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Bioassay-Guided Isolation of Topoisomerase I Poison from *Paphiopedilum callosum* (Rchb.f.) Stein

San Yoon Nwe[1], Chayapol Tungphatthong[1,2], Areerat Laorpaksa[3], Boonchoo Sritularak[1], Witchuda Thanakijcharoenpath[1], Somboon Tanasupawat[3] and Suchada Sukrong[1,2*]

[1]*Department of Pharmacognosy and Pharmaceutical Botany, Chulalongkorn University Drug and Health Products Innovation Promotion Center (CU.D.HIP), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, 10330 Thailand*

[2]*Research Unit of DNA Barcoding of Thai Medicinal Plants, Chulalongkorn University, 10330, Thailand*

[3]*Department Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, 10330, Thailand*

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Abstract: *Paphiopedilum callosum* (Rchb.f.) Stein belongs to the Orchidaceae family. The phytochemistry and bioactivities of this plant have not been reported. In this study, a bioassay-guided isolation with a yeast cell-based assay was performed to isolate topoisomerase I poison compounds from the roots of *P. callosum*. One new and five known compounds were isolated. According to their spectroscopic data (ESI-MS, NMR, IR and UV), the new compound (**1**) is 3′-hydroxy-2,6,5′-trimethoxystilbene. The known compounds (**2**-**6**) were identified by comparing their spectroscopic data with those in the literature. According to the yeast cell-based assay, all the compounds were topoisomerase I poisons based on their ability to inhibit the growth of yeast cells. As further confirmation, the cytotoxic activities of the isolated compounds were evaluated using human cancer cell lines (MCF-7 and NCI-H187). The compounds exhibited varying degrees of cytotoxicity on the representative cell lines. These isolated compounds could serve as new lead compounds in the development of cancer chemotherapeutics.

Keywords: *Paphiopedilum callosum*; Orchidaceae; yeast cell-based assay; topoisomerase I poison; stilbene

1. Introduction

Cancer is currently a major life-threatening disease. There are many types of cancer treatments available, including surgery, radiotherapy, immunotherapy, chemotherapy, cancer vaccinations, photodynamic therapy, stem cell transplantation or combinations of these [1]. The currently used chemotherapeutic agents target DNA topoisomerases, which are ubiquitous enzymes for DNA replication and transcription [2]. There are two main types of DNA topoisomerases, topoisomerase I (TopI) and topoisomerase II (TopII). TopI is the enzyme that is responsible for the relaxation of supercoiled DNA by the transient breaking and resealing of the single-stranded DNA, and TopII breaks and re-ligates double-stranded DNA [3]. Therefore, many chemotherapeutic agents target those

* Corresponding author: E-Mail: suchada.su@chula.ac.th

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enzymes and inhibit their normal function. Topoisomerase inhibitors can be divided into two classes depending on their mechanisms: topoisomerase poisons and catalytic inhibitors [4]. Topoisomerase poisons can stabilize the covalent complex between the enzyme and DNA, which are termed cleavable complexes, and prevent the re-ligation step of the reactions [5], while catalytic inhibitors act on the other steps in the catalytic cycle [6].

Many chemotherapeutic agents are derived from natural sources (plants, marine sources and microorganisms) [7]. Natural compounds have provided skeletons for therapeutically effective anticancer drugs. Orchids, plants in the Orchidaceae family, have been used as herbal remedies in China since 2800 BC because of their medicinal value [8]. A number of secondary metabolites, such as alkaloids, bibenzyl derivatives, flavonoids, and phenanthrenes, have been reported in many orchid species [9]. Their phytochemicals exert antimicrobial activities, antitumour activities, anti-inflammatory activities, antiviral activities, etc. [10]. *Paphiopedilum* (slipper orchids), a genus in Orchidaceae, comprising approximately 80 species, are mainly distributed in Southeast Asia, Thailand, Myanmar, New Guinea and some areas of India and China [11]. Slipper orchids are popular ornamental plants because they possess colourful flowers with characteristic shoe-shaped labellum [12]. Although many phylogenetic studies have been performed on members of the genus *Paphiopedilum*, phytochemical studies are very rare. The phytochemical constituents and biological activities of only one species, *P. godefroyae*, have been reported [13]. Almost all compounds isolated from *P. godefroyae* showed cytotoxicity against small cell lung cancer cells (NCI-H187) [13]. The reports of cytotoxic agents from *P. godefroyae* confirm that the genus *Paphiopedilum* is an interesting source of new lead compounds for the development of cancer chemotherapeutics. To date, there have been no previous reports concerning the chemical constituents of *P. callosum*, a terrestrial herb widely distributed in Thailand.

