THE STUDY OF *THUNBERGIA LAURIFOLIA* LINN. ON BEHAVIOR CHANGES AND DOPAMINERGIC SYSTEM IN RATS TREATED WITH ACUTE AND CHRONIC ALCOHOL

RATIRAT SANGPAYAP

Graduate School Srinakharinwirot University

2019

การศึกษารางจืดต่อการเปลี่ยนแปลงทางพฤติกรรมและระบบประสาทโดปามีนในหนูทดลองที่ได้รับแอลกอฮอล์อย่างเฉียบพลันและเรื้อรัง

รติรัตน์ แสงพยัพ

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปรัชญาดุษฎีบัณฑิต สาขาวิชาชีวภาพการแพทย์
คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2562

THE STUDY OF *THUNBERGIA LAURIFOLIA* LINN. ON BEHAVIOR CHANGES AND DOPAMINERGIC SYSTEM IN RATS TREATED WITH ACUTE AND CHRONIC ALCOHOL

RATIRAT SANGPAYAP

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of DOCTOR OF PHILOSOPHY

(Biomedical Sciences)

Faculty of Medicine, Srinakharinwirot University

2019

THE DISSERTATION TITLED

THE STUDY OF *THUNBERGIA LAURIFOLIA* LINN. ON BEHAVIOR CHANGES AND DOPAMINERGIC SYSTEM IN RATS TREATED WITH ACUTE AND CHRONIC ALCOHOL

BY

RATIRAT SANGPAYAP

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

(Assoc. Prof. Dr. Chatchai Ekpanyaskul, MD.)
Dean of Graduate School

ORAL DEFENSE COMMITTEE

..... Major-advisor	..... Chair
(Assoc. Prof. Dr.Watchareewan Thongsaard)	(Assoc. Prof. Dr.Rosama Pusoonthornthum)
..... Co-advisor	..... Committee
(Prof. Dr.Pansiri Phansuwan)	(Assoc. Prof. Dr.Chatsri Deachapunya)
..... Co-advisor	..... Committee
(Dr. Ratchadaporn Pramong)	(Assoc. Prof. Dr.Wisuit Pradidarcheep)

Title	THE STUDY OF *THUNBERGIA LAURIFOLIA* LINN. ON BEHAVIOR CHANGES AND DOPAMINERGIC SYSTEM IN RATS TREATED WITH ACUTE AND CHRONIC ALCOHOL
Author	RATIRAT SANGPAYAP
Degree	DOCTOR OF PHILOSOPHY
Academic Year	2019
Thesis Advisor	Associate Professor Dr. Watchareewan Thongsaard
Co Advisor	Professor Dr. Pansiri Phansuwan
Co Advisor	Dr. Ratchadaporn Pramong

Thunbergia laurifolia Linn. (*T. laurifolia*), “Rang-jert”, is a Thai traditional medicine that has been used as an antidote for poisons detoxification including ethanol. *T. laurifolia* can stimulate neuronal activity and dopamine release in striatum and nucleus accumbens which are correlated in motor and reward systems in a similar way as drug abuse. However, chronic administration of *T. laurifolia* can not induce addiction. Moreover, its antioxidant property and bioactive compound, iridoid glycoside, show effects against neurotoxics. The aims of this thesis were to investigate the potential protective effect of *T. laurifolia* on behavior changes and alterations in the nigrostriatal and mesolimbic dopaminergic pathways in acute and chronic ethanol administrations rats. The four groups of male Wistar rats (control, ethanol, *T. laurifolia* and *T. laurifolia* plus ethanol groups) were used to test of acute and chronic treatments followed by the determination of various behaviors (motor coordination impairment, anxiety, exploratory and addiction) and tyrosine hydroxylase expression. In acute ethanol administration studies, *T. laurifolia* showed protective effects on the ethanol-induced motor coordination impairment on balance beam. Acute ethanol produced a significant increase in tyrosine hydroxylase expression in the striatum but not in substantia nigra, nucleus accumbens, and ventral tegmental area. Administration of *T. laurifolia* before acute ethanol reversed the expression of tyrosine hydroxylase in the striatum back to the control level. Chronic administration of *T. laurifolia* prior to ethanol for 30 consecutive days demonstrated a potential protective effect to ethanol-induced motor coordination impairment and addiction. Moreover, the expression of the tyrosine hydroxylase in the striatum and nucleus accumbens were reversed to the control level by the action of *T. laurifolia*. From both acute and chronic experiments, it can be concluded that *T. laurifolia* extract showed the protective effects on ethanol-induced motor coordination impairment, addiction, and tyrosine hydroxylase expression changes in the striatum and nucleus accumbens in rats. The findings can be suggested that the effects of *T. laurifolia* may perhaps improve an ability to prevent the alcohol-related impairment of motor coordination and motor skill to drive, addiction, and neurodegenerative diseases.

Keyword : Thunbergia laurifolia Linn, Ethanol, Nigrostriatal pathway, Mesolimbic pathway, Tyrosine hydroxylase

ACKNOWLEDGEMENTS

I wish to express my thanks to Associate Professor Watchareewan Thongsaard, Professor Pansiri Phansuwan and Dr. Ratchadaporn Pramong for their encouragement and guidance throughout the course of this Ph.D. I also thanks Associate Professor Theekapun Charoenpong, Department of Biomedical Engineering, Srinakharinwirot University for the use of Microsoft visual studio 2010 for evaluation of the behaviors study in this thesis. I wish to thanks Assistant Professor Sunan Chainakul and Assistant Professor Maneekarn Namsa-aid, Department of Chemistry, Faculty of Science, Srinakharinwirot University for advice and the use of the facility for the Thunbergia laurifolia Linn extraction. There are many others who I wish to thanks particularly research assistances and other Ph.D. students for their technique guidance, animal handling in these experiments and friendship throughout my time as a Ph.D. student.

I also acknowledge the Faculty of Medicine, Srinakharinwirot University for my graduation research grants of and research presentation in Vienna, Austria. I also acknowledge the Graduate School, Srinakharinwirot University for financial support of my traveling grant for research presentation in Vienna.

Finally, I would like to thank my family, especially Dad, Mom, and brother, for taking great care and never ending supporting throughout my study without whom my study would never have success.

RATIRAT SANGPAYAP

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

Thunbergia laurifolia Linn. (*T. laurifolia*), commonly known Rang-jert in Thai, is a Thai traditional medicine in the Acanthaceae family. The profiling of *T. laurifolia* was suggested to have high contents of the phenolic and flavonoid compounds that act as an antioxidant agent [1, 2]. The different parts of the plant are used for various purposes in a Thai traditional treatment. For example, the leaves, root, and vine have been used in a local Thai traditional recipe for poisonous substances and toxic mushroom detoxification, thirsty, antipyretic, anti-diarrhea, abnormal menstruation and, anti-allergy. Based on the traditional recipe, several reports have suggested that *T. laurifolia* has an antinociceptive effect, wound healing, anti-diabetic, antioxidant property, hepatoprotective effect [3-6]. The effect of *T. laurifolia* detoxification of poisonous substances such as paraquat and insecticides in rats was reported [7-9]. Furthermore, *T. laurifolia* leaves extract has been reported to protect ethanol-induced hepatotoxicity using hepatic lipid peroxidation, blood ethanol concentration, alcohol dehydrogenase, and aldehyde dehydrogenase activity as indicators [10]. Generally, this plant commonly used as a Thai traditional recipe for drug addiction treatment [11]. *T. laurifolia* is a major ingredient in a mixture compound to treat drug addiction [11] and alcohol-dependent patients [12-14]. The extraction of *T. laurifolia* extract stimulates endogenous dopamine release from striatal tissue slides similar to amphetamine action [15]. From the study using functional magnetic resonance imaging (fMRI) technique, the administration of *T. laurifolia* extract to the rats increase the neuronal activity in brain areas responsible for reward and locomotion such as nucleus accumbens, striatum [caudate putamen globus pallidus], amygdala, frontal cortex, and hippocampus [16]. *T. laurifolia* shows an increase in cerebral blood flow in anesthetized rats in doses and timely manner [17]. Acute administration of *T. laurifolia* extract in rats induces an enhancement of extracellular dopamine release in the nucleus accumbens similar to cocaine stimulation using the microdialysis technique [18]. From all these results, it is indicated that *T. laurifolia* extract

was used in addicted drug treatment due to the property that it can stimulate endogenous dopamine release and similarly increase dopaminergic function as amphetamine and cocaine. However, chronic *T. laurifolia* administration does not induce addiction in rats [19].

Alcohol consumption is reported as a serious problem worldwide for health and society. According to the global status report from the World Health Organization (WHO) on alcohol and health in 2018, more than half of the global population (57%, or 3.1 billion people) aged 15 years and over drink alcohol. Total alcohol per capita consumption continued to elevate from 5.5 liters of pure alcohol in 2005 to 6.4 liters in 2010 and maintain the average value of 6.4 liters until the year 2016. The WHO suggests that alcohol consumption could increase to 6.6 liters in 2020 and may be increased up to 7.0 liters in 2025 [20]. The Center for Alcohol Studies (CAS) in Thailand reported that alcohol consumption in Thai drinkers ages 15 years and older is 16,992,017 person or 31.5 percent of the population in 2014 [21].

The adverse effect of alcohol drinking is under concerned as it has effect on society and health. Alcohol intoxication is a common cause of accidents, vandalism, fighting, and other forms of social interaction including family violence and breakdown [22]. National Statistical Office of Thailand (NSO) reported that most of road accidents from drivers ages 15 years and older (about 7,792 person in 2014) were caused by alcohol drinkers [23]. The road accidents caused by alcohol drinkers have greatly increased during long weekends or festivals. The Road Safety Operation Center (RSOC) in Thailand reported that the causes of accidents during festivals such as New Year festival and Songkran festival were drinking alcohol before driving [24, 25]. Moreover, Road Safety Thailand Road Safety Policy Foundation (RSPF) reported an increasing rate of injuries and deaths from drunk drivers during New Year and Songkran festivals in the year 2016-2019 [26].

Concerning the health problem, acute alcohol intoxication is clinically harmful. An abnormal behavioral or psychological disturbance usually present during or shortly after alcohol ingestion. Aggressive behavior, unstable mood, and impaired

judgment conditions are found in alcohol drinkers. Following signs after alcohol use are slurred speech, lack of coordination, impairment of attention or memory and stupor or coma which correlated with blood alcohol concentration (BAC) [27]. Alcohol drinkers are at risk for many diseases. WHO has shown the research that 1.7 million alcohol drinkers died from non-communication diseases including digestive, cardiovascular disease, and cancer. Globally, alcohol use disorder or alcohol addiction, developmental of chronic alcohol consumption, has 237 million men and 46 million women [28].

Ethanol is a major compound of alcoholic beverages. Ethanol absorption occurs mainly in the stomach and duodenum. The remaining occurs in other parts of the intestinal tract [29]. Gastric alcohol dehydrogenase (ADH), generally known as "first-pass metabolism", transforms ethanol to acetaldehyde. The remaining of ethanol (90%) is metabolized in the liver by three liver enzymes including liver ADH, microsomal ethanol oxidizing system and catalase [30]. Ethanol induces organ damage such as liver, heart, and respiratory system and promotes disease stages and neuropsychological disorders [31]. Ethanol suppresses the central nervous system (CNS) in a similar manner as some depressant drugs. It affects cellular and molecular levels and changes in synaptic functions of neurons. Acute ethanol intoxication either stimulates or inhibits the function of proteins implicated in synaptic transmission. Chronic ethanol intoxication presents opposing and /or compensatory/homeostatic effects on the expression, localization, and function of these proteins [32]. Many synaptic transmissions have been affected by acute and chronic ethanol intoxication and promoted many abnormal behaviors. Several channels, secondary messenger systems, and neurotransmitters such as dopamine (DA), γ-aminobutyric acid (GABA), glutamate, and serotonin (5-HT) implicate with ethanol actions [33]. Exposure of ethanol to the central nervous system causes behavioral changes such as motor coordination impairments, anxiety, exploratory behavior, and addiction behavior [34-36].

From many reports on the ethanol intoxication, the first neuronal circuit correlating with ethanol action is a dopaminergic system. The processes correlate with the dopaminergic system including acute intoxication, reinforcement, tolerance,

dependence, and withdrawal. The studies show the correlation between ethanol effect and the dopaminergic system. For example, alcohol consumption was blocked by injection of a low dose of dopamine antagonists which disturbed dopamine activity [37, 38]. Several publications suggest the acute and chronic ethanol exposure via several routes, i.e., intravenous injection, intraperitoneal injection, and oral administration, in rats and human. The acute ethanol intoxication increases dopaminergic neurons firing and dopamine release in rodents [39, 40] and human [41, 42]. Chronic ethanol intoxication on dopamine levels during ethanol withdrawal are found to have some controversial results i.e., a decrease dopamine release in adult female alcohol-preferring (P) rats [43], no change of dopamine level in adolescence male mice, [44] or increase dopamine release in adult female alcohol-preferring rats [45].

Dopamine is a monoamine neurotransmitter. The original dopaminergic neurons are in the ventral tegmental area, substantia nigra, midbrain, and the arcuate nucleus of the hypothalamus. Dopaminergic neuron projections have effects to regulate many behaviors. Nigrostriatal dopaminergic pathway is a projection of dopaminergic neurons in substantia nigra to striatum, caudate nucleus, and the putamen. This projection implicates movement, motor function, and learning new motor skills. The original dopaminergic neurons in the ventral tegmental area projects to nucleus accumbens also called mesolimbic dopaminergic pathway and regulates emotion and reward systems. Another projection from ventral tegmental dopaminergic neurons is called the mesocortical dopaminergic pathway. This pathway projects to prefrontal cortex and functions to regulate cognitive and emotional behavior. The tuberoinfundibular dopaminergic pathway originates in the arcuate nucleus of the hypothalamus and projects to pituitary gland. The dopamine in this pathway inhibits prolactin release [46].

Ethanol intoxication induces dopamine activity changes and promotes abnormal behaviors. The ethanol intoxication in the nigrostriatal dopaminergic pathway has been reported to have a different response dependent on the dose of ethanol used. Low dose of intravenous injection of ethanol excited the dopaminergic neurons in the substantia nigra of Male Sprague-Dawley rats but inhibited the activity when using a

high dose [47]. These findings were also found in the striatum by acute ethanol administration [48]. These imbalances of dopamine activities by ethanol cause abnormal behaviors that are regulated by dopaminergic pathways. Motor coordination was impaired within a few minutes after intraperitoneal injection of ethanol in both rats and mice and continued to impair toward a hangover [49, 50]. The electrophysiological studies, *in vivo* and *in vitro*, demonstrating ethanol effect on mesolimbic and mesocortical dopaminergic pathways that ethanol increases the firing rate of dopaminergic neurons in the ventral tegmental area [51, 52]. Exposure of chronic ethanol causes an increase in dopamine-releasing in the ventral tegmental area [53]. When the ventral tegmental area was activated, the brain areas which are the targets of its projection i.e., nucleus accumbens (of alcohol-preferring (P) rats [54] and prefrontal cortex (of the Long-Evans rat [55] exhibited the increase in dopamine release. However, the withdrawal effect of ethanol intoxication showed a decrease in dopamine level [56]. Acute and continuously exposure of ethanol increased dopamine activity and promoted the biphasic behavior response to ethanol. Low to moderate doses induced exploratory behavior, anxiolytic, and increased locomotor activity [57, 58]. High dose of ethanol caused sedation and decreased locomotor, and exploratory behaviors [59]. Moreover, continuous ethanol exposure was found to develop alcoholism in mice which is also known as alcohol addiction[60]. Furthermore, ethanol intoxication showed an effect in the tuberoinfundibular dopaminergic pathway. Prolactin release was observed in acute ethanol intoxication and chronic ethanol intoxication [61, 62].

As mentioned earlier, *T. laurifolia* is a traditional plant that can be used to treat for prevention and detoxification of drug toxicity and addiction. Many studies have reported that *T. laurifolia* can activate many brain areas [16] and dopamine [15, 18] in rewarding and motor areas similar to that of abusive drugs such as cocaine or amphetamine. However, chronic administration of *T. laurifolia* did not cause addiction in rats [19]. Therefore, it is interesting to investigate preventive effects of *T. laurifolia* on behaviors changes and dopaminergic system response in rat treated with acute and chronic ethanol administration through the following aspects:

1. The preventive effect of *T. laurifolia* on behavioral changes and dopaminergic system response in male Wistar rats induced by acute administration of *T. laurifolia*, ethanol, and *T. laurifolia* plus ethanol.

1.1 To determine motor coordination impairment, anxiety, and exploratory behavior changes in response to acute administrations by using balance beam, elevated plus maze, and hole board apparatus, respectively

1.2 To determine the expression and alteration of the tyrosine hydroxylase in mesolimbic and nigrostriatal dopaminergic pathways in response to acute administrations by using western blot analysis and immunohistochemistry method

2. The preventive effects of *T. laurifolia* on behavioral changes and dopaminergic system response in male Wistar rats induced by chronic administration of *T. laurifolia*, ethanol, and *T. laurifolia* plus ethanol

2.1 To determine ethanol addiction, motor coordination impairment, anxiety, and exploratory behavior changes in response to chronic administrations by using condition place preference test, balance beam, elevated plus maze, and hole board apparatus, respectively

2.2 To determine the expression and alteration of the tyrosine hydroxylase in mesolimbic and nigrostriatal dopaminergic pathways in response to chronic administrations by using western blot analysis and immunohistochemistry methods

Conceptual framework

In this study, the protection of *T. laurifolia* on the expression and alteration of tyrosine hydroxylase in mesolimbic and nigrostriatal dopaminergic pathways was studied in rats treated with *T. laurifolia* prior to acute and chronic ethanol administration. Behaviors that implicated with mesolimbic and nigrostriatal functions, changes in the administration of *T. laurifolia* prior to acute and chronic ethanol administration could explain the protective effect of *T. laurifolia* on the functional pathways of dopamine. Understanding the effects of *T. laurifolia* on dopaminergic response in these pathways of acute and chronic ethanol administration may explain the protective mechanism of *T. laurifolia* to ethanol administration.

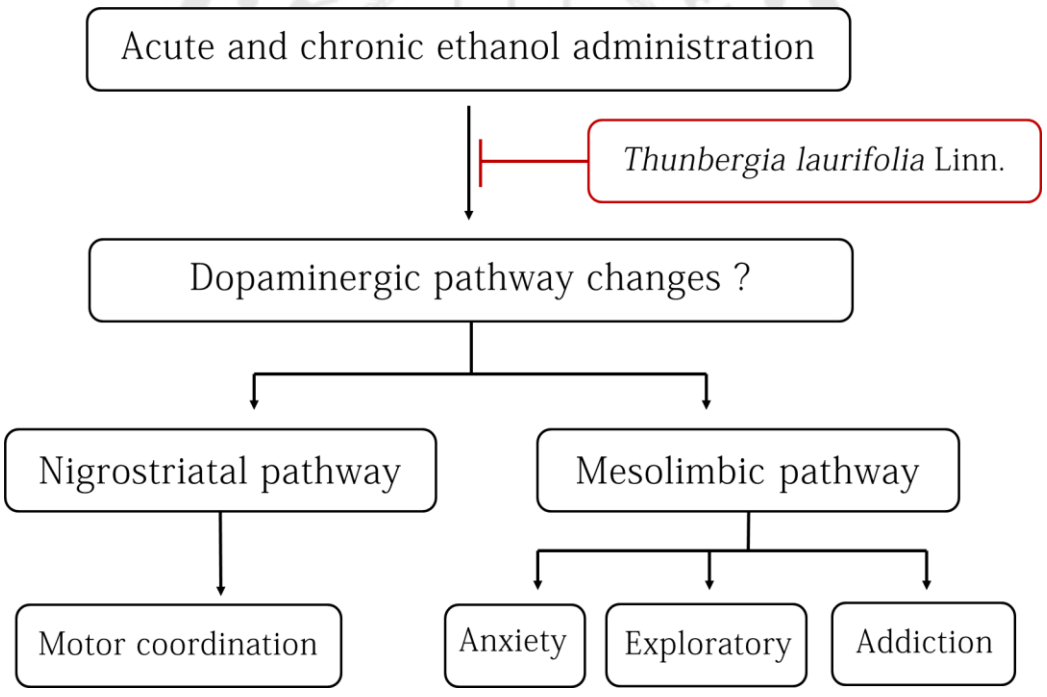

Figure 1 Conceptual framework.

CHAPTER II
REVIEW LITERATURE

Thunbergia laurifolia Linn.

Thunbergia laurifolia Linn. (*T. laurifolia*) is a Thai traditional herb that has been used as an antidote for poisonings detoxification. Different parts of *T. laurifolia* such as leaves, root, and vine are used for toxic substances and toxic mushroom detoxification, thirsty, antipyretic, anti-diarrhea, abnormal menstruation, and anti-allergy in a local Thai traditional recipe.

Thai traditional uses of *T. laurifolia*

T. laurifolia has long been used as a Thai traditional medicine which has no publications for its effects in use. In Thailand, *T. laurifolia* is used locally as an antidote for several detoxifications such as arsenic, strychnine, insecticide, and ethanol. In Thai tradition recipes, the dried leaves extraction with hot water is used for detoxification of acute paraquat administration. *T. laurifolia* leaves are pounded and extracted in water or rice wash water as known as nam sow kow in Thai for local use. After that, the extracts are squeezed before drinking as a detoxification treatment. Furthermore, the pounded root and vine are used to reduce the poisoning effect in the body.

Taxonomy

T. laurifolia is commonly known in Thai as “Rang-jert” or babbler’s bill, laurel clock vine, blue trumpet vine, or laurel-leaved in English name. *T. laurifolia* is classified in the Acanthaceae family and Genus, Thunbergia which distributed in Southeast Asia. *T. laurifolia* is a climbing plant that the woody green vine has a hairless and climbing with the stem (figure 2A). Its leaves are laurel or heart-shaped, dark green, opposite pairing, a pointed tip, and slightly serrated leaf (figure 2B). The leaves are bright and thin when young and tend to be dark green when mature. Its flower is a trumpet-shaped with 5 lobes purple-blue color shade and a yellow throat (figure 2C).

(A) (B) (C)

Figure 2 Characteristics of *T.laurifolia*.

T. laurifolia shows in figure 2A, its leaf in figure 2B and flower in figure 2C.

Phytochemical substances of *T. laurifolia*

T. laurifolia flower extract contains apigenin-7-o-β-D-glucopyranoside delphinidin 3:5-di-o-β-D-glucopyranoside, and apigenin [63]. Methanolic extract of the aerial part of *T. laurifolia* shows 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 [64]. The extractions of the leaves with boiling water, ethanol, and acetone contain the phenolic, caffeic acid, apigenin, carotenoids, gallic acid, chlorophyll a and b, pheophorbide a, pheophytin a, and lutein [1].

Pharmacological effects of *T. laurifolia*

Various publications have reported the studies of *T. laurifolia* both *in vitro* and *in vivo* in order to explain many effects of *T. laurifolia* in traditional uses. Several effects of *T. laurifolia* including neuronal and behavior, detoxifying, antioxidant, hepatoprotective, and other effects such as anti-inflammatory, anti-fibrosis, anti-cancer, and wound-healing.

Neuronal and behavior effects

In 2002, Thongsaard and colleagues studied the stimulation effect of *T. laurifolia* on endogenous dopamine release in rat striatal tissues slice comparing with amphetamine by using high-performance liquid chromatography system with electrochemical detection (HPLC-ECD). The *T. laurifolia* crude water extract (0.1 g/ml) showed a stimulating effect on potassium-stimulated dopamine release similar to that of amphetamine when compared to control [(15)]. Furthermore, Thongsaard W. *et al*. (2005) showed that 200 mg/kg of leaves *T. laurifolia* methanol extract increased cerebral activity in several brain areas responsible for reward and locomotion including striatum (caudate and putamen), nucleus accumbens, globus pallidus, amygdala, frontal cortex, and hippocampus by using functional magnetic resonance imaging (fMRI) technique while blood pressure, blood pH, partial pressure of CO_2 (pCO_2), and partial pressure of O_2 (pO_2) of rats did not change [(16)]. Intraperitoneal injections of *T. laurifolia* at the doses of 100, 200, and 300 mg/kg increased cerebral blood flow in a dose-related and timely manner in anesthetized rats at 5, 10, 20, and 30 minutes after injection of *T. laurifolia* [(17)]. A single injection of 200 mg/kg *T. laurifolia* methanol extract compared with 10 mg/kg cocaine showed that *T. laurifolia* increased dopamine release in rat nucleus accumbens in a similar manner as cocaine by using microdialysis technique. The increased dopamine exhibited within 20 minutes after *T. laurifolia* injection followed by a decrease after 40 minutes of injection by using HPLC-ECD [(18)]. Thongsaard W and co-workers (2015) exhibited that chronic administration of 1 and 10 g/kg *T. laurifolia* methanol extract for 30 consecutive days did not cause addiction when compared with 10 mg/kg cocaine administration using a standard behavior model for addiction called condition place preference test [(19)]. Furthermore, the oral administration of 250 and 500 mg/kg *T. laurifolia* for 3 weeks decreased cognitive, depression-like behavior deficits and reversed expression levels of choline acetyltransferase (ChAT) and muscarinic M1 receptor in hippocampus of Alzheimer's disease model mice (OBX mice) [(65)].

Detoxifying effects

Several publications suggest the detoxification effect of *T. laurifolia* on several poisons. *In vitro* study, *T. laurifolia* promotes detoxification effect on

organophosphorous (chlorpyrifos) inhibited neurotransmitter system damage. The organophosphorous (chlorpyrifos) inhibited activity of cholinesterase (ChE) was decreased by *T. laurifolia* (2 and 4 mg/ml) treated mouse brains and red blood cells [66]. The attenuation of paraquat toxicity showing an administration of *T. laurifolia* 1 hour before paraquat injection increased survivor rate of rats treated with mortality dose (70 mg/kg) of paraquat and decreased plasma concentration of malonaldehyde (MDA) in rats receiving 60 mg/kg paraquat [67]. Chronic feeding of *T. laurifolia* at a human dosage of 3 cups (250 ml/cup) for 20 consecutive days before injection of 1 mg/kg/day cadmium for 20 days reduced toxicity signs and also kidney and liver damages caused by cadmium[68]. A reducing lead concentration in liver and muscle of *Oreochromis niloticus* (Nile tilapia or Plah Nin in Thai) was observed for *T. laurifolia* leaf extract dietary supplement administration for 28 days[69].

Antioxidant effect

Antioxidant activity and total phenolic compounds of *T. laurifolia* were evaluated using free radical scavenging, ferric reducing antioxidant power assay (FRAP) and the Folin-Ciocalteu method. Water, ethanol, and acetone extracts of dried leaf of *T. laurifolia* showed 2430, 565, and 142 mg GAE/100 g of total phenolic content, respectively. A higher free radical scavenging at 0.13 mg GAE/ml was observed in water extraction whereas ethanol and acetone extract showed at 0.26 and 0.61 mg GAE/mL respectively. Besides, water, ethanol, and acetone had total antioxidant activity at 0.93, 0.18, and 0.04 mmol/g, respectively using FRAP assay[2]. Different drying methods of *T. laurifolia* show a difference in total phenolic containing. Freeze-dried, microwave dried, oven-dried, and fresh leaves of *T. laurifolia* had 3850 ± 127, 3080 ± 202, 1800 ± 57, and 577 ± 39 mg GAE/100 g of total phenolic content, respectively [70]. Furthermore, A decrease in hemolysis of mice infected with *Plasmodium berghei* (malaria infection) was suggested that its antioxidant property may reduce oxidative stress during malaria infection in erythrocytes [71]. Furthermore, *T. laurifolia* administration in a diet as a food supplement increased the intrinsic antioxidant (glutathione) and the activities of catalase, glutathione reductase, and glutathione

peroxidase. Lipid peroxidation in gill, kidney, and liver in Nile tilapia were also decreased after chronic exposure to lead [69].

Hepatoprotective activity

The hepatoprotective effect of *T. laurifolia* leaves extract was found in rats treated with ethanol both *in vitro* and *in vivo* studies. Rat hepatocytes treated with ethanol before 2.5, 5.0, 7.5, and 10.0 mg/ml concentrations of *T. laurifolia* leaves extract showed that *T. laurofolia* decreased the percentage of MTT and recovered transaminase enzymes, aspartate transaminase, and alanine aminotransferase in rat hepatocytes to baseline. Chronic ethanol administration for 14 days and followed by 7 consecutive days of *T. laurifolia* administration also showed the recovery of transaminase enzymes and hepatic triglyceride to the normal levels [72].

Other effects of *T. laurifolia*

Administration of *T. laurifolia* to male Sprague Dawley rats before inflammation induction for 1 hour reduced ear and paw edema by using ethyl phenylpropiolate (EPP)-induced ear edema, carrageenan or arachidonic acid-induced paw edema, and cotton pellet-induced granuloma formation test[73]. Lipopolysaccharide-induced nitric oxide production, inflammatory response molecule, and cell proliferation in murine macrophage cells were inhibited by *T. laurifolia* exposure [74]. *T. laurifolia* treatment reduced the inflammatory cells in the surrounding of the hepatic bile ducts of Syrian hamsters infected with *Opisthorchis viverrini* (Southeast Asian liver fluke)[3]. *T. laurifolia* exposure to hepatic stellate cells treated with transforming growth factor-beta 1 resulted in a suppression of the extracellular matrix (α-SMA) and collagen-I production, decreasing matrix metallopeptidase 9 activity, and inhibiting signal transduction pathways such as p38 MAPK and Erk1/2 kinases [75]. The observation of anti-cancer activity of *T. laurifolia* suggested that *T. laurifolia* exposure to breast cancer cells for 24 hours decreased cell growth, clonogenic cell survival, and cell viability in a dose-dependent manner. A significant increase in cell population in the G1 phase and decreased in the S phase of the breast cancer cells cycle at a higher dose of *T. laurifolia* indicated its effect to induce breast cancer cell cycle arrest [76]. Furthermore, Second-degree burn wound in male Wistar rats treated with leaves *T. laurifolia* extract

gel for 28 consecutive days was shown to abolish edema or congestion in second-degree burn, rapidly promote in epithelialization, fibroblast infiltration, and angiogenesis and increase wound healing rate and collagen content [4].

Toxicity of *T. laurifolia*

The observation of *T. laurifolia* toxicity was suggested by Chivapat S *et al* (2010). Water extract of *T. laurifolia* leaves was chronically administered to rats for 6 months at a dose of 20, 200, 1,000, and 2,000 mg/kg/day. The parameters used for *T. laurifolia* toxicity determination were hematological parameters, body weight, food consumption, behavior, or general health. The observations showed that *T. laurifolia* administration at any doses did not significantly change food consumption, physical appearance, or behavior and any toxic signs or mortality throughout the treatments. Chronic treatment of *T. laurifolia* showed a decrease in red blood cell and an increase in white blood cells. Besides, albumin and bilirubin levels were higher than that of the control group given drinking water. However, any doses of *T. laurifolia* used in this experiment did not induce any histopathological alterations of visceral organs in both sex rats [77].

Alcohol

Alcohol is an organic compound. The general formula of alcohol is $C_nH_{2n}OH$. The alcohol structure has a saturated carbon atom joined with a hydroxyl functional group (-OH). The hydroxyl group is an important part responsible for the physical and/or chemical properties of the alcohol molecule. Alcohol can be classified based on a carbon atom that joined with a hydroxyl group. The connection between the carbon atom that joins with the hydroxyl group and alkyl group represents a type of alcohol. The primary alcohol has only one alkyl group that connected, while secondary or tertiary has two and three alkyl groups that connect with carbon atom (figure 3) [78].

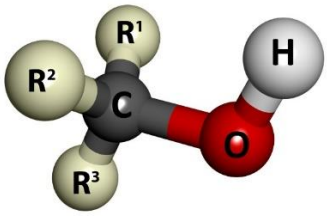

Figure 3 A ball-and-stick model of alcohol molecule.

The alcohol structure consists of hydroxyl functional group and alkyl groups.

Source: https://en.wikipedia.org/wiki/Alcohol[79]

Alcoholic beverages

An alcoholic beverage (or alcoholic drink) is a drink that contains ethanol. In industry, ethanol is prepared by acid-catalyze hydration of ethylene [80]. Generally, the production of alcoholic beverages uses different fermentation processes and materials. Types of alcoholic beverages such as beer, wine, whiskey, brandy, rum, gin, and vodka are difference in the percentage of ethanol, methods, and materials. Beer use starches mainly derived from cereal grains such as malted, barley, cereal, or corn for a fermentation process and stop the process when the fermentation contains 4 to 8 percent of ethanol. Wine has 10 to 12 percent of ethanol and commonly uses grapes for fermenting process. Distilled fermented juices such as whiskey, brandy, rum, and gin contain 40 to 55 percent of ethanol. Whiskey and brandy are distilled fermented juices brewed in wooden casks while rum and gin are not stored in wooden casks. Whiskey made from fermented grain mash i.e., barley, corn, rye, wheat, and leave for fermentation in wooden casks made of charred white oak. However, brandy is distilled from fermented fruit juices such as apple and grapes and stored in an oak cask.

Blood alcohol concentration (BAC) and symptoms

After alcohol consumption, alcohol is rapidly absorbed into the blood circulation and reaches maximum blood alcohol concentration (BAC) within 10 and 60 minutes after consumption. The blood alcohol concentrations are related to clinical

symptoms (Table 1). BAC less than 50 mg/dl causes increased talkativeness and relaxation while ataxia, poor motor coordination, mood, behavioral changes, and nystagmus occur at a BAC higher than 100 mg/dl. Respiratory depression, coma, and death present at BAC higher than 400 mg/dl [81].

Table 1 Main symptoms correlated blood alcohol concentrations (BAC) in alcohol consumption.

Symptoms	BAC
Impairment in some task requiring skill	BAC < 50 mg/dl
Increase in talkativeness	
Relaxation	
Altered perception of the environment	BAC > 100 mg/dl
Ataxia	
Hyper-reflexia	
Impaired judgment	
Lack of coordination	
Mood, personality, and behavioral changes, nystagmus	
Prolonged reaction time	
Slurred speech	
Amnesia	BAC > 200 mg/dl
Diplopia	
Dysarthria	
Hypothermia	
Nausea	
Vomiting	
Respiratory depression	BAC > 400 mg/dl
Coma	
Death	

Ethanol

Ethyl alcohol or commonly known as “ethanol” is simple alcohol with a chemical formula of C_2H_6O. It is naturally produced by the fermentation of sugars by yeasts or via petrochemical processes. Ethanol is an active gradient in alcoholic beverages.

After alcohol consumption, ethanol undergoes the pharmacokinetic process including absorption from the gastrointestinal tract, distribution to liver and other organs, metabolism in the liver or other cells and excretion from the body. Ethanol-induced symptoms exhibit a dose-dependent pharmacokinetic. The metabolism of ethanol by liver enzyme, hepatic alcohol dehydrogenase (ADH), is saturated at BAC above 15-20 mg/100 ml. This is a cause that has ethanol in blood circulation and penetrates into the cells. In the central nervous system, ethanol easily crosses the blood-brain barrier and interacts with nerve cell membranes and receptor proteins to cause many impairments of drinkers (figure 4) [82].

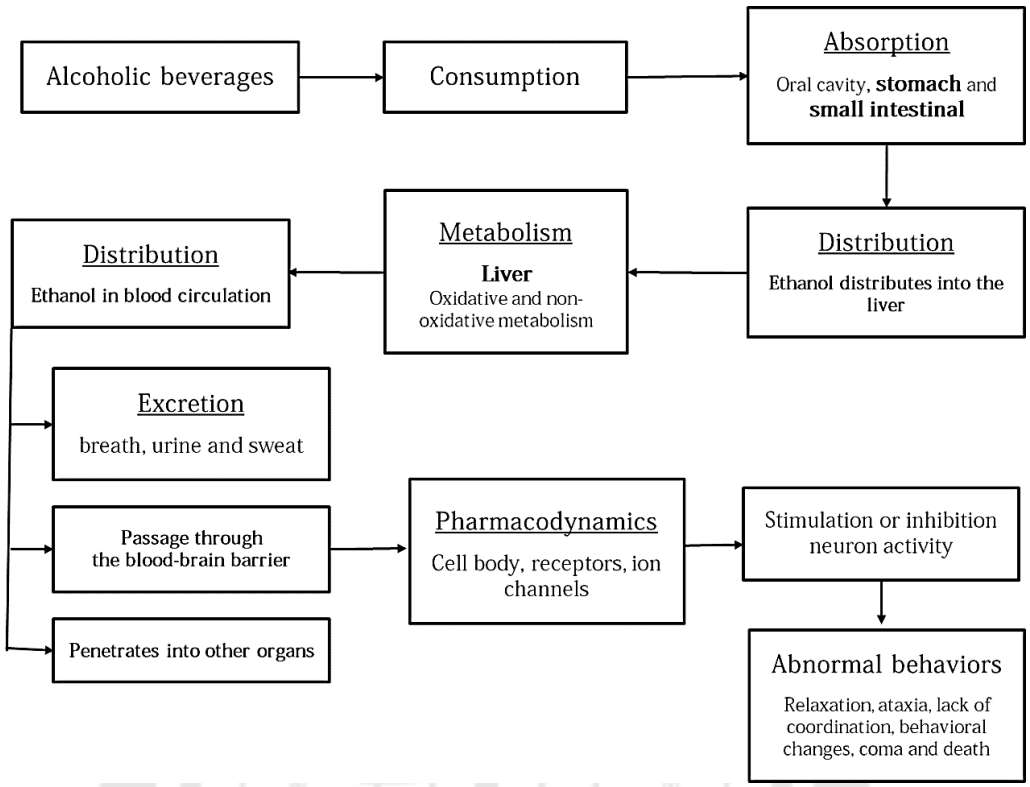

Figure 4 Diagram of ethanol in the body.

Source: Jones AW. (2019) [83]

The diagram exhibits ethanol consumption, pharmacokinetics, pharmacodynamics in the brain and, symptoms. After alcohol consumption, ethanol is absorbed into blood circulation via the oral cavity, stomach, and small intestinal. The liver is the first organ that ethanol distributes to. Ethanol is metabolized by oxidative and non-oxidative metabolism in the liver. The remaining ethanol penetrates into the heart and other organs. Ethanol in blood circulation crosses the blood-brain barrier and interacts with cell body, receptor, and ion channels. The interactions produce an inhibition or stimulation of neurons and result in abnormal behaviors.

Pharmacokinetics of ethanol

Absorption

The absorption process refers to uptake ethanol into blood circulation. Ethanol penetrates from the gastrointestinal tract into blood circulation by a hydrophilic molecule of ethanol, a hydroxyl group. An uncharged molecule of ethanol easily passes membranes by a passive diffusion process that depends on the concentration gradient [84, 85]. In the mouth, ethanol molecules can be absorbed by mucous surfaces in an oral cavity. The absorption areas of ethanol are stomach and small intestine. The study of an absorption rate in the stomach showed an absorption increased to 40 % of ethanol within 20 minutes [86] and up to 60 % in an hour [87] using rats pylorus ligation. Generally, ethanol consumption is mainly absorbed in the small intestine which has a large absorptive area i.e., villi and microvilli. Several factors are correlated with the rate of absorption in the gastrointestinal tract such as gastric emptying, food types, portal blood flow, and percent of alcohol contents [85, 88]. Drinking alcohol during meals or after meals delayed gastric emptying and slowed the absorption of ethanol into the blood circulation [89]. Protein and fat that induced cholecystokinin release from the duodenum resulted in an inhibition of gastric emptying in the stomach. The sympathetic effect and vasoconstriction of the portal vein in the liver caused the reduction of ethanol absorption by decreasing the flow rate in the portal vein. Percentage of ethanol in alcoholic beverages has been reported to have an effect on an absorption rate. The moderate percentage of ethanol in alcoholic beverages, 20-30 % ethanol, has been reported to have the fastest rate of absorption. High dose of ethanol induced irritation to the intestinal mucosa and mucus secretion and resulted in the decrease in gastric emptying [88].

Distribution

The heart pumps ethanol to the blood circulation and penetrates into extra- and intra-cellular compartments. The ethanol distribution within systemic blood circulation dose not bind to plasma proteins or biomolecules. The rate of ethanol equilibrium in the body depends on the cross-section of local capillary and blood flow per gram tissue [84]. Ethanol diffuses from systemic blood circulation into extra- and

intracellular by gradient equilibrium. High water containing organs such as brain, liver, and kidneys have a high distribution of ethanol. Furthermore, gender has a different effect on the relationship between water containing in the body and ethanol distribution. Men have more rate of ethanol distribution than women. This evidence suggests that women have a lower ethanol distribution into extra- and intracellular fluid than men leading to a higher blood alcohol concentration in systemic circulation in women than men [83].

Metabolism

The main metabolism process is in the liver by the action of two liver enzymes including hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase enzymes (ALDH). About 85% of ethanol ingestion is metabolized via oxidative metabolism. Other hepatic enzymes involved in ethanol metabolism are the microsomal ethanol-oxidizing system and catalase enzyme. Ethanol is metabolized via this system approximately 10 % of ethanol consumption. Oxidative metabolism metabolizes ethanol into acetate, entries into Kreb's cycle, and converts into CO_2 and H_2O while the microsomal ethanol-oxidizing system and catalase enzyme metabolize ethanol into acetaldehyde. Less than 1 % of the remaining ethanol is metabolized via non-oxidative metabolism [90].

Oxidative metabolism

The cytosolic enzyme, ADH, transforms ethanol to acetaldehyde, a highly reactive compound that can bind to macromolecule in stool. This is a rate-limiting step of ethanol metabolism. The acetaldehyde is transformed into acetate in mitochondria. The oxidative metabolism step of ethanol requires nicotinamide adenine dinucleotide (NAD^+) as a co-enzyme. Therefore, NAD^+ is reduced during the metabolism process. Apart from the first step of oxidative metabolism, the microsomal ethanol-oxidizing system uses the microsomal enzyme in a smooth endoplasmic reticulum called cytochrome P450 2E1 (CYP2E1) for a transformation of ethanol to acetaldehyde. This process is induced by chronic heavy drinking. Acetate is a final product of oxidation metabolism and enters into the Kreb's cycle to produce carbon dioxide and water. The multiple isoforms of ADH and ALDH with a difference in kinetic and variable

of polymorphism result in a different rate of ethanol metabolism. The ethanol metabolism in a different individual and between racial and ethnic found a variation in toxicity among racial groups. Normally, class I ADH and class II ALDH is considered as the main isoform responsible for ethanol metabolism [85]. The addition system is the catalase enzyme located within cell bodies called peroxisomes. The system is a hydrogen peroxide (H_2O_2)-generating system that converts ethanol into acetaldehyde. The oxidative metabolism of ethanol is shown in figure 5.

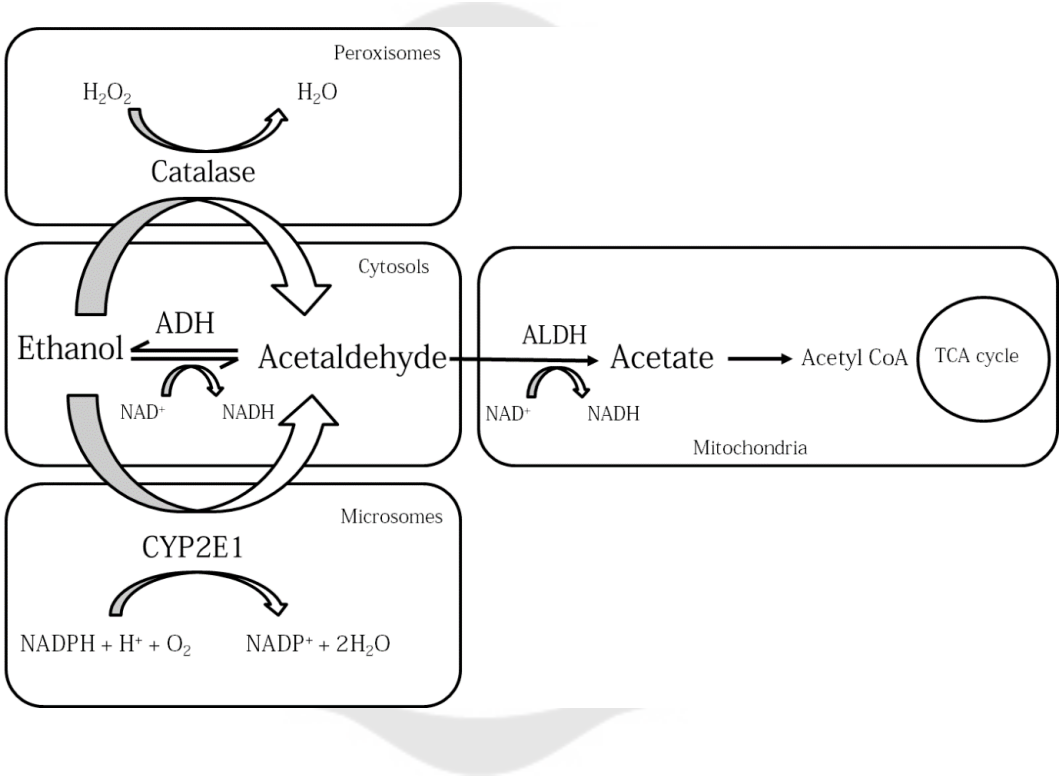

Figure 5 Oxidative pathways of ethanol metabolism.

