ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Screening of Thai medicinal plants for inhibitors of Ca^{2+} signaling using a yeast cell growth-based assay

Saipin Boonkerd[1], Chulee Yompakdee[2*], Tokichi Miyakawa[3] and Warinthorn Chavasiri[4*]

[1]Program in Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.
[2]Department of Microbiology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.
[3]Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter (AdSM), Hiroshima University, Higashi-Hiroshima, Japan.
[4]Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.

ABSTRACT

Calcium signaling is a fundamental control mechanism for many aspects of cellular function, and as such faults in these pathways underlie many disease states. In the search for potential inhibitors of Ca^{2+} signaling, we screened the ethanol extracts of 69 species of Thai medicinal plants for the ability to suppress the external $CaCl_2$-induced hyper activated Ca^{2+} signaling mediated growth defect of the Ca^{2+}-sensitive YNS17 (Δ*zds1*) mutant strain of *Saccharomyces cerevisiae*. Three of the 69 screened plant species, *Andrographis paniculata* (Acanthaceae), *Boesenbergia pandurata* (Zingiberaceae) and *Kaempferia parviflora* (Zingiberaceae), were found to exhibit potent biological activity in this assay.

Key words: Calcium signaling inhibitors, Thai medicinal plants, Yeast-based screening

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Ca^{2+} is one of the principal signal mediators that modulate a wide range of cellular functions in eukaryotic organisms (for a review, see [1]). Ca^{2+} signaling in mammals plays important roles in the regulation of diverse cellular processes, such as cell proliferation, T-cell activation, secretion, muscle contraction and the release of neurotransmitters in higher eukaryotes [1]. Hence, small-molecule inhibitors of Ca^{2+} signaling are of great medical importance. For example, the immunosuppressive compounds tacrolimus (FK506) and cyclosporine A (CsA) are potent and highly specific inhibitors of the calcineurin phosphatase [2], and have been widely used as immunosuppressants to prevent graft rejection after organ and tissue transplantations. Moreover, the use of these drugs has revealed important roles of calcineurin in various Ca^{2+}-dependent cellular processes; including lymphocyte activation, cardiac development and hypertrophy, learning and memory based neural development and angiogenesis [3, 4].

Ca^{2+} signaling is also important in the unicellular microorganism yeast, *Saccharomyces cerevisiae*, where it is involved in the regulation of various cell functions (for a review, see [5]). Since hyper-activation of Ca^{2+} signaling in the mutant Δ*zds1* strain yeast leads to defective cell growth, exogenous inhibitors of this regulatory mechanism can lead to suppression of the growth defect, allowing the affected cells to resume growth. Based on this assumption, a convenient drug-screening procedure was developed [5, 6]. The screening was conducted as follows. The Δ*zds1*mutant yeast cells are suspended and solidified in molten soft-agar containing a relatively high concentration of $CaCl_2$ (150 mM) in a Petri dish. The growth of the yeast cells is arrested due to the hyper-activation of the Ca^{2+} signaling induced by the external $CaCl_2$. When the test compound or extract, such as FK506 or cyclosporine A, are dotted on this plate, the inhibitors allow the growth of the assay cells on or around the spots, giving rise to a growth zone halo after 2 days of incubation. Because bioactive compounds are detected by the growth of the assay cells, the procedure was designated as “positive screening” [6]. Since many low-molecular-weight inhibitors exert their physiological effects by an evolutionally conserved manner throughout eukaryotic organisms, this convenient yeast-based positive screening test is considered to be suitable for the screening of the drugs of medicinal interest. Several potential target molecules of medicinal interest are implicated in this mechanism, such as calcineurin (immunosuppressant and anti-inflammatory agent), GSK-3 (drugs for type II diabetes and Alzheimer’s disease), protein kinase C (anti-cancer drugs) and HSP90 (anti-cancer drugs).

Thailand is situated in one of the world floral biodiversity hot spots. In addition, the Thai people have a long tradition of folklore medicine as an integral part of Thai culture and this can then serve as potentially suitable plants for screening. In this paper, we searched for inhibitors of the Ca^{2+}-mediated growth regulation from various Thai medicinal plants using the yeast-proliferation-based positive screening procedure. From the screening of 69 initially selected plant species with folklore medical applications, a potent inhibitory activity against Ca^{2+}-mediated growth arrest of the yeast was found in three species from two diverse plant families.

