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ORIGINAL ARTICLE

A flavonoid, 5-hydroxy-3,7-dimethoxyflavone, from *Kaempferia parviflora* Wall. Ex. Baker as an inhibitor of Ca^{2+} signal-mediated cell-cycle regulation in yeast

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Abstract Calcium (Ca^{2+}) signal transduction pathways play important roles in the regulation of diverse biological processes in eukaryotes ranging from unicellular (e.g., yeasts) to complex multicellular (e.g., humans) organisms. Small-molecule inhibitors of Ca^{2+}-signaling pathways in humans can be of great medical importance, as represented by the immunosuppressants FK506 and cyclosporine A. A high-throughput drug screening assay for inhibitors of Ca^{2+}-signaling has been developed on the basis of the ability of test compounds to restore the severe growth defect of a Ca^{2+}-sensitive *zds1* null-mutant strain YNS17 of *Saccharomyces cerevisiae* in a medium containing a high concentration of calcium ions. A previous screening of Thai medicinal plants using this yeast-based assay indicated that the crude extract of *Kaempferia parviflora* Wall. Ex. Baker contains a potent inhibitory activity. The aim of this study was to isolate and characterize the pure compound(s) responsible for this inhibitory activity against Ca^{2+}-mediated cell-cycle regulation in yeast. Dichloromethane and methanol extracts of *K. parviflora* rhizomes were subjected to bioassay-mediated chromatographic fractionation using this yeast [YNS17 (Δ*zds1*) strain]-based assay to screen for and select positive fractions. From the dichloromethane extract, four known flavonoid compounds with significant inhibitory bioactivity were obtained: compounds **1** (5-hydroxy-3,7-dimethoxyflavone), **2** (5-hydroxy-7-methoxyflavone), **3** (5-hydroxy-3,7,4'-trimethoxyflavone) and **4** (5,7-dimethoxyflavone). The inhibitory activity of all four compounds was dose-dependent. Compound **1** exhibited the highest activity and with no observed cytotoxic activity against the yeast. The Ca^{2+} induced severe growth defect, abnormal budding morphology, and G2 cell-cycle delay of the Δ*zds1* yeast strain were all alleviated or abrogated by 200 μM compound **1**. Therefore, we conclude that 5-hydroxy-3,7-dimethoxyflavone possesses a potent inhibitory activity against the Ca^{2+}-mediated cell-cycle regulation.

Keywords Ca^{2+}-signaling · Δ*zds1* yeast strain · *Kaempferia parviflora* · Flavonoid · Inhibitor

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Introduction

In eukaryotic organisms, elevation of the intracellular calcium ion (Ca^{2+}) concentration serves as a universal second messenger that plays key regulatory roles in numerous biological processes, including cell proliferation, muscle contraction, fertilization, development, motility, memory, and apoptosis (Clapham 1995). Generally, small molecule inhibitors of Ca^{2+}-dependent signaling pathways exert their physiological effects by an evolutionary conserved manner throughout eukaryotes (Mager and Winderickx 2005). Consequentially, inhibitors found to be functional in yeast may well be active in relevance to humans.

In *Saccharomyces cerevisiae*, Ca^{2+} signals have been implicated in the regulation of G2/M cell-cycle progression (Mizunuma et al. 1998). The Ca^{2+}-sensitive *zds1* null mutant (Δ*zds1*) of *S. cerevisiae* can grow apparently normally in yeast-peptone-dextrose [YPD; 1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] medium, but exhibits

severe growth defects when cultivated in YPD containing a high concentration of $CaCl_2$ (Mizunuma et al. 1998). Accordingly, a convenient drug-screening procedure for inhibitors of Ca^{2+} signaling pathways was developed using the *S. cerevisiae* Δ*zds1* strain YNS17 on the basis of the ability of test drugs to antagonize the highly activated Ca^{2+}-signals, leading to the recovery of growth of the Ca^{2+}-sensitive mutant in high-calcium medium (Shitamukai et al. 2000). According to this procedure, potential targets for the inhibitors of the Ca^{2+} signaling pathway are calcineurin (potential target of immunosuppressants and anti-inflammatory agents), GSK3-family protein kinases (potential target of drugs for type 2 diabetes and Alzheimer's disease), protein kinase C (potential target of anti-cancer drugs) and HSP90 (potential target of anti-cancer drugs) (Boonkerd et al. 2011). Therefore, small molecule Ca^{2+}-signaling inhibitors obtained by such screens have high potential as drugs of medical interest.