Source: Zakhari S. (2006)[91]

Non-oxidative metabolism

Non-oxidative metabolism of small fraction that contains a hydroxyl group. Non-oxidative biotransformation involves the conjugated enzyme of ethanol with endogenous substrates, fatty acids, phospholipids, sulfate, or glucuronic acid produce fatty acid ethyl esters (FAEEs), phosphadityl ethanol (PEth), ethyl sulfate (EtS), and ethyl

glucuronide (EtG), respectively. Ethanol in non-oxidative metabolism involves mainly two enzymes including fatty acid ethyl ester (FAEE) and phospholipase D (PLD). The reaction by catalyzed FAEE produces FAEEs molecules. The enzyme phospholipase D produces phosphadityl ethanol [90] (figure 6).

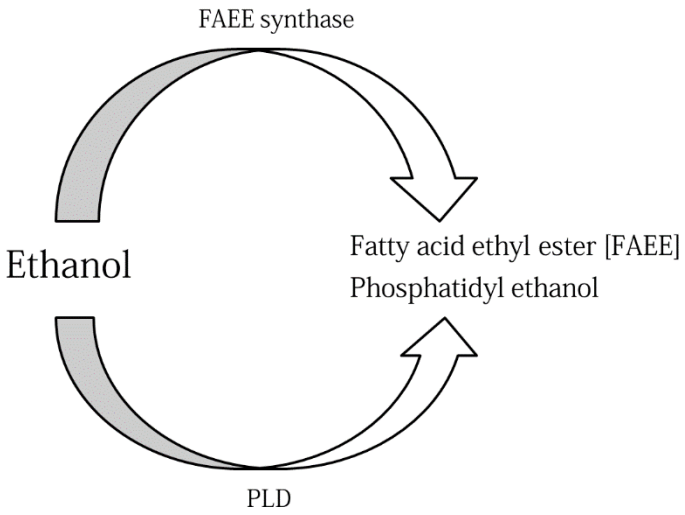

Figure 6 Non-oxidative metabolism of ethanol.

Source: Zakhari S. (2006) [91]

Elimination

The main ethanol elimination is an oxidative metabolism by converting ethanol to acetate following transferring into H_2O and CO_2 in Krebs's cycle. Less than 10% of total ethanol is eliminated unchanged via breath, urine, and sweat excretion [82, 88]. (figure 7).

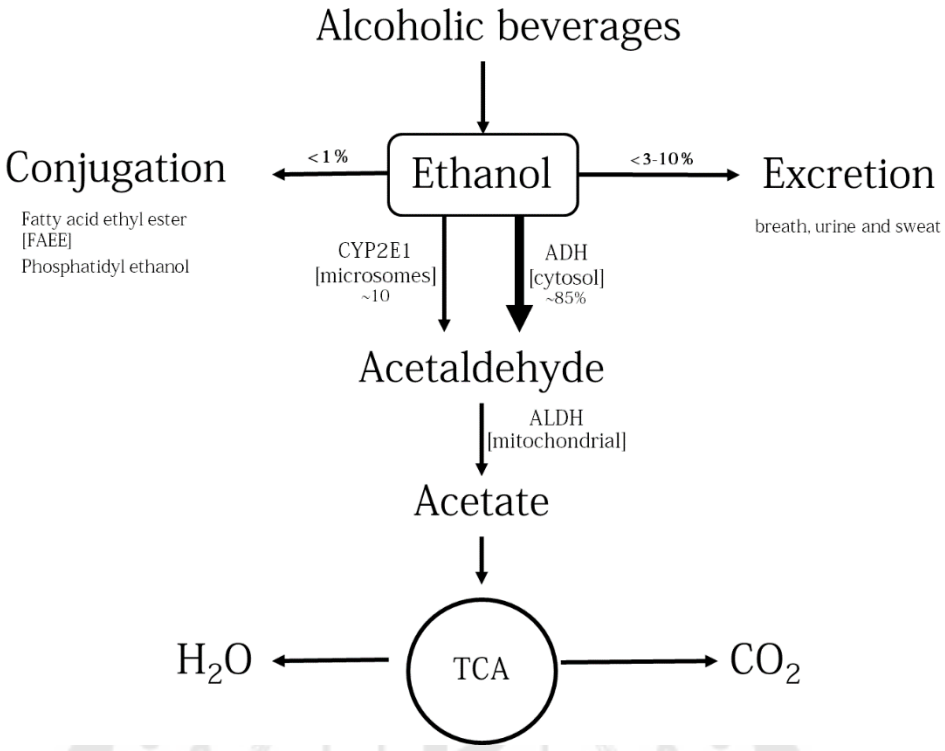

Figure 7 Ethanol elimination.

Source: Jones AW. (2011)[83]

Pharmacodynamics of ethanol in the brain

In the central nervous system, ethanol can pass the blood-brain barrier and directly trigger neurons. Ethanol exerts particular effects to induce an imbalance of neuronal activity and exhibits neuronal damages, neuronal dysfunctions, and abnormal behaviors.

Ethanol effects on brain activity

Several publications have been reported the acute effect of ethanol on several brain areas using many methodologies such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies. The PET scan is used for detecting brain activity with glucose conjugated radiation. A moderate dose of ethanol significantly decreased glucose metabolism in several brain areas but exhibited an increase of the glucose metabolism in the striatum, nucleus accumbens,

amygdala, insula, and mesencephalon [92]. A correlation between ethanol consumption and cerebral blood flow in alcohol drinker who consumed 1.0 g/kg of ethanol presented the increase in cerebral blood flow in the prefrontal and temporal lobes whereas cerebellum presented a reduction in cerebral blood flow using co-functional of PET scan and arterial spin labeling (ASL) study [93]. Furthermore, Ingvar *et al*. (1998) studied an ethanol effect on brain activity at rest in thirteen humans. The PET scan showed an increase of the cerebral blood flow in medial parts of the temporal lobes and a decrease of blood flow in the cerebellum during blood alcohol concentration steady-state in non-alcoholic volunteers infused with alcohol [94]. Administration of 0.7 g/kg ethanol increased activity of the visual network, auditory network, anterior cingulate cortex, and brainstem in healthy subjects using fMRI study [95].

Effects of ethanol on neurotransmitters and receptors

Effects of ethanol on dopamine

Dopamine is a neurotransmitter that has been suggested to involve in drug abuse and ethanol action. The injection of a low dose of ethanol stimulated the firing of dopaminergic neurons in the substantia nigra par compacta of unanesthetized paralyzed rats whereas a high dose of ethanol inhibited these firing in electrophysiological study [47]. The results were confirmed by the finding that low dose of ethanol stimulated dopamine release while high dose inhibited dopamine release from the rat striatums [48]. Ethanol has been reported to facilitate dopaminergic neuron firing and induced dopamine releases from rat ventral tegmental area [96]. The physiological mechanism of ethanol on dopaminergic neurons in the ventral tegmental area was suggested to stimulate directly onto neurons [97] and other channels [98, 99]. The firing of ventral tegmental dopaminergic neurons induced an increase of dopamine release in nucleus accumbens of ethanol-treated rats [100] and increased dopamine release and reduced dopamine clearance in the medial prefrontal cortex [101]. The observation of the correlation between dopamine release in the nucleus accumbens and blood ethanol concentration indicated that dopamine release in the nucleus accumbens did not respond to ethanol in a linear fashion. Dopamine release in the nucleus accumbens increased and followed by a decrease to basal level even when an ethanol blood

concentration is still high [102]. Repeated ethanol exposure in mice midbrain slices containing the ventral tegmental area and the substantia nigra pars compacta produced down-regulation of the hyperpolarization-activated cation current in dopaminergic neurons [103]. Whole-cell voltage-clamp on midbrain slices of mice previously given chronic administration of ethanol have been reported to enhance dopamine receptor type 2 autoreceptor-mediated inhibition of dopaminergic neurons in the ventral tegmental area [44]. Up-regulation of dopamine auto-inhibition may play an important role in mesolimbic dopaminergic output during ethanol withdrawal *in vivo* [104]. These suggested that the reduction in the stimulatory effect of ethanol may responsible for the increase in alcohol consumption in alcoholism [105].

Effects of ethanol on gamma-aminobutyric acid (GABA)

GABA is a main inhibitory neurotransmitter of the CNS. Previous reports have suggested the effects of ethanol on GABAergic pre- and postsynaptic terminal. At the presynaptic terminal, ethanol increased GABA function and GABA release in several brain areas [106, 107]. Ethanol caused an increase in spontaneous GABA release in the hippocampus [108], hypoglossal nucleus [109], amygdala, both basolateral and central nuclei, [110] and ventral tegmental area [111]. At the postsynaptic terminal neurons, the electrophysiological method showed an increase in the activity of GABA-A receptor by acute ethanol exposure in cerebellum tissue slices of the Sprague-Dawley rats [112]. Ethanol exposure to hippocampal tissue slices showed a reducing in the excitability of the dentate gyrus via alpha 5 subunit of GABA-A receptor [113]. Furthermore, ethanol exhibited a decrease in the current of thalamocortical neurons indicating decrease in neuronal excitability and firing rate in response to depolarization of neurons [114]. However, chronic ethanol consumption exhibited a different expression of GABA-A subunits at a transcription and translation processes[115]. The GABA-A subunits in the cerebral cortex have shown to decrease in the mRNA and protein expression of the α1, α2, and α3 subunits [116, 117].

Effects of ethanol on glutamate

Glutamate is a main excitatory neurotransmitter in the brain that excites ventral tegmental dopaminergic neuron cells [118]. Ethanol can alter glutamate

release and glutamate transmission. Acute ethanol administration blocked glutamate receptors such as *N*-methyl-D-aspartate receptors (NMDA) and non-NMDA receptors, as well as decreased glutamate release [119] while chronic ethanol consumption increased glutamate release [120] and receptors [115]. Zhu W *et al*. (2007)[121] studied glutamate synaptic transmission in CeA neurons of the central amygdala by whole-cell voltage-clamp recording. The results showed an acute effect of ethanol on the inhibition of synaptic transmission while a chronic ethanol exposure increased synaptic transmission of glutamate.

Effects of ethanol on opioid systems

An endogenous opioid is naturally produced in the body. This small molecule has long been reported to involve in drug abuse and alcohol. Three classes of endogenous opioid including endorphins, enkephalins, and dynorphins were altered by the effect of ethanol [122]. Ethanol can alter the opioid system in several levels such as synthesis, release, degradation of opioid peptides, and binding to opioid receptors [123]. Intraperitoneal injection of ethanol to male Sprague–Dawley rats caused an increase of β-endorphin and dynorphin A1–8 in the amygdala [124]. The ethanol administration for three consecutive days per week during 4 to 9 weeks of age enhanced Met-enkephalin-Arg^{6}Phe^{7} in the substantia nigra and the ventral tegmental area of the rats [125].

Effects of ethanol on serotonin (5-HT)

The serotonin neuron locates in the raphe nucleus where it produces and releases serotonin [126]. The function of serotonin involves emotion and motivation regulation. Acute ethanol consumption increased in serotonin levels within the brain [127] and serotonin receptor type 3 [115]. Chronic ethanol consumption and alcohol withdrawal resulted in neuro-adaptation of the serotonin system by decreasing serotonin release [128].

From the above reviews, it has been shown that ethanol affects many neurotransmission. The present study concentrates only on the effect of ethanol on dopaminergic system in order to understand the preventive effect of *T. laurifolia* on ethanol intoxication. There are many reports from our lab on the effect of *T. laurifolia* on

dopaminergic neurotransmission in response to drug addiction. Therefore, it was interesting to find out how *T. laurifolia* affected the brain dopaminergic neurotransmission in ethanol intoxication. The dopaminergic neurotransmission will be reviewed as followed.

Dopamine

Dopamine (3-hydroxytryptamine) is a neurotransmitter and hormone. Dopamine is classified as catecholamine similar to norepinephrine and epinephrine (figure 8). It is an intermediate in the synthesis of norepinephrine and epinephrine (figure 9). Dopamine plays a critical role in both the central nervous system and the peripheral nervous system functions. Dopamine was first synthesized in 1910 and used as an intermediate in the synthesis of noradrenaline. Until Blaschko H. (1957) who was the first to show that dopamine could lower the pressure and this effect was inhibited by monoamine oxidase which led to the suggestion that dopamine may have some physiologically significance [(129)]. The nuclei of dopaminergic neurons located in the substantia nigra and ventral tegmental area are projected to striatum, nucleus accumbens, prefrontal cortex, and other limbic structures [(130)]. In dopamine biosynthesis, dopamine is produced in the cytosol of dopaminergic neurons and stored in synaptic vesicles until the neurons are stimulated and endogenous dopamine release occurred. Dopamine released from presynaptic dopaminergic neurons binds to dopamine receptors and exert effects on postsynaptic neurons. The remaining of dopamine is reuptaked and being recycled by dopaminergic neurons or degraded after uptake by glial cells.

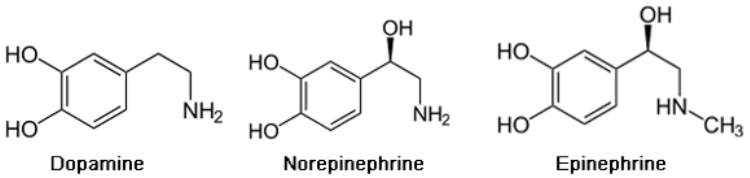

Figure 8 Chemical structures of catecholamines.

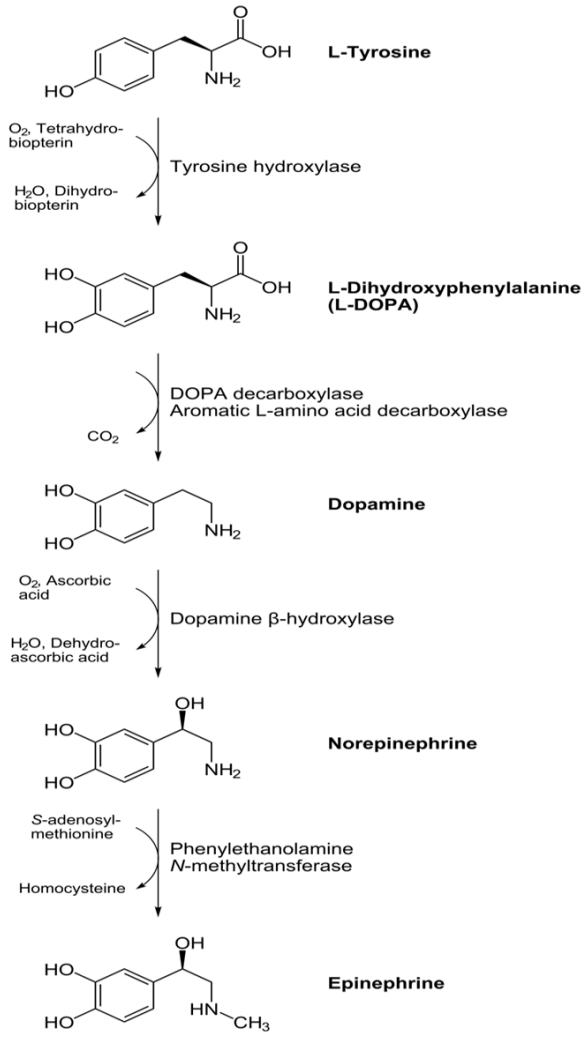

Figure 9 Catecholamine synthesis pathway.

Source: Sonne J *et al.* (2020) [131]

Dopamine biosynthesis

The biosynthesis of dopamine takes place in the cytosol of catecholamine neurons and dopaminergic neurons. Phenylalanine is transformed into tyrosine in the liver by phenylalanine hydroxylases and enters into dopaminergic neurons. The dopamine biosynthesis has two steps. The first step is a transformation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) with hydroxylation of tyrosine hydroxylase enzyme at the phenol ring of L-tyrosine. This step is accounted for a rate-limiting step enzyme and requires tetrahydrobiopterin (BH_4), which produced from guanosine triphosphate (GTP) by GTP cyclohydrolase (GTPCH) and molecular oxygen as co-factors. Final product, dopamine, is a molecule that regulates this step. Second step, L-DOPA is then transforming to dopamine by decarboxylation of L-aromatic amino acid decarboxylase. The decarboxylation of L-aromatic amino acid decarboxylase requires pyridoxal phosphate as a cofactor and this step is more rapid (figure 9). Dopamine is then entry into synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2) with secondary active transport [132, 133].

Dopamine receptors

The physiological action of the G-protein-coupled receptor can be divided dopamine receptors into two subdivisions including D_1 and D_2 like receptors. The classification of the dopamine receptor is based on the ability to modulate adenylyl cyclase activity and activate cyclic adenosine monophosphate (cyclic AMP)-dependent pathways, mainly protein kinase A (PKA) and other downstream signals.

D_1 like receptors include D_1 and D_5 receptors. The activation of adenylyl cyclase by D_1 like receptors via stimulating α subunit of G-protein ($G_{s/olf}$ proteins) enhances level of cyclic AMP and then induces phosphorylation of cellular proteins (figure 10). D_1 like receptor modulates Na^+, Ca^{2+}, and K^+ channels [134]. In the brain, the D_1 receptor mostly expresses in nigrostriatal, mesolimbic, and mesocortical areas such as the caudate-putamen (striatum), nucleus accumbens, substantia nigra, olfactory bulb, amygdala, and frontal cortex and less in the hippocampus, cerebellum, thalamic areas, and hypothalamic areas. D_5 receptor located in a lower level in pyramidal

neurons of the prefrontal cortex, premotor cortex, cingulate cortex, entorhinal cortex, substantia nigra, hypothalamus, hippocampus, and dentate gyrus.

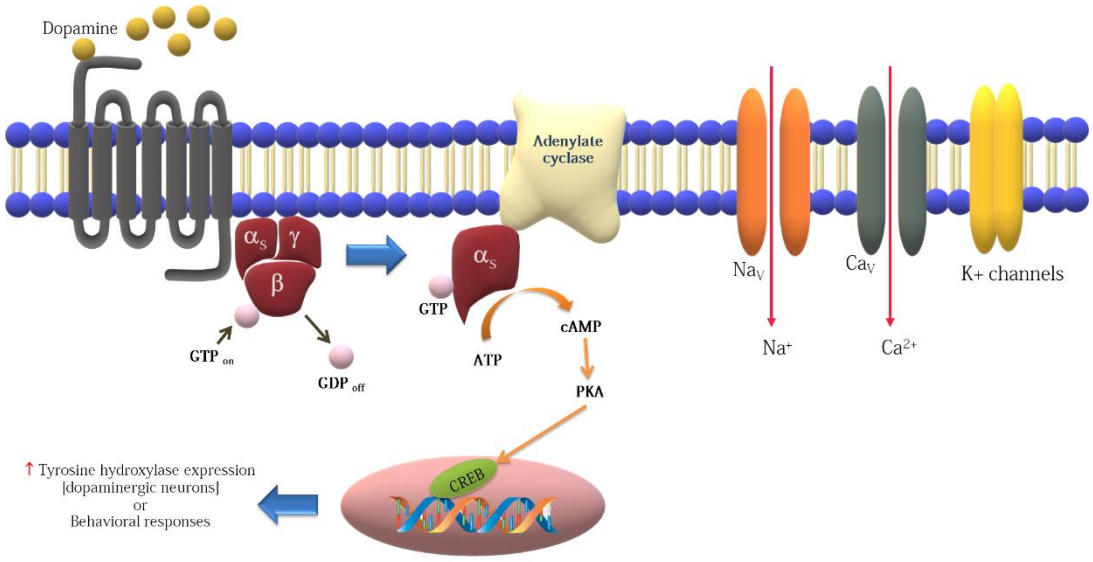

Figure 10 D_1-like receptor.

Source: Mishra A *et al*. (2018); Ledonne A *et al*. (2017)[135, 136]

D_2 like receptors includes D_2, D_3, and D_4 receptors. The activation of D_2 like receptor contrasts with D_1 like receptors. D_2 like receptors inhibit the induction of the α subunit of G-protein ($G_{i/o}$ proteins) resulting in an inactivation of adenylyl cyclase and cyclic AMP (figure 11). These inhibitions lead to the inhibition of PKA, as well as activation GIRK channel and closure Ca^{2+} channel [134]. The expression of D_2 and D_3 receptors are found in both presynaptic and postsynaptic neurons. The D_2 receptor in the presynaptic neuron of dopaminergic neurons acts as an autoreceptor and regulates extra-dopamine concentration. D_2 receptor located in the substantia nigra, ventral tegmental area, hypothalamus, cortical areas, septum, amygdala, and hippocampus. D_3 receptor distributed in limbic areas such as in the shell of the nucleus accumbens, olfactory tubercle, and the islands of Calleja. D_4 receptor expresses in the frontal cortex,

amygdala, hippocampus, hypothalamus, globus pallidus, substantia nigra pars reticulata, and thalamus [134, 137].

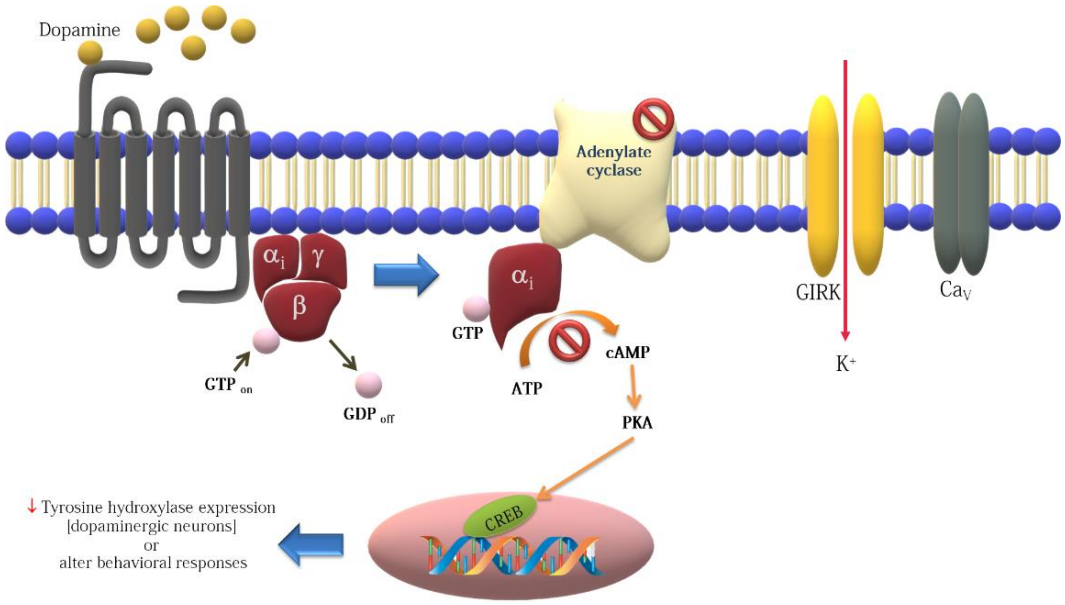

Figure 11 D_2-like receptor.

Source: Mishra A *et al.* (2018); Ledonne A *et al.* (2017) [135, 136]

Dopamine reuptake and metabolism

Upon excitation of dopaminergic neurons, dopamine is released from synaptic vesicle by exocytosis into the synaptic cleft. Dopamine binds to their receptors at postsynaptic neurons in order to exert effects. Some dopamine molecules bind to autoreceptor (D_2 like receptor) at presynaptic dopaminergic neurons which caused the inhibition of dopamine release. The extracellular dopamine is cycled into synaptic vesicles in dopaminergic neurons by the dopamine transporter (DAT) depending on Na^+ and Cl^- or uptaked into surrounding glial cells or astrocyte for degradation.

Dopamine reuptake by DAT is followed by recycling into the synaptic vesicle. The remaining is oxidized into hydrogen peroxide and the 3, 4

dihydroxyphenylacetaldehyde (DOPAL) by monoamine oxidase (MAO) in the outer mitochondrial membrane. The DOPAL is converted into 3, 4-Dihydroxyphenylacetic acid (DOPAC) by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH). The DOPAC is then metabolization into homovanillic acid (HVA) by cytosol enzyme, catechol-o-methyl transferase (COMT). On the other hand, dopamine uptake into surrounding glial cells or astrocyte is also metabolized into HVA by MAO and COMT in these cells [138, 139] (figure 12).

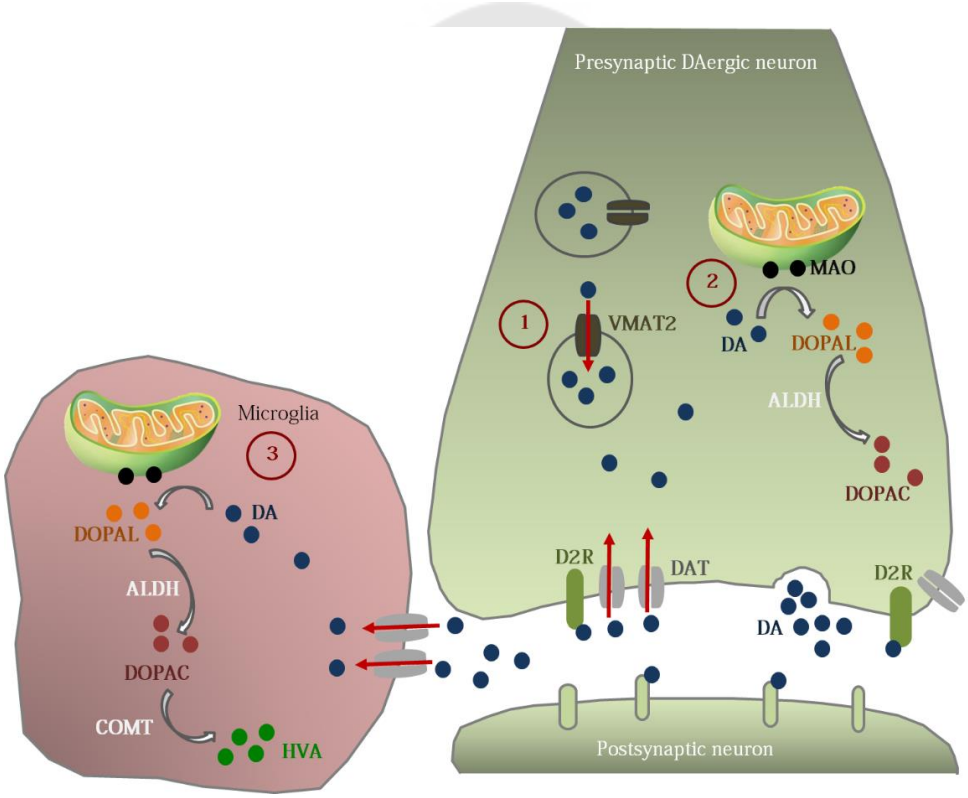

Figure 12 Dopamine metabolism.

Source: Meiser J *et al*. (2013)[132]

After dopamine reuptake by dopamine transporter (DAT), dopamine is cycled into synaptic vesicles in dopaminergic neurons (1). A few of dopamine is metabolized by MAO to DOPAC (2) in dopaminergic neurons. Besides, dopamine in the

synaptic cleft is also uptake into the surrounding cell such as microglia for metabolization into HVA by MAO and COMT enzymes (3).

Dopaminergic pathways and functions

Dopamine localization has both long and short projections that can be divided into four pathways of dopamine including three long projections and one short projection. The three long projections are called nigrostriatal, mesolimbic, and mesocortical pathways. The class of short projection is called the tuberoinfundibular pathway. Three long dopaminergic neurons projections have two original dopaminergic neurons areas including the substantia nigra and ventral tegmental area (VTA). The three long projections are called mesocortical, mesolimbic, and nigrostriatal pathways (figure 14).

The nigrostriatal dopaminergic pathway has the extending dopaminergic neurons of substantia nigra par compacta into the striatum. This pathway is important for the expression of basal ganglia mediated motor behavior and extrapyramidal system. Dopamine released from the nigrostriatal dopaminergic pathway modulates medium spiny neurons (MSNs) of the striatum and produces direct and indirect pathways of movement by binding D_1 and D_2 receptors, respectively [140]. In the direct pathway of movement, dopamine from substantia nigra binds to D_1 receptor in MSNs of striatum and produces activation of MSNs, GABAergic neurons, follows by release of GABA, an inhibitory neurotransmitter, into substantia nigra par reticular (SNr) and globus pillidus par interna (GPi). This inhibition in SNr and GPi leads to disinhibition of thalamic glutaminergic neurons that project to cortex resulting in movements and locomotor activation. Conversely, the binding of dopamine to D_2 receptor reduces movements and locomotor activation by indirectly stimulates SNr and GPi via the inhibition of globus pillidus par externa (GPe) and stimulates the subthalamic nucleus (STN) (figure 13) [140]. The dysfunction of this pathway causes motor coordination impairment and movement disorders such as parkinsonian syndrome, dystonia, and chorea [141].

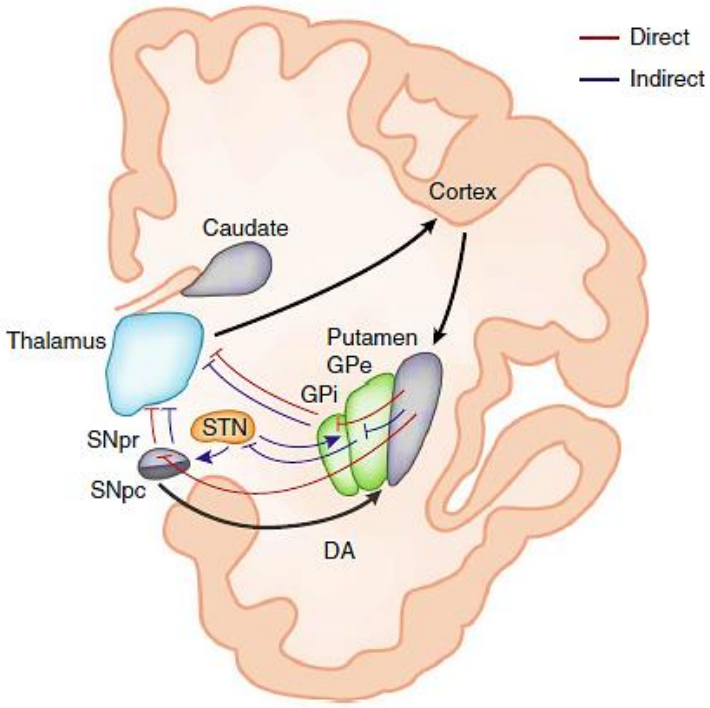

Figure 13 The direct and indirect pathways of movement.

Sourec: Calabresi P *et al*. (2014)[140]

The binding of dopamine from substantia nigra par compacta (SNpc) regulates movements and locomotor activation by direct (Red lines) and indirect pathways (Blue lines) via D_1 and D_2 receptors, respectively. The direct inhibition of substantia nigra par reticular (SNr) and globus pillidus par interna (GPi) activates movements and locomotion, while indirect stimulation of both brain areas inhibits movements and locomotion.

In the mesolimbic dopaminergic pathway, the axons from ventral tegmental area mainly project to nucleus accumbens and also project to amygdala, bed nucleus of stria terminal (BNST), hippocampus, and lateral septal area. This projection involves natural rewards such as food preference, sexual behavior, social interactions, and drug addiction. Alcohol addiction is suggested to cause dysfunction of the mesolimbic

dopaminergic pathway [142]. Furthermore, mesolimbic pathway and nucleus accumbens are suggested to specially involve in anxiety-like behavior [143]. Another projection of VTA dopaminergic neurons into the prefrontal cortex is called the mesocortical dopaminergic pathway. The mesocortical dopaminergic pathway involves a cognitive function and motivation. The dysfunction of the mesocortical dopaminergic pathway leads to abnormal functions in psychoses such as schizophrenia [144]. Furthermore, the projection of the VTA dopaminergic neurons into two main areas, nucleus accumbens, and prefrontal cortex, is called the mesocorticolimbic dopaminergic pathway which has been reported to correlate with the rewarding system and drug and ethanol addiction [145].

The short projection that projects dopamine from the arcuate nucleus of the hypothalamus into the pituitary gland and inhibits prolactin release from the pituitary gland. The prolactin regulates the last step of gametogenesis as final oocyte maturation, ovulation in females, and spermatogenesis in males.

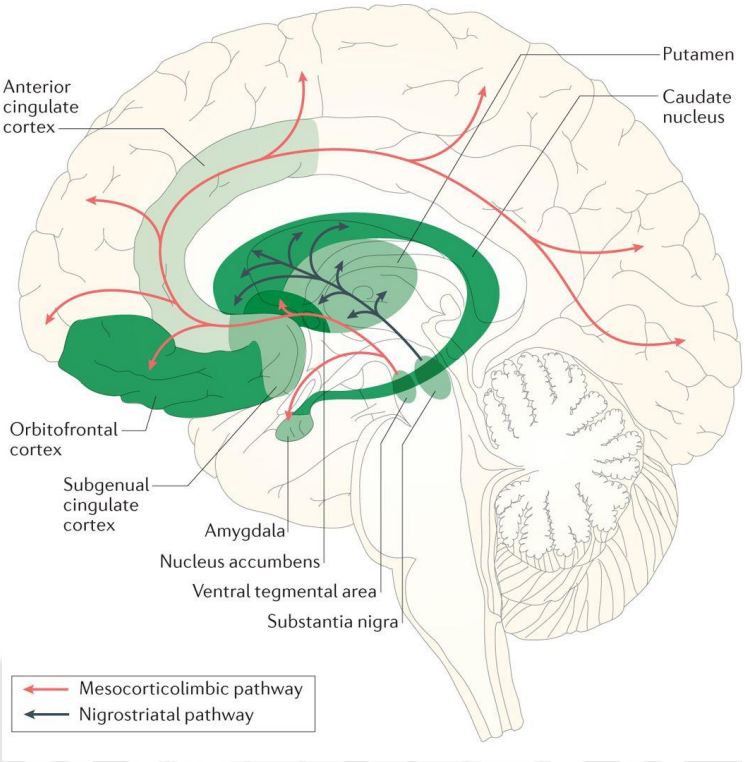

Figure 14 Three main dopaminergic pathways.

Source: Teicher MH *et al.* (2016)[146]

Dopamine neuron cell bodies are located primarily in substantia nigra par compacta and ventral tegmental area. The dopaminergic neuron projection of substantia nigra into the striatum is called the nigrostriatal dopamine pathway (black). Mesocorticolimbic pathway (red) consists of the projections of ventral tegmental area dopaminergic neurons into nucleus accumbens, as known as mesolimbic pathway and prefrontal cortex, as known as mesocortical pathway.

Brain areas correlated dopaminergic systems

Brian areas associated with dopaminergic pathways include substantia nigra, ventral tegmental area, striatum, nucleus accumbens, prefrontal cortex, and hypothalamus

Nigrostriatal pathway

Substantia nigra

The substantia nigra is a part of basal ganglia and located in the midbrain. The substantia nigra can be divided into two parts including par reticular (SNr) and par compacta (SNc). The SNc composes of dopaminergic neurons, as known as A9 group, whereas SNr consists of GABAergic neurons. The substantia nigra par compacta are compact more than par reticular [147]. The SNc dopaminergic neurons are called neuromelanin due to cutaneous melanin deposits in dopaminergic neurons [148]. Figure 15A shows tyrosine hydroxylase-immunoreactive neurons substantia nigra.

The SNc dopaminergic neurons mainly project dopamine onto striatum, as known as the nigrostriatal dopaminergic pathway and other areas such as the subthalamic nucleus (STN), and globus pallidus [149, 150]. The SNc dopaminergic neuron projection involves voluntary movement control.

Afferent input from various areas has been reported to either stimulate or inhibit the SNc dopaminergic neurons in order to release dopamine to the striatum. Generally, the GABAergic afferent input from various areas projects axons onto the SNc dopaminergic neurons in order to inhibit dopamine release. SNc dopaminergic neurons receive input from the striatum, globus pallidus and SNr GABAergic neurons, and local interneurons via binding to GABA-A receptor [151]. On the other hand, the SNc dopaminergic neurons receive strong excitatory input from glutamatergic neurons of the medial prefrontal cortex and STN via NMDA receptor. The strong excitation afferent input from glutamatergic neurons via the NMDA receptor induces the bursty firing pattern of the SNc dopaminergic neurons. Functionally, the bursty firing of the SNc dopaminergic neurons causes an increase of extracellular dopamine in the striatum [152]. Besides, SNc dopaminergic neurons also receive serotonergic projection from raphe

nuclei, acetylcholinergic input from pedunculopontine tegmentum, and local opioid input from peptidergic neurons [153].

Striatum

The dorsal striatum or neostriatum is located in the subcortical part of the forebrain. The striatum has two regions including the caudate nucleus and the putamen [154] The caudate nucleus is located in the medial dorsal striatum and putamen is located in the lateral dorsal striatum. The striatum consists of two classes of neuronal cell types including spiny projection neurons and aspiny interneurons. The spiny projection neurons, known as medium spiny neurons (MSNs), are GABAergic neurons and major neurons in the striatum. The MSNs can be subclassed into striatonigral and striatopallidal. The striatonigral MSNs project monosynapse directly to internal globus pallidus and the SNr. The striatopallidal MSNs indirectly connect into globus pallidus by polysynaptic neurons [155]. Aspiny interneurons consist of medium-size GABAergic neurons and large cholinergic cells.

The striatonigral MSNs involve the direct pathway of movement control that express high levels of D_1 receptor, opioid peptide, substance P, and muscarinic M_4 receptors. The striatopallidal MSNs involve in the indirect pathway of movement control and express D_2 receptor, opioid peptide, and adenosine A_{2A} receptors. The SNc dopaminergic neuron axons highly synapse to the dendrites and spine neck of the MSNs. Tyrosine hydroxylase staining showed positive immunolabelling within striatum (figure 15B)[156]. Under the light microscope, the striatum exhibits the patches-matrix organization. The patches form of the striatum, as called as striosome, consists of the MSNs that strongly express the μ-opioid receptor and substance P but less express the acetylcholine. The MSNs in this area receive input from the SNc dopaminergic neurons and involve the regulation of dopaminergic neurons of the SNc for movement control. The matrix form represents the MSNs that have strong connections of cholinergic neurons and an input from cortex and thalamus [157].

Mesolimbic and mesocortical dopaminergic pathways

Ventral tegmental area (VTA)

The VTA implicates in reward behaviors, cognitive functions, arousal, motivation, and memory. The VTA is derived from the mesencephalic floor plate and is located in the midbrain. The VTA consists of two regions including the anterior-lateral VTA (aVTA) and posterior-medial VTA (pVTA). The VTA neurons are loosely arranged and small. These neurons have an averages diameter between 10 to 53 μm. It mainly contains dopaminergic cell group A10 (~70%) and other neurons including GABAergic neurons (~30%) and glutamatergic neurons (~2-3%) [158]. Figure 15A shows tyrosine hydroxylase-immunoreactive neurons ventral tegmental area.

The output of VTA dopaminergic neurons innervates neurons in several areas such as nucleus accumbens, prefrontal cortex, septal area, amygdala, and lateral habenula. The projections of VTA dopamine in nucleus accumbens is called the mesolimbic dopaminergic pathway and in prefrontal cortex is called mesocortical dopaminergic pathway.

The mesolimbic and mesocortical dopaminergic pathways, known as the mesocorticolimbic system, regulate reward behaviors and cognitive functions. Furthermore, VTA GABAergic neurons and glutamatergic neurons also project their axon onto dopaminergic neurons in the VTA. However, VTA dopaminergic neurons are regulated the activation not only by input from various regions but also by other local VTA neuron types. The nucleus accumbens input axon onto VTA dopamine and GABAergic neurons in rats and mice [159]. A monosynaptic connection from medial prefrontal cortex projects neuron onto VTA dopaminergic neurons [160].

VTA dopaminergic neurons also regulate other VTA neurons locally through the expression of the D_2 receptor. Whereas, the VTA GABAergic neurons regulates the VTA dopaminergic neurons through the GABA-A receptor. The VTA glutamatergic neurons firing induces the activation of the VTA dopaminergic neurons which project axon onto nucleus accumbens [161].

Nucleus accumbens

The nucleus accumbens or ventral striatum is a subcortical brain structure that is located anterior to posterior border of anterior commissure. Functionally, this brain area involves psychological disorders such as addiction, obsessive-compulsive disorder, anxiety, and depression [162]. The nucleus accumbens can be divided into two sub-regions including a central core and a shell region. The central core of the nucleus accumbens is located surrounding in the middle region and the shell region is located in the ventral and lateral of the core region. Furthermore, the nucleus accumbens can be determined as an arrangement of patch-matrix compartment under microscope. 95% of the GABAergic medium spiny neurons in the nucleus accumbens neurons express with either D_1 or D_2 receptors. The remaining 5 % of the nucleus accumbens neuron composes of a heterogeneous population interneuron such as GABAergic interneuron and tonically activity of cholinergic neurons (TANs)[163].

Generally, the nucleus accumbens receives dopaminergic projection input via mesolimbic dopaminergic neurons of the ventral tegmental area which tyrosine hydroxylase staining shows positive immunolabelling within nucleus accumbens (figure 15B) [156] and glutamatergic projections from the prefrontal cortex, amygdala, thalamus, and hippocampus. The afferent input onto the patch and matrix compartment in both regions of nucleus accumbens receive an input from different regions. The patch compartment receives afferent input from prelimbic cortex and substantia nigra while the matrix receives afferent input from prefrontal, motor, and sensory cortex areas. Moreover, the patch has a high expression of μ-opioid receptor while the matrix exhibits a high connecting of cholinergic neuron axons and plexus of somatostatin fiber and less of μ-opioid receptor [164].

The output of GABAergic medium spiny neurons project to various region of mesencephalon and basal ganglia such as the substantia nigra-ventral tegmental area, the lateral hypothalamus, cingulum, thalamus, globus pallidus, subpallidal region, stria terminalis, preoptic region, nucleus parataenialis, nucleus mediodorsalis thalami, and lateral habenular nucleus [165].

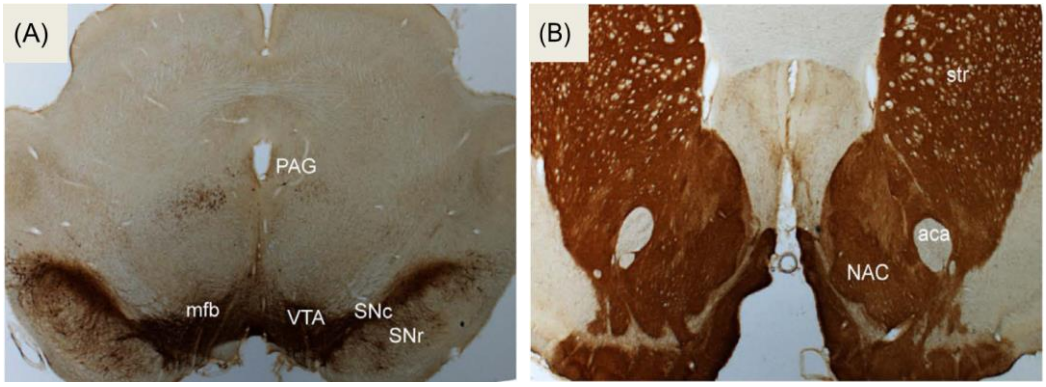

Figure 15 Tyrosine hydroxylase-immunoreactive in nigrostriatal and mesolimbic brain areas.

Source: Furlanetti LL *et al*. (2016) [156]

Tyrosine hydroxylase staining exhibits strong positive immunolabeling within dopaminergic neurons of substantia nigra (SNc and SNr) and ventral tegmental area (VTA) (A) and target brain areas of nigrostriatal and mesolimbic pathway including striatum (str) and nucleus accmbens (NAC) (B), respectively.

Prefrontal cortex

The prefrontal cortex is a cortex of frontal lobe. From the cytoarchitectonic map of Brodman, the prefrontal cortex is located in areas 8-13, 24, 32, 46, and 47 [166]. The prefrontal cortex can be divided into three regions including the orbital, medial, and lateral prefrontal cortex. The orbital and medial prefrontal cortexes involve emotional behavior. The lateral prefrontal cortex involves the cognitive that support the temporal organization of behavior, speech, and reasoning.

The neurons of the prefrontal cortex connect to several areas such as brainstem, thalamus, the limbic system, and basal ganglia. The prefrontal cortex neurons project axon and receive input from the subcortical brain areas neurons. The afferent input from the ventral tegmental area and the limbic system to the prefrontal cortex correlate with the internal environment, level of arousal, drives, and motives of

animals. Besides, the prefrontal cortex has a connection with the other areas of the cortex [167].

Ethanol effect on dopamine (DA)

Many neurotransmitter systems involve the behavioral effect of ethanol. The dopaminergic system is the main neurotransmitter system of acute ethanol stimulation [168]. The ethanol induces dopaminergic neuron firing, dopamine release, and leads to behavior changes in an animal including motor coordination, alcohol addiction, anxiety behavior, and exploratory behaviors.

Nigrostriatal pathway

The nigrostriatal pathway is a projection of dopaminergic neurons from substantia nigra par compacta to the striatum and responsible for the regulation of movement. Many studies have reported that ethanol administration exerted the effect on the SNc and striatal dopaminergic neurons and led to motor coordination impairment.

