH, glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) while decreasing malondialdehyde (MDA) concentrations.[32] Quercetin has been found to exhibit neuroprotective effects by inhibiting proinflammatory cytokines like NF-kB and iNOS, enhancing motor function, preventing synuclein fibrillization, preserving neurons in the hippocampus, and promoting neuroplasticity. It also shows potential in inhibiting β-amyloidosis, improving cognitive and emotional function, and reducing tauopathies, astrogliosis, and microgliosis in the hippocampus and amygdala.[33] In elderly adults aged 65-75, quercetin may inhibit the decline in cerebral blood flow and reduce the accumulation of amyloid β (Aβ).[34] Additionally, in rat models induced with neurotoxicity using Lipopolysaccharide (LPS), quercetin has been observed to inhibit gliosis and neuroinflammation in the cortex and hippocampus, suppress the apoptosis pathway in mitochondria, hinder neurodegeneration, improve memory function, and preserve synapses in these brain regions.[35]

S. androgynus contains the flavonoid kaempferol, demonstrating neuroprotective effects on striatal neurons in rat models induced with neurotoxicity via LPS. Its mechanisms of neuroprotection include suppressing neuroinflammation and preserving the integrity of the blood-brain barrier (BBB). Kaempferol has been observed to increase levels of tyrosine hydroxylase (TH) and postsynaptic density protein 95 (PSD95) in the rat striatum. Additionally, it inhibits the production of proinflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α). It reduces levels of monocyte chemotactic protein-1 (MCP-1), intercellular cell adhesion molecule-1 (ICAM-1), and cyclooxygenase-2 (COX-2) in the striatum.36 In a rat model of cerebral ischemia/reperfusion (I/R) injury, kaempferol administration improved neurological deficits, reduced infarct volume and brain water content, and inhibited cell apoptosis.[37,38] It also modulated protein expressions related to apoptosis, increasing anti-apoptotic Bcl-2 and Akt phosphorylation while decreasing pro-apoptotic Bax and P53 in the hippocampus and cortex.[37] Kaempferol enhanced neuron arrangement, distribution, and morphology, and inhibited neuronal apoptosis in this injury model. Furthermore, it exhibited antioxidant effects by increasing superoxide dismutase (SOD) and glutathione (GSH) activity while decreasing malondialdehyde (MDA) levels in serum and brain tissue.[38] In rat models of traumatic brain injury (TBI), kaempferol administration improved sensorimotor behavior, preserved mitochondrial function, prevented degeneration of dopaminergic neurons in Parkinson's models by inhibiting lipid peroxidation, mitigating mitochondrial damage, and enhancing lipophagy. It also inhibited motor dysfunction and increased dopamine levels in the striatum.[39] In SH-SY5Y cell culture models induced with apoptosis, kaempferol inhibited cell apoptosis by reducing Bax expression, increasing Bcl-2 expression, and decreasing caspase-3 expression.[40]

LIMITATIONS OF THE STUDY

The reduction in cell number can be caused by apoptosis. Apoptosis can be assessed from the increase in BAX and caspase-3 expression or the TUNEL assay. This study only assessed BAX expression. Further studies can assess BAX expression, Bcl2 expression, BAX/bcl2 ratio, caspase-3 expression, and TUNEL assay. The hippocampus includes the CA1, CA2, CA3, and dentate gyrus areas. In this study, only the CA1 area of the hippocampus and the dentate gyrus were examined, while the CA2 and CA3 areas were not evaluated. Further studies can assess the entire hippocampus area. This study has not evaluated the toxicity of S. androgynus. Further studies can assess the toxicity of S. androgynus.

CONCLUSION

Sauropus androgynus at doses of 75, 150, and 300 mg/kg body weight per day improves cognitive function and increases the number of neurons in the CA1

hippocampus, dentate gyrus, and prefrontal cortex of rats induced neurotoxicity using TMT. S. androgynus dose difference did not affect the increase in the number of neurons. S. androgynus at doses of 150 and 300 mg/kg body weight per day inhibits Bax expression. S. androgynus dose difference of 150 and 300 mg/kg body weight per day did not inhibit Bax expression. S. androgynus at 75 mg/kg body weight doses cannot inhibit Bax expression.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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