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Research Paper

Amelioration of Behavioral and Cognitive Impairment of Ethanolic Leaf Extract of Ziziphus Talanai Against Msg in Mice

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Abstract

Ziziphus talanai is an endemic plant species in the Philippines. Recently, this plant's phytochemical analysis revealed pivotal compounds with different pharmaceutical properties, including neuroprotective activity in the cerebellum of mice. Nonetheless, the scarcity of information on the neuroprotective potential of this plant has been a focal point. Thus, this study aimed to evaluate the neuroprotective potential of Z. talanai extract against MSG-induced aberrations in mice. A total of 24 mice were distributed and administered 9000mg/kg of MSG (T-), MSG and 1000mg/kg of L-taurine (T+), 100% of extract (T1), MSG, and 25% extract (T2), MSG and 50% of the extract, MSG and 75% extract, and MSG and 100% of the extract. The results of the test crawling along the rope showed that mice treated with MSG exhibited anxiety-like behavior, while mice treated with L-taurine managed to surpass the atrocious effect of MSG. In mice treated with the extracts, it is revealed that the anxiolytic effect directly relates to the concentration and results. The results of the Y-maze test obtained significant differences between the MSG-treated group versus extract alone, L-taurine, 50%, 75%, and 100% extract. Interestingly, a 75% concentration of the plant extract was the most promising of the group results. These justify the ameliorative potential of *Z. talanai* extract on the behavior deficits and cognitive impairment of mice treated with MSG.

Keywords: *anxiolytic, MSG, neuroprotective, Ziziphus talanai*

INTRODUCTION

Since ancient times, plants have been used as alternative therapeutic agents to remedy many diseases due to the presence of primary and secondary metabolites such as alkaloids, saponins, flavonoids, tannins, and volatile oils (Karthikeyan et al., 2009). Phytochemical analysis of the ethanolic leaf extract of *Ziziphus talanai* revealed the presence of flavonoids, saponins, tannins, and alkaloids (Reyes et al., 2016). Studies showed that secondary metabolites such as flavonoids are responsible for neuroprotection against different neurotoxic inducers (Taati et al., 2011).

Monosodium glutamate (MSG) is a widely used food additive to enhance the palatability of many culinary foods (Mondal et al., 2016). Despite the benefits of this food enhancer, it also has adverse effects, such as deleterious damage to the brain regions (Ogbuagu et al., 2019; Kardesler and Baskale, 2017; Shivasharan et al., 2012; Mesallam et al., 2017). This chemical causes oxidative stress in the brain, leading to neural damage in the context of apoptosis and necrosis (Abass and El-Haleem, 2011; Ankarcrona et al., 1998). Interestingly, the mechanisms of glutamate toxicity are still

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vague. Still, the atrocious effects of this agent are analogous to many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease by overproduction of reactive oxygen species (ROS) (John et al., 2015).

Currently, there is no study regarding the neuroprotective potential of *Z. talanai* against MSG in the hippocampus of male ICR mice. Ergo, this study aimed to assess the neuroprotective potential of ethanolic leaf extract of *Z. talanai* against MSG-induced oxidative stress in male ICR mice.

LITERATURE REVIEW

Secondary Metabolites of *Ziziphus* species

Several phytochemical analyses of different species of *Ziziphus* revealed the presence of flavonoids, alkaloids, tannins, saponins, phenolic compounds, glycosides, carbohydrates, proteins, and triterpenoids using various solvents. The studies of Alhakmani, Khan, and Ahmad (2014); Rathore et al. (2012); Asgarpanah and Haghighat (2012) revealed that *Ziziphus* plants commonly contained cyclopeptide and isoquinoline alkaloids, flavonoids, terpenoids, and their glycosides. In addition, they also found out that the leaves of these plants contain betulinic and ceanothic acids, various flavonoids, saponins, sterols, tannins, and triterpenes. Generally, these *Ziziphus* species studied include *Z. mauritiana*, *Z. spina-christi*, *Z. jujube*, and *Z. xyloperus* (Tanvir et al., 2014).

