รายงานวิจัยฉบับสมบูรณ์

โครงการ “การใช้ยีสต์เพื่อคัดกรองและศึกษาคุณสมบัติของสารออกฤทธิ์ทางชีวภาพในพืชสมุนไพรไทย”

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

จากที่มีการพบว่าลำดับเบสระหว่างยีนในมนุษย์และยีนในยีสต์มีความเหมือนกันอยู่สูง และมีการใช้เทคนิคทางพันธุศาสตร์ของยีสต์เข้ามาช่วยในการศึกษากระบวนการในสิ่งมีชีวิตชั้นสูง ทำให้ยีสต์ *S. cerevisiae* เป็นจุลินทรีย์หนึ่งที่มีบทบาทในการค้นหายาใหม่ๆ วิถีการส่งสัญญาณของแคลเซียมในเซลล์มีความเกี่ยวข้องกับการควบคุมกระบวนการทางชีวภาพต่างๆมากมายในสิ่งมีชีวิตชั้นสูง ยีสต์สายพันธุ์กลายหนึ่งที่ขาดยีน*ZDS1* (*zds1*Δ) ไม่สามารถเจริญได้เมื่อถูกเลี้ยงในอาหารเลี้ยงเชื้อที่มีความเข้มข้นของแคลเซียมสูง (150 มิลลิโมล่าร์) อันเป็นผลมาจากวัฏจักรการแบ่งเซลล์ได้หยุดอยู่ที่ระยะG2 จากลักษณะการเจริญที่มีเงื่อนไขดังกล่าว ทำให้ยีสต์*zds1*Δถูกนำมาใช้ในระบบคัดกรองสารที่มีโมเลกุลขนาดเล็กซึ่งสามารถออกฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมที่มีผลต่อการควบคุมการแบ่งเซลล์ ในการศึกษานี้ได้ใช้ระบบคัดกรองที่ใช้ยีสต์สายพันธุ์ดังกล่าวเพื่อคัดกรองหาสารออกฤทธิ์ยับยั้งการส่งสัญญาณของแคลเซียมจากพืชสมุนไพรไทย จากการทดสอบสารสกัดอย่างหยาบจำนวน 143 ตัวอย่าง พบสารสกัด 3 ตัวอย่างที่ให้ผลบวกในระบบคัดกรองดังกล่าวได้แก่สารสกัดอย่างหยาบจากฟ้าทะลายโจร (*Andrographis paniculata*) กระชายเหลือง (*Boesenbergia pandurata*)และกระชายดำ (*Kaempferia parviflora*) เฉพาะสารสกัดอย่างหยาบจากกระชายเหลืองได้ถูกเลือกมาศึกษาต่อ โดยสารสกัดอย่างหยาบถูกแยกเป็นส่วนย่อยและทำให้บริสุทธิ์โดยเทคนิคโครมาโตกราฟฟี่ และใช้เทคนิคโครมาโตกราฟฟี่แบบแผ่นบาง ร่วมกับระบบการทดสอบในยีสต์เพื่อติดตามฤทธิ์ สามารถแยกได้สารบริสุทธิ์ 3 ชนิด ได้แก่ pinostrobin alpinetin และ pinocembrin chalcone จากการทดสอบในระบบยีสต์พบว่า pinostrobin มีฤทธิ์ทางชีวภาพแรงที่สุด เมื่อเลี้ยงเซลล์ยีสต์ *zds1*Δ ในอาหารเลี้ยงเชื้อที่มี pinostrobin อยู่ก่อนที่จะนำเซลล์ดังกล่าวไปกระตุ้นวิถีการส่งสัญญาณของแคลเซียม พบว่า pinostrobin สามารถยับยั้งการหยุดการแบ่งเซลล์ที่ระยะ G2 ของเซลล์ยีสต์ *zds1*Δได้จากการใช้วิธีโฟลว์ไซโทเมททรี(flow cytometry) เพื่อติดตามระยะการแบ่งเซลล์ นอกจากนี้ pinostrobin ยังยับยั้งเซลล์ดังกล่าวในการเกิดรูปร่างที่ผิดปกติเมื่อแตกหน่อและการแบ่งนิวเคลียสที่ผิดปกติได้ การศึกษานี้แสดงให้เห็นว่าสาร pinostrobin จากกระชายเหลืองมีฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมในยีสต์

Abstract

Because of the high degree of gene conservation between humans and yeast, the rapid progress in molecular biology and the powerful of classical yeast genetics render the yeast *S. cerevisiae* to be a fascinating organism in drug discovery. Calcium (Ca^{2+}) signaling pathway is involved in regulation of diverse biological processes in eukaryotes. A mutant yeast strain, *zds1*Δ, grown in high Ca^{2+} containing medium exhibits a severe growth defect through the inhibition of the cell-cycle at G2 phase. Based on such phenotype observed, a novel drug screening system to detect small molecule inhibitors of Ca^{2+}-dependent cell-cycle regulation was purposed. This study aimed to employ such yeast-based assay to screen Thai medicinal plants, a rich resource of bioactive compounds, for small molecule Ca^{2+} signal inhibitors. Three out of 143 crude ethanol extracts, that is *Andrographis paniculata*, *Boesenbergia pandurata* and *Kaempferia parviflora*, showed positive results in the screens. A crude extract of *B. pandurata* was fractionated and purified using column chromatographic techniques and TLC. The biological activity of each column was monitored by the yeast based assay. Three isolated compounds were purified and characterized by nuclear magnetic resonance spectroscopy as pinostrobin, alpinetin and pinocembrin chalcone. Pinostrobin shows the highest biological activity among the three. The pinostrobin-treated yeast cells could alleviate the abnormal morphology and abnormal nuclear division of the budding cells suffering from Ca^{2+}-induced G2 phase cell-cycle arrest as confirmed by flow cytometry (FACS) profile. This study showed that pinostrobin from *B. Pandurata* contains inhibitory activity on the Ca^{2+} signal in the yeast, *S. cerevisiae*.

หน้าสรุปโครงการ (Executive Summary)

ชื่อโครงการ (ภาษาไทย) การใช้ยีสต์เพื่อคัดกรองและศึกษาสมบัติของสารออกฤทธิ์ทางชีวภาพในพืชสมุนไพรไทย

(ภาษาอังกฤษ) Screening and some characterization of bioactive compounds from Thai medicinal plants using yeast as a screening system

วัตถุประสงค์

เพื่อคัดกรองหาสารออกฤทธิ์ทางชีวภาพจากพืชสมุนไพรไทยโดยใช้ยีสต์เป็นระบบคัดกรอง และศึกษาสมบัติบางประการของสารออกฤทธิ์ที่ได้

สิ่งที่ได้ดำเนินการไป และผลที่ได้ ค้นพบอย่างย่อ

1. หาสภาวะที่เหมาะสมของระบบคัดกรองที่ใช้ยีสต์ ได้แก่ความเข้มข้นที่เหมาะสมของเซลล์ยีสต์ซึ่งใช้เป็นเซลล์บ่งชี้ในระบบคัดกรอง ความเข้มข้นที่เหมาะสมของ $CaCl_2$

   ผลที่ได้: พบว่าสภาวะที่เหมาะสมของระบบคัดกรองที่ใช้ยีสต์ ได้แก่ความเข้มข้นของเซลล์ยีสต์ซึ่งใช้เป็นเซลล์บ่งชี้ในระบบคัดกรองที่ 3-6 $X10^5$ เซลล์/ มล. ความเข้มข้นที่เหมาะสมของ $CaCl_2$ ที่ 150 มิลลิโมล่าร์

2. เปรียบเทียบวิธีการสกัดสารสกัดอย่างหยาบ 4 วิธี ได้แก่ การต้ม การดองใน 70% เอทานอล การสกัดด้วยไดคลอโรมีเทน และการสกัดด้วย 95% เอทานอล ตามลำดับ

   ผลที่ได้: จากการเปรียบเทียบวิธีการสกัด 4 วิธี ได้แก่ การต้ม การดองใน 70% เอทานอล การสกัดด้วยไดคลอโรมีเทน และการสกัดด้วย 95% เอทานอล ตามลำดับ พบว่าการต้ม เป็นวิธีการสกัดที่ให้ผลไม่ดีเท่าอีก 3 วิธีที่เหลือซึ่งให้ผลการทดสอบใกล้เคียงกัน จึงเลือกวิธีการดองใน 95% เอทานอล สำหรับเตรียมสารสกัดเพื่อใช้ในการคัดกรองหาสารออกฤทธิ์ทางชีวภาพจากพืชต่อไป

3. เปรียบเทียบสภาพของตัวอย่างพืชที่ใช้เตรียมสารสกัดอย่างหยาบ ระหว่างสภาพสดและสภาพแห้ง ที่มีต่อผลบวกในระบบคัดกรอง

   ผลที่ได้: พบว่าการใช้ตัวอย่างพืชในสภาพแห้งเพื่อใช้เตรียมสารสกัดอย่างหยาบ ให้ผลบวกที่แรงและชัดเจนกว่าการใช้ตัวอย่างพืชในสภาพสด เมื่อเปรียบเทียบในน้ำหนักแห้งของสารสกัดที่เท่ากัน

4. นำสภาวะที่เหมาะสมที่ได้จากการศึกษาเบื้องต้น มาใช้เพื่อการคัดกรองหาสารออกฤทธิ์ทางชีวภาพจากสารสกัดอย่างหยาบในพืช ในระบบยีสต์

ผลที่ได้: ได้เลือกใช้ตัวอย่างพืชในสภาพแห้ง และเตรียมสารสกัดอย่างหยาบโดยการดองใน 95% เอทานอล ทำการคัดกรองสารออกฤทธิ์ในระบบยีสต์ จากสารสกัดอย่างหยาบในพืชจำนวน 143 ชนิด พบสารสกัดอย่างหยาบในพืชที่ให้ผลบวกจำนวน 3 ชนิด คือ ฟ้าทะลายโจร (*Andrographis paniculata*) และกระชายเหลือง (*Boesenbergia pandurata*) และ กระชายดำ (*Kaempferia parviflora*)

5. เลือกสารสกัดอย่างหยาบในพืชที่ให้ผลบวกมา 1 ชนิด เพื่อแยกเป็นส่วนย่อย ติดตามฤทธิ์ในส่วยย่อยโดยระบบยีสต์

ผลที่ได้: ได้เลือกแยกสารสกัดอย่างหยาบจาก กระชายเหลืองให้เป็นส่วนย่อย และใช้ระบบยีสต์ในการติดตามฤทธิ์จากส่วนย่อย ได้สารออกฤทธิ์บริสุทธิ์ 3 ชนิด คือ pinostrobin alpinetin และ pinocembrin chalcone

6. ศึกษาลักษณะสมบัติบางประการของสารออกฤทธิ์บริสุทธิ์ที่ได้

ผลที่ได้: สารบริสุทธิ์ทั้ง 3 ชนิด แสดงลักษณะ dose dependent cytotoxicity กล่าวคือที่ความเข้มข้นสูงจะเป็นพิษต่อเซลล์ยีสต์ แต่จะให้ผลบวกต่อเซลล์ยีสต์เมื่อใช้ที่ความเข้มข้นที่เหมาะสม Pinostrobin มีความแรงของฤทธิ์ที่ให้ผลบวกในยีสต์ได้สูงสุด และมีความเป็นพิษต่อเซลล์ยีสต์ต่ำที่สุด เมื่อเทียบกับ alpinetin และ pinocembrin chalcone จึงได้นำ Pinostrobinที่ความเข้มข้นต่างๆ ไปกระตุ้นเซลล์ยีสต์สายพันธุ์กลาย *zds1*Δ และติดตามวัฏจักร (cell cycle) ของเซลล์ สัณฐานวิทยาของเซลล์ และของนิวเคลียสภายในเซลล์ที่กำลังแตกหน่อ ภายใต้กล้องจุลทรรศน์ และกล้อง fluorescence ตามลำดับของยีสต์ดังกล่าว โดยเปรียบเทียบกับ 500 nM FK506 ที่ใช้ เป็นชุดควบคุมบวก (positive control) หรือ DMSO ที่ใช้เป็นชุดควบคุมลบ (negative control) ผลการตรวจสอบ DNA content โดย Flow cytometer พบว่า pinostrobin ที่ความเข้มข้น 1 mM สามารถปกป้องเซลล์ยีสต์จากการหยุด/ ชะลออยู่ที่ระยะ G2 (G2 phase delay) และป้องกันการเกิดการแตกหน่อที่ผิดปกติ (daughter cell มีการยืดยาวออก และไม่มีการแบ่งนิวเคลียสมายัง daughter cell) อันเป็นผลมาจากการถูกกระตุ้นด้วยสัญญาณ Ca^{2+} ได้ ผลจากการทดลองนี้เป็นการยืนยันทั้งทางพันธุศาสตร์และชีวเคมี ว่า Pinostrobin จากกระชายเหลือง ทำหน้าที่เป็นสารยับยั้งการส่งสัญญาณของวิถีแคลเซียม (Ca^{2+} signal inhibitor) ในเซลล์ยีสต์

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Introduction

The search for novel drugs is still one of a major interest in the research especially in the health related area. This dues to so many problems of currently used drugs such as high side effects, drug resistance, not so effective drug or lack of drugs against newly evolved diseases. Screening has been one of the sources to search for new drugs for the pharmaceutical industry for several decades. Commonly employed screening procedures for bioactive compounds are negative screening which based on their ability to inhibit growth of various types of indicator cells such as pathogenic bacteria, fungi, viruses and cancer cells. The widely use of such screening procedures caused many serious problems such as isolation of the cytotoxic compounds and re-isolation of previously known drugs. Therefore, a screening procedure with a novel principle is critically important to improve the possibility of isolating novel bioactive compounds for further development as new drugs.

The yeast, *Saccharomyces cerevisiae* is the most extensively studied eukaryote dued to its characteristics of safety, rapid growth and its powerful genetics. In addition, the high degree of conservation with mammalian cells in terms of gene sequences and proteins and pathway functions make *S. cerevisiae* very appealing for drug discovery. This is because approximately 40% of yeast proteins share some conserved sequences with at least one known or predicted human protein, including several hundred genes implicated in human diseases (Hughes 2002., Parsons *et al.* 2003).

Ca^{2+}-signaling pathway is one of the signal transduction pathways extensively studied in this yeast (Shitamukai *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 the yeast *S. cerevisiae*, the Ca^{2+} signal is implicated in the regulation of the G2/M cell cycle progression. Swe1 kinase specifically inhibits a G2 form of Cdc28 cyclin dependent protein kinase by phosphorylating it at Tyr-19 and delays the cell cycle (Booher *et al.* 1993., Means 1994). Ca^{2+} signal transduces through two parallel pathways, calcineurin and Mpk1, which cooperatively activate Swe1 (Fig.1). A negative regulator of Swe1, Zds1(Bi and Pringle 1996), helps to balance the activity of Swe1 in Ca^{2+} induced condition.

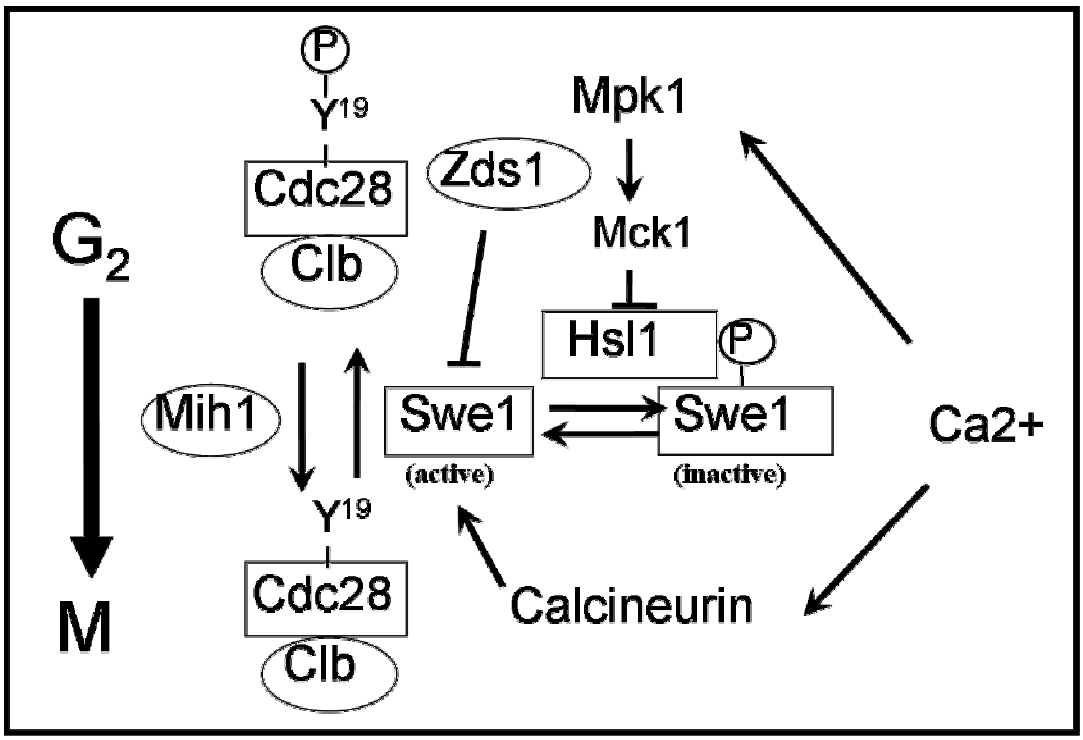


Fig.1 The Ca^{2+}-activation signaling pathway that regulates G2 cell-cycle progression in *S. cerevisiae* (Mizunuma *et al*. 1998)

Shitamukai *et. al*. (2000) developed a novel drug screening procedure based on the hyperactivation of the Ca^{2+}-signaling pathway by *S. cerevisiae* Δ*zds1* mutant strain. This mutant can not grow in Ca^{2+} induced condition, however, bioactive compounds that antagonize the signaling activity can promote growth of cells from the hyperactivated Ca^{2+} signal. The use of Δ*zds1* mutant strain for this screening, because of its distinct principle, renders isolation of novel bioactive compounds or re-isolation of the known compounds with novel activities.

A wide range of target molecules in Ca^{2+}-signaling pathway such as calcineurin, Mpk1(homolog human ERK1/2)(Levin 2005) and Mck1(homolog human GSK-3)(Kassir *et,al* 2006) which conserved from yeast to human, are potential targets of the drug that expected to be obtained by this screening (Table 1). Since the relationships between the drugs and their molecular targets so far characterized are relatively well conserved from yeast to mammalian cells, it seems reasonable to consider that the drugs isolated by the yeast screening system may be applicable to the mammalian cell systems.

