ฤทธิ์ของสารสกัดเอทานอลของกระชายดำและน้ำมันระเหยของลูกจันทน์เทศ
ต่อระดับสารสื่อประสาทกลุ่มโมโนเอมีนและโปรไฟล์โปรตีโอมิกส์ในสมองส่วนฮิปโปแคมปัส
ของหนูขาวใหญ่สายพันธุ์ Sprague Dawley

นางสาววาลุกา พลายงาม

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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF *KAEMPFERIA PARVIFLORA* ETHANOLIC EXTRACT AND *MYRISTICA FRAGRANS* SEED VOLATILE OIL ON THE LEVELS OF MONOAMINE NEUROTRANSMITTERS AND HIPPOCAMPAL PROTEOMIC PROFILES IN SPRAGUE DAWLEY RATS

Miss Waluga Plaingam

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
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CHAPTER 1

INTRODUCTION

1.1 Background and rationale

World Health Organization (WHO) predicted that by 2040 neurodegenerative diseases (NDs) will become the second leading cause of death in the world (Halliday and Mallucci, 2014, Mak and Caldeira, 2014, Freitas-Andrade and Naus, 2015, Garcia-Huerta et al., 2016) . NDs such as Alzheimer's disease (AD) and Parkinson's disease (HD) belong to the heterogeneous group of disorders that are characterized by progressive degeneration of the structure and function of the central nervous system or peripheral nervous system. Common NDs include psychiatric disorders such as depression and bipolar disorder. In addition, depression is the most prevalent symptom in PD and HD, occurring in approximately 40–60% of patients (Duff et al., 2007). The hippocampus is considered to be one of the most important brain regions for mood regulation. The monoamine neurotransmitters, including dopamine (DA), norepinephrine (NE), epinephrine (E), and serotonin (5-hydroxytryptamine, 5-HT) are produced from neurons both in the brain and peripheral nervous system(Duncan et al., 2012, Garcia-Miralles et al., 2016). The functions of monoamine neurotransmitters are considered to play a crucial role in arousal, emotion, and cognition. Drugs which augment the effects of monoamines on their target tissues are used to treat psychiatric disorders, including

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depression, anxiety, and schizophrenia (Swoboda and Hyland, 2002, Johnson et al., 2011). For these reasons, the measurement of monoamine neurotransmitters in the rat hippocampus is important for understanding of the effect of herbals on the secretion of neurotransmitters. Recently, proteomics is an important tool for identifying expression proteins that are important for comprehensive understanding ofthe pharmacological role of drug (Liu et al., 2012, Wang et al., 2012, Zhou et al., 2015). Many Medicinal plants have been identified and used for neuroprotective and neurotrophic agents that promote neuronal survival, differentiation, neuritogenesis and synaptic plasticity, both *in vitro* and *in vivo* models (Yende et al., 2014, Bhuiyan et al., 2015). *Kaempferia parviflora* Wall Ex. Baker or Black ginger in local name “Kra-chai-dam” is a plant from the family Zingiberaceae used for health promotion in Thai traditional medicine. The phytochemical studies revealed that the rhizomes of *K. parviflora* contained volatile oil (Wongsinkongman et al., 2012) , chalcones (Hawiset et al., 2011), phenolic glycosides (Azuma et al., 2008) and many flavonoids such as 5-hydroxy-7-methoxyflavonone, 5, 7-dimethoxyflavone and 3, 5, 7-trimethoxyflavone (Yenjai et al., 2004, Sutthanut et al., 2007). The rhizomes of this plant have been traditionally used for leucorrhea, oral diseases, abdominal pain, health promotion and as an aphrodisiac(Wutythamawech, 1997). In addition, the ethanolic extract of *K. parviflora* has been shown to induce relaxation of both aortic rings and ileum precontracted with phenylephrine and acetylcholine(Wattanapitayakul et al., 2008). It has been reported that alcoholic extract

of *K. parviflora* rhizomes contained numerous flavonoids (Sutthanut et al., 2007) which were previously reported to possess antioxidant activity, neuroprotective and cognitive enhancing effects (Spencer, 2009). Recent finding showed that the alcoholic extract of *K. parviflora* rhizome could mitigate depression-like behavior in aged rats (Wattanathorn et al., 2007) and its antidepressant activity in aged rats. We hypothesized that alcoholic extract of *K. parviflora* rhizome might also possess other neuropharmacological activities. *Myristica fragrans* Houtt. or Nutmeg (a tropical evergreen dioecious tree with a narrow range of distribution) is the source of the high value medicinal spices, nutmeg (endosperm) and mace (the reddish aril) with immense phytochemical diversity (Iyer et al., 2009). It contains volatile oils that include myristicin, elemicin, eugenol, isoeugenol, gerinol, pinese, cineole, borneol, and safrole. It has been reported on its antibacterial, antiviral, antidiabetic, antileukaemic effects and other biological activities (Sa-nguanmoo and Poovorawan, 2007, Iyer et al., 2009, Latha et al., 2012) indicate its enormous therapeutic potential. Medicinally, eugenol, and isoeugenol from nutmeg is known for its anti-inflammatory and antithrombotic (Olajide et al., 1999), as well as anti-rheumatic, carminative and stimulant properties (Prabuseenivasan et al., 2006). Nutmeg had reported that the psychoactive effects can cause hallucinations, feelings of unreality, euphoria, and delusions. Both myristicin and elemicin were metabolized to amphetamine-related compounds that had the effects on serotonergic systems and possibly have an antidepressant effect(Forrester, 2005), but this has not yet been

verified. There was a presence of an antidepressant activity in male rats forced swimming test (Moinuddin et al., 2012). In this study, we aim to investigate the effects of *Kaempferia parviflora* and *Myristica fragrans* on the levels of monoamine neurotransmitters (norepinephrine, serotonin, and dopamine), as well as on the proteomic profiles in rat hippocampus. Discoveries from this study could help us to obtain help us obtain more knowledge about the global effects of these herbs on the brain. Better understanding of molecular mechanisms, through which these herbs exert their neuro-protective effects, could lead to development of new herbal medications for neurodegenerative disease with less unwanted effects.

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1.2 Review of related literature

To support background and rationale of this study the articles are relate and categorized and review as follows:

1.2.1 Neurodegenerative disorders and proteomics of neurodegenerative disorders

Neurodegenerative disorders are serious public health that is an increasing amount of in the world. Neurodegenerative disorders range from rare to common illnesses (Zhang et al., 2008). Examples of neurodegenerative diseases including: Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD) and PD-related disorders, Prion disease, Motor neuron diseases (MND), Huntington's disease (HD), Spinocerebellar ataxia (SCA) and Spinal muscular atrophy (SMA).The data from the total number of disability-adjusted life years DALYs represent the total number of years lost to illness, disability, or premature death within a given population. The data showed neurological disorders and as percentage of total DALYs for two thousand five, fifteen and thirty. Neurological disorders increase approximately a 12% while Alzheimer and other dementias show a 66% increase from two thousand five to two thousand thirty (see in table 1). In addition, Neurological disorders are an important cause of mortality and constitute 12% of total deaths globally (Table 2). From the data present by WHO in scaling up care for mental, neurological and substance used disorder reported Thailand that is Lower middle incomes country have this problem 2400 per 100, 000 of population.

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Table 1 Number of DALYs for neurological disorders and as percentage of global DALYs projected for 2005, 2015 and 2030 (Organization, 2006)

category	2005		2015		2030	
	No. of DALYs	Percentage of total DALYs	No. of DALYs	Percentage of total DALYs	No. of DALYs	Percentage of total DALYs
Epilepsy	7308	0.50	7419	0.50	7442	0.49
Alzheimer and other dementias	11078	0.75	13540	0.91	18394	1.26
Parkinson's disease	1617	0.11	1762	0.12	2015	0.13
Multiple sclerosis	1510	0.10	1586	0.11	1648	0.11
Migraine	7660	0.52	7736	0.52	7596	0.50
Cerebrovascular disease	50785	3.46	53815	3.63	60864	3.99
Poliomyelitis	115	0.01	47	0.00	13	0.00
Meningitis	5337	0.36	3528	0.24	2039	0.13
Japanese encephalitis	561	0.04	304	0.20	150	0.01

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Table 2 Deaths attributable to neurological disorders as percentage of total deaths, 2005, 2015 and 2030 data from WHO, 2006

Category	2005 (%)	2015 (%)	2030 (%)
Epilepsy	0.22	0.21	0.19
Alzheimer and other dementias	0.73	0.81	0.92
Parkinson's disease	0.18	0.20	0.23
Multiple sclerosis	0.03	0.03	0.02
Migraine	0.00	0.00	0.00
Cerebrovascular disease	9.00	10.19	10.63
Poliomyelitis	0.00	0.00	0.00
Tetanus	0.33	0.23	0.13
Meningitis	0.26	0.17	0.10
Japanese encephalitis	0.02	0.01	0.01

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Neurodegenerative diseases are incurable and debilitating conditions that result in progressive degeneration and / or death of nerve cells. These disorders characterized by progressive loss of neuronal structure and function. This causes problems with movement (called ataxias), or mental functioning (called dementias). Neurodegenerative disorders are believed to be attributable to the deposition of abnormal toxic proteins in the brain (Taylor et al., 2002). The key points are abnormal of functional and structural connectivity, that are candidate biomarkers for neurodegenerative diseases (Pievani et

al., 2014). These diseases share certain common characteristics, including: alterations in energy metabolism, mitochondrial dysfunction, increased oxidative stress, failing proteostasis networks, aggregation of misfolded proteins, and neuronal cell death (Butterfield and Kanski 2001, Lin and Beal 2006) (see in figure 1). The evidence shows that proteins are connectivity biomarkers for neurodegenerative diseases. This might activate a cascade of biochemical, metabolic, functional and structural changes that precede clinical symptoms. Describe the extended time frame and the lack of clinical specificity, identification of biomarkers linking proteinopathies to symptoms is critical for early disease detection. Unfortunately, clinical symptoms are also nonspecific, in the sense that the same clinical phenotype can have different underlying molecular pathology (Villemagne et al., 2013). It is now possible to characterize brain cells, such as neurons and glial cells, or even their subcellular components, at the molecular level. This ability enables researchers to more closely examine brain cell specific molecular pathways to elucidate distinct brain functions. Application of proteomics to the human brain, cerebrospinal fluid and plasma has greatly hastened the unbiased and high-throughput searches for novel biomarkers (Shi et al., 2009). Proteomics are providing insight into the biochemical pathogenesis of neurodegeneration as well as fueling major efforts in biomarker discovery (Davidsson and Sjögren, 2005). Furthermore, the analyses often require the isolation of individual cell types or subcellular components to investigate specific questions. More than 15 years, there has been significant

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advancement in neuroscience research, as evidenced by the dramatic increase in the papers published. However, the great progress in understanding the molecular mechanisms underlying nervous system disorders, such findings have not been effectively translated into developing disease-modifying therapies for neurological and psychiatric diseases. Upstream determinants (genes and molecular pathology) and downstream effects (clinical phenotypes) are important role for the main neurodegenerative disorders understanding (Figure 2).

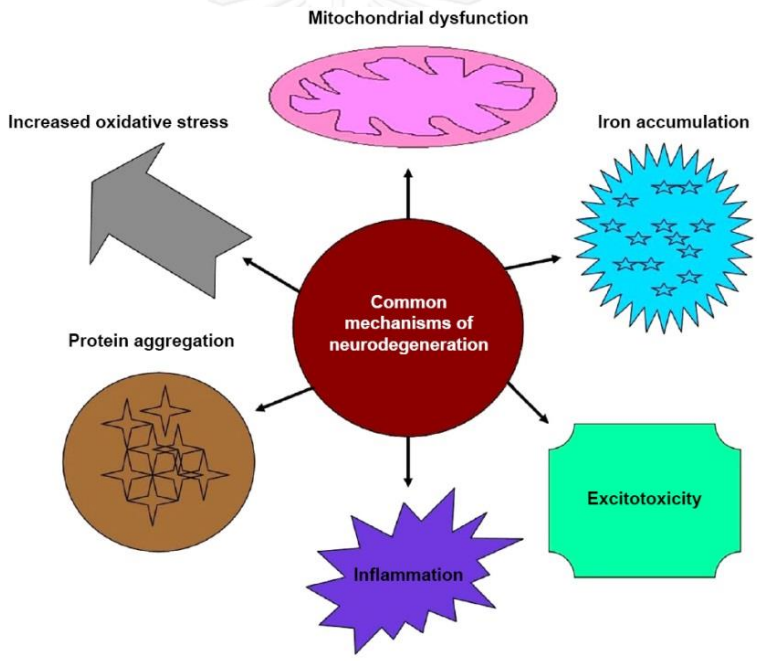

Figure 1 Common possible mechanism of neurodegeneration

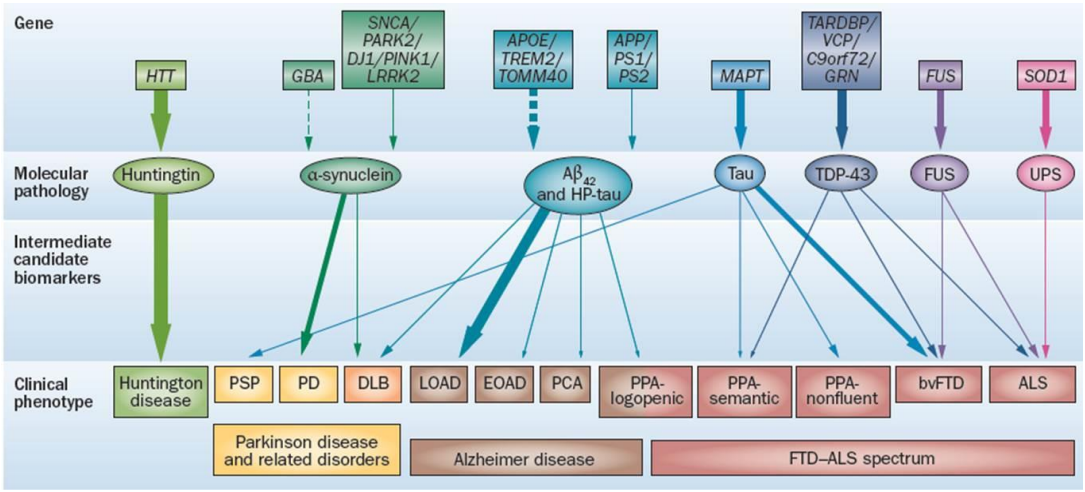

Figure 2 (Park et al., 2014) Upstream determinants and downstream for main neurodegenerative diseases. Functional and structural brain connectivity might act as intermediate biomarkers.

1.2. 1.1 neurodegenerative diseases and misfolding of protein

Many neurodegenerative diseases are associated with the misfolding of specific although structurally unrelated proteins that share a common tendency to misfold and form aggregates of proteins such as polyglutamine disorders (Huntington's disease, spinal and bulbar muscular atrophy, spinocerebellar ataxias and others) show in table 3 and figure 3 (Neef et al., 2011). Synaptic deficits are the pathophysiological hallmark of neurodegenerative diseases. According to Lewis and Sweet in 2009 regarding therapeutic approaches for schizophrenia (Lewis and Sweet, 2009) suggested that although targeting pathogenesis may be a suitable approach for prophylactic treatment, targeting the pathophysiology may be a better approach for therapeutic.

Protein degradation play important role in neurodegenerative diseases. Before, targeted for proteasomal degradation, most proteins are covalently modified with ubiquitin (Ub) (Korolchuk et al., 2010). Typically, three enzyme types are involved in this process — ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes. (Figure 4).

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Table 3 Neurodegenerative diseases that are associated with protein misfolding

Neurodegenerative diseases	Aggregation-prone protein	Neuronal population lost	Disease symptoms	Current therapies
Polyglutamine disorders: Huntington's disease, spinal and bulbar muscular atrophy, spinocerebellar ataxias and others	Polyglutamine tracts within distinct pathogenic proteins	Variable	Chorea, cognitive decline, depression and anxiety, dementia and ataxia	Tetrabenazine, amantadine, remacemide, antipsychotics and antidepressants
Parkinson's disease	α-synuclein	Substantia nigra pars compacta, ventral tegmental area, autonomic ganglia and neurons of the myenteric plexus	Tremors, bradykinesia, postural instability, rigidity, dementia, depression, anxiety, gastrointestinal dysfunction and hallucinations	Levodopa/carbidopa, dopamine agonists and monoamine oxidase inhibitors

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Table 3 Neurodegenerative diseases that are associated with protein misfolding (continue)

Neurodegenerative diseases	Aggregation-prone protein	Neuronal population lost	Disease symptoms	Current therapies
Alzheimer's disease	Amyloid-β, hyperphosphorylated tau	Cortical and subcortical neurons, locus coeruleus and cholinergic neurons	Learning and memory impairments, motor dysfunction, and irritability	Acetylcholinesterase inhibitors, memantine and antipsychotics
Amyotrophic lateral sclerosis/ Lou Gehrig's disease	Superoxide dismutase, TAR DNA binding protein 43 and others	Motor neurons of the cortex, brain stem and spinal cord	Muscular atrophy and motor dysfunction	Riluzole
Prion disorders: Creutzfeldt–Jakob disease, Gerstmann–Straussler syndrome, fatal familial insomnia	Various forms of the prion protein such as scrapie (PrP^{Sc})	Variable	Spongiform encephalopathy, personality changes, depression and insomnia	None

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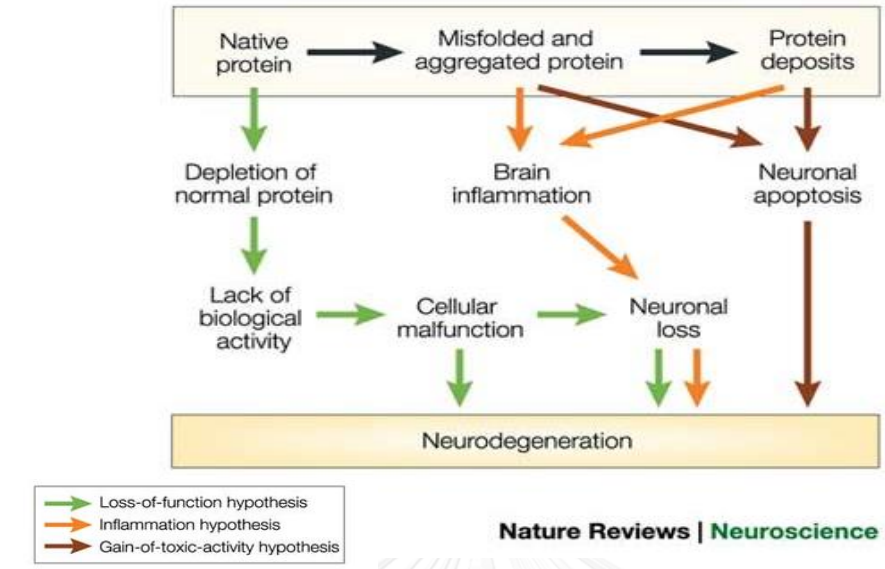

Figure 3 Models for the mechanism of neurodegeneration associated with protein misfolding and aggregation. Three models have been proposed. Although the beginning and the end of the process are the same in the three hypotheses, the events that induce neuronal death are different. In the loss-of-function model, the lack of activity of the native protein is the key step, whereas in the gain-of-toxic-activity hypothesis, the crucial process is the neurotoxicity of the misfolded and/or aggregated protein. In the inflammation model, neuronal death is indirectly mediated by activation of astroglial cells.

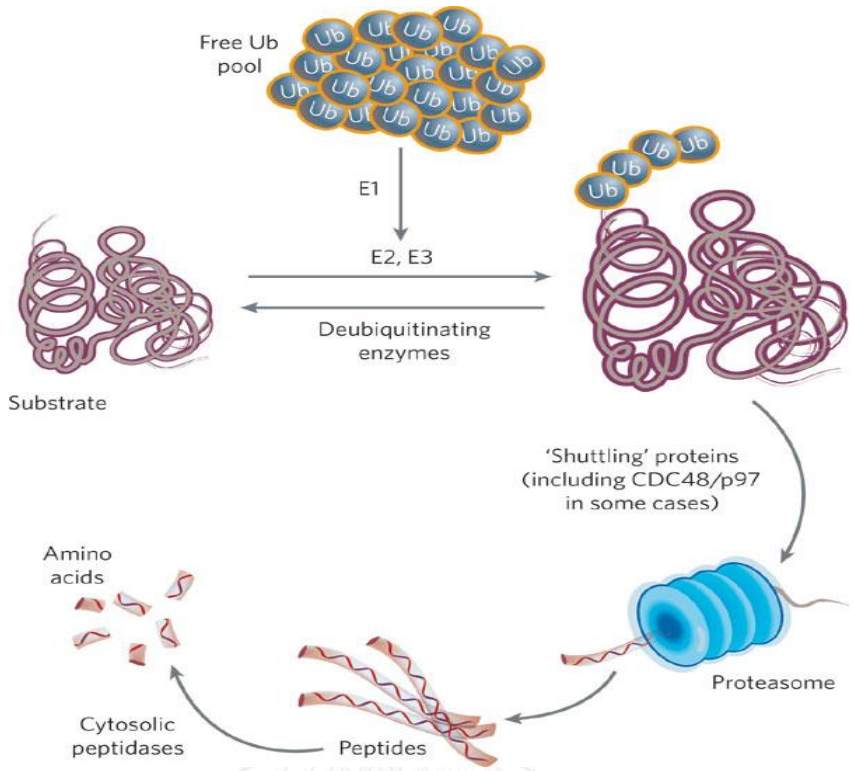

Figure 4 Proteasomal degradation. Proteins tagged with chains of four or more ubiquitins are shuttled to the the proteasome by various proteins such as CDC48/p97. In the proteasome, proteins are reduced to peptides, which are then released into the cytosol and further broken down by peptidases

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1.2.1.2 Mitochondrial impairment and neurodegenerative diseases

The mitochondrial respiratory chain, located on the inner mitochondrial membrane, is composed of five multi-subunit protein complexes, which generate ATP by electron (e-) mediated coupling of H+ and O_2 to form H_2O. The majority of energy generated by oxidative phosphorylation is spent maintaining ion gradients, propagating action potentials, releasing and recycling neurotransmitters; therefore, mitochondrial dysregulation can be detrimental to neuronal survival (Attwell and Laughlin, 2001). Oxidative stress is implicated in multiple neurodegenerative disorders(Uttara et al., 2009). An imbalance in this system can lead to oxidative damage to lipids, proteins and DNA. .An oxidatively-stressed environment leads to damaged biomolecules, such as lipid membranes and proteins, and can induce post translational modifications that may result in altered protein structure and function, protein unfolding and aggregation, among others (Butterfield et al., 2010). ROS and RNS are toxic species and free radicals that include: superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide, and peroxynitrite, among others. ROS and RNS are tightly controlled by antioxidant enzymes, e.g., superoxide dismutase (SOD), glutathione peroxidase, peroxiredoxins, catalase, and many heat shock proteins, and molecules(Gilgun-Sherki et al., 2001).

Mitochondrial dysfunction and oxidative/nitrosative stress is the main of physiological symptoms of degenerative diseases, It relate to protein misfolding/aggregation, synapse loss, and decreased neuronal survival (Finkel, 2011, Paus et al., 2013). The

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accumulation of oxygen and nitrogen species when neurons and immune cells are exposed to toxic proteins, a large amount of energy is needed to defend against that induce stress in the cells. This results in mitochondrial malfunction with the release of cytochrome C and other mitochondrial proteins leads to apoptosis of neuronal cells (Finkel, 2011). This overabundance of protein aggregation affects cellular signaling and neuronal function and is a key cause of neuronal loss (Nakamura and Lipton, 2007). The possible mechanism present in Figure 5.

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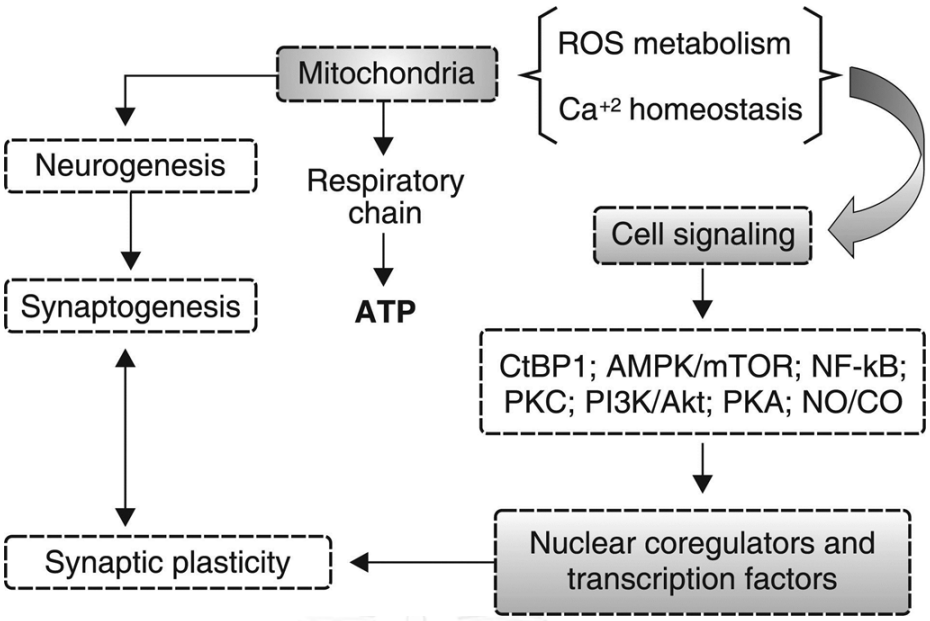

Figure 5 Mitochondrial function and integrity. These include abnormal elevation of Ca2+, glutamate excitotoxicity, glutathione depletion, and altered gene expression of electron transport chain complexes. Additionally, the mitochondria are the main intracellular source of ROS, and ROS production contributes to mitochondrial dysfunction. Furthermore, mitochondrial dysfunction decreases ATP levels, causes apoptosis and oxidative stress, and inhibits ion pumps, and these changes are associated with psychiatric disorders. Akt = protein kinase B; AMPK = monophosphate-activated protein kinase; CtBP1 = C-terminal-binding protein 1; CO = carbon monoxide; mTOR = mammalian target of rapamycin; NF-kB = nuclear factor - kappa B; NO = citric oxide; PKA = protein kinase A; PKC = protein kinase C; PI3K = phosphoinositide 3-kinase; ROS = reactive oxygen species (Streck et al., 2014).

A potential role for mitochondrial dysfunction in neurodegenerative diseases is gaining increasing (Beal, 1998). There is strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis Moreover, an impressive

number of disease-specific proteins interact with mitochondria and have recently been found to interact with many of the specific proteins implicated in genetic forms of neurodegenerative diseases (Table 4) (Lin and Beal, 2006). There is wide literature supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of neurodegenerative diseases especially in AD (Nunomura et al., 2001). Mitochondrial dysfunction produces over oxidative stress that may activate signaling pathways such as APP or tau processing. For example, increasing of expression of β-secretase via activated of c-Jun amino-terminal kinase and p38 mitogen-activated protein kinase (MAPK) (Tamagno et al., 2005). And increases tau phosphorylation by activation of glycogen synthase kinase 3 (Lovell et al., 2004). In Alzheimer's disease mitochondria dysfunction may be linked to the senile plaques and neuro¢brillary tangles. From previous studies showed that impairment of cytochrome oxidase *in vitro* leads to an increase in C-terminal fragments of the amyloid precursor protein, which contain the L-amyloid peptide (Hölscher, 2005). Huntington's disease is characterized clinically by chorea, psychiatric disturbances and dementia, and pathologically by loss of long projection neurons in manner, and is due to expansion of a CAG trinucleotide repeat in the huntingtin (HTT) gene, which gives rise to an expanded polyglutamine stretch in the corresponding protein. Various of evidence demonstrate the involvement of mitochondrial dysfunction in HD. Nuclear magnetic resonance spectroscopy reveals increased lactate in the cortex and basal ganglia (Jenkins et al., 1993). Biochemical

studies show decreased activities of complexes II and III of the electron-transport chain in the human HD brain88. In striatal cells mitochondrial respiration and ATP production are significantly impaired (Milakovic and Johnson, 2005). Moreover, 3-nitropropionic acid and malonate mitochondrial toxins that selectively inhibit succinate dehydrogenase and complex II induce a clinical and pathological phenotype that closely resembles HD(Brouillet et al., 1995). In striatal neurons expressing the first 171 amino acids of HTT with an insertion of 82 glutamines, overexpression of complex-II subunits restored complex-II activity and blocked mitochondrial dysfunction and cell death(Benchoua et al., 2006). Thus, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria.

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Table 4 Proteins that have a function in major neurodegenerative diseases with mitochondrial involvement

Disease	Genetic causes	Function
Alzheimer's disease	APP	Gives rise to Aβ, the primary component of senile plaques
	PS1 and PS2	A component of γ-secretase, which cleaves APP to yield Aβ
Parkinson's disease	α-Synuclein	The primary component of Lewy bodies
	Parkin	A ubiquitin E3 ligase
	DJ-1	Protects the cell against oxidant-induced cell death
	PINK1	A kinase localized to mitochondria. Function unknown. Seems to protect against cell death
	LRRK2	A kinase, Function unknown
	HTRA2	A serine protease in the mitochondrial intermembrane space. Degrades denatured proteins within mitochondria. Degrades inhibitor of apoptosis proteins and promotes apoptosis if released into the cytosol

Table 4 Proteins that have a function in major neurodegenerative diseases with mitochondrial involvement (continue 1)

Disease	Genetic causes	Function
Amyotrophic lateral	SOD1	Converts superoxide to hydrogen peroxide. Disease-causing mutations seem to confer a toxic gain of function sclerosis
Huntington's disease	Huntingtin	Function unknown. Disease-associated mutations produce expanded polyglutamine repeats
Friedreich's ataxia	Frataxin	A mitochondrial iron chaperone that promotes the biogenesis of enzymes with Fe–S clusters and detoxifies excess iron. Frataxin deficiency causes iron accumulation and impairs the activity of Fe–S cluster-containing enzymes (complexes I and II, and aconitase). It may also perturb manganese balance and impair MnSOD activity

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Table 4 Proteins that have a function in major neurodegenerative diseases with mitochondrial involvement (continue 2)

Disease	Genetic causes	Function
Hereditary spastic paraplegia (HSP)	SPG7	An inner mitochondrial membrane m-AAA metalloprotease. It functions as a chaperone and is involved in the assembly of respiratory-chain complexes. SPG7 deficiency causes recessive HSP with mitochondrial myopathy.
Neurodegeneration with brain iron accumulation (NBIA)	PANK2	PANK2 is localized to mitochondria and catalyses the first step in coenzyme A synthesis. PANK2 deficiency is the most common cause of NBIA, accounting for ~50% of cases

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1.2.3 Monoamine neurotransmitters

Selected classical neurotransmitters can be classified by system into 4 groups including cholinergic, aminoacidergic, monoaminergic and purinergic. In this research interest in 3 kind of monoamine group such as dopamine and norepinephrine are catecholamines group which synthesized from tyrosine and serotonin (Indolamines) that is synthesized from tryptophan see in figure 6 and 7. Action of catecholamines and indolamines on target cells is terminated; they are removed from synaptic cleft by reuptake. The major enzymes involved in the catabolism of catecholamines are monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT). Dysfunction of catecholamine or indolamine pathways in brain contributes to affective disorders and schizophrenia. Some antidepressants inhibit affinity of reuptake system for prolongs monoamine neurotransmitters action. Moreover, psychotic illness focuses on dysfunction at dopaminergic synapses too (Castrén, 2005). Neurotransmitters must bind to uptake systems and metabolic enzymes effect to enzymes such as adenylyl cyclase or phospholipase, which generate secondary messengers such as cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), diacylglycerol (DG), inositoltriphosphate (IP3), calcium (Ca^{2+}) and second messengers activate protein kinases A, G, C or CaM-II (Hroudová et al., 2014). Selective serotonin reuptake inhibitors (SSRI) (citalopram, fluvoxamine, fluoxetine, sertraline, paroxetine) are the most frequently used antidepressants.

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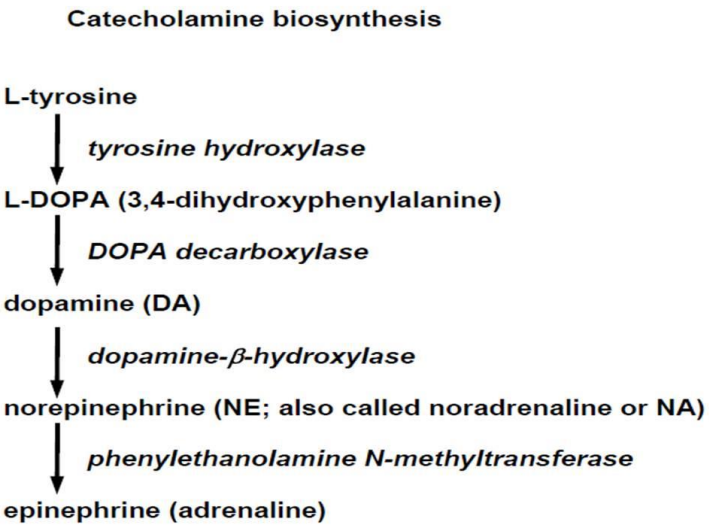

Figure 6 Catecholamine biosynthesis. Dopamine (and norepinephrine) belongs to a subcategory of monoamines called catecholamines. The precursor for dopamine is the amino acid tyrosine that is essential amino acid. In the neuron, the enzyme tyrosine hydroxylase adds a hydroxyl group to tyrosine, which converts it into L-DOPA (short for L-3,4-dihydroxyphenylalanine). Then, another enzyme called aromatic L-amino acid decarboxylase removes a carboxyl group L-DOPA and becomes dopamine. Once dopamine is synthesized and stored in synaptic vesicles, an enzyme called dopamine-β-hydroxylase further hydroxylates dopamine into norepinephrine.

