# การตรวจหาและคัดเลือกสารเครื่องหมายจากสารสกัดกะทกรกด้วยวิธีไฮเพอร์ฟอร์มานซ์ลิควิด-โครมาโทกราฟีและการทดสอบการจับกับตัวรับและตัวขนส่งโดปามีน

นางสาว ดวงกมล ภูมิราช



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# SCREENING AND SELECTION OF MARKER(S) OF *PASSIFLORA FOETIDA* EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DOPAMINE RECEPTORS/TRANSPORTER BINDING ASSAY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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| Thesis Title | SCREENING AND SELECTION OF MARKER(S) OF *PASSIFLORA FOETIDA* EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DOPAMINE RECEPTORS/TRANSPORTER BINDING ASSAY |
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ดวงกมล ภูมิราช : การตรวจหาและคัดเลือกสารเครื่องหมายจากสารสกัดกะทกรกด้วยวิธีไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีและการทดสอบการจับกับตัวรับและตัวขนส่งโดปามีน. (SCREENING AND SELECTION OF MARKER(S) OF *PASSIFLORA FOETIDA* EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DOPAMINE RECEPTORS/ TRANSPORTER BINDING ASSAY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. บุญศรี องค์พิพัฒนกุล, 107 หน้า

กะทกรกเป็นพืชสมุนไพรที่มีการใช้เป็นยาพื้นบ้านมาเป็นเวลานานในหลายวัตถุประสงค์ได้แก่ ลดอาการปวด ช่วยการนอนหลับและคลายอาการวิตกกังวล ผลการศึกษาเบื้องต้นพบว่าสารสกัดกะทกรกที่ได้จากการแยกด้วยไดคลอโรมีเทน (PF003) มีความสามารถในการแย่งจับตัวรับโดปามีน ดังนั้นวัตถุประสงค์ของการศึกษาในครั้งนี้จึงมุ่งเน้นการคัดกรองสารองค์ประกอบในสารสกัดกะทกรกที่สามารถจับตัวรับโดปามีน 1, 2 หรือตัวขนส่งโดปามีนได้และคัดเลือกสารเครื่องหมายเพื่อใช้ในงานควบคุมคุณภาพต่อไป วิธีการตรวจสอบโดยใช้ไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีที่มีตัวตรวจหาเป็นไดโอดแอเรย์ (HPLC-DAD)ได้ถูกพัฒนาขึ้นเพื่อวิเคราะห์และตรวจเอกลักษณ์สารที่พบในสารสกัด PF003 เบื้องต้นพบพีคหลักอย่างน้อย 6 พีคปรากฏในโครมาโทแกรมของสารสกัด PF003 จากนั้น PF003 ถูกสกัดแยกย่อยออกเป็นสารสกัดย่อย A ถึง F ด้วยวิธีการเตรียมจากไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีและสารสกัดย่อยทั้ง 6 ถูกนำมาศึกษาความชอบจับต่อตัวรับ/ตัวขนส่งโดปามีนด้วยวิธี radioligand receptor binding assay ผลการทดลองพบว่าสารสกัดย่อย D สามารถแย่งจับกับตัวรับโดปามีน 1 และ 2 ที่ร้อยละการยับยั้ง 12 ± 1.2 และ 61 ± 2.6 ตามลำดับ สารหลักที่พบในสารสกัดย่อย D ถูกตรวจเอกลักษณ์ด้วยวิธี HPLC-DAD โดยเปรียบเทียบเวลาที่สารถูกหน่วงเหนี่ยวในคอลัมน์และสเปกตรัมของสารกับสารมาตรฐาน สารหลักที่สามารถจับกับตัวรับโดปามีน 1 และ 2 ได้คือ luteolin และยืนยันความชอบจับต่อตัวรับโดปามีน 2 โดยให้ค่า $IC_{50}$ ที่ 14 ไมโครกรัมต่อมิลลิลิตร (48.9 ไมโครโมลาร์) จากข้อมูลการทดลองดังกล่าวแสดงให้เห็นว่า luteolin มีความเป็นไปได้ที่จะใช้เป็นสารเครื่องหมายของสารสกัดกะทกรก นอกจากนี้ในโครมาโทแกรมของ PF003 ยังพบสาร vitexin และ apigenin ซึ่งเคยมีรายงานว่าเป็นสารหลักที่พบในพืชสกุลนี้และถูกใช้เป็นสารเครื่องหมายด้วย การตรวจสอบสารเครื่องหมายจะถูกนำมาประยุกต์เพื่อใช้ในการควบคุมคุณภาพของสารสกัด PF003

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....ดวงกมล ภูมิราช.....

สาขาวิชา.....ชีวเวชเคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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## 4976568733: MAJOR BIOMEDICINAL CHEMISTRY
KEYWORDS: *PASSIFLORA FOETIDA*/ DOPAMINE RECEPTOR/ DOPAMINE TRANSPORTER/ HPLC

DUANGKAMOL PHUMMIRATCH: SCREENING AND SELECTION OF MARKER(S) OF *PASSIFLORA FOETIDA* EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DOPAMINE RECEPTORS/TRANSPORTER BINDING ASSAY. ADVISOR: ASST. PROF. BOONSRI ONGPIPATTANAKUL, Ph.D., 107 pp.

*Passiflora foetida* has long been used in traditional medicine for several purposes such as analgesic, sedative and anxiolytic effect. Preliminary studies showed that the dichloromethane fraction of *P. foetida* (PF003) potentially bound to dopamine receptor. Therefore, this study aimed to further screen for constituents in this extract that could bind with dopamine $D_1$, $D_2$ receptors or dopamine transporter (DAT) and to select marker(s) for using in quality control. High-performance liquid chromatography–diode array detection (HPLC–DAD) method has been developed for the components analysis and characterization. From preliminarily study, six major peaks were present in the PF003 chromatogram. Later, PF003 was fractionated to fraction A-F by preparative HPLC and six fractions were further studied for the affinity to dopamine $D_1$, $D_2$ and DAT by radioligand receptor binding assay. The results showed that only fraction D was able to bind to dopamine $D_1$ and $D_2$ receptor with 12 ± 1.2 and 61 ±2.6 % inhibition, respectively. The main component in fraction D was identified by HPLC based on retention time and UV adsorption spectra, and compared with standard. Main compound which could bind to dopamine $D_1$ and $D_2$ receptors was luteolin. The affinity of luteolin to dopamine $D_2$ receptor was confirmed with an $IC_{50}$ value of 14 μg/ml (48.9 μM). Luteolin showed potentially to be a biomarker. Moreover, vitexin and apigenin were also found in PF003 chromatogram. They were the dominant compounds described in this genus and were served as the chemical markers as well. The detection of the markers would be applied for the herbal medicine quality control for PF003.



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## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | percentage |
| °C | degree Celsius (centigrade) |
| µg | microgram (s) |
| µL | microlitre (s) |
| µm | Micrometer (s) |
| $^{3}H$ | tritium |
| ACN | acetronitrile |
| AcOH | acetic acid |
| ATCC | American Type Culture Collection |
| $B_{max}$ | density of binding sites |
| BSA | bovine serum albumin |
| BuOH | butanol |
| cAMP | adenosine 3′,5′-cyclic monophosphate |
| $CH_2Cl_2$ | dichloromethane |
| $Cl^-$ | chloride ion |
| $cm^2$ | square centimeters |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| DAD | diode array detector |
| DAT | dopamine transporter |
| DMEM | dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulfoxide |
| ELSD | evaporative laser scattering detector |
| EMEA | the European Agency for the Evaluation of Medicinal Products |
| et al. | *et alii*, and others |
| etc. | *et cetera*, and other things |
| EtOH | ethanol |
| FBS | fetal bovine serum |
| g | gram (s) |

| | |
|---|---|
| GABA | gamma amino butyric acid |
| $G_i$ | inhibitory guanosine triphosphate-binding protein |
| $G_s$ | stimulatory guanosine triphosphate-binding protein |
| h | hour |
| HCl | hydrochloride |
| HPLC | High performance liquid chromatography |
| i.d. | internal diameter |
| IP | interfering peaks |
| $K_d$ | receptor dissociation constant |
| $K_i$ | inhibition constant |
| M | molar |
| MAO | monoamine oxidase |
| mAU | milli absorbance unit |
| MeOH | methanol |
| mg | milligram (s) |
| $MgCl_2$ | magnesium chloride |
| min | minute (s) |
| mL | millilitre (s) |
| mM | millimolar (s) |
| mm | millimeter (s) |
| mRNA | messenger ribonucleic acid |
| MS | Mass spectrometry |
| NA | not applicable |
| $Na^+$ | sodium ion |
| NaCl | sodium chloride |
| nm | nanometer (s) |
| NMR | Nuclear Magnetic Resonance |
| OT | olfactory tubercle |
| PBS | phosphate buffer saline |
| pH | the negative logarithm of hydrogen ion concentration |
| PTZ | pentylenetetrazole |
| RPA | relative peak area |
| rpm | round per minute |

| | |
|---|---|
| RRT | relative retention time |
| RSD | relative standard deviation |
| S.E.M. | standard error of mean |
| SNc | substantia nigra compacta |
| TM | transmembrane |
| UV | ultraviolet |
| WHO | the World Health Organization |

# CHAPTER I

# INTRODUCTION

*Passiflora*, comprising about 500 species, is the largest genus in the family Passifloraceae (Dhawan *et al.*, 2004). Plants of this genus are found in both temperate and tropical regions. Various species of *Passiflora* have been used extensively in traditional therapy in many countries throughout the world *e.g. P. alata*, *P. edulis* and *P. foetida* in South American countries, *P. incarnata* and *P. caerulae* in European countries, and *P. edulis* in Southeast and Middle East Asian. The plants are often said to possess sedative, anxiolytic, analgesic, and anti-convulsant effects (Dhawan *et al.*, 2004; Muller, 2005; Santos *et al.*, 2005). In Thailand, *P. foetida* (ka-tok-rok) is native species which can be found growing as climbing weed all over the country. This plant was used as a folk medicine for treatment of anxiety, stress and insomnia (Pongpan *et al.*, 2007). Recently, this plant was selected as one of 52 plants that were studied for the affinity to monoamine receptors by a National Research Council of Thailand supported research program entitled Development of Herbal Extract for the Treatment of CNS Stimulant Addicts in the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The results showed that *P. foetida* extract could strongly displace specific radioligand from dopamine $D_1$ receptor. *P. foetida* extract could be fractionated to yield five fractions namely the ethanol extract (PF001), hexane extract (PF002), dichloromethane extract (PF003), butanol extract (PF004) and aqueous extract (PF005). The dichloromethane extract at 100 µg/ml could displace the specific radioligand of dopamine $D_1$ receptor with more than 50%. Corresponding to the *in vivo* assays including open-space swimming (depressive behavior), elevated plus maze (anxious behavior), Y-maze and Morris water maze (learning and memory behavior) models, the dichloromethane extract at the dose of 50 mg/kg body weight could reduce significantly anxiety and depressant behaviors of rat when compared with the positive control group given amitriptyline. Furthermore, their effective dose did not affect mobility, learning and memory of the studied animals. The results of both *in vitro* and *in vivo* experiment were important evidence to support that *P. foetida* extract, especially the dichloromethane fraction, could potentially be

developed as a treatment for depressive disorder, anxiety and dopaminergic malfunction. To obtain higher purity, the dichloromethane extract of *P. foetida* was fractionated into sub-fractions PF003-1 to PF003-9, and then these sub-fractions were further isolated to provide at least nine isolated compounds namely, PF-2, PF-3, PF-A, PF-B, PF-C, PF-D, PF-E, PF-F and PF-G.

Herbal extract or preparation contains complex constituents which could fluctuate by harvest seasons, plant origins, drying processes and other factors (Calixto, 2000; Liang *et al.*, 2004). Therefore, one of the major concerns relating to herbal medicine development is to ensure the reproducibility and reliability of botanical materials. Quality control is an important requirement for herbal products and guidelines concerning the issue have been drawn by both the World Health Organization (WHO) and the European Agency for the Evaluation of Medicinal Products (EMEA) (WHO, 2000; EMEA, 2005). Thus, if PF003 extracts are to be proceeded for clinical investigation, more complete information on the phytochemical constituents is necessary and required as supportive data for the quality control of the plant extracts.

General methods used in quality control of herbal medicines depend on both visual inspection (macroscopic and microscopic examinations) and analytic inspection using instrumental techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) (Liang *et al.*, 2004). HPLC is the most popular tool for the analysis of herbal medicines because it is not limited by volatility or stability of the sample. In this thesis, HPLC was therefore selected for identifying the chemical constituents of the extract. Marker determination is the one approach often used for ensuring the herbal plant quality and is recommended by EMEA and WHO (WHO, 2000; EMEA, 2005). Marker(s) indicates constituents or groups of constituents of herbal substance which are of interest for control purposes.

The goal of the present thesis was to select suitable marker(s) for ensuring the quality of PF003 extract. Major chemical constituents of the extract would be identified by HPLC method and the selection of marker would be based on the interaction with dopamine $D_1$, $D_2$ receptors and dopamine transporter which are some of the key target molecules for pathological intervention relating to drug addiction, depression and anxiety.

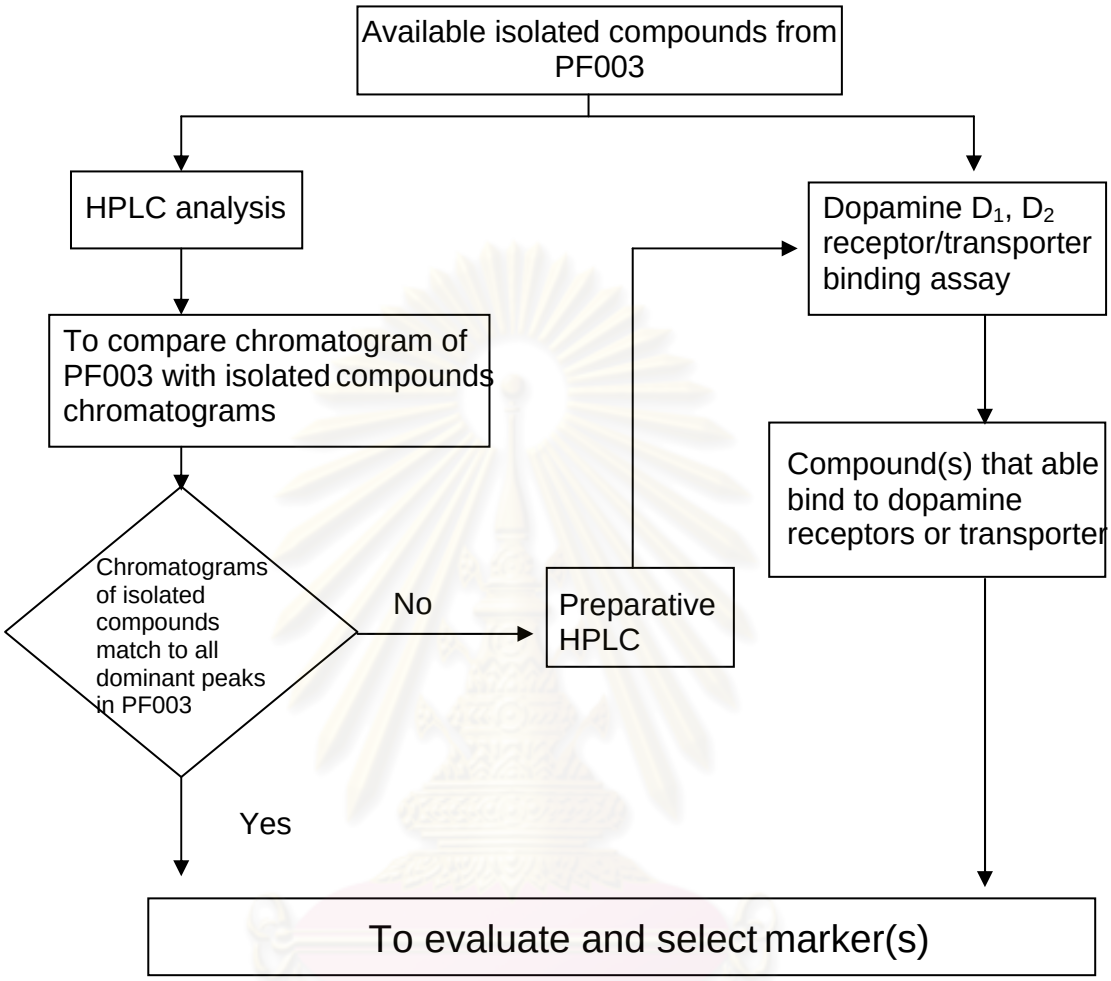
**Objectives**

1. To screen for the constituents of *P. foetida* extract that are able to bind to dopamine $D_1$, $D_2$ receptors or dopamine transporter (DAT) using radioligand receptor binding assay.
2. To select marker(s) for the quality control of *P. foetida* extract with HPLC technique.

**Contributions of the study**

1. The information of crucial constituent(s) and bioactive compound(s) from the *P. foetida* extract would be obtained.
2. The marker compound(s) for qualitative analysis and quality control of *P. foetida* extract would be selected.
3. The research would provide useful information for further development of *P. foetida* extract.

## Conceptual framework



# CHAPTER II

# LITERATURE REVIEW

## Dopamine system

Dopamine is a catecholamine transmitter in the mammalian brain. Until the mid-1950s, it was exclusively considered to be an intermediate in the biosynthesis of catecholamines including norepinephrine and epinephrine. Significant tissue levels of dopamine were first demonstrated in peripheral organs of ruminant species. A short time later, Montagu, Carlsson, and co-workers found that dopamine was also present in the brain in about equal concentrations to those of norepinephrine but with quite different distribution (Cooper, Bloom and Roth, 1996). Dopamine synthesis originates from tyrosine, and its rate-limiting step is the conversion of L-tyrosine to L-DOPA by the enzyme tyrosine hydroxylase. DOPA is subsequently converted to dopamine by L-aromatic amino acid hydroxylase (Brunton, Lazo and Parker, 2005).

The central dopaminergic neuron system is composed of 4 main pathways (Missale *et al.*, 1998; Vollone, Picetti and Borrelli, 2000):

(1) Nigro-striatal pathway arises from dopamine-synthesizing neurons of the midbrain nucleus, the substantia nigra compacta (SNc) which innervates the dorsal striatum (caudate-putamen). It is involved in the control of movement and its degeneration causes Parkinson's disease.

(2) The mesocortical pathway originates in the ventral tegmental area and innervates different regions of the frontal cortex. It is involved in aspect of memory learning and cognition.

(3) The mesolimbic pathway originates in the ventral tegmental area and projected to the ventral striatum (nucleus accumbens), the olfactory tubercle (OT) and parts of the limbic system. It influences the motivated behaviour.

(4) The tubuloinfundibular pathway arises from hypothalamus, and then projects to the hypophysis. This pathway is involved in neuroendocrine regulation.

As mentioned above, dopamine plays an important role in controlling movement, emotion and cognition. Consequently, dopaminergic dysfunction has been implicated in the pathophysiology of schizophrenia, mood disorders, attention-deficit disorder, Tourette's syndrome, substance dependency, Parkinson's disease and other disorders (Emilien *et al.*, 1999).

Dopamine receptors are the primary targets of drug action in the pharmacological treatment of various diseases as described above. Dopamine receptors belong to the family of seven transmembrane domain (TM) G-protein coupled receptors. They comprises transmembrane region, extracellular $NH_2$ terminal region and intracellular COOH terminal region. The structure of dopamine receptor was present in Figure 1. They were divided into two main groups as dopamine $D_1$-like receptor and dopamine $D_2$-like receptor on the basis of their biochemical and pharmacological properties. At least six different forms of the cloned dopamine receptors have been reported. Dopamine $D_1$ receptor type was coupled with $G_s$ and increased adenylate cyclase activity, while dopamine $D_2$ receptor type was coupled with $G_i$ and reduced the production of cAMP. The dopamine $D_1$ receptor-like subfamily comprises $D_1$- and $D_5$ receptors and the dopamine $D_2$ receptor-like includes $D_2$L-, $D_2$S, $D_3$- and $D_4$-receptors (see Table 1) (O'Dowd, 1993; Missale *et al.*, 1998; Vollone *et al.*, 2000; Sealfon and Olanow, 2000).

The dopamine $D_1$ receptor is the most widespread dopamine receptors and expressed at higher levels than any other dopamine receptors. Dopamine $D_1$ receptor mRNA and protein have been found in the striatum, the nucleus accumbens, olfactory tubercle, caudate putamen, septum, amygdale, and hippocampus (Cooper *et al.*, 1996), while dopamine $D_2$ receptors were detected in dorsal striatum, olfactory tubercle, nucleus accumbens, substantial nigra pars compacta and ventral tegmental area. The dopamine $D_3$, $D_4$, and $D_5$ receptor mRNAs are mostly present in tissues where the dopamine $D_1$ receptor and/or the dopamine $D_2$ receptor mRNAs are also expressed. However, their relative abundances are one to two orders of magnitude lower than that of the dopamine $D_1$ or $D_2$ receptor (Missale *et al.*, 1998).

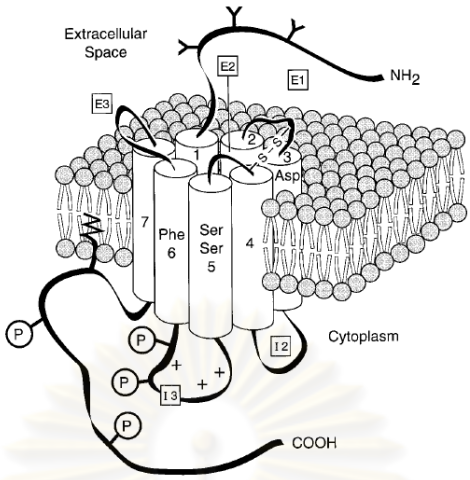



**Figure 1 Dopamine receptor structure**. Structural features of $D_1$- like receptors are represented. $D_2$-like receptors are characterized by a shorter COOH-terminal tail and by a bigger 3rd intracellutar loop. Residues involved in dopamine binding are highlighted in transmembrane domains. E1-E3, extracellular loops; 1-7, transmembrane domains; I2-I3, intracellular loops (Missale *et al.*, 1998).

**Table 1 Classification of the dopamine receptors** (Emilien *et al.*, 1999).

| | $D_1$ family | | $D_2$ family | | |
|---|---|---|---|---|---|
| | $D_1$ | $D_5$ | $D_2$ | $D_3$ | $D_4$ |
| Agonists | SKF-38393<br>*R*(+)SKF-81297<br>Dihydrexedine | Dopamine<br>SKF-38393 | Quinpirole<br>Bromocriptine<br>(+)PHNO | Quinpirole<br>Pergolide<br>7-OH-DPAT<br>PD-128907 | Dopamine |
| Antagonists | SCH-23390<br>α-Flupenthixol<br>SKF-83566<br>SCH-39166 | SCH-23390 | Spiperone<br>Raclopride<br>Sulpiride<br>Haloperidol<br>U-101958 | UH-232<br>Nafadotride<br>(+)*S*-14297 | Spiperone<br>Clozapine<br>U-101387 |
| Function | | | | | |
| Adenylate cyclase | Stimulates | Stimulates | Inhibits | ? | ? |
| Phosphoinositol turnover | ? | ? | Inhibits | ? | ? |
| Molecular structure | | | | | |
| Size (amino acid residues) | 446 | 447 | 414 (short)<br>443 (long) | 446 | 387 |
| mRNA size | 3.8 kb | 3 kb | 2.5 kb | 8.3 kb | 5.3 kb |
| Distribution | | | | | |
| Brain | Striatum<br>Nucleus accumbens | Hypothalamus<br>Hippocampus | Striatum<br>Substantia nigra | Olfactory tubercle<br>Hypothalamus | Frontal cortex<br>Midbrain |
| Periphery | — | — | Heart | — | Heart |
| Archetypic tissue | Parathyroid gland | — | Pituitary gland | — | — |

*R*(+)SKF-81297, *R*(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-benzazepine; (+)*S*-14297, (+)-7-(*N*,*N*-dipropylamino)-5,6,7,8-tetrahydronaphtho[2,3-*b*]dihydro-2,3-furane; 7-OH-DPAT, 7-hydroxy-diphenylaminotetralin; (+)PHNO, 9-hydroxy-4-propyl-naphthoxazine; PD-128907, *R*(+)-*trans*-3,4,4*a*,10*b*-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano[4,3-*b*]-1,4-oxazine-9-ol; U-101387, (*S*)-(−)-(4-[4-{isochroman-1-yl}ethyl]piperazin-1-yl)benzenesulphonamide; U-101958, 3-isopropoxy-*N*-methyl-*N*-(1-[phenylmethyl]-4-piperidinyl)-2-pyridinylamine.

