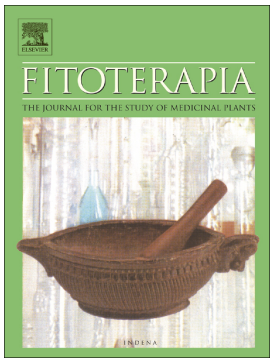

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Cytotoxic Lignans from Fruits of *Cleistanthus tonkinensis*

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ABSTRACT

Seven new lignans, cleistonkinins A– E (**1**–**5**), cleistonkisides A and B (**6**–**7**) were isolated from the fruits of *Cleistanthus tonkinensis* (Euphorbiaceae), together with five known aryltetralin lignans, cleisindoside B (**8**), cleistantoxin (**9**), cleisindoside D (**10**), neocleistantoxin (**11**) and polygamain (**12**). Their structures were established from spectral analysis, including mass

spectrometry and 2D-NMR. The absolute configurations of **4**–**7** were determined by analysis of their experimental CD spectra and comparison with calculated electronic circular dichroism (ECD) spectra. Compounds **2** and **6** had selective inhibition with moderate cytotoxicity against Pan C1 and A549 cell lines, respectively. Cleistantoxin (**9**) was significantly active against A549, HeLa, Hep3B, Pan C1 and MCF7 cell lines while it was less cytotoxic against HeLa cells. Neocleistantoxin (**11**) exhibited remarkable inhibition toward A549, HeLa, MCF7 and Pan C1. This is the first report for cytotoxicity of **9** and **11** against A549, Hep3B and Pan C1 cell lines.

1. Introduction

Cleistanthus genus belongs to the Euphobiaceae family and is distributed in tropical region of Africa, Asia and Australia [1]. An overview of literature revealed that so far, only eight species (*C. collinus*, *C. patulus*, *C. sumafranus*, *C. sumatranus*, *C. boivinianus*, *C. indochinensis*, *C. gracilis* and *C. schlechteri*) of the 140 species of *Cleistanthus* were examined for their chemical contents [1,2]. Lignans and terpenoids have been found to be major bioactive metabolites of plants of the *Cleistanthus* genus [3–8]. Previously, we reported the isolation and structural characterization of cleistanone, an unprecedented triterpenoid skeleton from the leaves [9], and a number of lignans from the fruits of *C. indochinensis* [10,11]. In the course of a screening program of the flora in Vietnam, the fruit extract of *C. tonkinensis* showed significant cytotoxicity against KB cell line (88.4% inhibition at the concentration of 1 μg/mL). This plant is used in traditional medicine to treat stomach pain, rheumatism and irregular menstruation [12]. Herein, we describe the isolation and structural elucidation of seven new lignans (**1**–**7**) and five known compounds, **8**–**12** from the fruits of *C. tonkinensis*.

2. Experimental section

2.1. General experimental procedures

Optical rotations were recorded on an Atago Polax-2L polarimeter, using a sodium (589, D line) lamp. IR spectra were measured on a Nicolet Impact-410 FT-IR spectrometer and CD spectra on a Chirascan CD spectrometer. NMR spectra were recorded on a Bruker AM500 FT-NMR spectrometer operating at 500.13 MHz and 125.76 MHz for ^{1}H NMR and ^{13}C NMR spectra, respectively. ^{1}H NMR chemical shifts were referenced to 7.27 ($CDCl_3$) and 3.31 ppm

(CD_3OD), respectively. Whereas, ^{13}C NMR chemical shifts to the central peak of $CDCl_3$ at 77.0 and CD_3OD at 49.0 ppm. The HMBC measurements were optimized to 7.0 Hz long-range couplings, and NOESY experiments were run with 150 ms mixing time. HR-ESIMS were recorded on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system.

2.2. Plant material

The fruits of *C. tonkinensis* was collected in Dai Tu, Thai Nguyen, Vietnam in June 2017 and identified by Dr. Nguyen The Cuong. A Voucher specimen (VN 2345B) was deposited at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST) in Hanoi.