The acute ethanol administration on the substantia nigra was demonstrated by Mereu G *et al*. (1984). The observation shows a difference of the SNc dopaminergic neuron firing to different doses of ethanol administration. The intravenous injection of 0.5 to 2.0 g/kg ethanol to male Sprague-Dawley CD rats increased the frequency and duration of spontaneous of dopaminergic neurons in the SNc. From the observation, the administration of 1 or 2 g/kg ethanol excited the overall neurons of the SNc dopaminergic neurons. In opposite, a high dose of ethanol (4 g/kg) inhibited the dopaminergic neurons firing. The study using anesthetic drugs including chloral hydrate and halothane found that the anesthetic drugs stimulated SNc dopaminergic neurons firing and these results were inhibited when combined anesthetics drugs with ethanol administration. 4 g/kg ethanol inhibited the SNc dopaminergic neurons firing in anesthetized rats without producing the initial stimulation of firing as found in unanesthetized rats [47]. Ethanol has been reported to stimulate extracellular dopamine release in caudate nucleus of striatum in freely moving rats [169]. From the study of chronic administration of ethanol, the administration of ethanol at doses of 2 or 4 g/kg for 30 consecutive days to male Wistar rats found that dopamine level of the striatum

decreased after the last administration and continuously decreased until 48 hours after last administrations [170].

The stimulation of ethanol on the SNc dopaminergic neurons and the striatum resulted in psychomotor disabilities. Low to moderate doses of ethanol caused motor stimulation while a high dose of ethanol showed an ataxic effect. For instance, Ornelas LC *et al*. (2015) [49] studied the correlation between doses of acute ethanol administration and ages of male Sprague-Dawley rats. The study used adolescent and adult male Sprague-Dawley rats for determining ethanol-induced motor coordination impairment using the rotarod apparatus. The rats were injected intraperitoneally with 1 or 2 g/kg ethanol and validated time in the apparatus. The observation found that ethanol-induced motor coordination impairment in ages and doses dependent manners. The rat received ethanol exhibited an impairment within 10 minutes after injections when compared with saline injection. Similarly, White AM *et al*. (2002) studied the motor impairment in adolescent and adult rats using the tilting plane test. The intraperitoneal injection of ethanol at the doses of 1, 2, and 3 g/kg to the rats was followed by motor coordination impairment test at 15, 30, 60, 120, and 180 minutes after ethanol injection. The study presented the motor coordination impairment in both ages at 2 and 3 g/kg of ethanol injections. The impairment on the tilting plane test was observed at every time point [34]. Moreover, the motor coordination impairment was found in alcohol hangover rats that received 3.8 g/kg of ethanol [50, 171]. The study of chronic ethanol administration for 55 days from adolescence to adulthood rats exhibited that the rats receiving daily administration of 6.5 g/kg showed the impairments of motor coordination and muscle strength [172].

Mesocorticolimbic system

Robinson and Berridge. (2008) [173] reported that the mesocorticolimbic system correlated with the ethanol sensation. The dopaminergic neurons project dopamine from the ventral tegmental area to the nucleus accumbens (NAc), known as mesolimbic dopaminergic pathway, and prefrontal cortex, known as mesocortical

dopaminergic pathway. These two projections involve with an alcohol addiction in chronic ethanol administration.

In vitro and *in vivo* studies with the electrophysiological method demonstrated that acute ethanol administration increased the firing rate of dopaminergic neurons in the ventral tegmental area. *In vitro* study, ethanol at 20 to 320 mM concentrations produced 89% excitation of dopaminergic neurons of the ventral tegmental area using tissue slides in involved in concentrations of ethanol used [52]. Intravenous injection of ethanol at the doses of 0.125 to 0.5 g/kg to unanesthetized rats stimulated 30-80% dopaminergic neurons firing of the ventral tegmental area [51]. Increase of dopaminergic neuron firing of the ventral tegmental area correlated with the dopamine release and tyrosine hydroxylase of the ventral tegmental area and nucleus accumbens [122, 174, 175]. The microdialysis study showed that ethanol administration produced dopamine release in nucleus accumbens [54]. Besides, an intravenous injection of 1 g/kg ethanol to male Long-Evans rats increased dopamine levels in the medial prefrontal cortex [55]. Ethanol administration to the alcohol preferring rats showed an increase in dopamine release more than that found in non- alcohol preferring rats [176].

The study of the effect of chronic ethanol administration on the firing rate of the ventral tegmental area dopaminergic neurons was performed using the ventral tegmental area tissue slices from the C57BL/6J mice treated with the twice a day injection of 3.5 g/kg ethanol for 21 consecutive days. The result showed an increase in dopaminergic neurons firing in the ventral tegmental area tissue slices [53]. Chronic alcohol administration leads to alcohol addiction. Alcohol addiction refers to the loss in control of consumption which normally follows by high alcohol consumption, alcohol-seeking behavior, and unable to drop its use [177]. A common behavioral test in animals for drug addiction behavior is a conditional place preference (CPP) test. Ciccocioppo R *et al.* (1999) reported that chronic administration of 1.5 g/kg ethanol caused an enhancement of the time spent in a non-preference compartment of CPP when compared with the time spent in the non-preference compartment before chronic ethanol administration [178] During chronic ethanol administration, the dopaminergic

system is suggested to be modulated in response to ethanol stimulation. The studies showed the modulation of dopaminergic neurons in response to chronic ethanol stimulation by decreased sensitivities of reinforcing in nucleus accumbens [179] and increased affinity of the D_2 receptor in nucleus accumbens and the prefrontal cortex in Fisher 344 rats [180]. These adaptations produced an inhibition of dopamine release of the nucleus accumbens and the ventral tegmental area. Thus, these adaptation produced a decrease in dopamine synthesis and dopamine release in the mesolimbic system. This is the reason why alcoholism has to enhance drinking in order to stimulate dopamine release up to the started level.

In anxiety behavior test, Cruz J *et al*. (2012) studied an anxiety and anxiolytic stage in male Swiss mice treated with acute and chronic ethanol administration. It was found that acute 1 and 1.2 g/kg ethanol produced the anxiolytic effect. Chronic administration of ethanol at the doses of 0.1, 0.2, 0.4, 2, or 4 g/kg for 14 consecutive days found to have anxiolytic effect doses of 0.2 and 0.4 g/kg but not found in high doses [181]. Ethanol administration to adolescent rats treated with acute stress using plastic flat-bottom restrainers found that ethanol administration reduced anxiety induced by acute stress [182]. On the other hand, an anxiety behavior was found during withdrawal [183] that occurred after stop drinking alcohol [184]. Stefka Valcheva-Kuzmanova S *et al*. (2013) demonstrated that the rats receiving chronic ethanol at the dose of 8 g/kg for 14 consecutive days and stopped ethanol administration for 16 hours exhibited an anxiety behavior tested on the elevated plus-maze test [185].

The response of exploratory behavior to ethanol administration showed dose-dependence effects. Exploratory behavior test commonly uses to test for locomotion, rearing, and head-dipping. The study of systemic ethanol administration at a series dose of 0.12 to 1.5 g/kg in alcohol-preferring and non-preferring rats showed that spontaneous motor activity was found to increase in alcohol-preferring rats [186]. Besides, the adolescence and adult rats receiving 2.5 g/kg of ethanol exhibited an increase in locomotor activity [187]. A high dose of ethanol administration exhibited a suppression of locomotor activity [188].

Tubuloinfundibular pathway

The tuberoinfundibular pathway is a dopamine projection from hypothalamus to the pituitary gland. The projection regulates prolactin release via an inhibitory effect of dopamine. Ethanol administration has been reported to stimulate dopamine release, therefore, ethanol should inhibit prolactin release. However, several studies also showed an opposing effect that the action of dopamine to inhibit prolactin release was reversed to stimulation of prolactin release by ethanol administration [(189)]. For instance, Fernández-Mateos P *et al*. (2012) reported that male Wistar rat receiving ethanol for 35 consecutive days exhibited an increase of prolactin [(62)]. Alfonso M *et al*. (1991) study showed that an inhibition effect of dopamine to regulate prolactin release was abolished by ethanol administration [(190)].

The dopaminergic transmission was determined by the levels of tyrosine hydroxylase and/or dopamine in the areas of dopaminergic neurons and/or projection targets. The tyrosine hydroxylase enzyme is a rate-limiting enzyme of catecholamine such as dopamine biosynthesis. The western blot is a basic protein determination. The dopaminergic transmission by determining tyrosine hydroxylase levels has long been used in animals. Tran S *et al*. (2017) studied the acute ethanol effect on the zebrafish dopaminergic system by focusing on tyrosine hydroxylase. Using western blot, they used the ratio of tyrosine hydroxylase and β-actin in the whole brain for explaining the ethanol effect on the dopaminergic system [(191)]. Furthermore, the ratio of tyrosine hydroxylase and β-tubulin levels in western blot analysis was used to determine the modulation of nicotinic acetylcholine receptors to the dopamine system in the nucleus accumbens and striatum of C57BL/6 mice [(192)]. In another drug abuse, the western blot and immunohistochemistry were used to determine the melanin effect on chronic methamphetamine-induced reduction of tyrosine hydroxylase in rats. The western blot analysis showed that melatonin attenuated methamphetamine effect using the ratio of tyrosine hydroxylase of the mesolimbic, mesocortical, and nigrostriatal dopamine pathway. The immunohistochemistry method was used to observe the tyrosine

hydroxylase expression and morphological dopaminergic neurons for confirming the western blot's result [193]. Thiele TE *et al*. (2000) used the tyrosine hydroxylase-immunoreactivity for examining the neuroanatomical location of cells and identifying the neurochemical identity of rat neurons that are activated after ethanol administration [194].

The abnormal behaviors in response to ethanol or drug abuse intoxication in animals can be verified using various behavioral tests. The motor coordination was assessed in mice and rats by using balance beam. The balance beam test was useful for detecting the deficits in motor skill, [195] motor coordination, and balance [196]. In 1998, Metz and colleagues reported that a lesion of the pyramidal tract in rodents caused an increase in the number of hindlimb hanging down from the balance beam indicating the sign of motor impairment [197]. da Silva FBR *et al*. (2018) used the time taken to traverse a balance beam and the number of slips as indicators for analysis motor coordination, ataxia and, dystonic movements in adolescent female Wistar rats treated with chronic ethanol administration [198]. The elevated plus-maze [199, 200] has been used to identify potential anxiolytic drug based on the observation that rats normally spend more time in the closed rather than open arms due to an aversion to open spaces and height [201, 202] but administration of anxiolytic drug such as diazepam increase time in the open arms [203, 204]. The time in each arm and the number of crossed in the open arms of the elevated plus-maze were used to examine an anxiety stage of male Wistar rats treated with acute ethanol intraperitoneal injection [205]. The hole board is used to determine the exploration in rats based on the observations that the curiosity and fear regulate behavior for an approach to a new environment. The repeated head-dip into the holes [206] and the rearing, temporarily stands on its hindlimbs and lifts both forelimbs, were used as indicators for investigating the exploratory behavior [207]. The exploratory behavior was used to determine anxiety stages by total number of head-dips of adolescent C57BL/6 mice treated with nicotine on the hole board apparatus [208]. Conditioned place preference (CPP) test is a standard behavior model that is used to determine the reward system. The different environments of CPP with drug treatment represent the basic determination in addiction behavior. The CPP consists of three

phases including pre-test, condition and test pahases. Pre-test phase refers to preference and non-preference compartment of each rat. The compartment where the rat spend longer time in is determined as its preference compartment. On the other hand, the compartment where the rat spend less time in is determined as a non-preference compartment. Condition phase, the rat is forced to stay in the non-preference compartment after given the addicted drug, tested drug or combination of drugs. This process is repeated for a certain period of time. On the testing phase, each rat is placed in the middle of the CPP again without given any drugs and allowed to move freely in the CPP without guillotine door. The comparison of time spent in the non-preference compartment between pre and post-drug administration is used to determine the possible effect of drug addiction [209]. Ethanol and drugs induced addiction can be assessed directly in laboratory animals by using the conditioned place preference test [210]. The time that animals spend more in a non-preference compartment before and after pairing with ethanol or drugs is used to determine the addiction behavior. If the time in the non-preference compartment after pairing is more than the time before pairing with ethanol or drugs, it means that ethanol or drugs induces addiction [211, 212].

CHAPTER III
MATERIALS AND METHODS

Experimental designs

Acute effect of *T. laurifolia* and ethanol administration in rats

Male Wistar rats were randomly divided into four groups including control, ethanol, *T. laurifolia*, and *T. laurifolia* plus ethanol groups. Each group was given the different combination of treatments. *T. laurifolia* was given by oral administration and ethanol was given by intraperitoneal injection (i.p) once at 8 a.m. on the experiment date.

Control group was given 0.3 ml of 0.9% normal saline via oral administration and after 1 hour, they were given 0.3 ml of 0.9% normal saline via intraperitoneal injection.

Ethanol group was given 0.3 ml of 0.9% normal saline via oral administration and after 1 hour, they were given 2 g/kg of 20% ethanol via intraperitoneal injection.

T. laurifolia group was given 200 mg/kg of *T. laurifolia* methanol extraction via oral administration and after 1 hour, they were given 0.3 ml of 0.9% normal saline via intraperitoneal injection.

T. laurifolia plus ethanol group was given 200 mg/kg of *T. laurifolia* methanol extraction via oral administration and after 1 hour, they were given 2 g/kg in 20% ethanol via intraperitoneal injection.

At 30 minutes after injection of different treatments, rats were tested for various behaviors such as motor coordination, anxiety, and exploratory behaviors. In tissue analysis, each group of rats were divided into 2 groups. The first group was used to determine the levels of tyrosine hydroxylase of striatum and nucleus accumbens using western blot. The second group was used to confirm the expression and determine an intensity of tyrosine hydroxylase of ventral tegmental area and substantia nigra. In the first group, striatum and nucleus accumbens were snap frozen and kept in -

80°C before evaluations. The second group was perfused with normal saline, 4% paraformaldehyde, and collected the whole brain in cryopreservation solution in 4°C before immunohistochemistry processes.

The purposes of each test are classified as follows:

1. To examine the preventive effect of *T. laurifolia* on motor coordination in acute ethanol intoxication using balance beam model

2. To examine the preventive effect of *T. laurifolia* on anxiety in acute ethanol intoxication using elevated plus-maze model

3. To examine the preventive effect of *T. laurifolia* on exploratory behavior in acute ethanol intoxication using hole board model

4. To examine the preventive effect of *T. laurifolia* on the expression levels of tyrosine hydroxylase in striatum and nucleus accumbens in acute ethanol intoxication using western blot analysis

5. To examine the preventive effect of *T. laurifolia* on the expression and localization of tyrosine hydroxylase in substantia nigra and ventral tegmental area in acute ethanol intoxication using immunohistochemistry method

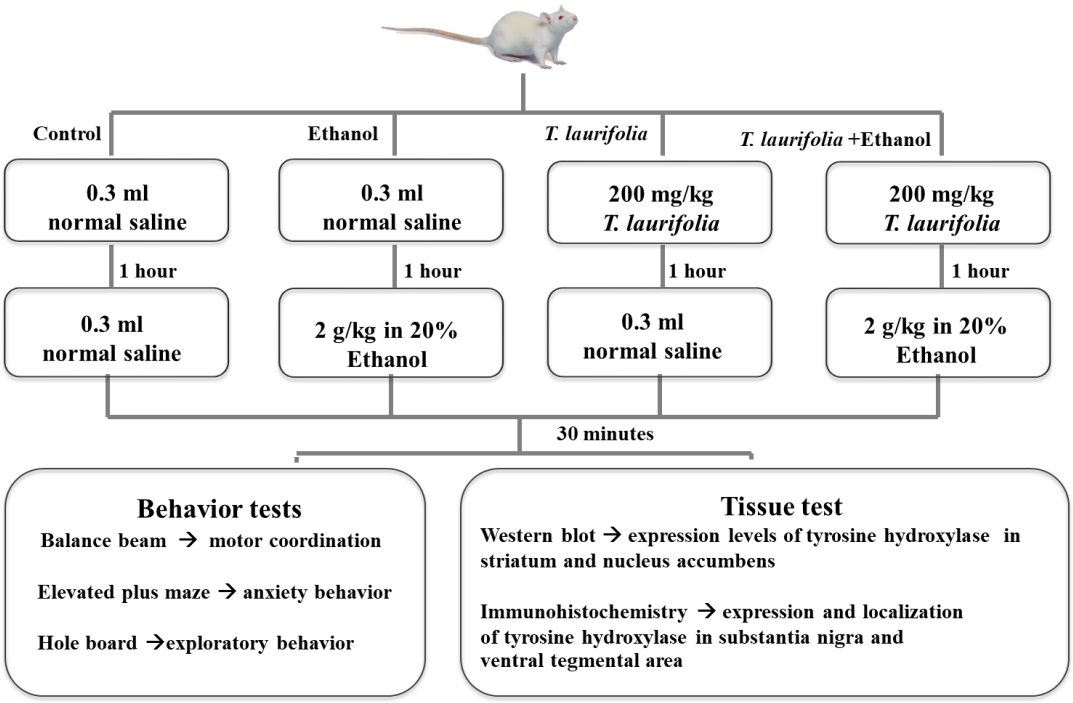

Figure 16 The acute study of *T. laurifolia* and ethanol effect on behaviors and tyrosine hydroxylase expression of nigrostriatal and mesolimbic dopamine pathways in rats.

Chronic effect of *T. laurifolia* and ethanol administration in rats

Male Wistar rats were randomly divided into four groups which are control, ethanol, *T. laurifolia*, and *T. laurifolia* plus ethanol groups. Each group received a different combination of treatments by oral administration following intraperitoneal injection (i.p), once at 8 a.m.

Control group received 0.3 ml 0.9% normal saline via oral administration and after 1 hour, they received 0.3 ml 0.9% normal saline via intraperitoneal injection.

Ethanol group received 0.3 ml 0.9% normal saline via oral administration and after 1 hour, they received 1 g/kg in 15% ethanol via intraperitoneal injection.

T. laurifolia group received 200 mg/kg of *T. laurifolia* methanol extraction via orally administration and after 1 hour, they received 0.3 ml 0.9% normal saline via intraperitoneal injection.

T. laurifolia plus ethanol group received 200 mg/kg of *T. laurifolia* methanol extraction via oral administration and after 1 hour, they received 1 g/kg in 15% ethanol via intraperitoneal injection

After 30 consecutive days of the different combination treatments, rats were examined to see the change in behaviors including ethanol addiction, motor coordination, anxiety, and exploratory behavior. In tissue analysis, each group of rats was divided into 2 groups. The first group was used to determine the levels of tyrosine hydroxylase of striatum and nucleus accumbens using western blot. The second group was used to confirm the expression and determine an intensity of tyrosine hydroxylase of ventral tegmental area and substantia nigra. In the first group, striatum and nucleus accumbens were snap frozen and kept in -80°C before evaluations. The second group was perfused with normal saline, 4% paraformaldehyde, and collected the whole brain in cryopreservation solution in 4°C before immunohistochemistry processes.

The purposes of each test are classified as follows:

1. To examine the protective effect of *T. laurifolia* on ethanol addiction in chronic ethanol intoxication using condition place preference test

2. To examine the protective effect of *T. laurifolia* on motor coordination in chronic ethanol intoxication using the balance beam

3. To examine the protective effect of *T. laurifolia* on anxiety in chronic ethanol intoxication using elevated plus-maze

4. To examine the protective effect of *T. laurifolia* on exploratory behavior in chronic ethanol intoxication using hole board

5. To examine the protective effect of *T. laurifolia* on the expression levels of tyrosine hydroxylase in the striatum and nucleus accumbens in chronic ethanol intoxication using western blot analysis

6. To examine the protective effect of *T. laurifolia* on the expression and localization of tyrosine hydroxylase in the substantia nigra and ventral tegmental area in chronic ethanol intoxication using immunohistochemistry method.

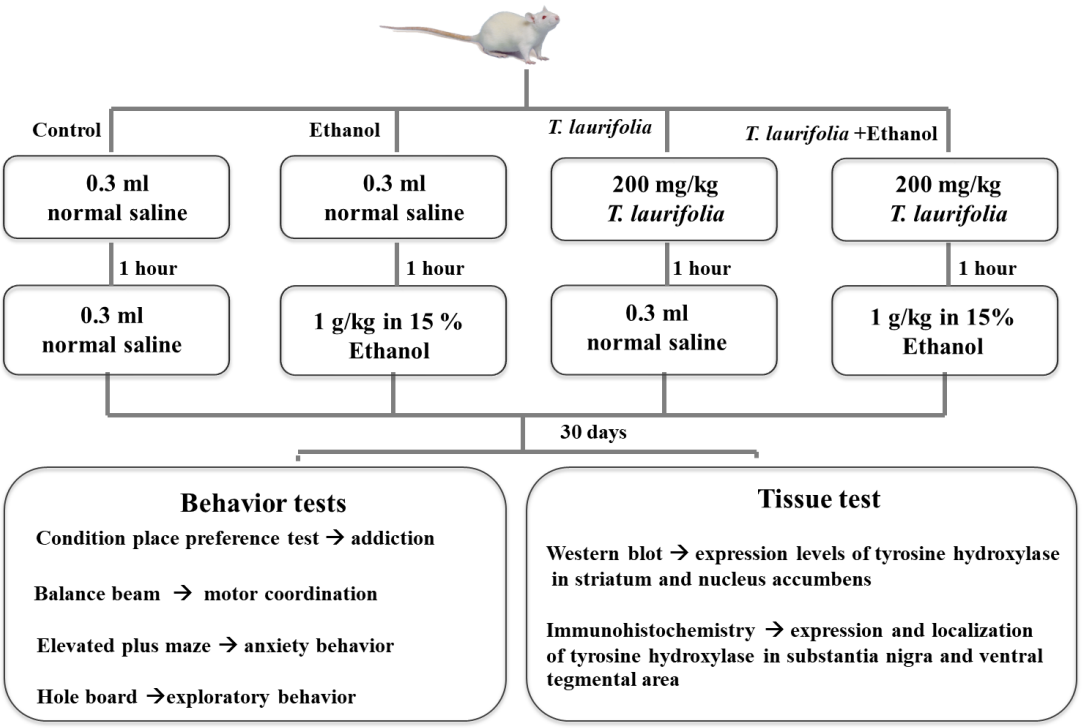

Figure 17 The chronic study of *T. laurifolia* and ethanol effect on behaviors and tyrosine hydroxylase expression of nigrostriatal and mesolimbic dopamine pathways in rats.

Chemical and antibody

Chemical

Sodium dodecyl sulfate (SDS), Ponceau-S red, and 3,3'-diaminobenzidine-tetrahydrochoride hydrate (DAB) were purchased from Sigma-Aldrich (St. Louis, MO). Protein assay kit was purchased from bio-Rad (Hercules, CA). The ECL Plus ™ Hyper film ECL was purchased from Amersham Biosciences (Piscataway, NJ).

Antibody

Mouse monoclonal antibody against tyrosine hydroxylase and β-actin were purchased from Millipore (Billerica, MA). Horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG antibody was purchased from Cell Signaling Technology (MA, USA). The biotinylated rabbit anti-mouse IgG was purchased from DAKO (Glostrup, Denmark). The avidin-biotin horseradish peroxidase (HRP) complex from Vector (Burlingame, CA) was purchased from Sigma.

T. laurifolia extraction and identification

Fresh *T. laurifolia* leaves were obtained from Tanusorn garden, Surin province and collected in September to December 2015. The leaves were washed before putting to dry on the controller oven at 60°C for 6 hours. Dried leaves were blended into powder and leave to soak in 80% methanol solution in a ratio of 1 g. of *T. laurifolia* powder to 20 ml of methanol-water overnight. *T. laurifolia* liquid was filtered with filter paper (Whatman No. 4) before removing out methanol solution by rotary evaporator on 337 mbar under 40°C. Sequentially, *T. laurifolia* extract was lyophilized into the powder with freeze dryer and kept at -20°C. In the identification of *T. laurifolia* pattern with the thin-layer chromatography (TLC) method, the *T. laurifolia* extract was dissolute in the solution containing 90% methanol/water and separated in a mobile phase of 10% methanol/ethyl acetate containing. After running the extract in chromatography, the visible light, UV light, and spay with anisaldehyde sulfuric acid were used to identify *T. laurifolia* patterns.

Experimental animals

Male Wistar rats weighing 300-350 g. were used to determine the acute effects of *T. laurifolia* and ethanol administration. The rats weighing 200-250 g were used to determine the chronic effect of *T. laurifolia* and ethanol administration. They were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Rats were housed in groups of four in a plexiglass-wall cage and given food and water *ad libitum* in a controlled temperature and humidity environment. The rats were maintained in a 12-h light/dark cycle.

Methods

Behavior studies

Balance beam apparatus

The acute and chronic effect of *T. laurifolia* and ethanol administration on motor coordination impairment was determined using balance beam. The balance beam has a square stainless steel beam of 180 cm in length that connected with a black box. The beam was elevated 50 cm above the floor (figure 18).

The motor coordination test consists of training and tested dates. On the training date, rats were trained to cross the balance beam at the distance of 30, 50, 80, and 100 centimeters from the start point toward the other end where the black box was located. The training was performed for 3 consecutive times in 3 consecutive days. On a tested date, each rat received four different combination treatments and placed on a start point. They were allowed to walk on balance beam at the distances of 30, 50, 80, and 100 centimeters for 3 times. The numbers of hindlimb slips in each rat were counted and compared between each group and used to determine the motor coordination impairment. A higher number of hindlimb slips was used to indicate the motor coordination impairment in rats.

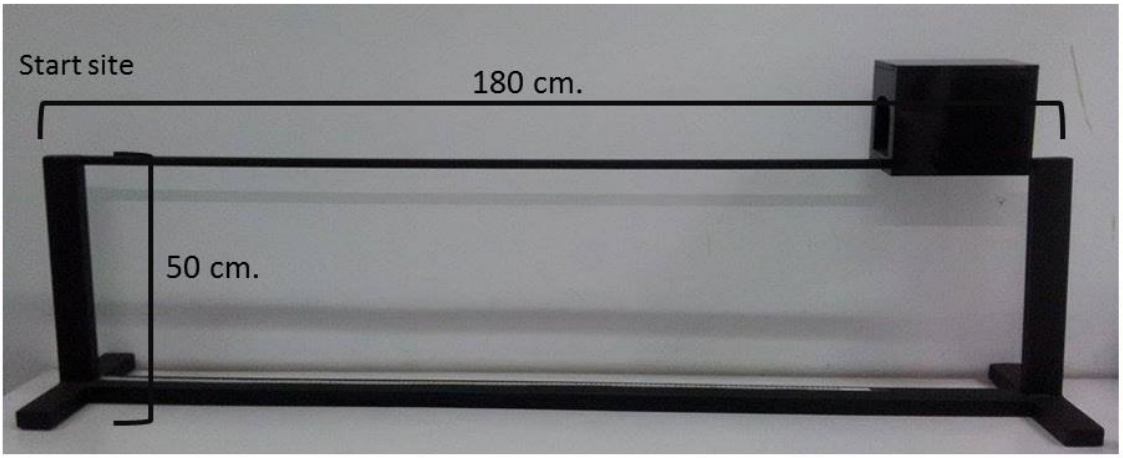

Figure 18 Balance beam.

Elevated plus-maze

The acute and chronic effect of *T. laurifolia* and ethanol administration on anxiety behavior was determined using elevated plus-maze. Elevated plus-maze consists of two open and closed arms that elevated above the ground. The elevation of open arms of the platform induces anxiety and fear in animals. The elevated plus-maze has two open and two closed arms. Each arm has 45 centimeters in length and 15 centimeters in width and has a wall high of 10 centimeters in the close arm. The platform of the apparatus is elevated 67 centimeters above the floor (figure 19).

In our experiment, male Wistar rats were used to determine the protection of *T. laurifolia* on ethanol-induced anxiety stage. After finishing the different combination treatments, each rat was placed on the center of the elevated plus-maze and allowed to freely travel in the platform for 5 minutes. The traveling on elevated plus maze was recorded on video and later analyzed the time entries, time in open arms, and time in end of open arms by using Microsoft visual studio 2010. The apparatus was cleaned with tap water and then 70% ethanol after the end of each experiment to avoid a bias based on olfactory cues.

The time entries, time in open arms, and time in end of open arms were used as indicators for anxiety determination. The longer time spent on each indicator on the open arm of the elevated plus-maze than that of the control value indicating that the rats present an anxiolytic effect. On the other hand, if the rat spent less time in these parameters than that of the control value, indicating that the rats present anxiety behavior i.e., more anxious.

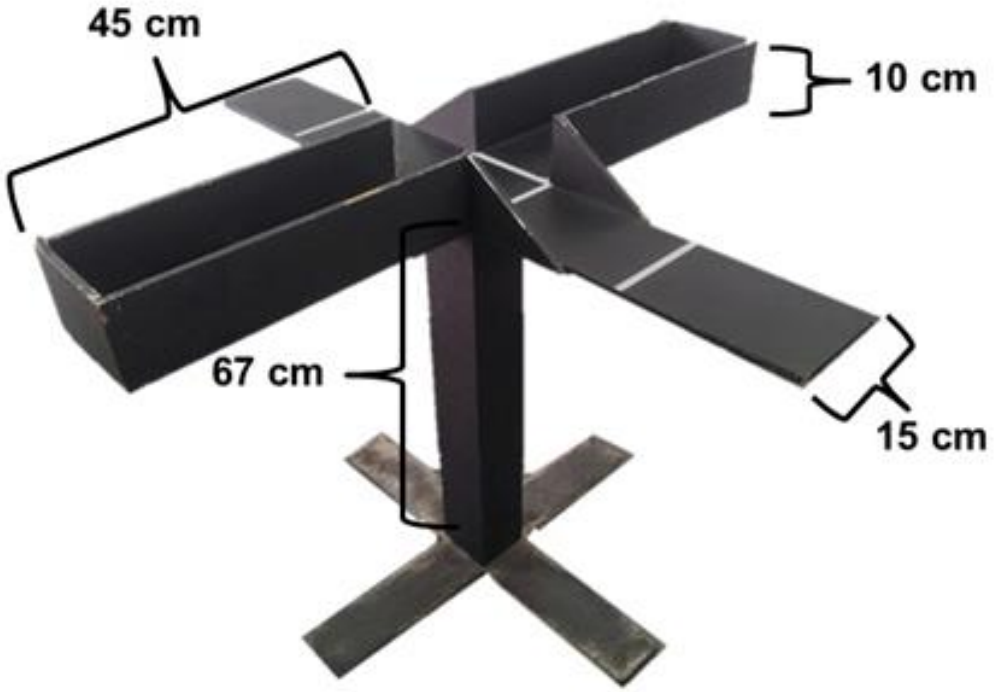

Figure 19 Elevated plus-maze.

Hole board

The acute and chronic effect of *T. laurifolia* and ethanol administration on exploratory behavior was determined using hole board. The hole board composes of 55 cm in width and 65 cm in length and surrounded by 45 cm walls height and four pores on the floor (figure 20).

After finished four combination treatments, the rats were placed on the center of the apparatus and let them search on the apparatus for 10 minutes. The number of rearing and head dipping under the hole was recorded by the video recorder. The apparatus was cleaned with tap water and then 70% ethanol after the end of the experiment of each rat to avoid a bias based on olfactory cues.

The number of rearing and head dipping were used to validate the exploratory behavior. The higher number of rearing and head dipping represent a high exploratory behavior in rats.

Figure 20 Hole board.

Conditioned place preference (CPP) test

The chronic effect of *T. laurifolia* and ethanol administration on addiction behavior was determined using the CPP The CPP has two compartments (30x30x40 cm.) with different designs of horizontal black and white thickness in the wall, thick (2.5 cm) or thin (1.0 cm), and each compartment is separated by guillotine door (figure 22). The CPP test for addiction behavior consists of 3 phases including pre-test, condition, and testing phases (figure 21).

During the pre-test phase, each rat was placed in the middle of the CPP apparatus without the guillotine door down i.e., allow to move freely in the model for 15 minutes and the time in each compartment was recorded on video for behavior analysis. The compartment where the rat spent longer time in was determined as its preference compartment. On the other hand, the compartment where the rat spent less time in was determined as a non-preference compartment. These were recorded for each individual rat for further study.

During the condition phase, each rat was given four different combination treatments and followed by placing in the non-preference compartment of CPP with the guillotine door down for 10 minutes. This process was repeated daily for 30 consecutive days.

On the testing phase, each rat was placed in the middle of the CPP again without given any treatments and allowed to move freely in the CPP without guillotine door 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 time spent in the non-preference compartment at post-drug administration more than pre-drug administration indicates that drug-induced addiction.

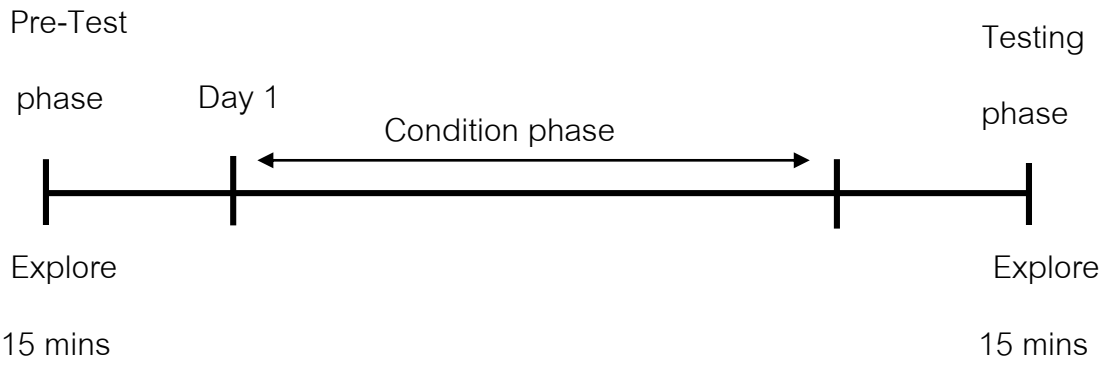

Figure 21 Diagram of conditioned place preference test.

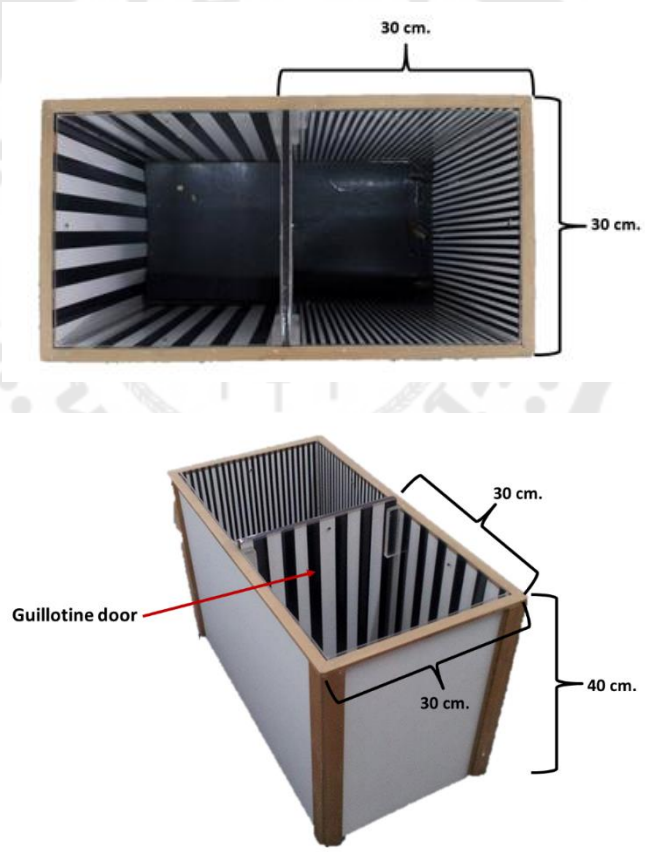

Figure 22 Conditional place preference apparatus.

Microsoft visual studio 2010

Microsoft visual studio 2010 is used to evaluate the movement duration and distance in elevated plus-maze model. The program was invented by Associate Professor Theekapun Charoenpong, Department of Biomedical Engineering, Srinakharinwirot University [213]. The process of analysis consists of two main steps: rat segmentation and parameters measurement. First, the movement of the rat is recorded and separated into image sequence format. The rat in each frame is extracted from the background by using background subtraction technique, i.e., difference in color of rat (white) and behavior model (black). Second, the center of the rat is computed from rat body. The center position of the rat is used to evaluate the distance that the rat move around on the model. Frame rate is an additional parameter to calculate the duration (time) of the movement of the rat.

Western blot assay

Tissue preparation

The acute and chronic effect of *T. laurifolia* and ethanol on the expression levels of tyrosine hydroxylase protein in the striatum and nucleus accumbens in rats was determined after finishing different combination of treatments. The rats were sacrificed, the striatum and nucleus accumbens were immediately dissected, frozen, and kept at -80°C.

Protein determination

Protein concentrations in the striatum and nucleus accumbens were determined by the Bradford method using bovine serum albumin (BSA) as the protein standard.

Western blotting

Striatum and nucleus accumbens were homogenized with RIPA buffer (50mM Tris-Cl, 150 mM NaCl, 1%Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1% protease inhibitor cocktail) followed by centrifuged 12,000 rpm at 4°C for 10 minutes. The supernatants were collected and kept at -80°C for further analysis. Sample proteins concentrations were determined using the Bradford method. Each sample was denatured in a mixture of loading buffer and β-mercaptoethanol at 65°C for 5 minutes.

For gel electrophoresis, 30 µg/lane of protein concentration was loaded into 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separating protein based on molecular weight. The resolved proteins were transferred onto polyvinylidene difluoride membranes (PVDF). The transfer potency was checked by Ponceau-S red solution. The membranes were blocked with 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 1 hour to prevent non-specific protein binding. The membranes were then washed with TBST followed by overnight incubation with the primary antibody; 1:10000 mouse monoclonal antibody against tyrosine hydroxylase (Millipore, Billerica, MA, #MAB318) or 1:40000 mouse monoclonal antibody against β-actin (Millipore, Billerica, MA, #MAB1501) diluted in TBST. After washing with TBST, the membranes were incubated for 1 hour at room temperature with the horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG secondary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA, #7076S) at a dilution of 1:10,000 for the detection of tyrosine hydroxylase or 1:40,000 for the detection of actin. The membranes were then washed with TBST, and the protein bands were visualized using enhanced chemiluminescence plus (ECL Plus™, Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film. The tyrosine hydroxylase band was exposed for 1 minute and 10 seconds for β-actin exposure. 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). The ratio of tyrosine hydroxylase and β-actin is used as an indicator for evaluation of tyrosine hydroxylase level in western blot study.

Histological study

Tissue preparation

The acute and chronic effect of *T. laurifolia* and ethanol on the expression and localization of tyrosine hydroxylase protein in the substantia nigra and ventral tegmental area in rats were determined after finishing different combination of treatments. Male Wistar rats were anesthetized with 46 mg/kg sodium pentobarbital and rapidly transcardially perfused with normal saline and followed by fixative (4% paraformaldehyde mixed with 0.35% glutaraldehyde in 0.1 M 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 rat brains were sectioned at a thickness of 50 µm with cryostat (Leica CM 1950; Leica, Watzlar, Germany). The tissue sections were collected in 0.1 M phosphate-buffered saline (PBS) and kept at 4°C.

Immunoperoxidase assay

The free-floating sections were washed in 0.1 M PBS following pretreated with 1 % hydrogen peroxide (H_2O_2) in PBS for 10 minutes. The sections were washed with PBS-B (PBS containing 0.1% Triton X-100 and 0.25% BSA) for 10 minutes and then 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. The sections were then incubated with mouse monoclonal antibody against tyrosine hydroxylase at a dilution of 1:10,000 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 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) 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 gelatinized slide, air-dried, and coverslipped with Permount®. The immunoperoxidase activity was visualized by light microscopy (Olympus BH2, Tokyo, Japan) and photographed with a digital camera (Olympus DP70, Tokyo Japan). The intensity of tyrosine hydroxylase was determined by image J.

Statistical analysis

The data were shown as mean ± SEM and analyzed statistically using analysis of variance (ANOVA) with Newman-Keuls multiple comparison test to compare differences among individual groups. The scientific statistic software Graphpad Prism 5 was employed. The significance levels were set at *p* values less than 0.05 and 0.01.

CHAPTER IV
RESULTS

T. laurifolia identification

T. laurifolia extraction used in the experiments was identified the consistency by TLC. *T. laurifolia* was dissolved in the solution containing of 90% methanol/water before spotting on the TLC. The spotting of *T. laurifolia* extract was isolated in a running buffer containing 10% methanol/ethyl acetate. The result of four time extractions of *T. laurifolia* showed the same pattern (figure 23). Figure 23A showed the distance of each band of *T. laurifolia* on TLC. Seven bands of *T. laurifolia* on TLC was identified by visible light, UV light, and spay with anisaldehyde sulfuric acid (figure 23B).

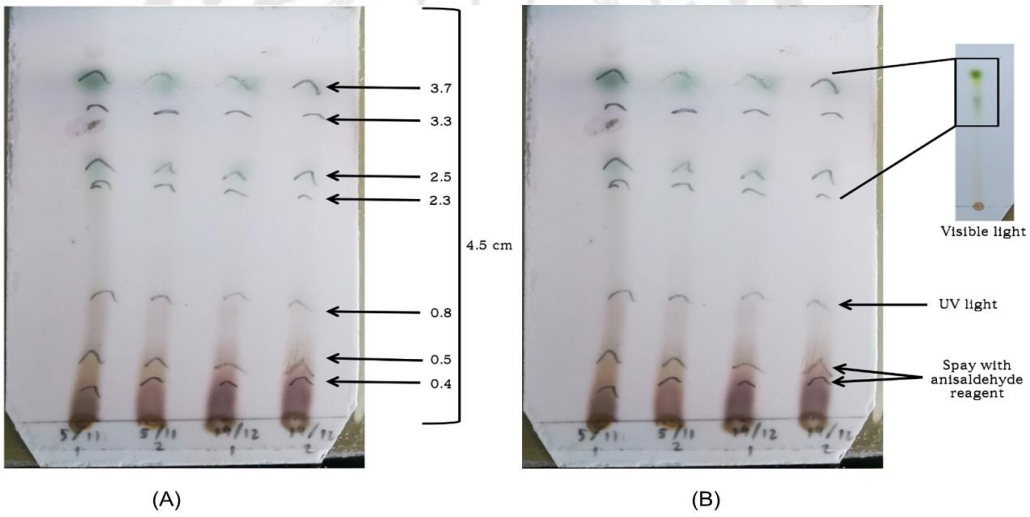

Figure 23 *T. laurifolia* extract pattern on thin-layer chromatography paper.

The TLC shows a distance of each substance of *T. laurifolia* extract that dissolved in a solution containing 90% methanol/water and isolated in a running buffer containing 10% methanol/ethyl acetate (A) and shows the components of *T. laurifolia* by visible light, UV light, and spay with anisaldehyde sulfuric acid (B).

The acute study of *T. laurifolia* and ethanol effect on behaviors of nigrostriatal dopamine pathway in rats

Motor coordination behavior

The acute administrations of *T. laurifolia* and ethanol on motor coordination in male Wistar rats were evaluated using the number of hindlimbs slips from the balance beam at 30, 50, 80, and 100 cm. distance from the started point as indicators. The higher number of hindlimbs slip indicates greater motor coordination impairment in rats.

A high number of hindlimbs slips of acute ethanol administration rats showed at all distances of the balance beam. On the other hand, *T. laurifolia* administration alone did not differ in the number of hindlimbs slips compared with the control group. *T. laurifolia* administration prior to ethanol administration diminished the effect of ethanol (figure 24). The significant increase the number of hindlimbs slips in acute ethanol administration rats displayed at 30 ($p < 0.05$), 50 ($p < 0.01$), 80 ($p < 0.05$), and 100 ($p < 0.01$) cm. of distance when compared with the control rats. The number of hindlimbs slips was reduced at 30 (figure 25), 50 (as shown in figure 26), 80 (figure 27), and 100 (figure 28) cm. distance, and significant at 50 ($p < 0.05$) and 100 ($p < 0.05$) cm. distances in *T. laurifolia* before acute ethanol administration. Furthermore, *T. laurifolia* administration alone did not observe the difference in the number of hindlimbs slips when compared to the control. The mean±SEM values of all group treatments were shown in Table 2.

These results indicated that pre-administration of *T. laurifolia* could protect acute ethanol administration-induced motor coordination impairment. Moreover, *T. laurifolia* did not exhibit the effects to induce motor coordination impairment.