The methanolic leaf extract of Z. *mauritiana* revealed the presence of alkaloids, carbohydrates, saponins, flavonoids, phenolic compounds, triterpenoids, proteins, amino acids, and glycosides. In contrast, its aqueous leaf extract showed the presence of carbohydrates, saponins, phenolic compounds, proteins, and amino acids (Abalaka et al., 2010). The fruit extract indicated the presence of high phenolic flavonoid and ascorbic acid that possessed antioxidant potential (Tanvir et al., 2014).

On the other hand, the ethanol leaf extract of *Z. spina-christi* contained secondary metabolites, namely, carbohydrates, reducing sugar, monosaccharides, terpenoids, and alkaloids (El-Kamali and Elshikh, 2015). The aqueous leaf extract had secondary metabolites such as carbohydrates, reducing sugar, tannins, saponins, terpenoids, and anthraquinones. Furthermore, *Z. spina-christi* leaf extract contains secondary metabolites such as alkaloids, tannins, saponins, glycosides, steroids, terpenoids, and especially flavonoids that possess antioxidant properties and showed neuroprotective activity against induced brain ischemia and inhibition of oxidative stress in brain of rats (Mahbubeh and Zahra, 2017).

The ethanol and aqueous seeds extract of *Ziziphus jujube* revealed the presence of secondary metabolites, which are alkaloids, saponins, flavonoids, phenols, glycosides, anthocyanin, betacyanin, and steroids (Abd-Alrahman et al., 2013). On another note, Washid and Ameeta (2011) reported that *Z. xyloperus* ethanol leaf extract contained glycosides, phenols/tannins, high concentrations of flavonoids, saponins, carbohydrates, and steroids using column chromatographic screening.

Ethnomedical Reports of *Ziziphus talanai* (Blanco) Merril

Different pharmaceutical potentials were recorded for *Z. talanai*. The accounts of its ethnomedicinal use involve the decoction of the bark used by folks in Antique province as an antimicrobial agent against UTI and skin diseases like scabies and ringworm. According to Anas et al. (2009), at 20,000

μg and 2,000 μg dosages, the plant's extract can be used against *Mycobacterium phlei, S. aureus*, and *B. subtilis.* In addition, other research includes the investigation in mice models on histoprotection against tetracyclin-induced hepatotoxicity and reprotoxicity (Reyes et al*.,* 2016) and amelioration of the cerebral region due to aberrations in motor behavior induced by postnatal exposure to valproic acid (Tejano, 2016).

Monosodium Glutamate and Its Influences on Animal Model Brain

In the early twenty-first century, MSG is claimed to be one of the most widely used food additives in China, Central and South America, North America, Europe, Africa, the Middle East, and other Asia countries (Tomescu, 2021) and Ajinomoto Corporation of Japan patented it in 1909 (Vivek and Rahul, 2015). The chemical formula for monosodium glutamate is C5H8NO4Na, with 78% glutamic acids and 22% sodium and water (Vivek and Rahul, 2015). MSG has no texture or smell of its own. However, it emphasizes the food's natural flavor rather than adding an independent flavor (Singh, 2005). Despite the contribution of MSG in the culinary field, it also has deleterious effects on different brain regions using animal models. It is reported that exposure to MSG in laboratory animals leads to brain lesions and neuroendocrine disorders. In addition, dosage-dependent MSG can cause deleterious effects on microglial reactions in the cerebral cortex and even cortical excitability in the developing brain of the organism. Moreover, MSG can induce neural necrosis in hypothalamic arcuate nuclei in neonatal rats. Nevertheless, the negative effect is not limited to this region (Vivek and Rahul, 2015).

Administration of 2g/kg in mice resulted in variable histopathological changes in hippocampal formation. These were neurodegenerative changes, with almost all nerve cells distorted in shape with deeply stained shrunken pyknotic nuclei surrounded by unstained pale areas with vacuolization of the neuropile surrounding the damaged neurons. There was also choroidal plexus congestion and focal aggregation of glial cells (Gliosis and satellitosis) (Abass and El-Haleem, 2011; Zhang *et al.*, 2012).

