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Original Research Article

Thunbergia laurifolia Linn. Extract Protects Ethanol Addiction and Increases Dopamine Synthesis

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ABSTRACT

Thunbergia laurifolia Linn. is a plant used in traditional medicine for the detoxification of several substances including ethanol. Previous studies showed that *T. laurifolia* increased neuronal activity and altered the dopaminergic system in various brain areas similar to the effect of amphetamine and cocaine, but did not produce addiction in a chronic study. In the present study, we investigated the protective effect of *T. laurifolia* on behavioural changes and alteration of the tyrosine hydroxylase expressions in the mesolimbic dopaminergic pathway in acute and chronic ethanol administration in adult male Wistar rats. The animals were divided into four groups i.e., control, ethanol, *T. laurifolia* (200 mg/kg, i.p.), and *T. laurifolia* (200 mg/kg, i.p.) with ethanol in both acute and chronic (30 days) administrations. The anxiety behaviour, exploratory behaviour, and addiction behaviour were tested and tyrosine hydroxylase expression alteration was determined. Chronic ethanol administration significantly ($p < 0.01$) induced addictive behaviour in the conditioned place preference (CPP) test and significantly ($p < 0.01$) decreased tyrosine hydroxylase expression in the nucleus accumbens. Chronic *T. laurifolia* administration alone did not cause any change in the behaviours and tyrosine hydroxylase expression. Interestingly, daily administration of *T. laurifolia* prior to ethanol administration prevented ethanol-induced addictive behaviour and significantly ($p< 0.01$) increased tyrosine hydroxylase expression in the nucleus accumbens. Therefore, it can be concluded from our results that daily pre-administration of *T. laurifolia* can protect against ethanol-induced addiction and a decrease in dopamine synthesis in the reward system of the mesolimbic dopaminergic pathway.

Keywords: *Thunbergia laurifolia* Linn., Ethanol intoxication, Mesolimbic dopaminergic pathway.

Introduction

Ethanol consumption is a widespread problem throughout the world. Ethanol mainly acts on dopaminergic system which is a major circuit in mediating the reward system of ethanol and other drugs leading to the change in behaviour.[1,2] Dopaminergic neurons in the ventral tegmental area (VTA) innervates several brain areas including the nucleus accumbens affected by acute and chronic ethanol exposure. Ethanol was shown to cause excitation of dopaminergic neurons in the VTA *in vitro* and *in vivo*.[3] The enhancement of the firing rate of the VTA dopaminergic neurons correlates with dopamine release and tyrosine hydroxylase of the VTA and nucleus accumbens.[4,5] On the other hand, chronic ethanol administration decreases the VTA dopaminergic neurons firing rate and activity.[6,7] The dopaminergic connection between the VTA and nucleus accumbens is associated with a critical role in addiction and anxiety-like behaviours.[8,9] Ethanol addicted rodents spent more time in the non-preference compartment of the conditioned place preference (CPP) behaviour model after chronic ethanol administration.[10,11] The changes of synaptic output induced by chronic ethanol administration caused ethanol addiction in rats.[12] Furthermore, acute and chronic ethanol administrations also produced emotional changes. For example, acute and chronic ethanol administrations caused anxiolytic behaviour by increasing the time spent and the number of entries to the open arms of the elevated plus-maze[13] and increased rearing time in the exploratory behaviour test.[14]

Thunbergia laurifolia Linn. (*T. laurifolia*), commonly known as Rang-jert in Thai and a trumpet vine in English, is a plant used in Thai traditional medicine. Different parts of *T. laurifolia* such as leaves, root, and bark have been used as antidotes for insecticide, paraquat, ethyl alcohol, arsenic, and strychnine-induced poisoning.[15] The leaf of *T. laurifolia* was shown to have analgesic, antipyretic, anti-inflammatory, antioxidant, and hepatoprotective effects.[16,17] Preparation of the extracts of *T. laurifolia* leaves with boiling water, ethanol, and acetone contains the phenolic, caffeic acid, apigenin, carotenoids, gallic acid, chlorophyll a and b, pheophorbide a, pheophytin a, and lutein.[18] These compounds consist of high content of the phenolic, flavonoid, and other compounds that act as antioxidant agents.[19] Methanol extract of *T. laurifolia* leaves showed two iridoid glucosides including 8-epi-grandifloric acid and 3'-*O*-*β*-glucopyranosyl-stilbericoside along with benzyl *β*-glucopyranoside, (*E*)-2-hexenyl *β*-glucopyranoside, 6-*C*-glucopyranosylapigenin, benzyl *β*-(2'-*O*-*β*-glucopyranosyl) glucopyranoside, hexanol *β*-glucopyranoside, grandifloric acid, and 6,8-di-*C*-glucopyranosylapigenin.[20] In the brain, methanol extract of *T. laurifolia* leaves stimulated the neuronal activity in brain areas responsible for reward and motor control such as nucleus accumbens, frontal cortex, amygdala, hippocampus, and striatum [caudate putamen globus pallidus].[21] Interestingly, the leaves extract of *T. laurifolia* could stimulate dopamine release in the nucleus accumbens

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similar to the action of abused drug.[22] The present study aimed to evaluate the protective effect of *T. laurifolia* on acute and chronic ethanol-induced alterations of behaviours and tyrosine hydroxylase expression of the mesolimbic dopaminergic pathway.