This study aimed to identify TopI poisons from the roots of *P. callosum*. The isolation processes will be carried out through chromatographic techniques based on a yeast cell-based assay-guided fractionation. The TopI poison activities of the isolated compounds will be tested and then their cytotoxicities will be assessed using human cancer cell lines.

2. Materials and Methods

2.1. General Experimental Procedures

^{1}H NMR, ^{13}C NMR and 2D-NMR spectra were obtained on either a 500 MHz Varian Unity INOVA-500 (Varian, Inc., Palo Alto, CA, USA) or a 300 MHz Bruker Avance DPX-300 NMR spectrometer (Bruker Corp., Billerica, MA, USA) in acetone-d_6 with tetramethylsilane as an internal standard. HR-ESI-MS data were recorded on a Bruker microTOF mass spectrometer. IR spectra were recorded on an FTIR Perkin-Elmer 1760X spectrophotometer (Perkin-Elmer, Inc., Waltham, MA, USA). UV spectra were obtained on an Agilent Cary 60 UV-Vis spectrophotometer in methanol. TLC separations were performed on precoated silica gel 60 F_{254} plates (Merck, KGaA, Darmstadt, Germany). Column chromatography (CC) separations were performed using either silica gel (230-400 mesh, Merck) or Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden).

2.2. Plant Material

P. callosum plants were purchased from Ao Luek, Krabi Province, Thailand, in September 2017. Plant identification was performed by Associate Professor Thatree Padungcharoen at The Chulalongkorn University. A voucher specimen (No. 190101-Suchada) was deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2.3. Yeast Cell-Based Assay

A yeast cell-based assay was developed by our colleagues [14]. In brief, complex media (YPD broth) was used to activate the propagation and growth of yeast. A yeast colony was taken from the

YPD growth media and then suspended in SC ura$^-$ media containing glucose. The suspension of yeast was incubated in a shaking incubator at 30°C (200 rpm) for 18 hours. The yeast suspension was adjusted to 0.3 OD_{600} and diluted as serial ten-fold dilutions (10^0, 10^{-1}, 10^{-2}, and 10^{-3}). Camptothecin (10, 5, and 2.5 µg/mL) was used as a positive control. DMSO (1%) was used as the vehicle control. The test compounds were dissolved in dimethylsulfoxide (DMSO) and then filtered through a Millipore filter (0.45 µm). The samples were added to selective SC ura$^-$ media containing 2% glucose and galactose. The serially diluted yeast suspensions (5 µL) were spotted on sample-containing media. Spotted plates were incubated at 30°C for 48 hours. The viability of the yeast cells was observed by comparing the viabilities of the colonies on the vehicle control culture (DMSO) and the positive control culture (camptothecin) to those on the two types of media (2% galactose and glucose agar media).

2.4. Yeast Cell-Based Bioassay-Guided Isolation

The dried roots of *P. callosum* (250 g) were powdered and macerated with methanol at room temperature three times (1.5 L x 3) until exhaustion. The extract was filtered and concentrated under reduced pressure using a rotatory evaporator at 45°C to yield the methanolic extract (30 g). The resulting crude extract was screened for TopI poison activity by a yeast cell-based assay. The extract exhibited growth inhibitory activity. The extract was then separated on a silica gel column (10 x 22 cm) eluted with a gradient mixture of *n*-hexane:acetone (1:0 to 0:1) followed by EtOAc:MeOH (20:1 to 1:1). Seven fractions (A-G) were obtained. Fractions B, C and D, which showed growth inhibitory activities on yeast cells, were subjected to further separations by CC. Fraction B (1 g) was separated on a silica gel column eluted with CH_2Cl_2:EtOAc (20:1) to give 13 subfractions (BA-BM). Subfractions BC and BJ were chromatographed on Sephadex LH-20 columns using MeOH as the eluent to give 14 (BC_1-BC_{14}) and (BJ_1-BJ_2) subfractions, respectively. Compound **2** (14.2 mg) was obtained from subfraction BC_{11} by purification on a silica gel column eluted with *n*-hexane:acetone (8:2), while **3** (7 mg) was directly obtained from subfraction BJ_2. Fraction C (0.2 g) was separated on a silica gel column eluted with CH_2Cl_2:MeOH (98:2) to give 7 subfractions (CA-CG). Subfraction CB was fractionated to give 6 subfractions (CB_1-CB_6) on a Sephadex LH20 column eluted with MeOH. Compound **1** (38.2 mg) was directly obtained from subfraction CB_4, while **4** (17.5 mg) was obtained from subfraction CB_6 following purification on a silica gel column eluted with *n*-hexane:EtOAc (9:1). The last fraction, D, was fractionated on a silica column eluted with *n*-hexane:EtOAc (3:2) to give 10 subfractions (DA-DJ). Subfraction DD was separated on a silica gel column eluted with CH_2Cl_2:MeOH (98:2) to give **5** (3 mg). Subfraction DE was chromatographed on a Sephadex LH-20 column eluted with MeOH to yield 6 subfractions (DE_1-DE_6). The purification of DE_3 by silica gel chromatography using CH_2Cl_2:MeOH (50:1) as the eluent gave **6** (15 mg).