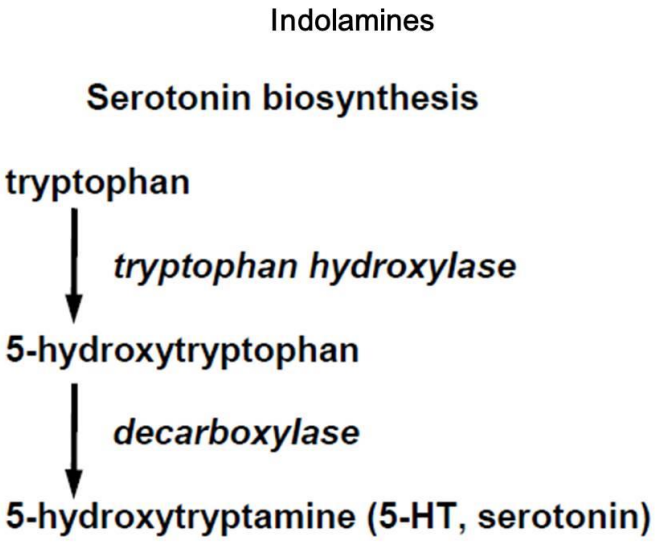

Figure 7 The synthesis of serotonin. Tryptophan is also the precursor to serotonin. Tryptophan is taken up by serotonergic neurons in restricted brain areas such as the raphe nucleus. Once it enters the neurons, an enzyme called tryptophan hydroxylase adds the hydroxyl group and produces 5-HTP (short for 5-hydroxytryptophan). 5-HTP is further decarboxylated by aromatic L-amino acid decarboxylase to produce serotonin.

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1.2.4 The hippocampus

The hippocampus is a major component of the human's brains. Humans and other mammals have two hippocampi, one in each side of the brain (Figure 8). It plays important roles in the limbic system and in the consolidation of information. It contains two main interlocking parts: the hippocampus proper (also called Ammon's horn) and the dentate gyrus (Figure 5 B) (Pearce, 2001).

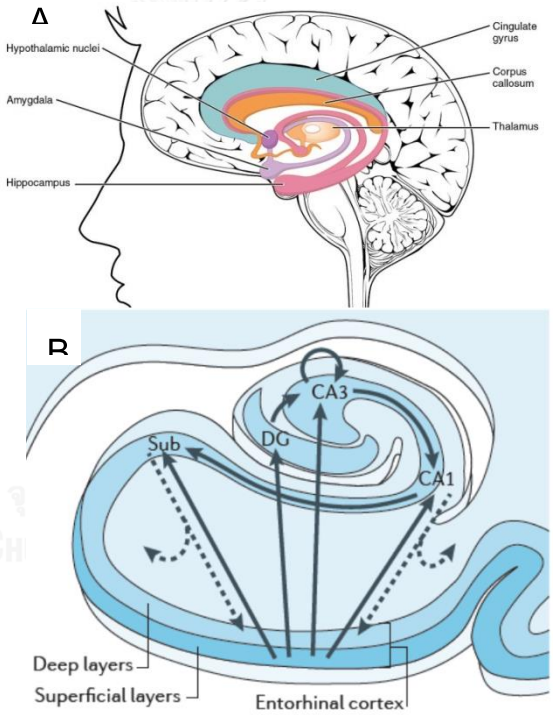

Figure 8 The hippocampus of brain (A) the area of hippocampus in human's brains

The hippocampal formation has been implicated in a growing number of neurodegenerative disorders. In addition, some disorders are associated with hippocampal hypometabolism, whereas others show evidence of hypermetabolism.

Interpreted in the context of the functional and molecular organization of the hippocampal circuit, these observations give rise to a unified pathophysiological framework of hippocampal dysfunction. Regional vulnerability and metabolic state differentiate disorders that affect the hippocampal formation. Although multiple hippocampal sub-regions can be affected in disorders, by comparing patterns of alterations that are observed by functional and structural MRI it is possible to isolate individual sub-regions differentially affected by each disorder. Furthermore, functional imaging techniques that are sensitive to metabolic state have suggested that some hippocampus-based disorders are characterized by hypometabolism (shown in blue), whereas others are abnormally hypermetabolic.

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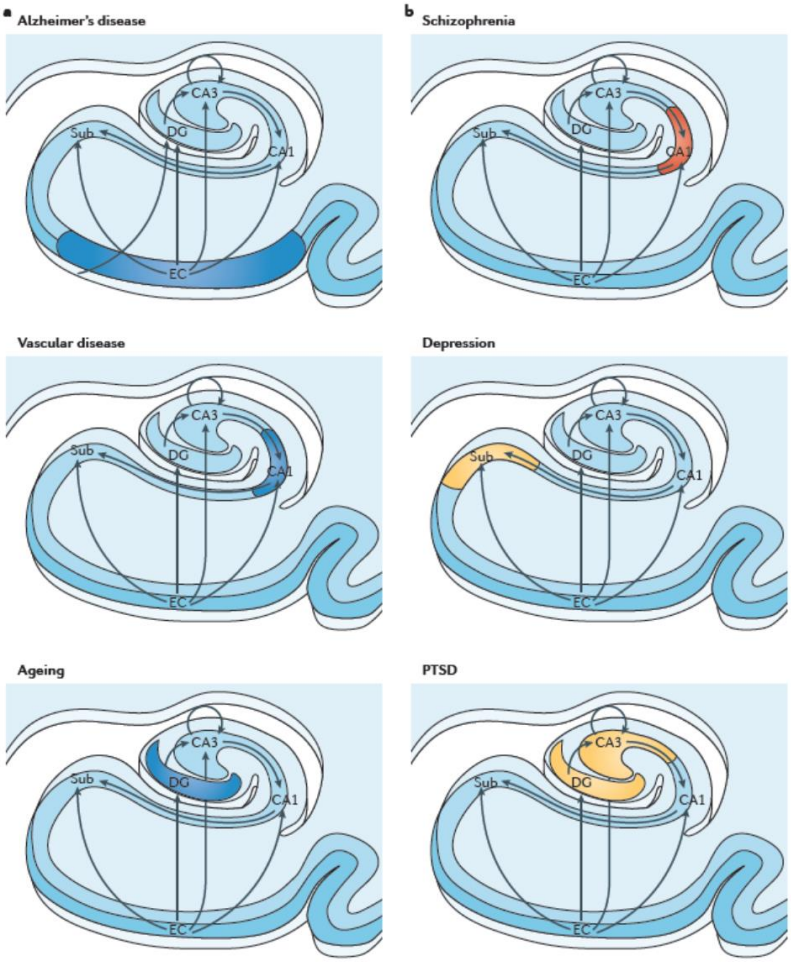

Figure 9 Regional vulnerability and metabolic state differentiate disorders that affect the hippocampal formation.

Nowadays, proteomics finds a wide application in neuroscience. It has mainly been used for protein screening in brain tissue in healthy and diseased states for the detection of drug targets and diagnostic markers. Proteomics is the ideal tool for studying protein–protein interactions and posttranslational modifications. Moreover, it has been applied in the generation of two-dimensional (2D) protein databases which are

essential in the quantification of alterations in the protein levels resulting from the various disorders or the effect of external factors. Recently, the database comprises 148 different gene products, which are in the majority enzymes, structural proteins and heat shock proteins. It also includes 39 neuron specific gene products. The database may be useful in animal model studies of neurological disorders (Fountoulakis et al., 2005).

1.2.5 Plant review

1.2.5.1 *Kaempferia parviflora* Wall Ex. Baker

Scientific Name: *Kaempferia parviflora* Wall Ex. Baker (Figure 10)

Thai local name: "Krachaidam"

Family: Zingiberaceae

Category: Tonic, carminative

Description of plant: Herbaceous/perennial plant, up to 25 cm tall, rhizome underground, light to dark purple within, with several succulent roots in fascicle. Leaves one to several: blades ovate or elliptic and deep green/violet color (5-10 cm wide, 10-15 cm length). Flower: inflorescence appears between the stem and the base of leaf, stalk 5-6 cm, petals fuse at base forming a tube, length 3-3.2 cm, split at tip. Stamen sterile, white, oblong, 3mm wide, 10-13mm long, purple lip (Chuthaputti, 2013).

Description: Odure, characteristic and aromatic; taste, slightly bitter

(Chuthaputti, 2013).

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Distribution: India, Myanmar, Thailand (Loei, Tak, Kanchana Buri and other northern provinces (Chuthaputti, 2013).

Figure 10 *Kaempferia parviflora* Wall Ex. Baker (THP, 2009). The plant habit; 2. A bract; 3. A bracteole; 4. A flower; 5. Ovary and calyx tube; 6. Apex of calyx tube; 7. A dorsal corolla lope; 8. A lateral corolla; 9. A lateral staminode; 10. A labellum; and 11. Anther, stigma and anther crest.

From the phytochemical studies revealed that the rhizomes of *K. parviflora* contained volatile oil (Wongsinkongman et al., 2012), chalcones, phenolic glycosides (Azuma et al., 2008) and many flavonoids such as 5-hydroxy-7-methoxyflavonone, 5, 7-dimethoxyflavone and 3, 5, 7-trimethoxyflavone (Yenjai et al., 2004, Sutthanut et al., 2007). Currently, the research on Thai *K. parviflora* appears to be 'healthy' and a good source of a class of bioflavonoids compounds with methoxy groups. The rhizomes of this plant have been traditionally used for leucorrhea, oral diseases, abdominal pain, health promotion and aphrodisiac(Wutythamawech, 1997). In addition, the ethanolic extract of *K. parviflora* has been shown to induce relaxation of both aortic rings and ileum precontracted with phenylephrine and acetylcholine (Wattanapitayakul et al., 2008). It is recommended for various ailments including allergy, fatigue, and sexual dysfunction, ulcer that it is used as longevity promoting substance and as nerve tonic. It has been reported that alcoholic extract of *K. parviflora* rhizomes contained numerous flavonoids (Sutthanut et al., 2007), which previously reported to possess antioxidant activity, neuroprotective and cognitive enhancing effects (Spencer, 2009). Recent finding showed that the alcoholic extract of *K. parviflora* rhizome could mitigate depression-like behavior in aged rats (Wattanathorn et al., 2007) and its antidepressant activity in aged rats, we hypothesized that alcoholic extract of *K. parviflora* rhizome might also possess other neuropharmacological activities. On the other hand the properties of *K. parviflora* have antifungal, antiplasmodial, antimycobacterial (Yenjai et

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al., 2004), anti-allergic (Tewtrakul and Subhadhirasakul, 2008, Tewtrakul et al., 2008) and anti-gastric ulcers (Rujjanawate et al., 2005). It has been reported that the ethanolic extract and 5-hydroxy-3,7,3',4',-tetramethoxyflavone of this plant exhibited appreciable inhibitory effects on nitric oxide and PGE2 release from murine macrophage cells (Tewtrakul et al., 2008) Moreover, *K. parviflora* has been report that the extract increases energy consumption through activation of BAT in mice(Yoshino et al., 2014). The ethanol extract of *K. parviflora* also increased the expression of catalase, that an antioxidant enzyme in skin. In addition, expression of inflammatory mediators, such as nuclear factor kappa B (NF-**K**B), interleukin-1β (IL-1β), and cyclooxygenase-2 (COX-2), was significantly decreased (Park et al., 2014).

1.2.5.2 ***Myristica fragrans*** **Houtt**

Scientific Name: *Myristica fragrans* Houtt (Figure 11)

Thai local name: "Chan Thet"

Family: Myristicaceae

Category: The tonic principle is Myristicin Oil of Nutmeg is used to conceal the taste of various drugs and as a local stimulant to the gastro-intestinal tract.

Description of plant: *M. fragrans*. or Nutmeg (a tropical evergreen dioecious tree with a narrow range of distribution) is the source of the high value medicinal spices, nutmeg (endosperm) and mace (the reddish aril) with immense phytochemical diversity (Latha

et al., 2012). The plant is about 25 feet high, has a greyish-brown smooth bark, abounding in a yellow juice. The branches spread in whorls - alternate leaves, on petioles about 1 inch long, elliptical, glabrous, obtuse at base - acuminate, aromatic, dark green and glossy above, paler underside and 4 to 6 inches long.

Figure 11 *Myristica fragrans* Houtt

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It contains volatile oils that include myristicin, elemicin, eugenol, isoeugenol, gerinol, pinese, cineole, borneol, and safrole. It have been reported on its anti-bacterial, anti-viral, anti-diabetic, antileukaemic effects and other biological activities (Yang et al., 2006, Sa-nguanmoo and Poovorawan, 2007, Latha et al., 2012) indicate its enormous therapeutic potential. Medicinally, eugenol, and isoeugenol from nutmeg is known for its anti-inflammatory and antithrombotic (Olajide et al., 1999) as well as anti-rheumatic, carminative and stimulant properties (Prabuseenivasan et al., 2006). Nutmeg had

reported the psychoactive effects and can cause hallucinations, feelings of unreality, euphoria, and delusions. Both myristicin and elemicin were metabolized to amphetamine-related compounds that had the effects on serotonergic systems and possibly have an antidepressant effect (Brenner et al., 1993, Forrester, 2005) but this has not yet been verified. And it has been antidepressant activity present in male rats forced swimming test (Moinuddin et al., 2012).

1.2.5.3 Overview of herbal psychopharmacology

Complementary and Alternative Medicine (CAM) or herbal medicine was used widespread among sufferers of mood and anxiety disorders. Eighty two, North American in patients were interviewed about psychiatry found that 44% of total patients had used herbal medicine (mainly for psychiatric purposes) during the previous 12 months (Elkins et al., 2005a, ELKINS et al., 2005b). The data represents sample of 2055 people using herbal medicine and CAM therapies during the previous 12 months to treat those suffering anxiety attacks (57% of patient) , and severe depression reported (54% of patient) (Fleming et al., 2007). In recent years, the modern research on herbal medicine in psychiatry has increased 50% of patient over 5 years up to 2008 (Garcia-Garcia et al., 2008). Over the last several decade It have evidence of beneficial therapeutic activity of the plants that may effect on central nervous system (CNS) has flourished, with an abundance of pre-clinical in vitro and in vivo studies validating many

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phytotherapies as biopsychological effects usually containing alkaloids such as cocaine from Erythroxylon coca (coca), morphine from Papaver somniferum (opium poppy), arecoline from Areca catechu (betel nut) and Hypericum perforatum (St John's wort) (Spinella, 2001, Kumar, 2006). Psychotropic herbal medicines were fairly safe, and presented with fewer side effects in comparison to conventional pharmacotherapies such as antidepressants (cholinergic symptoms, sexual dysfunction, insomnia, and withdrawal issues) and benzodiazepines (somnolence, dependence and withdrawal issues) (Baldwin and Polkinghorn, 2005, Papakostas, 2008, Schweitzer et al., 2009). For example in 2006, WHO showed a significant of depression will result in the second greatest increase in morbidity after cardiovascular disease, estimated that by the year 2020. Depression is a complex and multifactorial trait with important genetic and nongenetic contributory factors. In recent years, the main premise concerning the biopathophysiology of MDD has focused on monoamine impairment (dysfunction in monoamine expression and receptor activity), lowering of monoamine production or secondary messenger (e.g. G proteins or cyclic AMP) system malfunction (Hindmarch, 2001, Ressler and Nemeroff, 2001). In addition, attention has also focused on the role of neuroendocrinological abnormalities involving excess cortisol and its impeding effects on neurogenesis via reducing brain-derived neurotropic factor (Antonijevic, 2006). Several herbal medicines revealed an array of pre-clinical antidepressant activity, some antidepressant herbal medicines such as *H. perforatum*, *Rhodiola rosea* (rose root) and

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Crocus sativus (saffron) offer for the treatment of this disorder via known psychopharmacological actions including inhibition of monoamine re-uptake (such as serotonin, dopamine and noradrenaline), enhanced binding and sensitisation of serotonin receptors, monoamine oxidase inhibition, and neuro-endocrine modulation (Spinella, 2001, Kumar, 2006, Sarris, 2007). Some herbal medicines with increased mood effects such as *R. rosea* and *C. sativus* also display anxiolytic effects. This may be due to modulation of neurological pathways that have both antidepressant and anxiolytic effects (e.g. GABA, serotonin, and noradrenaline systems), or may be due to a "halo effect" whereby epression is effectively treated, anxiety may also be reduced (Nierenberg, 2001, Brady and Verduin, 2005). Most of the antidepressant mechanisms of action are not as clearly defined as with SSRIs, having a multitude of biological effects on reuptake and receptor binding of various monoamines, In addition to endocrine and psycho-neuro-immunological modulation (Butterweck and Schmidt, 2007, Sarris, 2007). The pathophysiology of anxiety disorders is still being solved, although it has the evidence indicates that the neurobiology involves abnormalities of serotonergic, noradrenergic, glutamatergic, and GABA-ergic transmission (Kalueff and Nutt, 2007). The involvement of these pathways is reflected in the efficacy of selective serotonin reuptake inhibitors (SSRIs), selective serotonin and noradrenalin reuptake inhibitors (SNRIs), and benzodiazepines (Baldwin and Polkinghorn, 2005). Phytotherapeutic

interventions that may benefit anxiety disorders such as *P. methysticum* are classed as “anxioly

1.2.5.4 Phytochemicals and neurodegenerative disorders

Many evidence data indicates that the phytochemicals may prevent or reverse neurodegenerative disease by many targeting pathway (Adibhatla and Hatcher, 2007, Woo et al., 2014, Venkatesan et al., 2015). However, most of traditional herbal medicines were commonly prepared from crude materials. Thus, there are many questions about concentration of active compound and the identity of the active ingredients as well as their specific medicinal effects and mechanism of action (Kim et al., 2010). Therefore, most research has focused on the specific components of an active herb, a number of active ingredients still need to be identified and characterized with regard to their potential therapeutic effects, particularly their effects on neurodegenerative diseases. Neuroprotective phytochemical substances such as fatty acids, phenols, alkaloids, flavonoids, saponins, terpenes etc (Kumar and Khanum, 2012). Many numbers of pharmaceutical compounds have been used for neuroprotective therapeutic to improve neurofunction, generally work by altering the balance of neurotransmitters in the brain(Capasso et al., 2000, Russo and Borrelli, 2005). Some acts by selective enhancement of cerebral blood flow, cerebral oxygen usage metabolic rate and cerebral glucose metabolic rate in chronic impaired human

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brain function. Numbers of medicines are derived from the medicinal plants and have shown memory enhancing properties by virtue of their bioactive phytochemical constituents. One of the mechanisms suggested to dementia is decreased cholinergic activity in brain. Therefore, cholinergic drugs (of plant origin) like: Muscarinic agonists (e.g. arecoline, pilocarpine etc.), nicotinic agonists (e.g. nicotine) and cholinesterase inhibitors (e.g. huperzine) can be employed for improving memory.[8] Some other classes of drugs used in dementia are: Stimulants or nootropics (e.g. piracetam, amphetamine), putative cerebral vasodilators (e.g. ergot alkaloids, papavarine), calcium channel blocker (e.g. nimodipine) (Harvey, 1999, Kumar and Khanum, 2012).

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1.3 Objectives

1. To determine protein changes developing in SD rat hippocampus after treat with ethanolic extract of *Kaempferia parviflora* rhizome and *Myristica fragrans* nutmeg volatile oil.

2. To study the effect of ethanolic extract of *Kaempferia parviflora* rhizome and *Myristica fragrans* nutmeg volatile oil on monoamine neurotransmitter (norepinephrine, dopamine and serotonin) level in SD rat hippocampus.

1.4 Hypothesis

Ethanolic extract of *Kaempferia parviflora* rhizomes and nutmeg volatile oil from *Myristica fragrans* cause changes in the levels of norepinephrine, dopamine, and serotonin, as well as proteomic profiles, in the hippocampus and the blood of SD rats.

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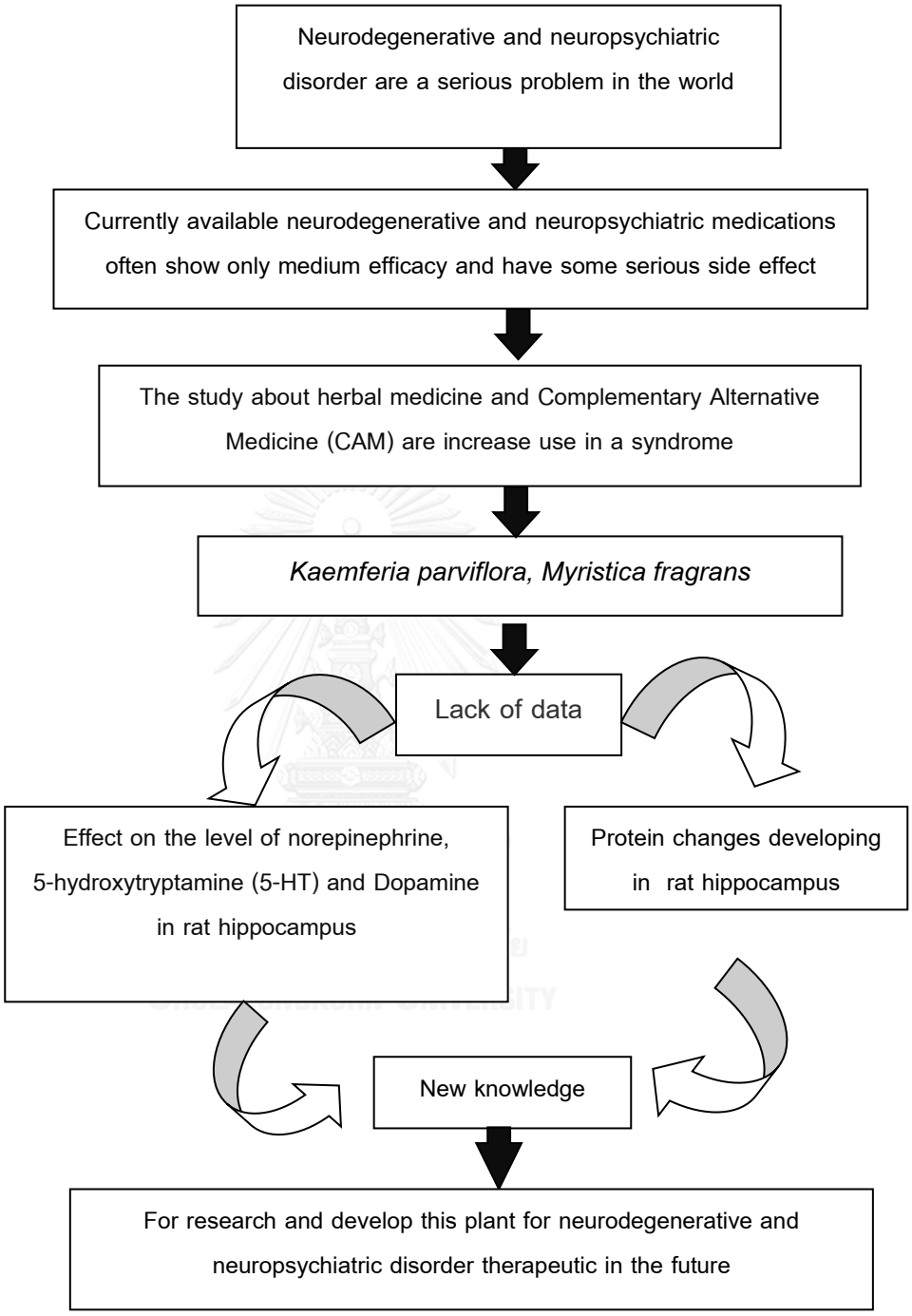
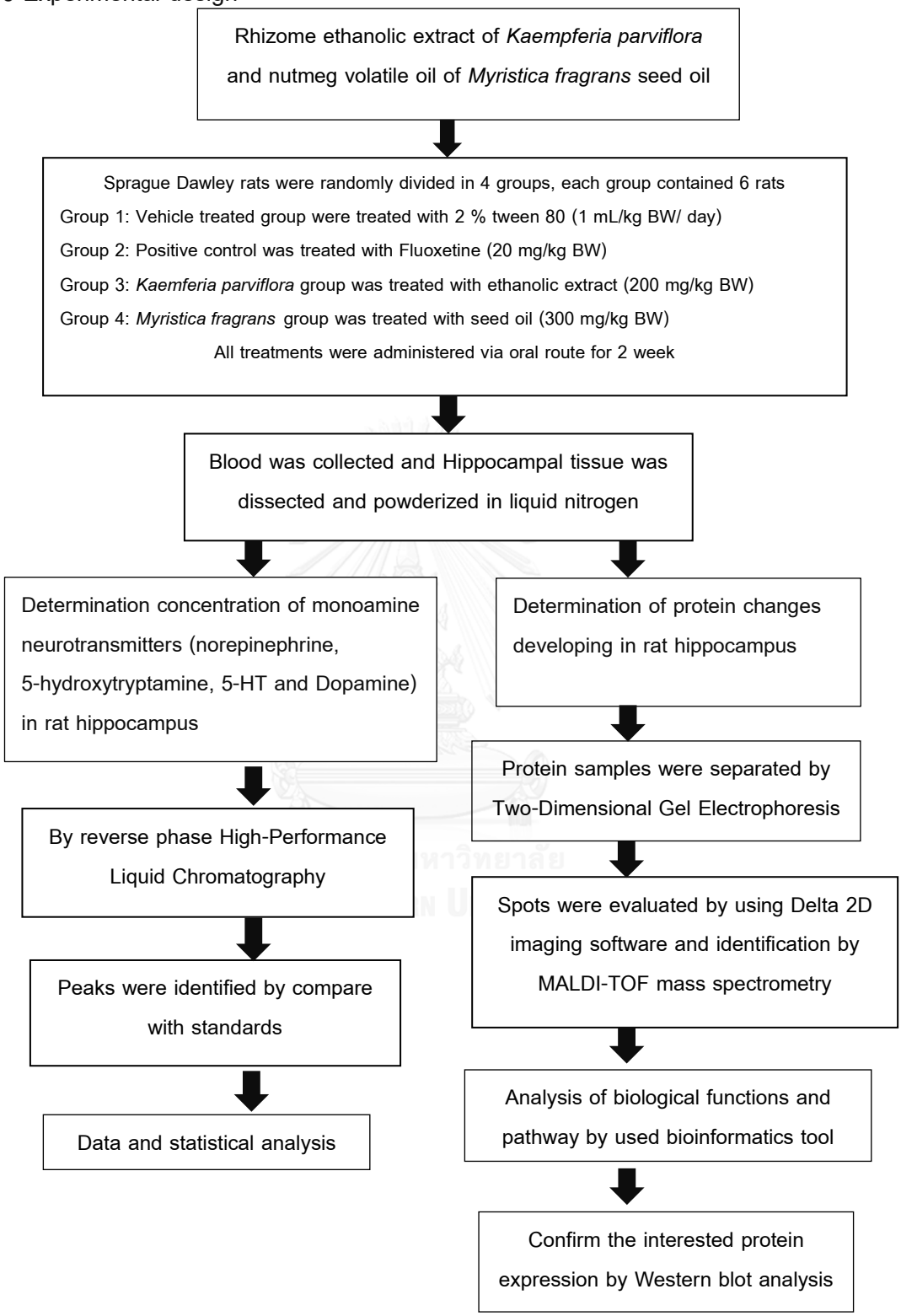
1.5 Conceptual framework



1.6 Experimental design



CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Chemicals

Name of chemicals	Company/Country
-30% Acrylamide and Bis-Acrylamide Solutions	BIO-RAD/ USA
-N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Omnipur/ Germany
-Sodium Luaryl Sulphate (SDS)	Bio basic inc/ Canada
-Ammonium persulfate	Omnipur/ Germany
-Dithiothreitol (DTT)	Omnipur/ Germany
-85% Phosphoric acid	Merck/ Germany
-Tris	Vivantis/ Malaysia
-Glysine	usb/ USA
-Glycerol	Bio basic inc/ Canada
-Iodoacetamide (IAA)	Sigma Aldrich/ USA
-IPG-buffer (pH 3-10)	GE Healthcare/ UK
- Ammonium sulfate ACS	Merck/ USA
- Acetic acid gracial 100% anhydrous	Merck/ USA
-Methanol	Merck/ Germany

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Name of chemicals	Company/Country
-Protein ladder (broad range)	Thermoscientific/ USA
-Trpsin TPCK treated from bovine pancreases	Sigma Aldrich/ USA
-Formic acid	Merck/ Germany
-Ammonium hydrogen carbonates	Merck/ Germany
-Acetonitrile, HPLC grade (for LC-MS/MS)	Merck/ Germany
-HPLC water (for LC-MS/MS)	Merck/ Germany
-Methanol, HPLC grade (for LC-MS/MS)	Merck/ Germany
- Acetonitrile, HPLC grade (for HPLC)	RCI labscan/ USA
-HPLC water (for HPLC)	RCI labscan/ USA
-Methanol, HPLC grade (for HPLC)	RCI labscan/ USA
-Ethanol, Analytical grade,	Merck/ Germany
-Trichloroacetic acid (TCA)	Sigma Aldrich/ USA
-Protease inhibitor cocktail tablet	Sigma Aldrich/ USA
-Perchloric acid	Sigma Aldrich/ USA
-Sodium acetate	Merck/ Germany
-Citric acid	Merck/ Germany
-Methanesulfonic acid	Sigma Aldrich/ USA

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Name of chemicals	Company/Country
-Ethylenediaminetetraacetic acid (EDTA)	Merck/ Germany
-Norepinephrine (Standard, HPLC grade)	Sigma Aldrich/ USA
-Dopamine hydrochloride (Standard, HPLC grade)	Sigma Aldrich/ USA
-5-hydroxytryptamine-5HT (Standard, HPLC grade)	Sigma Aldrich/ USA
-Caffeine (Standard, HPLC grade)	Sigma Aldrich/ USA
-Primary antibodies Anti-PIA3 (ERp57), Anti-GFAP, Anti- DPYSL2 (CRMP2), Anti- Phospho-DPYSL2 (Phospho-CRMP2) Anti-β-actin	cell signaling/ USA

Name of materials and instruments	Company/Country
-Immobiline drystrip (pH 3-10)	GE Healthcare/ UK
-Gel Electrophoresis Apparatus	BIO-RAD/ USA
-Electrophoresis power supply	GE Healthcare/ UK
-Ettan IPGphor 3	GE Healthcare/ UK
-HPLC	AgilentTechnologies/ Austria
-GC-MS (a model 7890A GC and 5978C MSD)	Agilent Technologies/ Austria
-HPLC column (Inertsil® ODS3 C18)	GL Sciences Inc./ USA
-HPLC column (Hypersil GoLD C18)	Thermo Scientific/ USA

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Name of materials and instruments	Company/Country
-LC–MS/MS system (Dionex Ultimate 3000)	Thermo Scientific/ USA
-Electrospray ionization (ESI)/ Quadrupole ion trap mass spectrometer	Bruker/ Germany

2.2 Plants material and extraction

K. parviflora was collected from Loei province in Northeast Thailand and *M. fragrans* from Nakhonpathom province in central Thailand (Figure 12). Plants were identified by an expert Ass. Prof. Dr. Nijsiri Ruangrungsi.

K. parviflora

M. fragrans

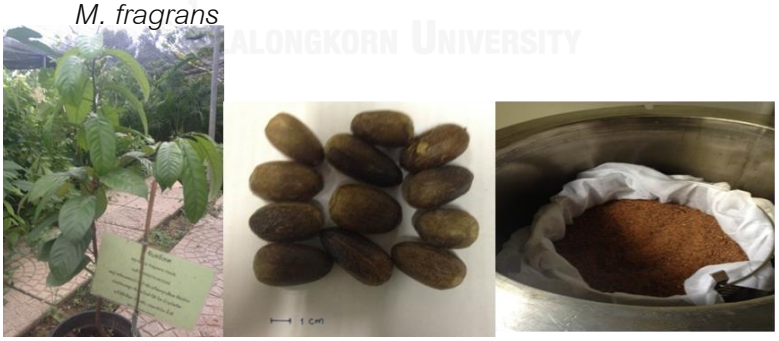

Figure 12 Plants material

K. parviflora, rhizomes were shade dried and agitated by using a blender and the powder was extracted with 95% ethanol by maceration for 4 days (1 kg sample: 3 liters) with occasional stirring. The extract was dissolved in 2% of tween 80 in water to a final concentration of 200 mg/ml (Hawiset et al., 2011). Crude extracts of were standardized by reversed-phase HPLC (Agilent 1260 Infinity, Austria). 5, 7-dimethyoxyflavone was used for the standard. The HPLC system consists of an analytical C18 reversed-phase column (ODS3 C18, 4.6X250 mm, diameter 5 μm) and UV detector (best condition at 280 nm). All analyses were performed with a mobile phase of 2% acetic acid in water and methanol, an injection volume of 10 μl and a flow rate of 1.01 mL/min. The optimized analytical separations were carried out using a mobile phase with a multi-step gradient showed in Table 5.