The binding site and binding interaction of both dopamine receptor types have been studied in order to further understand their mechanism and to increase the ability of appropriate ligand design. Currently, this knowledge is limited, causing lack of complete X-ray crystal structure of the receptors. Fortunately, Kalani's group has demonstrated the possible binding interaction of dopamine $D_2$ receptor by bioinformatic method and analysis with the reported mutation data (Kalani *et al.*, 2006). The prediction of the binding interaction of dopamine $D_2$ receptor was observed with three groups of ligands, the first group is dopamine $D_2$ agonist compounds such as dopamine; second group is class I dopamine $D_2$ antagonist *e.g.* clozapine and the third group is class II dopamine $D_2$ antagonist e.g. haloperidol. The results suggested that the binding interaction of agonist is critically involved with transmembrane (TM) domain 3-6. The carboxyl group of aspartate at the position 114 in TM3 forms a tight salt bridge with the primary amino group of dopamine (Figure 2). Whilst, serine 193 and serine 197 in TM5 hydrogen bond to the metahydroxyl and the parahydroxyl groups, respectively, of the catechol ring of dopamine, playing an essential role in recognizing dopamine. The last interaction force, phenylalanine 110 (TM3), methionine 117 (TM3), cysteine 118 (TM3), phenylalanine 164 (TM4), phenylalanine 189 (TM5), valine 190 (TM5), tryptophan 386 (TM6), phenylalanine 390 (TM6) and histidine 394 (TM6) form a mostly hydrophobic pocket for dopamine. Both classes of dopamine $D_2$ antagonists make contact to aspartate 114 in TM3 but does not form strong contact to either serine residues in TM5, which is contrast to the interactions of agonists. In summary, a salt bridge to TM3 and two hydrogen bondings to TM5 are essential for agonist activation, whereas the salt bridge and one hydrogen bonding are important for antagonists (Kalani *et al.*, 2006).

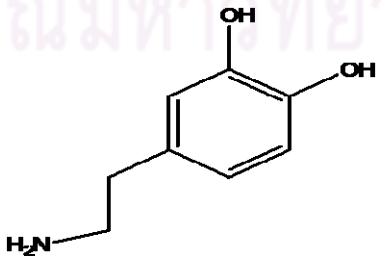



**Figure 2 Structure of dopamine.**

## Dopamine transporter

The dopamine transporters (DAT) are located on the plasma membrane of nerve terminals, and transports dopamine across the membrane by taking up synaptic dopamine into neurons. DAT plays a critical role in terminating the signals from neurons releasing the neurotransmitters and in maintaining dopamine homeostasis in the central nervous system (Kimmel *et al.*, 2001). The reuptake mechanism is $Na^+$ and $Cl^-$dependent, and follows a sequence of events where one dopamine molecule and two sodium ions initially bind to the transporter protein, followed by binding of one chloride ion to the transporter (Zahniser and Doolen, 2001). The inwardly directed sodium gradient provides energy for an inward movement of dopamine against a concentration gradient. The dopamine transporter has been identified from brains of various species. The mammalian dopamine transporters exhibit high sequence identity. Dopamine transporter is a glycoprotein consisting of 12 transmembrane segments connecting with extracellular and intracellular loops with the N- and C-terminals located in the cytosol. The density of distribution of DAT sites varies in different brain regions and agrees with immunohistochemical studies that report differing levels of DAT mRNA or DAT protein in different dopaminergic pathways. Generally, a higher level of DAT expression and transporter protein was found in the nigrostriatal and mesolimbic dopamine neurons (*e.g.* the caudate and putamen, the nucleus accumben, and cell body areas) and significantly less in the frontal cortex and the hypothalamus (Chen and Reith, 2000; Jiao *et al.*, 2001).

The structural requirements of dopamine transporters for the interaction with substrates have been examined by comparing the transport of phenethylamine derivatives. These studies indicate that the dopamine transporter requires molecules that possess a phenyl ring with a primary ethylamine side chain for optimal activity, and the beta-rotamer of the extended conformation of catecholamines is transported preferentially (Meiergerd and Schenk, 1994). It is proposed that the catechol moiety appears to mediate the recognition of the substrate, whereas the amine side chain apparently facilitates the conformational change of the transporter that results in movement of dopamine across the membrane. Almost all of dopamine carrier substrates are phenethylamine derivatives and positively charged at physiological pH.

It is reasoned that the positively charged amino group of catecholamine substrates might interact with the negatively charged carboxylic acid of aspartate79 of dopamine transporter pocket site. These features have been used as a guide to find residues of the substrate-binding site at the carrier.

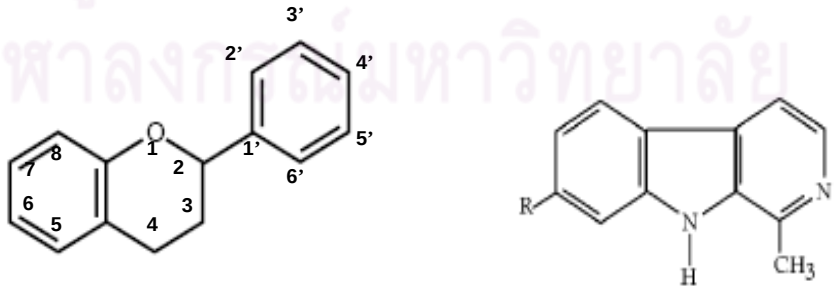
## *Passiflora* species

The genus *Passiflora*, comprising about 500 species, is the largest in the family Passifloraceae. The species of this genus are distributed in the warm temperate and tropical regions of the world. Several *Passiflora* species are grown in the tropics for their edible fruits and a number of species have been employed widely as folk medicine because of sedative and tranquilizer activities. Some species such as *P. quadrangularis*, *P. actinia*, *P. incarnata*, *P. edulis* and *P. alata* have been described to induce anxiolytic-like and sedative effects in rodents, as described by different laboratories worldwide (Barbosa *et al.*, 2008). In Brazil, *P. caerulae* has been used since the 17th century as a sedative and anxiolytic with similar effect to *P. incarnata.* In Italy, the plant has been used as an anti-spasmodic and sedative. In South America, *P. edulis* is used to relief insomnia and diarrhea. It has also been used as sedative, diuretic, anthelmintic, anti-diarrheal agent, stimulant, and treatments for hypertension, menopausal symptoms and colic of infants. In Asia, there are many reports on the use of *Passiflora* as traditional therapy. *P. incarnata* has been used for morphine de-addiction in the traditional Indian medicine (Dhawan *et al.*, 2004). The species is well known and popular in Europe and has been developed into medicinal products for relief of mild mental stress and to aid sleep.

Most of the pharmacological works have been carried out on the CNS depressant effects of various *Passiflora* species. A group of Brazilian researchers have studied *P. alata* leaves using mice as the experimental animals. On intraperitoneal administration to mice at a dose of 150 mg/kg, *P. alata* extract reduced amphetamine-induced spontaneous motor activity and prolonged pentobarbital-induced sleep time. The hydro-ethanol extracts of *P. alata* and *P. edulis* leaves have been evaluated at three dose levels (50, 100 and 150 mg/kg) to confirm the anxiolytic effects in accordance with the traditional use of both species. Pharmacological effects of chrysin, a flavonoid, occurring in *P. caerulea* were examined in mice. It induced

significant anxiolytic effect and was found to be a ligand for central benzodiazepine receptors (Medina *et al.,* 1990). Anti-anxiety effect of *P. incarnata* extract in mice was investigated and the results showed that benzoflavone nucleus was the basic moiety essential for the bioactivity of the plant extract (Dhawan *et al.,* 2002). In another report on its CNS depressant effect, the aqueous extract of *P. edulis* could prolong barbiturate-induced, as well as morphine-induced, sleep time in mice and also "partially" blocked the amphetamine-induced stimulant effects (Dhawan *et al.,* 2004). Intraperitoneal injection of the ethanolic extract of *P. incarnata* could prolong sleeping time and protect animals from convulsive effects of pentylenetetrazole by increasing the onset and the survival time in PTZ-treated mice, and decreasing the amphetamine-induced locomotor activity in a dose-dependent manner. Nonetheless, the compounds responsible for the reported pharmacological activities have not been clearly identified.

Flavonoids (Figure 3) are reported to be the major phyto-constituents of this plant. These include apigenin, kaempferol, quercetin, luteolin, chrysoeriol and C-glycosyl flavonoids *e.g.* isovitexin, vitexin, luteolin-7-β-D-glucoside, orientin, isoorientin, schaftoside and isoschaftoside. Besides flavonoids, alkaloids, and cyanogenic compounds are also known as constituents of *Passiflora.* The alkaloids reported to be present are simple indole alkaloids based on β-carboline ring system (Figure 3) namely harman, harmin, harmalin, harmol and harmalol. The other phyto-constituents found in this plant are cyanohydrin glycosides tetraphyllin A, tetraphyllin B, γ-benzo-pyrone derivative maltol, fatty acids linoleic acid, alpha-pyrones named passifloricins and *etc.* (Dhawan *et al.,* 2004).




flavonoid alkaloid

**Figure 3 Basic structure of flavonoid and alkaloid found in *Passiflora.***

## Quality control of herbal medicine

Presently, herbal medicine has gained increasing popularity worldwide for health promotion and adjuvant therapy. The main problem of natural products is the variation of constituents, depending on harvest season, plant origin and other factors. Thus, it is necessary to determine phytochemical constituents of herbal products in order to assure the reliability of each batch (Gong *et al.*, 2003; Liang *et al.*, 2004; Ong, 2004; Li *et al.*, 2008). Chromatographic methods were highly recommended for developing chemical profiles of extract (Liang *et al.*, 2004). Many types of chromatographic methods have been used as described below.

Thin layer chromatography (TLC) is the common method of choice for herbal analysis. The advantages of this method are its simplicity, versatility, high velocity and simple sample preparation (Liang *et al.*, 2004).

Gas chromatography (GC) is well known for the analysis volatile chemical components. The advantages of GC lie in its high sensitivity for the detection of almost all volatile compounds. However, the most serious disadvantage of the method is that it is not suitable for its analysis of non-volatile compounds (Liang *et al.*, 2004).

High performance liquid chromatography (HPLC) is one type of liquid chromatography (LC) that is a physical separation technique conducted in the liquid phase. Components of analytes are separated by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside column). HPLC is very popular technique for analysis of herbal extract because it is easy to use and is not limited by the volatility or stability of the samples. Furthermore, the qualitative analysis or structure elucidation of the chemical components in extract can be achieved by hyphenated HPLC systems such as HPLC-DAD, HPLC-MS and HPLC-NMR (Liang *et al.*, 2004; Dong, 2006).

Quality control of herbal medicine is concerned by the World Health Organization (WHO) and the European Agency for the Evaluation of Medicinal Products (EMEA). The guideline of WHO requires the quality appraisal of crude drug materials and plant materials. The guideline explains that the botanical definition including genus, species and authority, should be given to ensure correct

identification of a plant. The active and characteristic constituents should be specified. The crude plant materials which are processed with some techniques such as fractionation, purification or concentration are called plant preparation. The requirement of quality assessment in plant preparation concerns the identity of active compound(s). If identification of an active compound is not possible, it should be sufficient to identify a characteristic substance and mixture of substances (*e.g.* "chromatographic fingerprint") to ensure consistent quality of the preparation. Another guideline for herbal substance quality control widely used is drawn by EMEA. The indicated requirements in this guideline also pay attention to a comprehensive specification for each herbal substance such as scientific name of plant, chemotype (where applicable). In case of herbal substances with constituents of known therapeutic activity, assays of their content are demanded. If the bioactive compounds are not known, marker substance evaluation is needed. From the two major guidelines, quality control requirement of herbal material can be summarized into two aspects that are plant authentication and justification of the characteristic compound (marker). Marker was defined by EMEA as described below.

**Markers** are chemically defined constituents or groups of constituents of a herbal substance, a herbal preparation or a herbal medicinal product which are of interest for control purposes independent of whether they have any therapeutic activity. Markers serve to calculate the quantity of herbal substance(s) or herbal preparation(s) in the Herbal Medicinal Product if the marker has been quantitatively determined in the herbal substance or herbal preparations. There are two categories of markers (EMEA, 2006):

**Active markers** are constituents or groups of constituents which are generally accepted to contribute to the therapeutic activity.

**Analytical markers** are constituents or groups of constituents that serve for analytical purposes.

## Radioligand receptor binding assay

Radioligand binding assays are a relatively simple but extremely powerful tool for studying the affinity of unknown compounds to interested receptors (Jong *et al.*, 2005). They allow an analysis of the interactions of hormones, neurotransmitters,

growth factors, and related drugs with the receptors. Most of the commonly used techniques now available for measuring receptor-ligand interactions involve the use of radioisotope (Matthews, 1993). The principle of this technique is based on the competitive interaction between a labeled ligand and a test compound for the same specific receptor binding site. The general assay procedure involves the preparation of animal tissue rich in a particular receptor and the incubation of prepared receptor with a radiolabeled ligand in the absence and the presence of a test compound.

Most receptors are often membrane incorporated or membrane associated which are prepared from laboratory animals, notably rodents, or part of animals from slaughter house. At present, these sources still substantially account for the receptors used in binding assays. Other alternative source widely used in routine drug screening comes from genetically engineered cells with specifically expressed receptors for binding study. The advantage of cell lines transfected with cloned receptor gene are the higher ratio of specific to non-specific binding, a more consistent available binding sites in each experiments and freedom from ethical issues (Martin, 2006). The preparation of receptor for binding assay usually starts from tissue homogenization followed by a series of centrifugation steps, resulting in a preparation that can either be used immediately or stored frozen (at -80°C) until use.

The binding experiment starts when receptor, radioligand and test sample are brought together in an incubation buffer prepared at specific pH (mostly in the range of 7.0-8.0). Incubations are then carried out at a set temperature (in the range between 0°C - 37°C). Reaction times may vary from ten minutes up to several hours. For each different assay, incubation conditions have to be optimized. The reactions can be terminated by filtration and the quantity of receptor-ligand complex remaining on the filters were then determined. The amount of radioactivity in remained labeled ligand-receptor complex is directly measured, either by a Geiger counter or by a scintillation counter. In scintillation counting, the sample is mixed with a material that will fluoresce upon interaction with a particle emitted by radioactive decay. The scintillation counter quantifies the resulting flashes of light. (Matthews, 1993; Sweetnam *et al.*, 1993; Zhu *et al.*, 1996; Bylund and Murrin 2000; Kvernmo *et al.*, 2006; Tulp, 2006). The advantage of radioligand receptor binding assays is its

sensitivity, specificity and ease of use. In contrast, this technique has some disadvantages in the disposal of radioactive waste, relatively long read times, costs, health hazards, labour intensive in the step of separation of free from bound ligand and the requirement for special licences (Jong *et al.*, 2005).

# CHAPTER III

# MATERIALS AND METHODS

## Materials

### 1. Chemicals

Apigenin (Sigma-Aldrich, Germany)

Bovine serum albumin (Sigma-Aldrich, Germany)

(+)-Butaclamol HCl (Sigma-Aldrich, Germany)

Chrysin (Sigma-Aldrich, Germany)

Dimethyl sulfoxide (Fisher Scientific, England)

Di-sodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) (Merck, Germany)

Dulbecco's Modified Eagle's Medium (Gibco, USA)

Ethanol (J.T. Baker, Malaysia)

Fetal bovine serum (Hyclon, USA)

GBR 12909 dihydrochloride (Sigma-Aldrich, Germany)

Glacial acetic acid AR grade (Labscan Asia, Thailand)

Kaempferol (Sigma-Aldrich, Germany)

Liquid scintillant (Ultima Gold, Perkin Elmer, USA)

Luteolin (Fluka, Switzerland)

Magnesium Chloride ($MgCl_2 \cdot 6H_2O$) (Merck, Germany)

Methanol HPLC grade (Labscan Asia, Thailand)

Methoxy- $^3H$ Raclopride (Perkin Elmer Life Sciences, USA)

Methoxy- $^3H$ WIN 35,428 (Perkin Elmer Life Sciences, USA)

Naringin (Sigma-Aldrich, Germany)

N-methyl-$^3H$ SCH23390 (Amersham Biosciences GE Healthcare UK Limited, UK)

Nomifensine (Sigma-Aldrich, Germany)

Pennicillin-streptromycin (10,000units/ml penicillin G sodium and 10,000 µg/ml streptromycin sulfate) (Gibco, USA)

Polyethyleneimine (Sigma-Aldrich, Germany)

Quercetin dehydrate (Sigma-Aldrich, Germany)

R-(+)-SCH23390 HCl (Sigma-Aldrich, Germany)

S-(-)-Raclopride L(+)-tartrate salt (Sigma-Aldrich, Germany)

Sodium chloride (NaCl) (Merck, Damstadt, Germany)

Tris-Hydrochloride (Trizma hydrochloride™) (Sigma-Aldrich, Germany)

Vitexin (Fluka, Switzerland)

**2. Equipments**

Cellulose acetate membrane pore size 0.45µm (Sartorius, Germany)

GF/B Glass filter (Whatman, England)

Guard column (4.6 x 4 mm. particle size 5 µm) (Thermo Scientific, USA)

High Performance Liquid Chromatography instrument (Shimadzu, Japan) equipped with system controller (SCL-10 AVP), pump (LC-10ADVP), degasser (DGU-14A), diode array detector (SPD- M10 AVP), autoinjector (SIL-10 ADVP), column oven (CTO – 10ASVP), fraction collector (FRC – 10 A) and software version : 6.14 SP1

HIMAC centrifuge model SCR2013 (Hitachi Koki Co., Ltd, Japan)

Homogenizer (Glas–Col) (Cole-PARMER, USA)

Hypersil Gold C18 analytical column (4.6 x 250 mm. particle size 5 µm) (Thermo Scientific, USA)

Hypersil Gold C18 preparative column (10 x 250 mm. particle size 5 µm) (Thermo Scientific, USA)

Liquid Scintillation counter model WALLAC 1409 (WALLAC Oy, Finland)

Millipore model 1225 filtration manifolds. (Millipore corporation, USA)

Minisart filters pore size 0.2µm (Sartorius, Germany)

Nylon syringe filters pore size 0.45µm (National Scientific, USA)

Pump GAST model: DOA-V114-FD (Gast Manufacturing INC., USA)

Sartolon Polyamide membrane pore size 0.45µm (Sartorius, Germany)

Sonicator model Transsonic T890 (Elma Hans Schmidbauer GmbH & Co KG, Germany)

**3. Plant materials**

*Passiflora foetida* was collected from Rayong and Nonthaburi in June – August, 2003. The plants were extracted by Associate Professor Dr. Rutt Suttisri's group, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Dried *P. foetida* aerial parts were ground, then macerated with 95% ethanol. The filtrate was pooled and evaporated under reduced pressure at temperature not over 40°C to yield the ethanol extract (PF001). The ethanol extract was re-dissolved with 70% ethanol and partitioned with $CH_2Cl_2$. The $CH_2Cl_2$ layer was concentrated and further dissolved with 70% ethanol, then partitioned with hexane. Hexane layer was evaporated to provide the hexane extract (PF002) while the $CH_2Cl_2$ extract (PF003) was obtained by evaporating $CH_2Cl_2$ layer. The aqueous layer was concentrated under reduced pressure, and further partitioned with BuOH to afford the BuOH (PF004) and aqueous extracts (PF005). The PF003 extract was the focal composition investigated in this research due to the preliminary positive results of the *in vivo* anxiolytic effect and the *in vitro* dopamine $D_1$ receptor binding. Total extraction scheme is illustrated in Figure 4. PF003 could be further purified into at least 9 isolated compounds, namely, PF-2, PF-3, PF-A, PF-B, PF-C, PF-D, PF-E, PF-F and PF-G.

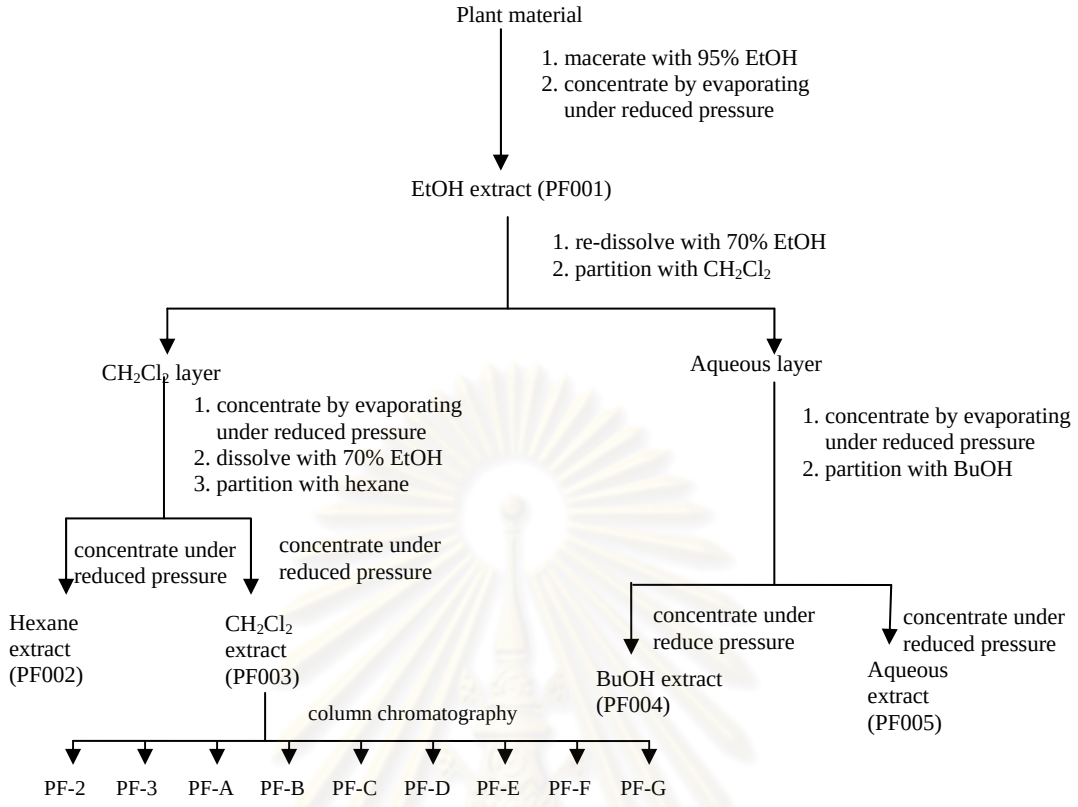



**Figure 4 Extraction scheme of *P. foetida*.**

## Methods

### I. High performance liquid chromatography (HPLC)

HPLC has become a broadly applicable and valuable tool for herbal analysis. For the present study, HPLC was applied to profiling and identifying the PF003 extract and related compounds, in addition to fractionating the extract for activity testing.

#### 1. HPLC sample preparation

PF003 extract was dissolved under a 30-minute sonication in a 1:1 mixture of 1% AcOH and MeOH. For profiling and identifying examination, a 5 mg/ml solution was prepared, while a 50 mg/ml solution was used for fractionation study.

Chrysin, a heterogenous flavonoid to *P. foetida*, was sometimes added to the crude extract as an internal standard. A brief sonication was required to prepare a 2 mg/ml stock chrysin solution in methanol. The chrysin stock solution was spiked to offer a final concentration of 0.02 mg/ml chrysin in the 5 mg/ml crude extract.

There were 9 isolated compounds obtained from the extract as described previously under the topic of plant materials. Due to limited availability of PF-F, only PF-2, PF-3, PF-A, PF-B, PF-C, PF-D, PF-E and PF-G were subjected to HPLC analysis. Other commercially available flavonoids, which have been reported as constituents of *Passiflora* spp., *e.g.* apigenin, chrysin, kaempferol, luteolin, naringin and vitexin were also analyzed for comparison. All these sample solutions were prepared in methanol and needed a brief sonication at room temperature to enhance complete dissolution.

Prior to HPLC column injection, all sample solutions were filtered through a 0.45 µm membrane filter. The filtrates were stored in amber vials until analysis.