2.5. Cytotoxicity Assay

A resazurin microplate assay (REMA) was used to investigate the cytotoxic activities of the isolated compounds against human cancer cell lines, namely, small cell lung cancer (NCI-H187) and breast adenocarcinoma (MCF-7). The experiments were performed according to the assay protocol developed by O'Brien et al. [15].

In brief, cell suspensions at the appropriate concentration (9 x 10^5 cells/mL) were plated and incubated at 37°C under 5% CO_2 overnight. Then, the test compounds were added. After the incubation period (5 days for NCI-H187 and 3 days for MCF-7), 12.5 µL of 62.5 µg/mL resazurin solution was added to each well, and the cells were further incubated at 37°C for 4 hours. The fluorescence signal was then measured at excitation and emission wavelengths of 530 nm and 590 nm, respectively. The percent inhibition of cell growth was calculated as follows.

$$\% \text{ inhibition} = [1-(FUT/FUC)] \times 100$$

FUT and FUC are the mean fluorescent units under treated and untreated conditions, respectively. Dose response curves were plotted for six concentrations of two-fold serially diluted test compounds, and the sample concentration that inhibited cell growth by 50% (IC_{50} value) was derived using SOFTMax pro software (Molecular device, USA).

3. Results and Discussion

3.1. Yeast Bioassay-Guided Isolation

The budding yeast *Saccharomyces cerevisiae* has been shown to be suitable for the study of DNA-topoisomerase targeting antitumour drugs [16]. This yeast is used as a model system for biological studies because of its rapid growth, flexibility in its DNA transformation system and ease of its replication and mutation [17]. A yeast cell-based assay was successfully utilized for the screening of TopII poisons from plant extracts according to a previous report [18].

In the present study, the fractionation and isolation of compounds from *P. callosum* extract were guided by a yeast cell-based assay using genetically modified *S. cerevisiae*. To prepare the transgenic yeast for screening, the *Top*I gene of *Arabidopsis thaliana* was cloned into the expression vector pYES-DEST52, and the respective constructs were transformed into *S. cerevisiae* strain RS190 (MATa, top1Δ) under the control of the GAL1 promoter [14]. The transformant yeast was used to explore TopI poisons from *P. callosum*. Two types of media, 2% galactose and glucose, were used as carbon sources for yeast growth. Camptothecin was used as a positive control because it is a potent TopI poison that targets TopI by stabilizing the cleavable complex through the inhibition of the re-ligation step [19]. DMSO was used as the vehicle control.