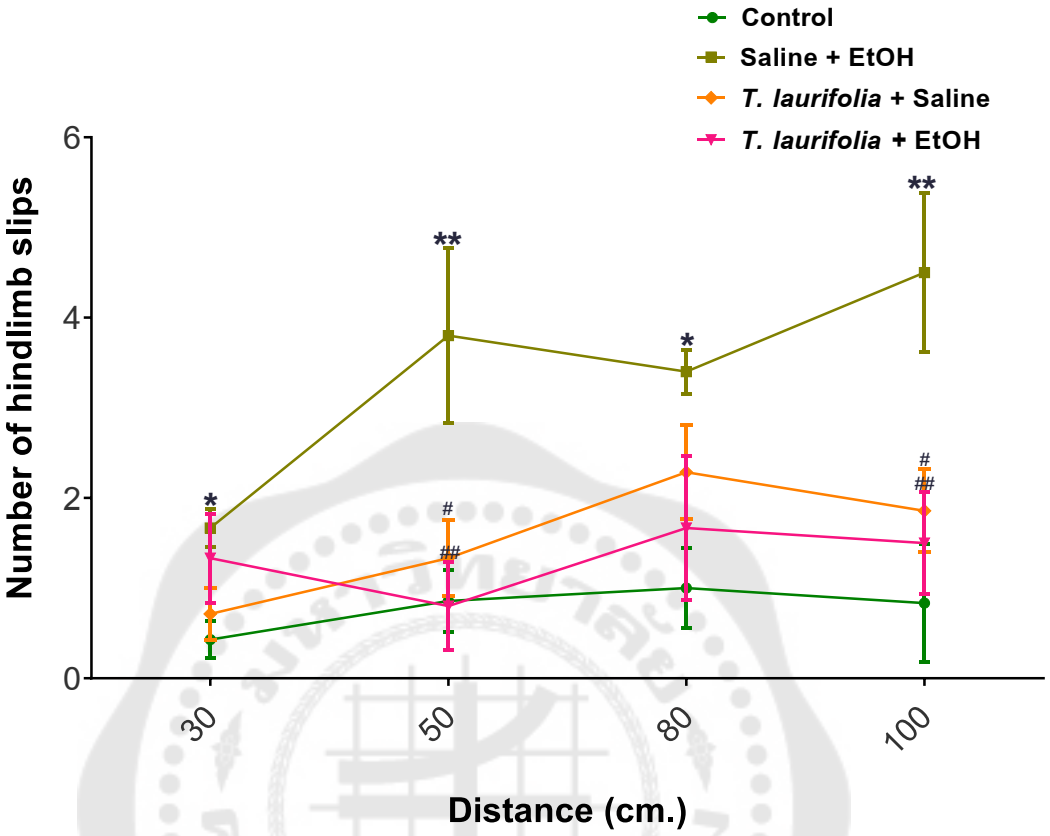

Figure 24 The number of hindlimbs slips of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups on the balance beam.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined 30 minutes after drugs administration. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

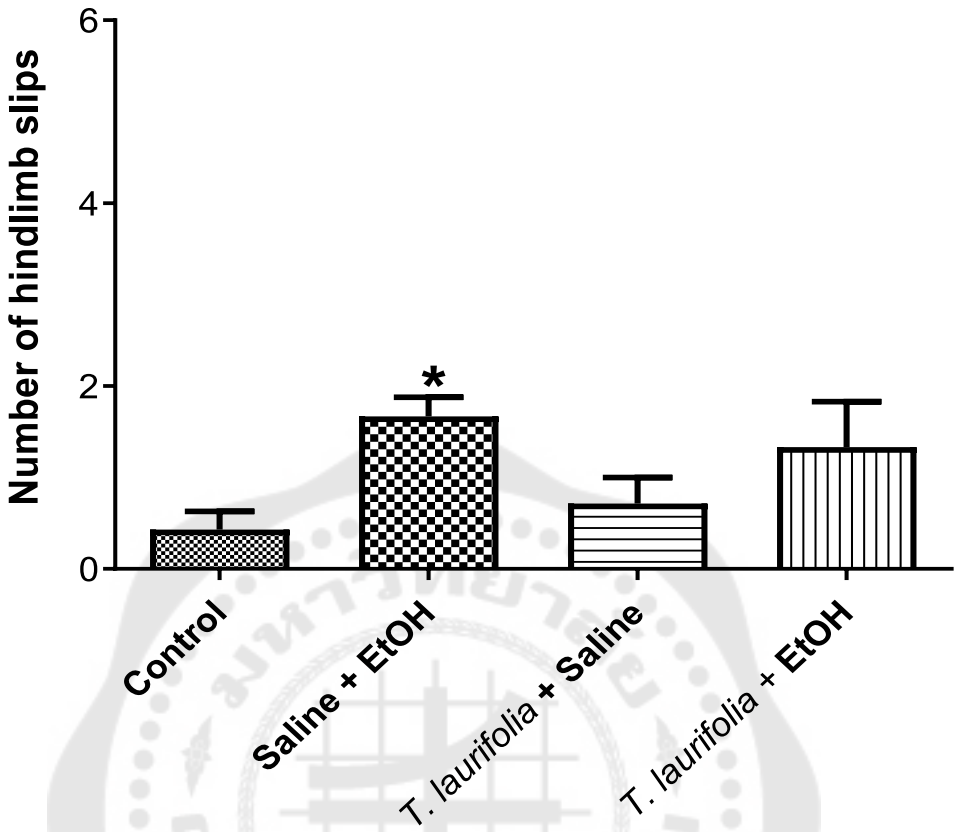

Figure 25 The number of hindlimbs slips of four groups on the balance beam at **30 cm.** distance.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined 30 minutes after drugs administration. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 7, EtOH n = 6, *T. laurifolia* n = 7, *T. laurifolia* + EtOH n = 6). *$p < 0.05$ compared with control group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

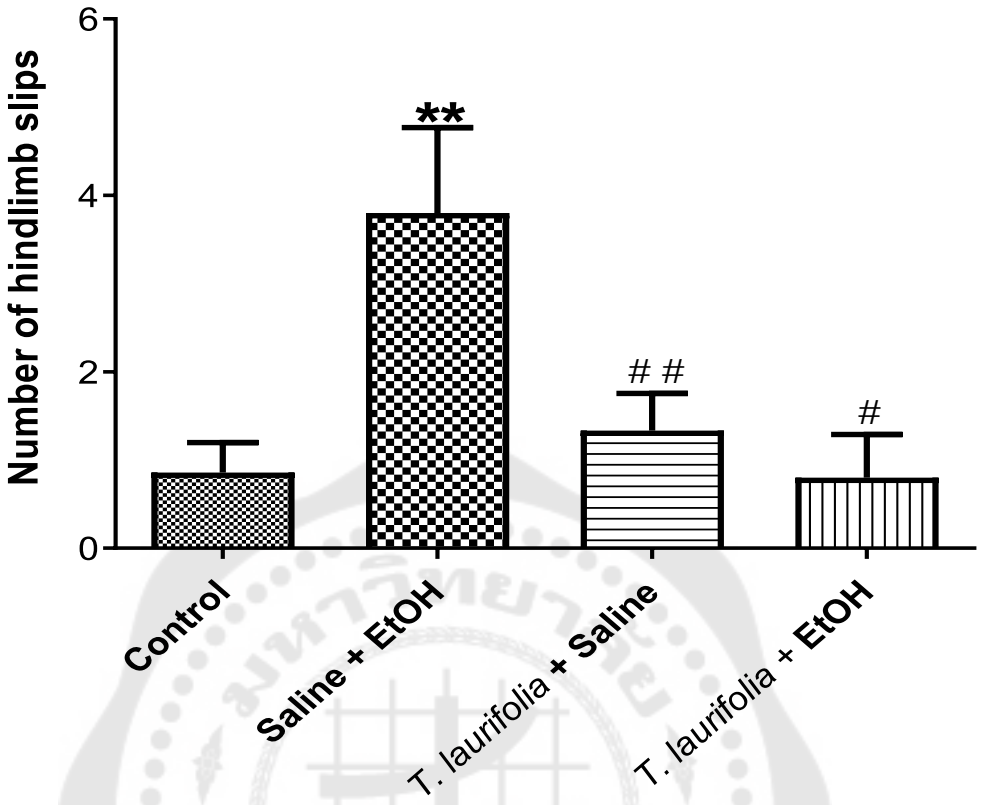

Figure 26 The number of hindlimbs slips of four groups on the balance beam at **50 cm.** distance.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined 30 minutes after drugs administration. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 7, EtOH n = 5, *T. laurifolia* n = 5, *T. laurifolia* + EtOH n = 5). $^{**}p < 0.01$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

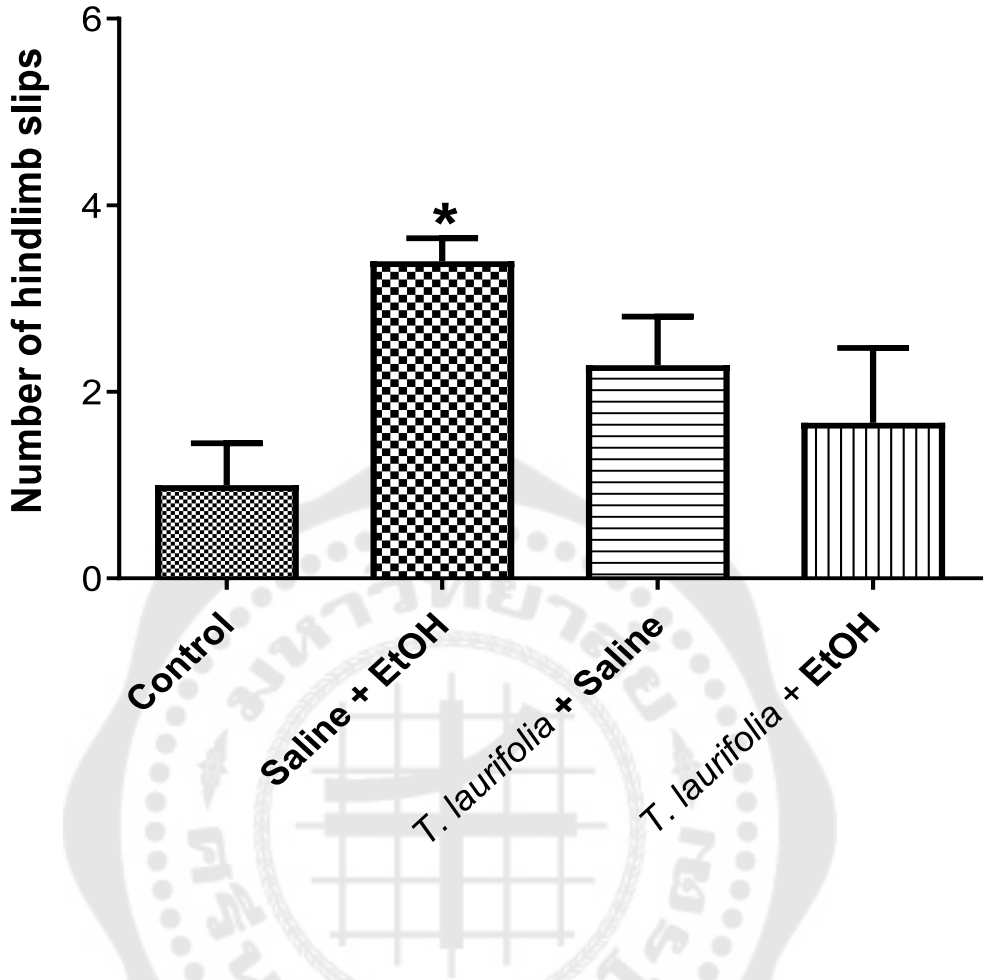

Figure 27 The number of hindlimbs slips of four groups on the balance beam at **80 cm.** distance.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined 30 minutes after drugs administration. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 6, EtOH n = 5, *T. laurifolia* n = 7, *T. laurifolia* + EtOH n = 6). *$p < 0.05$ compared with control group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

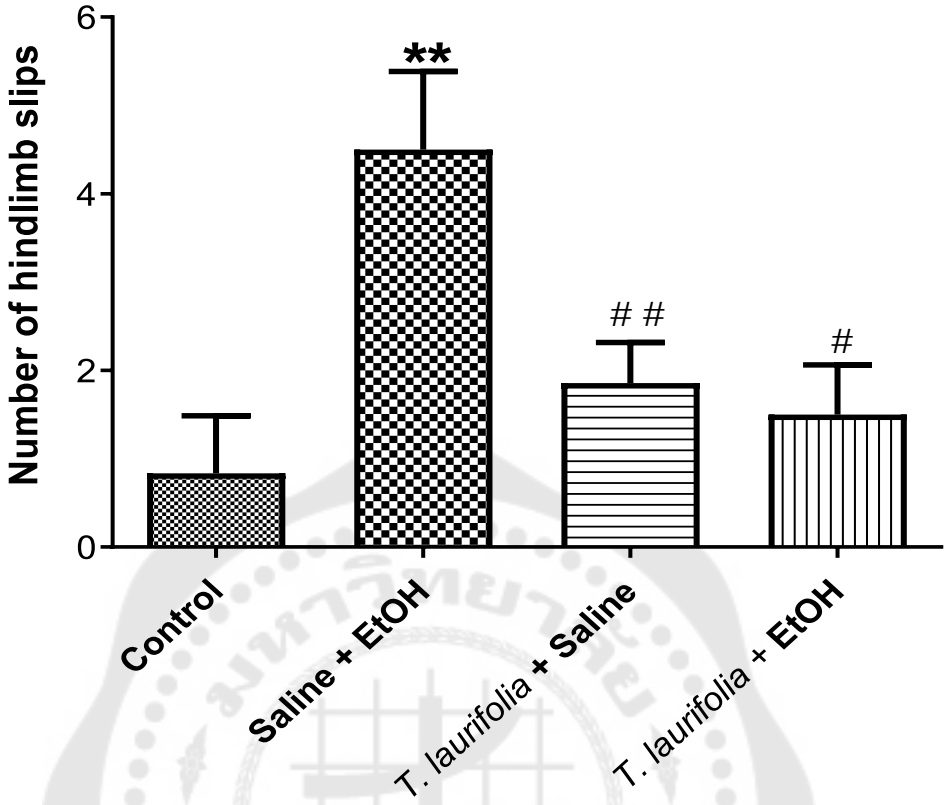

Figure 28 The number of hindlimbs slips of four groups on the balance beam at **100 cm.** distance.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined 30 minutes after drugs administration. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 6, EtOH n = 6, *T. laurifolia* n = 7, *T. laurifolia* + EtOH n = 6). $^{**}p < 0.01$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 2 The number of hindlimbs slips of control, ethanol, *T. laurifolia* and *T. laurifolia* + ethanol groups on the balance beam.

Groups	Number of hind-limb slips (Mean±SEM)			
	30 cm	50 cm	80 cm	100 cm
Control	0.43 ± 0.20 (n = 7)	0.867 ± 0.34 (n = 7)	1.00 ± 0.45 (n = 6)	0.83 ± 0.65 (n = 6)
Saline + Ethanol	1.67 ± 0.21 * (n = 6)	3.80 ± 0.97 ** (n = 5)	3.40 ± 0.240* (n = 5)	4.50 ± 0.89** (n = 6)
T. laurifolia + Saline	0.71 ± 0.29 (n = 7)	1.33 ± 0.42$^{\#\#}$ (n = 6)	2.29 ± 0.52 (n = 5)	1.86 ± 0.46$^{\#\#}$ (n = 7)
T. laurifolia + Ethanol	1.33 ± 0.50 (n = 5)	0.80 ± 0.49$^{\#}$ (n = 5)	1.67 ± 0.80 (n = 6)	1.50 ± 0.56$^{\#}$ (n = 6)

*$p < 0.05$, **$p < 0.01$ compared with the control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group.

The acute study of *T. laurifolia* and ethanol effect on behaviors of mesolimbic dopamine pathway in rats

Anxiety behavior

The acute effect of *T. laurifolia* and ethanol on anxiety behavior in male Wistar rats was determined using time entries to open arms, %time in open arms, and time in end open arms in the elevated plus-maze as indicators. A high value of these parameters represents anxiolytic behavior while the low value of these parameters represents anxiety behavior.

Acute ethanol administration did not significantly alter the time entries (figure 29), % time in open arms (figure 30), and time in end open arms (figure 31) when compared with the control group. However, *T. laurifolia* administration prior to acute ethanol administration did not show a significant difference in these parameters when compared with the control group. *T. laurifolia* administration alone did not observe the difference of the time entries to open arms, %time in open arms, and time in end open arms when compared with the control group. The mean±SEM of all group treatments are shown in Table 3.

These results indicated that acute ethanol administration with the dose used did not induce anxiolytic or anxiety behavior in rats. The administration of *T. laurifolia* before acute ethanol administration is not able to show a protective effect on acute ethanol administration intoxication. However, *T. laurifolia* administration alone did not induce anxiolytic or anxiety behavior in rats.

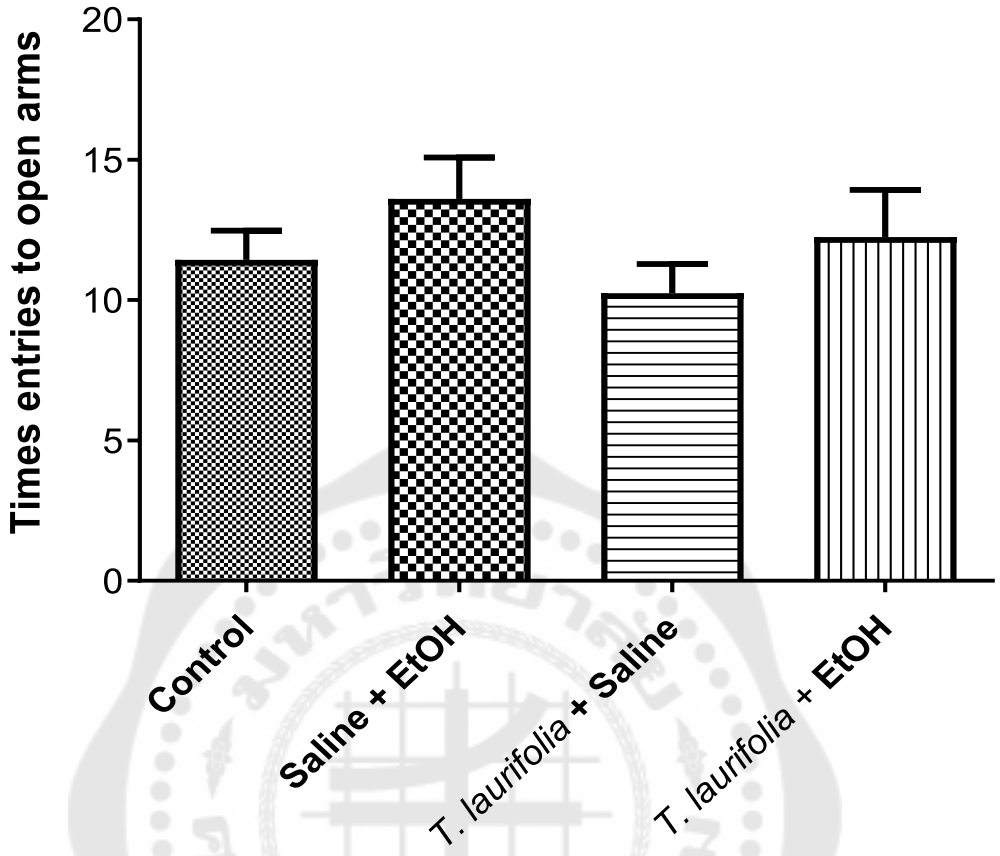

Figure 29 Time entries to open arms of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined at 30 minutes after drug administration. Each group was counted the time entries to open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 7, EtOH n = 10, *T. laurifolia* n = 8, *T. laurifolia* + EtOH n = 8). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

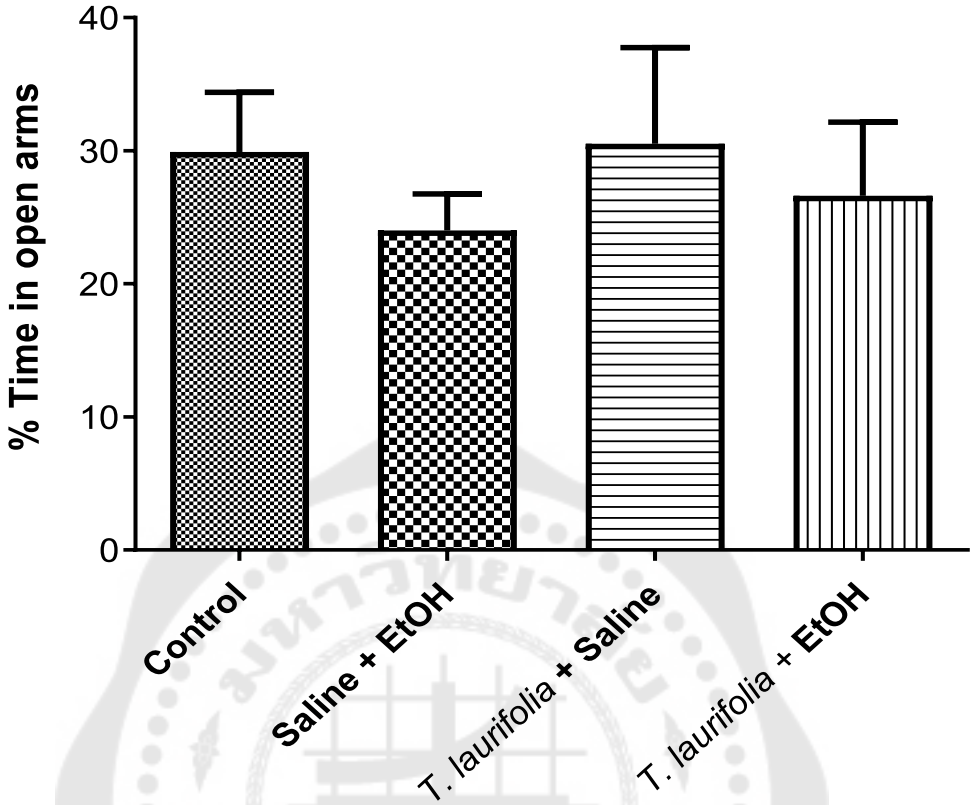

Figure 30 Percent time spent in open arms of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol group.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined at 30 minutes after drug administration. Each group was counted the percent that rats spent in open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 7, EtOH n = 10, *T. laurifolia* n = 8, *T. laurifolia* + EtOH n = 8). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

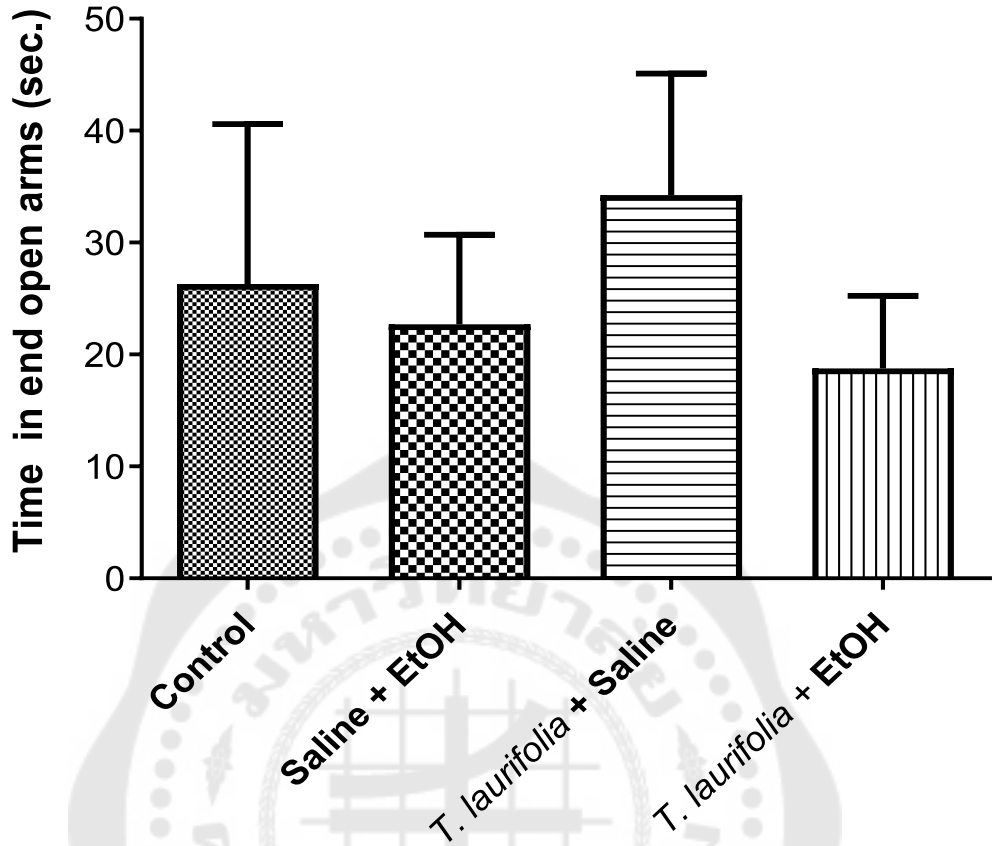

Figure 31 Time in end open arms of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined at 30 minutes after drug administration. Each group was counted the time that rats spent in end open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 7, EtOH n = 10, *T. laurifolia* n = 8, *T. laurifolia* + EtOH n = 8). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 3 Time entries to open arms, %time in open arms and time in end open arms on the elevated plus-maze (Mean±SEM).

Groups	Time entries	%time in open arms	time in end open arms
Control	11.43 ± 1.04 (n = 7)	29.92 ± 4.48 (n = 7)	26.29 ± 14.31 (n = 7)
Saline + Ethanol	13.60 ± 1.48 (n = 10)	24.00 ± 2.74 (n = 10)	22.70 ± 8.01 (n = 10)
T. laurifolia + Saline	10.25 ± 1.03 (n = 8)	30.53 ± 7.22 (n = 8)	34.25 ± 10.86 (n = 8)
T. laurifolia + Ethanol	12.25 ± 1.68 (n = 8)	26.63 ± 5.53 (n = 8)	18.75 ± 6.51 (n = 8)

Exploratory behavior

The acute effect of *T. laurifolia* and ethanol on exploratory behavior in male Wistar rats was determined using the numbers of rearing and head dipping into the holes of the hole board as indicators.

Acute ethanol administration did not induce the difference in the number of rearing (figure 32) and head dipping (figure 33) when compared with the control group. *T. laurifolia* administration before ethanol administration and *T. laurifolia* administration alone did not show a significant difference in the number of rearing and head dipping when compared with the control or ethanol groups. However, *T. laurifolia* administration alone was found to significantly decrease ($p < 0.05$) the number of rearing in rats when compared with *T. laurifolia* administration prior to ethanol administration. These results were shown as mean±SEM in Table 4.

These results suggested that acute ethanol administration did not alter exploratory behavior. *T. laurifolia* administration did not show the protective effect on exploratory behavior when given prior to ethanol administration.

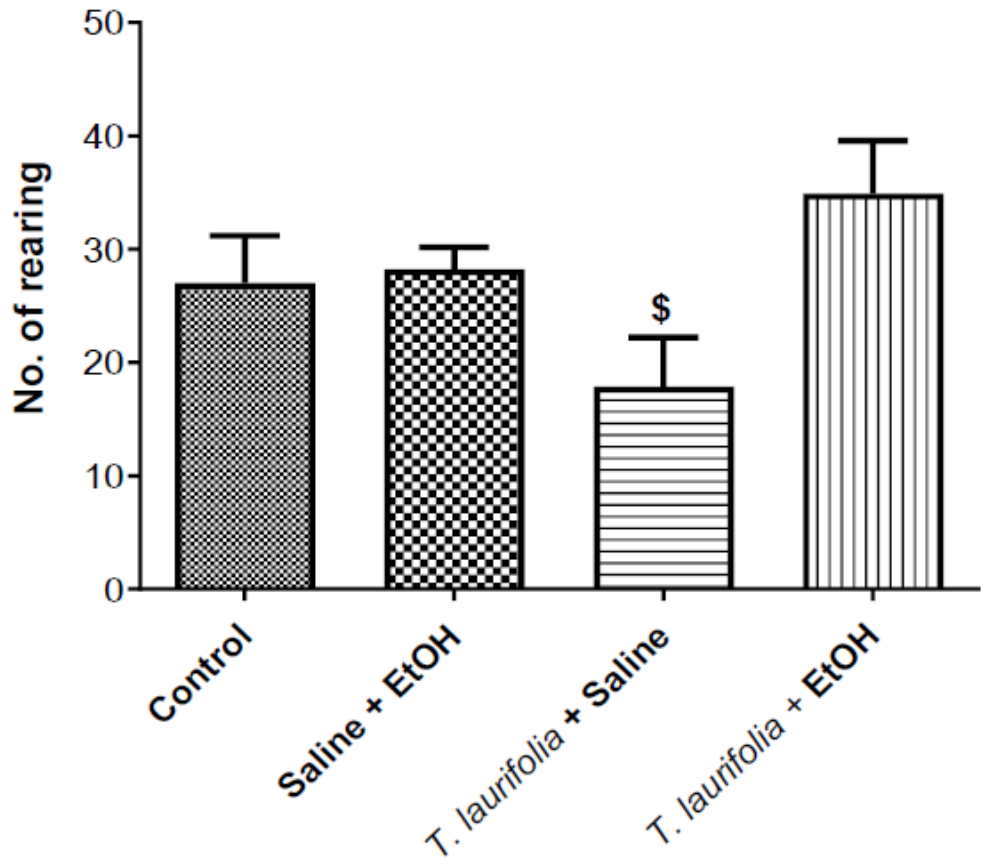

Figure 32 The number of rearing in the exploratory behavior test of control, ethanol, *T. laurifolia,* and *T. laurifolia* + ethanol group.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Exploratory behavior in the hole board was determined at 30 minutes after drug administration. Each group was counted the number of rearing on hole board for 10 minutes. The data were mean±SEM (control n = 10, EtOH n = 10, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n= 9). $^{\$}p < 0.05$ compared with the *T. laurifolia* + ethanol using a one-way ANOVA and Newman-Keuls multiple comparison test.

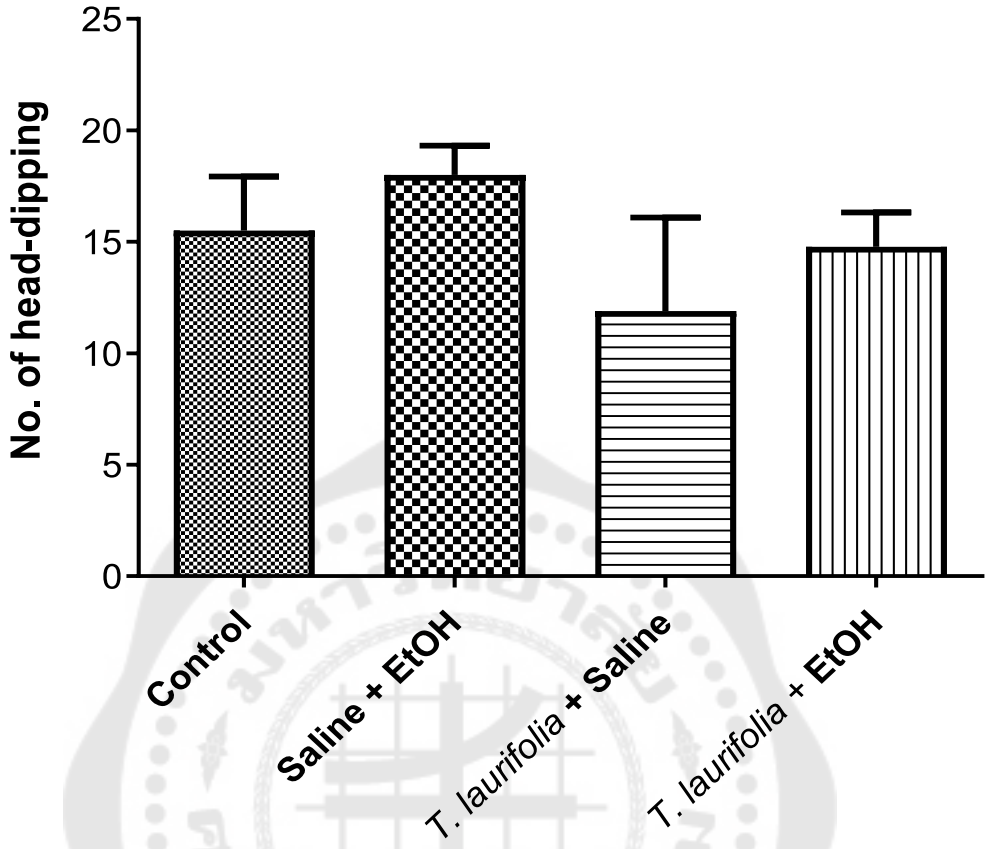

Figure 33 The number of head dipping in the exploratory behavior test of control, ethanol, *T. laurifolia,* and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Exploratory behavior in the hole board was determined at 30 minutes after drug administration. Each group was counted the number of head-dipping on hole board for 10 minutes. The data were mean±SEM (control n = 10, EtOH n = 10, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n= 9). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 4 The number of rearing and head dipping in hole board (Mean±SEM).

Groups	Rearing	Head dipping
Control	27.00 ± 4.18 (n = 10)	15.50 ± 2.43 (n = 10)
Saline + Ethanol	28.20 ± 1.98 (n = 10)	18.00 ± 1.32 (n = 10)
T. laurifolia + Saline	17.89 ± 4.34 (n = 9)$^{\$}$	11.89 ± 4.20 (n = 9)
T. laurifolia + Ethanol	34.89 ± 4.69 (n = 9)	14.78 ± 1.54 (n = 9)

$^{\$}p < 0.05$ compared with the *T. laurifolia* +EtOH group.

The acute study of *T. laurifolia* and ethanol effect on tyrosine hydroxylase levels of nigrostriatal dopamine pathway in rats

The expression levels of tyrosine hydroxylase level in the striatum altered by acute administration of *T. laurifolia* and ethanol were determined by western blot. The expression and localization of tyrosine hydroxylase in the substantia nigra used the immunohistochemistry method to determine. The mean±SEM of all group treatments is shown in Table 5.

Tyrosine hydroxylase levels on the striatum (figure 34) displayed a significant ($p < 0.05$) increase by 44% in acute ethanol administration when compared with control group. *T. laurifolia* administration alone showed no different effect in tyrosine hydroxylase level when compared to the control group but significantly ($p < 0.01$) showed lower tyrosine hydroxylase level when compared with acute ethanol administration group. *T. laurifolia* administration prior to ethanol administration significantly ($p < 0.01$) reduced the level of tyrosine hydroxylase in the striatum when compared to acute ethanol administration group.

The tyrosine hydroxylase- immunoreactivity in the substantia nigra was used to validate the expression and localization in all groups. The results showed the tyrosine hydroxylase localization in the substantia nigra neurons in all groups (figure 35). The expression of tyrosine hydroxylase of the substantia nigra (figure 36) did not exhibit any difference in all groups. The acute ethanol administration did not induce an increase in tyrosine hydroxylase intensity.

These results indicated that the acute ethanol administration could up-regulate the level of tyrosine hydroxylase in the striatum but not in the substantia nigra. *T. laurifolia* showed protective effect of acute ethanol administration by maintaining the tyrosine hydroxylase level in the striatum to the control level. However, *T. laurifolia* administration alone had not effect on the expression of tyrosine hydroxylase in both areas.

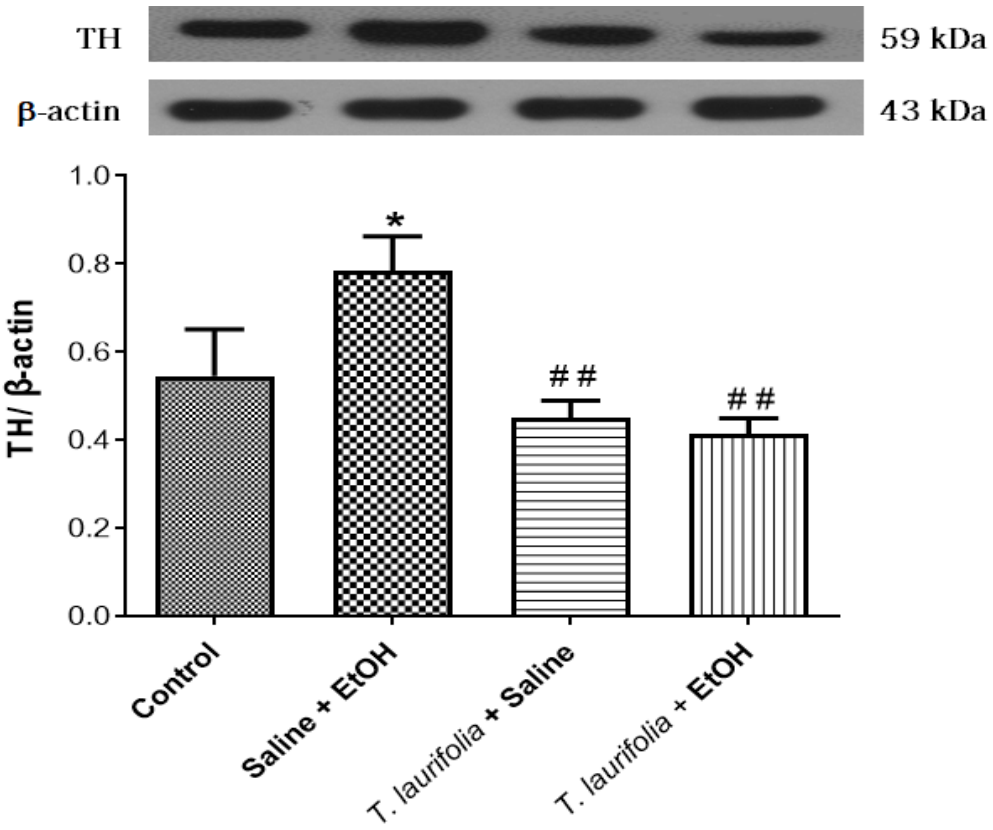

Figure 34 Tyrosine hydroxylase expression levels of striatum of four groups (acute study).

The expression ratio of tyrosine hydroxylase enzyme (TH) to β-actin in the striatum of control, ethanol, *T. laurifolia,* and *T. laurifolia* + Ethanol group. Each group received saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Tyrosine hydroxylase expression was determined by **western blot analysis**. Representative bands from different four groups were shown. The protein band was quantified via densitometry and normalized by β-actin. The results are mean±SEM (control n = 4, EtOH n = 5, *T. laurifolia* n = 5, *T. laurifolia* + EtOH n= 5). $^{*}p < 0.05$ compared with control group; $^{\#\#}p < 0.01$ compared with the ethanol group using a one-way ANOVA and Newman-Keuls multiple comparison test.

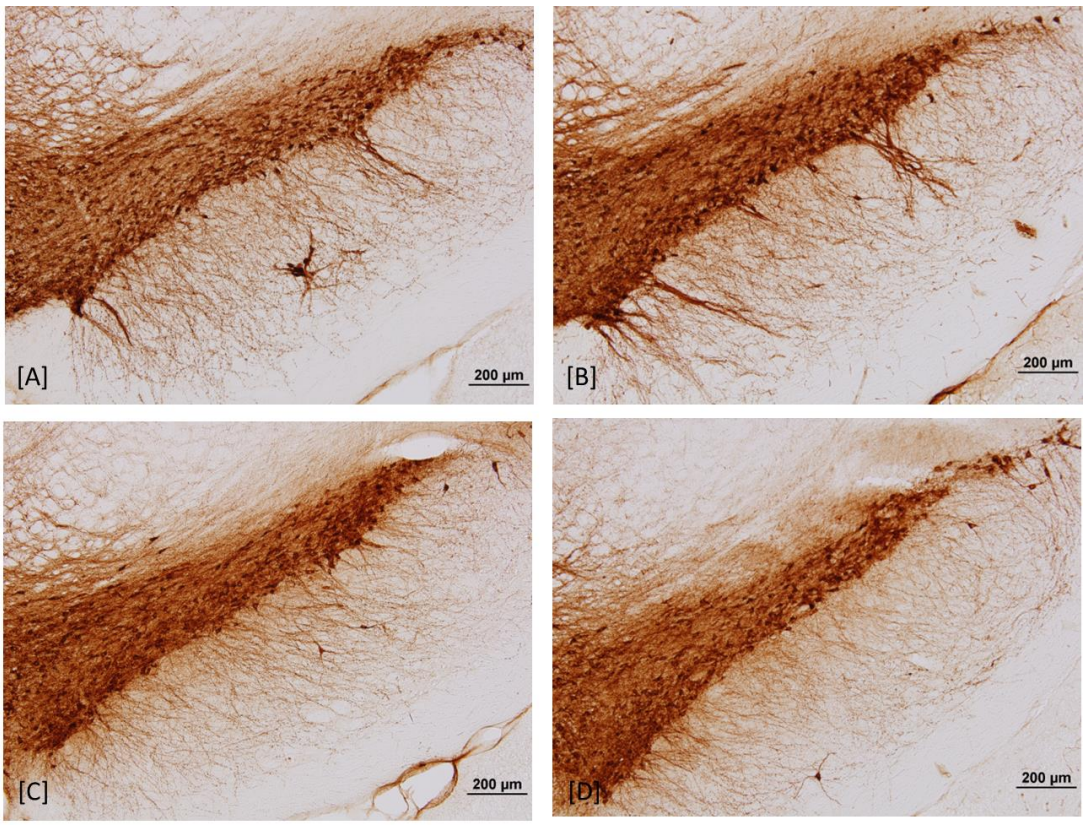

Figure 35 Tyrosine hydroxylase localization in the substantia nigra of four groups (acute study).

Photomicrographs showing tyrosine hydroxylase localization in dopaminergic neurons. The tyrosine hydroxylase-immunoreactive neurons in the substantia nigra of control (A), ethanol (B), *T. laurifolia* (C), and *T. laurifolia* + EtOH groups (D), Scale bar, 200 µm. (n=3).

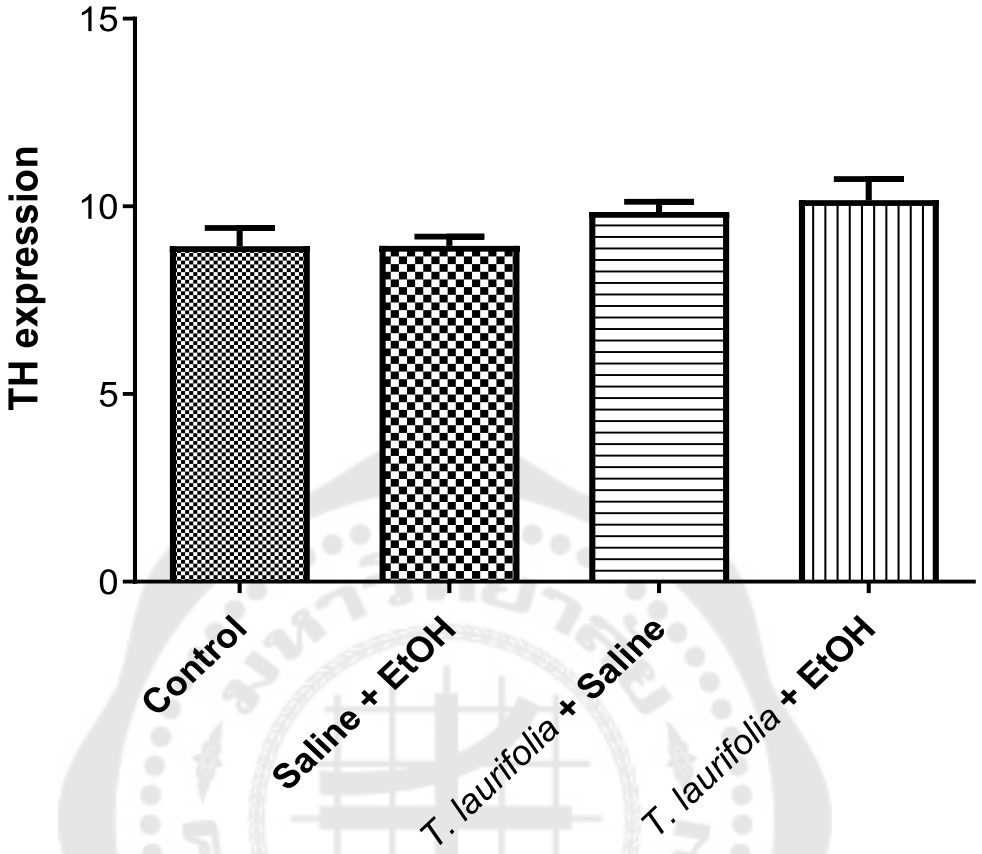

Figure 36 Tyrosine hydroxylase expression in the substantia nigra of four groups (acute study).

Semiquantitative densitometric analysis of tyrosine hydroxylase-immunoreactivity in the substantia nigra was performed in control, ethanol, *T. laurifolia*, and *T. laurifolia* + Ethanol groups. Each group received saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol, respectively. The expression was quantified by image J. Statistical analysis all data are expressed as mean±SEM (n=3). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

The acute study of *T. laurifolia* and ethanol effect on tyrosine hydroxylase levels of mesolimbic dopamine pathway in rats

The tyrosine hydroxylase expression level in the nucleus accumbens altered by acute *T. laurifolia* and ethanol administration was determined by western blot. The expression and localization of tyrosine hydroxylase of the ventral tegmental area were determined by immunohistochemistry method. The mean±SEM of all group treatments is shown in Table 6.

