การค้นหาสารออกฤทธิ์ทางชีวภาพจากกระชายเหลือง *Boesenbergia pandurata* (Roxb.) Schltr. และกระชายดำ *Kaempferia parviflora* Wall Ex. Baker

โดยยีสต์กลายพันธุ์ Δ*zds1* เบสแอสเสย์

นางสาวสายพิณ บุญเกิด

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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SEARCHING FOR BIOACTIVE COMPOUNDS FROM *Boesenbergia pandurata* (Roxb.) Schltr. AND *Kaempferia parviflora* Wall Ex. Baker USING Δ*zds1* MUTANT YEAST-BASED ASSAY

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Thesis Title	SEARCHING FOR BIOACTIVE COMPOUNDS FROM *Boesenbergia pandurata* (Roxb.) Schltr. AND *Kaempferia parviflora* Wall Ex. Baker USING Δ*zds1* MUTANT YEAST-BASED ASSAY
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สายพิณ บุญเกิด : การค้นหาสารออกฤทธิ์ทางชีวภาพจากกระชายเหลือง *Boesenbergia pandurata* (Roxb.) Schltr. และกระชายดำ *Kaempferia parviflora* Wall Ex. Baker โดยยีสต์กลายพันธุ์ *Δzds1* เบสแอสเสย์. (SEARCHING FOR BIOACTIVE COMPOUNDS FROM *Boesenbergia pandurata* (Roxb.) Schltr. AND *Kaempferia parviflora* Wall Ex. Baker USING *Δzds1* MUTANT YEAST-BASED ASSAY) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. วรินทร ชวศิริ, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. ชุลี ยมภักดี, 110 หน้า.

งานวิจัยนี้เป็นการค้นหาสารออกฤทธิ์ทางชีวภาพที่แยกได้จากพืชสมุนไพรไทยโดยยีสต์กลายพันธุ์ *Δzds1* เบสแอสเสย์ ซึ่งเป็นวิธีคัดกรองเชิงบวกที่มีลักษณะจำเพาะเพื่อค้นหายา จากการคัดกรองเบื้องต้น พบว่าสารสกัดหยาบจากพืช 4 สายพันธุ์ จากทั้งหมด 50 สายพันธุ์ที่แสดงฤทธิ์สูงในการยับยั้งวิถีการส่งสัญญาณแคลเซียมในยีสต์สายพันธุ์กลาย *Δzds1* ได้แก่ สารสกัดหยาบของฟ้าทะลายโจร กระชายเหลือง กระชายดำและเสม็ดแดง ในงานวิจัยนี้ได้เลือกพืชที่มีศักยภาพในการยับยั้งวิถีการส่งสัญญาณแคลเซียมในยีสต์ในสกุลขิงข่า 2 ชนิด คือ กระชายเหลืองและกระชายดำมาทำการแยกสารบริสุทธิ์โดยการติดตามฤทธิ์ด้วยวิธีการทดสอบในยีสต์ สารสกัดด้วยไดคลอโรมีเทนของพืชทั้ง 2 ชนิดถูกนำมาแยกให้บริสุทธิ์โดยเทคนิคโครมาโทกราฟีและพิสูจน์โครงสร้างทางเคมีด้วยข้อมูลทางสเปคโทรสโคปีคได้สารในกลุ่มฟลาโวนอยด์ที่เคยมีรายงานแล้ว 7 ชนิด ได้แก่ pinostrobin (**A**), alpinetin (**B**) และ pinostrobin chalcone (**C**) ซึ่งแยกได้จากกระชายเหลือง และ 5-hydroxy-3,7-dimethoxyflavone (**D**), 5-hydroxy-7-methoxyflavone (**E**), 5-hydroxy-3,7,4′-trimethoxyflavone (**F**) และ 5,7-dimethoxyflavone (**G**) ซึ่งแยกได้จากกระชายดำ สาร **A** และสาร **D** ให้ผลที่ดีและไม่เป็นพิษต่อการเจริญของเซลล์ยีสต์จึงถูกเลือกไว้ใช้ในการศึกษาฤทธิ์ทางชีวภาพต่อไป สำหรับการหาโมเลกุลเป้าหมายของสารทั้ง 2 ชนิดในเซลล์ยีสต์ พบว่าสาร **A** และสาร **D** มีโมเลกุลเป้าหมาย คือ Swe1 (เหมือนกับ Wee1 ในสัตว์เลี้ยงลูกด้วยนม) และ *MCK1* (เหมือนกับ *GSK3* ในสัตว์เลี้ยงลูกด้วยนม) ตามลำดับ ส่วนการศึกษาฤทธิ์ในการต้านการอักเสบ พบว่าสารทั้ง 2 ชนิดมีฤทธิ์ในการต้านการอักเสบโดยวิธี carrageenin-induced paw edema อยู่ในระดับปานกลาง คือมีค่าเปอร์เซ็นต์การยับยั้งอาการบวมของอุ้งเท้าหนูเท่ากับ 43.71 และ 42.2 ตามลำดับ นอกจากนี้ สาร **A** แสดงความเป็นพิษต่อเซลล์มะเร็งในมนุษย์ (A375 และ Kato III) ด้วยวิธี MTT ที่ค่า IC_{50} เท่ากับ 56, 67 และ 86 ไมโครโมลาร์ ตามลำดับ ในการทดสอบการยับยั้งการเจริญของเชื้อ *Helicobacter pylori* 6 สายพันธุ์ พบว่าสาร **D** มีฤทธิ์ค่อนข้างดีในการยับยั้งเชื้อ *H. pylori* โดยมีค่า MIC อยู่ระหว่าง 0.84-6.71 ไมโครโมลาร์

สาขาวิชา..........เทคโนโลยีชีวภาพ......ลายมือชื่อนิสิต..

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SAIPIN BOONKERD: SEARCHING FOR BIOACTIVE COMPOUNDS FROM *Boesenbergia pandurata* (Roxb.) Schltr. AND *Kaempferia parviflora* Wall. Ex. Baker USING Δ*zds1* MUTANT YEAST-BASED ASSAY. THESIS ADVISOR : ASSISTANT PROFESSOR WARINTHORN CHAVASIRI, Ph.D., THESIS CO-ADVISOR : ASSISTANT PROFESSOR CHULEE YOMPAKDEE, Ph.D., 110 pp.

The purpose of this research was to search for bioactive compound isolated from Thai medicinal plants using Δ*zds1* yeast-based assay, a unique positive-drug screening system. In the preliminary screening, four crude extracts of 50 medicinal herbs (*Andrographis paniculata*, *B. pandurata*, *K. parviflora* and *Syzygium cinereum*) exhibited strong positive results on calcium signaling inhibition in the assay. Two potent positive plants of Zingiberaceae family, *B. pandurata* and *K. parviflora*, were chosen for further fractionation followed by activity guided yeast-based assay. The CH_2Cl_2 extracts of these plants were isolated by chromatographic techniques and seven known flavonoid compounds were obtained. Structures of the compounds were elucidated by spectroscopic techniques. Pinostrobin (**A**), alpinetin (**B**) and pinostrobin chalcone (**C**) were isolated from *B. pandurata* and 5-hydroxy-3,7-dimethoxyflavone (**D**), 5-hydroxy-7-methoxyflavone (**E**), 5-hydroxy-3,7,4′-trimethoxyflavone (**F**) and 5,7-dimethoxyflavone (**G**) were found in *K. parviflora*. Compound **A** and **D** exhibited strong positive and non-toxic to the yeast cells so they were chosen for further biological studies. For the study of **A** and **D** on finding the target molecules in the calcium signaling pathway, the target molecules of these two compounds were Swe1 (ortholog of mammalian Wee1) and *MCK1* (homolog of mammalian *GSK3*), respectively. The two compounds exhibited moderate anti-inflammatory activity on carrageenin-induced paw edema in rat with % inhibition values of 43.71 and 42.2, respectively. Moreover, Compound **A** also exhibited the cytotoxicity with MTT assay on Jukat, A375 and Kato III human cancer cell lines with IC_{50} value of 56, 67 and 86 μM, respectively. Compound **D** moderately inhibited 6 strains of *Helicobacter pylori* with the MIC value of 0.84-6.71 μM.

Field of Study : Biotechnology

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Student's Signature

Advisor's Signature W. Chavasiri

Co-Advisor's Signature

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LIST OF ABBREVIATIONS

^{o}C	=	degree Celsius
$CaCl_2$	=	calcium chloride
$CDCl_3$	=	deuterated chloroform
CD_3OD	=	deuterated methanol
CH_2Cl_2	=	methylene chloride
CH_3OH	=	methanol
δ	=	chemical shift
d	=	day
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublet (for NMR spectral data)
DMSO	=	dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
et al.	=	and other
EPP	=	Ethyl phenylpropiolate
EtOAc	=	ethyl acetate
g	=	gram
µg	=	microgram
hrs	=	hours
^{1}H NMR	=	proton nuclear magnetic resonance
Hz	=	Hertz
IC_{50}	=	inhibitory concentration require for 50% inhibition of growth
J	=	coupling constant
L	=	liter
µL	=	microliter

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M	=	Molar
MH	=	Muller Hinton
μM	=	micromolar
m	=	multiplet (for NMR spectral data)
M^+	=	Molecular ion
mg	=	milligram
MIC	=	minimum inhibitory concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
MS	=	mass spectroscopy
m/z	=	mass to charge ratio
N	=	normal
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
NMR	=	nuclear magnetic resonance
ONPG	=	*o*-nitrophenyl-β-D-galactosidase
ppm	=	part per million
psi	=	pound per square inch
rpm	=	round per minute
s	=	singlet (for NMR spectral data)
SC medium	=	Synthetic complete medium
SDS	=	sodium dodecyl sulfate
sp.	=	species

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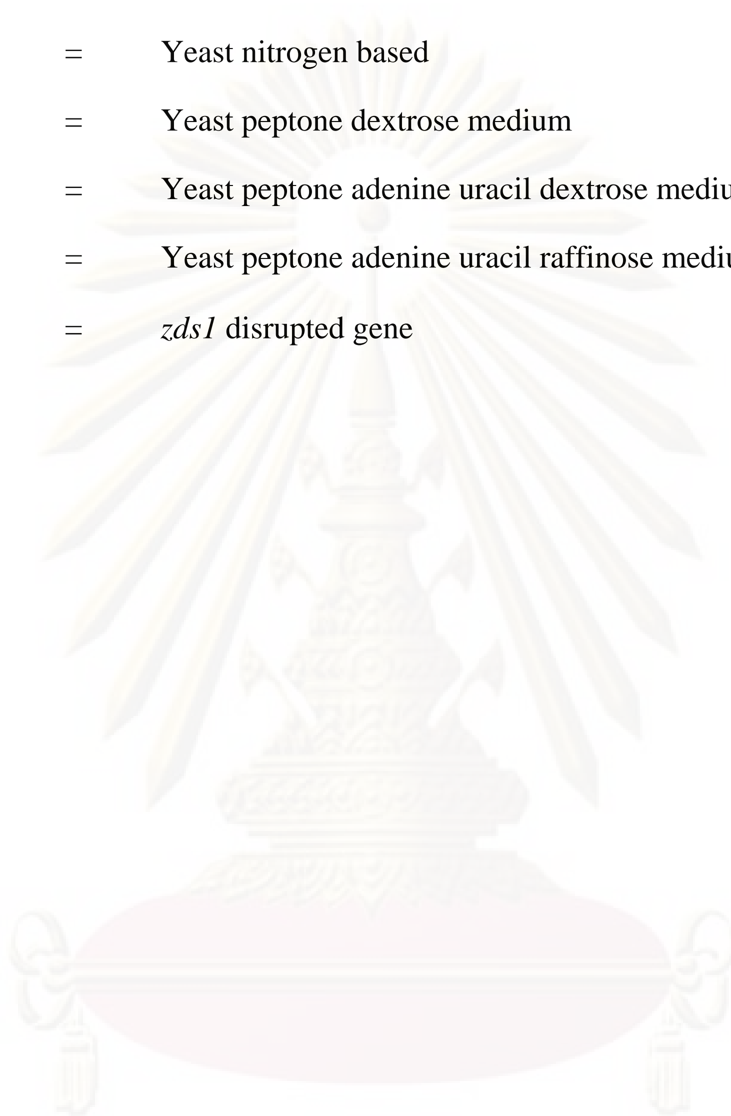
TLC	=	thin layer chromatography
UV	=	ultraviolet
YNB	=	Yeast nitrogen based
YPD	=	Yeast peptone dextrose medium
YPAUD	=	Yeast peptone adenine uracil dextrose medium
YPAUR	=	Yeast peptone adenine uracil raffinose medium
Δz*ds1*	=	*zds1* disrupted gene



CHAPTER I

INTRODUCTION

Recent advances in exploiting yeast molecular biology are utilized for a promising technology directed to the 3Rs (replacing, reducing, refining) of animal experimentation. Yeast as a tool and a model for target discovery, drug screening for leads and test bed for toxicity screens enable the routine design of robust, high-throughput capabilities throughout academia and industry (http://www.multiplex-network.de/home.html). The unicellular bakers' yeast *Saccharomyces cerevisiae* is a proven model eukaryote for molecular and cellular biology studies. Yeast growth and division can be controlled efficiently and effectively by adjusting environmental conditions. Yeast is also genetically well defined: its entire genome sequence has been elucidated and the corresponding databases are generally accessible. Moreover, it is a genetically tractable organism, amenable to modifications such as gene disruption, gene marking, mutations or gene-dosage effects. Because of these advantageous features, yeast has become the model organism of choice for medicine-related research. For example, studies with yeast have contributed greatly to our knowledge of the regulation of eukaryotic cell division, including the cancer-related disturbances thereof. Up to now, yeast has maintained its role as a useful model system in fundamental studies of disease processes. Indeed, recent studies revealed the presence of a regulatory network of apoptosis in yeast that encompasses many of the crucial events that occur in mammalian cells. As a model of fundamental cellular processes and metabolic pathways of the human, yeast has improved our understanding and facilitated the molecular analysis of many disease genes. It has long been recognized as a valuable model organism for studying of eukaryotic cells since many of basic cellular processes between yeast and humans are highly conserved. These so-called humanized yeast systems hold great promise for the dissection of disease-related molecular processes and the discovery of novel medicinal compounds.

1.1 Yeast-based screening assay

The hyperactive-activated Ca^{2+}-signaling pathways in yeast cause defective cell growth, it was assumed that exogenous inhibitors of the regulatory pathway can lead to the suppression of the deleterious effects of the Ca^{2+}-signaling, allowing the cells to resume growth. This idea are apply to drug screening and tested its feasibility using known calcineurin inhibitors (Shitamukai *et al*., 2000). The growth of the assay cells (*Saccharomyces cerevisiae*, *zds1*Δ strain) suspended and solidified in molten soft-agar containing a high concentration of $CaCl_2$ (150 mM) in a petri dish was blocked by hyper-active Ca^{2+}-signals. When filter-paper discs containing FK506 or cyclosporine A were placed on this plate, the growth of the assay cells around the paper disc was restored, giving rise to a halo (Shitamukai *et al*., 2000). Based on this observation, a unique and convenient procedure for drug screening was developed, in which the inhibitory substances of this pathway can be detected positively, as it were, by their ability to give rise to cell growth. Generally, many low-molecular-weight inhibitors act universally throughout eukaryotic organisms in an evolutionally conserved manner, inhibiting target molecules that share common features procedures appear to provide a very convenient drug screening system. The unique features of this screening system may be summarized as follows:

1) Because detection of the active substances is based on their positive effects on the growth of assay cells, cytotoxic compounds are not selected by the screening. Every crude samples, such as culture filtrates of microorganisms or plant extracts, are applicable for screening.
2) Because the sole cause for growth inhibition of the assay cells is the hyper-activation of Ca^{2+}-signaling, the molecules giving a hit (restoration of growth) are presumably highly oriented toward this pathway.
3) Because the hit substances obtained by the screening may be targeted towards one of several potential target molecules of the pathway, the chances of obtaining hits by screening are high. The potential targets of medical importance so far identified include calcineurin (immunosuppressants, anti-

inflammatory agents), GSK-3 (drugs for type II diabetes, Alzheimer's disease), protein kinase C (anticancer drugs).

4) Once the active substance in a hit sample is identified, the powerful genetic techniques available in the use of yeast provide valuable tools to identify the drug target of this pathway.

Ca^{2+} is an universal signal transduction element in cells modulating cell growth and differentiation (Lodish *et al.*, 2000). Ca^{2+}-signals play roles in the regulation of diverse cellular processes such as cell proliferation, T-cell activation, secretion, muscle contraction and the release of neurotransmitter in higher eukaryote (Clapham, 1995). In these processes, camodulin (CaM), a small Ca^{2+}-binding protein highly conserved among all eukaryotic cell, serve as a major intracellular Ca^{2+} receptor that mediates the effect of Ca^{2+}.

Calcineurin (protein phosphatase 2B), the only serine/threonine phosphatase under the control of Ca^{2+}/calmodulin, is an important mediator in signal transmission, connecting the Ca^{2+}dependent signaling to a wide variety of cellular responses. Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin, a protein phosphatase type 2B, is one of four principal types of serine/threonine-specific protein phosphatases present in mammalian tissues (Cohen, 1989). Although it was originally discovered as a neural tissue-enriched calmodulin-binding protein (Klee and Krinks 1978; Wang and Desai, 1977), calcineurin is broadly distributed in other tissues, and highly conserved from yeast to humans. The phosphatase is a heterodimer of the catalytic (calcineurin A) and the regulatory (calcineurin B) subunits (Klee *et al.*, 1979). The regulation of gene expression in response to Ca^{2+} stimuli is one of the most explored functions of calcineurin. Importantly, the critical target of calcineurin is the NF-AT family of transcription factors, during T-cell activation (Crabtree and Clipstone, 1994). In resting cells, NF-AT proteins are phosphorylated and are retained in the cytoplasm. Upon binding of antigen to T-cell receptor, intracellular Ca^{2+} is elevated through the action of protein tyrosine kinases and phospholipase C. Calcineurin is then activated through binding of Ca^{2+}/CaM and dephosphorylates the cytosolic forms of the NF-AT proteins (O'Keefe *et al.*, 1992), which are co-transported with calcineurin to the nucleus (Shibasaki *et al.*, 1996), where either alone

or in co-operation with AP-1 family members bind with specific *cis* elements in the promoter/enhancer regions of cytokine genes, such as IL-2. The immunosuppressants CsA and FK506 bind to inhibit calcineurin, thereby preventing the dephosphorylation, nuclear translocation and the activation of NF-AT, thus leading to the suppression of T-cell receptor activated signal transduction pathway by CsA and FK506 (Liu *et al.*, 1991).

Two important immunosuppressive drugs, tacrolimus (FK506) and cyclosporin A (CsA), are potent and specifically inhibit calcineurin phosphatase activity (Liu *et al.*, 1991). These drugs have been widely used to prevent graft rejection after organ and tissue transplantations. The use of these drugs has revealed the role of calcineurin in various cellular processes, including lymphocyte activation, cardiac development and hypertrophy, learning and memory, and angiogenesis (Aramburu *et al.*, 2000; Sugiura *et al.*, 2001).

1.2 Positive screening for drugs that specifically inhibit the Ca^{2+}-signaling activity

In the yeast *S. cerevisiae*, the Ca^{2+}-signal is implicated in the regulation of the G2/M cell-cycle progression **(Figure 1-1)**. Swe1 kinase specifically inhibit a G_2 form of the Cdc28 cycline-dependent protein kinase by phosphorylating it at Tyr-19 and delays the entry into mitosis. The cell-cycle regulation by Ca^{2+} is executed through the activation of the two parallel pathways, calcineurin and Mpk1 mitogen-activated protein (Mpk1 MAP) kinase cascade, and these two pathways cooperatively activate Swe1. The effect of $CaCl_2$ added to the medium, which leads to the activation of the cellular Ca^{2+}-signaling pathway, on the cell-cycle regulation is particularly obvious in the genetic background of Δ*zds1* strain. The activation of Ca^{2+}-signaling eventually leads to severe growth retardation which is accompanied by polarized bud growth and G_2 cell-cycle arrest. These physiological effects are caused by the activation of Swe1, a negative regulatory kinase that phosphorylates Cdc28 of the G_2 cell-cycle engine (Mizunuma *et al.*, 1998). Based on the mechanism, yeast-based screening assay will be a novel and convenient drug-screening procedure in yeast (designated as '*positive screening*') (Shitamukai *et al.*, 2000). Many small-molecule inhibitors elicit their

physiological effects through the common mechanisms from yeast to mammalian cells by acting on the evolutionally conserved target molecules, suggesting that the yeast-based screening systems serve as a convenient and powerful means for drug discovery. Screening of microbial metabolites for activity alleviating the deliterious physiological effects of external $CaCl_2$ identified the Hsp90 inhibitor radicicol as an inhibitor of Ca^{2+}-signal-dependent cell-cycle regulation in yeast (Chanklan *et al.,* 2008). Radicicol alleviated analogous physiological effects due to the expression of a constitutively active form of calcineurin or overexpression of Swe1, the negative regulatory kinase of the Cdc28-Clb complex. Western blot analysis indicated that radicicol inhibited Ca^{2+}-induced accumulation of Swe1and Clb2.

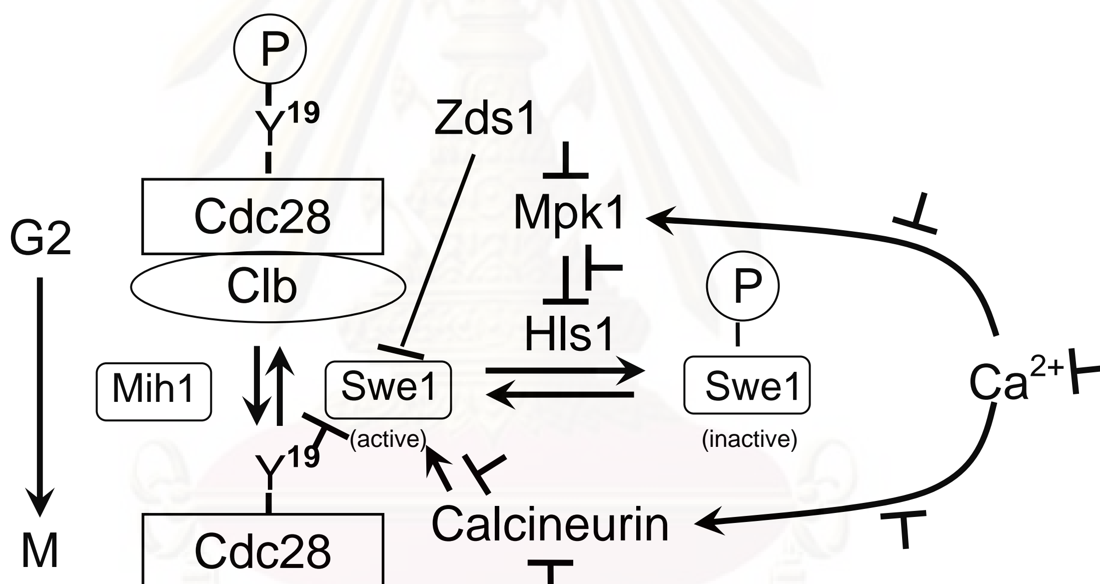


Figure 1-1 The Ca^{2+}-activated signaling pathway that regulates G2 cell-cycle progression in *S. cerevisiae* (Modified from Mizunuma *et al.*, 1998)

1.3 The scope of this research

This research aims to study Thai medicinal plants for calcium signaling inhibition activity, for example, *Alpinia galanga* (L.) Sw., *Andrographis paniculata* (Burm.f.) Nees, *Boesenbergia pandurata* (Roxb.) Schltr. *Curcuma longa* L., *Erythrina variegate* L., *Kaempferia parviflora* Wall.ex Baker, *Kaempferia galanga* L., *Melodorum fruticosum* Lour., *Eugenia cumini* Druce., *Zingiber montanum* Roscoe.

These plants were extracted and screened for preliminary activity using budding yeast, *S. cerevisiae* mutant strain (Δ*zds1*) as indicator cells. Two species of plants from the screens were chosen for isolation, structural elucidation and the evaluation of biological activity such as anti-inflammatory activity of pure compounds were performed.

1.4 The main objectives of this research are as follows:

1. To screen for Thai medicinal plants using the yeast-based assay
2. To isolate, purify and chemically characterize bioactive compounds from selected plants
3. To evaluate biological activities of the isolated bioactive compounds

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CHAPTER II

EXPERIMENTALS

2.1 Culture media and chemicals

2.1.1 Culture media

Culture media used for yeast-based assay were yeast peptone dextrose agar (YPDA), yeast peptone adenine uracil dextrose agar (YPAUDA), yeast peptone dextrose broth (YPDB), yeast peptone adenine uracil dextrose broth (YPAUDB), yeast peptone adenine uracil raffinose broth (YPAURB), uracil drop out synthetic complete medium (SC-U broth), uracil drop out synthetic complete agar (SC-U agar); the formula of culture media used for cultivation of the yeast cells are shown in Appendix.

2.1.2 Chemicals

Chemicals used in yeast-based assay are as follows: glucose (Difco Laboratories, USA), D-galactose (Difco Laboratories, USA), raffinose (Difco Laboratories, USA), yeast nitrogen based (YNB) (Difco Laboratories, USA), yeast extract (Difco Laboratories, USA), peptone (Difco Laboratories, USA), adenine hemisulfate salt, minimum 99% (Sigma, USA), uracil, minimum 99% (Sigma, USA), agar powder (Merck, Germany), calcium chloride anhydrous (Merck, Germany), Tacrolimus (FK506, Fujimycin, Japan), Radiciciol (Sigma, USA), absolute ethanol (Merck, Germany), 95% ethanol (industrial grade, Lab-scan, Thailand), dimethyl sulfoxide (DMSO) (Merck, Germany), glycerol (Carlo ERBA, France), sodium hydroxide (NaOH) (Merck, Germany), hydrochloric acid (HCl) (LAB-SCAN, Ireland), sodium dodecyl sulfate (SDS, $C_{12}H_{25}OSO_3$) (Sigma, USA), *o*-nitrophenyl-β-D-galactosidase (ONPG) (Bio Basic, USA), disodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) (Merck, Germany), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) (Merck, Germany), potassium chloride (KCl) (Merck, Germany), magnesium sulfate heptahydrate ($MSO_4.7H_2O$) (Merck, Germany), β-

mercaptoethanol (Merck, Germany), sodium carbonate (Na_2CO_3) (Merck, Germany), sodium chloride (NaCl) (Merck, Germany), sodium hydrogen phosphate ($NaHPO_4$) (Merck, Germany), potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany).