**2. Chemical analysis of PF003 and isolated compounds**

Although the bioactivity of PF003 was preliminarily supported by the anxiolytic effect observed in the *in vivo* elevated plus maze model and by the positive dopamine $D_1$ receptor binding, the knowledge of PF003 chemical composition was limited. The attempt to illustrate the chemical information of the extract was therefore performed with HPLC analysis. The analysis condition was optimized by adjusting key separation and detection factors such as column temperature, wavelength for detection and gradient system. The evaluation of optimal condition was based on the characteristics of good resolution, reproducibility and the duration of analysis time.

**2.1 Chromatographic condition**

Chromatographic separations were carried out on a Hypersil gold C18 column (250 × 4.6 mm; i.d. 5 µm) with a guard column (4.6 x 4 mm; 5 µm). The mobile phase consisted of 1% acetic acid (A) and methanol (B) using a gradient program of 20% B in 0-20 min, 20-50% B in 20-50 min, 50-75% B in 50-63 min, 75% B in 63-68 min. The flow rate was 1.0 ml/min and the column temperature was maintained at 45 ºC. Injection volumes of sample solutions were 30 µl. DAD was set at 320 nm for monitoring chromatographic profile. The absorbance spectra for every chromatographic run were acquired from 200 to 550 nm.

## 2.2 Method validation

The chromatographic method in this experiment was developed for potential application as HPLC fingerprint analysis for plant extract. The parameters used to evaluate and validate are thus different from those of a general quantitative method. The requirement of a fingerprint analytical method is the ability to authenticate and identify each sample from different batches using the chromatographic profile. Considering this demanding application of a chromatographic pattern, the relative retention time (RRT) and the relative peak area (RPA) of dominant peaks (>2% of total peak area) were used to form the basic characterization of the *P. foetida* samples. RRT and RPA were calculated as follows.

$$\text{RRT} = \frac{\text{Retention time of peak of interest}}{\text{Retention time of reference peak}}$$

$$\text{RPA} = \frac{\text{Peak area of peak of interest}}{\text{Peak area of reference peak}}$$

Externally introduced chrysin was used as reference. The rigorousness of HPLC method was validated using the following parameters: precision, repeatability, and sample stability. The procedures to obtain each validating parameter were modified from the protocol of Ji group and Jin group (Ji *et al.*, 2005; Jin *et al.*, 2006) and described as follows.

**Precision**

The method precision was determined by replicate injection of the same sample solution for six times within a day. The precision was evaluated by observing the relative standard deviation (RSD) of RRT and RPA**.**

**Repeatability**

Six independently prepared sample solutions were analyzed with described HPLC condition and the RSD values of RRT and RPA were calculated to evaluate repeatability.

**Sample stability test**

The sample stability test was performed by injection of the same sample solution everyday for 3 days. During such period, the sample was kept at room temperature. The RSD values of RRT and RPA were used to verify the stability of the sample solutions during analysis.

### 3. The fractionation of extract by preparative HPLC

To examine the active composition of *P. foetida*, the extracts were fractionated by HPLC and tested for bioactivity using radioligand receptor binding assay. Chromatography was performed on a C18 preparative column (250 mm x 10 mm, 5 μm) with a flow rate of 4.0 ml/min. A 300 μl of extract solution (50 mg/ml) was injected to the column. The fractionation was achieved by using 1%AcOH (A) and MeOH (B) as mobile phase and adjusting the gradient accordingly:- 20% B in 0-20 min, 40-75% B in 20-30 min, and 70-75% B in 30-35 min. The column temperature was maintained at 45 °C and peak elution was monitored at 320 nm. Six fractions from the extract were collected: fraction A from 2.5 to 6 min, fraction B from 6 to 7 min, fraction C from 7.5 to 11.5 min, fraction D from 16 to 20 min, fraction E from 26 to 29.5 min and fraction F from 30 to 38.5 min. All fractions were concentrated by evaporation under vacuum and the final weight was noted. Each fraction was stored at -20°C in amber glass bottle until use.

## II. Radioligand receptor binding technique

The ability of a test sample to bind to target receptor is determined and described as the inhibition percentage to illustrate the potency of the test sample to displace radiolabeled ligands from binding sites. If the test sample has high affinity to target receptor, it will potentially displace high numbers of occupied radioligands and accordingly exhibit high inhibition percentage.

## 1. Dopamine receptor/transporter preparation

The receptors and transporter used in the present study were acquired from either isolated rat brains except for dopamine $D_2$ receptor which obtained from the culture of expressed cell lines available commercially.

### 1.1 Animals

Adult male Wistar rats (National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom) weighing 250-300 g were used. Upon arrival, the animals were housed two per cage, and maintained at ambient with 60% relative humidity and a 12-hour light/dark cycle with free access to food and water. The animal care and the study protocol were approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, under the project reference number 0833001.

### 1.2 Dissection of the brain areas

The animal was sacrificed by decapitation and the desired body part (head) was removed with scissors. After the skull was opened, the forebrain was rapidly removed and cooled in ice-cold phosphate buffered saline. The brain was then dissected to collect striatum which was immersed in liquid nitrogen for 2 min and stored at -80°C until use.

### 1.3 Membrane preparation for dopamine $D_1$ receptor binding assay

The membrane enriched with dopamine $D_1$ receptor was prepared according to the method by Pengsuparp group (Pengsuparp *et al.,* 2004). After striatum was dissected from rat brain, the tissue was homogenized at 70 rpm in 10 volumes of ice-cold 50 mM Tris–HCl buffer using a glass teflon homogenizer. Homogenates were centrifuged for 10 min at 3,300 rpm. The supernatant was re-centrifuged at 18,000 rpm for 15 min. Pellets were collected and re-suspended in 10 volumes of ice-cold Tris-HCl buffer and centrifuged at 18,000 rpm for 15 min. This step was done twice. Final pellets were gently homogenized in 3 volumes of ice-cold 50 mM Tris-HCl, 120 mM NaCl and 2 mM $MgCl_2$ (pH 7.4), then immersed in liquid

nitrogen and stored at -80°C until use. All centrifugation was performed at 4°C. Protein concentrations of the homogenates were determined using Bradford method.

**1.4 Membrane preparation for dopamine transporter (DAT) binding assay**

The membrane for DAT binding assay was prepared according to the methods of Avor group (Avor *et al.*, 1998) with some modifications. Striatum was dissected from rat brain and homogenized in 10 volumes of 50 mM Tris–HCl with 0.32 M sucrose at 70 rpm. Homogenates were centrifuged at 3,300 rpm for 10 min. The resultant supernatant was centrifuged at 13,800 rpm for 20 min. Pellets were collected, re-suspended and centrifuged once more at 13,800 rpm. The final pellets were homogenized and stored in 3 volumes of 50 mM Tris–HCl with 0.32 M sucrose. Degradation was prevented by steeping the final homogenates in liquid nitrogen for 2 min and stored at -80 °C until needed. Centrifugation process was controlled at 4°C. The membrane suspension was used for the dopamine transporter binding assay. Total protein concentration of the suspension was measured by Bradford method.

**1.5 Membrane preparation for dopamine $D_2$ receptor binding assay**

A9LhD2L cell lines (ATCC®Catalog no. CRL 10225) stably transfected with human dopamine $D_2$ receptor were grown in 144-$cm^2$ plastic culture dishes in complete DMEM medium containing 10% heat-activated fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37°C in humidified atmosphere containing 5% $CO_2$ and grown until confluency was reached. Cell harvest was achieved by gentle scraping and cell passages were performed every 3-4 days. Cell viability was monitored using trypan blue exclusion method.

To prepare membrane for dopamine $D_2$ receptor binding assay, the A9LhD2L cell lines at least $10^8$ cells were gently scraped from culture plates and then homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) at 70 rpm. Pellets were collected after centrifugation at 18,000 rpm for 15 min. Then, the pellets were further homogenized and centrifuged again following the aforementioned procedure. Lastly, the pellets were re-suspended in ice-cold 50 mM Tris-HCl, 120 mM NaCl and 2 mM $MgCl_2$ (pH 7.4) followed by a gentle homogenization, steeped in liquid nitrogen, then

kept at -80°C until use. Temperature of centrifugation process was set at 4°C. Protein concentrations were measured using Bradford method.

### 1.6 Protein determination by Bradford method

In order to quantify total protein using Bradford dye binding assay, a standard absorbance curve was developed using a series of bovine serum albumin (BSA) solutions in the range of 0.02 to 0.25 mg/ml. Two hundred and fifty microliters of BSA standards were added with 2.5 ml of Bradford reagent and then kept in dark area for 10 min. The UV absorbance of each standard solution was subsequently measured in duplicate at 595 nm. For cell or tissue homogenates, each test sample was initially diluted 50-100 folds preceding the introduction of Bradford reagent. The reaction begun when 250 µl of sample were incubated with 2.5 ml of Bradford reagent. After a 10-minute incubation without light exposure, the UV absorbance of each test sample solution was read and the concentration of each test sample was determined from the BSA standard curve.

## 2. Preparation of sample for radioligand binding assay

PF003 extract, PF-D, PF-E and HPLC fractionated samples (fractions A-F) were examined for the affinity to dopamine receptor/transporter compared with common *Passiflora* flavonoids, *e.g.* luteolin, vitexin, apigenin, chrysin and quercetin. The stock solution of each test substance was prepared at 20 mg/ml in DMSO, except for apigenin which was prepared at 10 mg/ml due to its limited solubility. All stock solutions were stored at -20 °C. Prior to the binding evaluation, each sample solution was diluted ten folds with distilled water. The mixtures of fractions D-F were also prepared by adding 10 µl of 20 mg/ml solution of each fraction in DMSO into 270 µl of distilled water. The final concentration of DMSO in the assay was 0.5 %.

## 3. Radioligand receptor/transporter binding assays

The ability of any test compounds to compete with specific radioligand in binding to target receptors can be evaluated and described as the inhibition percentage. The assay generally comprises the determination of the radioligand binding to the studied receptor by observing the radioactivity remained in the

membrane preparation, with and without a test sample. To account for any non-specific binding, the radioligand binding is also separately determined in the presence of excessive specific unlabeled ligand. The high concentration of the specific unlabeled ligand is expected to entirely displace bound radioligand from the receptor. The remaining radioactivity in the assay membrane is thus from the binding of radioligand to non-specific sites. Specific binding of radioligand to the receptor can then be calculated by subtracting the non-specific binding estimate from the original binding result and used to determine the percent inhibition of the test sample as follows:

% Inhibition = $100 - [(\text{specific binding})_{\text{test sample}} / (\text{specific binding})_{\text{total binding}}] \times 100]$, where $(\text{specific binding})_{\text{test sample}}$ is the radioligand binding when the test sample is present and non-specific binding is excluded; $(\text{specific binding})_{\text{total binding}}$ is the total radioligand binding and non-specific binding is excluded.

A positive control may be used to confirm the quality of the membrane preparations. Measurements of radioligand dopamine receptor/transporter bindings were prepared according to the methods of Zhu group and Pengsuparp group (Zhu *et al.*, 1996; Pengsuparp *et al.*, 2004) with some modifications. The details of specific conditions used for each receptor/transporter binding assay were described below.

### 3.1 Dopamine $D_1$ receptor binding assay

The assay was performed at 25°C in a test tube containing 750 µg protein of membrane preparation in binding buffer consisting of 50 mM Tris-HCl, 120 mM NaCl and 2 mM $MgCl_2$ (pH 7.4). One hundred microliters of 5 nM [$^3$H]SCH23390 were added to the membrane preparation to achieve a final concentration of 0.5 nM. Fifty microliters of 2 mg/ml test sample were then added to the membrane tube with a final concentration of 100 µg/ml. After a 30-minute incubation, the mixture was rapidly filtered under vacuum through GF/B glass filters pre-soaked with 0.3% polyethylenimine. The filters were washed three times with 3 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4), then placed in vials containing 5 ml of scintillation fluid and agitated with a vortex mixer for 3 min. The radioactivity remaining on filters was determined by liquid scintillation counter. Non-specific binding was assessed using similar procedure and $5 \times 10^{-6}$ M butaclamol was used as specific unlabeled ligand. The total radioligand binding was evaluated when only

vehicle (0.5% DMSO) was present. The experiment also used SCH23390 at a concentration of a 5 x $10^{-10}$ M as the positive control. Percent inhibition was calculated by the aforementioned equation.

### 3.2 Dopamine $D_2$ receptor binding assay

Each assay tube contained 100 µl of [$^3$H]raclopride to achieve a final concentration of 2 nM, 50 µl of 2 mg/ml test sample, 200 µg protein of membrane preparation and the final volume was adjusted to 1,000 µl with 50 mM Tris-HCl, 120 mM NaCl and 2 mM $MgCl_2$ buffer (pH 7.4). The tubes were incubated at 25°C in a shaker water bath. After incubation period of 30 min, the mixture was rapidly filtered under vacuum through Whatman GF/B glass fiber filters previously soaked in 0.3% polyethyleneimine. Then, the filters were rinsed with ice-cold 50 mM Tris-HCl (pH 7.4) three times and put in vials containing 5 ml of liquid scintillant. After mixing, the radioactivity bound to filters was measured by liquid scintillation counter. Non-specific binding was estimated in the presence of 1 x $10^{-4}$ M butaclamol. The incubation of dopamine $D_2$ receptor membranes with only 0.5% DMSO was used to determine total radioligand binding. Positive control in the experiment was 1x$10^{-8}$ M raclopride. Percent inhibition was calculated as previously described.

### 3.3 Dopamine transporter (DAT) binding assay

The experiment started by mixing 50 µl of 2 mg/ml test sample (a final concentration of 100 µg/ml) with 100 µl of 36 x $10^{-9}$ M [$^3$H]WIN35428 (a final concentration of 3.6 nM), and 750 µg protein of the vesicle preparation and adjusted the final volume to 1,000 µl with 32 mM sodium phosphate buffer (pH 7.4). Incubating condition for DAT binding assay was 25°C for duration of 15 min. Afterwards, the binding experiment was stopped by rapid vacuum filtration through GF/B glass fiber filters which were soaked in 0.3% polyethyleneimine prior to use. Filters were washed with ice-cold 32 mM sodium phosphate buffer three times, then placed in glass tubes containing 5 ml of liquid scintillant and agitated for 3 min. Radioactivity was measured using liquid scintillation counter. Non-specific binding was estimated in the presence of 1 x $10^{-4}$ M nomifensine and the total radioligand binding was evaluated when only 0.5% DMSO was present. GBR12909 at a

concentration of 1 x $10^{-7}$ M was used as the positive control. Percent inhibition was calculated by the above equation.

**4. Scatchard analysis of [$^3$H]raclopride binding to dopamine $D_2$ receptor**

Scatchard analysis was performed to derive $K_d$ and $B_{max}$ of [$^3$H]raclopride binding to dopamine $D_2$ receptor. Membrane preparation (750µg protein/assay) was incubated for 30 min at 25˚C in 50 mM Tris-HCl, 120 mM NaCl and 2 mM $MgCl_2$ buffer (pH 7.4) with various concentration of [$^3$H]raclopride in the range of 0.5-8 nM. Non-specific binding was defined as the residual binding observed in the presence of 1 x $10^{-4}$ M butaclamol. Total radioactivity of each radioligand concentration was determined when only [$^3$H]raclopride was present without dopamine $D_2$ receptor membrane. When the incubation ended, the membrane-bound radioligand was recovered by rapid filtration through GF/B glass fiber filters pre-soaked in 0.3 % polyethyleneimine. The filters were washed repeatedly 3 times with 3 ml of ice-cold 50 mM Tris HCl buffer (pH 7.4) and were then added to 5 ml of liquid scintillation. The remaining radioactivity on the filter was counted. This procedure was modified from Kokey and Macer protocol (Kokey and Macer, 1996). Scatchard plot was constructed between the bound versus free radioligand ratios (Y-axis) and the free radioligand (X-axis). Bound value was conversed from specific binding while free value were obtained by subtracting bound from total radioactivity at the same concentration. $K_d$ (receptor dissociation constant) and $B_{max}$ (the density of binding sites) were calculated from Scatchard plot as described below (Foreman, 2004).

$K_d$ = -1/ Slope

$B_{max}$ = the intercept on the X-axis

**5. Determination of the $IC_{50}$ value and inhibition constant ($K_i$) of luteolin to dopamine $D_2$ receptor**

The concentration of luteolin that exhibited 50 % inhibition ($IC_{50}$) against [$^3$H]raclopride binding to the dopamine $D_2$ receptors was analyzed from the dose-response curve using CurveExpert 1.3 program. The curve was generated from the results of a competitive binding experiment between [$^3$H]raclopride and luteolin. The

concentration of [$^3$H]raclopride was fixed at 2 nM while the luteolin concentrations were varied from 0.78 to 100 µg/ml. $K_i$ was calculated from the following equation:

$K_i = IC_{50}/(1 + L/K_d)$, where $L$ represented the chosen [$^3$H]raclopride concentration and $K_d$ represented the dissociation constant of [$^3$H]raclopride (Nencini *et al.*, 2006).

**6. Statistical analysis**

For binding experiments, the data were presented as the mean ± S.E.M. The reproducibility of the results was confirmed with two or three repeated experiments, and duplicate or triplicate readings in each analysis.

# CHAPTER IV

# RESULTS

## I. Chromatographic analysis of dichloromethane extract of *P. foetida* (PF003) and isolated compounds

### 1. Optimization of HPLC conditions

A set of initial HPLC conditions was obtained from the preliminary work of the Development of Herbal Prototype for Suppression of Addictive CNS Stimulants research project. The PF003 extract was separated on a reversed phase C18 column and eluted with 1% AcOH (solvent A) and MeOH (solvent B) using the following mobile phase scheme: 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-100% B) in 50-75 min, and 100% (B) in 75-85 min. The chromatographic profile (Figure 5) was observed at 280 nm. The elution peaks from this condition were fairly resolved but the analysis time was lengthy and the reproducibility of retention times was mostly unsatisfactory. The optimization was thus performed with an aim to shorten chromatographic run time and to enhance the reproducibility. Firstly, the effect of column temperature on the separation performance was examined. The chromatograms obtained with column temperature set at 25, 35 and 45 °C were compared and shown in Figure 6. The results showed that the temperature at 45 °C could improve peak resolution, shorten peak elution time, and enhance the consistency of chromatographic profile. The improvement of peak resolution could be observed with the separation between peaks at retention time of 44 and 45 min (in Figure 6, the arrow-pointed peak position). The resolution value of these two peaks in PF003 chromatogram when column temperature at 25°C was 0.8 while the value of 1.9 and 2.8 were obtained at temperature 35°C and 45°C, respectively. Additionally, higher temperature was also able to reduce the elution time of most peaks. For example, the highest peak in the chromatogram was eluted at retention time of 47.4 min at column temperature of 25°C, but when the temperature was increased to 35°C and 45°C, the retention time was reduced to 44.4 and 42.3 min, respectively.

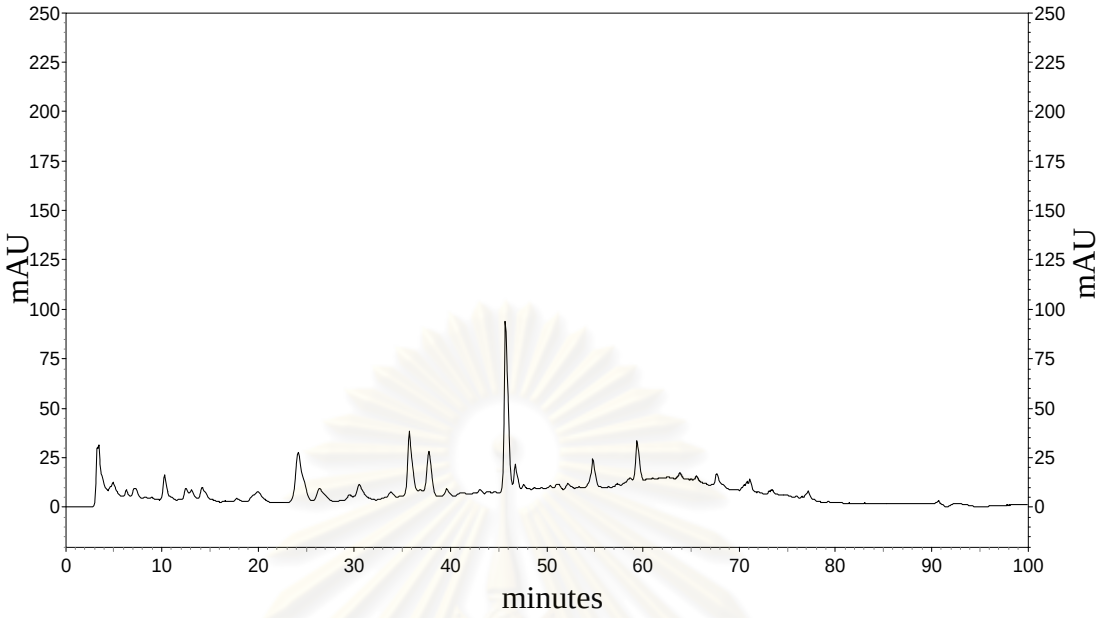



**Figure 5 HPLC chromatogram of PF003 (5 mg/ml) obtained from initial condition.** HPLC conditions - column: Hypersil gold C18, 5 µm, 250 mm x 4.6 mm i.d.; the mobile phase: 1% AcOH (A) and MeOH (B), with a gradient program of 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-100% (B) in 50-75 min, 100% (B) in 75-85 min; flowing rate: 1.0 ml/min; ambient temperature; detection wavelength: 280 nm.

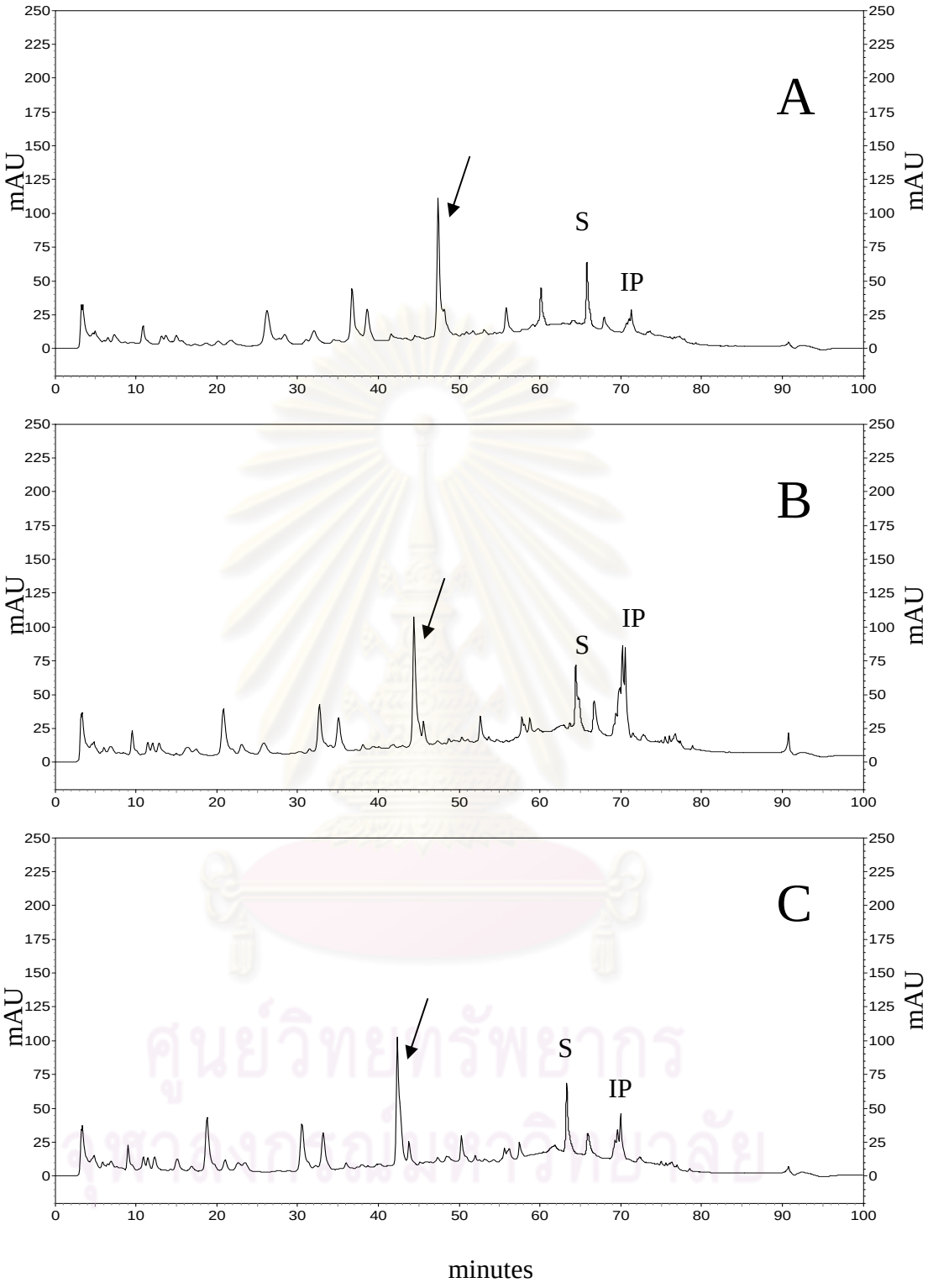



**Figure 6 HPLC chromatograms of PF003 (5 mg/ml) obtained from different column temperature.** A, B and C as chromatograms at temperature of 25, 35 and 45°C, respectively. S = peak of 0.008 mg/ml chrysin standard. IP = the interfering peak group from column materials.