The crude extract of *P. callosum* exhibited inhibited the growth of yeast cells and was separated by column chromatography (Figure 1). In the fractionation process, fractions (A-G) were collected according to polarity, and then the TopI poison activity of each fraction was determined by a yeast assay. The results indicated that fractions B, C and D showed growth inhibitory activities against yeast cells. Chromatographic separation coupled with the yeast assays of the three fractions led to the isolation of six compounds. Compounds **2** and **3** were isolated from fraction B. Compounds **1** and **4** were obtained from fraction C, while **5** and **6** were obtained from fraction D. Compound **1** was isolated for the first time as a new compound. Compounds **2**, **5**, and **6** were isolated from the roots of *P. godefroyae* in a previous study [13]. All the pure compounds (**1**-**6**) obtained from *P. callosum* were subjected to a yeast assay to determine their TopI poison activity by observing the growth of the yeast in galactose and glucose media. Our group has reported that the expression of the *Top*I gene of *Arabidopsis thaliana*, which is homologous to the human gene, in *S. cerevisiae* strain RS190 also conferred TopI poison sensitivity [14]. The wild-type yeast *Top*I gene was deleted from the RS190 strain, and the *Top*I gene of *A. thaliana* was inserted into an expression vector using a strong inducible promoter, pGAL1. TopI is expressed and suppressed in yeast grown in galactose and glucose, respectively. Normal yeast cell growth could be observed in the glucose media because the cleavage complex could not form even in the presence of TopI poisons [14]. The expression of TopI in the galactose media caused an enzyme/DNA complex to form with the TopI poisons, and this covalent complex led to cell death. In the assay, the isolated compounds at different concentrations (100, 50, and 25 μM) showed variable degrees of growth inhibitory activity towards the yeast cells in galactose media (Figure 1). Compounds **1**-**3** and **5**-**6** caused yeast cell death in galactose media. Less cell death was observed with lower concentrations of the test compounds. The yeast was not able to grow in the presence of compound **4**, galangin, at concentrations greater than 50 μM. Our results for galangin were in agreement with a previous report of its TopI inhibitory activity [20]. The TopI poison activities of the tested compounds were compared to DMSO and camptothecin as the vehicle and positive control, respectively. Thus, the results suggested that this yeast system can be used for screening TopI poisons.

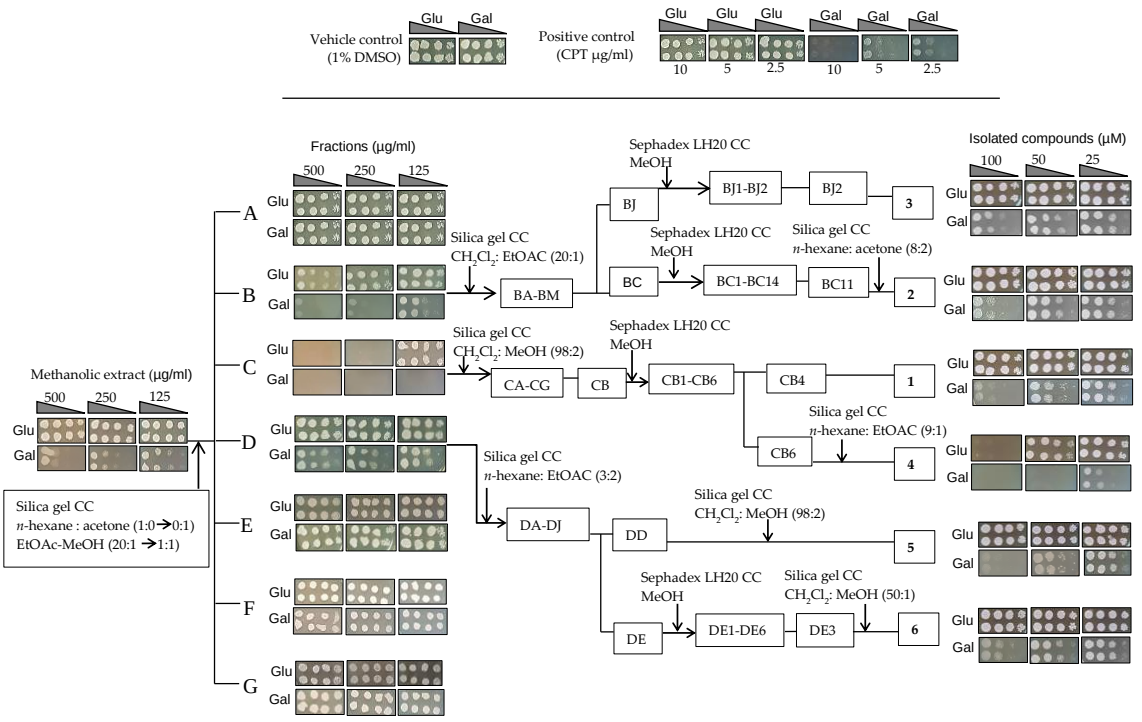


Figure 1. Scheme for the bioassay-guided isolation of *P. callosum* by a yeast cell-based assay. Ten-fold serially diluted *S. cerevisiae* samples were grown in culture media containing various concentrations of extract (500, 250, and 125 µg/mL), Fractions, A-G (500, 250, and 125 µg/mL), compounds **1-6** (100, 50, and 25 µM), camptothecin (10, 5 and 2.5 µg/mL) and 1% DMSO. The results were observed and photographed