Chemicals used for plant extraction, isolation and structure elucidation are shown as follows: *n*-hexane (commercial grade), dichloromethane (CH_2Cl_2) (commercial grade), ethyl acetate (EtOAc) (commercial grade), methanol (CH_3OH) (commercial grade). All solvents were pre-distilled before use, acetone (commercial grade), chloroform-D ($CDCl_3$, 99.9 atom %D) (Merck, Germany), acetone-$d_{6'}$ (99.9 atom %D) (Aldrich), DMSO-$d_{6'}$ (99.9 atom %D) (Aldrich).

2.2 Instrumentations

2.2.1 Apparatus used for extraction, isolation and structure elucidation

Rotary evaporator was used for the efficient and gentle removal of solvents from plant extract by evaporation, performed on Buchi®, Switzerland. Sinter glass frit (∅, 17 cm) was used for quick column chromatography, performed on silica gel No. 7729 (Merck, Germany). Column chromatography was performed on silica gel 60 G No. 7734 (Merck, Germany). Thin layer chromatography (TLC) was performed on aluminum sheets pre-coated with silica gel, Kieselgel 60 F_{254} (Merck, Germany). Mass spectrometer was used for the determination of the elemental composition of a pure compound, performed on Micromass UK Limited (Manchester, UK). All NMR spectra (^1H and ^{13}C-NMR) were performed in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal reference on Varian (Palo Alto, USA) nuclear magnetic resonance spectrometer, mercury plus 400 NMR spectrometer with operated at 399.84 MHz for ^1H and 100.54 MHz for ^{13}C nuclei. The chemical shifts (δ) are assigned by comparison with residue solvent protons.

2.2.2 Apparatus used for yeast-based screening assay

Autoclave-HVE-50 (HIRAYAMA, Japan) was set the temperature to 121 ^oC, 15 psi for 15 min and used for sterilization of culture media. Hot air oven-UE 600 (Memmert, Germany) was set to 180 ^oC for 2 hrs and used for sterilization of glass

wares. Incubator (Memmert-BE60, Germany) was set to 30 ^{o}C for incubation of yeast cells. Laminar flow-Clean model V4 (LAB service, Thailand) was used for sterile work. Water bath-(Memmert, Germany) was set to 55 ^{o}C for prewarm of YPD soft agar media. Water bath shaker-NST (EYELA 2000, Japan) was set to 300 ^{o}C for incubation of yeast cells. The pH meter-S-20K (Mettler-Toledo, Switzerland) was used for measurement the pH (acidity or alkalinity) of media. Precision weighing balances-AG285 (Mettler-Toledo, Switzerland) was used for the measurement of mass to a very high degree of precision and accuracy. Vortex mixer (Geniell G-560E, Scientific Industries, USA) was used to mix a small vials of liquid. Conventional microscope (Olympus, USA) was used for observing living cells on a glass slide. Refrigerated micro-centrifuge (KUBOTA-6500, Japan) was used for the sedimentation of yeast cells. Sonicator-RK100 (BANDELIN, Germany) was used for speed dissolution of pure compounds. Haemacytometer was used for determining the number of cells in a given quantity of cell suspension and micropipette-P20, P200, P1000 (Gilson, France) were used for accurately measurement and dispense small volumes of liquid.

2.3 Collection of plant samples

Fifty Thai medicinal plants were examined. The fresh plants were collected from local areas in Thailand, while the dried plants were bought locally at the medical herbal shop. The selection of these plants was based on their activities reported from previous studies as shown in **Table 2-1**.

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Table 2-1 The list of plants for preliminary screening

List	Scientific Name	Plant codes	References
	ACANTHACEAE		
1	*Acanthus ilicifolius* (L.) (เหงือกปลาหมอ)	AIL	Kiem *et al.,* 2008
2	*Andrographis paniculata* (Burm.f.) Nees. (ฟ้าทะลายโจร)	APA	Singh *et al*., 2009
	AMARANTHACEAE		
3	*Amaranthus viridis* (L.) (ผักโขม)	AVI	Ashok Kumar *et al.*, 2009
	ANNONACEAE		
4	*Melodorum fruticosum* Lour. (ลำดวน)	MFR	Chaichantipyuth *et al.,* 2001
	ARALIACEAE		
5	*Panax ginseng* C.A. Mey. (โสม)	PGI	Chang *et al.* (2003)
	ASCLEPIADACEAE		
6	*Calotropis gigantea* R. Br. (ดอกรัก)	CGI	Ajay *et al.*, 2008
7	*Gymnema indorum* Decne. (ผักเชียงดา)	GIN	Shimizu *et al.*, 2001
	ASTERACEAE (COMPOSITAE)		
8	*Verbesina (Eclipta) alba* (L.) (กะเม็ง)	VAL	Sawant *et al.*, 2004
9	*Pluchea indica* (L.) Less. (ขลู่)	PIN	Sen *et al.*, 2006
10	*Chromolaena odorata* (L.) (สาบเสือ)	COD	Pierangeli *et al.*, 2009
11	*Tagetes erecta* (L.) (ดาวเรือง)	TER	Chatterjee *et al.*, 2009
	BIGNONIACEAE		
12	*Oroxylum indicum* (L.) Vent. (เพกา)	OIN	Nakahara *et al.*, 2001
	BORAGINACEAE		
13	*Heliotropium indicum* (L.) (หญ้างวงช้าง)	HIN	Srinivas *et al.*, 2000

Table 2-1 (continued)

List	Scientific Name	Plant codes	References
	CAPPARIDACEAE		
14	*Cleome viscosa* (L.) (ผักเสี้ยนผี)	CVI	Williams, 2003
	CYPERACEAE		
15	*Cyperus rotundus* (L.) (แห้วหมู)	CRO	Uddin, 2006
	EBENACEAE		
16	*Diospyros rhodocalyx* Kurz. (ตะโกนา)	DRH	Sutthivaiyakit *et al.*, 1995
	FLACOURTIACEAE		
17	*Hydnocarpus anthelminthicus* Pierre. (กระเบา)	HAN	Prachya *et al.*, 2007
	FLAGELLARIACEAE		
18	*Flagellaria indica* (L.) (หวายลิง)	FIN	-
	GRAMINEAE		
19	*Cymbopogon nardus* (L.) Rendle. (ตะไคร้หอม)	CNA	Abena *et al.*, 2007
	GUTTIFERAE		
20	*Garcinia mangostana* (L.) (มังคุด)	GMA	Pothitirat, *et al.*, 2009
	LEGUMINOSAE		
21	*Bauhinia sirindhorniae* K. & S.S. Larsen (สิรินธรวัลลี)	BSI	Athikomkulchai *et al.*, 2005
22	*Cassia siamea* (Lam.) Irwin et Barneby. (ขี้เหล็ก)	CSI	Ntandou *et al.*, 2010
23	*Clitoria ternatea* (L.) (อัญชัน)	CTE	Daisy *et al.*, 2009
24	*Erythrina variegata* (L.) (ทองหลาง)	EVA	Zhang *et al.*, 2007

Table 2-1 (continued)

List	Scientific Name	Plant codes	References
25	**LORANTHACEAE** *Dendrophthoe pentandra* (L.) Miq. (กาฝากมะม่วง)	DPE	Hanafi *et al.*, 2006
26	**MAGNOLIACEAE** *Michelia champaca* (L.) (จำปา)	MCH	-
27	**MALVACEAE** *Hibiscus sabdariffa* (L.) (กระเจี๊ยบแดง)	HSA	Sarr *et al.*, 2009
28	**MORINGACEAE** *Moringa oleifera* Lam. (มะรุม)	MOL	Sulaiman *et al.*, 2008
29 30	**MYTACEAE** *Syzygium cumini* Druce. (หว้า) *Syzygium cinereum* (เสม็ดแดง)	SCU SCI	Jagetia and Baliga, 2002 -
31	**PANDANACEAE** *Pandanus amaryllifolus* Roxb. (เตยหอม)	PAM	Takayama *et al.*, 2002
32	**PIPERACEAE** *Piper nigrum* (L.) (พริกไทย)	PNI	Singh and Rao, 1993
33	**PTERIDACEAE** *Acrostichum aureum* (L.) (ปรงทะเล)	AAU	Lai *et al.*, 2009
34	**RUBIACEAE** *Morinda citrifolia* (L.) (ยอ)	MCI	Sang *et al.*, 2003
35	**TILIACEAE** *Corchorus olitorius* (L.) (ปอกระเจาฝักยาว)	COL	Zakaria *et al.*, 2005
36	**VERBINACEAE** (Labiatae) *Clerodendrum inerme* (L.) Gaertn. (สำมะงา)	CIN	Manoharan *et al.*, 2006

Table 2-1 (continued)

List	Scientific Name	Plant codes	References
	EUPHORBIACEAE		
37	*Jatropha curcas* (L.) (สบู่ดำ)	JCU	David *et al.*, 2009
	VITIDACEAE		
38	*Parthenocissus quinquefolia* Planch. (เถาคันแดง)	PQU	-
	ZINGIBERACEAE		
39	*Alpinia (Languas) conchigera* Griff. (ข่าลิง)	ACO	Lee *et al.*, 2006
40	*Alpinia galanga* (L.) willd. (ข่า)	AGA	Al-Yahya *et al.*, 2006
41	*Alpinia mutica* Roxb. (ข่าน้ำ)	AMU	Jantan *et al.*, 2005
42	*Alpinia purpurata* (Vielle.) Schum. (ขิงแดง)	APU	-
43	*Amomum villosum* Lour.var *xanthioide* T. L. Wu & S. J. Chen. (เร่ว)	AVI	Lee *et al.*, 2007
44	*Boesenbergia pandurata* (Roxb.) Schltr. (กระชายเหลือง)	BPA	Cheenpracha *et al.*, 2006
45	*Curcuma longa* (L.) (ขมิ้นชัน)	CLO	Lee, 2006
46	*Hedychium coronarium* J.Konig. (มหาหงส์)	HCO	Braga *et al.*, 2000
47	*Kaempferia galanga* (L.) (เปราะหอม)	KGA	Sulaiman *et al.*, 2008
48	*Kaempferia parviflora* Wall. Ex Baker (กระชายดำ)	KPA	Tewtrakul and Subhadhirasakul, 2008
49	*Zingiber montanum* (*cassumunar* Roxb.) (Koen.) Thelade (ไพล)	ZMO	Ozaki *et al.*, 1991
50	*Zingiber officinale* Roscoe. (ขิง)	ZOF	Blessy *et al.*, 2009

2.4 Preliminary screening of medicinal plants for calcium signaling inhibition using the yeast-based assay

2.4.1 Yeast strains and plasmids

The yeast strains used in this study were shown in **Table 2-2**. Yeast mutant strain, *Saccharomyces cerevisiae*, mutant *zds1*Δ strain, YNS17, (*MAT*a *zds1*::*TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) were cultivated on YPAUD agar and incubated at 30 ^{o}C for 2 d (Chanklan *et al*., 2008). Then, the cells were subcultured into YPAUD broth and incubated at 30 ^{o}C with shaking at 200 rpm for 18-24 hrs. To preserve the yeast strains, aliquot of 50 μL of culture broth was placed into each cryotube and added 50 μL of 30% glycerol in YPAUD broth and mixed with vortex mixer. The aliquot containing the yeast cells were stored at -80 ^{o}C.

Table 2-2 *S. cerevisiae* strains used in this study

Strain	Genotype	Source of reference
W303	*trp1 leu2 ade2 ura3 his3 can1-1*	Rothstein, R., 1983
Δ*zds1*, YNS17	Same as W303 except *zds1::TRP1 syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	Chanklan *et al*., 2008
Δ*mpk1*	Same as W303 except *TRP1 mpk1::HIS3 syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	Miyakawa, T., Hiroshima University
Δ*cnb1*	Same as W303 except *TRP1 cnb1::HIS syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	
swe1::GAL1p-SWE1	Same as W303 except *TRP1 swe1::GAL-SWE1-HA::LEU2 TRP1 syr1::HIS3 pdr1::hisG-ura3-hisG pdr3::hisG-URA3-hisG*	

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Table 2-2 (continued)

mck1::GAL1p-MCK1	Same asW303 except *TRP1* *pYES2::GAL1p-MCK1*	Wangkangwan, W. (2007)

Plasmid used in this study, pYES2 was purchased from Invitrogen (UK), pYES2::*GAL1*p-MCK1 was constructed by Wangkangwan, W. (2007) and pKC190 obtained from Miyakawa, T.

2.4.2 Yeast-based screening assay

The assay was modified from (Shitamukai *et al.*, 2000). *Saccharomyces cerevisiae*, mutant *zds1*Δ strain was used as an indicator cell. The cells from -80 ^{o}C were recultured on YPAUD agar at 30 ^{o}C for 2 d then inoculated in 5 mL YPAUD broth at 30 ^{o}C with shaking at 200 rpm until cell density reached approximately 1-5x10^{7} cells/mL. The YPAUD soft agar (8,000 μL) was prewarmed at 55 ^{o}C, then 300 μL of 4 M $CaCl_2$ (150 mM) was added and indicator cells (yeast cells) were mixed to get appropriate concentration before pouring to the plates (6.0x10^{5} cells/mL). Five microlitres of 5 mg/mL crude plant extracts and absolute ethanol (as a negative control), and three microlitres of 100 nM FK506 (as a positive control) were dotted onto the screening plates. The plates were incubated at 30 ^{o}C for 40 hrs. After this time plates were examined and scored, compared with the positive control which showed a growth zone at or around the application spots.

2.4.3 Plant extraction and preliminary screening results of yeast-based assay

Fresh plants were chopped into small pieces and dried in open air for a few days. Then the dried plants were powdered with an electric blender. Fifty grams of dried powder were macerated three times with CH_2Cl_2 (3x50 mL) at room temperature for five days and filtered. The plant residue was similarly extracted with CH_3OH. The extracts were concentrated by rotary evaporator under reduced pressure. Then both extracts were assayed using the yeast-based assay (**Table 2-3**).

Table 2-3 Preliminary screening results of the extract from Thai medicinal plants for calcium signal inhibition activity using the yeast-based assay.

List	Plant codes	Plant parts	Preliminary results of plant extracts	
			CH_2Cl_2	CH_3OH
1	AIL	whole plant/ seed	-/-	-/-
2	APA	whole plant	++++	+
3	AVI	whole plant	-	-
4	MFR	flower	-	-
5	PGI	root	-	-
6	CGI	flower	-	-
7	GIN	whole plant	-	-
8	VAL	whole plant	-	-
9	PIN	whole plant	-	-
10	COD	whole plant	-	-
11	TER	flower	-	-
12	OIN	leaf/ stem bark	-/-	-/-
13	HIN	whole plant	-	-
14	CVI	whole plant	-	-
15	CRO	whole plant	-	-
16	DRH	leaf	-	-
17	HAN	seed	-	-
18	FIN	whole plant	-	-
19	CNA	whole plant	-	-
20	GMA	pericarp	-	-
21	BSI	leaf	-	-
22	CSI	Leaf/ stem	-/-	-/-
23	CTE	flower	-	-
24	EVA	leaf/ stem bark	-/-	-/-
25	DPE	Leaf/ stem	-/-	-/-

Table 2-3 (continued)

List	Plant codes	Plant parts	Preliminary results of plant extracts	
			CH_2Cl_2	CH_3OH
26	MCH	leaf	-	-
27	HSA	flower	-	-
28	MOL	stem	-	-
29	SCU	Leaf/ stem bark	-/-	-/-
30	SCI	leaf	+++	-
31	PAM	leaf	-	-
32	PNI	fruit	-	-
33	AAU	leaf	-	-
34	MCI	fruit	-	-
35	COL	whole plant	-	-
36	CIN	leaf	-	-
37	JCU	leaf/ seed	-/-	-/-
38	PQU	whole plant	-	-
39	ACO	rhizome	-	-
40	AGA	rhizome	-	-
41	AMU	rhizome	-	-
42	APU	rhizome	-	-
43	AVI	rhizome	-	-
44	BPA	rhizome	+++*	-
45	CLO	rhizome	-	-
46	HCO	rhizome	-	-
47	KGA	rhizome	-	-
48	KPA	rhizome	+++*	-
49	ZMO	rhizome	-	-
50	ZOF	rhizome	-	-

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to positive control using FK 506, which gave +++)

* = positive (ring like growth)

Table 2-3 shows calcium signal inhibition activity of Thai medicinal plant extracts using the yeast-based assay. The yeast-based assay is a novel procedure for detecting the bioactive compounds that the positive result show the growth of the yeast indicator cells allowing the cytotoxic effect could be excluded. Fifty species of Thai medicinal plants were chosen for screening. Among them *Andrographis paniculata*, *Boesenbergia pandurata*, *Kaempferia parviflora* and *Syzygium cinereum* gave a strong positive on the yeast growth while the other plants exhibited the negative results **Figure 2-1**.

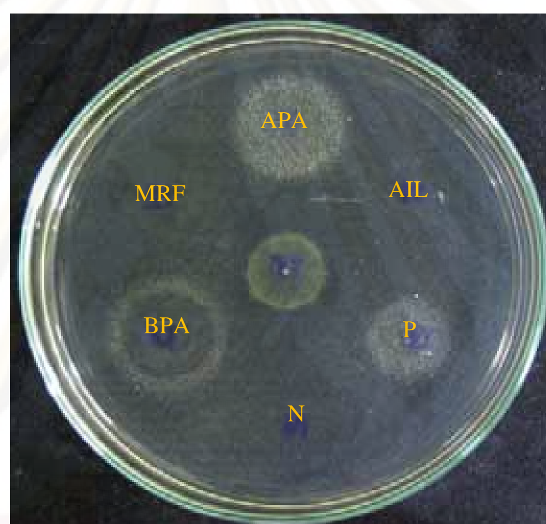
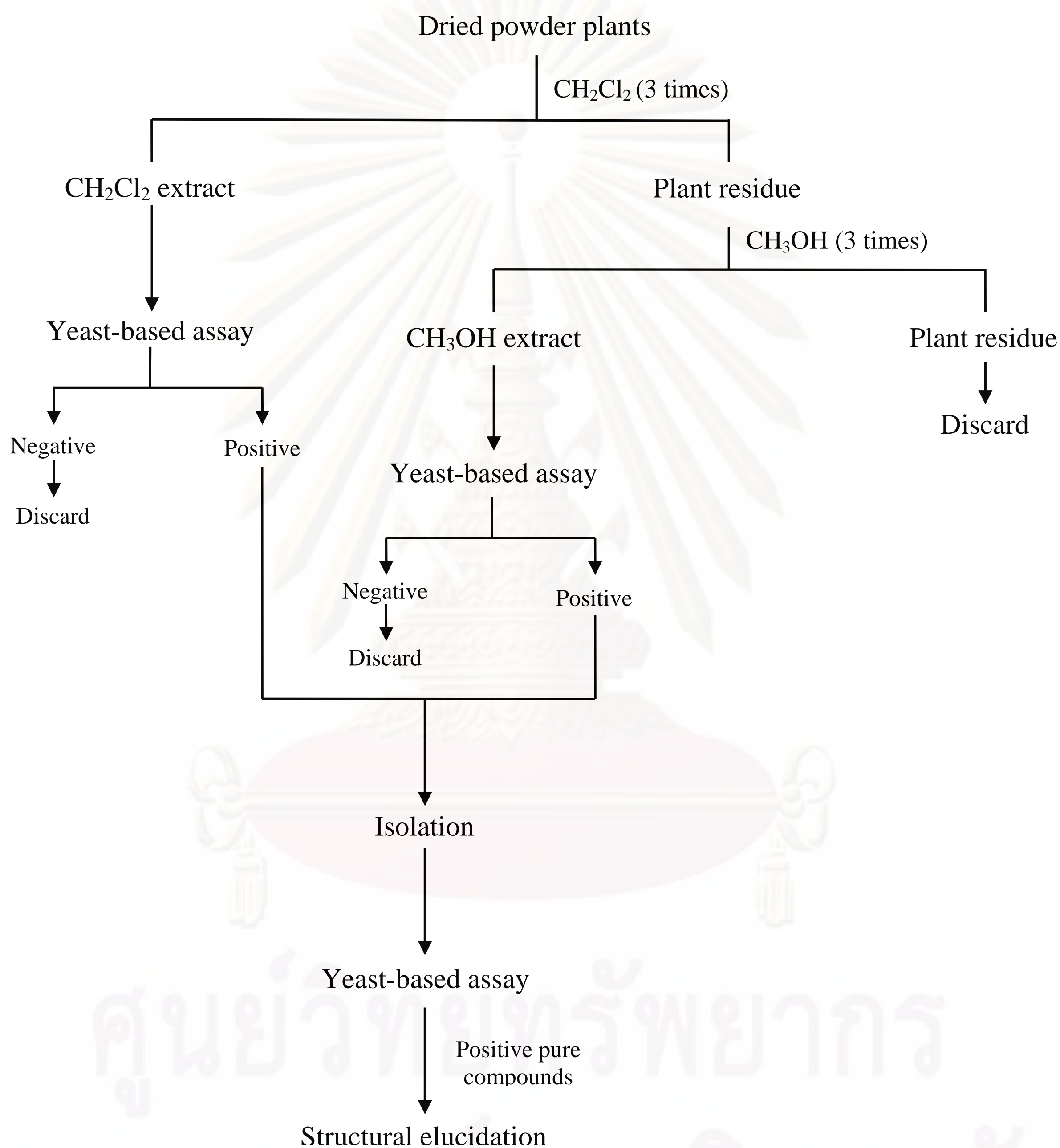


Figure 2-1 The preliminary screening of medicinal plants using the yeast-based assay.

2.5 Extraction, isolation and purification of selected plants

Potent plants: *B. pandurata* (Roxb.) Schlt. and *K. parviflora* Wall.Ex Baker. were chosen for further investigation. The extraction was perform by maceration the plant sample with CH_2Cl_2 (3x3L) at room temperature for 5 d (three times) and filtered. The plant residue was similarly extracted with CH_3OH. The extract was concentrated by rotary evaporator under reduced pressure. Both plant extracts were preliminary screened with the yeast-based assay. The positive extracts on the yeast growth were fractionated and isolated following the activity guided on the yeast-based assay. The structure of pure isolated compounds obtained from the plant extracts were

elucidated by spectroscopic technique such as NMR and mass spectrometry **Scheme 2-1** summarizes the whole extraction process and the yeast based-assay.



Scheme 2-1 General procedure for extraction and yeast-based screening assay.

2.6 Biological activity of the isolated compounds on the yeast growth

The isolated compounds were diluted with DMSO to appropriate concentrations then the diluted compounds were assayed using the yeast-based assay.

2.7 Other biological activities of the isolated compounds

2.7.1 Study on finding the target molecules in the yeast Ca^{2+}-signaling pathway of the isolated compounds

Base on Ca^{2+}-signal-dependent growth regulation in yeast, we developed a unique drug-screening procedure by which small-molecule inhibitors of Ca^{2+}-dependent growth regulation can be detected by their growth-promoting effect on the compromised the mutant cells growth due to excess $CaCl_2$. Further study the growth promoting/inhibiting effect of isolated compound was examined using liquid culture (YPAUD/SC) medium containing with 100-200 mM $CaCl_2$. The growth of the mutant yeast cell was monitored every two or four hours up to 14-24 hrs by counting cells with haemacytometer.

2.7.2 Anti-inflammatory activity on EPP-induced ear edema in rat

All bioactive compounds that gave a strong positive result on the yeast-based assay were tested for anti-inflammatory activity on EPP-induced ears edema in rat. The compounds were suspended in 5% Tween-80 and induced by topical application of EPP at a dose 1 mg/20μL/ear to the inner and outer surfaces of both ears by means of an automatic microliter pipette (Brattsand, *et al.*, 1982). Tested drugs were applied topically in volumes of 20 μL just before the irritant. The control (acetone) received vehicle only. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear were measured by vernier calipers. The percent inhibition of the edema formation of the tested substances were calculated as below.

$$\%\ \text{inhibition} = \frac{(C-T) \times 100}{C}$$

C = edema thickness of control rat T = edema thickness of tested rat

2.7.3 Anticancer activity (Suksawatamnuay *et al*., 2009)

The MTT assay (Mosmann, 1983) was applied for the evaluation of cytotoxicity against HepG2, SW620, KATO III, A375, BT474, MDA-MB-231, HeLa, Ca-Ski, SiHa, THP-1, HL-60 and Jurkat cancer cell lines. Cells were plated in a 96-well microplate (100 µL/well at a density of $5x10^4$ to $1x10^5$ cells/well), and incubated at 37 ^oC under 5% CO_2 for 18-24 hrs. Fifty microliters of the tested compound were mixed with culture media at various concentrations then added to the cell lines and incubated further for 4 d. Cell viability was determined by staining with MTT. The MTT stock solution (5mg/mL) was prepared in PBS, which was diluted (1:10) with a culture media prior to use. After removing the culture medium, the diluted MTT solution (50 µL) was added to the adhesive cells, and plates were incubated at 37 ^oC under 5% CO_2 for 4 hrs. After that times 100 µL of 0.04 N HCl in isopropanol was added, then the tested plates were read on a microplate reader using a test wavelength of 540 nm. Percentage of cell viability (% cell viability) was calculated by the following equation.

$$\%Viability = \frac{(\text{OD test average-OD blank average}) \times 100}{\text{OD control cell average-OD blank average}}$$

OD test average = average absorbance of cells plus tested compound or DMSO
OD control cell average = average absorbance of cells
OD blank average = average absorbance of culture media without serum

CHAPTER III

Bioactive flavonoids from *Boesenbergia pandurata* (Roxb.) Schltr.

3.1 Literature reviews of *B. pandurata*

3.1.1 Botanical description

B. pandurata (Roxb.) Schltr. (Syn. *Kaempferia pandurata*, *B. rotunda* L.), common name: finger root, temu kunci, gazhutu, kachai, krachai, kasay, Chinese ginger, Thai ginger, suo shi, chinese key, fingerwarr. It is a perennial herb of the family Zingiberaceae (ginger family), origin in Southern China and Southeastern Asia, has been known as 'kra-chai in Thailand. Finger-root is a tall ginger with large beautiful pink-purple flowers **(Figure 3-1 A and B),** the long tubers sprouting in the same direction from the middle of the rhizome **(Figure 3-1 C)** like a finger. It has a strong typical odor. There are culinary applications as a spice in Indonesian and Thai kitchen. It contains 1 to 3% of an essential oil. Several aroma components have been identified, namely, 1,8-cineol, camphor, *d*-borneol and methyl cinnamate being the most important components. Trace components are *d*-pinene, zingiberene, zingiberone, curcumin, zedoarin and others. In other context, the rose-flavored monoterpenoid alcohols are identified: geraniol and nerol. Among non-volatile constituents, flavones and flavonoids (pinostrobin, alpinetin, pinocembrin), chalcones (cardamonin) and dihydrochalcones (boesenbergin A) have been identified. Cardamonin is under investigation because of its anti-tumor properties. Finger root contains a significant amount of several polyphenols as important constituents that show an antimutagenic effect. These antimutagenic phytochemicals may play an important role in the prevention of cancer. In folk medicine in Suriname and Southeast Asia, the rhizome is used for swelling, wounds and diarrhea (Katzer, 2003; http://www.uni-graz.at/~katzer/engl/Boes_pan.html).

