PHARMACOLOGICAL INVESTIGATION ON ALPHA-ASARONE IN EXPERIMENTAL MODEL OF NICOTINE WITHDRAWAL INDUCED DEPRESSION IN MICE

RANJITH KUMAR CHELLIAN

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

PHARMACOLOGICAL INVESTIGATION ON ALPHA-ASARONE IN EXPERIMENTAL MODEL OF NICOTINE WITHDRAWAL INDUCED DEPRESSION IN MICE

RANJITH KUMAR CHELLIAN

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Ranjith kumar Chellian [redacted]

Matric No: MHA140058

Name of Degree: Doctor of Philosophy

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Pharmacological investigation on alpha-asarone in experimental model of nicotine withdrawal induced depression in mice.

Field of Study: Pharmacology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature [redacted] Date: 25/01/2018

Subscribed and solemnly declared before,

[redacted] Date: 25/01/2018

Name: Dr. VIJAYAPANDI PANDY.

Designation: Assoc. Professor.

Dr. Vijayapandi Pandy
Associate Professor
Department of Pharmacology
Faculty of Medicine
University of Malaya

PHARMACOLOGICAL INVESTIGATION ON ALPHA-ASARONE IN EXPERIMENTAL MODEL OF NICOTINE WITHDRAWAL INDUCED DEPRESSION IN MICE

ABSTRACT

Depression is one of the major withdrawal symptoms during smoking cessation. The cAMP response element protein binding (CREB) and brain-derived neurotrophic factor (BDNF) signalling pathways have been implicated in the neuroplasticity during nicotine-withdrawal in mice. Traditionally, *Acorus calamus* Linn (Acoraceae) rhizomes were used to discontinue the habit of smoking cigarettes. Alpha-asarone is one of the bioactive phytochemicals present in the rhizomes of *Acorus calamus* Linn and possesses an antidepressant-like activity in ICR mice. In addition, α-asarone was shown to interact with CREB and BDNF pathways. Hence, it is hypothesized that α-asarone could be effective in alleviating depression-like behaviour during nicotine-withdrawal in mice.

In this study, the dose-dependent effect of α-asarone in the tail suspension test and locomotor activity in ICR mice was studied. In addition, the possible monoaminergic mechanism(s) involved in the antidepressant effect of α-asarone was studied in alpha-methyl-para-tyrosine (AMPT, a catecholamine synthesis inhibitor) and 4-chloro-DL-phenylalanine (PCPA, a serotonin synthesis inhibitor) pre-treated ICR mice, respectively, using tail suspension test. In a separate study, the effect of α-asarone or bupropion on nicotine-induced hypomotility and hypothermia in ICR mice was investigated. In the nicotine-withdrawal study, the effect of repeated α-asarone or bupropion treatment in naïve and nicotine-withdrawn C57BL6 mice was studied in forced swim test and spontaneous locomotor activity test. In addition, the effects of repeated α-asarone or bupropion treatment on the hippocampal CREB, phosphorylated-CREB (pCREB), and BDNF levels in nicotine-withdrawn C57BL6 mice were measured.

The study results showed that the acute treatment of α-asarone at lower doses (15 and 20 mg/kg, i.p.) significantly decreased the duration of immobility time in the tail suspension test. Moreover, AMPT and PCPA pre-treatment significantly reversed the anti-immobility effect of α-asarone (20 mg/kg, i.p.) in the tail suspension test. The effect of α-asarone on nicotine's pharmacological effect revealed that the α-asarone (5, 10, 20 and 30 mg/kg, i.p.) did not reverse or block the nicotine-induced hypomotility and hypothermia in mice. However, bupropion (20 mg/kg, i.p.) significantly reversed the nicotine-induced hypomotility and hypothermia in mice. In the nicotine-withdrawal study, the immobility time of nicotine-withdrawn mice was significantly attenuated with repeated α-asarone (5, 10 and 20 mg/kg, i.p. × 8 days) or bupropion (10 mg/kg, i.p. × 8 days) pretreatment in the forced swim test. However, repeated α-asarone and bupropion treatment did not significantly alter the immobility time in the forced swim test or spontaneous locomotor activity in naïve mice. In addition, repeated α-asarone or bupropion pre-treatment significantly attenuated the hippocampal pCREB levels in nicotine-withdrawn mice.

Taken together, these results suggest that α-asarone possess an antidepressant-like activity only at lower doses through its interaction with noradrenergic, dopaminergic and serotonergic systems in ICR mice. Furthermore, unlike bupropion, α-asarone was not capable of interacting with nicotinic acetylcholine receptors. Besides, α-asarone treatment attenuated the nicotine-withdrawal induced depression-like behaviour through the modulation of hippocampal pCREB levels in nicotine-withdrawn C57BL6 mice. Therefore, α-asarone may have a place in the treatment of depression upon cessation of nicotine-containing products mainly in the form of cigarettes.

Keywords: Alpha-asarone, depression, nicotine-withdrawal, neuroplasticity

PENYIASATAN FARMAKOLOGIKAL TERHADAP ALPHA-ASARONE SEBAGAI MODEL EXPERIMENTAL PEMBERHENTIAN NIKOTIN DI DALAM TIKUS MENCIT

ABSTRAK

Kemurungan adalah salah satu daripada gejala utama semasa berhenti merokok. Laluan signal oleh elemen tindak balas cAMP yang mengikat protein (CREB) dan faktor neurotropik yang berasal dari otak (BDNF) telah terlibat dalam neuroplasticity semasa pemberhentian-nikotin di dalam tikus mencit. . Secara tradisinya, rizom *Acorus calamus* Linn (Acoraceae) digunakan untuk menghentikan tabiat menghisap rokok. Alpha-asarone adalah salah satu komponen fitokimia bioaktif yang terdapat dalam rizom *Acorus calamus* Linn dan mempunyai aktiviti seperti ubat anti-kemurungan pada tikus mencit ICR. Di samping itu, α-asarone berupaya untuk berinteraksi dengan laluan signal CREB dan BDNF. Oleh sebab itu, ianya telah dihipotesiskan bahawa α-asarone boleh memberi kesan dalam mengurangkan tingkah laku yang menyerupai kemurungan semasa pemberhentian-nikotin dalam tikus mencit.

Dalam kajian ini, kesan kebergantungan-dos α-asarone dalam ujian penggantungan ekor dan aktiviti locomotor pada tikus mencit ICR telah dikaji. Di samping itu, mekanisme monoaminergik yang mungkin terlibat dalam kesan ubat anti-kemurungan dari α-asaron telah dikaji dalam pra-rawatan tikus ICR yang telah menerima alpha-methyl-para-tyrosine (AMPT), dan 4-chloro-DL-phenylalanine (PCPA), secara berasingan, dengan menggunakan ujian penggantungan ekor. Dalam kajian yang berasingan, kesan α-asarone atau bupropion terhadap nikotin-meransang hipomotiliti dan hipotermia, dalam tikus mencit ICR telah dikaji. Dalam kajian pemberhentian-nikotin , kesan pengambilan α-asarone atau bupropion yang berulang kali terhadap kumpulan tikus mencit naif dan tikus mencit pemberhentian-nikotin dari strain C57BL6 telah dikaji melalui ujian paksa-renang dan ujian aktiviti locomotor spontan. Di samping itu, kesan

pengambilan α-asarone atau bupropion yang berulang terhadap CREB hippocampal, fosforilasi-CREB (pCREB), dan paras BDNF dalam tikus mencit C57BL6 pemberhentian-nikotin, telah diukur.

Hasil kajian menunjukkan bahawa rawatan akut α-asarone pada dos yang agak rendah (15 dan 20 mg / kg, i.p)mengurangkan dengan ketara terhadap tempoh masa imobiliti dalam ujian penggantungan ekor. Selain itu, pra-rawatan AMPT dan PCPA secara signifikan membalikkan kesan anti-imobiliti α-asarone (20 mg / kg, i.p) dalam ujian penggantungan ekor. Kesan α-asarone terhadap nikotin menunjukkan bahawa α-asarone (5, 10, 20 dan 30 mg / kg, i.p.) tidak menghalang hipomotiliti dan hipotermia dalam tikus. Walau bagaimanapun, bupropion (20 mg / kg, i.p) mampu membuat sedemikian. Dalam kajian pemberhentian-nikotin, tempoh masa imobiliti dalam tikus pemberhentian-nikotin, telah dikurangkan dengan memberi pra-rawatan α-asarone secara berulang (5, 10 dan 20 mg / kg, ip × 8 hari) atau bupropion (10 mg / kg, ip × 8 hari) dalam ujian paksa renang. Walau bagaimanapun, pengunaan α-asarone dan bupropion secara berulangan tidak banyak mengubah masa imobilasi dalam ujian berenang-paksa atau aktiviti locomotor spontan dalam tikus mencit naif. Tambahan pula, pra-rawatan α-asarone atau bupropion berulang kali telah melemahkan dengan ketara paras pCREB hippocampal dalam tikus mencitpemberhentian-nikotin.

Secara keseluruhan, keputusan kajian ini menunjukkan bahawa α-asarone hanya mempunyai kesan aktiviti seakan anti-kemurungan pada dos yang agak rendah melalui interaksi dengan sistem noradrenergik, dopaminergik dan serotonergik pada tikus mencit ICR. Tambahan pula, tidak seperti bupropion, α-asarone tidak berupaya berinteraksi dengan reseptor asetilkolin nikotinik. Di samping itu, rawatan α-asarone mengurangkan tingkah laku menyerupai kemurungan yang diransang olehpemberhentian-nikotin melalui modulasi paras pCREB hippocampal dalam tikus mencit C57BL6. Oleh sebab itu, α-

asarone mungkin berupaya membantu dalam rawatan kemurungan akibat penghentian pengambilan produk yang mengandungi nikotin, terutamanya dalam bentuk rokok.

Kata kunci: Alpha-asarone, kemurungan, nikotin-pengeluaran, neuroplasticity

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to individuals that have made this thesis possible. My foremost appreciation to my supervisors Assoc/Prof. Dr. Vijayapandi Pandy and Prof. Dr. Zahurin Mohamed for their patience, invaluable guidance, critical comments, insightful ideas and motivation throughout my research. I am very grateful to Dr. Jayakumar Rajarajeswaran, Department of Molecular Medicine, for providing invaluable guidance related to molecular biology techniques. Also, appreciation to my colleagues for their continuous support throughout my research. My sincere thanks to Department of Pharmacology for providing an opportunity with the good learning environment. Also, I would like to extend my thanks to Department of Pharmacology and Medical Biotechnology Laboratory for providing the necessary infrastructure and facilities to conduct this research. I would like to express my special gratitude to my beloved parents, brother and friends for their love, invaluable support and encouragement throughout my doctoral journey. This research was supported by the University of Malaya Research Grant (H-20001-E000088 and PG008-2015A). Special thanks to the International Brain Research Organization (IBRO) for awarding me the IBRO International travel grant-2017 to attend the Society for Research on Nicotine & Tobacco Annual Meeting -2017.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	alpha
β	:	beta
γ	:	gamma
%	:	percentage
µg	:	microgram
µL	:	microlitre
µM	:	micromolar
°C	:	degree Celsius
cm	:	centimetre
h	:	hour
kg	:	kilogram
L	:	litre
mg	:	milligram
min	:	minute
mL	:	millilitre
nm	:	nanometre
nmol	:	nanomole
s	:	second
v/v	:	volume/volume
w/w	:	weight/weight
AMPT	:	alpha-methyl-para-tyrosine
Asr	:	alpha-asarone
ANOVA	:	analysis of variance

BCA	:	bicinchoninic acid
BDNF	:	brain-derived neurotrophic factor
b.i.d	:	twice daily
Bup	:	bupropion
C57BL6	:	C57 black 6
cAMP	:	cyclic adenosine monophosphate
Ca^{2+}	:	calcium ions
CaM	:	calmodulin
CaMKIV	:	calcium/calmodulin-dependent protein kinase type IV
CRE	:	cAMP response element
CREB	:	cAMP response element binding protein
cRNA	:	complementary ribonucleic acid
CNS	:	central nervous system
CYP450	:	cytochrome P450
DOPAC	:	3,4-dihydroxyphenylacetic acid
EAAC1	:	excitatory amino acid carrier 1
ELISA	:	enzyme linked immunosorbent assay
ERK	:	extracellular signal-regulated kinases
FDA	:	food and drug administration
GABA	:	gamma-aminobutyric acid
HRP	:	horseradish peroxide
HVA	:	homovanillic acid
IC_{50}	:	half maximal inhibitory concentration
ICR	:	institute of cancer research
IgG	:	immunoglobulin

i.p.	:	intraperitoneal injection
LD_{50}	:	median lethal dose
mAb	:	monoclonal antibody
MAO	:	monoamine oxidase
MAPK	:	mitogen-activated protein kinases
NAc	:	nucleus accumbens
nAChRs	:	nicotinic acetylcholine receptors
NGF	:	nerve growth factor
NMDA	:	N-methyl D-aspartate
NRT	:	nicotine replacement therapy
o.d.	:	once daily
PCPA	:	para-chlorophenylalanine
pCREB	:	phosphorylated-CREB
PI3K	:	phosphatidylinositol 3-kinase
PKA	:	protein kinase A
PKAc	:	catalytic subunits of PKA
PKA_R	:	regulatory subunits of PKA
PLCγ	:	phospholipase Cγ
p.o.	:	per oral route
RNA	:	ribonucleic acid
RSK	:	ribosomal s6 kinase
s.c.	:	subcutaneous injection
Ser 133	:	serine 133
$t_{1/2}$	:	half-life
TMB	:	3,3', 5,5'-Tetramethylbenzidine
TrkB	:	tropomyosin receptor kinase B

T80	:	tween 80
VTA	:	ventral tegmental area
5-HT	:	5-hydroxytryptamine

CHAPTER 1: INTRODUCTION

1.1 Overview

In humans, the chronic use of tobacco products causes nicotine addiction. The activation of neuronal nicotinic acetylcholine receptors (nAChRs) by nicotine stimulates the release of dopamine in the mesolimbic regions and produces pleasurable effects (N. L. Benowitz, 2009; Govind, Vezina, & Green, 2009; Laviolette & van der Kooy, 2004). The activation of nAChRs by nicotine also releases other neurotransmitters such as acetylcholine, glutamate, noradrenaline, serotonin, and gamma-aminobutyric acid (GABA), which results in modification of numerous physiological processes such as locomotion, anxiety, depression, nociception, learning and memory, and also produces physical dependence (N. L. Benowitz, 2008; Neal L. Benowitz, 2010; M. R. Picciotto & Mineur, 2014; Xi, Spiller, & Gardner, 2009). Cessation of tobacco products mainly smoking cigarettes induce withdrawal symptoms such as depression, anxiety, anhedonia, dysphoria, hyperalgesia and cognitive deficits (Kenny & Markou, 2001; Markou, 2008). In particular, depression is one of the predominant withdrawal symptoms in smoking cessation and contribute to smoking relapse (Lembke, Johnson, & DeBattista, 2007; Leventhal, Ameringer, Osborn, Zvolensky, & Langdon, 2013; Reid & Ledgerwood, 2016).

Monoamine neurotransmitters (dopamine, noradrenaline and serotonin) plays a key role in developing withdrawal symptoms during nicotine cessation. In rodents, nicotine- withdrawal decreases brain dopamine, noradrenaline, and serotonin levels and produce withdrawal symptoms (Benwell & Balfour, 1979; Gäddnäs, Pietilä, & Ahtee, 2000). The phosphorylated- cAMP response element-binding protein (pCREB) and brain-derived neurotrophic factor (BDNF) are two well-characterized biomarkers of neuronal plasticity in nicotine addiction and withdrawal. Interestingly, the activation of CREB (elevated pCREB level) signalling pathway in the nucleus accumbens is required for the

development of nicotine reward in mice (Brunzell, Mineur, Neve, & Picciotto, 2009; Walters, Cleck, Kuo, & Blendy, 2005). In addition, both pCREB and BDNF levels is increased in several brain regions (prefrontal cortex, ventral tegmental area, nucleus accumbens, striatum, substantia nigra or hippocampus) of mice or rats during chronic nicotine administration and nicotine-withdrawal (Brunzell, Russell, & Picciotto, 2003; Fisher, LeMalefant, Zhou, Huang, & Turner, 2016; Kenny, File, & Rattray, 2000; Kivinummi, Kaste, Rantamaki, Castren, & Ahtee, 2011; Roni & Rahman, 2014; Turner et al., 2014; Walters et al., 2005).

Acorus calamus Linn (Acoraceae) is an indigenous medicinal plant used in traditional Indian and Chinese system of medicine for the treatment of central nervous system (CNS) disorders (Rajput, Tonge, & Karuppayil, 2014). Traditionally, the powder made from the rhizomes of *Acorus calamus* is smoked or chewed for treatment of tobacco addiction and had been claimed to be helpful in smoking cessation (Motley, 1994). Alpha-asarone is one of the bioactive phytochemical presents in the rhizomes of *Acorus calamus* Linn (Acoraceae) (Rajput et al., 2014). Interestingly, the extract of *Acorus calamus* rhizomes and α-asarone in the animal models of CNS disorders shares similar pharmacological activities such as antianxiety, antidepressant, antiepileptic, neuroprotective, anti-dementia and anti-Alzheimer's effects (Han, Han, Peng, & Wang, 2013; Kumar et al., 2012; Lee et al., 2014; Liu et al., 2012; Rajput et al., 2014; Shin et al., 2014). Furthermore, α-asarone is found to be effective against the depression-like behaviour in two well-validated mouse models of depression such as tail suspension test and forced swim test in ICR mice (Han et al., 2013). At present, the dose-dependent effect of α-asarone on depression-like behaviour and the possible contribution of the monoaminergic mechanism (s) which underlie its antidepressant-like effect is unknown. Recently, it has been found that α-asarone treatment increased the pCREB expressions through protein kinase-A signalling pathway in the cultured pheochromocytoma (PC12)

cells (Lam et al., 2016). In addition, α-asarone treatment showed an interaction with hippocampal BDNF signalling pathways in exogenous corticosterone injected rats (Lee et al., 2014). Based on these existing literature, it has been hypothesised that the α-asarone could alleviate depression-like behaviour during nicotine-withdrawal through its modulatory activity on neuroplasticity during nicotine-withdrawal. Besides, the interaction of α-asarone with nicotinic acetylcholine receptors is unknown.

CHAPTER 2: LITERATURE REVIEW

2.1 Nicotine addiction

Tobacco consumption is one of the main global public health problems and contributes to preventable deaths in most developed countries. Tobacco use is a major risk factor for serious health consequences such as cancer, cardiovascular disease and, pulmonary disease and was projected to kill 1 billion people during the 21st century (Neal L. Benowitz, 2010; Dome, Lazary, Kalapos, & Rihmer, 2010; Jha & Peto 2014; O'Connor, 2012). Nicotine is a most abundant alkaloid ((*S*)-nicotine, 95% of total alkaloid content) and a primary addictive compound present in the leaves of tobacco plant, which is a CNS stimulant and known to cause tobacco addiction (N. L. Benowitz, 2009; Berrendero, Robledo, Trigo, Martin-Garcia, & Maldonado, 2010; Dome et al., 2010; Matta et al., 2007). Tobacco leaves also contain minor concentration of other alkaloids such as (*R*)-nicotine, nornicotine, anatabine, and anabasine, which is pharmacologically active and less potent than nicotine ((*S*)-nicotine) (Armstrong, Wang, & Ercal, 1998; N. L. Benowitz, 2009; B. Cai, Jack, Lewis, Dewey, & Bush, 2013; Matta et al., 2007). Tobacco leaves are available as smoked and non-smoked products. Smoked tobacco products include manufactured cigarettes, cigars, kreteks, pipes, water pipes, bidis, and cheroot. Smokeless forms of tobacco are chewing tobacco, moist snuff and, dry snuff, however, all of these forms of tobacco is addictive due to the presence of nicotine (O'Connor, 2012).

Smoking cigarettes account for 98 % of tobacco use, which is a highly effective way of nicotine intake other than nicotine-containing products (Dome et al., 2010; Mitrouska, Bouloukaki, & Siafakas, 2007). Importantly, > 80 % of smokers attempt to quit cigarette smoking, however, relapse occurs within the first few months of abstinence and only ~3% remain abstinent in six months (N. L. Benowitz, 2009; Dwoskin et al., 2009). This epidemic reflects the strong addictive and reinforcement nature of nicotine.

2.1.1 Neuropharmacology of nicotine addiction

Nicotine is a potent agonist at nicotinic acetylcholine receptors (nAChRs) (Dani, 2015; Whiting & Lindstrom, 1988). The nAChRs are the ligand-gated ion channel proteins (pentameric), highly permeable to calcium (Ca^{2+}) ions and consist of a different combination of α_1-α_{10}, β_1-β_4, γ, δ and ε subunits which are homomeric, and heteromeric in nature (Dani, 2015; Grando, 2014). These receptors are activated by nicotine or acetylcholine and the pharmacological activities of nicotine depend on the combination of subunits formed as nAChRs (D'Souza & Markou, 2011; Dani, 2015; Grando, 2014; Marina R. Picciotto, Caldarone, King, & Zachariou, 2000). To date, several neuronal [homomeric subunits: $(\alpha_7)_5$; heteromeric subunits: (α_2, α_3, α_4, α_5, or $\alpha_6)_{2\text{-}3}$, (β_2 and/or β_3 and/or $\beta_4)_{2\text{-}3}$] and non-neuronal or muscle [heteromeric subunits: $(\alpha_1)_2$, β_1, γ and δ or ε] types of human nAChRs were identified (Dani, 2015; Grando, 2014).

The activation of neuronal $\alpha_4\beta_2$ and α_7 subunits containing neuronal nAChRs by nicotine facilitates the release of dopamine neurotransmitter in the mesolimbic dopaminergic region mainly ventral tegmental area and nucleus accumbens regions, produces rewarding, motivation and reinforcing effect of nicotine (**Figure 2.1**) (N. L. Benowitz, 2009; Govind et al., 2009; Laviolette & van der Kooy, 2004). Moreover, nucleus accumbens receive limbic information from the amygdala, hippocampus, and frontal cortex regions which are also involved in the reinforcement of nicotine (Nestler, 2005; Marina R. Picciotto et al., 2000; M. R. Picciotto & Mineur, 2014).

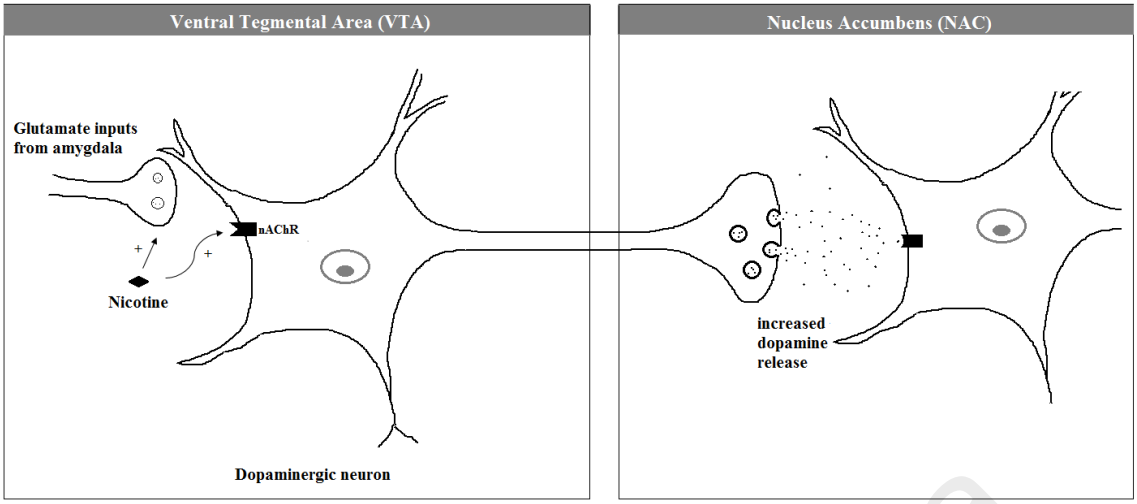

Figure 2.1: Mesolimbic reward pathway in nicotine addiction. The mesolimbic dopamine pathway includes dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their projections in the limbic forebrain, especially the nucleus accumbens (NAc). The mesolimbic dopamine pathway (VTA-NAc) is the primary substrate for the rewarding effect of nicotine. Nicotine directly activates the VTA dopaminergic neurons through the activation (+) of nicotinic acetylcholine receptors (nAChR) and indirectly stimulates the glutamate nerve terminals from the amygdala, resulting in evoked dopamine release in the NAc and produces a rewarding effect. This nicotine-evoked dopamine release causes addiction. Diagram adapted from Nestler (2005).

The activation of neuronal nAChRs by nicotine also enhances the release of other neurotransmitters such as acetylcholine, noradrenaline, serotonin, glutamate and GABA, which results in the modification of numerous physiological processes such as locomotion, anxiety, depressed mood, nociception, learning and memory and produces physical dependence (**Figure 2.2**) (N. L. Benowitz, 2008; M. R. Picciotto & Mineur, 2014; Xi et al., 2009). In humans, the reinforcing effect of nicotine contributes to initiation and maintenance of using tobacco products (e.g. smoking cigarettes) (Jasinska, Zorick, Brody, & Stein, 2014; B. Le Foll & Goldberg, 2005). In other species such as rats and mice, the reinforcing effect of nicotine is evidenced from intravenous self-administration of nicotine (Corrigall, 1991; DeNoble & Mele, 2006; Fowler & Kenny, 2011).

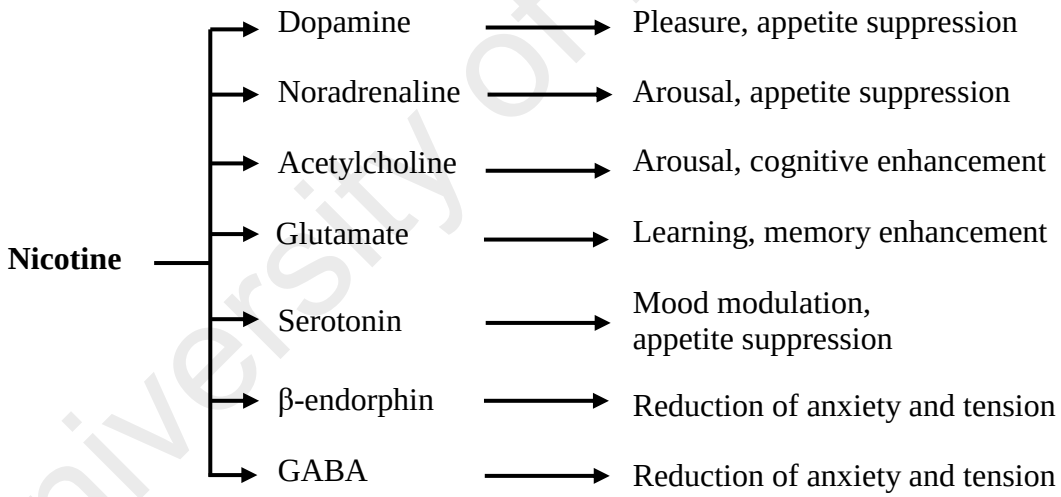

Figure 2.2: Effect of nicotine on brain neurotransmitter release and its physiological processes. Nicotine also activates the nicotinic acetylcholine receptors in various brain regions, releases neurotransmitters such as dopamine, noradrenaline, acetylcholine, glutamate, serotonin, beta-endorphin and GABA, which produces behavioural effects of nicotine and contributes to reinforcement action of nicotine. A schematic diagram adapted from N. L. Benowitz (1999).

2.2 Nicotine-withdrawal

Chronic nicotine use causes neuroadaptive changes in the mesolimbic pathway, which results in nicotine dependence. In humans, cessation of chronic use of nicotine-containing products mainly smoking cigarettes produces aversive abstinence syndrome due to the imbalance in the brain neurochemicals caused by the absence of nicotine. The severity of nicotine withdrawal symptoms mainly depends on how nicotine is consumed. The nicotine withdrawal symptoms are classified as somatic, affective and cognitive symptoms (Kenny & Markou, 2001; Markou, 2008; McLaughlin, Dani, & De Biasi, 2015). Somatic symptoms manifest as bradycardia, increased appetite, gastrointestinal discomfort, and tremors. Affective symptoms during nicotine-withdrawal include depressed mood, anxiety, dysphoria, anhedonia, hyperalgesia and irritability. Cognitive symptoms include difficulty in concentrating and impaired memory (Heishman, Kleykamp, & Singleton, 2010; Wesnes, Edgar, Kezic, Salih, & de Boer, 2013). Moreover, these withdrawal symptoms peak within the first week of withdrawal and last for 2-4 weeks (Hughes, 2007; Hughes, Gust, Skoog, Keenan, & Fenwick, 1991). Thus, to avoid these negative withdrawal symptoms the nicotine intake should be maintained. For that, compulsive use of nicotine-containing products mainly smoking cigarettes is continued and this could be one of the contributing factors for smoking relapse. Besides, the contribution of the affective symptoms is much higher than somatic signs in smoking relapse (K. J. Jackson, Muldoon, De Biasi, & Damaj, 2015; McLaughlin et al., 2015; Paolini & De Biasi, 2011).

2.2.1 Nicotine-withdrawal in rodents

In rodents, nicotine withdrawal manifests as somatic, and affective symptoms and cognitive deficits exhibits as those observed in humans. Nicotine-withdrawal induced somatic signs are mediated by both central, and peripheral nAChRs and affective symptoms are mediated through the central nAChRs in mice or rats (K. J. Jackson, Martin, Changeux, & Damaj, 2008; Watkins, Stinus, Koob, & Markou, 2000). The experimental methods such as repeated subcutaneous or intraperitoneal nicotine injection (Bagosi et al., 2016; Isola, Vogelsberg, Wemlinger, Neff, & Hadjiconstantinou, 1999; Zaniewska, McCreary, Wydra, & Filip, 2010), nicotine infusion through subcutaneous osmotic minipump implantation containing nicotine solution (Damaj, Kao, & Martin, 2003; Malin et al., 1992) and chronic oral nicotine self-administration (Grabus et al., 2005; Roni & Rahman, 2014) methods are carried out to induce nicotine dependence in mice or rats. Followed by the cessation of chronic nicotine administration, nicotine-withdrawal symptoms were assessed.

The nicotine-withdrawal in mice and/or rats induced somatic signs includes tremors (fore paw, body, facial), shakes (head, body, forelimb, wet dog), jumping, backing, chewing, rearing, scratching, grooming, circling, digging, abdominal constriction, licking (paw, genital), piloerection, ptosis, teeth chattering, yawns or changes in locomotor activity (Bagosi et al., 2016; Cryan, Bruijnzeel, Skjei, & Markou, 2003; Damaj et al., 2003; Isola et al., 1999; Kotagale, Chopde, Umekar, & Taksande, 2015; Malin et al., 1992; Zhao-Shea, Liu, Pang, Gardner, & Tapper, 2013). The affective symptoms during nicotine-withdrawal shows hyperalgesia, anxiety, dysphoria, anhedonia and depression-like behaviour in nicotine-withdrawn mice and/or rats (Biala, Polak, Michalak, Kruk-Slomka, & Budzynska, 2014; Damaj et al., 2003; Igari et al., 2014; K. J. Jackson et al., 2008; Johnson, Hollander, & Kenny, 2008; Jonkman, Henry, Semenova, & Markou, 2005; Mannucci, Tedesco, Bellomo, Caputi, & Calapai, 2006; Roni &

Rahman, 2014; Zaniewska et al., 2010). Furthermore, during nicotine-withdrawal cognitive deficit was observed in nicotine-withdrawn mice (Davis, James, Siegel, & Gould, 2005; Kenney, Adoff, Wilkinson, & Gould, 2011). Importantly, these withdrawal symptoms in mice or rats occur within the first week of nicotine-withdrawal, however, anxiety-like behaviour, learning and memory deficits, hyperalgesia last up to 2 weeks of nicotine-withdrawal, and depression like-behaviour last up to 60 days of nicotine-withdrawal and which mimics the occurrence of nicotine-withdrawal symptoms in humans (Damaj et al., 2003; Gould et al., 2012; Johnson et al., 2008; Mannucci et al., 2006; Roni & Rahman, 2014).