A previous extensive screening study using this Δ*zds1* mutant yeast-based assay revealed that the crude extract of *Kaempferia parviflora* Wall. Ex. Baker contained a potent inhibitory activity (Boonkerd et al. 2011). *K. parviflora* is a member of Zingiberaceae (ginger) family, locally known in Thailand as Black Galingale or Kra-chai-dam. Members of this family, including *K. parviflora*, are traditionally very popular for health promotion (Putiyanan et al. 2004). The rhizomes of *K. parviflora*, locally known as Thai ginseng, have been used as a traditional medicine for various purposes, including the treatment of leucorrhea, oral diseases (Chomchalow et al. 2003; Sudwan et al. 2006), stomach ache, flatulence, digestive disorders, gastric ulcer as well as diuresis and tonic (Wattanapitayakul et al. 2007).

The aim of this study was to isolate pure active compound(s) from *K. parviflora* that display a significant ability to inhibit the Ca^{2+}-induced cell-cycle regulation of yeast.

Materials and methods

Plant materials

Fresh rhizomes of *K. parviflora* were purchased from a Thai market in Pratumthani. A voucher specimen (BKF 152278) has been deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Chatuchak, Bangkok, Thailand.

Extraction and fractionation of the *K. parviflora* rhizome extract

Fresh rhizomes of *K. parviflora* were sliced and dried in the open air for a few days and then powdered into small pieces with an electrical blender. The dried powder (5 kg) was extracted with dichloromethane (CH_2Cl_2) at a 1:1 (w/v) ratio in a soxhlet apparatus and then filtered. The plant residue was then similarly extracted with a 1:1 (w/v) ratio of methanol (CH_3OH). The two solvent extracts were concentrated separately in a rotary evaporator under reduced pressure to give a brown solid residue of 470.0 g and 90.0 g [9.4 % and 1.8 % (w/w) of the rhizome mass] from the CH_2Cl_2 and CH_3OH extracts, respectively. Fractionation of the extracts was performed by sequential chromatography with increasing solvent polarity, with bioselection of positive fractions from each stage being guided by the Δ*zds1* yeast-based assay (rescue from Ca^{2+}-mediated inhibition of proliferation), until pure compounds were obtained. Chemical structures of the pure isolated compounds were elucidated by NMR (Varian Mercury Plus 400, Palo Alto, CA) and mass spectrometry (MS) (Micromass UK Limited, Manchester, UK) and compared with those reported in the literature (Sutthanut et al. 2007).

Yeast strains, cultivation and the yeast-based screening assay

The Ca^{2+}-sensitive Δ*zds1* strain YNS17 (*MAT*a *zds1*::*TRP1 erg3*::*HIS3 pdr1*::*hisG URA3 hisG pdr3*::*hisG*), in which several genes responsible for drug resistance have been deleted (Chanklan et al. 2008), was used as the indicator cell for the bioassay. YNS17 cells were cultivated on YPD agar plates at 30 °C.

The growth-promoting and cytotoxic effects of compound **1** on the Δ*zds1* strain were evaluated by assays in liquid YPD and solid YPD agar culture as described by Wangkangwan et al. (2009). In brief, with respect to using solid medium, YNS17 cells (6×10^5 cells) were dispersed in molten soft YPD-agar and then overlaid on top of a YPD agar plate containing 150 mM $CaCl_2$. Five microliters each of various concentrations of compound **1** (500, 250, 125, 62.5, 31.3 and 15.6 μM) was applied by spotting onto the surface of the agar plate. In addition, 500 nM FK506 and absolute ethanol were used as the positive and negative (solvent only) controls, respectively. Plates were then cultured at 30 °C for 2 days prior to examination for cell growth.

The growth-promoting effect of 200 μM compound **1** was confirmed in liquid culture in YPG medium containing 75 mM $CaCl_2$. YNS17 cells were seeded at 5×10^6 cells/mL in YPD broth and cultured at 30 °C with or without compound **1** (or FK506 or DMSO as positive and negative controls) for 30 min before the addition of $CaCl_2$ to 75 mM (preincubation was to ensure the effect of the drug) and cultured at 30 °C. Samples were taken at 2 h intervals for measurement of the cell density with a hemacytometer under light microscopy following suitable dilution.