A B C

Figure 3-1 *Boesenbergia pandurata* (Roxb.) Schltr.
A: whole plant **B**: flower **C**: rhizome and roots

3.1.2 Chemical constituents and biological activities

In Thailand, *B. pandurata* occurs in four varieties described by colors presented in the rhizome as yellow, white, black and red. The chemical constituents found in yellow rhizomes are given in **Table 3-1** and their chemical structures are shown in **Figure 3-2**. The white rhizome contained 2′-hydroxy-4,4′,6′-trimethoxy-chalcone, isopimaric acid, boesenboxide, crotepoxide and (+)-zeylenol (Pancharoen *et al.*, 1984; Tuntiwachwuttikul *et al.*, 1987). Eleven flavonoids were reported as the constituents of the black rhizome variety; 5-hydroxy-7-methoxyflavanone (pinostrobin), 5,7-dimethoxyflavanone, 5-hydroxy-7-methoxyflavone, 5-hydroxy-7,4′-dimethoxy-flavone, 5,7-dimethoxyflavone, 5,7,4′-trimethoxyflavone, 5,7,3′,4′-tetramethoxyflavone, 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-3,7,4′-trimethoxyflavone, 3,5,7-trimethoxy-flavone and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (Jaipetch *et al.*, 1983). Chemical investigation of the hexane extract of the red rhizome of *B. pandurata* resulted in the isolation of panduratin A, boesenbergin A and rubranine (Tuntiwachwuttikul *et al.*, 1984), sakuranetin and dihydro-5,6-dehydrokawain (Tuchinda *et al.,* 2002).

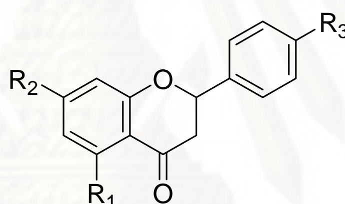
Table 3-1 Chemical constituents from the yellow rhizome of *B. pandurata*

Compound groups	Chemical constituents	References
Flavanones	pinostrobin (**3-1**)	Mongkolsuk and Dean, 1964
	pinocembrin (**3-2**)	Mahidol *et al*., 1984
	alpinetin (**3-3**)	Jaipetch *et al*., 1983
	5,7-dimethoxyflavanone (**3-4**)	Tuchinda *et al.,* 2000
	sakuranetin (**3-5**)	
Flavones	5,7-dimethoxyflavone (**3-6**)	Jaipetch *et al*., 1983
	3′,4′,5,7-tetramethoxyflavone (**3-7**)	
Chalcones	2′,6′-dihydroxy-4′-methoxychalcone (**3-8**)	Jaipetch *et al*., 1982
	2′-hydroxy-4,4′,6′-trimethoxy chalcone (**3-9**)	Mahidol *et al*., 1984
	flavokawain C (**3-10**)	Wang *et al*., 2001
	cardamonin (**3-11**)	Trakoontivakorn *et al*., 2001
	pinocembrin chalcone (**3-12**)	Tuntiwachwuttikul *et al*., 1984
	panduratin A (**3-13**)	Pancharoen *et al*., 1984
	panduratin B (**3-14**)	
	(-)-hydroxypanduratin A (**3-15**)	
	(-)-panduratin C (**3-16**)	
	(-)-isopanduratin A1 (**3-17**)	
	(-)-isopanduratin A2 (**3-18**)	
	(-)-nicolaioidesin B (**3-19**)	
	boesenbergin A (**3-20**)	
	boesenbergin B (**3-21**)	
	rubranine (**3-22**)	
Monoterpenes	Geranial (**3-23**)	Panji *et al*., 1993
	neral (**3-24**)	
Diterpene	pimaric acid (**3-25**)	Tuntiwachwuttikul *et al*., 1984

Table 3-1 (continued)

Compound groups	Chemical constituents	References
Alicyclic	boesenboxide (**3-26**) crotepoxide (**3-27**) (+)-zeylenol (**3-28**)	Tuntiwachwuttikul *et al*., 1984 Pancharoen *et al*., 1989
Pyrone	dihydro-5,6-dehydrokawain (**3-29**)	Tuchinda *et al.,* 2002

Flavanone



3-1 R_1 = OH, R_2 = OCH_3, R_3 = H

3-2 R_1 = R_2 = OH, R_3 = H

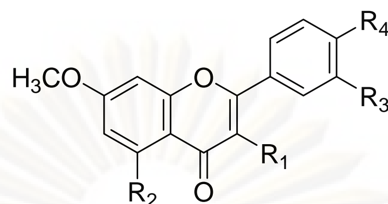
3-3 R_1 = OCH_3, R_2 = OH, R_3 = H

3-4 R_1 = R_2 = OCH_3, R_3 = H

3-5 R_1 = R_3 = OH, R_2 = OCH_3,

Figure 3-2 Structure of flavonoid compounds from the yellow rhizome of *B. pandurata*

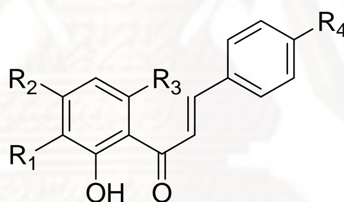
Flavones



3-6 $R_1 = R_3 = R_4 = H$, $R_2 = OCH_3$,

3-7 $R_1 = H$, $R_2 = R_3 = R_4 = OCH_3$

Chalcones



3-8 $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$

3-9 $R_1 = H$, $R_2 = R_3 = R_4 = OCH_3$

3-10 $R_1 = H$, $R_2 = R_3 = OCH_3$, $R_4 = OH$

3-11 $R_1 = R_4 = H$, $R_2 = OH$, $R_3 = OCH_3$

3-12 $R_1 = R_4 = H$, $R_2 = R_3 = OH$

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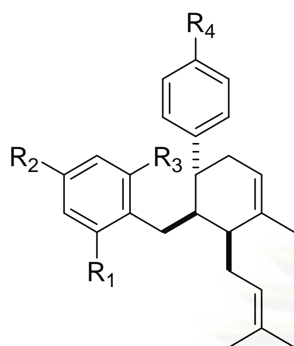
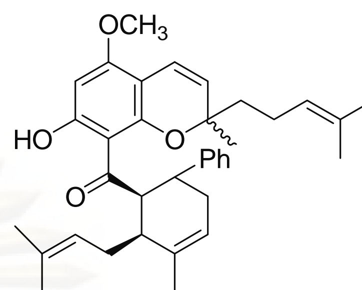
Figure 3-2 (continued)



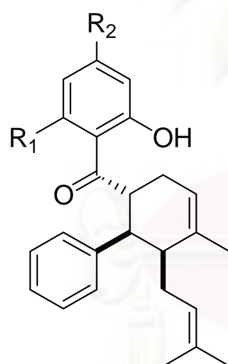


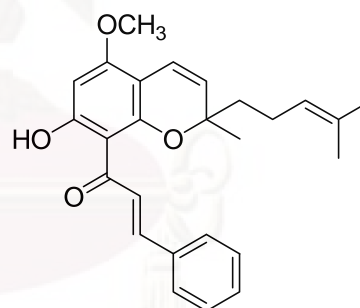
3-13 $R_1 = R_3 = OH$, $R_2 = OCH_3$, $R_3 = H$ (**3-14**)

3-15 $R_1 = R_2 = R_3 = OH$, $R_4 = H$

3-16 $R_1 = R_2 = R_4 = OH$, $R_3 = OCH_3$

3-18 $R_1 = OCH_3$, $R_2 = R_3 = OH$, $R_4 = H$





3-17 $R_1 = OCH_3$, $R_2 = OH$ (**3-20**)

3-19 $R_1 = OCH_3$, $R_2 = R_3 = OH$, $R_4 = H$

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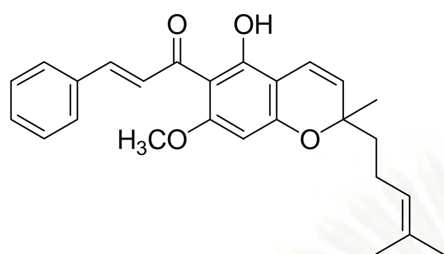
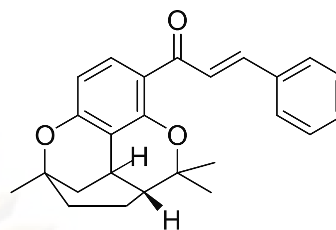
Figure 3-2 (continued)

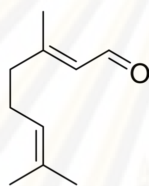


(**3-21**)

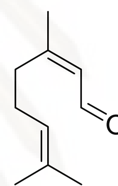


(**3-22**)

Monoterpenes

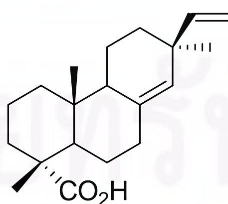


(**3-23**)



(**3-24**)

Diterpene



(**3-25**)

Figure 3-2 (continued)

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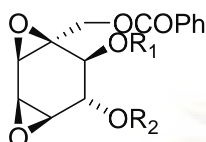
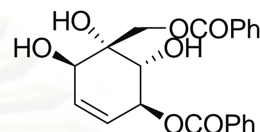
Alicyclic

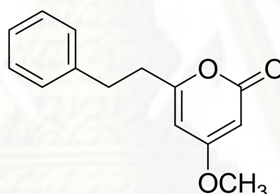




3-26 R_1 = Ac, R_2 = COPh (**3-28**)

3-27 R_1 = Ac, R_2 = Ac

Pyrone



(**3-29**)

Figure 3-2 (continued)

Pinostrobin (**3-1**) from *K. pandurata* revealed the inhibitory activity against DNA topoisomerase I which was isolated from human tomor. It may be interfered with DNA breakge-reunion reaction by stabilizing a key covalent intermediate between DNA and the enzyme, resulting in the clevage DNA (Sukardiman *et al.*, 2000).

Six compounds: pinostrobin (**3-1**), pinocembrin (**3-2**), cadamonin (**3-11**), pinocembrin chalcone (**3-12**), (2,4,6-trihydroxy-phenyl)-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl] methanone and panduratin A (**3-13**) were isolated

from the fresh rhizomes of *B. pandurata*. These compounds displayed strong antimutagens toward 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in *Salmonella typhimurium* TA98. They also similarly inhibited the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 2-amino-1-methyl-6-phenyl-limidazo[4,5-*b*]pyridine (PhIP). Antimutagenic activity was mainly due to the inhibition of the first step of enzymatic activation of heterocyclic amines (Trakoontivakorn *et al*., 2001).

In 2002, chalcone derivatives: (-)-panduratin A (**3-13**) and (-)-hydroxy-panduratin A (**3-15**) isolated from the $CHCl_3$ extract of the red rhizome variety of *B. pandurata* were assayed for topical anti-inflammatory activity in the experimental model of TPA-induced ear edema in rats. Challenge of the rat ear with the inflammogen TPA (12-O-tetradecanoylphorbol-13-acetate; 4 mg/ear) provoked maximum edematous response 8 hrs after application. Pretreatment of the rat ear by topical application of the two compounds (20–2,000 mg/ear) were significantly ($P<0.01$) inhibited TPA-induced ear edema formation in a dose-dependent manner. The ID_{50}-values of those compounds were determined as 84 and 12 mg/ear, respectively. The presence of these anti-inflammatory compounds in *B. pandurata* may very well be related to the use of this plant in traditional medicine (Tuchinda *et al.,* 2002).

Four flavonoids: pinostrobin (**3-1**), pinocembrin (**3-2**), alpinetin (**3-3**) and cardamonin (**3-11** isolated from the methanol extract of *B. pandurata* Holtt. (yellow rhizome) were tested for activity against HIV-1 protease (HIV1 PR). The result showed that **3-11** exhibited an appreciable anti-HIV1 PR activity with an IC_{50} value of 31μg/ml (Tewtrakul *et al*., 2003).

Panduratin A (**3-13**) isolated from the CH_3OH extract of *K. pandurata* displayed a strongly inhibition for both nitric oxide, NO (IC_{50} : 0.75 μM) and postaglandin E_2, PGE_2 (IC_{50}: 0.0195 μM) production and suppressed both nitric oxidase synthase (iNOS) and cyclooxygenase-2 (COX-2) enzyme expression without any appreciable cytotoxic effect on RAW264.7 cells. The inhibitory effects on the production of NO and PGE_2 , indicating its potential for use as an anti-inflammatory agent (Yun and Hwang, 2003).

Isopanduratin A (**3-17**) isolated from *K. pandurata* exhibited specific activity against the oral bacteria *Streptococcus mutans*, *S. sobrinus*, *S. sanguinis* and *S. salivarius*. In particular, isopanduratin A showed high activity against *S. mutans* at 20 mg/mL in one minute. These data suggest that it could be employed as a natural anticariogenic agent for preventing dental caries caused by the growth of *Streptococcus* spp. (Hwang *et al*., 2004).

Panduratin A (**3-13**) and hydroxypanduratin A (**3-15**) isolated from *B. pandurata* rhizome responsible for potent anti-HIV-1 PR activity with an IC_{50} values of 5.6 and 18.7 μM, respectively. The structure-activity relationships of these compounds required the hydroxylation at position 4 and the prenylation of chalcone. This study also supports the use of *B. pandurata* by AIDS patients of Thailand (Cheenpacha *et al*., 2006).

Cyclohexenyl chalcone derivatives, panduratin A (**3-13**) and 4-hydroxy-panduratin A (**3-15**), from *B. rotunda* L. showed good competitive inhibitory activities toward dengue-2 virus NS3 protease with the *ki* values of 21 and 25 μM, respectively. Flavanone pinostrobin (**3-1**) showed non-competitive inhibition towards DEN-2 protease (Kiat *et al.*, 2006).

In 2006, the CH_2Cl_2-CH_3OH (1:1) extract from the rhizome of *B. pandurata* Schult. was found to possess potent antioxidant activity in rat brain homogenate model. Bioassay-guided isolation of the active compounds from the extract led to the isolation of 5-hydroxy-7-methoxyflavanone (**3-1**), 5,7-dimethoxyflavanone (**3-4**), 2′,6′-dihydroxy-4′-methoxychalcone (**3-8**) and panduratin A (**3-13**) which were found to exert neuroprotective effects (Shindo *et al*., 2006).

Panduratin A (**3-13**), a chalcone derivative isolated from *B. pandurata*, was found to inhibit the growth of MCF-7 human breast cancer and HT-29 human colon adenocarcinoma cells with an IC_{50} of 3.75 and 6.56 μg/mL, respectively. Compound **3-13** arrested cancer cells labelled with Annexin-V and propidium iodide in the G0/G1 phase and induced apoptosis in a dose-dependent manner (Kirana *et al*., 2007).

Pinostrobin (**3-1**), alpinetin (**3-3**) and pinocembrin chalcone (**3-12**) isolated from the CH_2Cl_2 extract of *B. pandurata* exhibited strong positive inhibitory activity

on the Ca^{2+}-signaling pathway using the *zds1*Δ yeast proliferation based assay. Further biochemical experiments confirmed that **3-1** possess inhibitory activity on the Ca^{2+}-signals involved in the control of G2/M phase cell cycle progression in *Saccharomyces cerevisiae* (Wangkangwan *et al*., 2009).

The compounds isolated from the rhizomes of *B. pandurata* were examined for inhibitory activities against nitric oxide (NO) production. Regarding NO inhibitory activity of *B. pandurata*, panduratin A (**3-13**) displayed the most potent effect against NO production, with an IC_{50} value of 5.3 μM, followed by hydroxyl-panduratin A (**3-15**), IC_{50} = 13.3 μM and cardamonin (**3-11**), IC_{50} = 24.7 μM), whereas other compounds showed moderate or mild effects (62.3–74.7 μM). The NO inhibition activity of **3-13** (IC_{50} = 5.3 μM) was comparable to that of caffeic acid phenethylester (CAPE, IC_{50} = 5.6 μM) and very much higher than that of L-nitroarginine (L-NA, IC_{50} = 61.8 μM) (Tewtrakul *et al.*, 2009). For the tests on PGE_2 and TNF-α production, compound **3-13** and **3-15** showed strong activities against PGE_2 release, with IC_{50} values of 10.5 and 12.3 μM, respectively, and moderate effects on TNF-α (IC_{50} = 60.3 and 57.3 μM, respectively.

4-Hydroxypanduratin A (**3-15**) isolated from *K. pandurata* Roxb. in the range of 0.001–0.1 mM significantly reduced the expression of matrix metalloproteinases (MMP-1) levels and inhibited UV-induced mitogen-activated protein kinases (MAPKs) activation. Moreover, inhibition of MAPKs by this compound resulted in decreasing c-Fos expression and c-Jun phosphorylation induced by UV, which led to inhibiting activator protein-1 (AP-1) DNA binding activity. The results suggest that **3-15** be a potential candidate for the prevention and treatment of skin aging brought about by UV (Shim *et al.*, 2009).

K. pandurata significantly decreased MMP-9 expression at both protein and mRNA levels in a dose-dependent manner. *K. pandurata* interfered *Porphyromonas gingivalis* supernatant induced MMP-9 expression in KB cells by downregulating MAPK phosphorylation (extracellular signal related kinase 1/2, p38 kinase and c-Jun N-terminal kinase), inhibiting transcriptional expression (Elk1, c-Jun and c-Fos), and blocking AP-1 and NF-κB activities. *K. pandurata* could be employed as a candidate

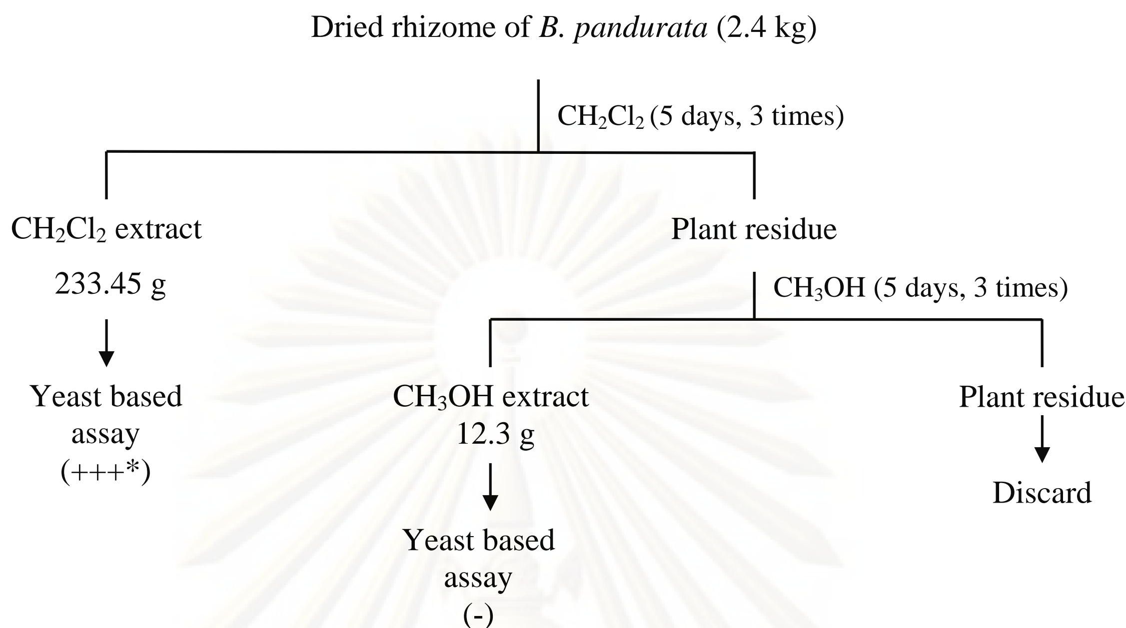
for MMP-9 inhibitor with therapeutic potential for treatment of periodontal inflammation (Yanti *et al.*, 2009).

3.1.3 Ethnomedicinal uses

Kra-chai is generally utilized as a folk medicine in Southeast Asia. In Thailand, the fresh rhizomes and roots are used in cooking, also in folk medicine as an aphrodisiac, dysentery, anti-inflammatory, the treatment of colic disorder and for health promotion (Farnsworth and Bunyapraphatsara, 1992). In Indonesia, the plant is used for thrush be chewed with areca, cough and throat (Hirschhorn, 1983). In Malaysia, it is used as a stomachic and decoction given to woman after childbirth (Ilham *et al.*, 1995).

3.2 Extraction and activity guided fractionation of *B. pandurata*

The fresh rhizomes of *B. pandurata* were purchased from Hua-Trakae market, Ladkrabang, Bangkok in 2006. The voucher specimen (BKF 152279) has been deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Chatuchak, Bangkok, Thailand. The fresh rhizomes (10.0 kg) were sliced and dried in open air for a few days and powdered into small pieces with an electrical blender. The dried powder (2.4 kg) was extracted with CH_2Cl_2 by maceration technique three times (3x3L) at room temperature for five days and filtered. The plant residue was similarly extract with CH_3OH. The extracts were concentrated by rotary evaporator under reduced pressure to give brown crude 233.45 g (9.73%, wt by wt) of CH_2Cl_2 extract and 12.3 g (0.51%, wt by wt) of CH_3OH extract. Both extracts were preliminary tested with yeast-based assay as shown in **Scheme 3-1**.



Scheme 3-1 The extraction of *B. pandurata* rhizomes and preliminary yeast-based assay

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

* = positive (ring like growth)

3.3 Fractionation of CH_2Cl_2 extract of *B. pandurata* and yeast-based assay

The fractionation of bioactive extract from *B. pandurata* was carried out using the yeast-based assay as an activity guided. According to the biological activity of yeast-based assay, the CH_2Cl_2 extract gave strong positive result on yeast growth. Thus, the CH_2Cl_2 extract (100.0 g) was subjected to silica gel quick column chromatograph with sinter glass frit, firstly eluting with *n*-hexane. The *n*-hexane extracts were combined and evaporated to dryness. The silica residue was then exhaustively eluted using solvents with increasing polarity by mixing with EtOAc and CH_3OH, successively. The fractions were collected and combined according to TLC furnishing 5 fractions (**Table 3-2**). Each fraction was further assayed using the yeast-based assay.

Table 3-2 The separation of the CH_2Cl_2 extract of *B. pandurata* by silica gel quick column

Fraction	Solvent system	Remarks	Yeast-based assay	Weight (g)
BPA1	100% Hexane	yellow oil	C	2.31
BPA2	5-20% EtOAc-Hex	yellow crystal	++*	29.5
BPA3	40% EtOAc-Hex	brownish oil	+++*	30.17
BPA4	60% EtOAc-Hex	brownish wax	C	3.72
BPA5	80-100% EtOAc-Hex 5-50% MeOH-EtOAc	brownish wax	-	13.52

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth) C = clear zone (cytotoxic/suspect)

3.3.1 Isolation and purification

According to the preliminary biological activity on the yeast-based assay, two positive fractions (**BPA2** and **BPA3**) were reisolated using silica gel column. The yellow solid obtained from **BPA2** fraction (29.0 g) was further chromatographed on silica gel column eluting with *n*-hexane and a gradient solvent of *n*-hexane and EtOAc. The fractions were collected and combined according to TLC furnishing 4 fractions (**Table 3-3**). The white plate of **BPA2/2-A** was recrystallized from **BPA2/2** (**Scheme 3-2**) with hexane yielding Compound **A** (9.65 g), the major composition of *B. pandurata*. Each fraction and the pure compound were assayed using the yeast-based assay. Compound **A** exhibited strong Ca^{2+} -signal inhibition on the Δ*zds1* mutant yeast growth.

Table 3-3 The reseparation of **BPA2** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weight (g)
BPA2/1	5-10% EtOAc-Hex	yellow solid	-	0.5
BPA2/2-A	10% EtOAc-Hex	white plates	+++*	9.65
BPA2/2-B	10% EtOAc-Hex	yellow oil	-	7.54
BPA2/3	20-40% EtOAc-Hex	yellow solid	-	7.17

+ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

The **BPA3** fraction **(Table 3-2)** also displayed a positive growth on the yeast-based assay. Thus, the brownish oil of this fraction (30.0 g) was subjected to silica gel column eluting with *n*-hexane and a gradient of *n*-hexane and EtOAc. The fractions were collected and combined according to TLC furnishing 4 fractions (**Table 3-4**). The cream solid of **BPA3/1-B** was crystallized from **BPA3/1** (**Scheme 3-2**). This fraction was then recrystallized with EtOAc-Hex which gave cream powder of Compound **B** (0.60 g)**.** Each fraction and the pure compound were assayed using the yeast-based assay.

Table 3-4 The reseparation of **BPA3** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weight (g)
BPA3/1-A	5-20% EtOAc-Hex	Brownish oil	+*	14.25
BPA3/1-B	5-20% EtOAc-Hex	Cream powder	++*	0.60
BPA3/2	40-60% EtOAc-Hex	Brownish solid	++*	13.16
BPA3/3	80-100% EtOAc-Hex	Brownish wax	-	1.31

+ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

Fractions **BPA3/1-A** and **BPA3/2** also exhibited the positive result on the yeast growth. Thus the brownish oil of **BPA3/1-A** (14.0 g) was chromatographed on silica gel column eluting with gradient solvent of *n*-hexane and EtOAc. Based on the

TLC results, 5 fractions (**Table 3-5**) could be collected. The TLC result of **BPA3/1/1-B** showed double spots. This fraction was then recrystallized with EtOAc-Hex to gain yellow solid of Compound **A** (0.85 g) **(Scheme 3-2**). Each fraction and the pure compound were assayed using the yeast-based assay.

Table 3-5 The reseparation of **BPA3/1** by silica gel column

Fraction	Solvent system	Remarks	Yeast based-assay	Weight (g)
BPA3/1/1-A	5-20% EtOAc-Hex	Yellow oil	-	0.29
BPA3/1/1-B	5-20% EtOAc-Hex	Yellow solid	+++*	0.85
BPA3/1/2	40-60% EtOAc-Hex	Brownish oil	-	7.40
BPA3/1/3	80-100% EtOAc-Hex	Brownish oil	-	4.65
BPA3/1/4	80-100% EtOAc-Hex	Brownish wax	-	0.81

\+ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

The brownish solid of **BPA3/2** (13.0 g) was reseparated by silica gel column eluting with EtOAc-Hex. The fractions were collected and combined according to TLC resulting 4 subfractions. The results of the separation are presented in **Table 3-6**. The yellow crystal of **BPA3/2/1-A** was crystallized from **BPA3/2** (**Scheme 3-2**) with hexane to furnish Compound **A** (0.35 g). Each fraction and the pure compound were assayed using the yeast-based assay.