Thermostated column could also enhance reproducibility of chromatographic profile by decreasing a retention time shift of most peaks in PF003 chromatogram.

Secondly, the selection of appropriate detection wavelength was also a key to a reliable and reproducible chromatogram. DAD was applied to identify the optimal wavelength for monitoring most chemical constituents in the chromatogram of the extract. A full-scan chromatogram was therefore obtained between 220 to 500 nm. The analytical wavelength desirable for peak detection would be decided by main peak maximization and minimization of interfering peaks. The interfering peaks were a group of peaks inconsistently appeared at the end of chromatogram (IP peak in Figure 7). They could be ruled out from the interested peaks or an incomplete elution of previous run by injecting blank without sample after the column was washed. This occurrence was commonly observed with phenyl type stationary phases such as a C18 column. Williams (2004) suggested that the extraneous peaks could stem from the increased "bleed" of stationary phase under the late and stronger eluting power of the gradient. For PF003 chromatogram, maximal absorbance was observed around 280 to 350 nm. Summary of detectable peak areas in the PF003 chromatograms obtained at 280, 320 and 350 nm was presented in Figure 7 and Table 2. The results demonstrated that major peaks were most prominent at 320 nm concurrent with a smooth baseline, while undesirable peaks were reduced.

Lastly, gradient elution was adjusted with an aim to lower the use of organic solvent as much as possible while the separation of major peaks was still satisfactory. In the PF003 chromatogram obtained from the original gradient program, the last peak was eluted when the ratio of methanol was around 75 – 80 % and at the highest percentage (100%) of methanol, the chromatogram did not show any peaks. Therefore, a new gradient program was established by reducing the highest percentage of methanol from 100 to 75 and hold for 5 min. The increasing rate of the methanol in gradient step was kept in the same of the initial gradient condition but the run time was shortened. Comparison of gradient steps between the new and original gradient programs was shown in Figure 8. The new gradient program was of 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-75% (B) in 50-63 min, 75% (B) in 63-68 min. The optimal HPLC condition was summarized in Table 3.

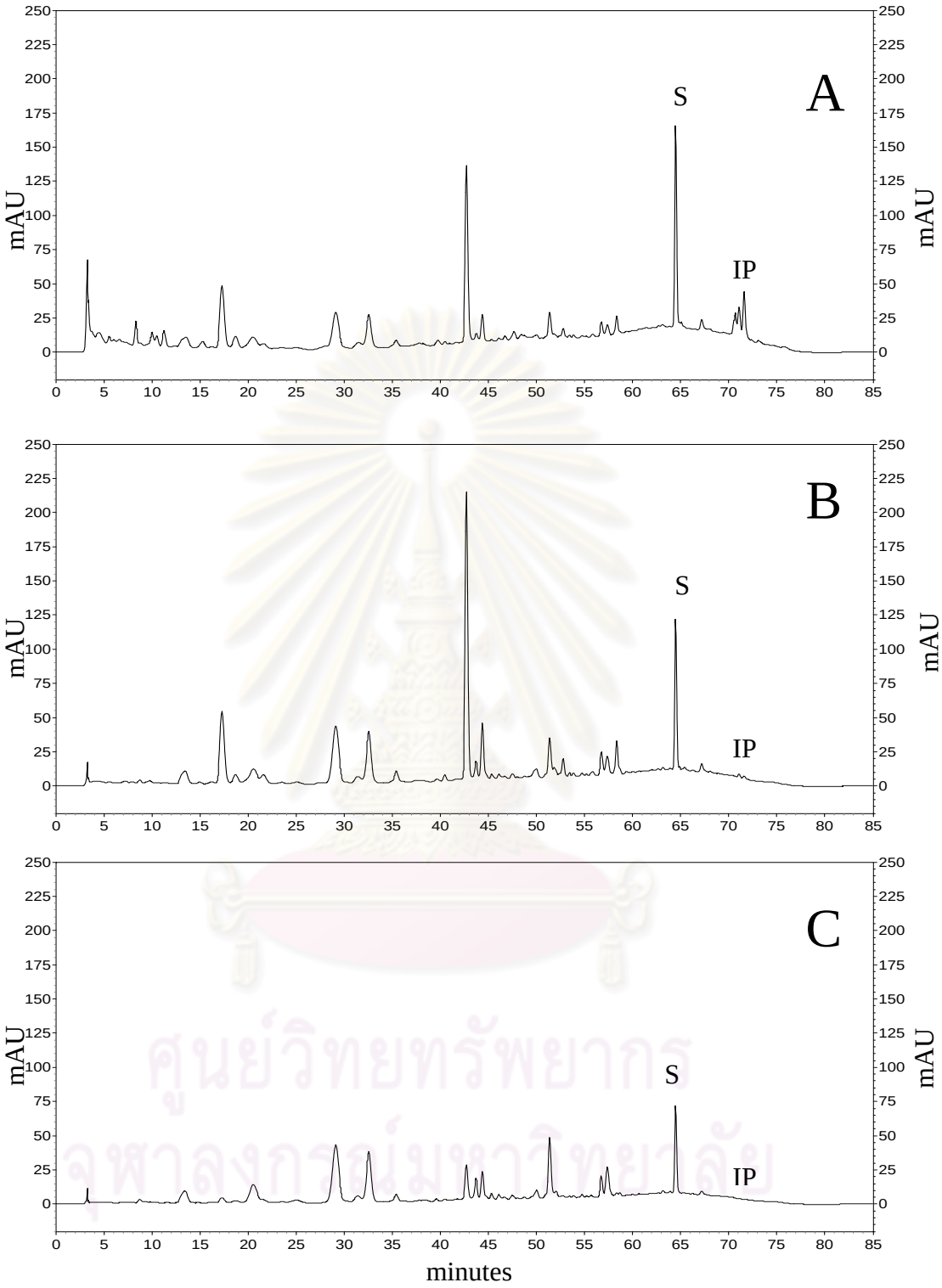



**Figure 7 HPLC chromatograms of PF003 (5.2 mg/ml) obtained at different wavelength.** A, B and C as chromatograms at 280, 320 and 350 nm, respectively. S = peak of 0.02 mg/ml chrysin standard. IP = the interfering peak group from column materials.

**Table 2 The peak area of PF003 chromatogram at 280, 320 and 350 nm.**

| Peak | Retention time | Peak area at 280 nm | Peak area at 320 nm | Peak area at 350 nm |
|---|---|---|---|---|
| 1 | 3 | + + | - | - |
| 2 | 4.5 | + | - | - |
| 3 | 8 | + | - | - |
| 4 | 14 | + | + | + |
| 5 | 17 | + + | + + + | - |
| 6 | 20.5 | + | + | + |
| 7 | 29 | + + | + + + | + + + |
| 8 | 32 | + | + + | + + |
| 9 | 43 | + + + | + + + + | + |
| 10 | 44 | + | + | + |
| 11 | 51 | + | + | + |
| 12 | 57 | - | + | + |
| 13 | 58 | - | + | + |
| 14 (chrysin) | 64.5 | + + + | + + | + + |

The illustration of symbols

+ = peak area in the range of 200,000 – 800,000 mAU-min

++ = peak area in the range of 800,001 – 1,500,000 mAU-min

+++ = peak area in the range of 1,500,000 – 3,000,000 mAU-min

++++ = peak area of more than 3,000,000 mAU-min

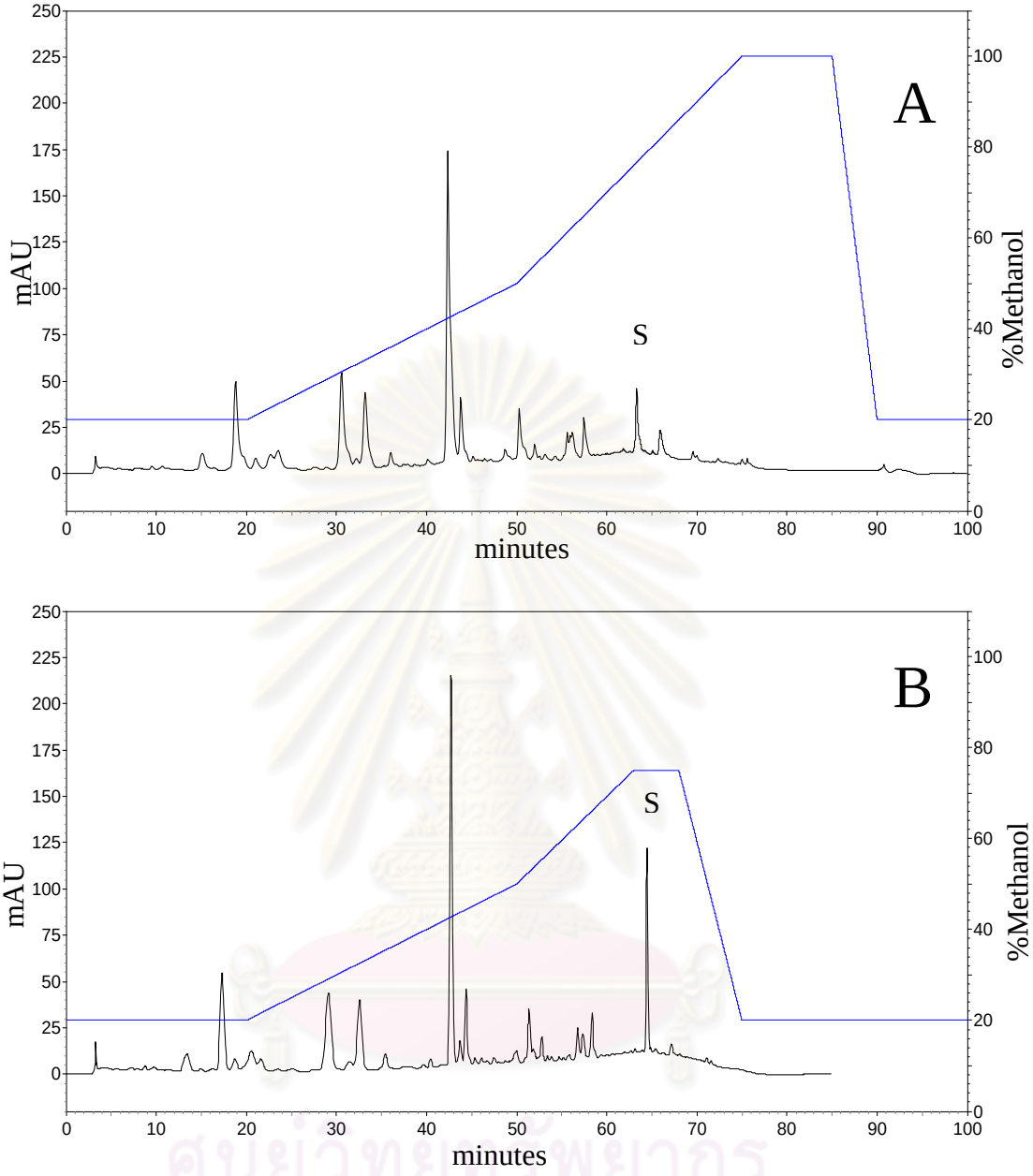



**Figure 8 PF003 chromatogram overlaid with its gradient program.**

Chromatogram A; HPLC condition: the mobile phase: 1% AcOH (A) and MeOH (B), with a gradient program of 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-100% (B) in 50-75 min, 100% (B) in 75-85 min; flowing rate: 1.0 ml/min; temperature: 45 °C; detection wavelength: 320 nm. Chromatogram B; HPLC condition: the mobile phase: 1% AcOH (A) and MeOH (B), with a gradient program of 20% B in 0-20 min, 20-50% B in 20-50 min, 50-75% B in 50-63 min, 75% B in 63-68 min; flowing rate: 1.0 ml/min; temperature: 45 °C; detection wavelength: 320 nm. S = chrysin standard, in chromatogram A with 0.008 mg/ml and 0.02 mg/ml in chromatogram B.

**Table 3 Optimization for qualitative analysis of constituents in PF003 extract.**

| HPLC Parameters | Optimized condition |
|---|---|
| Stationary phase | Reversed phase C18 column (250 x 4.6 mm) |
| Mobile phase | 1% AcOH (A) and MeOH (B) |
| Gradient condition | 20% B in 0-20 min, 20-50% B in 20-50 min, 50-75% B in 50-63 min, 75% B in 63-68 min |
| Column temperature | 45 °C |
| Injection volume | 30 microliters |
| Flow rate | 1 ml/min |
| Wavelength for detection | Photodiode array detection, 320 nm |

**2. Method validation**

The reproducibility, repeatability and sample stability of the optimized HPLC method for PF003 were evaluated. Six major peaks, each possessing over 2% of total peak area, were selected as characteristic peaks of the extract (Figure 9). The method precision, represented by the relative standard deviations (RSD), were below 0.62 % (n = 6) for RRT and 0.76 % (n=6) for RPA. Repeatability assessed by analyzing six independently prepared samples gave the highest RSD values of RRT and RPA at 3.99 and 5.82 %, respectively. Stability of sample during analysis was examined by determining the variations of RRT and RPA from repeated HPLC runs of the same sample for three consecutive days. The RSD of RRT and RPA found in the stability test were less than 3.23 and 6.68%, respectively, and the summary of all results was shown in Tables 4 and 5.

**3. Analysis of PF003 and isolated compounds from PF003**

To illustrate the chemical information of PF003 extract and the isolated compounds, the HPLC analysis was applied. The chromatographic profile of PF003 showed more than 10 peaks. Six peaks were marked and named as major peaks based on their significant contribution (not less than 2%) to the total peak area, as shown Figure 9. It was noted that only PF-E, PF-D, PF-3 and PF-2 appeared on the chromatogram with retention times of 29, 50.5, 56.8 and 57.3 min, respectively. Other isolated compounds, namely, PF-A, PF-B, PF-C and PF-G, could not be detected with HPLC at studied sample concentration of higher than 100µg/ml. Attempts were made to detect the peaks of PF-A, PF-B, PF-C and PF-G by scanning each chromatogram within the range of 200 – 800 nm with contour screen program in HPLC-DAD. The contour picture of each chromatogram was an integrate data plotted between three parameters, namely, retention time, detected wavelength and intensity of peak. The contour pictures of PF-A, PF-B, PF-C and PF-E chromatograms did not exhibit any dominant peak within 200 – 800 nm.

Another objective of this analysis was to observe the association of the chromatographic profiles of isolated compounds from PF003 with the original extract chromatogram. When compared, only the chromatograms of isolated PF-E and PF-D

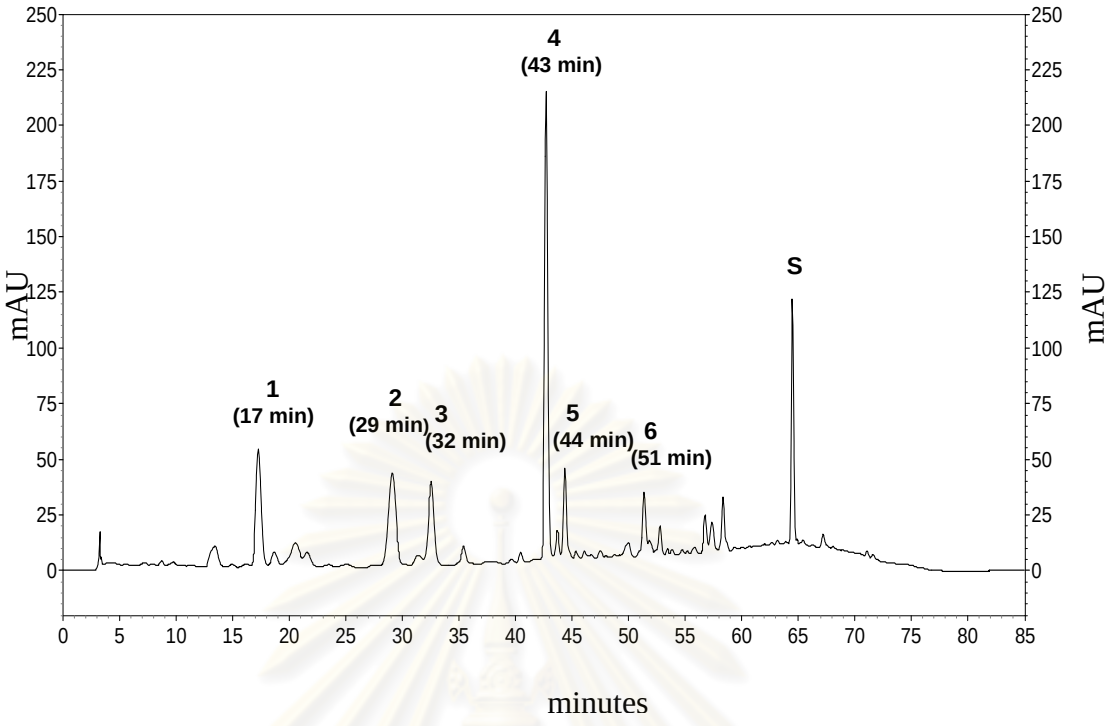



**Figure 9 Six major peaks in HPLC chromatogram of PF003 at 320 nm.** HPLC condition: the mobile phase: 1% AcOH (A) and MeOH (B) with gradient program, 20% B in 0-20 min, 20-50% B in 20-50 min, 50-75% B in 50-63 min, 75% B in 63-68 min; flowing rate: 1.0 ml/min; temperature: 45 °C; detection wavelength: 320 nm. S = peak of chrysin standard.

**Table 4 The percentage of Relative Standard Deviation of relative retention time**.

| Peak | Retention time (min) | RRT | %RSD precision | %RSD repeatability | %RSD sample stability |
|---|---|---|---|---|---|
| 1 | 17 | 0.26 | 0.35 | 2.88 | 2.11 |
| 2 | 29 | 0.45 | 0.62 | 3.99 | 3.23 |
| 3 | 32 | 0.50 | 0.39 | 2.41 | 1.98 |
| 4 | 43 | 0.67 | 0.08 | 0.87 | 0.72 |
| 5 | 44 | 0.68 | 0.08 | 0.71 | 0.59 |
| 6 | 51 | 0.79 | 0.04 | 0.49 | 0.42 |
| chrysin | 64.5 | 1 | 0 | 0 | 0 |

**Table 5 The percentage of Relative Standard Deviation of relative peak area**.

| Peak | Peak area (mAU-min) | RPA | %RSD precision | %RSD repeatability | %RSD sample stability |
|---|---|---|---|---|---|
| 1 | 1583543 | 0.97 | 0.58 | 5.82 | 0.56 |
| 2 | 1874768 | 1.15 | 0.58 | 5.02 | 1.04 |
| 3 | 1262360 | 0.77 | 0.15 | 3.90 | 1.20 |
| 4 | 4003026 | 2.45 | 0.39 | 5.13 | 0.54 |
| 5 | 655246 | 0.40 | 0.60 | 5.72 | 6.68 |
| 6 | 579490 | 0.35 | 0.76 | 4.70 | 1.11 |
| chrysin | 1633800 | 1 | 0 | 0 | 0 |

could be match with peaks 2 and 6, respectively, of the extract (Figure 10). Additionally, similar peaks to those of PF-2 and PF-3 also appeared in PF003 chromatogram but were not assigned as dominant peaks. Four major peaks, peak 1, 3, 4 and 5, in the extract chromatogram, therefore, have not been isolated by column chromatography.

## II. Fractionation of PF003 by preparative HPLC and fraction analysis

In order to isolate the four remaining major peaks in chromatogram of the extract preparative HPLC was employed. A suitable condition for fractionation was developed by taking into account the increased diameter of a preparative column compared with that of an analytical column (10 mm versus 4.6 mm). Due to the increased surface area, the flow rate used to elute compounds had to be increased accordingly to around four times of the rate used for analytical condition. The optimized condition consisted of a gradient elution using 1%AcOH (A) and MeOH (B) as mobile phase together with the following gradient profile: 20% B in 0-20 min, 40-75% B in 20-30 min, 70-75% B in 30-35 min with a flow rate of 4 ml/min. The column temperature was controlled at 45˚C. The total elution time was 35 min. The preparative HPLC condition was summarized in Table 6. PF003 extract was separated into 6 major fractions, namely, fractions A to F. As shown in Figure 11, fractions A, B, C, D, E and F were collected during the elution time of 2.5 – 6, 6 – 7, 7.5 – 11.5, 16 – 20, 26 – 29.5 and 30 – 38.5 min, respectively. Each fraction was analyzed by HPLC and compared with the PF003 chromatogram. The results showed that they represented the chromatographic profiles of PF003 at following elution times, 15 - 20, 25 - 35, 40 - 45, 46 - 55, 55 - 60 and 60 - 80 min (Figure 12). The yields of five fractions collected from 18 rounds of a 300 µl of 50 mg/ml PF003 extract injection were 7.7, 4.7, 20.5, 4.8, 15 and 186.8 mg for fractions A to F, respectively. The calculated percent yields are summarized in Table 7. All fractions were investigated for their binding activities to dopamine receptors and transporter.