Materials and Methods

T. laurifolia preparation

Fresh *T. laurifolia* leaves were collected from Surin province, Thailand, in the late rainy season and early winter i.e., during September to December 2015 and the identification confirmed by comparison with the specimen deposited in Herbarium Department of Pharmaceutical Botany, Mahidol University, Thailand. The voucher number of the batch used in this study is PBM-005558. The *T. laurifolia* leaves were cleaned before drying in the oven at 60°C for 6 hours. Dried leaves were blended into powder and left to extract in 80% methanol in a ratio of 1 g of *T. laurifolia* powder to 20 mL of methanol-water at room temperature, overnight. The extraction was filtered with filter paper (Whatman No. 4, Whatman, USA). The filtrate was concentrated using a rotary evaporator, and then lyophilised using a freeze dryer, and kept at -20°C until used. The compounds of *T. laurifolia* were identified based on molecular mass and fragmentation pattern using liquid chromatography-mass spectrometry analysis.

Animal handling and experimental design

Male Wistar rats weighing 300 - 350 g were obtained from the National Laboratory Animal Centre, Mahidol University, Thailand. Rats were housed in metallic cages and given commercial food (Nomura Siam International, Thailand) and water *ad libitum*. The environments were maintained in a 12-h light/dark cycle, temperature of 26-30°C, and humidity of 50-60%. All animals were housed, handled, and manipulated according to the guidelines of the Office of National Standard for Research Animal (ONSRA) Thailand. All protocol for animal experiments performed in this study was approved by Srinakharinwirot University-Institutional Animal Care and Use Committee (SWU-IACUC). Animal license number SWU-MED 1/2558 and 1/2560. Animal handling licence was approved by Institute of Animal for Scientific Purposes Development (IAD), National Research Council of Thailand (NRCT), Thailand. Animal handling licence number U1-00310-2558.

The rats were divided into four groups (n = 10 in each group) including control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups. Deionized sterile water was used to dilute *T. laurifolia* and ethanol before administration. The oral administration of *T. laurifolia* at a dose of 200 mg/kg was used as this dose has been reported to stimulate neuronal activity and dopamine release in the nucleus accumbens.[21,22] In the acute experiment, control group was given normal saline (0.3 mL, orally) followed by normal saline (0.3 mL, i.p.). Ethanol group was given normal saline followed by 20% ethanol (2 g/kg, i.p.). *T. laurifolia* group was given *T. laurifolia* (200 mg/kg, orally) followed by normal saline (0.3 mL, i.p.). *T. laurifolia* + ethanol group was given *T. laurifolia* (200 mg/kg, orally) followed by 20% ethanol (2 g/kg, i.p.). The behaviour tests and tyrosine hydroxylase expression alteration determination in the VTA and nucleus accumbens were performed 30 minutes after administration of the treatments. In the chronic experiment, rats were given treatments for 30 consecutive days. Control group received normal saline (0.3 mL, orally) followed by normal saline (0.3 mL, i.p.). Ethanol group received normal saline followed by 15% ethanol (1 g/kg, i.p.). *T. laurifolia* group received *T. laurifolia* (200 mg/kg, orally) followed by normal saline (0.3 mL, i.p.). *T. laurifolia* + ethanol group received *T. laurifolia* (200 mg/kg, orally) followed by 15% ethanol (1 g/kg, i.p.). A dose of ethanol administration in chronic experiment was reduced for protecting hypoglycemic condition in male Wistar rats.[23]

The behaviour tests and tyrosine hydroxylase expression alteration determination in the VTA and nucleus accumbens were performed on the last day of administration of the treatments.

Elevated plus-maze behaviour test

Anxiety behaviour following acute and chronic *T. laurifolia* and ethanol administrations was determined using the elevated plus-maze test.[24] The apparatus consists of two open (length: 45 cm, width: 15 cm) and closed arms (wall height: 10 cm) that is elevated above the ground (67 cm). Each rat was placed on the centre of the apparatus and allowed to freely travel in the platform for 5 minutes. The traveling on elevated plus-maze was recorded in a video and later the time of entry, time in open arms, and time in end of open arms ~~was~~ were analysed using Microsoft visual studio 2010. The longer the time spent on each parameter indicates that the rats present an anxiolytic effect. On the other hand, if the rat spent less time in these parameters than the control value, indicates that the rats present anxiety behaviour.

Hole board behaviour test

The acute and chronic effect of *T. laurifolia* and ethanol administrations on exploratory behaviour was determined using the hole board.[24] The apparatus composes of a wooden, black box (length: 65 cm, width: 55 cm, surrounding wall height: 45 cm), and four ~~pores~~ holes on the floor. The rats were placed on the centre of the apparatus and observed for 10 minutes. The number of rearing and head dipping under the hole was recorded by the video recorder. The higher number of rearing and head dipping represents a high exploratory behaviour in rats.

Conditioned place preference (CPP) test

The chronic effect of *T. laurifolia* and ethanol administrations on addiction behaviour was determined using the CPP test.[25] The apparatus has two compartments (length: 30 cm, width: 30 cm, surrounding wall height: 40 cm) with different designs of horizontal black and white thickness in the wall, and each compartment was separated by a guillotine door. The timeline of the CPP test is described in figure 1. The test consists of 3 phases including pre-test, condition, and testing phases. During the pre-test phase, each rat was allowed to freely explore in the CPP for 15 minutes to determine the individual preference compartment. The following condition phase was performed for 30 consecutive days. Each animal from each group was given certain treatment and followed by placing in the non-preference compartment of CPP with the guillotine door down for 10 minutes. In the testing phase, each rat was placed in the middle of the CPP again without given any treatments for 15 minutes. The time in each compartment was recorded. The comparison of time spent in the non-preference compartment between pre- and post-drug administration was used to determine the possible effect of drug addiction. An increase in the time spent in the non-preference compartment at post-drug administration compared to that at pre-drug administration indicates drug-induced addiction.