2.2.2 Depression during nicotine-withdrawal

Depressed mood is one of the predominant withdrawal symptoms in smoking cessation and contributes to smoking relapse (Lembke et al., 2007; Leventhal et al., 2013; Reid & Ledgerwood, 2016). Besides, individual with history of depression shows high rates of nicotine dependence, the high degree of depressed mood was observed during nicotine withdrawal and makes quitting smoking unsuccessful (Covey, Glassman, & Stetner, 1997; Lembke et al., 2007; Leventhal et al., 2013). In rodents, depression-like behaviour upon nicotine cessation is commonly measured using forced swim test. During nicotine withdrawal, the immobility time in forced swim test is increased on day 1, 3, 4, 8, 10, 14, 15, 30, 45 and 60 of nicotine-withdrawal in mice or rats, which indicates depression-like behaviour persist up to 60 days of nicotine-withdrawal (Bagosi et al., 2016; Biala et al., 2014; Kotagale et al., 2015; Mannucci et al., 2011; Mannucci et al., 2006; Roni & Rahman, 2014; Zaniewska et al., 2010).

The monoamine theory of depression states that the depression is caused by the functional deficit of the brain monoamine neurotransmitters mainly noradrenaline and/or serotonin (Schildkraut, 1965). In addition, both preclinical and clinical evidence suggest

that the involvement of down-regulation of brain monoamine neurotransmitters (noradrenaline, serotonin and dopamine) in the pathophysiology of depression (Moret & Briley, 2011). Besides, nicotine administration in mice and rats increased the brain neurochemicals such as dopamine, noradrenaline and serotonin levels (Gäddnäs, Piepponen, & Ahtee, 2002; Gäddnäs et al., 2000; Singer et al., 2004). These nicotine-mediated neurochemical changes in brain relieve stress and, improve mood and because of these behavioural effects smoking cigarettes is very hard to quit. However, the neurotransmitter (noradrenaline, serotonin and dopamine) release by nicotine resembles the mechanism of currently available antidepressant drugs such as noradrenaline reuptake inhibitors, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors, serotonin and noradrenaline reuptake inhibitors, and tricyclic antidepressants, which are effective in the treatment of depression by enhancing the brain dopamine, noradrenaline and/or serotonin levels (N. L. Benowitz, 2008; Berton & Nestler, 2006; Moret & Briley, 2011). In recent preclinical and clinical studies, nicotine showed antidepressant activity (Salin-Pascual, Rosas, Jimenez-Genchi, Rivera-Meza, & Delgado-Parra, 1996; Tizabi et al., 1999; Vazquez-Palacios, Bonilla-Jaime, & Velazquez-Moctezuma, 2005), however, it is not recommended clinically for the treatment of depression due to its highly addictive liability. Nicotine cessation modulates the brain monoaminergic neurotransmission, leads to withdrawal symptoms such as irritability and depressed mood (K. J. Jackson et al., 2015; Kenny & Markou, 2001).

2.2.3 Brain monoaminergic systems during nicotine-withdrawal

Chronic nicotine administration in rodents elevates the brain monoamine (dopamine, noradrenaline and serotonin) levels. During nicotine withdrawal, the metabolites of dopamine [3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA)], noradrenaline and serotonin levels in the brain regions (striatum, hypothalamus, cortex or hippocampus) of mice and rats was decreased in the absence of nicotine (Benwell &

Balfour, 1979; Gäddnäs et al., 2000). For instance, in preclinical studies, the drugs which elevates the brain dopamine, noradrenaline and/or serotonin including bupropion, atomoxetine and desipramine attenuates the nicotine-withdrawal symptoms such as somatic signs, depression-like behaviour, anxiety-like behaviour or learning and memory deficits during nicotine cessation in rodents (Damaj et al., 2010; Davis & Gould, 2007; Paterson, Semenova, & Markou, 2008; Placzek, Zhang, & Dani, 2009; Roni & Rahman, 2014).

Similarly, in clinical studies, the positive neuromodulators of brain monoaminergic systems such as bupropion, nortriptyline, atomoxetine and fluoxetine attenuate the withdrawal symptoms during smoking cessation (Gonzales et al., 2006; Jorenby et al., 2006; Prochazka et al., 1998; Saules et al., 2004; Silverstone & Dadashova, 2012). Interestingly, most of these drugs are approved as antidepressants. Thus, these evidences highlight that the imbalance in the brain monoamine levels upon nicotine cessation produces withdrawal symptoms and if any compounds which have positive modulatory activity on monoaminergic system mainly noradrenergic and/or serotonergic systems could improve the depressed mood during nicotine withdrawal.

2.3 Neuronal plasticity during nicotine-withdrawal

In the mammalian brain, neuroplasticity plays a key factor in normal neuronal development and activity throughout the lifespan. The neuronal activity generated by experiences (learning, stress, or consumption of psychoactive substances), impact the brain by modifying the activity and organization of specific neuronal circuitry and thereby modifies subsequent thoughts, feelings and behaviour, that is, neuroplasticity (Citri & Malenka, 2008; N. Sharma, Classen, & Cohen, 2013). At the cellular level, neuronal circuits consist of synaptic connections between axons and dendrites. A ubiquitous mechanism by which the neuronal activity produced by experiences, changes brain

function through modification of the strength of synaptic transmissions, that is synaptic plasticity (Citri & Malenka, 2008; N. Sharma et al., 2013). Importantly, impairments in the synaptic plasticity contribute to several neuropsychiatric disorders.

Chronic use of addictive drugs produces neuroadaptive changes. The activation of neuronal nAChRs (ligand-gated cation channels) by nicotine induce a depolarization and calcium (Ca^{2+}) influx. This Ca^{2+} influx induces subsequent Ca^{2+} release from the intracellular stores and initiates intracellular cascades, whereas, depolarization electrically excites the neuronal cells. Moreover, the entry of Ca^{2+} ions into neurons release neurotransmitters including dopamine, noradrenaline, serotonin, glutamate and, GABA from the vesicles, which produces behavioural effects such as pleasure, stimulation and improved mood. Chronic nicotine exposure produces increased expressions of nAChRs which arise from nAChRs desensitisation. Thereby, neuroadaptive changes in dopaminergic, noradrenergic, serotoninergic, glutamatergic and, GABAergic circuit occurs, which strengthen the communication between neurons and facilitates the development of new neuronal circuits (synaptic plasticity), and plays a key role in the rewarding and reinforcing effect of nicotine (Neal L. Benowitz, 2010; Dani, Ji, & Zhou, 2001). In the absence of nicotine, due to the synaptic plasticity of nicotine-mediated neuronal circuits, an imbalance in brain neurotransmitters produces withdrawal symptoms and contributes to craving and relapse. Besides, associative learning contributes to the development of nicotine addiction, which is the association between stress, unpleasant mood or environmental factors (smoking-related cues) and reinforcing, and the rewarding effects of nicotine, which causes neuroadaptation (synaptic plasticity). The brain regions mainly involved in associative learning includes nucleus accumbens, amygdala and hippocampus. For instance, an unpleasant or depressed mood is conditional cues for smoking, and a smoker may learn that without smoking cigarettes increases irritability while smoking cigarettes gives relief (Neal L. Benowitz, 2010; Dani

et al., 2001). In rodents, long-term nicotine exposure facilitates synaptic plasticity in different brain regions including nucleus accumbens, ventral tegmental area, cortex, and hippocampus which contributes to the reward and withdrawal signs (Brunzell et al., 2009; Brunzell et al., 2003; Walters et al., 2005).

2.3.1 cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF)

The chronic use of addictive drugs causes changes in the brain CREB and BDNF levels, which promotes long-term changes in synaptic strength and contributes to drug reward, seeking and withdrawal symptoms (Autry & Monteggia, 2012; Kalivas & O'Brien, 2007). The CREB and BDNF signalling are required for the development, survival and behaviour-related plasticity of the neurons (Autry & Monteggia, 2012; Lonze & Ginty, 2002). The CREB and BDNF are two well-characterized biomarkers of neuronal plasticity in nicotine addiction, and withdrawal and which is required for the nicotine-mediated behavioural effect and withdrawal symptoms.

2.3.1.1 Involvement of CREB in nicotine addiction and withdrawal

Extracellular stimuli such as excitatory neurotransmitters, ligands for G-protein coupled receptors, and stress inducers (activity-dependent survival) and neuronal growth factors (BDNF-dependent survival) activates intracellular protein kinase [protein kinase-A (PKA), calcium/calmodulin-dependent protein kinase type IV (CaMKIV), mitogen-activated protein kinases (MAPK) and ribosomal s6 kinase (RSK)] cascades which then activates the nuclear transcription factor CREB. These protein kinases translocate to the nucleus and activate CREB by the phosphorylation at serine residue 133 (Ser 133) of CREB. Within the nucleus, pCREB binds to cAMP response element (CRE) and activates gene transcription. CREB promotes the transcription of genes involved in addiction such as c-fos, delta-fos. Moreover, CREB is a nuclear transcription factor which regulates the

gene expression of BDNF. The CREB mediated gene expressions contributes to growth, and survival of neurons, neuroprotection, synaptic plasticity and regulates physiological, and behavioural effects with respect to environmental factors (e.g. smoking-related cues) (Autry & Monteggia, 2012; Lonze & Ginty, 2002; Nestler, 2004; Shaywitz & Greenberg, 1999).

In nicotine addiction, the influx of Ca^{2+} ions through the activation of calcium-permeable nAChRs by nicotine activates the CaMKIV cascades, then phosphorylates the transcription factor CREB, which results in neurotransmitter release and contributes to synaptic plasticity (**Figure 2.3**). It is evident that nicotine administration in mice increased the CaMKIV expressions in nucleus accumbens (Kia J. Jackson, Sanjakdar, Chen, & Damaj, 2012). Interestingly, nicotine reward is not produced in CaMKIV knockout mice, using nicotine conditioned place preference test. Furthermore, during nicotine withdrawal CaMKIV knockout mice did not produce affective symptoms (anxiety-like behaviour), however, CaMKIV knockout develops somatic signs, which infers CaMKIV is mainly involved in the nicotine-withdrawal induced affective symptoms (Kia J. Jackson et al., 2012).

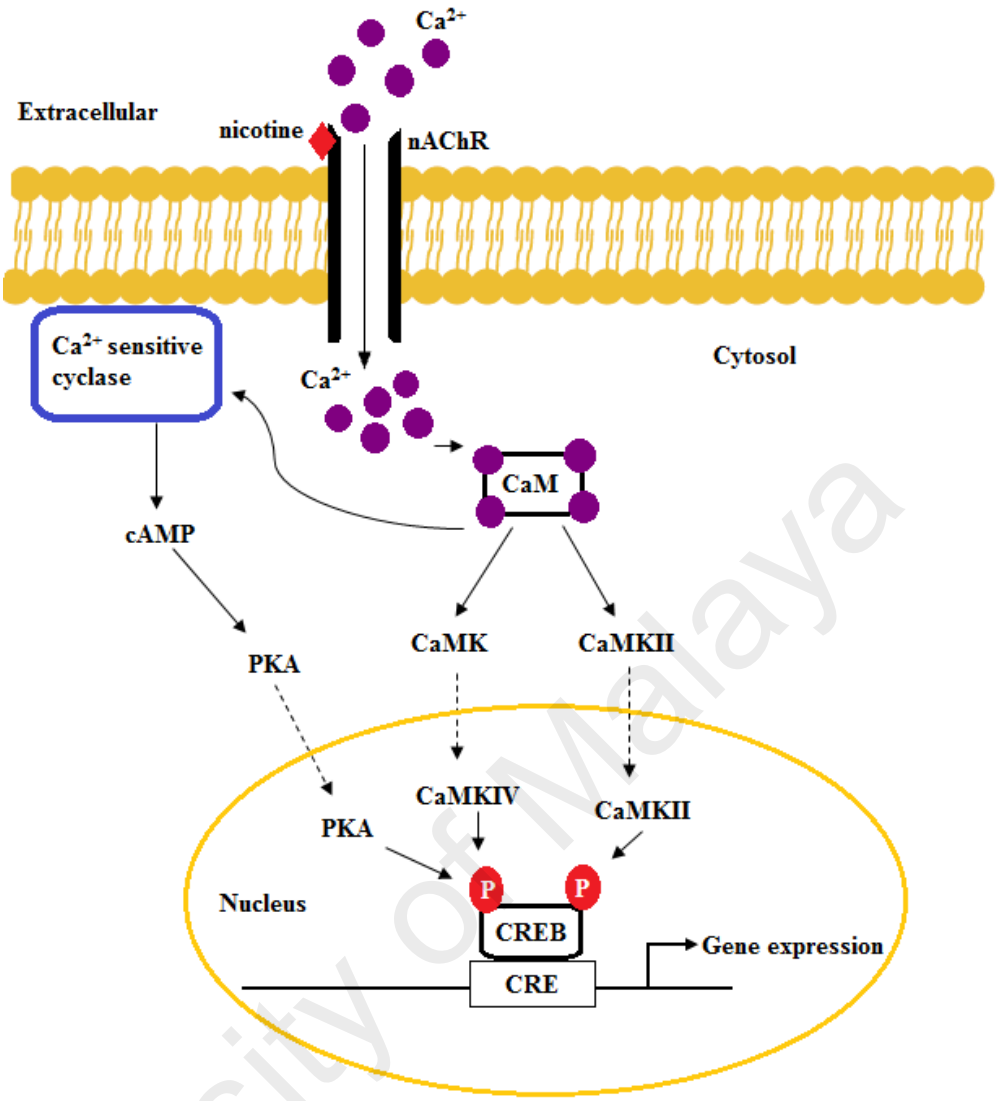

Figure 2.3: Activation of CREB-mediated gene expression by nicotine. The activation of nicotinic acetylcholine receptors (nAChRs) by nicotine causes Ca^{2+} influx and upon entering the neuronal cell, Ca^{2+} binds to a protein calmodulin (CaM). This Ca^{2+} /CaM complex directly activates calcium/calmodulin-dependent protein kinases (CaMK), then translocate into the nucleus and phosphorylates CREB at Ser 133. Besides, Ca^{2+} /CaM complex also activate the protein kinase-A (PKA) pathway by stimulating the Ca^{2+} - sensitive cyclase, results in the production of cyclic adenosine monophosphate (cAMP) and activates PKA. Then the PKA translocate into the nucleus and phosphorylates CREB (pCREB) at Ser 133, binds to cAMP response element (CRE) and initiates CREB mediated gene expressions. Diagram adapted from Shaywitz and Greenberg (1999).

The activation of nAChRs by nicotine primarily releases dopamine which binds to dopaminergic receptors and regulates the CREB-mediated gene expressions (McPherson & Lawrence, 2007; Shaywitz & Greenberg, 1999). The dopaminergic receptors are classified as D_1-like family (D_1 and D_5) and D_2-like family (D_2, D_3 and D_4) receptors. Both D_1- and D_2-like family receptors are G-protein coupled receptors. The dopamine D_1-like family receptors are coupled to G protein Gs alpha subunit, which activates adenylyl cyclase and thereby increasing the intracellular concentration of second messenger cAMP and activates the PKA. Then, the activated PKA translocate into the nucleus and activates CREB-mediated gene expression (**Figure 2.4**) (McPherson & Lawrence, 2007; Shaywitz & Greenberg, 1999). The dopamine D_2-like family receptors are coupled to G protein Gi alpha subunit, which inhibits the formation of second messenger cAMP by inhibiting adenylyl cyclase.

The involvement of mesocorticolimbic or hippocampal CREB activity plays a key role in producing nicotine reward and withdrawal signs in rodents. In *in vitro* study, nicotine treatment increased the expression of pCREB expressions in PC12 cells (Nakayama, Numakawa, Ikeuchi, & Hatanaka, 2001). Besides, *in vivo* studies revealed that chronic nicotine treatment increased the pCREB levels in several brain regions of the mice including the prefrontal cortex, ventral tegmental area, nucleus accumbens, striatum and hippocampus (Brunzell et al., 2003; Walters et al., 2005). Interestingly, the elevated pCREB levels in the nucleus accumbens is required for the development of nicotine reward (Brunzell et al., 2009; Walters et al., 2005). Similarly, during nicotine withdrawal, increase in pCREB levels was evident in the ventral tegmental area, nucleus accumbens and hippocampus of mice (Brunzell et al., 2003; Fisher et al., 2016; Kivinummi et al., 2011; Roni & Rahman, 2014).

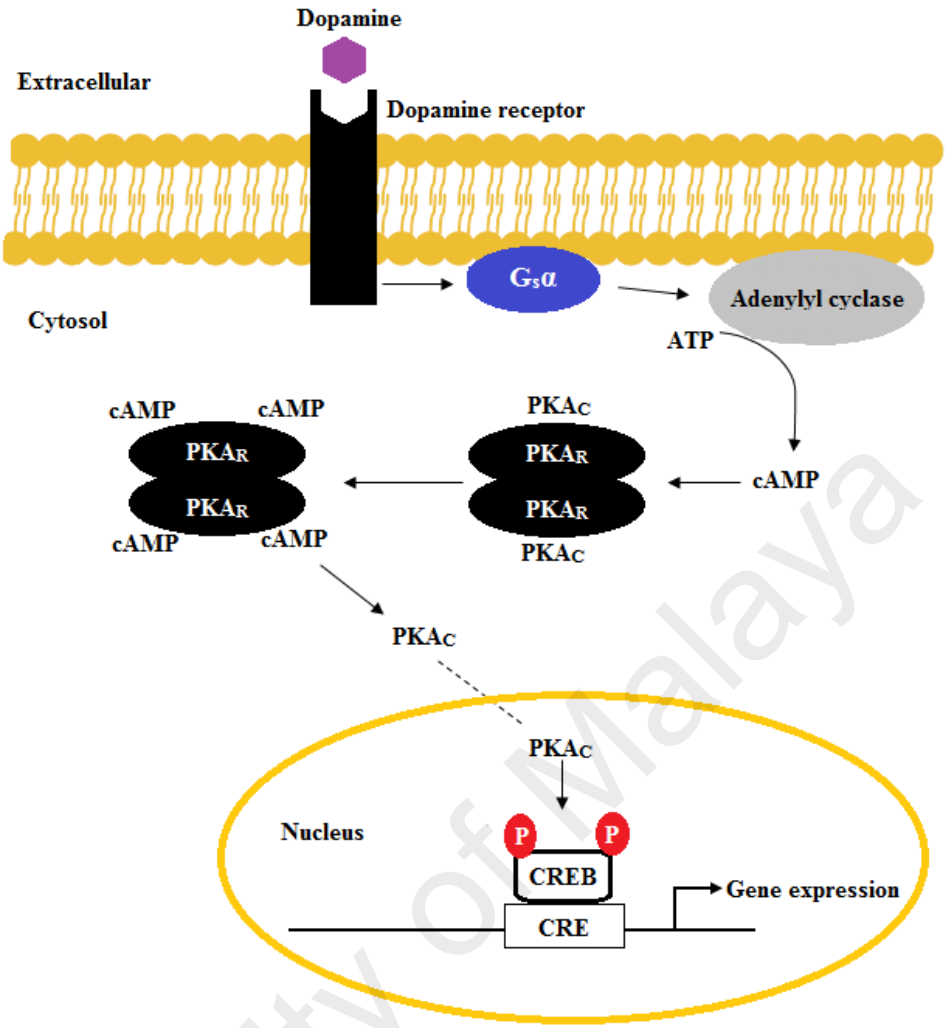

Figure 2.4: Activation of CREB-mediated gene expressions by dopamine. The nicotine activates the release of dopamine in several brain regions. The released dopamine binds to the dopamine receptors and activates heteromeric G-proteins (Gs alpha subunit) and thereby stimulates adenyl cyclase which catalyses adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Protein kinase-A (PKA) consists of a tetramer of two catalytic subunits of PKA (PKA_C) and two regulatory PKA subunits (PKA_R). PKA is inactive while cAMP is absent. When intracellular cAMP levels increased, four molecules of cAMP binds to the dimer of PKA_R and releases active PKA_C. This active PKA_C translocate into the nucleus, phosphorylates CREB (pCREB) at Ser 133, bind to cAMP response element (CRE) and initiates CREB mediated gene expressions. Diagram adapted from Shaywitz and Greenberg (1999).

2.3.1.2 **Involvement of BDNF in nicotine addiction and withdrawal**

Neurotrophins are an important class of signalling protein in the brain and responsible for axon targeting, neuronal growth, maturation of synapses during development, and synaptic plasticity. Neurotrophins includes nerve growth factor, BDNF, neurotrophins-3 and -4 (Autry & Monteggia, 2012). The role of BDNF is also implicated in the nicotine addiction and withdrawal. BDNF-signalling is mediated through tropomyosin receptor kinase B (TrkB) receptors and this activates the CREB-mediated gene expressions. Moreover, the nuclear transcription factor CREB regulates the gene expression of BDNF (Autry & Monteggia, 2012; Lonze & Ginty, 2002). The activation of TrkB receptors by BDNF leads to phosphorylation at various sites of TrkB receptor and activates three signal transduction protein kinase pathways including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), phospholipase Cγ (PLCγ), leading to survival, and growth of neurons and synaptic plasticity. Moreover, these protein kinases activate CREB (pCREB) mediated gene expressions (**Figure 2.5**) (Autry & Monteggia, 2012). In the preclinical study, the involvement of BDNF-signalling is reported in both nicotine-exposure and nicotine-withdrawal. The chronic nicotine administration in mice increased the BDNF levels in the hippocampus (CA1 region) of rats (Kenny et al., 2000). Moreover, nicotine cessation in mice increased the BDNF levels in the nucleus accumbens, substantia nigra and hippocampus regions (Fisher et al., 2016; Kivinummi et al., 2011; Roni & Rahman, 2014).

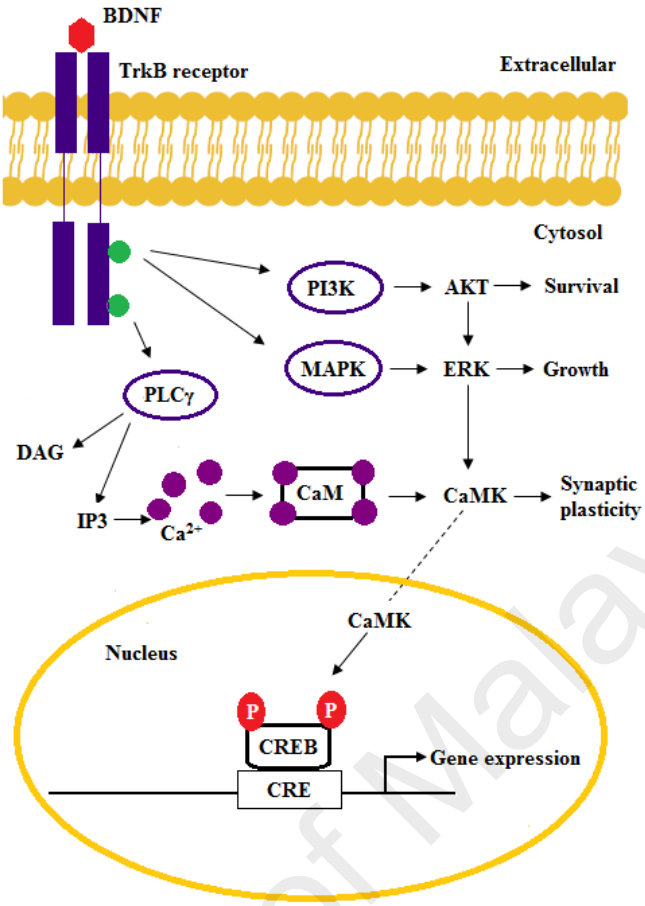

Figure 2.5: BDNF-CREB pathway. The BDNF binds to tropomyosin receptor kinase B (TrkB) receptors, induces dimerization and autophosphorylates TrkB receptors. The phosphorylation at various sites of TrkB receptors leads to an activation of intracellular protein kinase cascades. The activation of phosphatidylinositol 3-kinase (PI3K) activates serine/threonine kinase AKT (protein kinase B) and contributes to neuronal survival. The activation of mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinases (ERK) leads to growth and development of neurons. The activation of phospholipase Cγ (PLCγ) activates inositol triphosphate (IP3), leading to an activation of calcium/calmodulin-dependent protein kinases (CaMK) which then translocate into the nucleus, phosphorylate CREB at Ser 133, binds to cAMP response element (CRE) contributes to the CREB-mediated synaptic plasticity. The AKT and ERK also phosphorylates CREB at Ser 133and initiate CREB-mediated gene expressions. Diagram adapted from Autry and Monteggia (2012).

2.4 Treatment of nicotine addiction

The pharmacotherapies approved by food and drug administration (FDA) for first-line treatment of nicotine addiction includes nicotine replacement therapy (NRT), bupropion and varenicline (Casella, Caponnetto, & Polosa, 2010; Crooks, Bardo, & Dwoskin, 2014; Patel, Feucht, Reid, & Patel, 2010; Xi et al., 2009). It is estimated that the success rate of smoking cessation with the aid of pharmacotherapies is 20%, however, without pharmacotherapies 10% abstinence rate in smoking cessation was observed. The second-line pharmacotherapies such as nortriptyline and clonidine are not approved by FDA, however, it is considered for patients who unable to use first-line medications because of contraindication or for patients in which first-line medications are not effective (Fiore et al., 2008).

2.4.1 Nicotine replacement therapy

The nicotine replacement therapy (NRT) was clinically approved for the treatment of smoking cessation in the early 1980s. The NRT is available as the transdermal patch, chewing gum, nasal spray, lozenge, sublingual tablets and vapour inhaler (Casella et al., 2010; Patel et al., 2010). The NRT delivers the nicotine to replace the nicotine obtained from tobacco smoke and thereby facilitate smoking cessation in a person who has been dependent on nicotine. Besides, the NRT is effective in reducing the rewarding effect of nicotine from tobacco smoke and attenuated the somatic, and affective withdrawal symptoms during smoking cessation (Mitrouska et al., 2007; Patel et al., 2010; Xi et al., 2009). However, the efficacy of NRT is low with only 20% of smokers maintain long-term abstinence and the relapse rate is about 80% within a year of abstinence (Patel et al., 2010; Xi et al., 2009). Besides, NRT in combination with bupropion is helpful for the treatment of highly dependent smokers with severe withdrawal symptoms (Mitrouska et al., 2007; Patel et al., 2010).

2.4.2 Bupropion

Bupropion is an antidepressant drug approved for the treatment of the major depressive disorder in 1989 and later approved for the treatment of smoking cessation in 1997. Bupropion is a neuronal noradrenaline and dopamine reuptake inhibitor, which results in increased brain noradrenaline and dopamine levels and contributes to its effectiveness in the treatment of depression. In addition, bupropion showed a nicotinic antagonist effect at $\alpha_3\beta_2$, $\alpha_4\beta_2$ and, α_7 nAChRs, which prevents the binding of nicotine to neuronal $\alpha_4\beta_2$ nAChRs, blocks the reinforcing action of nicotine and reduce craving (Slemmer, Martin, & Damaj, 2000). Moreover, the inhibitory activity of bupropion at brain noradrenaline and dopamine transporter enhances the noradrenaline and dopamine levels, resembles the effect of nicotine on these neurotransmitters and prevents the withdrawal symptoms upon smoking cessation (N. L. Benowitz, 2009; Crooks et al., 2014).

In preclinical studies, bupropion increased the noradrenaline and dopamine in the microdialysis of brain regions including, hypothalamus, nucleus accumbens, prefrontal cortex and hippocampus of freely moving rats (Li, Perry, & Wong, 2002; Nomikos, Damsma, Wenkstern, & Fibiger, 1992; Piacentini et al., 2003). Moreover, bupropion at higher doses decreases the intravenous nicotine self-administration in rats (Rauhut, Neugebauer, Dwoskin, & Bardo, 2003). During nicotine withdrawal, bupropion was effective in alleviating nicotine-withdrawal induced somatic signs, depression-like behaviour (forced swim test) and learning and memory deficits (contextual fear conditioned test) in mice (Damaj et al., 2010; Portugal & Gould, 2007; Roni & Rahman, 2014). In human studies, bupropion is effective for the treatment of smoking cessation by alleviating craving to smoke and withdrawal symptoms, including depressed mood, difficulty concentrating, increased appetite, and irritability (Hurt et al., 1997; Mooney & Sofuoglu, 2006; Shiffman et al., 2000). Abstinence rate for bupropion treatment after one year was 15% and 10% for placebo (Gonzales et al., 2006; Jorenby et al., 2006). In 2009,

FDA issued a public health advisory notice due to serious post-marketing neuropsychiatric symptoms with bupropion treatment, including depressed mood, suicidal thoughts, suicidal actions and seizures (Crooks et al., 2014; Patel et al., 2010).

2.4.3 Varenicline

Varenicline is approved for smoking cessation in 2006. Varenicline is a potent partial agonist at neuronal $\alpha_4\beta_2$ nAChRs and a full agonist at α_7 nAChRs (Mihalak, Carroll, & Luetje, 2006). Varenicline is a synthetic analogue of cytisine, a plant alkaloid presents in the seeds of *Laburnum anagyroids* Medik. (Leguminosae) and a weak partial agonist at nAChRs (Coe et al., 2005; Papke & Heinemann, 1994). However, cytisine is available for clinical use for smoking cessation for 40 years in Eastern Europe (Crooks et al., 2014; Zatonski, Cedzynska, Tutka, & West, 2006). Nicotine is a full agonist at neuronal $\alpha_4\beta_2$ nAChRs, activation of nAChRs by nicotine increases the release of dopamine in the mesolimbic region. Varenicline competitively binds to the neuronal $\alpha_4\beta_2$ nAChRs, through its partial activation causes a moderate and sustained release of dopamine in the mesolimbic regions and thereby reduce craving, and withdrawal symptoms during smoking cessation. In addition, competitive binding of varenicline at $\alpha_4\beta_2$ nAChRs prevents the binding of nicotine to $\alpha_4\beta_2$ nAChRs, thus, inhibits the nicotine-induced mesolimbic dopaminergic activation and blocks the reinforcing action of nicotine, prevents relapse (Coe et al., 2005).