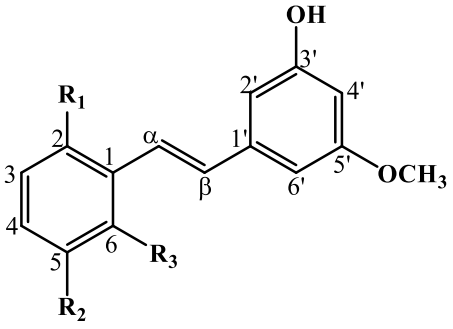
3.2. Structure Elucidation and Identification of the Isolated Compounds

Compound **1** was obtained as a yellowish-brown semisolid. Its molecular formula ($C_{17}H_{18}O_4$) was established on the basis of its negative HR-ESI-MS data, which showed an $[M-H]^-$ ion at *m/z* 285.1126 (calculated for $C_{17}H_{17}O_4$, 285.1126). Its IR spectrum displayed an absorbance for a hydroxyl moiety at 3360 cm^{-1}. The ^1H NMR spectrum of **1** showed one hydroxyl signal at δ_H 8.30 (1H, *s*, 3′-OH), two methoxy signals at δ_H 3.77 (3H, *s*, 5′-OCH_3) and 3.88 (6H, *s*, 2-OCH_3, 6-OCH_3), and olefinic proton signals corresponding to a *trans*-double bond at δ_H 7.41 (1H, *d*, *J* = 16.5 Hz, H-α) and 7.49 (1H, *d*, *J* = 16.5 Hz, H-β). In the aromatic region of δ_H 6.31 - 7.18, two sets of signals related to reciprocal coupled protons were observed. One set was identified as three triplets, corresponding to three *meta*-coupled protons, at δ_H 6.31 (1H, *t*, *J* = 1.8, H-4′), 6.56 (1H, *t*, *J* = 1.8, H-6′) and 6.64 (1H, *t*, *J* = 1.8, H-2′). The other set, which including signals of three *ortho*-coupled protons, appeared as a two-proton doublet at δ_H 6.67 (2H, *d*, *J* = 8.4, H-3, H-5) and a triplet at δ_H 7.18 (1H, *t*, *J* = 8.4, H-4). The former indicated the presence of a 1,3,5-trisubstituted benzene ring in the structure of **1,** while the latter suggested the presence of a 1,2,3-trisubstituted benzene ring in which the 1- and 3-substituents are identical. In the ^{13}C NMR spectrum, signals of three methyl, eight methine and six quaternary carbons were observed. All the above spectroscopic data were indicative of a *trans*-stilbene containing two methoxy groups at positions 2 and 6 on one ring, as well as a methoxy and a hydroxy group at positions 3′ and 5′ on the other ring (Table 1). The location of the four substituents on the stilbene skeleton was also confirmed by the HMBC spectrum, which exhibited three-bond correlations of C-2/C-6 (δ_C 159.6) with H-4 (δ_H 7.18) and H-α (δ_H 7.41) and two-bond correlations of C-3′ (δ_C 159.5) with H-2′ (δ_H 6.64) and H-4′ (δ_H 6.31) as well as correlations of C-5′ (δ_C 162.1) with H-4′ (δ_H 6.31) and H-6′ (δ_H 6.56). In addition, the HMBC correlations of all oxygenated carbons with their corresponding methoxy or hydroxy protons were clearly observed. Therefore, the structure of **1** was elucidated as 3′-hydroxy-2,6,5′-trimethoxystilbene. The assignments of its proton and carbon signals were accomplished on the basis of HSQC and HMBC experiments.

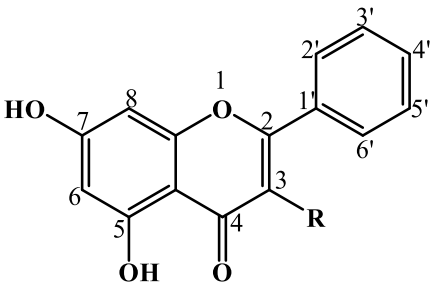
Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **1** in acetone-d_6