Table 1. Potential target molecules of bioactive compounds expected to obtain by this assay.

Potential target molecules	**The expected effects**
Calcineurin	Immunosuppressant, Allergy remedy
MPK1(ERK1, ERK2)	Anticancer agent, Antiinflammatory agent
MCK1 (GSK-3)	Alzheimer disease, Type II diabetes

The activity of Calcineurin is depended on calcium signals and it plays an essential role in T cell signal transduction by controlling the nuclear import of the nuclear factor of activated T cells (NFAT) family of transcription factors. Calcineurin is activated through binding of Ca^{2+}/Calmodulin and then dephosphorylates the cytosolic forms of the NFAT proteins resulting in transportation of NFAT transcription factor into the nucleus. Hence, various cytokine genes, such as IL-2 were then activated (Surgiura *et al*. 2002).

The regulation of a large number of cellular processes depends on the activation state of ERK. Blockage of the RAS MAPK pathway has conceivably been used against a multitude of human diseases and conditions including cancer and inflammatory diseases. Ras is frequently mutated in many tumors, and the mutated Ras that leads to constitutive activation of ERK1/2 can promote tumor cell proliferation (Qi and Elion 2005). ERK1/2 regulates the activating protein 1 (AP-1) family of transcription factors by phosphorylation. Members of the AP-1 family that are phosphorylated by ERK1/2 include c-Jun, c-Fos and activating transcription factor 2 (ATF-2)(Hommes *et al* 2003).

GSK-3 interacts with several neuronal proteins that are directly linked with Alzheimer's disease (AD). The microtubule-associated protein tau, is hyperphosphorylated by GSK-3 kinase on serine and threonine residues, and this is believed to be the primary cause of the generation of AD tangles. GSK-3 has been shown to phosphorylate tau protein both *in vitro* and in intact cells on multiple sites, some of which are aberrant in the abnormally hyperphosphorylated tau. On the other hand, GSK-3's activity has been found higher than normal level in the insulin-sensitive tissue which

directly involved in the pathogenesis of type 2 diabetes. This increased in GSK-3 ’s activity presumably contributes to the impairment of insulin action (Eldar-Finkelman 2002).

Nature has been a source of medical agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources. More than 25% of modern medicine comes from natural products and another 25% are structural modification of the lead compounds from natural source (Pootakham 2005).

Bioactive compounds could be screened from many natural sources such as bacteria, actinomyces, fungi, animals as well as plants. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. Because of the structural and biological diversity of their constituents, terrestrial plants offer unique and renewable resources for the discovery of potential new drugs and biological entities. Herbal medicine has a long history of use by the indigenous populations of many countries. Moreover, in developing countries, medicinal plants continue to be the main source of medication. It has been estimated that approximately 80% of the world’s inhabitants and 88% of the inhabitants of underdeveloped countries rely mainly on traditional medicine for their primary health care (Farnsworth *et al*. 1985., Pezzuto 1997). Our country, Thailand, due to its unique geographical location has an innumerable variety of plants. It has a great diversity of indigenous medicinal plants. The Thais have a long tradition of folklore medicine, utilizing medicinal herbs and plants which have been used for centuries as an integral part of Thai culture.

This study aimed at using a particular novel positive screening system of yeast to screen for bioactive compounds from Thai medicinal plants.

Materials and Methods

1. Collection of plants

Plants were collected in natural places or bought at medicinal herb stores. Fresh plants were dried in shelter for a few days.

Table 2 List of plants used in this study (Smitinand 1980)

Family	Code*	Species	Common name	Part of plants
Acanthaceae	AEB	*Acanthus ebracteatus* Vahl	Ngueak plaa mo dok khaao	Whole plant
	AIL	*Acanthus ilicifolius.* L	Ngueak plaa mo dok muang	Whole plant
	AVA	*Adhatoda vasica* Nees	Saniat	Root
	APA	*Andrographis paniculata*	Fa thalaai	Whole plant
	BPU	*Barleriaiu pulina* Lindl.	Salet phangphon	Whole plant
	CNU	*Clinacanthus nutans* (Burm.F.) Lindau	Phayaa yo	Whole plant
	RNA	*Rhinacanthus nasutus* (L.)Kurz.	Thong phan chang	Whole plant
	TLA	*Thunbergia laurifolia* L.	Raang chuet	Trunk

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
Aizoaceae	GOP	*Glinus oppositifolius* A.DC.	Phak khuang	Whole plant
Alliceae	ALSA	*Allium sativum* Linn.	Krathiam	Leaves
Amaranthaceae	IHE	*Iresine herbstii* Hook f.	Phak phaeo daeng	Leaves
Amaryllidaceae	CAS	*Crinum asiaticum* Linn.	Phlap phlueng	Leaves
Anacardiaceae	MUS	*Melanorrhoea usitata* Wall.	Rak yai	Leaves
Apiaceae	CEAS	*Centella asiatica* Urb.	Bua bok	Leaves
Apocynaceae	AGMA	*Aganosma marginata*	Maduea din	Trunk
	ASC	*Alstonia scholaris* (Linn.) R. Br.	Sattaban	Trunk
Araceae	AIN	*Alocasia indica* Schott var. metallica Schott	Kradaat daeng	Bark
	AOD	*Alocasia odorata C. Koch*	Uttaphit	Rhizome
	ABL	*Amorphophallus companulatus* Blume.ex Dene	Buk	Rhizome
	LSP	*Lasia spinosa* Thw.	Phak naam	Root

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
Araceae	TRO	*Typhonium roxburghii*	Uttaphit	Root
Asclepiadaceae	HOV	*Hoya ovalifolia* W. & A.	Nom tamlia	Whole plant
Asparagaceae	ARA	*Asparagus racemosus* willd	Saamsip	Root
Asteraceae	PIN	*Pluchea indica* (Linn.) Less.	Khluu	Whole plant
	SAC	*Spilanthes acmella* Murr.	Phak khraat huawaen	Whole plant
Avicenniaceae	AAL	*Avicennia alba Bl.*	Samae khaao	Leaves
Bixaceae	BOR	*Bixa orellana* L.	Kham saet	Seed
Boraginaceae	HIN	*Heliotropium indicum*	Yaa nguang chaang	Whole plant
Caesalpiniaceae	CGA	*Cassia garrettiana* Craib.	Samae saan	Trunk
	CSA	*Caesalpinia sappan* Linn.	Faang	Trunk

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
	CTO	*Cassia tora Linn.*	Chumhet thai	Trunk
Caricaceae	CPAA	*Carica papaya Linn.*	Malako	Trunk
Celastraceae	SCE	*Siphonodon celastrineus* Griff.	Ma duuk	Trunk
Cleomaceae	CVI	*Cleome viscosa* Linn.	Phak sian phee	Whole plant
Clusiaceae	GAC	*Garcinia acuminated* Planch. & Triana	Rong thong	Resin
Combretaceae	LLI	*Lumnitzera littorea Voigt*	Faat daeng	Leaves
	TBE	*Terminalia bellirica* (Gaertn.) Roxb.	Samo phi phek	Seed
	TCH	*Terminalia chebula* Retz. var. *chebula*	Samo thai	Seed
Compositae	ACO	*Ageratum conyzoides Linn.*	Saapreaeng saapkaa	Whole plant
	ASCO	*Artemisia scoparia*	Thian	Seed

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
		Waldst. & Kit.	Thianyaowapha anee	
Compositae	CTI	*Carthamus tinctorius Linn.*	Kham foi	Flower
	CAN	*Centratherum anthelminthicum (Willd.) Kuntz.*	Thian loat	Seed
	CGR	*Coccinia grandis* (Linn.) Voigt.	Tam-lueng	Trunk
	EPR	*Eclipta prostrata* Linn.	Kameng	Whole plant
	EST	*Eupatorium stoechadosnum* Ham	San phraa hom	Whole plant
	GPS	*Gynura pseudo* – china hispida	Waan mahaakan	Rhizome
	TCU	*Trichosanthes cucumerrina* Linn.	Buap khom	Seed
	VCI	*Vermonia cinerea*	Yaa dok khaao	Whole plant

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
		Less.		
Cucurbitaceae	GIN	*Gymnopetalum integriforium* Kurz.	Kheekaa daeng	Whole plant
	MCH	*Momordica charantia* L.	Mara	Whole plant
	MOCO	*Momordica cochinensis* (Lour.) Spreng.	Fak khaao	Trunk
Dioscoreaceae	DHI	*Dioscorea hispida Dennst.*	Kloi	Rhizome
Euphorbiaceae	BMO	*Baliospermum montanum Muell. Arg.*	Tong tae	Leaves
	BOV	*Bridelia ovata* Decne	Makaa	Leaves
	COR	*Cladogynos orientalis* Zipp.ex Span.	Chettaphang-khee	Root
	CTIG	*Croton tiglium Linn.*	Salot	Seed

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
	EHI	*Euphorbia hirta Linn.*	Nam nom raatchasee thale	Flower
	ECO	*Excoecarice cochinueuse*	Krabue chet tua	Whole plant
Euphorbiaceae	GMU	*Gelonium multiflorum* A. Juss.	Khan thong phayaabatt	Trunk
	COB	*Croton oblongifolius* Roxb.	Plao yai	Trunk
	PAC	*Phyllanthus acidus* Skeels.	Ma yom	Trunk
	PEM	*Phyllanthus emblica* Linn.	Ma khaam pom	Seed
	PAM	*Phyllanthus* amarus Schum. & Thonn.	Luuk tai bai	Whole plant
	SIN	Sapium indicum Willd.	Samo thale	Seed
Flacourtiaceae	FIN	*Flacourtia indica Merr.*	Ta khop paa	Trunk
	HAN	*Hydnoccarpus anthelminthicus*	Kra bao	Seed

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
		Pierre.		
Goodeniaceae	STA	*Scaevola taccada Roxb.*	Phakchee farang	Whole plant
Gramineae	CDA	*Cynodon dactylon* Pers	Yaa phraek	Whole plant
Guttiferae	GMA	*Garcinia mangostana L.*	Mangkhut	Fruit
	GCO	*Garcinia cowa Roxb.*	Cha muang	Leave
Iridaceae	BCH	*Belamcanda chinensis* (Linn.) DC.	Waan haanginiifolia	Rhizome
Labiatae	OSA	*Ocimum sanctum Linn.*	Ka phrao	Whole plant
	OBA	*Ocimum basilicum Linn.*	Maeng lak	Whole plant
Lauraceae	CBE	*Cinnamomum bejolghota* Sweet	Op choei	Trunk
	LGL	*Litsea glutinosa* (Lour.) C.B. Robinson	Mee men	Trunk
Leguminosae	APR	*Abrus precatorius* Linn.	Ma klam taanuu	Whole plant
	CFI	*Cassia agnes* Brenan	ratchaphruek	Pod

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
	EVA	*Erythrina variegata* L.	Thong laang bai mon	Whole plant
	MPI	*Mimosa pigra* L.	Maiyaraap	Whole plant
Liliaceae	GSU	*Gloriosa superba* Linn.	Dong dueng	Pod
Menispermaceae	CPA	*Cissampelos pareira* Linn.	Krung khemaa	Root
	SPI	*Stephania pierrei* Diels.	Boraphet phungchaang	Rhizome
Menispermaceae	TTI	*Tiliacora triandra Diels.*	Yaa naang	Leaves
	TTU	*Tinospora tuberculata* Beumee	Boraphet	Trunk
Melastomataceae	MPO	*Melastoma polyanthum Bl.*	Khlong-khleng	Leaves
Malvaceae	HAS	*Hibiscus sabdariffa* linn.	Kra-chiap	Flower
Meliaceae	AIN	*Azadirachta indica* A.Juss var.siamensis *Valet*	Sadao	Leaves

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
Milacaceae	SMI	*Smilax* spp. S	Hua khaao yen	Rhizome
Mimosoideae	ARU	*Acacia rugata* Merr.	Som poi	Whole plant
	ACA	*Acacia catechu* Willd.	Seesiat	Resin
	FPU	*Ficus pubigera* Wall.	Maa krathuep rong	Trunk
	MCO	*Maclura cochinchinensis* Corner	Kae lae	Trunk
Moraceae	MAL	*Morus alba L.*	Mon	Leaves
	SAS	*Streblus asper* Lour.	Khoi	Trunk
Myristicaceae	MFR	*Myristica fragrans* Houtt.	Chan thet	Seed
Myrtaceae	SAR	*Syzygium aromaticum* (L.) Merr. & Perry	Kaan phluu	Seed
Nelumbonaceae	NNU	*Nelumbo nucifera Gaertn*	Bua luang	Pollen
Orchidaceae	LDI	*Ludisia discolor* (Ker-Gawl.) A.Rich.	Waan ron thong	Rhizome
Pandanaceae	POD	*Pandanus odoratissimus* L.f.	Lam chiak	Flower
Papilionaceae	DEL	*Derris elliptica* (Roxb.)	Haang lai daeng	Trunk

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
		Benth.		
	GGL	*Glycyrrhiza glabara*	Cha ame thet	Trunk
Piperaceae	PCH	*Piper chaba* Hunter.	Dee plee	Seed
	PINI	*Piper nigrum* L.	Phrik thai	Seed
	PRI	*Piper ribesoides* Wall.	Sakhaan	Trunk
	PSA	*Piper sarmentosum* Roxb.	Chaa phluu	Whole plant
Plantaginaceae	PMA	*Plantago major Linn.*	Mo noi	Whole plant
Plumbaginaceae	PRO	*Plumbago rosea* Linn.	Chettamuun phloeng daeng	Root
	PZE	*Plumbago zeylanica* Linn.	Chettamuun phloeng khaao	Root
Poaceae	CNA	Cymbopogon nardus Rendle	Ta khrai hom	Leaves
Punicaceae	PGR	*Punica granatum* Linn.	Thap thim	Trunk
Rhizophoraceae	RMU	*Rhizophora mucronata* Poir.	Kong kaang baiyai	Leaves

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
Rubiaceae	HFO	*Hydnophytum formicarum* Jack	Kra-chao pheemot	Trunk
Rubiaceae	MCI	*Morinda citrfolia Linn.*	Yo	fruit
	PFO	*Paederia foetida* Linn.	Phang hom	Trunk
Rutaceae	AEMA	*Aegle marmelos* (L.) Corr.	Matuum	Seed
Salvadoraceae	ASA	*Azima sarmentosa* Benth. & Hook.	Naam phungdo	Trunk
Sapindaceae	CHA	*Cardiospermum halicacabum* L.	Khok kra om	Whole plant
Saururaceae	HCO	*Houttuynia cordata* Thunb.	Phluu khaao	Whole plant
Scrophulariaceae	AHI	*Adenosma hirsuta* (Miq.) Kurz	Thong theng	Whole plant
	SDU	*Scoparia dulcis* L.	Mafia dueanhaa	Whole plant
Simaroubaceae	BJA	*Brucea javanica Merr.*	Ratchadat	Seed

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
	ELO	*Eurycoma longifolia* Jack.	Plaa-lai phueak	Trunk
Solanaceae	SST	*Solanum stramonifolium Jacq.*	Ma uek	Whole plant
Sonneratlaceae	SOV	*Sonnertia ovata Back.*	Lam phean	Leaves
Sterculiaceae	AAU	*Abroma augusta Linn.f.*	Thian dam	Seed
	SMA	*Scaphium macropodum Beaumee*	Samrong	Seed
Umbelliferae	AGR	*Anethum graveolens Linn.*	Thian khaao plueak	Seed
	CASI	*Centella asiatica Linn.*	Bau bok	Leaves
	CCY	*Cuminum cyminum Linn.*	Thian khaao	Seed
	EFO	*Eryngium foetidum Linn.*	Phakchee farang	Whole plant
Umbelliferae	HSI	*Heracleum siamicum Craib var. gracilius Craib*	Thian takkataen	Seed
Verbenaceae	VTR	*Vitex trifolia* Linn.	Khon thiso	Leaves
Zingiberaceae	AKR	*Amomum krervanh* Pierre	Krawaan khaao	Seed
	AVI	*Amomum villosum* Lour. var.xanthioides *(Wall)*	Reo yai	Seed

Table 2 List of plants used in this study (cont.)

Family	Code*	Species	Common name	Part of plants
	ANI	*Alpinia nigra* (Gaertn.)	Khaa	Rhizome
Zingiberaceae	AOF	*Alpinia officinarum* Hance.	Khaa ling	Rhizome
	BPA	*Boesenbergia pandurata*	Kra chaai	Rhizome
	CLO	*Curcuma longa* Linn.	Khamin chan	Rhizome
	CXA	*Curcuma xanthorrhiza* Roxb.	Waan chak-motluuk	Rhizome
	CZE	*Curcuma zedoaria* Rosc.	Khaminoi	Rhizome
	KPA	*Kaempferia parviflora Wall.*	Kra chaai dam	Rhizome
	ZZE	*Zingiber zerumbet Smith*	Ka thue	Rhizome

* Code of plants consists of three characters. The first character came from the first character of genus name and the second and third characters were from the first and second characters of species name. In case of three characters gave repetitive code, four characters was assigned: the first and second characters were from the first and second characters of genus name and the third and fourth characters were from species name.

2. Preparation of crude extracts from plants

Four different methods were first compared and screened for the bioactive compounds.

2.1 Reflux with Dichloromethane

2.2 Reflux with 95% Ethanol

Dried plants were blended into small pieces by blender. Fifty gram of each blended plant was extracted by dichloromethane using reflux method for 3 hours. Crude extracts were obtained after filtration followed by evaporation and stored at 4 °C or -20 °C until use.

2.3 Boiling

Ten gram of each blended plants was soaked with 75 ml water for 10 min.and boiled until 2/3 of the aqueous remained from the started volume. The crude extracts were recovered by filtration followed by lyophillization. Dried crude extracts were stored at 4 °C or -20 °C until use.

2.4 Soaking in 70% ethanol

Ten gram of blended plants was submerged in 70% ethanol at room temperature for 1 day, followed by sonication for 1 minute. After filtration, the solid parts were repeatedly soaked in fresh

70% ethanol for twice. The liquid parts were evaporated and the crude extracts were stored at 4 °C or -20 °C until use.

3. Yeast strain & cultivation

Yeast mutant *zds1*Δ strain, YNS17 (*MAT*a *zds1*::*TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) were obtained from Prof. Tokichi Miyakawa, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Japan. The mutant yeast cells were cultivated on YPD agar and incubated at 30 °C for 2 days. Then were subcultured into YPD broth and incubated at 30°C with shaking at 200 rpm for 18-24 hours. Aliquot of 500 µl of culture broth was placed into a cryotube and 500 µl of 30% glycerol in YPD broth were added and mixed. The aliquot containing the yeast cells were stored at -80 °C.