The tyrosine hydroxylase expression level in the nucleus accumbens (figure 37) was unchanged in the acute ethanol administration group when compared with the control group. *T. laurifolia* administration alone groups did not show the difference in the expression level of tyrosine hydroxylase when compared to the control. *T. laurifolia* administration prior to acute ethanol administration showed no significant effect.

The tyrosine hydroxylase- immunoreactivity in the ventral tegmental area was used to validate the expression and localization in all groups. The results showed the tyrosine hydroxylase localization in the ventral tegmental area neurons in all groups (figure 38). The expression of tyrosine hydroxylase in the ventral tegmental area did not change by acute ethanol administration when compared with the control group. *T. laurifolia* administration alone and administration of *T. laurifolia* prior to acute ethanol administration did not show any difference in the tyrosine hydroxylase expression with the other groups. (figure 39).

These results suggested that an acute ethanol administration did not change the tyrosine hydroxylase expression level in the nucleus accumbens or ventral tegmental area, respectively. *T. laurifolia* administration had no effect to either stimulate or inhibit tyrosine hydroxylase expression in both areas. This result shows an unclear protective effect on the tyrosine hydroxylase level in both areas.

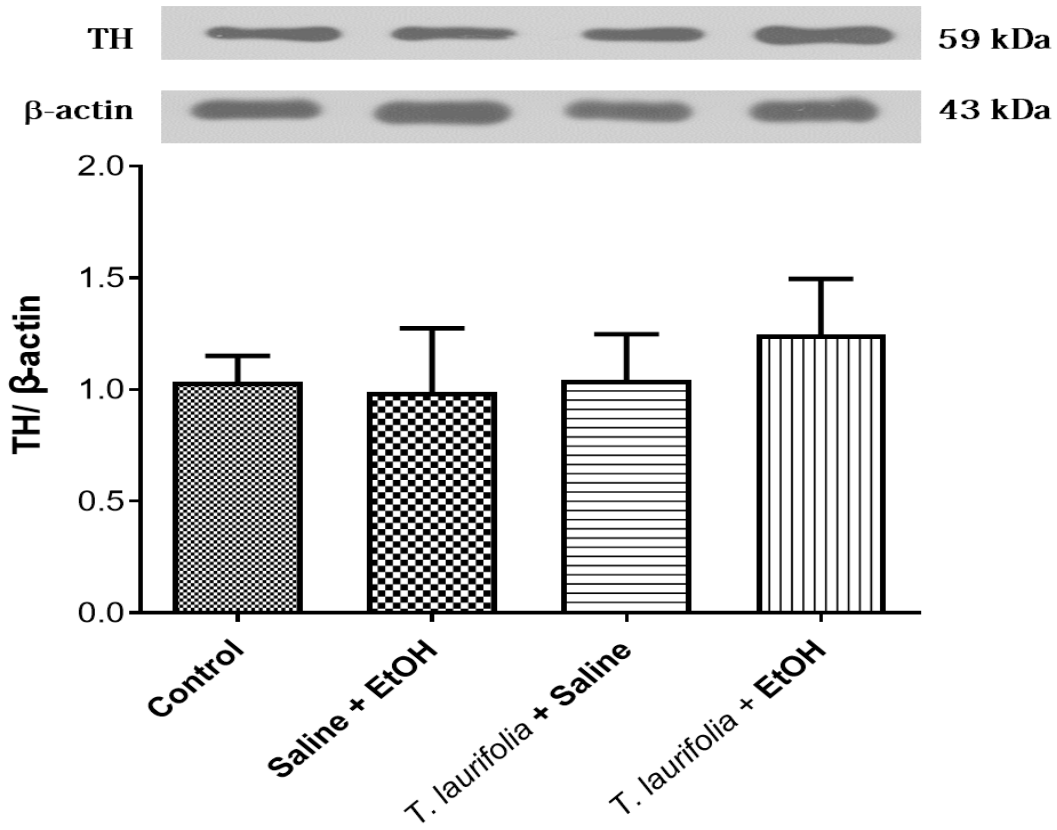

Figure 37 Tyrosine hydroxylase expression levels of nucleus accumbens of four groups (acute study).

The expression ratio of tyrosine hydroxylase enzyme (TH) to β-actin in the nucleus accumbens of control, ethanol, *T. laurifolia,* and *T. laurifolia* + Ethanol group. Each group received saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Tyrosine hydroxylase expression was determined by **western blot analysis**. Representative bands from different four groups were shown. The protein band was quantified via densitometry and normalized by β-actin. The results are mean±SEM (control n = 4, EtOH n = 5, *T. laurifolia* n = 5, *T. laurifolia* + EtOH n= 6). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

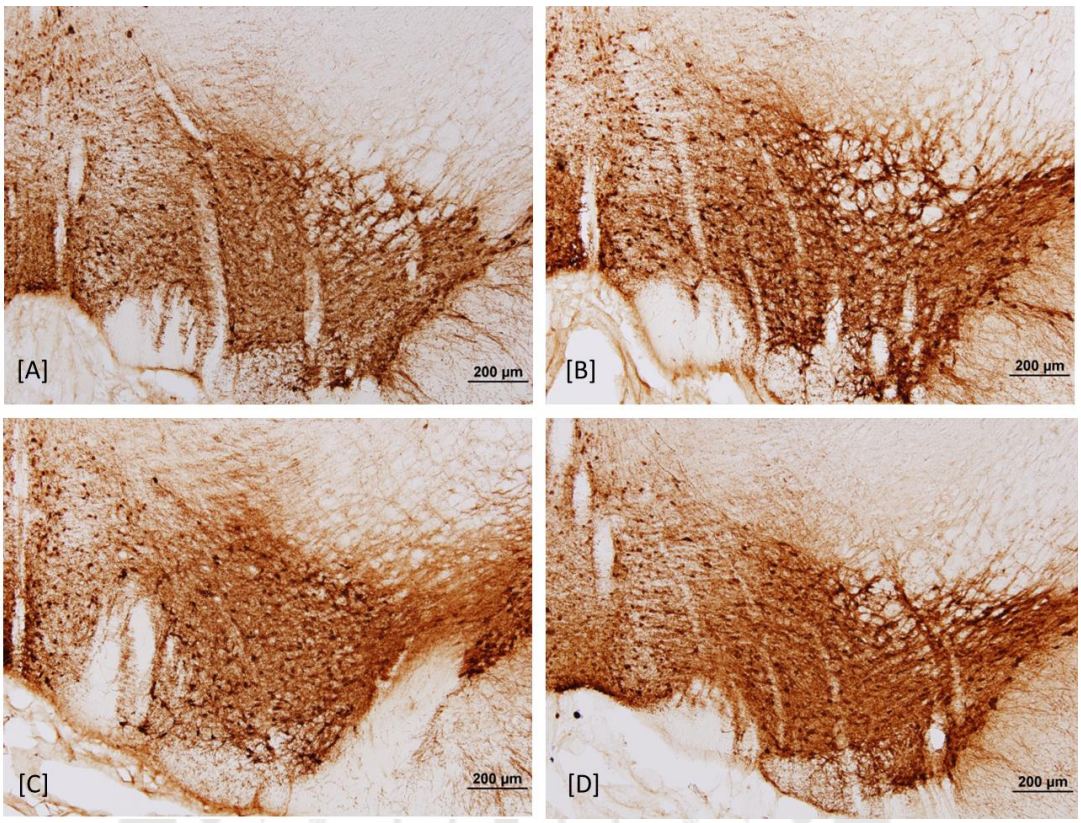

Figure 38 Tyrosine hydroxylase localization in the ventral tegmental area of four groups (acute study).

Photomicrographs showing tyrosine hydroxylase localization in dopaminergic neurons. The tyrosine hydroxylase-immunoreactive neurons in the ventral tegmental area of control (A), ethanol (B), *T. laurifolia* (C), and *T. laurifolia* + EtOH groups (D), Scale bar, 200 µm. (n=3).

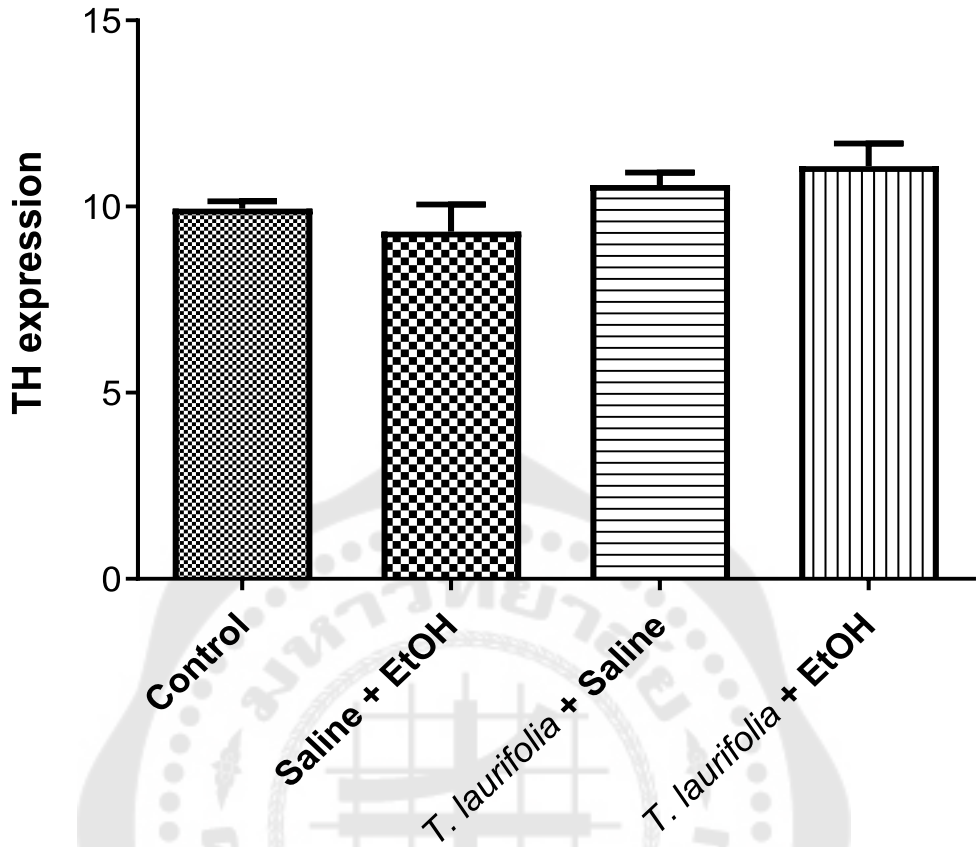

Figure 39 Tyrosine hydroxylase expression in the ventral tegmental area of four groups (acute study).

Semiquantitative densitometric analysis of tyrosine hydroxylase-immunoreactivity in the ventral tegmental area was performed in control, ethanol, *T. laurifolia*, and *T. laurifolia* + Ethanol groups. Each group received saline, ethanol (2 g/kg, 20% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol, respectively. The expression was quantified by image J. Statistical analysis all data are expressed as mean±SEM (n=3). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 5 The expression of tyrosine hydroxylase of nigrostriatal pathway (Mean±SEM).

Groups	Striatum	Substantia nigra
Control	0.55 ± 0.11 (n = 4)	8.94 ±0.85 (n = 3)
Saline + Ethanol	0.78 ± 0.08* (n = 5)	8.95 ±0.43 (n = 3)
T. laurifolia + Saline	0.45 ± 0.04$^{\#\#}$ (n = 5)	9.85 ± 0.47 (n = 3)
T. laurifolia + Ethanol	0.41 ± 0.04$^{\#\#}$ (n = 5)	10.17 ± 0.97 (n = 3)

*$p < 0.05$ compared with the control group

$^{\#\#}p < 0.01$ compared with the ethanol group

Table 6 The expression of tyrosine hydroxylase of mesolimbic pathway (Mean±SEM).

Groups	Nucleus accumbens	Ventral tegmental area
Control	0.88 ± 0.11 (n = 4)	9.95 ± 0.34 (n = 3)
Saline + Ethanol	1.11 ± 0.22 (n = 5)	9.33 ± 1.26 (n = 3)
T. laurifolia + Saline	1.16 ± 0.25 (n = 5)	10.58 ± 0.59 (n = 3)
T. laurifolia + Ethanol	1.22 ± 0.19 (n = 6)	11.08 ± 1.06 (n = 3)

The chronic study of *T. laurifolia* and ethanol effect on behaviors of nigrostriatal dopamine pathway in rats

Motor coordination

The motor coordination of chronic *T. laurifolia* and ethanol administration in male Wistar rats was evaluated using the number of hindlimbs slips from the balance beam at 30, 50, 80, and 100 cm. distances from the started point as indicators. The higher number of hindlimbs slip indicates greater motor coordination impairment in rats.

Chronic ethanol administration induced a high number of hindlimb slips at all distances on the balance beam. The number of hindlimbs slips was significantly increased at 30 (figure 40) ($p < 0.05$) and 50 ($p < 0.01$) (figure 41) cm distances by chronic ethanol administrations when compared to the control group. Chronic *T. laurifolia* administration alone showed no significant difference in the number of hindlimb slips when compared to the control group (figure 40). Daily administration of *T. laurifolia* prior to ethanol administration showed a protective effect of ethanol-induced motor coordination impairment by significantly decrease the number of hindlimb slips when compared with chronic ethanol administration alone at 30 ($p < 0.05$) and 50 ($p < 0.01$) cm distances (figure 41-44). The mean±SEM of all group treatments is shown in Table 7.

These results indicated that chronic ethanol administration produced a sign of motor coordination impairment. Daily administration of *T. laurifolia* prior to ethanol administration could reduce a sign of motor coordination impairment caused by chronic ethanol administration. Moreover, chronic *T. laurifolia* administration alone did not produce motor coordination impairment.

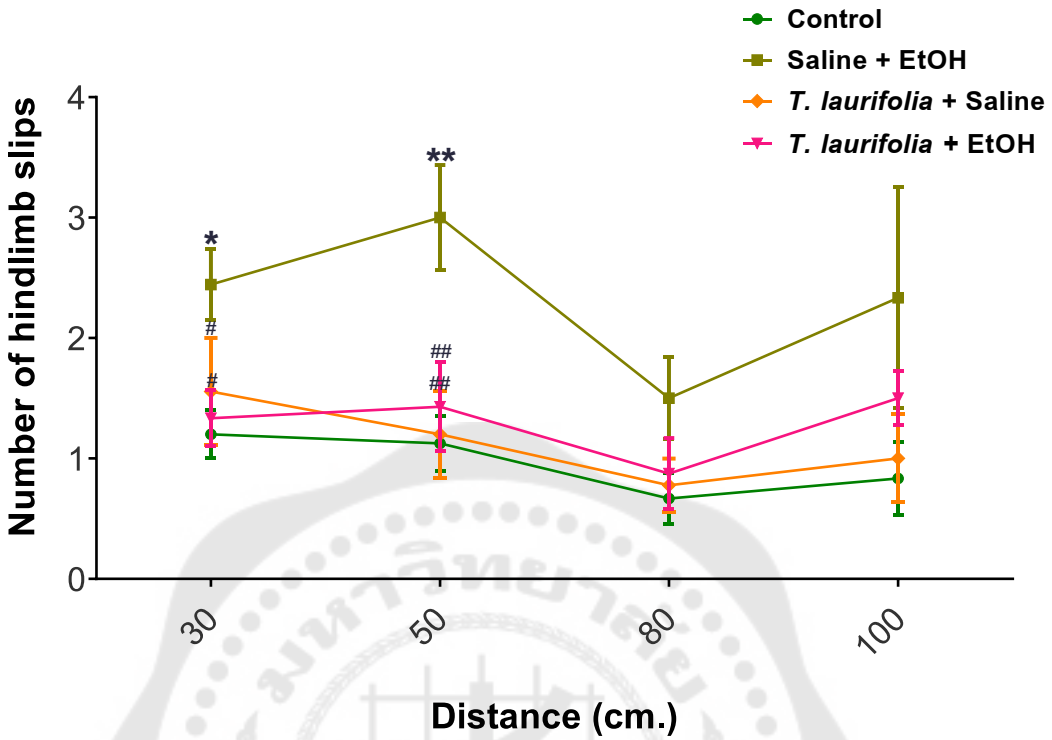

Figure 40 The number of hindlimbs slips of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups on the balance beam.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined after 30 consecutive days of four different combination treatments. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

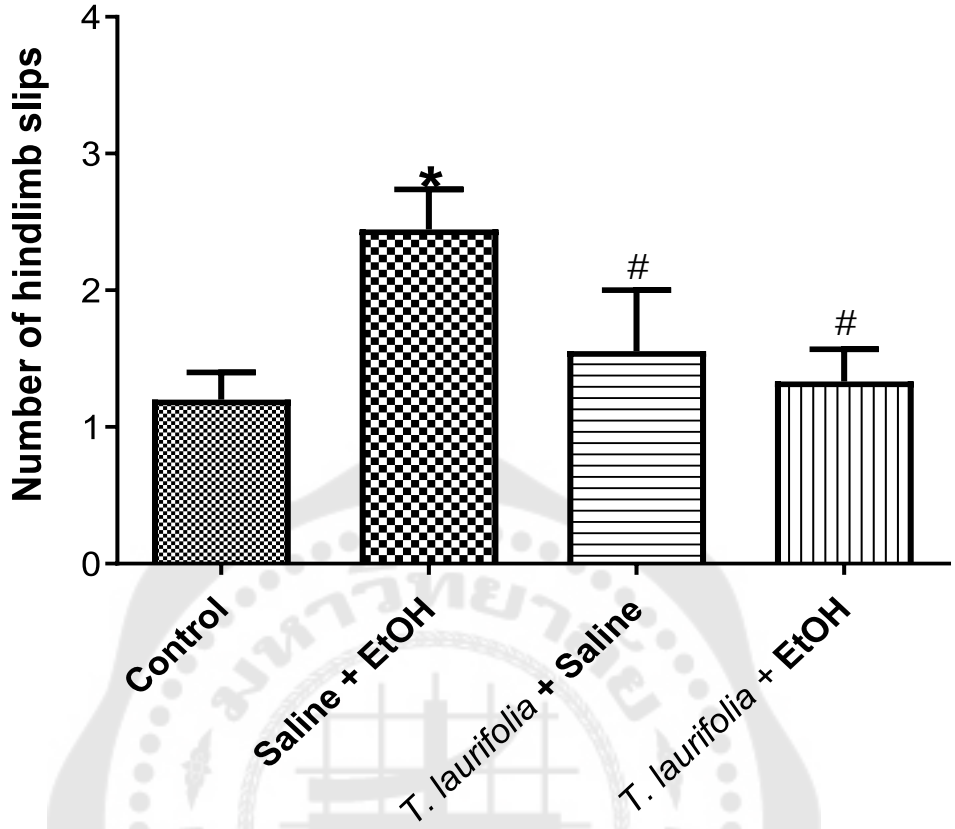

Figure 41 The number of hindlimbs slips of four groups on the balance beam at **30 cm.** distance.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined after 30 consecutive days of four different combination treatments. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 10, EtOH n = 9, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n = 9). $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

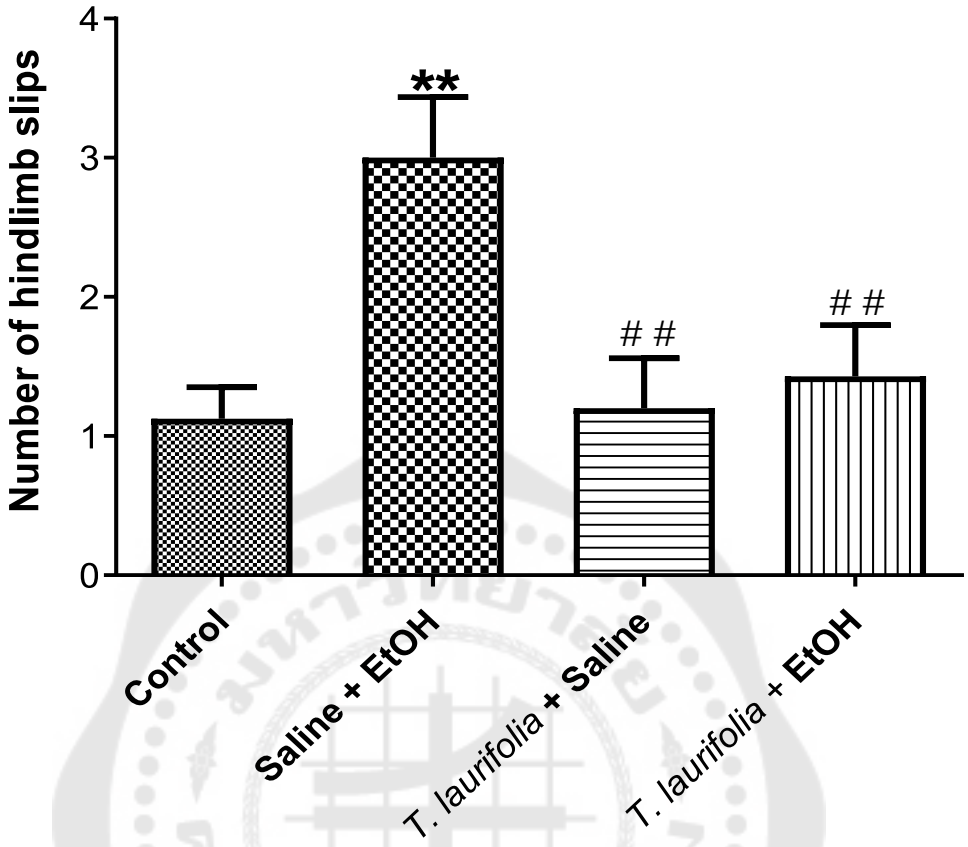

Figure 42 The number of hindlimbs slips on the balance beam at **50 cm**. distance.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined after 30 consecutive days of four different combination treatments. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 8, EtOH n = 7, *T. laurifolia* n = 10, *T. laurifolia* + EtOH n = 7). $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with the ethanol group at each distance using a one-way ANOVA and Newman-Keuls multiple comparison test.

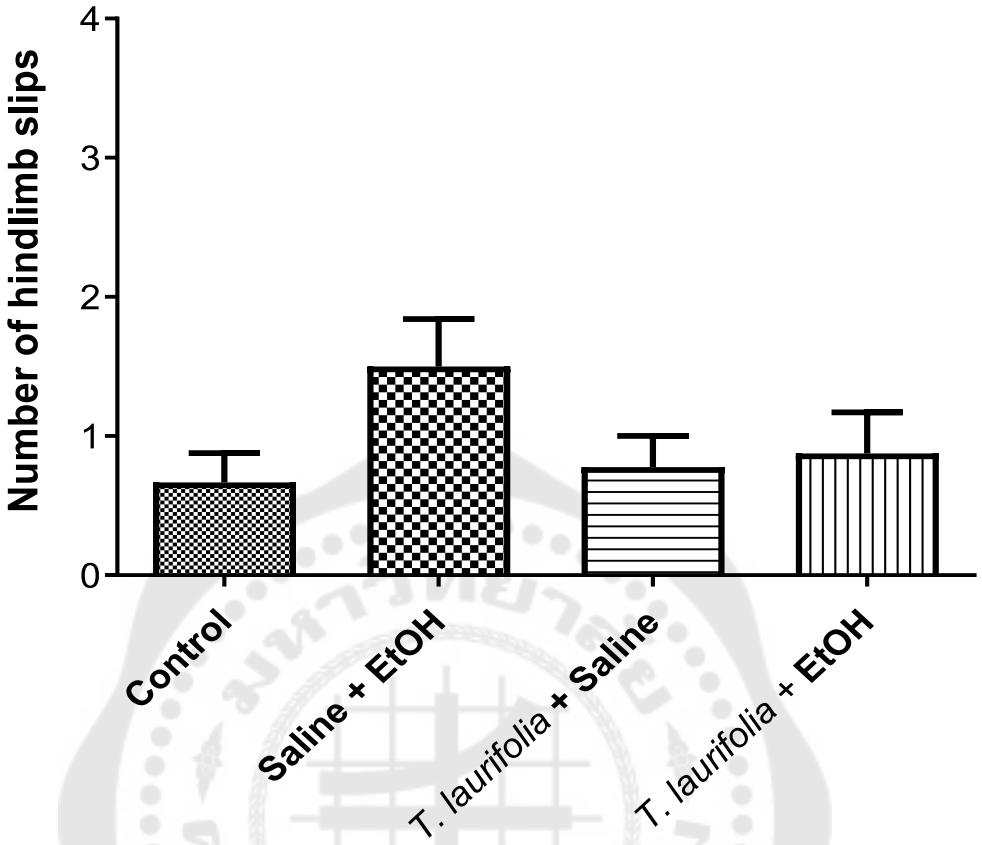

Figure 43 The number of hindlimbs slips on the balance beam at **80 cm**. distance.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined after 30 consecutive days of four different combination treatments. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 6, EtOH n = 6, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n = 8). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

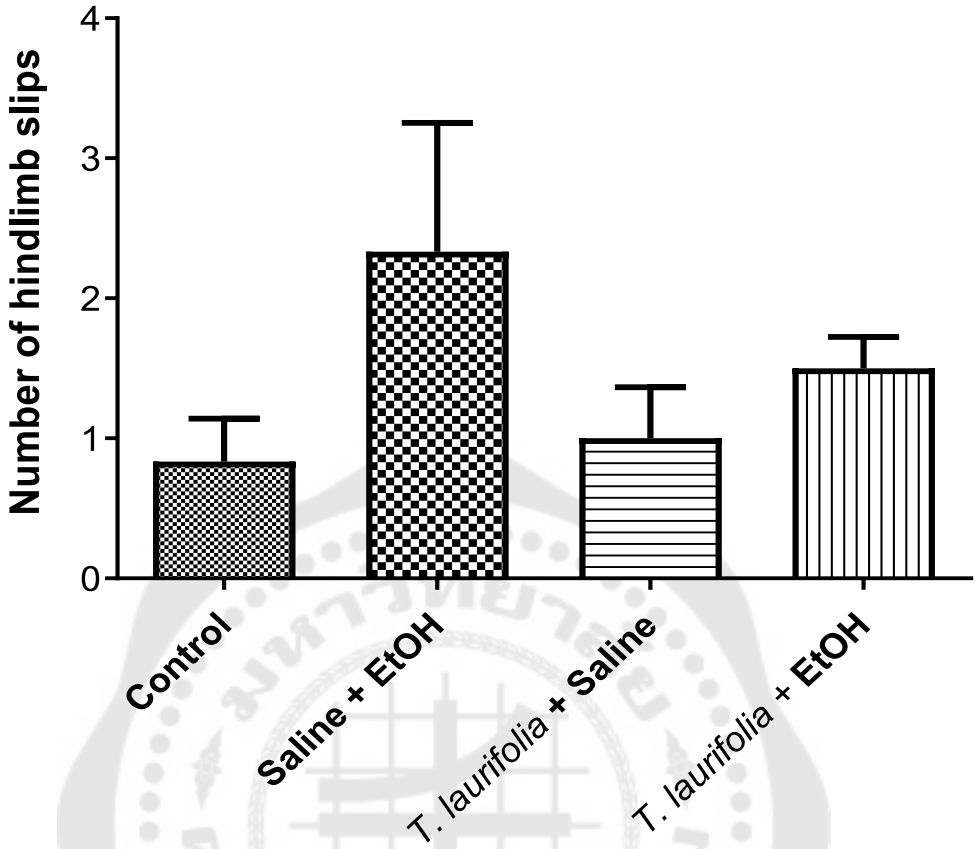

Figure 44 The number of hindlimbs slips on the balance beam at **100 cm.** distance.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Motor coordination impairment was determined after 30 consecutive days of four different combination treatments. The number of hindlimbs slips on the balance beam was counted at 30, 50, 80, and 100 cm. distances from the started point. The data were mean±SEM (control n = 6, EtOH n = 6, *T. laurifolia* n = 6, *T. laurifolia* + EtOH n = 6). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 7 The number of hindlimbs slips of control, ethanol, *T. laurifolia* and *T. laurifolia* + ethanol groups on the balance beam.

Groups	Number of hind-limb slips (Mean±SEM)			
	30 cm	50 cm	80 cm	100 cm
Control	1.20 ± 0.20 (n = 10)	1.13 ± 0.22 (n = 8)	0.67 ± 0.21 (n = 6)	0.83 ± 0.31 (n = 6)
Saline+ Ethanol	2.44± 0.29* (n = 9)	3.00 ± 0.44** (n = 7)	1.50 ± 0.34 (n = 6)	2.33 ± 0.92 (n = 6)
T. laurifolia + Saline	1.56 ± 0.44$^{\#}$ (n = 9)	1.20 ± 0.36$^{\#\#}$ (n = 10)	0.78 ± 0.22 (n = 9)	1.00 ± 0.37 (n = 6)
T. laurifolia + Ethanol	1.33 ± 0.24$^{\#}$ (n = 9)	1.43 ± 0.37$^{\#\#}$ (n = 8)	0.88 ± 0.30 (n = 8)	1.50 ± 0.22 (n = 6)

*$p < 0.05$, **$p < 0.01$ compared with the control group

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group

The chronic study of *T. laurifolia* and ethanol effect on behaviors of mesolimbic dopamine pathway in rats

Addiction behavior

The chronic *T. laurifolia* and ethanol administration on addiction behavior in male Wistar rats was evaluated using the comparison of the time spent in the non-preference compartment of CPP between pre-and post-treatments as an indicator. If the time spent in the non-preference compartment after chronic drug administration combined with forcing the animal to stay in the non-preferred compartment is higher than that of the pre-treatment, this means that this drug exhibit addiction behavior in rats.

The time spent in the non-preference compartment of pre-drug treatment did not show any difference between each group. The time spent in the non-preference compartment before and after chronic drugs treatment was compared between each group. Chronic ethanol administration significantly ($p < 0.01$) increased the time spent in the non-preference compartment when compared with pre-drug treatment. The value increased up to 41.02±12.96% of that of the pre-drug administration value. Chronic administration of *T. laurifolia* alone did not increase the time spent in the non-preference compartment when compared to that of the pre-drug treatment. Daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days inhibited the effect of chronic ethanol intoxication (figure 45). The mean±SEM of all group treatments is shown in Table 8.

These results indicated that chronic ethanol administration produced ethanol addiction. Daily administration of *T. laurifolia* prior to ethanol administration prevented ethanol- addiction behavior. Chronic *T. laurifolia* administration did not produce addiction behavior.

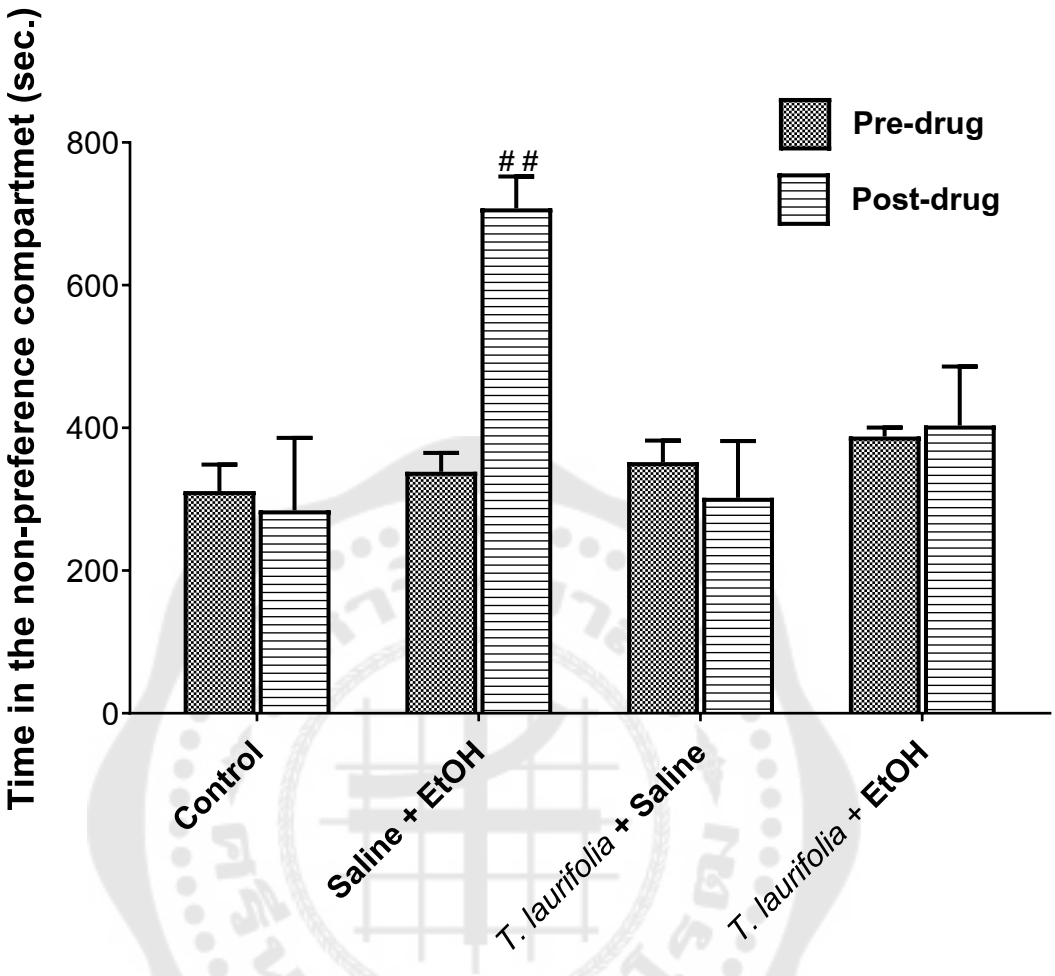

Figure 45 The time spent in the non-preference compartment in CPP of control, EtOH, *T. laurifolia*, and *T. laurifolia* + EtOH groups.

Each rat received saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or *T. laurifolia* + EtOH for 30 consecutive days. The addiction was determined after 30 consecutive days of four different combination treatments. The comparison of the time spent between pre-and post-drug in the non-preference compartment of each rat used as an indicator. The data were mean±SEM (n = 5). $^{\#\#}p < 0.01$ compared with the ethanol group using a two-way ANOVA and Newman-Keuls multiple comparison test.

Table 8 Time spent in non-preference compartment of CPP between pre- and post-drug treatment in each group (Mean±SEM).

Groups	Time spent in non-preference compartment of CPP	
	Pre-drug treatments	Post-drug treatments
Control	311.60 ± 37.05 (n = 5)	284.40 ± 101.70 (n = 5)
Saline+ Ethanol	338.60 ± 26.33 (n = 5)	707.80 ± 44.60$^{\#\#}$ (n = 5)
T. laurifolia + Saline	352.00 ± 30.33 (n = 5)	302.20 ± 79.50 (n = 5)
T. laurifolia + Ethanol	388.00 ± 12.49 (n = 5)	403.40 ± 82.66 (n = 5)

$^{\#\#}$ $p<0.01$ compared with the ethanol group

Anxiety behavior

The chronic effect of *T. laurifolia* and ethanol administration anxiety behavior in male Wistar rats was determined using time entries to open arms, %time in open arms, and time in end open arms in the elevated plus-maze as indicators. A high value of these parameters represents anxiolytic behavior while the low value of these parameters represents anxiety behavior.

Chronic ethanol administration did not significantly change these indicators when compared to that of the control group. The time entries to open arms (figure 46), %time in open arms (figure 47), and time in end open arms (figure 48) were not significantly different from that of the control group. These results were the same in the group with daily administration of *T. laurifolia* prior to ethanol administration and chronic *T. laurifolia* administration groups. All parameters measured, i.e., the time entries to open arms, %time in open arms, and time in end open arms were not significantly altered. Table 9 presents the value of mean±SEM in all group treatments.

These results indicated that chronic ethanol administration did not produce anxiolytic or anxiety behaviors in rats. Therefore, the daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days could not clearly identify the protective effect on chronic ethanol administration intoxication. However, chronic *T. laurifolia* administration alone did not produce anxiolytic or anxiety behavior in rats.

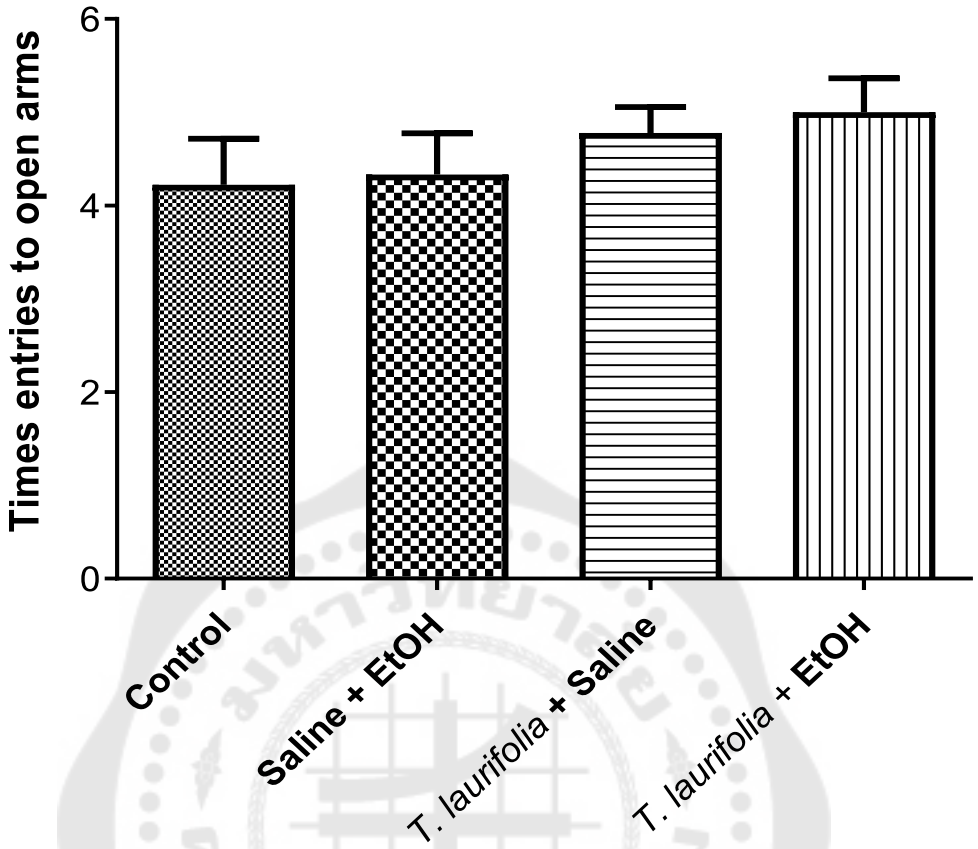

Figure 46 Time entries to open arms of control, ethanol, *T. laurifolia* and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined after 30 consecutive days of combination treatments. Each group was counted the time entries to open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 9, EtOH n = 9, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n = 10). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

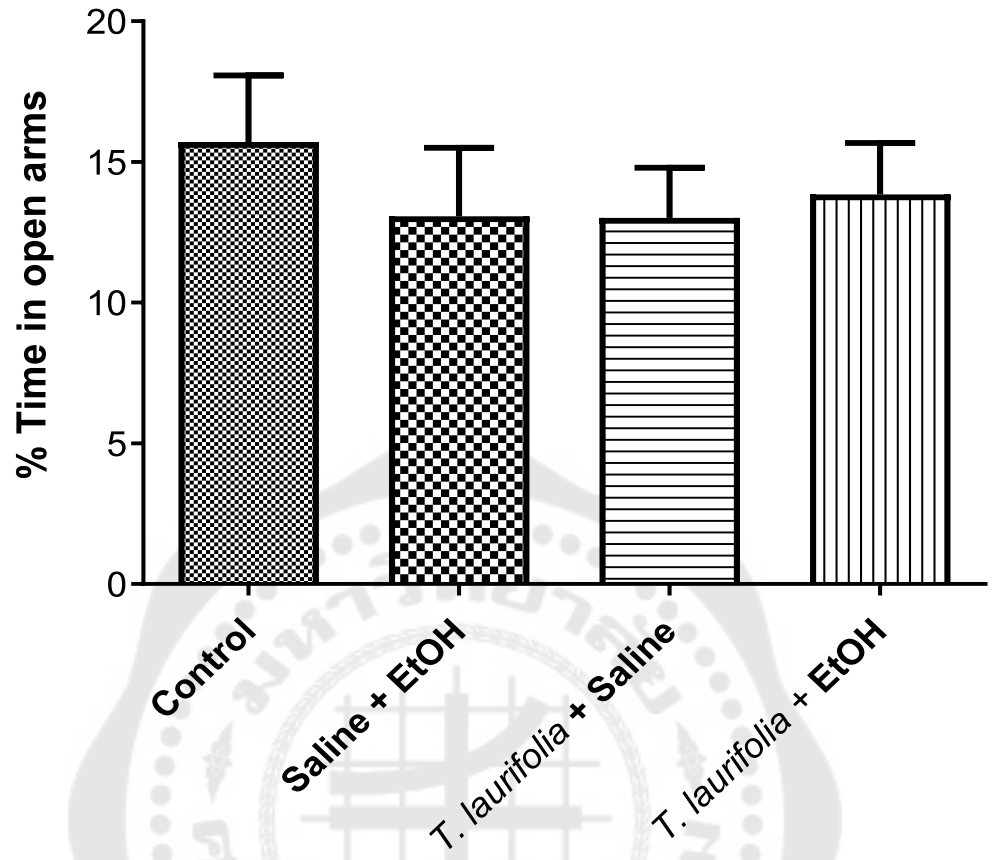

Figure 47 Percent time spent in open arms of control, ethanol, *T. laurifolia* and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined after 30 consecutive days of combination treatments. Each group was counted the percent that rats spent in open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 9, EtOH n = 9, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n = 10). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

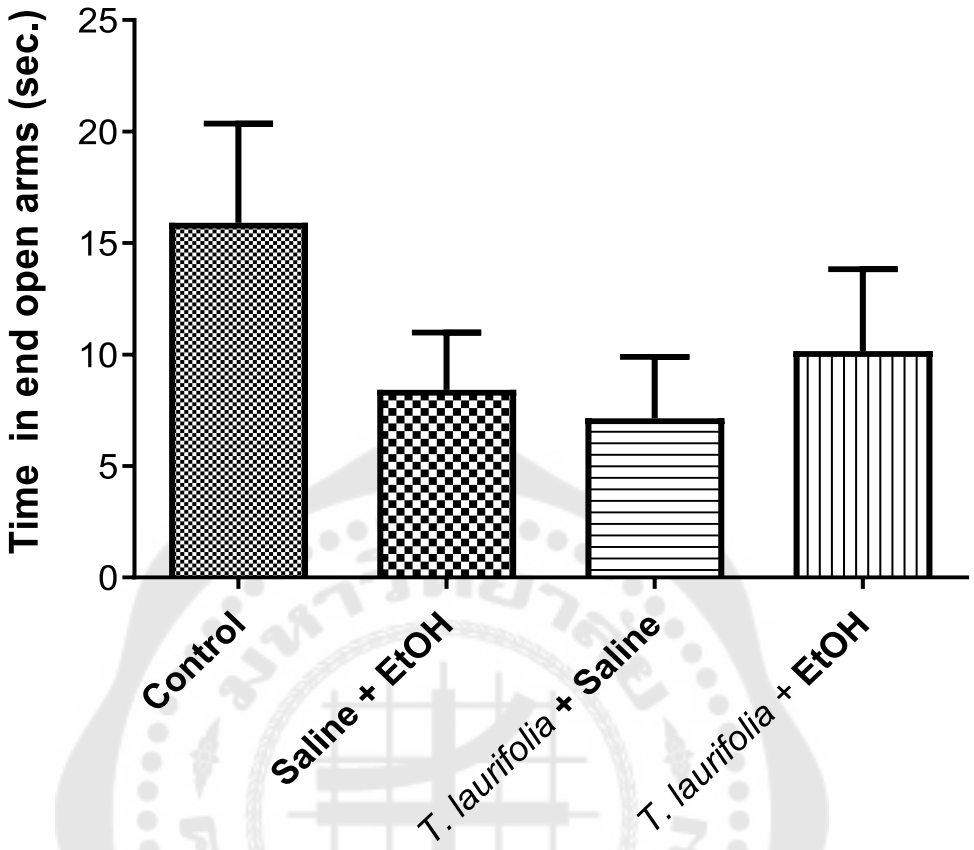

Figure 48 Time in end open arms of control, ethanol, *T. laurifolia,* and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Anxiety behavior in the elevated plus-maze was determined after 30 consecutive days of combination treatments. Each group was counted the time that rats spent in end open arms on elevated plus-maze for 5 minutes. The data were mean±SEM (control n = 9, EtOH n = 9, *T. laurifolia* n = 9, *T. laurifolia* + EtOH n = 10). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 9 Time entries to open arms, %time in open arms and time in end open arms on the elevated plus-maze (Mean±SEM).

Groups	Time entries	%time in open arms	time in end open arms
Control	4.22 ± 0.49 (n = 9)	15.71 ± 2.37 (n = 9)	15.91 ± 4.44 (n = 9)
Saline+ Ethanol	4.33 ± 0.44 (n = 9)	13.07 ± 2.42 (n = 9)	8.42 ± 2.56 (n = 9)
T. laurifolia + Saline	4.78 ± 0.28 (n = 9)	13.01 ± 1.78 (n = 9)	7.13 ± 2.76 (n = 9)
T. laurifolia + Ethanol	5.00 ± 0.37 (n = 10)	13.85 ± 1.82 (n = 10)	10.16 ± 3.67 (n = 10)

Exploratory behavior

The chronic effect of *T. laurifolia* and ethanol administration on exploratory behavior in male Wistar rats was determined using the numbers of rearing and head-dipping into the holes of the hole board as indicators.