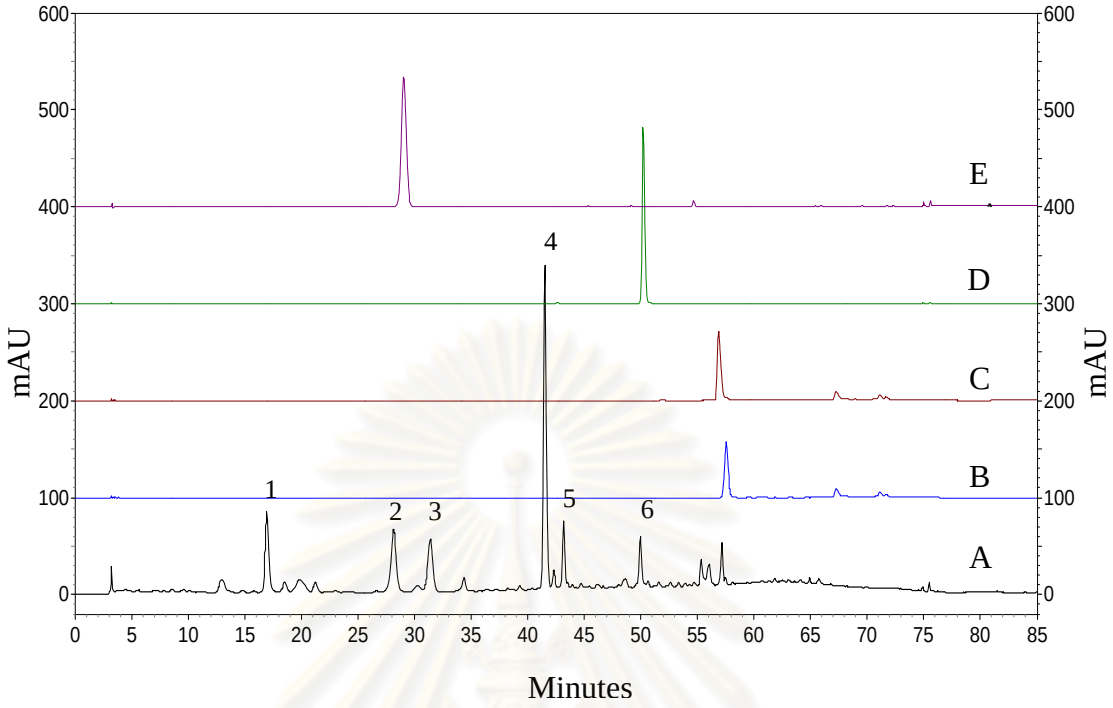



**Figure 10 The overlaid HPLC chromatogram of PF003 (A), PF-2 (B), PF-3 (C), PF-D (D) and PF-E (E) at 320 nm.**

**Table 6 Preparative HPLC condition for PF003 fractionation.**

| HPLC Parameters | Optimized condition |
|---|---|
| Stationary phase | Reversed phase C18 column (250 x 10 mm) |
| Mobile phase | 1% AcOH (A) and MeOH (B) |
| Gradient condition | 20% B in 0-20 min, 20-70% B in 20-30 min, 70-75% B in 30-35 min |
| Column temperature | 45 degree celcius |
| Injection volume | 300 microliters |
| Flow rate | 4 ml/min |
| Wavelength for detection | Photodiode array detection, 320 nm |

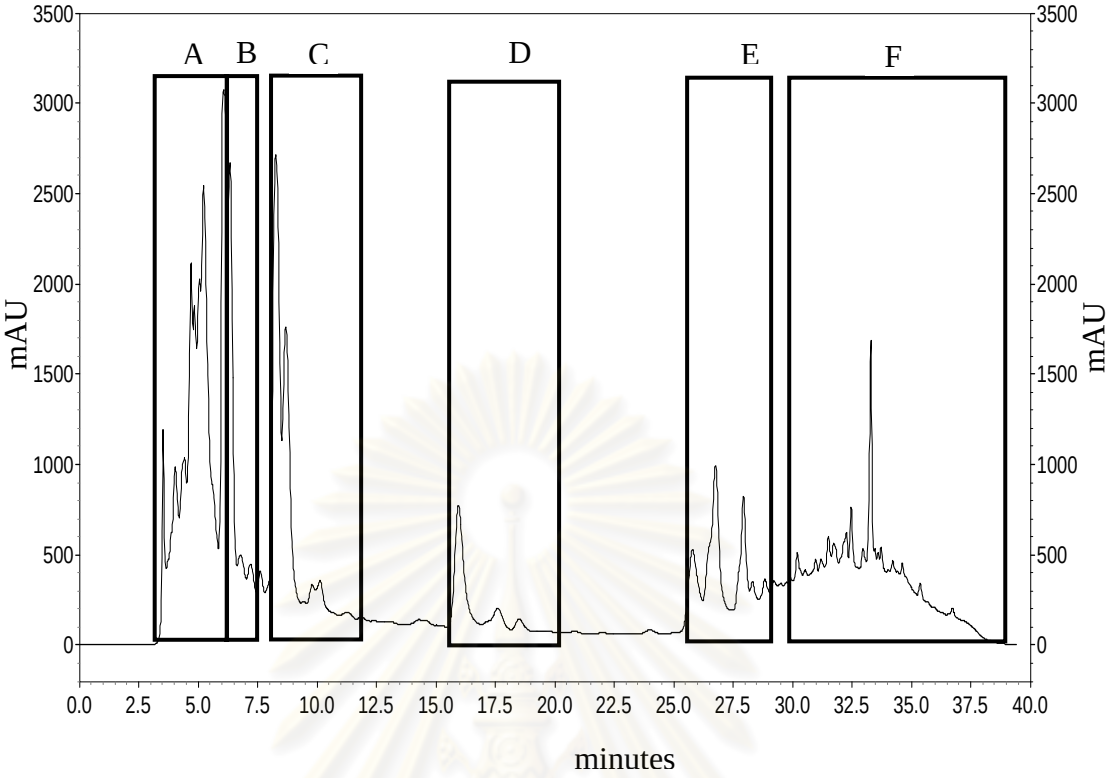



**Figure 11 The HPLC chromatogram of PF003 from preparative HPLC at 320 nm.** Six fractions collected from column; A from 2.5 – 6 min, B from 6 – 7 min, C from 7.5 – 11.5 min, D from 16 – 20 min, E from 26 – 29.5 min and F from 30 – 38.5 min.

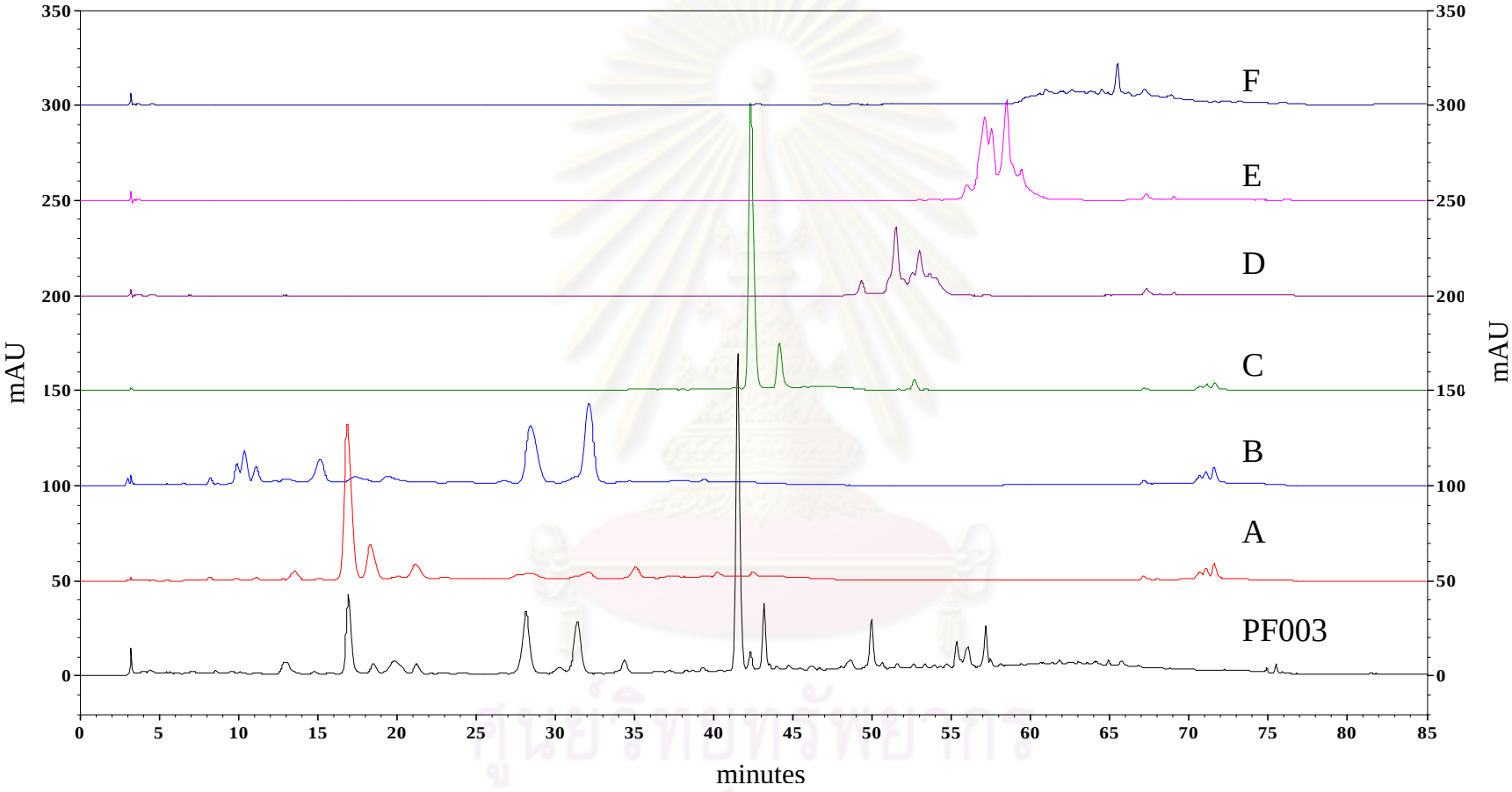



**Figure 12 The overlaid HPLC chromatogram of PF003 and fraction A-F at 320 nm.**

**Table 7 The percent yield of six fractions isolated from PF003 extract by using preparative HPLC.**

| Fraction | Percent yield |
|---|---|
| A | 2.8 |
| B | 1.4 |
| C | 7.6 |
| D | 1.6 |
| E | 5.5 |
| F | 69.2 |

## III Dopamine receptors/transporter binding assay

To find out the bioactive substances based on the affinity to dopamine $D_1$, $D_2$ and dopamine transporter (DAT), six fractions obtained from preparative HPLC were evaluated and compared with the binding performance of PF003.

### 1. Dopamine $D_1$ receptor binding assay

The affinity of PF003 to dopamine $D_1$ receptor was represented by percent inhibition value of 26 ± 2.1, tested at the PF003 concentration of 100 µg/ml. The dopamine $D_1$ receptor binding of six fractions from semi-preparative HPLC were also evaluated. As shown in Figure 13, it is noted that fractions A-C, E and F at concentration of 100 µg/ml did not appear to bind to the dopamine $D_1$ receptors. Fraction D (100 µg/ml) showed weak binding with an inhibition value of 12 ± 1.2 %, while the combination of fractions D, E and F at concentration of 100 µg/ml could exhibit higher inhibition than individual fraction with a value of 22 ± 3.8 %. In addition, some of isolated compounds from PF003 (PF-D and PF-E) were assessed as well. The results showed that only PF-D was able to bind to dopamine $D_1$ receptor with an inhibition value of 26 ± 6.4 %, whereas no affinity was observed for PF-E (2 ± 1.7%).

### 2. Dopamine $D_2$ receptor binding assay

The PF003 extract at the concentration of 100 µg/ml showed moderate affinity to dopamine $D_2$ receptor with percent inhibition of 30 ± 4. To identify the active fractions of PF003 for binding to dopamine $D_2$ receptor, six isolated fractions from preparative HPLC were evaluated. As shown in Figure 14, fractions A, B, C and F appeared to lack the ability to displace the radioligand from dopamine $D_2$ receptor, thus minimal percent inhibition was obtained. Astonishingly, fraction-D (100 µg/ml) exhibited a very high percent inhibition of 61 ± 2.6 % which indicated that the fraction likely possessed high affinity to dopamine $D_2$ receptor. Lastly, fraction E also showed some ability to displace the radioligand with a percent inhibition of 11 ± 2.1 at the same concentration. The combination of fractions D-F at concentration of 100 µg/ml gave an inhibition percentage of 43 ± 2.3. The isolated compound PF-D was also assessed and showed a high percent inhibition of 66 ± 3.1 %, while PF-E seemed to lack the binding ability to dopamine $D_2$ receptor.

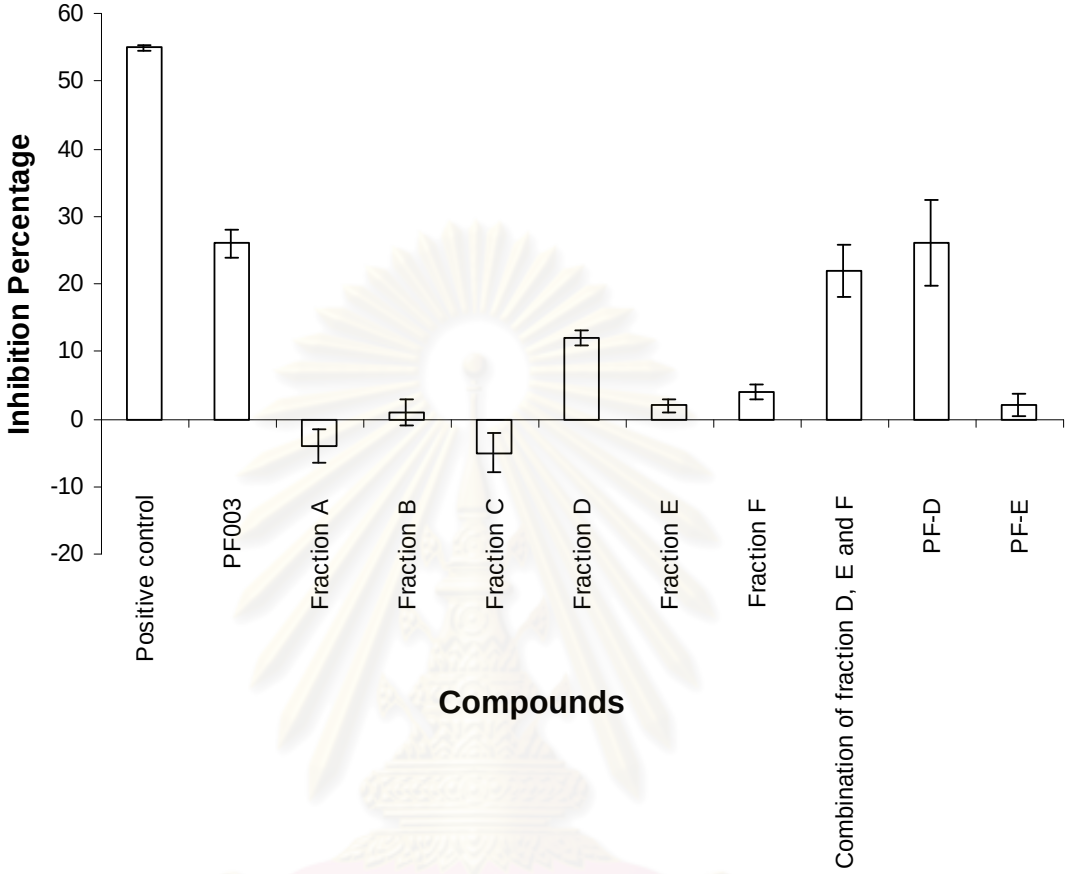



**Figure 13** **The Inhibition of radioligand specific binding on dopamine $D_1$ receptor of the extract, fractions and isolated compounds.** Each compound is incubated with 0.5 nM [$^3$H]SCH23390, and 750 µg of dopamine $D_1$ receptor protein. The 5 x $10^{-10}$ M SCH23390 is used as positive control. The tested concentration of each compound is 100 µg/ml. Each column represents the mean value ± S.E.M. of three independent experiments, with duplicate replication in each experiment.

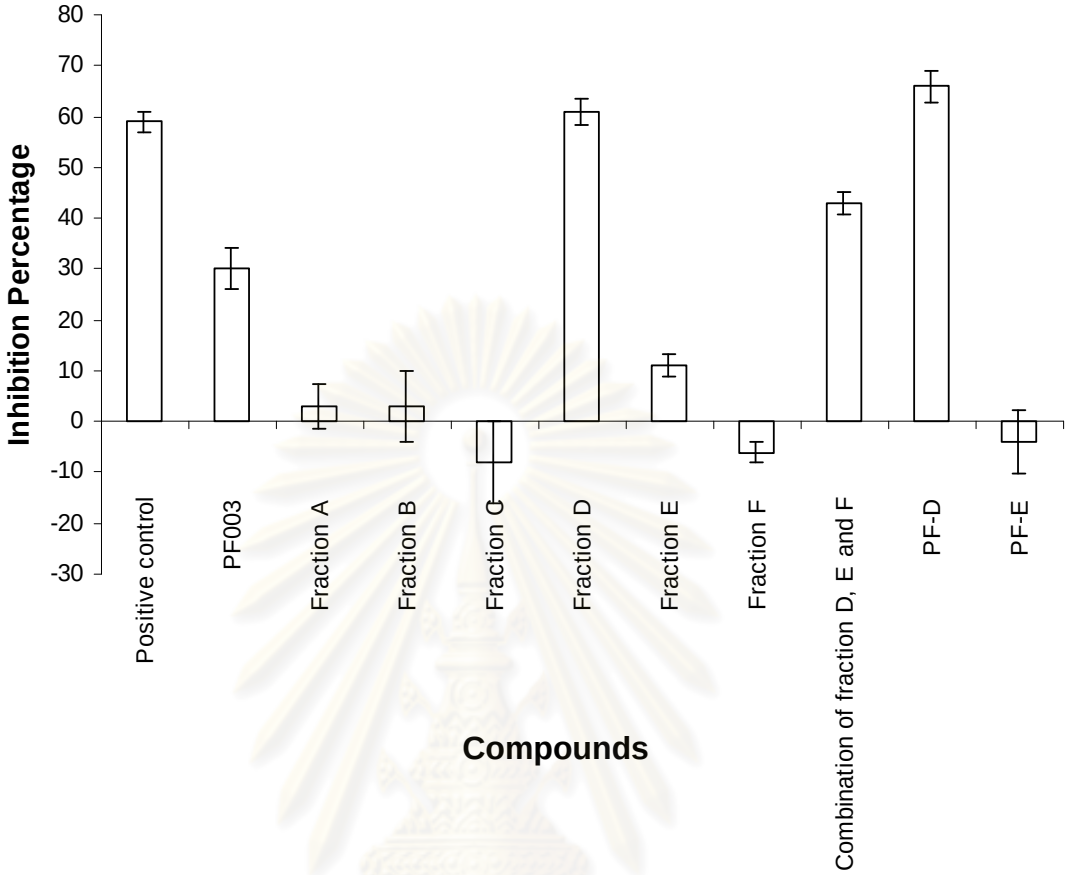



**Figure 14** **The Inhibition of radioligand specific binding on dopamine $D_2$ receptor of the extract, fractions and isolated compounds**. Each compound (100 µg/ml) is incubated with 2 nM [$^3$H]raclopride, and 200 µg of dopamine $D_2$ receptor protein. The $1x10^{-8}$ M of raclopride is used as positive control. The tested concentration of each compound was 100 µg/ml. Each column represents the mean value ± S.E.M. of three independent experiments, with duplicate replication in each experiment.

### 3. Dopamine transporter (DAT) binding assay

The evaluation of PF003 and its isolated compounds for the binding to DAT was performed. Results from initial experiment were shown in Figure 15. PF003 appeared to bind to DAT with a high percent inhibition of 70; while fractions A, B and C showed some degrees of binding with percent inhibition ranging from 11 to 24 at the concentration of 100 µg/ml. The binding of isolated compounds, PF-D and PF-E to DAT was found to be minimal. In addition, the DAT binding assay had inherently higher variations due to the low density of DAT in collected rat striatum. The effect of non-specific binding was dominant which was approximately 40%, therefore some fractions were not evaluated due to unreliable readings.

### 4. The binding of flavonoids to dopamine $D_1/D_2$ receptors

Five flavonoids which were reported founding in *Passiflora* spp. were assessed the binding capability to dopamine $D_1$ and $D_2$ receptor. As shown in Figure 16, only luteolin and quercetin were able to bind to both receptors. Luteolin at concentration of 100 µg/ml could moderately displace the radioligand from dopamine $D_1$ receptor with 17 ± 2.3 % and showed a high affinity to dopamine $D_2$ receptor with the inhibition percentage of 56.4 ± 0.52. The binding capabilities of quercetin (100 µg/ml) to dopamine receptors were present with inhibition percentage of 39 ± 2.8 and 40 ± 4.6 for dopamine $D_1$ and $D_2$ receptor, respectively. In contrast, vitexin, apigenin and chrysin at the same concentration could not compete with the specific radioligands of both receptors.

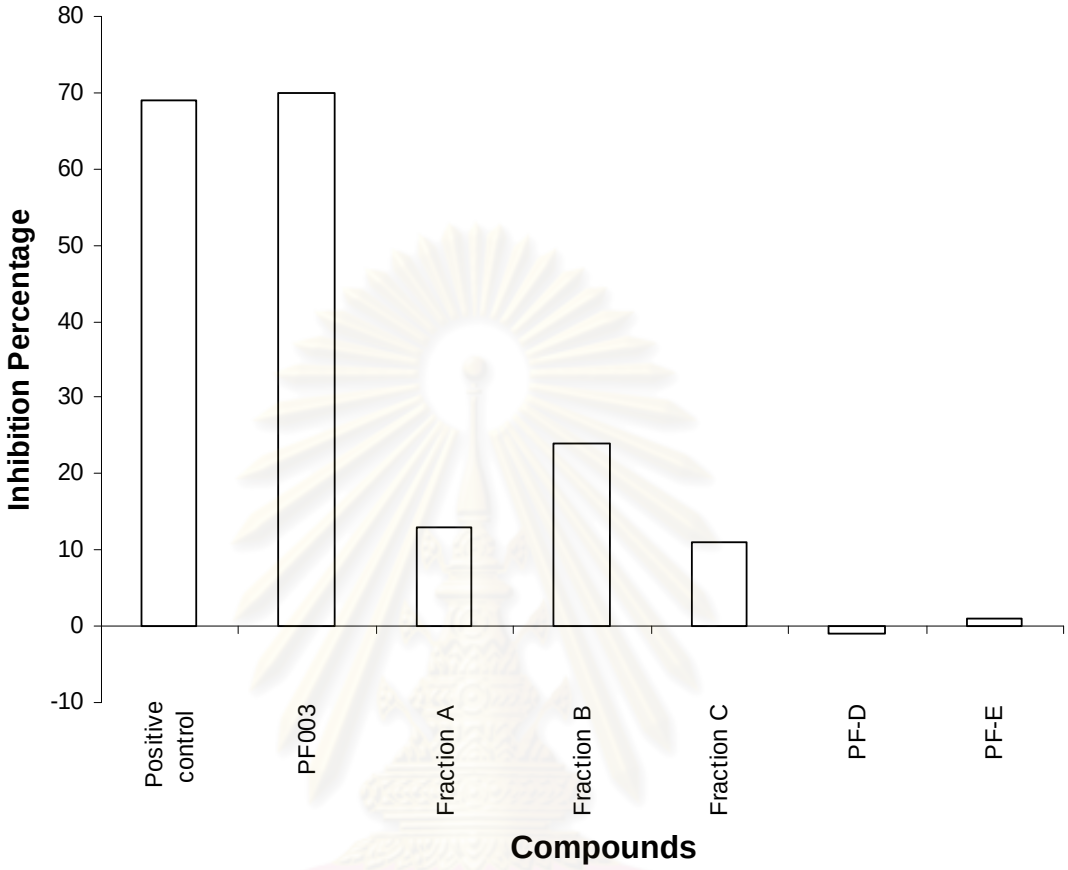



**Figure 15** **The Inhibition of radioligand specific binding on dopamine transporter of the PF003 extract, fractions and isolated compounds.** Each sample with the concentration of 100 µg/ml is incubated with 3.6 nM [$^{3}$H]WIN35428 and 750 µg of DAT protein. The $1x10^{-7}$ M of GBR12909 is used as positive control. Each column represents the mean value of two independent experiments, with duplicate replication in each experiment.

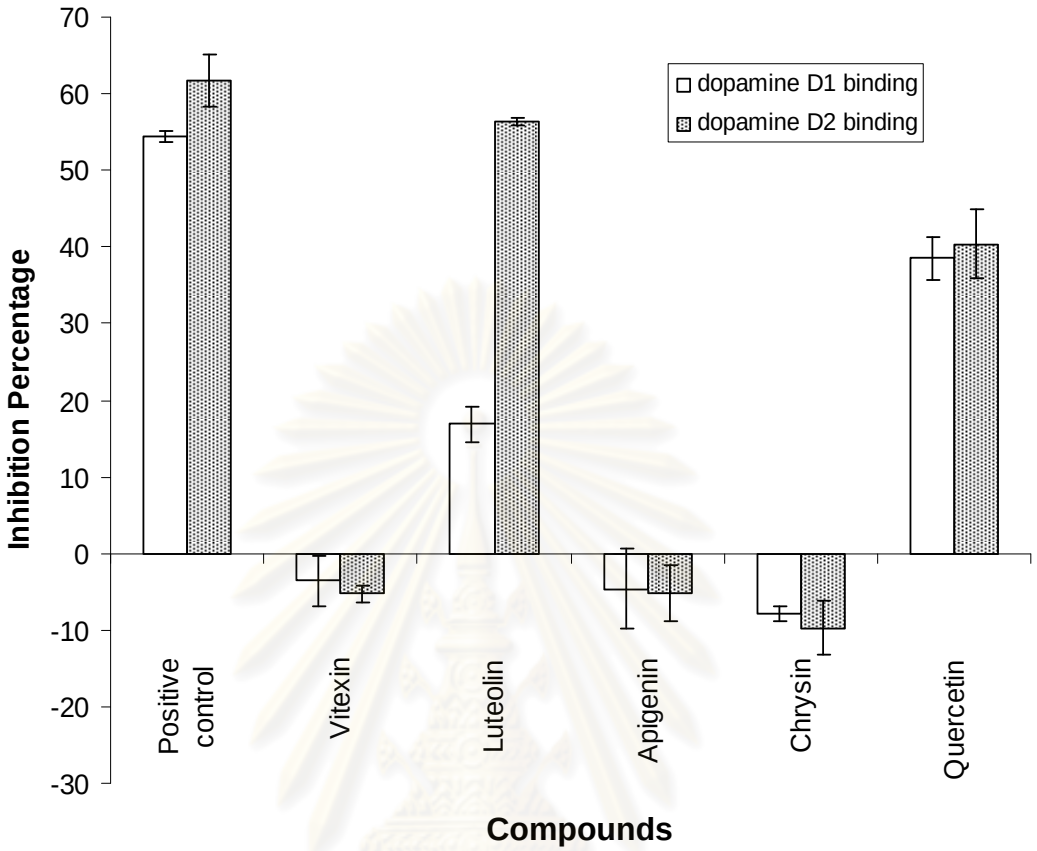



**Figure 16** **The Inhibition of radioligand specific binding on dopamine $D_1$ and $D_2$ receptor of flavonoid standards.** The tested concentration of each compound was 100 µg/ml, whereas the apigenin concentration is only 50 µg/ml because of solubility limitation. The positive controls are the 5 x $10^{-10}$ M SCH23390 for dopamine $D_1$ receptor and the 1x$10^{-8}$ M of raclopride for dopamine $D_2$ receptor. Each column represents the mean value ± S.E.M of three independent experiments, with duplicate replication in each experiment.