Extensive accumulation of MSG in brain regions such as synaptic clefts causes excitotoxicity which may lead to severe neurochemical damage and neurotoxic effects in some brain regions. This occurs when receptors for the excitatory neurotransmitter glutamate, such as the N-methyl- D- aspartate (NMDA) receptor, are over-activated. Excitotoxins, namely NMDA and kainic acid, bind to these receptors. Pathologically, high levels of glutamate can cause excitotoxicity by allowing high levels of calcium ions (Ca2+) to enter the cell. Ca2+ influx into cells can activate several enzymes, including phospholipases, endonucleases, and proteases enzymes. These expressions of different enzymes may lead to cell damage or organelle degeneration of cells like the mitochondria (Sadasiyan et al., 2010).

Neuroprotective Potential of Ziziphus Species

The antioxidant properties of secondary metabolites extracted from the Ziziphus plant were reported. Antonio and Druse (2008) established that the antioxidant properties of secondary metabolites, such as flavonoid compounds extracted from the plant, have neuroprotective potential against alcohol-induced injury. This was supported by the research of Kumar et al.(2009); and Khalili and company (2009).

Based on the findings of Hossain et al. (2015), an ethanol root extract of *Z. rugosa* could be a potential natural source of antioxidants. It could have greater importance as a therapeutic agent in preventing or slowing oxidative stress-related degenerative diseases. From the studies of Aruoma (2003), Hemati and company (2010), as cited by Asgarpanah and Haghighat (2012), the antioxidant properties of plants protect the human body against damages induced by reactive free radicals generated in atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and even in the aging process. Further, Zhang et al. (2003) reported that Jujuboside in the *Z. jujube* plant inhibited glutamate-mediated excitatory signal pathway in the hippocampus by anti-calmodulin action. Hence it possessed a neuroprotective effect. In addition, according to Devi et al. (2021), the presence of flavonoid compounds in *Z. jujube* fruit, namely quercetin, kaempferol, and phloretin, have a pivotal contribution to decreasing neurodegeneration and lipid peroxidation induced by ethanol. Based on the findings of Shou et al. (2002) and Kumar et al. (2015), Z. *jujube* can potentially be an anxiolytic herb that reduces the anxiety stress of mice. Also, the inhibitory potential of this plant made the hippocampus less hyperactive, thus lowering the anxiety stress driven by the inducer.

In addition, from the research of Tanvir et al. (2014), *Z. mauritiana* fruit extract indicates the presence of high phenolic flavonoid and ascorbic, which possess antioxidant potential. The secondary metabolites of *Z. spina-christi*, notably its flavonoids, showed neuroprotective activity against induced brain ischemia and inhibition of oxidative stress in the brain of rats (El-Kamali and Elshikh, 2015; Mahbubeh and Zahra, 2017). More studies on the antioxidant and neuroprotective effects of secondary metabolites in other *Ziziphus* species and other plants supported the effectivity of flavonoids (Abd-Alrahman and company, 2013; Zhang et al., 2003; Shou *et al.*, 2002; Kumar *et al.*, 2015; Antonio and Druse (2008) and Khalili and company (2009).

METHODOLOGY

Research Design

The study utilized a Complete Randomized Design (CRD). The albino mice were randomly distributed to different treatments regardless of their body weight and characteristics. In addition, this study employed a Post-Test Only Control Group Design. All controls and treated groups had three (3) replicates. This study focused on ameliorating behavioral and cognitive impairment of ethanolic leaf extract of *Z. talanai* against MSG, specifically, the anxiety-like behavior and memory consolidation.

Treatment Groups

Table 1 comprehensively describes the controls and treated groups with their corresponding concentration relative to body weight (gram)(bw).