ISSN: 0975-8585

The details of screening and the solvent extractability of the bioactivity with four different solvents of increasing polarity are described.

MATERIALS AND METHODS

Plant Materials

The fresh medicinal plants, as leaves, rhizomes or whole plants, were collected from the Royal Research and Development Centre of Khao Hin Son, Panomsarakarm, Chachoengsao, Thailand. Voucher specimens were deposited at either Chulalongkorn University Herbarium, Bangkok, Thailand, or at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Bangkok, Thailand. The names of plants, part used and the specimen voucher numbers are shown in Table 1.

Table 1. List of medicinal plants and the tissue parts used for screening with yeast growth-based assay

List	Plant family/ Species	Code	Plant Part	Voucher number[a]	Activity
	Acanthaceae				
1	Andrographis paniculata (Burm.f.) Nees.	APA	Whole plant	BKF 152277	+++
2	Barleria inpulina Lindl.	BIN	Whole plant	-	-
3	Barleria strigosa Willd.	BST	Leaf	CY & NP 31	-
4	Justicia adhatoda L.	JAD	Leaf	-	-
5	Justicia grossa C.B. Clarke.	JGR	Leaf	-	-
6	Justicia betonica L.	JBE	Leaf	-	-
	Annonaceae				
7	Anaxagorea javanica Blume.	AJA	Leaf	CY & NP 18	-
8	Uvaria vietnamensis Ast. ex.C. meade.	UVI	Leaf	-	-
9	Polyalthia suberosa (Roxb) Thwaites.	PSU	Leaf	-	-
10	Mitrephora tomentosa Hook. F Thomsan.	MTO	Leaf	-	-
	Araceae				
11	Aglaonema tenuipes Engl.	ATE	Leaf	-	-
	Araliaceae				
12	Polyscias fruticosa (L) Harms.	PFR	Leaf	-	-
13	Scheffiera leucantha R. Vig.	SLE	Leaf	-	-
	Apocynaceae				
14	Holarrhna pubescens Wall. ex. G. Don	HPU	Leaf	-	-
	Bignoniaceae				
15	Oroxylum indicum (L.) Vent.	OIN	Leaf	-	-
16	Crecentia cujete L.	CCU	Leaf	-	-
	Bixaceae				
17	Rixa orellana L.	ROE	Leaf	-	-
	Cieaceae				
18	Myxopryrum smilacifolium (Wall) Blume	MSM	Leaf	-	-
	Compositae				
19	Elephantopus mollis Kunth	EMO	Leaf	-	-

20	Eupatorium stoechadosmum Hance	EST	Leaf	-	-
21	Artemisia lactiflora Wall. ex D.C.	ALA	Leaf	-	-
	Elaegnaceae				
22	Elaegnus latifolia L.	ELA	Leaf	-	-
	Euphorbiaceae				
23	Croton stellatopilosus Ohba	CST	Leaf	-	-
24	Croton caudutus Geieslen	CCA	Leaf	-	-
25	Alchornea rugosa (Lour). Mull. Arg.	ARU	Leaf	-	-
26	Excoeoaria cochinchinesis Lour. Var. cochinchinensis	ECO	Leaf	-	-
27	Antidesma bunius (L) spreng var. bunius	ABU	Leaf	-	-
28	Phyllanthus pulcher Wall. Ex Mill. ARG.	PPU	Leaf	CY & NP 42	-
29	Sauropus thorelii Beille	STH	Leaf	-	-
	Flacourtiaceae				
30	Hydnocarpus anthelminthicus Pierre.	HAN	Leaf	-	-
31	Hydnocarpus ilicifolius King.	HIL	Leaf	-	-
	Guttiferae				
32	Garcinia gracilis Pierre	GRA	Leaf	-	-
	Labiatae				
33	Vitex negundo L.	VNE	Leaf	-	-
34	Clerodendrum paniculatrum L. var paniculatum	CPA	Leaf	CY & NP 46	-
	Lauraceae				
35	Cinnamomum porrectum (Roxb.) Kostrem	CPO	Leaf	-	-
36	Cinnamomum camphoa (L.) J. Presl	CCM	Leaf	-	-
	Leeaceae				
37	Leea rubra Blume ex Speng	LRU	Leaf	-	-
	Leguminosae				
38	Caesalpinia digyna Rottler	CDI	Leaf	-	-
	Magnoliaceae				
39	Magnolia rajanjana (Craib) Figlar	MRA	Leaf	-	-
40	Bauhinia sirindhorn K. & S.S. Larsen	BSI	Leaf	CY & NP 4	-
	Moracese				
41	Antiaris toxicaria lesch. Subsp. toxicaria	ATO	Leaf		
42	Ficus tinctoria G. Forst. subsp. gibbesa (Blume). Coiner	FTI	Leaf		
	Myrsinaceae				
43	Maesa ramentaceae (Roxb) A.D.C.	MRA	Leaf	-	-
44	Ardisia crenata Sims var. crenata	ACR	Leaf	-	-
	Mytaceae				
45	Syzygium cumini (L.) Skeels	SCU	Leaf	-	-
	Ochnaceae				
46	Ochna intergerrima (Lour.) Mer.	OIN	Leaf	-	-
	Rubiaceae				
47	Oxyceros horridus Lour.	OHO	Leaf	-	-
	Palmaceae				
48	Caryota mitis Lour.	CMI	Leaf	-	-
	Phormiaceae				
49	Dianella ensifolia (L.) DC.	DEN	Leaf	-	-
	Sapindaceae				