Table 3-6 The separation of **BPA3/2** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weight (g)
BPA3/2/1-A	10-20% EtOAc-Hex	Brownish solid	++*	7.89
BPA3/2/1-B	10-20% EtOAc-Hex	Yellow crystal	+++*	0.35
BPA3/2/2	40-60% EtOAc-Hex	Brownish wax	-	2.38
BPA3/2/3	80-100% EtOAc-Hex	Brownish wax	-	1.49

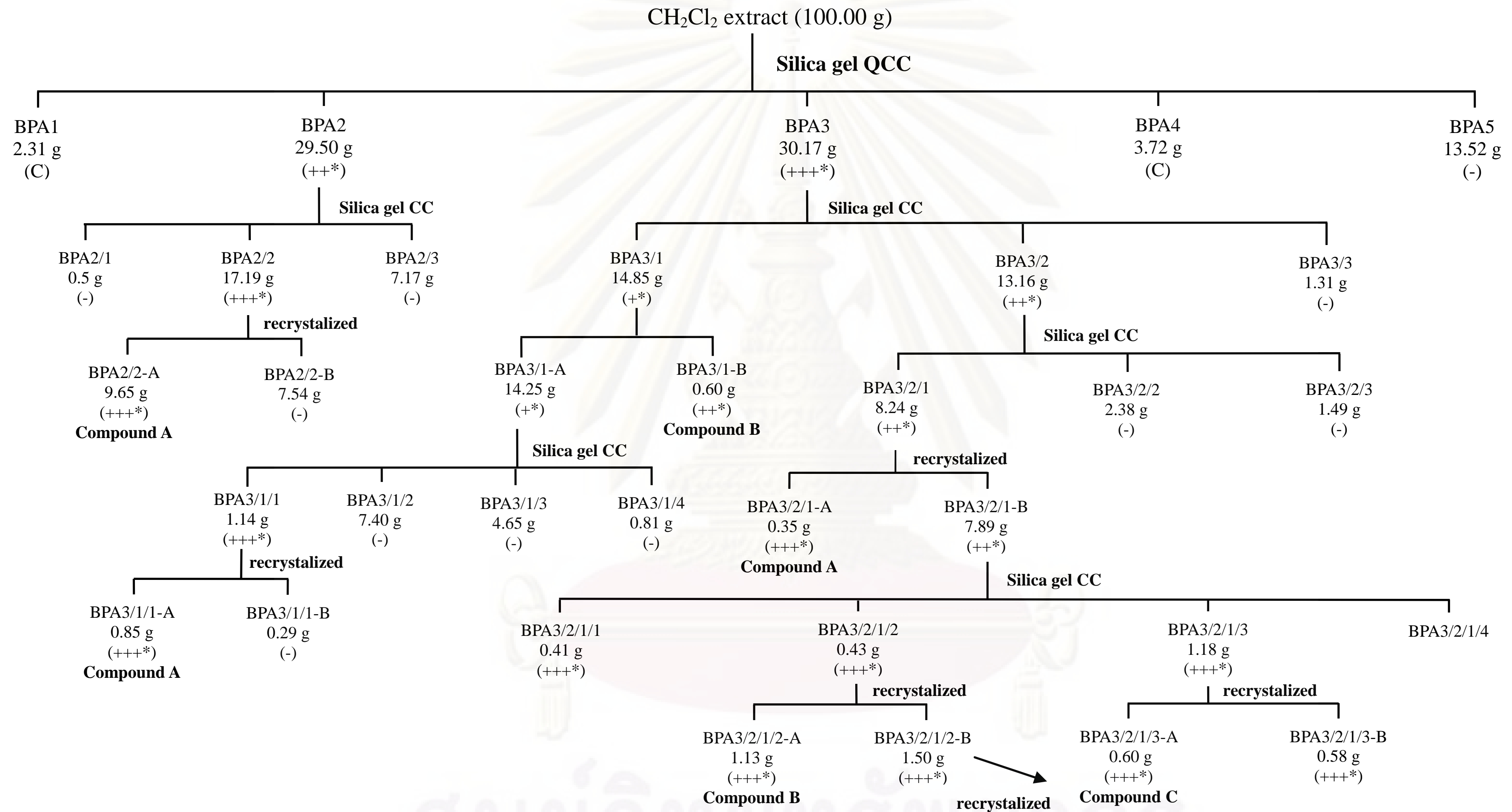
\+ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

The brownish solid of **BPA3/2/1** (7.0 g) was reseparated by silica gel column eluting with EtOAc-Hex. The fractions were collected and combined according to TLC resulting 6 subfractions. The results of the separation are shown in **Table 3-7**. The **BPA3/2/1/2-A** was recrystallized with EtOAc-Hex to afford cream powder of compound **B** (1.13 g). The red solid of **BPA3/2/1/3-A** was crystallized from **BPA3/2/1/3** with EtOAc-Hex to yield red solid of compound **C** (0.60 g) **Scheme 3-2**. Each fraction and the pure compounds were assayed using the yeast-based assay. The brown solid of **BPA3/2/1/2-B** also disclosed the positive result on the yeast-based assay. After recrystallization with EtOAc-Hex, red solid Compound **C** was achieved (**Scheme 3-2**).

Table 3-7 The separation of **BPA3/2/1** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weight (g)
BPA3/2/1/1	10-20% EtOAc-Hex	Yellow oil	+++*	0.41
BPA3/2/1/2-A	20-60% EtOAc-Hex	Cream powder	+++*	1.13
BPA3/2/1/2-B	20-60% EtOAc-Hex	Brown solid	+++*	1.50
BPA3/2/1/3-A	60-80% EtOAc-Hex	Red solid	+++*	0.60
BPA3/2/1/3-B	60-80% EtOAc-Hex	Red brown solid	+++*	0.58
BPA3/2/1/4	60-80% EtOAc-Hex	Brownish solid	C	2.37

+++ = positive (growth)   - = negative (no growth
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)   C = clear zone (cytotoxic/suspect)

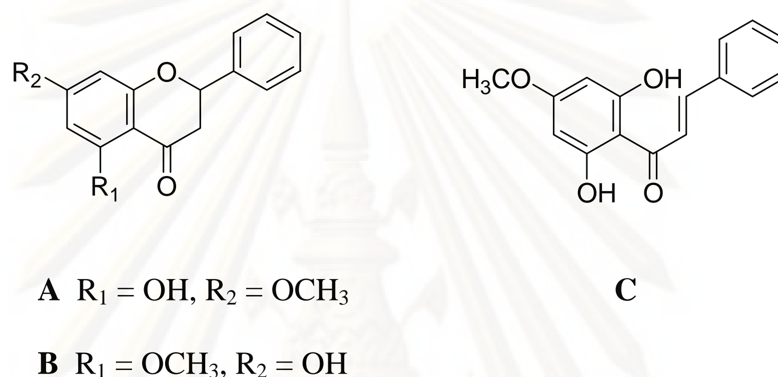


Scheme 3-2 The separation of the CH_2Cl_2 extract of *B. pandurata* by silica gel quick column and column chromatographs

+ = positive (growth)
- = negative (no growth)
* = positive (ring like growth)
C = clear zone (cytotoxic/suspect)
+++, ++, + = positive (compared to FK506 used as a positive control which gave +++)

3.4 Structural elucidation of the isolated compounds

Compounds **A**, **B** and **C**, three known flavonones could be isolated from this plant using the yeast-based assay as an activity guided fractionation. The structural elucidation of these compounds were described below. The structures of three flavonoid compounds are shown in **Figure 3-3**.



A $R_1 = OH, R_2 = OCH_3$ **C**

B $R_1 = OCH_3, R_2 = OH$

Figure 3-3 Structure of flavonoid compounds obtained from *B. pandurata*

3.4.1 Structural elucidation of Compound A

Compound **A**, fully characterized as pinostrobin was isolated as colorless crystal. The molecular formula was suggested as $C_{16}H_{14}O_4$. Its ^{1}H-NMR spectrum **(Figure 3-4** and **Table 3-8)** reveals the signal of a methoxy group as a singlet at δ_H 3.80. A one-proton singlet signal at δ_H 12.00, interchangeable with D_2O, could be ascribed for the free hydroxyl proton at the position 5 with a H-bridge to O at the carbonyl at the position 4. Two doublets at δ_H 6.08 (J = 2.2 Hz) and 6.06 (J = 2.2 Hz) attributable to *meta* H-6 and H-8, could be assigned for the methoxy group on C-7. The multiplet signals of five protons were detected at δ_H 7.37-7.47 confirmed the non-substitution of the B-ring. Thus Compound **A** was identified by NMR as pinostrobin, with spectral data in agreement with Ching *et al*., (2007).

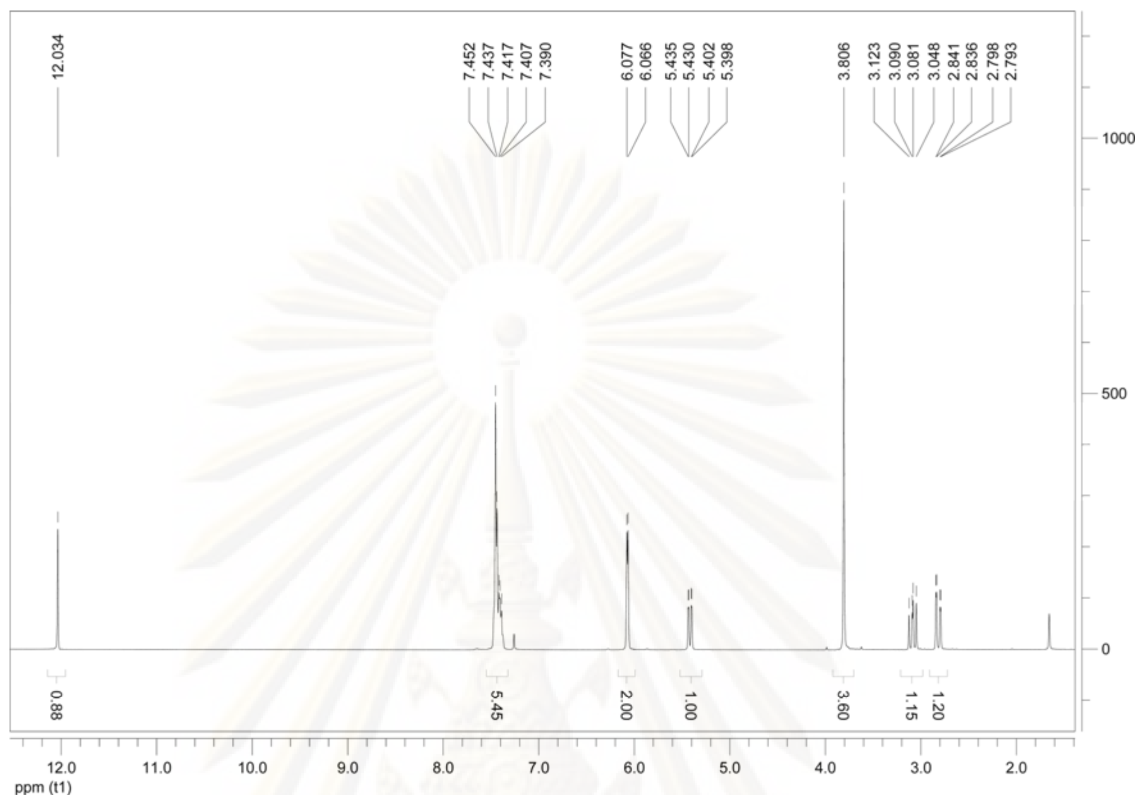


Figure 3-4 The ^{1}H NMR ($CDCl_3$) spectrum of Compound **A**

Table 3-8 ^{1}H NMR data of Compound **A** (400 MHz, $CDCl_3$)

Position	**Pinostrobin**	**Compound A**
	δ_H (multiplicity *J* in Hz)*	δ_H (multiplicity *J* in Hz)
2	5.39, dd, *J* = 12.84, 2.76	5.41, dd, *J* = 14.80, 2.00
3-*cis*	2.79, dd, *J* = 14.68, 2.76	2.82, dd, *J* = 17.20, 2.00
3-*trans*	3.06, dd, *J* = 15.14, 12.84	3.08, dd, *J* = 15.00, 13.29
6	6.05, d, *J* = 2.72	6.08, d, *J* = 2.20
8	6.05, d, *J* = 2.72	6.06, d, *J* = 2.20
2′, 3′, 4′, 5′, 6′	7.41, m	7.45, m
5-OH	12.00, s	12.03, s
7-OMe	3.79, s	3.80, s

*Ching, *et al*., (2007)

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3.4.2 Structural elucidation of Compound B

Compound **B** was isolated as colorless crystal. The molecular formula was suggested as $C_{16}H_{14}O_4$. In addition, the comparison of the ^{1}H-NMR of this compound with that of alpinetin was tabulated in **Table 3-9** (Ching *et al*., 2007).

The ^{1}H NMR spectrum (**Figure 3-5** and **Table 3-9**) displays the singlet signal belonging to a methoxy group at δ_H 3.40 (5-OMe). The one-proton singlet at δ_H 12.00, interchangeable with D_2O, could be assigned for the free hydroxyl at the position 5 with a H-bridge to O at the carbonyl at the position 4. Two doublets at δ_H 6.08 (J = 2.3 Hz) and 6.06 (J = 2.3 Hz) attributable to *meta* H-6 and H-8 could be placed for the methoxy group on C-7. The multiplet signal of five protons at δ_H 7.30 confirmed the non-substitution of the B-ring. The ^{1}H-NMR spectral data of Compound **B** showed similar pattern to that of alpinetin.

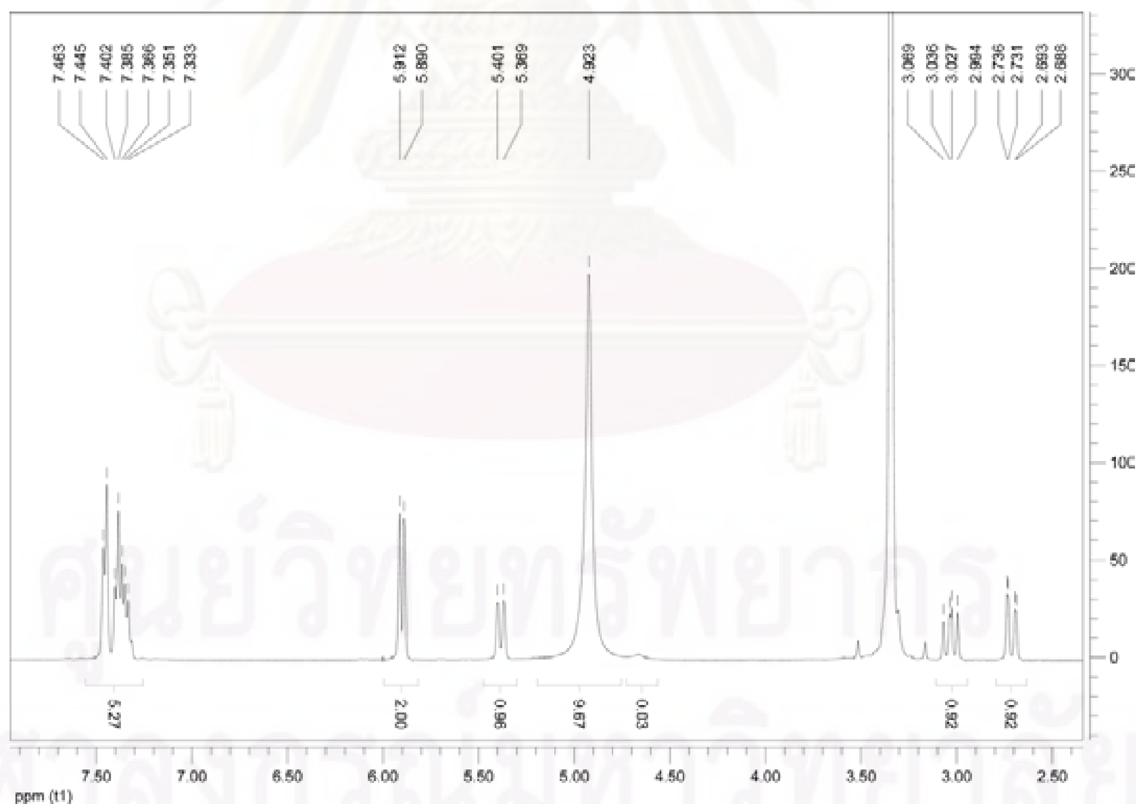

Figure 3-5 The ^{1}H NMR (CD_3OD) spectrum of Compound **B**

Table 3-9 The comparison of the ^{1}H-NMR data between alpinetin and Compound **B**

Position	**Alpinetin**	**Compound B**
	δ_H (multiplicity *J* in Hz)*	δ_H (multiplicity *J* in Hz)
2	5.33, dd, *J* = 10.08, 2.84	5.38, dd, *J* = 12.8, 2.73
3-*cis*	2.63, dd, *J* = 12.84, 3.68	2.71, dd, *J* = 13.28, 2.00
3-*trans*	2.90, dd, *J* = 12.84, 4.60	3.03, dd, *J* = 13.28, 3.60
6	5.96, d, *J* = 2.76	5.89, d, *J* = 2.9
8	6.01, d, *J* = 1.84	5.91, d, *J* = 1.90
2′, 3′, 4′, 5′, 6′	7.32, m	7.41, m
7-OH	-	-
5-OMe	3.74, s	3.68, s

*Ching, *et al*., (2007)

3.4.3 Structural elucidation of Compound C

Compound **C** was isolated as red prisms. The molecular formula was suggested as $C_{16}H_{14}O_4$. In addition, the comparison of the ^{1}H-NMR of this compound with that of pinostrobin chalcone was tabulated in **Table 3-10**. The ^{1}H-NMR spectrum data CD_3OD (**Figure 3-6**), showed a broad one-proton singlet at δ_H 7.29, doublets at δ_H 8.10 (1H, *J* = 16.00 Hz) and 7.63 (1H, *J* = 15.60 Hz), multiplets at δ_H 7.51 (2H) and 7.29 (3H), and singlets at δ_H 5.86 (2H), 3.68 (3H) and δ_H 4.56 was a hydroxyl group. Thus Compound **C** was identified by NMR as pinostrobin chalcone, with spectral data in agreement with (Cooksey *et al*., 1982).

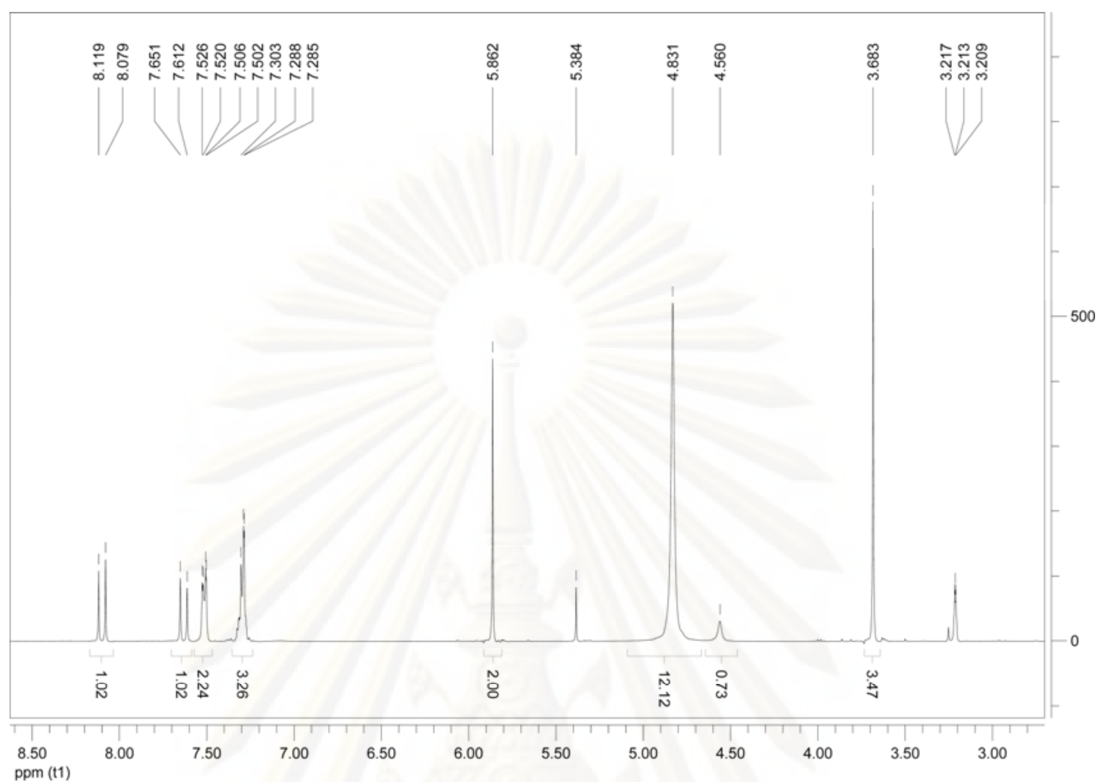

Figure 3-6 The ^{1}H NMR (CD_3OD) spectrum of Compound **C**

Table 3-10 The comparison of the ^{1}H-NMR data between pinostrobin chalcone and Compound **C**

Position	Pinostrobin chalcone	Compound C
	δ_H (multiplicity J in Hz)*	δ_H (multiplicity J in Hz)
2, 6	7.70, m	7.51, m
3, 5	7.45, m	7.29, m
4	10.1, s	7.29
α-H	8.04, d, J = 15.6	8.10, d, J = 16.00
β-H	7.82, d, J = 15.6	7.63, d, J = 15.60
3′,5′	5.99, s	5.86, s
2′, 6′-OH	-	4.56, s
4′-OMe	3.81	3.68, s

*Cooksey *et al*., (1982)

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3.5 Biological activities of isolated compounds on the yeast-based assay

The pure isolated compounds were diluted with DMSO at concentration of 2,000, 1,000, 500, 250, and 125 μM, then the dilutions were assayed using the yeast-based assay. The activity assay was examined, scored and compared with that of the positive control (FK506) which showed a growth zone at or around the application spots.

3.5.1 Effect of the isolated compounds from *B. pandurata* on the Δ*zds1* yeast growth

Biological activities of the isolated compounds on the Δ*zds1* yeast growth were evaluated. The yeast cells proliferation assay on YPAUD soft agar plate containing 150 mM $CaCl_2$ using a Ca^{2+}-sensitive Δ*zds1* strain suggested that the isolated compounds rescue compromised growth and inhibit the cell growth at various dose-dependent concentrations. The ring like growth pattern on the plate assay was the result from the dose effect on growth. At higher concentration (dotted area), it displayed toxicity to the indicator cells, a clear zone or ring like growth. At the diluted concentration, the compound was well diffused far away from the dotted area resulting in no toxicity, promoting the cells growth. From the results, Compounds **B** and **C** displayed the ring like growth on the growth of Δ*zds1* mutant yeast cells at the concentration of 1 mM while Compound **A** did not. This suggested that Compounds **B** and **C** at high concentration caused toxicities on the growth of the mutant yeast cells. (**Figure 3-7 and Table 3-11**).

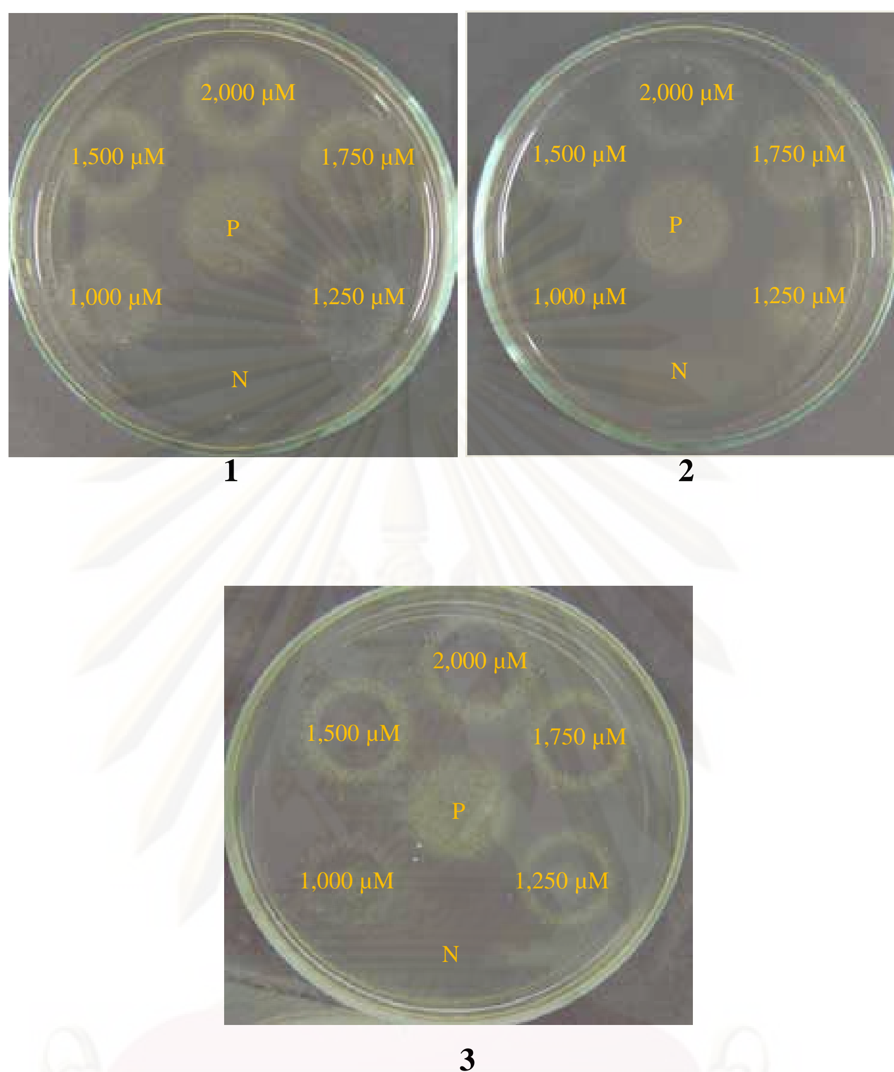

Figure 3-7 Dose dependent effect of the isolated compounds from *B. pandurata* on the growth of Δ*zds1* cells on YPAUD soft agar containing 6.0×10^5 cells/mL indicator cells 150 mM $CaCl_2$ at 30 °C for 2 d. Various concentrations of isolated compounds: **1**, **2 and 3**; Compounds **A**, **B and C** at 2,000, 1,750, 1,500, 1,250 and 1,000 µM, whereas, P = 500 nM FK506 (positive control), N = absolute ethanol (negative control)

Table 3-11 The dose dependent effect of the isolated compounds from *B. pandurata* on the growth of Δ*zds1* cells on agar containing 150 mM $CaCl_2$*

Concentration (μM)	Isolated compounds		
	Compound **A**	Compound **B**	Compound **C**
2,000	+++*	++*	+++*
1,750	+++*	++*	+++*
1,500	+++*	++*	++*
1,250	++	+*	++*
1,000	++	+	++*
500	+	-	+*
250	+	-	-
125	-	-	-

\+ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

* The Δ*zds1* mutant cells were cultivated on YPAUD soft agar containing 150 mM $CaCl_2$ and incubated at 30 ^oC for 2 d.