Flow cytometric analysis

Analysis of DNA content in the cells was performed by flow cytometric evaluation of cell DNA content as described by

Mizunuma et al. (1998), using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The yeast cell suspensions were fixed, permeabilized (Mizunuma et al. 1998), and then stained with propidium iodide for DNA analysis.

Results and discussion

Purification of the extract from *K. parviflora*

Fresh rhizomes of *K. parviflora* were extracted with CH_2Cl_2 and the 300.0 g of the CH_2Cl_2 extract was further fractionated using solvents with increasing polarity to obtain four pure compounds, designated as **1**, **2**, **3** and **4**, that are likely to be known flavonoids.

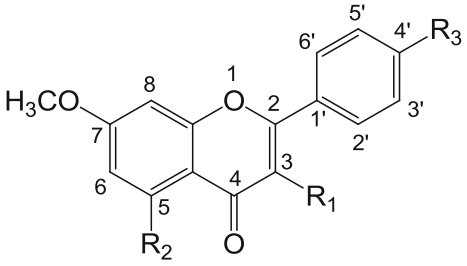
Compound **1** was obtained as yellow solid (6.27 g; 2.09 % yield). The molecular formula was suggested as $C_{17}H_{14}O_5$ and confirmed by ^{1}H-NMR. The ^{1}H-NMR spectrum (Table 1) clearly showed two sets of methoxy group resonating at δ_H 3.87 (3H, s) and 3.88 (3H, s) while a methylene group appears at δ_H 6.37 (1H, d, J=2.0 Hz), 6.46, (1H, d, J=2.4 Hz), aromatic protons at δ_H 7.52 (3H, m), 8.07 (2H, m) and hydroxyl group at δ_H 12.59 (1H, s). The ^{1}H-NMR spectral data of compound **1** showed a pattern similar to that of 5-hydroxy-3,7-dimethoxyflavone (Table 1, see Sutthanut et al. 2007).

Compound **2** was isolated as a yellow crystal (1.02 g; 0.34 % yield). The molecular formula was suggested as $C_{16}H_{12}O_4$, and confirmed by ^{1}H-NMR, which showed an identical pattern to that of 5-hydroxy-7-methoxyflavone (Sutthanut et al. 2007).

Compound **3** was obtained as a yellow crystal (3.45 g; 1.15 % yield). The molecular formula was suggested as $C_{18}H_{16}O_6$ and its ^{1}H-NMR spectral data was identical to that of 5-hydroxy-3,7,4′-trimethoxyflavone (Sutthanut et al. 2007).

Compound **4** was isolated as a yellow crystal (7.92 g; 2.64 % yield). The molecular formula was suggested as $C_{17}H_{14}O_4$ and its ^{1}H-NMR spectrum showed the pattern expected from that of 5,7-dimethoxyflavone (Sutthanut et al. 2007).

Accordingly, the likely structures of the four flavonoids (**1**–**4**) are shown in Fig. 1. When the biological activities of the serial dilutions of each compound were compared, compound **1** showed the highest activity against the Ca^{2+}-induced growth arrest of the YNS17 strain yeast (Table 2). Therefore, compound **1** was chosen for further characterization.



Compound	R_1	R_2	R_3
1	OCH_3	OH	H
2	H	OH	H
3	OCH_3	OH	OCH_3
4	H	OCH_3	H

1, 5-hydroxy-3,7-dimethoxyflavone; **2**, 5-hydroxy-7-methoxyflavone; **3**, 5-hydroxy-3,7,4′-trimethoxyflavone; **4**, 5,7-dimethoxyflavone

Fig. 1 Structure of the four flavonoid compounds obtained from *Kaempferia parviflora* (compounds **1**–**4**), shown as the 7-methoxyflavone backbone and the three variable substituent groups (R_1–R_3). In addition the ring numbering of the cyclic atom positions is shown for reference

Effect of compound **1** on growth of the Δ*zds1* mutant yeast strain YNS17

The growth-promoting effect of compound **1** on the Ca^{2+}-sensitive Δ*zds1* strain YNS17 was evaluated by assays in solid and liquid YPD culture. For solid medium, the assay cells suspended in molten soft agar containing $CaCl_2$ were solidified. The samples were spotted on the surface of the medium. After 2 days of incubation, no visible growth of the YNS17 yeast cells on the agar plate was evident, but their growth was

Table 1 Comparison of the ^{1}H-NMR data between 5-hydroxy-3,7-dimethoxyflavone and compound **1**