## IV. Identification of main compound in active fraction and PF003 extract

In an attempt to identify main compounds in both active fraction and PF003 extract, HPLC retention times of reported flavonoids found in *Passiflora* spp. were obtained. Selected flavonoids were vitexin, naringin, quercetin, luteolin, kaempferol, apigenin, and chrysin. Their peaks, retention times and maximum wavelength were shown in Figure 17 and Table 8. Figure 18 showed that the main peak in fraction D was matched with that of PF-D, peak 6 of PF003 including that of luteolin and additionally, the spectral pattern of major peak in fraction D, PF-D, peak 6 of PF003 and luteolin were also similar. The chemical structure of PF-D was further confirmed with NMR technique by Associate Professor Dr. Rutt Suttisri's group to be luteolin. Other compounds that could be in PF003 were vitexin, apigenin, acacetin and chrysoeriol. Peak of vitexin was present on the chromatogram at the same retention time with peak 2 and PF-E peak (Figure 19). As shown in Figure 20, the retention time of apigenin appeared to be close to those of PF-2 and PF-3 around 57-59 min. PF-2 and PF-3 were previously identified as chrysoeriol and acacetin, respectively, by NMR. These data were in line with their close structures and polarity. Kaempferol, chrysin, naringin was likely not present in PF003 extract.

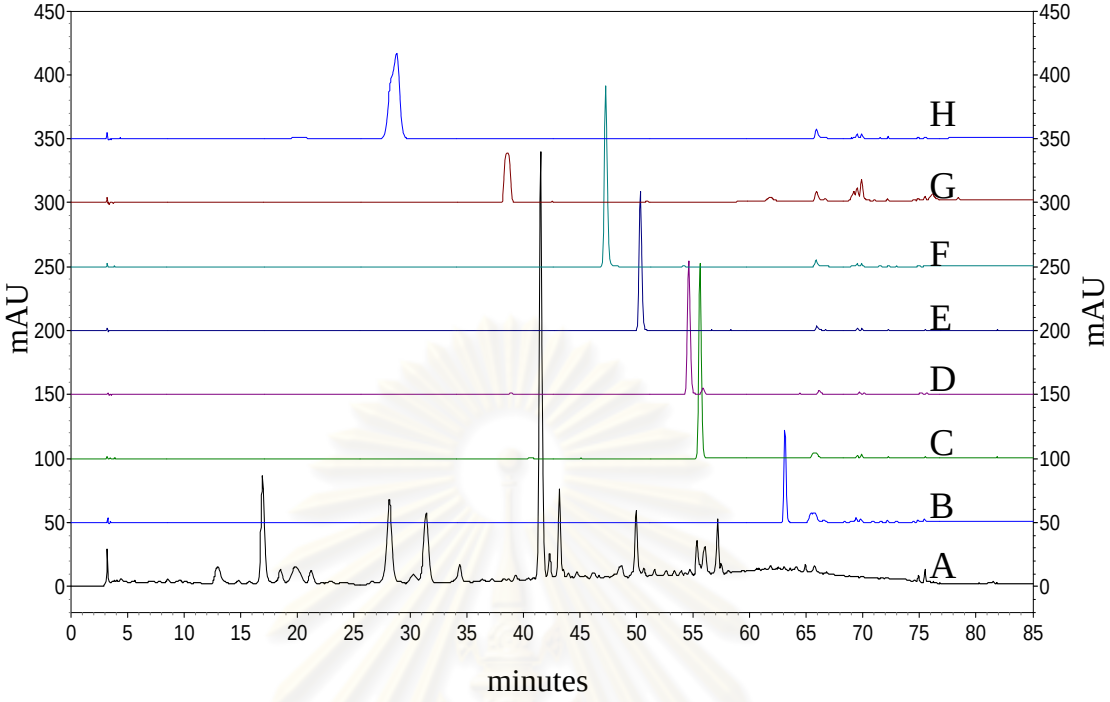



**Figure 17 The overlaid HPLC chromatogram of PF003 (A), chrysin (B), apigenin (C), kaempferol (D), luteolin (E), quercetin (F), naringin (G) and vitexin (H) at 320 nm.**

**Table 8 The chromatographic characteristics of major peaks in *P. foetida* extract and reference standards by HPLC-DAD.**

| Elution peak/ Reference standard | Retention time | Maximum wavelengths(nm) | Remark |
|---|---|---|---|
| 1 | 17 | 309, 219 | |
| 2 | 29 | 338, 268 | Similar retention time and maximum wavelengths to those of vitexin |
| vitexin | 29 | 338, 267 | |
| 3 | 32 | 335, 268 | |
| naringin | 37 | 282, 323 | |
| 4 | 43 | 313, 216 | |
| 5 | 44 | 245, 327 | |
| quercetin | 47.5 | 369, 254 | |
| 6 | 51 | 347, 253 | Similar retention time and maximum wavelengths to those of luteolin |
| luteolin | 51 | 348, 253 | |
| kaempferol | 54.4 | 365, 264 | |
| - | 56.5 | 336, 268 | Similar retention time and maximum wavelengths to those of apigenin |
| apigenin | 56.1 | 337, 266 | |
| chrysin | 64 | 313, 267 | |

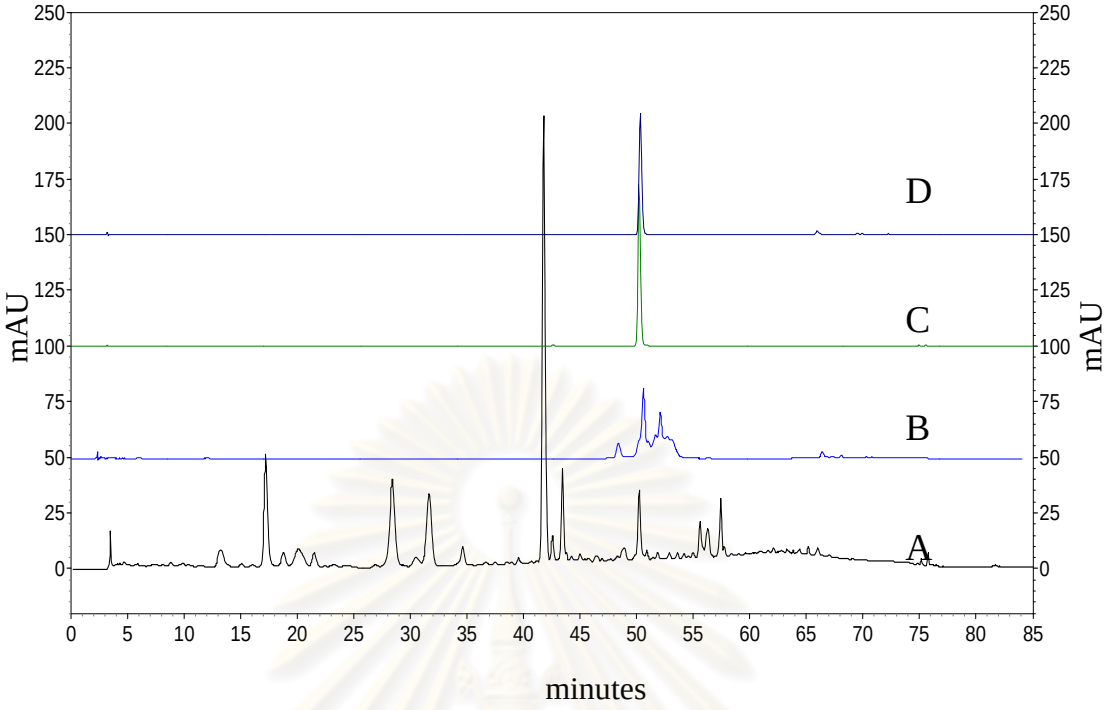



**Figure 18 The overlaid HPLC chromatogram of PF003 (A), fraction D (B), PF-D (C), and luteolin (D) at 320 nm.**

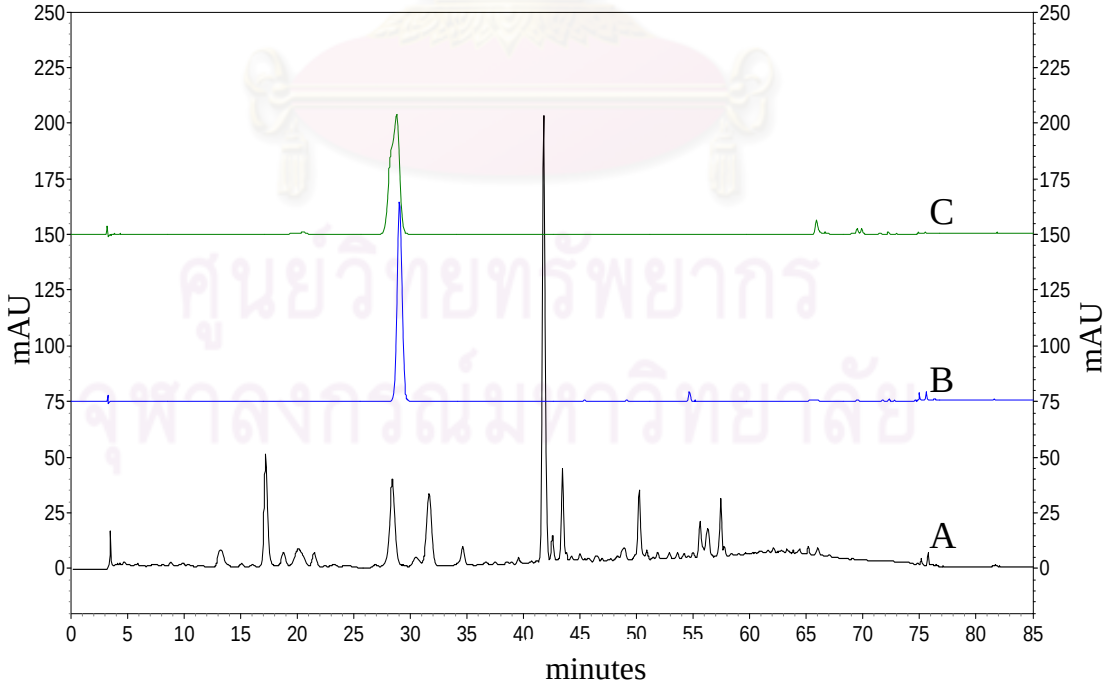



**Figure 19 The overlaid HPLC chromatogram of PF003 (A), PF-E (B) and vitexin (C) at 320 nm.**

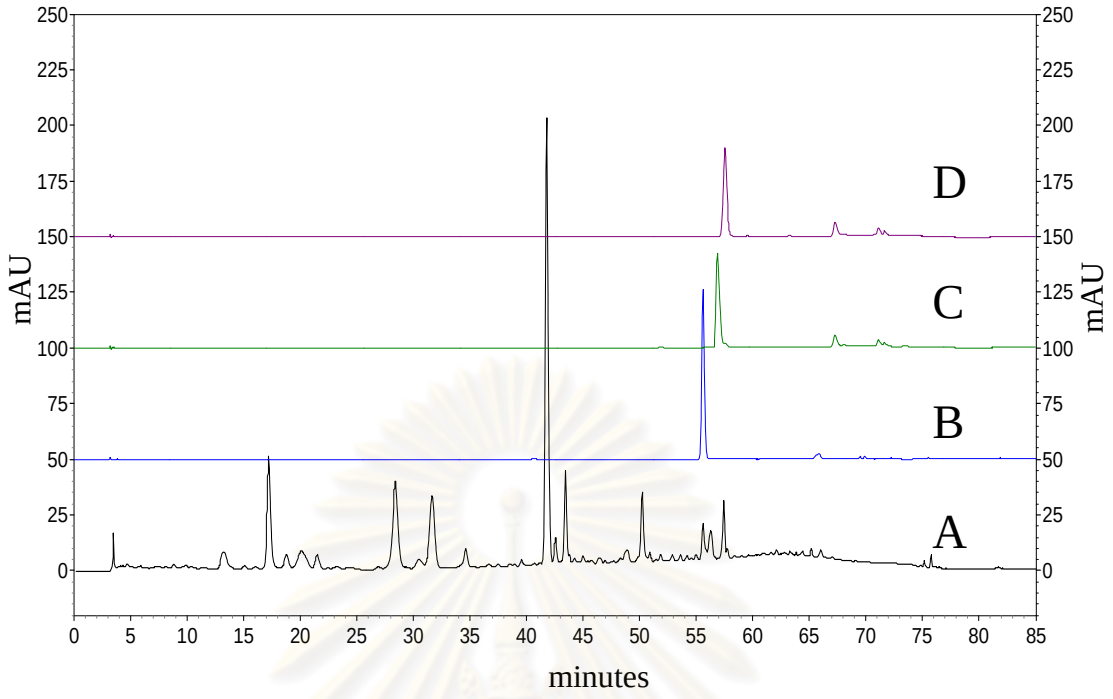



**Figure 20 The overlaid HPLC chromatogram of PF003 (A), apigenin (B) acacetin (C) and chrysoeriol (D) at 320 nm.**

## V. Dopamine receptor binding of luteolin

Since luteolin could bind to dopamine $D_2$ receptor with more than 50 percent inhibition, it was further studied to assess its $IC_{50}$ and $K_i$ values. The $IC_{50}$ value of luteolin was derived from the curve between the dopamine $D_2$ binding ability (Y-axis) and luteolin concentration (X-axis) using CurveExpert 1.3 program. $K_i$, which is the inhibition constant referring to affinity, was calculated from the equation of $K_i = IC_{50}/(1 + L/K_d)$, where *L* represents concentration of the ligand and $K_d$, the dissociation constant. $K_d$ value could be obtained from the Scatchard plot which yielded a straight line. Assuming a single class for all binding sites (Figure 21), the apparent $K_d$ for this experiment was calculated to be 1.1 nM. The density of [$^3$H]raclopride binding sites was estimated to be 348 fmol/mg protein. Subsequently, $K_d$ value derived from Scatchard analysis was used to calculate the $K_i$ of luteolin. The concentration of luteolin used to obtain the $IC_{50}$ value was ranged from 0.78 to 100 µg/ml. The results showed that luteolin bound to dopamine $D_2$ receptor in a concentration-dependent manner with an $IC_{50}$ value of 48.9 µM and a $K_i$ value of 17 µM as shown in Figure 22.

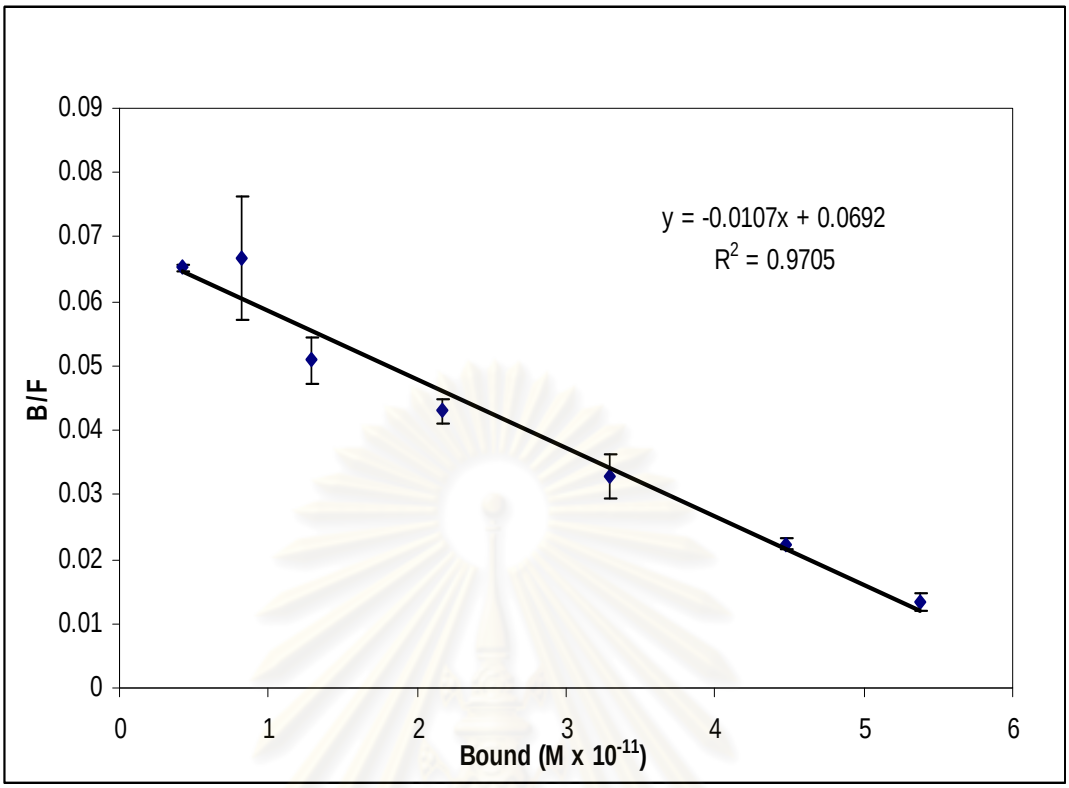



**Figure 21** **Scatchard plot curve of specific [$^3$H]raclopride binding to dopamine $D_2$ binding sites in homogenates of hD2L cell line.** Data points are the means of two experiments, with duplicate replication in each experiment. Specific binding is calculated by subtracting nonspecific binding (binding in the presence of $1x10^{-4}$ M butaclamol) from total binding. The binding parameters are $K_d$ = 1.1 nM, $B_{max}$ = 348 fmol/mg membrane protein.

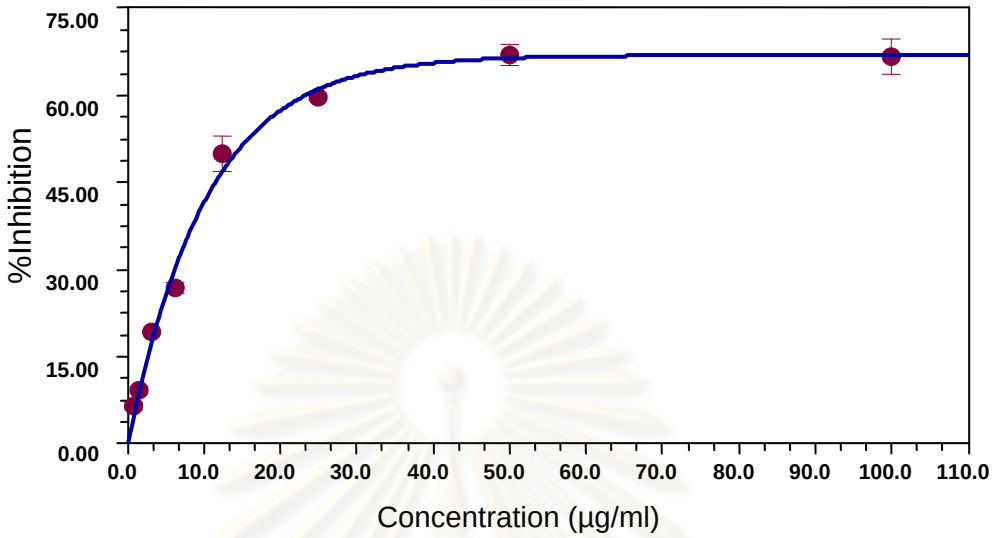



**Figure 22** **The concentration-dependent curve of luteolin binding to dopamine $D_2$ receptor binding.** Luteolin is tested at various concentrations ranging 0.8 to 100 µg/ml. Luteolin is incubated with 2 nM [$^3$H]raclopride, and 200 µg of dopamine $D_2$ receptor protein. The $1x10^{-8}$ M of raclopride is used as positive control. Each point represents the mean value ± S.E.M. of three independent experiments, with duplicated replication in each experiment.

# CHAPTER V

# DISCUSSION AND CONCLUSION

Chromatographic analysis is a well known method for herbal extract assessment (Drasar and Moravcova, 2004; Liang *et al.,* 2004; Fan *et al.,* 2006). Especially, HPLC is a popular method because it is easy to use and is not limited by the volatility or stability of the sample compounds (Xie *et al.,* 2006). Method development was still a trial-and-error approach by a logical sequence of exploring runs and fine adjusting step to achieve the needed resolution and method performance. The most common initial conditions are reversed phased chromatography using C18 column with MeOH or ACN and aqueous buffer (Dong *et al.,* 2006). The chromatographic optimization experiment for PF003 extract was based on a reversed-phase liquid chromatography method with C18 column and a mobile phase composed of 1% AcOH (solvent A) – MeOH (solvent B). The initial chromatographic condition was a gradient elution program consisting 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-100% (B) in 50-75 min, 100% (B) in 75-85 min. The chromatogram from the initial condition was able to resolve peaks fairly but showed some problems which were an inconsistency of peak elution time and a quite long run time. Optimizing the separation condition was performed by exploring the effect of column temperature, mobile phase gradient and the detection wavelength. At higher column temperature, the separation performance was increased because of the reduction in mobile phase viscosity, thus improving mass transfer. Analysis time could also be shortened by the increased diffusion coefficients (Meyer, 1994; Chen *et al.,* 2008). In addition, thermostated condition for the HPLC column gives higher reproducibility. The other modification on the chromatographic condition was the reduction of the final ratio of the organic modifier, methanol (B), from 100% to 75%. A shortened analysis time was further observed with the following new gradient program: 20% (B) in 0-20 min, 20-50% (B) in 20-50 min, 50-75% (B) in 50-63 min, 75% (B) in 63-68 min. Lastly, the selection of the detection wavelength was one of the key factors contributing to a reliable and reproducible chromatogram. DAD is a preferred detector for method development of complex mixture and was applied to

select the optimal wavelength (Springfield *et al.,* 2005; Yan *et al.,* 2005; Columbo, Lanças and Yariwake, 2006). 3D-plots of PF003 chromatograms showed that the maximal absorbance of most main peaks was not at the same wavelength. Most compounds in the PF003 chromatogram possessed strong UV absorbance at 320 nm. Selecting 320 nm as the monitoring wavelength therefore increased the signal to noise ratio of the chromatogram.

Validation of an analytical procedure is usually performed to ensure the suitability for intended purposes. Typical validation characteristics are accuracy, precision, specificity, detection limit, quantitation limit, linearity and range. Each type of method requires different validation schemes according to their purposes. For example, an identification test requires validating in specificity while a quantitative analytical method for impurities or active compounds pays attention to accuracy, precision, specificity, quantitation limit, linearity and range. The aim of this thesis focused on finding out the marker of the plant extract in order to use for standardization. The HPLC method was developed to be a tool supporting the marker selection by profiling the whole chemical constituents of PF003 extract. Preliminarily, the constituents in chromatogram of the extract had not been characterized. Suitable optimized HPLC condition should produce close chromatographic results obtained from several measurements of samples under the same condition. Hence, the precision test was selected for ensuring this developed HPLC condition that was applicable. The relative standard deviation (RSD) of RRT and RPA in the injection precision, the repeatability and the sample stability was used to evaluate the reliability of the method (Ji *et al.,* 2005; Jin *et al.,* 2006; Chen *et al.,* 2007). The injection precision and the repeatability test displayed low RSD value, indicating consistency of the analysis. The same low RSD of sample stability test means that the sample remained stable throughout the time of analysis. All results showed that the optimized HPLC condition was valid and reliable. However, after suitable markers for quality control were identified, more validation on the quantitative aspects such as specificity, detection limit, quantitation limit, linearity and range, should be performed.