Chronic ethanol administration did not induce significant changes in the number of rearing (figure 49) and head-dipping (figure 50) when compared to that of the control group. Chronic *T. laurifolia* administration alone and daily administration of *T. laurifolia* prior to ethanol administration showed no significant changes in the number of rearing and head-dipping. These results show as mean±SEM in Table 10.

These results suggested that chronic ethanol administration did not produce an increase or suppress exploratory behavior. Therefore, it is unable to show the protective effect of *T. laurifolia* on chronic ethanol intoxication. However, chronic *T. laurifolia* administration did not induce an increase or suppress exploratory behavior.

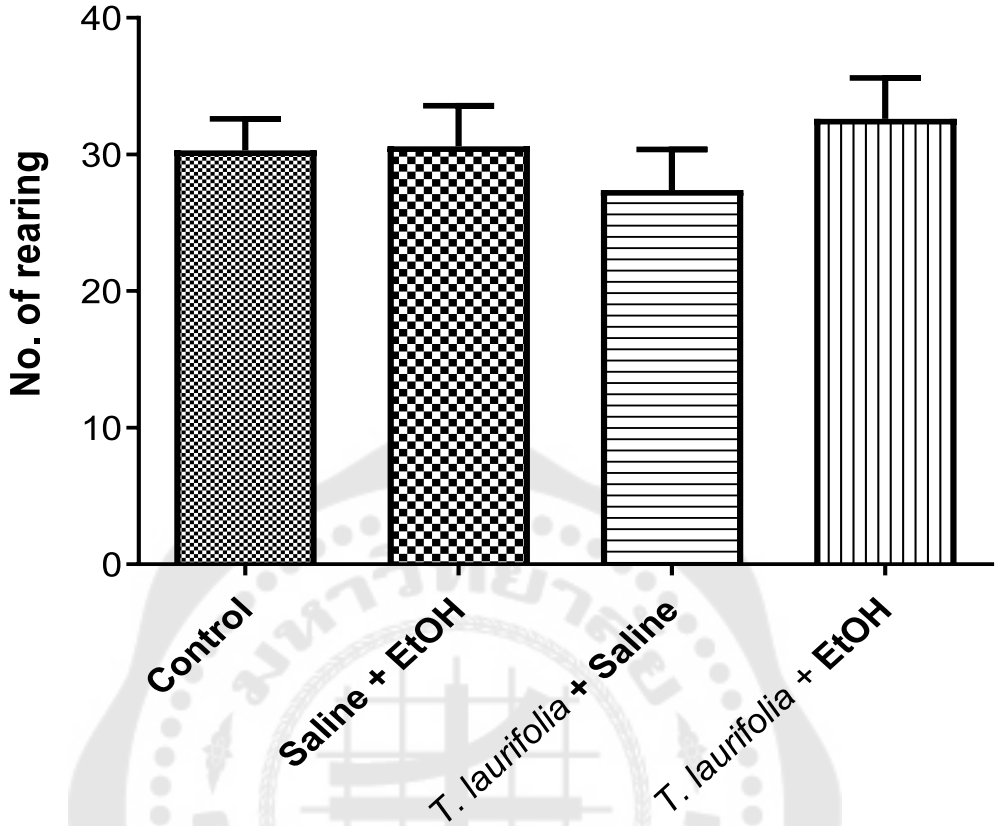

Figure 49 The number of rearing in the exploratory behavior test of control, ethanol, *T. laurifolia*, and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. The exploratory behavior was determined after 30 consecutive days of different combination treatments by the hole board apparatus. Each group was counted the number of rearing on hole board for 10 minutes. The data were mean±SEM (control n = 10, EtOH n = 10, *T. laurifolia* n = 10, *T. laurifolia* + EtOH n = 10). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

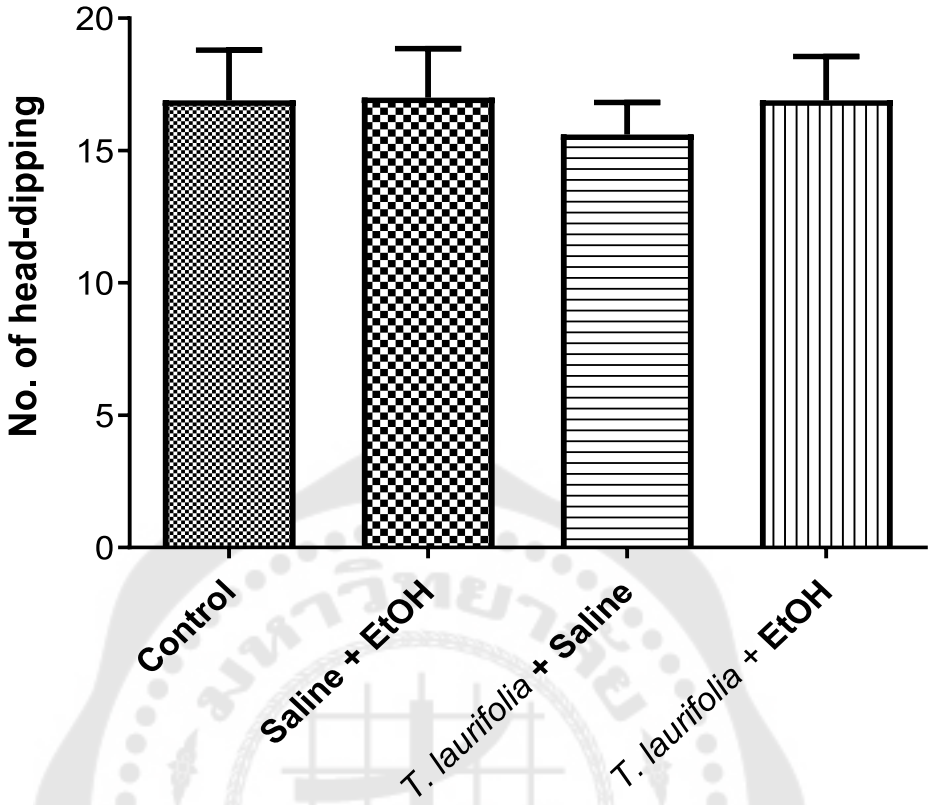

Figure 50 The head dipping in the exploratory behavior test of control, ethanol, *T. laurifolia,* and *T. laurifolia* + ethanol groups.

Each group received either the saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. The exploratory behavior was determined after 30 consecutive days of different combination treatments by the hole board apparatus. Each group was counted the number of head-dipping on hole board for 10 minutes. The data were mean±SEM (control n = 10, EtOH n = 10, *T. laurifolia* n = 10, *T. laurifolia* + EtOH n = 10). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 10 The number of rearing and head-dipping in hole board (Mean±SEM).

Groups	Rearing	Head-dipping
Control	30.30 ± 2.31 (n = 10)	16.90 ± 1.90 (n = 10)
Saline+ Ethanol	30.60 ± 2.98 (n = 10)	17.00 ± 1.86 (n = 10)
T. laurifolia + Saline	27.40 ± 2.96 (n = 10)	15.60 ± 1.22 (n = 10)
T. laurifolia + Ethanol	32.60 ± 3.00 (n = 10)	16.90 ± 1.66 (n = 10)

The chronic study of *T. laurifolia* and ethanol effect on tyrosine hydroxylase of nigrostriatal dopamine pathway in rats

The chronic effect of *T. laurifolia* and ethanol administration on tyrosine hydroxylase expression level in the striatum was determined by western blot. The expression and localization of tyrosine hydroxylase in the substantia nigra used the immunohistochemistry to determine. The mean±SEM of all group treatments is shown in Table 11.

Tyrosine hydroxylase expression level in the striatum (figure 51) displayed a significant ($p < 0.05$) decreased in the chronic ethanol administration group. The result showed that chronic ethanol administration reduced the level of tyrosine hydroxylase up to 79% when compared to that of the control value. Chronic *T. laurifolia* administration alone did not exhibit a decrease of the tyrosine hydroxylase expression level when compared to that of the control group. Daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days significantly ($p < 0.05$) protected a decrease in tyrosine hydroxylase expression level in the striatum. The tyrosine hydroxylase expression level in the *T. laurifolia* plus ethanol group exhibited a higher level than that of the chronic ethanol administration up to 77%.

The tyrosine hydroxylase-immunoreactive of the substantia nigra was used to validate the expression and localization of tyrosine hydroxylase in all groups. The localization of tyrosine hydroxylase exhibited in substantia nigra dopaminergic neurons in all groups (figure 52). The expression of tyrosine hydroxylase of the substantia nigra remained unchanged when compared to the control group. However, *T. laurifolia* prior to ethanol administration for 30 consecutive days significantly ($p < 0.05$) increased the expression of tyrosine hydroxylase of the substantia nigra compared with the ethanol group. (figure 53)

These results indicated that chronic ethanol administration induced a decrease of the tyrosine hydroxylase expression level in the striatum but not in the substantia nigra. The results also indicated that the daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days protected the decrease in the tyrosine hydroxylase expression level in the striatum induced by chronic ethanol administration.

Chronic *T. laurifolia* administration showed no effect on the tyrosine hydroxylase expression level of the striatum and substantia nigra.

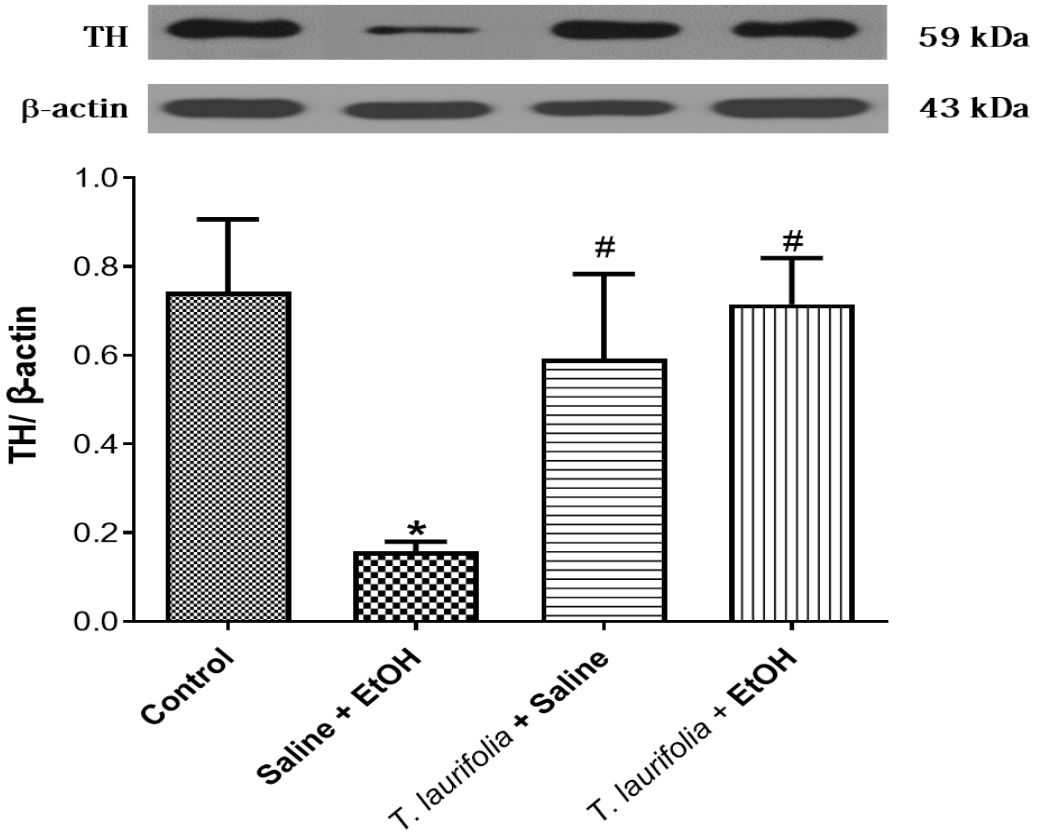

Figure 51 The tyrosine hydroxylase expression levels in the striatum of four groups (chronic study).

The expression ratio of tyrosine hydroxylase enzyme (TH) to β-actin in the striatum of control, ethanol, *T. laurifolia,* and *T. laurifolia* + Ethanol group. Each group received saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Tyrosine hydroxylase expression was determined by **western blot analysis**. Representative bands from different four groups were shown. The protein band was quantified via densitometry and normalized by β-actin. The results are mean±SEM (control n = 5, EtOH n = 5, *T. laurifolia* n = 5, *T. laurifolia* + EtOH n= 4). $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with the ethanol group using a one-way ANOVA and Newman-Keuls multiple comparison test.

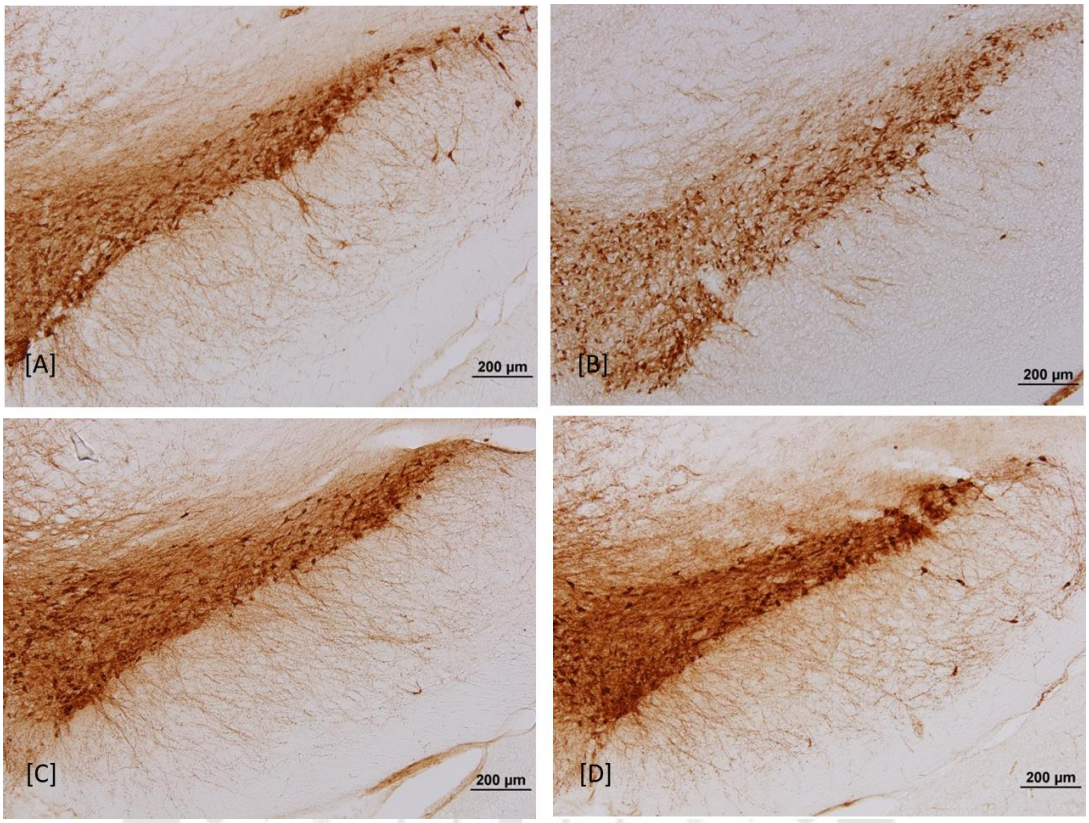

Figure 52 Tyrosine hydroxylase localization in the substantia nigra of four groups (chronic study).

Photomicrographs showing tyrosine hydroxylase localization in dopaminergic neurons. The tyrosine hydroxylase-immunoreactive neurons in the substantia nigra of control (A), ethanol (B), *T. laurifolia* (C), and *T. laurifolia* +EtOH groups (D), Scale bar, 200 µm. (n=3).

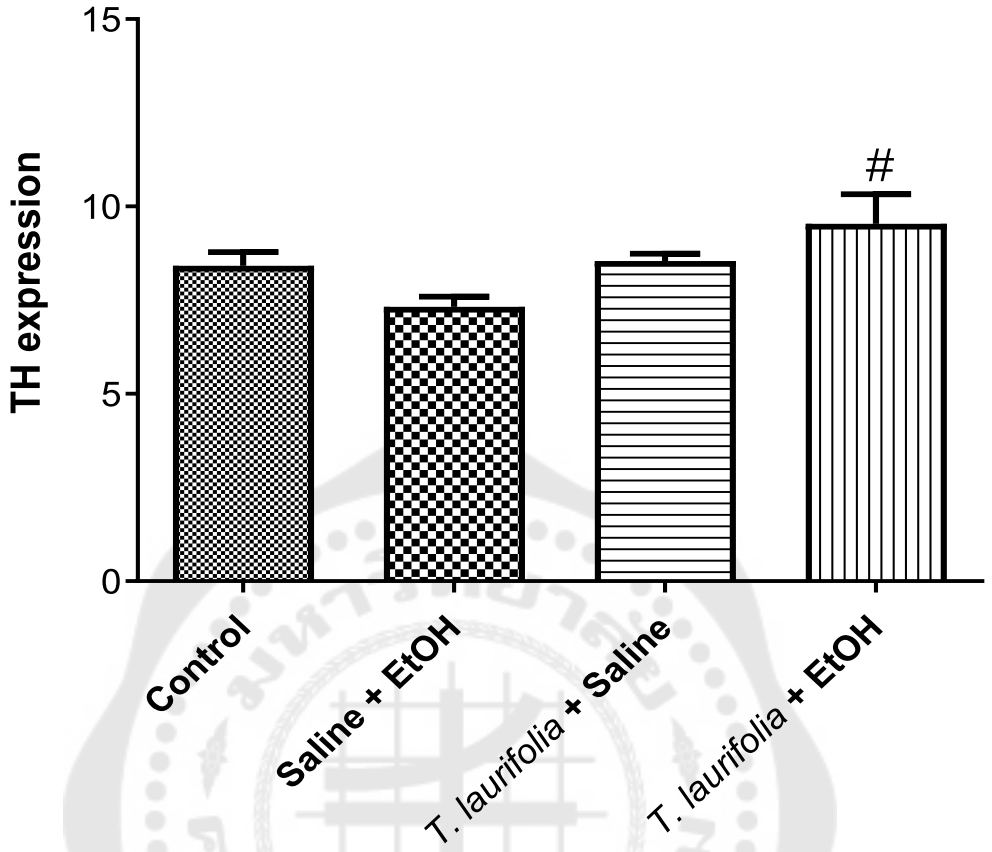

Figure 53 Tyrosine hydroxylase expression in the substantia nigra of four groups (chronic study).

Semiquantitative densitometric analysis of tyrosine hydroxylase-immunoreactivity in the substantia nigra was performed in control, ethanol, *T. laurifolia*, and *T. laurifolia* + Ethanol groups. Each group received saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol, respectively. The expression was quantified by image J. Statistical analysis all data are expressed as mean±SEM (n=3). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

The chronic study of *T. laurifolia* and ethanol effect on tyrosine hydroxylase of mesolimbic dopamine pathway in rats

The tyrosine hydroxylase expression level in the nucleus accumbens altered by chronic *T. laurifolia* and ethanol administration was determined by western blot. The expression and localization of tyrosine hydroxylase of the ventral tegmental area were determined by immunohistochemistry method. The mean±SEM of all group treatments is shown in Table 12.

Tyrosine hydroxylase expression level of the nucleus accumbens (figure 54) displayed a significant ($p < 0.01$) decreased in the chronic ethanol administration when compared to that of the control group. The result exhibited a decrease to 66% when compared to that of the control value. Chronic *T. laurifolia* administration showed no significant difference in the tyrosine hydroxylase expression level when compared to that of the control group. Daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days showed a significant ($p < 0.01$) protective effect on chronic ethanol administration intoxication. The tyrosine hydroxylase expression level increased up to 63% of the chronic ethanol administration group.

The tyrosine hydroxylase-immunoreactive of the ventral tegmental area was used to determine the expression and localization of tyrosine hydroxylase in all groups. The tyrosine hydroxylase localization was exhibited in ventral tegmental dopaminergic neurons in all groups (figure 55). The expression of tyrosine hydroxylase of the ventral tegmental area was not significantly different in all groups. The daily administration of *T. laurifolia* before ethanol and chronic *T. laurifolia* administration for 30 consecutive days did not show any effect on the tyrosine hydroxylase expression (figure 56).

These results indicated that chronic ethanol administration produced a decrease in the tyrosine hydroxylase expression in the nucleus accumbens but not in the ventral tegmental area. Daily administration of *T. laurifolia* before ethanol administration for 30 consecutive days produced the protective effect on the tyrosine hydroxylase of the nucleus accumbens induced by chronic ethanol administration. Furthermore, chronic *T. laurifolia* administration did not decrease the tyrosine hydroxylase expression in the nucleus accumbens or ventral tegmental area.

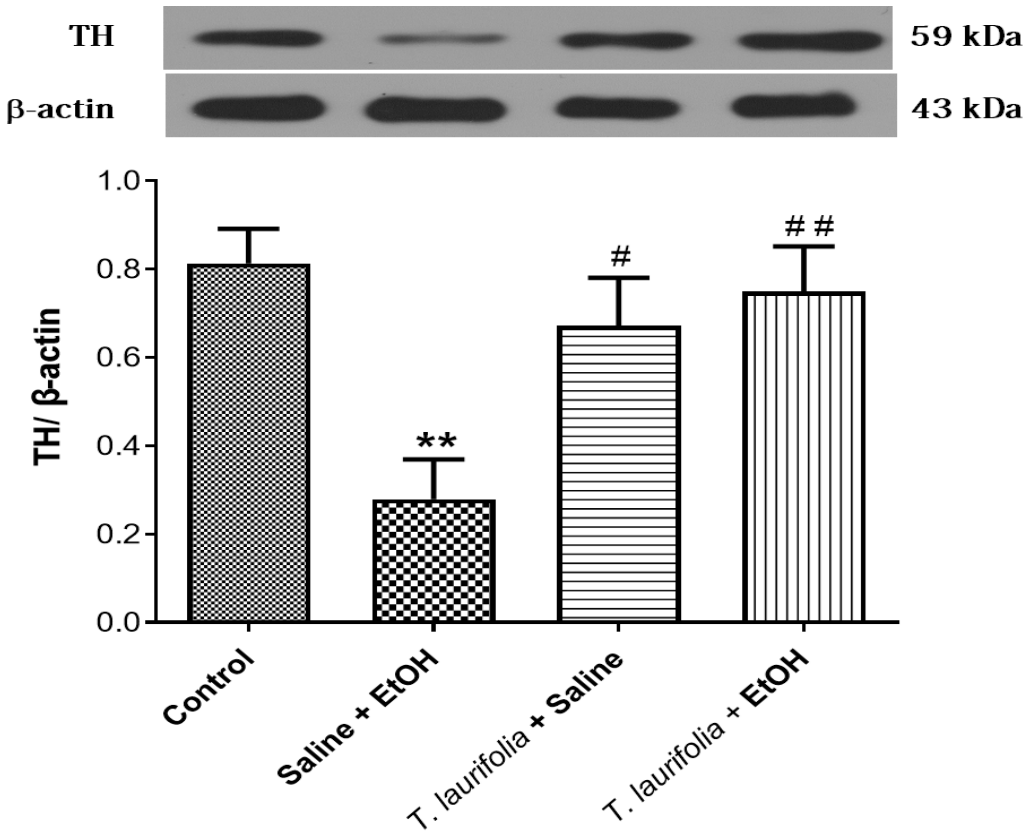

Figure 54 Tyrosine hydroxylase expression levels of nucleus accumbens of four groups (chronic study).

The expression ratio of tyrosine hydroxylase enzyme (TH) to β-actin in the nucleus accumbens of control, ethanol, *T. laurifolia,* and *T. laurifolia* + Ethanol group. Each group received saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol. Tyrosine hydroxylase expression was determined by **western blot analysis**. Representative bands from different four groups were shown. The protein band was quantified via densitometry and normalized by β-actin. The results are mean±SEM (control n = 5, EtOH n = 5, *T. laurifolia* n = 5, *T. laurifolia* + EtOH n= 4). $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with the ethanol group using a one-way ANOVA and Newman-Keuls multiple comparison test.

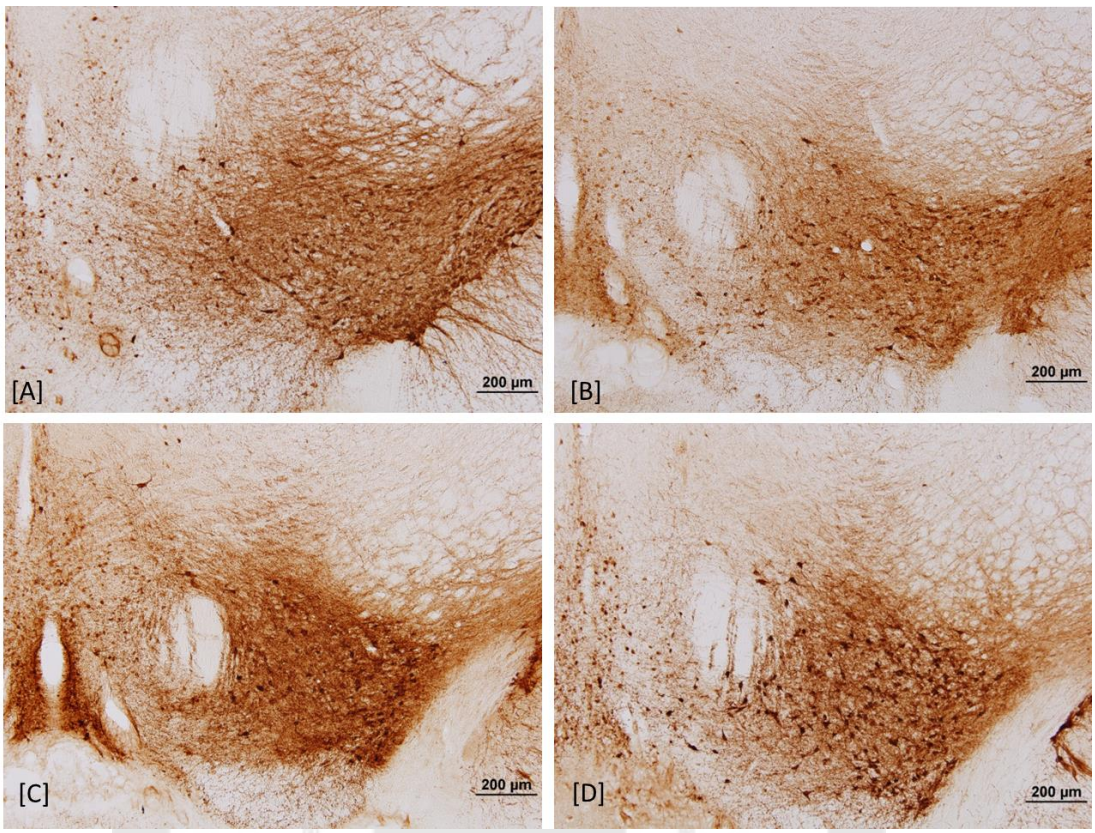

Figure 55 Tyrosine hydroxylase localization in the ventral tegmental area of four groups (chronic study).

Photomicrographs showing tyrosine hydroxylase localization in dopaminergic neurons. The tyrosine hydroxylase-immunoreactive neurons in the ventral tegmental area of control (A), ethanol (B), *T. laurifolia* (C), and *T. laurifolia* +EtOH groups (D), Scale bar, 200 µm. (n=3).

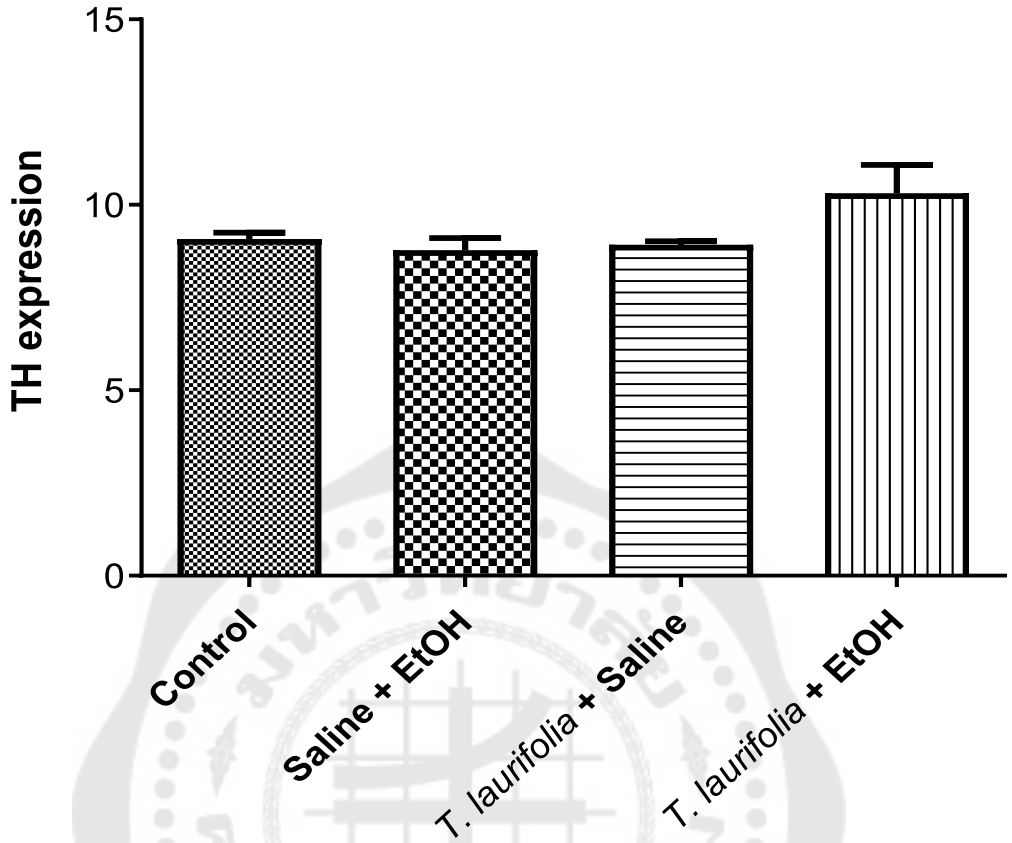

Figure 56 Tyrosine hydroxylase expression in the ventral tegmental area of four groups (chronic study).

Semiquantitative densitometric analysis of tyrosine hydroxylase-immunoreactivity in the ventral tegmental area was performed in control, ethanol, *T. laurifolia*, and *T. laurifolia* + Ethanol groups. Each group received saline, ethanol (1 g/kg, 15% ethanol), *T. laurifolia* (200 mg/kg), or pre- *T. laurifolia* + ethanol, respectively. The expression was quantified by image J. Statistical analysis all data are expressed as mean±SEM (n=3). Statistical analysis was examined by using a one-way ANOVA and Newman-Keuls multiple comparison test.

Table 11 The expression of tyrosine hydroxylase of nigrostriatal pathway (Mean±SEM).

Groups	Striatum	Substantia nigra
Control	0.74 ± 0.16 (n = 5)	8.42 ±0.63 (n = 3)
Saline + Ethanol	0.16 ± 0.02* (n = 5)	7.32 ± 0.46 (n = 3)
T. laurifolia + Saline	0.59 ± 0.19$^{\#}$ (n = 5)	8.54 ± 0.35 (n = 3)
T. laurifolia + Ethanol	0.71 ± 0.10$^{\#}$ (n = 4)	9.54 ± 1.38$^{\$}$ (n = 3)

*$p < 0.05$ compared with the control group

$^{\#}p < 0.05$ compared with the ethanol group

Table 12 The expression of tyrosine hydroxylase of mesolimbic pathway (Mean±SEM).

Groups	Nucleus accumbens	Ventral tegmental area
Control	0.81 ± 0.08 (n = 5)	9.07 ±0.29 (n = 3)
Saline+ Ethanol	0.28 ± 0.09** (n = 5)	8.78 ± 0.56 (n = 3)
T. laurifolia + Saline	0.67 ± 0.11$^{\#}$ (n = 5)	8.93 ± 0.16 (n = 3)
T. laurifolia + Ethanol	0.75 ± 0.10$^{\#\#}$ (n = 4)	10.31 ± 1.3 (n = 3)

**$p < 0.01$ compared with the control group

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the ethanol group

CHAPTER V
DISCUSSIONS AND CONCLUSIONS

T. laurifolia extract identify

In the present study, *T. laurifolia* leaves were collected from the same place in September to December 2015. The four extractions that were used in our experiment were collected and extracted in November and December 2015. These extractions were identified by using a thin-layer chromatography method. The pattern of *T. laurifolia* showed seven bands in each extraction. Each band of each extraction presented the same distance on TLC paper. This indicates that four times of extraction showed the same components of *T. laurifolia* and the rats in our experiments were given the same components of *T. laurifolia*.

Acute and chronic ethanol induced nigrostriatal and mesolimbic dopaminergic pathways alterated

Ethanol effect on nigrostriatal dopaminergic pathway

Nigrostriatal dopaminergic pathway, substantia nigra dopaminergic neurons project axons and release dopamine to striatum. The binding to these receptors induce different responses of striatal MSNs followed by stimulating or inhibiting movement or locomotor activation [(140)]. In the present study, we used 2 g/kg, 20% ethanol in an acute study and 1 g/kg, 15% ethanol in a chronic study [(49)]. Both doses of ethanol presented a steady increase in blood alcohol concentration of male Wistar rats treated [(214)].

Acute ethanol-induced motor coordination impairment and increased tyrosine hydroxylase expression level in striatum.

In this present study, the investigation showed that acute ethanol administration produced motor coordination impairment in rats. The motor coordination impairment induced by acute ethanol has been reported both in adolescent and adult rats within 10 minutes after 2 g/kg ethanol administration [(49)] and continue to impair at the time of 15, 30, 60, 120, and 180 minutes [(34)] after ethanol administration and hangover [(50)] using tilting plane test. There is a strong suggestion in the relation of acute ethanol

produced impairment of motor coordination and increase in locomotor of humans and rodents [169]. However, there is a controversy of motor coordination impairment responding to ethanol. The previous study showed no response to acute ethanol at a dose of 0.5 g/kg [215].

Tyrosine hydroxylase-immunoreactive in substantia nigra neurons and these axon fibers in striatum were presented [193]. Our immunohistochemistry observation in substantia nigra showed the controversy of previous reports that ethanol did not induce tyrosine hydroxylase expression enhancement. Some evidence showed unchanged in tyrosine hydroxylase activity unchanged in substantia nigra at 15 minutes following ethanol intraperitoneally injection in male short sleep and long sleep mice [216] and continue to see at 1 hour following ethanol administration in male Sprague-Dawley albino rats treated [217]. These investigations indicate a difference of brain sensitivity for ethanol stimulation. However, our finding by using western blot analysis showed an increase in the expression level of tyrosine hydroxylase protein in axon fibers of substantia nigra dopaminergic neurons extending into striatum that correlated with motor coordination impairment in rats. Many reports found to increase tyrosine hydroxylase by acute ethanol. Tyrosine hydroxylase gene expression was found in acute ethanol exposure to N1E-115 neuroblastoma cells [218]. Acute intraperitoneal injection of ethanol caused the enhancement of tyrosine hydroxylase activity in the striatum and other brain areas [216]. These enhancements of gene expression, tyrosine hydroxylase protein and its activity via phosphorylation, and dopamine synthesis and release produced by acute ethanol corresponded to an increase in locomotor activity [191], speed of movement [219] and motor hyperactivity [220]. These investigations may indicate a relationship between motor coordination impairment and an increase in tyrosine hydroxylase expression in our findings. Ethanol-induced an increase in tyrosine hydroxylase protein expression in striatum may induce an imbalance of tyrosine hydroxylase activity and production of dopamine. This imbalance may produce impairment of motor coordination in rats. Another possible reason for explaining is that

ethanol may alter the function of cerebellum such as Purkinje cell function and induce an impairment of motor coordination and balance [221]

Chronic ethanol-induced motor coordination impairment and decreased tyrosine hydroxylase expression level in striatum

Our finding showed that chronic ethanol administration for 30 consecutive days produced motor coordination impairment by using the number of hindlimb slips as an indicator. Motor coordination impairment has been found in acute [34, 49], chronic administration and withdrawal stage. Chronic ethanol administration for 55 days from adolescence to adulthood rats exhibiting that the rats receiving daily administration of 6.5 g/kg showed the impairments of motor coordination and muscle strength [172]. Additionally, chronic ethanol administration produced an impairment of motor coordination function [222] and continued toward the withdrawal stage [223] by enhancing the number of foot slip and latency time to climb on the walking beam.

Chronic ethanol administration was found to decrease tyrosine hydroxylase-immunoreactive in substantia nigra dopaminergic neurons and significantly decreased tyrosine hydroxylase expression in axon fibers of substantia nigra dopaminergic neurons extending into striatum. Down level of dopamine system showed a decrease in tyrosine hydroxylase [224], dopamine release [225, 226], and input/output function in response to stimulation intensity in striatum [227] in chronic ethanol administration. These findings may implicate in ethanol intoxication-induced dopaminergic neurons of substantia nigra and their fibers extending into striatum damages and neuronal cell death. Reactive oxygen species (ROS) act as toxic molecules, oxidizing membrane lipids, changing the conformation of proteins, damaging nucleic acids, and causing deficits in synaptic plasticity [228] leading to neurodegeneration [229]. Ethanol metabolism via cytochrome P450-2E1 (CYP2E1) produced ROS production such as superoxide anions (O^{-}_{2}) and hydrogen peroxide (H_2O_2) [230]. Dopamine metabolism by monoamine oxidase stimulated Ca^{2+} signal which produces ROS, induces lipid peroxidation [231], and damages dopaminergic neurons [232]. Additionally, ROS could increase the abnormal accumulation of neurofilaments which appear in several neurodegenerative diseases such as Parkinson's disease [233].

Furthermore, a report on an effect of ethanol to induce cytokine releasing such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8, IL-10, and transforming growth factor-beta 1, TGF-β1 which associate with the promotion of pro-apoptotic mechanisms [234].

Ethanol effect on mesolimbic dopaminergic pathway

Mesolimbic dopaminergic pathway, dopaminergic neurons of ventral tegmental extend their axons into nucleus accumbens. The dopamine release into nucleus accumbens regulates rewarding system and involve in addiction behavior [145], and anxiety behavior [143]

Acute ethanol administration did not induce mesolimbic dopaminergic function altered

Our finding showed the conversely results to previous reports that acute ethanol did not induce an alteration of anxiety and exploratory behaviors in rats. These findings may be explained that many factors may involve in the production of ethanol-induced anxiety and exploratory behavior changes. For instance, the different doses of ethanol showed different anxiety responses. Previous report suggested that a low dose (1 g/kg) of ethanol did not induce anxiety or anxiolytic effect whereas a moderate dose (2 and 2.25 g/kg) exhibited the anxiolytic effect. The high doses (2.5 and 3 g/kg) of ethanol produced sedation [235]. These studies were performed using adolescent male Sprague Dawley (SD) rats. Sex and gene induced a diversity of anxiety responses to ethanol were shown [236]. A difference of Wistar rat strains was observed diversity of anxiety behavior in response to ethanol administration. Anxiolytic behavior was exhibited in Wistar–Harlan strain but could not in Wistar–BgVV strain [237]. From our results of the exploratory behavior test, the number of rearing and head-dipping in hole board apparatus test was not found to change by acute ethanol administration. There are controversial results in the exploratory behavior studies in different animal models. The head-dipping response to ethanol administration of 2 strains mice were reported in responses to 0.8, 1.6, and 2.4 g/kg ethanol administration. The difference of the number of head-dipping in C57Bl/6 mice was not observed while the head-dipping of DBA/2 mice was decreased after treated with ethanol at a dose of 2.4 g/kg [238]. These

evidences may suggest that the different behavioral responses to ethanol administration depend on doses of ethanol administration, sex of animals, or animal strains.

Immunoreactive of tyrosine hydroxylase and expression level did not increase in the ventral tegmental area and nucleus accumbens, respectively. We considered that these results exhibit the difference of brain sensitivity for ethanol stimulation. The tyrosine hydroxylase was not observed to increase in the ventral tegmental area by acute ethanol administration [239]. Furthermore, there was an increase in the dopamine outflow in the core but not the shell of the nucleus accumbens [240]. Besides, various studies have been reported for the detection time of ethanol stimulating tyrosine hydroxylase changes of the dopaminergic neurons. The level of tyrosine hydroxylase gene expression of the ventral tegmental area did not observe the linear fashion but found the increase following by the decrease depending on time [241]. Moreover, ethanol stimulated dopamine enlargement in the ventral tegmental area within 40 minutes before returned to baseline within 60 minutes after administration. Meanwhile, dopamine in nucleus accumbens was found to increase within the first 20 minutes of ethanol administration. The researchers have a suggestion that an increase followed by a decrease may mediating with activation of a negative feedback system to the ventral tegmental area and different mechanisms on dopaminergic neuronal activity and terminal dopamine release [242].

Chronic ethanol-induced addiction and decreased tyrosine hydroxylase expression in nucleus accumbens.

Our behavior findings exhibited ethanol addiction but did not change in anxiety and exploratory behavior in chronic ethanol administration. Several publications have been suggested the addiction behavior and enhancement of ethanol preference in long-term ethanol administration using variation of sex, ages, stains, gene, or dose of ethanol [243-245]. Anxiety and exploratory behavior changes by chronic ethanol administration were not found in our studies which is in consistence with previous studies [246, 247]. However, several reports were showed the difference behavioral responses. For example, anxiety behavior was examined in chronic ethanol administration and withdrawal rats. They found that anxiety or anxiolytic effect were not

observed in chronic ethanol administration rats but found in withdrawal rats [248]. The alteration of exploratory behavior was not presented in female rats treated with ethanol for 42 days [249].

Tyrosine hydroxylase-immunoreactive in ventral tegmental area did not decrease whereas expression in axon fibers of dopaminergic neurons in ventral tegmental area extending into nucleus accumbens decreased. This finding may involve a local regulation of ventral tegmental area dopaminergic neurons by intrinsic GABA neurons via the D_2 receptor [250] or by GABAergic inputs from the rostromedial tegmental nucleus [251]. That regulation may result in disinhibition of GABA release to ventral tegmental area and reduce in dopaminergic neurons of ventral tegmental area response to ethanol stimulation. Moreover, there was strongly reported a decrease in ventral tegmental area dopaminergic neurons firing in chronic ethanol [252]. Disinhibition of GABA release into ventral tegmental area and a decrease in dopaminergic neurons firing may explain our finding that a decrease in tyrosine hydroxylase expression in axon fibers of dopaminergic neurons in ventral tegmental area extending into nucleus accumbens. Previous investigation has shown a decrease in tyrosine hydroxylase [253, 254], dopamine release [255], presynaptic dopamine transmission [256], or enhancement of dopamine reuptake by the dopamine transporter (DAT) [257].

Acute and chronic prevention of *T. laurifola* on ethanol-induced nigrostriatal and mesolimbic dopaminergic pathways altered.

Acute and chronic *T. laurifolia* administration alone

In the present study, we used 200 mg/kg *T. laurifolia* for giving to the rats in acute and chronic studies. *T. laurifolia* at a dose of 200 mg/kg was reported to increase dopamine release in the rat striatal tissue slides [15] and nucleus accumbens [18]. In our experiments, behaviors and tyrosine hydroxylase expression were analyzed at 90 minutes after *T. laurifolia* oral administration in an acute study and analyzed on day 31 after 30 consecutive days of treatments in a chronic study. We found that acute and chronic *T. laurifolia* administrations alone did not change behaviors and tyrosine hydroxylase expression in the nigrostriatal and mesolimbic dopaminergic pathways. Our

behavior results are correlated with the previous report that the *T. laurifolia* administrations at doses of 1 and 10 g/kg for 30 consecutive days did not cause addiction behavior in male Wistar rats using CPP [19].

The results from the functional magnetic resonance imaging study in anesthetized rats reported that *T. laurifolia* stimulated the neuronal activity of several brain areas such as the striatum and nucleus accumbens but not in the ventral tegmental area and substantia nigra [16]. Additionally, *T. laurifolia* administration was reported to increase dopamine release in the rat striatal tissue slides [15] and nucleus accumbens [18]. These observations indicate that *T. laurifolia* may stimulate dopamine release at the presynaptic axon terminal of dopaminergic neurons of substantia nigra and ventral tegmental area but not at the cell body. This can explain our result that *T. laurifolia* could not change the expression of tyrosine hydroxylase in substantia nigra and ventral tegmental area. In our finding, tyrosine hydroxylase expression in striatum and nucleus accumbens exhibited the controversy results with previous reports. This can be explained a previous report suggesting that an oral administration of *T. laurifolia* also stimulated extracellular dopamine release in the nucleus accumbens within 20 minutes and decreased at 40 minutes after administration [18]. This evidence may explain why *T. laurifolia* administration alone could not observe the increases of tyrosine hydroxylase expression of the nucleus accumbens and striatum in *T. laurifolia* administration alone.