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Table 5 The optimized analytical separations gradient

Condition gradient		
Time (Min)	MeOH (%)	2% Acetic acid (%)
0-19	30	70
20-40	80	20
41-50	100	0
51-65	30	70

For *M. fragrans*, 1 kg of powder from dry seeds was extracted by stream distillation for 12 hours, the volatile oil were dissolved in 2% of tween 80 in water to final concentration 300 mg/ml. (Moinuddin et al., 2012). The *M. fragrans* nutmeg volatile oil was analyzed by GC-MS on gas chromatograph, a model 7890A GC and 5978C MSD, mass selective detector (EIMS, electron energy, 70 eV), and an Agilent ChemStation data system following.

-GC-MS VOC 2_SPLIT50_Liq_inlet 300

-Column=HP-5MS (30X250X0.25 μm), 1.3 ml/min

-Solvent delay=6 min

-Scan 30-1000 mass, scan/sec=1.55

-Inlet=300 °C

-MS source 230 ° C

-MS quad 150 ° C

-AUX heater 270 ° C

-Inject 1 μl, split 1:200

Drug preparation, Fluoxetine (20 mg tablet) was dissolved in 2% of tween 80 in water (Hawiset et al., 2011, Moinuddin et al., 2012)

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2.3 Experimental protocol for animals

Two months old healthy male Sprague Dawley rats (300-400 g) were obtained and approved from National Laboratory Animal Centre Animal Care and Use Committee (NLAC-ACUC), Mahidol University, Salaya, Nakornpathom, Thailand (Appendix B). The rats were housed in individually (one per cage) in standard stainless steel (WXLXH, 11X18.5X8 inch). Rats were housed under standard conditions (NLAC-MU; SOP-VM.VCP-01.10.) at 22±1°C on standard fluorescent 12:12 hours light:dark cycle and with changing of bedding once a week. Rats were given access to diet and water (hyperchorinate 10-12 ppm) ad libitum. Animal's data including body weight, food and water consumption can see in Appendix A. The number of animals in this study is based on the following equation from many standard text books in statistics. Determination of sample size:

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$$N = 2s^2 (Z_\alpha + Z_\beta)^2 / (\mu_1 - \mu_2)^2$$

N = sample size of each group in experiment

S = standard deviation is 1.00

μ_1 = mean of previous experiment (experiment I)

μ_2 = mean of previous experiment (experiment II)

α = the level of significance is 5% (Z_α = 1.96)

β = the risk (probability) of erroneously concluding that the treatments are not significantly difference. It is 5% ($Z\beta$ = 1.65)

N = 2 x 1 (1.96 + 1.65)2 / (8.5-6)2

= 2 (13.03) / 4.17

= 6.25 mice per group

The resource equation (E): (Acceptable values=10-20)

N (Number of animal per treatment X number of treatment)-T (number of treatment)

(6X4)-4=20

All rats were randomly divided into 4 groups, each group contain 6 rats.

Group 1: Vehicle treated group was treated with 2 % tween 80, 1 mL/kg BW/day.

Group 2: Fluoxetine group was treated with fluoxetine 20 mg/kg BW.

Group 3: *Kaemferia parviflora* group was treated with ethanolic extract 200 mg/kg BW

Group 4: *Myristica fragrans* group was treated with seed oil 300 mg/kg BW.

All treatments were administered via oral route for 12 days

After treatments blood were corrected. The haematological parameters and Chemical values of the blood were analysed by using standard method. Every parameters compare with control group and using reference from Clinical Laboratory Parameters for Crl:WI (Han) in 2008 see table 6 and 7. And data about chemical values

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in healthy male Sprague-Dawley rat in (The National Laboratory Animal Center, Mahidol University; 2002, 2016) see table 8.

Table 6 Hematology range data of male rat (8-16 weeks old)

Test	Unit	Range
MPV	fL (μm^3)	6.2-9.4
Platelets	$10^3/\mu L$	638-1177
RDW	%	11.1-15.2
MCHC	g/dL	32.9-37.5
MCH	pg	17.1-20.4
Hematocrit	%	39.6-52.5
Hemoglobin	g/dL	13.7-17.6
Red Blood Cells	$10^6/\mu L$	7.27-9.65
White Blood Cells	$10^3/\mu L$	1.96-8.25
MCV	fL (μm^3)	48.9-57.9
Neutrophils	$10^3/\mu L$	0.22-1.57
Lymphocytes	$10^3/\mu L$	1.41-7.11
Monocytes	$10^3/\mu L$	0.03-0.18
Eosinophils	$10^3/\mu L$	0.01-0.16

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Table 6 Hematology range data of male rat (8-16 weeks old) (continue)

Test	Unit	Range
Basophils	$10^3/\mu L$	0-0.05
Reticulocytes	$10^9/L$	152.3-381.5
Large unstained cells	$10^3/\mu L$	0.01-0.06
CHCM	g/dL	0.01-0.16
CH	pg	33-37.7
HDW	g/dL	17.4-20.3
PDW	%	43.2-64.3
%Neutrophils	%	6.2-26.7
%Lymphocytes	%	66.6-90.3
%Monocytes	%	0.8-3.8
%Eosinophils	%	0.2-3.5
%Basophils	%	0-0.8
%Reticulocytes	%	1.7-4.9
%Large unstained cells	%	0.1-1.1

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Table 7 Blood chemistry range data of male rat (8-16 weeks old)

Test	Unit	Range
Phosphorus	mg/dL	5.58-10.41
Calcium	mg/dL	9.5-11.5
Total protein	g/dL	5.2-7.1
Triglycerides	mg/dL	20-114
Cholesterol	mg/dL	37-85
Glucose	mg/dL	70-208
Creatinine	mg/dL	0.2-0.5
Indirect Bilirubin	mg/dL	0.01-0.12
Direct Bilirubin	mg/dL	0.03-0.05
Total Bilirubin	mg/dL	0.05-0.15
Alkaline Phosphatase	U/L	62-230
Aspartate Aminotransferase	U/L	74-143
Alanine Aminotransferase	U/L	18-45
Creatine Kinase	U/L	162-1184
Albumin	g/dL	3.4-4.8

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Table 7 Blood chemistry range data of male rat (8-16 weeks old) (continue)

Test	Unit	Range
Globulin	g/dL	1.5-2.5
A/G Ratio	ratio	1.58-2.67
Urea	mg/dL	12.3-24.6
Sodium	mmol/L	142-151
Potassium	mmol/L	3.82-5.55
Chloride	mmol/L	100-106

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Table 8 Chemical values in healthy male Sprague-Dawley rat (The National Laboratory Animal Center, Mahidol University)

Chemical parameter	Unit	4 weeks old		10 weeks old	
		Dilok et. al., 2016	Wannee et. al., 2002	Dilok et. al., 2016	Wannee et. al., 2002
GLU	mg/dl	56.6-182.6	36.2-86.4	105.6-206.8	80.3-156.7
BUN	mg/dl	16.3-27.7	10.2-18.7	15.6-20.1	10.6-18.5
CREA	mg/dl	0.18-.39	0.35-0.50	0.37-0.47	0.46-0.65
CHOL	mg/dl	72-94	97-129	73-95	69-104
TG	mg/dl	42-139	100-249	64-112	39-92
URIC	mg/dl	1.95-4.61	0.75-1.28	1.98-4.38	0.84-1.53
TP	g/dl	4.63-5.78	4.86-5.60	5.70-7.09	5.51-6.74
ALB	g/dl	3.5-4.0	3.6-4.6	4.1-4.5	3.8-4.3
GLOB	g/dl	1.0-1.9	1.0-1.4	1.5-2.7	1.4-2.7
Bill-T	mg/dl	0.08-0.12	0.05-0.11	0.07-0.11	0.04-0.08
AST	U/L	95-171	129-248	82-127	82-145
ALT	U/L	26-77	25-51	36-64	32-53
ALP	U/L	132-240	170-272	82-112	102-168

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2.4 Tissue preparation and protein extraction

Dissected brains were frozen in liquid nitrogen at -80 °C until extraction (see in figure 13). Hippocampal tissue was powderized in liquid nitrogen, protein extract with buffer containing 40 mM Tris, 7M Urea, 2M Thiourea, 4% CHAPS, 65 mM DTT, 0.3 mg/ml EDTA, 35 µg/ml PMSF and 1 tablet of protease inhibitor cocktail. The protein was precipitated with 20% of Trichloroacetic acid (TCA) in acetone plus with 20 mM Dithiothreitol (DTT). The protein was re-suspended in lysis buffer and the protein concentration was measured by Bradford assay. For Bradford assay, Prepare dye reagent by diluting 1:4 of deionizes water and filter through Whatman No. 1 filter paper. The protein standard was diluted to 5 concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg/ml) and protein samples were diluted with lysis solution (1:1). Pipet 10 µL of standard and sample into separate microtiter well plate, add 200 µL of dye reagent to each well. Mix the sample and reagent, incubate at room temperature for at least 5 min but not more than 1 hour. The absorbance was measured at 595 nm.

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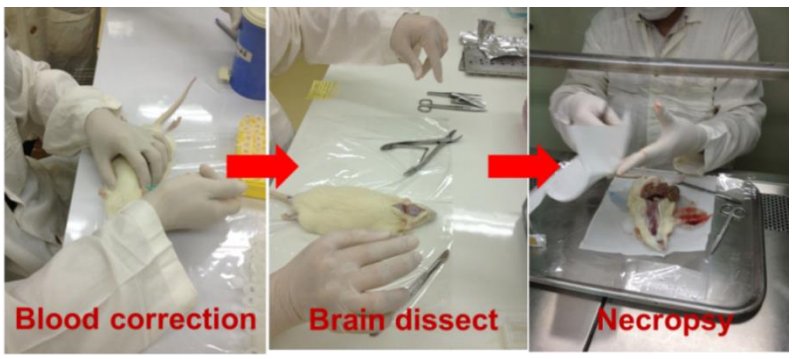


Figure 13 Present the animal processing

2.5 Determination concentration of monoamine neurotransmitters including norepinephrine, Serotonin (5-hydroxytryptamine, 5-HT), Dopamine by Reverse phase High-Performance Liquid Chromatography (HPLC)

The hippocampal tissue was extracted with 0.1 M perchloric acid and sonicated for 30 min on ice-cold. Homogenates were centrifuged at 12, 000 g for 15 min at 4°C while clear supernatants were decanted and filtered through 0.45 μm filter. This filtrate was determined by the concentration of monoamine neurotransmitters. The HPLC system consists of the analytical C18 reversed-phase column (ODS3 C18, 4.6X250 mm, diameter 5 μm) and UV detector (best condition at 220 nm). The mobile phase is 0.02 M sodium acetate, 0.0125 M citric acid buffer pH 4, 0.042 Metanesuffonic acids, 0.1 mM EDTA. The flow rate was set at 1 mL/min. The working standard solutions were prepared fresh in 0.05 M perchloric acid containing 0.1 mM Na_2EDTA on ice and stored at -20°C before using. Peaks were identified by comparing the retention time of each peak in the sample solution, where each individual peak was further compared in the standard solution of norepinephrine, Serotonin (5-hydroxytryptamine, 5-HT), Dopamine and caffeine (Sigma-Aldrich, USA) was used for internal standards.

2.6 Determination of protein changes developing in SD rat hippocampus by two-dimensional gel electrophoresis (2-DE)

2-D electrophoresis can be used to resolve complex mixtures of thousands of proteins. In the first dimension, proteins are separated based on differences in isoelectric point (pI). In the second dimension, they are separated according to molecular weight (Rabilloud et al., 2010). The basic concept of 2D electrophoresis is schematized in figure 14. Although the name of 2D electrophoresis suggests that it is a two-step process, it is indeed a five-step process starting from (1) protein sample preparation prior to the first separation (2) first-dimension Separation; the technique is called isoelectric focusing (IEF) which is the first proteins separation on the basis of pI, the pH at which a protein carries no net charge and thus will not move in an electrical field. (3) The second separation; In the next step of 2-D electrophoresis, an anionic surfactant such as sodium dodecyl sulfate (SDS) is typically added to impart a uniform negative charge to the proteins per unit mass and so insure uniform separation based on their molecular weights. (4) Gel Staining, to visualize proteins in gels then finally (5) 2D Gel Image Analysis (Rabilloud and Lelong, 2011).

In this study, 400 ug of protein was mixed with 340 μl of rehydration buffer (8 M urea, 4% CHAPS, 0.001% bromphenol blue and 3 mM dithiothreitol) containing 1% 3-10 L IPG buffer. The sample was loaded onto 13^{-cm} IPG strips with pH range of 3-10 L of an isoelectric focusing system (Ettan IPGphoreIII). Samples were run through steps of strip

rehydration (20 ºC, 16 h) and isoelectric focusing (500 volts for 500 volt^{-h}, 1,000 volts for 800 volt^{-h}, and 10,000 volts to reach 27,000 volt^{-h}). The maximum current was maintained at 75 µA per strip. After the completion of the process, the strips were equilibrated twice (15 min each) in equilibration buffer supplemented with 65 mM DTT and 135 mM iodoacetamide. Each strip was subjected to the second dimensional separation (Ettan DALTsix) using a SDS-polyacrylamide gel (12.5%). Separation of protein was executed under the applied voltage of 10 watt per gel at 20ºC until the bromophenol blue dye front reached 0.5 cm from the bottom of the gel. The gels were stained with colloidal Coomassie blue staining according to standard recommendation. After staining, gel images were acquired using Image scanner III (GE Health Care, USA). Differential analysis was performed by Image Master 2D Platinum version 7.0 (GE Health Care, USA) software tool and spots of interest excised for identification by mass spectrometry.

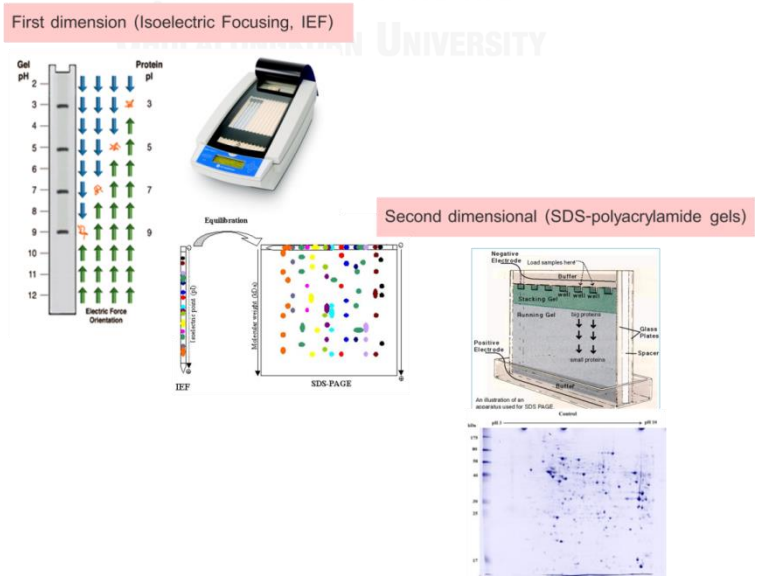


Figure 14 Scheme of principle of two-dimensional gel electrophoresis (2-DE) assay

2.7 Protein identification with liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The spots of interest were carefully cut and destained in a solution containing 50 % ACN in 100 µL of 25 mM ammonium bicarbonate ($(NH_4)HCO_3$) and then submitted to in gel trypsin digestion overnight at 37 °C in 25 mM ($(NH_4)HCO_3$). Peptides were extracted in 50 mL of 5% Formic acid/ 50% ACN and then put into the ultrasonic bath for 15 min and dried in a speed-vac. Peptide samples were dissolved in 98% H_2O, 2% ACN and 0.1% formic acid. The LC–MS/MS system consists of a liquid chromatography part (Dionex Ultimate 3000, Thermo Scientific) in combination with an electrospray ionization (ESI)/ Quadrupole ion trap mass spectrometer (Model amazon SL, Bruker, Germany). The LC separation was performed on a reversed phase column (Hypersil GoLD 50×0.5 mm, 5 µm C18), protected by a guard column, eluted at a flow rate of 100 µl/min under gradient conditions of 5-80%B over 50 min. Mobile phase A consists of water/formic acid (99.9:0.1, v/v), and B consists of acetonitrile (100, v). Mass spectral data from 150 to 1500 m/z was collected in the positive ionization mode.

Concept of sequence database searching resembles a generic bottom-up MS experiment, as for each stage of the experiment follow by figure 15. For Protein identification, MS/MS data was performed by Mascot search engine, against Swiss-Prot database. Mascot score was taken into consideration and reported after manual verification of the fragmentation spectrum (figure 16). Mascot is a search engine which

uses mass spectrometry data to identify proteins from sequence databases. These different search methods can be categorized as follows; Peptide Mass Fingerprint, Sequence Query and MS/MS Ion Search. In this study, the sequence databases that were searched on public Mascot server including EST, NCBInr, cRAP and SwissProt. In this study we should SwissProt, that high quality and curated protein database. The Taxonomy parameter allows searches to be limited to entries from particular species or groups of species. Mascot supports modification of specific residue including, fixed modifications are applied to every instance of the specified residue(s) or terminus. For example, selecting carboxymethyl (C) means that all calculated use 161 Da as the mass of cysteine. Oxidation (M) is selected a peptide contains 3 methionines. Mascot was tested for a match with the experimental data for that peptide containing 0, 1, 2, or 3 oxidised methionine residues. Variable modifications can be a very powerful means of finding a match, but there are also be aware a single variable modification will generate many possible additional peptides to be tested.

The Mascot generic format for a data file submitted to Mascot is square brackets indicate optional elements. In the case of a peptide mass fingerprint, each query is just a single peptide m/z value, with an optional second value for peak area or intensity. A peptide mass fingerprint data file can only contain peptide mass fingerprint. Sequence queries or MS/MS datasets are not permitted. For an MS/MS Ions Search, each query represents a complete MS/MS spectrum, and is delimited by a pair of statements:

BEGIN IONS and END IONS. Parameters within an MS/MS query only apply locally, to the one spectrum. A summary report is displayed that provides an overview of the results that showed in Figure 17.

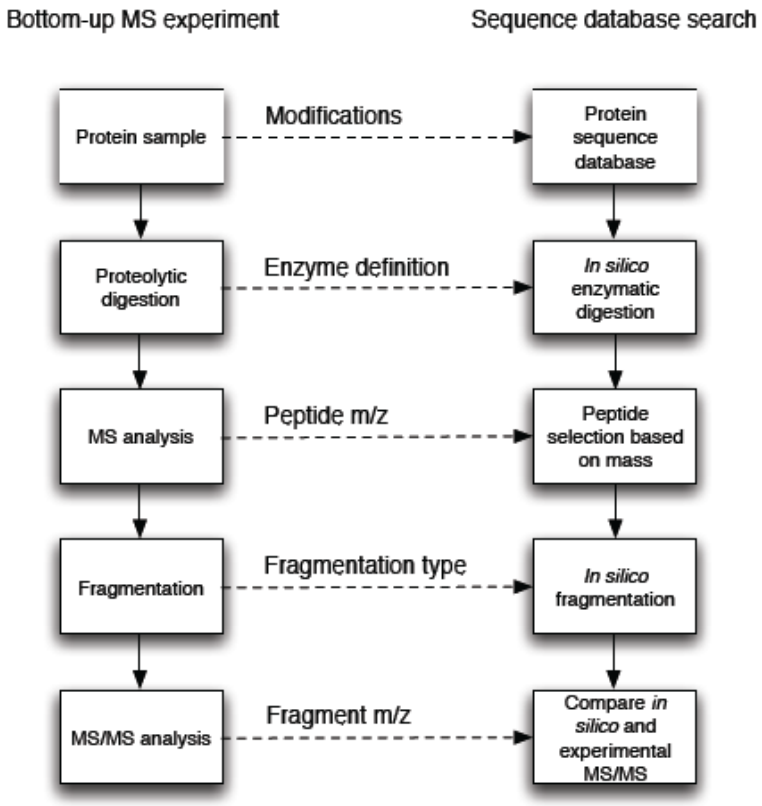


Figure 15 Concept of database searching resembles a generic bottom-up MS experiment (From Markus Brosch, 2009)

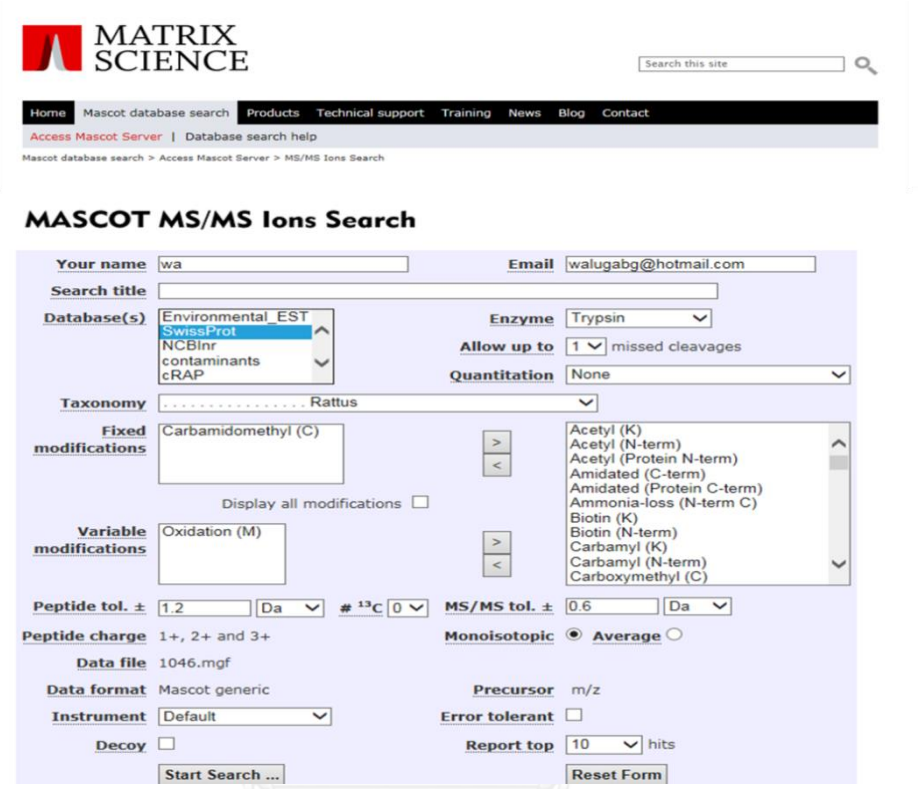
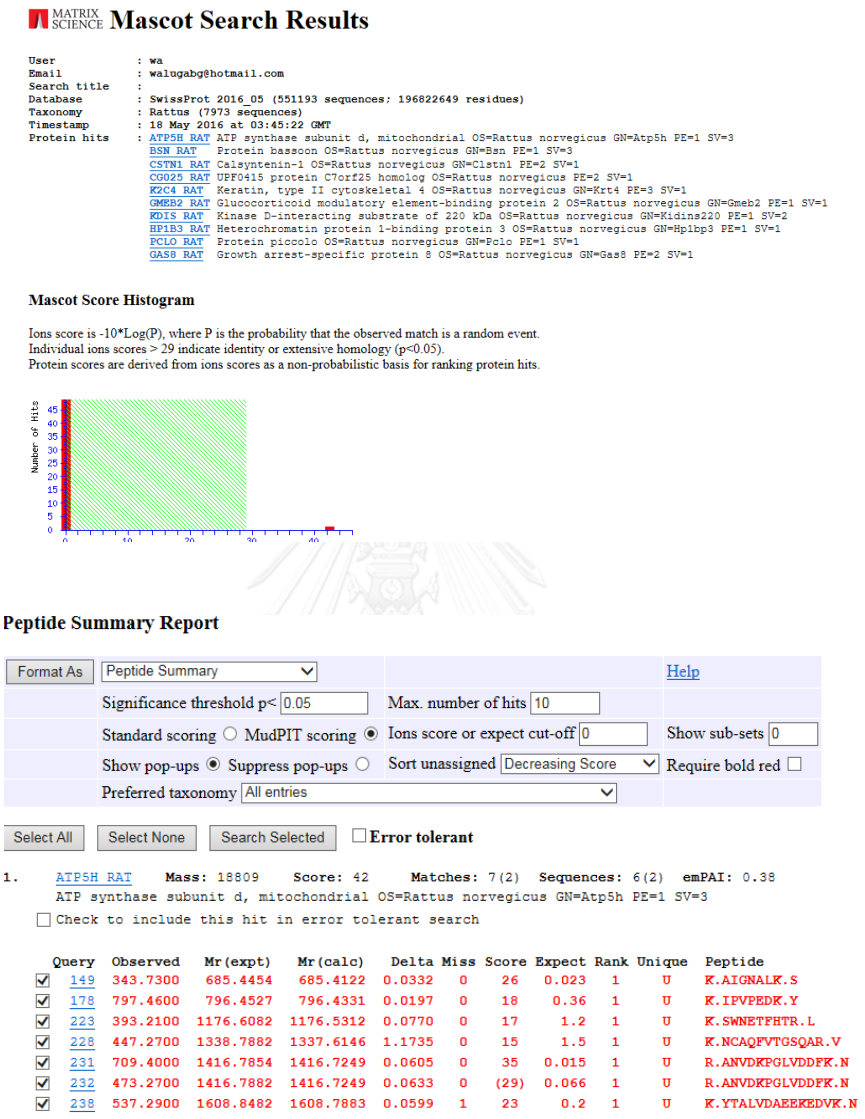


Figure 16 MASCOT analysis condition

(From http://www.matrixscience.com/search_form_select.html)



MATRIX SCIENCE **Mascot Search Results**

```
User          : wa
Email         : walugabg@hotmail.com
Search title  :
Database      : SwissProt 2016_05 (551193 sequences; 196822649 residues)
Taxonomy      : Rattus (7973 sequences)
Timestamp     : 18 May 2016 at 03:45:22 GMT
Protein hits  : ATP5H_RAT ATP synthase subunit d, mitochondrial OS=Rattus norvegicus GN=Atp5h PE=1 SV=3
                BSN_RAT   Protein bassoon OS=Rattus norvegicus GN=Bsn PE=1 SV=3
                CSTN1_RAT Calsyntenin-1 OS=Rattus norvegicus GN=Clstn1 PE=2 SV=1
                CG025_RAT UPF0415 protein C7orf25 homolog OS=Rattus norvegicus PE=2 SV=1
                KZC4_RAT  Keratin, type II cytoskeletal 4 OS=Rattus norvegicus GN=Krt4 PE=3 SV=1
                GMEB2_RAT Glucocorticoid modulatory element-binding protein 2 OS=Rattus norvegicus GN=Gmeb2 PE=1 SV=1
                KDIS_RAT  Kinase D-interacting substrate of 220 kDa OS=Rattus norvegicus GN=Kidins220 PE=1 SV=2
                HP1B3_RAT Heterochromatin protein 1-binding protein 3 OS=Rattus norvegicus GN=Hp1bp3 PE=1 SV=1
                PCLO_RAT  Protein piccolo OS=Rattus norvegicus GN=Pclo PE=1 SV=1
                GAS8_RAT  Growth arrest-specific protein 8 OS=Rattus norvegicus GN=Gas8 PE=2 SV=1
```

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 29 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits 10
Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned Decreasing Score — Require bold red
Preferred taxonomy: All entries

Select All | Select None | Search Selected | Error tolerant

1. ATP5H_RAT **Mass:** 18809 **Score:** 42 **Matches:** 7(2) **Sequences:** 6(2) **emPAI:** 0.38
ATP synthase subunit d, mitochondrial OS=Rattus norvegicus GN=Atp5h PE=1 SV=3
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
149	343.7300	685.4454	685.4122	0.0332	0	26	0.023	1	U	K.AIGNALK.S
178	797.4600	796.4527	796.4331	0.0197	0	18	0.36	1	U	K.IPVPEDK.Y
223	393.2100	1176.6082	1176.5312	0.0770	0	17	1.2	1	U	K.SWNETFHTR.L
228	447.2700	1338.7882	1337.6146	1.1735	0	15	1.5	1	U	K.NCAQFVTGSQAR.V
231	709.4000	1416.7854	1416.7249	0.0605	0	35	0.015	1	U	R.ANVDKPGLVDDFK.N
232	473.2700	1416.7882	1416.7249	0.0633	0	(29)	0.066	1	U	R.ANVDKPGLVDDFK.N
238	537.2900	1608.8482	1608.7883	0.0599	1	23	0.2	1	U	K.YTALVDAEEKEDVK.N

Figure 17 A summary reports is displayed from MASCOT

(From http://www.matrixscience.com/search_form_select.html)

Many interactions and functional partnerships between proteins, that occurs at the core of cellular and molecular systems. Knowledge about its specific interaction partners is important (Szklarczyk et al., 2014). Protein–protein interaction and signaling pathway analysis can also be predicted computationally. In this study we used the Ingenuity Pathways Analysis (IPA) and STRING that offered us some additional valuable clues about the complex interactive link of the various identified proteins within their commonly known interactive protein networks also obtained from other cellular metabolic information. The basic interaction unit in STRING is the functional association such as a specific and productive functional relationship between two or multiple proteins. The interactions are predicted *de novo* by number of algorithms by using genomic information (Lewis et al., 2010). The current version is STRING V10 covers human disease associations and tissue annotations. A protein or set of proteins is identified the network see in Figure 18. IPA (Ingenuity System Inc, USA) was used to interpret the data in the context of biological processes, pathways relationships, mechanisms, functions and networks powerful that relevant to changes observed in an analyzed dataset and algorithms identify regulators (Jiménez-Marín et al., 2009) see in Figue 19. The process of IPA is: Mapping the data to the IPA Knowledge Base (KB) and molecular networks creating (algorithmically generated pathways). IPA pathway analysis was used to explain expression patterns for Identifying the important key regulators and activity. Moreover, IPA can be predicted downstream effects biological and disease

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processes providing targeted data on genes, proteins, chemicals, and drugs. Furthermore, we can build the models of interaction of experimental systems. The example of basic IPA core analysis making showed in Figure 19. In addition, IPA can used for determine the signaling and metabolic canonical pathways.