The PF003 chromatogram was analyzed with the optimized condition exhibiting six major peaks which have not been characterized. The presence of glycosyl flavonoids, alkaloids, phenols and cyanogenic compounds has been reported for *Passiflora* plants (Dhawan *et al.,* 2004). Some reports pointed out the flavonoid compounds as main components in *Passiflora* spp. (Soulimani *et al.,* 1997) especially, C-glycosyl derivatives of the flavonoid apigenin and luteolin (*e.g.* vitexin, isovitexin, orientin, isoorientin, schaftoside) were suggested to be the principal bioactive compounds and were employed as quality markers for *P. edulis, P. incarnata and P. alata* (Rehwald *et al.,* 1994; Abourashed *et al.,* 2002; Muller *et al.,* 2005). Moreover, many flavonoids such as apigenin, chrysin and quercetin were indicated to be neuroactive based on their abilities to induce behavioral effects in animal models of anxiety, sedation and anti-convulsion (Marder and Paladini, 2002). From the suggested biological roles of flavonoids, we thus set out to investigate the existence of flavonoids in PF003 chromatogram by HPLC method. Liquid chromatography coupling with photodiode array detector is an efficient tool for compound screening using spectroscopic properties. Flavonoids are polyphenolic compounds that present a common benzo-γ-pyrone structure and all flavonoid aglycones contain at least one aromatic ring (Markham, 1892). Consequently, they are able to absorb UV light and generally show 2 ranges of maximum wavelength. The first maximum is found within 240-285 nm, originating from the A-ring. The second maximum is 300–550 nm and is influenced by the substitution pattern and conjugation of the C-ring (Rijke *et al.,* 2006). Based on the general flavonoid spectroscopic properties, any flavonoids in *P. foetida* were preliminarily screened by observing the persistence of all elution peaks in the extract chromatogram when the monitoring wavelength was varied among 260, 280, 350 or 370 nm. Our screening test confirmed 5 persistent elution peaks at the retention times of 28.8, 32.4, 51.1, 56.5 and 57.1 min. All those peaks possessed UV absorbance maxima: one at around 250-280 nm and the other at around 335-350 nm. The observation that the second maximum wavelength was lower than 370 nm suggested that these eluted flavonoids were likely to be flavones. Further confirmation and identification of the flavonoids were achieved by deriving the absorbance spectrum of potential flavonoid peaks from chromatogram of the extract and comparing with those of reference flavonoids. The peak areas of all predicted flavonoids accounted for 40% of the total peak area of PF003 chromatogram at 320

nm. The total flavonoid peak area could therefore be considered to be used for quality control of PF003 extract.

When comparing between isolated compounds from PF003 extract and whole chromatogram of PF003, the results showed that PF-2, PF-3, PF-D and PF-E could not account for all the peaks in PF003 chromatogram. The researcher aimed to select the biomarker by screening the constituents in PF003 that could bind to dopamine receptor and dominant transporter. Therefore, the compounds in PF003 should be isolated before investigating in the binding assay. The PF003 extract was fractionated by preparative HPLC into different polarity fractions (A-F). Fraction A represented the most polar compounds and the others in descending alphabetical order represented lesser polarity. To screen the active part of PF003 extract, all six fractions, PF-D and PF-F were inspected for their binding ability to dopamine $D_1$, $D_2$ receptors and dopamine transporter. The affinity of PF003 extract at concentration of 100 µg/ml to dopamine $D_1$ and $D_2$ receptor could be expressed as inhibition percentage of 25 ± 2.1 and 30 ± 4. For isolated compounds PF-D and PF-E, only PF-D showed the binding ability to both receptors at the same concentration. Nonetheless, PF-D could displace radioligand from dopamine $D_2$ receptor better than $D_1$ receptor. Among six fractions obtained from preparative HPLC, fractions A-C representing the flavonoid glycosides or the polar compounds did not show a binding capability in both receptors, whereas fraction D could displace radioligand from both dopamine receptors, with higher $D_2$ receptor inhibition. On the other hand, fractions E and F were able to trivially bind to the receptors. In summary, Fraction D and PF-D were notable in the two receptor binding. Fraction D comprised luteolin as the main compound and was potentially active compound and a biomarker. This hypothesis was supported by the binding results of luteolin standard and isolated compound PF-D, later confirmed as luteolin by NMR technique.

Percentage of luteolin content in PF003 extract calculated from the percent yield of PF-D was 0.09 %. When comparing the ratio of luteolin content in PF003 and their binding activities, the flavonoid seemed not to be the only active compound in the extract. The results suggested that some other bioactive compounds might not have been identified or there might be synergistic action of several constituents. The

effect was preliminarily investigated by combination of fractions D-F which showed higher dopamine $D_1$ receptor inhibition percentage than individual fraction but similar effect was not observed with dopamine $D_2$ receptor.

Luteolin was able to inhibit radioligand binding of both dopamine $D_1$ and $D_2$ receptors and appeared to preferentially bind to dopamine $D_2$ than $D_1$ receptor. In contrast, vitexin, chrysin and apigenin were unable to compete with the specific radioligands of either receptors. No inhibition on either dopamine $D_1$ or dopamine $D_2$ receptor was observed for vitexin and chrysin even at the concentration of 100 µg/ml. Due to the limited solubility of apigenin, negative inhibition was observed at 50 µg/ml. The affinity of luteolin to dopamine $D_2$ receptor was confirmed with an $IC_{50}$ value of 48.9 µM. Despite a similar structure consisting of benzopyran and benzene ring, luteolin, chrysin, vitexin, apigenin flavones (Figure 23) exhibited different capacity in displacing specific radioligand. This result appeared to emphasize the role of the catechol group, the two hydroxylation of benzene ring at meta- and para- position, which is present in luteolin. Moreover, this distinct part is also similar to catechol group of dopamine structure. Report by Kalani group (Kalani *et al.*, 2004) on the interaction of dopamine to dopamine $D_2$ receptor has also implied that the pocket site residues at transmembrane 5 of dopamine $D_2$ receptor hydrogen-bond to the metahydroxyl and parahydroxyl groups of the catechol ring of dopamine, playing an essential role in recognizing dopamine. Nonetheless, information on the interaction between dopamine and dopamine $D_1$ receptor has not been elucidated. If the ability of luteolin binding to dopamine receptors mimics the receptor-neurotransmitter interaction of dopamine, the selectivity of luteolin to dopamine $D_2$ receptor might be explained and appeared consistent with the observation that dopamine $D_1$ receptors are activated by micromolar concentrations of dopamine, while the dopamine $D_2$ receptors are nanomolar sensitive (Carvey, Bloom and Roth, 1998; Cooper, 2003) which means dopamine had a higher affinity to dopamine $D_2$ than dopamine $D_1$ receptor. Moreover, the hypothesis on the importance of catechol unit in the flavonoid structure was also supported by the positive result of quercetin on the dopamine $D_1$ and $D_2$ receptor binding assays. Quercetin is a flavonol compound that also have catechol group and showed the inhibition percentage as 39 (± 2.7, n=2) and 40 (± 4.6, n=2) for dopamine $D_1$ and $D_2$ receptor, respectively.

Following this line of thought, only one or no hydroxylation on the benzene ring (ring B of flavonoids) as in the structures of apigenin and chrysin may be unfit to bind to the pocket site. Similar explanation might be applied to vitexin. Furthermore, the sugar moiety of vitexin is likely cleaved by phase I de-glycosylation to apigenin which is its aglycone (Spencer, Mohsen and Rice-Evans, 2004; Walle, 2004). Biotransformation of glycosyl flavonoids might be produced and their bioactivity seemed to depend on their aglycone form.

In 2006, Coleta and co-workers evaluated the anxiolytic effect of *Passiflora edulis* extract with the elevated plus-maze model and then bioguided its fractionation to explore the active compounds (Coleta *et al.,* 2006). Luteolin-7-O-[2-rhamnosylglucoside] was isolated and characterized which showed an anxiolytic activity. Two year later, this group attempted to study the neurological mechanism of luteolin on the neurotransmitter system (Coleta *et al.,* 2008). Their results implied that luteolin has CNS activity with anxiolytic effect but did not dominantly involve with GABA receptor. They suggested a possible interaction with other neurotransmitters. Therefore, our research may answer at least one of its mechanisms via dopamine $D_1$ and $D_2$ receptor interaction.

A summary of association between peaks in PF003 chromatographic profile and their binding activities was outlined in Table 10. Peaks which appeared between the elution time of 0 to 20 min represented polar compounds which could be glycosides. They possessed no affinity to both dopamine $D_1$ and $D_2$ receptors, but showed slight affinity to dopamine transporter. Group of compounds between 20 to 38 min likely included vitexin as a main component. They did not bind to both dopamine receptors but moderately bound to the dopamine transporter. The most prominent peak present around 38 – 45 min was unlikely flavonoid based on their UV spectral pattern. Compounds in this dominant peak also showed no affinity to both receptors, although they possessed some ability to bind to the dopamine transporter. Compounds at the elution time of 45 -52 min exhibited high affinity to only dopamine $D_2$ receptor. Luteolin was observed as the main component in this region. Compounds at the elution time of 52 – 64 min likely consisted of apigenin, acacetin and chrysoeriol. These compounds slightly bound to dopamine $D_2$ receptor. Lastly,

nonpolar or wax-like components at the elution time later than 64 min did not bind to both dopamine receptors. Their binding to dopamine transporter was not determined due to unacceptable non-specific binding of the assay system.

Although we did not identify all the active constituents in dichloromethane extract of *P. foetida*, this research exhibited that this plant's extracts had an ability to bind dopamine $D_1$ and $D_2$ receptors and at least one of its compositions, luteolin, can elicit the same kind of action. The results suggested that luteolin can be used as a biomarker for monitoring the quality of the extract. To confirm the bioactivity of luteotin, it should be investigated in the *in vivo* assay for the anxiolytic effect with plus maze model (Walf and Frye, 2007) and antidepressant activity by forced swimming test (Petit-Demouliere *et al.*, 2005). In order to elucidate the action of luteolin via dopaminergic system, the behaviors of rat from the above two models were observed when rats were treated with luteolin together with SCH23390 (dopamine $D_1$ receptor antagonist) or butaclamol (dopamine $D_2$ receptor antagonist).

However the ineffective results of some flavonoids such as apigenin and vitexin measured in the dopamine receptor binding assay might be not implied that they have no anxiolytic or anti-depressant activities. Some evidences with the *in vivo* model showed that some flavonoids were potential compounds playing a role in CNS modulation. Apigenin showed the anti-depressant like effect by significantly decreasing the duration of immobility in forced swimming test (Nakazawa *et al.*, 2003; Yi *et al.*, 2008). Chrysin was able to reduce locomotor activity when injected in rats at a minimal effective dose of 25 mg/kg (Zanoli *et al.*, 2000) and induced significant anxiolytic behavior in mice by increasing the number of entries as well as the time spent by mice in open arms of the elevated plus maze apparatus but they did not exhibit myorelaxant effect in the horizontal wire test (Wolfram *et al.*, 1994). The mechanism of two compounds was known via GABA receptor system. They have been identified as a new type of ligand of benzodiazepine binding site on GABA receptor (Paladini *et al.*, 1999). Not only two substances but also the other compounds in flavone subtypes such as baicalain, amentoflavone, norwoganin or acacetin were known as ligands for GABA receptors as well (Medina *et al.*, 1997; Paladini *et al.*, 1999; Huang *et al.*, 2001; Fernandez *et al.*, 2004). Moreover some

flavonoids such as apigenin, kaempferol, luteolin and quercetin were also possessed the neuroactive effect by increasing monoamine neurotransmitter via blockade of monoamine oxidase (MAO) enzyme (Sloley *et al.*, 2000; Chimenti *et al.*, 2006; Han *et al.*, 2007). The inactive flavonoids in dopamine binding assay might give the anxiolytic effect with the other mechanisms described above. Consequently, the anxiolytic effect of PF003 found *in vivo* which comprised main compounds as flavonoid group is possibly exhibited from the combination of many mechanisms.

In conventional method for quality control, one or two markers or bioactive components in herbal preparation was engaged for evaluating the quality of them and determining the quantitative herbal composition of herbal product. In many cases, these markers or components are not unique to a specific herb. For example, in this experiment, luteolin was defined as the marker for PF003 extract and it was also found in the other herbal such as pasley. In reality, the activity of herbal preparation is due to more than one single chemical such as St. John's wort. They could hardly be separated into active part. Therefore, only one or two markers may seem not to be sufficient to represent the whole information and the impacts of other inherent components on the safety and efficacy of the herbal preparation should be considered. The full herbal product could be regarded as the active compound. The concept of phytoequivalence was usually developed in order to ensure the consistency of herbal product. According to the above concept, a chemical profile, such as fingerprint of herbal product should be preferably employed and were recommended by FDA and EMEA guideline. Fingerprint could be classified into three main methods namely "multi-component approach", "pattern approach" and "multi-pattern approach". Multi-component approach uses the relative compositions of many identified components to represent sample. While the pattern approach concern whole chromatographic profile. And the last, multi-pattern approach uses the same sample information from different techniques (Mok and Chau, 2006). From our results, mass of fraction F collected from PF003 was high, although the chromatogram of this fraction did not show noticeable peak when it detected with DAD. It might cause from a limit of chromophoric moiety. That implied the entire components of PF003 extracts possibly could not completely determined by HPLC-DAD. This occurrence was explained that generally, natural products have many different classes and

properties of compounds, consequently the only one technique cannot characterize all or most of components. Therefore, in the future, if the HPLC method needed to develop to be a fingerprint analysis, the whole information of PF003 was necessarily expressed. Multi-pattern approach was recommended. It was suggested that the pattern one was investigated with DAD and two was simultaneously estimated with universal detector such as Evaporative Light Scattering Detector (ELSD). ELSD is increasingly being used in liquid chromatography (LC). Its operation principle was to nebulize the HPLC eluent to eliminate the mobile phase and measure the scattered radiation of a laser beam by the particle stream of all nonvolatile analytes. In the field of pharmaceutical analysis, it has already been proposed as an effective alternative for the determination of the compounds which lacking of chromatophoric moiety. The multi-dimensional data give a better picture of extract. However, the fingerprint analysis would be complete when analyzing with several different samples such as difference in harvest seasons, plant origins, and other factors. This condition should be used to analyze the *Passiflora* in other species and the other plant to determine the specificity of method to determine *P. foetida* extract.

In conclusion, PF003 was investigated by HPLC method in this research work. The results showed at least 5 possible flavones presented in extract, three of which were vitexin, luteolin and apigenin. Chrysoeriol and acacetin were also found in low concentrations. Only luteolin showed obvious affinity to both of dopamine receptors and selectivity toward dopamine $D_2$ receptor. Hence, the appearance of luteolin readily was utilized as a marker for quality control and moreover, vitexin and apigenin, the dominant bioactive compounds described in this genus (Quimin *et al.*, 1991; Rehwald *et al.*, 1994; Abourashed *et al.*, 2002; Muller *et al.*, 2005), were also observed. In developing countries seeking to promote the rational use of herbal medicines, correct plant identification is important to quality assurance. Almost all herbal raw materials are obtained from natural sources. Consequently, mis-identification can easily occur. This methodology, which observed with a marker(s) together with a combination of taxonomy, classical microscopy is applied to the herbal medicine quality control for the traditional medicine in the country.

The present study is the first to report the selective affinity of luteolin to dopamine $D_2$ receptor. Since the dopamine $D_2$ receptor plays an important role in psychological disorders and Parkinson's disease. The information showed the potential of the compound to be used for dopamine system malfunction treatment. Although, the bioactivity of luteolin was performed only with an *in vitro* assay system, this finding substantiates that dopamine receptors could be one of the mechanistic sites of many putative neuroactive flavonoids which possess similar aglycone structure to luteolin. In addition, examination of its effects *in vivo* system seems to be required for clinical application.

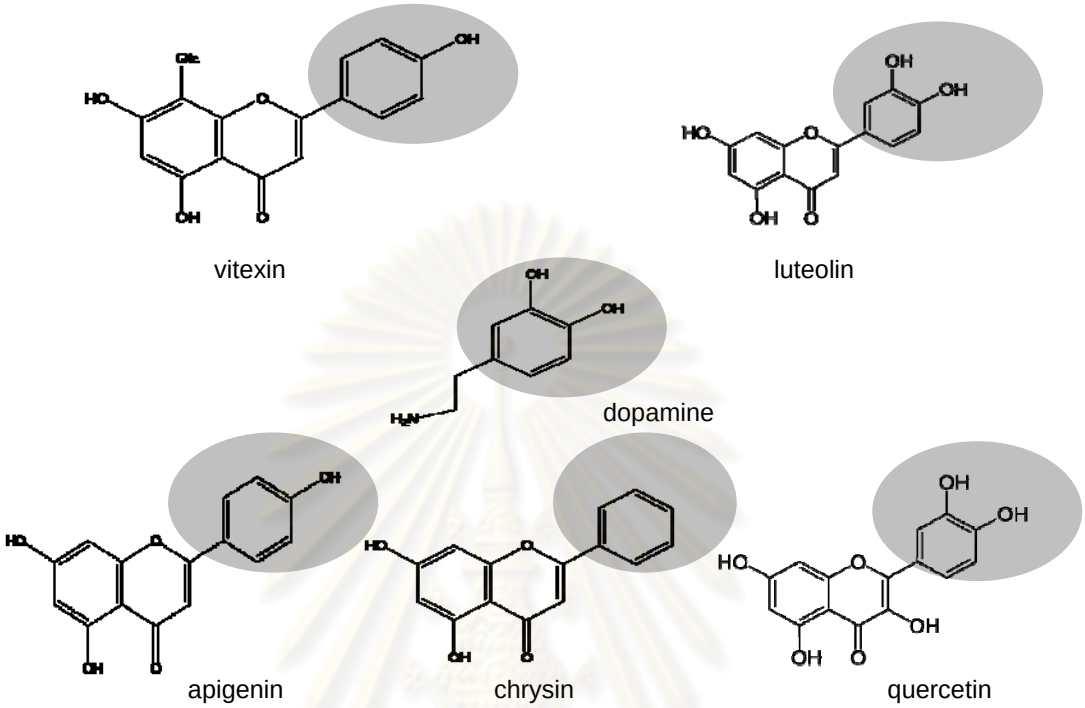
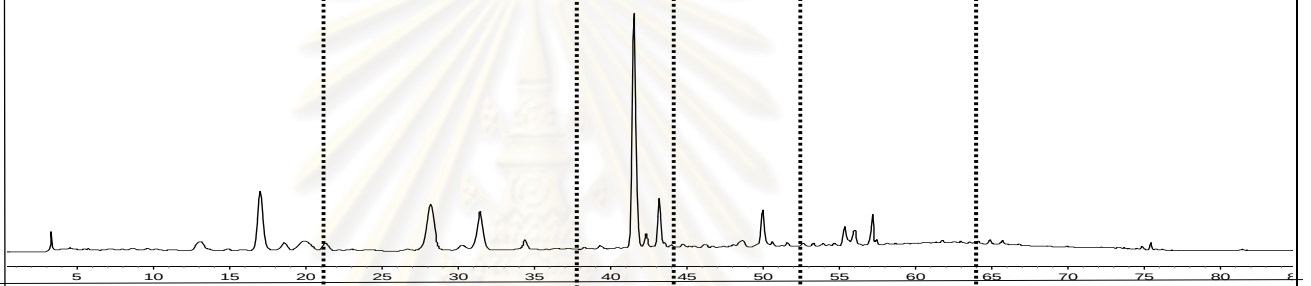
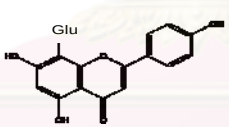
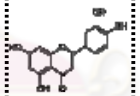
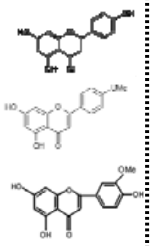



**Figure 23 The structures of vitexin, luteolin, apigenin, chrysin, quercetin and dopamine.**

**Table 9 The summary of the association of chromatographic profile and binding activities.**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bioactivity** | $D_1$ receptor | - | - | - | + | - | - |
| | $D_2$ receptor | - | - | - | ++++ | + | - |
| | DAT | + | ++ | + | NA | NA | NA |
| **Chromatogram** | |  | | | | | |
| **Possible main compound** | | Polar compounds such as glycoside compounds | Vitexin and related-compounds | unknown | Luteolin | Apigenin<br>Acacetin<br>Chrysoeriol | Non polar compounds,<br>Wax-like compounds |
| **Possible main compound structure** | | NA |  | NA |  |  | NA |



The illustration of bioactivity symbols

+ = the inhibition percentage in range of 5-15 ++ = inhibition percentage in range of 16-30 +++ = the inhibition percentage in range of 31-45

++++ = inhibition percentage of more than 45

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# APPENDICES

# APPENDIX A

# PREPARATION OF REAGENT

**Growth medium of A9L cell line hD2L (ATCC®Catalog no. CRL 10225)**

DMEM powder was dissolved with deionized distilled water and the 3.7 g/l sodium bicarbonate was added. The solution was mixed well and adjusted pH to 7.2 with 2N HCL. Then, the solution was adjusted volume to 1,000 ml. This solution was sterilized by filtration (0.2 µm Millipore filter membrane). Before use, this solution was supplemented with 10% FBS.

**Heat-inactivated FBS**

The desired amount of FBS was thawed at ambient temperature. The bottle of FBS was placed into the water bath which was adjusted to 56 ± 2°C so that the entire contents of the bottle are immersed in water. The bottle was heated for 30 minutes, and swirled periodically. The bottle was removed from the water bath, and allowed to cool. FBS was aliquoted in sterile bottles or conical tube and stored at -20°C or 2-8°C.

**Phosphate buffer saline (PBS)**

To proper 1 liter of PBS, the ingredients including 8 g NaCl, 0.2 g KCl, 0.2 g $KH_2PO_4$, and 1.44 g $Na_2HPO_4$ were dissolved in deionized water. The solution was mixed well and adjusted the pH to 7.4 with 2 N NaOH. The solution was adjusted volume to 1,000 ml and autoclaved for 20 min at 121°C.

**50 mM Tris-HCl**

To make 1 liter of 50 mM Tris-HCl, 7.88 g of Tris HCl powder were weighed and dissolved in deionized water. The solution was mixed well and adjusted pH to

7.2-7.4 with 2N NaOH. Then, the solution was adjusted volume to 1,000 ml. The solution was kept in bottle and stored in 2-8°C.

**50 mM Tris-HCl, 120 mM NaCl, 2 mM $MgCl_2$**

To proper 1 liter of 50 mM Tris-HCl, 120 mM NaCl, 2 mM $MgCl_2$, the ingredients including 7.01 g NaCl and 0.4 g $MgCl_2 \cdot H_2O$ were dissolved in 50 mM Tris-HCl. The solution was mixed well and adjusted pH to 7.2-7.4 with 2N NaOH. Then, the solution was adjusted volume to 1,000 ml. The solution was kept in bottle and stored in 2-8°C.

**Bradford reagent**

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

# APPENDIX B

# TABLES OF EXPERIMENTAL RESULTS

**Table 1** The inhibition percentage of PF003 extract, fraction and isolated compounds for dopamine $D_1$ receptor.

| Sample | Concentration | % Inhibition |
|---|---|---|
| SCH23390 | $5x10^{-10}$ M | 55 ± 0.4 |
| PF003 | 100 µg/ml | 26 ± 2.1 |
| Fraction A | 100 µg/ml | -4 ± 2.5 |
| Fraction B | 100 µg/ml | 1 ± 1.9 |
| Fraction C | 100 µg/ml | -5 ± 2.8 |
| Fraction D | 100 µg/ml | 12 ± 1.2 |
| Fraction E | 100 µg/ml | 2 ± 0.9 |
| Fraction F | 100 µg/ml | 4 ± 1.1 |
| Combination of fraction D, E and F | 100 µg/ml | 22 ± 3.8 |
| Vitexin | 100 µg/ml | -4 ± 3.3 |
| Apigenin | 50 µg/ml | -7 ± 6.1 |
| PF-D | 100 µg/ml | 26 ± 6.4 |
| PF-E | 100 µg/ml | 2 ± 1.7 |

Each value represented the mean value with S.E.M. of three independent experiments, with duplicate replication in each experiment.