In preclinical studies, pretreatment of varenicline increased the extracellular dopamine levels in the nucleus accumbens of rats lesser than the effect of nicotine. Additionally, varenicline blocked the nicotine-induced increased dopamine levels in the nucleus accumbens of rats (Coe et al., 2005). It has also been reported that the varenicline treatment attenuates the intravenous nicotine-self administration and inhibits the nicotine-mediated electrical brain-stimulation in rats through the activation of $\alpha_4\beta_2$ nAChRs

(Bernard Le Foll et al., 2012; Spiller et al., 2009). Furthermore, varenicline and cytisine attenuate the nicotine-withdrawal induced anhedonia (increased brain reward threshold in intracranial self-stimulation test) in rats (Igari et al., 2014). Moreover, varenicline reversed the nicotine-withdrawal induced learning and memory deficits in mice using contextual fear conditioned test (Raybuck, Portugal, Lerman, & Gould, 2008). Besides, varenicline and cytisine showed antidepressant activity in the mouse tail suspension test and forced swim test (Igari et al., 2014; Mineur, Somenzi, & Picciotto, 2007; Rollema et al., 2009) and anxiolytic effect in mice using marble burying test and novelty-induced hypophagia test (Turner et al., 2013). These pharmacological effects of varenicline could contribute to its effectiveness against nicotine-withdrawal symptoms in humans.

In clinical trials, varenicline is effective for the treatment of smoking cessation by reducing the craving and withdrawal symptoms such as irritability, difficulty in concentration, depressed mood and anxiety. Interestingly, the efficacy of varenicline is comparatively superior to bupropion and NRT. After 1 year of varenicline and placebo treatment, abstinence was observed at 23% and 10%, respectively (Gonzales et al., 2006; Jorenby et al., 2006). Moreover, FDA has issued a public health advisory notice regarding a serious post-marketing neuropsychiatric symptom with varenicline treatment, including depressed mood, suicidal thoughts, suicidal actions and increased risk of cardiovascular adverse effects in patients with cardiovascular disease (Crooks et al., 2014; Patel et al., 2010).

2.4.4 Nortriptyline

Nortriptyline is an active metabolite of amitriptyline and belongs to a tricyclic class of antidepressant medication approved for the treatment of major depression. The antidepressant effect of nortriptyline is primarily mediated through its inhibitory activity on serotonin and noradrenaline reuptake mechanisms. In preclinical studies, nortriptyline

attenuates the intravenous nicotine self -administration and nicotine withdrawal-induced somatic signs in rats (Wing & Shoaib, 2007, 2012). In clinical studies, nortriptyline is effective for the treatment of nicotine addiction by increasing the smoking cessation rate and alleviating the nicotine-withdrawal symptoms including depressed mood, anxiety, difficulty concentrating and irritability (Hall et al., 2002; Prochazka et al., 1998).

2.4.5 Clonidine

Clonidine is a centrally acting α_2-adrenergic agonist that decreases central sympathetic outflow and originally approved for the treatment of hypertension. Recently, it has been approved by FDA for the treatment of the attention deficit hyperactive disorder. In preclinical studies, clonidine decreased the somatic signs during nicotine-withdrawal in rats. However, clonidine did not attenuate the nicotine withdrawal-induced elevation in brain reward threshold (anhedonia) in rats in the intracranial self-stimulation test (Bruijnzeel et al., 2010).

In humans, clonidine improved the smoking cessation rate and reduced craving, anxiety, and irritability upon smoking cessation (Glassman et al., 1988; Prochazka et al., 1992). However, clonidine is not effective in attenuating depressed mood and cognitive deficits during smoking cessation (Prochazka et al., 1992). It has been postulated that calming and anti-anxiety effect of clonidine could contribute its effectiveness in smoking cessation (Gourlay & Benowitz, 1995).

2.5 *Acorus calamus* Linn

Acorus calamus Linn (Family: Acoraceae) commonly known as "sweet flag", a herbaceous perennial and has long, erect, narrow, aromatic leaves grown from underground rhizomes (**Figure 2.6**) (Motley, 1994; Rajput et al., 2014). It has been widely used alone or in combination with other herbs in traditional Indian and Chinese medicine for centuries (Rajput et al., 2014). Traditionally, a powder made from *Acorus calamus* rhizomes has been smoked or chewed to discontinue the habit of smoking cigarettes (tobacco) and as a treatment for tobacco addiction (Motley, 1994). The main phytochemicals present in the extract from the dried rhizomes of the *Acorus calamus* Linn was identified as α-asarone and β-asarone (Rajput et al., 2014; Verma, Padalia, & Chauhan, 2015).

Figure 2.6: *Acorus calamus* Linn plant specimen showing leaves, rhizome and roots.

2.6 Alpha-asarone an investigational compound for the potential treatment of depression during nicotine-withdrawal in mice

In China, numerous clinical reports have indicated the effectiveness of α-asarone (**Figure 2.7**) against respiratory disorders and epilepsy (Feng, Yu, Qin, Gao, & Yao, 2015; Zhang Q, Ma WC. Li H, & SJ, 2010). Interestingly, in preclinical studies, α-asarone has been reported to possess numerous pharmacological activities such as antidepressant, antianxiety, anti-Parkinson's, anticancer, antihyperlipidemic, anticholestatic, antiplatelet, antithrombotic, radioprotective and antioxidant activities. Interestingly, evidences suggest that the extract of *Acorus calamus* rhizomes or α-asarone shares similar pharmacological activities in central nervous system such as anticonvulsant (Bhat, Ashok, Acharya, & Ravishankar, 2012; Pages et al., 2010), neuroprotective (Gu et al., 2010; Limon et al., 2009; Muthuraman & Singh, 2012; Shukla et al., 2006), anti-dementia, anti-Alzheimer's (Kumar et al., 2012; Limon et al., 2009; Muthuraman & Singh, 2012; Shin et al., 2014) and antidepressant (Han et al., 2013; Ilaiyaraja, Singsit., & Khanum, 2012; Pawar Vinod, Akhade., Baokar., & H, 2012) activities. Thus, it has been postulated that α-asarone could be responsible for the traditional claim of *Acorus calamus* rhizomes for the treatment of nicotine addiction.

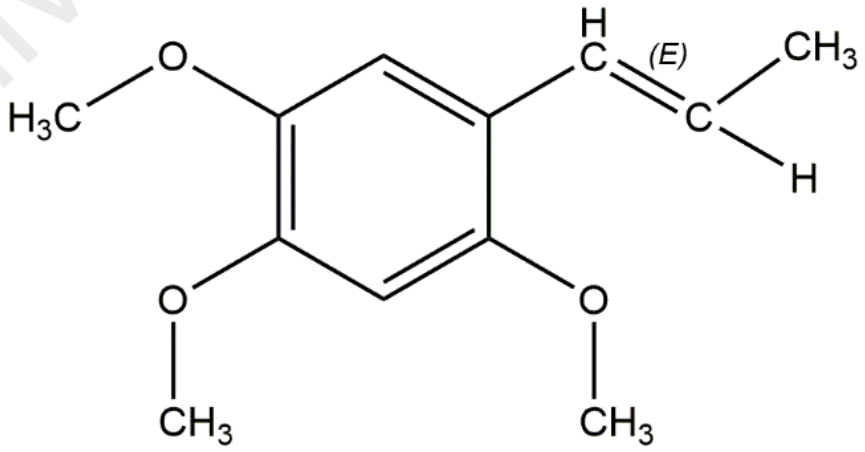

Figure 2.7: Chemical structure of α- asarone (1, 2, 4-trimethoxy-5-[(E)-prop-1-enyl] benzene).

2.6.1 Pharmacokinetic profile of alpha-asarone

Alpha-asarone has been extensively studied on pharmacokinetic aspects preclinically and reported in the literature. It has been reported that the plasma and brain half-life ($t_{1/2}$) of intraperitoneal administration of α-asarone (10 mg/kg) in male C57BL6 mice was found to be ≤11 min and ≤ 29 min, respectively (Kim et al., 2015). Moreover, α-asarone was found to be extensively distributed in the brain regions of rats and mice, which indicated its ability to permeate through the blood-brain barrier, a positive characteristic when considering the use of the compounds for the treatment of any CNS disorders (Kim et al., 2015; Lu et al., 2014). The metabolism of α-asarone occurs mainly through its interaction with cytochrome P450 (CYP450) enzymes in rat hepatocytes and liver microsomes (Hasheminejad & Caldwell, 1994; Pandit, Mukherjee, Ponnusankar, Venkatesh, & Srikanth, 2011). Furthermore, α-asarone prepared in dimethyl sulfoxide or ethanol inhibits the CYP isoenzymes, namely CYP2D6 (IC_{50}: 42.15 ± 2.45 or 55.17 ± 1.62 µg/ml) and CYP3A4 (IC_{50}: 57.46 ± 3.34 or 65.16 ± 2.37 µg/ml), using fluorometric assay (Pandit et al., 2011). Besides, *in vitro* metabolism of α-asarone was extensively studied in rat, bovine, porcine and, human liver microsomal preparations (Cartus & Schrenk, 2016) and it is illustrated in **Figure 2.8**.The rate of metabolism of α-asarone was found to be directly proportional to the CYP450 concentration. The overall ranking of metabolites yield of α-asarone in different species of liver microsomes with respect to the CYP450 concentration was found to be Aroclor 1254 (CYP450 inducer)-pretreated liver microsomes (1.34-1.67 nmol/mg protein) > human liver microsomes (0.34 nmol/mg protein)> bovine liver microsomes (0.88 nmol/mg protein) > porcine liver microsomes (0.39 nmol/mg protein) > rat liver microsomes (0.28-0.34 nmol/mg protein) (Cartus & Schrenk, 2016). In another study, 2, 4, 5-trimethoxycinnamic acid was identified to be the major metabolite of α-asarone upon incubation in the rat hepatocytes (Hasheminejad & Caldwell, 1994).

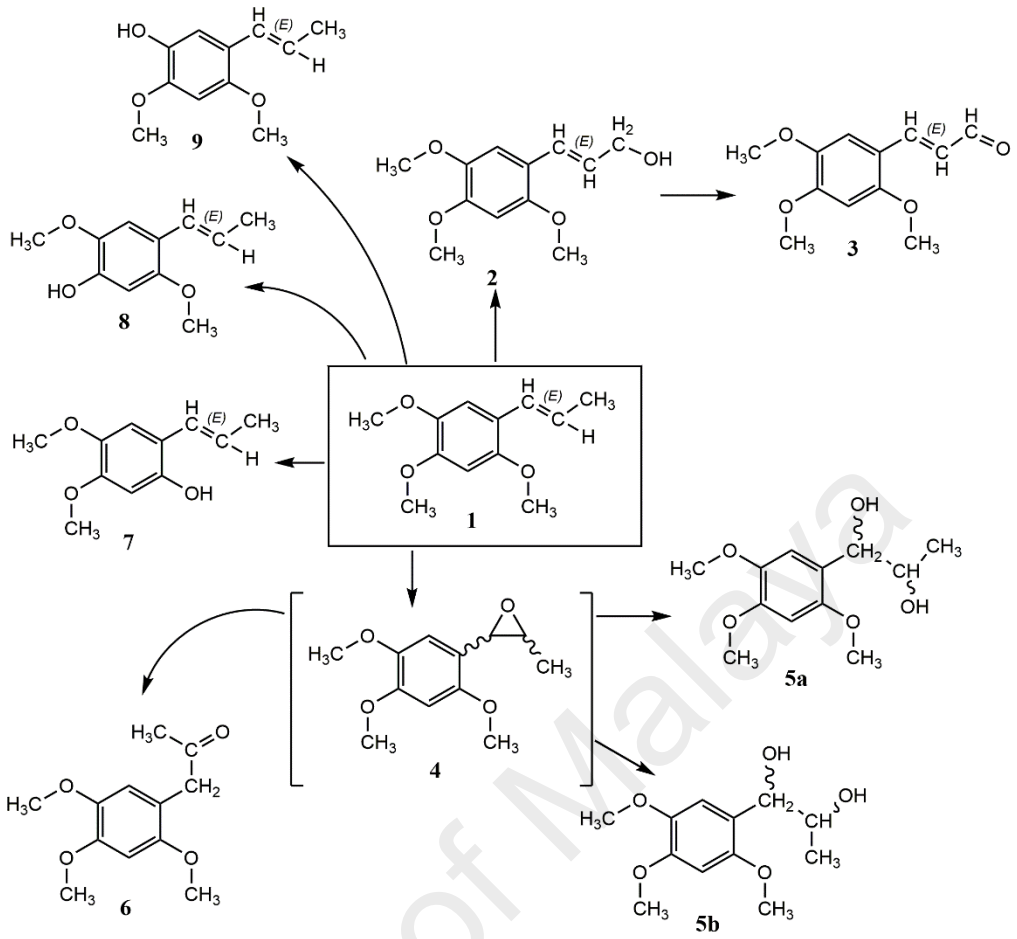

Figure 2.8: *In vitro* metabolism of α- asarone (1, 2, 4-trimethoxy-5-[(E)-prop-1-enyl] benzene; 1) in the rat, bovine, porcine and, human liver microsomal preparations. (E)-3′-hydroxyasarone (**2**) is the major metabolite of α-asarone. (E)-asarone-1′,2′-epoxide (**4**) is the unstable intermediate metabolite of α-asarone. Threo and erythro-1′,2′-dihydro-dihydroxy asarone (**5a and 5b**) are the minor metabolites of α-asarone. (E)-3′-oxoasarone (**3**), 3,4,6-trimethoxy-phenylacetone (**6**), (E)-6-hydroxyasarone (**7**), (E)-4-hydroxyasarone, (**8**) and (E)-3-hydroxyasarone (**9**) are the minor metabolites of α-asarone.

2.6.2 Preclinical pharmacology of α-asarone on the central nervous system

2.6.2.1 Alpha-asarone on locomotor activity, motor coordination and body temperature

The acute treatment of α-asarone at lower doses (<50 mg/kg, i.p) did not affect the spontaneous locomotor activity, motor coordination and body temperature in mice. However, hypomotility (decrease in the locomotor activity), impaired motor coordination and hypothermia was observed in the mice treated with higher doses (≥50 mg/kg, i.p) of α-asarone (Dandiya & Sharma, 1961; Han et al., 2013; Pages et al., 2010) (**Table 2.1**).

2.6.2.2 Alpha-asarone on gamma-aminobutyric acid modulators-induced sleeping time

Alpha-asarone at higher doses (≥50 mg/kg, i.p) potentiates the gamma-aminobutyric acid ($GABA_A$) receptor positive allosteric modulators (pentobarbital, hexobarbital, ethanol)-induced sleeping time in mice, which indicates the positive interaction of α-asarone with the GABAergic system (Dandiya & Sharma, 1961; Pages et al., 2010) (**Table 2.1**).

Table 2.1: Effect of α-asarone on general behaviour in mice.

General behaviour	Strain/Sex	Method used	Dose Used	Pharmacological effect	References
			α- asarone (mg/kg, i.p.)	**α- asarone (mg/kg, i.p.)**	
Spontaneous locomotor activity	ICR mice/ male	Open field test	5, 10 and 20	No effect on locomotor activity	(Han et al., 2013)
	Swiss albino mice/ female	Infrared-Actimeter	22, 60 and 100	100: decreased the locomotor activity	(Pages et al., 2010)
	Mice	Infrared-Actimeter	50	50: decreased the locomotor activity	(Dandiya & Sharma, 1961)
Motor Coordination	Swiss albino mice/ female	Rotarod test	22, 60 and 100	100: affects the motor coordination	(Pages et al., 2010)
Sleeping time	Swiss albino mice/ female	Pentobarbital-induced sleeping time	22, 60 and 100	60 and 100: potentiate the sleeping time	(Pages et al., 2010)
	Mice	Pentobarbital-induced sleeping time	50	50: potentiates the sleeping time	(Dandiya & Sharma, 1961)
Body temperature	Swiss albino mice/ female	Rectal temperature	22, 60 and 100	60 and 100: produced hypothermia	(Pages et al., 2010)
	Mice	Rectal temperature	50	50: produced hypothermia	(Dandiya & Sharma, 1961)

i.p.: intraperitoneal route of administration.

2.6.2.3 **Neuroprotective effect of α-asarone**

Alpha-asarone was found to have neuroprotective effect by inhibiting the glutamatergic neurotransmission. In *ex vivo* study, α-asarone (IC_{50}: 18.2 µg/ml for NMDA or 89.7 µg/ml for glutamate) inhibits the N-methyl-D-aspartate (NMDA) or glutamate-induced excitotoxicity in rat cortical preparations (Cho, Kim, Kong, Yang, & Park, 2002). In radioligand binding assay, the binding of [^{3}H]-MK-801 (dizocilpine; an uncompetitive NMDA receptor antagonist) to NMDA receptor was displaced by α-asarone, whereas, the binding of [^{3}H]-MDL105519 (a potent and selective antagonist of glycine binding site in NMDA receptor) was not displaced by α-asarone in the presence of glutamate and glycine in crude synaptic membrane preparations from the forebrain preparation of SD rats (Cho et al., 2002). In another study, α-asarone increased the uptake of [^{3}H]-labelled glutamate and decreased the excitatory amino acid carrier 1 (EAAC1) mediated current in the *Xenopus laevis* Oocytes expressed with mouse EAAC1 cRNA, using two-electrode voltage clamp method (Gu et al., 2010). Besides, α-asarone potentiates the nerve growth factor-induced neuronal differentiation through the phosphorylation of CREB by the activation of protein kinase-A pathway in PC12 cells (Lam et al., 2016). Thus, the neuroprotective effect of α-asarone could contribute its effectiveness in the delay or progression of neuropsychiatric and neurodegenerative disorders.

2.6.2.4 **Antidepressant effect of α-asarone**

The acute treatment of α-asarone in mice showed an antidepressant-like activity in the forced swim test and tail suspension test (Han et al., 2013) (**Table 2.2**). In *in vitro* enzymatic assay, α-asarone inhibits both the isoforms of human recombinant monoamine oxidase (MAO-A: IC_{50} = 124±16 µM; MAO-B: IC_{50} = 338±52 µM) enzyme (Tao, Irie, Li, & Keung, 2005). This MAO inhibitory effect of α-asarone could contribute its effectiveness against depression through enhancing the brain monoamine levels.

2.6.2.5 **Antianxiety effect of α-asarone**

Alpha-asarone showed an anxiolytic effect in the animal models of anxiety such as elevated plus maze (EPM) test, light-dark transition test, novel food consumption test and marble burying test (Liu et al., 2012). Similarly, α-asarone treatment attenuated the exogenous corticosterone-induced anxiety in rats using elevated plus maze test, open-field test, and hole-board test by modulating corticotrophin-releasing factor and BDNF-tropomyosin receptor kinase B (TrkB) pathways (Lee et al., 2014) (**Table 2.2**).

2.6.2.6 **Antiepileptic effect of α-asarone**

The activation of $GABA_A$ receptors by α-asarone was evidenced from electrophysiological studies and this activation was not mediated through inhibition of GABA uptake or GABA transaminase activity (Huang et al., 2013; Wang, Levinson, Sun, & Heinbockel, 2014). *In vivo* studies revealed that the mice treated with α-asarone showed a protective effect on $GABA_A$ receptor antagonists such as picrotoxin or pentylenetetrazol-induced seizures (Chen et al., 2013; Dandiya & Sharma, 1961; Huang et al., 2013; Pages et al., 2010). Furthermore, α-asarone was found to be effective against lithium-pilocarpine induced status epilepticus in rats through its positive modulatory activity on GABAergic systems (Chen et al., 2013; Jing-Kun Miao et al., 2013). Moreover, α-asarone protects the N-methyl D-aspartate (a specific NMDA receptor agonist) or kainate (a kainate receptor agonist)- induced seizures in mice (Huang et al., 2013; Pages et al., 2010). Similarly, the radio-ligand binding assay confirmed the antagonistic effect of α-asarone at NMDA receptors (Cho et al., 2002). In another study, α-asarone showed antiepileptic activity in rat or mice models of epilepsy such as maximal electroshock-induced seizures (Chen et al., 2013; Dandiya & Menon, 1963; Dandiya & Sharma, 1961; Pages et al., 2010) and magnesium deficiency-dependent audio-genic seizures (Pages et al., 2010) (**Table 2.2**).

2.6.2.7 **Anti-Alzheimer's effect of α-asarone**

Alpha-asarone was effective against the animal models of dementia. In *ex vivo* study, acetylcholinesterase (AChE) inhibitory activity of α-asarone (IC_{50} 46.38±2.69 μM) was observed (Mukherjee, Kumar, Mal, & Houghton, 2007). *In vivo* studies revealed that α-asarone pretreatment improved the spatial learning and memory in scopolamine or lipopolysaccharide-induced memory-impaired mice (Kumar et al., 2012; Shin et al., 2014) and noise-stress-induced memory-impaired rats (Sundaramahalingam, Ramasundaram, Rathinasamy, Natarajan, & Somasundaram, 2013) through the inhibition of AChE in the brain regions (cerebral cortex and/or hippocampus) of mice or rats (Kumar et al., 2012; Sundaramahalingam et al., 2013). Recently, the locomotor hyperactivity exhibited by the fragile-X-mental retardation gene (Fmr1) knockout mice was reversed with α-asarone treatment through the inhibition of striatal AChE, thereby increased the expression of acetylcholine levels and muscarinic M_1 cholinergic receptors (Qiu, Chen, Guo, Wu, & Yi, 2016). Interestingly, α-asarone (10 mg/kg, p.o; od × 16 days) treatment improved the spatial learning and memory (eight-arm radial maze test) in β-amyloid peptides ($A\beta_{(25-35)}$)-induced memory-impaired rats by attenuating the $A\beta_{(25-35)}$-induced neuronal damages in the hippocampus and temporal cortex regions (Limon et al., 2009) (**Table 2.2**).

2.6.2.8 **Anti-Parkinson's effect of α-asarone**

The pretreatment of α-asarone completely abolished tremorine (a muscarinic agonist)-induced parkinsonism-like signs such as tremors of head and limbs, hypomotility and muscular rigidity (Dandiya & Menon, 1965). Moreover, α-asarone (10 mg/kg, p.o; o.d. × 15 days) pretreatment reversed the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced parkinsonism-like signs in mice such as motor deficits (Y-maze test) and bradykinesia (pole test) through the reversal of MPTP-induced loss of dopaminergic neurons (decreased tyrosine hydroxylase positive immunostaining) and decreased

DOPAC levels in the substantia nigra pars compacta and striatum (Kim et al., 2015). Interestingly, the MAO-B (human recombinant isoenzymes) inhibitory activity of α-asarone (IC_{50} = 338±52 μM) might involve in the protective effect against Parkinson's disease by enhancing brain dopamine levels (Tao et al., 2005) (**Table 2.2**).

Table 2.2: Pharmacological effects of α-asarone in the animal models of CNS disorders.

Pharmacological effect	Animal strain/sex	Method used	Treatment	Pharmacologically active dose	References
			α-asarone (mg/kg)	**α-asarone (mg/kg)**	
Antidepressant	ICR mice/ male	Forced swim test	5, 10, and 20; i.p.	10 and 20; i.p.	(Han et al., 2013)
		Tail suspension test	5, 10, and 20; i.p.	10 and 20; i.p.	
Antianxiety	Swiss albino mice/male	Elevated plus-maze test	1. 7, 3.5, 7, and 14; p.o.; o.d. × 7 days	3.5, and 7; p.o.; o.d. × 7 days	(Liu et al., 2012)
		Light-dark transition test	1. 7, 3.5, 7, and 14; p.o.; o.d. × 7 days	7; p.o.; o.d. × 7 days	
		Novel food consumption test	1. 7, 3.5, 7, and 14; p.o.; o.d. × 7 days	3.5, 7, and 14 p.o.; o.d. × 7 days	
		Marble burying test	3.5, 7 ,14, and 28; p.o.; o.d. × 7 days	14, and 28; p.o.; o.d. × 7 days	
	SD rats/male	Corticosterone-induced anxiety	50, 100 and 200; i.p.; o.d. × 21 days	200; i.p.; o.d. × 21 days	(Lee et al., 2014)

b.i.d., twice daily; i.p., intraperitoneal route; o.d., once daily; p.o., oral route.

Table 2.2, continued

Pharmacological effect	Animal strain/sex	Method used	Treatment	Pharmacologically active dose	References
			α-asarone (mg/kg)	**α-asarone (mg/kg)**	
Antiepileptic	Albino rats	Maximal electroshock seizure	20; i.p.	20; i.p.	(Dandiya & Sharma, 1961)
	Albino mice	Maximal electroshock seizure	25; i.p.	25; i.p.	(Dandiya & Menon, 1963)
	Swiss mice/ female	Maximal electroshock seizure	30,60, and 80; i.p.	30,60, and 80; i.p.	(Pages et al., 2010)
	Swiss mice/ male	Maximal electroshock seizure	50,100, and 200; p.o.; b.i.d × 28 days	50,100, and 200; p.o.; b.i.d × 28 days	(Chen et al., 2013)
	Swiss mice/ female	Picrotoxin-induced seizure	22, 60, and 100; i.p.	60, and 100; i.p.	(Pages et al., 2010)
	Albino rats	Pentylenetetrazol-induced seizures	50; i.p.	50; i.p.	(Dandiya & Sharma, 1961)
	C57BL6 mice/male	Pentylenetetrazol-induced seizures	50; i.p.; o.d. × 3 days	50; i.p.; o.d. × 3 days.	(Huang et al., 2013)

b.i.d., twice daily; i.p., intraperitoneal route; o.d., once daily; p.o., oral route.

Table 2.2, continued

Pharmacological effect	Animal strain/sex	Method used	Treatment	Pharmacologically active dose	References
			α-asarone (mg/kg)	**α-asarone (mg/kg)**	
Antiepileptic	Swiss mice/ female	Pentylenetetrazol-induced seizures	22, 60, and 100; i.p.	60, and 100; i.p.	(Pages et al., 2010)
	Swiss mice/ female	N-methyl-d-aspartate - induced seizures	22, 60, and 100; i.p.	22, 60, and 100; i.p.	(Pages et al., 2010)
	C57BL6 mice/ male	Kainate-induced seizures	50; i.p.; o.d. × 3 days	50; i.p.; o.d. × 3 days	(Huang et al., 2013)
	Swiss mice/ female	Magnesium deficiency-dependent audiogenic seizures	15, 20,30 and 90; i.p.	15, 20,30 and 90; i.p.	(Pages et al., 2010)
	Wistar rats/ male	Lithium-pilocarpine induced status epilepticus	50, 100, and 200; p.o.	50, 100, and 200; p.o.	(Chen et al., 2013; Jing-Kun Miao et al., 2013)
			50,100, and 200; p.o.; b.i.d × 28 days	50,100, and 200; p.o.; b.i.d × 28 days	

b.i.d., twice daily; i.p., intraperitoneal route; o.d., once daily; p.o., oral route.

Table 2.2, continued

Pharmacological effect	Animal strain/sex	Method used	Treatment	Pharmacologically active dose	References
			α-asarone (mg/kg)	**α-asarone (mg/kg)**	
Anti-Alzheimer's	ICR mice	Scopolamine -induced amnesia; passive avoidance test	3, 10, and 30; i.p.; o.d. × 15 days	10, 30; i.p.; o.d. × 15 days	(Kumar et al., 2012)
		Scopolamine -induced amnesia; Y-maze test	3, 10, and 30; i.p.; o.d. × 15 days	3, 10, 30; i.p.; o.d. × 15 days	
	Wistar albino rats/male	Noise-stress-induced amnesia; eight-arm radial maze test	9; i.p.; o.d. × 30 days	9; i.p.; o.d. × 30 days	(Sundaramahalingam et al., 2013)
	C57BL6 mice/male	Lipopolysaccharide-induced memory impairment; Morris water maze test	7.5, 15, and 30; p.o.; o.d. × 3 days	30; p.o.; o.d. × 3 days	(Shin et al., 2014)
	Wistar albino rats/male	$A\beta_{(25-35)}$ -induced memory impairment; eight-arm radial maze test	10; p.o.; o.d. × 16 days	10; p.o.; o.d. × 16 days	(Limon et al., 2009)

b.i.d., twice daily; i.p., intraperitoneal route; o.d., once daily; p.o., oral route.

Table 2.2, continued

Pharmacological effect	Animal strain/sex	Method used	Treatment	Pharmacologically active dose	References
			α-asarone (mg/kg)	**α-asarone (mg/kg)**	
Anti-Parkinson's	Mice	Tremorine-induced Parkinson's-like signs	3, and 10; i.p.	3, and 10; i.p.	(Dandiya & Menon, 1965)
	C57BL6 mice/male	MPT- induced Parkinson's-like syndrome; Y-maze test and pole test	10; p.o.; o.d. × 15 days	10; o.d. × 15 days	(Kim et al., 2015)

b.i.d., twice daily; i.p., intraperitoneal route; o.d., once daily; p.o., oral route.

2.6.3 Preclinical toxicology of α-asarone

2.6.3.1 Acute and sub-acute toxicity test

In mice, the median lethal dose (LD_{50}) of acute oral and intraperitoneal administration of α- asarone was found to be >1000 mg/kg and 245.2 mg/kg, respectively (Chen et al., 2013; Morales-Ramirez et al., 1992). In recent study, the Swiss albino mice (male) treated with α-asarone (50, 100 mg/kg, p.o.; o.d. × 28 days) did not show changes in behavioural (walking, rearing and grooming) and righting reflex (Chen et al., 2013). However, α-asarone at a higher dose (200 mg/kg, p.o., o.d. × 28 days) significantly decreased spontaneous locomotor activity and no mortality was observed (Chen et al., 2013). The morphology of adult rat hepatocytes exposed (1 or 2 weeks) to α-asarone was altered (Lopez, Hernandez, Chamorro, & Mendoza-Figueroa, 1993). However, *in vivo* α-asarone (9 mg/kg, i.p., once daily for 30 days) treatment in Wistar albino rats (male) did not produce any morphological changes of the liver (parenchyma and sinusoids) (Manikandan & Devi, 2005).

2.6.3.2 Mutagenicity test

Mutagenicity is defined as the induction of irreversible transmissible changes in the structure of genetic materials of cells or organisms with a mutation in a single gene or a block of genes (Daniela Maurici et al., 2005). Ames test is the most widely used test to predict the mutagenicity of any compounds. In Ames test, the mutagenic effect of α-asarone was observed in the *Salmonella typhimurium* strain [TA-100 only in the presence of S9 metabolic activation (a S9 fraction obtained from rat liver homogenates contains both phase-I (microsomes mostly contains CYP450 isoforms) and phase-II (cytosol portion which contains mainly transferases)] enzymes (Berg, Bischoff, Stegmuller, Cartus, & Schrenk, 2016; Cassani-Galindo et al., 2005; Marczewska, Drozd, Anuszewska, Chilmonczyk, & Lozowicka, 2013). However, 3′- hydroxylated metabolites of α-asarone did not possess mutagenicity in the human sulphotransferase-expressing

Salmonella strains (TA100-hSULT1A1 and TA100-hSULT1C2) with or without S9 activation (Berg et al., 2016).

The dominant lethal test is used to assess the mutagenic effect of test compounds, whereby mutation in the germ cell does not affect the gamete but lethal to the fertilized egg or developing embryo (OECD, 1984). The α-asarone treatment did not cause germinal mutation, no changes were observed on pre- and post- implantations in untreated pregnant female CF1 mice cohabitated with α-asarone (10 and 20 mg/kg, p.o., 5 days a week for 8 consecutive weeks) treated male CF1 mice and have no effect on sperm count, testicular weight, epididymal weight and testicular histology (G. Chamorro, Salazar, Tamariz, Diaz, & Labarrios, 1999; G. A. Chamorro, M.Salazar, S.Salazar, & J.Tamariz, 1995). These results indicated that repeated treatment of α-asarone *in vivo* did not possess germinal mutation.