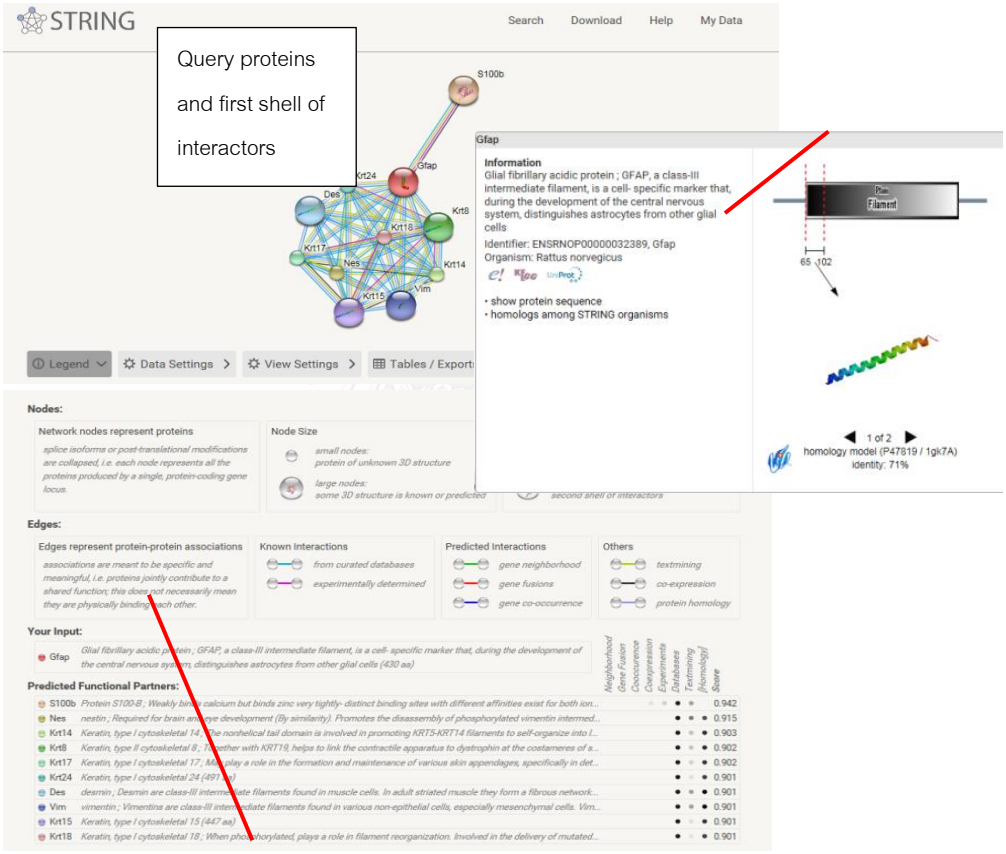

Figure 18 The STRING network view combined with screenshots. The cored lines between proteins indicate the various types of interaction evidence. (From http://string-db.org)

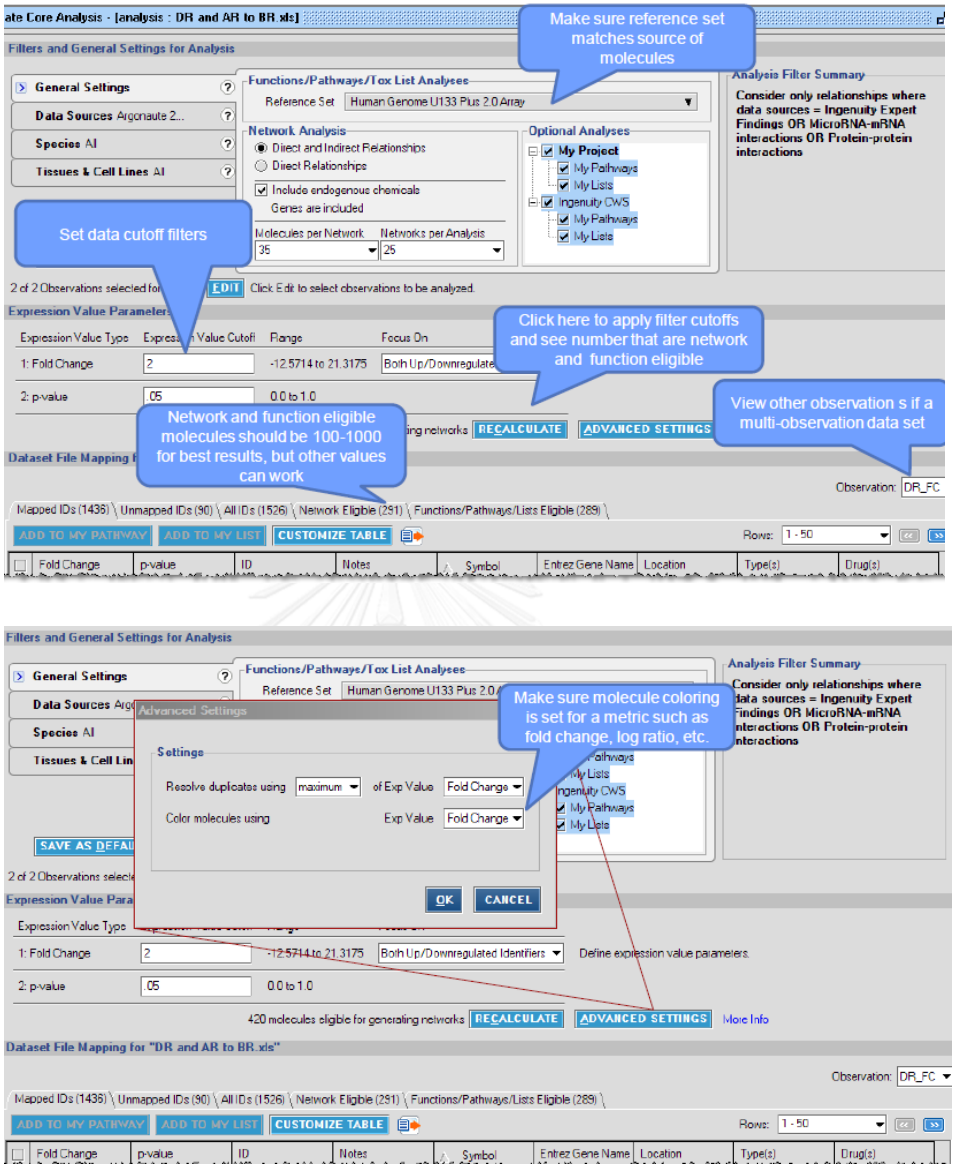

Figure 19 The IPA core analysis procedure

(From www.usc.edu/hsc/.../IPA/Data%20Analysis%20training%20Handouts.pdf)

2.8 Determination of protein expression by Western blot analysis

Proteins were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) in transfer buffer (192mM Glycine, 25mM TrisHCl, 20% methanol). Membranes are washed in TBS-T (20mM TrisHCl, 150 mM NaCl, 1% Tween at pH 7.5) and then blocked with non-fat dried milk in Tris-buffered saline containing 0.01% Tween-20 for 1 hr. Primary antibodies were incubated in following 1:8000 Anti-PIA3 (ERp57), Anti-GFAP, Anti- DPYSL2 (CRMP2), Anti- Phospho-DPYSL2 (Phospho-CRMP2) and Anti-β-actin (cell signaling, USA) in 1% BSA in TBS-T for 1h at room temperature to overnight on ice. After washed with TBS-T 3 time for 10 minutes, membranes were incubated with secondary antibody (HRP159 linked antibody). The signal was developed with a chemiluminescence reagent (GE healthcare, USA) and band images were detected with X-ray film. A western blot analysis was presented in figure 20.

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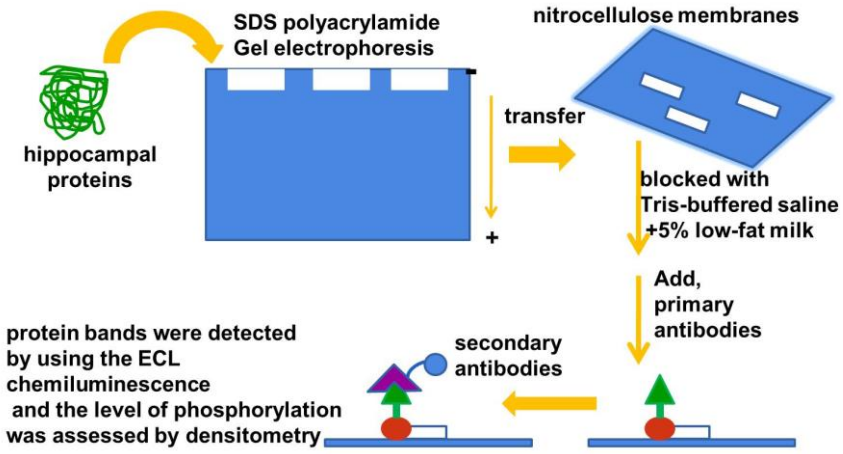

Figure 20 A western blot analyses

2.9 Data analysis and statistical method

Raw data were presented in mean and standard deviation and the data were performed to find significantly *P*-values <0.05 and 0.01. Differences between the groups were established by using an unpaired Student's *t-test* while within-group comparisons were performed by using the paired Student's *t-test.* The spot densities were further compared with control by ANOVA, *P*-values <0.05.

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CHAPTER 3

RESULTS

3.1 Standardized crude extract of *K. parviflora* and *M. fragrans* nutmeg volatile oil

The content of 5,7-dimethyoxyflavone in *K. parviflora* extract was analyzed by reversed-phase HPLC. The sample contained 5,7-dimethoxyflavone 13.4618±0.0145 mg/g (R^2=0.9997) of dry sample, representative HPLC fingerprint as shown in figure 21. The chemical composition of *M. fragrans* nutmeg volatile oil was analyzed by using GC–MS technique (figure 22). Total of 26 volatile compounds of the nutmeg were tentatively identified and reported. The data describing all compounds are shown in Table 9. This research demonstrated that the crude extract of *K. parviflora* and nutmeg volatile oil are of good quality with a high potential.

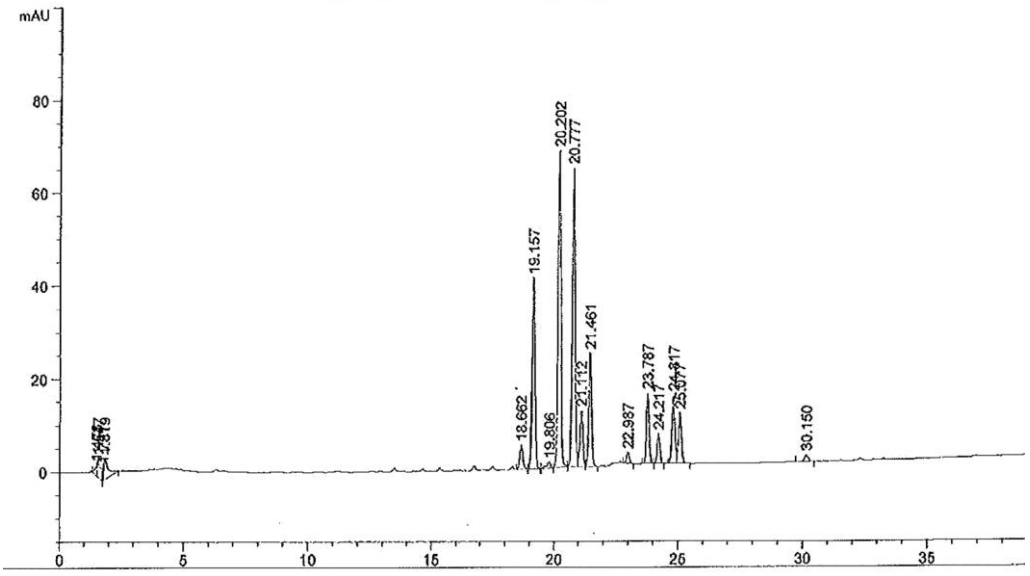

Figure 21 Representative HPLC fingerprint of *K. parviflora* ethanolic extract.

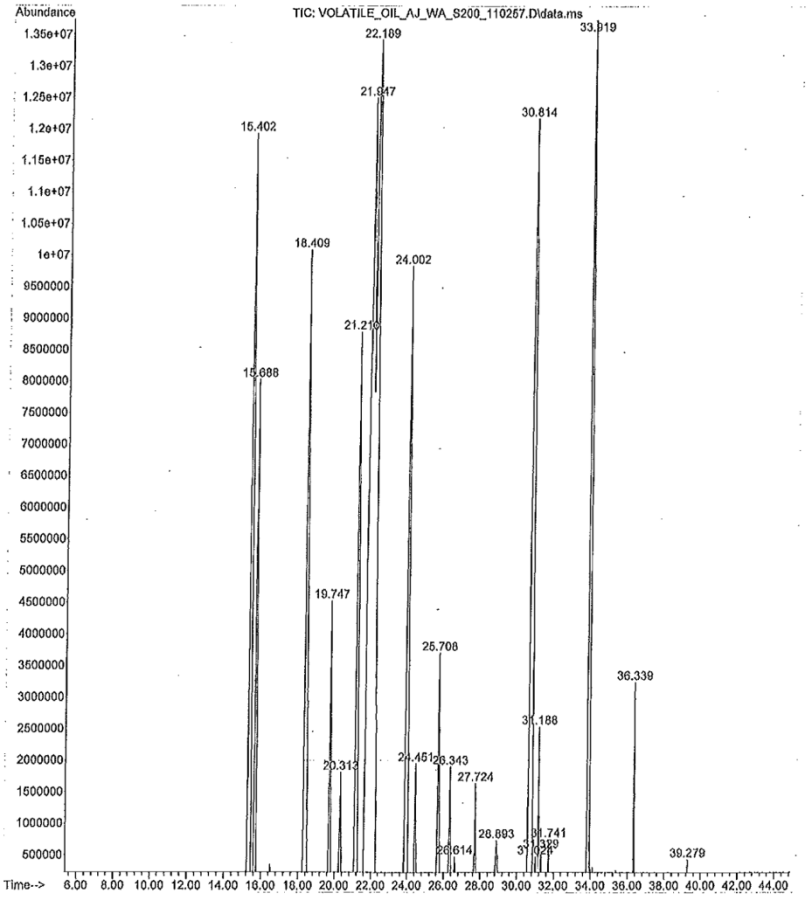

Figure 22 GC–MS chromatogram of *M. fragrans* nutmeg volatile oil

Table 9 Chemical composition of nutmeg volatile oil obtained by GC–MS

NO.	RT	Area%	Compound
1	14.428	1.97	1R-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene
2	15.434	6.17	Bicyclo [3.1.1] hex-2-ene, 2-methyl-5(1-methyethyl)
3	15.729	2.68	1R-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene
4	16.522	0.12	Camphene
5	18.462	5.13	Cyclohexene, 4-methylene-(1-methylethyl)
6	19.066	0.52	Beta-Myrcene
7	20.646	1.59	4-Carene
8	21.266	7.09	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)
9	22.024	14.89	o-cymene
10	22.702	10.04	Limonene
11	23.574	1.95	Gamma-terpinene
12	24.059	5.28	Gamma-terpinene
13	25.396	0.46	Cyclohexene, 1-methl-4-(1-methylethylidene)
14	28.767	0.03	(+)-2-Bornanone
15	30.696	5.67	Terpinen-4-ol
16	30.885	6.20	3-Cyclohexene-1-ol, 4-methyl-1-(1-methylethyl)

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Table 9 Chemical composition of nutmeg volatile oil obtained by GC–MS (continue)

17	31.051	0.16	Benzenemethanol, alpha., 4-trimethyl
18	31.228	0.61	Alpha-terpineol
19	31.217	0.78	Alpha-terpineol
20	31.347	0.15	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl), cis
21	31.761	0.23	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl), cis
22	33.961	8.88	Safrole
23	35.369	0.18	Eugenol
24	36.334	0.78	Methyleugenol
25	36.695	0.01	Caryophyllene
26	38.168	0.05	Benzen,1,2-dimethoxy-4-(1-propenyl)
27	38.665	0.90	Meristicin
28	41.320	0.03	Ar-tumerone
29	43.024	0.06	Tetradecanoic

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3.2 Effects of *K. parviflora* rhizome ethanolic extract and *M. fragrans* nutmeg volatile oil on the levels of monoamine neurotransmitters including norepinephrine, Serotonin (5-hydroxytryptamine, 5-HT), Dopamine

Reverse phase High Performance Liquid Chromatography (HPLC) is a commonly used method which can accurately determine content of neurotransmitters upon *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) and Fluoxetine (20 mg/kg BW) compared with the control group (2 % tween 80, 1 mL/kg BW) for 12 days. Concentration of monoamine neurotransmitters were reported in Table 10 and figure 23A, the representative HPLC fingerprints are show in figure 23B-F. Application of *K. parviflora* group and *M. fragrans* group via oral route once per day for 12 days led to an significant increase level of serotonin (5-HT), norepinephrine and dopamine when compared with control figure 10A ($P<0.01$). However, *M. fragrans* treated rat exhibited a non-significant improvement of serotonin compared with fluoxetine treated group shown in figure 10A. All results were expressed as mean±SD student's paired *t-test* and were utilized to compare the different groups. The statistical significance was taken at a *P*-value less than 0.01.

Table 10 Concentration of neurotransmitter (μg/g wet weight) in the hippocampal homogenates from control and treated rat

Group	Neurotransmitter (μg/g wet weight)		
	NE (Mean±SD)	DA (Mean±SD)	5-HT (Mean±SD)
Control (*n*=6)	0.1789 ±0.0153	0.2594±0.1077	0.3695±0.0262
K. parviflora (*n*=5)	0.3316± 0.0365	0.3337±0.0674	0.5132±0.0137
M. fragrans (n=5)	0.2825±0.0133	0.4517±0.0259	0.5897±0.0189
Fluoxetine (*n*=5)*	0.2408±0.0171	0.3463±0.0221	0.5811±0.0208

* At the final treatments group one rat was died. Data from the liver and kidney function and histology in dead rat show non-toxicity but the data did not shown. NE, norepinephrine; DA, dopamine; 5-HT, serotonin

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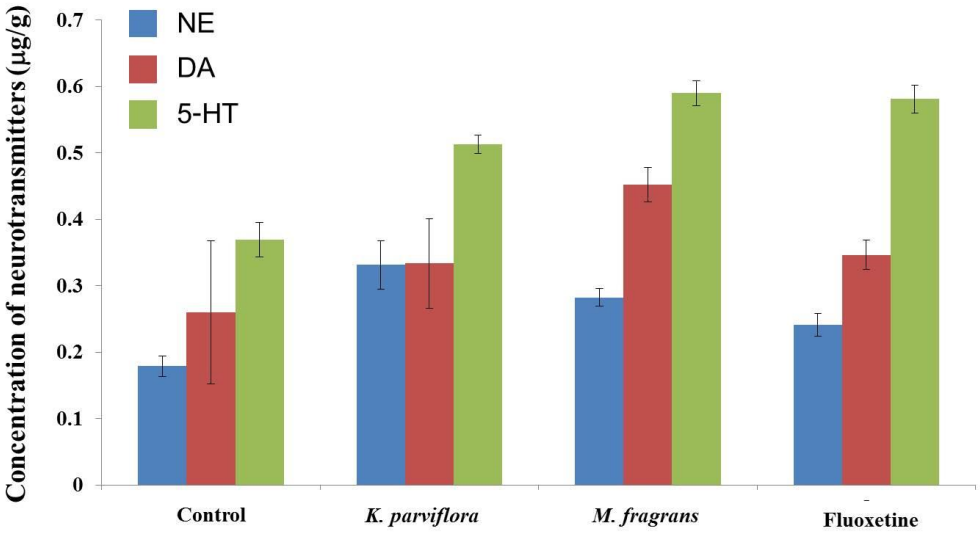

Figure 23 (A) The increase level of monoamine neurotransmitters including norepinephrine (NE), Serotonin (5-hydroxytryptamine, 5-HT) and Dopamine (DA) in the rat hippocampal when treated with *K. parviflora* rhizome ethanolic extract, *M. fragrans* nutmeg volatile oil and fluoxetine. The statistical analysis showed a significant difference between the control and each treatment groups at $P<0.05$ (control: n=6, *K. parviflora*: n=5, *M. fragrans*: n=5, fluoxetine: n=5). Data are expressed as the mean ± standard deviation (S.D.)

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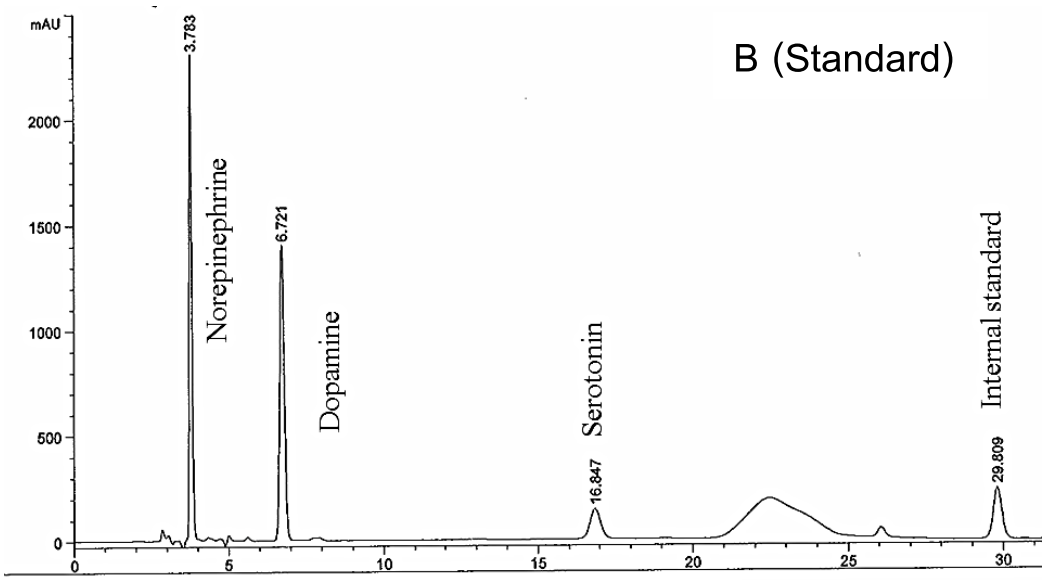
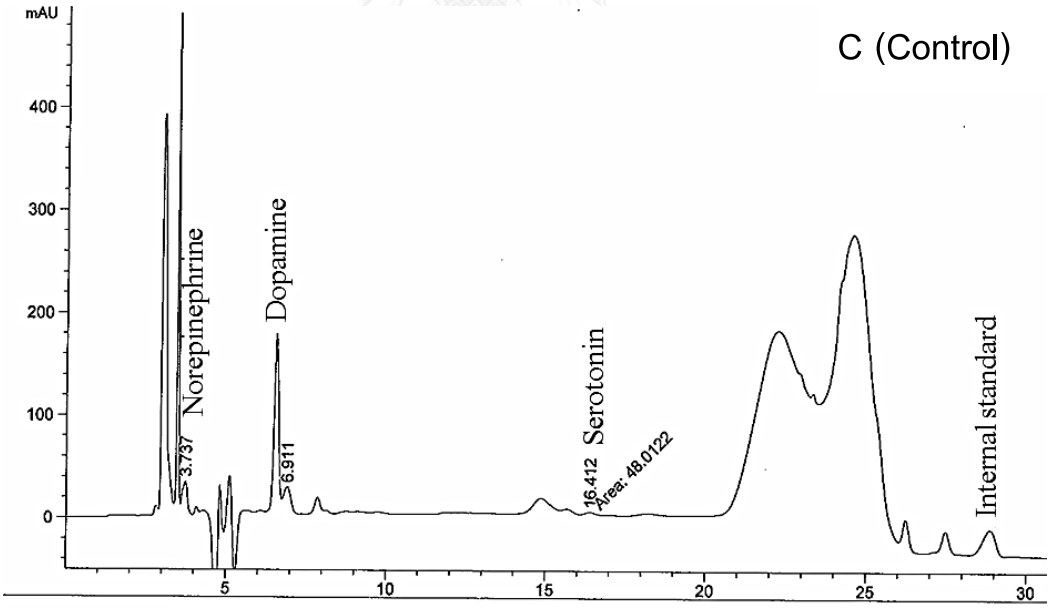
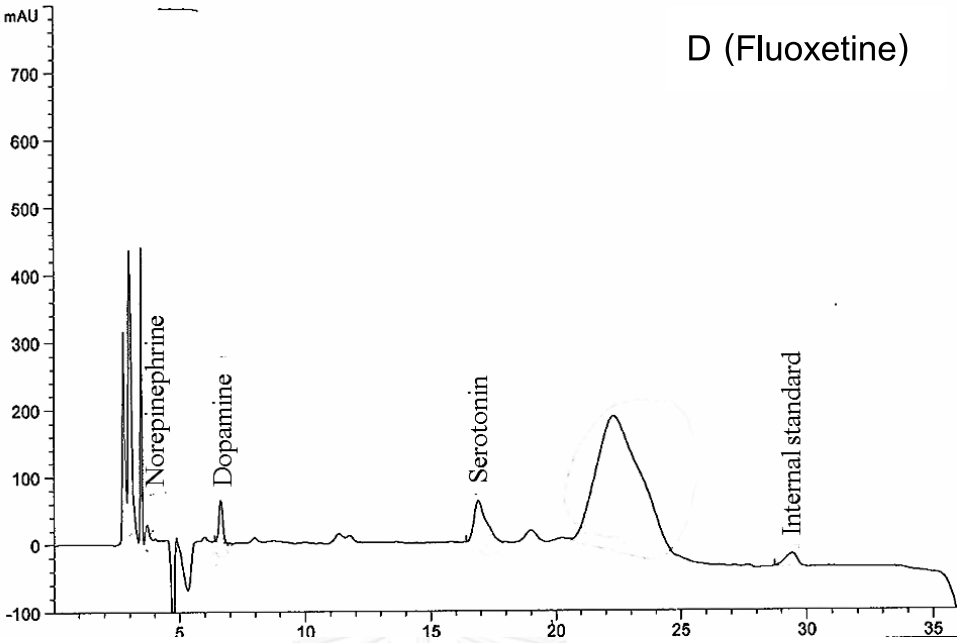
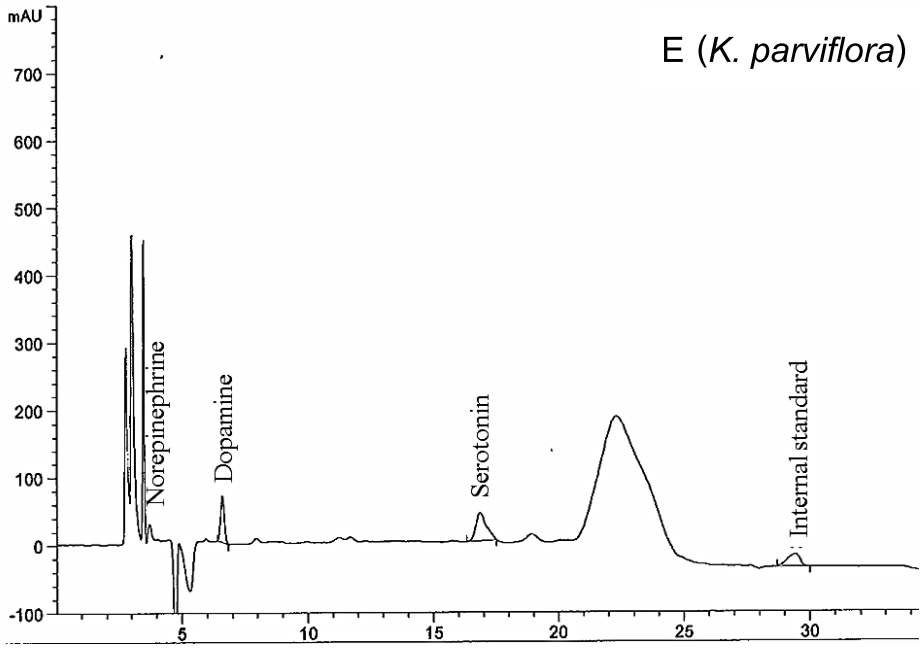

B (Standard)
mAU
2000
1500
1000
500
0
3.783
Norepinephrine
6.721
Dopamine
Serotonin
16.847
Internal standard
29.809
0
5
10
15
20
25
30


C (Control)
mAU
400
300
200
100
0
3.737
Norepinephrine
6.911
Dopamine
16.412
Area: 48.0122
Serotonin
Internal standard
5
10
15
20
25
30


D (Fluoxetine)
mAU
700
600
500
400
300
200
100
0
-100
Norepinephrine
Dopamine
Serotonin
Internal standard
5
10
15
20
25
30
35


E (*K. parviflora*)
mAU
700
600
500
400
300
200
100
0
-100
Norepinephrine
Dopamine
Serotonin
Internal standard
5
10
15
20
25
30

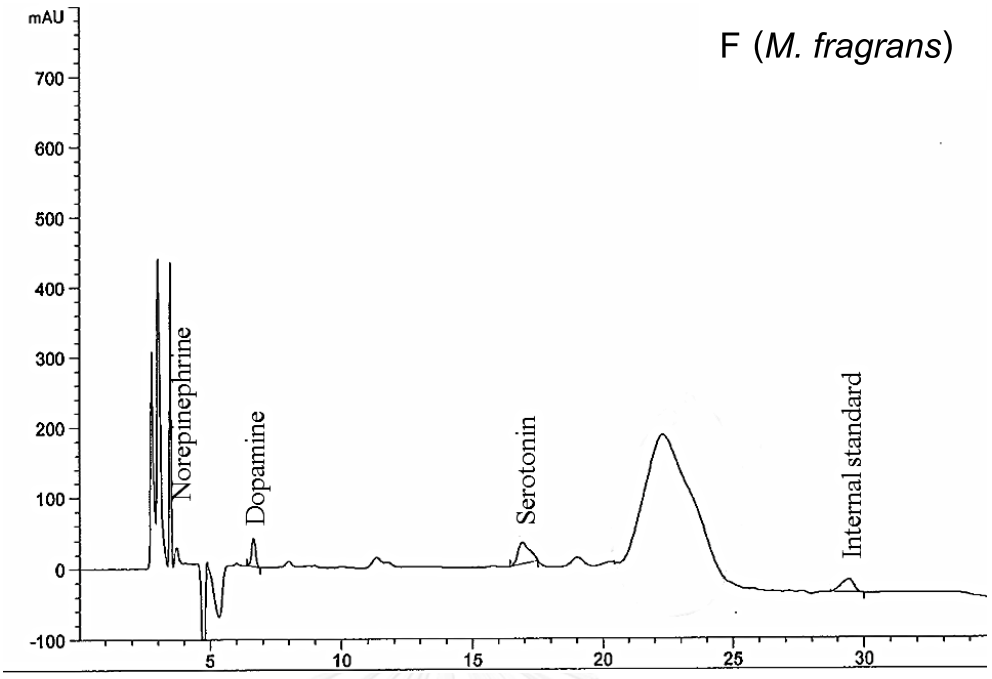


Figure 23 (B-F) HPLC Chromatograms of monoamine neurotransmitter after rats treated with *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) and Fluoxetine (20 mg/kg BW).

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3.3 Proteomic identification of proteins modulated by K. parviflora, M. fragrans and fluoxetine in SD rat hippocampus

The interpretation of high-throughput proteins expression data is greatly facilitated by the consideration of prior biological knowledge. We have extended our study to identify possible molecular candidates for *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) and Fluoxetine (20 mg/kg BW) treated on hippocampal proteomic profiles in SD rats. The hippocampal protein extracts of rats from control and treated groups were analyzed by 2-DE. For each experimental group, gels were made in triplicate (12 gels in total) as the representative 2D gels. The gels are presented in figure 6. The image analysis of gels demonstrated a similar number and pattern distribution of spots in twelve 2D gels by using Image Master 2D Platinum version 7. Approximately 1050 spots have been detected on each gel (as shown in figure 24A-D).

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We have been focusing our interest on the proteins involved in a fold change$\geq$1.5 in 3 times of each treatment groups compared with the control group (P<0.05). The table 3 presents the functional classification and regulation of differentially expressed proteins in each group. Ninety proteins were accepted in total as significantly different and were analyzed with LC-MS/MS. Mass spectrometry gave the identified information within 79 of 90 spots (87.78 % success rate). However, we found the

presence of the same in different spot number, which our data did not display. After that each group was compared with control group, where the analysis indicated that 37 proteins were up-regulated while 14 were down-regulated in *K. parviflora* group. *M. fragrans* shows that 27 proteins were up-regulated while 16 were down-regulated. In fluoxetine treated group we found 29 proteins were up-regulatedand 14 were down-regulated.

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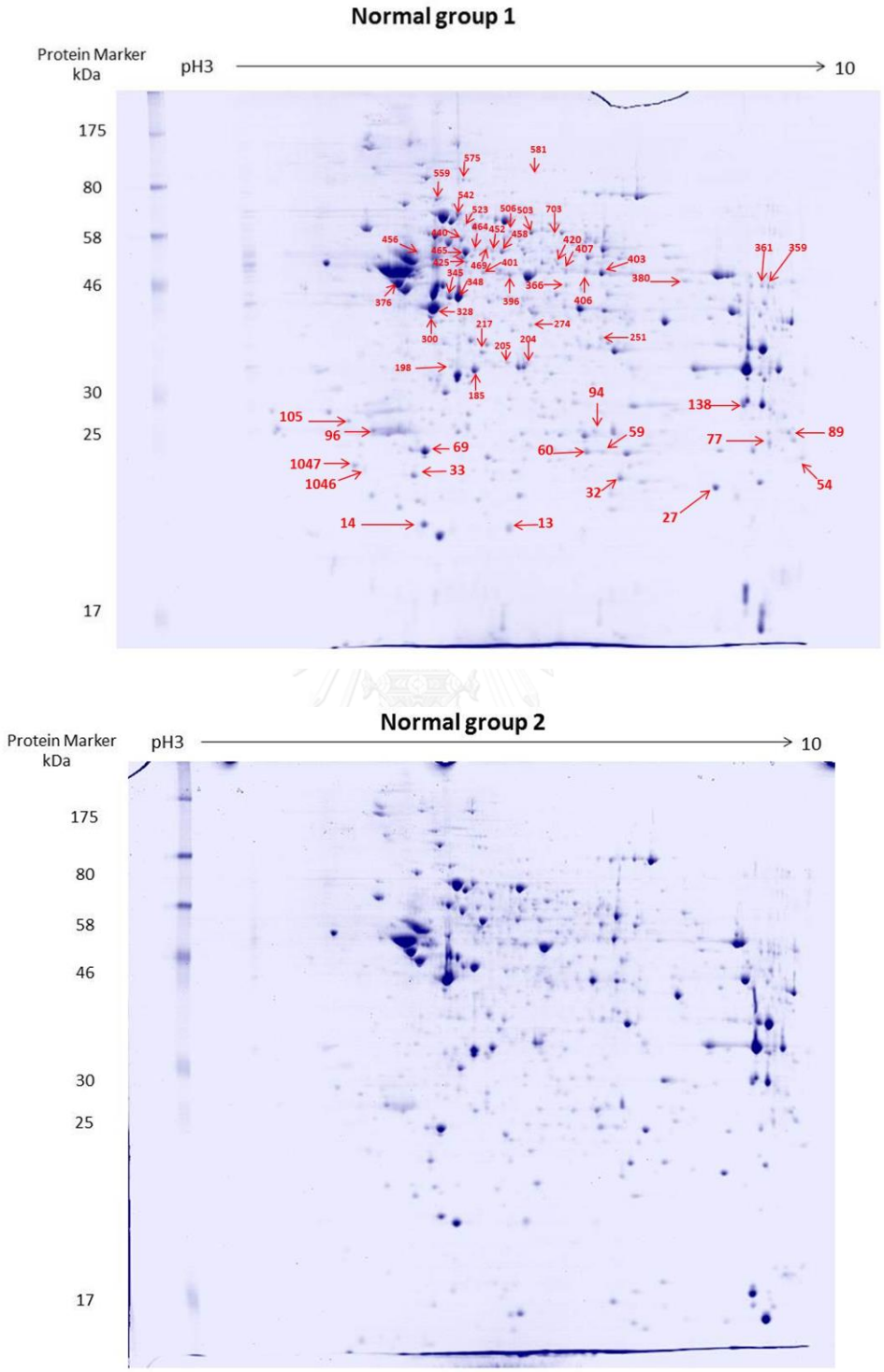
Normal group 1
Protein Marker
kDa
pH3
10
175
80
58
46
30
25
17
581
575
559
542
523
506
503
703
464
452
458
440
456
465
425
469
401
420
407
403
380
361
359
345
348
366
376
396
406
328
217
274
251
300
205
204
198
185
94
138
105
96
59
77
89
69
60
1047
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pH3
10
175
80
58
46
30
25
17

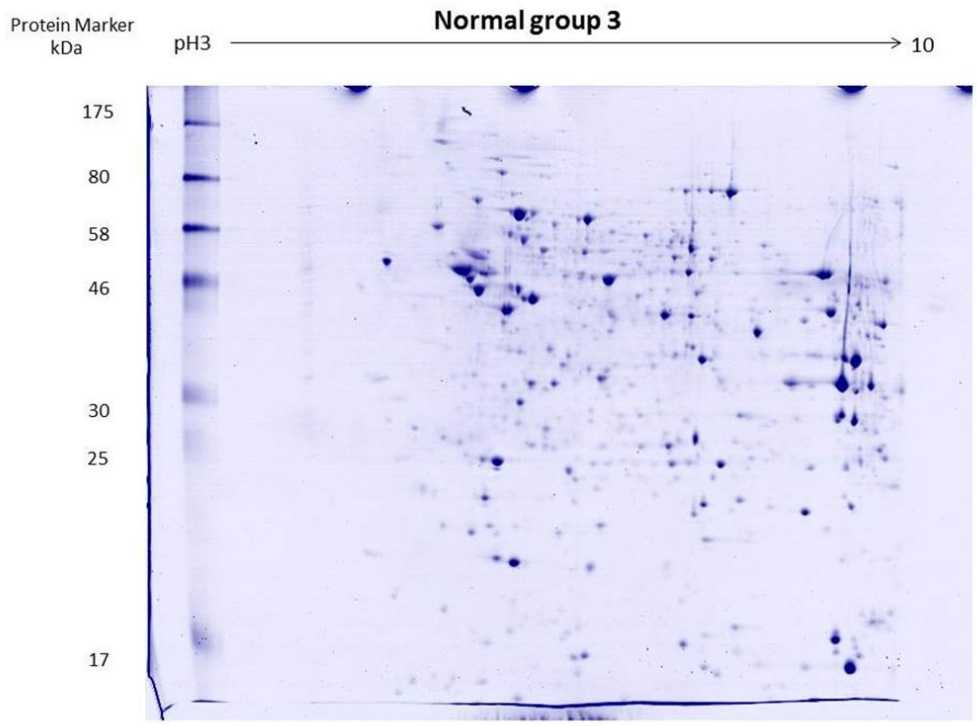

Figure 24 A. Gels showing the two-dimensional gel analysis profile of the rat hippocampus protein of control group (2 % tween 80, 1 mL/kg BW). Gels were made triplicate after that gels were stained with Coomassie blue. The image analysis of gels demonstrated a similar number and pattern distribution of spots in 2D gels by using Image Master 2D Platinum version 7.