3.6 Other biological activities of the isolated compounds

3.6.1 Study on finding the target molecules of Compound A in the Ca^{2+}-signaling pathway using mutant yeast strains

Compound **A** was chosen for investigating on the target molecule because it is nontoxic on the yeast growth (Wangkangwan *et al.,* 2007). The growth promoting/ inhibiting effect of the isolated compound was examined in liquid culture (YPAUD/SC) medium containing 100-200 mM $CaCl_2$. The growth of mutant yeast cells was monitored every two or four hrs up to 14-24 hrs by counting the cell numbers using haemacytometer.

3.6.1.1 Effect of Compound A on free intracellular Ca^{2+}-levels in the mutant yeast cells (Wangkangwan *et al.*, 2007)

The assay for detecting free Ca^{2+} in mutant yeast cells under hyperactivation of high Ca^{2+} was modified from Tutulan-Cunita *et al.* (2005), to determine the levels of β-galactosidase activity. The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190 by carrying the *PMR2A*-LacZ reporter under the activation of four tandem copies of the calcineurin-dependent response element (CDRE) (**Figure 3-8**). The mutant cells were subcultured on SC agar lacking uracil (Appendix) and incubated at 30 °C for 1-2 d. The colonies of mutants strain were grown in SC medium lacking uracil and incubated at 30 °C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase was close to 2-5x10^{7} cells/mL. The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with sterile water and separated at 7,500 rpm for 5 min. The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUD, the cell suspension was counted with haemacytometer and adjusted to the final concentration of 1.0x10^{7} cells/mL. The assay cell suspension of 3 mL was transferred into each of test tube, 200 mM of Compound **A** was added to final concentration of 2 mM, 0.5% DMSO and 500 nM of FK506 were added as a negative and positive control, respectively. The test samples were incubated at 30 °C, for 30 min. After that, 4 M $CaCl_2$ was added to the final concentration of 150 mM except for the treatment of negative control. The assays were incubated at 30°C for 4 hrs. β-galactosidase activity was measured using ONPG assay (Miller, 1972). The assay cell suspension was diluted and measured the cell density at OD_{600} using spectrophotometer (blank against YPAUD). For enzyme activity, 1 mL of the cell suspension was divided into eppendorf tube and centrifuged at 12,000 rpm for 2 min. The pellet cells were suspended with 1 mL Z buffer (Appendix) and re-centrifuged at 12,000 rpm for 2 min. The pellet cells were mixed with 150 µL Z buffer and 50 µL $CHCl_3$, 20µL 0.1% SDS was added, vortex (each sample exactly alike) for 15 min. After that time, 700 µL ONPG (4 mg/mL) (Appendix) was added as a substrate, vortex and incubated at 30 °C. Record the time of addition precisely with timer. Stop the reaction after sufficient yellow color has

developed by adding 500 μL 1 M Na_2CO_3, vortex and note the time of addition precisely. The test samples were centrifuged at 12,000 rpm for 2 min (remove derbris and $CHCl_3$). The supernatant was measured for OD_{420} (blank against 700 μL ONPG plus 500 μL Na_2CO_3). The unit of β-galactosidase activity was calculated by the following equation.

$$\textbf{Miller Units} = (OD_{420} \times 1{,}000)/(OD_{600} \times T \times V)$$

OD420 = OD of the reaction mixture

OD600 = OD of the culture (as measured of biomass)

T = time of the reaction in minutes

V = volume of culture used in the assay in mLs

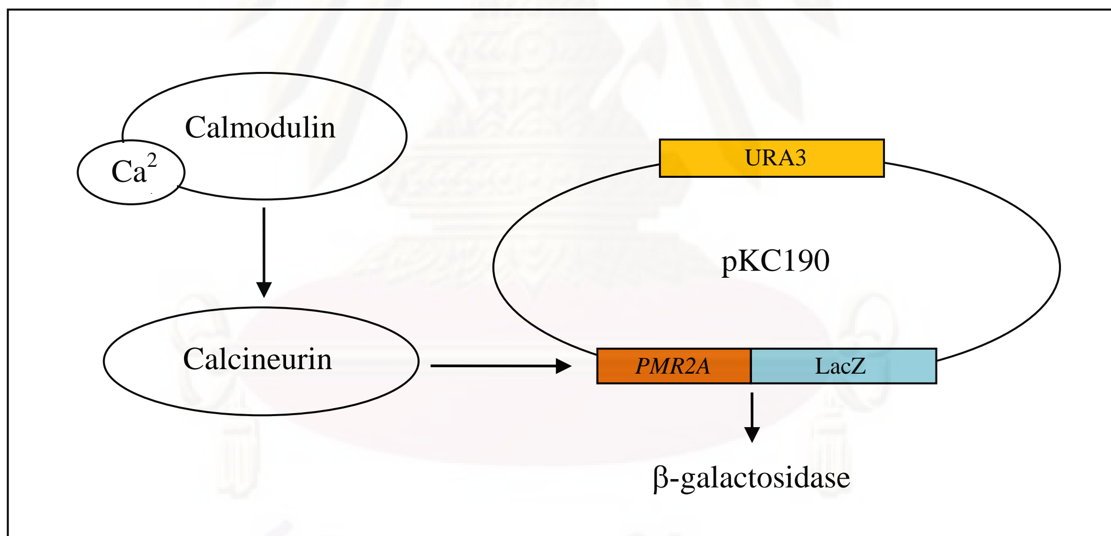

Figure 3-8 The component of a pKC190 plasmid which carrying *PMR2A*-lacZ reporter gene of β-galactosidase (Cunningham and Fink, 1996).

From the experiment, the mutant strain was cultured in YPAUD containing 150 mM $CaCl_2$ then incubated with and without Compound **A**. As shown in **Figure 3-9**, Compound **A** displayed no significant effect on β-galactosidase activity compared with control (YPAUD + 150 mM $CaCl_2$). This suggests that the cytosolic

Ca^{2+}-level is not influenced by the inhibitor and that Compound **A** inhibited the Ca^{2+}-signal mediated cell-cycle regulation at a step following elevation of the cytosolic Ca^{2+} concentration (Chanklan *et al*., 2008).

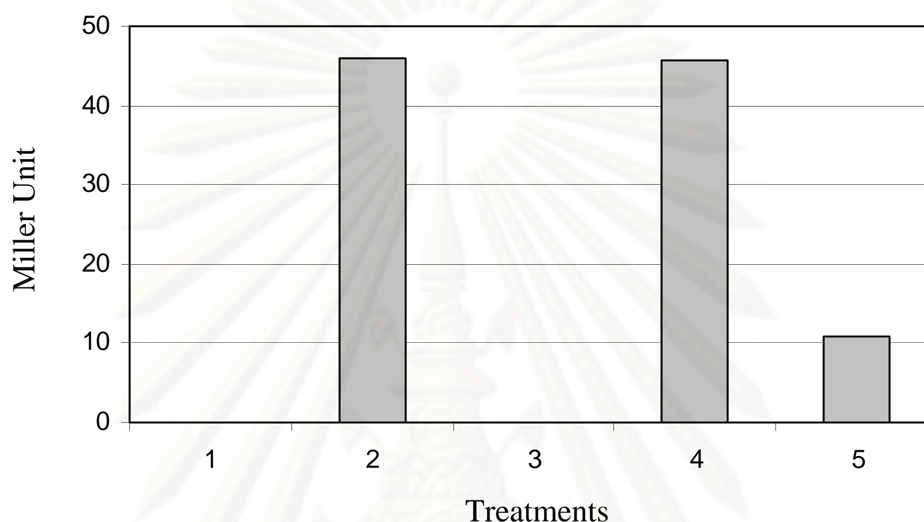

Figure 3-9 The effect of Compound **A** from *B. pandurata* on free intracellular Ca^{2+}-levels. The wild-type yeast (strain W303) was transformed with plasmid pKC190 carrying *PMR2A–lacZ,* grown in YPAUD medium at 30 ^{o}C for 4 hrs: **1**, YPAUD + 1.5% DMSO; **2**, YPAUD + 150 mM $CaCl_2$; **3**, YPAUD + 150 mM $CaCl_2$ + 500 nM FK506; **4,** YPAUD + 150 mM $CaCl_2$ + 2 mM Compound **A**; **5**, YPAUD + 150 mM $CaCl_2$ + 1 mM $MgCl_2$. (Wangkangwan *et al*., 2009).

3.6.1.2 Effect of Compound A on Cnb1 and Mpk1 (Wangkangwan *et al*., 2007)

From the assay results, Compound **A** had no effect on free intracellular Ca^{2+} in mutant yeast cells. Therefore, the next experiment aims to examine the effect of Compound **A** on Ca^{2+}-signal inhibitor by inhibiting the activities of Mpk1 and calcineurin protein.

The mutant strains of Δ*cnb1* and Δ*mpk1* (kindly supplied by Prof. Tockichi Miyakawa, Department of Molecular Biology, Graduate school of Advance Science and Matter, Hiroshima University, Japan) were subculture on YPUAD agar and incubated at 30 ^{o}C for 1-2 d. The colonies of the mutant strains were grown in

YPAUD broth and incubated at 30 °C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase was 2-7x10^7 cells/mL. The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with sterile water and separated at 7,500 rpm for 5 min (2 times). The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUD, the cells suspension was counted with haemacytometer and adjust to the final concentration of 5.0x10^6 cells/mL. The assay cell suspension 2 mL was transferred into each test tube, 200 mM of Compound **A** was added to the final concentration of 2 mM, 0.5% DMSO and 500 nM of FK506 were added as a negative and positive control. The assays were incubated at 30 °C, the growth of yeast cells were monitored every 2 hrs up to 14 hrs by counting the cells with haemacytometer.

In a previous report, the Ca^{2+}-signals activated two parallel pathways; Calcineurin and MAPK pathway. The deletion of either gene had no effect on cell viability. When the double disruptant of the two genes (*CNB1* and *MPK1*), the mutant showed lethality to the cell (Nakamura *et al*., 1996). This report mainly examined Calcineurin or Mpk1 which was a possible target molecule of Compound **A** by using the synthetic lethality test as a model. Synthetic lethality arises when a combination of mutations in two or more genes leads to cell death, whereas a mutation in only one of these genes does not, and by itself is said to be viable (Tucker and Fields, 2003). In a synthetic lethal genetic screens, it is necessary to begin with a mutation that does not kill the cell, although may confer a phenotype (for example, slow growth), and then systematically test other mutations at additional loci to determine which confer lethality. If Compound **A** could inhibit one of the two genes in the single disruptants of *cnb1* or *mpk* (Δc*nb1* or Δ*mpk1*), the mutant cells would grow. From the experiment, the cells Δc*nb1* or Δ*mpk1* could be grown very well in the medium in the presence of Compound **A**, whereas Δ*mpk1* strain could not grow in the presence of FK506, a potent calcineurin inhibitor. The results suggested that Mpk1 and calcineurin are not the target molecule of Compound **A** (Wangkangwan *et al*., 2009).

3.6.1.3 Effect of Compound A on Δ*zds1* overexpress *mck1* (Wangkangwan *et al.*, 2007)

The mutant strain of Δ*zds1* was subcultured on SC agar lacking uracil and incubated at 30 °C for 1-2 d. The colonies of mutant strain was grown in SC medium lacking uracil and incubated at 30 °C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase was 2-5x10^7 cells/mL. The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The cell precipitate was collected and washed with SG (with 2% galactose) and separated at 7,500 rpm for 5 min. The supernatant was discarded and the precipitated was resuspended with 5 mL SC medium (with 1% raffinose) lacking uracil, the cells suspension were counted with haemacytometer and adjusted to the final concentration of 1.0x10^7 cells/mL. The assay cell suspension 2 mL was transferred into each test tube, 600 mM of Compound **A** was added to the final concentration of 3 mM, 0.5% DMSO and 500 nM FK506 were added as a negative and positive control. Then the treatments were incubated at 30 °C, for 30 min. After that times 20% (w/v) galactose was added to the final concentration of 1% except for the treatment of negative control. The assays were incubated at 30 °C, the growth of yeast cells were monitored every 12 hrs up to 72 hrs by counting the cells with haemacytometer.

This examination was carried out to search for the next target molecule in the calcineurin or the Mpk1 branch of the Ca^{2+}-signaling pathways whether Compound **A** inhibited. The activation of one of these branches by overexpression of Mpk1, Mck1 or Cmp2ΔC, a hyperactived form of the calcineurin catalytic subunit, (Garrett-Engele, *et al.*, 1995) on a Δ*zds1* background leads to polarized bud growth and G2 delay in mutant yeast cells (Mizunuma *et al.*, 2001). If a mutation located at the downstream of the activated pathway, the physiological effects could not be induced by overexpression due to the interruption of this signal. On the basis of this assumption, the plasmid containing *MPK1* (pGAL::MPK1), *MCK1* (pGAL::MCK1) or *CMP2ΔC* (pGAL::CMPΔ2C) placed under the control of the galactose-inducible *GAL1* promoter was introduced into the Δ*zds1* strain (Mizunuma, *et al.*, 2001). Cells with overproduction grew normally in raffinose medium (in which the *GAL1* promoter is turned off), whereas in galactose medium (in which the *GAL1* promoter is turned on)

cell growth was severely inhibited and G2-delayed cells accumulated. However, the overexpression of Mpk1, Mck1 and Cmp2ΔC induced the physiological changes by Compound **A**. This suggested that Compound **A** inhibited the Ca^{2+}-signal at a step after the activation of downstream of the Mpk1, Mck1 and calcineurin (Wangkangwan *et al*., 2007).

3.6.1.4 Effect of Compound A on Swe1 (Suksawatamnuay *et al*., 2009)

The mutant strain of YRC2 (*trp1 leu2 ade2 ura3 his3 can1-1 TRP1 swe1::GAL-SWE1-HA::LEU2 TRP1 syr1::HIS3 pdr1::hisG-ura3-hisG pdr3::hisG-URA3-hisG*) was subcultured on YPUAD agar and incubated at 30 ^{o}C for 1-2 d. The colonies of mutant strain were grown in YPAUD broth and incubated at 30 ^{o}C with shaking at 200 rpm for 18-24 h until the concentration of cells in early log phase was close to 0.5-1x10^{7} cells/mL. The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with YPAUR and separated at 7,500 rpm for 5 min (2 times). The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUR, the cell suspension was counted with haemacytometer and adjusted to the final concentration of 5.0x10^{6} cells/mL. The assay cell suspension 2 mL was transferred into each test tube, 200 mM of Compound **A** was added to final concentration of 2 mM, 0.5% DMSO and 1 μM Radicicol were added as a negative and positive control. Then the treatments were incubated at 30 ^{o}C, for 30 min. After that time, 20% (w/v) galactose was added to the final concentration of 1% except the treatment of negative control. The assays were incubated at 30 ^{o}C, the growth of yeast cells were monitored every 4 hrs up to 24 hrs by counting the cells with haemacytometer.

Ca^{2+}-induced physiological consequences such as growth inhibition, cell-cycle arrest, and polarized bud growth are due to transcriptional activation of the *SWE1* gene. Using genetic analysis, Compound **A** could be suppressed the analogous effects elicited by expressing a constitutive active same variant of the calcineurin catalytic subunit (Cmp2ΔC), suggesting that Compound **A** acted at the step downstream of calcineurin. The overexpressed *SWE1* yeast strain displayed the phenotype analogous to that of the Ca^{2+} hyperactivation. From the experiment, Compound **A** suppressed the

effects of *SWE1* overexpression. It would be suggested that it inhibited the Ca^{2+}-signals at Swe1 protein, one of the key components of the Ca^{2+}-signaling pathway.

3.6.2 Anti-inflammatory activity on EPP-induced ear edema in rat

All bioactive compounds that exhibited strong positive results on the yeast-based assay were tested for anti-inflammatory activity on EPP-induced ear edema in rat. The compounds were suspended in 5% Tween-80 and induced by topical application of EPP at a dose 1 mg/20µL/ear to the inner and outer surfaces of both ears by means of an automatic microliter pipette (Brattsand, *et al.*, 1982). Test drugs were applied topically in volumes of 20 µL just before the irritant. The control (acetone) received vehicle only. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear were measured by vernier calipers. The percent inhibition of the edema formation of the test substances was calculated as below.

$$\% \text{ Inhibition} = \frac{(C-T) \times 100}{C}$$

C = edema thickness of control rat T = edema thickness of tested rat

The results of all bioactive compounds isolated from *B. pandurata* for anti-inflammatory activity on EPP-induced ear edema in rat are reported in **Table 3-12**.

Table 3-12 The effect of isolated compounds from *B. pandarata* on EPP-induced ear edema in rat.

Compounds	Dose (mg/ear)	Edema thickness		% Inhibition	
		30 min	**60 min**	**30 min**	**60 min**
Vehicle (acetone)	-	147 ± 12	210 ± 15	-	-
Phenylbutazone	1	57 ± 3***	117 ± 3*	61	44
Compound **A**	1	53 ± 12***	127 ± 34*	64	40
Compound **B**	1	30 ± 15***	103 ± 9*	80	51
Compound **C**	1	43 ± 15***	110 ± 20*	71	48

Ethyl phenylpropiolate (EPP)-induced rat ear edema model
Values are mean ± SEM (n = 3: just for first screening)
Significant from control * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Compound **B** displayed the highest activity on EPP-induced ear edema in rat at 30 min after EPP-induced, followed by Compounds **C** and **A** with % inhibition of 80, 71 and 64, respectively.

3.6.3 Anticancer activity of Compound A (Suksawatamnuay *et al*., 2009)

The MTT assay (Mosmann, 1983) was applied for the evaluation of cytotoxicity against HepG2, SW620, KATO III, A375, BT474, MDA-MB-231, HeLa, Ca-Ski, SiHa, THP-1, HL-60 and Jurkat cancer cell lines. Cells were plated in a 96-well microplate (100 µL/well at a density of $5x10^4$ to $1x10^5$ cells/well), and incubated at 37 ^oC under 5% CO_2 for 18-24 hrs. Fifty microliters of tested compounds mixed with culture media at various concentrations were added to the cell lines, which were incubated further for 4 d. Cell viability was determined by staining with MTT. The MTT stock solution (5 mg/mL) was prepared in PBS, which was diluted (1:10) with a culture media prior to use. After removing the culture medium, the diluted MTT solution (50 µL) was added to the adhesive cell, and plates were incubated at 37 ^oC under 5% CO_2 for 4 hrs. After that time, 100 µL of 0.04 N HCl in *iso*-propanol was added, then the tested plates were read on a microplate reader at 540 nm. Percentage of cell viability was calculated by the following equation.

$$\%\ \text{Viability} = \frac{(\text{OD test average-OD blank average}) \times 100}{\text{OD control cell average-OD blank average}}$$

OD test average = average absorbance of cells plus tested compound or DMSO

OD control cell average = average absorbance of cells

OD blank average = average absorbance of culture media without serum

The MTT assay (Mosmann, 1983) was applied for the evaluation of cytotoxicity against HepG2, SW620, Kato III, A375, BT474 and Jurkat cancer cell lines. From the report of Suksawatanuay *et al*., (2009) six human cancer cell lines were treated with Compound **A** at various concentrations for 4 d and measured the cells viability by MTT assay. Among them, Jukat, A375 and Kato III showed

sensitivity to Compound **A** with IC_{50} value of 56, 67 and 86 μM, respectively (Data not shown).

3.7 Conclusion

Compound (**A**) 5-hydroxy-7-methoxyflavanone, (**B**) 7-hydroxy-5-methoxy-flavanone and (**C**) 2,6-dihydroxy-4-methoxychalcone were isolated from *B. pandurata* The target molecule of the isolated bioactive compounds, (**A**) in the Ca^{2+}-signaling pathway in yeast *S. cerevisiae* Swe1p, the protein kinase which is ortholog to Wee1 in human (Asano *et al*., 2005) and the Compound **A** was also exhibited the cytoxicity on Jukat, A375 and Kato III human cancer cell lines with IC_{50} value of 56, 67 and 86 μM, respectively. Compound **A** showed partially in anti-inflammatory activity on carrageenin-induced paw edema in rat with % inhibition of 43.71.

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CHAPTER IV

Bioactive flavonoids from *Kaempferia parviflora* Wall. Ex. Baker

4.1 Literature reviews of *K. parviflora*

4.1.1 Botanical description

K. parviflora Wall. Ex. Baker is a member of Zingiberaceae family (ginger family), locally known in Thailand as Black Galingale, Krachaidam, Krachaidum or Kra-chai-dam. It is very popular for health promotion. The rhizomes of this plant, well-known as Thai ginseng have been used as traditional medicine for various medicinal purposes including a tonic for rectifying male impotence, body pains and gastrointestinal disorders. The characteristics of this genus as: fleshy rhizomes, tuberous; roots often bearing small tubers (**Figure 4-1 C)**. Pseudostem short or obsolete (**Figure 4-1 A**). Leaves 1 to few; ligule usually small or absent; petiole short; leaf blade suborbicular to filiform, sometimes variegated or abaxially purple. Inflorescences terminal on pseudostems or on separate shoots arising from rhizomes (when appearing before pseudostems), capitate, spirally few to many flowers; bracts 1-flowered; bracteoles small, apically 2-lobed or sometimes 2-cleft to base (**Figure 4-1 B**). Calyx tubular, split on 1 side, apex unequally 2-or 3-toothed. Corolla tube equaling or much longer than calyx; lobes spreading or reflexed, lanceolate, subequal. Lateral staminodes petaloid. Labellum usually white or lilac, sometimes marked with different color near base, showy, apically 2-lobed to 2-cleft to base. Filament very short or absent; connective extended into crest exserted from throat of flower, entire or 2-cleft. Ovary 3-loculed. Capsule globose or ellipsoid; pericarp thin. Seeds subglobose to ellipsoid; aril lacerate (Wu *et al*., 1981; http://www.zipcodezoo.com/Plant/K/Kaempferia_parviflora/# Taxonomy).

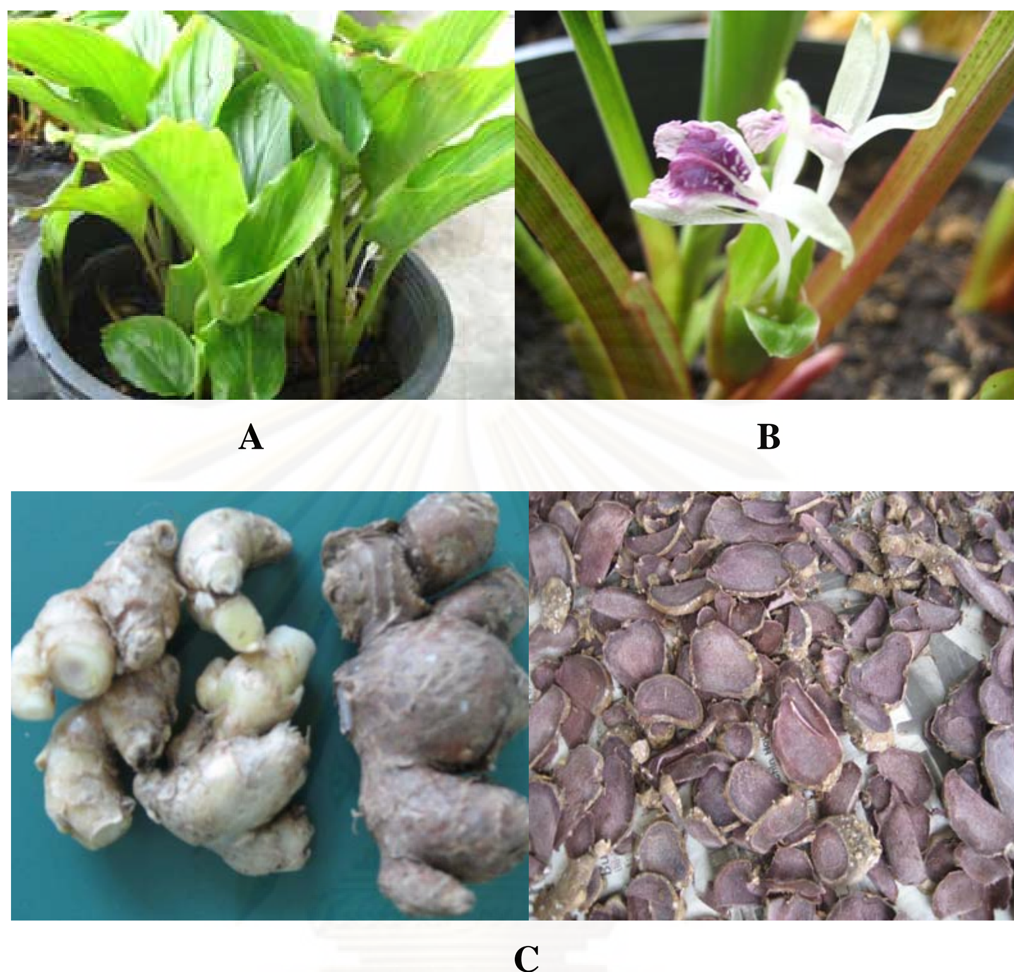


Figure 4-1 *Kaempferia parviflora* Wall. Ex. Baker

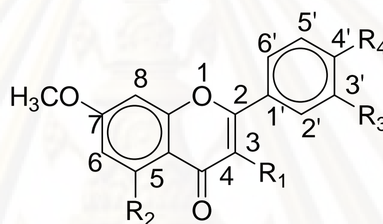
A: Whole plant B: Flowers C: Rhizomes

4.1.2 Chemical constituents and biological activities

A number of reports concerning chemical constituents of *K. parviflora* rhizome were investigated. Nine flavonoids were isolated from *K. parviflora*: 5-hydroxy-3,7-dimethoxyflavone (**4-1**, 0.2%), 5-hydroxy-7-methoxyflavone (**4-2**, 1.3%), 5-hydroxy-3,7,4′-trimethoxyflavone (**4-3**, 0.08%), 5-hydroxy-7,4′-dimethoxyflavone (**4-4**, 0.3%), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**4-5**, 1.5%), 3,5,7-trimethoxyflavone (**4-6**, 0.14%), 3,5,7,4′-tetramethoxyflavone (**4-7**, 0.6%), 5,7,4′-trimethoxyflavone (**4-8**, 1.6%) and 5,7,3′,4′-tetramethoxyflavone (**4-9**, 1.01%) **(Table 4-1)**. Among these

mentioned flavonoids, compounds **4-8** and **4-9** exhibited antiplasmodial activity against *Plasmodium falciparum*. Compounds **4-7** and **4-8** possessed anticandidal activity against *Candida albicans*, and also showed mild antimycobacterial activity. On the other hand, these isolated flavonoids possessed no cytotoxicity against KB, BC and NCi-H187 cell lines, and this cytotoxic information suggests that the rhizomes of *K. parviflora* may be safe when using as an ingredient in traditional medicine (Yenjai *et al*., 2004).