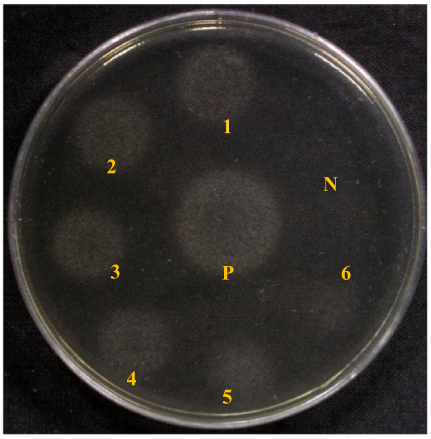
Position	5-hydroxy-3,7-dimethoxyflavone δ_H (multiplicity J in Hz)[a]	Compound **1** δ_H (multiplicity J in Hz)
6	6.28, d, J=2.0	6.37, d, J=2.0
8	6.38, d, J=2.0	6.46, d, J=2.4
2′, 3′, 4′, 5′, 6′	7.48, 8.03, m	7.52, 8.07, m
5-OH	12.55, s	12.59, s
7-OCH_3	3.82, 3.85, s	3.87, 3.88, s

[a] Sutthanut et al. 2007

Table 2 Relative activity of the four isolated compounds (**1**–**4**) from *Kaempferia parviflora* rhizomes in suppressing the growth defect due to Ca^{2+}-induced G2 cell-cycle arrest of the YNS17 (Δ*zds1*) yeast cells. Relative activity of each compound is indicated by +, ++, +++, and ++++ in increasing order of activity as estimated by the diameter of the cell-growth zone in the yeast-based assays (as shown in Fig. 2a for compound **1**). The result shown are representative of those seen in three independent assays

Concentration (μM)	Isolated compound			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
1,000	++++	++	+	++*
750	+++	++	+	+*
500	+++	++	+	+
250	+++	+	-	-
125	++	-	-	-
62.5	+	-	-	-
31.3	+	-	-	-

[a] Clear zone in the center of the growth zone suggesting the growth-inhibitory effect



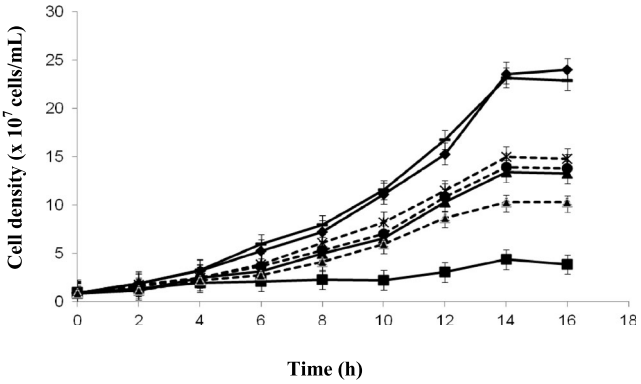


Fig. 2 Effect of compound **1** on the Ca^{2+}-induced growth inhibition of the Ca^{2+}-sensitive YNS17 yeast strain (Δ*zds1*) in **a** solid-plate and **b** liquid culture assays. **a** *1–6* represent the application of 5 μL of 500, 250, 125, 62.5, 31.3 and 15.6 μM compound **1**, respectively, whilst 500 nM FK506 (*P*) and absolute ethanol (*N*) were the positive and negative controls. Plates were evaluated after 2 days at 30 °C for YNS17 growth, and the image shown is representative of two independent trials. **b** YNS17 cells (seeded at 5×10^6 cells/mL) were cultivated in YPD broth at 30 °C and samples were taken at 2 h intervals for measurement of cell density with a hemacytometer. Data are shown as the mean±1 SD, and are derived from three independent repeats. Symbols: ♦ YPD medium; – YPD medium with 200 μM compound **1**; ▲ YPD medium with 75 mM $CaCl_2$ and 250 nM FK506; ■ YPD medium with 75 mM $CaCl_2$; –*– YPD medium with 75 mM $CaCl_2$ and 400 μM compound **1**; –●– YPD medium with 75 mM $CaCl_2$ and 200 μM compound **1**; –▲– YPD medium with 75 mM $CaCl_2$ and 100 μM compound **1**

restored in a dose-dependent manner around the spot where compound **1** was applied (Fig. 2a). In the liquid culture assay, YNS17 yeast cells grew well in YPD medium and the addition of 200 μM compound **1** showed no discernible inhibition of this growth (Fig. 2b). In the presence of 75 mM Ca^{2+} the YNS17 cells hardly grew at all, as expected, but this was reversed in a dosage-dependent manner by the addition of compound **1** (400, 200 and 100 μM, respectively) or the calcineurin inhibitor FK506 (500 nM), to a similar extent as each other (Fig. 2b). These results suggest that compound **1** alleviated the Ca^{2+}-induced growth defect of Δ*zds1* cells, whilst compound **1** itself had no significant toxic effect on the growth of YNS17 yeast cells (Fig. 2b).