Western blot assay

Western blots were used to determine the level of tyrosine hydroxylase in the nucleus accumbens.[26] The fresh nucleus accumbens were collected according to the atlas of Paxinos and Watson[27] and homogenized with RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1%Triton X-100, 1% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO) and 1% protease inhibitor cocktail). The brain homogenised was centrifuged at 12,000 rpm, 4°C for 10 minutes, the supernatant was collected, and kept at -80°C for further analysis.

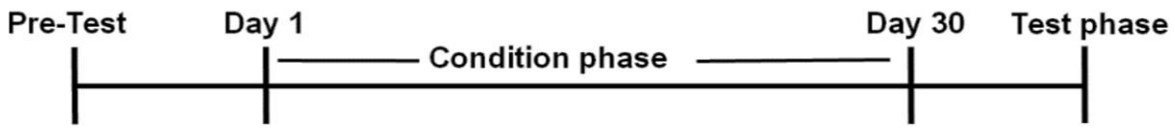

Figure 1: Diagram of the conditioned place preference test

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Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA). Each brain sample was denatured in a mixture of loading buffer and β-mercaptoethanol at 65°C for 5 minutes. Each sample was loaded into 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (PVDF). The membranes were blocked for non-specific proteins with 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 1 hour. Primary antibodies, 1:10,000 mouse monoclonal antibody against tyrosine hydroxylase (Millipore, Billerica, MA, #MAB318) and 1:40,000 mouse monoclonal antibody against β-actin (Millipore, Billerica, MA, #MAB1501) diluted in TBST were used. Secondary antibody, 1:10,000 horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG antibody (Cell Signaling Technology, Inc., Danvers, MA, USA, #7076S) for the detection of tyrosine hydroxylase or 1:40,000 for the detection of β-actin were used. The protein bands were visualised using enhanced chemiluminescence plus (ECL Plus™, Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film. The immunoblot bands were quantified by measuring the density of each band using densitometry with the Scion Image program (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

The immunohistochemistry method was used to determine the expression and localisation of tyrosine hydroxylase in the VTA.[28] Male Wistar rats were anaesthetised with 46 mg/kg sodium pentobarbital intraperitoneally and rapidly transcardially perfused with normal saline and followed by fixation with 4% paraformaldehyde mixed with 0.35% glutaraldehyde in 0.1 M ~~PBS~~ phosphate-buffered saline (PBS) buffer. After dissection, each rat brain was removed and post-fixed with the same fixative at 4°C overnight and then transferred to 30% sucrose for 1 day to ensure cryoprotection. The VTA slices were sectioned according to the atlas of Paxinos and Watson[27] at a thickness of 50 µm with a cryostat (Leica CM 1950; Leica, Watzlar, Germany). The tissue sections were collected in 0.1 M PBS and kept at 4°C. The free-floating sections were washed in 0.1 M PBS following pre-treatment with 1% hydrogen peroxide (H_2O_2) in PBS for 10 minutes. The sections were incubated with 5% normal rabbit serum in PBS-A (PBS containing 0.3% Triton X-100 and 1% BSA) for 30 minutes at room temperature after washing with PBS-B (PBS containing 0.1% Triton X-100 and 0.25% BSA). The sections were then incubated with 1:1,000 mouse monoclonal antibody against tyrosine hydroxylase for 12 hours at 4°C. After washing with PBS-B, they were incubated with secondary antibody (biotinylated rabbit anti-mouse IgG antibody) at a dilution of 1:400 for 1 hour at room temperature. After washing with PBS-B and PBS, the sections were treated with avidin-biotin HRP complex (Vector, Burlingame, CA) diluted 1:250 in PBS for 1 hour and then washed sequentially with PBS and 0.05 M Tris-HCl buffer (pH = 7.6). The sections were reacted with the solution of 3,3'-diaminobenzidine-tetrahydrochloride hydrate (DAB; Sigma-Aldrich, St. Louis, MO) in 0.05 M Tris–HCl buffer containing 0.01% H_2O_2 and were then washed with distilled water. The sections were mounted on a gelatinised slide, air-dried, and coverslipped with Permount®. The immunoperoxidase activity was visualised by light microscopy (Olympus BH2, Tokyo, Japan) and photographed with a digital camera (Olympus DP70, Tokyo Japan). The expression of tyrosine hydroxylase was determined by image J (NIH & LOCI, University of Wisconsin, USA).

Statistical analysis

The data were presented as mean ± SEM and analyzed statistically using analysis of variance (ANOVA) with Newman-Keuls multiple comparison tests to compare differences among individual groups. The Graphpad Prism 5 statistic software was employed. The significance levels were set at p values less than 0.05.