Table 4-1 Isoflavonoids isolated from the rhizome of *K. parviflora*



Compound	R_1	R_2	R_3	R_4
4-1	OCH_3	OH	H	H
4-2	H	OH	H	H
4-3	OCH_3	OH	H	OCH_3
4-4	H	OH	H	OCH_3
4-5	OCH_3	OH	OCH_3	OCH_3
4-6	OCH_3	OCH_3	H	H
4-7	OCH_3	OCH_3	H	OCH_3
4-8	H	OCH_3	H	OCH_3
4-9	H	OCH_3	OCH_3	OCH_3
4-10	OCH_3	OCH_3	OCH_3	OCH_3
4-11	OCH_3	H	H	H

The ethanolic extract of *K. parviflora* possessed an anti-gastric ulcer effect, which was related partly to a prevention of gastric mucus secretion and unrelated to the inhibition of gastric acid secretion (Rujjanawate *et al*., 2005).

The effects of flavone derivatives derived from this particular species on P-glycoprotein function were reported in 2006. Among six flavonoids tested, compound (**4-10**) was the most potent to increase the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells in a concentration dependent manner (Patanasethanont *et al*., 2006).

The inhibitory potency of the ethanolic extract from *K. parviflora* rhizome for MRP function was greater than that of the aqueous extract. Among six flavone derivatives isolated, 5,7-dimethoxyflavone (**4-11**) exhibited a maximal stimulatory effect on the accumulation of doxorubicin in A549 cells. The accumulation of doxorubicin was increased by four flavone derivatives without 5-hydroxy group, but not by the other two flavone derivatives with 5-hydroxy group. In addition, compounds **4-10** and **4-11** decreased resistance to doxorubicin in A549 cells. These findings indicate that the extracts and flavone derivatives from the rhizome of *K. parviflora* suppress MRP function, and therefore may be useful as modulators of multidrug resistance in cancer cells (Patanasethanon *et al*., 2007).

In 2008, the ethanolic extract of *K. parviflora* (5-100 mg/mL) was reported to suppress HL-60 cell growth and decrease cell viability in dose- and time-dependent manner. Apoptotic cell death was demonstrated by changes in cell morphology, externalization of phosphatidylserine on the cell surface, loss in mitochondrial transmembrane potential and activation of caspase 3 (Banjerdpongchai *et al*., 2008).

Seven methoxyflavones were isolated from the hexane fraction of *K. parviflora* and were tested for their anti-inflammatory effects. Among those isolated compounds, 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**4-5**) exhibited the highest activity against NO release with an IC_{50} value of 16.1 μM, followed by 5-hydroxy-7,4′-dimethoxyflavone (**4-4**) (IC_{50} 24.5 μM) and 5-hydroxy-3,7,4′-trimethoxyflavone (**4-3**) (IC_{50} 30.6 μM). Compound **5** was also tested on LPS-induced prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) release from

RAW264.7 cells and revealed appreciable inhibitory effect on PGE_2 release (IC_{50} = 16.3 μM), but inactive on TNF-α (IC_{50} > 100 μM) (Tewtrakul *et al.*, 2008).

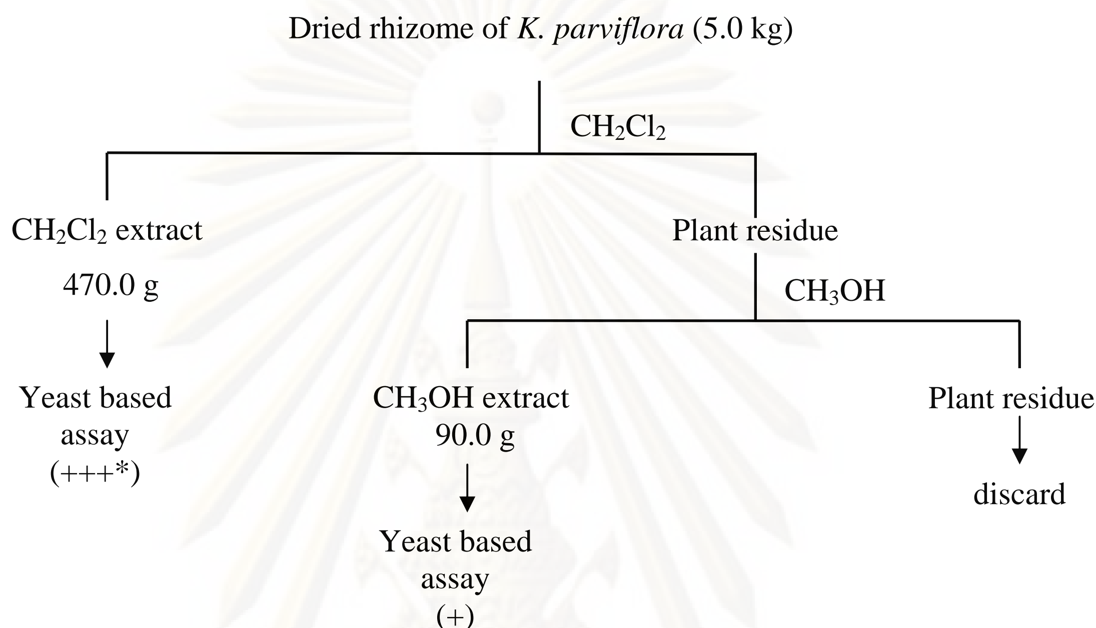
The anti-inflammatory mechanism of *K. parviflora* extract and compound **4-5** against inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expressions. The ethanolic extract of *K. parviflora* markedly inhibited PGE_2 release with IC_{50} value of 9.2 μg/mL. This plant extract and **4-5** also suppressed mRNA expression of iNOS in dose-dependent manners, whereas COX-2 mRNA expression was partly affected. According to the *in vivo* study, the chloroform and hexane fractions decreased rat paw edema greater than the ethanolic, ethyl acetate and water fractions (Sae-wong *et al.*, 2009).

The compounds isolated from the rhizomes of *K. parviflora* were examined for their inhibitory activities against nitric oxide (NO) production. Compound **4-5** exhibited the highest activity against NO inhibitory effect with an IC_{50} value of 16.1 μM, followed by **4-4** (IC_{50} = 24.5 μM) and **4-3** (IC_{50} = 30.6 μM), whereas other compounds possessed moderate or weak activity. The NO inhibition activity of **4-5** (IC_{50} = 16.1 μM) was three times weaker than that of caffeic acid phenethylester (CAPE, IC_{50} = 5.6 μM), an NF-κB inhibitor, but four times higher than that of L-nitroarginine (L-NA, IC_{50} = 61.8 μM), a nitric oxide synthase inhibitor (Tewtrakul *et al.*, 2009). For the tests on PGE_2 and TNF-α production, **4-5** exhibited a potent inhibitory effect on PGE_2 production (IC_{50} = 16.3 μM), but a mild effect on TNF-α (IC_{50} > 100 μM).

4.2 Extraction and activity guided fractionation of *K. parviflora*

The fresh rhizomes of *K. parviflora* were purchased from Thai-market, Patumthani in 2007. The voucher specimen (BKF 152278) has been deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Chatuchak, Bangkok, Thailand. The fresh rhizomes were sliced and dried in open air for a few days and powdered to small pieces with an electrical blender. The dried powder (5 kg) was extracted with CH_2Cl_2 using soxhlet apparatus. The plant residue was similarly extracted with CH_3OH. The extracts were concentrated by rotary evaporator under

reduced pressure to give brown crude 470.0 g (9.40 %, wt by wt) of CH_2Cl_2 extract and 90.0 g (1.80 %, wt by wt) of CH_3OH extract. Both extracts were preliminarily assayed with yeast-based assay as shown in **Scheme 4-1.**



Scheme 4-1 The extraction of *K. parviflora* rhizomes and preliminary yeast-based assay

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

* = positive (ring like growth)

4.3 Fractionation of CH_2Cl_2 extract of *K. parviflora* and yeast-based assay

According to the biological activity on the yeast-based assay, the CH_2Cl_2 extract gave strong positive result on yeast growth. Thus, the CH_2Cl_2 extract (300.0 g) was subjected to silica gel quick column with sinter glass frit, firstly eluting with *n*-hexane. The *n*-hexane extracts were combined and evaporated to dryness. The column was then exhaustively eluted with the solvent with increasing polarity by mixing with EtOAc and CH_3OH, successively. The fractions were collected and combined according to TLC furnishing 5 fractions (**Table 4-2**). Each fraction was assayed using the yeast-based assay.

Table 4-2 The separation of the CH_2Cl_2 extract of *K. parviflora* by silica gel quick column

Fraction	Solvent system	Remarks	Yeast-based assay	Weights (g)
KPA1	100% Hexane-40% EtOAc-Hex	yellow solid	++++	30.50
KPA2	60-80% EtOAc-Hex	yellow solid	+++	6.85
KPA3	60-80% EtOAc-Hex	yellow solid	+++*	20.63
KPA4	100% EtOAc-5%MeOH	yellow solid	+++*	70.29
KPA5	10% MeOH	brownish solid	-	87.48

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

* = positive (ring like growth)

4.3.1 Isolation and purification

According to the biological activity on the yeast-based assay, the positive fractions (**KPA1-KPA4**) were isolated using silica gel column. Firstly, the yellow solid of **KPA1** fraction (29.0 g) was chromatographed on silica gel column eluting with *n*-hexane and accomplished with a gradient of *n*-hexane and EtOAc. The fractions were collected and combined according to TLC furnishing 5 fractions (**Table 4-3**). Each fraction was assayed using the yeast-based assay. The yellow needle of **KPA1/1-A** was crystallized from fraction **KPA1/1** (**Scheme 4-2**) which gave a single spot of Compound **D** on TLC plate when developing with CH_2Cl_2. Compound **D** showed strong Ca^{2+}-signal inhibition (++++) on the Δ*zds1* mutant yeast growth.

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Table 4-3 The reseparation of **KPA1** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weights (g)
KPA1/1-A	10-20% EtOAc-Hex	yellow needle	++++	0.67
KPA1/1-B		yellow solid	++	2.0
KPA1/2	10-20% EtOAc-Hex	yellow solid	+++	2.63
KPA1/3	20-40% EtOAc-Hex	yellow solid	+++	10.30
KPA1/4	20-40% EtOAc-Hex	yellow solid	++	12.52

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

According to the TLC and the yeast based-assay results, the yellow solid of **KPA1/2** and **KPA1/3** (12.93 g) also revealed strong Ca^{2+}-signaling inhibition (+++). Upon recrystalization with Hex-EtOAc, the cream solid of Compound **E** (0. 53 g) and yellow crystal of **F** (2.48 g) were obtained, respectively. In addition, the yellow solid of **KPA1/4** exhibited a positive test on the yeast-based assay. Thus it was recrystalized with EtOAc-Hex to yield Compounds **D** (3.57 g) and **E** (0.49 g) **Scheme 4-2**.

KPA2 fraction also reveals a positive result on the yeast-based assay. Thus, the yellow solid of this fraction (6.0 g) was reseparated by silica gel column eluting with a gradient of EtOAc-Hex. The fractions were collected and combined according to TLC furnishing 4 fractions (**Table 4-4**). Each fraction was assayed using the yeast-based assay. The yellow needle of **KPA2/1-A** was recrystalized with EtOAc-Hex furnishing Compound **D** (1.16 g) **Scheme 4-2**.

Table 4-4 The separation of **KPA2** by silica gel column

Fraction	Solvent system	Remarks	Yeast-based assay	Weights (g)
KPA2/1-A	60% EtOAc-Hex	yellow needle	++++	1.16
KPA2/1-B		yellow solid	+++	1.32
KPA2/2	60-80% EtOAc-Hex	brownish solid	-	1.73
KPA2/3	80% EtOAc-Hex	cream solid	-	1.25

\+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

The yellow solid of **KPA2/1-B** was recrystalized with EtOAc-Hex to give the yellow needle of Compound **D** (0.87 g) **Scheme 4-2**.

Next, the yellow solid of **KPA3** (15.0 g) which gave a positive test was chromatographed on silica gel column eluting with gradient solvent between EtOAc-Hex. The TLC results of each fraction were collected into 4 fractions (**Table 4-5**). Each fraction was assayed using the yeast-based assay. The yellow crystal of **KPA3/1-A** was recrystalized yielding Compound **F** (0.97 g) **Scheme 4-2**.

Table 4-5 The separation of **KPA3** by silica gel column

Fractions	Solvent system	Remarks	Yeast based-assay	Weights (g)
KPA3/1-A	60% EtOAc-Hex	yellow crystal	+	0.97
KPA3/1-B		brownish solid	-	2.30
KPA3/2	60-80% EtOAc-Hex	brownish solid	-	5.55
KPA3/3	80% EtOAc-Hex	brownish solid	++*	4.69

+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

* = positive (ring like growth)

Finally, the yellow solid of **KPA4** (20.0 g) was separated by silica gel column eluting with EtOAc and CH_3OH. The fractions were collected and combined according to TLC resulting 3 fractions. The results of the separation are shown in **Table 4-6**. Each fraction was assayed using the yeast-based assay. The brownish solid of **KPA4/1-A** was recrystalized with CH_3OH to gain the white solid of Compound **G** (6.85 g).

Table 4-6 The separation of **KPA4** by silica gel column

Fractions	Solvent system	Remarks	Yeast-based assay	Weights (g)
KPA4/1	100% EtOAc	Brownish solid	-	1.13
KPA4/1-A		White solid	+++*	6.85
KPA4/2	5% MeOH-EtOAc	Brownish solid	-	1.38
KPA4/3	5-10% MeOH-EtOAc	Brownish solid	-	1.49
		Yellow solid	-	8.20

+ = positive (growth) - = negative (no growth)

+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)

* = positive (ring like growth)

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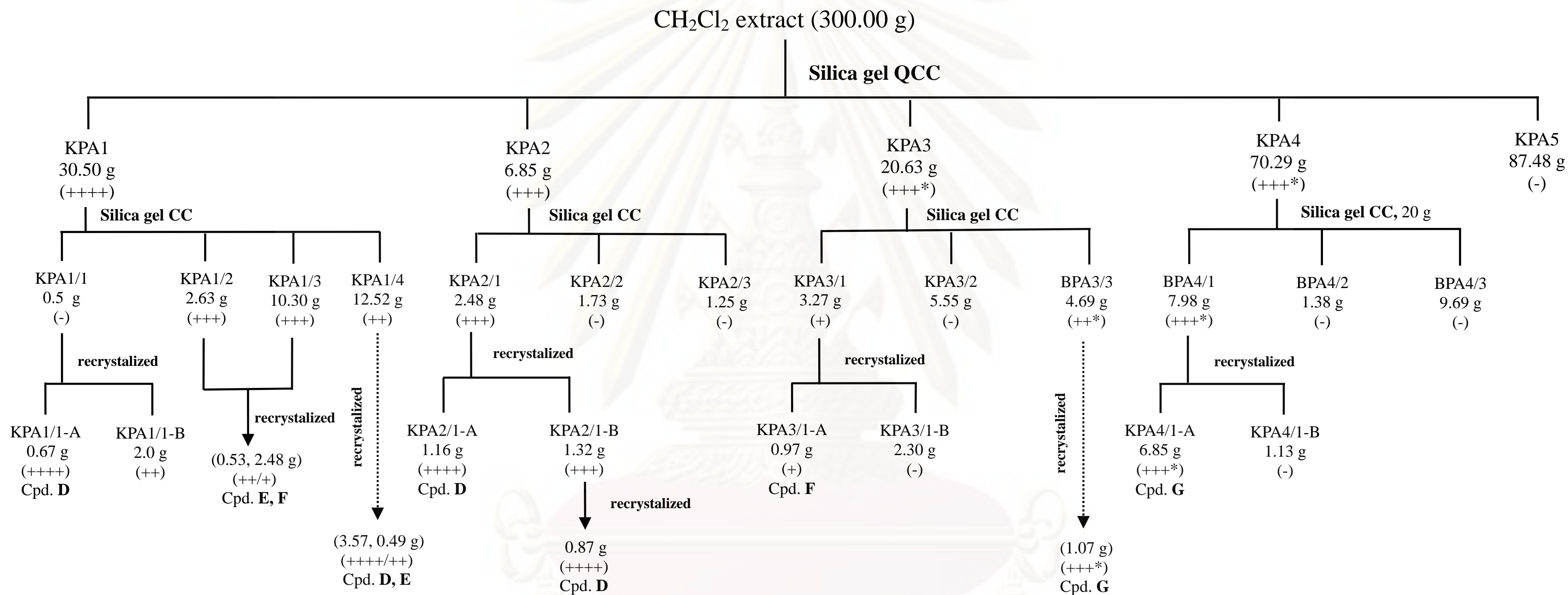
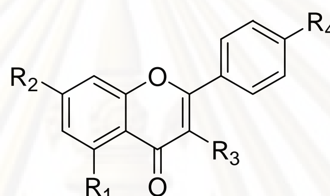


Scheme 4-2 The separation of the CH_2Cl_2 extract of *K. parviflora* by silica gel quick column and column chromatographs

+ = positive (growth)
- = negative (no growth)
* = positive (ring like growth)
C = clear zone (cytotoxic/suspect)
+++, ++, + = positive (compared to FK506 used as a positive control which gave +++)

4.4 Structural elucidation of the isolated compounds

The fresh rhizomes of *K. parviflora* were extracted with CH_2Cl_2 and the plant extract was further fractionated using solvents with increasing polarity to obtain 4 known flavonoids: Compounds **D**, **E, F** and **G**. The structural elucidation of these compounds was described below. The structures of three flavonoid compounds are shown in **Figure 4-2**.



D R_1 = OH, R_2 = R_3 = OCH_3, R_4 = H

E R_1 = OH, R_2 = OCH_3, R_3 = R_4 = H

F R_1 = OH, R_2 = R_3 = R_4 = OCH_3

G R_1 = R_2 = OCH_3, R_3 = R_4 = H

Figure 4-2 Structure of flavonoid compounds obtained from *K. parviflora*

4.4.1 Structural elucidation of Compound D

Compound **D** was obtained as yellow solid. The molecular formula was suggested as $C_{17}H_{14}O_5$ and confirmed by the ^{1}H-NMR spectral data. The ^{1}H-NMR spectrum (**Figure 4-3**) clearly showed 2 sets of methoxy group resonating at δ_H 3.87 (3H, s) and 3.88 (3H, s) while a methylene group appears at δ_H 6.37 (1H, d, J = 2.0 Hz), 6.46, (1H, d, J = 2.4 Hz), aromatic protons at δ_H 7.52 (3H, m), 8.07 (2H, m) and hydroxyl group at δ_H 12.59 (1H, s). The ^{1}H-NMR spectral data of Compound **D** showed similar pattern to that of 5-hydroxy-3,7-dimethoxyflavone (**4-1**) (**Table 4-7**).

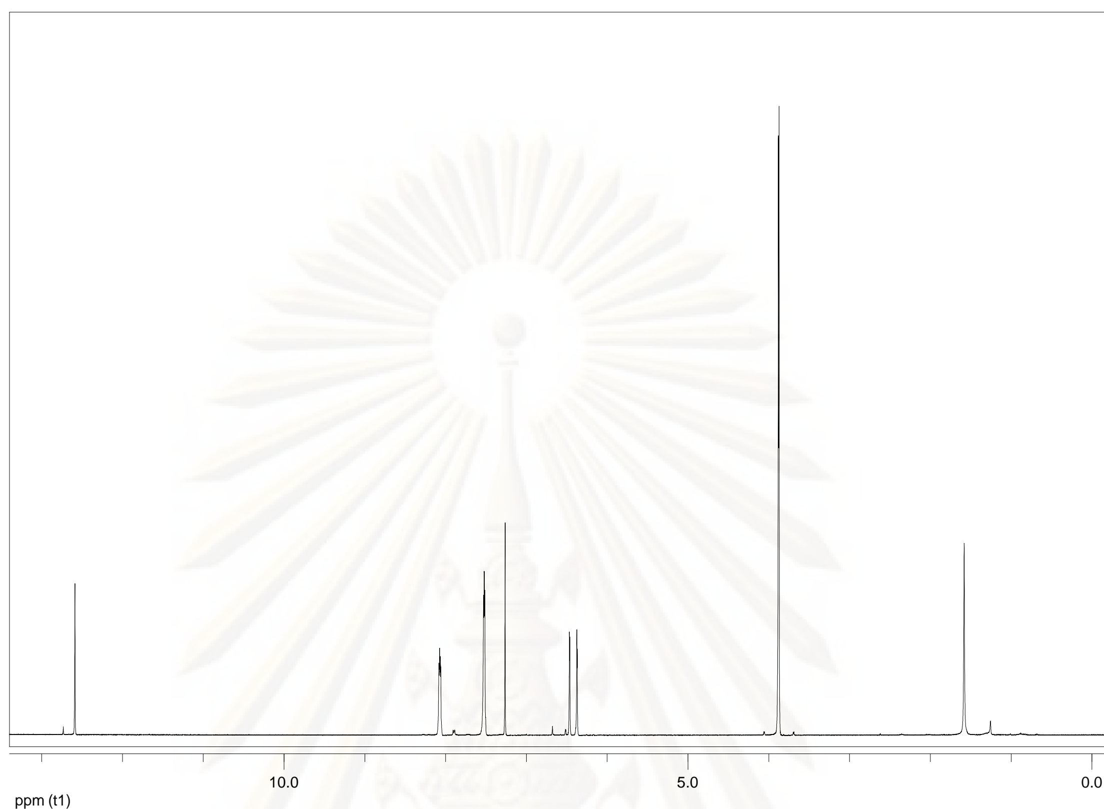


Figure 4-3 The ^{1}H-NMR ($CDCl_3$) spectrum of Compound **D**

Table 4-7 The comparison of the ^{1}H-NMR data between 5-hydroxy-3,7-dimethoxyflavone and Compound **D**

Position	5-hydroxy-3,7-dimethoxyflavone	Compound D
	δ_H (multiplicity *J* in Hz)*	δ_H (multiplicity *J* in Hz)
6	6.28, d, *J* = 2.0	6.37, d, *J* = 2.0
8	6.38, d, *J* = 2.0	6.46, d, *J* = 2.4
2′, 3′, 4′, 5′, 6′	7.48, 8.03, m	7.52, 8.07, m
5-OH	12.55, s	12.59, s
7-OCH_3	3.82, 3.85, s	3.87, 3.88, s

*Sutthanut, *et al.*, 2007

4.4.2 Structural elucidation of Compound E

Compound **E** was isolated as yellow crystal. The molecular formula was suggested as $C_{16}H_{12}O_4$, and confirmed by the ^{1}H-NMR spectral data. The ^{1}H-NMR spectrum (**Figure 4-4**) clearly shows the methoxy group at δ_H 3.93 (3H, s) while a methylene group appears at δ_H 6.68 (1H, s), 6.39, (1H, d, J = 2.4 Hz) and 6.57, (1H, d, J = 2.0 Hz), aromatic protons at δ_H 7.53 (3H, m), 7.90 (2H, m) and hydroxyl group at δ_H 12.73 (1H, s). The ^{1}H-NMR spectral data of Compound **E** shows similar pattern to that of 5-hydroxy-7-methoxyflavone (**4-2**) (**Table 4-8**).

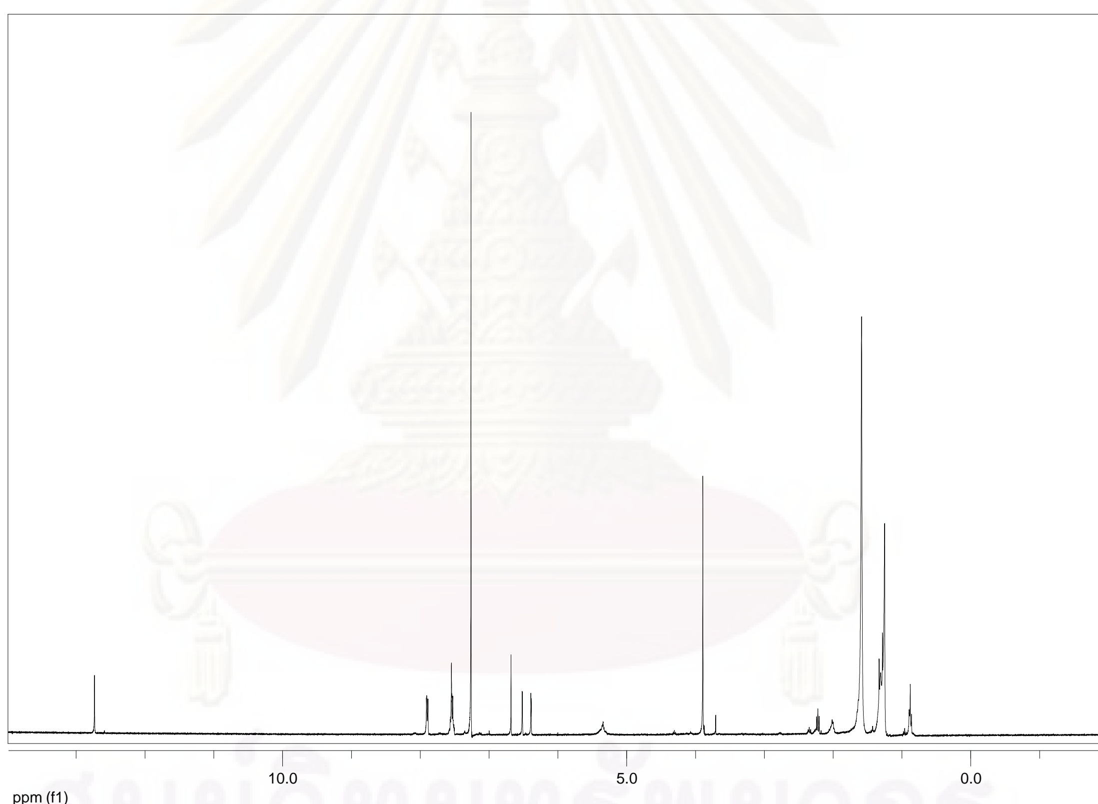


Figure 4-4 The ^{1}H NMR ($CDCl_3$) spectrum of Compound **E**

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Table 4-8 The comparison of the ^{1}H-NMR data between 5-hydroxy7-methoxy-flavone and Compound **E**

Position	5-hydroxy-7-methoxyflavone	Compound E
	δ_H (multiplicity *J* in Hz)*	δ_H (multiplicity *J* in Hz)
3	6.64, s	6.68, s
6	6.36, d, *J* = 2.0	6.39, d, *J* = 2.4
8	6.48, d, *J* = 2.0	6.52, d, *J* = 2.0
2′, 3′, 4′, 5′, 6′	7.52, 7.87, m	7.55, 7.90, m
5-OH	12.73, s	12.73, s
7-OCH_3	3.88, s	3.93, s

*Sutthanut, *et al.*, 2007

4.4.3 Structural elucidation of Compound F

Compound **F** was obtained as yellow crystal. The molecular formula was suggested as $C_{18}H_{16}O_6$ and confirmed by the ^{1}H-NMR spectrum data. The ^{1}H-NMR spectrum (**Figure 4-5**) clearly shows the methoxy group at δ_H 3.87 (3H, s) and 3.97 (3H, s) while a methylene group appears at δ_H 6.35 (1H, d, *J* = 2.0 Hz) and 6.46, (1H, d, *J* = 2.0 Hz), aromatic proton at δ_H 7.01 (3H, d, *J* = 9.0 Hz), 8.00 (2H, d, *J* = 9.0) and hydroxyl group at δ_H 12.65 (1H, s). The ^{1}H-NMR spectral data of Compound **F** was found very close to that of 5-hydroxy-3,7,4′-trimethoxyflavone (**4-3**) (**Table 4-9**).