Acute prevention of *T. laurifolia* on ethanol intoxication

In our studies on the protective effect of *T. laurifolia* on acute ethanol administration, acute ethanol giving to the rats after 1 hour of *T. laurifolia* administration was resemble to the study of Ussanawarong S and colleague studied a protective effect of *T. laurifolia* on rat treated with parathion in the year 2001 [67]. We found that *T. laurifolia* can improve motor coordination impairment caused by acute ethanol administration. *T. laurifolia* administration prior to acute ethanol administration could protect the effect of the acute ethanol produced motor coordination impairment and an increase in tyrosine

hydroxylase expression level in axon fibers of dopaminergic neurons of substantia nigra extending into the striatum.

We suggest that *T. laurifolia* may increase ethanol elimination and decrease acetaldehyde production to reduce their intoxication. The previous report exhibited an increase of P- glycoprotein and a decrease in some cytochrome P450 isoenzymes such as cytochrome P450 2E1 or CYP2E1 in HepG2 cells treated with *T. laurifolia* [(258)]. P-glycoprotein expresses in hepatocytes and the blood-brain barrier [(259)] and has a function as a biological barrier by extruding toxins and xenobiotics out of cells [(260)]. An increase in P-glycoprotein expression by *T. laurifolia* located at the biliary canalicular membrane of hepatocytes may increase excretion of ethanol out of the cell resulting in a decrease in systemic ethanol concentration and ethanol entering into the brain. Moreover, an increase in P-glycoprotein expression at the blood-brain barrier may reduce the penetration of ethanol into neirons leading to the reduction of ethanol intoxication-induced neuronal activity, functions, and behaviors. The CYP2E1 plays a significant role in ethanol metabolism in the brain by oxidizing ethanol to acetaldehyde [(261)]. A decrease in CYP2E1 may reduce acetaldehyde production in dopaminergic neurons and other neurons leading to reduce acetaldehyde intoxication-induced abnormal neuronal activity, functions, and behaviors. Therefore, a decrease of ethanol in systemic ethanol concentration, entering into the brain and acetaldehyde production in the dopaminergic neurons may protect dopaminergic neurons stimulated [(262)] and abnormal behavior produced, such as euphoria, anxiolytic, hypnotic, amnesiac, aggression, reinforcement, or aversion to voluntary ethanol consumption as well as motor coordination impairment, by ethanol and acetaldehyde [(263)].

Chronic prevention of *T. laurifolia* on ethanol intoxication

The daily administration of *T. laurifolia* prior to ethanol administration for 30 consecutive days can protect ethanol addiction and motor coordination impairment in rats. We have also found the effect of *T. laurifolia* on the tyrosine hydroxylase expression level in axon fibers of dopaminergic neurons of the substantia nigra and ventral tegmental area extending into the striatum and nucleus accumbens recovering the

levels up to the normal control levels. The daily protective effect of *T. laurifolia* on chronic ethanol administration may assume that *T. laurifolia* may decrease ethanol and acetaldehyde intoxication, protect hepatocytes and dopaminergic neurons damaged by ethanol and its metabolism agent, and/or indirectly regulate D_3 receptor, which implicates with the functions of nigrostriatal and mesolimbic dopaminergic pathways, via brain-derived neurotrophic factor (BDNF).

The first possible effect is that *T. laurifolia* may decrease ethanol and acetaldehyde intoxication to induce neuronal activity and behaviors abnormal by an increase in ethanol excretion and a decrease in acetaldehyde production in hepatocytes and brain. The report found that *T. laurifolia* could increase the expression of P-glycoprotein activity and cytochrome P450 (CYP450) but decreased some isoform of cytochrome P450, such as cytochrome P450 2E1 or CYP2E1 [258], that involved the efflux of drugs or several toxics and ethanol metabolism in hepatocytes and neurons.

The next possible effect of *T. laurifolia* is that *T. laurifolia* may protect hepatocytes and dopaminergic neurons damaged by ethanol and its metabolism induction. *T. laurifolia* effect on protecting ethanol intoxication is may be due to its antioxidant property. *T. laurifolia* has been reported to exhibit large quantities of phenolic compounds such as delphinidin 3:5-di-O-β-d-glucopyranoside, chlorogenic acid, apigenin, and 7-O-β-d-glucopyranoside [1]. These phenolic compounds act as antioxidant agents [1, 70, 264, 265]. In the liver, *T. laurifolia* effect on hepatocytes in *in vitro* and *in vitro* studies using aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as indicators. The finding showed the reduction in AST and ALT in hepatocytes after ethanol exposure and *T. laurifolia* recovered liver enzyme to the normal level in male Wistar rat treated with ethanol [72] and exhibited unchanged in pathological of the glomerulus in cadmium exposure rats [68]. In the brain, its antioxidant property protects neuron abnormality and dysfunction from various toxic substances. For example, *T. laurifolia* administration once a day prior to lead consumption for 8 weeks in male ICR mice found that total antioxidants in the rat brains were maintained normal. These results were in agreement with the finding of the reduction in neuronal cell

death, apoptosis activity by decreasing caspase-3 activity and cognitive impairment [266]. A combination between *T. laurifolia* and lead has shown to increase the acetylcholinesterase activity as well as decrease the cognitive impairment in the Morris water maze swimming test [267]. At the doses of 250 and 500 mg/kg/day, *T. laurifolia* improving effects on non-spatial short-term memory in olfactory bulbectomized mice (OBX) mice, an Alzheimer's disease model mice, which could be mediated by the normalization of central cholinergic systems due to the effects to reverse choline acetyltransferase and the muscarinic acetylcholine receptor M1 mRNA expression [96]. Furthermore, iridoid glycoside, the bioactive compound of *T. laurifolia* may act as a protective and treatment agent in neurotoxic and neurodegeneration conditions. Catapol is an iridoid glycoside that was extracted from the roots of Radix Rehmanniae. The study of catapol effect to protect and treatment in PC12 cells and rats induced Alzheimer's disease symptoms by beta-amyloid fragment 23–35 ($A\beta_{25-35}$) showed that catapol protected and recovered expression of choline acetyl-transferase expression and activity in PC12 cells and rats [268]. The density of muscarinic acetylcholine receptor M2 in CHO cell found to increase in the treatment of catapol [269]. Furthermore, the antioxidant property and the action of iridoid glycoside may protect abnormal protein expressions, neuronal cell death, and behaviors expression by ethanol and other toxic substances. Some researches have reported an increase BDNF after chronic consumption of an antioxidant dietary supplement [270, 271]. Furthermore, male Sprague–Dawley aged rats received iridoid glycoside, catapol, for 10 consecutive days found an up-regulation of BDNF and signaling molecule, protein kinase A (PKA) [272]. BDNF is an important factor to support differentiation of the neurons [273], involved in survival of neurons, [274] and exhibits a neuroprotective effect [275] under adverse conditions such as neurotoxicity from ethanol [276].

Additionally, BDNF increased by antioxidant property and iridoid glycoside may regulate D_3 receptor functions resulting to protect ethanol effect on the functions of nigrostriatal and mesolimbic dopaminergic pathways. The BDNF effect on the D_3 receptor expression in reward and motor areas and its involvement in

pathophysiological conditions such as drug addiction, schizophrenia, or Parkinson's disease [277-281]. Generally, the D_3 receptor is located both at pre- and post-synapses of dopaminergic neurons [282, 283]. The function of the D_3 receptor has been suggested to modulate dopamine synthesis and release [284]. Furthermore, several studies suggested that there was an up-regulation of the D_3 receptor in alcohol and drug abuse [285-289]. The studies of D_3 receptor functions have reported via D_3 receptor antagonist agents. The findings showed that the reduction in the movement was produced by actions of the D_3 receptor whereas blocking of the D_3 receptor increased of movement [290]. The selective D_3 receptor antagonist, SB-277011-A, injection to the non-alcohol preferring rats, the wild type rats, and the alcohol-preferring rats showed to decrease seeking behavior and ethanol intake. The alcohol-preferring rats showed to be more sensitive to SB-277011-A than the non-alcohol preferring rats [291, 292]. Other D_3 receptor antagonists, SB-277011-A and SR-21502, demonstrated either decrease or abolish drugs abuse induced addiction behavior using the conditioned place preference behavior model [293, 294]. D_3 receptors implicated with an increase in the number of intrinsic tyrosine hydroxylase-positive striatal neurons of CD1 mice [295].

The proposed explanation of *T. laurifolia* acute and chronic preventive effect on ethanol-induced behaviors and tyrosine hydroxylase expression in nigrostriatal and mesolimbic dopaminergic pathways changes.

Ethanol is a common drug abuse that affects in cellular and molecular levels and changes in the synaptic functions of dopaminergic pathway. Acute ethanol intoxication either stimulates or inhibits the function of proteins implicated in synaptic transmission [32]. Acute ethanol administration produces an alteration in nigrostriatal dopaminergic pathway by inducing motor coordination impairment. The impairment is correlated with an increase in tyrosine hydroxylase expression levels in axon fibers of substantia nigra dopaminergic neurons extending into the striatum. The mesolimbic dopaminergic pathway dose not respond to acute ethanol stimulation (figure 57A). The impairment of motor coordination and enhancements of dopaminergic neuron activity such as the increase in gene expression, protein, activity of tyrosine hydroxylase via

phosphorylation, dopamine synthesis and dopamine release by acute ethanol administration in previous investigations indicated that ethanol directly stimulates dopaminergic neuron activities and their functions. When *T. laurifolia* was administered prior to ethanol administration, the attenuation of the motor coordination impairment and the enhancement of tyrosine hydroxylase expression are exhibited in an acute preventive effect of *T. laurifolia* to ethanol intoxication (figure 57B). The traditional use and several studies strongly suggest the detoxification effects of *T. laurifolia*. Previous report showed that *T. laurifolia* increased P-glycoprotein and decreased CYP2E1 in HepG2 cells, hepatocytes [(258)]. These may leads to an increase in ethanol elimination and acetaldehyde production and decrease their intoxications to dopaminergic neurons (figure 60).

Chronic ethanol intoxication presents opposing and /or compensatory /homeostatic effects on the expression, localization, and function of proteins [(32)]. The administration of chronic ethanol for 30 consecutive days induces impairment of nigrostriatal dopaminergic function and damages of dopaminergic neurons in substantia nigra. These result in motor coordination impairment and decreases tyrosine hydroxylase expression in axon fibers of substantia nigra dopaminergic neurons extending into the striatum (figure 58A). The down-regulation of mesolimbic dopaminergic pathway including the reduction of dopaminergic neuronal activities resulting in abnormal behavior in chronic ethanol administration. In an addiction behavior study, unchanged of tyrosine hydroxylase expression in ventral tegmental area dopaminergic neurons, and decreased tyrosine hydroxylase expression levels in axon fibers of ventral tegmental area dopaminergic neurons extending into the nucleus accumbens displayed a down-response to chronic ethanol stimulation (figure 59A). The daily administration of *T. laurifolia* prior to chronic ethanol administration protects dopaminergic neurons stimulation and damaged by ethanol via detoxification effect (figure 58B, 59B) via detoxification effect [(258)], antioxidation properties [(1, 70, 264, 265)], and its bioactive compound actions [(268, 269)] (figure 60) which leads normal behaviors. Therefore, the acute and chronic preventive effect of *T. laurifolia* on ethanol-induced motor

coordination impairment may be useful to decrease road accidents from drivers in alcohol drinkers. An administration of *T. laurifolia* before alcohol drinking may decrease the risk to become alcoholism in heavy and social drinkers and neurodegenerative diseases.

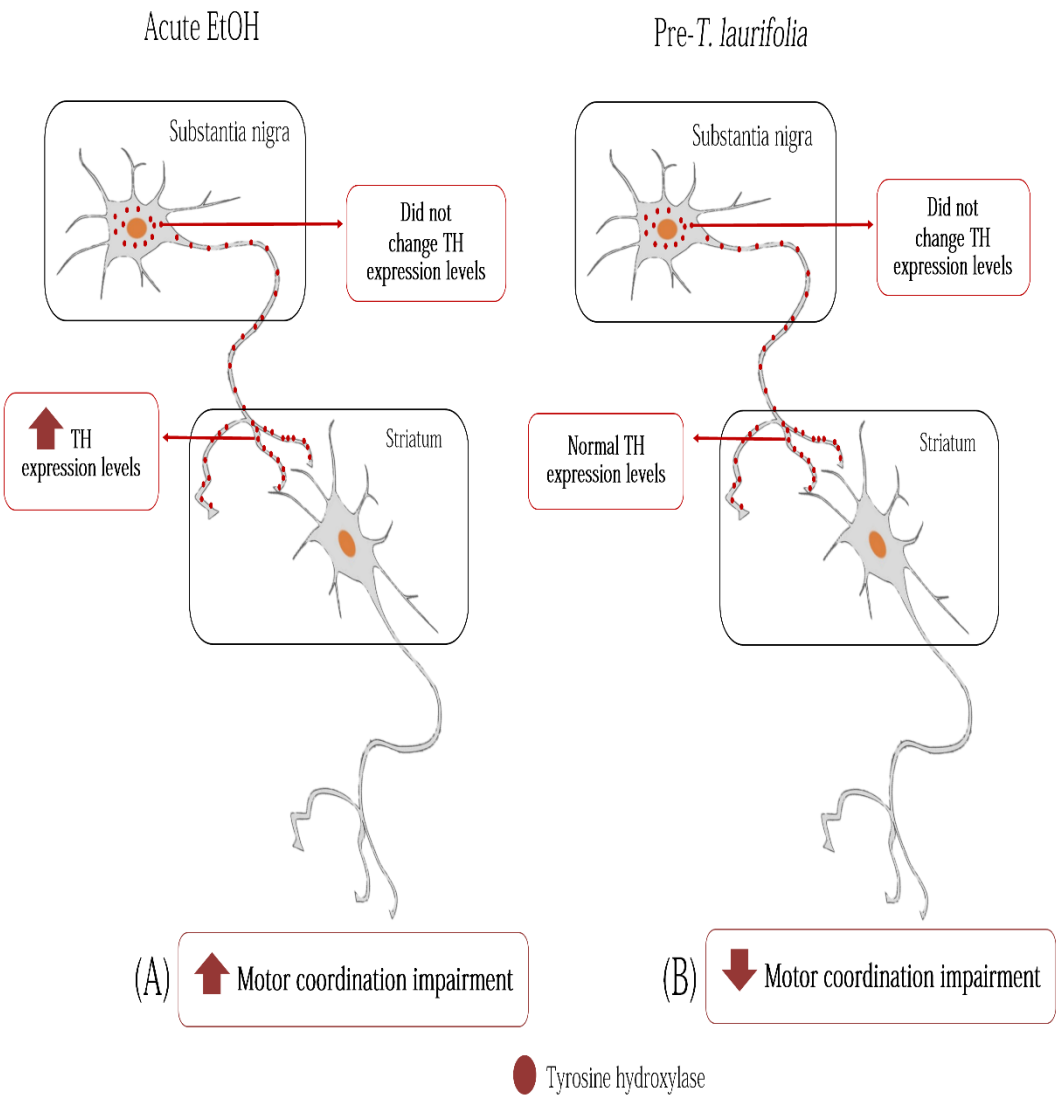

Figure 57 Acute ethanol intoxication and preventive effect of *T. laurifolia* on nigrostriatal pathway.

Acute ethanol-induced tyrosine hydroxylase expression levels in axon fiber of dopaminergic neurons enhancement and motor coordination impairment (A). *T. laurifolia* reverse tyrosine hydroxylase expression and abnormal behavior to normal (B).

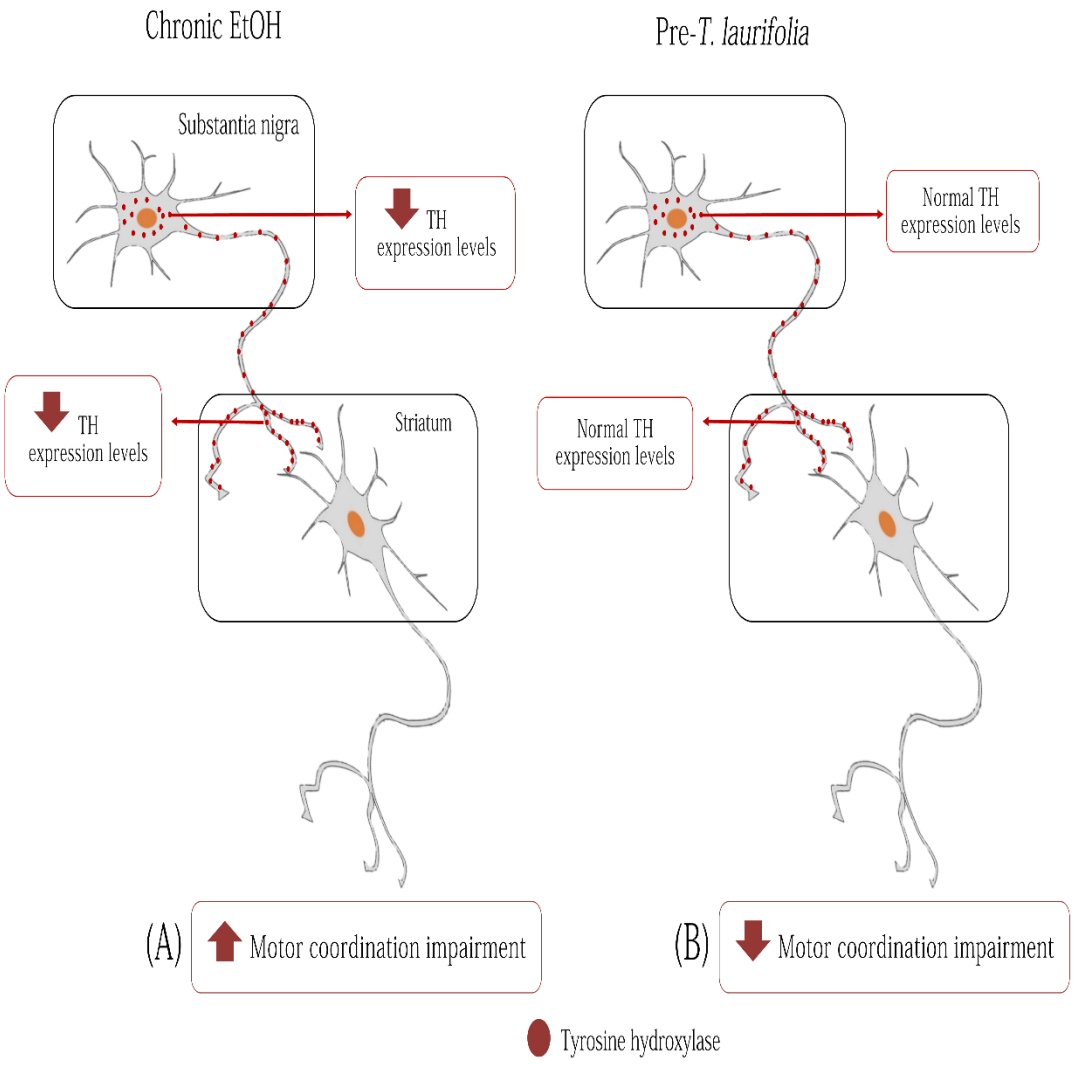

Figure 58 Chronic ethanol intoxication and preventive effect of *T. laurifolia* on nigrostriatal pathway.

The reduction of tyrosine hydroxylase expression level in substantia nigra and thier axon fiber extending into striatum results in motor coordination impairment in chronic ethanol (A). A daily *T. laurifolia* administration protects a decrease in tyrosine hydroxylase expression and an impairment of motor coordination by chronic ethanol (B).

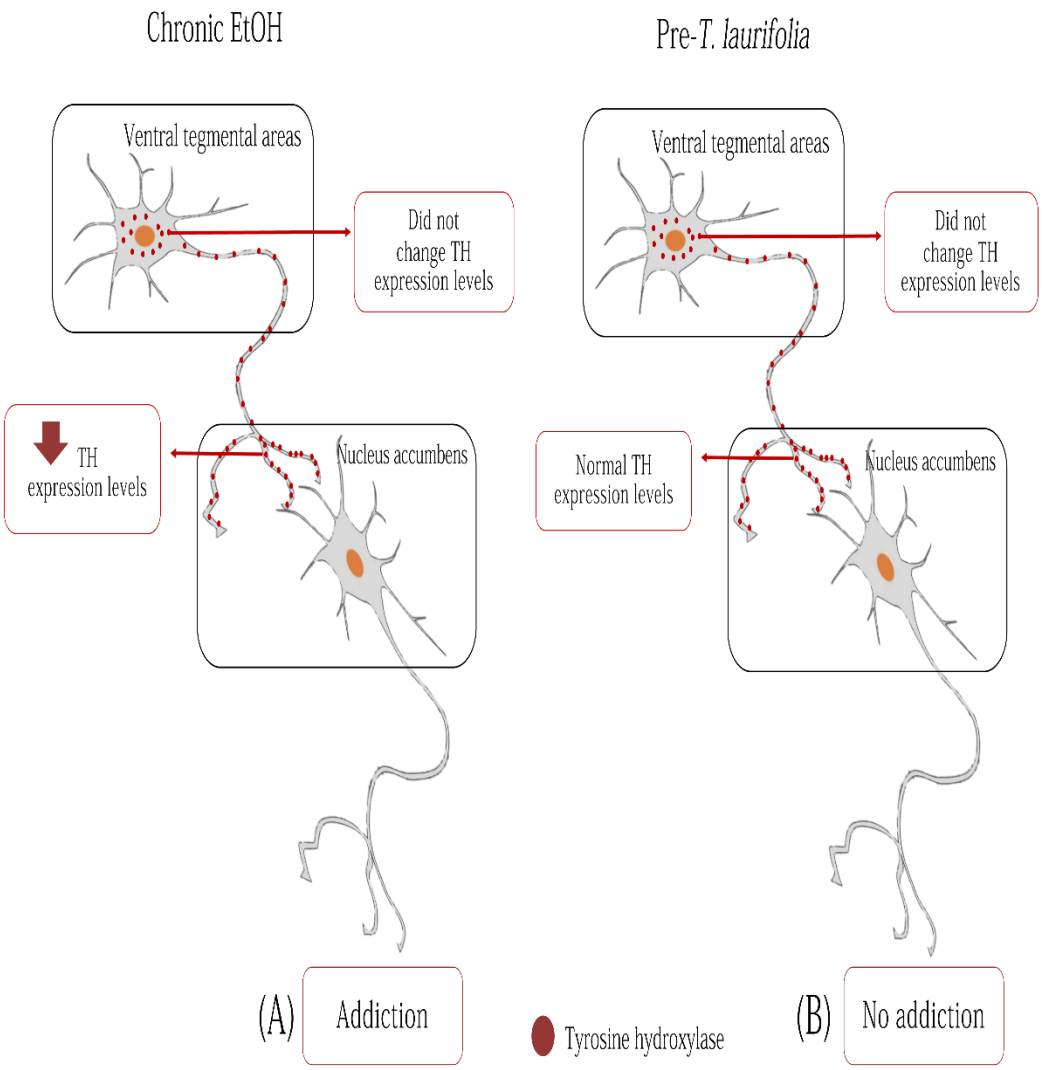

Figure 59 Chronic ethanol intoxication and preventive effect of *T. laurifolia* on mesolimbic pathway.

A reduction of tyrosine hydroxylase expression level in axon fiber of ventral tegemental area dopaminergic neurons and an addiction behavior induced by chronic ethanol (A). A daily *T. laurifolia* administration protects a decrease in tyrosine hydroxylase expression and an addiction behavior (B).

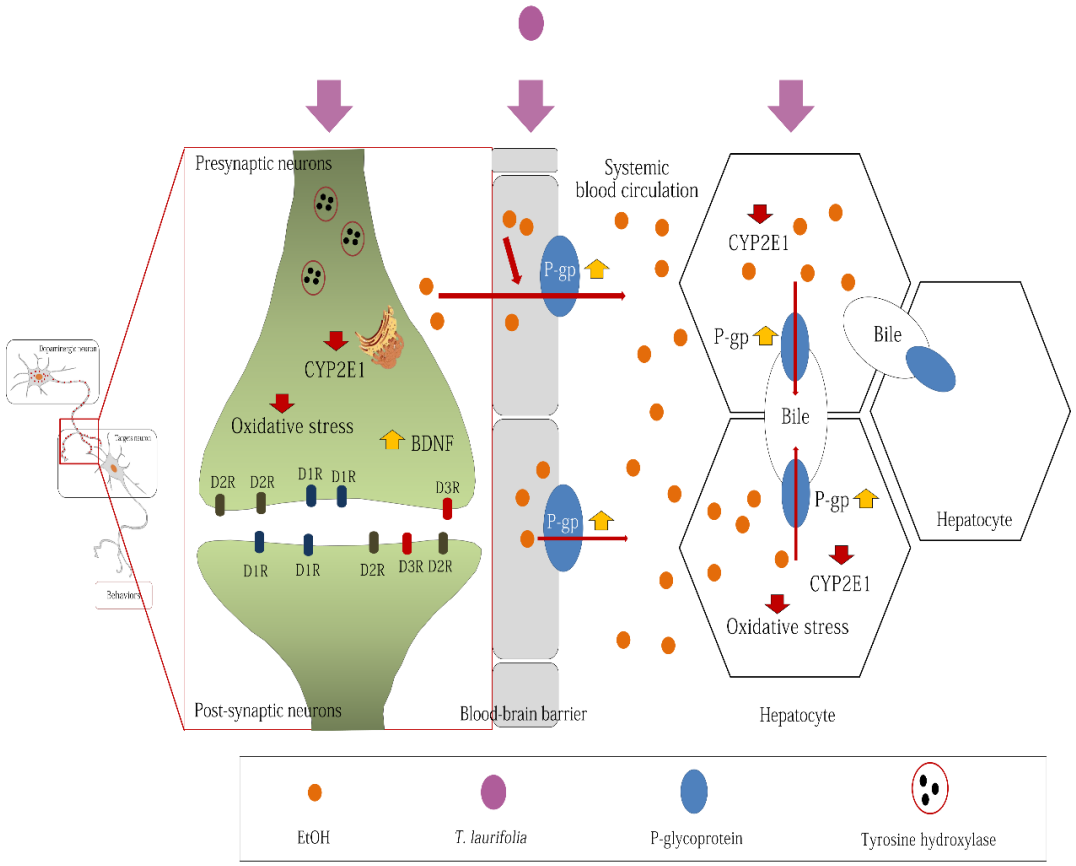

Figure 60 The expectation of *T. laurifolia* on protection from acute and chronic ethanol intoxication.

An acute preventive effect of *T. laurifolia* mainly decreases ethanol concentration and acetaldehyde production by induced ethanol excretion and reduced acetaldehyde production in the hepatocytes and blood-brain barrier via an increase in P-glycoprotein and decrease in CYP2E1. Chronic *T. laurifolia* administration affects not only P-glycoprotein and CYP2E1 but also oxidative stress, as well as induces an increase in BDNF to protect dopaminergic pathways from chronic ethanol intoxication.

Conclusion

The present study demonstrates the alterations of behaviors and tyrosine hydroxylase expression in nigrostriatal and mesolimbic dopaminergic pathways by acute and chronic ethanol administrations. These indicate the ethanol intoxication induces changes in their activities and functions. Furthermore, *T. laurifolia* administration presents the acute and chronic preventive effects in ethanol-induced the alteration of behaviors and tyrosine hydroxylase expression in both dopaminergic pathways. The results from this present study are concluded as follows

Ethanol induced alterations of behaviors and tyrosine hydroxylase expression in nigrostriatal and mesolimbic dopaminergic pathways.

1. Acute and chronic ethanol produces motor coordination impairment, behavior regulated by nigrostriatal dopaminergic pathway.

2. Acute ethanol increases tyrosine hydroxylase expression in axon fiber of dopaminergic neurons in the substantia nigra extending into the striatum.

3. Chronic ethanol decreases tyrosine hydroxylase expression in axon fiber of dopaminergic neurons in the substantia nigra extending into the striatum.

4. Chronic ethanol tends to decrease tyrosine hydroxylase expression of dopaminergic neurons in the substantia nigra.

5. Chronic ethanol causes an addictive behavior regulated by mesolimbic dopaminergic pathway.

6. Chronic ethanol decreases tyrosine hydroxylase expression in axon fiber of dopaminergic neurons in the ventral tegmental area extending into the nucleus accumbens.

Acute and chronic prevention of *T. laurifolia* on ethanol intoxications on nigrostriatal and mesolimbic dopaminergic pathways.

1. *T. laurifolia* protects motor coordination impairment by acute and chronic ethanol intoxications.

2. *T. laurifolia* protects tyrosine hydroxylase expression in axon fiber of dopaminergic neurons in the substantia nigra extending into the striatum enlargement in an acute ethanol stimulation and reduction in a chronic ethanol stimulation.

3. *T. laurifolia* protects ethanol addiction behavior induced by chronic ethanol administration.

4. *T. laurifolia* protects the reduction of tyrosine hydroxylase expression in axon fiber of dopaminergic neurons in the ventral tegmental area extending into the nucleus accumbens in a chronic ethanol stimulation.

REFERENCES

1. Oonsivilai R, Cheng C, Bomser J, Ferruzzi MG, Ningsanond S. Phytochemical profiling and phase II enzyme-inducing properties of *Thunbergia laurifolia* Lindl. (RC) extracts. J Ethnopharmacol. 2007;114(3):300-6.

2. Oonsivilai R, Mario G. Antioxidant activity and cytotoxicity of rang chuet (*Thunbergia laurifolia* Lindl.) extracts. As J Food Ag-Ind. 2008;1.

3. Boonyarikpunchai W, Sukrong S, Towiwat P. Antinociceptive and anti-inflammatory effects of rosmarinic acid isolated from *Thunbergia laurifolia* Lindl. Pharmacol Biochem Behav. 2014;124:67-73.

4. Kwansang J, Itthipanichpong C, Limpanasithikul W. Evaluation of wound healing activity of *Thunbergia laurifolia* supercritical carbon dioxide extract in rats with second-degree burn wounds. J Adv Pharm Technol Res. 2015;6(3):103-7.

5. Wonkchalee O, Boonmars T, Aromdee C, Laummaunwai P, Khunkitti W, Vaeteewoottacharn K, et al. Anti-inflammatory, antioxidant and hepatoprotective effects of *Thunbergia laurifolia* Linn. on experimental *opisthorchiasis*. Parasitol Res. 2012;111(1):353-9.

6. Aritajat S, Wutteerapol S, Saenphet K. Anti-diabetic effect of *Thunbergia laurifolia* Linn. aqueous extract. Southeast Asian J Trop Med Public Health. 2004;35 Suppl 2:53-8.

7. Ussanawarong S, Thesiri T, Mahakunakorn T, Parasupattana S. Effect of *Thunbergia laurifolia* Linn. on detoxication of paraquat. KKU Res 2000;5:11–7.

8. Ussanawarong S, Thesiri T. Effect of *Thunbergia laurifolia* Linn. on detoxication of parathion in rat. KKU Res J 2001;6:3–13.

9. Tejasen P, Thongthapp C. The study of the insecticide antitoxicity of *Thunbergia laurifolia* Linn. Chiang Mai Med Bull. 1980;19:105-14.

10. Chanawirat A. The protective effect of *Thunbergia laurifolia* extract on ethanol-induced hepatotoxicity in mice. Bangkok, Thailand: Mahidol University.; 2000.

11. Wutythamawech W. Encyclopedia of Thai herbs. Bangkok: O S Printing House; 1998. 390-497 p.

12. Chanprasit C, Wongkwanklom M, Sethabouppha H. Isan local wisdom for health care among persons with alcohol dependence. Nursing Journal 2011;38(122-133).

13. Chanprasit C, Wanbun P, Sethabouppha H. Thaksin local wisdom for health care among persons with alcohol dependence. Nursing Journal 2011;38:134-45.

14. Chanprasit C, Tongseaw N, Sethabouppha H. “Lanna” local wisdom for health care among persons with alcohol dependence. Nursing Journal 2011;38:110-21.

15. Thongsaard W, Marsden CA. A herbal medicine used in the treatment of addiction mimics the action of amphetamine on in vitro rat striatal dopamine release. Neurosci Lett. 2002;329(2):129-32.

16. Thongsaard W, Marsden CA, Morris P, Prior M, Shah YB. Effect of *Thunbergia laurifolia*, a Thai natural product used to treat drug addiction, on cerebral activity detected by functional magnetic resonance imaging in the rat. Psychopharmacology (Berl). 2005;180(4):752-60.

17. Thongsaard W, Jariyapongskul A, Areebambud C. Effect of *Thunbergia laurifolia* extract on cerebral blood flow in rats. J Med Assoc Thai 2017;100(10):13-8.

18. Thongsaard W, Marsden C. Effect of *Thunbergia laurifolia* extract on extracellular dopamine level in rat nucleus accumbens. J Med Assoc Thai. 2013;96 Suppl 1:S85-9.

19. Thongsaard W, Sangpayap R, Marsden C. Chronic effect of *Thunbergia laurifolia* extract and cocaine in rats using behavior model of addiction. J Med Assoc Thai. 2015;98 Suppl 9:S48-52.

20. Organization WH. Global status report on alcohol and health 2018 [internet] 2018 [cited 2019 Nov 03]. Available from: https://www.who.int/substance_abuse/publications/global_alcohol_report/en/.

21. The Center for Alcohol Studies (CAS). Provincial alcohol report 2011[internet] 2016 [cited 2016 Feb 15]. Available from: http://cas.or.th/publication-types/books.

22. BBC. Problems with alcoholic drinks 2014 [internet] 2016 [cited 2016 April 18]. Available from: http://www.bbc.co.uk/schools/gcsebitesize/science/edexcel_pre_2011/designerproducts/foodanddrinkrev2.shtml.

23. National Statistical Office. The smoking and drinking behavior survey 2014 [internet] 2014 [cited 2016 Feb 29]. Available from: http://service.nso.go.th/nso/nsopublish/themes/files/smokeFullReport57-1.pdf.

24. Road safety directing center. The statistic of accidents in songkarn festival 2016 [internet] 2016 [cited 2016 May 29]. Available from: http://www.roadsafetythailand.com/main/files/songkran%2059/7/report.pdf.

25. Road safety directing center. The statistic of accidents in New Year festival 2016 [internet] 2016 [cited 2016 May 29]. Available from: http://www.roadsafetythailand.com/main/files/4-01-59/3-4(1).pdf.

26. Foundation RSTRSP. Key facts on road safety situation in Thailand 2016-2017 [internet] 2019 [cited 2019 Oct 07]. Available from: http://www.roadsafetythai.org/edoc/doc_20190805151113.pdf.

27. Guze SB. Diagnostic and satistical manual of mental disorders. 4th ed. Washington DC: American Psychiatry Association; 2000.

28. World Health Organization. Global status report on alcohol and health 2014 [internet] 2016 [cited 2019 Nov 03]. Available from: https://www.who.int/substance_abuse/publications/global_alcohol_report/en/.

29. Marco CA, Kelen GD. Acute intoxication. Emerg Med Clin North Am. 1990;8(4):731-48.

30. Lieber CS. Hepatic, metabolic and toxic effects of ethanol: 1991 update. Alcohol Clin Exp Res. 1991;15(4):573-92.

31. Brust JC. Ethanol and cognition: indirect effects, neurotoxicity and neuroprotection: a review. Int J Environ Res Public Health. 2010;7(4):1540-57.

32. Lovinger DM, Roberto M. Synaptic effects induced by alcohol. Curr Top Behav Neurosci. 2013;13:31-86.

33. Melis M, Diana M, Enrico P, Marinelli M, Brodie MS. Ethanol and acetaldehyde action on central dopamine systems: mechanisms, modulation, and relationship to stress. Alcohol (Fayetteville, NY). 2009;43(7):531-9.

34. White AM, Truesdale MC, Bae JG, Ahmad S, Wilson WA, Best PJ, et al. Differential

effects of ethanol on motor coordination in adolescent and adult rats. Pharmacol Biochem Behav. 2002;73(3):673-7.

35. Sharko AC, Kaigler KF, Fadel JR, Wilson MA. Individual differences in voluntary ethanol consumption lead to differential activation of the central amygdala in rats: relationship to the anxiolytic and stimulant effects of low dose ethanol. Alcohol Clin Exp Res. 2013;37 Suppl 1(Suppl 1):E172-E80.

36. Zeng K, Xie A, Zhang X, Zhong B, Liu X, Hao W. Chronic alcohol treatment-induced GABA-Aα5 histone H3K4 trimethylation upregulation leads to increased GABA-Aα5 expression and susceptibility to alcohol addiction in the offspring of Wistar rats. Front Psychiatry. 2018;9:468-.

37. Rassnick S, Pulvirenti L, Koob GF. Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. Psychopharmacology (Berl). 1992;109(1-2):92-8.

38. Hodge CW, Samson HH, Chappelle AM. Alcohol self-administration: further examination of the role of dopamine receptors in the nucleus accumbens. Alcohol Clin Exp Res. 1997;21(6):1083-91.

39. Sullivan JM, Risacher SL, Normandin MD, Yoder KK, Froehlich JC, Morris ED. Imaging of alcohol-induced dopamine release in rats:preliminary findings with [(11) C]raclopride PET. Synapse. 2011;65(9):929-37.

40. Yoshimoto K, McBride WJ, Lumeng L, Li TK. Alcohol stimulates the release of dopamine and serotonin in the nucleus accumbens. Alcohol. 1992;9(1):17-22.

41. Boileau I, Assaad JM, Pihl RO, Benkelfat C, Leyton M, Diksic M, et al. Alcohol promotes dopamine release in the human nucleus accumbens. Synapse. 2003;49(4):226-31.

42. Aalto S, Ingman K, Alakurtti K, Kaasinen V, Virkkala J, Nagren K, et al. Intravenous ethanol increases dopamine release in the ventral striatum in humans: PET study using bolus-plus-infusion administration of [(11)C]raclopride. J Cereb Blood Flow Metab. 2015;35(3):424-31.

43. Engleman EA, Keen EJ, Tilford SS, Thielen RJ, Morzorati SL. Ethanol drinking

reduces extracellular dopamine levels in the posterior ventral tegmental area of nondependent alcohol-preferring rats. Alcohol. 2011;45(6):549-57.

44. Perra S, Clements MA, Bernier BE, Morikawa H. In vivo ethanol experience increases D(2) autoinhibition in the ventral tegmental area. Neuropsychopharmacology. 2011;36(5):993-1002.

45. Ding ZM, Ingraham CM, Rodd ZA, McBride WJ. Alcohol drinking increases the dopamine-stimulating effects of ethanol and reduces D2 auto-receptor and group II metabotropic glutamate receptor function within the posterior ventral tegmental area of alcohol preferring (P) rats. Neuropharmacology. 2016;109:41-8.

46. Ayano G. Dopamine: receptors, functions, synthesis, pathways, locations and mental disorders: review of literatures. Ment Disord Treat 2016;2.

47. Mereu G, Fadda F, Gessa GL. Ethanol stimulates the firing rate of nigral dopaminergic neurons in unanesthetized rats. Brain Res. 1984;292(1):63-9.

48. Barbaccia ML, Reggiani A, Spano PF, Trabucchi M. Ethanol effects on dopaminergic function: modulation by the endogenous opioid system. Pharmacol Biochem Behav. 1980;13 Suppl 1:303-6.

49. Ornelas LC, Novier A, Van Skike CE, Diaz-Granados JL, Matthews DB. The effects of acute alcohol on motor impairments in adolescent, adult, and aged rats. Alcohol 2015;49(2):121-6.

50. Asorey LG, Carbone S, Gonzalez BJ, Cutrera RA. Behavioral effects of the combined use of alcohol and energy drinks on alcohol hangover in an experimental mice model. Neurosci Lett. 2018;670:1-7.

51. Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Res. 1985;348(1):201-3.

52. Brodie MS, Shefner SA, Dunwiddie TV. Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area *in vitro*. Brain Res. 1990;508(1):65-9.

53. Brodie MS. Increased ethanol excitation of dopaminergic neurons of the ventral tegmental area after chronic ethanol treatment. Alcohol Clin Exp Res. 2002;26(7):1024-30.

54. Melendez RI, Rodd-Henricks ZA, Engleman EA, Li TK, McBride WJ, Murphy JM.

Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation and operant self-administration of ethanol. Alcohol Clin Exp Res. 2002;26(3):318-25.

55. Schier CJ, Dilly GA, Gonzales RA. Intravenous ethanol increases extracellular dopamine in the medial prefrontal cortex of the Long-Evans rat. Alcohol Clin Exp Res. 2013;37(5):740-7.

56. Barak S, Carnicella S, Yowell QV, Ron D. Glial cell line-derived neurotrophic factor reverses alcohol-induced allostasis of the mesolimbic dopaminergic system: implications for alcohol reward and seeking. J Neurosci. 2011;31(27):9885-94.

57. Frye GD, Breese GR. An evaluation of the locomotor stimulating action of ethanol in rats and mice. Psychopharmacology (Berl). 1981;75(4):372-9.

58. Ferreira VM, Takahashi RN, Morato GS. Dexamethasone reverses the ethanol-induced anxiolytic effect in rats. Pharmacol Biochem Behav. 2000;66(3):585-90.

59. Koo MW. Effects of ginseng on ethanol induced sedation in mice. Life Sci. 1999;64(2):153-60.

60. Sajja RK, Rahman S. Cytisine modulates chronic voluntary ethanol consumption and ethanol-induced striatal up-regulation of Delta FosB in mice. Alcohol. 2013;47(4):299-307.

61. Frias J, Torres JM, Miranda MT, Ruiz E, Ortega E. Effects of acute alcohol intoxication on pituitary-gonadal axis hormones, pituitary-adrenal axis hormones, beta-endorphin and prolactin in human adults of both sexes. Alcohol Alcohol. 2002;37(2):169-73.

62. Fernandez-Mateos P, Jimenez-Ortega V, Cano Barquilla P, Cardinali DP, Esquifino AI. Discontinuous versus continuous drinking of ethanol in peripubertal rats: effect on 24-hour pattern of hypophyseal-gonadal axis activity and anterior pituitary oxidative stress. Neuroendocrinology. 2012;96(3):194-203.

63. Purnima M, Gupta P. Colouring matters from the flowers of *Thunbergia laurifolia*. Indian Chem Soc 1978;55(6):622-3.

64. Kanchanapoom T, Kasai R, Yamasaki K. Iridoid glucosides from *Thunbergia*

laurifolia. Phytochemistry. 2002;60(8):769-71.

65. Rojsanga P, Sithisarn P, Tanaka K, Mizuki D, Matsumoto K. *Thunbergia laurifolia* extract ameliorates cognitive and emotional deficits in olfactorectomized mice. Pharm Biol. 2015;53(8):1141-8.

66. Tayeh M, Nawarat J, Phyu M, Tangpong J. Protective effects of *Thunbergia laurifolia* (linn.) on organophosphorous (chlorpyrifos)-induced cholinesterase dysfunction. Walailak J Sci & Tech. 2018;15(8):569-78.

67. Ussanawarong S, Thesiri T. Effect of *Thunbergia Laurifolia* Linn on detoxification of parathion in rat. KKU RJ 2001;6:3–13.

68. Ruangyuttikarn W, Chattaviriya P, Morkmek N, Chuncharunee S, Lertprasertsuke N. *Thunbergia laurifolia* leaf extract mitigates cadmium toxicity in rats. ScienceAsia. 2013;39:19.

69. Palipoch S, Tansatit T, Preyavichyapugdee N, Jaikua W, Kosai P. Effect of *Thunbergia laurifolia* (Linn) leaf extract dietary supplement against lead toxicity in Nile tilapia (*Oreochromis niloticus*). World J Fish & Marine Sci 2011;3:1-9.

70. Chan E, Eng SY, Tan Y, Wong Z, Lye P, Tan L. Antioxidant and sensory properties of Thai herbal teas with emphasis on *Thunbergia laurifolia* Lindl. Chiang Mai J Sci 2012;39:599.

71. Khobjai W, Jaihan U, Watcharasamphankul W, Somsak V. Protective effect of *Thunbergia laurifolia* extract on hemolysis during *Plasmodium berghei* infection. Parasitol Res. 2014;113(5):1843-6.

72. Pramyothin P, Chirdchupunsare H, Rungsipipat A, Chaichantipyuth C. Hepatoprotective activity of *Thunbergia laurifolia* Linn extract in rats treated with ethanol: in vitro and in vivo studies. J Ethnopharmacol. 2005;102(3):408-11.

73. Nanna U, Chiruntanat N, Jaijoy K, Rojsanga P, Sireeratawong S. Effect of *Thunbergia laurifolia* Lindl. extract on anti-inflammatory, analgesic and antipyretic activity. J Med Assoc Thai. 2017;100 (6):98.

74. Junsi M, Siripongvutikorn S, Yupanqui C, Usawakesmanee W. Efficacy of *Thunbergia laurifolia* (Rang Jued) aqueous leaf extract for specific biological activities

using RAW 264.7 macrophage cells as test model. IFRJ. 2017;24:2317-29.