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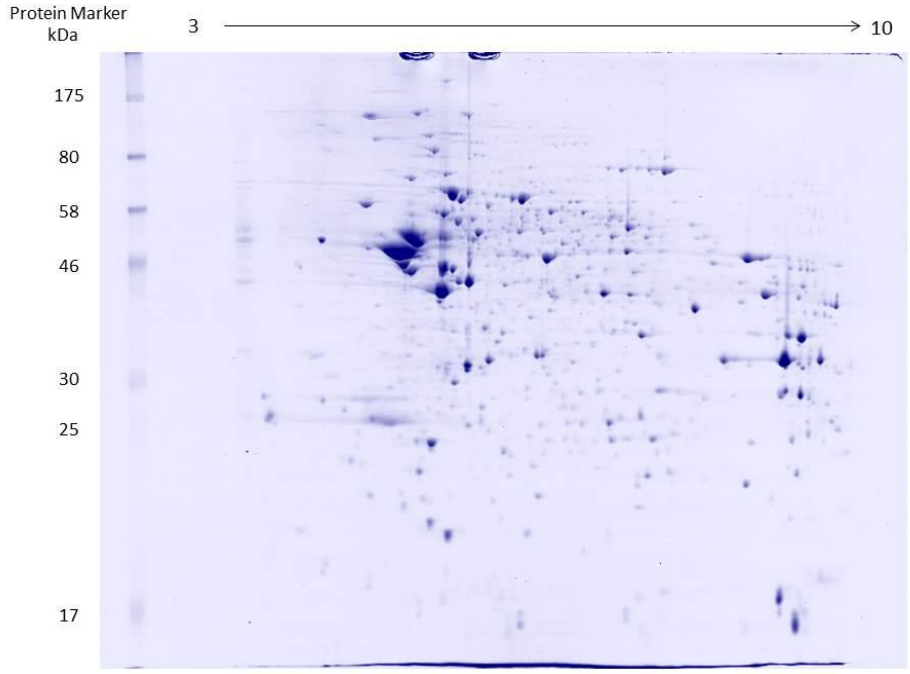
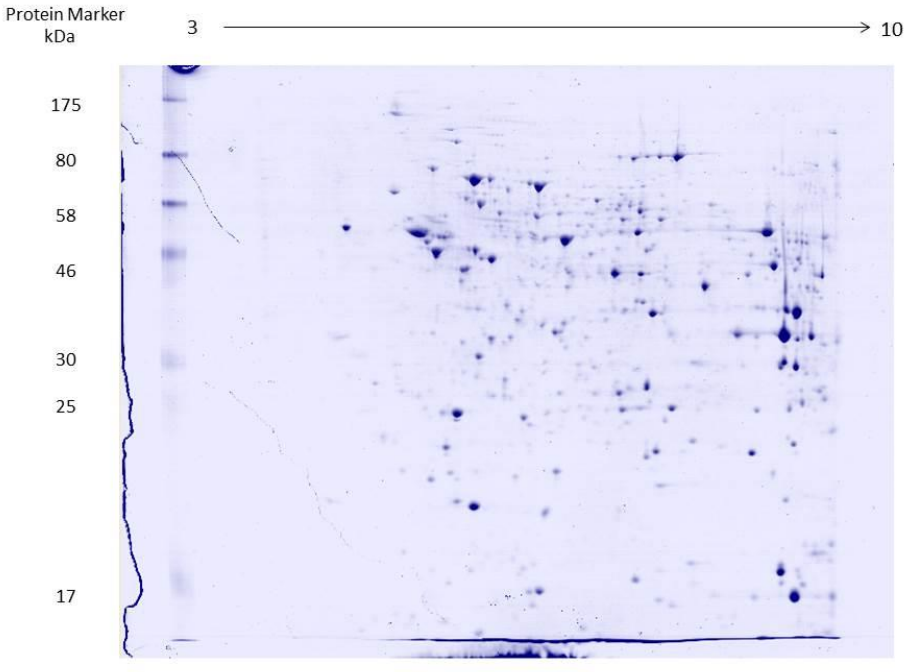
***Kaemferia parviflora* treatment group 1**



***Kaemferia parviflora* treatment group 2**



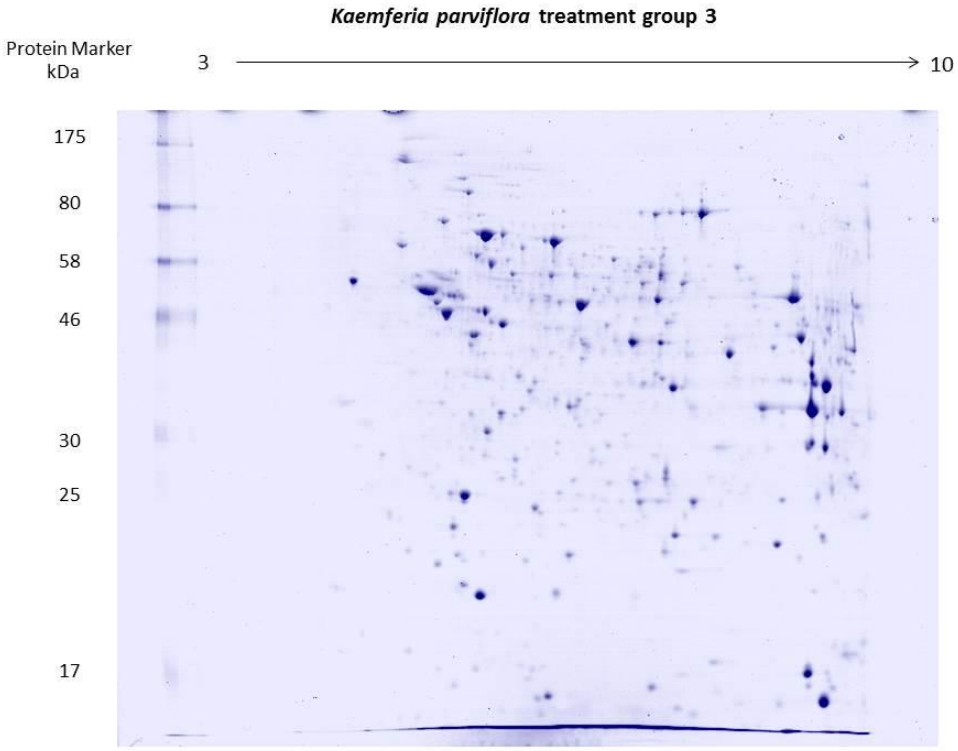


Figure 24B. Gels showing the two-dimensional gel analysis profile of the rat hippocampus protein of *K. parviflora* rhizome ethanolic extract (200 mg/kg BW). Gels were made triplicate after that gels were stained with Coomassie blue. The image analysis of gels demonstrated a similar number and pattern distribution of spots in 2D gels by using Image Master 2D Platinum version 7.

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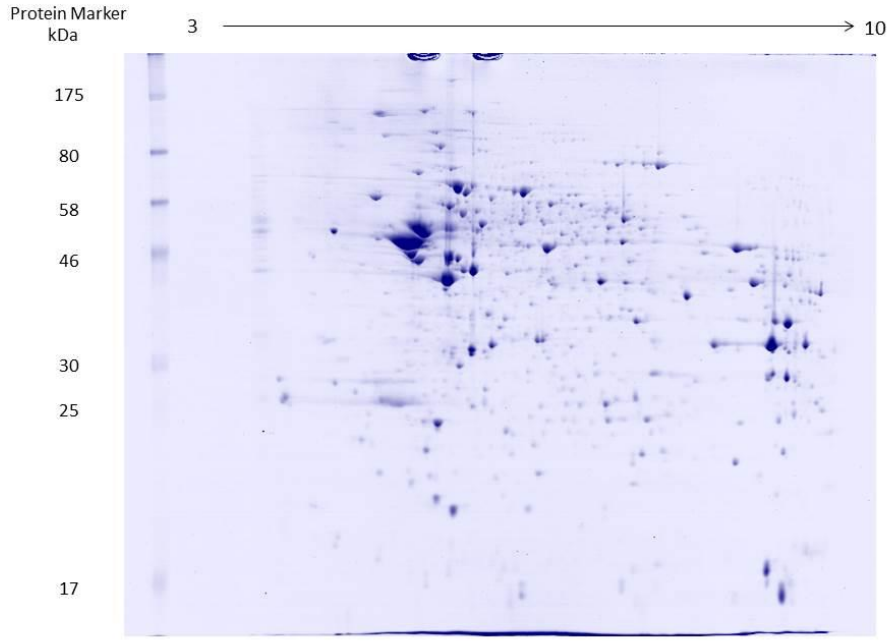
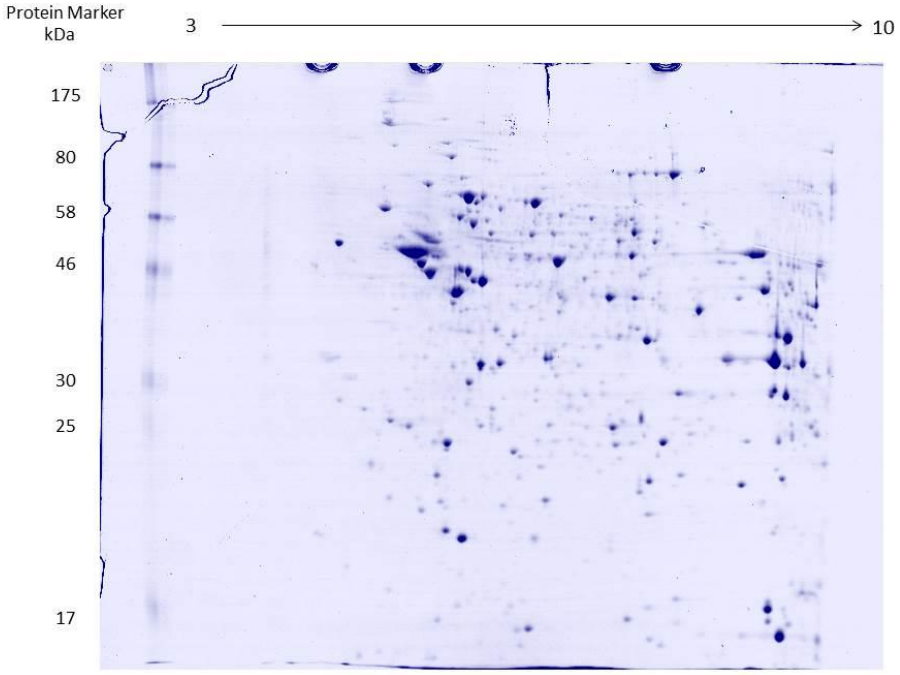
***Myristica fragrans* treatment group 1**



***Myristica fragrans* treatment group 2**



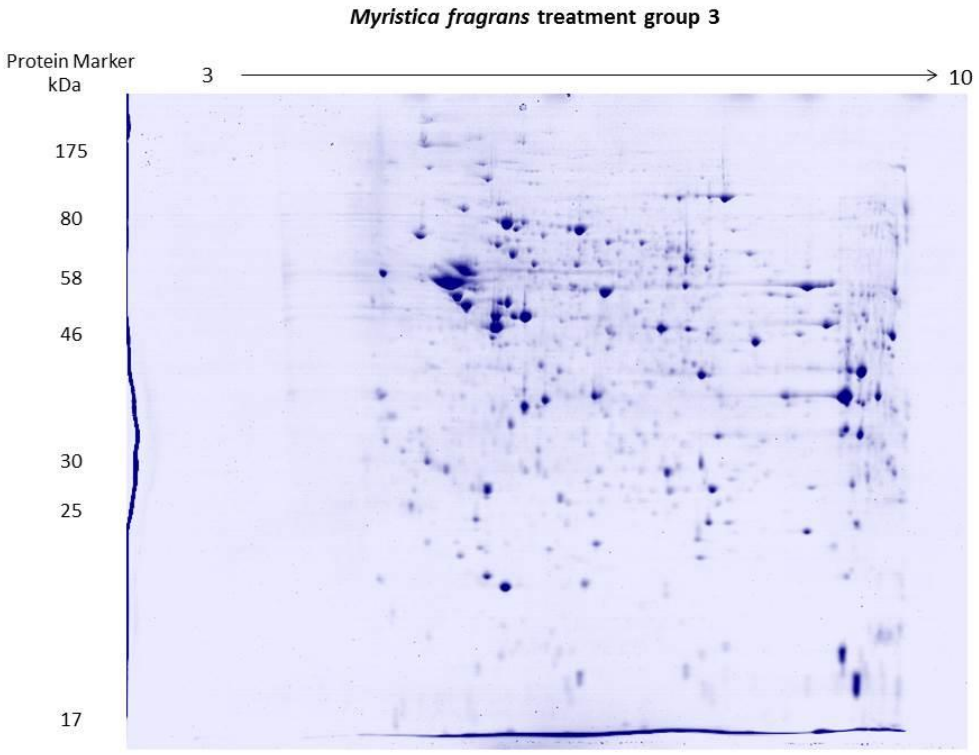


Figure 24C. Gels showing the two-dimensional gel analysis profile of the rat hippocampus protein of *M. fragrans* nutmeg volatile oil (300 mg/kg BW). Gels were made triplicate after that gels were stained with Coomassie blue. The image analysis of gels demonstrated a similar number and pattern distribution of spots in 2D gels by using Image Master 2D Platinum version 7.

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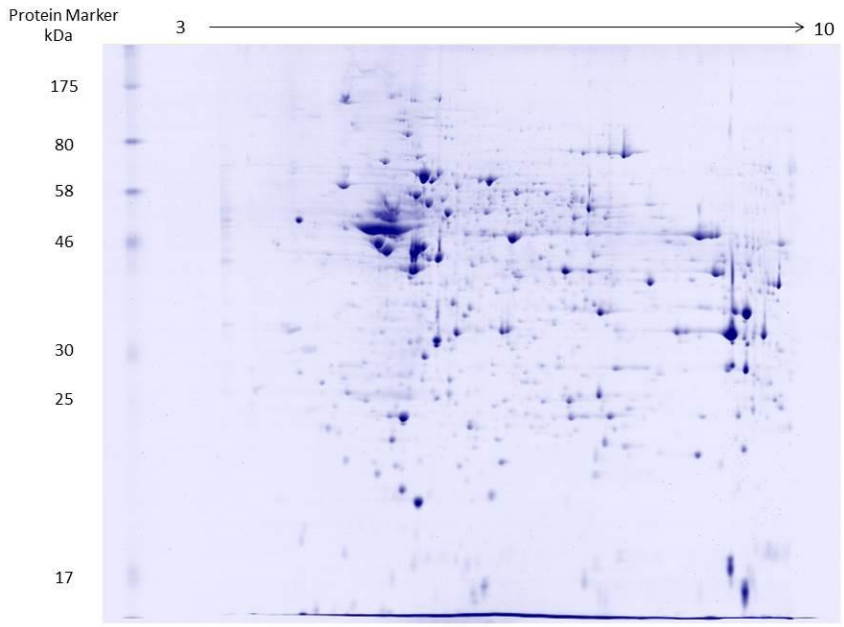
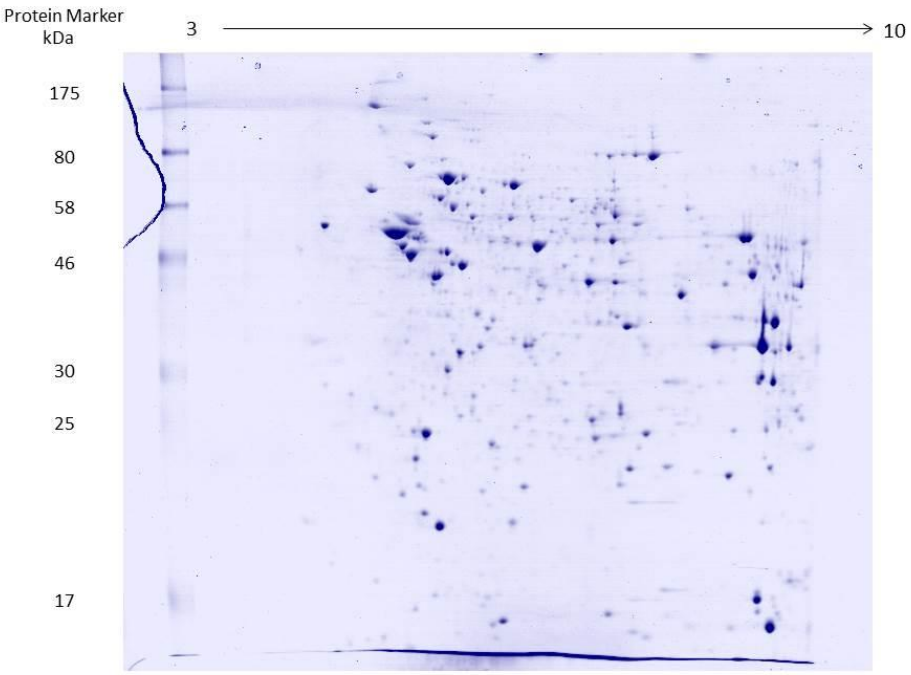
Fluoxetine treatment group 1



Fluoxetine treatment group 2



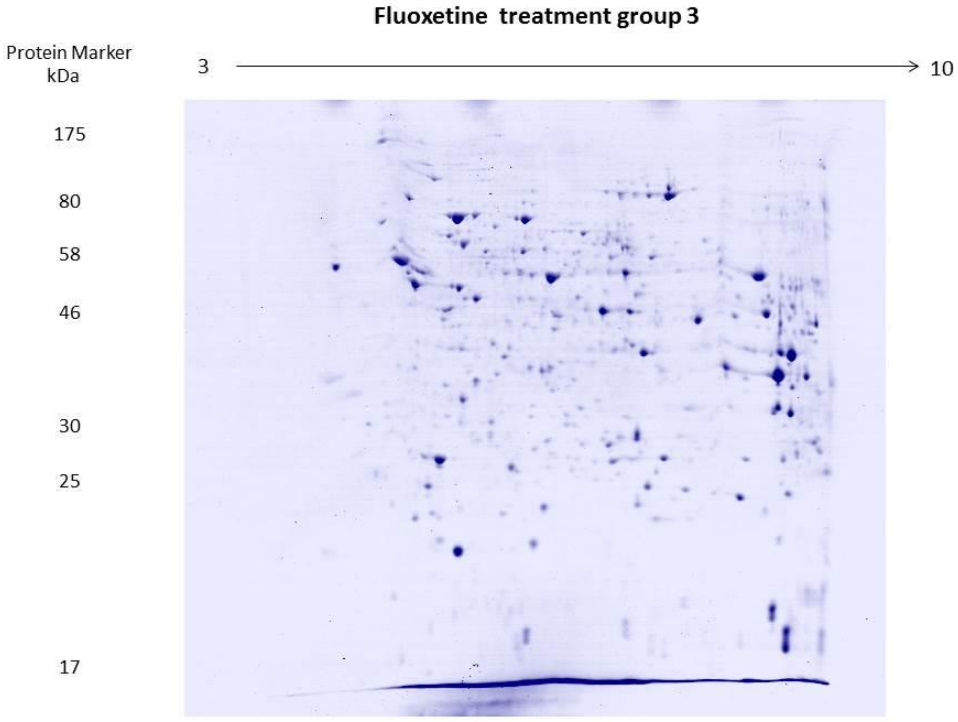


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Figure 24D. Gels showing the two-dimensional gel analysis profile of the rat hippocampus protein of Fluoxetine (20 mg/kg BW) Gels were made triplicate after that gels were stained with Coomassie blue. The image analysis of gels demonstrated a similar number and pattern distribution of spots in 2D gels by using Image Master 2D Platinum version 7.

3.4 Bioinformatics analysis

Bioinformatics analysis (SwisProt, UniProtKB, IPA and STRING-10.0) have been used for pathway analysis, functional classification and regulation of the differentially expressed proteins (see table 11). Considering their biological function, the proteins were classified by the functional process into nine different cellular or subcellular localizations. This analysis revealed that most of the entries corresponded to cytoplasm localization (47% of total), to the nucleus (14%), to the cytoskeleton (12%), to the cell membrane (8%), to the mitochondrial matrix (7%), to the mitochondrion (5%), to the mitochondrial membrane (4%), (see figure 25). Moreover, IPA analysis shows that the numerous proteins participate in the many pathways that important to nervous system development, functioning and neurological disease. The protein-protein interaction data (STRING and IPA), integrate information that provides the understanding of cellular physical and functional interactions (see figure 26). IPA analysis could demonstrate the involvement of identified proteins and their interactive pathways within a neuronal network especially important for neuro-regenerative processes such as neural plasticity (see figure 27 and table 12-17). In addition, proteins which have a potential antioxidative role, which is important for neuronal protection in the brains were up-regulated, while proteins that are rather known for their inhibitory effects on neuro-regenerative processes were down-regulated as show in table 3.

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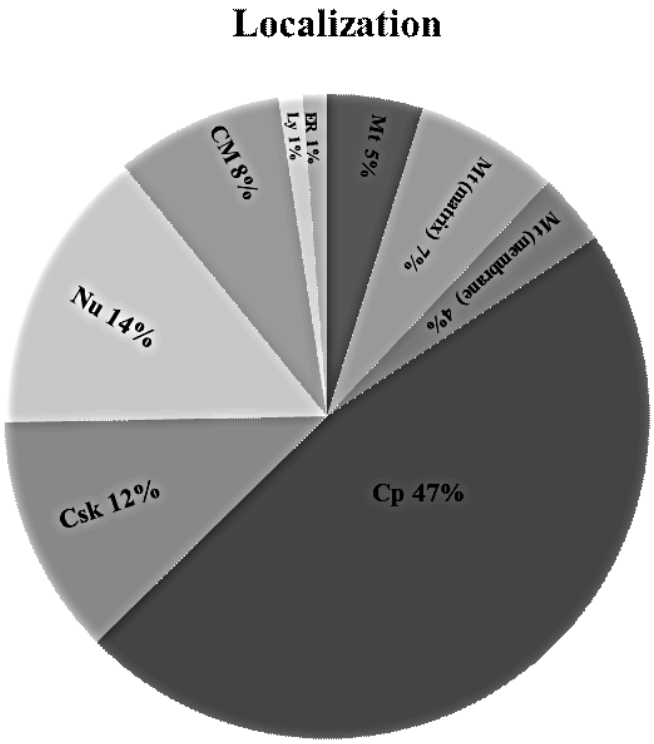

Figure 25 Pie chart represents the percentage of nine different cellular/subcellular localizations by using the Protein Knowledgebase UniProtKB.

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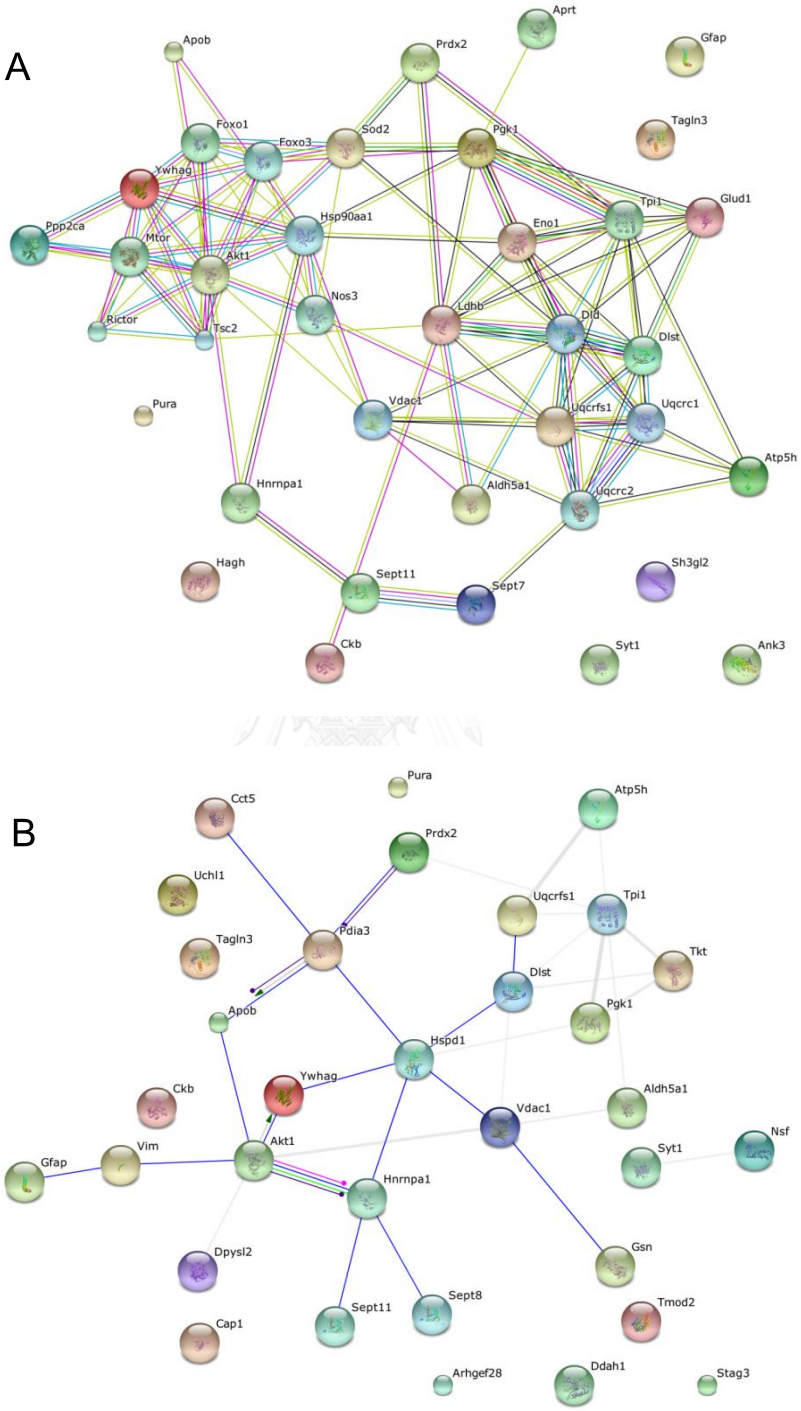
3.4.1 STRING protein–protein interaction analysis of treatments-activated proteins

STRING is a database resource dedicated to protein–protein interactions, including both physical and functional interactions. It integrates the information from many sources, such as computational prediction methods, experimental and public text collections of genomes and proteins. Thus, this analysis (default settings were used) provided us with essential neural systems-level understanding of cellular events activated by *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) and Fluoxetine (20 mg/kg BW). (see in figure 26A-C)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Aprt
Apob
Prdx2
Gfap
Foxo1
Foxo3
Sod2
Pgk1
Tagln3
Ywhag
Tpi1
Glud1
Ppp2ca
Hsp90aa1
Eno1
Mtor
Akt1
Nos3
Ldhb
Dld
Rictor
Tsc2
Dlst
Pura
Vdac1
Uqcrfs1
Uqcrc1
Atp5h
Hnrnpa1
Aldh5a1
Uqcrc2
Hagh
Sept11
Sept7
Sh3gl2
Ckb
Syt1
Ank3
B
Pura
Cct5
Atp5h
Prdx2
Uchl1
Uqcrfs1
Tpi1
Pdia3
Tagln3
Tkt
Dlst
Apob
Pgk1
Hspd1
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Vdac1
Nsf
Akt1
Vim
Syt1
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Cap1
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Ddah1
Stag3

C

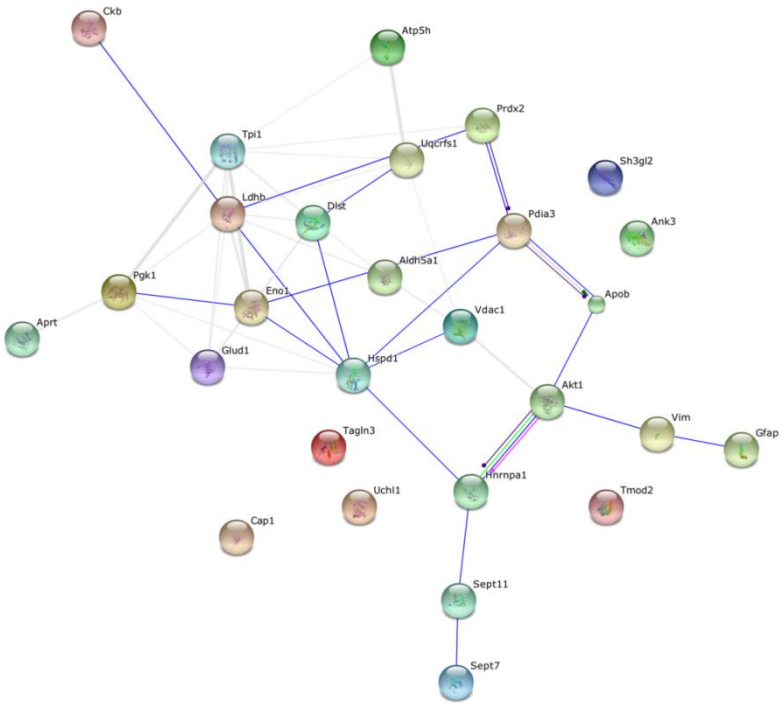

Figure 26 A-C. STRING analysis of fluoxetine, *K. parviflora* and *M. fragrans* modulated proteins in rat hippocampus. Different line colors represent the types of evidence for the association. (A) *K. parviflora* treated group, (B) *M. fragrans* treated group and (C) fluoxetine treated group.

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3.4.2 IPA signaling pathway analysis of treatments-activated proteins

Further bio-computational network analysis of *K. parviflora, M. fragrans* and fluoxetine treatment using the Ingenuity Pathways Analysis (IPA) offered us additional valuable clues about the complex interactive link of the various identified proteins within their commonly known interactive protein networks also obtained from other cellular metabolic information (Figure 27A-C and Table 12-17). Moreover, IPA analysis demonstrate the interactive pathways such as nervous system (development, function and morphology), neuronal diseases, especially important for the proper functions of active neurons in the central nervous system (CNS) during neuro-regenerative processes such as neural plasticity neural-specific. In addition, IPA could demonstrate the network among proteins from various intracellular localizations with important roles in cell survival and neuro-regeneration (Table 12-17).