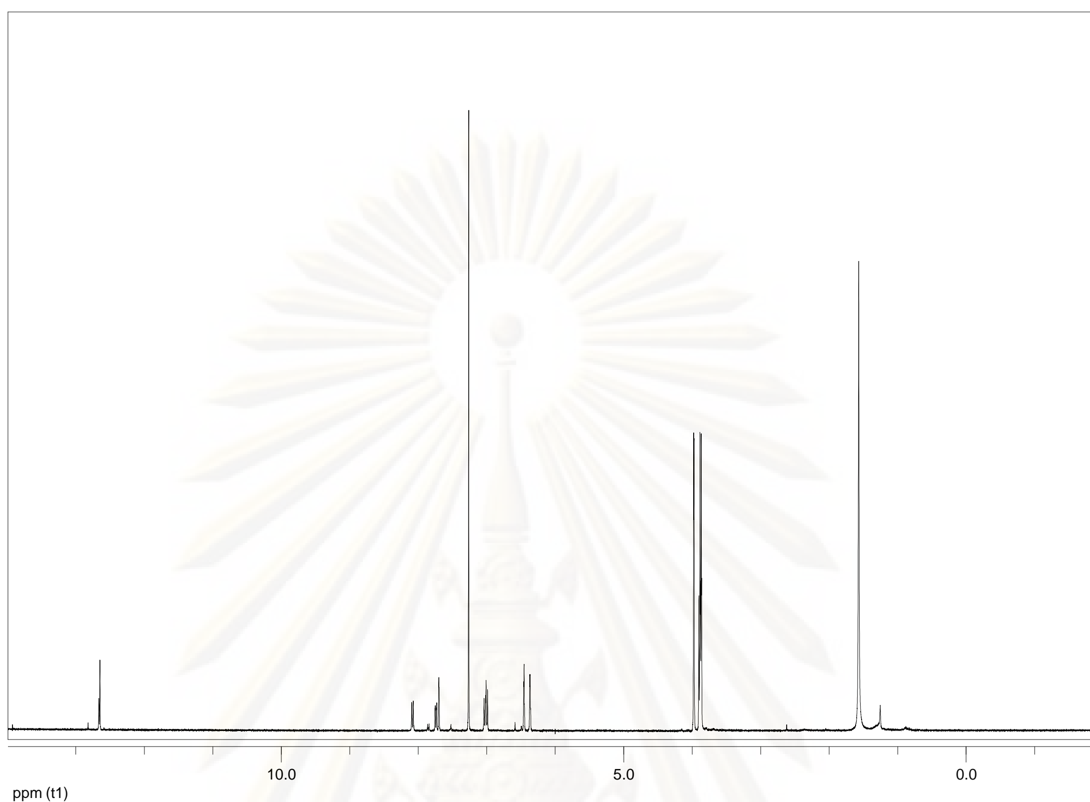

Figure 4-5 The ^{1}H NMR ($CDCl_3$) spectrum of Compound **F**

Table 4-9 The comparison of the ^{1}H-NMR data between 5-hydroxy-3,7,4′-trimethoxyflavone and Compound **F**

Position	**5-hydroxy-3,7,4′-trimethoxyflavone**	**Compound F**
	δ_H (multiplicity *J* in Hz)*	δ_H (multiplicity *J* in Hz)
6	6.32, d, *J* = 2.0	6.37, d, *J* = 2.0
8	6.42, d, *J* = 2.0	6.46, d, *J* = 2.0
2′, 3′, 5′, 6′	7.01, 8.06, d, J = 9.0	7.01, 8.08, d, J = 9
5-OH	12.65, s	12.66, s
7-OCH_3	3.86, 3.89, s	3.87, 3.88, 3.97, s

*Sutthanut, *et al.*, 2007

4.4.4 Structural elucidation of Compound G

Compound **G** was isolated as yellow crystal. The molecular formula was suggested as $C_{17}H_{14}O_4$, and confirmed by the ^{1}H-NMR spectrum data. The ^{1}H-NMR (**Figure 4-6**) clearly shows 2 sets of methoxy group resonating at δ_H 3.90 (3H, s) and 3.93 (3H, s) while a methylene group appears at δ_H 6.68 (1H, s), 6.38, (1H, d, J = 2.0 Hz) and 6.57, (1H, d, J = 2.0 Hz) and aromatic protons at δ_H 7.50 (3H, m), 7.88 (2H, m). The ^{1}H-NMR spectrum of Compound **G** was very similar to that of 5,7-dimethoxyflavone (**4-11**) (**Table 4-10**).

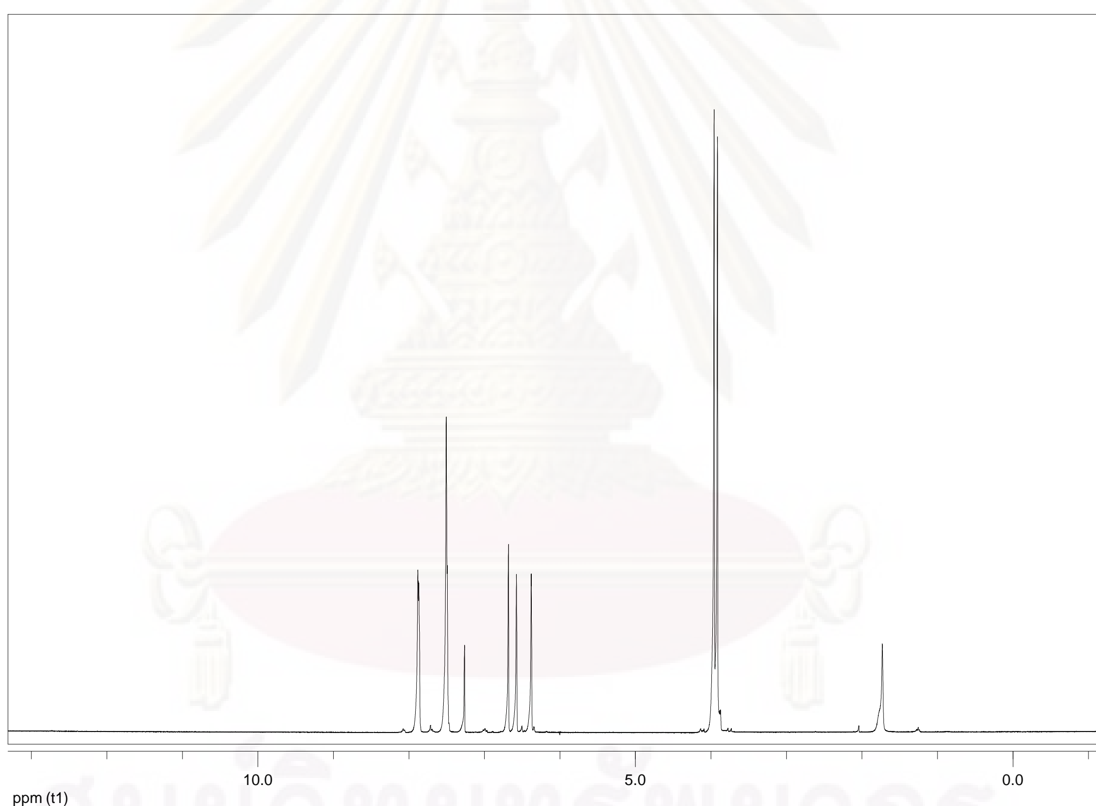

Figure 4-6 The ^{1}H NMR ($CDCl_3$) spectrum of Compound **G**

Table 4-10 The comparison of the ¹H-NMR data between 5,7-dimethoxyflavone and Compound **G**

Position	5,7-dimethoxyflavone	Compound G
	δ_H (multiplicity J in Hz)*	δ_H (multiplicity J in Hz)
3	6.67, s	6.68, s
6	6.37, d, J = 2.2	6.38, d, J = 2.0
8	6.56, d, J = 2.2	6.57, d, J = 2.0
2′, 3′, 4′, 5′, 6′	7.49,7.86, m	7.50,7.88, m
7-OCH_3	3.90, 3.94, s	3.91, 3.96, s

*Sutthanut, *et al.*, 2007

4.5 Biological activities of the isolated compounds on the yeast-based assay

The pure isolated compounds were diluted with DMSO at concentration of 500, 250, 125, 62.5 and 31.25 μM then the dilutions were assayed using the yeast-based assay. The activity assay was examined and scored, compared to the positive control (FK506) which showed a growth zone at or around the application spots.

The dose dependent effect of isolated compounds from *K. parviflora* on the growth of Δ*zds1* cells on YPAUD soft agar containing with 150 mM $CaCl_2$ were examined. From the experiment, Compound **G** showed clear zones of growth of Δ*zds1* mutant yeast cells at the concentration of 500 μM while the other compounds did not, This suggested that Compound **G** at highly concentration cause toxicity on the yeast growth (**Figure 4-7** and **Table 4-11**). Compound **D** could rescue the compromise growth at the dose-dependent concentration (**Figure 4-6 A**).

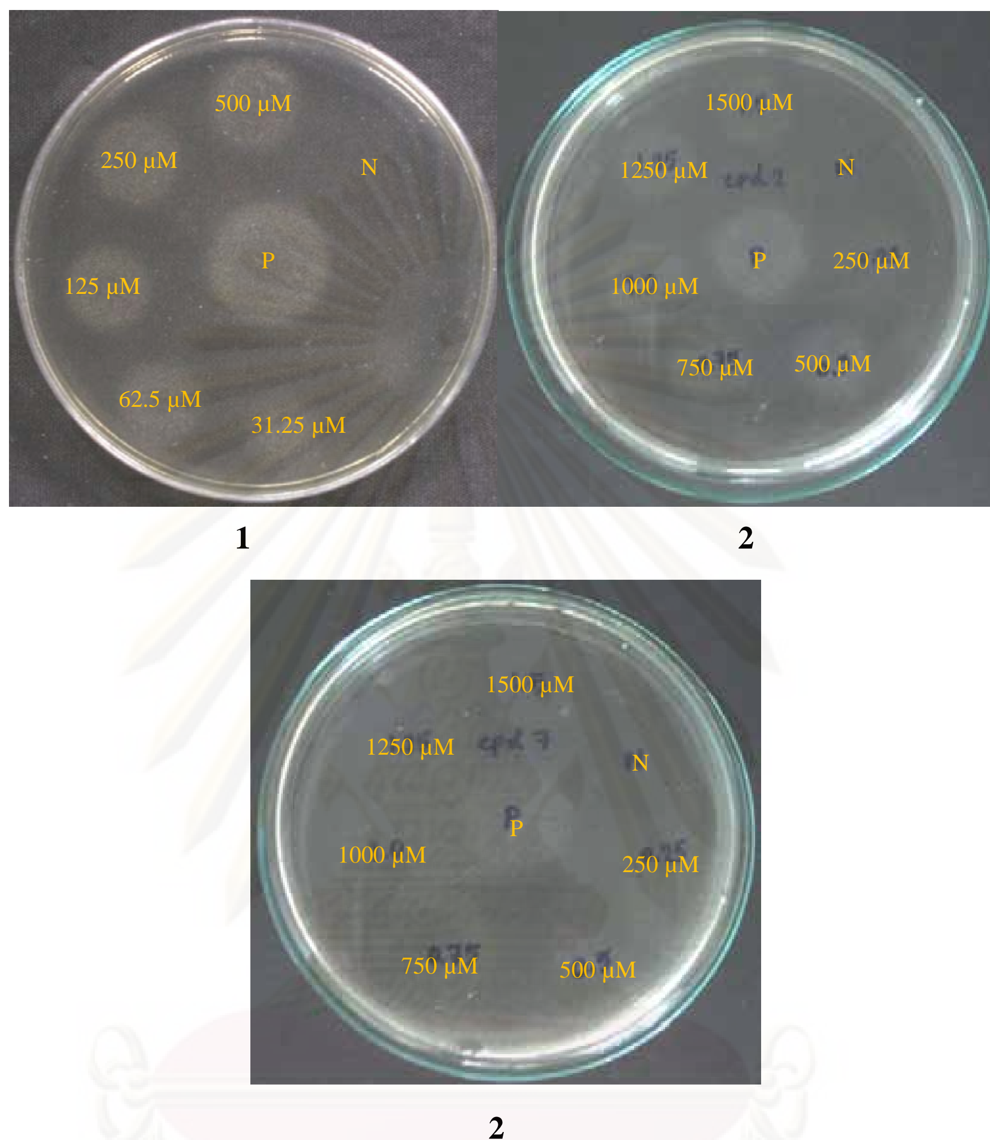

Figure 4-7 Dose dependent effect of isolated compounds from *K. parviflora* on the growth of Δ*zds1* cells on YPAUD soft agar containing 6.0×10^5 cells/mL indicator cells 150 mM $CaCl_2$ at 30 °C for 2 d. Various concentrations of isolated compounds: **1**; Compound **D** at 500, 250, 125, 62.5 and 31.25 µM, **2** and **3**; Compounds **E** and **G** at 1,500, 1,250, 1,125, 750 and 250 µM, whereas, P = 500 nM FK506 (a positive control), N = absolute ethanol (a negative control)

Table 4-11 The dose dependent effect of the isolated compounds from *K. parviflora* on the growth of Δ*zds1* cells on agar containing 150 mM $CaCl_2$*

Concentration (µM)	Isolated compounds			
	Compound **D**	Compound **E**	Compound **F**	Compound **G**
500	+++	++	-	+
250	+++	+	-	-
125	++	-	-	-
62.5	+	-	-	-
31.25	+	-	-	-

+++ = positive (growth) - = negative (no growth)
+++, ++, + = positive (compared to FK506 used as a positive control, which gave +++)
* = positive (ring like growth)

* The Δ*zds1* mutant cells were cultivated on YPAUD soft agar containing 150 mM $CaCl_2$ and incubated at 30 °C for 2 d.

4.6 Other biological activities of the isolated compounds

4.6.1 Study on finding the target molecules of Compound D on the Ca^{2+}-signaling pathway in yeast

Compound **D** was chosen for finding its target molecule in the yeast Ca^{2+}-signaling pathway because it is nontoxic to the yeast growth. The growth promoting/inhibiting effect of isolated compounds were examined using liquid culture (YPAUD/SC) medium containing with 100-200 mM $CaCl_2$. Then the growth of mutant yeast cell was monitored every 2 or 4 hrs up to 14-24 hrs by counting the cells with haemacytometer.

The growth promoting and cytotoxic effects of Compound **D** on Δ*zds1* strain were confirmed in liquid culture medium. The Δ*zds1* mutant yeast cell was grown in YPAUD medium containing 100 mM $CaCl_2$. This result found that Compound **D** showed nontoxic on the growth of mutant yeast cells. After pretreatment of the yeast cells with 0.5 mM of Compound **D** or 500 nM FK506 (a positive control) before the cultivation which contained 100 mM $CaCl_2$, the cell could be grown in the presence of Compound **D** or FK506. On the other hand, the cell could not grow in the presence

of only $CaCl_2$ (**Figure 4-8**). This could be concluded that the Ca^{2+}-dependent growth inhibition in yeast was accompanied by polarized the bud growth and cell-cycle arrest in the G2 phase (Mizunuma *et al*., 1988). Moreover, Compound **D** could suppress abnormal morphology of the yeast cells cultivate in high Ca^{2+}-activation.

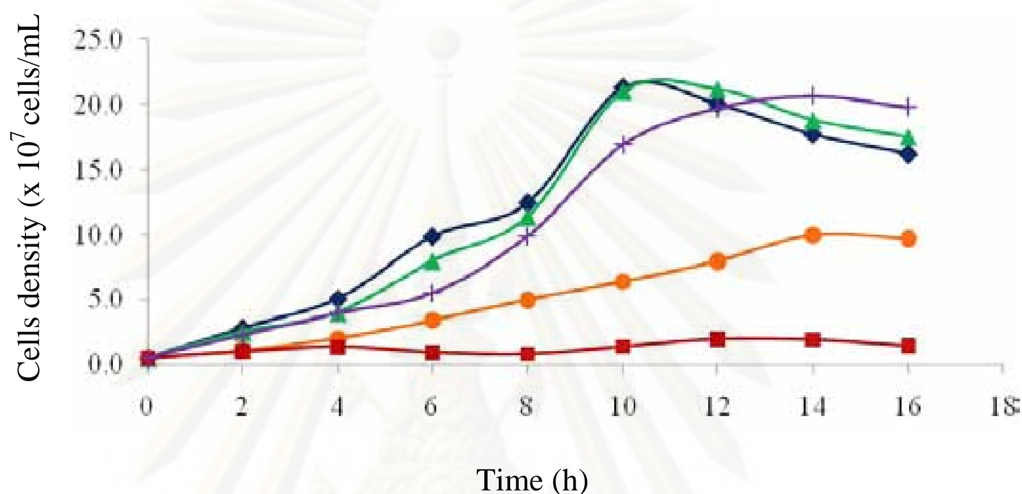

Figure 4-8 The effect of Compound **D** on the growth of Δ*zds1* mutant yeast cells. The Δ*zds1* of $5x10^6$ cells/mL were cultivated in YPAUD broth at 30 °C with shaking at 120 rpm. The cells density was measured every 2 hours until 16 hrs of incubation times. The symbols are as follow: ◆,YPAUD medium; + ,YPAUD medium + 0.5 mM Compound **D**; ▲,YPAUD medium + 100 mM $CaCl_2$ + 500 nM FK506; ■,YPAUD medium + 100 mM $CaCl_2$; ●,YPAUD medium + 100 mM $CaCl_2$ + 0.5 mM Compound **D**.

4.6.1.1 Effect of Compound D on free intracellular Ca^{2+}-levels in mutant yeast cells

The assay for detecting of free Ca^{2+} in mutant yeast cells under the hyperactivation was modified from Tutulan-Cunita *et al.* (2005) in order to determine the levels of β-galactosidase activity. The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190, carrying *PMR2A*-lacZ reporter under the activation of four tandem copies of the calcineurin-dependent response element (CDRE). The mutant cells were subcultured on SC agar lacking uracil and incubated

at 30 ^{o}C for 1-2 d. The colonies of mutants strain were grown in SC medium lacking uracil and incubated at 30 ^{o}C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase (2-5x10^{7} cells/mL). The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with sterile water and separated at 7,500 rpm for 5 min. The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUD, the cells suspension was counted with haemacytometer and adjust to the final concentration of 1.0x10^{7} cells/mL. The assay cells suspension 3 mL was transferred into each test tube, 100 mM of Compound **D** was added to final concentration of 0.5 mM, 0.5% DMSO and 500 nM FK506 were added as a negative and positive control. Then the test samples were incubated at 30 ^{o}C, for 30 min. After that 4 M $CaCl_2$ was added to the final concentration of 150 mM except the treatment of negative control. The assays were incubated at 30°C for 4 hrs. β-Galactosidase activity was measured using ONPG assay (Miller, 1972). The assay cell suspension were diluted and measured the OD_{600} (as a measurement of biomass) using spectrophotometer (blank against YPAUD). For enzyme activity, 1 mL of the cell suspension was divided into eppendorf tube and centrifuged at 12,000 rpm for 2 min. The pellet cells were suspended with 1 mL Z buffer (Appendix) and re-centrifuged at 12,000 rpm for 2 min. The pellet cells were mixed with 150 µL Z buffer and 50 µL $CHCl_3$, 20µL 0.1% SDS was added, vortex (each sample exactly alike) for 15 min. After that 700 µL ONPG (4 mg/mL) (Appendix) was added as a substrate, vortex and incubated at 30 ^{o}C. The time was recorded precisely with the timer. Stop the reaction after sufficient yellow color has developed by adding 500 µL 1 M Na_2CO_3, vortex and note the times of addition precisely. The test samples were centrifuged at 12,000 rpm for 2 min (remove derbris and $CHCl_3$). The supernatant was measured OD_{420} (blank against 700 µL ONPG plus 500 µL Na_2CO_3). The unit of β-galactosidase activity was calculated by the following equation.

Miller Units = $(OD_{420} \times 1{,}000)/(OD_{600} \times T \times V)$

OD420 = OD of the reaction mixture

OD600 = OD of the culture (as measure of biomass)

T = time of the reaction in minutes

V = volume of culture used in the assay in mLs

The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190, carrying *PMR2A*-lacZ reporter under the activation of four tandem copies of the calcineurin-dependent response element (CDRE). The CDRE-driven LacZ reporter gene was activated in response to the external $CaCl_2$ (100 mM), Cunningham and Fink, (1996). In this experiment the mutant strain was cultured in YPAUD containing 150 mM $CaCl_2$, then incubated with and without Compound **D**. As shown in **Figure 4-9**, Compound **D** showed no significant effect on β-galactosidase activity when compared with control (YPAUD+150 mM $CaCl_2$). This was suggested that the cytosolic Ca^{2+}-level was not influenced by the inhibitor and that Compound **D** inhibited the Ca^{2+}-signal mediated cell-cycle regulation at a step following elevation of the cytosolic Ca^{2+} concentration (Chanklan, *et al*., 2008).

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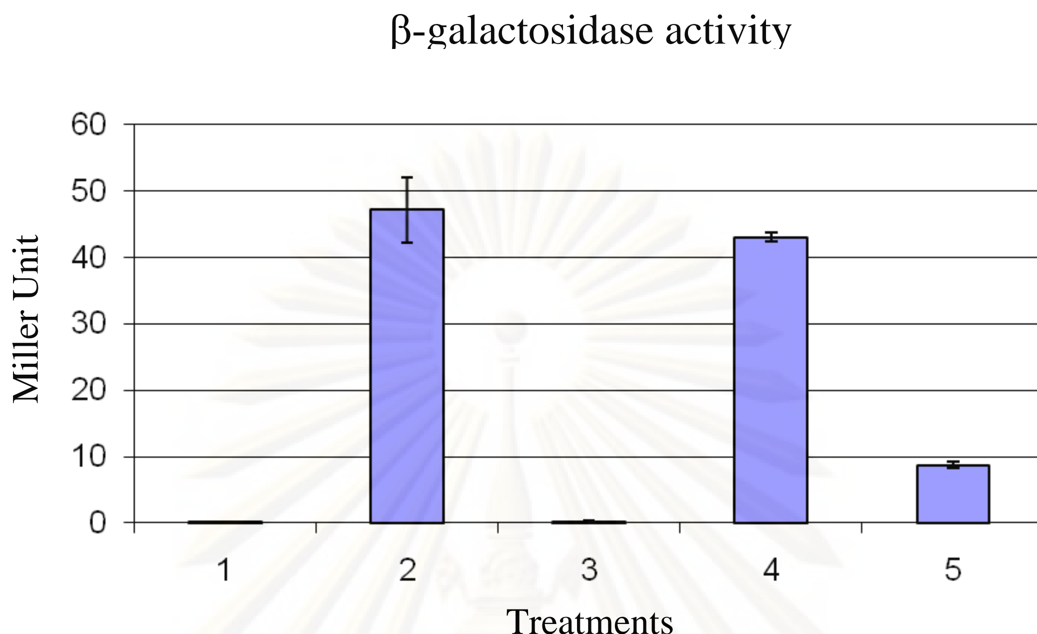

Figure 4-9 The effect of Compound **D** from *K. parviflora* on intracellular Ca^{2+}-levels. Wild-type yeast (strain W303) was transformed with plasmid pKC190 carrying *PMR2A–lacZ,* grown in YPAUD medium at 30 °C for 4 hrs: **1**, YPAUD + 1.5% DMSO; **2**, YPAUD + 150 mM $CaCl_2$; **3**, YPAUD + 150 mM $CaCl_2$ + 500 nM FK506; **4,** YPAUD + 150 mM $CaCl_2$ + 0.5mM Compound **D**; **5**, YPAUD + 150 mM $CaCl_2$ + 1 mM $MgCl_2$.

4.6.1.2 Effect of Compound D on Cnb1 and Mpk1

From the assay results, Compound **D** had no effected on free intracellular Ca^{2+} in the mutant yeast cells. Therefore, the next experiment aims to study on the effect of Compound **D** on the Ca^{2+}-signal inhibitory activity of Mpk1 and calcineurin protein.

The mutant strains of Δ*cnb1* and Δ*mpk1* (obtained from Prof. Tockichi Miyakawa, Department of Molecular Biology, Graduate school of Advance Science and Matter, Hiroshima University, Japan) were subcultured on YPUAD agar and incubated at 30 °C for 1-2 d. The colonies of mutant strains were grown in YPAUD broth and incubated at 30 °C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase (2-7x10^7 cells/mL). The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with sterile water and separated at 7,500

rpm for 5 min (2 times). The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUD, the cells suspension was counted with haemacytometer and adjust to the final concentration of 5.0×10^6 cells/mL. The assay cells suspension 2 mL was transferred into each test tube, 100 mM of Compound **D** was added to final concentration of 0.5 mM, 0.5% DMSO and 500 nM FK506 were added as a negative and positive control. The assays were incubated at 30 ^oC, the growth of yeast cells were monitored every 2 hrs up to 14 hrs by counting the cells with haemacytometer.