2.6.3.3 **Genotoxicity test**

Genotoxicity refers to the ability of a compound interact with DNA and/or the cellular components such as the spindle apparatus and topoisomerase enzymes that regulates the fidelity of the genome (Daniela Maurici et al., 2005). In recent study, Unger and Melzig (2012) found that α-asarone did not cause changes in the number of micronuclei formation in human hepatoma (Hep-G2) cell in the presence and absence of S9-mix, using acridine orange staining under fluorescence microscopy. Similarly, α-asarone (50 mg/kg, p.o.) pretreatment in Swiss albino mice did not affect the micronucleus formation in the peripheral blood reticulocytes, using acridine orange staining (Sandeep & Nair, 2011). In addition, the bone marrow samples of α-asarone (50 mg/kg, p.o.) treated Swiss albino mice did not cause the DNA damage or chromosomal abbreviation using Comet assay or geimsa staining, respectively (Sandeep & Nair, 2011), indicating the absence of genotoxicity with α-asarone at this tested dose.

2.6.3.4 **Teratogenicity test**

The α-asarone treated eggs survived in the chicken embryo test, which indicates the absence of teratogenicity (European-commission, 2002; JECFA, 1981). Furthermore, α-asarone did not produce developmental defects in zebrafish embryos (Q. Cai, Li, Mao, & Pei, 2016). In addition, no teratogenicity was observed in organogenesis of pregnant rats administered with α-asarone (G. Chamorro et al., 1999; Jimenez, Chamorro, Salazar, & Pages, 1988).

2.7 Research hypothesis: Alpha-asarone for the treatment of depression-like behaviour during nicotine-withdrawal in mice

The currently available pharmacotherapy for the treatment of nicotine addiction and withdrawal symptoms is limited, very expensive and a common man who wish to quit smoking cigarettes would usually not be able to afford it. During nicotine withdrawal, depressed mood is one of the primary affective symptoms which contributes the most to relapse within a few days after quitting tobacco products mainly in the form of cigarettes, which make the treatment for nicotine addiction more difficult and even unsuccessful. In this aspect, it is important to discover and develop novel pharmacotherapy (non-nicotine based) with greater efficacy against depressed mood during nicotine-withdrawal is necessary to increase the success rate of quitting tobacco products. Besides, the phytochemical based research could lead a discovery of novel agents for the treatment of nicotine addiction and withdrawal symptoms.

It is evident that the brain monoamines (dopamine, noradrenaline and serotonin) of nicotine-withdrawn mice was decreased in several brain regions (striatum, hypothalamus, cortex or hippocampus) and leading to irritability and depressed mood during nicotine-withdrawal (Neal L. Benowitz, 2010; Benwell & Balfour, 1979; Gäddnäs et al., 2000). For instance, hippocampal synaptic plasticity is implicated in associated learning, and memory (e.g. smoking-related cues) and pathophysiology of depression. Besides, nAChRs are widely expressed in the hippocampus, activation of nAChRs facilitate the releases neurotransmitters including GABA, glutamate and noradrenaline leading to synaptic plasticity (Placzek et al., 2009). Importantly, chronic nicotine administration followed by withdrawal in mice increased the biomarkers of neuroplasticity including pCREB (ventral tegmental area, nucleus accumbens and hippocampus) and BDNF (nucleus accumbens, substantia nigra and hippocampus) in different brain regions (Brunzell et al., 2003; Fisher et al., 2016; Kivinummi et al., 2011;

Roni & Rahman, 2014). Furthermore, upon chronic nicotine cessation in mice showed depression-like behaviour in forced swim test through increased hippocampal pCREB and BDNF levels (Roni & Rahman, 2014). The clinically approved antidepressant and smoking cessation drug bupropion (a noradrenaline, and dopamine reuptake inhibitor and a neuronal nAChRs antagonist) in rodents and humans alleviates depression during nicotine-withdrawal (Hurt et al., 1997; Mooney & Sofuoglu, 2006; Roni & Rahman, 2014; Shiffman et al., 2000). Thus, these evidences highlight that the imbalance in the brain monoamine levels upon nicotine cessation produce withdrawal symptoms and if any compounds which have positive modulatory activity on monoaminergic system mainly noradrenergic, dopaminergic and serotonergic symptoms could improve depressed mood during nicotine-withdrawal

Alpha-asarone was demonstrated for antidepressant activity in two well-validated mouse models of depression such as tail suspension test and forced swim test (Han et al., 2013), however, dose-dependent effect of α-asarone on the animal models of depression and its interaction with monoaminergic systems is not studied. In addition, it has been postulated that α-asarone could interact with the brain monoaminergic system through its monoamine oxidase inhibitory activity (Tao et al., 2005) and thereby produce anti-depressant effect. Moreover, in recent studies, the interaction of α-asarone with the CREB (Lam et al., 2016) and BDNF (Lee et al., 2014) signalling pathways has been reported. However, the interaction of α-asarone with nicotinic acetylcholine receptors is unknown. In the light of this evidences, it has been hypothesised that α-asarone could be effective in attenuating nicotine withdrawal-induced depression-like behaviour by modulating the neuroplasticity upon nicotine-withdrawal in mice.

2.8 Aim and objectives

The general aim of this study is to evaluate the effect of α-asarone on nicotine withdrawal-induced depression-like behaviour in mice. The specific objectives of this study are as follows

i. To investigate the dose-dependent effect of α-asarone on depression-like behaviour in the tail suspension test in ICR mice.
ii. To evaluate the involvement of monoaminergic systems in the antidepressant-like effect of α-asarone in the tail suspension test in ICR mice.
iii. To study the interaction of α-asarone with nicotinic acetylcholine receptors on nicotine's pharmacological effects in ICR mice.
iv. To study the effect of α-asarone on nicotine-withdrawal induced depression-like behaviour in C57BL6 mice.
v. To examine the effect of α-asarone on neuronal plasticity during nicotine-withdrawal in C57BL6 mice.

CHAPTER 3: METHODOLOGY

3.1 Animals

ICR mice (male, 8 to 10 weeks old) and C57BL6 mice (male, 6 to 7 weeks old) were obtained from Animal Experimental Unit, Faculty of Medicine, University of Malaya, Malaysia and Monash University, Malaysia, respectively. Animals were housed (3-4 mice per individually ventilated cages) in a humidity-controlled environment (22 ±2°C, 50-70% humidity) and 12 h light/dark (lights on at 7.00 AM) animal care facility (Satellite Animal Facility, Department of Pharmacology, Faculty of Medicine, University of Malaya, Malaysia) with food and water available *ad libitum*. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All the experimental protocols were approved by the Faculty of Medicine-Institutional Animal Care and Use Committee, University of Malaya (Ethics Approval no: 2014-10-14/PHAR/R/VP) and conducted according to the National Institutes of Health guide for the care and use of Laboratory animals (Garber et al., 2011). All the behavioural experiments were performed during the light cycle from 09.00 to 17.00 h.

3.2 Drugs and preparations

Alpha-asarone (lot # S18779V; purity 98% w/w), α-methyl-p-tyrosine (AMPT; lot # STBD4408V; purity 98% w/w), para-chlorophenylalanine methyl ester hydrochloride (PCPA or Fenclonine; lot # SHBD9164V; purity 97% w/w) and (-)-nicotine hydrogen tartrate (lot # SLBD5902V; purity ≥98% w/w) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Bupropion hydrochloride (lot #2596608; purity ≥99.5% w/w) and fluoxetine hydrochloride (lot #2597489; purity 99.8% w/w) were obtained from LKT laboratories Inc., St. Paul, MN, USA.

Alpha-asarone was suspended in 5% v/v Tween 80 (polyethylene sorbitan monooleate) prepared in normal saline. Bupropion, fluoxetine and PCPA were dissolved in normal saline and AMPT was suspended in 10% v/v Tween 80 prepared in normal saline. All the drugs were administered intraperitoneally (i.p.) and nicotine was injected subcutaneously (s.c.) at a constant dose volume of 10 mL/kg body weight of mice, respectively. For oral nicotine administration, nicotine solution (free base) was prepared in drinking water and chronically exposed to mice. Furthermore, in this study, the clinically approved antidepressant and smoking cessation drug bupropion was used as reference standard.

3.3 Experimental design

The overview of experimental design is showed in **Figure 3.1**. The behavioural experiment such as tail suspension test and forced swim test were video recorded, monitored using a Logitech webcam (C270) connected to a personal computer and then the immobility time was measured. All the behavioural test apparatus was cleaned with 70 % v/v ethanol between each test.

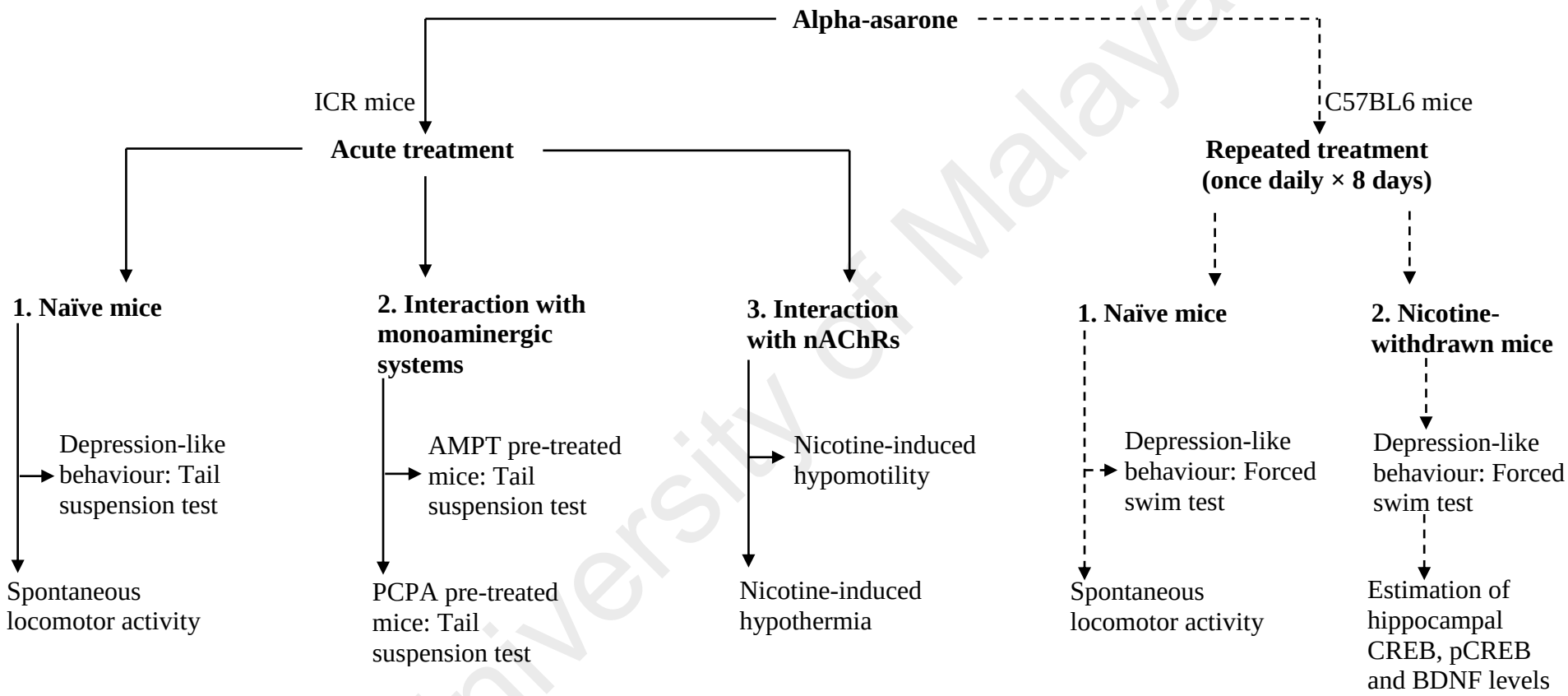

Figure 3.1: Overview of experimental design.

AMPT: α-methyl-p-tyrosine, a catecholamine synthesis inhibitor; PCPA: para-chlorophenylalanine, a serotonin synthesis inhibitor; nAChRs: nicotine acetylcholine receptors; CREB: cAMP response element-binding protein; pCREB: phosphorylated-CREB; BDNF: brain-derived neurotrophic factor.

3.3.1 Effect of α-asarone in the tail suspension test and spontaneous locomotor activity in ICR mice

3.3.1.1 Tail suspension test

The tail suspension test was performed as described previously (Steru, Chermat, Thierry, & Simon, 1985). Briefly, ICR mice (n=10/group) were treated with vehicle (5% v/v Tween 80), bupropion (20 mg/kg, i.p.) or α-asarone (10, 15, 20, 30, 50 and 100 mg/kg, i.p.). Thirty minutes after treatment, the mouse was acoustically and visually isolated, suspended 25 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail and the immobility time was measured using a digital stopwatch during the 6min test session. The mouse was considered immobile when they hung passively and completely motionless (depression-like behaviour).

3.3.1.2 Spontaneous locomotor activity

The spontaneous locomotor activity was assessed using actimeter (Model: ACT-01, Orchid's Scientific, Nasik, India), fabricated with clear square Plexiglas arena (50 cm x 50 cm), equipped with 32-infrared sensors. Briefly, ICR mice (n=8-10/group) were treated with vehicle (5% v/v Tween 80; n=10), bupropion (20 mg/kg, i.p.; n=8) or α-asarone (10, 15, 20, 30, 50 and 100 mg/kg, i.p.; n=9-10). Thirty minutes after treatment, the mouse was placed in the centre of an arena of actimeter and the locomotor activity was measured for the duration of 10 min. The data were expressed as the total light beam interruptions (locomotor counts).

3.3.2 Monoaminergic mechanism(s) involved in the antidepressant effect of α-asarone in the tail suspension test in ICR mice

3.3.2.1 Investigation of the involvement of noradrenergic and dopaminergic systems in the antidepressant effect of α-asarone

ICR mice were divided into two groups, injected with saline (n=24) and AMPT (100 mg/kg, i.p., a catecholamine synthesis inhibitor; n=24). Four hours after saline and AMPT treatment, mice were further divided (n=8/group) and treated either vehicle (5% v/v Tween 80), bupropion (20 mg/kg, i.p) or α-asarone (20 mg/kg, i.p.), respectively. Thirty minutes after vehicle, bupropion or α-asarone treatment, the mouse was acoustically and visually isolated, suspended 25cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail and the immobility time was measured using a digital stopwatch during the 6 min tail suspension test session (Kwon et al., 2010; Machado et al., 2008).

3.3.2.2 Investigation of the involvement of serotonergic system in the antidepressant effect of α-asarone

ICR mice were divided into two groups, treated with saline (n=24) and PCPA (100 mg/kg, i.p., a serotonin synthesis inhibitor, once daily for 4 consecutive days; n=24). On day 5, saline or PCPA (24 hours after last PCPA treatment) pretreated mice were further divided (n=8/group) and received either vehicle (5% v/v Tween 80), fluoxetine (30 mg/kg, i.p) or α-asarone (20 mg/kg, i.p.) at 30 min prior to the tail suspension test, respectively. Then the mouse was acoustically and visually isolated, suspended 25 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail and the immobility time was measured using a digital stopwatch during 6min tail suspension test (Kwon et al., 2010; Machado et al., 2009).

3.3.3 Effect of α-asarone on nicotine's pharmacological effect in ICR mice

3.3.3.1 Nicotine-induced hypomotility

This study was conducted as described previously (Freitas, Negus, Carroll, & Damaj, 2013; Slemmer et al., 2000). ICR mice (n=6/group) were injected with vehicle (5% v/v Tween 80, i.p.), α-asarone (5, 10, 20 and 30 mg/kg, i.p.) or bupropion (20 mg/kg, i.p.). Thirty minutes after treatment the mice were injected either with vehicle (normal saline, s.c.) or nicotine (1mg/kg, s.c.). Five minutes after saline or nicotine injection, the mouse was placed in the centre of the arena of actimeter (Model # ACT-01, Orchid's Scientific, Nasik, India) fabricated with clear square Plexiglas arena (50 cm × 50 cm), equipped with 32-infrared sensors and the total light beam interruption (locomotor counts) was measured for 10 min.

3.3.3.2 Nicotine-induced hypothermia

In this study, the rectal temperature (pretreatment) of ICR mice (n=72) were measured using a digital thermometer (Model # TERM-01; Orchid's Scientific, Nasik, India). The ambient temperature of the laboratory was maintained at 25±1 °C. The mice (n=6/group) were treated with vehicle (5% v/v Tween 80, i.p.), α-asarone (5, 10, 20 and 30 mg/kg, i.p.) or bupropion (20 mg/kg, i.p.), 30 min after treatment, either vehicle (normal saline, s.c.) or nicotine (2.5 mg/kg, s.c.) was injected. The rectal temperature (post-treatment) of mice was measured 30 min after saline or nicotine administration. The change in body temperature was calculated from the difference between post and pretreatment measurements (Alajaji, Bowers, Knackstedt, & Damaj, 2013; Freitas et al., 2013; Ignatowska-Jankowska, Muldoon, Lichtman, & Damaj, 2013).

3.3.4 Effect α-asarone on nicotine withdrawal-induced depression-like behaviour in C57BL6 mice

3.3.4.1 Effect of repeated α-asarone treatment in the forced swim test in naïve C57BL6 mice

The forced swim test was performed as described previously (Porsolt, Bertin, & Jalfre, 1977). Briefly, C57BL6 mice (n=8/group) were administered once daily with vehicle (5% v/v Tween 80), bupropion (10 mg/kg, i.p.) or α-asarone (5, 10 and 20 mg/kg, i.p.) for eight consecutive days. Thirty minutes after last treatment, the mouse was gently placed in the 5 L glass transparent beaker (27 cm height, 19 cm internal diameter) containing fresh tap water (up to 15 cm height at 24±1°C), forced to swim for 6 min. The duration of immobility was measured using a digital stopwatch during last 4 min of the test. The mouse was considered to be immobile when it remained floating motionless (depression-like behaviour) in water, making only simple limb movements necessary to keep its head above water (Can et al., 2012). The beaker was cleaned and filled with fresh water between each test.

3.3.4.2 Effect of repeated α-asarone treatment in the spontaneous locomotor activity in naïve C57BL6 mice

In this study, C57BL6 mice (n=8/group) were treated once daily with vehicle (5% v/v Tween 80), bupropion (10 mg/kg, i.p.) or α-asarone (5, 10 and 20 mg/kg, i.p.) for eight consecutive days. Thirty minutes after last treatment, the locomotor activity was assessed using an actimeter (model#ACT-01, Orchid's Scientific, Nasik, India) fabricated with clear square Plexiglas arena (50cm × 50cm), equipped with 32-infrared sensors. The mouse was placed in the centre of the arena and the locomotor activity was measured for the duration of 10 min. The data were expressed as the total light beam interruptions (locomotor counts).

3.3.4.3 Effect of repeated α-asarone treatment in the forced swim test in nicotine-withdrawn C57BL6 mice

(a) ***Induction of nicotine dependence: Chronic oral nicotine administration***

C57BL6 mice were grouped into control (received drinking water *ad libitum*; n=6) and nicotine group (received nicotine solution *ad libitum*, 10-200 µg/mL for forty days as an only source of drinking; n=33) (**Figure 3.2**). In nicotine group, the concentration of nicotine was gradually increased from 10 to 100 µg/mL for every three days (day 1-12) and followed by 200 µg/mL for the next four weeks (day 13-40). The concentration of nicotine and duration of nicotine exposure was selected based on the previous studies (Grabus et al., 2005; Kivinummi et al., 2011; Robinson, Marks, & Collins, 1996; Roni & Rahman, 2014; Zhao-Shea et al., 2015). During this period, daily fluid consumption, food intake and body weight were recorded.

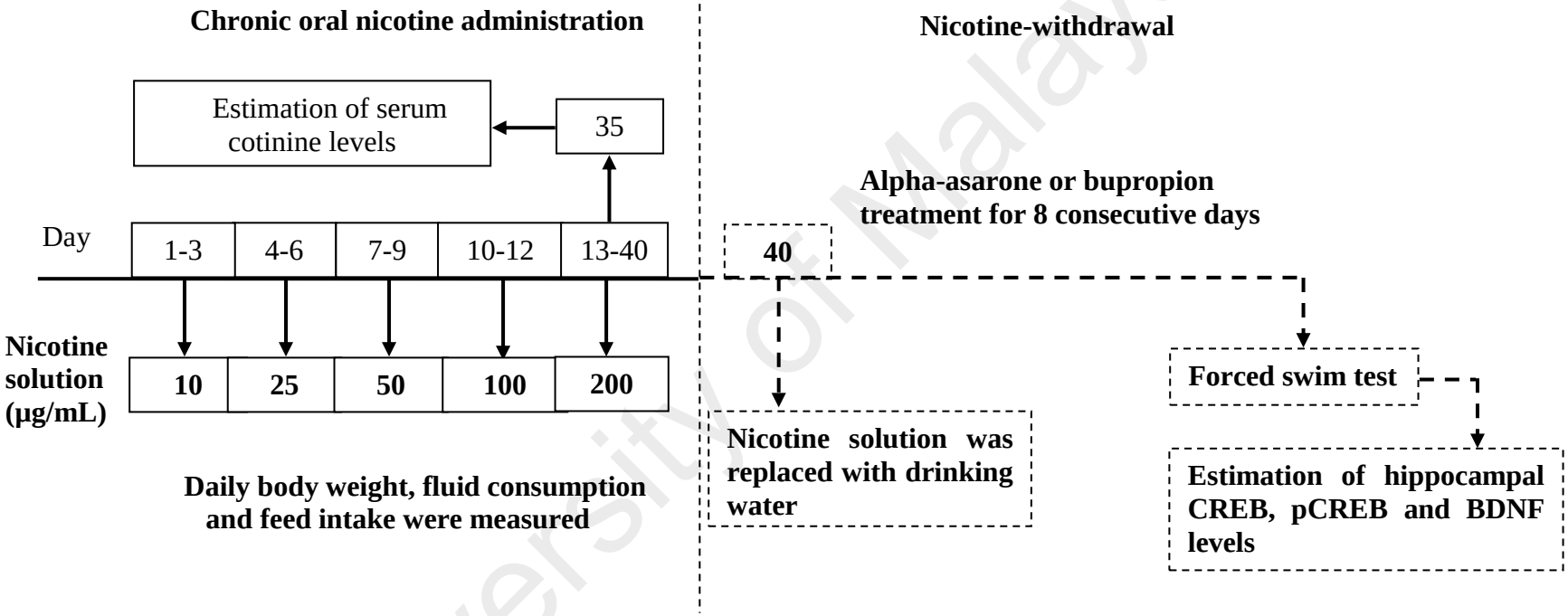

Figure 3.2: Experimental timeline of nicotine-withdrawal induced depression-like behaviour in the forced swim test in C57BL6 mice.

C57BL6 mice were exposed to nicotine solution (10-200 µg/ml) *ad libitum* for forty days and serum cotinine levels (a major metabolite of nicotine) were measured five days before nicotine-withdrawal. During nicotine-withdrawal, 5% v/v Tween 80, α-asarone or bupropion was administered for 8 consecutive days. Forced swim test was performed 30 min after drug treatment. Followed by forced swim test, the hippocampus samples were collected for the estimation of CREB, pCREB and BDNF levels.

(b) ***Serum cotinine measurement***

Five days before nicotine-withdrawal (day 35), ~50 µL of blood sample was collected from the mouse tail vein using Microvette® 100 tubes (Sarstedt, Germany). The blood samples were kept undisturbed and allowed to clot at room temperature for 30 min and centrifuged (model # 2-16PK, Sigma, Germany) at 2000 g for 10 min at 4°C. The resulting supernatant (serum) was analyzed for cotinine levels using enzyme-linked immunosorbent assay (Cotinine Direct ELISA kit, Calibiotech, CA, USA) as per the manufacturer's instructions (catalogue # CO096D-100).

Briefly, the serum samples from the water-exposed (n=6) and nicotine-exposed mice (n=33) were diluted with ELISA sample diluent (1:4). Ten microliters of cotinine standard (5, 10, 25, 50 and 100 ng/mL) and serum samples were pipetted into microwells coated with polyclonal antibody (Ab) to cotinine in duplicates. Then, 100 µL of cotinine horseradish peroxide (HRP) enzyme conjugate was added to each well, the plate was gently shaken for 30sec and incubated at room temperature in dark for 60 min. After incubation, the plate contents were discarded and washed 6 times with 300 µL distilled water and the plate was strike on fresh adsorbent paper towel to remove the residual solution in each well. Followed by this step, 100 µL of 3,3', 5,5'-Tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature in dark for 30 min. Immediately after incubation, 100 µL of stop solution was added to each well and the absorbance was measured at 450nm using plate reader. The unknown serum concentration of cotinine level was interpolated from the standard curve.

(c) ***Nicotine-withdrawal and treatment***

On day 40 of nicotine exposure, nicotine-withdrawal was initiated by replacing the nicotine solution with drinking water. Nicotine-withdrawn mice (n=33) were randomised based on serum cotinine levels and treated once daily with vehicle (5 % v/v Tween 80, i.p.; n=6), α-asarone (5, 10 and 20 mg/kg, i.p.; n=7/group) or bupropion (10 mg/kg, i.p.; n=6) for eight consecutive days, respectively. Besides, the control group (received drinking water *ad libitum*) was treated with vehicle (5 % v/v Tween 80, i.p.; n=6) for eight consecutive days.

(d) ***Forced swim test in nicotine-withdrawn C57BL6 mice***

Thirty minutes after final treatment, the forced swim test was conducted as described previously (Porsolt et al., 1977). Briefly, the mouse was gently placed in the 5L glass transparent beaker (27 cm height, 19 cm internal diameter) containing fresh tap water (up to 15 cm height at 24±1°C) and forced to swim for 6 min. The duration of immobility was measured using a digital stopwatch during last 4 min of the test. The mouse was considered to be immobile when it remained floating motionless (depression-like behaviour) in water, making only simple limb movements necessary to keep its head above water (Can et al., 2012). The beaker was cleaned and filled with fresh water between each test.

3.3.4.4 Effect of repeated α-asarone treatment on the hippocampal CREB, pCREB and BDNF levels during nicotine-withdrawal in C57BL6 mice

C57BL6 mice were sacrificed by cervical dislocation immediately after the forced swim test and the hippocampus samples were collected as described previously (Spijker, 2011). Briefly, the brain was carefully removed and placed over the ice chilled stainless-steel plate. The cerebellum was removed, and the cortex was opened. Then the hippocampus was isolated and freed from the attached cortex. The whole procedure was done within

4-6 min. The isolated hippocampus was immediately transferred into pre-chilled polypropylene tubes and stored at -80°C until analysis. Moreover, the hippocampus samples were weighed and homogenized in a pre-chilled 1 mL Dounce homogenizer with 1:20 ice-cold cell lysis buffer (catalogue # 7018, Cell signalling technology, Inc., MA, USA) containing 1mM phenylmethylsulfonyl fluoride (PMSF, catalog#8553, Cell signaling technology, Inc., MA, USA) added just prior to tissue homogenization. After homogenization, the samples were centrifuged (model # 2-16PK, Sigma, Germany) at 16000 g for 20 min at 4° C, the supernatant were transferred into pre-chilled polypropylene tubes and stored at -80° C until further use. These samples were thawed on ice, centrifuged at 10000 g for 5 min at 4° C before analysis.

(a) ***Estimation of hippocampal total protein levels***

The total protein concentration was measured using a bicinchoninic acid assay (BCA assay) protein quantification kit (catalogue # ab102536, Abcam, Cambridge, UK) as per manufacturer's instructions. Briefly, BCA standard (0.01, 0.02, 0.04, 0.08, 0.016, 0.32 and 0.64mg/mL or 0.5, 1, 2, 4, 8, 16 and 32 µg/mL/ 50 µL) was prepared in cell lysis buffer and the samples were diluted with cell lysis buffer (1:20). Pipette 50 µL of standard and samples into microwells as duplicates. Then, 100 µL of working solution (copper reagent and BCA reagent; 1:50) was added to each well and incubated at 37 ºC for 60 min at 200 rpm (VorTemp 56 shaking incubator, Labnet International Inc, New Jersey, USA). After incubation, the absorbance was measured at 562 nm using plate reader. The unknown total protein concentration was interpolated from the standard curve.

(b) ***Estimation of hippocampal CREB and pCREB levels***

In this study, the samples were diluted with ELISA sample diluent to obtain the total protein concentration of 0.25 µg/mL for the estimation of phosphorylated CREB (pCREB) and CREB levels using PathScan® pCREB (Serine 133) sandwich ELISA kit (catalog # 7385C, Cell signaling technology, Inc., MA, USA), PathScan® total CREB

sandwich ELISA kit (catalog # 7390C, Cell signaling technology, Inc., MA, USA) as per the manufacturer's instructions. Briefly, 100 µL of samples were added to CREB rabbit monoclonal antibody (mAb) coated microwells and incubated at 37 ºC for 2 h (VorTemp 56 shaking incubator, Labnet International Inc, New Jersey, USA). After incubation, the plate content was discarded and washed 4 times with 200 µL of wash buffer to each well. Then, strike plates on the fresh absorbent paper towel to remove the residual solution in each well. After this step, 100 µL of CREB mouse detection mAb or phospho-CREB (Ser 133) mouse detection mAb, respectively, into each well of the corresponding plate and incubated at 37 ºC for 1 h. Followed this step, the plate content was discarded and washed 4 times with 200 µL of wash buffer to each well. Then, the plates were strike on the fresh absorbent paper towel to remove the residual solution in each well. Immediately, 100 µL of anti-mouse immunoglobulin G (IgG), the HRP-linked secondary antibody was added and incubated for 30 min at 37 ºC. Followed this step, the plate content was discarded and washed 4 times with 200 µL of wash buffer to each well. Then, 100 µL TMB substrate was added to each well and incubated for 10 min at 37 ºC. Immediately after incubation, 100 µL of stop solution was added to each well and the absorbance was measured at 450 nm using plate reader.

(c) ***Estimation of hippocampal BDNF levels***

In another study, the samples were analysed for BDNF levels using a BDNF sandwich ELISA kit (catalog#SEA011Mu, Cloud-Clone Corp, Houston, USA) as per the manufacturer's instructions. Briefly, the samples (1:16) were diluted with ELISA sample diluent, 100 µL of sample or standard (0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 ng/mL) was added to each well and incubated at 37 ºC for 2 h (VorTemp 56 shaking incubator, Labnet International Inc, New Jersey, USA). After incubation, the plate content was discarded, 100 µL of detection reagent A was added and incubated at 37 ºC for 1 hr. Then, the plate was washed three times with 300 µL of wash buffer to each well. After this step,

plates were strike on the fresh absorbent paper towel to remove the residual solution in each well and 100 µL of detection reagent B was added and incubated at 37 ºC for 30 min. The plate was washed five times with 300 µL of wash buffer to each well and strike plates on the fresh absorbent paper towel to remove the residual solution in each well. Then, 100 µL TMB substrate was added to each well and incubated for 15 min at 37 ºC. Immediately after incubation, 50 µL of stop solution was added to each well and the absorbance was measured at 450 nm using plate reader.

3.4 Statistical analysis

Statistical analysis and standard curve were performed using GraphPad Prism 5 (Graphpad Software, Inc., USA). The results were analysed by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test or two-way ANOVA followed by *post hoc* Bonferroni multiple comparison test. Data were expressed as mean ± SEM and $p<0.05$ were considered statistically significant.