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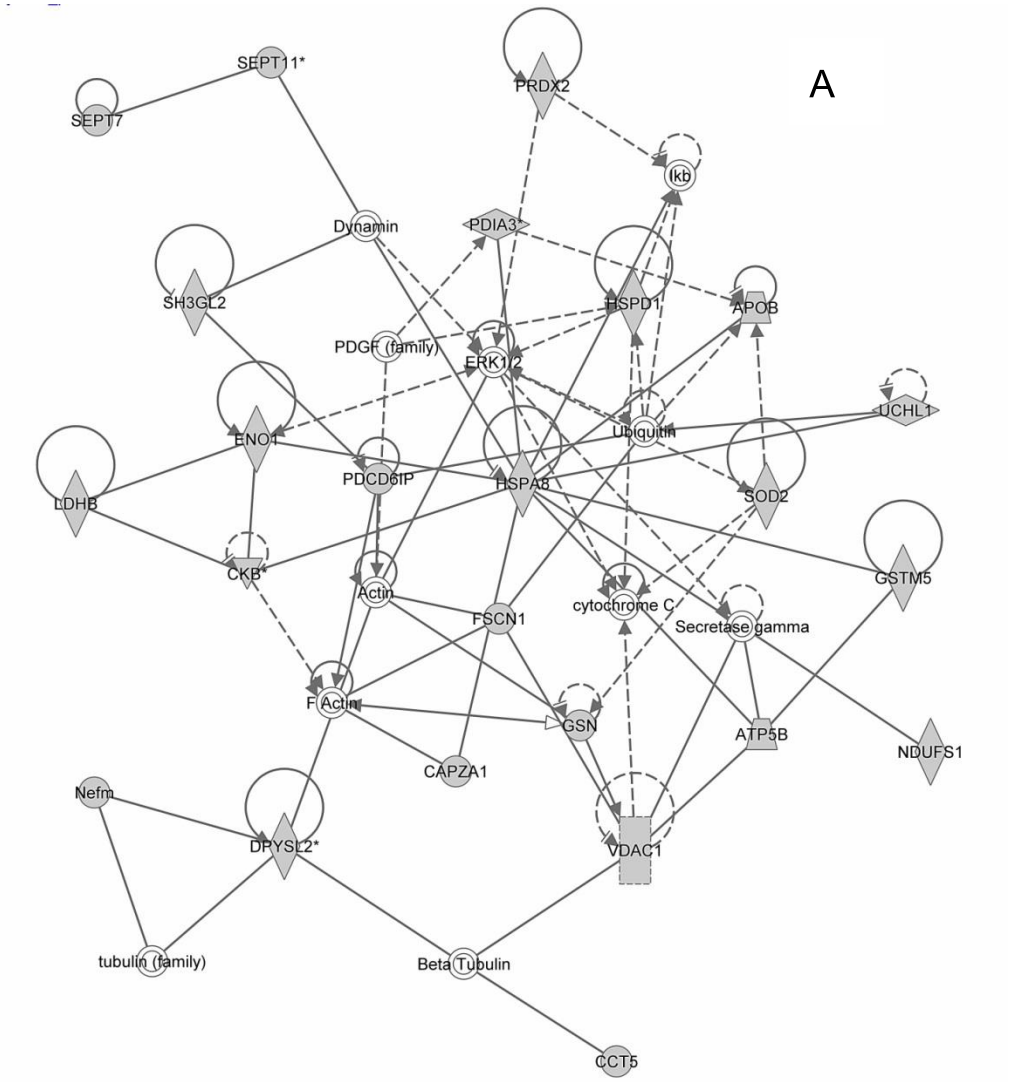

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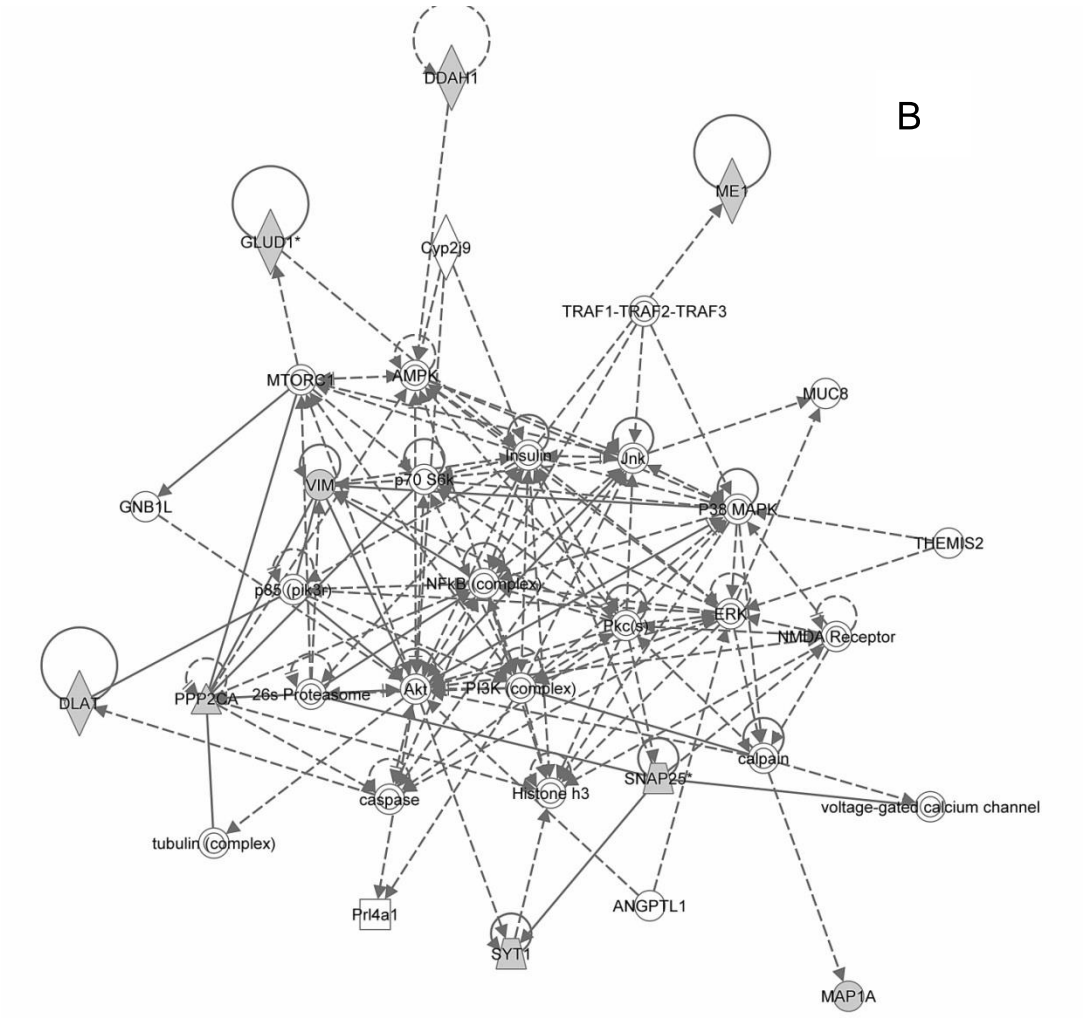

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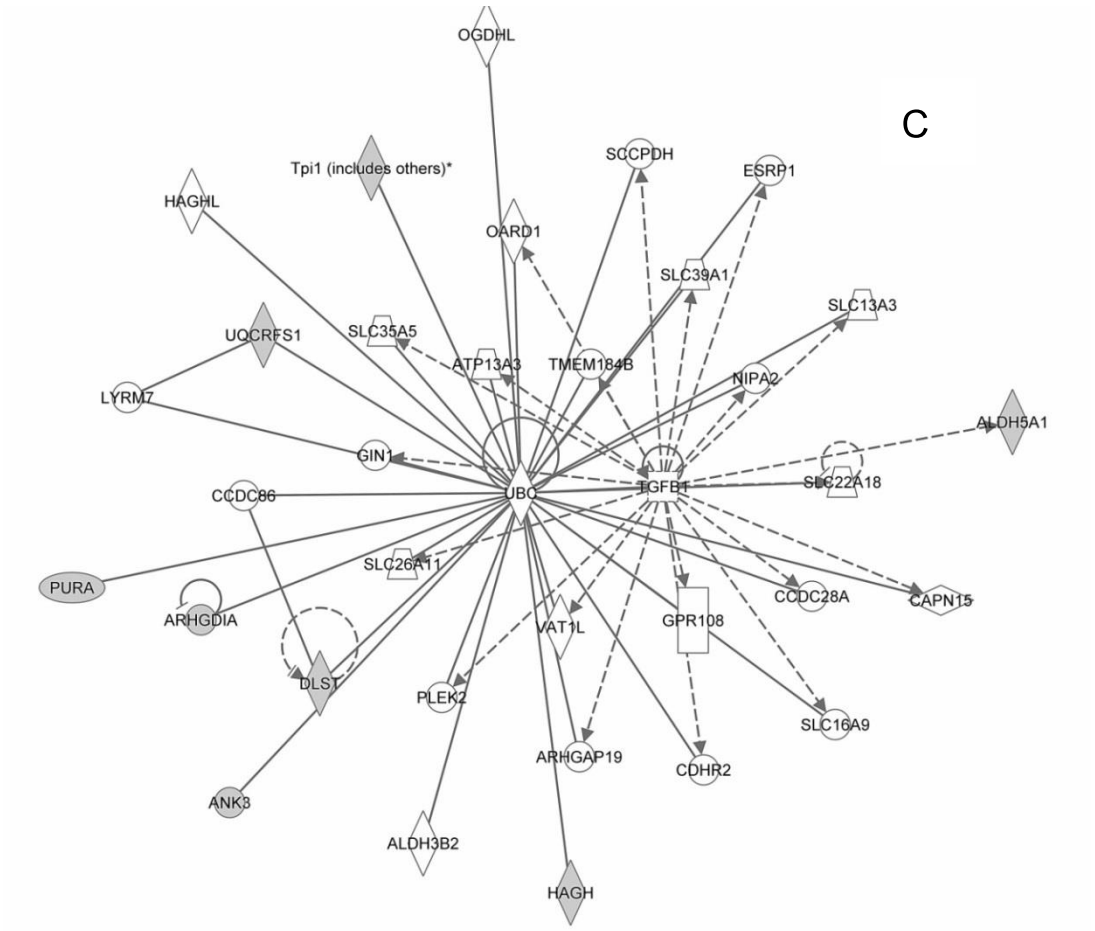

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Figure 27 A-C. IPA analysis of fluoxetine, *K. parviflora* and *M. fragrans* modulated proteins in rat hippocampus. Different line colors represent the types of evidence for the association. (A) *K. parviflora* treated group, (B) *M. fragrans* treated group and (C) fluoxetine treated group.

Table 11 Functional classification and regulation of differentially expressed proteins in treated rat hippocampal

Table 11 Functional classification and regulation of differentially expressed proteins in treated rat hippocampal

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotS core	localization	Molecular function	Regulated		
								KP	MP	Flu
12	Tagln3	Transgelin-3	8.96	24887	388	Mt (matrix)	Development of central nervous system.	↑	↓	↑
13	Atp5H	ATP synthase subunit d	6.79	18491	112	Mt	Enzyme, produces ATP from ADP.	↑	↓	↑
14	Prdx2	Peroxiredoxin-2	5.34	21941	143	Cp	Enzyme, redox regulation of the cell.	↑	↑	↑
15	Aprt	Adenine phospho-ribosyltransferase	6.17	19761	86	Cp	Enzyme, catalyzes a salvage reaction in the formation of AMP.	↓	-	↑
27	Sod2	Superoxide dismutase [Mn]	8.96	24887	299	Mt (matrix)	Enzyme, destroys superoxide anion radicals.	↑	-	-

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
32	Uqcrfs1	Cytochrome b-c1 complex subunit Rieske	9.04	29712	148	Mt (Inner membrane)	Enzyme, generates an electrochemical potential coupled to ATP synthesis.	↑	↓	↑
33	Ddir1	Rho GDP-dissociation inhibitor 1	5.12	23450	349	Cp	Controls the homeostasis of Rho proteins.	-	↓	-
59	Tpi1	Triosephosphate isomerase	6.89	27345	278	Cp	Enzyme, involved in the pathway gluconeogenesis and in Carbohydrate biosynthesis.	↑	↓	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
69	Uchl1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5.14	25165	357	Cp, ER (membrane)	Enzyme, involved both in the processing of ubiquitin precursors and of ubiquitinated proteins.	-	↑	↑
77	Gstm	Glutathione S-transferase	8.78	25360	216	Cp	Enzyme, Conjugation of reduced glutathione to exogenous and endogenous hydrophobic electrophiles.	↑	↑	↑
89	Map	Microtubule-associated protein	4.87	300831	265	Cp, Csk	Bind to the tubulin subunits that regulate microtubule stability.	↑	-	-

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
94	Hagh	Hydroxyacyl glutathione hydrolase	7.64	34544	148	Cp	Enzyme, catalyzes the hydrolysis of S-D-lactoyl-glutathione to form glutathione and D-lactic acid.	↑	-	-
96	Ywhag	Tryptophan 5-monooxygenase activation protein, gamma (14-3-3 protein gamma)	4.80	28456	503	Cp	Intracellular signaling and cell cycle.	↓	↑	-

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
105	Ank3	Ankyrin-3	7.94	285692	84	Cp, Csk, CM	Maintenance/targeting of ion channels and cell adhesion molecules at axonal initial segments and the nodes of Ranvier.	↑	-	↑
138	Vdac1	Voltage-dependent anion-selective channel protein 1	8.62	30851	330	Mt (outer membrane)	Forms a channel through the mitochondrial outer membrane and also the plasma membrane.	↑	↓	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
169	Hnrnpa1	Heterogeneous nuclear ribonucleoprotein A1	9.20	34362	89	Cp, Nu	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm.	↑	↑	↑
185	Ldhb	L-lactate dehydrogenase B chain	5.70	36874	560	Cp	Involved in the subpathway that synthesizes (S)-lactate from pyruvate.	↓	-	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
194	Ppp1B	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	5.84	37961	50	Cp, Nu	Cell division, role in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase.	↑	↑	↑
204	Syt1	Synaptotagmin-1	9.43	43385	267	Cp (vesicel)	A regulatory role in the membrane interactions of synaptic vesicles at the active synapse zone.	↑	↑	-

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
205	Akt	Serine/threonine-protein phosphatase	5.94	38229	205	Cp, Nu	Regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis.	↑	↓	↑
217	Ddah1	N(G)-dimethylarginine dimethylaminohydrolase	5.75	31805	325	Cp	A role in the regulation of nitric oxide generation, Inhibit NOS	-	↑	-
227	Ark72	Acetyl-CoA carboxylase 1	5.97	266678	95	Cp	Role in controlling fatty acid metabolism.	↑	↑	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
245	Ak1A1	Alcohol dehydrogenase 1	6.84	36711	70	Cp, Nu	Catalyzes the oxidation of long-chain primary alcohols and the oxidation of S-(hydroxymethyl) glutathione.	↑	↓	↑
251	LOC102548564	Peptidyl-prolyl cis-trans isomerase D	6.73	41139	426	Cp, Nu	Catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.	↑	-	↑
262	Tmod2	Tropomodulin 2	5.34	39468	211	Cp, Csk	Blocks the elongation and depolymerization of the actin filaments at the pointed end.	-	↑	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
274	Pura	Transcriptional activator protein Pur-alpha (Fragments)	4.69	15370	223	Nu	Role in the initiation of DNA replication and in recombination.	↑	↓	-
300	Sh3gl2	Endophilin-A1	5.26	40045	397	Cp, CM	Implicated in synaptic vesicle endocytosis.	↓	-	↓
317	Pgk1	Phosphoglycerate kinase 1	8.02	44909	78	Cp	Role as a glycolytic enzyme.	↑	↑	↑
331	Gfap	Glial fibrillary acidic protein	5.35	49984	362	Cp	Role in astrocyte-neuron and many important processes in CNS such as development of CNS.	↑	↑	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
345	Ckb	Creatine kinase B-type	5.39	42983	285	Cp	Catalyzes the transfer of phosphate between ATP and various phosphogens.	↓	↑	↓
354	Eftu	Elongation factor Tu,mitochondrial	7.23	49890	53	Mt	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes in protein synthesis.	-	↓	↓
359	Sept7	Septin-7	8.82	50818	393	Nu, Cp	Normalize the organization of the actin cytoskeleton, role in ciliogenesis and collective cell movements.	↑	-	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
361	Sept11	Septin-11	6.24	50005	442	Cp, Csk	Play important role in cytokinesis, cytoarchitecture of neurons (dendritic arborization and dendritic spines), and in GABAergic synaptic connectivity.	↑	↓	↑
366	Aldh5a1	Succinate-semialdehyde dehydrogenase	8.35	56723	606	Mt	Catalyzes the degradation of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA).	↓	↓	↓
396	Eno1	Alpha-enolase	6.16	47440	302	Cp, CM	Play role in glycolysis.	↑	-	↑

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
401	Dlst	dihydrolipoyllysine-residue succinyltransferase	8.76	67637	37	Mt (matrix)	Convert 2-oxoglutarate to succinyl-CoA and CO2.	↑	↑	↑
403	Glud1	Glutamate dehydrogenase 1	8.05	61719	850	Mt (matrix)	Converts L-glutamate into alpha-ketoglutarate and increas the turnover of the excitatory neurotransmitter glutamate	↑	-	↑
442	Tba4a	Tubulin alpha-4A chian	4.95	50634	52	Cp, Csk	A major constituent of microtubules	↓	↑	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
450	Cap1	Adenylyl cyclase-associated protein 1	7.61	51899	207	CM	Regulates filament dynamics and play role in a number of complex developmental and morphological processes, (mRNA localization and cell polarity).	↑	↑	↑
452	Pdia3	Protein disulfide-isomerase A3	4.74	64400	367	ER	Plays a role in protein folding by promoting the formation of disulfide bonds.	↑	↑	↑
456	Vim	Vimentin	5.06	53757	644	Nu (matrix)	The major IF protein in mesenchymal cells.	-	↑	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
464	Hspd1	60 kDa heat shock protein	5.91	61088	143	Mt (matrix)	Prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides.	-	↑	↑
465	Dpysl2	dihydropyrimidinase-related protein 2	5.95	62638	535	Cp, Csk, CM	Involved in neuronal differentiation and axonal guidance.	↑	↑	↑
490	Cct5	T-complex protein 1 subunit epsilon	5.51	59955	918	Cp, Csk, MT	Molecular chaperone, help the folding of proteins upon ATP hydrolysis.	↓	↑	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
559	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit	8.51	51499	1345	Mt (Inner membrane)	Role in the transfer of electrons from NADH to the respiratory chain.	↓	-	↓
574	Gsn	Gelsolin	5.76	86413	287	Cp, Csk	Promote the assembly of monomers into filaments.	↓	↓	↓
581	Pdcd6ip	Programmed cell death 6-interacting protein	6.15	97141	221	MT	Play a role in the regulation of both apoptosis and cell proliferation	↓	-	-
641	Pura	Transcriptonal activator protein Pur-alpha	4.69	15370	90	Nu	Play important role in the initiation of DNA replication and recombination.	↑	↓	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
687	Sept8	Septin 8	5.74	51562	45	Cp, Csk	Role in cytokinesis	↑	↑	↑
703	Me1	NADP-dependent malic enzyme	6.49	64589	139	Cp	Generates NADPH for fatty acid biosynthesis and reverse malate to pyruvate.	-	-	↓
819	Stag3	Cohesin subunit SA-3	5.81	143628	34	Nu	The component of cohesin complex.	↑	↑	↑
846	Hyou1	Hypoxia up-regulated protein 1	8.73	63702	37	Cp, Nu	Role in cytoprotective, mechanisms triggered by oxygen deprivation.	-	-	↓
848	Nsf	Vesicle-fusing ATPase	6.55	83170	189	Cp	Catalyzes the fusion of transport vesicles within the Golgi cisternae	↓	↓	↓

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
849	Dldh	Dihydrolipoyl dehydrogenase	7.96	54574	48	Mt (matrix)	A component of the glycine cleavage system and alpha-ketoacid dehydrogenase complexes	↑	↑	↓
873	Hnrpm	Heterogeneous nuclear ribonucleoprotein M	8.90	74076	86	Nu (matrix)	Important role in p53/TP53 response to DNA damage.	↓	-	↓
882	Tkt	Transketolase	7.23	68342	68	Ly	Catalyzes the transfer of a two-carbon ketol group from a ketose donor to an aldose acceptor	↑	↑	_

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
890	Arhgef28	Rho guanine nucleotide exchange factor 28	5.47	192748	165	Cp, CM	Functions in axonal branching, synapse formation and dendritic morphogenesis. Regulate numerous cellular responses such as proliferation, differentiation and movement.	↑	↑	↓
918	Bin1	Myc box-depent interacting protein 1	4.95	64721	49	Cp, Nu	Role in the regulation of synaptic vesicle endocytosis.	↑	↑	-

Spot No.	Gene symbol	Proteins Name	*pI*	MW (Da)	MascotScore	localization	Molecular function	Regulated		
								KP	MP	Flu
960	Sptn1	Spectrin alpha chain	5.20	285261	81	Cp, Csk	Role in secretion and interaction of calmodulin in a calcium-dependent manner, candidate for the calcium-dependent movement of the cytoskeleton.	↓	↓	↓
1046	Snap25	Synaptosomal-associated protein 25	4.66	23528	100	Cp, CM	Important role in the synaptic function including molecular regulation of neurotransmitter release, vesicle docking and membrane fusion.	↑	-	-

The table reports the list of spots, SwissProt protein, full name, theoretical *pI* and MW (Da), molecular function and protein regulation. The main localization: Cp, Cytoplasm; Nu, Nucleus; Csk, Cytoskeleton; CM, Cell Membrane; Mt, Mitochondrion; Ly, Lysosome; ER, Endoplasmic Reticulum.

Table 12 **The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora*.**

Ingenuity Canonical Pathways	Molecules
14-3-3-mediated Signaling	GFAP,PDCD6IP
Creatine-phosphate Biosynthesis	CKB
Thymine Degradation	DPYSL2
4-aminobutyrate Degradation I	ALDH5A1
Uracil Degradation II (Reductive)	DPYSL2
Glutamate Degradation III (via 4-aminobutyrate)	ALDH5A1
Pentose Phosphate Pathway (Non-oxidative Branch)	TKT
Telomere Extension by Telomerase	HNRNPA1
Signaling by Rho Family GTPases	SEPT8,GFAP
Pentose Phosphate Pathway	TKT
Mechanisms of Viral Exit from Host Cells	PDCD6IP
Glycolysis I	PGK1
Gluconeogenesis I	PGK1
Semaphorin Signaling in Neurons	DPYSL2
GABA Receptor Signaling	ALDH5A1
Apoptosis Signaling	SPTAN1
Crosstalk between Dendritic Cells and Natural Killer Cells	FSCN1
Death Receptor Signaling	SPTAN1

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Table 12 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* (continue).

Ingenuity Canonical Pathways	Molecules
Oxidative Phosphorylation	NDUFS1
RhoA Signaling	SEPT8
Atherosclerosis Signaling	APOB
LXR/RXR Activation	APOB
FXR/RXR Activation	APOB
IL-12 Signaling and Production in Macrophages	APOB
Aryl Hydrocarbon Receptor Signaling	ALDH5A1
Tight Junction Signaling	SPTAN1
Dendritic Cell Maturation	FSCN1
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	APOB
Sertoli Cell-Sertoli Cell Junction Signaling	SPTAN1
Mitochondrial Dysfunction	NDUFS1
Clathrin-mediated Endocytosis Signaling	APOB
LPS/IL-1 Mediated Inhibition of RXR Function	ALDH5A1
Xenobiotic Metabolism Signaling	ALDH5A1
Axonal Guidance Signaling	DPYSL2

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Table 13 The **IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *M. fragran***

Ingenuity Canonical Pathways	Molecules
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	DLAT,DLD
Glycolysis I	PGK1,Tpi1 (includes others)
Aldosterone Signaling in Epithelial Cells	HSPA8,PDIA3,HSPD1
Gluconeogenesis I	PGK1,ME1
GABA Receptor Signaling	NSF,ALDH5A1
Protein Ubiquitination Pathway	UCHL1,HSPA8,HSPD1
TR/RXR Activation	ACACA,ME1
Regulation of Actin-based Motility by Rho	ARHGDIA,GSN
PRPP Biosynthesis I	PRPS1
Glutamate Biosynthesis II	GLUD1
Glutamate Degradation X	GLUD1
Creatine-phosphate Biosynthesis	CKB
2-ketoglutarate Dehydrogenase Complex	DLD
Branched-chain α-keto acid Dehydrogenase Complex	DLD
Adenine and Adenosine Salvage I	APRT
Thymine Degradation	DPYSL2
4-aminobutyrate Degradation I	ALDH5A1
14-3-3-mediated Signaling	PDIA3,VIM
Uracil Degradation II (Reductive)	DPYSL2
Glutamate Degradation III (via 4-aminobutyrate)	ALDH5A1

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Table 13 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *M. fragran* (continue).

Ingenuity Canonical Pathways	Molecules
Glycine Cleavage Complex	DLD
Biotin-carboxyl Carrier Protein Assembly	ACACA
Parkinson's Signaling	UCHL1
2-oxobutanoate Degradation I	DLD
Sucrose Degradation V (Mammalian)	Tpi1 (includes others)
Dendritic Cell Maturation	PDIA3,FSCN1
Arginine Biosynthesis IV	GLUD1
Clathrin-mediated Endocytosis Signaling	HSPA8,SH3GL2
Lipid Antigen Presentation by CD1	PDIA3
Isoleucine Degradation I	DLD
Valine Degradation I	DLD
Huntington's Disease Signaling	HSPA8,NSF
Signaling by Rho Family GTPases	VIM,SEPT11
Retinoate Biosynthesis I	ADH1C
Antigen Presentation Pathway	PDIA3
Mechanisms of Viral Exit from Host Cells	SH3GL2
TCA Cycle II (Eukaryotic)	DLD
Ethanol Degradation II	ADH1C
Noradrenaline and Adrenaline Degradation	ADH1C
Semaphorin Signaling in Neurons	DPYSL2
Unfolded protein response	HSPA8

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Table 13 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *M. fragran* (continue).

Ingenuity Canonical Pathways	Molecules
Wnt/Ca+ pathway	PDIA3
Superpathway of Methionine Degradation	DLD
Phospholipases	PDIA3
Melatonin Signaling	PDIA3
Serotonin Degradation	ADH1C
Leptin Signaling in Obesity	PDIA3
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	PDIA3
RANK Signaling in Osteoclasts	GSN
UVA-Induced MAPK Signaling	PDIA3
Crosstalk between Dendritic Cells and Natural Killer Cells	FSCN1
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	PDIA3
Antioxidant Action of Vitamin C	PDIA3
Neuropathic Pain Signaling In Dorsal Horn Neurons	PDIA3
Axonal Guidance Signaling	DPYSL2,PDIA3
Type I Diabetes Mellitus Signaling	HSPD1
Sphingosine-1-phosphate Signaling	PDIA3
RhoA Signaling	SEPT11
p70S6K Signaling	PDIA3
Synaptic Long Term Potentiation	PDIA3

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Table 13 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *M. fragran* (continue).

Ingenuity Canonical Pathways	Molecules
LXR/RXR Activation	ACACA
P2Y Purigenic Receptor Signaling Pathway	PDIA3
Cellular Effects of Sildenafil (Viagra)	PDIA3
Aryl Hydrocarbon Receptor Signaling	ALDH5A1
AMPK Signaling	ACACA
Synaptic Long Term Depression	PDIA3
Glioblastoma Multiforme Signaling	PDIA3
eNOS Signaling	HSPA8
Germ Cell-Sertoli Cell Junction Signaling	GSN
Tight Junction Signaling	NSF
Gap Junction Signaling	PDIA3
Dopamine-DARPP32 Feedback in cAMP Signaling	PDIA3
CREB Signaling in Neurons	PDIA3
RhoGDI Signaling	ARHGDIA
RAR Activation	ADH1C
PPARα/RXRα Activation	PDIA3
Endothelin-1 Signaling	PDIA3
Role of NFAT in Cardiac Hypertrophy	PDIA3
ILK Signaling	VIM
Thrombin Signaling	PDIA3
Actin Cytoskeleton Signaling	GSN

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Table 13 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with *M. fragran* (continue).

Ingenuity Canonical Pathways	Molecules
LPS/IL-1 Mediated Inhibition of RXR Function	ALDH5A1
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	GSN
Cardiac Hypertrophy Signaling	PDIA3
Glucocorticoid Receptor Signaling	HSPA8
Xenobiotic Metabolism Signaling	ALDH5A1
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	PDIA3
Protein Kinase A Signaling	PDIA3

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Table 14 **The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine.**

Ingenuity Canonical Pathways	Molecules
Mitochondrial Dysfunction	NDUFS1,SOD2,ATP5B,UQCRFS1, VDAC1
14-3-3-mediated Signaling	PDIA3,VIM,PDCD6IP
Oxidative Phosphorylation	NDUFS1,ATP5B,UQCRFS1
Mechanisms of Viral Exit from Host Cells	SH3GL2,PDCD6IP
Glycolysis I	ENO1,Tpi1 (includes others)
Gluconeogenesis I	ENO1,ME1
Aldosterone Signaling in Epithelial Cells	HSPA8,PDIA3,HSPD1
Clathrin-mediated Endocytosis Signaling	HSPA8,APOB,SH3GL2
Huntington's Disease Signaling	HSPA8,ATP5B,SNAP25
Signaling by Rho Family GTPases	SEPT7,VIM,SEPT11
Glutamate Biosynthesis II	GLUD1
Glutamate Degradation X	GLUD1
Protein Ubiquitination Pathway	UCHL1,HSPA8,HSPD1
TR/RXR Activation	ENO1,ME1
Regulation of Actin-based Motility by Rho	ARHGDIA,GSN
Creatine-phosphate Biosynthesis	CKB
Methylglyoxal Degradation I	HAGH

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Table 14 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine (continue).

Ingenuity Canonical Pathways	Molecules
Superoxide Radicals Degradation	SOD2
Xenobiotic Metabolism Signaling	GSTM5,PPP2CA,ALDH5A1
2-ketoglutarate Dehydrogenase Complex	DLST
Pyruvate Fermentation to Lactate	LDHB
Thymine Degradation	DPYSL2
4-aminobutyrate Degradation I	ALDH5A1
Uracil Degradation II (Reductive)	DPYSL2
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	DLAT
Glutamate Degradation III (via 4-aminobutyrate)	ALDH5A1
RhoA Signaling	SEPT7,SEPT11
p70S6K Signaling	PPP2CA,PDIA3
Parkinson's Signaling	UCHL1
Aryl Hydrocarbon Receptor Signaling	GSTM5,ALDH5A1
Synaptic Long Term Depression	PPP2CA,PDIA3
Sucrose Degradation V (Mammalian)	Tpi1 (includes others)
Tight Junction Signaling	PPP2CA,SNAP25
Dopamine-DARPP32 Feedback in cAMP Signaling	PPP2CA,PDIA3
Arginine Biosynthesis IV	GLUD1
Dendritic Cell Maturation	PDIA3,FSCN1
NRF2-mediated Oxidative Stress Response	SOD2,GSTM5

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Table 14 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine (continue).

Ingenuity Canonical Pathways	Molecules
Lipid Antigen Presentation by CD1	PDIA3
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	APOB,PPP2CA
ILK Signaling	PPP2CA,VIM
Cell Cycle Regulation by BTG Family Proteins	PPP2CA
LPS/IL-1 Mediated Inhibition of RXR Function	GSTM5,ALDH5A1
Antigen Presentation Pathway	PDIA3
Glutathione-mediated Detoxification	GSTM5
TCA Cycle II (Eukaryotic)	DLST
Semaphorin Signaling in Neurons	DPYSL2
Unfolded protein response	HSPA8
Role of CHK Proteins in Cell Cycle Checkpoint Control	PPP2CA
Wnt/Ca+ pathway	PDIA3
Mitotic Roles of Polo-Like Kinase	PPP2CA
Phospholipases	PDIA3
PEDF Signaling	SOD2
GABA Receptor Signaling	ALDH5A1
Melatonin Signaling	PDIA3
Leptin Signaling in Obesity	PDIA3
Cyclins and Cell Cycle Regulation	PPP2CA

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Table 14 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine (continue).

Ingenuity Canonical Pathways	Molecules
Ceramide Signaling	PPP2CA
HIPPO signaling	PPP2CA
CTLA4 Signaling in Cytotoxic T Lymphocytes	PPP2CA
RANK Signaling in Osteoclasts	GSN
UVA-Induced MAPK Signaling	PDIA3
Crosstalk between Dendritic Cells and Natural Killer Cells	FSCN1
Dopamine Receptor Signaling	PPP2CA
Telomerase Signaling	PPP2CA
CDK5 Signaling	PPP2CA
Antioxidant Action of Vitamin C	PDIA3
HIF1α Signaling	LDHB
Neuropathic Pain Signaling In Dorsal Horn Neurons	PDIA3
Type I Diabetes Mellitus Signaling	HSPD1
Sphingosine-1-phosphate Signaling	PDIA3
Axonal Guidance Signaling	DPYSL2,PDIA3
Atherosclerosis Signaling	APOB
Synaptic Long Term Potentiation	PDIA3
LXR/RXR Activation	APOB
PI3K/AKT Signaling	PPP2CA
P2Y Purigenic Receptor Signaling Pathway	PDIA3

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Table 14 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine (continue).