Table 1. Treatment Groups

Treatment Groups	Treatment Description
T0 (Normal Control)	Male ICR mice were treated with distilled water (0.3 mL/20 g bw)
T- (Negative Control)	Male ICR mice treated with MSG (9000mg/kg)
T+ (Positive Control)	Male ICR mice treated with L-Taurine (1000mg/kg) and MSG (9000mg/kg)
T1 (Extract Control)	Male ICR mice were treated with 100% ethanol leaf extract of *Z. talanai* (0.3 mL/20 g bw).
T2	Male ICR mice were treated with 25% ethanol leaf extract of *Z. talanai* (0.3 mL/20 g bw) and MSG (9000mg/kg).

T3	Male ICR mice were treated with 50% ethanol leaf extract of *Z. talanai* (0.3 mL/20 g bw) and MSG (9000mg/kg).
T4	Male ICR mice were treated with 75% ethanol leaf extract of *Z. talanai* (0.3 mL/20 g bw) and MSG (9000mg/kg).
T5	Male ICR mice were treated with 100% ethanol leaf extract of *Z. talanai* (0.3 mL/20 g bw) and MSG (9000mg/kg).

Plant material

Matured leaves of *Z. talanai* were collected at Xevera, Tabun, Mabalacat City, Pampanga, authenticated by Reyes (2016) at the University of the Philippines, Diliman, Quezon City, Philippines. The City Environment and Natural Resources Office (CENRO) of Mabalacat City, Pampanga, Philippines, approved the permit to collect.

Animal models

A total of 24 mice were purchased at the Zoology Department, Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, Philippines, which were utilized as animal models in the study. Mice were distributed randomly per treatment; three mice were represented in triplicate. All mice were acclimatized for two weeks before administration of chemicals. Each mouse was kept in a cage measuring 6 x 6 x 6 inches with shredded paper as their substrate. Mice were maintained in an environment with a 12/12 hour light/dark cycle at room temperature. They were fed using Integra 3000 and water *ad libitum*. Hygienic conditions and proper ventilation were conserved, along with weekly disinfection of the mice. Enforcement of protocols and animal ethics we adopted from Handling Methods of Laboratory Mice and Rats, 1st edition and Methods of Philippine Association for Laboratory Animal Sciences (PALAS) (Tejano et al., 2020; Donovan and Brown, 2013; Ago et al., 2002; O'Malley et al., 2022).

Preparation of Ethanol Leaf Extract

The procured leaves were washed using tap water and air-dried at room temperature for seven days without exposure to sunlight. The dried leaves were homogenized using an electric blender. Powered leaves were soaked using industrial-grade ethanol with a concentration of 95% for 72 hours with a ratio of 350 g of powdered leaves soaked in 1,400 ml of ethanol (25:75) (Hussain *et al.* 2014). The solution was filtered using Whatman filter paper no. 2. The solution was sent to the Angeles University Foundation, Angeles City Pampanga, for rotary evaporation. Concentrations of *Z. talanai* were generated using 5 g of extract diluted into 15 ml of distilled water (25%), 10 g of extract diluted into 10ml of distilled water (50%), and 15 g of extract diluted into 5 ml of distilled water. Stock solutions were lyophilized until the last day of the administration period (Tejano *et al.* 2017).

Percentage Yield

(M2/M1) x 100, wherein M_1 is the mass of the powdered leaves of *Z. talanai* before extraction, and M_2 is the mass of the semi-solid portion of the ethanol extract. (294.94 g /1,443 g) x 100 = 20.44%.

Preparation of Monosodium glutamate

150g of MSG was purchased at SM hypermarket Dau, Mabalacat, Pampanga, Philippines. The 9000 mg/kg dosage was utilized and diluted to 0.2 ml/20 g b.w. of distilled water (Oghenesuvwe et al., 2014; Zhang et al., 2012a) and administered via oral gavage technique lasting for seven days to induce behavioral and cognitive impairment in mice. The ethical consideration and procedure of PALAS adopted protocols on mouse straining and oral administration.

Preparation of L-taurine

L-Taurine was purchased at Bambang Enterprise, Manila, Philippines. 1000 g of L-taurine was diluted using 20 ml of distilled water to generate the stock solution. 0.2 ml/20 g b.w. was used as a dosage, which is equivalent to 20 mg/20 g b.w. and administered to mice via oral gavage technique

for seven days to neutralize the damage of MSG in mice (Oghenesuvwe et al., 2014; Jae-Seong et al., 2010).