**Table 2** The inhibition percentage of PF003 extract, standard flavonoids and isolated compounds for dopamine $D_2$ receptor.

| Sample | Concentration | % Inhibition |
|---|---|---|
| Raclopride | $1x10^{-8}$ M | 59 ± 2 |
| PF003 | 100 µg/ml | 30 ± 4 |
| Fraction A | 100 µg/ml | 3 ± 4.3 |
| Fraction B | 100 µg/ml | 3 ± 6.9 |
| Fraction C | 100 µg/ml | -8 ± 7.9 |
| Fraction D | 100 µg/ml | 61 ± 2.6 |
| Fraction E | 100 µg/ml | 11 ± 2.1 |
| Fraction F | 100 µg/ml | -6 ± 2.1 |
| Combination of fraction D, E and F | 100 µg/ml | 43 ± 2.3 |
| Vitexin | 100 µg/ml | -10 ± 6.1 |
| Apigenin | 50 µg/ml | -6 ± 5.4 |
| PF-D | 100 µg/ml | 66 ± 3.1 |
| PF-E | 100 µg/ml | -4 ± 6.1 |

Each value represented the mean value with S.E.M. of two or three independent experiments, with duplicate replication in each experiment.

**Table 3** The inhibition percentage of PF003 extract and isolated compounds for dopamine $D_2$ receptor.

| Sample | Concentration | % Inhibition (1) | % Inhibition (2) | % Inhibition (average) |
|---|---|---|---|---|
| GBR 12909 | $1x10^{-7}$ M | 62 | 76 | 69 |
| PF003 | 100 µg/ml | 56 | 83 | 70 |
| Fraction A | 100 µg/ml | 3 | 23 | 13 |
| Fraction B | 100 µg/ml | 19 | 28 | 24 |
| Fraction C | 100 µg/ml | 7 | 14 | 11 |
| PF-D | 100 µg/ml | -6 | 4 | -1 |
| PF-E | 100 µg/ml | -8 | 10 | 1 |

Each value represented the mean value of two independent experiments, with duplicate replication in each experiment.

**Table 4** The inhibition percentage of flavonoids for dopamine $D_1$ receptors.

| Sample | Concentration | % Inhibition |
|---|---|---|
| SCH23390 | $5x10^{-10}$ M | 54.5 ± 0.73 |
| Vitexin | 100 µg/ml | -4 ± 3.3 |
| Luteolin | 100 µg/ml | 17 ± 2.3 |
| Apigenin | 50 µg/ml | -5 ± 5.1 |
| Chrysin | 100 µg/ml | -8 ± 1.0 |
| Quercetin | 100 µg/ml | 39 ± 2.8 |

Each value represented the mean value with S.E.M. of two or three independent experiments, with duplicate replication in each experiment.

**Table 5** The inhibition percentage of flavonoids for dopamine $D_2$ receptors

| Sample | Concentration | % Inhibition |
|---|---|---|
| Raclopride | $1x10^{-8}$ M | 65 ± 3.4 |
| Vitexin | 100 µg/ml | -5 ± 1.2 |
| Luteolin | 100 µg/ml | 56.4 ± 0.52 |
| Apigenin | 50 µg/ml | -5 ± 3.7 |
| Chrysin | 100 µg/ml | -10 ± 3.6 |
| Quercetin | 100 µg/ml | 40 ± 4.6 |

Each value represented the mean value with S.E.M. of two or three independent experiments, with duplicate replication in each experiment.

**Table 6** The inhibition percentage of luteolin for dopamine $D_2$ receptor in a concentration-dependent manner.

| Luteolin concentration (µg/ml) | % Inhibition |
|---|---|
| 100 | 67 ± 3.1 |
| 50 | 67 ± 1.9 |
| 25 | 59.8 ± 0.82 |
| 12.5 | 50 ± 3.3 |
| 6.3 | 27 ± 1.1 |
| 3.1 | 19.3 ± 0.40 |
| 1.6 | 9.3 ± 0.67 |
| 0.8 | 6.5 ± 0.84 |

Each value represented the mean value with S.E.M. of three independent experiments, with duplicate replication in each experiment.

## Experiment data of the dopamine $D_1$ receptor binding assay

### Experiment data 1

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 7649.6 | 100.0 | 6787.3 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 862.4 | 11.3 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 4027.2 | 52.6 | 3164.9 | 46.6 | 53.4 |
| PF003 (100 µg/ml) | 5763.4 | 75.3 | 4901.0 | 72.2 | 27.8 |
| Fraction A (100 µg/ml) | 7897.3 | 103.2 | 7034.9 | 103.7 | -3.7 |
| Fraction B (100 µg/ml) | 7755.5 | 101.4 | 6893.1 | 101.6 | -1.6 |
| Fraction C (100 µg/ml) | 7834.2 | 102.4 | 6971.8 | 102.7 | -2.7 |
| Fraction D (100 µg/ml) | 6793.1 | 88.8 | 5930.7 | 87.4 | 12.6 |
| Fraction E (100 µg/ml) | 7572.9 | 99.0 | 6710.5 | 98.9 | 1.1 |
| Fraction F (100 µg/ml) | 7435.8 | 97.2 | 6573.4 | 96.9 | 3.2 |
| Combination of fractions D, E and F (100 µg/ml) | 6576.5 | 86.0 | 5714.1 | 84.2 | 15.8 |
| PF-D (100 µg/ml) | 6235.8 | 81.5 | 5373.4 | 79.2 | 20.8 |
| PF-E (100 µg/ml) | 7640.1 | 99.9 | 6777.7 | 99.9 | 0.1 |

%INH = % inhibition

**Experiment data 2**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 6783.5 | 100.0 | 5927.0 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 856.5 | 12.6 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3471.1 | 51.2 | 2614.6 | 44.1 | 55.9 |
| PF003 (100 µg/ml) | 5150.0 | 75.9 | 4293.5 | 72.4 | 27.6 |
| Fraction A (100 µg/ml) | 7283.1 | 107.4 | 6426.6 | 108.4 | -8.4 |
| Fraction B (100 µg/ml) | 6811.9 | 100.4 | 5955.4 | 100.5 | -0.5 |
| Fraction C (100 µg/ml) | 7392.2 | 109.0 | 6535.7 | 110.3 | -10.3 |
| Fraction D (100 µg/ml) | 6184.3 | 91.2 | 5327.8 | 89.9 | 10.1 |
| Fraction E (100 µg/ml) | 6592.7 | 97.2 | 5736.2 | 96.8 | 3.2 |
| Fraction F (100 µg/ml) | 6639.5 | 97.9 | 5783.0 | 97.6 | 2.4 |
| Combination of fractions D, E and F (100 µg/ml) | 5072.4 | 74.8 | 4215.9 | 71.1 | 28.9 |
| PF-D (100 µg/ml) | 4511.1 | 66.5 | 3654.6 | 61.7 | 38.3 |
| PF-E (100 µg/ml) | 6571.9 | 96.9 | 5715.4 | 96.4 | 3.6 |

%INH = % inhibition

**Experiment data 3**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 8815.2 | 100.0 | 7866.6 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 948.7 | 10.8 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 4542.8 | 51.5 | 3594.1 | 45.7 | 54.3 |
| PF003 (100 µg/ml) | 7122.4 | 80.8 | 6173.7 | 78.5 | 21.5 |
| Fraction A (100 µg/ml) | 8808.2 | 99.9 | 7859.5 | 99.9 | 0.1 |
| Fraction B (100 µg/ml) | 8454.2 | 95.9 | 7505.5 | 95.4 | 4.6 |
| Fraction C (100 µg/ml) | 8920.7 | 101.2 | 7972.0 | 101.3 | -1.3 |
| Fraction D (100 µg/ml) | 7691.9 | 87.3 | 6743.2 | 85.7 | 14.3 |
| Fraction E (100 µg/ml) | 8794.8 | 99.8 | 7846.1 | 99.7 | 0.3 |
| Fraction F (100 µg/ml) | 8341.7 | 94.6 | 7393.0 | 94.0 | 6.0 |
| Combination of fractions D, E and F (100 µg/ml) | 7209.7 | 81.8 | 6261.0 | 79.6 | 20.4 |
| PF-D (100 µg/ml) | 7393.8 | 83.9 | 6445.1 | 81.9 | 18.1 |
| PF-E (100 µg/ml) | 8673.7 | 98.4 | 7725.0 | 98.2 | 1.8 |

%INH = % inhibition

**Experiment data 4**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 6994.7 | 100.0 | 6293.4 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 701.3 | 10.0 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3635.9 | 52.0 | 2934.6 | 46.6 | 53.4 |
| Vitexin (100 µg/ml) | 7130.4 | 101.9 | 6429.1 | 102.2 | -2.2 |
| Luteolin (100 µg/ml) | 5649.3 | 80.8 | 4948.0 | 78.6 | 21.4 |
| Apigein (50 µg/ml) | 7162.7 | 102.4 | 6461.4 | 102.7 | -2.7 |
| Chrysin (100 µg/ml) | 7554.4 | 108.0 | 6853.1 | 108.9 | -8.9 |

%INH = % inhibition

**Experiment data 5**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 6783.5 | 100.0 | 5927.0 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 856.5 | 12.6 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3471.1 | 51.2 | 2614.6 | 44.1 | 55.9 |
| Vitexin (100 µg/ml) | 7371.5 | 108.7 | 6515.0 | 109.9 | -9.9 |
| Luteolin (100 µg/ml) | 5963.2 | 87.9 | 5106.7 | 86.2 | 13.8 |
| Apigein (50 µg/ml) | 7630.2 | 112.5 | 6773.7 | 114.3 | -14.3 |
| Chrysin (100 µg/ml) | 7298.9 | 107.6 | 6442.4 | 108.7 | -8.7 |

%INH = % inhibition

**Experiment data 6**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 7642.2 | 100.0 | 6999.1 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 643.1 | 8.4 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3840.9 | 50.3 | 3197.8 | 45.7 | 54.3 |
| Vitexin (100 µg/ml) | 7552.7 | 98.8 | 6909.6 | 98.7 | 1.3 |
| Luteolin (100 µg/ml) | 6560.0 | 85.8 | 5916.9 | 84.5 | 15.5 |
| Apigein (50 µg/ml) | 7416.3 | 97.0 | 6773.2 | 96.8 | 3.2 |
| Chrysin (100 µg/ml) | 8046.8 | 105.3 | 7403.7 | 105.8 | -5.8 |

%INH = % inhibition

**Experimental data 7**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 5506.8 | 100.0 | 4867.6 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 639.2 | 11.6 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3354.4 | 60.9 | 2715.2 | 55.8 | 44.2 |
| Quercetin (100 µg/ml) | 3764.2 | 68.4 | 3125.0 | 64.2 | 35.8 |

%INH = % inhibition

**Experiment data 8**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 5479.1 | 100.0 | 4590.8 | 100.0 | 0.0 |
| 5 x $10^{-6}$ M butaclamol | 888.3 | 16.2 | 0.0 | 0.0 | 100.0 |
| 5 x $10^{-10}$ M SCH23390 | 3265.4 | 59.6 | 2377.1 | 51.8 | 48.2 |
| Quercetin (100 µg/ml) | 3583.1 | 65.4 | 2694.8 | 58.7 | 41.3 |

%INH = % inhibition

## Experiment data of the dopamine $D_2$ receptor binding assay

### Experiment data 1

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 3123.0 | 100.0 | 2821.2 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 301.8 | 9.7 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 1515.5 | 48.5 | 1213.7 | 43.0 | 57.0 |
| PF003 (100 µg/ml) | 2476.9 | 79.3 | 2175.1 | 77.1 | 22.9 |
| Fraction A (100 µg/ml) | 3220.0 | 103.1 | 2918.2 | 103.4 | -3.4 |
| Fraction B (100 µg/ml) | 3216.7 | 103.0 | 2914.9 | 103.3 | -3.3 |
| Fraction C (100 µg/ml) | 3742.8 | 119.8 | 3441.0 | 122.0 | -22.0 |
| Fraction D (100 µg/ml) | 1405.2 | 45.0 | 1103.4 | 39.1 | 60.9 |
| Fraction E (100 µg/ml) | 2718.2 | 87.0 | 2416.4 | 85.7 | 14.4 |
| Fraction F (100 µg/ml) | 3374.1 | 108.0 | 3072.3 | 108.9 | -8.9 |
| Combination of fractions D, E and F (100 µg/ml) | 1788.0 | 57.3 | 1486.2 | 52.7 | 47.3 |
| PF-D (100 µg/ml) | 1086.4 | 34.8 | 784.6 | 27.8 | 72.2 |
| PF-E (100 µg/ml) | 3297.9 | 105.6 | 2996.1 | 106.2 | -6.2 |

%INH = % inhibition

**Experiment data 2**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 3173.9 | 100.0 | 2893.8 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 280.0 | 8.8 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 1262.6 | 39.8 | 982.6 | 34.0 | 66.0 |
| PF003 (100 µg/ml) | 2111.8 | 66.5 | 1831.8 | 63.3 | 36.7 |
| Fraction A (100 µg/ml) | 3159.0 | 99.5 | 2879.0 | 99.5 | 0.5 |
| Fraction B (100 µg/ml) | 3316.2 | 104.5 | 3036.2 | 104.9 | -4.9 |
| Fraction C (100 µg/ml) | 3429.6 | 108.1 | 3149.6 | 108.8 | -8.8 |
| Fraction D (100 µg/ml) | 1287.3 | 40.6 | 1007.3 | 34.8 | 65.2 |
| Fraction E (100 µg/ml) | 2920.3 | 92.0 | 2640.3 | 91.2 | 8.8 |
| Fraction F (100 µg/ml) | 3386.8 | 106.7 | 3106.8 | 107.4 | -7.4 |
| Combination of fractions D, E and F (100 µg/ml) | 1946.8 | 61.3 | 1666.8 | 57.6 | 42.4 |
| PF-D (100 µg/ml) | 1396.1 | 44.0 | 1116.1 | 38.6 | 61.4 |
| PF-E (100 µg/ml) | 3011.7 | 94.9 | 2731.7 | 94.4 | 5.6 |

%INH = % inhibition

**Experiment data 3**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 3898.0 | 100.0 | 3485.7 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 412.3 | 10.6 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 1978.8 | 50.8 | 1566.5 | 44.9 | 55.1 |
| PF003 (100 µg/ml) | 2817.1 | 72.3 | 2404.8 | 69.0 | 31.0 |
| Fraction A (100 µg/ml) | 3515.6 | 90.2 | 3103.3 | 89.0 | 11.0 |
| Fraction B (100 µg/ml) | 3320.8 | 85.2 | 2908.5 | 83.4 | 16.6 |
| Fraction C (100 µg/ml) | 3705.6 | 95.1 | 3293.3 | 94.5 | 5.5 |
| Fraction D (100 µg/ml) | 1880.8 | 48.3 | 1468.5 | 42.1 | 57.9 |
| Fraction E (100 µg/ml) | 3580.1 | 91.8 | 3167.8 | 90.9 | 9.1 |
| Fraction F (100 µg/ml) | 3971.2 | 101.9 | 3558.9 | 102.1 | -2.1 |
| Combination of fractions D, E and F (100 µg/ml) | 2516.3 | 64.6 | 2104.0 | 60.4 | 39.6 |
| PF-D (100 µg/ml) | 1615.6 | 41.4 | 1203.3 | 34.5 | 65.5 |
| PF-E (100 µg/ml) | 4260.5 | 109.3 | 3848.2 | 110.4 | -10.4 |

%INH = % inhibition

**Experiment data 4**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 3123.0 | 100.0 | 2821.2 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 301.8 | 9.7 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 1515.5 | 48.5 | 1213.7 | 43.0 | 57.0 |
| Vitexin (100 µg/ml) | 3770.2 | 120.7 | 3468.4 | 122.9 | -22.9 |
| Luteolin (100 µg/ml) | 1625.9 | 52.1 | 1324.1 | 46.9 | 53.1 |
| Apigein (50 µg/ml) | 3526.7 | 112.9 | 3224.9 | 114.3 | -14.3 |
| Chrysin (100 µg/ml) | 3173.7 | 101.6 | 2871.9 | 101.8 | -1.8 |

%INH = % inhibition

**Experiment data 5**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 5461.8 | 100.0 | 5129.2 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 332.6 | 6.1 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 2117.6 | 38.8 | 1785.0 | 34.8 | 65.2 |
| Vitexin (100 µg/ml) | 5792.1 | 106.0 | 5459.5 | 106.4 | -6.4 |
| Luteolin (100 µg/ml) | 2596.8 | 47.5 | 2264.2 | 44.1 | 55.9 |
| Apigein (50 µg/ml) | 5909.4 | 108.2 | 5576.8 | 108.7 | -8.7 |
| Chrysin (100 µg/ml) | 5778.9 | 105.8 | 5446.3 | 106.2 | -6.2 |

%INH = % inhibition

**Experiment data 6**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 5775.8 | 100.0 | 5002.2 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 773.6 | 13.4 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 3021.7 | 52.3 | 2248.1 | 44.9 | 55.1 |
| Vitexin (100 µg/ml) | 5923.2 | 102.6 | 5149.6 | 102.9 | -2.9 |
| Luteolin (100 µg/ml) | 2903.7 | 50.3 | 2130.1 | 42.6 | 57.4 |
| Apigein (50 µg/ml) | 5667.1 | 98.1 | 4893.5 | 97.8 | 2.2 |
| Chrysin (100 µg/ml) | 6618.5 | 114.6 | 5844.9 | 116.8 | -16.8 |

%INH = % inhibition

**Experimental data 7**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 4860.0 | 100.0 | 4374.1 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 485.9 | 10.0 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 2038.7 | 41.9 | 1552.8 | 35.5 | 64.5 |
| Quercetin (100 µg/ml) | 3337.8 | 68.7 | 2851.9 | 65.2 | 34.8 |

%INH = % inhibition

**Experiment data 8**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 4652.3 | 100.0 | 4102.7 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 549.6 | 11.8 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 2064.6 | 44.4 | 1515.0 | 36.9 | 63.1 |
| Quercetin (100 µg/ml) | 2764.2 | 59.4 | 2214.6 | 54.0 | 46.0 |

%INH = % inhibition

**Experiment data 9**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 4821.2 | 100.0 | 4208.8 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 612.4 | 12.7 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 2540.0 | 52.7 | 1927.6 | 45.8 | 54.2 |
| Luteolin (100 µg/ml) | 2035.0 | 42.2 | 1422.6 | 33.8 | 66.2 |
| Luteolin (50 µg/ml) | 1988.7 | 41.2 | 1376.3 | 32.7 | 67.3 |
| Luteolin (25 µg/ml) | 2304.3 | 47.8 | 1691.9 | 40.2 | 59.8 |
| Luteolin (12.5 µg/ml) | 2725.2 | 56.5 | 2112.8 | 50.2 | 49.8 |
| Luteolin (6.3 µg/ml) | 3693.2 | 76.6 | 3080.8 | 73.2 | 26.8 |
| Luteolin (3.1 µg/ml) | 4008.9 | 83.2 | 3396.5 | 80.7 | 19.3 |
| Luteolin (1.6 µg/ml) | 4421.4 | 91.7 | 3809.0 | 90.5 | 9.5 |
| Luteolin (0.8 µg/ml) | 4551.8 | 94.4 | 3939.4 | 93.6 | 6.4 |

%INH = % inhibition

**Experiment data 10**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 5776.8 | 100.0 | 4839.6 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 937.2 | 16.2 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 3456.4 | 59.8 | 2519.2 | 52.1 | 47.9 |
| Luteolin (100 µg/ml) | 2282.9 | 39.5 | 1345.7 | 27.8 | 72.2 |
| Luteolin (50 µg/ml) | 2380.2 | 41.2 | 1443.0 | 29.8 | 70.2 |
| Luteolin (25 µg/ml) | 2815.7 | 48.7 | 1878.5 | 38.8 | 61.2 |
| Luteolin (12.5 µg/ml) | 3068.1 | 53.1 | 2130.9 | 44.0 | 56.0 |
| Luteolin (6.3 µg/ml) | 4566.9 | 79.1 | 3629.7 | 75.0 | 25.0 |
| Luteolin (3.1 µg/ml) | 4877.4 | 84.4 | 3940.2 | 81.4 | 18.6 |
| Luteolin (1.6 µg/ml) | 5272.0 | 91.3 | 4334.8 | 89.6 | 10.4 |
| Luteolin (0.8 µg/ml) | 5390.2 | 93.3 | 4453.0 | 92.0 | 8.0 |

%INH = % inhibition

**Experiment data 11**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 4362.1 | 100.0 | 3810.1 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M butaclamol | 552.0 | 12.7 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-8}$ M raclopride | 2815.2 | 64.5 | 2263.2 | 59.4 | 40.6 |
| Luteolin (100 µg/ml) | 2021.5 | 46.3 | 1469.5 | 38.6 | 61.4 |
| Luteolin (50 µg/ml) | 1932.4 | 44.3 | 1380.4 | 36.2 | 63.8 |
| Luteolin (25 µg/ml) | 2139.6 | 49.0 | 1587.6 | 41.7 | 58.3 |
| Luteolin (12.5 µg/ml) | 2661.5 | 61.0 | 2109.5 | 55.4 | 44.6 |
| Luteolin (6.3 µg/ml) | 3259.6 | 74.7 | 2707.6 | 71.1 | 28.9 |
| Luteolin (3.1 µg/ml) | 3601.5 | 82.6 | 3049.5 | 80.0 | 20.0 |
| Luteolin (1.6 µg/ml) | 4053.1 | 92.9 | 3501.1 | 91.9 | 8.1 |
| Luteolin (0.8 µg/ml) | 4168.4 | 95.6 | 3616.4 | 94.9 | 5.1 |

## Experiment data of the dopamine transporter binding assay

### Experiment data 1

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 2591.3 | 100.0 | 1375.9 | 100.0 | 0.0 |
| 1 x $10^{-4}$ M nomifensine | 1215.4 | 46.9 | 0.0 | 0.0 | 100.0 |
| 1 x $10^{-7}$ M GBR12909 | 1742.6 | 67.2 | 527.2 | 38.3 | 61.69 |
| PF003 | 1814.2 | 70.0 | 598.8 | 43.5 | 56.48 |
| Fraction A | 2555.8 | 98.6 | 1340.4 | 97.4 | 2.6 |
| Fraction B | 2326.9 | 89.8 | 1111.5 | 80.8 | 19.2 |
| Fraction C | 2498.8 | 96.4 | 1283.4 | 93.3 | 6.7 |
| PF-D | 2668.4 | 103.0 | 1453.0 | 105.6 | -5.6 |
| PF-E | 2701.4 | 104.2 | 1486.0 | 108.0 | -8.0 |

%INH = % inhibition

**Experiment data 2**

| Sample | CPM | % | Specific binding | %Bind | %INH |
|---|---|---|---|---|---|
| 10%DMSO | 3040.1 | 100.0 | 1877.3 | 100.0 | 100.0 |
| 1 x $10^{-4}$ M nomifensine | 1162.9 | 38.3 | 0.0 | 0.0 | 0.0 |
| 1 x $10^{-7}$ M GBR12909 | 1618.7 | 53.2 | 455.9 | 24.3 | 75.7 |
| PF003 | 1476.0 | 48.5 | 313.2 | 16.7 | 83.3 |
| Fraction A | 2599.9 | 85.5 | 1437.1 | 76.6 | 23.5 |
| Fraction B | 2516.3 | 82.8 | 1353.5 | 72.1 | 27.9 |
| Fraction C | 2772.0 | 91.2 | 1609.2 | 85.7 | 14.3 |
| PF-D | 2966.9 | 97.6 | 1804.1 | 96.1 | 3.9 |
| PF-E | 2856.1 | 93.9 | 1693.3 | 90.2 | 9.8 |

%INH = % inhibition

# VITA

Miss Duangkamol Phummiratch was born on September 13, 1982 in Petchaburi province, Thailand. She received her Bachelor degree in Pharmacy in 2005 from the Faculty of Pharmaceutical Sciences, Hauchiew Chalermprakiet University, Thailand.