Ingenuity Canonical Pathways	Molecules
PI3K Signaling in B Lymphocytes	PDIA3
Sperm Motility	PDIA3
FXR/RXR Activation	APOB
IL-12 Signaling and Production in Macrophages	APOB
Cardiac β-adrenergic Signaling	PPP2CA
Cellular Effects of Sildenafil (Viagra)	PDIA3
AMPK Signaling	PPP2CA
Regulation of eIF4 and p70S6K Signaling	PPP2CA
Glioblastoma Multiforme Signaling	PDIA3
eNOS Signaling	HSPA8
Germ Cell-Sertoli Cell Junction Signaling	GSN
Gap Junction Signaling	PDIA3
Wnt/β-catenin Signaling	PPP2CA
Acute Phase Response Signaling	SOD2
CREB Signaling in Neurons	PDIA3
RhoGDI Signaling	ARHGDIA
PPARα/RXRα Activation	PDIA3
Endothelin-1 Signaling	PDIA3
Role of NFAT in Cardiac Hypertrophy	PDIA3
ERK/MAPK Signaling	PPP2CA

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Table 14 The IPA conical pathway analysis classification of differentially expressed proteins in rat hippocampal treated with Fluoxetine (continue).

Ingenuity Canonical Pathways	Molecules
mTOR Signaling	PPP2CA
Thrombin Signaling	PDIA3
Breast Cancer Regulation by Stathmin1	PPP2CA
Actin Cytoskeleton Signaling	GSN
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	GSN
Cardiac Hypertrophy Signaling	PDIA3
Glucocorticoid Receptor Signaling	HSPA8
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	PDIA3
Protein Kinase A Signaling	PDIA3

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Table 15 **Functional classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* from IPA**

Category	Molecules
Neurological Disease	ANK3,PGK1,DPYSL2,APOB,HYOU1, CKB,NDUFS1,HNRNPA1,CCT5,GFAP,SPTAN1,PURA,CA1,ALDH5A1,PRDX2
Nervous System Development and Function	CKB,DPYSL2,ANK3,APOB,TAGLN3, FSCN1,TKT,GFAP,SPTAN1,PURA, ALDH5A1
Organ Morphology	ANK3,CKB,APOB,TKT,GFAP,PURA, PRDX2
Tissue Morphology	ANK3,DPYSL2,CKB,APOB,FSCN1,TKT, GFAP,PURA,PRDX2
Organismal Development	ANK3,CKB,APOB,PURA,PRDX2
Cell Morphology	ANK3,CKB,DPYSL2,NDUFS1,FSCN1, GFAP,SPTAN1,PURA,PDCD6IP,PRDX2
Infectious Disease	SEPT8,APOB,TKT,FSCN1,SPTAN1,PURA,PDCD6IP,CA1,ALDH5A1,PRDX2
Cancer	ANK3,PGK1,DPYSL2,APOB,HYOU1,CKB,NDUFS1,HNRNPA1,FSCN1,STAG3, CCT5,GFAP,SPTAN1,PDCD6IP,PURA, CA1,ALDH5A1,PRDX2
Cell Death and Survival	ANK3,APOB,HNRNPA1,HYOU1,CCT5, GFAP,PDCD6IP,PURA,PRDX2
Cell-To-Cell Signaling and Interaction	ANK3,CKB,DPYSL2,APOB,HYOU1, CCT5,GFAP,SPTAN1,PDCD6IP, ALDH5A1

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Table 15 Functional classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* from IPA (continue)

Category	Molecules
Cellular Assembly and Organization	CKB,ANK3,DPYSL2,NDUFS1,FSCN1,STAG 3,CCT5,GFAP,SPTAN1,PDCD6IP,PURA
Cellular Compromise	ANK3,DPYSL2,HNRNPA1,SPTAN1, PDCD6IP,CA1
Cellular Development	ANK3,DPYSL2,TAGLN3,HNRNPA1,FSCN1, GFAP, SPTAN1,PURA,PRDX2
Cellular Function and Maintenance	CKB,ANK3,DPYSL2,NDUFS1,FSCN1, HYOU1,GFAPSPTAN1,PDCD6IP
Connective Tissue Disorders	PGK1,HNRNPA1,GFAP,PURA,CA1,PRDX2
Developmental Disorder	ANK3,CKB,NDUFS1,APOB,HNRNPA1,TKT, GFAP,PURA,CA1,ALDH5A1
Drug Metabolism	PGK1,NDUFS1,GFAP,ALDH5A1
Hematological Disease	PGK1,APOB,TKT,CA1,PRDX2
Hereditary Disorder	ANK3,CKB,PGK1,NDUFS1,APOB,HNRNPA 1,CCT5,GFAP,SPTAN1,CA1,ALDH5A1, PRDX2
Inflammatory Disease	CKB,PGK1,APOB,HNRNPA1,TKT,GFAP, PURA,CA1,PRDX2
Metabolic Disease	DPYSL2,NDUFS1,APOB,HNRNPA1,HYOU1, GFAP,CA1,ALDH5A1,PRDX2
Nucleic Acid Metabolism	PGK1,TKT
Organismal Injury and Abnormalities	ANK3,PGK1,APOB,TKT,HYOU1,CKB, NDUFS1,CCT5,GFAP,SPTAN1,PURA,CA1,A LDH5A1,PRDX2

Table 15 Functional classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* from IPA (continue)

Category	Molecules
Renal and Urological System Development and Function	TKT,PDCD6IP
Reproductive System Disease	PGK1,ANK3,CKB,SEPT8,NDUFS1, APOB,CCT5,GFAP,SPTAN1,PDCD6IP, PURA,CA1
Skeletal and Muscular Disorders	CKB,PGK1,HNRNPA1,GFAP,PURA,CA1, PRDX2
Small Molecule Biochemistry	PGK1,CKB,DPYSL2,NDUFS1,APOB, TKT,GFAP,PDCD6IP,ALDH5A1,PRDX2
Tissue Development	ANK3,DPYSL2,APOB,FSCN1,GFAP, SPTAN1,PDCD6IP,PURA
Hematological System Development and Function	DPYSL2,HYOU1,PURA,PRDX2
Hematopoiesis	PURA,PRDX2
Cardiovascular Disease	PGK1,APOB,TKT,HYOU1,CA1, ALDH5A1,PRDX2
Amino Acid Metabolism	CKB,DPYSL2,ALDH5A1
Cardiovascular System Development and Function	ANK3,APOB,TKT,HYOU1,GFAP,PRDX2
Hepatic System Development and Function	APOB,TKT
Lipid Metabolism	APOB,PDCD6IP,ALDH5A1,PRDX2
Molecular Transport	DPYSL2,NDUFS1,APOB,GFAP,PDCD6I, PRDX2
Tumor Morphology	HNRNPA1,HYOU1

Table 15 Functional classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* from IPA (continue)

Category	Molecules
Cell Cycle	SPTAN1,PURA
Connective Tissue Development and Function	APOB,TKT,PURA
Embryonic Development	DPYSL2,ANK3,APOB,FSCN1, PDCD6IP,PURA
Free Radical Scavenging	NDUFS1,APOB,PRDX2
Protein Trafficking	ANK3,APOB
Visual System Development and Function	APOB,TKT
Inflammatory Response	DPYSL2,CKB,APOB,TKT,HYOU1, GFAP,CA1
Cellular Growth and Proliferation	DPYSL2,PGK1,ANK3,TAGLN3, HNRNPA1,FSCN1,CCT5,GFAP, SPTAN1,PURA,PRDX2
DNA Replication, Recombination, and Repair	HNRNPA1,STAG3,PURA
RNA Post-Transcriptional Modification	HNRNPA1
Nutritional Disease	APOB,HNRNPA1,CA1,ALDH5A1, PRDX2
Reproductive System Development and Function	APOB,CCT5
Respiratory Disease	TKT,CA1,PRDX2
Gastrointestinal Disease	DPYSL2,ANK3,APOB,HNRNPA1, PRDX2
Hepatic System Disease	APOB
Ophthalmic Disease	GFAP,CA1
Immunological Disease	PGK1,HNRNPA1,TKT,GFAP,CA1, PRDX2
Dermatological Diseases and Conditions	FSCN1,CCT5,SPTAN1

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Table 15 Functional classification of differentially expressed proteins in rat hippocampal treated with *K. parviflora* from IPA (continue)

Category	Molecules
Organismal Survival	ANK3,APOB,TKT,FSCN1,HYOU1,GFAP, PURA,ALDH5A1
Respiratory System Development and Function	PDCD6IP
Organismal Functions	ANK3,PURA
Cellular Movement	DPYSL2,ANK3,APOB,FSCN1,HYOU1,GFAP, PRDX2
Organ Development	ANK3,APOB,GFAP
Endocrine System Disorders	HYOU1,CA1,PRDX2
Skeletal and Muscular System Development and Function	ANK3,APOB
Behavior	CKB
Protein Synthesis	APOB,ALDH5A1
Renal and Urological Disease	CA1
Post-Translational Modification	GFAP
Cell Signaling	APOB
Hair and Skin Development and Function	FSCN1,PDCD6IP
Lymphoid Tissue Structure and Development	PURA,PRDX2
Vitamin and Mineral Metabolism	APOB
Digestive System Development and Function	APOB
Immune Cell Trafficking	DPYSL2,APOB,HYOU1,GFAP
Cell-mediated Immune Response	DPYSL2,HYOU1
Gene Expression	HNRNPA1

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Table 16 **Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis.**

Category	Molecules
Amino Acid Metabolism	CKB,DDAH1,GLUD1,ME1,ALDH5A1
Small Molecule Biochemistry	PGK1,CA2,PDIA3,HYOU1,ADH1C,VIM, GLUD1,HSPD1,ME1,GSN,APRT,HSPA8,CK B,NSF,PRPS1,DLAT,DDAH1,DLD,ACACA,A LDH5A1
Cell Morphology	DPYSL2,Nefm,PDIA3,VIM,SH3GL2,SEPT11, HSPD1,GSN,BIN1,CKB,UCHL1,NSF,FSCN1, DDAH1,DLD,ARHGEF28,ACACA, ARHGDIA
Cellular Assembly and Organization	DPYSL2,Nefm,PDIA3,VIM,SH3GL2,SEPT11, GSN,BIN1,CKB,UCHL1,HSPA8,NSF,FSCN1, DDAH1,ARHGEF28,ACACA,ARHGDIA, TMOD2
Cellular Function and Maintenance	DPYSL2,CA2,Nefm,PDIA3,HYOU1,VIM, SH3GL2,SEPT11,HSPD1,ME1,GSN,BIN1, CKB,HSPA8,UCHL1,NSF,FSCN1,DDAH1, DLD,ARHGEF28,ACACA,ARHGDIA, TMOD2
Organismal Injury and Abnormalities	PGK1,CA2,Nefm,ADH1C,HYOU1,VIM, SH3GL2,GSN,BIN1,APRT,CKB,UCHL1, PRPS1,DLD,ARHGDIA, CA1,ALDH5A1
Reproductive System Disease	UCHL1,CKB,CA2,CA1

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Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Cellular Movement	HSPA8,DPYSL2,NSF,PDIA3,FSCN1, HYOU1,VIM,SEPT11,SH3GL2,HSPD1, GSN,BIN1
Nervous System Development and Function	DPYSL2,Nefm,PDIA3,VIM,SEPT11, SH3GL2,GSN,BIN1,HSPA8,CKB, UCHL1,NSF,FSCN1,DDAH1, ARHGEF28,TMOD2
Lipid Metabolism	HSPA8,PDIA3,DLAT,DLD,ADH1C,VI M,ACACA, ME1,GSN,ALDH5A1
Nucleic Acid Metabolism	PGK1,DPYSL2,HSPA8,NSF,PRPS1, DLAT,DLD,ACACA,HSPD1,ME1, APRT
Neurological Disease	TUFM,PGK1,DPYSL2,CA2,Nefm, PDIA3,ADH1C,HYOU1,VIM,ME1, SH3GL2,HSPD1,GSN,BIN1, HSPA8,CKB,UCHL1,NSF,PRPS1, DLAT,CA1,ALDH5A1
Renal and Urological Disease	CA2,DLD,ARHGDIA,CA1,APRT
Cellular Development	DPYSL2,CA2,Nefm,PDIA3,VIM,ME1, SEPT11,HSPD1,GSN,BIN1,UCHL1, HSPA8,FSCN1,DDAH1, ARHGEF28,ACACA
Tissue Development	UCHL1,DPYSL2,NSF,Nefm,PDIA3, FSCN1,DDAH1, VIM,ARHGEF28,ARHGDIA,ACACA, SEPT11,GSN,BIN1

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Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Developmental Disorder	CA2,PRPS1,DLD,VIM,GLUD1,ARHGDIA, ACACA,GSN,BIN1,CA1,APRT,ALDH5A1
Hereditary Disorder	TUFM,PGK1,CA2,GLUD1,VIM,HSPD1,GSN, BIN1,APRT,HSPA8,CKB,UCHL1,PRPS1, DLAT,DLD,ACACA,ARHGDIA,CA1, ALDH5A1
Skeletal and Muscular Disorders	PGK1,CA2,NONO,PDIA3,ADH1C,VIM, HSPD1,SH3GL2,GSN,BIN1,UCHL1,CKB, HSPA8,PRPS1,CA1
Psychological Disorders	DPYSL2,PGK1,CA2,ADH1C,VIM,ME1, SH3GL2,HSPD1,GSN,BIN1,HSPA8,CKB, UCHL1,NSF,DLAT,CA1,ALDH5A1
Metabolic Disease	TUFM,DPYSL2,CA2,HYOU1,GLUD1,VIM,S H3GL2,HSPD1,GSN,BIN1,APRT,UCHL1,NSF ,PRPS1,DLAT,DDAH1,DLD,ARHGDIA, ACACA,CA1,ALDH5A1
Cellular Compromise	DPYSL2,Nefm,HYOU1,VIM,SH3GL2,SEPT11 ,HSPD1,GSN,BIN1,UCHL1,DLD,ARHGEF28, ARHGDIA,CA1
Cell-To-Cell Signaling and Interaction	DPYSL2,Nefm,PDIA3,VIM,HSPD1,SH3GL2, GSN,BIN1,UCHL1,CKB,NSF,ARHGDIA, TMOD2,ALDH5A1

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Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Cancer	DPYSL2,CA2,PDIA3,HYOU1,ADH1C,ME1, SH3GL2,BIN1,UCHL1,CKB,NSF,PRPS1,DDAH1, CA1,ALDH5A1,PGK1,NONO,GLUD1,VIM, HSPD1,GSN,HNRNPM,HSPA8,DLAT,FSCN1, DLD,ARHGEF28,ARHGDIA,ACACA
Gastrointestinal Disease	DPYSL2,PGK1,CA2,ADH1C,HYOU1,GLUD1, VIM,HSPD1,SH3GL2,ME1,GSN,BIN1,HNRNPM, HSPA8,UCHL1,NSF,PRPS1,FSCN1,DLAT, DDAH1,DLD,ARHGEF28,ARHGDIA,ACACA, CA1
Hepatic System Disease	PGK1,CA2,ADH1C,GLUD1,VIM,ACACA, SH3GL2,BIN1
Cell Signaling	DDAH1,HYOU1,HSPD1,GSN,CAPZA1
Post-Translational Modification	HSPA8,PDIA3,DLD,GLUD1,ACACA,ME1, HSPD1,CAPZA1,GSN,ALDH5A1
Protein Synthesis	TUFM,UCHL1,PDIA3,DLD,ARHGDIA,ACACA, SEPT11,ME1,HSPD1,CAPZA1,GSN,ALDH5A1
Tissue Morphology	DPYSL2,CKB,Nefm,FSCN1,HYOU1,VIM, ARHGDIA,SH3GL2,GSN,BIN1
Inflammatory Disease	PGK1,CA2,NONO,PDIA3,VIM,HSPD1,GSN, APRT,UCHL1,HSPA8,CKB,ARHGDIA,CA1

Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Cellular Growth and Proliferation	DPYSL2,PGK1,Nefm,PDIA3,VIM,HSPD1, SH3GL2,GSN,BIN1,HNRNPM,HSPA8, UCHL1,FSCN1,DDAH1,DLD,ARHGDIA, ACACA,CAPZA1
Organ Morphology	VIM,ARHGDIA,GSN,APRT
Renal and Urological System Development and Function	ARHGDIA,ALDH5A1,APRT
Infectious Disease	CA2,PRPS1,FSCN1,HSPD1,CA1,ALDH5A1, HNRNPM
Ophthalmic Disease	CA2,HSPD1,GSN,CA1
Nutritional Disease	CA2,VIM,CA1
Immunological Disease	HSPA8,PGK1,CA2,NONO,PDIA3,VIM, HSPD1,GSN,CA1
Respiratory Disease	CKB,CA2,ADH1C,VIM,HSPD1,GSN,CA1
Cardiovascular Disease	PGK1,CA2,HYOU1,VIM,ARHGDIA,ACACA, HSPD1,GSN,BIN1,CA1,ALDH5A1
Organismal Development	UCHL1,PGK1,DDAH1,DLD,VIM,ACACA, ARHGDIA,GSN,BIN1,APRT
Protein Folding	HSPA8,HSPD1
Connective Tissue Disorders	HSPA8,PGK1,CA2,NONO,PDIA3,VIM, HSPD1,GSN,CA1

Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Organismal Survival	PDIA3,ADH1C,HYOU1,VIM,SH3GL2,GSN, BIN1,APRT,FSCN1,DDAH1,DLD,ARHGDI A,ACACA,ALDH5A1
Inflammatory Response	DPYSL2,CKB,CA2,PDIA3,HYOU1,VIM, ARHGDIA,HSPD1,GSN,BIN1,CA1,APRT
Cell Death and Survival	TUFM,Nefm,PDIA3,HYOU1,GLUD1,VIM, HSPD1,SH3GL2,GSN,BIN1,APRT,UCHL1, HSPA8,NSF,PRPS1,ARHGDIA,ACACA
Endocrine System Disorders	UCHL1,CKB,CA2,NSF,DDAH1,HYOU1, GLUD1,VIM,ACACA,HSPD1,CA1
Hematological Disease	PGK1,CA2,NONO,VIM,GLUD1,ARHGDIA, GSN,CA1
Protein Trafficking	ARHGDIA,GSN
Cardiovascular System Development and Function	PGK1,NSF,DDAH1,HYOU1,VIM, ARHGDIA,HSPD1,GSN,BIN1
Auditory Disease	PRPS1
DNA Replication, Recombination, and Repair	PDIA3,VIM
Drug Metabolism	HSPA8,PGK1,NSF,PDIA3,ADH1C,GSN
Molecular Transport	HSPA8,NSF,CA2,ADH1C,VIM,ACACA, GSN,APRT
Reproductive System Development and Function	NSF

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Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Tumor Morphology	UCHL1,HYOU1,VIM
Embryonic Development	UCHL1,DPYSL2,Nefm,PDIA3,FSCN1, DLD,VIM,ACACA,SEPT11,GSN,BIN1
Dermatological Diseases and Conditions	HSPA8,FSCN1,VIM,ARHGDIA,ME1, GSN
Hematological System Development and Function	HSPA8,DPYSL2,HYOU1,HSPD1,GSN, BIN1
Connective Tissue Development and Function	VIM,ACACA,ARHGDIA,ME1,GSN
Carbohydrate Metabolism	PGK1,PRPS1,Tpi1 (includes others), GSN
Cell Cycle	VIM
Digestive System Development and Function	VIM,ACACA
Energy Production	ADH1C,ACACA,HSPD1,ME1
Organ Development	UCHL1,VIM,GSN,BIN1
Skeletal and Muscular System Development and Function	UCHL1,NSF,VIM,GSN,BIN1
Vitamin and Mineral Metabolism	HSPA8,ADH1C,VIM
Organismal Functions	HYOU1,VIM,GSN
Free Radical Scavenging	GSN
Protein Degradation	UCHL1,PDIA3,DLD,HSPD1,GSN
Hepatic System Development and Function	ACACA

Table 16 Functional classification of differentially expressed proteins in rat hippocampal treated with *M. fragrans* from IPA analysis (continue)

Category	Molecules
Humeral Immune Response	HSPD1
Immune Cell Trafficking	DPYSL2,HYOU1,HSPD1
Cell-mediated Immune Response	DPYSL2,HYOU1,HSPD1
RNA Post-Transcriptional Modification	APRT
Lymphoid Tissue Structure and Development	ARHGDIA
Endocrine System Development and Function	UCHL1,VIM
Hair and Skin Development and Function	VIM,APRT
Behavior	CKB

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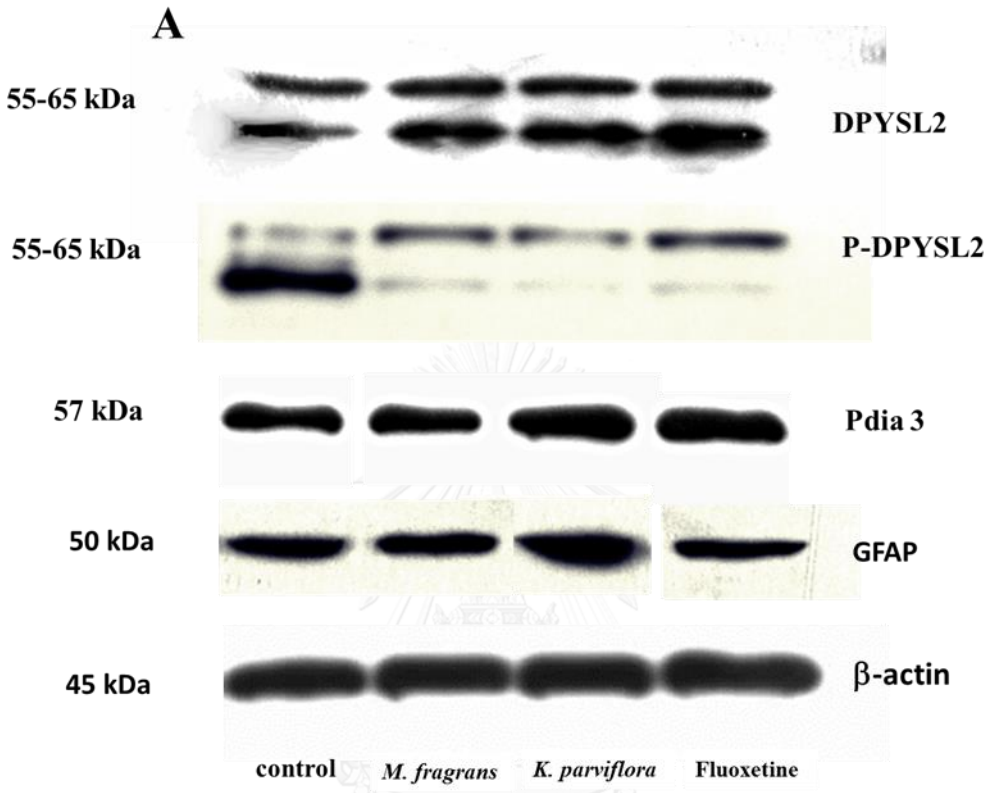
3.5 Validation level of protein differences from 2D results by western blot analysis.

We validated the expression level of protein by choosing from representative of down and upregulation in 2D gels. Western blot analysis was performed with specific antibodies which detect bands of PDIA3 at 57 kDa, GFAP at 50 kDa, DPYSL2 and p-DPYSL2 at 55-65 kDa. The intensity of bands was normalized by β-actin and the value was obtained in sample groups, which were normalized to the corresponding value in control group. Semi-quantitative analysis of the western blot confirmed the tendency observed in the 2D gel image analysis. Representative western blot of hippocampal samples were presented in figure 28A. In detail, GFAP protein level was significantly increased in hippocampus of *K. parviflora* and *M. fragrans* treated groups but non-significant in fluoxetine-treated rat when compared to control group (figure 28B). The PDIA3 level was significantly increased in *M. fragrans* and fluoxetine but irrelevant in *K. parviflora* group (see in figure 28C). The statistical analysis demonstrated significant differences of DPYSL2 and p-DPYSL2 between experimental and control groups (see in figure 28D, 28E). The expression level of DPYSL2 was exhibited increasingly in two herbal groups when compared with the control group (figure 28D). However, no difference was observed in fluoxetine treated group. In this study we are interested in p-DPYSL2 as a role protein in neuronal development. Western blotting revealed that p-DPYSL2 was significantly decreased in experimental group compared to control group

(figure 28E). The expression level of proteins was in accordance with the data from proteomics analysis.



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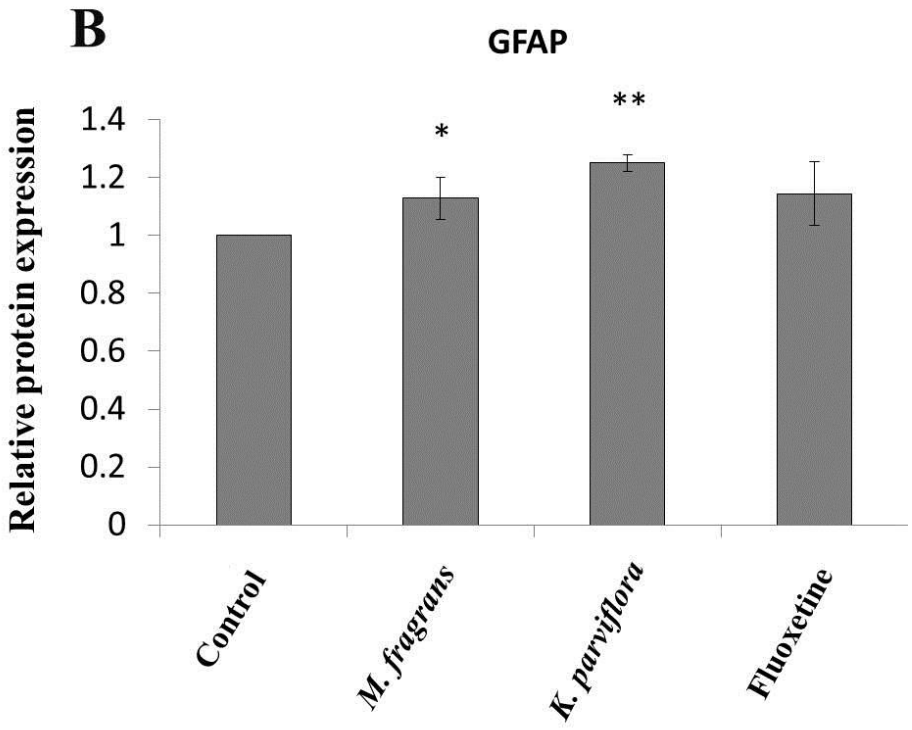
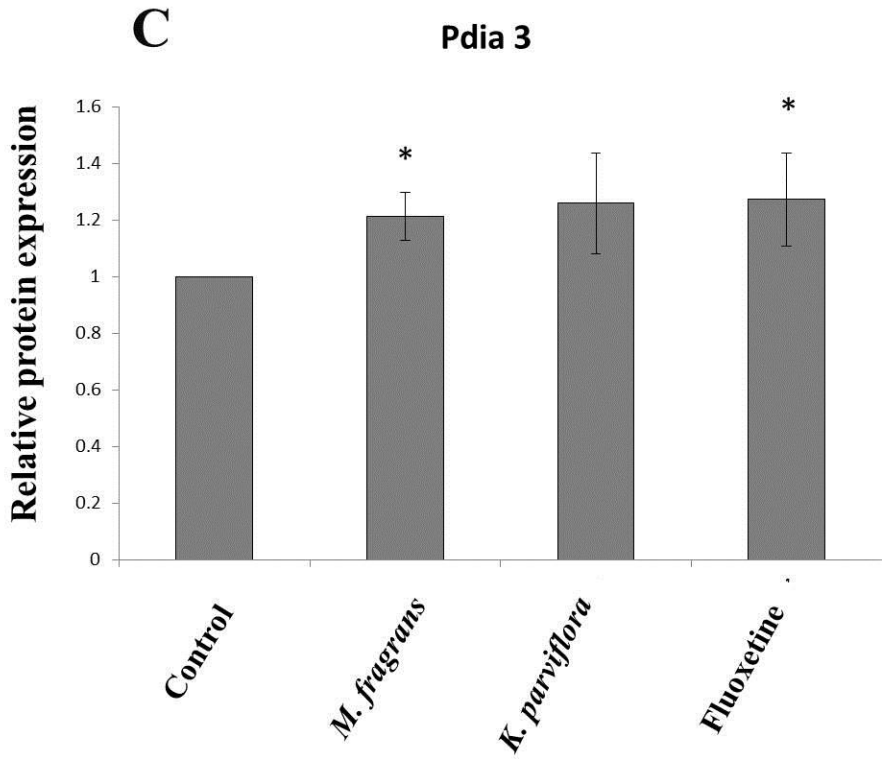

B
GFAP
Relative protein expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
Control
M. fragrans
K. parviflora
Fluoxetine


C
Pdia 3
Relative protein expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
M. fragrans
K. parviflora
Fluoxetine

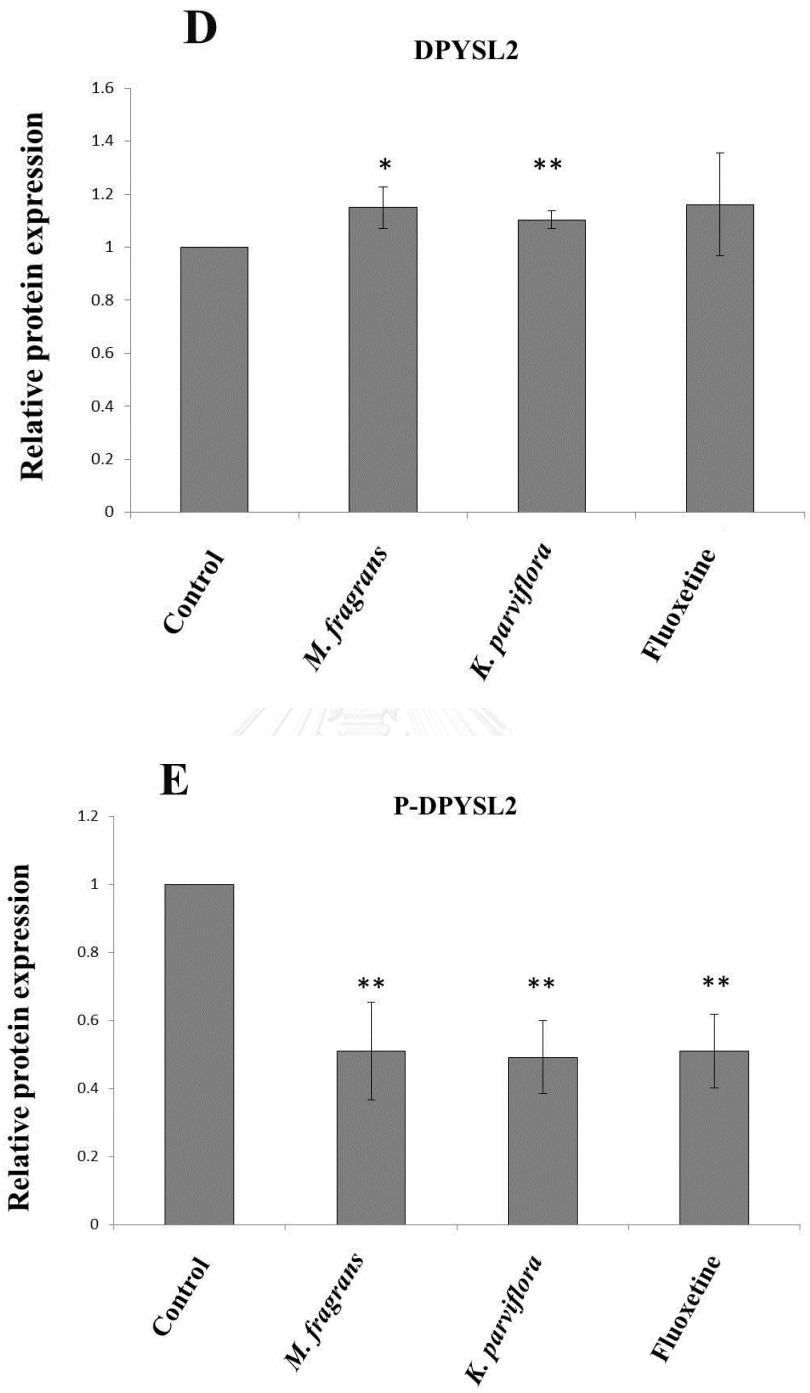


Figure 28 (A-E) Representative image of western blots evaluating expression of GFAP, PDIA3, DPYSL2 and p-DPYSL2 from rat hippocampal. Data are expressed as the mean± standard deviation (S.D.), statistically significant difference analyzed by *t-test* (P<0.05).

3.6 Effects of treatments on some haematological parameters and chemistry values.

The results about haematology showed in table 17 in which the mean, SD and SEM of WBC (NEU, LYM, EO, BASO MONO), RBC, HGB, HCT, MCV, MCH, MCHC, PLT, RDW, PDW, MPV and PCT. Eash treatments group were compared with the control. The statistical significance was taken at a *P*-value less than 0.05 by using ANOVA. A significant increase was observed in percentage of neutrophil of fluoxetine (6.483±1.356) group when compare with control (10.860±1.770), while no difference in herbals groups. However, a non-significant increase and decrease in another haematological parameters respectively when compared to the control (see in table 17). The results can summarize that *K. parviflora*, *M. fragrans* and fluoxetine showed non-cytotoxicity to blood cells. Moreover, this study has shown non changes of blood morphology both RBC and WBC.