Effect of compound **1** on YNS17 cell morphology, nuclear division and cell-cycle progression

The hyperactivation of Ca^{2+} signaling in YNS17 cells by external $CaCl_2$ leads not only to a severe growth defect, but also to the G2/M cell-cycle arrest/delay and polarized bud growth (Mizunuma et al. 1998). Accordingly, the ability of compound **1** to overcome these physiological effects induced by Ca^{2+} was examined in liquid YPD broth containing 100 mM $CaCl_2$. YNS17 cells grown in YPD plus 100 mM $CaCl_2$ medium mostly (> 70 %) displayed an abnormal morphology with an elongated bud (Fig. 3, left) and defects in nuclear division (Fig. 3, right), due to the hyperactivation of Ca^{2+} signaling. In contrast, when cotreated with 200 μM compound **1**, only 25 % of the cells showed an abnormal morphology (Fig. 3, left) with an equal distribution of nuclei between mother and daughter cells (Fig. 3, right). These results were broadly similar to those obtained with 500 nM FK506 (Fig. 3), and revealed that compound **1** could suppress Ca^{2+}-induced abnormal Δ*zds1* yeast cell morphology and the defect in nuclear division.

We further examined the effect of compound **1** on Ca^{2+}-induced G2 cell-cycle arrest/delay by flow cytometric analysis of propidium iodide-stained YNS17 cells. YNS17 cells grown for 3 h in YPD plus 100 mM $CaCl_2$ revealed a 2.5-fold higher 2C DNA content than those with a 1C DNA content, indicating that a G2 phase delay was induced by Ca^{2+} (Fig. 4). In contrast, in the presence

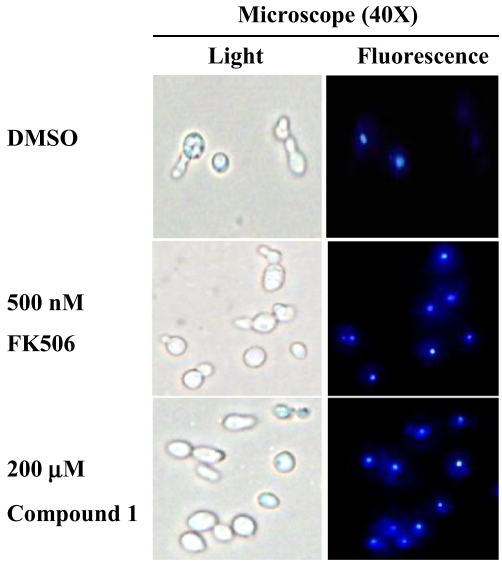


Fig. 3 Effect of compound **1** on Ca^{2+}-induced abnormal YNS17 cell morphology and defect of nuclear division. YNS17 cells in YPD were pretreated for 30 min with either DMSO as a negative (solvent) control, 500 nM FK506 as a positive control, or 200 μM compound **1**. Then, 100 mM $CaCl_2$ was added and the treated cells were cultivated for 6 h. Cell morphology was examined after Hoechst 33342 staining under a phase contrast light (*left*) and a fluorescence microscope (*right*). Images shown (× 40 magnification) are representative of those seen in at least 4–5 such fields of view (>100 cells) per sample and three independent samples