4. Yeast-based screening assay

The indicator cells (*zds1*Δ) from -80 °C were cultured on YPD plate at 30°C for 2 days. Then inoculated in 5 ml YPD broth and incubated with shaking at 200 rpm at 30°C until cell density reached approximately 5-7 x 10^7 cell/ml. Prewarmed 55 °C of 0.7 % YPD soft agar, prewarmed $CaCl_2$ and indicator cells were mixed to get appropriated concentrations and poured the plates to get the screening plates. Five microlitres of plants crude extracts or solvent (as negative control) or FK506 or Cyclosporine A (as positive controls) were dotted onto the screening plates. The plates were incubated at 30 °C for 40 hours.

5. Fractionation and purification of a crude plant extract

The crude positive plant extract were prepared in large scale (1 kg.) and fractionated with several chromatographic columns. The biological activity in each fraction was monitored by the yeast based assay as in 4. The positive fractions were further purification using chromatographic technique and precipitation until crystals were obtained. NMR and mass spectroscopic techniques were employed to elucidate the structure of the pure compounds compared to the database.

6. Some characterizations on a pure compound from 5.

6.1 Effect of a pure compound on protecting the *zds1*Δ cells from G2 to M phase delay as monitoring by Flow cytometer

6.1.1 Optimal conditions for monitoring the phases of yeast cell by flow cytometer

The mutant yeast cells, *zds1*Δ, were cultivated in YPD medium containing varying concentration of $CaCl_2$ from 50 -100 mM for 3-6 hrs prior to being fixed and nuclear stained with propidium iodide for flow cytometry analysis.

6.1.2 Sample preparation

Weighed a pure compound in microfuge tube and dissolved in dimethysulfoxide (DMSO) to get final concentration at 100 or 150 mM. Vortex mixer and ultrasonicator were used to facilitate better dissolve and get homogenous solution.

6.1.3 Preparation of the mutant yeast cells, *zds1*Δ for being activated by Ca^{2+} signals. (modified from Mizunuma *et. al*. 1998).

6.1.3.1 Streak the mutant yeast cells *zds1*Δ onto Yeast Peptone Dextrose (YPD) agar and incubated at 30 °C for 1-2 days.

6.1.3.2 Subculture the cells from YPD agar plates of 5.1.3.1 into 50 ml of YPD broth and incubated at 30 °C at 200 rpm for 18-24 hrs. Harvested the cells when the concentration of the culture reached early log phase (0.5 - 1.0 X 10^7 cells/ml.) by centrifugation at 7,500 rpm for 5 min. After decanting the supernatant, YPD broth supplemented with 400 mg/l adenine and 200 mg/l uracil (YPAUD) were added to resuspend the cell pellet to get final cell concentration of 5 X10^6 cells/ ml. Either 1 mM Pinostrobin or 500 nM FK506 or 1.5% DMSO were preincubated with the yeast cells suspension at 30 °C for 30 min. prior to activate the yeast cells with $CaCl_2$ at final concentration of 100 mM and further incubation at 30 °C for 3 hrs. Cells were harvested and the pellets were washed with 0.2 M Tris-HCl. The treated cells were resuspended in 250 µl of 0.2 M Tris-HCl, followed by addition of 750 µl absolute ethanol and placed at - 20 °C at least overnight.

6.1.4. Yeast cells preparation for flow cytometry analysis

Before harvesting the cells from from 6.1.3.2, 200 µl of 0.2 M Tris-HCl were added. The cell pellets were resuspended with 100 µl of 1 mg/ml RNaseA and incubated at 37 °C for 3 hrs. The RNase A treated cells were washed once with 200 µl of 0.2 M Tris-HCl and followed by resuspending in 100 µl of 50 mg/ml 4 mM Citrate buffer pH 8.0, 10 mM NaCl, 0.1% NP-40 and incubated at 4 °C overnight. Then, the cell suspension was transferred into a fresh Falcon tube and were added with 900 µl of 4 mM Citrate buffer pH 8.0, 10 mM NaCl, 0.1% NP-40. The ultrasonic probe was used to dissociate the cells with the setting at medium level for 5 s before the flow cytometry analysis (FACS caliber, BD FACSCalibur™).

6.2 Effect of a pure positive compound on protecting the *zds1*Δ cells from abnormal bud emergence suffering from the hyperactivation of the calcium signals.

The cell pellets from 6.1.3.2 were resuspended with 250 µl of 0.2 M Tris-HCl. The cell suspension was observed by 40X magnification of light microscope. Then, 20 µl of cells suspension were added with 2 µl of 50 µM Hoechst 33342 and incubated in dark for 5 min. before being analyzed by fluorescence microscope.

Results and Discussion

1. Setting up and optimization of the yeast screening assay

To get the clear growth result in the screening plates, the optimal concentration of indicator cells and $CaCl_2$ were first optimized. Various concentrations of indicator cells ranging from 5 X 10^4 – 6 X 10^5 cells/ml. and those of $CaCl_2$ in the range of 150-250 mM were used to prepared the screening plates. FK506 and cyclosporin A were used as positive growth control. Absolute ethanol as the solvent for the crude extracts was used as no growth control. The results were shown in Fig. 2.

The results revealed that the optimal conditions for the screening plate was at 150 mM $CaCl_2$ and indicator cells concentration at 3 X 10^5 cells/ml. showed the best results of growth of the positive control while no growth of negative control and no background growth

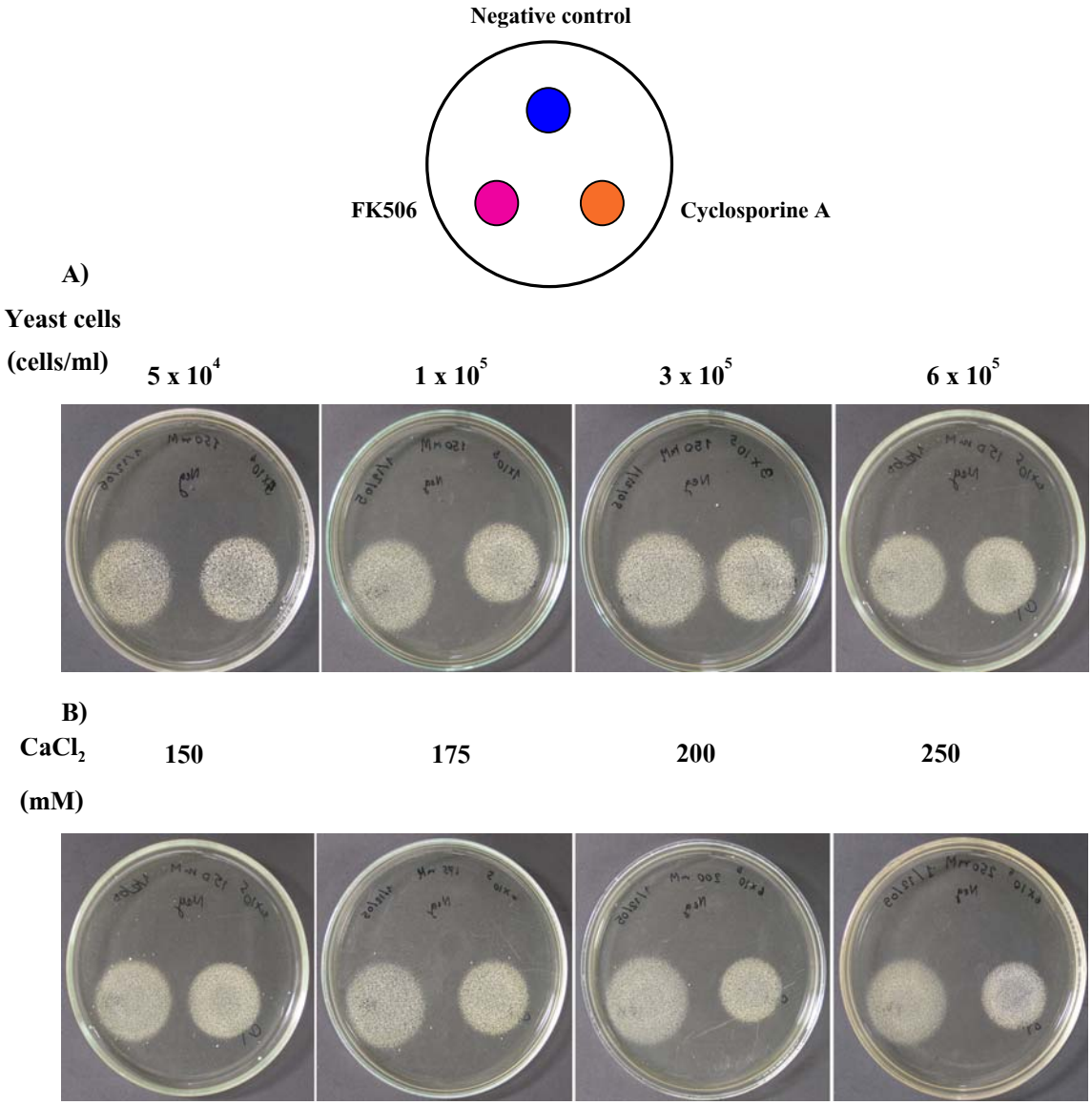


Fig. 2 Growth of indicator cells. A) in the screening plates containing 150 mM $CaCl_2$ with varying concentrations of yeast cells from 5 x 10^4- 6 x 10^5 cells/ml. B) in the screening platescontaining the indicator cells concentration at 6 x10^5 cells/ml with varying concentrations of $CaCl_2$ from 150 - 250 mM.

2. Comparison on methods for crude extract preparations

To choose the appropriate methods for crude extract preparation from plants, four different methods were performed: method 1 reflux with dichloromethane, method 2 reflux with 95% ethanol, method 3 boiling and method 4 soaking in 70% ethanol. Fifty-one medicinal plants were collected and blended. Each blended plants were extracted by 4 different methods. Three mg/ml of each crude extracts obtained from each methods were assayed in the yeast screening system. The results were shown in Table 3.

Table 3 Screening results of plant crude extracts prepared by four different methods.

Family	Code name	Reflux with Dichlorome-thane (1)	Reflux with 95% ethanol (2)	Boiling (3)		Soaking with 70% ethanol (4)
				3 mg/ml	20 mg/ml	
Acanthaceae	AEB	-	-	-	-	-
	AIL	-	-	-	-	-
	APA	++++	++++	-	+	+++
	CNU	-	-	-	-	-
	RNA	-	-	-	-	-
Amaryllidaceae	CAS	-	-	-	-	-

Table 3 Screening results of plant crude extracts prepared by four different methods. (cont.)

Family	Code name	Reflux with Dichlorome-thane (1)	Reflux with 95% ethanol (2)	Boiling (3)		Soaking with 70% ethanol (4)
				3 mg/ml	20 mg/ml	
Apocynaceae	AGMA	-	-	-	-	-
Asclepiadaceae	HOV	-	-	-	-	-
Asteraceae	PIN	-	-	-	-	-
	SAC	-	-	-	-	-
Boraginaceae	HIN	-	-	-	-	-
Caesalpiniaceae	CGA	-	-	-	-	-
	CSA	-	-	-	-	-
Celastraceae	SCE	-	-	-	-	-
Clusiaceae	GAC	-	-	-	-	-
Combretaceae	TBE	-	-	-	-	-
	TCH	-	-	-	-	-
Compositae	CGR	-	-	-	-	-
	EPR	-	-	-	-	-
	TCU	-	-	-	-	-

Table 3 Screening results of plant crude extracts prepared by four different methods. (cont.)

Family	Code name	Reflux with Dichlorome-thane (1)	Reflux with 95% ethanol (2)	Boiling (3)		Soaking with 70% ethanol (4)
				3 mg/ml	20 mg/ml	
Euphorbiaceae	COR	-	-	-	-	-
	ECO	-	-	-	-	-
	GMU	-	-	-	-	-
	COB	-	-	-	-	-
	PAM	-	-	-	-	-
	SIN	-	-	-	-	-
Flacourtiaceae	FIN	-	-	-	-	-
Iridaceae	BCH	-	-	-	-	-
Lauraceae	CBE	-	-	-	-	-
	CIN	-	-	-	-	-
	LGL	-	-	-	-	-
Liliaceae	GSU	-	-	-	-	-
Menispermaceae	SPI	-	-	-	-	-
	TTU	-	-	-	-	-

Table 3 Screening results of plant crude extracts prepared by four different methods. (cont.)

Family	Code name	Reflux with Dichlorome-thane (1)	Reflux with 95% ethanol (2)	Boiling (3)		Soaking with 70% ethanol (4)
				3 mg/ml	20 mg/ml	
Moraceae	FHI	-	-	-	-	-
	FPU	-	-	-	-	-
	MCO	-	-	-	-	-
	SAS	-	-	-	-	-
Myristicaceae	MFR	-	-	-	-	-
Myrtaceae	SAR	-	-	-	-	-
Papilionaceae	DEL	-	-	-	-	-
	GGL	-	-	-	-	-
Piperaceae	PCH	-	-	-	-	-
	PINI	-	-	-	-	-
	PRI	-	-	-	-	-
	PSA	-	-	-	-	-
Plumbaginaceae	PRO	-	-	-	-	-
	PZE	-	-	-	-	-

Table 3 Screening results of plant crude extracts prepared by four different methods. (cont.)

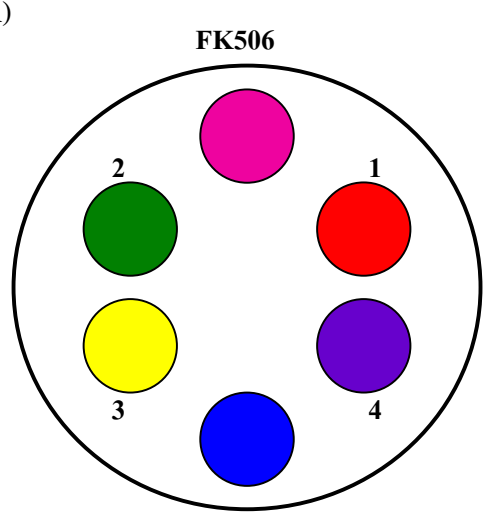
Family	Code name	Reflux with Dichlorome-thane (1)	Reflux with 95% ethanol (2)	Boiling (3)		Soaking with 70% ethanol (4)
				3 mg/ml	20 mg/ml	
Punicaceae	PGR	-	-	-	-	-
Rubiaceae	HFO	-	-	-	-	-
	PFO	-	-	-	-	-
Rutaceae	AEMA	-	-	-	-	-
Salvadoraceae	ASA	-	-	-	-	-
Saururaceae	HCO	-	-	-	-	-
Scrophulariaceae	AHI	-	-	-	-	-
Zingiberaceae	AKR	-	-	-	-	-
	ANI	-	-	-	-	-
	BPA	++++*	++++*	-	+	+++*
	CLO	-	-	-	-	-
	CXA	-	-	-	-	-
	CZE	-	-	-	-	-

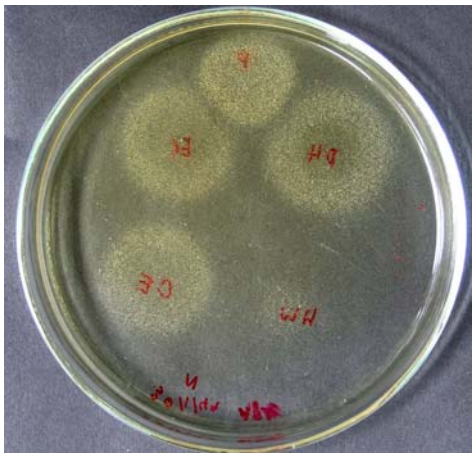
- = no growth; **+ =** growth; **+* =** ring-like growth

number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave +++

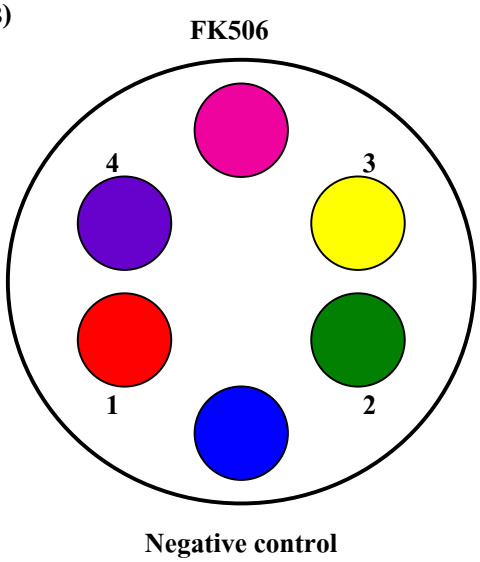
The crude extracts from two out of 51 medicinal plants, *Andrographis paniculata* and *Boesenbergia pandurata*, showed positive results when were used at 3 mg/ml from method 1,2 and 4. None of the crude extracts prepared by method 3 (boiling) gave positive result when used at 3 mg/ml. However, when increased the concentration of the crude extracts to 20 mg/ml, weak positive results were obtained from those of *A. paniculata* and *B. pandurata* (Table 3 and Fig. 3).

A)





B)



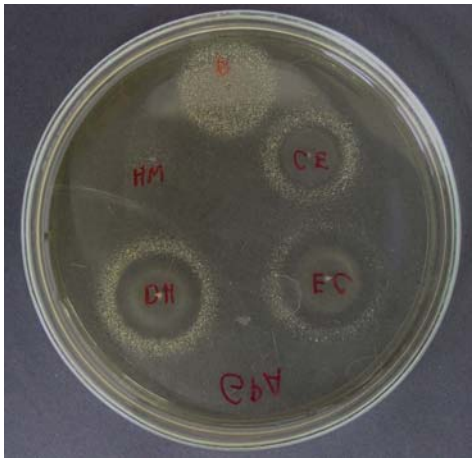


Fig 3. Growth of indicator cells tested with 4 different crude extracts of *Andrographis paniculata* (A) and *Boesenbergia pandurata* (B). Three mg/ml of each crude plant extracts prepared by method 1, 2, 4 and 20 mg/ml of that prepared by method 3 were dotted 5 µl each onto the assay plates. The plates were incubated at 30°C for 40 h. Positive control: 0.5 µM FK506; negative control: absolute ethanol; 1: Reflux with CH_2Cl_2; 2: Reflux with 95% ethanol; 3: Boiling; 4: Soaking in 70% ethanol

Crude extracts at 3 mg/ml prepared by method 1, 2 and 4 of *Andrographis paniculata* and those of *Boesenbergia pandurata* showed rather strong positive results while only weak growth could be detected in 20 mg/ml of the crude extracted prepared by method 3. This suggested that the bioactive compounds extracted by method 3 were rather in minute amount when compared to those obtained by the other methods. Therefore, boiling may not be a suitable method for preparing the crude extracts for the assay. In addition, the crude extracts obtained by method 1, 2 and 4 from *B. pandurata* showed ring-like growth on the assay plate. This ring-like growth pattern could be explained by 2 possibilities. First, it may due to the concentration effect in which at high concentration (near the dotted area) showed toxic effect to indicator cells while at lower concentration (far from dotted area) can be able to promote cell growth. The other possibility is that the crude extracts contain more than one bioactive compound in which one compound is toxic to the cells and another one promotes cell growth.