CHAPTER 4: RESULTS

4.1 Effect of α-asarone in the tail suspension test and spontaneous locomotor activity in ICR mice

4.1.1 Tail suspension test

Alpha-asarone and bupropion treatment showed a significant effect ($F_{6, 62} = 15.30$, $p<0.0001$) on the immobility time in the tail suspension test in ICR mice. Dunnett's *post hoc* analysis showed that the acute treatment of α-asarone at lower doses (15 and 20 mg/kg, i.p.) and bupropion (20 mg/kg, i.p.) significantly reduced the immobility time in tail suspension test as compared with vehicle-treated group. In contrast, α-asarone at higher doses (50 and 100 mg/kg, i.p.) significantly increased the immobility time in the tail suspension test as compared with vehicle-treated group **(Figure 4.1).**

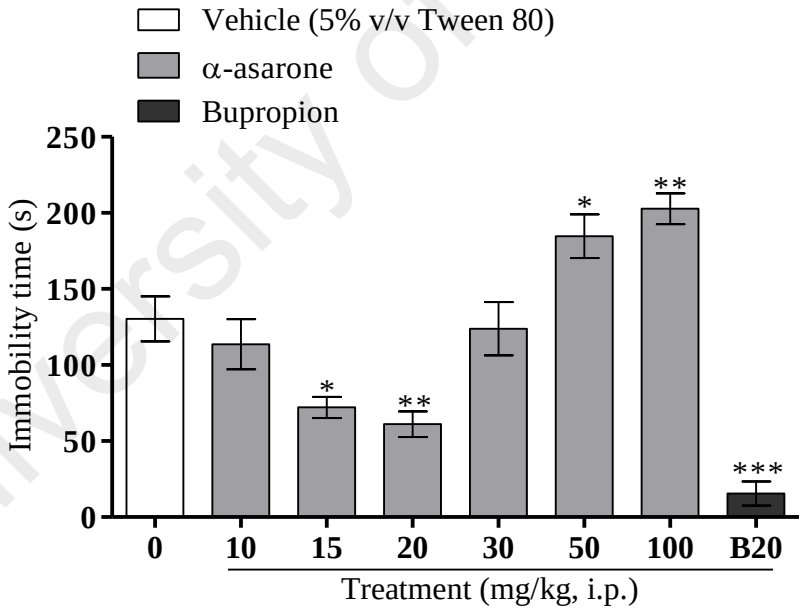

Figure 4.1: Effect of acute α-asarone and bupropion treatment on the immobility time of mice in the tail suspension test in ICR mice. Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 as compared with vehicle-treated group.

4.1.2 Spontaneous locomotor activity

Alpha-asarone treatment showed a significant effect ($F_{7, 67} = 6.953$, $p<0.0001$) on the spontaneous locomotor activity in ICR mice. Dunnett's *post hoc* analysis revealed that the α-asarone at higher doses (50 and 100 mg/kg, i.p.) significantly decreased the locomotor activity when compared with vehicle-treated group. However, α-asarone at lower doses (10, 15, 20 and 30 mg/kg, i.p.) and bupropion (20 mg/kg, i.p.) did not affect the spontaneous locomotor activity when compared with vehicle-treated group. **(Figure 4.2)**.

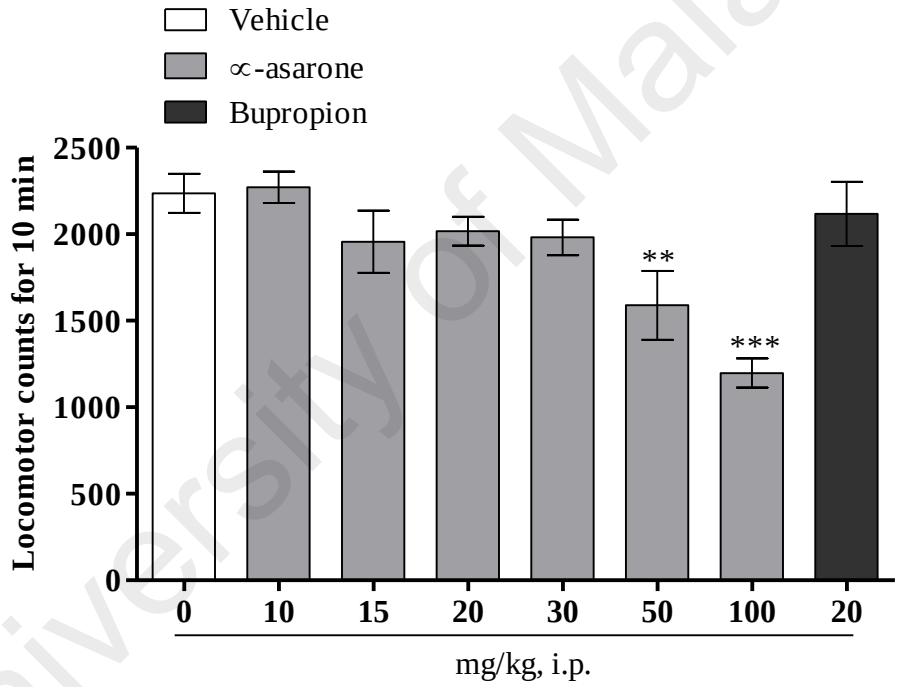

Figure 4.2: Effect of acute treatment of α-asarone and bupropion on the spontaneous locomotor activity in ICR mice. Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean ± SEM (n= 8-10). **p<0.01 and ***p<0.001 as compared with vehicle-treated group (5% v/v Tween 80).

4.2 Involvement of the noradrenergic and dopaminergic systems in the antidepressant effect of α-asarone in AMPT pretreated ICR mice

The pretreatment of AMPT (100 mg/kg, i.p.) significantly blocked the anti-immobility effect of α-asarone (20 mg/kg, i.p.) and bupropion (20 mg/kg, i.p.) in mice, using tail suspension test (**Figure 4.3**). In addition, the immobility time of AMPT/α-asarone treated mice was significantly increased when compared with the AMPT/bupropion treated. Two-way ANOVA results revealed a significant effect on AMPT pretreatment ($F_{2, 42}$ = 12.82, $p<0.0001$), α-asarone or bupropion treatment ($F_{1, 42}$ =72.92, $p<0.0001$) and α-asarone or bupropion × AMPT interaction ($F_{2, 42}$ =6.20, $p<0.0044$).

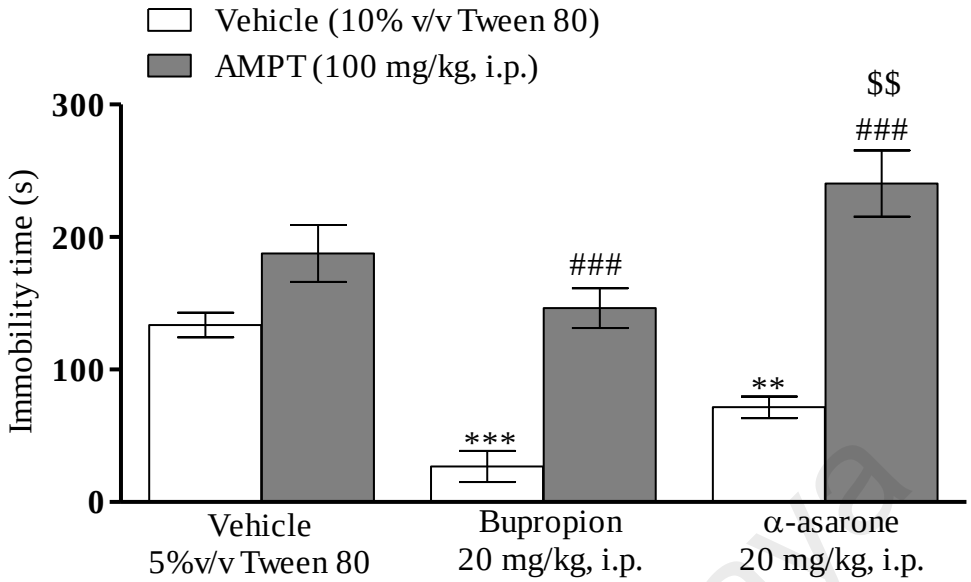

Figure 4.3: Effect of AMPT (100 mg/kg, i.p., a catecholamine synthesis inhibitor) pretreatment on α-asarone (20 mg/kg, i.p.) induced anti-immobility in the tail suspension test in ICR mice. The results were analysed using two-way ANOVA followed by *post hoc* Bonferroni test, expressed as mean ± S.E.M. (n=8). **p<0.01, 10% v/v Tween 80/α-asarone treated group *vs* 10% v/v Tween 80/5 % v/v Tween 80 treated group. ***p<0.001, 10% v/v Tween 80/bupropion treated group *vs* 10% v/v Tween 80/5 % v/v Tween 80 treated group. ###p<0.001, AMPT/bupropion treated group *vs* 10% v/v Tween 80/bupropion treated group or AMPT/α-asarone treated group *vs* 10% v/v Tween 80/ α-asarone treated group. $$ p<0.01**,** AMPT/α-asarone treated group *vs* AMPT/bupropion treated group

4.3 Involvement of the serotonergic system in the antidepressant effect of α-asarone in PCPA pretreated ICR mice

The pretreatment of mice with PCPA (100 mg/kg, i.p., once daily for 4 consecutive days) reversed the anti-immobility effect of α-asarone (20 mg/kg, i.p.) or fluoxetine (30 mg/kg, i.p.) in the tail suspension test **(Figure 4.4)**. Two-way ANOVA results showed a significant effect of α-asarone or fluoxetine treatment ($F_{1, 42} = 17.98$, $p<0.0001$), α-asarone or fluoxetine × PCPA interaction ($F_{2, 42} = 5.02$, $p<0.0111$), and not with PCPA pretreatment ($F_{2, 42} = 1.38$, $p = 0.2630$).

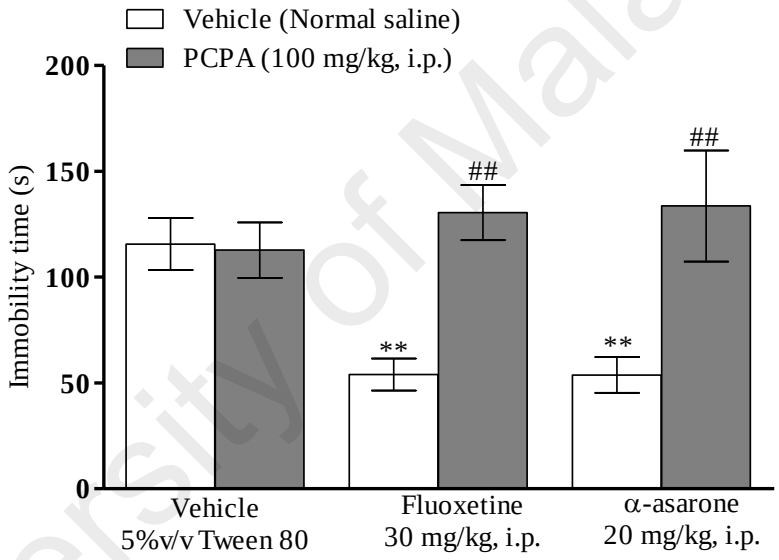

Figure 4.4: Effect of PCPA (100 mg/kg, i.p., once daily for 4 consecutive days, a serotonin synthesis inhibitor) pretreatment on α-asarone (20 mg/kg, i.p.) induced anti-immobility in the tail suspension test in ICR mice. Data were analysed using two-way ANOVA followed by *post hoc* Bonferroni test, expressed as mean ± S.E.M. (n=8). **p<0.01, normal saline/fluoxetine-treated group *vs* normal saline/5 % v/v Tween 80 treated group or normal saline/α-asarone treated group *vs* normal saline/5 % v/v Tween 80 treated group. ##p<0.01, PCPA/α-asarone treated group *vs* normal saline/ α-asarone treated group or AMPT/fluoxetine-treated group *vs* normal saline/fluoxetine-treated group.

4.4 Effect of α-asarone on nicotine's pharmacological effects in ICR mice

4.4.1 Nicotine-induced hypomotility in ICR mice

The present study results revealed that there was a significant effect of treatment on the nicotine-induced decrease in locomotor activity in mice ($F_{11,60} = 71.10$, $p<0.0001$). Dunnett's multiple comparison revealed that the mice treated with nicotine significantly ($p< 0.001$) decreased the locomotor activity (hypomotility) as compared with vehicle control group. Alpha-asarone (5, 10, 20 and 30 mg/kg, i.p.) or bupropion (20 mg/kg, i.p.) alone did not affect the locomotor activity in mice as compared with vehicle control group. Moreover, the reference standard bupropion (20 mg/kg, i.p.) treatment significantly ($p < 0.001$) reversed the nicotine-induced hypomotility in mice as compared with the nicotine alone treated group. However, alpha-asarone (5, 10, 20 and 30 mg/kg, i.p.) treatment did not reverse the nicotine-induced hypomotility as compared with the nicotine alone treated group (**Figure 4.5**).

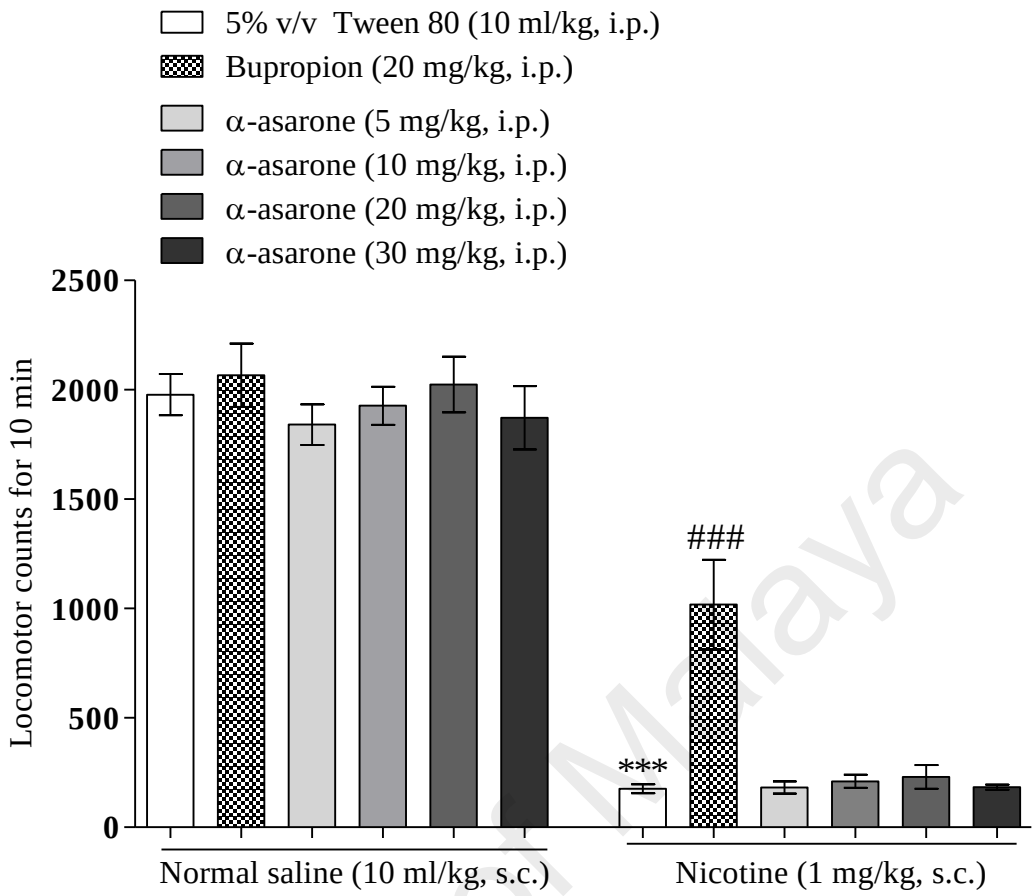

Figure 4.5: Effect of acute α-asarone and bupropion treatment on nicotine-induced hypomotility in ICR mice. Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean± SEM (n=6). ***p<0.001 as compared with vehicle-treated (5% v/v Tween 80/normal saline) group and ###p<0.001 as compared with nicotine (5% v/v Tween 80/nicotine) treated group.

4.4.2 Nicotine-induced hypothermia in ICR mice

In this study, one-way ANOVA results revealed that there was a significant effect of treatment on nicotine-induced hypothermia in mice ($F_{11,60} = 72.68$, $p<0.0001$). Dunnett's multiple comparison revealed that the mice treated with nicotine significantly ($p < 0.001$) decreased the body temperature (hypothermia) in mice as compared with vehicle control group. However, alpha-asarone (5, 10, 20 and 30 mg/kg, i.p.) or bupropion (20 mg/kg, i.p.) alone did not affect the normal body temperature in mice as compared with vehicle control group. Moreover, the reference standard bupropion (20 mg/kg, i.p.) treatment significantly ($p < 0.001$) reversed the nicotine-induced hypothermia in mice as compared with the nicotine alone treated group. In contrast, alpha-asarone (5, 10, 20 and 30 mg/kg, i.p.) treatment did not reverse the nicotine-induced hypomotility as compared with the nicotine alone treated group (**Figure 4.6**).

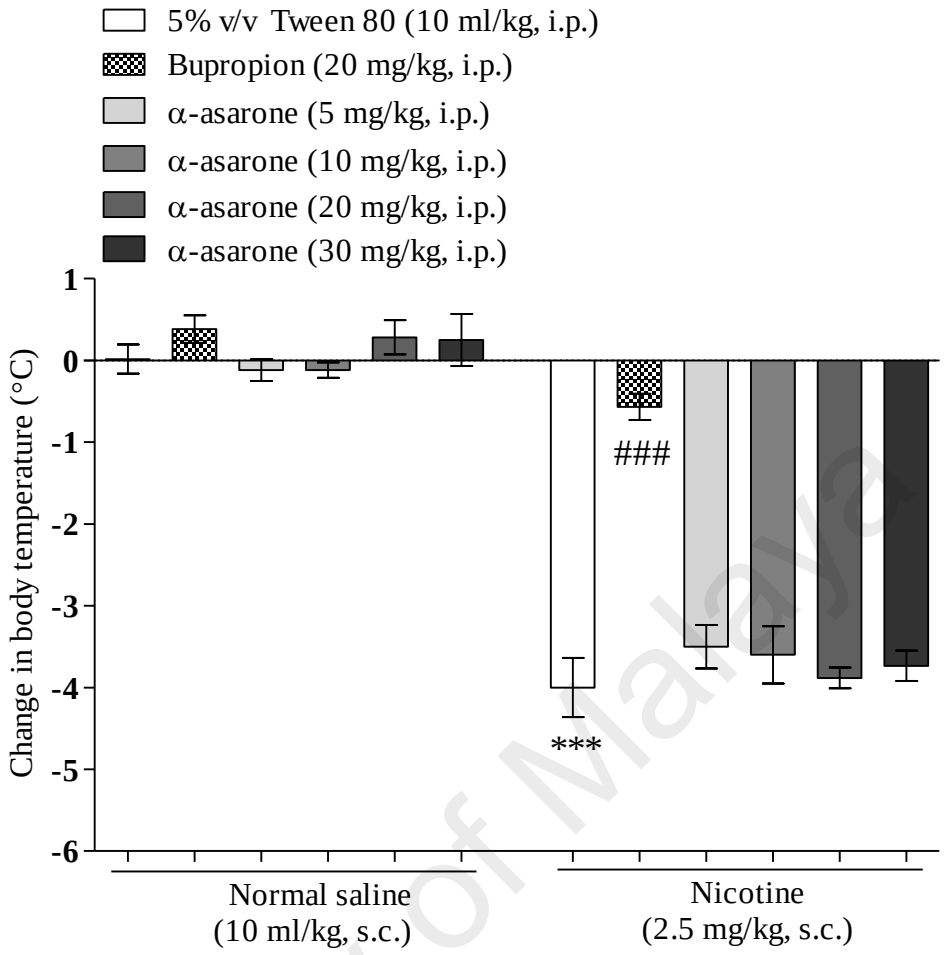

Figure 4.6: Effect of acute α-asarone and bupropion treatment on nicotine-induced hypothermia in ICR mice. Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean± SEM (n=6). ***p<0.001 as compared with vehicle-treated (5% v/v Tween 80/normal saline) group and ###p<0.001 as compared with nicotine (5% v/v Tween 80/nicotine) treated group.

4.5 Effect of repeated α-asarone administration in naïve C57BL6 mice in the forced swim test and spontaneous locomotor activity test

This study results showed that there was no significant effect of repeated α-asarone (5, 10 and 20 mg/kg, i.p.) and bupropion (10 mg/kg, i.p.) treatment on the immobility time [$F_{(4, 35)} = 1.911$, p=0.1303] in the FST (**Fig. 4.7**) or spontaneous locomotor activity [$F_{(4, 35)} = 1.911$, p=0.1303] (**Fig. 4.8**) in naïve mice, respectively.

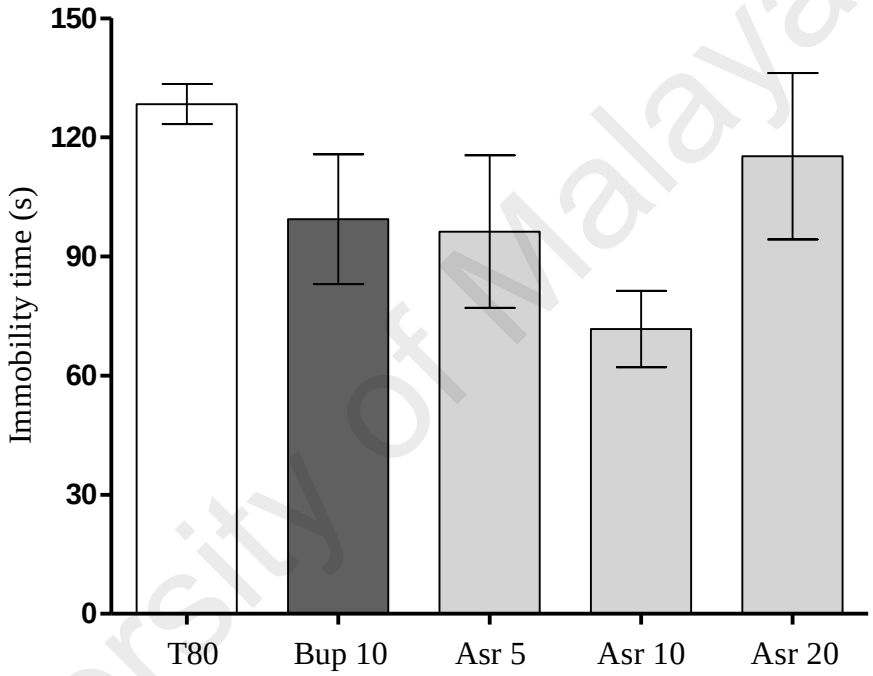

Figure 4.7: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) on the immobility time in the forced swim test in naïve C57BL6 mice. Control mice were treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean± SEM (n=8).

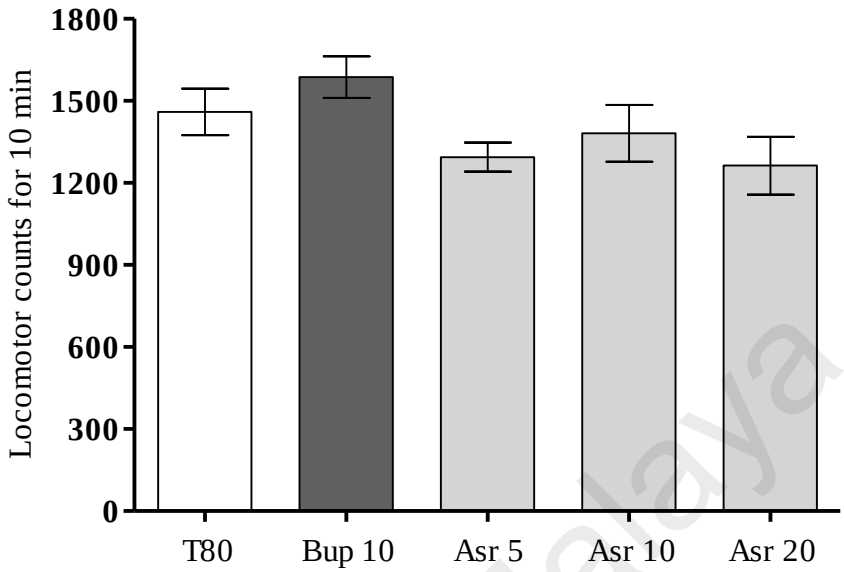

Figure 4.8: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) on the spontaneous locomotor activity in naïve C57BL6 mice. Control mice were treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean± SEM (n=8).

4.6 Effect of repeated α-asarone treatment on depression-like behaviour in nicotine-withdrawn C57BL6 mice

4.6.1 Body weight, fluid consumption and food intake during chronic nicotine exposure

The body weight gain of C57BL6 mice exposed to chronic oral nicotine solution was significantly ($p<0.01$) decreased when compared to the body weight gain of control mice (**Figure 4.9**). Two-way ANOVA results revealed that there was a significant effect of day ($F_{45, 1702} = 34.18$, $p<0.0001$), nicotine exposure ($F_{1, 1702}= 205.99$, $p<0.0001$) and day × nicotine exposure ($F_{45, 1702} = 2.54$, $p<0.0001$). However, the daily fluid consumption and food intake of mice were not significantly changed during nicotine exposure (**Figure 4.10 and 4.11**).

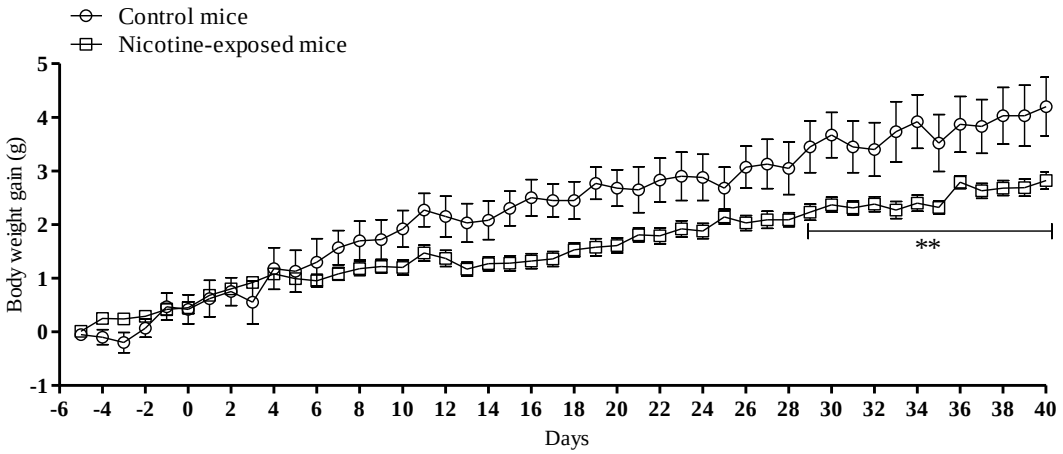

Figure 4.9: Effect of the chronic oral nicotine (10-200µg/mL) exposure on the change in daily body weight of C57BL6 mice. The change in body weight is calculated from the difference between daily and initial body weight. Control mice were exposed to drinking water *ad libitum* throughout the experiment. Nicotine solution *ad libitum* exposed mice (day 1-3, 10 µg/mL; day 4-6, 25 µg/mL; day 7-9, 50 µg/mL; day 10-12, 100 µg/mL and day 13-40, 200 µg/mL). Data were analysed using two-way ANOVA followed by *post hoc* Bonferroni multiple comparison test, expressed as mean ± SEM. **p<0.01, control mice (n=6) *vs* nicotine-exposed mice (n=33).

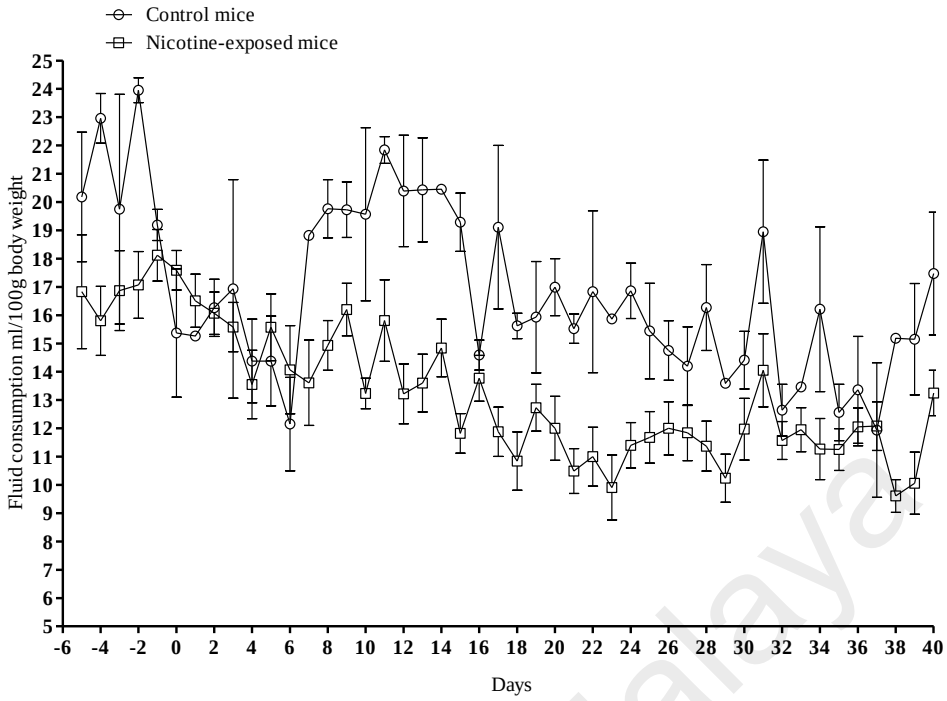

Figure 4.10: Effect of the chronic oral nicotine (10-200µg/mL) exposure on the daily fluid consumption of C57BL6 mice. Control mice were exposed to drinking water *ad libitum* throughout the experiment. Nicotine solution *ad libitum* exposed mice (day 1-3, 10 µg/mL; day 4-6, 25 µg/mL; day 7-9, 50 µg/mL; day 10-12, 100 µg/mL and day 13-40, 200 µg/mL). Data were analysed using two-way ANOVA followed by *post hoc* Bonferroni multiple comparison test, expressed as mean ± SEM., control mice (n=6) *vs* nicotine-exposed mice (n=33).