50	Allophylus cobbe (L.)	ACO	Leaf	-	-
51	Lepisanthes fruticosa (Roxb.) Leenh.	LFR	Leaf	-	-
52	Lepisanthes senegalensis Poiret	LSE	Leaf	-	-
	Simaroubaceae				
53	Picrasma jaranica Blume.	PJA	Leaf	-	-
54	Brucea javanica (L.) Merr.	BJA	Leaf	-	-
	Sterculiaceae				
55	Mansonia gagei J.R Drumm ex. Prain	MGA	Leaf	-	-
56	Helicteres isora L.	HIS	Leaf	CY & NP 70	-
	Vitaceae				
57	Cissus quadrangularis L.	CQU	Leaf	CY & NP 48	-
	Zingiberaceae				
58	Alpinia (Languas) conchigera Griff.	ACO	Rhizomes	-	-
59	Alpinia galanga (L.) Willd.	AGA	Rhizomes	-	-
60	Alpinia mutica Roxb.	AMU	Rhizomes	-	-
61	Alpinia purpurata (Vielle.) Schum.	APU	Rhizomes	-	-
62	Amomum villosum Lour. var xanthioide T. L. Wu & S. J. Chen.	AVI	Rhizomes	-	-
63	Boesenbergia pandurata (Roxb.) Schltr.	BPA	Rhizomes	BKF 152279	++*
64	Curcuma longa (L.)	CLO	Rhizomes	-	-
65	Hedychium coronarium J. Konig.	HCO	Rhizomes	-	-
66	Kaempferia galanga (L.)	KGA	Rhizomes	-	-
67	Kaempferia parviflora Wall. Ex Baker	KPA	Rhizomes	BKF 152278	++*
68	Zingiber montanum (cassumunar Roxb.) (Koen.) Thelade	ZMO	Rhizomes	-	-
69	Zingiber officinale Roscoe.	ZOF	Rhizomes	-	-

-, +, ++ and +++ indicate no growth, weak growth, medium growth and potent growth, respectively.

* indicates the formation of a ring-like growth zone

[a] The BKF voucher specimens were deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Bangkok, Thailand. The CY & NP voucher specimens were deposited at Chulalongkorn University Herbarium, Bangkok, Thailand.

* YNS17 (Δ*zds1* mutant) yeast cells were cultivated on YPAUD soft agar containing 150 mM $CaCl_2$ and incubated at 30 ^{o}C for 2 d.

Yeast Strain and Cell Culture

S. cerevisiae mutant YNS17, (*MAT*a *zds1*::*TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) was used as the indicator strain in this bioassay [6]. The YNS17 yeast cells were sub-cultured in YPAUD (YPD (10 g / L yeast extract, 20 g / L peptone and 20 g / L glucose) with 0.4 g / L adenine sulfate and 0.2 g / L uracil) broth at 30 ^{o}C with shaking at 200 rpm for 18 - 24 h. For the bioassay, the cells were cultivated on YPAUD soft-agar medium and incubated at 30 ^{o}C for 2 d, as described previously [7].