Results and Discussion

In the mesolimbic dopaminergic pathway, dopaminergic neurons of the VTA extend their axons into the nucleus accumbens. The dopamine transmission from the VTA into the nucleus accumbens regulates the rewarding system and involves addiction behaviour[29] and anxiety behaviour.[8] Previous report suggested that ethanol actions in the VTA increase in the rewarding responses. Chronic ethanol intoxication produces opposite effects of acute ethanol intoxication and/or compensatory/homeostatic effects on the expression, localisation, and function of proteins.[30] Besides, chronic ethanol intoxication produces an alteration of dopamine signalling resulting in deficits in function[31] and induces ethanol addiction.[32]

In this study, the acute and chronic effects of *T. laurifolia* and ethanol on anxiety or anxiolytic behaviour is shown in figure 2. In the acute study, the rats given ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol did not show significant difference in the time of entries into the open arms (figure 2A), % time in open arms (figure 2B), and time in end of open arms (figure 2C) when compared with the control group. The chronic administration in all groups also showed no significant difference in these parameters (figure 2D - 2F). These indicated that acute and chronic *T. laurifolia* and ethanol administrations did not produce anxiety or anxiolytic behaviour.

The acute and chronic effects of *T. laurifolia* and ethanol on exploratory behaviour is shown in figure 3. Rats in acute (figure 3A and B) and chronic (figure 3C and D) administrations of ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol did not show alteration of rearing and head dipping frequencies when compared with the control group. However, the rearing frequency in acute *T. laurifolia* administration alone was significantly ($p < 0.05$) reduced when compared with pre-administration of *T. laurifolia* prior to ethanol administration (figure 3A). The results indicated that acute and chronic *T. laurifolia* and ethanol did not alter exploratory behaviour.

The chronic effect of *T. laurifolia* and ethanol on addiction behaviour is shown in figure 4. Chronic ethanol administration showed a significant ($p < 0.01$) increase in the time spent in the non-preference compartment when compared with pre-drug treatment indicating that chronic administration of ethanol caused addiction. Chronic *T. laurifolia* administration did not increase the time spent in the non-preference compartment when compared to that of the pre-drug treatment indicating that *T. laurifolia* did not cause addiction. When *T. laurifolia* was given prior to ethanol, the addictive behaviour was abolished (figure 4), indicating that daily pre-administration of *T. laurifolia* protect against ethanol addiction in rats.

The acute effect of *T. laurifolia* and ethanol administrations on tyrosine hydroxylase alteration in the mesolimbic dopaminergic pathway was determined using the western blot analysis and immunohistochemistry. The localisation of tyrosine hydroxylase in the VTA is shown in figure 7A and the alteration of tyrosine hydroxylase expression is shown in figure 5A. The level of tyrosine hydroxylase in the nucleus accumbens is shown in figure 5B. Acute *T. laurifolia* administration alone and *T. laurifolia* prior to acute ethanol administration did not show any difference in the tyrosine hydroxylase expression in the VTA with the other groups (figure 5A). The level of tyrosine hydroxylase in the nucleus accumbens was unchanged in the acute ethanol, *T. laurifolia* alone, and *T. laurifolia* administration prior to acute ethanol administration groups when compared with the control group (figure 5B).

The alteration of tyrosine hydroxylase in the chronic study is shown by the localisation of tyrosine hydroxylase in the VTA (figure 7B), the tyrosine hydroxylase expression (figure 6A), and the level of tyrosine hydroxylase in the nucleus accumbens (figure 6B). The expression of tyrosine hydroxylase of the VTA was not significantly different in all groups (figure 6A). The level of tyrosine hydroxylase in the nucleus accumbens displayed a significant ($p < 0.01$) decrease in the chronic ethanol administration when compared to the control group (figure 6B). Chronic *T. laurifolia* administration showed no significant difference in the level of tyrosine hydroxylase when compared to the control group but significantly different ($p < 0.05$) when compared with chronic ethanol group. Daily administration of *T. laurifolia* prior to ethanol administration showed a significant ($p < 0.01$) increase in the level of tyrosine hydroxylase when compared with chronic ethanol group. These indicate that *T. laurifolia* prevented a decrease in the level of tyrosine hydroxylase in nucleus accumbens induced by chronic ethanol administration.

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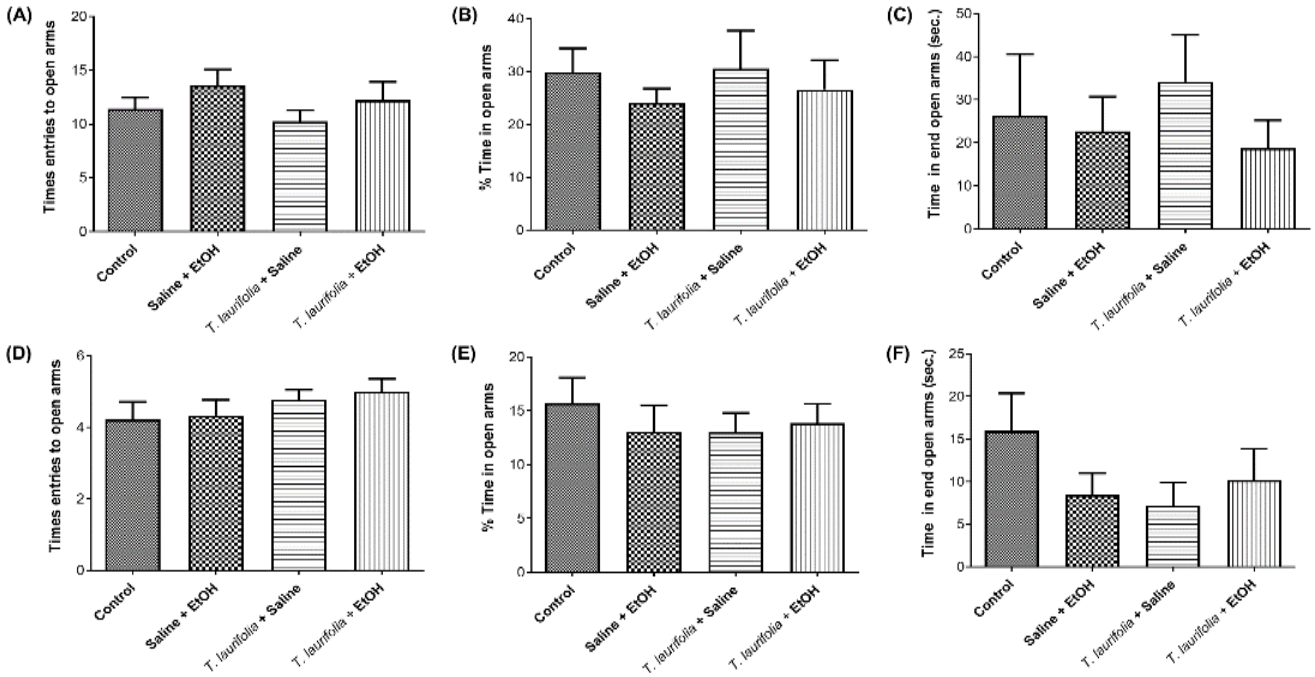