3. Comparison on the state of sample between fresh or dry plant to be used for crude extract preparations

Of 51 crude plant extract being screened, two gave positive activities in the assays. These samples were in dry form. We wondered the activity of active compounds in the positive samples whether in fresh or dry form will give better activities in the assay. To test this, fresh samples of the two positive plants, *Andrographis paniculata* and *Boesenbergia pandurata,* were collected. Each sample was divided into two equal weights: one for fresh and the other for dried form preparations of

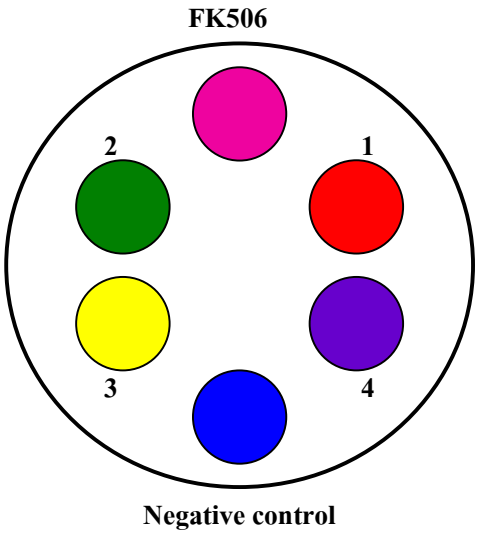
crude extracts. For fresh sample preparation, the sample was soaked in 95% ethanol and blended. After soaking for 3 days and followed by sonication for 1 minute prior to separate the liquid part by filtration, the liquid parts were collected. This soaking in 95% ethanol step was repeated three times for each sample. The liquid parts from each sample were mixed and evaporated. Five mg/ml of crude extracts prepared from either fresh or dry form preparation were assayed in the yeast screening system. The results were shown in Table 4.

Table 4 Comparison on activities in the assay between fresh and air-dried form of positive plant samples.

Positive plant samples	Growth of the indicator cells	
	Fresh form	Air-dried form
APA (*Andrographis paniculata*)	++	++
BPA (*Boesenbergia pandurata*)	+*	++*

- = no growth; **+** = growth; **+*** = ring-like growth

number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave +++ growth activity.



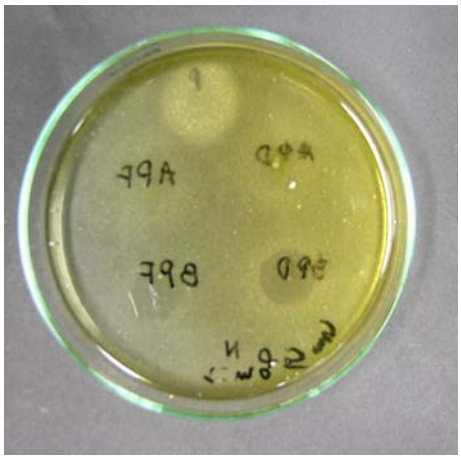


Fig 4. Growth of indicator cells tested with crude extracts of fresh and air-dried of *Andrographis paniculata* and *Boesenbergia pandurata.*The plates were incubated at 30°C for 40 h. Positive control: 0.5 μM FK506; negative control: absolute ethanol; 1: Fresh form of *Andrographis paniculata*; 2: Air-dried form of *Andrographis paniculata*; 3: Fresh form of *Boesenbergia pandurata*; 4: Air-dried form of *Boesenbergia pandurata.*

On comparison the activity in the yeast assays between the crude extract prepared from fresh form and the air-dried form of plants, the latter clearly gave better activities than the fresh one of the same plant samples (Fig 4). We, then, chose air-dried form of the plants and soaking in ethanol for further crude plant extract preparation for the rest of the screens.

4. Screening of bioactive compounds from plants in yeast screening system

To establish the yeast screening system, we had optimized several parameters in the assays, compared the methods of crude extract preparation and the condition of the samples to be tested. We

chose air-dried form of plants as a starting material and soaking in ethanol as a method for crude extract preparation for further screening of the bioactive compounds from the plants. However, crude extract preparation by soaking in 70% ethanol method resulted in expansion of volume of crude extract liquid which causes too long evaporation time. We decided to change from soaking in 70% ethanol to 95% ethanol to reduce the evaporation time for each sample preparation. The screening of bioactive compounds using yeast screening system was performed and the results were shown in table 5.

Table 5 Screening of bioactive compounds in Thai medicinal plants using the Δ*zds1* yeast mutant assay system

Family	Code*	Species	Yeast screening Results
Acanthaceae	AVA	*Adhatoda vasica* Nees	-
	BPU	*Barleriaiu pulina* Lindl.	-
	TLA	*Thunbergia laurifolia* L.	-
Aizoaceae	GOP	*Glinus oppositifolius* A.DC.	-
Alliceae	ALSA	*Allium sativum* Linn.	-
Amaranthaceae	IHE	*Iresine herbstii* Hook f.	-

Family	Code*	Species	Yeast screening Results
Anacardiaceae	MUS	*Melanorrhoea usitata* Wall.	-
Apiaceae	CEAS	*Centella asiatica* Urb.	-
	ASC	*Alstonia scholaris* (Linn.) R. Br.	-
Araceae	AIN	*Alocasia indica* Schott var. metallica Schott	-
	AOD	*Alocasia odorata C. Koch*	-
	ABL	*Amorphophallus companulatus* Blume.ex Dene	-
	LSP	*Lasia spinosa* Thw.	-
	TRO	*Typhonium roxburghii* Schott.	-
Asparagaceae	ARA	*Asparagus racemosus* willd	-
Avicenniaceae	AAL	*Avicennia alba Bl.*	-
Bixaceae	BOR	*Bixa orellana* L.	-
Caesalpiniaceae	CTO	*Cassia tora Linn.*	-
Caricaceae	CPAA	*Carica papaya Linn.*	-

Family	Code*	Species	Yeast screening Results
Cleomaceae	CVI	*Cleome viscosa* Linn.	-
Combretaceae	LLI	*Lumnitzera littorea Voigt*	-
Compositae	ACO	*Ageratum conyzoides Linn.*	-
	ASCO	*Artemisia scoparia Waldst. & Kit.*	-
	CTI	*Carthamus tinctorius Linn.*	-
	CAN	*Centratherum anthelminthicum (Willd.) Kuntz.*	-
	EST	*Eupatorium stoechadosnum* Ham	-
	GPS	*Gynura pseudo* – china hispida	-
	VCI	*Vermonia cinerea* Less.	-
Cucurbitaceae	GIN	*Gymnopetalum integriforium* Kurz.	-
	MCH	*Momordica charantia* L.	-
	MOCO	*Momordica cochinensis* (Lour.) Spreng.	-

Family	Code*	Species	Yeast screening Results
Euphorbiaceae	BOV	*Bridelia ovata* Decne	-
	PAC	*Phyllanthus acidus* Skeels.	-
	PEM	*Phyllanthus emblica* Linn.	-
Dioscoreaceae	DHI	*Dioscorea hispida Dennst.*	-
Euphorbiaceae	BMO	*Baliospermum montanum Muell. Arg.*	-
	CTIG	*Croton tiglium Linn.*	-
	EHI	*Euphorbia hirta Linn.*	-
Flacourtiaceae	HAN	*Hydnoccarpus anthelminthicus* Pierre.	-
Goodeniaceae	STA	*Scaevola taccada Roxb.*	-
Gramineae	CDA	*Cynodon dactylon* Pers	-
Guttiferae	GMA	*Garcinia mangostana L.*	-
	GCO	*Garcinia cowa Roxb.*	-
Labiatae	OSA	*Ocimum sanctum Linn.*	-
	OBA	*Ocimum basilicum Linn.*	-

Family	Code*	Species	Yeast screening Results
Leguminosae	APR	*Abrus precatorius* Linn.	-
	CFI	*Cassia agnes* Brenan	-
	EVA	*Erythrina variegata* L.	-
	MPI	*Mimosa pigra* L.	-
Malvaceae	HSA	*Hibiscus sabdariffa* linn.	-
Melastomataceae	MPO	*Melastoma polyanthum Bl.*	
Meliaceae	AIN	*Azadirachta indica* A.Juss var.siamensis *Valet*	-
Menispermaceae	CPA	*Cissampelos pareira* Linn.	-
	TTI	*Tiliacora triandra Diels.*	
Milacaceae	SMI	*Smilax* spp. S	-
Mimosoideae	ARU	*Acacia rugata* Merr.	-
	ACA	*Acacia catechu* Willd.	-
	MAL	*Morus alba L.*	-
Nelumbonaceae	NNU	*Nelumbo nucifera Gaertn*	-
Orchidaceae	LDI	*Ludisia discolor* (Ker-Gawl.) A.Rich.	-

Family	Code*	Species	Yeast screening Results
Pandanaceae	POD	*Pandanus odoratissimus* L.f.	-
Plantaginaceae	PMA	*Plantago major Linn.*	-
Poaceae	CNA	Cymbopogon nardus Rendle	-
Rhizophoraceae	RMU	*Rhizophora mucronata* Poir.	-
Rubiaceae	MCI	*Morinda citrfolia Linn.*	-
Sapindaceae	CHA	*Cardiospermum halicacabum* L.	-
Scrophulariaceae	SDU	*Scoparia dulcis* L.	-
Simaroubaceae	ELO	*Eurycoma longifolia* Jack.	-
Solanaceae	SST	*Solanum stramonifolium Jacq.*	-
Sonneratlaceae	SOV	*Sonnertia ovata Back.*	-
Sterculiaceae	AAU	*Abroma augusta Linn.f.*	-
	SMA	*Scaphium macropodum Beaumee*	-
Umbelliferae	AGR	*Anethum graveolens Linn.*	-
	CASI	*Centella asiatica Linn.*	-

Family	Code*	Species	Yeast screening Results
	CCY	*Cuminum cyminum Linn.*	-
	EFO	*Eryngium foetidum Linn.*	-
	HSI	*Heracleum siamicum Craib var. gracilius Craib*	-
Verbenaceae	VTR	*Vitex trifolia* Linn.	-
Zingiberaceae	AVI	*Amomum villosum* Lour. var.xanthioides *(Wall)*	-
	AOF	*Alpinia officinarum* Hance.	-
	KPA	*Kaempferia parviflora Wall.*	++*
	ZZE	*Zingiber zerumbet Smith*	-

- = no growth; **+ =** growth; **+* =** ring-like growth

number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave +++

On the screening of 143 crude ethanol extract from plants that can support growth of the yeast indicator cells *zds1*Δ, we found three that showed positive results (Table 3 and 5; Fig 5). All of

the positive crude extracts were retested and showed reproducibility of the results (data not shown). To see whether the three plant extracts show positive effect only in the presence of $CaCl_2$ in the assay plate or not. Five μl of three mg/ ml of each crude extracts of *A. paniculata* (APA), *B. pandurata* (BPA) and *K. parviflora* (KPA) were dotted in the assay plates containing 150mM $CaCl_2$ (A) or without $CaCl_2$(B). The results were shown in Fig 5.

A) 150 mM $CaCl_2$ **B) 0 mM $CaCl_2$**

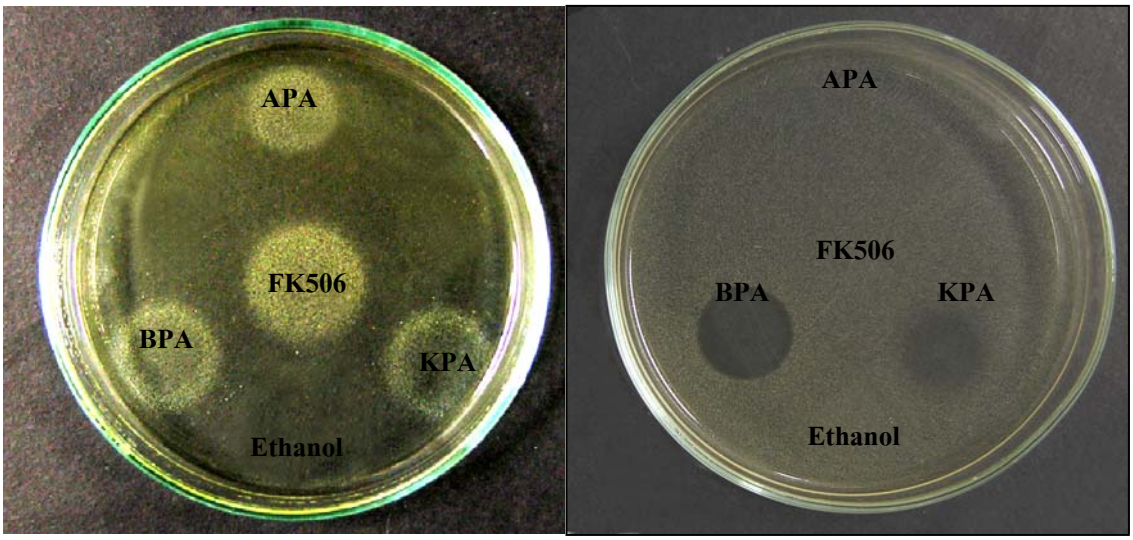


Fig 5. The three positive crude extracts showed activity only in the presence of Ca^{2+}.

Three mg/ml of each crude plant extracts of *Andrographis paniculata* (APA), *Boesenbergia pandurata* (BPA) and *Kaempferia parviflora* (KPA) were dotted for 5 µl each onto the assay plates; (A) containing 150 mM $CaCl_2$ (B) without $CaCl_2$. The plates were incubated at 30°C for 40 h. Positive control: 3 µl of 0.2 μM FK506; negative control: 5 µl absolute ethanol.

In the absence of $CaCl_2$, Δ*zds1*cells can grow and there was no difference of the yeast growth in the areas that were dotted with FK506 or absolute ethanol or APA (Fig. 5). For those of

BPA and KPA, no better growth of indicator cells were observed than those of background but clear zones were observed instead (Fig. 5). It could be concluded that the positive activity from the three plant extracts was specific to the presence of $CaCl_2$. There should be at least one toxic component in the crude extracts of BPA and KPA which results in clear zone at the dotted area.

5. Separation and purification of bioactive compounds using yeast based assay.

From the three positive samples obtained, we first chose to purify the crude extract from *B. pandurata* dued to the ease of its availability.

5.1. Large scale preparation of *B. pandurata* crude extract

Fresh *B. pandurata* were purchased from market and air-dried. The chopped-dried rhizomes (2.4 kg) were soaked in CH_2Cl_2 for 7 days at room temperature. After filtration, the filtrate was evaporated under reduced pressure until drying. This procedure was repeated three times to obtain CH_2Cl_2 crude extract 233.45 g. The residue was re-extracted with EtOAc and CH_3OH, respectively, in the same manner as that for CH_2Cl_2 extraction. The EtOAc and CH_3OH crude extracts were obtained in 28.66 g and 2.30 g, respectively. The extraction procedure and the results of the assay in the yeast system of each fraction are shown in Fig 6.

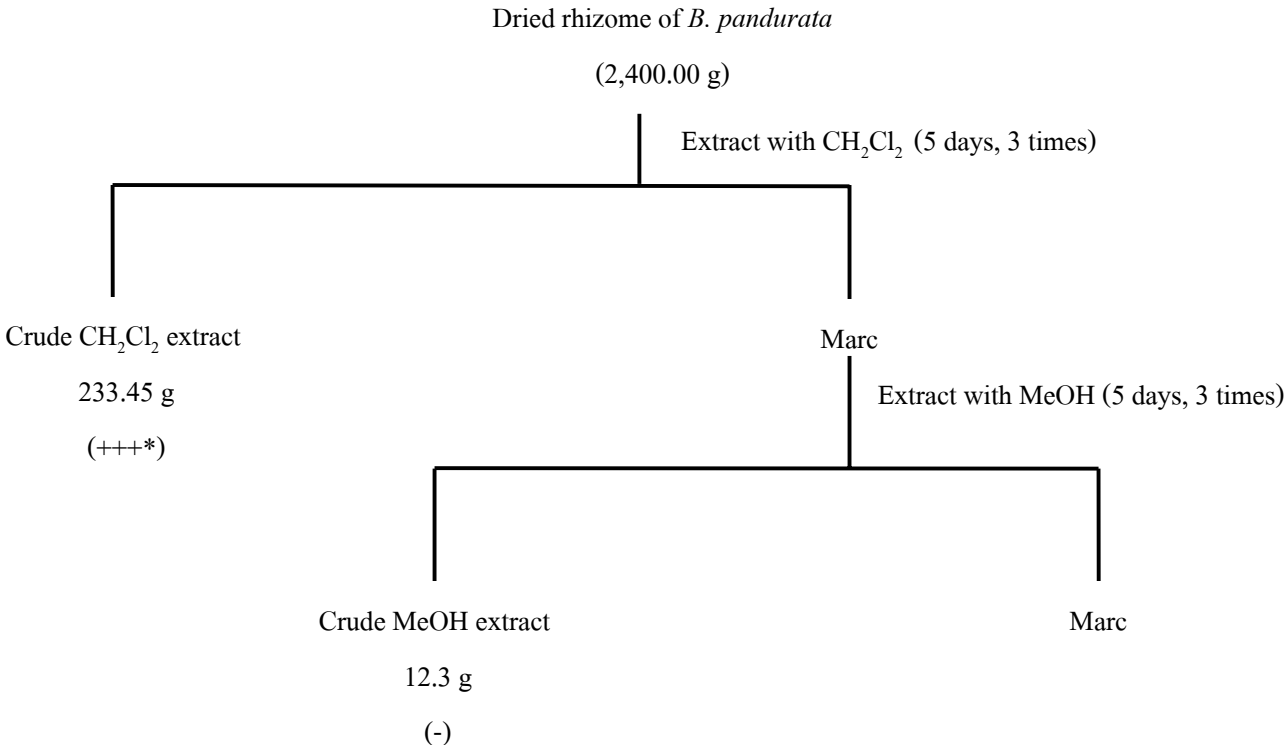


Fig 6 Yeast based assay activity guided fractionation of *B. pandurata*

Note: (-) = no growth; (+) = growth; (+*) = ring-like growth of yeast indicator cells in the bioassay

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave +++ of growth.