Preparation of Plant Extracts and Screening by Yeast-Based Assay

Each of 50 g of the medicinal plant extracts prepared from a total of 69 species was soaked in 200 mL of 95% ethanol for 3 d and the extraction was repeated three times. The extracts were combined and then the solvent removed by rotary evaporation under reduced pressure at 60 ^{o}C to dryness. Each dried extract was then dissolved in absolute ethanol to a final concentration of 3 g / L and examined for the desired bioactivity by the YNS17 yeast growth based positive screening method.

EXERIMENTAL WORK

Screening Assay

Detection of activity was performed according to the procedures of Shitamukai and co-workers (2000) with slight modifications. The YNS17 yeast cells (Δ*zds1*strain) were inoculated into YPD broth and incubated with shaking at 30 ^{o}C until the cell density reached 1 - 5 x 10^{7}cells / mL. To 8 ml of molten YPD soft-agar (7 g / L agar, kept at 55 ^{o}C), 4 M $CaCl_2$ and indicator cells were added to final concentrations of 150 mM and 6.0 x 10^{5} cells / mL, respectively, mixed well and poured into Petri dishes. Five L oeach assay sample (3 g / L) or sample diluents (ethanol or DMSO as a negative control) and 3 µl of 500 nM FK506 (as a positive control) were spotted onto agar plates. The plates were incubated at 30 °C for 40 h until a growth zone appeared around the spot of the samples. The plates were then examined for the presence, appearance and size of halos and scored for relative intensity.

Solvent Extraction of the Plant Bioactivity

A 100 g aliquot of the dried powder of each plant that showed the biological activity was extracted with *n*-hexane (1:1 (w/v) ratio) using a Soxhlet apparatus for 6 h. Following extraction, the residual insoluble plant material was dried of solvent and further extracted successively in the same manner with dichloromethane (1:1), ethyl acetate and finally with methanol. Each solvent extract was evaporated using an evaporator under vacuum at 40 ^{o}C to remove the solvent, and the residue was then resolvated in DMSO (for the *n*-hexane and dichloromethane extracts) or ethanol (for the ethyl acetate and methanol extracts) to 3 g / L, and then tested for the bioactivity in the YNS17 yeast growth-based assay. The (w/w) yield in terms of the amount of dry starting material and the activities are summarized in Table 2.

ISSN: 0975-8585

Table 2. Extraction of bioactivity with various solvents from the screened plants

Plants	Solvents	% Yield (w/w)	Activity
Andrographis paniculata (Burm.f.) Nees. (APA)	n-Hexane	0.57	+
	CH_2Cl_2	1.41	++
	EtOAc	1.37	+++
	MeOH	7.19	+
Boesenbergia pandurata (Roxb.) Schltr. (BPA)	n-Hexane	3.98	++*
	CH_2Cl_2	4.82	++*
	EtOAc	0.84	+++*
	MeOH	3.67	_
Kaempferia parviflora Wall. Ex Baker (KPA)	n-Hexane	1.49	++*
	CH_2Cl_2	12.29	++*
	EtOAc	2.84	+++*
	MeOH	2.47	+

-, +, ++ and +++ indicate no growth, weak growth, medium growth and potent growth, respectively.

* indicates the formation of a ring-like growth zone

RESULTS AND DISCUSSION

In order to search for bioactive compounds that inhibit Ca^{2+}-mediated signaling the YNS17 yeast growth-based positive drug-screening assay was performed on the ethanol extracts of 69 species of Thai medicinal plants. Of these 69 selected plant species, the samples prepared from three plants, *A. paniculata* (APA), *B. pandurata* (BPA) and *K. parviflora* (KPA) produced a halo zone of yeast cell growth around the spot of the sample, while those from the other 66 assayed plants did not show any such activity (Table 1).

As a preparative step for large-scale purification of the active components from the plant materials, we examined the effect of various solvents on the extraction of the bioactivity from the dried powder of the same three plants and tissue sources that had shown the positive bioactivity (*A. paniculata*, *B. pandurata* and *K. parviflora*). The dried powder of each plant tissue was extracted successively with *n*-hexane, dichloromethane, ethyl acetate, and methanol, representing an increasing solvent polarity of the solvent extract.