Figure 2: The time of entries to open arms **(A)**, % time in open arms **(B)**, and time in end of open arms **(C)** in the elevated plus-maze test in acute administration of control (n = 7), ethanol (EtOH) (n = 10), *T. laurifolia* (n = 8), or pre- *T. laurifolia* + EtOH (n = 8) groups and the time of entries to open arms (D), % time in open arms (E), and time in end of open arms (F) in the elevated plus-maze in chronic administration of control (n = 9), EtOH (n = 9), *T. laurifolia* (n = 9), or pre- *T. laurifolia* + EtOH (n = 10) groups. Data are presented as mean ± SEM.

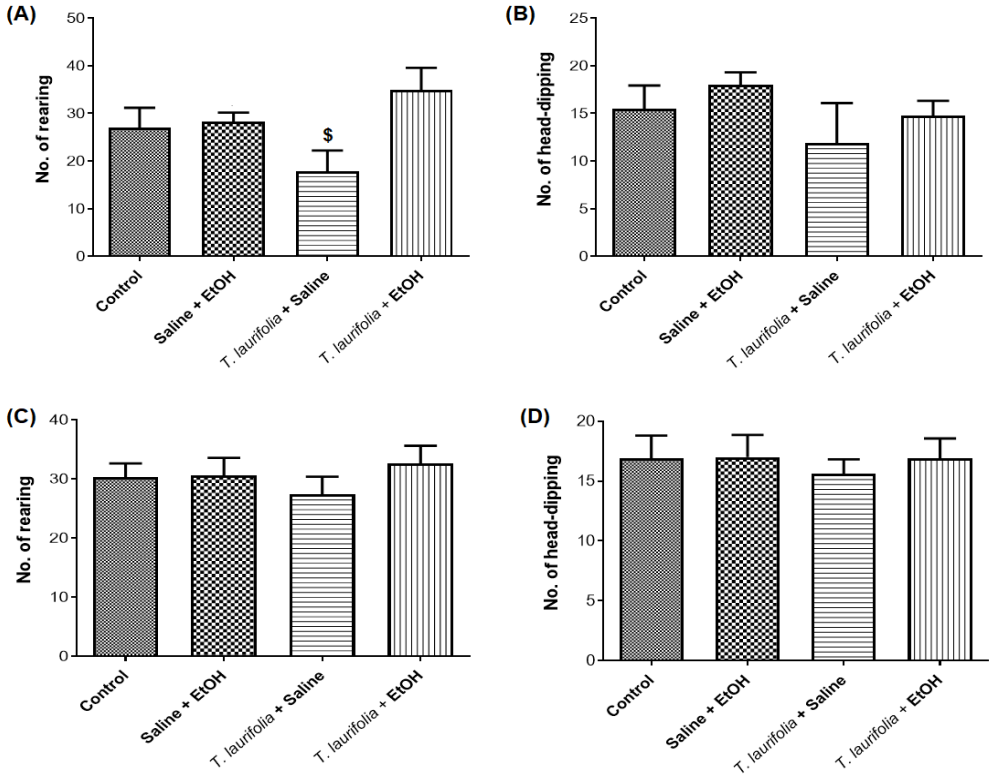

Figure 3: The numbers of rearing and head-dipping into the holes of the hole board in acute administration **(A-B)** of control (n = 10), EtOH (n = 10), *T. laurifolia* (n = 9), or pre- *T. laurifolia* + EtOH (n = 9) groups and in chronic administration **(C-D)** of control, ethanol, *T. laurifolia*, or *T. laurifolia* + EtOH groups. (n = 10). Data are presented as mean ± SEM. $^{\$}p < 0.05$ using analysis of variance (ANOVA) with Newman-Keuls multiple comparison test when compared with *T. laurifolia* + EtOH group.