The results clearly indicated that the biological activity was found in CH_2Cl_2 crude extract.

5.2. Separation and purification of the positive fractions of *B. pandurata*

5.2.1 Separation of CH_2Cl_2 crude extract

According to the biological activity on yeast based assay, 100 g of CH_2Cl_2 crude extract was fractionated by silica gel quick column. The column was initially eluted by hexane, followed by the addition of increasing polarity of solvent, EtOAc. Each fraction was monitored by TLC and fractions

containing the same or similar components were combined. Each fraction was tested with yeast based assay. The results of the separation of CH_2Cl_2 crude extract and yeast based assay of each fraction are shown in Table 6.

Table 6 The separation of CH_2Cl_2 extract by silica gel quick column and their biological activity in the yeast assay

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA1	100% hexane	Yellow oil	(clear zone)	2.31
BPA2	5-20% EtOAc	Yellow crystal	(++*)	29.5
BPA3	40% EtOAc	Brownish oil	(+++*)	30.17
BPA4	60% EtOAc	Brownish wax	(clear zone)	3.72
BPA5	80-100% EtOAc, 5-50% MeOH	Brownish wax	(-)	13.52

- = no growth; + = growth; +* = ring-like growth; clear zone = no growth and toxic to indicator cells

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

The results obtained showed that BPA3 fraction exhibited the strongest biological activity and differed from the others. Therefore, fractions BPA3 and BPA2 would be subjected for further separation.

5.2.1.1 Separation of fraction BPA2

The fraction BPA2 29.50 g was re-separated by silica gel column eluted with 5%EtOAc-hexane to 60%EtOAc-hexane as solvent. The fractions were collected and combined as monitoring by

TLC pattern resulted in 3 subfractions (BPA2/1-BPA2/3). The results of separation are shown in Table 7.

Table 7 The separation of subfraction BPA2 by silica gel column and their biological activity in the yeast assay.

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA2/1	5% EtOAc-hexane	Yellow oil	(-)	0.5
BPA2/2(s)	10-20% EtOAc-hexane	White crystal	(+++*)	9.65
BPA2/2		Yellow oil	(-)	7.54
BPA2/3	40-60% EtOAc-hexane	Brownish oil	(-)	7.17

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

The result from Table 7 revealed that a pure compound was obtained from BPA2/2 as white crystal (**Compound 1).**

5.2.1.2 Separation of fraction BPA3

The fraction BPA3 30.17 g was re-separated by silica gel column eluted with 5%EtOAc-hexane-100%EtOAc. The fractions were collected and combined according to TLC pattern resulted in 3 subfractions (BPA3/1-BPA3/3). The results of the separation of fraction BPA3 and the biological activity in yeast based assay of each subfractions are shown in Table 8.

Table 8 The separation of fraction BPA3 by silica gel column and their biological activity in the yeast assay.

Fractions	Solvent system	Remarks	Bioassay with yeast	Weights (g)
BPA3/1	5-20%EtOAc-hexane	Brownish oil	(+)	14.25
BPA3/1(s)		Cream powder	(++*)	0.60
BPA3/2	40-60%EtOAc-hexane	Brownish solid	(++*)	13.16
BPA3/3	80%EtOAc-hexane - 100% EtOAc	Brownish wax	(-)	2.31

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

A pure compound was obtained from BPA3/1 as cream powder (compound **2**).

The brownish oil of BPA 3/1 (14.25 g) was separated by silica gel column eluted with 5-20% EtOAc-hexane as eluent and the fractions were collected and combined according to TLC pattern resulted in 4 subfractions (BPA3/1/1-BPA3/1/4). The results of the separation are shown in Table 9.

Table 9 The results of the separation of BPA3/1 by silica gel column and their biological activity in the yeast assay.

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA3/1/1(s)	5-20%EtOAc-hexane	Yellow crystal	(+++*)	0.85
BPA3/1/1		Yellow oil	(-)	0.29
BPA3/1/2	40-60%EtOAc-hexane	Brownish oil	(-)	7.40
BPA3/1/3	80%EtOAc-hexane-100%EtOAc	Brownish oil	(-)	4.65
BPA3/1/4	80%EtOAc-hexane-100%EtOAc	Brownish wax	(-)	0.81

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

A pure compound was obtained from BPA3/1/1 as yellow crystal (compound **1**).

The brownish solid of BPA3/2 (13.16 g) was separated by silica gel column eluted with 10%EtOAc-hexane-100% EtOAc as solvent and the fractions were collected and combined according to TLC pattern resulted in 3 subfractions (BPA3/2/1-BPA3/2/3). The results of the separation are shown in Table 10.

Table 10 The separation of BPA3/2 by silica gel column and their biological activity in the yeast assay.

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA3/2/1(s)	10-20%EtOAc-hexane	Yellow crystal	(+++*)	0.35
BPA3/2/1		Brownish solid	(++*)	7.89
BPA3/2/2	40-60%EtOAc-hexane	Brownish wax	(-)	2.38
BPA3/2/3	80%EtOAc-hexane-100%EtOAc	Brownish wax	(-)	1.49

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

A pure compound was obtained from BPA3/2/1 as yellow crystal (compound **1**).

The brownish solid of BPA 3/2/1 (7.89 g) was further separated by silica gel chromatography eluting with 5%EtOAc-hexane-100%EtOAc as solvent and the fractions were collected and combined according to TLC pattern resulted in 5 subfractions (BAP3/2/1/1-BPA3/2/1/5). The results of the separation are shown in Table 11.

Table 11 The separation of BPA3/2/1 by silica gel column and their biological activity in the yeast assay.

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA3/2/1/1	5-10%EtOAc-hexane	Yellow crystal	(+++*)	0.41
BPA3/2/1/2 (s) **BPA3/2/1/2**	20-40%EtOAc-hexane	Cream powder Cream solid	(+++*) (+++*)	1.13 1.50
BPA3/2/1/3	20-40%EtOAc-hexane	Brown solid	(+++*)	0.43
BPA3/2/1/4 (s) **BPA3/2/1/4**	40-60%EtOAc-hexane	Red crystal Red solid	(+++*) (+++*)	0.60 0.15
BPA3/2/1/5 (s) **BPA3/2/1/5**	80%EtOAc-hexane - 100% EtOAc	Yellow powder Yellow solid	Clear zone Clear zone	0.06 2.31

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

Three pure compounds were obtained: BPA3/2/1/1, BPA3/2/1/3 as yellow crystal (compound **1**), BPA3/2/1/2 as cream powder (compound **2**), and BPA3/2/1/4 as red crystal (compound **3**). The structures of these compounds will be further elucidated.

5.2.1.3 Separation of fraction BPA4

The brownish wax of BPA4 (3.72 g) was separated by silica gel column eluted with 5%EtOAc-hexane-100%EtOAc as eluent and the fractions were collected and combined according to

TLC pattern resulted in 4 subfractions (BAP4/1-BPA4/4). The results of the separation are shown in Table 12.

Table 12 The separation of BPA4 by silica gel column and the activity guided fractionation by the yeast based assay.

Fractions	Solvent system	Remarks	Yeast based assay	Weights (g)
BPA4/1	5-10 % EtOAc	Yellow crystal	(++)	0.05
BPA4/2	20-40% EtOAc	Brownish oil	(++*)	0.15
BPA4/3 (s)	80-100% EtOAc	Yellow powder	Clear zone	0.06
BPA4/3		Yellow powder	Clear zone	0.06
BPA4	80-100% EtOAc	Brownish wax	(-)	2.78

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

The BPA4/1 and BPA4/2 fractions were positive in the yeast based assay, however, the yield obtained were rather low. Therefore, these two positive fractions were not further separated.

5.3 Structural elucidation of the active pure compounds from *B. pandurata*

5.3.1 Structural elucidation of compound **1**

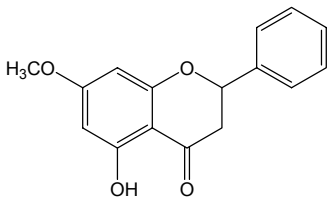
The fractions of BPA2/2, BPA3/1/1,BPA3/2/1 and BPA3/2/1/1 with 10-20% EtOAc-hexane gave pale-yellow crystal. After several recrystallization with a mixture of EtOAc and hexane, bright

pale-yellow crystal were obtained, designated as compound 1, m.p. 101-102 °C, R_f 0.51 (silica gel: CH_2Cl_2).

The ^{1}H-NMR ($CDCl_3$) displayed the proton chemical shifts at δ_H 2.82 (1H, d, *J* = 17.1 Hz), 3.09 (1H, t, *J* = 13.1 Hz), 3.80 (3H, s), 5.41 (1H, dd, *J* = 3.1, 3.1 Hz), 6.07 (2H, dd, *J* =2.5, 2.5 Hz), 7.46 (5H, m) and 12.02 (1H, s).

The ^{13}C-NMR ($CDCl_3$) exhibited thirteen carbon signals of sixteen carbons at δ_C 43.3, 55.6, 79.1, 94.2, 95.1, 103.1, 126.1(2C), 128.8(3C), 138.3, 162.7, 164.1, 167.9 and 195.7.

According to the spectroscopic data attained compared with those reported in the literature, compound **1** was identified as **pinostrobin** or **5-hydroxy-7-methoxyflavanone** (Burke and Nair, 1986; Tanaka *et. al*., 1985; Itokawa *et. al*., 1981); Cheenpracha, S., *et al.* 2006).



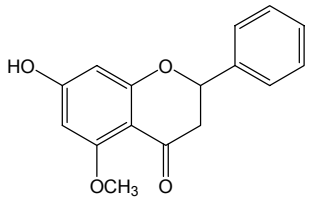
5.3.2 Structural elucidation of compound **2**

After the concentrated subfraction BPA3/1and BPA3/2/1/2 were left overnight, the yellow crystals were precipitated. After recrystallization from a mixture of methanol and dichloromethane for several times, compound **2** as white amorphous compound were obtained.

The ^{1}H-NMR ($CDCl_{3,}$ ppm) displayed the proton chemical shifts at δ_H 2.72 (1H, dd, J = 3.4, 3.1 Hz), 2.97 (1H, q, J = 12.5 Hz), 3.82 (3H, s), 5.41 (1H, dd, J = 3.1, 3.1 Hz), 6.08 (2H, dd, J = 2.4, 2.1 Hz), 7.34 (1H, m), 7.40 (2H, m) and 7.47 (2H, m)

The ^{13}C-NMR ($CDCl_{3,}$ ppm) exhibited thirteen carbon signals of sixteen carbons at δ_C 46.4, 56.2, 80.0, 94.5, 97.2, 105.9, 127.2(2C), 129.6(3C), 140.7, 164.4, 166.6, 167.1 and 191.8.

According to the spectroscopic data and compared with those reported in the literature, compound **2** was identified as **alpinetin** or **7-hydroxy-5-methoxyflavanone** (Burke and Nair, 1986; Tanaka *et. al*., 1985; Itokawa *et. al*., 1981; Shindo, K., *et al.,* 2006).

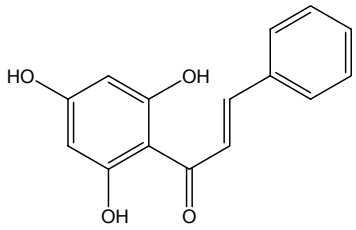


5.3.2 Structural elucidation of compound **3**

After the concentrated subfraction BPA3/2/1/4 was left overnight, the yellow crystals were precipitated. After recrystallization from a mixture of CH_3OH and CH_2Cl_2 for several times, compound **3** as read crystal was obtained.

The ^{1}H-NMR ($CDCl_3$) displayed the proton chemical shifts at 7.70 (m, 2,6-H), 7.45 (m, 3,4,5-H), 8.28 (d (br), J = 15.5 Hz, olefinic proton), 7.78 (d, J = 15.5 Hz, olefinic proton) and 6.00 (s, 3′, 5′H)

According to the spectroscopic data and compared with those reported in the literature, compound **3** was identified as pinocembrin chalcone or 2′,4′,6′-trihydroxychalcone (Bremner and Mayer, 1998).



To see whether the ring-like growth pattern in the yeast based assay is the result of concentration affected cell growth, pinostrobin, alpinetin and pinocembrin chalcone were tested in the yeast assay with various concentrations and the results were shown in Table 13.

Table 13 Minimal effective concentration of the three pure compounds from *B. pandurata* in the yeast based assay

Concentration (mM)	Yeast based assay		
	Pinostrobin	Alpinetin	Pinocembrin chalcone
2.00	+++*	++*	+++*
1.75	+++*	++*	+++*
1.50	+++*	++*	++*
1.25	++*	++	++*
1.00	++	+	+*
0.75	++	weak	+*
0.5	+	-	weak*
0.25	-	-	-

- = no growth; + = growth; +* = ring-like growth

Number of + indicates the strength of biological activity relative to that of positive control (FK506) which gave ++++ of growth.

Results from Table 13 revealed that pinostrobin exhibited the highest biological activity among the three. The minimal effective concentration of pinostrobin, alpinetin and pinocembrin chalcone in the yeast based assay was 0.5, 1 and 0.75 mM, respectively. The ring-like growth pattern on the yeast assay plates was the result of dosage effect on growth. At higher concentration (at center or near the dotted area) causes toxicity to the yeast indicator cells and as a result, clear zone was observed at and near dotted area. At lower concentrations where the compound was diffused far away from dotted area, causes no toxicity to the yeast cells and results in cell growth. At minimal effective

concentration, pinocembrin chalcone but not alpinetin and pinostrobin that showed the ring-like growth pattern. Therefore, the ring-like growth pattern observed in alpinetin and pinostrobin tested plate was the result of dosage effect that caused toxicity to the indicator cells. However, the ring-like growth pattern observed in pinocembrin chalcone tested plate suggested that this compound is the most toxic to the yeast cells.

Because of the highest yield obtained after purification, the highest biological activity in the yeast based assay and the lowest toxicity to the yeast cells, only pinostrobin was chosen for further characterizations.

6. Some characterizations on a pure compound, pinostrobin from *B. pandurata*

The hyperactivation of Ca^{2+} signals in the mutant yeast strain Δ*zds1* caused cells to arrest or delay at G2 phase of the cell cycle with abnormal bud morphology (Mizunuma *et. al*. 1998).
The three pure compounds of *B. pandurata* that are pinostrobin, alpinetin and pinocembrin chalcone showed growth recovery of *zds1*Δ cells cultivated on high calcium containing plates. The data suggested that the pure compounds can genetically inhibit the hyperactivation effect of Ca^{2+}. To see whether the pure compounds can alleviate the effect of Ca^{2+} hyperactivation caused the cells to arrest or delay at G2 phase and having abnormal bud morphology, Flow cytometry analysis and the cell morphology were examined.

6.1 Effect of Pinostrobin on alleviating the *zds1*Δ cells from G2 to M phase delay as monitoring by Flow cytometer

The phase of yeast cells could be determined by the number of DNA content in the cells. In the log phase of yeast cell suspension, two populations of cells appears as 1 and 2 copies of DNA content (1C and 2C), respectively. The 1C peak indicates the yeast cells in G1, early S, and M phase. The 2C peak indicates the yeast cells in late S or G2 phase. Here, we interested in determining the G2 and M phases of yeast cells, thus, the G2 cells contain in the 2C peak while the M phase cells correspond to the 1C peak in the flow cytometry profile.

6.1.1 Optimal condition for monitoring the phases of yeast cell by flow cytometer

To find the optimal condition for determining the phase of *zds1*Δ cell*s* after $CaCl_2$ activation, the yeast cells were cultivated in YPD with varying concentration of $CaCl_2$ and incubation time as 50-100 mM and 3-6 hrs, respectively. The treated cells were stained with propidium iodide prior to be analysed by flow cytometer. The results were shown in fig. 7.

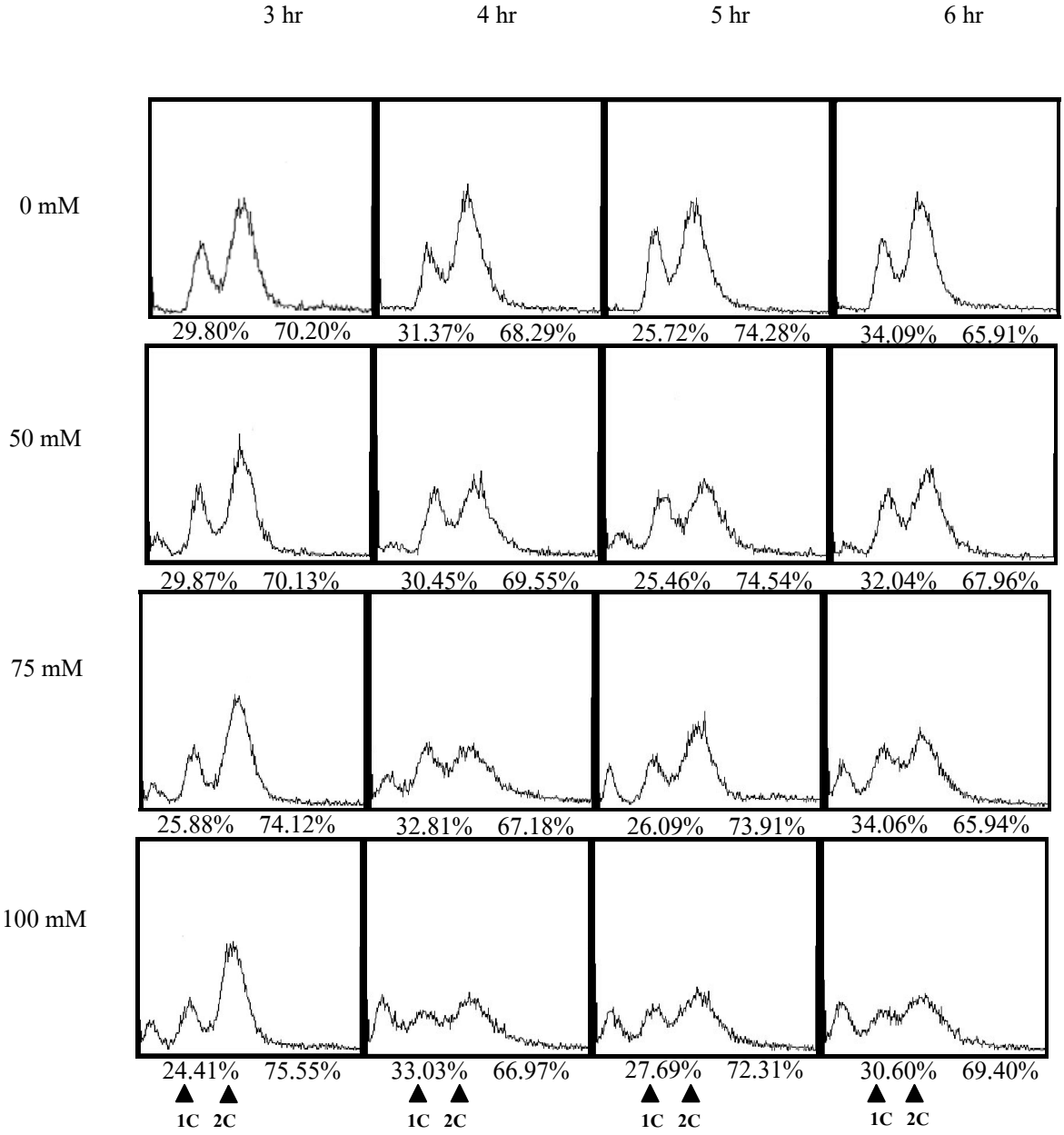


Fig. 7 Flow cytometry profile of *zds1*Δ cells activated by varying concentration of $CaCl_2$ and incubation time.