As shown in Figure 1 and Table 2, the extraction profile and the appearance of growth halo with each solvent varied by the plants, suggesting that different bioactive compounds may exist in each plant. The solvent extracts from APA showed a closed growth zone with the growth of assay cells throughout the halo. In contrast, the solvent extracts from BPA and KPA showed doughnut-like growth zones (except for the hexane extract of KPA) with the growth inhibitory zone inside (Figure 1). The latter feature may be explained either by the coexistence of the bioactive compound and cytotoxic compounds in the fraction or that the active compounds has both a growth-promoting activity and a growth inhibitory activity at high drug concentrations (*i.e.*, near the center of halo). These possibilities will be discriminated by the

ISSN: 0975-8585

subsequent purification of the active compound(s). From the result of the extraction experiments (Table 2), it was suggested that the ethyl acetate fraction of the three selected plants had the highest specific biological activity by the assay.

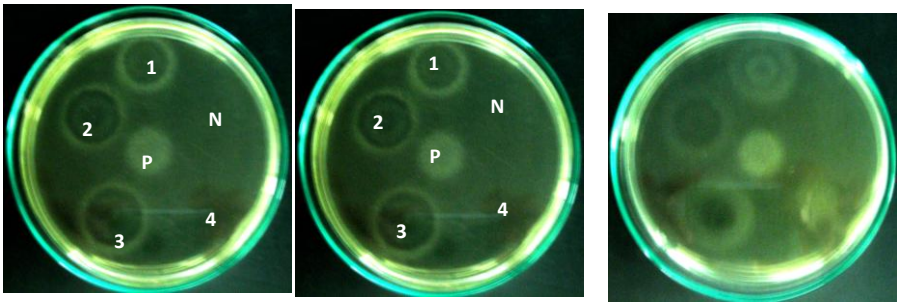

Figure 1. The effect of solvent extraction of bioactivity from *A. paniculata* (APA), *B. pandurata* (BPA), and *K. parviflora* (KPA). Dried powder of each plant was extracted successively with n-hexane (**1**), dichloromethane (**2**), ethyl acetate (**3**), and methanol (**4**) and subjected to yeast growth-based assay. **P**, FK506 (as a positive control); **N**, ethanol (as a negative control).

Of the three plant species that exhibited potent bioactivity (*A. paniculata*, *B. pandurata* and *K. parviflora*), pinostrobin has been identified as an active principle of *B. pandurata*. Pinostrobin has been shown to inhibit the Ca^{2+} signal-mediated control of cell- cycle progression in the G_2/M phase [8].

Small-molecule inhibitors generally elicit their physiological effects from the cells through acting on common mechanisms from yeast to mammalian cells by binding to evolutionarily conserved common target molecules, suggesting that the yeast-based screening is suitable as a convenient and powerful means for drug discovery. In fact, several interesting compounds have been identified using the positive screening assay [7, 8].

ACKNOWLEDGEMENTS

The authors would like to thank the National Research University Project of CHE and the Ratchadaphiseksomphot Endowment Fund (FW645A) for financial support, and the Royal Research and Development Center of Khoa Hin Son, Panomsarakarm, Chachoengsoa Province, for kindly providing the plant materials.

REFERENCES

[1] Clapham DE. Cell 1995; 80: 259-268.
[2] Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Cell 1991; 66: 807-815.
[3] Aramburu J, Rao A, Klee, CB. Curr Top Cell Regul 2000; 36: 237-295.
[4] Sugiura R, Sio SO, Shuntoh H, Kuno T. Cell Mol Life Sci 2001; 58: 278-288.

[5] Miyakawa T, Mizunuma M. Biosci Biotech Biochem 2007; 71(3): 633-645.
[6] Shitamukai A, Mizunuma M, Hirata D, Takahashi H, Miyakawa T. Biosci Biotechnol Biochem 2000; 64(9): 1942-1946.
[7] Chanklan R, Mizunuma M, Kongkathip N, Hasitapan K, Kongkathip B, Miyakawa T. Biosci Biotechnol Biochem 2008; 72(4): 1023-1031.
[8] Wangkangwan W, Boonkerd S, Chavasiri W, Sukapirom K, Pattanapanyasat K, Kongkathip N, Miyakawa T, Yompakdee C. Biosci Biotechnol Biochem 2009; 73(7): 1679-1682.