Administration of Treatments
The oral gavage technique was utilized in this study to introduce the chemicals in mice. The administration of chemicals was done every morning before refilling the food, which lasted seven days (Tejano et al., 2017; Zhang et al., 2012b).

Test Crawling Along the Rope
The test of crawling along a rope measures the animal's cooperation movement ability in high altitude stress in correlation to anxiety stress. On the $8^{th,}$ a day after the last administration of the chemicals, all mice were subjected to the test using a 2 m long rope with a thickness of 8 mm. The rope was elevated at the height of 1.5 m above the ground. Each mouse was subjected to the test for 3 minutes. The mouse that crawled the rope passed the test, and the mouse that did not crawl or drop failed the test. Pillows were utilized as cushions on mice that felt the rope (Zhang et al., 2012a; Zhang et al., 2012b).

Y-maze Test (Spontaneous Alternation Test)
The Guides Stanford Behavioral and Functional Neuroscience Laboratory Version 4 provided the required apparatus measurement. Y-maze apparatus was made of a plyboard with sizes 35 cm long, 15cm high, and 10 cm wide. All arms of the apparatus were rotated by 45 degrees to generate identical components. The apparatus was painted using non-toxic paint. Arms were labeled with A, B, and C to create the scoring. The Spontaneous Alternation percentage was evaluated as consecutive entries in 3 different arms (ABC, ACB, BAC, BCA, CAB, and CBA), divided by the total number of arm entries minus two, and multiplied by 100. Mice with less than eight arm entries during the 5-minute trial were excluded from the analysis, and the actual assessment was 10 minutes for each mouse to evaluate the spontaneous alternation. This behavioral test assessed the memory retrieval of mice in correlation to cognitive learning (Umukoro et al., 2015; Wolf et al., 2016; Casadesus et al., 2006)

Statistical Analysis
The quantitative results were presented as mean ± standard error of the mean (SEM). Statistical significance was analyzed using One-Way ANOVA and followed by Tukey's multiple comparison tests with a p-value of less than 0.05. All statistical analyses used GraphPad v6 (Tejano et al., 2017).

FINDINGS AND DISCUSSION

Test Crawling Along the Rope
Table 2 shows water alone with three mice that passed the test. All mice under treatment MSG failed the test. Further, the extract-alone group had three mice that passed the test, and L-taurine had two mice that passed the test. Interestingly, as the concentration of *Z. talanai* gets high, the number of mice that passed the test increases. The results of the test crawling along the rope showed that mice treated with MSG exhibited anxiety stress due to the amplification of MSG in the monoaminergic activities in the brain, which generate high altitude stress hormones in mice (Swamy et al., 2013)—on the one hand, mice treated with L-taurine experienced amelioration in the result of test crawling along the rope. The L-taurine acts as an antioxidant agent and neutralizes the oxidant generated by the MSG in the brain (Jong *et al.* 2012). Also, the anxiolytic property of L-taurine made the mouse pass the test (Tsvetkova et al., 2014).
On the other hand, mice treated with different concentrations of *Z. talanai* extract exhibited amelioration in their results. These happened because of the diverse secondary metabolites of *Z. talanai,* such as alkaloids, saponins, and flavonoids, which exert anxiolytic properties (Herrera-Ruiz et al., 2008; Chen et al., 2013; Chen et al., 2017). However, the 25% extract has a low score on the behavioral test. Theoretically, the concentration is not enough to neutralize the MSG's damage.