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From the present study, we found that chronic ethanol administration produced ethanol addiction and reduced tyrosine hydroxylase expression in the nucleus accumbens but not in the VTA. We induced addiction by daily ethanol administration for 30 consecutive days which repeatedly stimulates the reward system. Our chronic ethanol administration results are in agreement with several previous studies. For example, chronic ethanol self-administration in male and female adolescent rats exhibited an escalation of ethanol intake and preference.[33] Meanwhile, the repeated ethanol stimulation reduced the VTA dopaminergic neurons response to ethanol stimulation by reducing dopamine synthesis. We also found that daily *T. laurifolia* administration prior to ethanol administration could protect against ethanol addiction and decrease tyrosine hydroxylase expression in the nucleus accumbens. We suspect that *T. laurifolia* attenuates the chronic ethanol stimulated reward system and the VTA dopaminergic neurons by its detoxification property. A previous report exhibited an increase of P- glycoprotein and a decrease in some cytochrome P450 isoenzymes such as CYP2E1 in HepG2 cells treated with *T. laurifolia*.[34] P-glycoprotein expressed in hepatocytes and the blood-brain barrier[35] function as a biological barrier by extruding toxins and xenobiotics out of cells. In the brain, P-glycoprotein did not only increase the efflux but also inhibited the influx of the drug across the blood-brain barrier of nonhuman primates.[36] Moreover, P-glycoprotein expressed in liver and other organs prevent the accumulation of harmful and carcinogenic compounds.[37] Thus, *T. laurifolia* may produce a rise in P-glycoprotein located at the biliary canalicular membrane of hepatocytes and blood-brain barrier and may result in an increase in the excretion of ethanol out of the cell and a decrease in systemic ethanol concentration entering into the brain. The CYP2E1 plays a significant role in ethanol metabolism in the brain by oxidising ethanol to acetaldehyde.[38] A decrease in CYP2E1 may reduce acetaldehyde production in dopaminergic neurons and/or other systems that implicate motor control leading to a reduction of acetaldehyde-intoxication induced abnormal neuronal activity, functions, and behaviours such as dopaminergic neurons stimulated euphoria, anxiolytic, hypnotic, amnesiac, aggression, reinforcement, or aversion to voluntary ethanol consumption.[39, 40] Furthermore, we suspect that the antioxidant effect of *T. laurifolia* may protect ethanol-induced neuronal, liver, and kidney cell damage and dysfunctions.

There was a report that *T. laurifolia* administration once a day before lead consumption for 8 weeks in male ICR mice maintained the normal levels of total antioxidants in the rat brains and the reduction in neuronal cell death and apoptosis by decreasing caspase-3 activity and cognitive impairment.[41] *T. laurifolia* on ethanol-induced hepatotoxicity showed the restoration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in hepatocytes to normal level in male Wistar rat[42] and pathology of the glomerulus in cadmium-exposed rats.[43] Moreover, our finding that *T. laurifolia* administration alone did not induce any difference in behavioural score and tyrosine hydroxylase expression supports previous reports that chronic administration of *T. laurifolia* methanol extract does not cause addiction when compared with cocaine administration using the CPP test[24] and change in food consumption, physical appearance, or behaviour and any toxic signs or mortality throughout the treatment period.[44]

However, we could not observe any difference in anxiety and exploratory behaviours in acute and chronic ethanol administrations. Previous reports on acute ethanol administration showed converse results on ethanol-induced alteration of anxiety and exploratory behaviours in rats. These findings may be due to several factors involved in the alteration of ethanol-induced anxiety and exploratory behaviours. The different behavioural responses to ethanol administration depend on the doses of ethanol administered,[45] sex of the animals,[46] or animal strains.[47] In our finding, there was no alteration of the tyrosine hydroxylase expression in the VTA and nucleus accumbens and the preventive potential of *T. laurifolia* on the tyrosine expression. We consider that these results reveal the differences of the brain's sensitivity to ethanol stimulation. The tyrosine hydroxylase was not observed to increase in the VTA by acute ethanol administration.[48] Furthermore, there was an increase in the dopamine outflow in the core but not the shell of the nucleus accumbens.[49] Besides, the difference in the detection time of tyrosine hydroxylase expression has been reported. The level of tyrosine hydroxylase gene expression of the ventral tegmental area did not show the linear fashion but was found to increase followed by a decrease depending on time.[50] There has been a suggestion that an increase followed by a decrease may mediate the activation of a negative feedback system to the ventral tegmental area and different mechanisms on dopaminergic neuronal activity and terminal dopamine release.[51]

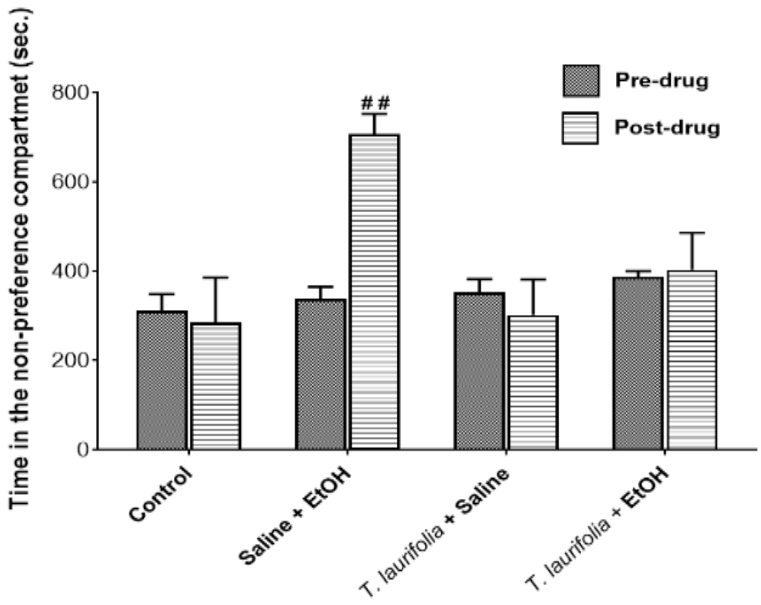

Figure 4: The time spent in the non-preference compartment in CPP of control, EtOH, *T. laurifolia*, and *T. laurifolia* + EtOH groups. (n = 5). Data are presented as mean ± SEM. $^{\#\#}p < 0.01$ using ANOVA with Newman-Keuls multiple comparison test when compared with the EtOH group.