The *zds1*Δ cells were cultivated in YPD medium with varying concentration of $CaCl_2$ from 50-100 mM and incubated for 3-6 hours, respectively. The harvested cells were fixed and stained with propidium iodide (PI) before flow cytometry analysis. The number under the peaks indicates percentage of cells with 1C and 2C DNA content, respectively.

With the increment increased in $CaCl_2$ concentration, 1C cells population of *zds1*Δ cells was gradually lower while the 2C cells population was slightly higher when compared to that of without $CaCl_2$ addition (Fig. 7 at the 100 mM$CaCl_2$ compared to o mM $CaCl_2$ at 3 hrs treatment). The *zds1*Δ cells die increasingly with the longer time of $CaCl_2$ exposure (Fig. 7). From the results obtained, the chosen condition for flow cytometer measurement was at 100 mM $CaCl_2$ incubated for 3 hrs.

Since the hyperactivation of Ca^{2+} signals causes cells to have abnormal bud emergence with elongated buds and unequaled nuclear division between mother and daughter cells. The morphology of *zds1*Δ treated cells at 100 mM $CaCl_2$ incubated for 3 hrs were observed under microscope with 40X magnification (Fig. 8). The elongated buds were only observed in the cells cultivated in 100 mM $CaCl_2$ condition (Fig. 8A) while normal bud morphology was shown in those grown in medium without $CaCl_2$ (Fig. 8B). Thus, the condition of 100 mM $CaCl_2$ with 3 hrs exposure is enough to activated the *zds1*Δ cells.

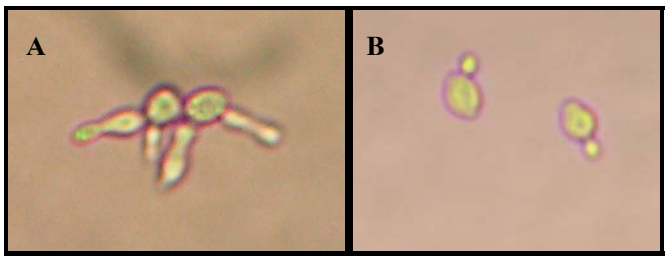


Fig. 8. Microscopic morphogy of the *zds1*Δ cells activated by 100 mM$CaCl_2$.
The *zds1*Δ cells were cultivated in YPAUD broth for 3 hrs A) In the presence of 100 mM $CaCl_2$ B) Without $CaCl_2$. The cells were observed under 40X light microscope.

6.2 Effect of pinostrobin on alleviating the *zds1*Δ cells from abnormal bud emergence caused by the hyperactivation of the calcium signals.

The physiological consequences such as G2-M cell cycle arrest and polarized bud growth were observed in Ca^{2+} hyperactivation of *zds1*Δ cells (Shitamukai *et al*, 2000). In order to confirm the genetic evidence which suggested that pinostrobin is an inhibitor of Ca^{2+} signaling pathway (Fig. 1), the phase of cell cycle and cell morphology were examined on the Ca^{2+} hyperactivated pinostrobin pretreated *zds1*Δ cells. The exponential phase of *zds1*Δ cells was harvested. Either 1 mM pinostrobin or 500 nM FK506 (as positive control) was preincubated with *zds1*Δ cells prior to addition of $CaCl_2$ (100 or 150 mM) and incubated for 3 hrs. The treated cells were harvested and stained with propidium iodide before flow cytometry analysis. The results were shown in Fig. 9.

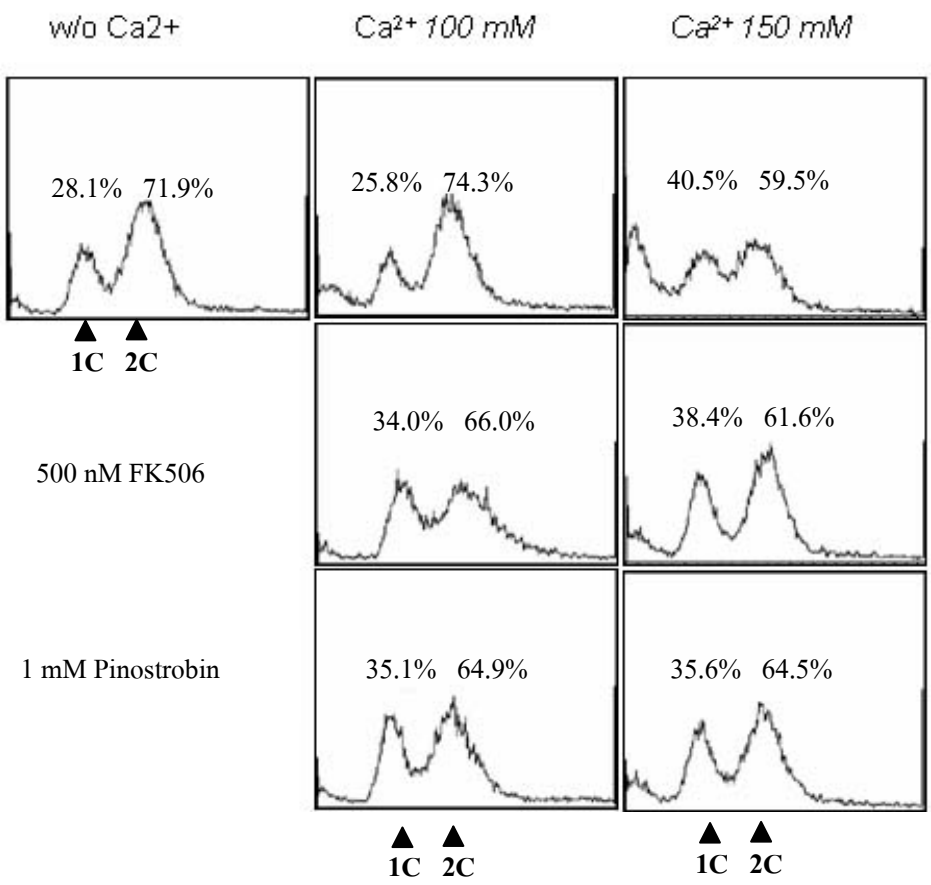


Fig. 9 Flow cytometry profile of pinostrobin treated *zds1*Δ cells under Ca^{2+} hyperactivation condition

The *zds1*Δ cells was pretreated with either 1 mM pinostrobin or 500 nM FK506 (as positive control) or DMSO (as negative control), incubated at 30°C for 30 min at room temperature prior to being cultivated in medium with either 100 or 150 mM $CaCl_2$ for 3 hrs.

At 100 mM $CaCl_2$, the yeast cells pretreated with solvent (DMSO; as negative control) showed higher population of cells with 2C DNA content (75.2%) than that of cells 1C DNA content (25.7%). However, in the pinostrobin pretreated cells prior to 100 mM $CaCl_2$ exposure showed decreased in number of cells with 2C DNA content (64.9%) and increased in that of cells with 1C DNA content (35.2%). The same pattern of flow cytometry profile could be observed in cells pretreated with 500

nM FK506 (as positive control). However, treatment of either pinostrobin or FK506 after $CaCl_2$ exposure could not alleviate the effect of G2 phase delay resulting from Ca^{2+} hyperactivation (data not shown). This result revealed that pretreatment of cells with 1 mM pinostrobin could alleviate the yeast cells from G2 delay a consequence from Ca^{2+} hyperactivation. This indicates that pinostrobin is a Ca^{2+} signal inhibitor in yeast.

The hyperactivation of Ca^{2+} signals not only causes the *zds1*Δ cells from G2 phase delay but abnormal bud emergence (Mizunuma *et. al.* 1998). To confirm the effect of pinostrobin pretreatment that can prevent G2 delay in the yeast cells cultivated in high $CaCl_2$ concentration, the morphology of the cells was examined in parallel. The treated cells from the experiments in Fig 9. were fixed and the morphology of the cells were observed (Fig. 10). Also the fixed cells were stained with Hoechst 33342, the nucleus staining dye. The stained cells were examined under 40X fluorescence microscope (Fig. 11).

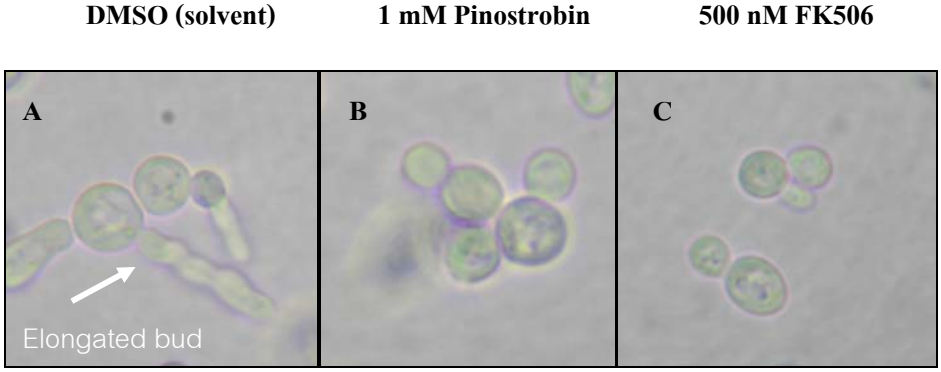


Fig. 10 The effect of pinostrobin pretreatment on morphology of Ca^{2+} hyperactivated *zds1*Δ cells

The Δ*zds1* cells were pretreated with either A) DMSO solvent B) 1 mM pinostrobin or C) 500 nM FK506 prior to being activated with 100 mM $CaCl_2$ for 3 hr at 30 °C. After being fixed, the cells were examined under normal light microscope (100X). The numbers indicate percentage of cells with an elongated bud at 6 h.

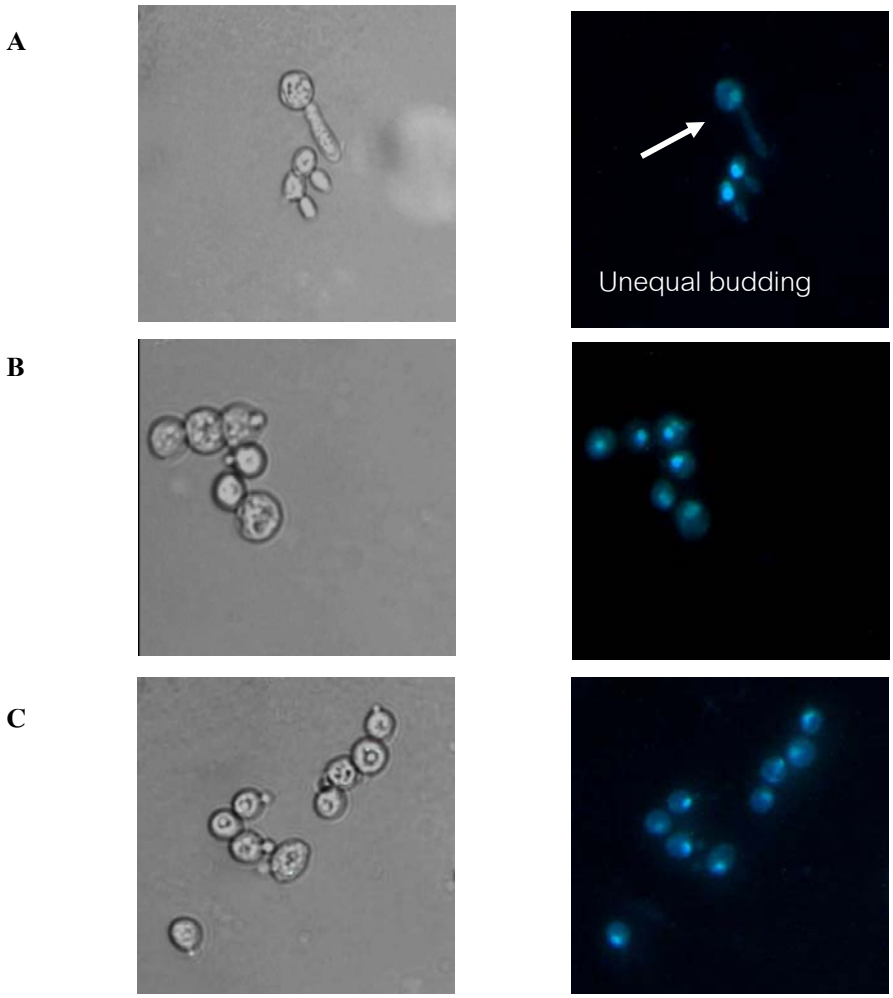


Fig. 11 The effect of pinostrobin pretreatment on the budding of Ca^{2+} hyperactivated *zds1*Δ cells

The *zds1*Δ cells were pretreated with either A) DMSO solvent B) 1 mM pinostrobin or C) 500 nM FK506 prior to being activated with 100 mM $CaCl_2$ for 3 hr at 30 °C. After the cells were fixed, nuclear stained with Hoechst 33342 dye and examined under fluorescence microscope (40X).

The Ca^{2+} hyperactivated cells pretreatment with DMSO solvent showed abnormal morphology with elongated buds (Fig. 10A) and have unequal budding (the nucleus was in only mother cell and not found in the daughter cell) as shown in fig. 11A. On the other hand, the cells

pretreated with either 1 mM pinostrobin or 500 nM FK506 showed normal morphology (Fig. 10B and 10C, respectively) with equal distribution of nucleus in mother and daughter cells (Fig. 11 B and 11C, respectively).

Conclusions

In this study, we have employed a novel drug screening system of the yeast, *Saccharomyces cereviciae* to search for an inhibitor of Ca^{2+} signaling pathway, using the growth dependent phenotype for detection. We first optimized the conditions for the assay system by varying the concentration of $CaCl_2$ and that of indicator cells. We found the optimal concentration of $CaCl_2$ was at 150 mM and that of indicator cells was at 3-6 X 10^5 cells/ml (Fig. 2) and we chose maceration in ethanol as a method for the crude extract preparation (Table 3; Fig. 3). As far as 143 crude Thai medicinal plant extracts were screened, only 3 positive samples were obtained, suggested that the screening is highly specific to the modulators of the Ca^{2+}- signaling pathways. The three positive plant extracts were *Andrographis paniculata*, *Boesenbergia pandurata* and *Kaempferia parviflora.*(Table 3; Table 5; and Fig. 5).

Andrographis paniculata (APA) or commonly known as "King of Bitters," is a member of the plant family *Acanthaceae*, and has been used as a medicinal herbs for centuries in Asia including Thai medical traditions, such as to treat GI tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases (http://www.altcancer.com/andcan.htm).

Boesenbergia pandurata (BPA) is member of the plant family Zingiberaceae. *B. pandurata* also known as finger root and was originally found in southern china and southest asia. In Thailand, it was consumed as a common vegetable. The rhizomes of *B. pandurata* are used as a food ingredient and a folk medicine for the treatment of colic disorder and as an aphrodisiac in southeat asian countries (Trakoontivakorn *et al*. 2001).

Kaempferia parviflora (KPA) is also a plant in family Zingiberaceae. In Thailand, it has been used as health promoting herb as well as for the treatment of colic disorder, peptic and duodenal ulcers. In addition, a tonic drink made from the rhizomes of *K. parvuflora* is believed to relieve impotent symptoms (Yenjai *et. al.* 2004).

Although, the three positive crude extracts were well known for their usages in traditional medicine, however, this screening procedure has employed a novel principle for bioactive compound detection, there will be a good possibility to rediscover previously identified compounds as possessing a novel activity.

Because of the ease on availability, the crude extract of *B. pandurata* was first chosen for further purification and some characterizations in this study. Column chromatography techniques in combination with TLC as well as biological activity in the yeast based assay were employed for monitoring fractionation and purification. Three isolated compounds were purified and characterized by nuclear magnetic resonance spectroscopy. The spectroscopic data revealed that all three active compounds were known compounds which were identified as pinostrobin, alpinetin (Burke and Nair 1986; Tanaka *et. al.* 1985; Itokawa *et.al*. 1981) and pinocembrin chalcone (Bremner and Mayer, 1998), respectively. All the three pure compounds have rather similar structures and contain dosage effect on cytotoxicity to the yeast indicator cells. The minimal effective concentration of pinostrobin, alpinetin and pinocembrin chalcone that could be detected for their biological activity in this yeast based assay was 0.5, 1 and 0.75 mM, respectively (Table 13). These three compounds showed

positive activity only in the presence of Ca^{2+} (Fig. 5) indicating that the positive results are Ca^{2+} dependency.

Among the three active compounds obtained in the assay, pinostrobin showed the highest activity in the yeast based assay and less toxicity to the yeast indicator cells, we therefore chose pinostrobin for further characterizations. The positive result of pinostrobin from *B. pandurata* in the yeast based assay indicates genetically that it can specifically attenuate the hyperactivation of Ca^{2+} signal in yeast (Fig. 5). To confirm the result from genetic observation, flow cytometry analysis as well as cell morphology and nuclear staining were performed. It was found that pretreatment of *zds1*Δ cells with 1 mM pinostrobin prior to being hyperactivated by Ca^{2+}signals could alleviate the cells from G2 phase delay as monitored by flow cytometry profile(Fig. 9) and abnormal bud emergence (Fig. 10). These pretreated cells with normal bud morphology also showed normal nuclear division between mother and dauther cells (Fig. 11). These data strongly indicates that pinostrobin from *B. pandurata* is an inhibitor of Ca^{2+} signaling activity in *S. cerevisiae*.