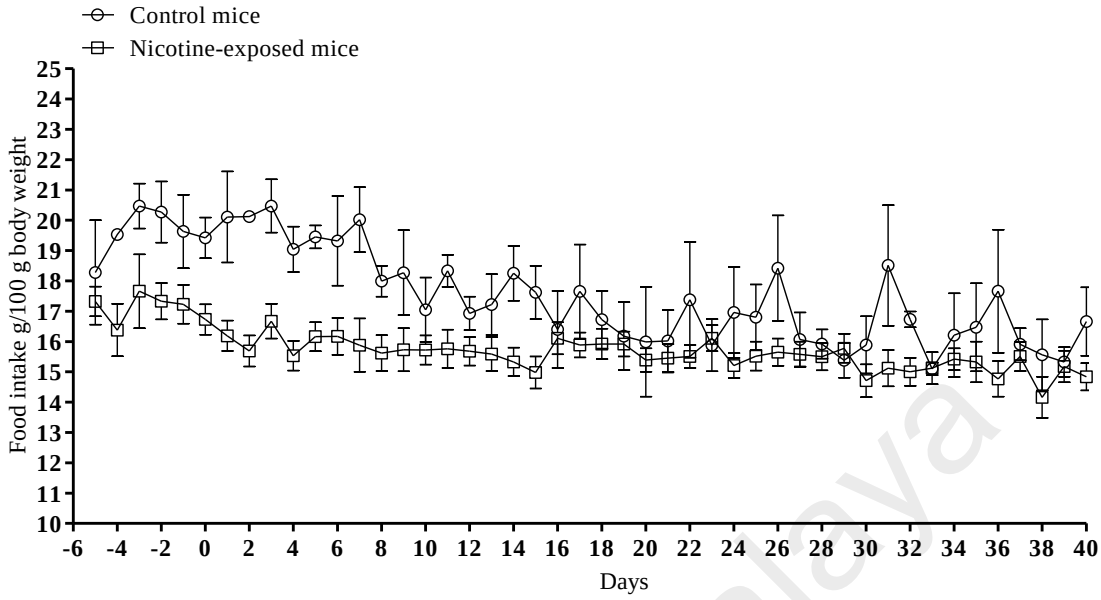

Figure 4.11: Effect of the chronic oral nicotine (10-200µg/mL) exposure on the daily food intake of C57BL6 mice. Control mice were exposed to drinking water *ad libitum* throughout the experiment. Nicotine solution *ad libitum* exposed mice (day 1-3, 10 µg/mL; day 4-6, 25 µg/mL; day 7-9, 50 µg/mL; day 10-12, 100 µg/mL and day 13-40, 200 µg/mL). Data were analysed using two-way ANOVA followed by *post hoc* Bonferroni multiple comparison test, expressed as mean ± SEM, control mice (n=6) *vs* nicotine-exposed mice (n=33).

4.6.2 Daily nicotine consumption and serum cotinine levels

The daily oral nicotine intake of mice during nicotine solution exposure is depicted in **Figure 4.12**. The average nicotine consumption of mice exposed to nicotine solution (200 µg/mL) was found to be 23.61±0.48 mg/kg/day. Furthermore, the serum cotinine was detected in the mice exposed to nicotine solution with an average of 159. 53 ± 13. 55 ng/mL.

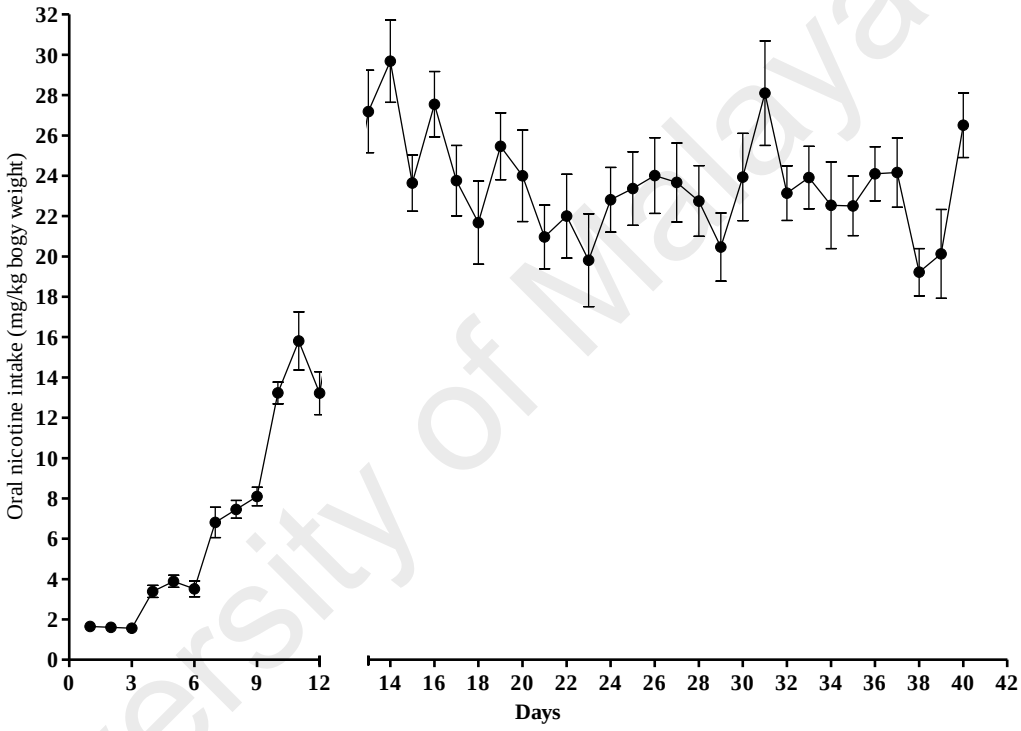

Figure 4.12: Daily nicotine-intake of chronic nicotine-exposed (10-200 µg/mL) C57BL6 mice. The daily nicotine intake was calculated from the daily fluid intake and body weight with respect to the concentration of nicotine solution exposed. Nicotine solution *ad libitum* exposed mice (day 1-3, 10 µg/mL; day 4-6, 25 µg/mL; day 7-9, 50 µg/mL; day 10-12, 100 µg/mL and day 13-40, 200 µg/mL). Data are expressed as mean ± SEM (n=33).

4.6.3 Forced swim test in nicotine-withdrawn C57BL6 mice

There was a significant ($F_{5, 32}$ =8.044; p<0.0001) effect of treatment on immobility time in the forced swim test. The immobility time of nicotine-withdrawn vehicle-treated mice (negative control) was significantly (p<0.01) increased when compared with control mice (water-exposed vehicle treated). Pretreatment of α-asarone (5, 10 and 20 mg/kg, i.p.) or bupropion (10 mg/kg, i.p.) significantly (p<0.001) decreased the immobility time when compared with the negative control mice (**Figure 4.13**).

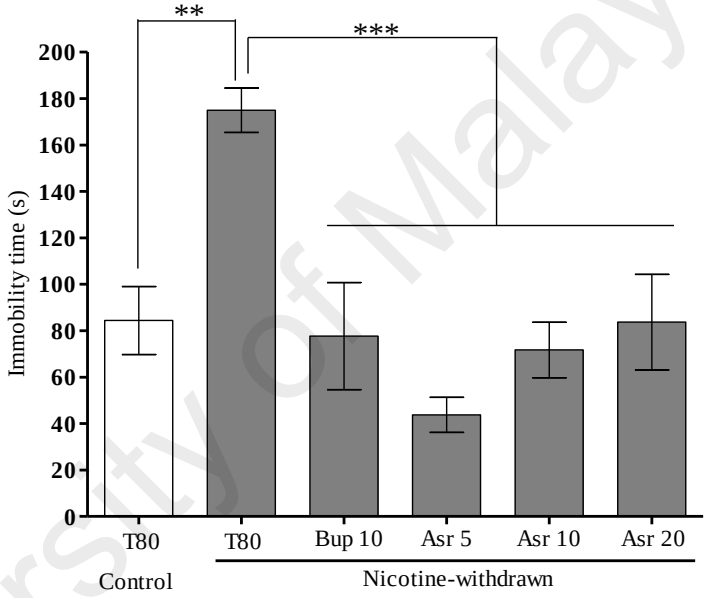

Figure 4.13: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) treatment on the immobility time in the forced swim test in nicotine-withdrawn C57BL6 mice. Negative control (nicotine-withdrawn) mice were treated with 5% v/v Tween 80 (T80). Control mice were exposed to drinking water *ad libitum* throughout the experiment and treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean ± SEM (n=6-7). The presence of statistical difference (**p<0.01; ***p<0.001) was observed in indicated groups.

4.6.4 Effect of repeated α-asarone treatment on the hippocampal pCREB, CREB and BDNF levels in nicotine-withdrawn C57BL6 mice

The main effect of treatment was significant ($F_{5, 32} = 5.731$; $p<0.0007$) on the hippocampal pCREB levels. Multiple comparisons revealed that the hippocampal pCREB levels of the nicotine-withdrawn vehicle-treated mice (negative control) were significantly ($p<0.01$) increased when compared to control mice (water-exposed vehicle treated). Moreover, α-asarone (5, 10 and 20 mg/kg, i.p.) or bupropion (10 mg/kg, i.p.) treatment significantly attenuated the nicotine-withdrawal-induced increase in the hippocampal pCREB levels when compared to nicotine-withdrawn vehicle-treated mice (**Figure 4.14**). However, no significant changes in the hippocampal CREB and BDNF levels were observed during nicotine-withdrawal or with α-asarone and bupropion treatment (**Figure 4.15 and 4.16)**.

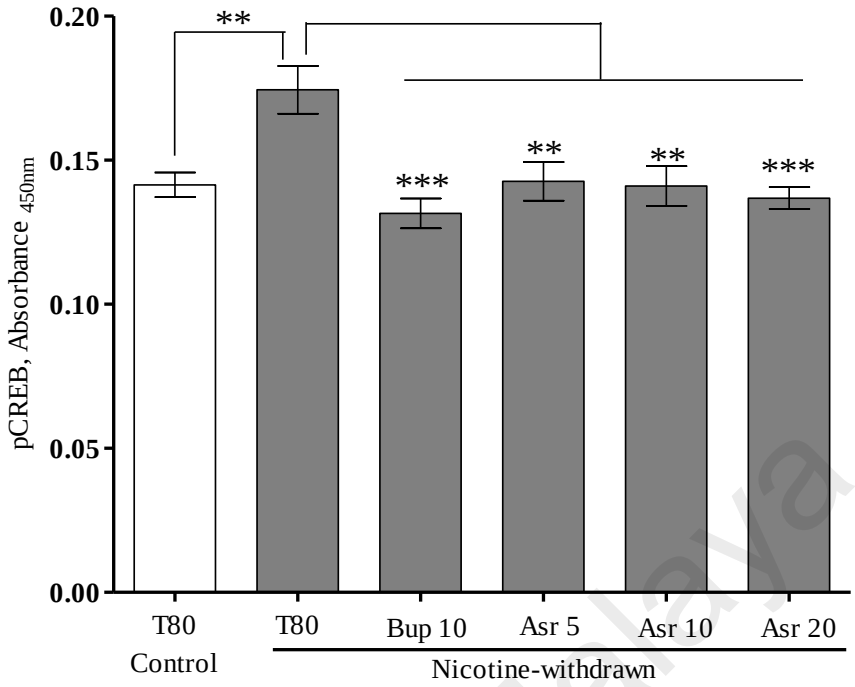

Figure 4.14: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) treatment on the pCREB levels in the hippocampus of nicotine-withdrawn C57BL6 mice. Negative control (nicotine-withdrawn) mice were treated with 5% v/v Tween 80 (T80). Control mice were exposed to drinking water *ad libitum* throughout the experiment and treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean ± S.E.M (n=6-7). The presence of statistical difference (**p<0.01; ***p<0.001) was observed in indicated groups.

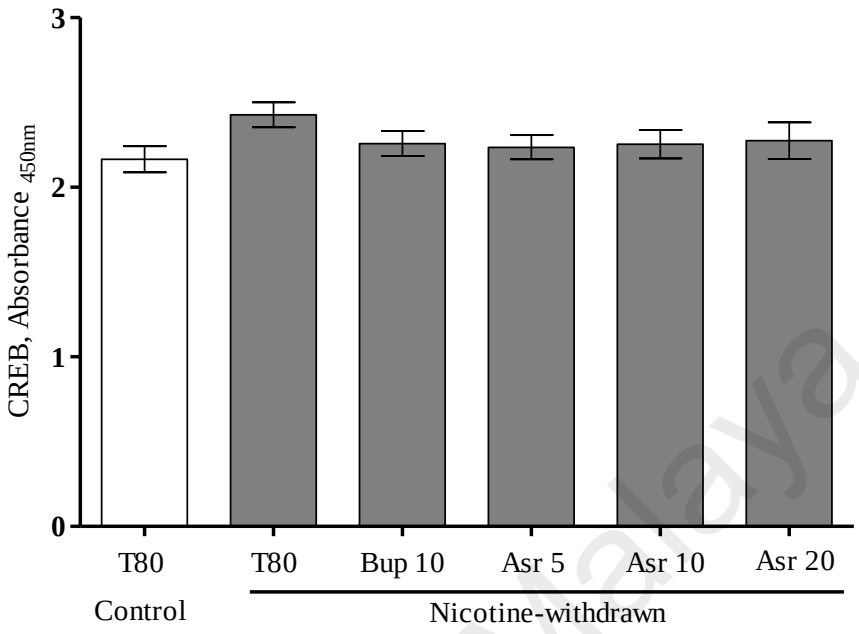

Figure 4.15: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) treatment on the CREB levels in the hippocampus of nicotine-withdrawn C57BL6 mice. Negative control (nicotine-withdrawn) mice were treated with 5% v/v Tween 80 (T80). Control mice were exposed to drinking water *ad libitum* throughout the experiment and treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean ± S.E.M (n=6-7).

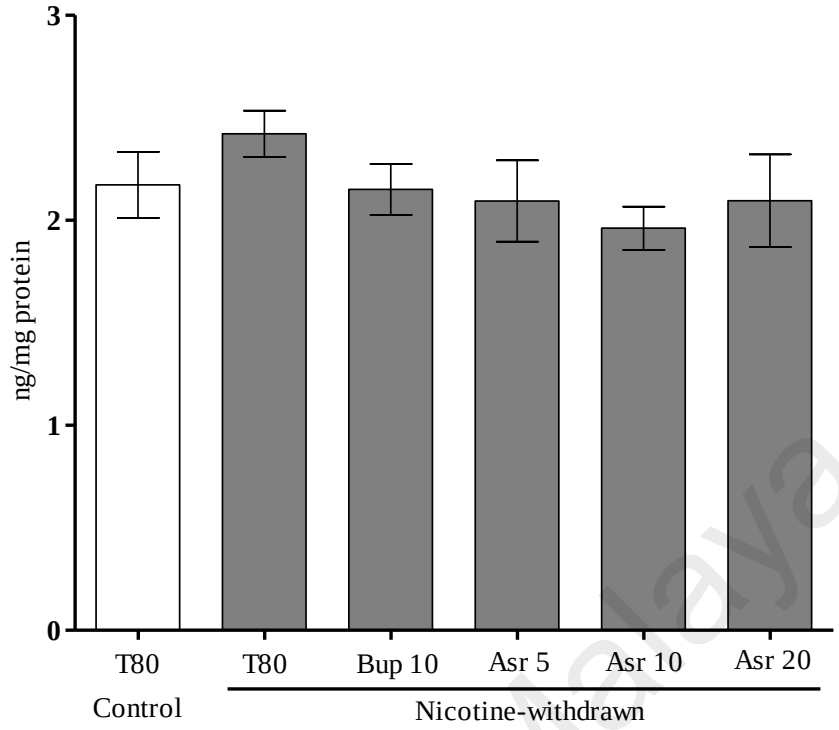

Figure 4.16: Effect of repeated α-asarone (Asr; 5, 10 and 20 mg/kg, i.p.) and bupropion (Bup; 10 mg/kg, i.p.) treatment on the BDNF levels in the hippocampus of nicotine-withdrawn C57BL6 mice. Negative control (nicotine-withdrawn) mice were treated with 5% v/v Tween 80 (T80). Control mice were exposed to drinking water *ad libitum* throughout the experiment and treated with 5% v/v Tween 80 (T80). Data were analysed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test, expressed as mean ± S.E.M (n=6-7).

CHAPTER 5: DISCUSSION

The tail suspension test and forced swim test are the two well-validated animal models of depression with high reliability and specificity. The principle of these two tests is due to an unavoidable and inescapable stress the mice became immobile and this immobility indicates depression-like behaviour. The currently available class of antidepressant drugs including noradrenaline reuptake inhibitors, selective serotonin reuptake inhibitors, serotonin, and noradrenaline reuptake inhibitors, monoamine oxidase inhibitors and tricyclic antidepressants are effective in both tail suspension test and forced swim test. However, CNS stimulants could produce false positive results in the tail suspension test and forced swim test and this could be ruled out by conducting complementary locomotor activity studies (Cryan, Markou, & Lucki, 2002; Cryan, Mombereau, & Vassout, 2005; Petit-Demouliere, Chenu, & Bourin, 2005).

In this study, the acute treatment of α-asarone at lower doses (15 and 20 mg/kg, i.p.) significantly decreased the immobility time in the tail suspension test in ICR mice, inferring antidepressant-like effect. Similarly, Han et al. (2013) reported that the acute treatment of α-asarone (10 and 20 mg/kg, i.p.) showed an antidepressant-like effect in both tail suspension test and forced swim test in ICR mice. At the same time, α-asarone at higher doses (50 and 100 mg/kg, i.p.) significantly increased the immobility time in the tail suspension test in ICR mice, indicated depressive-like effect. In locomotor activity study, the locomotor counts of α-asarone (10-30 mg/kg, i.p.) treated ICR mice was unaffected. Therefore, it is indicated that the anti-immobility effect of α-asarone (15 and 20 mg/kg, i.p.) in the tail suspension test is not mediated through stimulation of the CNS. Furthermore, α-asarone at higher doses (50 and 100 mg/kg, i.p.) significantly decreased the spontaneous locomotor activity in ICR mice. Similarly, in the previous studies, the

acute treatment of α-asarone (48, 50 and 100 mg/kg, i.p.) significantly decreased the locomotor activity in mice (Liu et al., 2012; Menon & Dandiya, 1967; Pages et al., 2010).

However, it is beyond the scope of the present to clarify the exact mechanism(s) involved in the depressive-like effect of α-asarone at higher doses in tail suspension test. In previous studies, it was observed that α-asarone mostly at higher doses (≥50 mg/kg, i.p.) potentiated the gamma-aminobutyric acid ($GABA_A$) receptor positive allosteric modulators (pentobarbital, hexobarbital or ethanol)-induced sleeping time in mice (Dandiya & Sharma, 1961; Pages et al., 2010). Moreover, α-asarone at higher doses (≥50 mg/kg, i.p.) showed a protective effect on $GABA_A$ receptor antagonists (pentylenetetrazol or picrotoxin)-induced seizures (Chen et al., 2013; Dandiya & Sharma, 1961; Huang et al., 2013; Pages et al., 2010). In addition, the positive neuromodulatory effect of α-asarone on $GABA_A$ receptors was evidenced from electrophysiological studies (Huang et al., 2013; Wang et al., 2014). Based on these reports, it has been postulated that α-asarone at higher doses could activate $GABA_A$ receptors in the CNS, thereby enhancing GABA-ergic neurotransmission and produce a decrease in locomotor activity or increase in immobility time in tail suspension test in mice.

The down-regulation of brain monoaminergic neurotransmission (adrenergic, serotonergic and dopaminergic) play a key factor in the pathophysiology of depression (Moret & Briley, 2011). Importantly, the clinically approved antidepressant class of drugs (monoamine oxidase inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors, noradrenaline reuptake inhibitors and serotonin and noradrenaline reuptake inhibitors) were effective in the treatment of depression mainly by increasing the brain's noradrenergic and/or serotonergic transmissions (Berton & Nestler, 2006; Moret & Briley, 2011). To investigate the involvement of noradrenergic and dopaminergic systems in the antidepressant effect of α-asarone, ICR mice were pretreated with AMPT and

subsequently the duration of immobility time in the tail suspension test in the presence of α-asarone was measured. AMPT, is an inhibitor of tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of noradrenaline and dopamine. In rodents (mice and rats), AMPT treatment reduces only the brain noradrenaline and dopamine levels without affecting serotonin levels (Mayorga et al., 2001; Widerlov & Lewander, 1978). Moreover, mice treated with AMPT showed a significant reduction in the brain noradrenaline (53%) and dopamine (57 %) levels without affecting serotonin levels (Mayorga et al., 2001). It is reported that AMPT pretreatment in mice significantly abolished the antidepressant activity of bupropion (a noradrenaline and dopamine reuptake inhibitor) in the tail suspension test (Kwon et al., 2010). To evaluate the contribution of the serotonergic system in the antidepressant effect of α-asarone, ICR mice were pretreated with PCPA before the duration of immobility was measured in the tail suspension test. PCPA, a selective serotonin synthesis inhibitor, inhibits tryptophan hydroxylase and depletes serotonin level in the brain without affecting the brain noradrenaline and dopamine levels (Mayorga et al., 2001; Redrobe, Bourin, Colombel, & Baker, 1998). Furthermore, mice treated with PCPA showed a significant reduction in the brain serotonin level (~60-70 %) without affecting noradrenaline and dopamine levels (Mayorga et al., 2001). Interestingly, the pretreatment of PCPA inhibited the antidepressant-like effect of fluoxetine (a selective serotonin reuptake inhibitor) in the tail suspension test and forced swim test (Kwon et al., 2010; Machado et al., 2009). In the present study, the ICR mice pretreated with AMPT and PCPA prevented the antidepressant-like effect of α-asarone, bupropion or fluoxetine. These results demonstrated the involvement of the monoaminergic (noradrenergic, dopaminergic and serotonergic) systems in the antidepressant effect of α-asarone. Besides, α-asarone exhibited a parabolic effect in the tail suspension test that could be due to a significant interaction with monoaminergic systems at lower doses ≤20 mg/kg, i.p., (produced

antidepressant-like effect) and a significant interaction with GABA-ergic system at higher doses ≥50 mg/kg, i.p. (produced depression-like effect). Furthermore, α-asarone showed a comparable antidepressant-like activity with a clinically approved antidepressant, bupropion (a noradrenaline and dopamine reuptake inhibitor) through its interaction with noradrenergic and dopaminergic systems.

Nicotine is known to produce the decrease in locomotor activity (hypomotility) and hypothermia in rodents through the activation of nAChRs and these effects are reversed or blocked by nAChRs antagonists (Freitas et al., 2013; Kota, Martin, Robinson, & Damaj, 2007). Several studies have demonstrated that the involvement of neuronal nAChRs in hypomotility and hypothermic effects of nicotine (Alajaji et al., 2013; Freitas et al., 2013; Ignatowska-Jankowska et al., 2013; Slemmer et al., 2000). Mice lacking α_3, α_5 or β_4 subunits of neuronal nAChRs subunit did not demonstrate nicotine-induced hypomotility in mice (K. J. Jackson et al., 2010; Ramiro Salas, Cook, Bassetto, & De Biasi, 2004; R. Salas et al., 2003). Moreover, the knockout of α_5 or β_4 subunits of neuronal nAChRs showed less sensitive to nicotine-hypothermia in mice (K. J. Jackson et al., 2010; Sack et al., 2005), which confirmed the involvement neuronal nAChRs in the nicotine-induced hypomotility and hypothermia in mice. Interestingly, the nicotine-induced hypomotility and hypothermia in mice is reversed or blocked by mecamylamine (a nonselective, non-competitive antagonist of the nAChRs) or bupropion (a noradrenaline and dopamine reuptake inhibitor and a neuronal nAChRs antagonist) (Damaj, Glassco, Dukat, & Martin, 1999; Damaj, Wiley, Martin, & Papke, 2005; Slemmer et al., 2000).

In this study, of α-asarone (5, 10, 20, and 30 mg/kg, i.p.) did not prevent the nicotine-induced hypomotility and hypothermia in ICR mice. In contrast, bupropion (20 mg/kg, i.p.) significantly reversed the nicotine-induced hypomotility and hypothermia in ICR mice and this result is agreeable with Slemmer et al. (2000). This result suggested

that α-asarone did not have any interaction with nAChRs. Besides, in preclinical studies, mecamylamine (a nonselective, non-competitive antagonist of the nAChRs) did not attenuate nicotine-withdrawal signs instead it precipitated the withdrawal signs in mice (Damaj et al., 2003; McLaughlin et al., 2015). Importantly, in clinical studies, mecamylamine alone or in combination with NRT was not very effective in smoking cessation (Glover et al., 2007; Nemeth-Coslett, Henningfield, O'Keeffe, & Griffiths, 1986). Thus, it is postulated that antagonism of nAChRs alone could not contribute to the effectiveness in attenuating nicotine withdrawal signs and symptoms, however, like bupropion, positive modulatory activity on the noradrenergic and dopaminergic system could attenuate nicotine withdrawal signs and symptoms. In addition, the immobility time of AMPT/α-asarone treated mice was significantly increased when compared with the AMPT/bupropion treated. Thus, this result showed a superior positive modulatory activity of α-asarone on brain noradrenergic and dopaminergic systems when compared with bupropion because α-asarone is devoid of its interaction with nAChRs at its antidepressant doses (15 and 20 mg/kg, i.p.), however, bupropion, showed its interaction with nAChRs at its tested antidepressant doses (20 mg/kg, i.p.).

It is evidenced that nicotine-mediated behavioural effects such as pleasure, arousal, relieves stress, and improved mood through an increase in brain monoamine (noradrenaline, dopamine and serotonin) levels. In mice and rats, nicotine administration increased the brain dopamine, noradrenaline and serotonin levels (Gäddnäs et al., 2002; Gäddnäs et al., 2000; Singer et al., 2004). During nicotine withdrawal, the brain dopamine, noradrenaline and serotonin levels were decreased in mice and rats (Benwell & Balfour, 1979; Gäddnäs et al., 2000) and these decrease in monoamine levels during nicotine cessation contributes to irritability and depressed mood (K. J. Jackson et al., 2015; Kenny & Markou, 2001).

Based on these existing results from the present study, unlike bupropion α-asarone did not show any interaction with nAChRs, however, α-asarone showed a positive modulatory activity on brain noradrenergic, dopaminergic and serotonergic systems. Hence, it is hypothesised that α-asarone might modulate the brain monoaminergic system during nicotine-withdrawal in mice and alleviate depression-like behaviour during nicotine withdrawal.

Researchers have adopted many experimental protocols to induce nicotine dependence and withdrawal in rodents. Administration of oral nicotine through drinking water is relatively stress-free in mice when compared to other experimental procedures such as chronic repeated nicotine injection (requiring 13-61 subcutaneous injections/mouse) (Biala et al., 2014; Kota et al., 2007; Kotagale et al., 2015; Mannucci et al., 2011; Rehni, Singh, & Arora, 2012) or surgical implantation of osmotic minipumps containing nicotine solution (Damaj et al., 2003; Stoker, Semenova, & Markou, 2008) for the induction of nicotine dependence in mice. Besides, it was reported that the C57BL6 mice consumed more oral nicotine from the nicotine solution as a source of drinking when compared to other inbred mouse strains (C3H, BUB, DBA/2, and ST/b mice) (Robinson et al., 1996). However, the presence of saccharin sodium (the sweetening agent was used to mask the bitter taste of nicotine) in the nicotine solution did not cause any difference in oral nicotine consumption (10 -200 µg/mL) of C57BL6 mice when compared to the C57BL6 mice exposed to the nicotine solution without saccharin sodium (Robinson et al., 1996; Wilking et al., 2012). Importantly, the chronic oral nicotine administration produces numerous peaks of plasma nicotine levels when the animal drinks oral nicotine solution (Pekonen, Karlsson, Laakso, & Ahtee, 1993; Pietila & Ahtee, 2000; Sparks & Pauly, 1999). Moreover, nicotine or cotinine (a major metabolite of nicotine) was detected in the blood samples of the chronic oral nicotine exposed mice, which confirmed the nicotine intake through oral nicotine solution (Grabus et al., 2005; Pietila, Laakso, &

Ahtee, 1995; Roni & Rahman, 2014; Sparks & Pauly, 1999). Interestingly, Gäddnäs et al. (2002) found that there was an increase in dopamine levels in nucleus accumbens dialysates of freely-moving mice exposed to chronic oral nicotine administration and this mimics the release of dopamine in the mesolimbic regions of human smokers (A. Sharma & Brody, 2009). In addition, mecamylamine treatment decreased the dopamine levels in nucleus accumbens of mice administered with chronic oral nicotine solution (Gäddnäs et al., 2002) which confirmed that the release of dopamine is regulated by oral nicotine administration through the activation of nAChRs. Besides, Sparks and Pauly (1999) found that there was an increase in neuronal α4, and α7 nAChRs expressions in the brain regions of C57Bl6 mice exposed to the chronic oral nicotine solution (200 µg/mL). Therefore, chronic oral nicotine administration in mice could be a reliable method to induce nicotine dependence.

In this study, the body weight gain of C57BL6 mice exposed to the chronic oral nicotine solution was significantly decreased and this might be due to the appetite suppressant effect of nicotine. Similarly, in previous studies, the chronic oral nicotine exposure significantly decreased the body weight gain of mice (Pekonen et al., 1993; Pietila & Ahtee, 2000). However, in this study, there were no significant changes in the fluid consumption and food intake during the forty days of chronic nicotine exposure and these results were consistent with a previous study (Robinson et al., 1996). Besides, the daily nicotine consumption from the oral nicotine solution was determined as 23.61 mg/kg/day. Moreover, the serum cotinine levels of the oral nicotine solution exposed C57BL6 mice were found to be 159. 53 ng/mL and a similar result were observed in the previous study (Roni & Rahman, 2014). Thus, this result confirms that oral nicotine intake of mice did occur during the period of chronic nicotine solution exposure and comparable to the plasma cotinine levels (100-200 ng/mL) of human smokers (10-15 cigarettes/day) (Lawson et al., 1998).

Furthermore, the results of the present study showed an increase in the immobility time of nicotine-withdrawn C57BL6 mice in forced swim test, which is a measure of depression-like behaviour upon nicotine-withdrawal. These results are in good agreement with earlier published reports (Kotagale et al., 2015; Mannucci et al., 2011; Mannucci et al., 2006; Roni & Rahman, 2014). Moreover, repeated α-asarone (5, 10 and 20 mg/kg, i.p.) or bupropion (10 mg/kg, i.p.) treatment significantly attenuated the depression-like behaviour in nicotine-withdrawn C57BL6 mice in the forced swim test. However, in this study, repeated α-asarone (5, 10 and 20 mg/kg, i.p.) or bupropion (10 mg/kg, i.p.) treatment in naïve C57BL6 mice did not significantly change the immobility time in forced swim test and did not significantly affect the locomotor activity, which infers repeated α-asarone or bupropion treatment did not possess CNS stimulant effect at the tested doses. In previous studies, acute α-asarone ≥ 50 mg/kg, i.p. treatment in mice showed the positive neuromodulatory effect of α-asarone on $GABA_A$ receptors (Chen et al., 2013; Dandiya & Sharma, 1961; Huang et al., 2013; Pages et al., 2010). In this study, α-asarone showed its interaction with brain noradrenergic, dopaminergic and serotonergic systems. Based on this results, it is postulated that repeated α-asarone treatment might interact with both brain monoaminergic and GABA-ergic systems, thus, did not produce antidepressant activity in naïve C57BL6 mice in forced swim test.

The activation of CREB (pCREB) and BDNF have been implicated to be biomarkers of neuroplasticity during nicotine withdrawal in mice (Brunzell et al., 2003; Kivinummi et al., 2011). The pCREB and BDNF levels are increased during nicotine cessation in the mesolimbic region (ventral tegmental area and nucleus accumbens) and hippocampus (Brunzell et al., 2003; Fisher et al., 2016; Kivinummi et al., 2011; Roni & Rahman, 2014). In this study, the hippocampal pCREB level was significantly increased during nicotine withdrawal and this agrees with previous studies (Roni & Rahman, 2014; Turner et al., 2014), which implies neuroplasticity during nicotine withdrawal.