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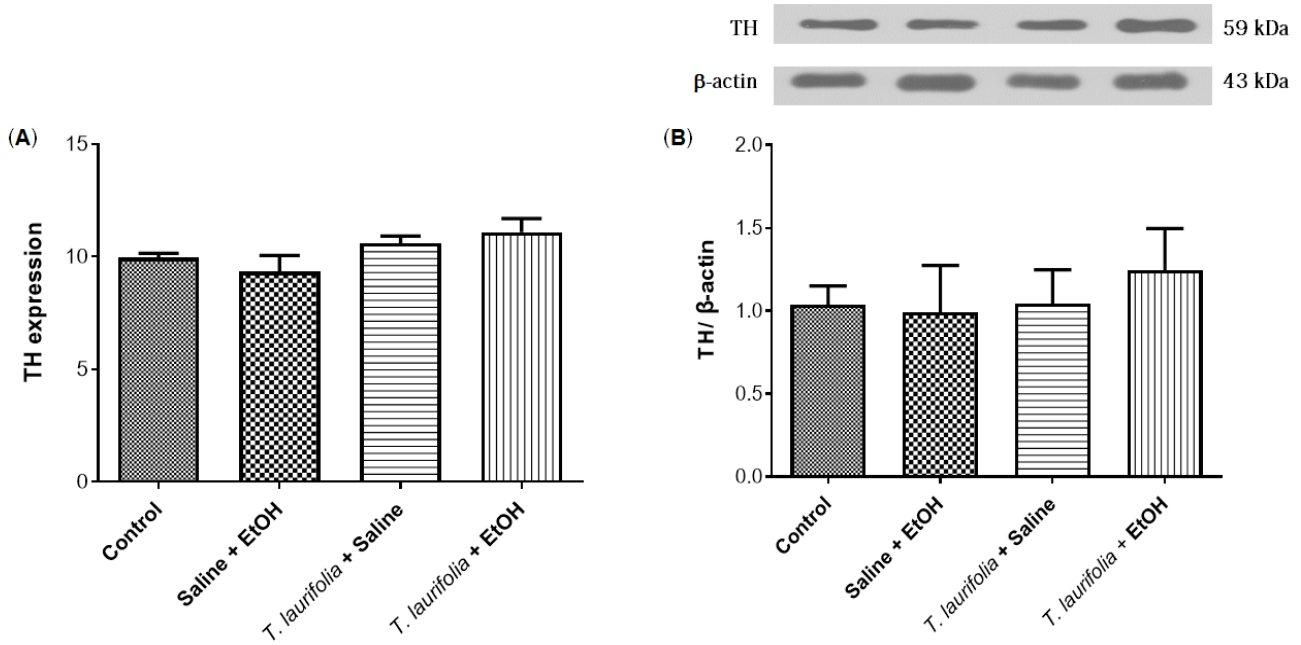

Figure 5: The expression of tyrosine hydroxylase-immunoreactivity in the VTA **(A)** (n = 3) and the ratio of tyrosine hydroxylase (TH) to *β*-actin in the nucleus accumbens **(B)** in acute administration of control (n = 4), EtOH (n = 5), *T. laurifolia* (n = 5), or *T. laurifolia* + EtOH groups (n = 6). Data are presented as mean ± SEM.