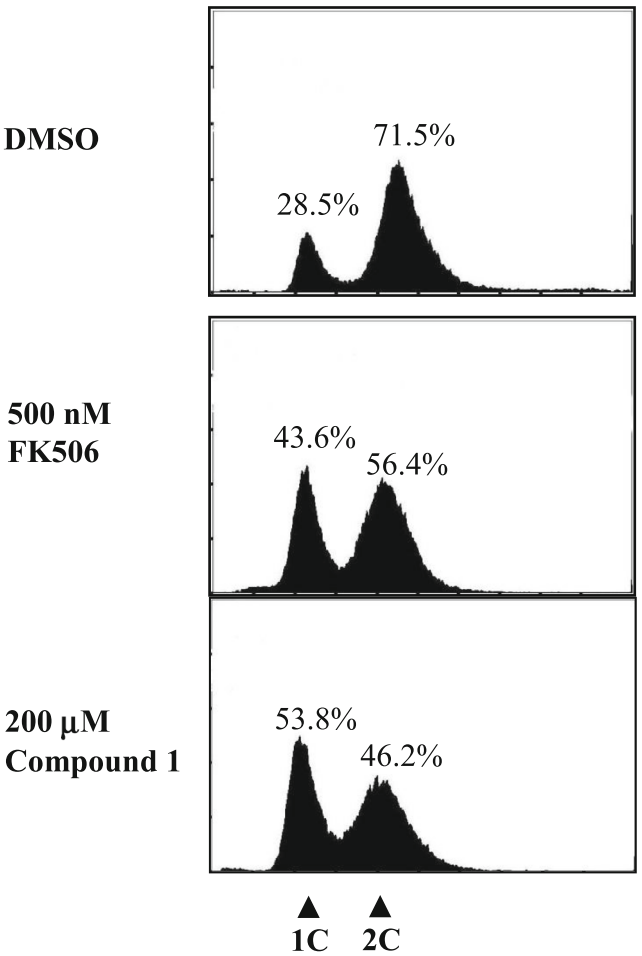


Fig. 4 Effect of compound **1** on Ca^{2+}-induced G2 cell-cycle delay. YNS17 yeast cells grown in YPD were treated as described in Fig. 4, except that cells were treated with the compounds for 3 h. The harvested cells were fixed, stained with propidium iodide, and subjected to flow cytometric analysis. The percentages shown are the proportion of cells with a DNA content of 1C and 2C, respectively, after exclusion of populations of<1C and>2C). Images shown are based upon 5,000 gated events (cells) and are representative of three independent repeats

of 200 μM compound **1**, the proportion of cells with a 2C DNA content decreased to about 0.9-fold that of those with a 1C DNA content (Fig. 4). A similar 1C / 2C cell DNA content profile was obtained with YNS17 cells treated with FK506 (Fig. 4). Thus, compound **1** alleviated the G2/M cell-cycle delay caused by hyperactivation of Ca^{2+}-signaling.

Previously, flavonoids (flavan-3-ols) and chalcones (phenyl styryl ketones) have been isolated from *K. parviflora* extract without the use of a bioassay-guided fractionation and purification (Jaipetch et al. 1983; Panthong et al. 1994; Yenjai et al. 2004). Subsequently, several studies on the biological activities of those flavonoids have been reported, including studies showing that compound **2** (5-hydroxy-7-methoxyflavone) has anti-mutagenic and anti-allergic activities (Azuma et al. 2011; Tewtrakul et al. 2008), and that compound **4** (5,7-dimethoxyflavone) exhibits an anti-inflammatory effect, in terms of the carrageenan-induced hind paw edema in rats (Panthong et al. 1994), and anti-mutagenic and anti-butyrylcholinesterase activities (Azuma et al. 2011; Sawasdee et al. 2009). However, to the best of our knowledge, the biological activities of compounds **1** (5-hydroxy-3,7-dimethoxyflavone) and **3** (5-hydroxy-3,7,4'-trimethoxyflavone) have not yet been clarified. Here, we showed that compound **1** from *K. parviflora* exhibited an inhibitory activity against the Ca^{2+}-induced G2 cell-cycle arrest, but a detailed mechanism for the action of compound **1** in yeast still needs to be investigated.

Conclusion

Four flavonoids, compounds **1**, **2**, **3** and **4**, were isolated from the CH_2Cl_2 extract of *K. parviflora* rhizomes through bioassay guided fraction selection, using inhibition of the Ca^{2+}-mediated inhibition of proliferation of Δ*zds1* yeast cells, to detect pure compound inhibitors of the Ca^{2+}-induced G2 cell-cycle arrest. Compound **1** (5-hydroxy-3,7-dimethoxyflavone) exhibited the highest activity against the Ca^{2+}-induced phenomena in YNS17 yeast cells, such as the growth defect, polarized bud growth, defect of nuclear division and

the G2/M phase delay, without any significant toxic effect on the cell growth. We are currently investigating its molecular target in yeast.

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