Ca^{2+} signals play pivotal roles in the regulation of diverse cellular processes, such as T-cell activation, secretion, muscle contraction, neurotransmitter release and probably many more (Clapham 1995). Therefore, the bioactive compounds that modulate the activity of the Ca^{2+} signals are expected to be of pharmacological interest since its potential target molecules cover a set of very wide range of well conserved pharmacologically interesting molecules such as calcineurin inhibitors, MAP kinase inhibitors, a glycogen synthetase kinase-3 family protein kinase inhibitor, protein kinase C inhibitor *etc* (Shitamukai *et. al*. 2000).

One of the crucial steps in drug development is the screening step. This study employed a novel yeast based assay with novel principle to screen for a bioactive compound from Thai plants. Because of several advantages of this yeast based screen such as *in vivo* system, positive screening, highly specific to Ca^{2+} signaling pathway, the pure compounds obtained from the screens suggested to be pharmacological interests. Here, as we found that pinostrobin from *B. pandurata* acts as an inhibitor of Ca^{2+} signal activity in yeast, it might be interesting to further evaluate its molecular target.

Acknowledgements

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Output ที่ได้จากโครงการ

1. Wangkangwan W, Boonkerd S, Chavasiri W**,** Sukapirom K, Pattanapanyasat K, Kongkathip N, Miyakawa T, **Yompakdee C**. Pinostrobin from *Boesenbergia pandurata* is an inhibitor of Ca^{2+}-signal-mediated cell-cycle regulation in the yeast *Saccharomyces cerevisiae*. Submitted to Bioscience Biotechnology and Biochemistry.

2. Boonkerd S, Chavasiri W, Miyakawa T, **Yompakdee C**. Screening of inhibitor of the Ca2+-signaling pathway from Thai medicinal plants using a zds1 null mutant yeast as a screening system. Manuscript in preparation.

3. Wangkangwan W, Chavasiri W, Kongkathip N, Miyakawa T, **Yompakdee C** (2006). Screening and some characterizations on bioactive compounds from Thai medicinal plants using yeast as a screening system: I. System optimizations. Poster presentation at the 18th Annual Meeting of the Thai Society for Biotechnology on "Biotechnology: Benefits & Bioethics " November 2-3. Bangkok, Thailand.

4. Wangkangwan W, Boonkerd S, Chavasiri W**,** Sukapirom K, Pattanapanyasat K, Kongkathip N, Miyakawa T, **Yompakdee C**. (2007) Screening and some characterizations of bioactive substances from Thai medicinal plants using a mutant yeast as a screening system. Poster presentation at the 6th Princess Chulabhorn International Science Congress. The interface of chemistry and biology in the "omics" era: Environment & health and drug discovery. November 25-29, Bangkok, Thailand.

ภาคผนวก

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3 Pinostrobin from *Boesenbergia pandurata* is an inhibitor of 4 Ca^{2+}-signal-mediated cell-cycle regulation in the yeast 5 *Saccharomyces cerevisiae*

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Abbreviations: NF-AT , nuclear factor of activated T cell; YPD, yeast extract peptone dextrose; TLC, thin layer chromatography; PI, propidium iodide

Abstract

Calcium signaling pathway is involved in regulation of diverse biological processes in eukaryotes. The high degree of gene conservation between humans and yeast, renders the yeast *S. cerevisiae* to be a fascinating organism in drug discovery. Upon screening for inhibitor of the Ca^{2+} signaling pathway using the *zds1*-null mutant yeast based assay as previously proposed[1)], a crude extract of *Boesenbergia pandurata* was one of the positive plant extracts obtained. Three pure compounds of pinostrobin, alpinetin and pinocembrin chalcone were isolated using the yeast-based assay and TLC pattern to guide fractionation and purification. Pinostrobin pretreated yeast cells could ameliorate the deleterious effect caused by Ca^{2+} dependent cell growth regulation such as G2/M phase cell cycle arrest as well as the abnormal morphology and budding of the yeast cells. This study confirms the genetic observation and indicates that pinostrobin involves in inhibitory of Ca^{2+} signaling activity in yeast.

Keywords: Yeast based assay; *Boesenbergia pandurata*; Pinostrobin; Ca^{2+} signal, inhibitor

Introduction

The search for novel drugs is still one of the major interests in the health related research. This dues to so many problems of currently used drugs such as high side effects, drug resistance, not so effective or lack of drug against newly evolved diseases. One critically important step in new drug discovery is to employ a new screening procedure with a novel principle to avoid re-isolation of the compounds being discovered by the previously used screening methods.

Genomic analysis not only revealed the high degree of gene conservation but most of the proteins especially those involved in basic cellular processes such as small-molecule metabolism, protein synthesis, cell division, DNA synthesis and repair, secretion and so on are conserved between human and other organisms including yeasts.[2)] In addition, by analyzing of the protein-protein interaction network in yeast and human, a strong positive correlation between evolutionary conserved and the number of interacting proteins was found.[3)] These evidences support the value of using yeast as a tool for drug target discovery. Cell based screening for small-molecule inhibitors that suppress a particular phenotype using model eukaryotic microorganisms such as yeast is important in discovering bioactive chemicals. The inhibitors thus found are potential candidates for drug development, and can also serve as valuable tools in investigating complex cellular processes.[4)] The potential of using yeast for drug discovery has been extensively reviewed.[1)]

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.[5] The pathway has been extensively studied in yeast and is implicated in the regulation of the G2/M cell cycle progression.[6] An inappropriate activation of a Ca^{2+}-signaling pathway in yeast cause a deleterious physiological effect and various defects inducing growth defects in the *zds1*-null mutant yeast.[6] According to such phenotype observed, a unique positive screening system utilizing the mutant yeast strain as indicator cells had developed.[1] The growth of a *zds1*-null mutant yeast strain was inhibited when the cells were cultivated in the medium containing $CaCl_2$.[6] The principle of the yeast based screening system based on the compromised cell growth in the *zds1*-null mutant yeast that can be rescued by small molecule chemicals on solid medium containing $CaCl_2$. The inhibition by small-molecule on the Ca^{2+} signaling pathway would result in failure of inducing the Ca^{2+} induced several physiological changes.[1] Small-molecule inhibitors of Ca^{2+} signaling pathway in human are of great medical importance, since Ca^{2+} signaling in mammalian cells plays pivotal roles in the regulation of diverse cellular processes, including T-cell activation, secretion, motility, apoptosis, and so on.[5,7] One example is calcineurin, a protein phosphatase involved in activation of the nuclear factor of activated T cell (NF-AT) which is a transcription factor required for the expression of cytokine gene in T cells.[8] The calcineurin inhibitors FK506 and cyclosporine A are widely used as potent immunosuppressants.[9] In addition,

several cell-cycle inhibitors have potential as anti-cancer agents.[10)]

The use of natural products especially medicinal plants still plays an important role on the basic health needs in developing countries. Medicinal plants were used as a rich source of therapeutic agents in traditional folk medicine since ancient time. The active principles of many drugs are found in plants or are produced as secondary metabolites. The remarkable contribution of plants to the drug industry was possible because of the large number of phytochemical and biological studies all over the world. Herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy, which might help overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine.[11)]

Upon searching for the inhibitor of the Ca^{2+} signaling pathway from the Thai medicinal plant extracts using the yeast-based positive drug-screening procedure, we found a crude extract of *Boesenbergia pandurata* as one of the positive samples from our screens (our unpublished data). Here, we report the use of such yeast based assay to guide fractionation and purification of the *B. pandurata* crude extract and three pure active compounds: pinostrobin, alpinetin and pinocembrin chalcone could be identified. Further biochemical experiments were conducted to confirm the genetic evidence and indicates

that pinostrobin, as one of pure compounds in the crude extract of *B. pandurata*, posses inhibitory activity on the Ca^{2+} signals in yeast *S. cerevisiae* by amelioration of the deleterious effects of external $CaCl_2$ on *zds1*Δ strain yeast.

Materials and Methods

Yeast strain and cultivation. Yeast mutant *zds1*Δ strain, YNS17 (*MAT*a *zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*)[12] was obtained from Prof. Tokichi Miyakawa, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Japan. The mutant yeast cells were cultivated on Yeast extract peptone dextrose (YPD) agar and incubated at 30 °C for 2 days.

Yeast-based screening assay. The assay was modified from Shitamukai *et.al.* (2000). An assay plate contained 6 X 10^5 cells/ml of the *zds1*Δ strain, YNS17 cells (*zds1*Δ strain), 150 mM $CaCl_2$ and 0.7 % YPD soft agar. Five microlitres of tested sample dissolved in ethanol (at concentration of 5 mg/ml) or ethanol (as a negative control) or 5 µl 100 nM FK506 (as a positive control) were dotted onto the assay plates. The plates were incubated at 30 °C for 2 days. A positive assay showed growth zone at or around the spots.

Plant material. B. pandurata was collected from Nakhon Pathom province, in the central part of Thailand. A voucher specimen (BKF 152279) has been deposited at the Bangkok

Forest Herbarium (BKF), Royal Forest Department, Chatuchak, Bangkok 10900, Thailand. The rhizomes of *B. pandurata* were harvested, sliced, dried in shed and grinded into powder.

Crude extract preparation and isolation of the bioactive constituents. The dried rhizome powder (2.4 kg) was extracted with dichloromethane by maceration process for 5 days with 3 time repeats. The dichloromethane fraction showed the strongest biological activity in the yeast assay. The concentrated dichloromethane extract (100.0 g) was resuspended in EtOAc and fractionated with silica gel quick column chromatography. Thin layer chromatography (TLC) and activity in the yeast based assay were used to guide the fractionation and purification processes. The positive fractions were further purified using chromatographic technique and precipitation until crystals were obtained. NMR and mass spectroscopic techniques were employed to elucidate the structure of the pure compounds compared to the database.

Flow cytometry analysis. The yeast cells were stained with propidium iodide (PI, Sigma, USA). The DNA content of the PI-stained cells was analyzed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) as described previously.[6)]

Yeast nuclear staining. Treated yeast cell suspension were stained with 5 μM Hoechst 33342 (Sigma, USA.) and incubated in dark for 5 min. before being analyzed by fluorescence microscope.

Results and discussion

Crude extract from B. pandurata can support growth of a yeast mutant strain, zds1Δ on high $CaCl_2$ containing medium

Upon the screens of crude extracts from Thai medicinal plants for small molecule inhibitor of the Ca^{2+} signaling pathway in the yeast based assay as proposed by Shitamukai *et. al.*, 2000, *B. pandurata* was one of the positive crude extracts we found (our unpublished data, Fig. 1). The positive result as in a positive control, FK506, showed growth of the yeast indicator cells on the spot area (Fig. 1A). Ethanol as a negative control showed no growth (Fig. 1C) while the spot of *B. pandurata* crude ethanolic extract showed growth around the spot area with halo zone in the middle.(Fig. 1B). Such halo growth pattern observed in Fig. 1B might be as a result from the mixture effects contained in the crude extract which consists of compounds that inhibit the Ca^{2+} signals and compounds that are cytotoxic to the yeast indicator cells. *B. pandurata* or finger root plant is a herb belongs to Zingiberaceae family. The fresh rhizomes are commonly used in Southeast and South asia as food ingredient.[13] In Thailand, the rhizomes of *B. pandurata* are used since ancient time both as food ingredient of unique gastronomic dishes and in Traditional Thai Medicine for several applications including functional food to maintain wellness[14], stomach discomfort, aphthous ulcer, anti-flatulence, diuresis, leukorrhea, treatment of oral diseases and anti-dysentery.[13,15] Many efforts were invested on active compounds isolations.[16-18] Several biological studies on the crude extract were reported including

anti-microbial activities[19-20], anti-mutagenicity[21]. The chloroform extract of rhizomes of *B. pandurata* showed marked preferential cytotoxicity against human pancreatic PANC-1 cancer cells in nutrient-deprived medium.[22]

Yeast based-activity guided fractionation and purification of a crude extract of B. pandurata

To search for pure compounds responsible for the inhibitory activity of the Ca^{2+} signals in yeast, the same yeast based assay was used for monitoring the fractionation and purification of the crude extract. Fresh rhizomes of *B. pandurata* collected from Nakhon Prathom province were extracted with CH_2Cl_2 or EtOAc or CH_3OH by maceration process at room temperature for 5 days with 3 time repeats. After filtration, the filtrate was evaporated under reduced pressure until dry. The biological activity of the yeast based assay was only found in CH_2Cl_2 crude extract (data not shown). One hundred gram of CH_2Cl_2 crude extract was fractionated by silica gel quick column. The column was initially eluted by hexane, followed by the addition of increasing polarity of solvent, EtOAc. Each fractions were monitored by TLC pattern and the activity in the yeast based assay. Spectroscopic techniques were used to identify the pure compounds. Three pure compounds were obtained: compound 1 as bright pale-yellow crystal, compound 2 as white amorphous powder, and compound 3 as red crystal. The yield obtained was 10.85, 1.73 and 1.2%, respectively (data not shown).

Structural elucidation of the positive pure compounds

The NMR spectroscopic data attained compared to those reported in the literatures, the compound 1 with bright pale-yellow crystal was identified as pinostrobin or 5-hydroxy-7-methoxyflavanone[13,23-25], the compound 2 with white amorphous powder was identified as alpinetin or 7-hydroxy-5-methoxyflavanone[23-26]and the compound 3 with red crystal was identified as pinocembrin chalcone or 2′,4′,6′-trihydroxychalcone.[27]

Pinostrobin shows the strongest biological activity in the yeast assay.

To compare biological activity of the three pure compounds of *B. pandurata* in the yeast based assay, pinostrobin, alpinetin and pinocembrin chalcone were separately tested in the yeast assay plates with varying concentrations of the compounds ranging from 0.5 - 1.5 mM with 0.25 mM increment using 500 nM FK506 and absolute ethanal as positive and negative controls, respectively. (Fig. 3A). The positive and negative controls exhibited growth of the yeast cells at the spot and no growth of the yeast cells, respectively (Fig. 3A). The three pure compounds exhibited dosage dependent of growth on YPD plate containing 150mM $CaCl_2$. Pinostrobin showed the highest biological activity among the three. The minimal effective concentration of pinostrobin, alpinetin and pinocembrin chalcone in the yeast based assay plates was <0.5, 1 and 0.5 mM, respectively (Fig. 3A). The positive effect from the three pure compounds showed growth with halo zone in the middle of the spot on the yeast assay plates when were tested at high

concentrations (from 1 mM or higher) and showed growth without halo zone in the middle like that in FK506, the positive control, when were tested at the lower concentrations (from 1mM or lower) (Fig 3A). The dosage dependent growth pattern might be explained as at the center of the spot where concentration of the pure compound is higher, such concentration causes cytotoxic to the yeast cells and as a result, halo zone in the middle of spot was observed. Whereas at the marginal area of the spot where concentration of the pure compound is lower, such concentration causes no toxicity to the yeast cells and could rescue the growth of the mutant yeast cells.

To evaluate the effect of the pure compounds on growth of *zds1*Δ cells in YPD broth containing 75 mM $CaCl_2$, we found that at 1 mM pinostrobin itself have undetectable toxic effect to cell growth (Fig 3B) while alpinetin and pinocembrin chalcone at 1 mM, respectively or at lower concentrations caused cytotoxic effect to the cells (data not shown). Pre-treatment of cells with either 1 mM pinostrobin or 500 nM FK506 (as a positive control) prior to cultivating in YPD containing 75 mM $CaCl_2$ could rescue the Ca^{2+} sensitive mutant yeast cells at the same extent (Fig 3B). The results emphasized that pinostrobin has highest biological activity among the three pure compound in the yeast based assay and showed the least toxic effect to the yeast indicator cells. Besides, the yield of pinostrobin obtained from the purification of the crude extract of *B. pandurata* was the highest among the three positive compounds. Therefore, only pinostrobin was further characterized.

Effect of pinostrobin on ameliorating the zds1Δ cells from G2 to M phase delay

The hyperactivation of Ca^{2+} signals in the mutant *zds1*Δ yeast strain caused cells to arrest or delay at G2 phase of the cell cycle.[6] Pinostrobin from *B. pandurata* showed growth recovery of *zds1*Δ cells cultivated on 150 mM $CaCl_2$ containing YPD plate (Fig. 3A). The genetics evidence showed that pinostrobin could suppress the Ca^{2+}-induced growth inhibition suggesting that pinostrobin can inhibit the Ca^{2+}-signaling activity. To see whether the pure compound could rescue the Ca^{2+} hyperactivated cells from cell cycle arrest or delay at G2 phase, flow cytometry analysis of the propidium iodide stained *zds1*Δ cells were examined. The exponential growth of *zds1*Δ cells was harvested. After resuspending in YPD medium, the cell suspensions were divided into 3 parts. One part as normal control, the *zds1*Δ cells were grown in YPD medium without $CaCl_2$. The second part as Ca^{2+} hyperactivated condition, the cells were cultivated in YPD medium with 100 mM $CaCl_2$ and incubated for 3 hrs at 30 °C. The last portion, the *zds1*Δ cells were pretreated with either 1 mM pinostrobin or 500 nM FK506 (as a positive control) or DMSO (as a negative control) for 30 min at 30 °C prior to addition of 100 mM $CaCl_2$ for 3 hrs at the same temperature. To examine the phase of cell cycle, the yeast cells were harvested and stained with propidium iodide before flow cytometry analysis. In the *zds1*Δ cells grew in without $CaCl_2$ condition, the population of cells with 2C DNA content had about 2.5 times more than that with 1C DNA content (71.9% and 28.1%, respectively) (Fig. 4A). The addition of 100 mM

$CaCl_2$ caused accumulation of population of cells with 2C DNA content (74.3%) and decreased in that with 1C DNA content (25.8%) (Fig. 4A). On the other hand, the treatment of *zds1*Δ cells with 150 mM $CaCl_2$ caused accumulation of dead cells (population of cells with less than 1C DNA content; Fig. 4). Pretreatment of cells with the calcineurin inhibitor, FK506 (as a positive control) prior to addition of 100 mM $CaCl_2$ into the medium caused decrease in cells with 2C DNA content and increased in those with 1C DNA content (66.0 and 34.0%, respectively) compared to those in negative control experiment (Fig. 4B VS 4A). The 1 mM pinostrobin pretreatment before adding 100 mM $CaCl_2$ into the medium showed the same pattern of flow cytometry profile with that of the FK506 pretreated cells (64.9% and 35.1%, respectively) (Fig. 4C). However, treatment of either 1 mM pinostrobin or 500 nM FK506 immediately or after $CaCl_2$ exposure could not rescue the cells from the deleterious effect on G2 phase delay caused by Ca^{2+} hyperactivation (data not shown). This result demonstrated that pretreatment of cells with 1 mM pinostrobin could prevent the yeast cells from G2 delay as a consequence from Ca^{2+} hyperactivation.