Position	δ_H (mult., *J* in Hz)	δ_C	Key HMBC correlations $^{13}C \rightarrow {}^{1}H$
1	-	115.1	H-3, H-5, H-β
2	-	159.6	H-4, H-α, 2-OCH_3, H-3,
3	6.67 (*d*, 8.4)	104.9	H-5
4	7.18 (*t*, 8.4)	129.3	-
5	6.67 (*d*, 8.4)	104.9	H-3
6	-	159.6	H-4,H-α, 6-OCH_3, H-5
α	7.41 (*d*, 16.5)	120.8	H-β
β	7.49 (*d*, 16.5)	132.9	H-2′, H-6′, H-α
1′	-	142.4	H-α, H-β
2′	6.64 (*t*, 1.8)	106.2	H-4′, H-6′, H-β, 3′-OH
3′	-	159.5	H-2′, H-4′, 3′-OH
4′	6.31 (*t*, 1.8)	101.2	H-2′,H-6′, 3′-OH
5′	-	162.1	5′-OCH_3, H-4′, H-6′
6′	6.56 (*t*, 1.8)	104.5	H-2′, H-4′, H-β
2-OCH_3	3.88 (*s*)	56.1	-
6-OCH_3	3.88 (*s*)	56.1	-
5′-OCH_3	3.77 (*s*)	55.4	-
3′-OH	8.30 (*s*)	-	-

The other isolated compounds were identified by comparing their spectroscopic data with those reported in the literature. Compounds **3** and **4** are flavonoids and were identified as 5,7-dihydroxy-3-methoxyflavone (**3**) [21] and galangin (**4**) [22], respectively. Compounds **2**, **5** and **6** are stilbenes and were identified as 3′- hydroxy-2,5′-dimethoxystilbene (**2**) [13], 2,3′-dihydroxy-5,5′-dimethoxystilbene (**5**) and 2,3′-dihydroxy-5′-methoxystilbene (**6**) [23], respectively (Figure 2).



	R_1	R_2	R_3
1	OCH_3	H	OCH_3
2	OCH_3	H	H
5	OH	OCH_3	H
6	OH	H	H



	R
3	OCH_3
4	OH

Figure 2. Chemical structures of compounds **1**-**6**

3.3. In Vitro Cytotoxicity Assay

The cytotoxicity of each isolated compound was examined against human cancer cell lines, including small cell lung cancer (NCI-H187) and breast adenocarcinoma (MCF-7), by using the resazurin assay (Table 2). TopI poison drug, camptothecin, was used as the positive control. Compounds **1**, **2**, **4**, and **6** showed cytotoxic activities against both cancer cell lines (NCI-H187 and MCF-7). Compounds **4** and **6** exhibited higher cytotoxicities against NCI-H187 (IC_{50}=74.10 and 77.94 µM, respectively) than against MCF-7 (IC_{50}=182.48 and 142.63 µM, respectively). New compound **1** showed stronger cytotoxicity against MCF-7 (IC_{50}=82.45 µM) than against NCI-H187 (IC_{50}=170.24), whereas **2** exhibited cytotoxicity against both NCI-H187 and MCF-7 (IC_{50}=62.82 and 74.30 µM, respectively). The IC_{50} values of these compounds against these cancer cells were highly dependent on the type of cell line. Compounds **2, 5** and **6** have been reported to have moderate cytotoxic activity against NCI-H187 in a previous study [13]. In this study, compounds **3** and **5** exhibited no cytotoxicity on the representative cell lines. We postulated that higher doses of **3** and **5** may be required or that the cytotoxicity activity may be specific to certain types of cancer cells. Overall, our study showed that compounds isolated from *P. callosum* roots can be developed as lead candidates for combating cancer via a TopI poisoning mechanism.

Table 2. IC_{50} values for the cytotoxicity of the isolated compounds from *P. callosum* roots.

Compound	**IC_{50} (µM)**	
	NCI-H187	**MCF7**
1	170.24	82.45
2	62.82	74.30
3	Inactive	Inactive
4	74.10	182.48
5	Inactive	Inactive
6	77.94	142.63
Camptothecin	0.0112±0.003	0.0170±0.004

This study demonstrated the yeast cell-based bioassay-guided isolation of TopI poisons from the roots of *P. callosum*. Secondary metabolites of this species with their biological activities are reported for the first time. The investigation yielded a new compound, 3′-hydroxy-2,6,5′-trimethoxystilbene, and 5 known compounds. These compounds were tested against representative human cancer cell lines, and their cytotoxic activities were determined. These newly discovered TopI poisons are structurally diverse and could be considered a good starting point for the development of new anticancer lead compounds.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID

San Yoon Nwe : 0000-0003-4811-2258
Chayapol Tungphatthong : 0000-0001-9695-5044
Areerat Laorpaksa : 0000-0001-5648-1067
Boonchoo Sritularak : 0000-0001-8352-4122
Witchuda Thanakijcharoenpath : 0000-0003-3839-8010
Somboon Tanasupawat : 0000-0002-7149-5341
Suchada Sukrong : 0000-0003-4062-2632

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