Interestingly, α-asarone (5, 10 and 20 mg/kg, i.p.) and bupropion (10 mg/kg, i.p.) treatment significantly attenuated the elevated hippocampal pCREB levels in nicotine-withdrawn C57BL6 mice, which infers the modulatory effect of α-asarone and bupropion against neuroplasticity. Besides, Lam et al. (2016) reported that α-asarone treatment increased the pCREB expressions through protein kinase-A signalling pathway in the cultured PC12 cells. Based on this evidence, it is postulated that α-asarone might interact with nicotine-mediated activation of protein kinase-A cascades, thereby, attenuates the elevated pCREB levels during nicotine-withdrawal in mice. The present study, however, did not show any significant changes in the hippocampal BDNF levels of nicotine-withdrawn mice. This result suggests that the increased pCREB levels in the hippocampus of nicotine-withdrawn mice might be due to the activation of intracellular protein kinases signalling cascades by nicotine but not by BDNF. These results indicate that the alleviation of depression-like behaviour upon nicotine-withdrawal with α-asarone treatment is mediated through the modulation of hippocampal neuroplasticity in nicotine-withdrawn mice.

CHAPTER 6: CONCLUSION AND FUTURE PERSPECTIVES

6.1 Summary and conclusion

In summary, the result of this study indicates that the α-asarone treatment showed an antidepressant-like activity only at lower doses (15 and 20 mg/kg, i.p.) in ICR mice. The antidepressant-like effect of α-asarone (20 mg/kg, i.p.) in mice is mediated by its interaction with noradrenergic, dopaminergic and/or serotonergic systems in ICR mice. Besides, unlike bupropion, α-asarone (5, 10, 20 and 30 mg/kg, i.p.) treatment in ICR mice did not interact with nicotinic acetylcholine receptors. Interestingly, α-asarone (5, 10 and 20 mg/kg, i.p.) treatment alleviates the nicotine withdrawal-induced depression-like behaviour through the modulation of pCREB activity in the hippocampus of nicotine-withdrawn C57BL6 mice. In conclusion, α-asarone could be potentially effective in the treatment of depression upon cessation of tobacco products mainly through smoking cigarettes and might help to prevent relapse.

6.2 Limitation of the study

This study highlights the therapeutic potential of α-asarone only on depression-like behaviour in nicotine-withdrawn C57BL6 mice. It is realised that other symptoms such as anxiety-like behaviour, cognitive deficits, hyperalgesia, dysphoria and anhedonia can also be present during nicotine-withdrawal. However, in the present study, the effect of α-asarone on these nicotine-withdrawal symptoms were not investigated.

6.3 Future perspectives

In humans and rodents, nicotine withdrawal also produces affective symptoms including anxiety and cognitive deficits (Bagosi et al., 2016; Damaj et al., 2003; Davis et al., 2005; Heishman et al., 2010; Stoker et al., 2008; Wesnes et al., 2013). Interestingly, α-asarone treatment in mice and rats possess anti-anxiety and anti-dementia effects (Kumar et al., 2012; Lee et al., 2014; Liu et al., 2012; Shin et al., 2014; Sundaramahalingam et al.,

2013). Based on this aspect, α-asarone could be further studied on nicotine withdrawal-induced anxiety and learning and memory deficits in mice. In addition, the effect of α-asarone in combination with the clinically approved nicotine cessation drugs such as varenicline and bupropion need to be further investigated using the animal models of nicotine withdrawal symptoms. Thereby, α-asarone could be developed as a drug for the treatment of nicotine addiction and withdrawal.

REFERENCES

Alajaji, M., Bowers, M. S., Knackstedt, L., & Damaj, M. I. (2013). Effects of the beta-lactam antibiotic ceftriaxone on nicotine withdrawal and nicotine-induced reinstatement of preference in mice. *Psychopharmacology (Berl), 228*(3), 419-426. doi: 10.1007/s00213-013-3047-3

Armstrong, D. W., Wang, X., & Ercal, N. (1998). Enantiomeric composition of nicotine in smokeless tobacco, medicinal products, and commercial reagents. *Chirality, 10*(7), 587-591. doi: 10.1002/(SICI)1520-636X(1998)10:7<587::AID-CHIR6>3.0.CO;2-#

Autry, A. E., & Monteggia, L. M. (2012). Brain-Derived Neurotrophic Factor and Neuropsychiatric Disorders. *Pharmacol Rev, 64*(2), 238-258. doi: 10.1124/pr.111.005108

Bagosi, Z., Palotai, M., Simon, B., Bokor, P., Buzas, A., Balango, B., . . . Szabo, G. (2016). Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice. *Brain Res, 1652*, 21-29. doi: 10.1016/j.brainres.2016.09.044

Benowitz, N. L. (1999). Nicotine addiction. *Prim Care, 26*(3), 611-631.

Benowitz, N. L. (2008). Neurobiology of nicotine addiction: implications for smoking cessation treatment. *Am J Med, 121*(4 Suppl 1), S3-10. doi: 10.1016/j.amjmed.2008.01.015

Benowitz, N. L. (2009). Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol, 49*, 57-71. doi: 10.1146/annurev.pharmtox.48.113006.094742

Benowitz, N. L. (2010). Nicotine Addiction. *The New England journal of medicine, 362*(24), 2295-2303. doi: 10.1056/NEJMra0809890

Benwell, M. E., & Balfour, D. J. (1979). Effects of nicotine administration and its withdrawal on plasma corticosterone and brain 5-hydroxyindoles. *Psychopharmacology (Berl), 63*(1), 7-11.

Berg, K., Bischoff, R., Stegmuller, S., Cartus, A., & Schrenk, D. (2016). Comparative investigation of the mutagenicity of propenylic and allylic asarone isomers in the Ames fluctuation assay. *Mutagenesis*. doi: 10.1093/mutage/gew007

Berrendero, F., Robledo, P., Trigo, J. M., Martin-Garcia, E., & Maldonado, R. (2010). Neurobiological mechanisms involved in nicotine dependence and reward: participation of the endogenous opioid system. *Neurosci Biobehav Rev, 35*(2), 220-231. doi: 10.1016/j.neubiorev.2010.02.006

Berton, O., & Nestler, E. J. (2006). New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci, 7*(2), 137-151. doi: 10.1038/nrn1846

Bhat, S. D., Ashok, B. K., Acharya, R. N., & Ravishankar, B. (2012). Anticonvulsant activity of raw and classically processed Vacha (Acorus calamus Linn.) rhizomes. *Ayu, 33*(1), 119-122. doi: 10.4103/0974-8520.100328

Biala, G., Polak, P., Michalak, A., Kruk-Slomka, M., & Budzynska, B. (2014). Influence of calcium channel antagonists on nonsomatic signs of nicotine and D-amphetamine withdrawal in mice. *Pharmacol Rep, 66*(2), 212-222. doi: 10.1016/j.pharep.2014.02.003

Bruijnzeel, A. W., Bishnoi, M., van Tuijl, I. A., Keijzers, K. F. M., Yavarovich, K. R., Pasek, T. M., . . . Yamada, H. (2010). Effects of prazosin, clonidine, and propranolol on the elevations in brain reward thresholds and somatic signs associated with nicotine withdrawal in rats. *Psychopharmacology (Berl), 212*(4), 485-499. doi: 10.1007/s00213-010-1970-0

Brunzell, D. H., Mineur, Y. S., Neve, R. L., & Picciotto, M. R. (2009). Nucleus accumbens CREB activity is necessary for nicotine conditioned place preference. *Neuropsychopharmacology, 34*(8), 1993-2001. doi: 10.1038/npp.2009.11

Brunzell, D. H., Russell, D. S., & Picciotto, M. R. (2003). In vivo nicotine treatment regulates mesocorticolimbic CREB and ERK signalling in C57Bl/6J mice. *J Neurochem, 84*(6), 1431-1441.

Cai, B., Jack, A. M., Lewis, R. S., Dewey, R. E., & Bush, L. P. (2013). (R)-nicotine biosynthesis, metabolism and translocation in tobacco as determined by nicotine demethylase mutants. *Phytochemistry, 95*, 188-196. doi: 10.1016/j.phytochem.2013.06.012

Cai, Q., Li, Y., Mao, J., & Pei, G. (2016). Neurogenesis-Promoting Natural Product α-Asarone Modulates Morphological Dynamics of Activated Microglia. *Frontiers in Cellular Neuroscience, 10*, 280. doi: 10.3389/fncel.2016.00280

Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C., & Gould, T. D. (2012). The Mouse Forced Swim Test. (59), e3638. doi: doi:10.3791/3638

Cartus, A. T., & Schrenk, D. (2016). Metabolism of the carcinogen alpha-asarone in liver microsomes. *Food Chem Toxicol, 87*, 103-112. doi: 10.1016/j.fct.2015.11.021

Casella, G., Caponnetto, P., & Polosa, R. (2010). Therapeutic advances in the treatment of nicotine addiction: present and future. *Therapeutic Advances in Chronic Disease, 1*(3), 95-106. doi: 10.1177/2040622310374896

Cassani-Galindo, M., Madrigal-Bujaidar, E., Chamorro, G., Diaz, F., Tamariz, J., & Espinosa-Aguirre, J. J. (2005). In vitro genotoxic evaluation of three alpha-asarone analogues. *Toxicol In Vitro, 19*(4), 547-552. doi: 10.1016/j.tiv.2005.01.007

Chamorro, G., Salazar, M., Tamariz, J., Diaz, F., & Labarrios, F. (1999). Dominant lethal study of alpha-asarone in male and female mice after sub-chronic treatment. *Phytother Res, 13*(4), 308-311. doi: 10.1002/(SICI)1099-1573(199906)13:4<308::AID-PTR449>3.0.CO;2-X

Chamorro, G. A., M.Salazar, S.Salazar, & J.Tamariz. (1995). Evaluation of the Dominant lethal potential of Alpha-asarone in male mice. *Toxicology letters, 78*, 24.

Chen, Q. X., Miao, J. K., Li, C., Li, X. W., Wu, X. M., & Zhang, X. P. (2013). Anticonvulsant activity of acute and chronic treatment with a-asarone from Acorus gramineus in seizure models. *Biol Pharm Bull, 36*(1), 23-30.

Cho, J., Kim, Y. H., Kong, J. Y., Yang, C. H., & Park, C. G. (2002). Protection of cultured rat cortical neurons from excitotoxicity by asarone, a major essential oil component in the rhizomes of Acorus gramineus. *Life Sci, 71*(5), 591-599.

Citri, A., & Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology, 33*(1), 18-41. doi: 10.1038/sj.npp.1301559

Coe, J. W., Brooks, P. R., Vetelino, M. G., Wirtz, M. C., Arnold, E. P., Huang, J., . . . O'Neill, B. T. (2005). Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem, 48*(10), 3474-3477. doi: 10.1021/jm050069n

Corrigall, W. A. (1991). Regulation of Intravenous Nicotine Self-Administration Dopamine Mechanisms. In F. Adlkofer & K. Thurau (Eds.), *Effects of Nicotine on Biological Systems* (pp. 423-432). Basel: Birkhäuser Basel.

Covey, L. S., Glassman, A. H., & Stetner, F. (1997). Major depression following smoking cessation. *Am J Psychiatry, 154*(2), 263-265. doi: 10.1176/ajp.154.2.263

Crooks, P. A., Bardo, M. T., & Dwoskin, L. P. (2014). Nicotinic Receptor Antagonists as Treatments for Nicotine Abuse. *Advances in pharmacology (San Diego, Calif.), 69*, 513-551. doi: 10.1016/B978-0-12-420118-7.00013-5

Cryan, J. F., Bruijnzeel, A. W., Skjei, K. L., & Markou, A. (2003). Bupropion enhances brain reward function and reverses the affective and somatic aspects of nicotine withdrawal in the rat. *Psychopharmacology (Berl), 168*(3), 347-358. doi: 10.1007/s00213-003-1445-7

Cryan, J. F., Markou, A., & Lucki, I. (2002). Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci, 23*(5), 238-245.

Cryan, J. F., Mombereau, C., & Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev, 29*(4-5), 571-625. doi: 10.1016/j.neubiorev.2005.03.009

D'Souza, M. S., & Markou, A. (2011). Neuronal mechanisms underlying development of nicotine dependence: implications for novel smoking-cessation treatments. *Addict Sci Clin Pract, 6*(1), 4-16.

Damaj, M. I., Glassco, W., Dukat, M., & Martin, B. R. (1999). Pharmacological characterization of nicotine-induced seizures in mice. *J Pharmacol Exp Ther, 291*(3), 1284-1291.

Damaj, M. I., Grabus, S. D., Navarro, H. A., Vann, R. E., Warner, J. A., King, L. S., . . . Carroll, F. I. (2010). Effects of Hydroxymetabolites of Bupropion on Nicotine Dependence Behavior in Mice. *J Pharmacol Exp Ther, 334*(3), 1087-1095. doi: 10.1124/jpet.110.166850

Damaj, M. I., Kao, W., & Martin, B. R. (2003). Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. *J Pharmacol Exp Ther, 307*(2), 526-534. doi: 10.1124/jpet.103.054908

Damaj, M. I., Wiley, J. L., Martin, B. R., & Papke, R. L. (2005). In vivo characterization of a novel inhibitor of CNS nicotinic receptors. *Eur J Pharmacol, 521*(1-3), 43-48. doi: 10.1016/j.ejphar.2005.06.056

Dandiya, P. C., & Menon, M. K. (1963). Effects of asarone and beta-asarone on conditioned responses, fighting behaviour and convulsions. *Br J Pharmacol Chemother, 20*, 436-442.

Dandiya, P. C., & Menon, M. K. (1965). Interaction of asarone with mescaline, amphetamine and tremorine. *Life Sci, 4*(17), 1635-1641.

Dandiya, P. C., & Sharma, J. D. (1961). Pharmacodynamical effect of Asarone and β-asarone. *Nature, 192*, 1299-1300.

Dani, J. A. (2015). Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine. *International review of neurobiology, 124*, 3-19. doi: 10.1016/bs.irn.2015.07.001

Dani, J. A., Ji, D., & Zhou, F.-M. (2001). Synaptic Plasticity and Nicotine Addiction. *Neuron, 31*(3), 349-352. doi: 10.1016/S0896-6273(01)00379-8

Daniela Maurici, Aardema, M., Corvi, R., Kleber, M., Krul, C., Laurent, C., . . . Vanparys, P. (2005). Establishment of timetables for the phasing out of animal experiments for cosmetics. *Alternatives to Lab Animals, 33*, 117-130.

Davis, J. A., & Gould, T. J. (2007). Atomoxetine reverses nicotine withdrawal-associated deficits in contextual fear conditioning. *Neuropsychopharmacology, 32*(9), 2011-2019. doi: 10.1038/sj.npp.1301315

Davis, J. A., James, J. R., Siegel, S. J., & Gould, T. J. (2005). Withdrawal from chronic nicotine administration impairs contextual fear conditioning in C57BL/6 mice. *J Neurosci, 25*(38), 8708-8713. doi: 10.1523/jneurosci.2853-05.2005

DeNoble, V. J., & Mele, P. C. (2006). Intravenous nicotine self-administration in rats: effects of mecamylamine, hexamethonium and naloxone. *Psychopharmacology (Berl), 184*(3-4), 266-272. doi: 10.1007/s00213-005-0054-z

Dome, P., Lazary, J., Kalapos, M. P., & Rihmer, Z. (2010). Smoking, nicotine and neuropsychiatric disorders. *Neurosci Biobehav Rev, 34*(3), 295-342. doi: 10.1016/j.neubiorev.2009.07.013

Dwoskin, L. P., Smith, A. M., Wooters, T. E., Zhang, Z., Crooks, P. A., & Bardo, M. T. (2009). Nicotinic Receptor-Based Therapeutics and Candidates for Smoking Cessation. *Biochemical pharmacology, 78*(7), 732-743. doi: 10.1016/j.bcp.2009.06.002

European-commission. (2002). Opinion of the Scientific Committee on Food on the presence of β-asarone in flavourings and other food ingredients with flavouring properties. *Scientific Committee on Food*, 1-15.

Feng, X.-L., Yu, Y., Qin, D.-P., Gao, H., & Yao, X.-S. (2015). Acorus Linnaeus: a review of traditional uses, phytochemistry and neuropharmacology. *RSC Advances, 5*(7), 5173-5182. doi: 10.1039/C4RA12049C

Fiore, M., Jaén, C., Baker, T., Bailey, W., Bennett, G., Benowitz, N., . . . Williams, C. (2008). A clinical practice guideline for treating tobacco use and dependence: 2008 update. A U.S. Public Health Service report. *Am J Prev Med, 35*(2), 158-176. doi: 10.1016/j.amepre.2008.04.009

Fisher, M. L., LeMalefant, R. M., Zhou, L., Huang, G., & Turner, J. R. (2016). Distinct Roles of CREB Within the Ventral and Dorsal Hippocampus in Mediating Nicotine Withdrawal Phenotypes. *Neuropsychopharmacology*. doi: 10.1038/npp.2016.257

Fowler, C. D., & Kenny, P. J. (2011). Intravenous nicotine self-administration and cue-induced reinstatement in mice: effects of nicotine dose, rate of drug infusion and prior instrumental training. *Neuropharmacology, 61*(4), 687-698. doi: 10.1016/j.neuropharm.2011.05.012

Freitas, K., Negus, S. S., Carroll, F. I., & Damaj, M. I. (2013). In vivo pharmacological interactions between a type II positive allosteric modulator of alpha7 nicotinic ACh receptors and nicotinic agonists in a murine tonic pain model. *Br J Pharmacol, 169*(3), 567-579. doi: 10.1111/j.1476-5381.2012.02226.x

Gäddnäs, H., Piepponen, T. P., & Ahtee, L. (2002). Mecamylamine decreases accumbal dopamine output in mice treated chronically with nicotine. *Neurosci Lett, 330*(3), 219-222. doi: 10.1016/S0304-3940(02)00734-6

Gäddnäs, H., Pietilä, K., & Ahtee, L. (2000). Effects of chronic oral nicotine treatment and its withdrawal on locomotor activity and brain monoamines in mice. *Behav Brain Res, 113*(1–2), 65-72. doi: 10.1016/S0166-4328(00)00201-1

Garber, J. C., barbee, R. W., bielitzki, J. T., Clayton, L. A., Donovan, J. C., Kohn, D. F., . . . Würbel, H. (2011) *Guide for the Care and Use of Laboratory Animals* (8th ed.). Washington (DC).

Glassman, A. H., Stetner, F., Walsh, B. T., Raizman, P. S., Fleiss, J. L., Cooper, T. B., & Covey, L. S. (1988). Heavy smokers, smoking cessation, and clonidine. Results of a double-blind, randomized trial. *JAMA, 259*(19), 2863-2866.

Glover, E. D., Laflin, M. T., Schuh, K. J., Schuh, L. M., Nides, M., Christen, A. G., . . . Strnad, J. V. (2007). A randomized, controlled trial to assess the efficacy and safety of a transdermal delivery system of nicotine/mecamylamine in cigarette smokers. *Addiction, 102*(5), 795-802. doi: 10.1111/j.1360-0443.2007.01763.x

Gonzales, D., Rennard, S. I., Nides, M., Oncken, C., Azoulay, S., Billing, C. B., . . . Reeves, K. R. (2006). Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking

cessation: a randomized controlled trial. *JAMA, 296*(1), 47-55. doi: 10.1001/jama.296.1.47

Gould, T. J., Portugal, G. S., Andre, J. M., Tadman, M. P., Marks, M. J., Kenney, J. W., . . . Adoff, M. (2012). The duration of nicotine withdrawal-associated deficits in contextual fear conditioning parallels changes in hippocampal high affinity nicotinic acetylcholine receptor upregulation. *Neuropharmacology, 62*(5-6), 2118-2125. doi: 10.1016/j.neuropharm.2012.01.003

Gourlay, S. G., & Benowitz, N. L. (1995). Is clonidine an effective smoking cessation therapy? *Drugs, 50*(2), 197-207.

Govind, A. P., Vezina, P., & Green, W. N. (2009). Nicotine-induced Upregulation of Nicotinic Receptors: Underlying Mechanisms and Relevance to Nicotine Addiction. *Biochemical pharmacology, 78*(7), 756-765. doi: 10.1016/j.bcp.2009.06.011

Grabus, S. D., Martin, B. R., Batman, A. M., Tyndale, R. F., Sellers, E., & Damaj, M. I. (2005). Nicotine physical dependence and tolerance in the mouse following chronic oral administration. *Psychopharmacology (Berl), 178*(2-3), 183-192. doi: 10.1007/s00213-004-2007-3

Grando, S. A. (2014). Connections of nicotine to cancer. *Nat Rev Cancer, 14*(6), 419-429. doi: 10.1038/nrc3725

Gu, Q., Du, H., Ma, C., Fotis, H., Wu, B., Huang, C., & Schwarz, W. (2010). Effects of alpha-asarone on the glutamate transporter EAAC1 in Xenopus oocytes. *Planta Med, 76*(6), 595-598. doi: 10.1055/s-0029-1240613

Hall, S. M., Humfleet, G. L., Reus, V. I., Munoz, R. F., Hartz, D. T., & Maude-Griffin, R. (2002). Psychological intervention and antidepressant treatment in smoking cessation. *Arch Gen Psychiatry, 59*(10), 930-936.

Han, P., Han, T., Peng, W., & Wang, X. R. (2013). Antidepressant-like effects of essential oil and asarone, a major essential oil component from the rhizome of Acorus tatarinowii. *Pharm Biol, 51*(5), 589-594. doi: 10.3109/13880209.2012.751616

Hasheminejad, G., & Caldwell, J. (1994). Genotoxicity of the alkenylbenzenes alpha- and beta-asarone, myristicin and elimicin as determined by the UDS assay in cultured rat hepatocytes. *Food Chem Toxicol, 32*(3), 223-231.

Heishman, S. J., Kleykamp, B. A., & Singleton, E. G. (2010). Meta-analysis of the acute effects of nicotine and smoking on human performance. *Psychopharmacology (Berl), 210*(4), 453-469. doi: 10.1007/s00213-010-1848-1

Huang, C., Li, W. G., Zhang, X. B., Wang, L., Xu, T. L., Wu, D., & Li, Y. (2013). alpha-asarone from Acorus gramineus alleviates epilepsy by modulating A-type GABA receptors. *Neuropharmacology, 65*, 1-11. doi: 10.1016/j.neuropharm.2012.09.001

Hughes, J. R. (2007). Effects of abstinence from tobacco: valid symptoms and time course. *Nicotine Tob Res, 9*(3), 315-327. doi: 10.1080/14622200701188919

Hughes, J. R., Gust, S. W., Skoog, K., Keenan, R. M., & Fenwick, J. W. (1991). Symptoms of tobacco withdrawal. A replication and extension. *Arch Gen Psychiatry, 48*(1), 52-59.

Hurt, R. D., Sachs, D. P., Glover, E. D., Offord, K. P., Johnston, J. A., Dale, L. C., . . . Sullivan, P. M. (1997). A comparison of sustained-release bupropion and placebo for smoking cessation. *N Engl J Med, 337*(17), 1195-1202. doi: 10.1056/nejm199710233371703

Igari, M., Alexander, J. C., Ji, Y., Qi, X., Papke, R. L., & Bruijnzeel, A. W. (2014). Varenicline and Cytisine Diminish the Dysphoric-Like State Associated with Spontaneous Nicotine Withdrawal in Rats. *Neuropsychopharmacology, 39*(2), 455-465. doi: 10.1038/npp.2013.216

Ignatowska-Jankowska, B. M., Muldoon, P. P., Lichtman, A. H., & Damaj, M. I. (2013). The cannabinoid CB2 receptor is necessary for nicotine-conditioned place preference, but not other behavioural effects of nicotine in mice. *Psychopharmacology (Berl), 229*(4), 591-601. doi: 10.1007/s00213-013-3117-6

Ilaiyaraja, N., Singsit., D., & Khanum, F. (2012). Effect of rhizome extract of Acorus calamus on depressive condition induced by forced swimming in mice. *International Journal of phytomedicine, 4*, 319-325.

Isola, R., Vogelsberg, V., Wemlinger, T. A., Neff, N. H., & Hadjiconstantinou, M. (1999). Nicotine abstinence in the mouse. *Brain Res, 850*(1-2), 189-196.

Jackson, K. J., Marks, M. J., Vann, R. E., Chen, X., Gamage, T. F., Warner, J. A., & Damaj, M. I. (2010). Role of alpha5 nicotinic acetylcholine receptors in pharmacological and behavioural effects of nicotine in mice. *J Pharmacol Exp Ther, 334*(1), 137-146. doi: 10.1124/jpet.110.165738

Jackson, K. J., Martin, B. R., Changeux, J. P., & Damaj, M. I. (2008). Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *J Pharmacol Exp Ther, 325*(1), 302-312. doi: 10.1124/jpet.107.132977

Jackson, K. J., Muldoon, P. P., De Biasi, M., & Damaj, M. I. (2015). New mechanisms and perspectives in nicotine withdrawal. *Neuropharmacology, 96*(Pt B), 223-234. doi: 10.1016/j.neuropharm.2014.11.009

Jackson, K. J., Sanjakdar, S. S., Chen, X., & Damaj, M. I. (2012). Nicotine Reward and Affective Nicotine Withdrawal Signs Are Attenuated in Calcium/Calmodulin-Dependent Protein Kinase IV Knockout Mice. *PLoS ONE, 7*(11), e51154. doi: 10.1371/journal.pone.0051154

Jasinska, A. J., Zorick, T., Brody, A. L., & Stein, E. A. (2014). Dual role of nicotine in addiction and cognition: A review of neuroimaging studies in humans. *Neuropharmacology, 84*, 111-122. doi: 10.1016/j.neuropharm.2013.02.015

JECFA. (1981). (Joint FAO/WHO Expert Committee on Food Additives). Monograph on β-asarone. *In: WHO Food Additive Series No. 16.*, 33-44.

Jha , P., & Peto , R. (2014). Global Effects of Smoking, of Quitting, and of Taxing Tobacco. *New England Journal of Medicine, 370*(1), 60-68. doi: 10.1056/NEJMra1308383

Jimenez, L., Chamorro, G., Salazar, M., & Pages, N. (1988). [Teratogenic evaluation of alpha-asarone in the rat]. *Ann Pharm Fr, 46*(3), 179-183.

Jing-Kun Miao, Qi-Xiong Chen, Chun Li, Xiao-Wen Li, Xiao-Mei Wu, & Zhang, X.-p. (2013). Modulation Effects of α-Asarone on the GABA homeostasis in the Lithium-Pilocarpine Model of Temporal Lobe Epilepsy. *International Journal of Pharmacology, 9*(1).

Johnson, P. M., Hollander, J. A., & Kenny, P. J. (2008). Decreased brain reward function during nicotine withdrawal in C57BL6 mice: evidence from intracranial self-stimulation (ICSS) studies. *Pharmacol Biochem Behav, 90*(3), 409-415. doi: 10.1016/j.pbb.2008.03.024

Jonkman, S., Henry, B., Semenova, S., & Markou, A. (2005). Mild anxiogenic effects of nicotine withdrawal in mice. *Eur J Pharmacol, 516*(1), 40-45. doi: 10.1016/j.ejphar.2005.04.032

Jorenby, D. E., Hays, J. T., Rigotti, N. A., Azoulay, S., Watsky, E. J., Williams, K. E., . . . Reeves, K. R. (2006). Efficacy of varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. *JAMA, 296*(1), 56-63. doi: 10.1001/jama.296.1.56

Kalivas, P. W., & O'Brien, C. (2007). Drug Addiction as a Pathology of Staged Neuroplasticity. *Neuropsychopharmacology, 33*(1), 166-180.

Kenney, J. W., Adoff, M. D., Wilkinson, D. S., & Gould, T. J. (2011). The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice. *Psychopharmacology (Berl), 217*(3), 353-365. doi: 10.1007/s00213-011-2283-7

Kenny, P. J., File, S. E., & Rattray, M. (2000). Acute nicotine decreases, and chronic nicotine increases the expression of brain-derived neurotrophic factor mRNA in rat hippocampus. *Brain Res Mol Brain Res, 85*(1-2), 234-238.

Kenny, P. J., & Markou, A. (2001). Neurobiology of the nicotine withdrawal syndrome. *Pharmacol Biochem Behav, 70*(4), 531-549.

Kim, B. W., Koppula, S., Kumar, H., Park, J. Y., Kim, I. W., More, S. V., . . . Choi, D. K. (2015). alpha-Asarone attenuates microglia-mediated neuroinflammation by inhibiting NF kappa B activation and mitigates MPTP-induced behavioural deficits in a mouse model of Parkinson's disease. *Neuropharmacology, 97*, 46-57. doi: 10.1016/j.neuropharm.2015.04.037

Kivinummi, T., Kaste, K., Rantamaki, T., Castren, E., & Ahtee, L. (2011). Alterations in BDNF and phospho-CREB levels following chronic oral nicotine treatment and its withdrawal in dopaminergic brain areas of mice. *Neurosci Lett, 491*(2), 108-112. doi: 10.1016/j.neulet.2011.01.015

Kota, D., Martin, B. R., Robinson, S. E., & Damaj, M. I. (2007). Nicotine dependence and reward differ between adolescent and adult male mice. *J Pharmacol Exp Ther, 322*(1), 399-407. doi: 10.1124/jpet.107.121616

Kotagale, N. R., Chopde, C. T., Umekar, M. J., & Taksande, B. G. (2015). Chronic agmatine treatment prevents behavioural manifestations of nicotine withdrawal in mice. *Eur J Pharmacol, 754*, 190-198. doi: 10.1016/j.ejphar.2015.02.033

Kumar, H., Kim, B. W., Song, S. Y., Kim, J. S., Kim, I. S., Kwon, Y. S., . . . Choi, D. K. (2012). Cognitive enhancing effects of alpha asarone in amnesic mice by influencing cholinergic and antioxidant defence mechanisms. *Biosci Biotechnol Biochem, 76*(8), 1518-1522. doi: 10.1271/bbb.120247

Kwon, S., Lee, B., Kim, M., Lee, H., Park, H. J., & Hahm, D. H. (2010). Antidepressant-like effect of the methanolic extract from Bupleurum falcatum in the tail suspension test. *Prog Neuropsychopharmacol Biol Psychiatry, 34*(2), 265-270. doi: 10.1016/j.pnpbp.2009.11.015

Lam, K. Y., Chen, J., Lam, C. T., Wu, Q., Yao, P., Dong, T. T., . . . Tsim, K. W. (2016). Asarone from Acori Tatarinowii Rhizoma Potentiates the Nerve Growth Factor-Induced Neuronal Differentiation in Cultured PC12 Cells: A Signaling Mediated by Protein Kinase A. *PLoS ONE, 11*(9), e0163337. doi: 10.1371/journal.pone.0163337

Laviolette, S. R., & van der Kooy, D. (2004). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci, 5*(1), 55-65. doi: 10.1038/nrn1298

Lawson, G. M., Hurt, R. D., Dale, L. C., Offord, K. P., Croghan, I. T., Schroeder, D. R., & Jiang, N. S. (1998). Application of serum nicotine and plasma cotinine concentrations to assessment of nicotine replacement in light, moderate, and heavy smokers undergoing transdermal therapy. *J Clin Pharmacol, 38*(6), 502-509.