Effect of pinostrobin on alleviating the zds1Δ cells from abnormal bud emergence caused by the hyperactivation of the calcium signals.

The hyperactivation of Ca^{2+} signals not only causes the *zds1*Δ cells from G2 phase delay or arrest but abnormal bud emergence.[6] To confirm the effect of pinostrobin pretreatment

before cultivating in high $CaCl_2$ concentration that could alleviate the yeast cells with G2 phase delay or arrest, the morphology of the cells was examined. The treated cells from the experiments in Fig. 4 were fixed and the morphology of the cells was observed. The fixed cells were also stained with Hoechst 33342, the nucleus staining dye and were examined under 40X fluorescence microscope. While the Ca^{2+} hyperactivated cells pretreatment with DMSO solvent showed fifty four percent of cells with abnormal morphology with elongated buds and had unequal nuclear budding (the nuclei were in only mother cell and not found in the daughter cell) (Fig. 5A), the cells pretreated with either 500 nM FK506 or 1 mM pinostrobin prior to exposure to 100 mM $CaCl_2$ showed normal morphology (Fig. 5B and 5C, respectively, left panel) with equal distribution of nuclei in mother and daughter cells and showed only 7 and 11% elongated buds, respectively (Fig. 5B and 5C, respectively, right panel).

Taken together, the flow cyctometry profiles, cell morphology and nuclear staining experiments indicate that pinostrobin can relieve the hyperaction of Ca^{2+} signal in yeast. Also, these evidences could confirm the genetic evidence which suggested that pinostrobin is an inhibitor of Ca^{2+} signals in yeast, *S. cerevisiae*.

Three pure compounds: pinostrobin, alpinetin and pinocembrin chalcone from *B. pandurata*, a well known Thai herb, could be detected in a yeast based drug screening system using *zds1*Δ mutant yeast strain as indicator cells. To support the genetic evidence that these compounds could inhibit the Ca^{2+}

signals in the *zds1*Δ cells, here we demonstrated that pinostrobin could ameliorate the mutant yeast cells suffering from Ca^{2+} hyperactivation. Pretreatment of *zds1*Δ cells with 1 mM pinostrobin can relieve the cells from G2/M arrest and the abnormal budding. We concluded that pinostrobin from *B. pandurata* contains inhibitory effect against the Ca^{2+} signaling in yeast *S. cerevisiae*. This study provides additional support to the positive screening system for drug discovery. Pinostrobin could be isolated from many natural sources such as from *Polygonum lapathifolium* L. ssp. Nodosum which exhibited an anti-leukemic activity against Jurkat and HL-60 cell lines[28], from *Piper lanceaefolium* with anti-*Candida albicans*.[29] Pinostrobin and pinocembrin chalcone from *B. Pandurata* showed potent anti-mutagenic activity against Trp-P-1 in the Ames test.[30] Pinostrobin from *B. Pandurata* also was reported to contain antioxidant activities.[26] It will be of interest to further focus on the molecular target of pinostrobin.

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Growth of *zds1*Δ cells in 150 mM $CaCl_2$ YPD plate

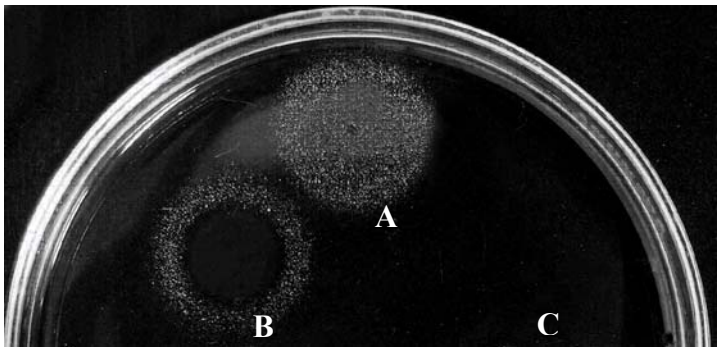
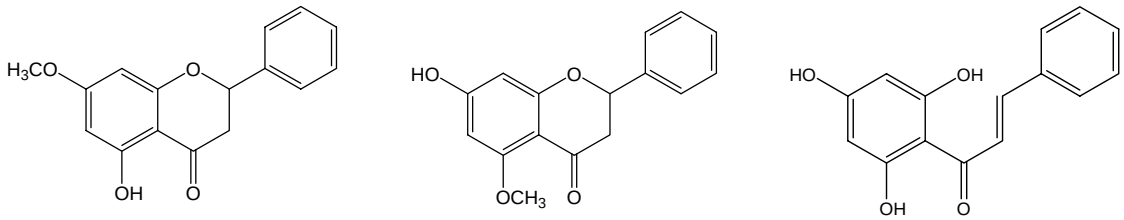


Fig. 1 A positive effect on growth in the yeast based assay by a crude extract from *B. pandurata*. A yeast based assay plate containing 6 x 10^5 of *zds1*Δ cells in YPD soft-agar medium (0.7 % agar) containing 150 mM $CaCl_2$ was spotted with A) 5µl of 100 nM FK506 (as a positive control) B) 5 µl of 5 mg/ml crude extract of B. pandurata C) 5 µl of absolute ethanol (as a negative control). The plate was incubated at 30°C for 2 days.

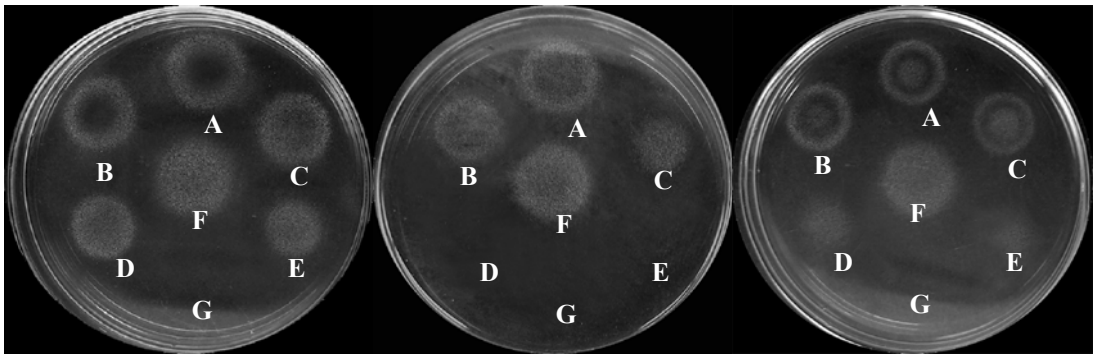


(1) (2) (3)

Fig. 2 Chemical structure of the three pure active compounds obtained from *B. pandurata* that exhibit inhibitory activity for Ca^{2+} signals in the yeast based assay. The compounds were identified as 1) Pinostrobin or 5-hydroxy-7-methoxyflavanone 2) Alpinetin or 7-hydroxy-5-methoxyflavanone and 3) Pinocembrin chalcone or 2′,4′,6′-trihydroxychalcone

A

Growth of *zds1*Δ cells in 150 mM $CaCl_2$ YPD plate



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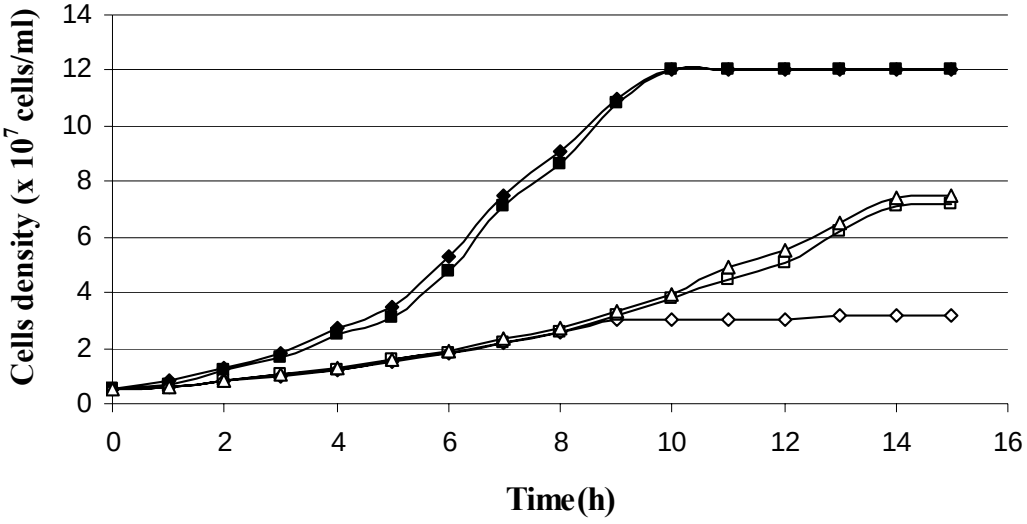

Fig 3. Effect of the pure compounds from *B. pandurata* on growth of *zds1*Δ mutant yeast cells.

A) Five µl of each the three pure compounds: pinostrobin, alpinetin and pinocembrin chalcone at varying concentrations: 1.5 mM (A), 1.25 mM (B), 1 mM (C), 0.75mM (D) and 0.5 mM (E) along with 5 µl of 100 nM FK506 (as a positive control) (F) and 5 µl of absolute ethanol (as a negative control) (G) were

applied on the yeast assay plate containing 6 x 10^5 cells/ml of *zds1*Δ cells, 0.7% YPD soft agar medium and 150 mM $CaCl_2$. The plates were incubated at 30°C for 2 days. B) Effect of pinostrobin pretreatment on growth of *zds1*Δ cells in YPD broth containing 75 mM $CaCl_2$. The *zds1*Δ of 5 x 10^6 cells/ml were cultivated in YPD broth (◆) or in YPD broth containing 1 mM pinostrobin (■) or in YPD broth containing 75 mM $CaCl_2$ (◇) or were preincubated at 30°C for 30 min with either 0.5 µM FK506 as a positive control (△) or 1 mM pinostrobin (□) prior to addition of 75 mM $CaCl_2$. The cultures were incubated at 30 °C with shaking at 120 rpm and the cell density was measured every hour until 15 h of incubation.

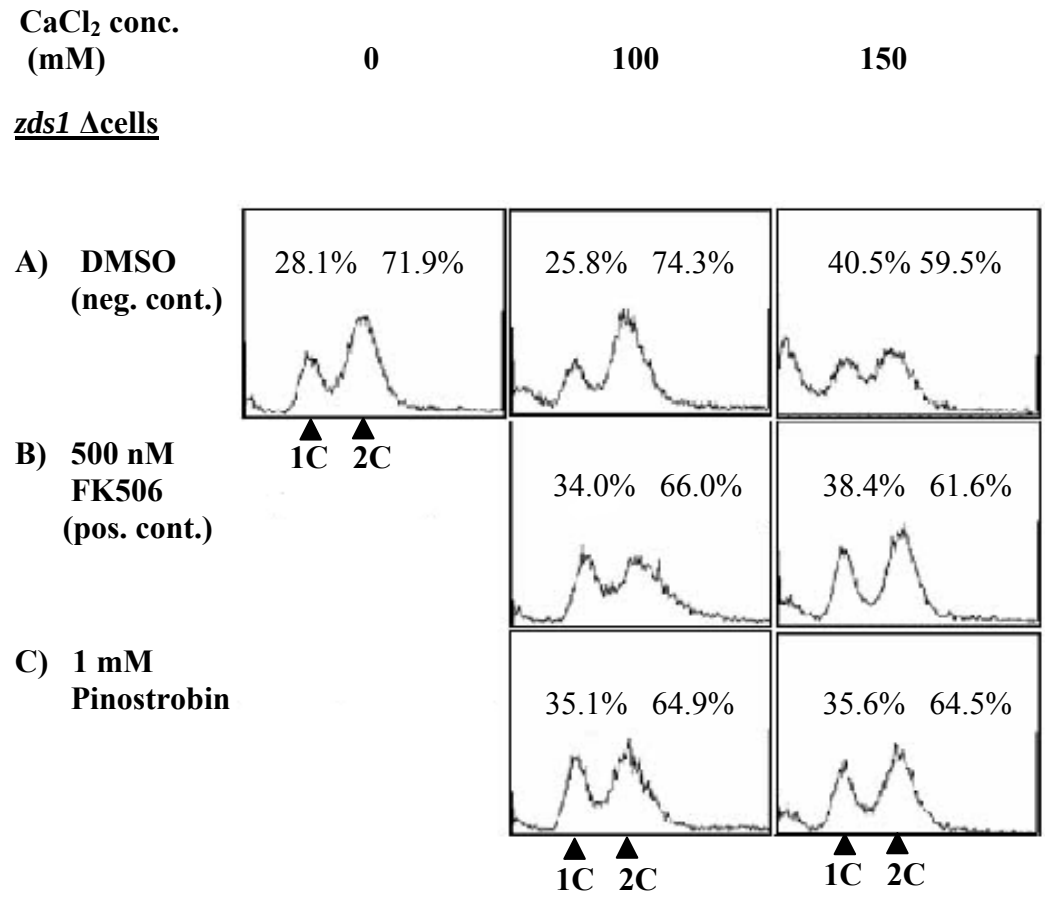

Fig. 4. The effect of pinostrobin pretreatment on flow cytometry profile of Ca^{2+} hyperactivated *zds1*Δ cells

The *zds1*Δ cells of 5 x 10^6 cells/ml were pretreated with either A) DMSO (as a negative control) or B) 500 nM FK506 (as a positive control) or C) 1 mM pinostrobin prior to being activated with 100 mM or 150 mM $CaCl_2$ for 3 h at 30 °C. The harvested cells were fixed and stained with propidium iodide (PI) before flow cytometry analysis. The numbers in percentage were those of 1C and 2C cells, respectively.

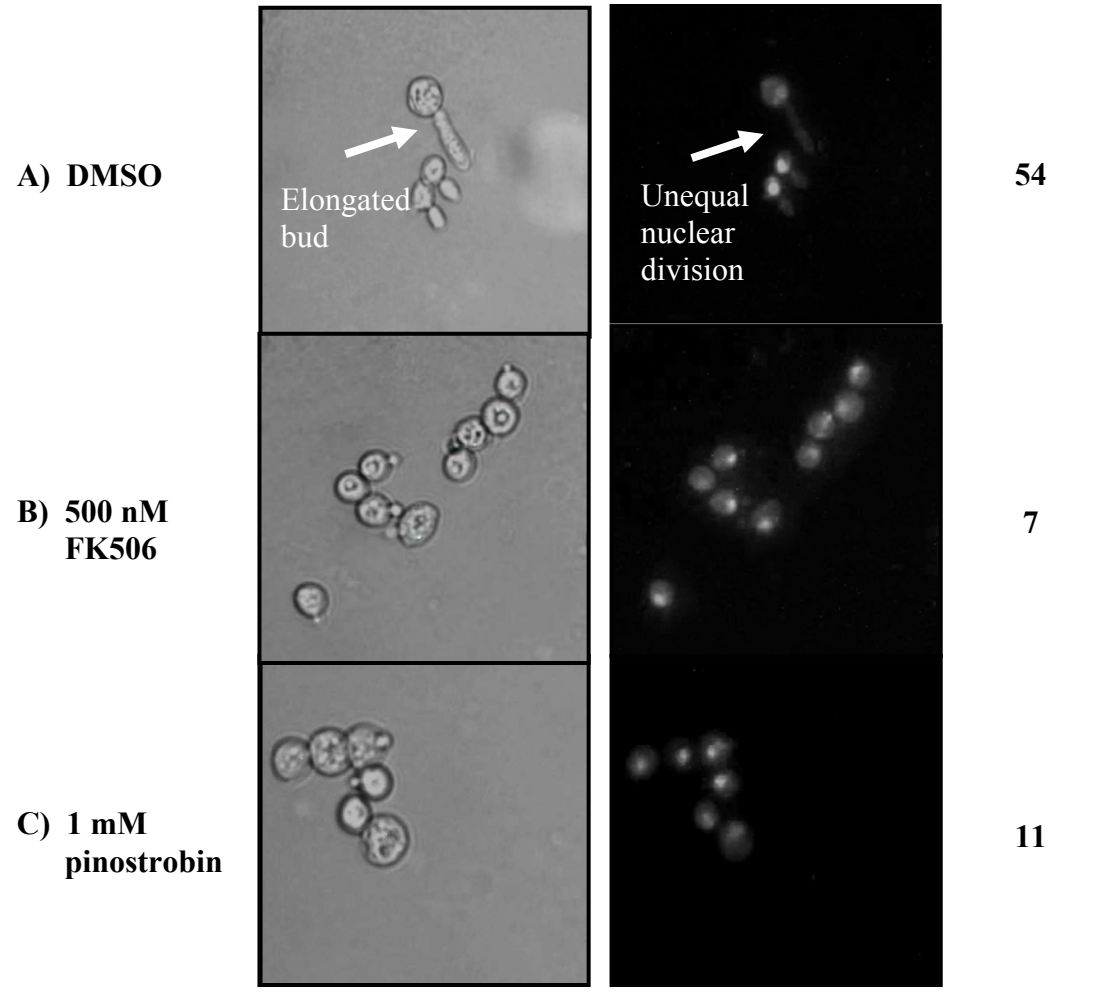

Fig. 5. The effect of pinostrobin pretreatment on cell morphology and budding of Ca^{2+} hyperactivated *zds1*Δ cells

The *zds1*Δ cells of 5 x 10^6 cells/ml were pretreated with either A) DMSO solvent (as a negative control) or B) 500 nM FK506 (as a positive control) or C) 1 mM pinostrobin prior to being activated with 100 mM $CaCl_2$ for 3 h at 30 °C. After being fixed, the cells were examined under normal light microscope (40X; left panel) and nuclear stained with Hoechst 33342 dye and examined under fluorescence microscope (40X; right panel).

1 The percentage of elongated buds were examined at 6 h after
2 addition of 100 mM $CaCl_2$.