Table 2. Results of Test Crawling Along the Rope

Treatments	Mouse 1	Mouse 2	Mouse 3
Water Alone	Passed	Passed	Passed
MSG	Failed	Failed	Failed
Extract alone	Passed	Passed	Passed
L-taurine	Passed	Failed	Passed
25% extract	Passed	Failed	Failed
50% extract	Passed	Failed	Passed
75% extract	Passed	Passed	Passed
100% extract	Passed	Passed	Passed

Results of the Y-maze Behavioral Test

Figure 1 shows that 75%, 100%, and extract alone almost have the same results in the spontaneous alternation, followed by 50% extract and L-taurine with close results. MSG and 25% extract have the lowest score in spontaneous alternation, and water alone has the expected results in spontaneous alternation. The results of ANOVA, with a p-value of <0.0001 revealed a significant difference in the spontaneous alternations of the treatment groups. The effects of MSG in spontaneous alterations of the treatments are due to oxidative damage in the hippocampus and other regions of the brain involved in behavioral deficits and cognitive impairment. Damages in these regions compromise the brain's memory activities, leading to a low score in spontaneous alteration (Umukoro et al., 2015). Interestingly, the mice treated with L-taurine showed amelioration in spontaneous alteration. This is due to the neuroprotective potential of L-taurine, which regulates the MSG's damage in the mice's cerebral cortex and hippocampus. The mice treated with different concentrations of *Z. talanai* showed increased spontaneous alternation scores. This phenomenon explains that the property of the extract of being a memory enhancer is directly proportional to its concentrations.

Furthermore, the flavonoid content of the *Z. talanai* extract enhances the memory activities of the hippocampus, such as rapid and slow acquisition, short-term memory, working memory, long-term memory, reversal learning, and memory retention/retrieval (Spencer, 2010). Nevertheless, the mice treated with 25 extracts showed low scores in spontaneous alternation. This indicates that the concentration of *Z. talanai* extract is low; hence, memory amplification did not occur.

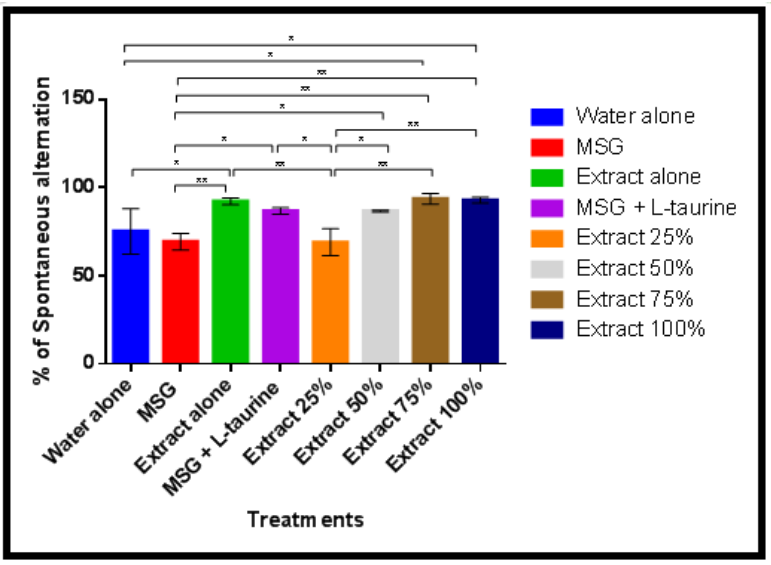

Figure 1. The score of spontaneous alternation among the treatment groups a day after the last administration. * indicates significance

CONCLUSIONS

Based on the preceding results of the parameters, this can be concluded that ethanolic leaf extract of *Z. talanai* at 75% concentration has the most promising result and has the highest ameliorative effects on the behavioral and cognitive task performed by the mice exposed to MSG compared to 25%, 50%, and 100% concentrations. Future research related to this study can be conducted to identify the specific compound and elucidate the neuroprotective mechanism of flavonoids against the deleterious effect of MSG in mice brains.

LIMITATION & FURTHER RESEARCH

The study was limited to the parameters, namely, behavioral and cognitive impairment using test crawling along the rope and spontaneous alteration test. Utilization of various behavioral tests such as the elevated plus maze test, light/dark transition test, and Morris water maze test is highly recommended to obtain robust data. In addition, increase the number of mice to be used in the experiment to gather variations in data. Moreover, phytochemical analysis of the extract and isolation of specific compounds are crucial in determining the extract's potency.

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