This experiment whether Calcineurin or Mpk1 was a target molecule of Compound **D** was carried out using the synthetic lethality test. From the experiment, the cells Δ*cnb1* or Δ*mpk1* could grow very well in the presence of Compound **D** different from FK506, a potent calcineurin inhibitor (**Figure 4-10** and **Figure 4-11**). The results suggested that neither the Mpk1 nor cancineurin is the target molecule of Compound **D**.

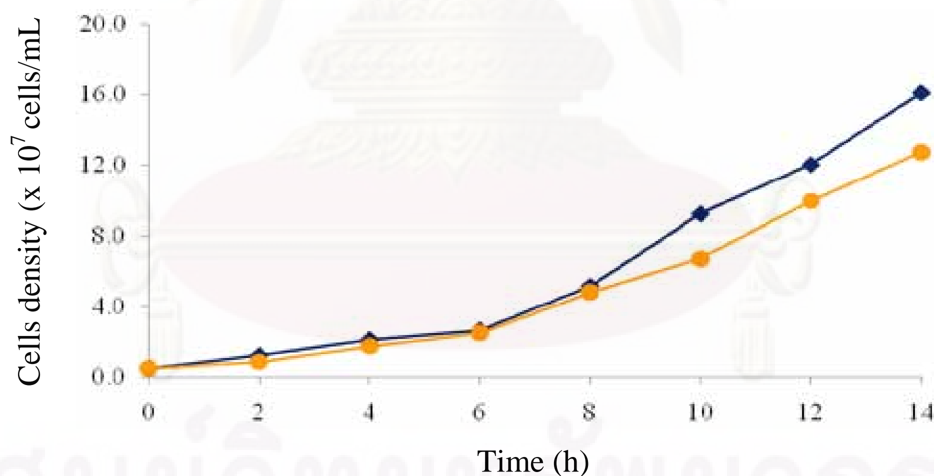

Figure 4-10 The effect of Compound **D** on the growth of Δ*cnb1* mutant yeast cells. The Δ*cnb1* of 5×10^6 cells/mL was cultivated in YPAUD broth at 30 °C with shaking at 120 rpm. The cells density was measured every 2 hrs until 14 hrs of incubation times. The symbols are as follow: ◆, YPAUD medium; ●, YPAUD medium + 0.5 mM Compound **D**.

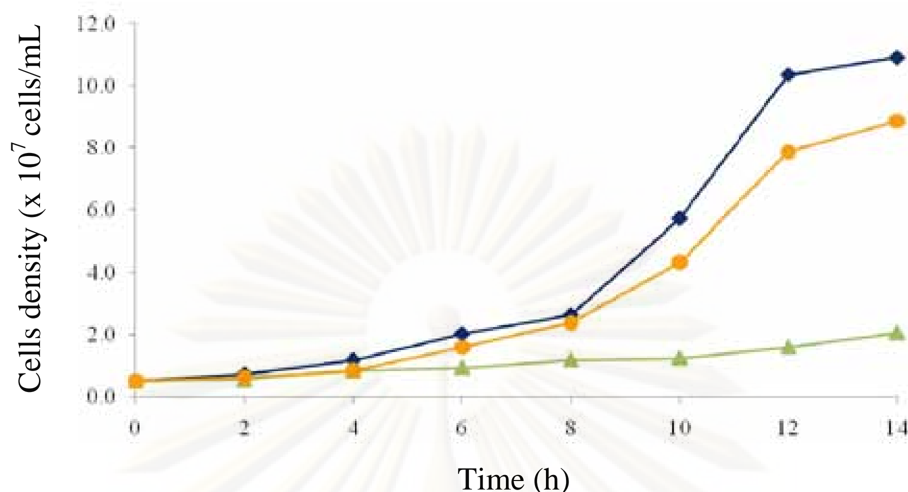

Figure 4-11 The effect of Compound **D** on the growth of Δ*mpk1* mutant yeast cells. The Δ*mpk1* of $5x10^6$ cells/mL was cultivated in YPAUD medium at 30 °C with shaking at 120 rpm. The cells density was measured every 2 hrs until 14 hrs of incubation times. The symbols are as follow: ◆, YPAUD medium ; ▲, YPAUD medium + 500 nM FK506; ●, YPAUD medium + 0.5 mM Compound **D**.

4.6.1.3 Effect of Compound D on Δ*zds1* overexpress *MCK1*

The mutant strain of Δ*zds1* was subcultured on SC agar lacking uracil (Appendix) and incubated at 30 °C for 1-2 d. The colonies of mutant strain was grown in uracil drop out SC medium and incubated at 30°C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in exponential phase (2-$5x10^7$ cells/mL). The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with SG (with 2% galactose) (Appendix) and separated at 7,500 rpm for 5 min. The supernatant was discarded and the precipitated was resuspended with 5 mL uracil drop out SC medium (with 1% raffinose) (Appendix), the cells suspension was counted with haemacytometer and adjust to the final concentration of $1.0x10^7$ cells/mL. The assay cell suspension 2 mL was transferred into each of test tubes, 100 mM of Compound **D** was added to final concentration of 0.5 mM, 0.5% DMSO and 500 nM FK506 were added as a negative and positive control. Then the treatments were incubated at 30 °C, for 30 min. After that times 20% (w/v) galactose was added to the final concentration of 1% except the

treatment of negative control. The assays were incubated at 30 °C, the growth of yeast cells were monitored every 12 hrs up to 72 hrs by counting the cells with haemacytometer.

Next experiment was performed whether Compound **D** inhibited at the step of Mck1 or not. The morphology and growth of Δ*zds1* overexpress MCK1 cells was observed. If the inhibition occurs downstream of the activated pathway, the physiological effects will not be induced by overexpression due to the interruption of this signal. On the basis of this assumption, the plasmid containing *MCK1* (pGAL::MCK1) placed under the control of the galactose-inducible *GAL1* promoter was introduced into the Δ*zds1* strain (Mizunuma, *et al.*, 2001). In this experiment the mutant growth was severely inhibited the G2-delayed cells accumulated and induced the physiological changes by Compound **D** (**Figure 4-12).** This is suggested that Compound **D** inhibit at a step after the activation of downstream of the Mpk1 and calcineurin, so Mck1 is a possible target molecule of Compound **D**.

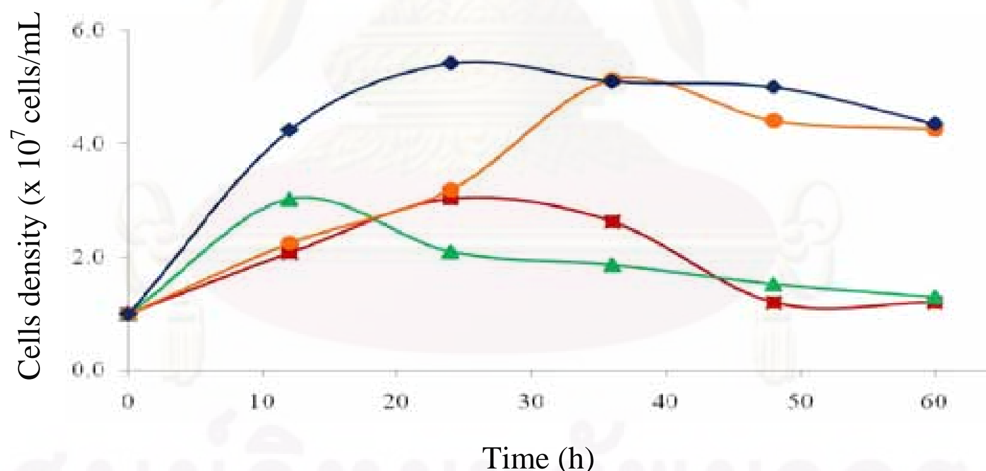

Figure 4-12 The effect of Compound **D** on the growth of Δ*zds1* overexpress *MCK1* mutant yeast cells. The *mck1* of 1x10^7 cells/mL was cultivated in uracil drop out SC medium (SC-U) at 30 °C with shaking at 120 rpm. The cell density was measured every 12 hours until 60 hrs of incubation times. The symbols are as follow: ◆, SC-U + 2% raffinose ; ■, SC-U + 2% galactose; ▲, SC-U + 2% galactose + 500 nM FK506; ●, SC-U + 2% galactose + 0.5 mM Compound **D**.

4.6.1.4 Effect of Compound D on Swe1

The mutant strain of YRC2 (*swe1::GAL1p*-SWE1) was subcultured on YPUAD agar and incubated at 30 ^{o}C for 1-2 d. The colonies of mutant strain was grown in YPAUD broth and incubated at 30 ^{o}C with shaking at 200 rpm for 18-24 hrs until the concentration of cells in early log phase (0.5-1x10^{7} cells/mL). The culture broth was transferred into microfuge tube and centrifuged at 7,500 rpm for 5 min. The precipitate cells were collected and washed with YPAUR and separated at 7,500 rpm for 5 min (2 times). The supernatant was discarded and the precipitated was resuspended with 5 mL YPAUR, the cells suspension was counted with haemacytometer and adjust to the final concentration of 5.0x10^{6} cells/mL. The assay cells suspension 2 mL was transferred into each of test tubes, 100 mM of Compound **D** was added to final concentration of 0.5 mM, 0.5% DMSO and 1 μM Radicicol were added as a negative and positive control. Then the treatments were incubated at 30 ^{o}C, for 30 min. After that times 20% (w/v) galactose was added to the final concentration of 1% except the treatment of negative control. The assays were incubated at 30 ^{o}C, the growth of yeast cells were monitored every 4 hrs up to 24 hrs by counting the cells with haemacytometer.

Recently, Compound **A**, a flavonoid from *B. pandurata* could be alleviated the growth defect due to Swe1 overexpression, the swe1 mutant yeast cells could grow well in YPG medium containing of 1% galactose (Suksawatumnuay *et al*., unplublished). This was suggested that Swe1p is the target molecule of Compound **A**. In this experiment, Compound **D** was also investigated for the target molecule in the Ca^{2+}-signaling pathway. In the presence of Compound **D** in YPAUR medium containing 1% galactose, the Swe1 overexpression strain could not grow (**Figure 4-13)** which was different from that in the presence of Radicicol, the mutant strain could grow (Chanklan *et al*., 2008). From the result, it could be concluded that the Swe1 was not the target molecule of Compound **D**.

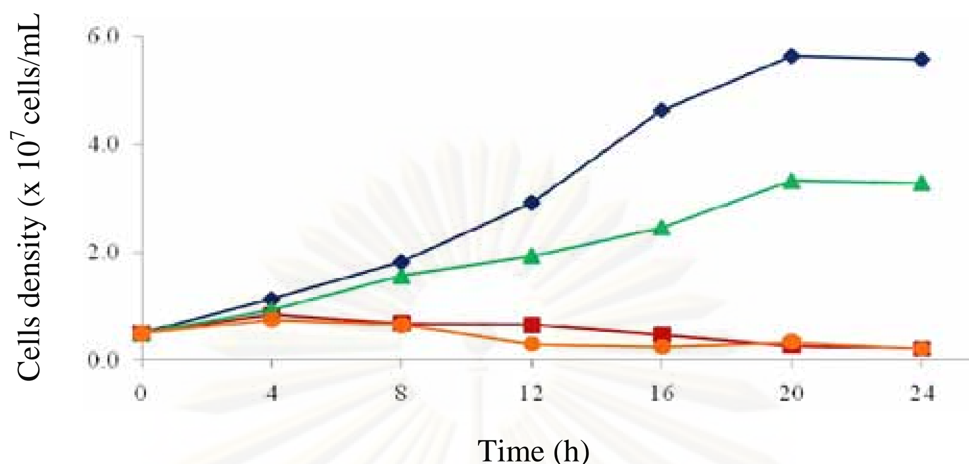

Figure 4-13 The effect of Compound **D** on growth of Δ*swe1*::*GAL1p-SWE1* cells that overexpress Swe1p. The Δ*swe1*::*GAL1p-SWE1* of 0.5x10^7 cells/mL was cultivated in YPAUR medium at 30 °C with shaking at 120 rpm. The cells density was measured every 4 hrs until 24 hrs of incubation times. The symbols are as follow: ◆, YPAUR medium; ■, YPAUR medium + 1% galactose; ▲, YPAUR medium + 1% galactose + 1 µM radicicol; ●, YPAUR medium + 1% galactose + 0.5 mM Compound **D**.

4.6.2 Anti-inflammatory activity on carrageenin-induced paw edema in rat

In previous report, four flavonoids were assayed for oral anti-inflammatory activity using carrageenan-induced hind paw edema in rat. The structural features necessary for anti-inflammatory activity appear to be the presence of methoxyl groups at C5 and C7 of ring A and the pyrano ring B of the flavonoid molecule. The difference in the efficacy of the flavonoids tested possibly depends on their pharmacokinetic properties (Panthong *et al*., 1984).

Compound **D,** the flavonoid compound revealing a strong positive result on the yeast-based assay was tested for anti-inflammatory activity on carrageenan-induced paw edema, according to Winter *et al.*, (1962). The animals were divided into eight groups of six rats. The negative control group received distilled water (0.5 ml/kg, p.o.), the positive control group received the acetyl salicylic acid (300 mg/kg, p.o.) and the test groups received the active compounds at the doses of

300 and 600 mg/kg p.o. The test was conducted using an electric plethysmometer 7140 (Ugo Basile, Italy). Carrageenan 2.5% (0.05 ml) was injected subcutaneously in the plantar surface of the rat's left hind paw 1 hr after oral administration of drugs to induce a progressive swelling of the paw. The paw volume, up to the tibiotarsal articulation, was measured at 0 hr (before the irritant).

The anti-inflammatory activity on carrageenin-induced paw edema in rat was revealed that the Compound **D** partially posses anti-inflammatory with %inhibition of 42.22 as well as Compound **A** was 43.71 while the control (ASA) was 71.56%.

4.6.3 Determination of minimum inhibitory concentration of Compound D on the growth of *Helicobacter pylori*

Kaempferia parviflora is one of the effective herbs for potential prevention and treatment of *H. pylori* infection. The EtOAc extract of this plant could inhibit the invasion of HEp-2 cells by *H. pylori* strains harboring *cag*A gene more effectively strains without *cag*A gene (Amonyingcharoen, S., 2007).

The minimum inhibitory concentration (MIC) value for the microorganism was determined as sensitivity to the active compounds on agar plate assay. The MIC assay was applied for the evaluation of sensitivity against six strains of *H. pylori*, ATCC43504, ATCC43526, ATCC51932, C7, HP001 and HP742. The active compound was initially prepared at the concentration of 50 μg/mL with DMSO. Then, the dilution was diluted in 1 mL of Mueller-Hinton broth as a 2 folded dilutions and mixed with 125 mL of Mueller-Hinton agar (supplement with 5% sheep red blood cells) to final concentration of 2, 1, 0.5, 0.25, 0.125, 0.0625 μg/mL/tested plate, respectively. The various strains of *H. pylori* were diluted with 0.85% NaCl to a concentration of $1x10^7$ to $1x10^8$ CFU/mL and dotted (3 μL) onto the surface of the dilution plate. The assay plates were incubated at 37^oC for 24 hrs in Anaerobe jar with Campy*Gen*™ (adjust to the condition of 3-7% O_2, 5-10% CO_2 and 80-85% H_2). The MIC values of Compound **D** on the various strains of *H. pylori* were determined.

Recently, the minimum inhibitory concentrations against 11 clinical isolates and 2 reference strains of *H. pylori* were examined using an agar dilution method. The

EtOAc extracts of *K. parviflora* exhibited significant *H. pylori* activities at MIC of 107.38 μM (Amonyingcharoen, S., 2007). In this experiment, Compound **D** isolated from the CH_2Cl_2 extract of this plant which showed a strong Ca^{2+}-signal inhibition was chosen for preliminary assay against six strains of *H. pylori* (ATCC43504, ATCC43526, ATCC51932, C7, HP001 and HP742) using an agar dilution method. Compound **D** moderately inhibited 6 strains of *H. pylori* with the MIC value of 0.84-6.71 μM (**Table 4-12**).

Table 4-12 The MIC values of Compound **D** on the various strains of *H. pylori*

Strains	Derived from	MIC (μM)
43504	ATCC	0.84
43526	ATCC	6.71
51932	ATCC	6.71
C7	Clinical strain	0.84
HP001	Clinical strain	0.84
HP742	Clinical strain	0.84

4.7 Conclusions

Compound **D** (5-hydroxy-3,7-dimethoxyflavone), **E** (5-hydroxy-7-methoxyflavone), **F** (5-hydroxy-3,7,4′-trimethoxyflavone) and **G** (5,7-dimethoxyflavone) were isolated from *K. parviflora* by using the Δ*zds1* yeast-based guided fractionation. Compound **D** showed no toxicity on the yeast growth so it was chosen for further study on finding its target molecule in the Ca^{2+}-signaling pathway in yeast. Compound **D** was a possibility target molecule of Mck1p, the protein kinase which is ortholog of GSK-3 kinase in human (Cohen and Goedert, 2004).

GSK-3 (Glycogen Synthase Kinase-3) is a ubiquitously expressed, highly conserved serine/threonine protein kinase found in all eukaryotes. Identified originally as a regulator of glycogen metabolism, GSK-3 acts as a downstream regulatory switch for numerous signaling pathways, including cellular responses to WNT, Growth Factors, Insulin, RTK (Receptor Tyrosine Kinases), Hedgehog pathways, and GPCR

(G-Protein-Coupled Receptors) and is involved in a wide range of signal transduction cascades involving cellular processes, ranging from glycogen metabolism, cell development, gene transcription, protein translation to cytoskeletal organization, cell cycle regulation, proliferation and apoptosis. Unlike most protein kinases involved in signaling, GSK-3 is active in un-stimulated, resting cells and its activity is diminished during cellular responses. Another peculiarity compared with other protein kinases is its preference for primed substrates, that is, substrates previously phosphorylated by another kinase (Doble and Woodgett, 2003).

There are two mammalian GSK-3 isoforms encoded by distinct genes: GSK-3 Alpha and GSK-3 Beta. GSK3-Beta is particularly abundant in the CNS (Central Nervous System) and directly phosphorylates several neuronal MAPs (Microtubule-Associated Proteins), involved in microtubule stabilization (Sayas *et al*., 2002).

GSK-3 is a key regulator in several physiological processes, such as cell cycle, oncogenesis and apoptosis in neuronal cells and VSMC (Vascular Smooth Muscle Cells) during hypoxia (Woodgett, 2001). Increased cAMP levels promote survival of neuronal cells by inactivating GSK-3 via a PKA-dependent mechanism (Hardt and Sadoshima, 2002). Many of the pathways that use GSK-3 as a regulator have links to human diseases. GSK-3 has been implicated in non-insulin-dependent Diabetes Mellitus and generation of NFT (Neurofibrilliary Tangles) associated with Alzheimer’s Disease (Doble and Woodgett, 2003) as well as several hallmarks of Alzheimer's Disease including neurodegeneration, reactive astrocytosis, microgliosis, and the formation of apoptotic bodies (Woodgett, 2001). Recently, a number of potent and selective GSK-3 inhibitors have been developed having several therapeutic uses, including the treatment of neurodegenerative disease, bipolar disorder, and inflammatory disease bodies (Woodgett, 2001). However, the best-characterized inhibitor of GSK-3 is lithium. Although inhibition of GSK-3 may be desirable in one context (e.g. in preventing neuronal apoptosis), it could have serious implications for another—for example, it might accelerate hyperplasia by deregulating Beta-Ctnn (Beta-Catenin). Given the involvement of GSK-3 in many pathophysiological processes and diseases, GSK-3 is a tempting therapeutic target (Doble and Woodgett, 2003).

Compound **D** exhibited moderate anti-inflammatory activity on carrageenin-induced paw edema in rat with 42.22%. In addition, **D** was chosen for preliminary assay against six strains of *H. pylori* (ATCC43504, ATCC43526, ATCC51932, C7, HP001 and HP742) using an agar dilution method and it showed moderately inhibited 6 strains of *H. pylori* with the MIC value of 0.84-6.71 μM.

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CHAPTER V

CONCLUSIONS

In preliminary screening four species of 50 Thai medicinal herbs (*Andrographis paniculata*, *Boesenbergia pandurata*, *Kaempferia parviflora* and *Syzygium cinereum*) exhibited strong positive calcium signal inhibition on the Δ*zds1* mutant yeast-based assay. Among them the dichloromethane extract of medicinal plants in the *Zingiberaceae* family, *B. pandurata* and *K. parviflora* were chosen for further study of the isolated compounds. There is a great need to identify novel lead compounds and the current study showed that the *Zingiberaceae* family can be a potential source for such compounds. Consequently, medicinal plants can continue to be a rich source of novel compounds for the treatment of human disease.

In conclusion, 5-hydroxy-7-methoxyflavanone (**A**), 7-hydroxy-5-methoxy-flavanone (**B**) and 2,6-dihydroxy-4-methoxychalcone (**C**) were isolated from *B. pandurata* and 5-hydroxy-3,7-dimethoxyflavone (**D**), 5-hydroxy-7-methoxyflavone (**E**), 5-hydroxy-3,7,4′-trimethoxyflavone (**F**) and 5,7-dimethoxyflavone (**G**) were isolated from *K. parviflora* by using the yeast-based guided fractionation. 5-hydroxy-7-methoxyflavanone. (**A**) and 5-hydroxy-3,7-dimethoxyflavone (**D**) showed no toxicity on the yeast growth, so they were chosen for further study on finding their target molecules in mutant yeast cells. The target molecule of the isolated bioactive compounds, (**A**) was Swe1p, the protein kinase which is the ortholog of Wee1 in human (Asano *et al*., 2005) and the Compound **A** was also exhibited the cytoxicity on Jukat, A375 and Kato III human cancer cell lines with IC_{50} value of 56, 67 and 86 μM, respectively. Compound **A** showed partial anti-inflammatory activity on carrageenin-induced paw edema in rat with % inhibition of 43.71. On the other hand, Compound **D** from *K. parviflora* was possibility the target on Mck1p, the protein kinase which is the ortholog of GSK-3 kinase in human (Cohen and Goedert, 2004). The inhibitor of GSK-3 has implicated in several therapeutic uses including the treatment of neurodegenerative disease, bipolar disorder. Compound **D** exhibited moderate anti-inflammatory activity on carrageenin-induced paw edema in rat with

42.22%. In addition, Compound **D** showed moderated anti-*Helicobacter pylori* activity against six strains of *H. pylori* (ATCC43504, ATCC43526, ATCC51932, C7, HP001 and HP742) using an agar dilution method with the MIC value of 0.84-6.71 μM.

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ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

1. Media

1.1 Yeast peptone dextrose (YPD)

Yeast extract	10 g
Peptone	20 g
Glucose	20 g

Dissolved in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

1.2 Yeast peptone adenine uracil dextrose broth (YPAUD broth)

Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Adenine	400 mg
Uracil	200 mg

Dissolved in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

1.3 Yeast peptone adenine uracil dextrose agar (YPAUD agar)

Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Adenine	400 mg
Uracil	200 mg
Agar	20 g

Dissolved in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

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1.4 Yeast peptone adenine uracil dextrose soft agar (YPAUD soft agar)

Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Adenine	400 mg
Uracil	200 mg
Agar	7 g

Dissolved in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

1.5 Synthetic complete medium (SC medium)

Yeast nitrogen base without amino acid	6.7 g
Glucose	20 g
Distilled water	1 L

Adjust pH up to pH 4.5 with 1 N NaOH and autoclaved at 121°C, 15 psi for 20 min, then supplemented with 100 mL 10 x amino acid without uracil.

1.6 Synthetic complete medium agar (SC medium agar)

Yeast nitrogen base without amino acid	6.7 g
Glucose	20 g
Agar	20 g
Distilled water	1 L

Adjust pH up to pH 4.5 with 1 N NaOH and autoclaved at 121°C, 15 psi for 20 min, then supplemented with 100 mL 10 x amino acid without uracil.

1.7 SG medium

Yeast nitrogen base without amino acid	6.7 g
Galactose	20 g
Distilled water	1 L

Adjust pH up to pH 4.5 with 1 N NaOH and autoclaved at 121°C, 15 psi for 20 min, then supplemented with 100 mL 10 x amino acid without uracil.

1.8 1% Raffinose SC medium

Yeast nitrogen base without amino acid	6.7 g
Raffinone	10 g
Distilled water	1 L

Adjust pH up to pH 4.5 with 1 N NaOH and autoclaved at 121°C, 15 psi for 20 min, then supplemented with 100 mL 10 x amino acid without uracil.

1.9 20% Galactose SC medium

Yeast nitrogen base without amino acid	6.7 g
Galactose	200 g
Distilled water	1 L

Adjust pH up to pH 4.5 with 1 N NaOH and autoclaved at 121°C, 15 psi for 20 min, then supplemented with 100 mL 10 x amino acid without uracil.

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1.0 10 x amino acid without uracil

Adenine sulfate	200 mg
L-Tryptophan	200 mg
L-Histidine HCl	200 mg
L-Arginine HCl	200 mg
L-Methionine	200 mg
L-Tyrosine	300 mg
L-Leucine	1,000 mg
L-Isoleucine	300 mg
L-Lysine HCl	300 mg
L-Phenylalanine	500 mg
L-Glutamic acid	1000 mg
L-Aspartic acid	1,000 mg
L-valine	1,500 mg
L-Threonine	2000 mg
L-Serine	4,000 mg

Dissolved in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2. Reagent and buffer

2.1 10% SDS

Dissolved sodium dodecyl sulfate 10 g in distilled water up to 100 mL and autoclaved at 121°C, 15 psi for 20 min.

2.2 4 mg/mL *o*-nitrophenyl-β-D-galactosidase (ONPG)

Dissolved ONPG 4 mg in Z buffer up to 10 mL (prepare as much as will need, and fresh prepared before used)

2.3 Z buffer

$Na_2HPO_4.7H_2O$ 16.1 g

$NaH_2PO_4.2H_2O$ 5.5 g

KCl 0.75 g

$MgSO_4.7H_2O$ 0.246 g

β-mercaptoethanol 2.7 mL

Dissolved in distilled water up to 1 L and adjusted pH up to 7.0 with 1N HCl

2.4 1 M Na_2CO_3

Dissolved Na_2CO_3 105.99 mg in distilled water up to 1 L and autoclaved at 121°C, 15 psi for 20 min.

2.5 Phosphate buffer saline (PBS, Ca^{2+}, Mg^{2+} free) pH 7.4

NaCl 8 g

KCl 0.2 g

$NaHPO_4$ 1.44 g

KH_2PO_4 0.24 g

Dissolved in distilled water up to 1 L and adjusted pH up to 7.4 with 1N HCl or 1 N NaOH

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จุฬาลงกรณ์มหาวิทยาลัย

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Oral Presentation

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จุฬาลงกรณ์มหาวิทยาลัย