75. Namsen R, Rojanasthien N, Sireeratawong S, Rojsanga P, Nimlamool W, Potikanond S. *Thunbergia laurifolia* exhibits antifibrotic effects in human hepatic stellate cells. Evid Based Complement Alternat Med. 2017;2017:3508569.

76. Jetawattana S, Boonsirichai K, Charoen S, Martin SM. Radical intermediate generation and cell cycle arrest by an aqueous extract of *Thunbergia Laurifolia* Linn. In human breast cancer cells. Asian Pac J Cancer Prev. 2015;16(10):4357-61.

77. Chivapat S, Chavalittumrong P, Attawish A, Bansiddhi J, Padungpat S. Chronic toxicity of *Thunbergia laurifolia* Lindl. extract. J Thai Trad Altern Med. 2010;7.

78. Bruice PY. Organic chemistry. 7th ed. United States of America: Pearson Education; 2014.

79. Wikipedia. Alcohol [internet] 2018 [cited 2019 Jul 5]. Available from: https://en.wikipedia.org/wiki/Alcohol.

80. Klein D. Organic chemistry. 2nd ed. United States of America: John Wiley and Sons; 2015.

81. Vonghia L, Leggio L, Ferrulli A, Bertini M, Gasbarrini G, Addolorato G. Acute alcohol intoxication. Eur J Intern Med. 2008;19(8):561-7.

82. Jones A. Alcohol, its absorption, distribution, metabolism, and excretion in the body and pharmacokinetic calculations. WIREs Forensic Sci. 2019:e1340.

83. Jones AW. Pharmacokinetics of ethanol - issues of forensic importance. Forensic Sci Rev. 2011;23(2):91-136.

84. Kalant H. Pharmacokinetics of ethanol: absorption, distribution, and elimination. The pharmacology of alcohol and alcohol dependence. Alcohol and alcoholism, No. 2. New York, NY, US: Oxford University Press; 1996. p. 15-58.

85. Kent W. The pharmacokinetics of alcohol in healthy adults. WebmedCentral PHARMACOLOGY 2012;3(5).

86. Karel L, Fleisher JH. Gastric absorption of ethyl alcohol in the rat. Am J Physiol. 1948;153(2):268-76.

87. Haggard H, Greenberg L, Lolli G. The absorption of alcohol with special reference

to its influence on the concentration of alcohol appearing in the blood. Quart Studies Ale 1941;1:684.

88. Paton A. Alcohol in the body. BMJ (Clinical research ed). 2005;330(7482):85-7.

89. Jones AW, Jonsson KA. Food-induced lowering of blood-ethanol profiles and increased rate of elimination immediately after a meal. J Forensic Sci. 1994;39(4):1084-93.

90. Zelner I, Koren G. Pharmacokinetics of ethanol in the maternal-fetal unit. J Popul Ther Clin Pharmacol. 2013;20(3):e259-65.

91. Zakhari S. Overview: how is alcohol metabolized by the body? Alcohol Res Health. 2006;29(4):245-54.

92. Gilman JM, Ramchandani VA, Davis MB, Bjork JM, Hommer DW. Why we like to drink: a functional magnetic resonance imaging study of the rewarding and anxiolytic effects of alcohol. J Neurosci. 2008;28(18):4583-91.

93. Sano M, Wendt PE, Wirsen A, Stenberg G, Risberg J, Ingvar DH. Acute effects of alcohol on regional cerebral blood flow in man. J Stud Alcohol. 1993;54(3):369-76.

94. Ingvar M, Ghatan PH, Wirsen-Meurling A, Risberg J, Von Heijne G, Stone-Elander S, et al. Alcohol activates the cerebral reward system in man. J Stud Alcohol. 1998;59(3):258-69.

95. Esposito F, Pignataro G, Di Renzo G, Spinali A, Paccone A, Tedeschi G, et al. Alcohol increases spontaneous BOLD signal fluctuations in the visual network. Neuroimage. 2010;53(2):534-43.

96. Deehan GA, Jr., Knight CP, Waeiss RA, Engleman EA, Toalston JE, McBride WJ, et al. Peripheral administration of ethanol results in a correlated increase in dopamine and serotonin within the posterior ventral tegmental area. Alcohol Alcohol. 2016;51(5):535-40.

97. Morikawa H, Morrisett RA. Ethanol action on dopaminergic neurons in the ventral tegmental area: interaction with intrinsic ion channels and neurotransmitter inputs. Int Rev Neurobiol. 2010;91:235-88.

98. Herman MA, Sidhu H, Stouffer DG, Kreifeldt M, Le D, Cates-Gatto C, et al. GIRK3 gates activation of the mesolimbic dopaminergic pathway by ethanol. Proc Natl Acad Sci U S A. 2015;112(22):7091-6.

99. Nimitvilai S, You C, Arora DS, McElvain MA, Vandegrift BJ, Brodie MS, et al. Differential effects of toluene and ethanol on dopaminergic neurons of the ventral tegmental area. Front Neurosci. 2016;10:434.

100. Ericson M, Blomqvist O, Engel JA, Soderpalm B. Voluntary ethanol intake in the rat and the associated accumbal dopamine overflow are blocked by ventral tegmental mecamylamine. Eur J Pharmacol. 1998;358(3):189-96.

101. Shnitko TA, Kennerly LC, Spear LP, Robinson DL. Ethanol reduces evoked dopamine release and slows clearance in the rat medial prefrontal cortex. Alcohol Clin Exp Res. 2014;38(12):2969-77.

102. Yim HJ, Robinson DL, White ML, Jaworski JN, Randall PK, Lancaster FE, et al. Dissociation between the time course of ethanol and extracellular dopamine concentrations in the nucleus accumbens after a single intraperitoneal injection. Alcohol Clin Exp Res. 2000;24(6):781-8.

103. Okamoto T, Harnett MT, Morikawa H. Hyperpolarization-activated cation current (Ih) is an ethanol target in midbrain dopamine neurons of mice. J Neurophysiol. 2006;95(2):619-26.

104. Diana M, Pistis M, Muntoni AL, Gessa GL. Ethanol withdrawal does not induce a reduction in the number of spontaneously active dopaminergic neurons in the mesolimbic system. Brain Res. 1995;682(1-2):29-34.

105. Schuckit MA. Low level of response to alcohol as a predictor of future alcoholism. Am J Psychiatry. 1994;151(2):184-9.

106. Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR. Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. Proc Natl Acad Sci U S A. 2003;100(4):2053-8.

107. Dahchour A, De Witte P. Effect of repeated ethanol withdrawal on glutamate microdialysate in the hippocampus. Alcohol Clin Exp Res. 1999;23(10):1698-703.

108. Ariwodola OJ, Weiner JL. Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic GABA(B) receptors. J Neurosci. 2004;24(47):10679-86.

109. Ziskind-Conhaim L, Gao BX, Hinckley C. Ethanol dual modulatory actions on spontaneous postsynaptic currents in spinal motoneurons. J Neurophysiol. 2003;89(2):806-13.

110. Zhu PJ, Lovinger DM. Ethanol potentiates GABAergic synaptic transmission in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala. J Neurophysiol. 2006;96(1):433-41.

111. Theile JW, Morikawa H, Gonzales RA, Morrisett RA. Ethanol enhances GABAergic transmission onto dopamine neurons in the ventral tegmental area of the rat. Alcohol Clin Exp Res. 2008;32(6):1040-8.

112. Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. Nat Neurosci. 2005;8(3):339-45.

113. Wei W, Faria LC, Mody I. Low ethanol concentrations selectively augment the tonic inhibition mediated by delta subunit-containing GABAA receptors in hippocampal neurons. J Neurosci. 2004;24(38):8379-82.

114. Jia F, Chandra D, Homanics GE, Harrison NL. Ethanol modulates synaptic and extrasynaptic GABAA receptors in the thalamus. J Pharmacol Exp Ther. 2008;326(2):475-82.

115. Roberto M, Varodayan FP. Synaptic targets: chronic alcohol actions. Neuropharmacology. 2017;122:85-99.

116. Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Bidirectional alterations of GABA(A) receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. J Neurochem. 1997;69(1):126-30.

117. Devaud LL, Smith FD, Grayson DR, Morrow AL. Chronic ethanol consumption differentially alters the expression of gamma-aminobutyric acid A receptor subunit mRNAs in rat cerebral cortex: competitive, quantitative reverse transcriptase-polymerase chain reaction analysis. Mol Pharmacol. 1995;48(5):861-8.

118. Suaud-Chagny MF, Chergui K, Chouvet G, Gonon F. Relationship between dopamine release in the rat nucleus accumbens and the discharge activity of

dopaminergic neurons during local in vivo application of amino acids in the ventral tegmental area. Neuroscience. 1992;49(1):63-72.

119. Selim M, Bradberry CW. Effect of ethanol on extracellular 5-HT and glutamate in the nucleus accumbens and prefrontal cortex: comparison between the Lewis and Fischer 344 rat strains. Brain Res. 1996;716(1-2):157-64.

120. Dahchour A, Hoffman A, Deitrich R, de Witte P. Effects of ethanol on extracellular amino acid levels in high-and low-alcohol sensitive rats: a microdialysis study. Alcohol Alcohol. 2000;35(6):548-53.

121. Zhu W, Bie B, Pan ZZ. Involvement of non-NMDA glutamate receptors in central amygdala in synaptic actions of ethanol and ethanol-induced reward behavior. J Neurosci. 2007;27(2):289-98.

122. Gilpin NW, Koob GF. Neurobiology of alcohol dependence: focus on motivational mechanisms. Alcohol Res Health. 2008;31(3):185-95.

123. Mendez M, Morales-Mulia M. Role of mu and delta opioid receptors in alcohol drinking behaviour. Curr Drug Abuse Rev. 2008;1(2):239-52.

124. Lam MP, Marinelli PW, Bai L, Gianoulakis C. Effects of acute ethanol on opioid peptide release in the central amygdala: an in vivo microdialysis study. Psychopharmacology (Berl). 2008;201(2):261-71.

125. Granholm L, Segerström L, Nylander I. Episodic ethanol exposure in adolescent rats causes residual alterations in endogenous opioid peptides. Front Psychiatry. 2018;9:425-.

126. LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and dependence: findings of animal studies. Biol Psychiatry. 1994;36(6):395-421.

127. Thielen RJ, Bare DJ, McBride WJ, Lumeng L, Li TK. Ethanol-stimulated serotonin release in the ventral hippocampus: an absence of rapid tolerance for the alcohol-preferring P rat and insensitivity in the alcohol-nonpreferring NP rat. Pharmacol Biochem Behav. 2002;71(1-2):111-7.

128. Pistis M, Muntoni AL, Gessa G, Diana M. Effects of acute, chronic ethanol and withdrawal on dorsal raphe neurons: electrophysiological studies. Neuroscience.

1997;79(1):171-6.

129. Blaschko H. Metabolism and storage of biogenic amines. Experientia. 1957;13(1):9-13.

130. Feldman RS, Meyer JS, Quenzer LF. Principles of neuropsychopharmacology. Sunderland, MA, US: Sinauer Associates; 1997.

131. Sonne J, Goyal A, Bansal P, Lopez-Ojeda W. Dopamine. Treasure Island (FL): StatPearls Publishing; 2020 [cited 2020 Feb 3]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK535451/

132. Meiser J, Weindl D, Hiller K. Complexity of dopamine metabolism. Cell Commun Signal. 2013;11(1):34-.

133. Chaudhry FA, Edwards RH, Fonnum F. Vesicular neurotransmitter transporters as targets for endogenous and exogenous toxic substances. Annu Rev Pharmacol Toxicol. 2008;48:277-301.

134. Ledonne A, Mercuri NB. Current concepts on the physiopathological relevance of dopaminergic receptors. Front Cell Neurosci. 2017;11(27).

135. Mishra A, Singh S, Shukla S. Physiological and functional basis of dopamine receptors and their role in neurogenesis: possible implication for Parkinson's disease. J Exp Neurosci. 2018;12:1179069518779829-.

136. Ledonne A, Mercuri NB. Current Concepts on the Physiopathological Relevance of Dopaminergic Receptors. Front Cell Neurosci. 2017;11:27.

137. Kienast T, Heinz A. Dopamine and the diseased brain. CNS Neurol Disord Drug Targets. 2006;5(1):109-31.

138. Halbach O, Dermietzel R. Neurotransmitters and neuromodulators: handbook of receptors and biological effects. 2nd ed. Germany: Wiley-Blackwell; 2006.

139. Webster RA. Neurotransmitter, drug and brain function. United Kingdom: John wiley & sons; 2001.

140. Calabresi P, Picconi B, Tozzi A, Ghiglieri V, Di Filippo M. Direct and indirect pathways of basal ganglia: a critical reappraisal. Nat Neurosci. 2014;17(8):1022-30.

141. Prediger R, Bortolanza M, Issy A, Lopes B, Del-Bel E, Raisman-Vozari R.

Dopaminergic neurons in Parkinson's disease. 2014.

142. National institure of drug abuse. The reward pathway [internet] 2016 [cited 2016 March 29]. Available from: https://www.drugabuse.gov/publications/teaching-packets/understanding-drug-abuse-addiction/section-i/4-reward-pathway.

143. Zarrindast MR, Khakpai F. The modulatory role of dopamine in anxiety-like behavior. Arch Iran Med. 2015;18(9):591-603.

144. Seeman P. Targeting the dopamine D2 receptor in schizophrenia. Expert Opin Ther Targets. 2006;10(4):515-31.

145. Pistillo F, Clementi F, Zoli M, Gotti C. Nicotinic, glutamatergic and dopaminergic synaptic transmission and plasticity in the mesocorticolimbic system: focus on nicotine effects. Prog Neurobiol. 2015;124:1-27.

146. Teicher MH, Samson JA, Anderson CM, Ohashi K. The effects of childhood maltreatment on brain structure, function and connectivity. Nat Rev Neurosci. 2016;17(10):652-66.

147. Neuroscientifically challenged. Know your brain: substantia nigra [internet] 2014 [cited 2016 March 29]. Available from: http://www.neuroscientificallychallenged.com/blog/know-your-brain-substantia-nigra.

148. Zecca L, Tampellini D, Gerlach M, Riederer P, Fariello RG, Sulzer D. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol Pathol. 2001;54(6):414-8.

149. Lindvall O, Bjorklund A. Dopaminergic innervation of the globus pallidus by collaterals from the nigrostriatal pathway. Brain Res. 1979;172(1):169-73.

150. Cossette M, Levesque M, Parent A. Extrastriatal dopaminergic innervation of human basal ganglia. Neurosci Res. 1999;34(1):51-4.

151. Lee CR, Tepper JM. Basal ganglia control of substantia nigra dopaminergic neurons. J Neural Transm Suppl. 2009(73):71-90.

152. Lee CR, Abercrombie ED, Tepper JM. Pallidal control of substantia nigra dopaminergic neuron firing pattern and its relation to extracellular neostriatal dopamine levels. Neuroscience. 2004;129(2):481-9.

153. Guatteo E, Cucchiaroni ML, Mercuri NB. Substantia nigra control of basal ganglia nuclei. J Neural Transm Suppl. 2009(73):91-101.

154. Ferre S, Lluis C, Justinova Z, Quiroz C, Orru M, Navarro G, et al. Adenosine-cannabinoid receptor interactions. Implications for striatal function. Br J Pharmacol. 2010;160(3):443-53.

155. Kreitzer AC. Physiology and pharmacology of striatal neurons. Annu Rev Neurosci. 2009;32:127-47.

156. Furlanetti LL, Coenen VA, Dobrossy MD. Ventral tegmental area dopaminergic lesion-induced depressive phenotype in the rat is reversed by deep brain stimulation of the medial forebrain bundle. Behav Brain Res. 2016;299:132-40.

157. Graybiel AM, Ragsdale CW, Jr. Histochemically distinct compartments in the striatum of human, monkeys, and cat demonstrated by acetylthiocholinesterase staining. Proc Natl Acad Sci U S A. 1978;75(11):5723-6.

158. Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA. Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience. 2008;152(4):1024-31.

159. Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO. Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. J Neurosci. 2011;31(21):7811-6.

160. Beier KT, Steinberg EE, DeLoach KE, Xie S, Miyamichi K, Schwarz L, et al. Circuit architecture of VTA dopamine neurons revealed by systematic input-output mapping. Cell. 2015;162(3):622-34.

161. Wang HL, Qi J, Zhang S, Wang H, Morales M. Rewarding effects of optical stimulation of ventral tegmental area glutamatergic neurons. J Neurosci. 2015;35(48):15948-54.

162. Harris HN, Peng YB. Evidence and explanation for the involvement of the nucleus accumbens in pain processing. Neural Regen Res. 2020;15(4):597-605.

163. Li Z, Chen Z, Fan G, Li A, Yuan J, Xu T. Cell-type-specific afferent innervation of

the nucleus accumbens core and shell. Front Neuroanat. 2018;12:84.

164. Zahm DS, Brog JS. On the significance of subterritories in the "accumbens" part of the rat ventral striatum. Neuroscience. 1992;50(4):751-67.

165. Salgado S, Kaplitt MG. The nucleus accumbens: a comprehensive review. Stereotact Funct Neurosurg. 2015;93(2):75-93.

166. Dharani K. The biology of thought: a neuronal mechanism in the generation of thought - a new molecular model. 1st ed: Academic Press; 2015.

167. Fuster JM. The prefrontal cortex--an update: time is of the essence. Neuron. 2001;30(2):319-33.

168. Yoder KK, Constantinescu CC, Kareken DA, Normandin MD, Cheng TE, O'Connor SJ, et al. Heterogeneous effects of alcohol on dopamine release in the striatum: a PET study. Alcohol Clin Exp Res. 2007;31(6):965-73.

169. Phillips TJ, Shen EH. Neurochemical bases of locomotion and ethanol stimulant effects. Int Rev Neurobiol. 1996;39:243-82.

170. Vasconcelos SM, Cavalcante RA, Aguiar LM, Sousa FC, Fonteles MM, Viana GS. Effects of chronic ethanol treatment on monoamine levels in rat hippocampus and striatum. Braz J Med Biol Res. 2004;37(12):1839-46.

171. Karadayian AG, Cutrera RA. Alcohol hangover: type and time-extension of motor function impairments. Behav Brain Res. 2013;247:165-73.

172. Teixeira FB, Santana LN, Bezerra FR, De Carvalho S, Fontes-Junior EA, Prediger RD, et al. Chronic ethanol exposure during adolescence in rats induces motor impairments and cerebral cortex damage associated with oxidative stress. PLoS One. 2014;9(6):e101074.

173. Robinson TE, Berridge KC. Review. The incentive sensitization theory of addiction: some current issues. Philos Trans R Soc Lond B Biol Sci. 2008;363(1507):3137-46.

174. World Health Organization. Neuroscience of psychoactive substance use and dependence [internet] 2004 [cited 2018 Feb 20]. Available from: https://www.who.int/substance_abuse/publications/en/Neuroscience.pdf.

175. Brodie MS, Pesold C, Appel SB. Ethanol directly excites dopaminergic ventral

tegmental area reward neurons. Alcohol Clin Exp Res. 1999;23(11):1848-52.

176. Katner SN, Weiss F. Neurochemical characteristics associated with ethanol preference in selected alcohol-preferring and -nonpreferring rats: a quantitative microdialysis study. Alcohol Clin Exp Res. 2001;25(2):198-205.

177. Planeta CS. Animal models of alcohol and drug dependence. Braz J Psychiatry. 2013;35 Suppl 2:S140-6.

178. Ciccocioppo R, Panocka I, Froldi R, Quitadamo E, Massi M. Ethanol induces conditioned place preference in genetically selected alcohol-preferring rats. Psychopharmacology (Berl). 1999;141(3):235-41.

179. Rodd ZA, Bell RL, McQueen VK, Davids MR, Hsu CC, Murphy JM, et al. Chronic ethanol drinking by alcohol-preferring rats increases the sensitivity of the posterior ventral tegmental area to the reinforcing effects of ethanol. Alcohol Clin Exp Res. 2005;29(3):358-66.

180. Tajuddin NF, Druse MJ. Effects of chronic alcohol consumption and aging on dopamine D2 receptors in Fischer 344 rats. Alcohol Clin Exp Res. 1996;20(1):144-51.

181. Cruz J, Delwing-de Lima D, Magro D, Cruz J. Anxiolytic effects of swimming exercise and ethanol in two behavioral models: beneficial effects and increased sensitivity in mice. Rev Ciênc Farm Básica Apl 2012;33:115-23.

182. Varlinskaya EI, Spear LP. Increases in anxiety-like behavior induced by acute stress are reversed by ethanol in adolescent but not adult rats. Pharmacology, biochemistry, and behavior. 2012;100(3):440-50.

183. Hall W, Zador D. The alcohol withdrawal syndrome. Lancet. 1997;349(9069):1897-900.

184. McKeon A, Frye MA, Delanty N. The alcohol withdrawal syndrome. J Neurol Neurosurg Psychiatry. 2008;79(8):854-62.

185. Valcheva-Kuzmanova S, Eftimov M, Kuzmanov K. An experimental model of alcohol-induced anxiety and depressive behaviour in rats. SSM. 2013;45:48.

186. Waller MB, Murphy JM, McBride WJ, Lumeng L, Li TK. Effect of low dose ethanol on spontaneous motor activity in alcohol-preferring and -nonpreferring lines of rats.

Pharmacol Biochem Behav. 1986;24(3):617-23.

187. Acevedo MB, Nizhnikov ME, Spear NE, Molina JC, Pautassi RM. Ethanol-induced locomotor activity in adolescent rats and the relationship with ethanol-induced conditioned place preference and conditioned taste aversion. Dev Psychobiol. 2013;55(4):429-42.

188. Karlsson O, Roman E. Dose-dependent effects of alcohol administration on behavioral profiles in the MCSF test. Alcohol. 2016;50:51-6.

189. Zimmermann US, Buchmann AF, Spring C, Uhr M, Holsboer F, Wittchen HU. Ethanol administration dampens the prolactin response to psychosocial stress exposure in sons of alcohol-dependent fathers. Psychoneuroendocrinology. 2009;34(7):996-1003.

190. Alfonso M, Marco J, Balvis IA, Pazo JA. Direct action of ethanol on pituitary prolactin secretion *in vitro*. Rev Esp Fisiol. 1991;47(3):133-40.

191. Tran S, Facciol A, Nowicki M, Chatterjee D, Gerlai R. Acute alcohol exposure increases tyrosine hydroxylase protein expression and dopamine synthesis in zebrafish. Behav Brain Res. 2017;317:237-41.

192. Wang Y, Lee J-W, Oh G, Grady SR, McIntosh JM, Brunzell DH, et al. Enhanced synthesis and release of dopamine in transgenic mice with gain-of-function α6* nAChRs. J Neurochem. 2014;129(2):315-27.

193. Kaewsuk S, Sae-ung K, Phansuwan-Pujito P, Govitrapong P. Melatonin attenuates methamphetamine-induced reduction of tyrosine hydroxylase, synaptophysin and growth-associated protein-43 levels in the neonatal rat brain. Neurochem Int. 2009;55(6):397-405.

194. Thiele TE, Cubero I, van Dijk G, Mediavilla C, Bernstein IL. Ethanol-induced c-fos expression in catecholamine- and neuropeptide Y-producing neurons in rat brainstem. Alcohol Clin Exp Res. 2000;24(6):802-9.

195. Luong TN, Carlisle HJ, Southwell A, Patterson PH. Assessment of motor balance and coordination in mice using the balance beam. J Vis Exp. 2011(49).

196. Goldstein LB, Davis JN. Beam-walking in rats: studies towards developing an animal model of functional recovery after brain injury. J Neurosci Methods. 1990;31(2):101-7.

197. Metz GA, Dietz V, Schwab ME, van de Meent H. The effects of unilateral pyramidal

tract section on hindlimb motor performance in the rat. Behav Brain Res. 1998;96(1-2):37-46.

198. da Silva FBR, Cunha PA, Ribera PC, Barros MA, Cartágenes SC, Fernandes LMP, et al. Heavy chronic ethanol exposure from adolescence to adulthood induces cerebellar neuronal loss and motor function damage in female rats. Front Behav Neurosci. 2018;12:88-.

199. Handley SL, Mithani S. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn Schmiedebergs Arch Pharmacol. 1984;327(1):1-5.

200. File SE. The contribution of behavioural studies to the neuropharmacology of anxiety. Neuropharmacology. 1987;26(7B):877-86.

201. Treit D, Menard J, Royan C. Anxiogenic stimuli in the elevated plus-maze. Pharmacol Biochem Behav. 1993;44(2):463-9.

202. Pellow S. Can drug effects on anxiety and convulsions be separated? Neurosci Biobehav Rev. 1985;9(1):55-73.

203. Wright IK, Heaton M, Upton N, Marsden CA. Comparison of acute and chronic treatment of various serotonergic agents with those of diazepam and idazoxan in the rat elevated X-maze. Psychopharmacology (Berl). 1992;107(2-3):405-14.

204. File SE. What can be learned from the effects of benzodiazepines on exploratory behavior? Neurosci Biobehav Rev. 1985;9(1):45-54.

205. Blatt SL, Takahashi RN. Experimental anxiety and the reinforcing effects of ethanol in rats. Braz J Med Biol Res. 1999;32:457-61.

206. File SE, Wardill AG. Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacologia. 1975;44(1):53-9.

207. Sturman O, Germain PL, Bohacek J. Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. Stress. 2018;21(5):443-52.

208. Abreu-Villaca Y, Queiroz-Gomes Fdo E, Dal Monte AP, Filgueiras CC, Manhaes AC. Individual differences in novelty-seeking behavior but not in anxiety response to a new environment can predict nicotine consumption in adolescent C57BL/6 mice. Behav Brain

Res. 2006;167(1):175-82.

209. Prus AJ, James JR, Rosecrans JA. Chapter 4 conditioned place preference. In: Buccafusco JJ, editor. Methods of behavior analysis in neuroscience. 2nd ed: CRC Press/Taylor & Francis; 2009.

210. Sanchis-Segura C, Spanagel R. Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. Addict Biol. 2006;11(1):2-38.

211. Lynch WJ, Nicholson KL, Dance ME, Morgan RW, Foley PL. Animal models of substance abuse and addiction: implications for science, animal welfare, and society. Comp Med. 2010;60(3):177-88.

212. Pildervasser JVN, Abrahao KP, Souza-Formigoni MLO. Distinct behavioral phenotypes in ethanol-induced place preference are associated with different extinction and reinstatement but not behavioral sensitization responses. Front Behav Neurosci. 2014;8:267-.

213. Charoenpong T, Promworn Y, Thangwiwatchinda P, Senavongse W, Thongsaard W. An experimental setup for measuring distance and duration of rat behavior2012. 1-5 p.

214. Walker BM, Ehlers CL. Age-related differences in the blood alcohol levels of Wistar rats. Pharmacol Biochem Behav. 2009;91(4):560-5.

215. Alinaghipour A, Mazoochi T, Ardjmand A. Low-dose ethanol ameliorates amnesia induced by a brief seizure model: the role of NMDA signaling. Neurol Res. 2019;41(7):624-32.

216. Baizer L, Masserano JM, Weiner N. Ethanol-induced changes in tyrosine hydroxylase activity in brains of mice selectively bred for differences in sensitivity to ethanol. Pharmacol Biochem Behav. 1981;15(6):945-9.

217. Masserano JM, Takimoto GS, Weiner N. Tyrosine hydroxylase activity in the brain and adrenal gland of rats following chronic administration of ethanol. Alcohol Clin Exp Res. 1983;7(3):294-8.

218. Gayer GG, Gordon A, Miles MF. Ethanol increases tyrosine hydroxylase gene expression in N1E-115 neuroblastoma cells. J Biol Chem. 1991;266(33):22279-84.

219. Puttonen H, Sundvik M, Rozov S, Chen Y-C, Panula P. Acute ethanol treatment

upregulates th1, th2, and hdc in larval zebrafish in stable networks. Front Neural Circuits. 2013;7(102).

220. Nowicki M, Tran S, Chatterjee D, Gerlai R. Inhibition of phosphorylated tyrosine hydroxylase attenuates ethanol-induced hyperactivity in adult zebrafish (Danio rerio). Pharmacol Biochem Behav. 2015;138:32-9.

221. Jaatinen P, Rintala J. Mechanisms of ethanol-induced degeneration in the developing, mature, and aging cerebellum. Cerebellum. 2008;7(3):332-47.

222. Oliveira GB, Fontes Ede A, Jr., de Carvalho S, da Silva JB, Fernandes LM, Oliveira MC, et al. Minocycline mitigates motor impairments and cortical neuronal loss induced by focal ischemia in rats chronically exposed to ethanol during adolescence. Brain Res. 2014;1561:23-34.

223. Rico-Barrio I, Penasco S, Puente N, Ramos A, Fontaine CJ, Reguero L, et al. Cognitive and neurobehavioral benefits of an enriched environment on young adult mice after chronic ethanol consumption during adolescence. Addict Biol. 2019;24(5):969-80.

224. Rothblat DS, Rubin E, Schneider JS. Effects of chronic alcohol ingestion on the mesostriatal dopamine system in the rat. Neurosci Lett. 2001;300(2):63-6.

225. Chatterjee D, Shams S, Gerlai R. Chronic and acute alcohol administration induced neurochemical changes in the brain: comparison of distinct zebrafish populations. Amino Acids. 2014;46(4):921-30.

226. Bailey CP, Andrews N, McKnight AT, Hughes J, Little HJ. Prolonged changes in neurochemistry of dopamine neurones after chronic ethanol consumption. Pharmacol Biochem Behav. 2000;66(1):153-61.

227. Adermark L, Jonsson S, Soderpalm B, Ericson M. Region-specific depression of striatal activity in Wistar rat by modest ethanol consumption over a ten-month period. Alcohol. 2013;47(4):289-98.

228. Beckhauser TF, Francis-Oliveira J, De Pasquale R. Reactive oxygen species: physiological and physiopathological effects on synaptic plasticity. J Exp Neurosci. 2016;10(Suppl 1):23-48.

229. Kim GH, Kim JE, Rhie SJ, Yoon S. The role of oxidative stress in neurodegenerative

diseases. Exp Neurobiol. 2015;24(4):325-40.

230. Ingelman-Sundberg M, Johansson I. Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. J Biol Chem. 1984;259(10):6447-58.

231. Vaarmann A, Gandhi S, Abramov AY. Dopamine induces Ca^{2+} signaling in astrocytes through reactive oxygen species generated by monoamine oxidase. J Biol Chem. 2010;285(32):25018-23.

232. Yamato M, Kudo W, Shiba T, Yamada KI, Watanabe T, Utsumi H. Determination of reactive oxygen species associated with the degeneration of dopaminergic neurons during dopamine metabolism. Free Radic Res. 2010;44(3):249-57.

233. Kyeong IG, Eum WS, Choi SY, Kang JH. Oxidative modification of neurofilament-L and neuronal cell death induced by the catechol neurotoxin, tetrahydropapaveroline. Toxicol Lett. 2013;217(1):59-66.

234. McVicker BL, Tuma DJ, Casey CA. Effect of ethanol on pro-apoptotic mechanisms in polarized hepatic cells. World J Gastroenterol. 2007;13(37):4960-6.

235. Sakharkar AJ, Tang L, Zhang H, Chen Y, Grayson DR, Pandey SC. Effects of acute ethanol exposure on anxiety measures and epigenetic modifiers in the extended amygdala of adolescent rats. Int J Neuropsychopharmacol. 2014;17(12):2057-67.

236. Tanchuck-Nipper MA, Ford MM, Hertzberg A, Beadles-Bohling A, Cozzoli DK, Finn DA. Sex differences in ethanol's anxiolytic effect and chronic ethanol withdrawal severity in mice with a null nutation of the 5alpha-reductase type 1 gene. Behav Genet. 2015;45(3):354-67.

237. Langen B, Dietze S, Fink H. Acute effect of ethanol on anxiety and 5-HT in the prefrontal cortex of rats. Alcohol. 2002;27(2):135-41.

238. Lister RG. The effects of ethanol on exploration in DBA/2 and C57Bl/6 mice. Alcohol. 1987;4(1):17-9.

239. Szot P, White SS, Veith RC, Rasmussen DD. Reduced gene expression for dopamine biosynthesis and transport in midbrain neurons of adult male rats exposed prenatally to ethanol. Alcohol Clin Exp Res. 1999;23(10):1643-9.

240. Pelkonen A, Hiltunen M, Kiianmaa K, Yavich L. Stimulated dopamine overflow and alpha-synuclein expression in the nucleus accumbens core distinguish rats bred for differential ethanol preference. J Neurochem. 2010;114(4):1168-76.

241. Oliva JM, Ortiz S, Perez-Rial S, Manzanares J. Time dependent alterations on tyrosine hydroxylase, opioid and cannabinoid CB1 receptor gene expressions after acute ethanol administration in the rat brain. Eur Neuropsychopharmacol. 2008;18(5):373-82.

242. Kohl RR, Katner JS, Chernet E, McBride WJ. Ethanol and negative feedback regulation of mesolimbic dopamine release in rats. Psychopharmacology (Berl). 1998;139(1-2):79-85.

243. Roger-Sanchez C, Aguilar MA, Rodriguez-Arias M, Aragon CM, Minarro J. Age- and sex-related differences in the acquisition and reinstatement of ethanol CPP in mice. Neurotoxicol Teratol. 2012;34(1):108-15.

244. Cunningham CL. Genetic relationships between ethanol-induced conditioned place aversion and other ethanol phenotypes in 15 inbred mouse strains. Brain Sci. 2019;9(8).

245. Wille-Bille A, de Olmos S, Marengo L, Chiner F, Pautassi R. Long-term ethanol self-administration induces ΔFosB in male and female adolescent, but not in adult, Wistar rats. Prog Neuropsychopharmacol Biol Psychiatry. 2017;74:15-30.

246. Sena MC, Nunes FC, Salvadori MG, Carvalho CC, Morais LC, Braga VA. Chronic consumption of distilled sugarcane spirit induces anxiolytic-like effects in mice. Clinics (Sao Paulo). 2011;66(5):873-8.

247. Hansen AW, Almeida FB, Bandiera S, Pulcinelli RR, Fragoso ALR, Schneider R, Jr., et al. Taurine restores the exploratory behavior following alcohol withdrawal and decreases BDNF mRNA expression in the frontal cortex of chronic alcohol-treated rats. Pharmacol Biochem Behav. 2017;161:6-12.

248. Gong MF, Wen RT, Xu Y, Pan JC, Fei N, Zhou YM, et al. Attenuation of ethanol abstinence-induced anxiety- and depressive-like behavior by the phosphodiesterase-4 inhibitor rolipram in rodents. Psychopharmacology (Berl). 2017;234(20):3143-51.

249. Moloney B, Leonard BE. Pre-natal and post-natal effects of alcohol in the rat--I.

Changes in body weight and exploratory activity. Alcohol Alcohol. 1984;19(2):131-6.

250. Ludlow KH, Bradley KD, Allison DW, Taylor SR, Yorgason JT, Hansen DM, et al. Acute and chronic ethanol modulate dopamine D2-subtype receptor responses in ventral tegmental area GABA neurons. Alcohol Clin Exp Res. 2009;33(5):804-11.

251. You C, Vandegrift B, Brodie MS. Ethanol actions on the ventral tegmental area: novel potential targets on reward pathway neurons. Psychopharmacology (Berl). 2018;235(6):1711-26.

252. Hoffman PL, Tabakoff B. Alcohol dependence: a commentary on mechanisms. Alcohol Alcohol. 1996;31(4):333-40.

253. Zhou FC, Zhang JK, Lumeng L, Li TK. Mesolimbic dopamine system in alcohol-preferring rats. Alcohol. 1995;12(5):403-12.

254. Spiga S, Talani G, Mulas G, Licheri V, Fois GR, Muggironi G, et al. Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. Proc Natl Acad Sci U S A. 2014;111(35):E3745-54.

255. Dohrman, Dp, Reiter CK. Chronic ethanol reduces nicotine-induced dopamine release in PC12 cells. Alcohol Clin Exp Res. 2003;27(11):1846-51.

256. Karkhanis AN, Rose JH, Huggins KN, Konstantopoulos JK, Jones SR. Chronic intermittent ethanol exposure reduces presynaptic dopamine neurotransmission in the mouse nucleus accumbens. Drug Alcohol Depend. 2015;150:24-30.

257. Carroll MR, Rodd ZA, Murphy JM, Simon JR. Chronic ethanol consumption increases dopamine uptake in the nucleus accumbens of high alcohol drinking rats. Alcohol. 2006;40(2):103-9.

258. Rocejanasaroj A, Tencomnao T, Sangkitikomol W. *Thunbergia laurifolia* extract minimizes the adverse effects of toxicants by regulating P-glycoprotein activity, CYP450, and lipid metabolism gene expression in HepG2 cells. Genet Mol Res. 2014;13(1):205-19.

259. Kumar YS, Adukondalu D, Sathish D, Vishnu YV, Ramesh G, Latha AB, et al. P-glycoprotein- and cytochrome P-450-mediated herbal drug interactions. Drug Metabol Drug Interact. 2010;25(1-4):3-16.

260. Lin JH, Yamazaki M. Role of P-glycoprotein in pharmacokinetics: clinical

implications. Clin Pharmacokinet. 2003;42(1):59-98.

261. Vasiliou V, Ziegler TL, Bludeau P, Petersen DR, Gonzalez FJ, Deitrich RA. CYP2E1 and catalase influence ethanol sensitivity in the central nervous system. Pharmacogenet Genomics. 2006;16(1):51-8.

262. Arizzi-LaFrance MN, Correa M, Aragon CM, Salamone JD. Motor stimulant effects of ethanol injected into the substantia nigra pars reticulata: importance of catalase-mediated metabolism and the role of acetaldehyde. Neuropsychopharmacology. 2006;31(5):997-1008.

263. Heit C, Dong H, Chen Y, Thompson DC, Deitrich RA, Vasiliou VK. The role of CYP2E1 in alcohol metabolism and sensitivity in the central nervous system. Subcell Biochem. 2013;67:235-47.

264. Chan E, Lim Y. Antioxidant activity of *Thunbergia Laurifolia* tea. J Trop For Sci. 2006;18.

265. Chan E, Lye P, Eng SY, Tan Y. Antioxidant properties of herbs with enhancement effects of drying treatments: A synopsis. Free Radicals and Antioxidants. 2013;3:2–6.

266. Tangpong J, Satarug S. Alleviation of lead poisoning in the brain with aqueous leaf extract of the *Thunbergia laurifolia* (Linn.). Toxicol Lett. 2010;198(1):83-8.

267. Phyu MP, Tangpong J. Protective effect of *Thunbergia laurifolia* (Linn.) on lead induced acetylcholinesterase dysfunction and cognitive impairment in mice. Biomed Res Int. 2013;2013:186098.

268. Wang JH, Xie H, Zhao TK, Kang B. Catalpol regulates cholinergic nerve system function through effect on choline acetyl-transferase not M receptor affinity. Biomed Pharmacother. 2015;69:291-6.

269. Wang JH, Sun QX, Xia ZQ, Hu YE. Regulatory effect of catalpol from *Radix Rehmanniae* on M2 receptor density in M2 receptor transfected CHO cells. Chin Pharmacol Bull. 2006;22:1462-6.

270. Sechi S, Chiavolelli F, Spissu N, Di Cerbo A, Canello S, Guidetti G, et al. An antioxidant dietary supplement improves brain-derived neurotrophic factor levels in serum of aged dogs: preliminary results. J Vet Med. 2015;2015:412501-.

271. Fahnestock M, Marchese M, Head E, Pop V, Michalski B, Milgram WN, et al. BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. Neurobiol Aging. 2012;33(3):546-54.

272. Liu J, He QJ, Zou W, Wang HX, Bao YM, Liu YX, et al. Catalpol increases hippocampal neuroplasticity and up-regulates PKC and BDNF in the aged rats. Brain Res. 2006;1123(1):68-79.

273. Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors. 2004;22(3):123-31.

274. Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci. 2001;24:677-736.

275. Maisonpierre PC, Le Beau MM, Espinosa R, 3rd, Ip NY, Belluscio L, de la Monte SM, et al. Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics. 1991;10(3):558-68.

276. Pandey SC. A critical role of brain-derived neurotrophic factor in alcohol consumption. Biol Psychiatry. 2016;79(6):427-9.

277. Guillin O, Griffon N, Bezard E, Leriche L, Diaz J, Gross C, et al. Brain-derived neurotrophic factor controls dopamine D3 receptor expression: therapeutic implications in Parkinson's disease. Eur J Pharmacol. 2003;480(1-3):89-95.

278. Guillin O, Diaz J, Carroll P, Griffon N, Schwartz JC, Sokoloff P. BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. Nature. 2001;411(6833):86-9.

279. Liang J, Zheng X, Chen J, Li Y, Xing X, Bai Y, et al. Roles of BDNF, dopamine D(3) receptors, and their interactions in the expression of morphine-induced context-specific locomotor sensitization. Eur Neuropsychopharmacol. 2011;21(11):825-34.

280. Bobadilla AC, Garcia-Keller C, Chareunsouk V, Hyde J, Medina Camacho D, Heinsbroek JA, et al. Accumbens brain-derived neurotrophic factor (BDNF) transmission inhibits cocaine seeking. Addict Biol. 2019;24(5):860-73.

281. Sokoloff P, Le Foll B. The dopamine D3 receptor, a quarter century later. Eur J Neurosci. 2017;45(1):2-19.

282. Bouthenet ML, Souil E, Martres MP, Sokoloff P, Giros B, Schwartz JC. Localization of dopamine D3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D2 receptor mRNA. Brain Res. 1991;564(2):203-19.

283. Booze RM, Wallace DR. Dopamine D2 and D3 receptors in the rat striatum and nucleus accumbens: use of 7-OH-DPAT and [125I]-iodosulpride. Synapse. 1995;19(1):1-13.

284. Levant B. The D3 dopamine receptor: neurobiology and potential clinical relevance. Pharmacol Rev. 1997;49(3):231-52.

285. Ciudad-Roberts A, Camarasa J, Ciudad CJ, Pubill D, Escubedo E. Alcohol enhances the psychostimulant and conditioning effects of mephedrone in adolescent mice; postulation of unique roles of D3 receptors and BDNF in place preference acquisition. Br J Pharmacol. 2015;172(20):4970-84.

286. Staley JK, Mash DC. Adaptive increase in D3 dopamine receptors in the brain reward circuits of human cocaine fatalities. J Neurosci. 1996;16(19):6100-6.

287. Segal DM, Moraes CT, Mash DC. Up-regulation of D3 dopamine receptor mRNA in the nucleus accumbens of human cocaine fatalities. Brain Res Mol Brain Res. 1997;45(2):335-9.

288. Le Foll B, Frances H, Diaz J, Schwartz JC, Sokoloff P. Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. Eur J Neurosci. 2002;15(12):2016-26.

289. Le Foll B, Diaz J, Sokoloff P. Increased dopamine D3 receptor expression accompanying behavioral sensitization to nicotine in rats. Synapse. 2003;47(3):176-83.

290. Gyertyan I, Saghy K. Effects of dopamine D3 receptor antagonists on spontaneous and agonist-reduced motor activity in NMRI mice and Wistar rats: comparative study with nafadotride, U 99194A and SB 277011. Behav Pharmacol. 2004;15(4):253-62.

291. Thanos PK, Katana JM, Ashby CR, Jr., Michaelides M, Gardner EL, Heidbreder CA, et al. The selective dopamine D3 receptor antagonist SB-277011-A attenuates ethanol consumption in ethanol preferring (P) and non-preferring (NP) rats. Pharmacol Biochem Behav. 2005;81(1):190-7.

292. Vengeliene V, Leonardi-Essmann F, Perreau-Lenz S, Gebicke-Haerter P, Drescher K, Gross G, et al. The dopamine D3 receptor plays an essential role in alcohol-seeking and relapse. Faseb j. 2006;20(13):2223-33.

293. Ashby CR, Jr., Rice OV, Heidbreder CA, Gardner EL. The selective dopamine D(3) receptor antagonist SB-277011A attenuates drug- or food-deprivation reactivation of expression of conditioned place preference for cocaine in male Sprague-Dawley rats. Synapse. 2015;69(6):336-44.

294. Rice OV, Heidbreder CA, Gardner EL, Schonhar CD, Ashby CR, Jr. The selective D(3) receptor antagonist SB-277011A attenuates morphine-triggered reactivation of expression of cocaine-induced conditioned place preference. Synapse. 2013;67(8):469-75.

295. Busceti CL, Bucci D, Molinaro G, Di Pietro P, Zangrandi L, Gradini R, et al. Lack or inhibition of dopaminergic stimulation induces a development increase of striatal tyrosine hydroxylase-positive interneurons. PLoS One. 2012;7(9):e44025.

VITA

NAME	RATIRAT SANGPAYAP
DATE OF BIRTH	05 APR 1989
PLACE OF BIRTH	Surin
INSTITUTIONS ATTENDED	Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Wattana, Bangkok, Thailand
HOME ADDRESS	522/163 Meechai mansion, Soi Songprang, Asoke-Dindaeng Rd., Dindaeng, Bangkok 10400
PUBLICATION	Thongsaard W, Sangpayap R, Marsden C. Chronic effect of Thunbergia laurifolia extract and cocaine in rats using behavior model of addiction. J Med Assoc Thai. 2015;98 Suppl 9:S48-52.
AWARD RECEIVED	-