Le Foll, B., Chakraborty-Chatterjee, M., Lev-Ran, S., Barnes, C., Pushparaj, A., Gamaleddin, I., . . . Goldberg, S. R. (2012). Varenicline decreases nicotine self administration and nicotine- and cue-induced reinstatement of nicotine-seeking behaviour in rats when a long pretreatment time is used. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 15*(9), 1265-1274. doi: 10.1017/S1461145711001398

Le Foll, B., & Goldberg, S. R. (2005). Control of the reinforcing effects of nicotine by associated environmental stimuli in animals and humans. *Trends Pharmacol Sci, 26*(6), 287-293. doi: 10.1016/j.tips.2005.04.005

Lee, B., Sur, B., Yeom, M., Shim, I., Lee, H., & Hahm, D. H. (2014). Alpha-Asarone, a Major Component of Acorus gramineus, Attenuates Corticosterone-Induced Anxiety-Like Behaviours via Modulating TrkB Signaling Process. *Korean J Physiol Pharmacol, 18*(3), 191-200. doi: 10.4196/kjpp.2014.18.3.191

Lembke, A., Johnson, K., & DeBattista, C. (2007). Depression and smoking cessation: Does the evidence support psychiatric practice? *Neuropsychiatric Disease and Treatment, 3*(4), 487-493.

Leventhal, A. M., Ameringer, K. J., Osborn, E., Zvolensky, M. J., & Langdon, K. J. (2013). Anxiety and Depressive Symptoms and Affective Patterns of Tobacco Withdrawal. *Drug and alcohol dependence, 133*(2), 324-329. doi: 10.1016/j.drugalcdep.2013.06.015

Li, S. X., Perry, K. W., & Wong, D. T. (2002). Influence of fluoxetine on the ability of bupropion to modulate extracellular dopamine and norepinephrine concentrations in three mesocorticolimbic areas of rats. *Neuropharmacology, 42*(2), 181-190.

Limon, I. D., Mendieta, L., Diaz, A., Chamorro, G., Espinosa, B., Zenteno, E., & Guevara, J. (2009). Neuroprotective effect of alpha-asarone on spatial memory and nitric oxide levels in rats injected with amyloid-beta((25-35)). *Neurosci Lett, 453*(2), 98-103. doi: 10.1016/j.neulet.2009.02.011

Liu, S., Chen, S. W., Xu, N., Liu, X. H., Zhang, H., Wang, Y. Z., & Xu, X. D. (2012). Anxiolytic-like effect of alpha-asarone in mice. *Phytother Res, 26*(10), 1476-1481. doi: 10.1002/ptr.4596

Lonze, B. E., & Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron, 35*(4), 605-623.

Lopez, M. L., Hernandez, A., Chamorro, G., & Mendoza-Figueroa, T. (1993). alpha-Asarone toxicity in long-term cultures of adult rat hepatocytes. *Planta Med, 59*(2), 115-120.

Lu, J., Fu, T., Qian, Y., Zhang, Q., Zhu, H., Pan, L., . . . Zhang, M. (2014). Distribution of alpha-asarone in brain following three different routes of administration in rats. *Eur J Pharm Sci, 63*, 63-70. doi: 10.1016/j.ejps.2014.06.006

Machado, D. G., Bettio, L. E., Cunha, M. P., Capra, J. C., Dalmarco, J. B., Pizzolatti, M. G., & Rodrigues, A. L. (2009). Antidepressant-like effect of the extract of Rosmarinus officinalis in mice: involvement of the monoaminergic system. *Prog Neuropsychopharmacol Biol Psychiatry, 33*(4), 642-650. doi: 10.1016/j.pnpbp.2009.03.004

Machado, D. G., Bettio, L. E., Cunha, M. P., Santos, A. R., Pizzolatti, M. G., Brighente, I. M., & Rodrigues, A. L. (2008). Antidepressant-like effect of rutin isolated from the ethanolic extract from Schinus molle L. in mice: evidence for the involvement of the serotonergic and noradrenergic systems. *Eur J Pharmacol, 587*(1-3), 163-168. doi: 10.1016/j.ejphar.2008.03.021

Malin, D. H., Lake, J. R., Newlin-Maultsby, P., Roberts, L. K., Lanier, J. G., Carter, V. A., . . . Wilson, O. B. (1992). Rodent model of nicotine abstinence syndrome. *Pharmacol Biochem Behav, 43*(3), 779-784.

Manikandan, S., & Devi, R. S. (2005). Antioxidant property of alpha-asarone against noise-stress-induced changes in different regions of rat brain. *Pharmacol Res, 52*(6), 467-474. doi: 10.1016/j.phrs.2005.07.007

Mannucci, C., Navarra, M., Pieratti, A., Russo, G. A., Caputi, A. P., & Calapai, G. (2011). Interactions between endocannabinoid and serotonergic systems in mood disorders caused by nicotine withdrawal. *Nicotine Tob Res, 13*(4), 239-247. doi: 10.1093/ntr/ntq242

Mannucci, C., Tedesco, M., Bellomo, M., Caputi, A. P., & Calapai, G. (2006). Long-term effects of nicotine on the forced swimming test in mice: an experimental model for the study of depression caused by smoke. *Neurochem Int, 49*(5), 481-486. doi: 10.1016/j.neuint.2006.03.010

Marczewska, J., Drozd, E., Anuszewska, E., Chilmonczyk, Z., & Lozowicka, B. (2013). Assessment of the genotoxic activity of alpha-asarone and its derivatives in the comet assay. *Acta Pol Pharm, 70*(2), 349-354.

Markou, A. (2008). Neurobiology of nicotine dependence. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*(1507), 3159-3168. doi: 10.1098/rstb.2008.0095

Matta, S. G., Balfour, D. J., Benowitz, N. L., Boyd, R. T., Buccafusco, J. J., Caggiula, A. R., . . . Zirger, J. M. (2007). Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl), 190*(3), 269-319. doi: 10.1007/s00213-006-0441-0

Mayorga, A. J., Dalvi, A., Page, M. E., Zimov-Levinson, S., Hen, R., & Lucki, I. (2001). Antidepressant-like behavioural effects in 5-hydroxytryptamine(1A) and 5-hydroxytryptamine(1B) receptor mutant mice. *J Pharmacol Exp Ther, 298*(3), 1101-1107.

McLaughlin, I., Dani, J. A., & De Biasi, M. (2015). Nicotine Withdrawal. *Current topics in behavioural neurosciences, 24*, 99-123. doi: 10.1007/978-3-319-13482-6_4

McPherson, C. S., & Lawrence, A. J. (2007). The Nuclear Transcription Factor CREB: Involvement in Addiction, Deletion Models and Looking Forward. *Current Neuropharmacology, 5*(3), 202-212. doi: 10.2174/157015907781695937

Menon, M. K., & Dandiya, P. C. (1967). The mechanism of the tranquillizing action of asarone from Acorus calamus Linn. *J Pharm Pharmacol, 19*(3), 170-175.

Mihalak, K. B., Carroll, F. I., & Luetje, C. W. (2006). Varenicline is a partial agonist at alpha4beta2 and a full agonist at alpha7 neuronal nicotinic receptors. *Mol Pharmacol, 70*(3), 801-805. doi: 10.1124/mol.106.025130

Mineur, Y. S., Somenzi, O., & Picciotto, M. R. (2007). Cytisine, a partial agonist of high affinity nicotinic acetylcholine receptors, has antidepressant-like properties in male C57BL/6J mice. *Neuropharmacology, 52*(5), 1256-1262. doi: 10.1016/j.neuropharm.2007.01.006

Mitrouska, I., Bouloukaki, I., & Siafakas, N. M. (2007). Pharmacological approaches to smoking cessation. *Pulmonary Pharmacology & Therapeutics, 20*(3), 220-232. doi: 10.1016/j.pupt.2005.10.012

Mooney, M. E., & Sofuoglu, M. (2006). Bupropion for the treatment of nicotine withdrawal and craving. *Expert Rev Neurother, 6*(7), 965-981. doi: 10.1586/14737175.6.7.965

Morales-Ramirez, P., Madrigal-Bujaidar, E., Mercader-Martinez, J., Cassini, M., Gonzalez, G., Chamorro-Cevallos, G., & Salazar-Jacobo, M. (1992). Sister-chromatid exchange induction produced by in vivo and in vitro exposure to alpha-asarone. *Mutat Res, 279*(4), 269-273.

Moret, C., & Briley, M. (2011). The importance of norepinephrine in depression. *Neuropsychiatric Disease and Treatment, 7*(Suppl 1), 9-13. doi: 10.2147/NDT.S19619

Motley, T. (1994). The ethnobotany of sweet flag, Acorus Calamus (Araceae). *Economic Botany, 48*(4), 397-412. doi: 10.1007/BF02862235

Mukherjee, P. K., Kumar, V., Mal, M., & Houghton, P. J. (2007). In vitro acetylcholinesterase inhibitory activity of the essential oil from Acorus calamus and its main constituents. *Planta Med, 73*(3), 283-285. doi: 10.1055/s-2007-967114

Muthuraman, A., & Singh, N. (2012). Neuroprotective effect of saponin rich extract of Acorus calamus L. in rat model of chronic constriction injury (CCI) of sciatic nerve-induced neuropathic pain. *J Ethnopharmacol, 142*(3), 723-731. doi: 10.1016/j.jep.2012.05.049

Nakayama, H., Numakawa, T., Ikeuchi, T., & Hatanaka, H. (2001). Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. *J Neurochem, 79*(3), 489-498.

Nemeth-Coslett, R., Henningfield, J. E., O'Keeffe, M. K., & Griffiths, R. R. (1986). Effects of mecamylamine on human cigarette smoking and subjective ratings. *Psychopharmacology (Berl), 88*(4), 420-425.

Nestler, E. J. (2004). Molecular mechanisms of drug addiction. *Neuropharmacology, 47, Supplement 1*, 24-32. doi: 10.1016/j.neuropharm.2004.06.031

Nestler, E. J. (2005). Is there a common molecular pathway for addiction? *Nat Neurosci, 8*(11), 1445-1449.

Nomikos, G. G., Damsma, G., Wenkstern, D., & Fibiger, H. C. (1992). Effects of chronic bupropion on interstitial concentrations of dopamine in rat nucleus accumbens and striatum. *Neuropsychopharmacology, 7*(1), 7-14.

O'Connor, R. J. (2012). Non-cigarette tobacco products: What have we learned and where are we headed? *Tobacco control, 21*(2), 181-190. doi: 10.1136/tobaccocontrol-2011-050281

OECD. (1984). *Test No. 478: Genetic Toxicology: Rodent Dominant Lethal Test, OECD Guidelines for the Testing of Chemicals, Section 4*. Paris: OECD Publishing.

Pages, N., Maurois, P., Delplanque, B., Bac, P., Stables, J. P., Tamariz, J., . . . Vamecq, J. (2010). Activities of alpha-asarone in various animal seizure models and in biochemical assays might be essentially accounted for by antioxidant properties. *Neurosci Res, 68*(4), 337-344. doi: 10.1016/j.neures.2010.08.011

Pandit, S., Mukherjee, P. K., Ponnusankar, S., Venkatesh, M., & Srikanth, N. (2011). Metabolism mediated interaction of alpha-asarone and Acorus calamus with CYP3A4 and CYP2D6. *Fitoterapia, 82*(3), 369-374. doi: 10.1016/j.fitote.2010.11.009

Paolini, M., & De Biasi, M. (2011). Mechanistic insights into nicotine withdrawal. *Biochemical pharmacology, 82*(8), 996-1007. doi: 10.1016/j.bcp.2011.07.075

Papke, R. L., & Heinemann, S. F. (1994). Partial agonist properties of cytisine on neuronal nicotinic receptors containing the beta 2 subunit. *Mol Pharmacol, 45*(1), 142-149.

Patel, D. R., Feucht, C., Reid, L., & Patel, N. D. (2010). Pharmacologic agents for smoking cessation: A clinical review. *Clinical pharmacology : advances and applications, 2*, 17-29.

Paterson, N. E., Semenova, S., & Markou, A. (2008). The effects of chronic versus acute desipramine on nicotine withdrawal and nicotine self-administration in the rat. *Psychopharmacology (Berl), 198*(3), 351-362. doi: 10.1007/s00213-008-1144-5

Pawar Vinod, S., Akhade., A., Baokar., S., & H, S. (2012). Antidepressant-like effects of Acorus calamus in forced swimming and tail suspension test in mice. *Asian Pacific Journal of Tropical Biomedicine*, S17-S19.

Pekonen, K., Karlsson, C., Laakso, I., & Ahtee, L. (1993). Plasma nicotine and cotinine concentrations in mice after chronic oral nicotine administration and challenge doses. *European Journal of Pharmaceutical Sciences, 1*(1), 13-18. doi: 10.1016/0928-0987(93)90013-Z

Petit-Demouliere, B., Chenu, F., & Bourin, M. (2005). Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology (Berl), 177*(3), 245-255. doi: 10.1007/s00213-004-2048-7

Piacentini, M. F., Clinckers, R., Meeusen, R., Sarre, S., Ebinger, G., & Michotte, Y. (2003). Effect of bupropion on hippocampal neurotransmitters and on peripheral hormonal concentrations in the rat. *J Appl Physiol (1985), 95*(2), 652-656. doi: 10.1152/japplphysiol.01058.2002

Picciotto, M. R., Caldarone, B. J., King, S. L., & Zachariou, V. (2000). Nicotinic Receptors in the Brain: Links between Molecular Biology and Behavior. *Neuropsychopharmacology, 22*(5), 451-465.

Picciotto, M. R., & Mineur, Y. S. (2014). Molecules and circuits involved in nicotine addiction: The many faces of smoking. *Neuropharmacology, 76 Pt B*, 545-553. doi: 10.1016/j.neuropharm.2013.04.028

Pietila, K., & Ahtee, L. (2000). Chronic nicotine administration in the drinking water affects the striatal dopamine in mice. *Pharmacol Biochem Behav, 66*(1), 95-103.

Pietila, K., Laakso, I., & Ahtee, L. (1995). Chronic oral nicotine administration affects the circadian rhythm of dopamine and 5-hydroxytryptamine metabolism in the striata of mice. *Naunyn Schmiedebergs Arch Pharmacol, 353*(1), 110-115.

Placzek, A. N., Zhang, T. A., & Dani, J. A. (2009). Nicotinic mechanisms influencing synaptic plasticity in the hippocampus. *Acta Pharmacol Sin, 30*(6), 752-760. doi: 10.1038/aps.2009.39

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther, 229*(2), 327-336.

Portugal, G. S., & Gould, T. J. (2007). Bupropion Dose-Dependently Reverses Nicotine Withdrawal Deficits in Contextual Fear Conditioning. *Pharmacol Biochem Behav, 88*(2), 179-187. doi: 10.1016/j.pbb.2007.08.004

Prochazka, A. V., Petty, T. L., Nett, L., Silvers, G. W., Sachs, D. P., Rennard, S. I., . . . Heim, C. (1992). Transdermal clonidine reduced some withdrawal symptoms but did not increase smoking cessation. *Arch Intern Med, 152*(10), 2065-2069.

Prochazka, A. V., Weaver, M. J., Keller, R. T., Fryer, G. E., Licari, P. A., & Lofaso, D. (1998). A randomized trial of nortriptyline for smoking cessation. *Arch Intern Med, 158*(18), 2035-2039.

Qiu, G., Chen, S., Guo, J., Wu, J., & Yi, Y. H. (2016). Alpha-asarone improves striatal cholinergic function and locomotor hyperactivity in Fmr1 knockout mice. *Behav Brain Res, 312*, 212-218. doi: 10.1016/j.bbr.2016.06.024

Rajput, S. B., Tonge, M. B., & Karuppayil, S. M. (2014). An overview on traditional uses and pharmacological profile of Acorus calamus Linn. (Sweet flag) and other Acorus species. *Phytomedicine, 21*(3), 268-276. doi: 10.1016/j.phymed.2013.09.020

Rauhut, A. S., Neugebauer, N., Dwoskin, L. P., & Bardo, M. T. (2003). Effect of bupropion on nicotine self-administration in rats. *Psychopharmacology (Berl), 169*(1), 1-9. doi: 10.1007/s00213-003-1450-x

Raybuck, J. D., Portugal, G. S., Lerman, C., & Gould, T. J. (2008). Varenicline ameliorates nicotine withdrawal-induced learning deficits in C57BL/6 mice. *Behav Neurosci, 122*(5), 1166-1171. doi: 10.1037/a0012601

Redrobe, J. P., Bourin, M., Colombel, M. C., & Baker, G. B. (1998). Psychopharmacological profile of the selective serotonin reuptake inhibitor, paroxetine: implication of noradrenergic and serotonergic mechanisms. *J Psychopharmacol, 12*(4), 348-355.

Rehni, A. K., Singh, T. G., & Arora, S. (2012). SU-6656, a selective Src kinase inhibitor, attenuates mecamylamine-precipitated nicotine withdrawal syndrome in mice. *Nicotine Tob Res, 14*(4), 407-414. doi: 10.1093/ntr/ntr228

Reid, H. H., & Ledgerwood, D. M. (2016). Depressive symptoms affect changes in nicotine withdrawal and smoking urges throughout smoking cessation treatment: Preliminary results. *Addiction research & theory, 24*(1), 48-53. doi: 10.3109/16066359.2015.1060967

Robinson, S. F., Marks, M. J., & Collins, A. C. (1996). Inbred mouse strains vary in oral self-selection of nicotine. *Psychopharmacology (Berl), 124*(4), 332-339.

Rollema, H., Guanowsky, V., Mineur, Y. S., Shrikhande, A., Coe, J. W., Seymour, P. A., & Picciotto, M. R. (2009). Varenicline has antidepressant-like activity in the forced swim test and augments sertraline's effect. *Eur J Pharmacol, 605*(1-3), 114-116. doi: 10.1016/j.ejphar.2009.01.002

Roni, M. A., & Rahman, S. (2014). The effects of lobeline on nicotine withdrawal-induced depression-like behaviour in mice. *Psychopharmacology (Berl), 231*(15), 2989-2998. doi: 10.1007/s00213-014-3472-y

Sack, R., Gochberg-Sarver, A., Rozovsky, U., Kedmi, M., Rosner, S., & Orr-Urtreger, A. (2005). Lower core body temperature and attenuated nicotine-induced hypothermic response in mice lacking the beta4 neuronal nicotinic acetylcholine receptor subunit. *Brain Res Bull, 66*(1), 30-36. doi: 10.1016/j.brainresbull.2005.02.032

Salas, R., Cook, K. D., Bassetto, L., & De Biasi, M. (2004). The α3 and β4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomotion in mice. *Neuropharmacology, 47*(3), 401-407. doi: 10.1016/j.neuropharm.2004.05.002

Salas, R., Orr-Urtreger, A., Broide, R. S., Beaudet, A., Paylor, R., & De Biasi, M. (2003). The nicotinic acetylcholine receptor subunit alpha 5 mediates short-term effects of nicotine in vivo. *Mol Pharmacol, 63*(5), 1059-1066.

Salin-Pascual, R. J., Rosas, M., Jimenez-Genchi, A., Rivera-Meza, B. L., & Delgado-Parra, V. (1996). Antidepressant effect of transdermal nicotine patches in nonsmoking patients with major depression. *J Clin Psychiatry, 57*(9), 387-389.

Sandeep, D., & Nair, C. K. (2011). Radioprotection by alpha-asarone: prevention of genotoxicity and hematopoietic injury in mammalian organism. *Mutat Res, 722*(1), 62-68. doi: 10.1016/j.mrgentox.2011.03.005

Saules, K. K., Schuh, L. M., Arfken, C. L., Reed, K., Kilbey, M. M., & Schuster, C. R. (2004). Double-blind placebo-controlled trial of fluoxetine in smoking cessation treatment including nicotine patch and cognitive-behavioural group therapy. *Am J Addict, 13*(5), 438-446. doi: 10.1080/10550490490512762

Schildkraut, J. J. (1965). The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am J Psychiatry, 122*(5), 509-522.

Sharma, A., & Brody, A. L. (2009). In vivo brain imaging of human exposure to nicotine and tobacco. *Handb Exp Pharmacol*(192), 145-171. doi: 10.1007/978-3-540-69248-5_6

Sharma, N., Classen, J., & Cohen, L. G. (2013). Neural plasticity and its contribution to functional recovery. *Handbook of clinical neurology, 110*, 3-12. doi: 10.1016/B978-0-444-52901-5.00001-0

Shaywitz, A. J., & Greenberg, M. E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem, 68*, 821-861. doi: 10.1146/annurev.biochem.68.1.821

Shiffman, S., Johnston, J. A., Khayrallah, M., Elash, C. A., Gwaltney, C. J., Paty, J. A., . . . DeVeaugh-Geiss, J. (2000). The effect of bupropion on nicotine craving and withdrawal. *Psychopharmacology (Berl), 148*(1), 33-40.

Shin, J. W., Cheong, Y. J., Koo, Y. M., Kim, S., Noh, C. K., Son, Y. H., . . . Sohn, N. W. (2014). alpha-Asarone Ameliorates Memory Deficit in Lipopolysaccharide-Treated Mice via Suppression of Pro-Inflammatory Cytokines and Microglial Activation. *Biomol Ther (Seoul), 22*(1), 17-26. doi: 10.4062/biomolther.2013.102

Shukla, P. K., Khanna, V. K., Ali, M. M., Maurya, R., Khan, M. Y., & Srimal, R. C. (2006). Neuroprotective effect of Acorus calamus against middle cerebral artery occlusion-induced ischaemia in rat. *Hum Exp Toxicol, 25*(4), 187-194.

Silverstone, P. H., & Dadashova, R. (2012). Atomoxetine treatment for nicotine withdrawal: a pilot double-blind, placebo-controlled, fixed-dose study in adult smokers. *Annals of General Psychiatry, 11*, 6-6. doi: 10.1186/1744-859X-11-6

Singer, S., Rossi, S., Verzosa, S., Hashim, A., Lonow, R., Cooper, T., . . . Lajtha, A. (2004). Nicotine-induced changes in neurotransmitter levels in brain areas associated with cognitive function. *Neurochem Res, 29*(9), 1779-1792.

Slemmer, J. E., Martin, B. R., & Damaj, M. I. (2000). Bupropion is a nicotinic antagonist. *J Pharmacol Exp Ther, 295*(1), 321-327.

Sparks, J. A., & Pauly, J. R. (1999). Effects of continuous oral nicotine administration on brain nicotinic receptors and responsiveness to nicotine in C57Bl/6 mice. *Psychopharmacology (Berl), 141*(2), 145-153. doi: 10.1007/s002130050818

Spijker, S. (2011). Dissection of Rodent Brain Regions. In K. W. Li (Ed.), *Neuroproteomics* (pp. 13-26). Totowa, NJ: Humana Press.

Spiller, K., Xi, Z.-X., Li, X., Ashby, C. R., Callahan, P. M., Tehim, A., & Gardner, E. L. (2009). Varenicline attenuates nicotine-enhanced brain-stimulation reward by activation of α4β2 nicotinic receptors in rats. *Neuropharmacology, 57*(1), 60-66. doi: 10.1016/j.neuropharm.2009.04.006

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl), 85*(3), 367-370.

Stoker, A. K., Semenova, S., & Markou, A. (2008). Affective and somatic aspects of spontaneous and precipitated nicotine withdrawal in C57BL/6J and BALB/cByJ mice. *Neuropharmacology, 54*(8), 1223-1232. doi: 10.1016/j.neuropharm.2008.03.013

Sundaramahalingam, M., Ramasundaram, S., Rathinasamy, S. D., Natarajan, R. P., & Somasundaram, T. (2013). Role of Acorus calamus and alpha-asarone on hippocampal dependent memory in noise stress exposed rats. *Pak J Biol Sci, 16*(16), 770-778.

Tao, G., Irie, Y., Li, D. J., & Keung, W. M. (2005). Eugenol and its structural analogues inhibit monoamine oxidase A and exhibit antidepressant-like activity. *Bioorg Med Chem, 13*(15), 4777-4788. doi: 10.1016/j.bmc.2005.04.081

Tizabi, Y., Overstreet, D. H., Rezvani, A. H., Louis, V. A., Clark, E., Jr., Janowsky, D. S., & Kling, M. A. (1999). Antidepressant effects of nicotine in an animal model of depression. *Psychopharmacology (Berl), 142*(2), 193-199.

Turner, J. R., Ray, R., Lee, B., Everett, L., Xiang, J., Jepson, C., . . . Blendy, J. A. (2014). Evidence from mouse and man for a role of neuregulin 3 in nicotine dependence. *Mol Psychiatry, 19*(7), 801-810. doi: 10.1038/mp.2013.104

Turner, J. R., Wilkinson, D. S., Poole, R. L., Gould, T. J., Carlson, G. C., & Blendy, J. A. (2013). Divergent functional effects of sazetidine-a and varenicline during nicotine withdrawal. *Neuropsychopharmacology, 38*(10), 2035-2047. doi: 10.1038/npp.2013.105

Unger, P., & Melzig, M. F. (2012). Comparative study of the cytotoxicity and genotoxicity of alpha- and Beta-asarone. *Sci Pharm, 80*(3), 663-668. doi: 10.3797/scipharm.1204-21

Vazquez-Palacios, G., Bonilla-Jaime, H., & Velazquez-Moctezuma, J. (2005). Antidepressant effects of nicotine and fluoxetine in an animal model of depression induced by neonatal treatment with clomipramine. *Prog Neuropsychopharmacol Biol Psychiatry, 29*(1), 39-46. doi: 10.1016/j.pnpbp.2004.08.008

Verma, R. S., Padalia, R. C., & Chauhan, A. (2015). Chemical Composition of Root Essential Oil of Acorus calamus L. *National Academy Science Letters, 38*(2), 121-125. doi: 10.1007/s40009-014-0304-x

Walters, C. L., Cleck, J. N., Kuo, Y.-c., & Blendy, J. A. (2005). μ-Opioid Receptor and CREB Activation Are Required for Nicotine Reward. *Neuron, 46*(6), 933-943. doi: 10.1016/j.neuron.2005.05.005

Wang, Z. J., Levinson, S. R., Sun, L., & Heinbockel, T. (2014). Identification of both GABAA receptors and voltage-activated Na(+) channels as molecular targets of anticonvulsant alpha-asarone. *Front Pharmacol, 5*, 40. doi: 10.3389/fphar.2014.00040

Watkins, S. S., Stinus, L., Koob, G. F., & Markou, A. (2000). Reward and somatic changes during precipitated nicotine withdrawal in rats: centrally and peripherally mediated effects. *J Pharmacol Exp Ther, 292*(3), 1053-1064.

Wesnes, K. A., Edgar, C. J., Kezic, I., Salih, H. M., & de Boer, P. (2013). Effects of nicotine withdrawal on cognition in a clinical trial setting. *Psychopharmacology (Berl), 229*(1), 133-140. doi: 10.1007/s00213-013-3089-6

Whiting, P. J., & Lindstrom, J. M. (1988). Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies. *The Journal of Neuroscience, 8*(9), 3395.

Widerlov, E., & Lewander, T. (1978). Inhibition of the in vivo biosynthesis and changes of catecholamine levels in rat brain after alpha-methyl-p-tyrosine; time- and dose-response relationships. *Naunyn Schmiedebergs Arch Pharmacol, 304*(2), 111-123.

Wilking, J. A., Hesterberg, K. G., Nguyen, V. H., Cyboron, A. P., Hua, A. Y., & Stitzel, J. A. (2012). Comparison of nicotine oral consumption and baseline anxiety measures in adolescent and adult C57BL/6J and C3H/Ibg mice. *Behav Brain Res, 233*(2), 280-287. doi: 10.1016/j.bbr.2012.05.022

Wing, V. C., & Shoaib, M. (2007). Examining the clinical efficacy of bupropion and nortriptyline as smoking cessation agents in a rodent model of nicotine withdrawal. *Psychopharmacology (Berl), 195*(3), 303-313. doi: 10.1007/s00213-007-0902-0

Wing, V. C., & Shoaib, M. (2012). Translating the smoking cessation properties of the antidepressant nortriptyline using reinforcing, discriminative and aversive stimulus effects of nicotine in rats. *Psychopharmacology (Berl), 219*(3), 847-857. doi: 10.1007/s00213-011-2413-2

Xi, Z. X., Spiller, K., & Gardner, E. L. (2009). Mechanism-based medication development for the treatment of nicotine dependence. *Acta Pharmacol Sin, 30*(6), 723-739. doi: 10.1038/aps.2009.46

Zaniewska, M., McCreary, A. C., Wydra, K., & Filip, M. (2010). Effects of serotonin (5-HT)2 receptor ligands on depression-like behaviour during nicotine withdrawal. *Neuropharmacology, 58*(7), 1140-1146. doi: 10.1016/j.neuropharm.2010.02.006

Zatonski, W., Cedzynska, M., Tutka, P., & West, R. (2006). An uncontrolled trial of cytisine (Tabex) for smoking cessation. *Tobacco control, 15*(6), 481-484. doi: 10.1136/tc.2006.016097

Zhang Q, Ma WC. Li H, & SJ, M. (2010). Asarone injection in treating respiratory disease: a meta-analysis. *Chinese Journal of Evidence-Based Medicine, 10* (10), 1174-1181.

Zhao-Shea, R., DeGroot, S. R., Liu, L., Vallaster, M., Pang, X., Su, Q., . . . Tapper, A. R. (2015). Increased CRF signalling in a ventral tegmental area-interpeduncular nucleus-medial habenula circuit induces anxiety during nicotine withdrawal. *Nat Commun, 6*, 6770. doi: 10.1038/ncomms7770

Zhao-Shea, R., Liu, L., Pang, X., Gardner, P. D., & Tapper, A. R. (2013). Activation of GABAergic neurons in the interpeduncular nucleus triggers physical nicotine withdrawal symptoms. *Curr Biol, 23*(23), 2327-2335. doi: 10.1016/j.cub.2013.09.041

LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Publications

1. *Ranjithkumar Chellian,* Vijayapandi Pandy and Zahurin Mohamed (2018). Alpha-asarone attenuates the nicotine-withdrawal induced depression-like behaviour in C57BL6 mice: Evidence for the modulation of hippocampal pCREB. European Journal of Pharmacology, 818, 10-16. doi.org/10.1016/j.ejphar.2017.10.025
2. *Ranjithkumar Chellian*, Vijayapandi Pandy and Zahurin Mohamed (2017). Pharmacology and Toxicology of α- and β- Asarone: A Review of Preclinical Evidence. Phytomedicine, 32, 41-58. doi.org/10.1016/j.phymed.2017.04.003
3. *Ranjithkumar Chellian,* Vijayapandi Pandy and Zahurin Mohamed (2016). Biphasic effects of α-Asarone on Immobility in the Tail Suspension Test: Evidence for the involvement of the noradrenergic and monoaminergic systems in its Antidepressant-Like activity. Frontiers in Pharmacology; 7:72. doi.org/10.3389/fphar.2016.00072

B. Conference presentation

1. Oral presentation: *Ranjithkumar Chellian*, Vijayapandi Pandy. Nicotine withdrawal-induced anxiety and depression-like behaviour in C57BL6 mice. An animal model for nicotine physical dependence (PA6-4). Society for Research on Nicotine & Tobacco Annual meeting March 8-11, 2017, Florence, Italy.
2. Poster presentation: *Ranjithkumar Chellian*, Vijayapandi Pandy and Zahurin Mohamed. Antidepressant-Like Effect of α-Asarone in mice: Involvement of α_1 and α_2 adrenoreceptors. Research Week, Faculty of Medicine, University of Malaya, Malaysia, 2015.