The effect of *K. parviflora*, *M. fragrans* and fluoxetine on various chemistry values is presented in Table 18. The data represent the mean, SD and SEM of Billi-T, ALP, TP, ALB, BUN, CREA, ALT, AST, URIC and GLOB. Increases or decreases in parameters were statistically significant ($p < 0.05$) by using ANOVA. fluoxetine (20 mg/ml) did not cause significant changes in all of parameters compared with control. However, *K. parviflora* (200mg/ml) has been found to increase the Billi-T (0.112±0.028) than its control (0.072±0.027) but non significant in normal range. In rats treated with *M. fragrans* (300 mg/ml) increasing of TP (7.820±0.460, control 6.6±0.126), ALB

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(5.720±0.217, control 4.817±0.098), BUN(29.200±4.061, control 22.867±1.989), and CREA (0.233±0.052, control 0.34±0.055), were found but non significant in maximum of normal range. All of treatments group were proved the histological parameter of liver and kidney. The results showed no changes histological parameters in all of treatmented. However, differences in haematology and serum chemistry values related to site and methodology.

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Table 17 Summary of hematological parameter of rats treated with K. parviflora, M. fragrans and fluoxetine

Table 17 Summary of hematological parameter of rats treated with *K. parviflora, M. fragrans* and fluoxetine

Test	I (2% tween80)			II (Fluoxetine 20 mg/ml)			III (*K. parviflora* 200mg/ml)			IV(*M. fragrans* 300 mg/ml)		
Parameter	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
WBC $10^6/\mu l$	12.317	1.458	0.595	12.068	3.387	1.515	12.620	2.303	1.152	11.442	2.060	0.921
RBC $10^3/\mu l$	7.415	0.391	0.160	7.644	0.299	0.134	7.393	0.134	0.067	7.278	0.203	0.091
HGB g/dl	14.933	0.981	0.401	15.460	0.835	0.374	14.925	0.457	0.229	14.900	0.255	0.114
HCT %	42.850	1.776	0.725	43.820	1.798	0.804	42.925	0.776	0.388	42.500	0.930	0.416
MCV ft	57.817	1.240	0.506	57.360	0.654	0.293	58.100	1.551	0.776	58.420	0.476	0.213
MCH pg	20.117	0.397	0.162	20.200	0.689	0.308	20.175	0.680	0.340	20.460	0.666	0.298
MCHC g/dl	34.800	1.064	0.434	35.240	0.902	0.403	34.750	0.843	0.421	35.060	0.921	0.412
PLT $10^3/\mu l$	964.333	104.305	42.582	823.200	72.513	32.429	1101.750	133.854	66.927	856.000	66.843	29.893

Table 17 Summary of hematological parameter of rats treated with *K. parviflora*, *M. fragrans* and fluoxetine (continue)

Test Parameter	I (2% tween80)			II (Fluoxetine 20 mg/ml)			III (*K. parviflora* 200mg/ml)			IV(*M. fragrans* 300 mg/ml)		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
RDW %	16.150	1.037	0.423	16.120	0.390	0.174	16.175	0.287	0.144	16.360	1.088	0.486
PDW ft	17.783	0.534	0.218	17.600	0.292	0.130	17.350	0.342	0.171	18.300	0.316	0.141
MPV ft	7.387	0.258	0.105	7.514	0.414	0.185	7.083	0.367	0.184	7.258	0.392	0.176
PCT %	0.714	0.100	0.041	0.617	0.044	0.020	0.779	0.086	0.043	0.621	0.055	0.025
NEU %	6.483	1.356	0.553	10.860	1.770	0.792	8.525	1.524	0.762	8.560	2.606	1.166
LYM %	81.133	4.509	1.841	78.340	3.071	1.374	80.600	3.873	1.936	77.360	6.383	2.855
EO %	0.900	0.297	0.121	1.180	0.327	0.146	0.850	0.191	0.096	1.020	0.377	0.168
BASO %	4.150	1.471	0.600	3.200	1.158	0.518	3.600	1.472	0.736	4.900	1.963	0.878
MONO %	7.317	2.558	1.044	6.380	2.935	1.313	6.450	1.682	0.841	8.160	3.392	1.517

Table 18 Summary of chemistry values of rats treated with *K. parviflora*, *M. fragrans* and fluoxetine

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Test	I (2% tween80)			II (Fluoxetine 20 mg/ml)			III (*K. parviflora* 200mg/ml)			IV(*M. fragrans* 300 mg/ml)		
Parameter	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Bili-T mg/dl	0.072	0.027	0.011	0.07	0.033	0.015	0.112	0.028	0.012	0.084	0.03	0.013
ALP U/L	227.333	25.097	10.246	229.8	27.308	12.212	266.8	33.056	14.783	247.8	22.742	10.171
TP g/dl	6.600	0.126	0.052	6.660	1.001	0.448	7.500	0.781	0.349	7.820	0.460	0.206
ALB g/dl	4.817	0.098	0.040	4.820	0.642	0.287	5.460	0.513	0.229	5.720	0.217	0.097
BUN mg/dl	22.867	1.989	0.812	24.360	3.589	1.605	26.380	4.020	1.798	29.200	4.061	1.816
CREA mg/dl	0.233	0.052	0.021	0.260	0.055	0.024	0.280	0.045	0.020	0.340	0.055	0.024
ALT U/L	57.500	8.313	3.394	71.000	9.950	4.450	61.800	7.328	3.277	72.400	23.180	10.366
AST U/L	93.667	11.483	4.688	110.600	21.173	9.469	86.000	6.364	2.846	118.400	30.550	13.662
URIC mg/dl	3.467	0.792	0.323	2.920	0.850	0.380	4.480	0.939	0.420	3.380	0.763	0.341
GLOB	1.767	0.052	0.021	1.860	0.358	0.160	2.040	0.251	0.112	2.080	0.259	0.116

Chapter 4

Discussion

Monoamine neurotransmitters level in hippocampus of the brain is involved in the regulation of cognitive processes such as mood, attention, sleep, arousal, and certain types of memory (Ng et al., 2015). It has been found that monoamine neurotransmitters play an important role in neurological disorders. Drugs which augment the effects of monoamines on their target tissues are used to treat psychiatric disorders, including depression, anxiety, and schizophrenia. This study is a first time that demonstrates the effect of *K. parviflora, M. fragrans* on NE, DA and 5-HT. Our results showed that *K. parviflora*, *M. fragrans* and fluoxetine have improved the levels of serotonin (5-HT), norepinephrine and dopamine in hippocampus of SD rat when compared with control group. Disturbances DA, 5-HT and NA neurotransmitter systems have been suggested to be involved in the pathogenesis of mood disorders (Kumar et al., 2003, Kamiński et al., 2013). In addition, the concentrations of norepinephrine (NE) are markedly decreased in various regions of the Parkinson's disease brain (LeWitt, 2012). The experiments in animal showed NE inhibits gene expression leading to pro-inflammatory molecules especially in cytokines in microglia, astroglia, and endothelial cells (Gesi et al., 2000). In our research show the increasingly of norepinephrine, the one of impossible mechanism may from increased of astroglia. (Luellen et al., 2003).

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GFAP that is the marker of mature astrocyte protein was increased; it may importance role in the secretion of other neurotransmitters such as dopamine and serotonin. On the other hand the increasing of neurotransmitters can induce GFAP expression. Moreover, the downstream mechanisms of noradrenergic control of progenitor cell proliferation in the adult brain have not been extensively examined. Nevertheless, activation of β3-AR has been shown to induce increased levels of intracellular cAMP, which in turn is known to regulate proliferation of progenitor cells (Nakagawa et al., 2002, Jhaveri et al., 2010). Interestingly the present study showed *K. parviflora*, *M. fragrans* induce serotonin as well as fluoxetine, it may the powerful therapeutic for depression. For many years, a deficiency of monoamines including serotonin has been the prevailing hypothesis on depression (Gardner and Boles, 2011). In the part of proteomic we founded many kinds of proteins that relate to energy metabolism in mitochondria. Previously study showed monoamines, energy metabolism and inflammatory pathways are inter-related in many complex manners. For example, the major categories of drugs used to treat depression have been demonstrated to exert effects on mitochondria and inflammation, as well as on monoamines(Gardner and Boles, 2011). The exact nature (agonist or antagonist) of the action of SSRI agents is not known, and the effects of them on mitochondria are largely untested. Fluoxetine has been found to exert multiple effects on the energy metabolism rat mitochondria and to affect electron transport and ATPase activity inhibiting ATP production in isolated rat brain mitochondria (Nahon et al., 2005, da Silva

et al., 2015). Moreover, we found the upregulation of proteins, that are involved the synaptic function and endocytosis including BIN1 and SYT1 in *K. parviflora* and *M. fragrans* group and SNAP25 only in *K. parviflora* treated group. Interestingly, the previously study demonstrates that norepinephrine triggers release of glial ATP to increase postsynaptic efficacy (Gordon et al., 2005) are related to our result in proteomic path. The results showed that *K. parviflora*, *M. fragrans* and fluoxetine are regulating the seven proteins, which are involved in the ATP synthesis: ATP5H, APRT, URCRFS1, CKB, ENO1, NDUFS and NSF. Several findings were discovered in the rat model: selective serotonin reuptake inhibitors (SSRI), such as fluoxetine increased the extracellular 5-HT levels in rat hippocampus (Robert et al., Malagié et al., 2002, Brooks et al., 2003, Ng et al., 2015). The observation indicates that *K. parviflora* and *M. fragrans* increase the level of 5-HT, but the mechanism remains the same, whether fluoxetine is included.

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Neurodegenerative diseases are a collection of disorders characterized by selective, progressive loss of neuronal structure and function. The present work we focused on the expression of GFAP, PDIA3, DPYSL2 and p-DPYSL2. Glial fibrillary acidic protein (GFAP), which is the intermediate filament protein in mature astrocytes, in addition to vimentin, nestin and synemin (Pekny et al., 2007, Middeldorp and Hol, 2011, Hol and Pekny, 2015, Cobb et al., 2016). There is a mounting evidence suggesting the aberrant astrocytic function in neurodegenerative disease such as dementia,

Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Middeldorp and Hol, 2011). Moreover, the expression of both GFAP mRNA and protein was reduced in hippocampus in major depressive disorder (MDD). Astrocyte pathology in depression is less well known in the human hippocampus, but the literature suggests the involvement of hippocampal astrocytes in blood depressed subjects at the time of death without antidepressant medication (Cobb et al., 2016). Recently, GFAP antibodies demonstrated the protective effect on oxidative stressed neuroretinal cells via interaction with PDIA3 (Wilding et al., 2015). Our study presents an upregulation of GFAP and PDIA3 in rat hippocampus when treated with *K. parviflora* and *M. fragrans*. Furthermore, we present that *M. fragrans* increase of the level of Vimentin (VIM) is a type III intermediate filament (IF). Thus, *M. fragrans* may be effective for neuroregeneration in plasticity. In addition, we found that the proteins play important role in neuronal differentiation and morphology of neuronal cells including microtubule-associated protein (MAP), binding to the tubulin subunits which regulate microtubule stability; Tubulin alpha-4A chian (Tba4a), a major constituent of microtubules; dihydropyrimidinase related protein 2 (Dpysl2) are involved in neuronal differentiation and axonal guidance.

Considering Dpysl2, beside its role on cytoskeleton organization, being a component of the dynein complex acting on intracellular retrograde motility of vesicles and organelles along with microtubules also at synaptic level (van den Berg and

Hoogenraad, 2012). Whereas DPYSL2 downregulation might be associated with neuronal and synaptic loss, as the protein is involved in axon guidance and growth, neurite development, and neuronal polarization (Ciavardelli et al., 2010). The expression of DPYSL2 is increase significantly in *K. parviflora* and *M. fragrans* group, although in fluoxetine group appears to be in no significance. The previously research identified, that DPYSL2 had a crucial function in axon formation of hippocampal neurons, thereby establishing and maintaining neuronal polarity (Koide et al., 2010). DPYSL2 interacts with tubulin heterodimers and promotes the microtubule assembly *in vitro*. Thus, DPYSL2 seems to promote neurite elongation and axon specification by regulating microtubule assembly, endocytosis of adhesion molecules and reorganization of actin filaments (Arimura and Kaibuchi, 2007). In addition, we found that *K. parviflora* upregulate Microtubule-associated protein (MAP) that plays an important role in microtubule stability. A more direct link altering a synaptic activity is also suggested by our discovery, that upregulation of SNAP25 has a key role in synaptic functioning and synaptic formation. We also found that *M. fragrans* group indicated an expression increase of Tubulin alpha-4A chain (TBA4A) that is a major constituent of microtubules as we all know, phosphorylated DPYSL1 and DPYSL2 are localized in the dendrites of cortical neurons where they regulate dendritic branch trajectories (Quach et al., 2015). The previous study in mice revealed the abnormal phosphorylation of DPYSL1 and DPYSL2 presenting a curling dendrite phenotype and suggesting that these two

molecules are critical for normal dendrite patterning in cortical neurons (Niisato et al., 2013). Dysregulation of DPYSL2 was also evident in the AD brain and associated with the decreased synapses present in AD brain; increased DPYSL2 activities were associated with negligible senescence. The most important mechanism of action (non-phosphorylated DPYSL2) is strongly binded to tubulin, which is leading to microtubule formation. Considering its phosphorylation by Rho kinase suppresses and it binding to tubulin and Numb. Surprisingly, our study found that p-DPYSL2 was significantly decreased in rat hippocampus treated with *K. parviflora*, *M. fragrans* and fluoxetine. This is the first time that their activity is reported on the effect of DPYSL2 and p-DPYSL2 expression. Brain plasticity is critical to healthy brain functioning.

Moreover, we found that the PDIA3 level was increased in *M. fragrans* and fluoxetine, but indicated a non-significant change in *K. parviflora* group ERp57, also known as Pdia3, Grp58, ER60 and 1,25D_3-MARRS, which is a member of the protein disulfide isomerase (PDI) family and a lumenal protein of the endoplasmic reticulum (ER). In order to enter the secretory pathway, proteins are cotranslationally translocated across the membrane of the endoplasmic reticulum (ER) as extended polypeptide chains (Erickson et al., 2005, He et al., 2014). ERp57 can interact with calreticulin and calnexin to ensure the correct folding of newly synthesized glycoproteins as a molecular chaperone, that is associated with a number of diseases including cystic fibrosis, prion diseases, Huntington's disease and Alzheimer's disease (He et al., 2014).

In fact, impaired mitochondrial function is commonly observed in many types of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, alcoholic dementia, brain ischemia-reperfusion related injury, and others. Although many of these neurological disorders contain unique etiological factors, however may share common factors of increased nitroxidative stress and mitochondrial dysfunction, likely resulting from the protein post-translational modifications (PTMs) including oxidation, nitration, hyperphosphorylation, acetylation, glycosylation and formation of various protein adducts (Narayan et al., 2015, Akbar et al., 2016). Depletion of ATP is a major factor in the cascade leading to apoptosis. In brain of neurodegenerative diseases subjects, declines in levels and/or activity of energy-related proteins associated with the mitochondria.

From IPA in this study we found *K. parviflora* regulate many proteins that relate to; Neurological Disease (ANK3,PGK1,DPYSL2,APOB,HYOU1,CKB,NDUFS1,HNRNPA1, CCT5, GFAP,SPTAN1,PURA,CA1,ALDH5A1,PRDX2), Nervous System Development and Function (CKB,DPYSL2,ANK3,APOB,TAGLN3,FSCN1,TKT,GFAP,SPTAN1,PURA, ALDH5A1). In *M. fragrans* we found the regulation of importance proteins in cell morphology (DPYSL2,Nefm,PDIA3,VIM,SH3GL2,SEPT11,HSPD1,GSN,BIN1, CKB,UCHL1,NSF,FSCN1,DDAH1,DLD, ARHGEF28,ACACA,ARHGDIA) and cellular function and maintenance (DPYSL2,CA2,Nefm,PDIA3,HYOU1,VIM,SH3GL2, SEPT11,HSPD1,ME1,GSN,BIN1,CKB,HSPA8,UCHL1,NSF,FSCN1,DDAH1,DLD,ARHGEF

28,ACACA,ARHGDIA,TMOD2). The data can summarized that two of plants may have the neuroprotective efficiency therapeutic and very interesting for study the underlying mechanism in future study.

Interestingly, *K. parviflora* improve the level of Superoxide dismutase [Mn] or SOD2, which is the enzyme to destroying superoxide anion radicals. In addition, *K. parviflora, M. fragrans* and fluoxetine are increasing the level of Glutathione S-transferase. We expect an increase approval of some of these beneficial agents in treatment of mitochondrial dysfunction-related organ damage preventing some of the neurodegenerative conditions. Because these mitochondria-targeted antioxidants were effective in preventing mitochondrial dysfunction and nitroxidative tissue injuries in various disease models (Ng et al., 2015). Lastly, these findings may lead to development of new herbal medications for neurological disorders including neurodegenerative disease and psychiatric disorders.

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Chapter 5

Conclusion

In summary, we revealed the association between *K. parviflora*, *M. fragrans* and fluoxetine increase of the level of serotonin (5-HT) norepinephrine and dopamine in rat hippocampus when compared to the control group. In proteomic analysis the data revealed that 37 proteins were up-regulated while 14 were down-regulated in *K. parviflora* group. In *M. fragrans* group 27 proteins were up-regulated while 16 were down-regulated. In fluoxetine treated group we found that 29 proteins were up-regulated and 14 were down-regulated. The level of GFAP, PDIA3, DPYSL2 and p-DPYSL2 were up and down-regulated in hippocampus of *K. parviflora*, *M. fragrans* and fluoxetine treated SD rat. The data suggest that *K. parviflora*, *M. fragrans* can target and regulate multiple pathways helpful in understanding the molecular therapeutic underlying mechanism pathways, especially involved in the level of GFAP, PDIA3, DPYSL2 and p-DPYSL2. In this study, *K. parviflora* and *M. fragrans* are exerting the synergistic therapeutic effect on the prevention and treatment of neurodegenerative diseases. A potential global therapy may target to key proteins in one or more of these common neurodegenerative pathways to prevent and promote a healthy lifespan.

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix A Animals data including body weight, food and water consumption.

Day 1 (19 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2% tween80	1	272	26	7	19	418	389	29	
	2	263	26	10	16	413	389	24	
	3	278	27	5	22	424	392	32	
	4	243	26	3	23	439	394	45	
	5	266	26	7	19	427	397	30	
	6	258	27	6	21	410	379	31	
II Fluoxetine 20 mg/ml	7	264	25	11	14	429	409	20	
	8	250	25	15	10	436	424	12	
	9	272	25	9	16	440	419	21	
	10	269	26	9	17	436	417	19	
	11	263	26	11	15	422	406	16	
	12	262	25	5	20	435	406	29	
III *Kaemferia parviflora* 200mg/ml	13	260	26	4	22	433	406	27	
	14	263	25	2	23	425	393	32	
	15	269	26	2	24	437	408	29	
	16	272	25	2	23	426	394	32	
	17	262	25	5	20	420	391	29	
	18	255	26	5	21	434	408	26	
IV *Myristica fragrans* 300 mg/ml	19	261	26	10	16	414	391	23	
	20	255	25	2	23	422	389	33	
	21	254	26	6	20	424	395	29	
	22	260	25	4	21	427	398	29	
	23	256	26	3	23	432	405	27	
	24	262	25	3	22	425	394	31	

Day 2 (20 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	279	26	3	23	389	357	32	
	2	259	26	0	26	389	346	43	
	3	283	27	4	23	392	358	34	
	4	267	26	2	24	394	351	43	
	5	279	26	7	19	397	370	27	
	6	265	27	9	18	379	346	33	
II Fluoxetine 20 mg/ml	7	263	25	9	16	409	386	23	
	8	246	25	15	10	424	406	18	
	9	274	25	10	15	419	401	18	
	10	271	26	9	17	417	395	22	
	11	263	26	10	16	406	388	18	
	12	271	26	5	21	406	377	29	
III *Kaemferia parviflora* 200mg/ml	13	269	26	3	23	406	377	29	
	14	271	25	3	22	393	359	34	
	15	281	26	6	20	408	380	28	
	16	285	25	2	23	394	368	26	
	17	268	25	2	23	391	354	37	
	18	266	26	5	21	408	383	25	
IV *Myristica fragrans* 300 mg/ml	19	257	26	9	17	391	367	24	
	20	266	25	5	20	389	349	40	
	21	266	26	7	19	395	369	26	
	22	269	25	6	19	398	368	30	
	23	269	26	3	23	405	380	25	
	24	274	26	3	23	394	364	30	

Day 3 (21 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	291	27	5	22	442	409	33	
	2	283	27	3	24	429	389	40	
	3	295	27	3	24	441	402	39	
	4	280	27	2	25	440	403	37	
	5	286	27	10	17	434	413	21	
	6	273	27	10	17	429	396	33	
II Fluoxetine 20 mg/ml	7	269	27	12	15	430	412	18	
	8	251	27	27	0	427	425	2	dead
	9	276	27	9	18	428	406	22	
	10	275	27	8	19	432	407	25	
	11	267	27	10	17	448	425	23	
	12	281	27	7	20	430	399	31	
III *Kaemferia parviflora* 200mg/ml	13	279	27	3	24	439	407	32	
	14	283	27	5	22	448	414	34	
	15	288	27	3	24	436	411	25	
	16	292	27	2	25	441	409	32	
	17	283	27	6	21	439	405	34	
	18	270	27	7	20	433	403	30	
IV *Myristica fragrans* 300 mg/ml	19	266	27	7	20	426	399	27	
	20	274	27	3	24	430	387	43	
	21	273	27	7	20	435	405	30	
	22	278	27	9	18	451	425	26	
	23	277	27	2	25	446	413	33	
	24	281	27	3	24	444	409	35	

Day4 (22 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	303	27	7	20	409	379	30	
	2	298	27	4	23	389	347	42	
	3	306	27	3	24	402	365	37	
	4	296	27	2	25	403	367	36	
	5	290	27	8	19	413	385	28	
	6	277	27	9	18	396	355	41	
II Fluoxetine 20 mg/ml	7	272	27	13	14	412	393	19	
	9	283	27	13	14	406	385	21	
	10	285	27	11	16	407	386	21	
	11	279	27	8	19	425	407	18	
	12	291	27	13	14	399	379	20	
III *Kaemferia parviflora* 200mg/ml	13	290	27	4	23	407	372	35	
	14	291	27	2	25	414	376	38	
	15	297	27	3	24	411	381	30	
	16	303	27	3	24	409	379	30	
	17	295	27	4	23	405	369	36	
	18	279	27	7	20	403	374	29	
IV *Myristica fragrans* 300 mg/ml	19	279	27	12	15	399	375	24	
	20	285	27	3	24	387	343	44	
	21	285	27	13	14	405	378	27	
	22	289	27	5	22	425	395	30	
	23	292	27	7	20	413	386	27	
	24	294	27	7	20	409	377	32	

Day5 (23 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	308	27	2	25	379	343	36	
	2	304	27	3	24	347	308	39	
	3	316	27	5	22	365	329	36	
	4	305	27	0	27	367	331	36	
	5	298	27	8	19	385	356	29	
	6	280	27	5	22	355	311	44	
II Fluoxetine 20 mg/ml	7	275	27	12	15	393	369	24	
	8								
	9	283	27	7	20	385	361	24	
	10	287	27	8	19	386	362	24	
	11	282	27	7	20	407	384	23	
	12	285	27	7	20	379	348	31	
III *Kaemferia parviflora* 200mg/ml	13	298	27	3	24	372	339	33	
	14	305	27	5	22	376	341	35	
	15	306	27	3	24	381	353	28	
	16	312	27	2	25	379	346	33	
	17	298	27	6	21	369	332	37	
	18	285	27	3	24	374	339	35	
IV *Myristica fragrans* 300 mg/ml	19	277	27	8	19	375	349	26	
	20	293	27	3	24	405	363	42	
	21	280	27	12	15	378	345	33	
	22	298	27	8	19	395	366	29	
	23	293	27	2	25	386	355	31	
	24	299	27	5	22	377	341	36	

Day6 (24 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	321	27	3	24	377	342	35	
	2	310	27	3	24	384	341	43	
	3	319	27	0	27	370	331	39	
	4	314	27	2	25	372	332	40	
	5	304	27	8	19	384	357	27	
	6	289	27	5	22	373	328	45	
II Fluoxetine 20 mg/ml	7	279	27	14	13	382	362	20	
	9	288	27	10	17	380	356	24	
	10	289	27	7	20	375	350	25	
	11	290	27	10	17	396	379	17	
	12	294	27	7	20	371	339	32	
III *Kaemferia parviflora* 200mg/ml	13	304	27	0	27	376	343	33	
	14	306	27	2	25	389	351	38	
	15	313	27	2	25	385	351	34	
	16	317	27	2	25	408	377	31	
	17	303	27	3	24	406	368	38	
	18	297	27	5	22	380	351	29	
IV *Myristica fragrans* 300 mg/ml	19	284	27	8	19	374	348	26	
	20	299	27	5	22	381	344	37	
	21	285	27	10	17	369	339	30	
	22	304	27	6	21	394	364	30	
	23	299	27	4	23	392	364	28	
	24	307	27	5	22	405	369	36	

Day7 (25 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	330	26	3	23	436	407	29	
	2	317	27	0	27	434	394	40	
	3	331	30	7	23	449	411	38	
	4	320	26	0	26	434	395	39	
	5	307	27	7	20	429	404	25	
	6	295	26	7	19	446	407	39	
II Fluoxetine 20 mg/ml	7	275	25	16	9	425	413	12	
	9	290	25	9	16	433	410	23	
	10	295	25	8	17	437	415	22	
	11	291	25	5	20	434	416	18	
	12	301	26	12	14	432	407	25	
III *Kaemferia parviflora* 200mg/ml	13	313	30	4	26	425	392	33	
	14	315	25	3	22	443	416	27	
	15	321	25	2	23	432	404	28	
	16	326	25	2	23	437	409	28	
	17	313	25	3	22	427	392	35	
	18	300	26	8	18	435	407	28	
IV *Myristica fragrans* 300 mg/ml	19	286	25	7	18	438	412	26	
	20	303	25	2	23	449	416	33	
	21	286	25	6	19	434	399	35	
	22	312	26	7	19	438	409	29	
	23	303	25	3	22	444	418	26	
	24	313	25	5	20	439	415	24	

Day8 (26 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	332	30	9	21	407	374	33	
	2	325	30	2	28	394	354	40	
	3	337	30	5	25	411	375	36	
	4	327	30	0	30	395	356	39	
	5	311	30	12	18	404	378	26	
	6	301	30	8	22	407	365	42	
II Fluoxetine 20 mg/ml	7	269	25	12	13	413	393	20	
	9	291	25	3	22	410	384	26	
	10	293	25	7	18	415	389	26	
	11	294	25	6	19	416	394	22	
	12	296	25	3	22	407	379	28	
III *Kaemferia parviflora* 200mg/ml	13	324	27	1	26	392	361	31	
	14	320	27	3	24	416	381	35	
	15	326	27	3	24	404	376	28	
	16	331	27	3	24	409	379	30	
	17	319	27	2	25	392	353	39	
	18	299	27	7	20	407	368	39	
IV *Myristica fragrans* 300 mg/ml	19	294	27	8	19	412	385	27	
	20	312	27	12	15	416	388	28	
	21	295	27	10	17	399	370	29	
	22	316	27	8	19	409	383	26	
	23	308	27	2	25	418	386	32	
	24	318	27	9	18	415	393	22	

Day9 (27 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	338	30	3	27	426	389	37	
	2	334	30	2	28	431	393	38	
	3	342	30	2	28	434	393	41	
	4	337	30	3	27	431	389	42	
	5	315	30	10	20	434	407	27	
	6	308	30	10	20	443	401	42	
II Fluoxetine 20 mg/ml	7	275	25	8	17	431	407	24	
	9	301	25	6	19	440	416	24	
	10	299	25	7	18	432	408	24	
	11	302	25	4	21	435	414	21	
	12	308	25	7	18	439	408	31	
III *Kaemferia parviflora* 200mg/ml	13	329	30	5	25	430	394	36	
	14	329	30	6	24	441	412	29	
	15	332	30	5	25	437	405	32	
	16	337	30	6	24	444	414	30	
	17	329	30	5	25	432	395	37	
	18	310	30	10	20	439	403	36	
IV *Myristica fragrans* 300 mg/ml	19	300	30	12	18	431	410	21	
	20	307	30	7	23	443	409	34	
	21	299	30	12	18	443	413	30	
	22	317	30	9	21	436	405	31	
	23	314	30	30	0	441	441	0	dead
	24	316	30	17	13	438	414	24	

Day10 (28 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	348	30	5	25	389	351	38	
	2	344	30	5	25	393	352	41	
	3	354	30	7	23	393	358	35	
	4	345	30	0	30	389	349	40	
	5	323	30	10	20	407	381	26	
	6	316	30	10	20	401	365	36	
II Fluoxetine 20 mg/ml	7	284	26	11	15	407	383	24	
	9	304	26	10	16	416	397	19	
	10	306	26	15	11	408	389	19	
	11	309	26	7	19	414	394	20	
	12	313	26	7	19	408	383	25	
III *Kaemferia parviflora* 200mg/ml	13	337	30	5	25	394	360	34	
	14	335	30	5	25	412	379	33	
	15	334	30	8	22	405	376	29	
	16	344	30	7	23	414	408	6	
	17	337	30	11	19	395	358	37	
	18	316	30	26	4	403	366	37	
IV *Myristica fragrans* 300 mg/ml	19	303	25	5	20	410	384	26	
	20	322	25	10	15	409	382	27	
	21	305	25	5	20	413	382	31	
	22	326	25	8	17	405	380	25	
	24	318	25	7	18	414	387	27	

Day11 (29 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	355	30	3	27	430	394	36	
	2	357	30	0	30	442	396	46	
	3	358	30	3	27	445	409	36	
	4	356	30	0	30	441	403	38	
	5	327	30	11	19	429	404	25	
	6	320	30	8	22	439	398	41	
II Fluoxetine 20 mg/ml	7	290	25	2	23	424	398	26	
	9	298	25	5	20	442	413	29	
	10	297	25	4	21	434	401	33	
	11	312	25	0	25	458	433	25	
	12	316	25	0	25	440	403	37	
III *Kaemferia parviflora* 200mg/ml	13	346	27	0	27	436	398	38	
	14	346	27	5	22	459	430	29	
	15	348	27	3	24	447	416	31	
	16								dead
	17	342	27	3	24	438	401	37	
	18	323	27	0	27	447	408	39	
IV *Myristica fragrans* 300 mg/ml	19	311	25	3	22	439	413	26	
	20	318	25	0	25	438	405	33	
	21	315	25	3	22	453	422	31	
	22	324	25	0	25	440	408	32	
	24	324	25	3	22	443	423	20	

Day12 (30 Sep 12)									
Gr.	ID No	BW (g)	Food weight (g)			Water weight (g)			Note
			give	remain	consume	give	remain	consume	
I 2%tween80	1	362	30	3	27	394	356	38	
	2	365	30	0	30	396	356	40	
	3	364	30	5	25	409	373	36	
	4	359	30	0	30	403	362	41	
	5	328	30	8	22	404	377	27	
	6	329	30	8	22	398	361	37	
II Fluoxetine 20 mg/ml	7	300	30	12	18	398	369	29	
	9	310	30	8	22	413	380	33	
	10	307	30	10	20	401	365	36	
	11	321	30	8	22	433	407	26	
	12	328	30	8	22	403	368	35	
III *Kaemferia parviflora* 200mg/ml	13	353	30	2	28	398	356	42	
	14	348	30	3	27	430	395	35	
	15	355	30	5	25	416	383	33	
	17	347	30	5	25	401	362	39	
	18	329	30	10	20	408	363	45	
IV *Myristica fragrans* 300 mg/ml	19	317	30	8	22	413	384	29	
	20	327	30	5	25	405	370	35	
	21	321	30	11	19	422	393	29	
	22	338	30	8	22	408	375	33	
	24	328	30	10	20	423	392	31	

Appendix B Animal protocol approve

ใบรับรองโครงการวิจัยในสัตว์ทดลอง

คณะกรรมการกำกับดูแลการเลี้ยงและการใช้สัตว์ทดลอง

ศูนย์สัตว์ทดลองแห่งชาติ มหาวิทยาลัยมหิดล

รหัสโครงการ #.... RA 2012-06.....

ชื่อข้อเสนอการวิจัย ... Identification of differentially regulated protein in hippocampal Sprague Dawley rats treated with *Kaempferia parviflora* and *Myristica fragrans*...

ชื่อ-สกุล ผู้เสนอข้อเสนอการวิจัย .....นางสาววรรณี อังคศิริสรรพ.....

หน่วยงานที่สังกัด (คณะ/กอง) ..... ศูนย์สัตว์ทดลองแห่งชาติ.....

(มหาวิทยาลัย/กรม) .....มหาวิทยาลัยมหิดล.....

(กระทรวง) .....กระทรวงศึกษาธิการ.....

ข้อเสนอการวิจัยนี้ได้ผ่านการพิจารณาจากคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ทดลองแล้ว จึงเห็นสมควรให้ดำเนินการเลี้ยงและใช้สัตว์ตามข้อเสนอการวิจัยนี้ได้

ลงนาม .....

(นางระพี อินปั่นแก้ว)

ตำแหน่ง ประธานคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์

วัน /เดือน /ปี 31/ธ.ค./55

ลงนาม .....

(นางกาญจนา เข่งคุ้ม)

ตำแหน่ง ผู้อำนวยการศูนย์สัตว์ทดลองแห่งชาติ

วัน /เดือน /ปี ๓๑ ธ.ค. ๕๕

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University