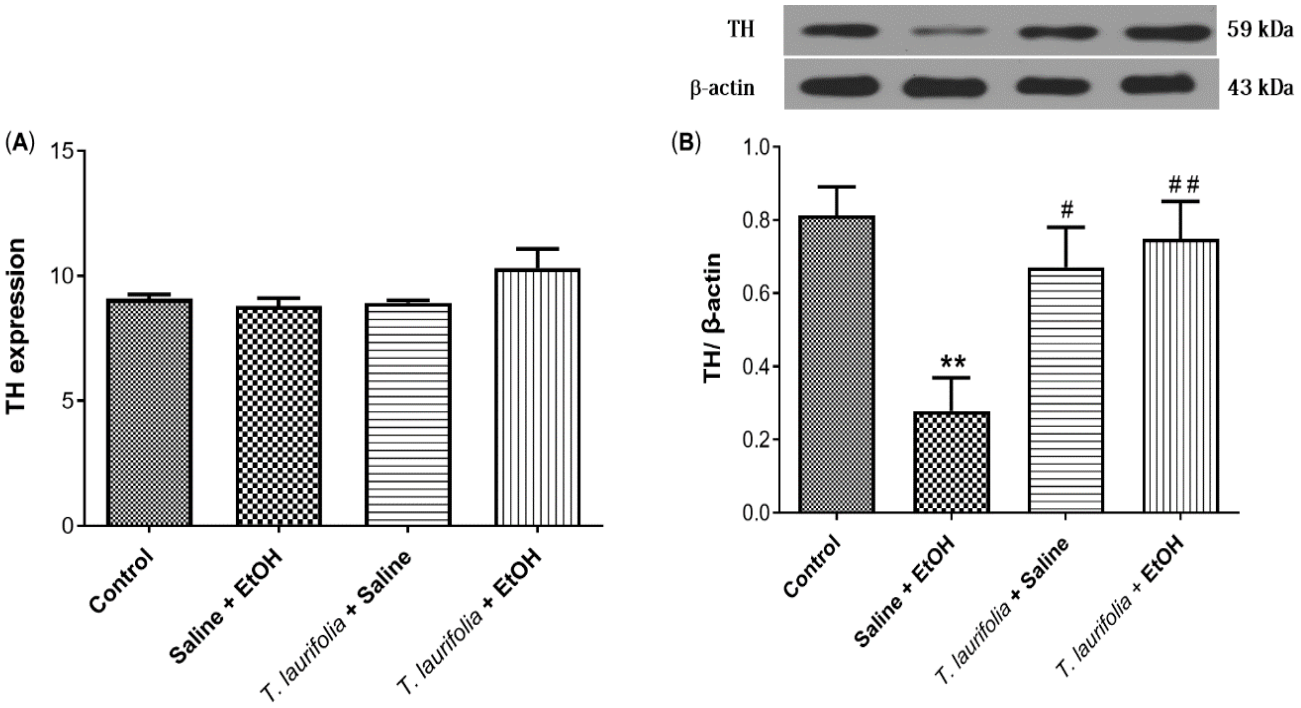

Figure 6: The expression of tyrosine hydroxylase-immunoreactivity in the VTA **(A)** (n = 3) and the ratio of tyrosine hydroxylase (TH) to *β*-actin in the nucleus accumbens **(B)** in chronic administration of control (n = 5), EtOH (n = 5), *T. laurifolia* (n = 5), or *T. laurifolia* + EtOH groups (n = 4). Data are presented as mean ± SEM. $^{**}p < 0.01$ using ANOVA with Newman-Keuls multiple comparison test compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ using ANOVA with Newman-Keuls multiple comparison test compared with the EtOH group.

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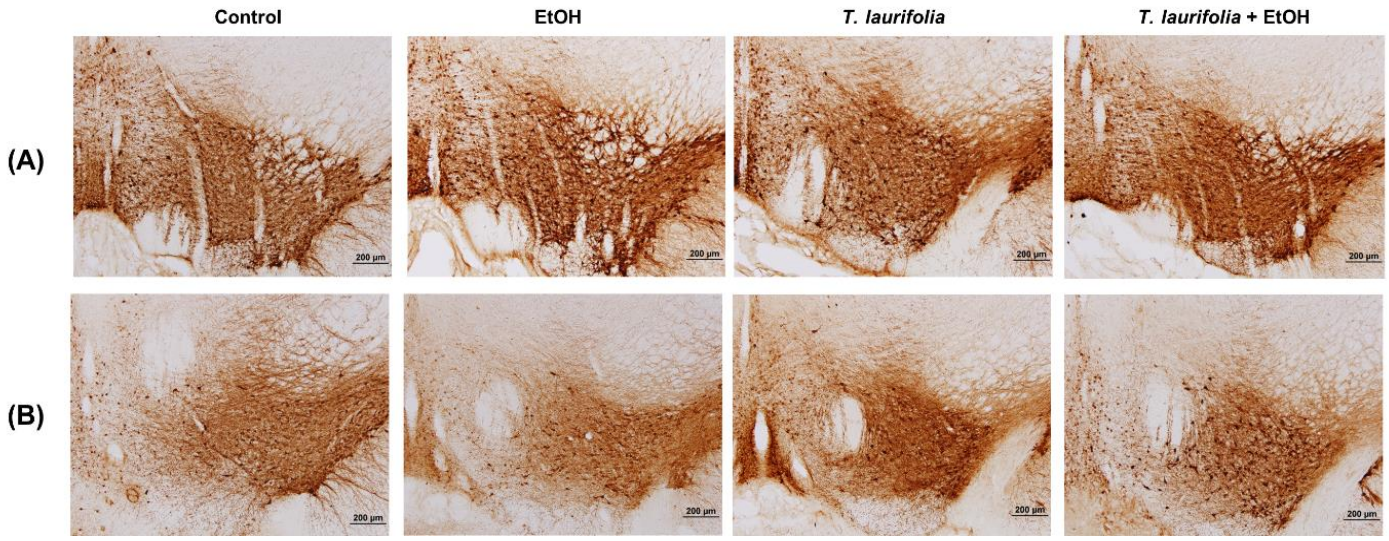

Figure 7: Semiquantitative densitometric analysis of tyrosine hydroxylase-immunoreactivity of the VTA neurons in acute **(A)** and chronic **(B)** administrations. (n = 3)

Conclusion

These findings show the potential neuroprotective property of *T. laurifolia* against chronic ethanol-induced addictive behaviour, by restoring the low level of tyrosine hydroxylase in mesolimbic dopaminergic system caused by chronic ethanol consumption back to normal level. There are possibilities that *T. laurifolia* may act on neuronal cells through various mechanism. However, further studies need to be performed to confirm the possible mechanism of action of *T laurifolia.*

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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