2.3. Extraction and isolation

Dried and ground fruits of *C. tonkinensis* (5 kg) were extracted with EtOH (5 × 12 L, 18 h each) at room temperature. The extracts were combined and concentrated under diminished pressure. The residue was suspended in water (350 mL) and extracted with *n*-hexane (3 × 500 mL). The aqueous solution was freeze-dried to afford a residue of 168 g, which was subjected to column chromatography (CC) on silica gel eluting with CH_2Cl_2/MeOH (from 0% to 100% MeOH in CH_2Cl_2) to yield 23 fractions. Fraction 1 (469 mg) was purified by CC on silica gel, eluted with 0 to 100% EtOAc in *n*-hexane affording **12** (10.1 mg). Fraction 7 (6.53 g) was chromatographed on silica gel column (0 to 100% acetone in *n*-hexane) giving 10 subfractions. Subfraction 8 (4.1 g) was recrystallized in a mixture of *n*-hexane/CH_2Cl_2 (3/7, v/v) yielded **9** (93.3 mg). Fraction 8 (4.2 g) was separated by CC on silica gel (0 to 100% acetone in *n*-hexane) to yield 5 subfractions. Subfraction 3 (1.9 g) was subjected to CC on Sephadex LH-20 (CH_2Cl_2/MeOH: 1/9), to provide **9** (2.0 g) and **4** (251.1 mg). Fraction 11 (8.64 g) was chromatographed on a silica gel CC (0 to 100% MeOH in CH_2Cl_2), giving 7 subfractions. Subfraction 3 (1.09 g) was purified by CC on silica gel (0 to 60% of acetone in CH_2Cl_2), followed by CC on Sephadex LH-20 (MeOH) to afford **5** (65.0 mg) and **11** (5.5 mg). Fraction 16 (6.5 g) was separated by CC on silica gel (0 to 100% MeOH in CH_2Cl_2), followed by CC on Sephadex LH-20 (CH_2Cl_2/MeOH: 1/9), to provide **10** (3.3 g). Fraction 17 (26.2 g) was subjected to a CC on silica gel (0% to 100% MeOH in CH_2Cl_2) giving 10 subfractions. Subfraction 4 (216 mg) was purified by CC on Sephadex LH-20 (MeOH/CH_2Cl_2: 9/1), followed by preparative TLC (CH_2Cl_2/MeOH: 90/10) affording **6** (18.4 mg). Fraction 18 (19.9 g) was separated by CC on

silica gel (0 to 100% MeOH in CH_2Cl_2), followed by CC on Sephadex LH-20 (CH_2Cl_2/MeOH: 1/9), to furnish **3** (8.2 mg). Fraction 20 (13.8 g) was purified by CC on silica gel (0 to 100% acetone in *n*-hexane) to yield 7 subfractions. Subfraction 2 (292 mg) was chromatographed on Sephadex LH-20 CC (MeOH), followed by preparative TLC (CH_2Cl_2/MeOH: 9.5/0.5) giving **1** (1.0 mg) and **2** (1.2 mg). Subfraction 5 (230 mg) was subjected to a CC on silica gel (0 to 90% MeOH in CH_2Cl_2) to provide **7** (10.8 mg). Finally, subfraction 6 (640 mg) was purified by Sephadex LH-20 CC (MeOH) affording **8** (60.7 mg).

Cleistonkinin A (**1**): white powder; mp 131–134 ^{o}C; IR (KBr) ν_{max} 3341, 2901, 1502, 1495, 1465, 1231, 1102, 1033, 933 cm^{-1}; UV (MeOH): λ_{max} nm (logε) 204 (4.78), 285 (3.70), 309 (3.57); NMR data see Table 1; HRESIMS (negative mode) *m/z* 321.0753 [M - H]$^-$ (calcd for $C_{19}H_{13}O_5$, 321.0763).

Cleistonkinin B (**2**): white powder; mp 154–156 ^{o}C; IR (KBr) ν_{max} 3341, 2901, 1502, 1495, 1465, 1231, 1102, 1033, 932 cm^{-1}; UV (MeOH): λ_{max} nm (logε) 204 (4.78), 285 (3.70), 309 (3.57); NMR data see Table 1; HRESIMS (negative mode) *m/z* 351.0887 [M - H]$^-$ (calcd for $C_{20}H_{15}O_6$, 351.0869).

Cleistonkinin C (**3**): white microcrystalline, mp 328–329 ^{o}C; IR (KBr) ν_{max} 2920, 1759, 1616, 1531, 1489, 1446, 1398, 1336, 1257, 1228, 1197, 1082, 1037, 1010, 927, 873 cm^{-1}; UV (MeOH): λ_{max} nm (logε) 204 (4.30), 230 (4.22), 257 (4.07), 332 (3.67), 344 (3.62); NMR data see Table 2; HRESIMS (positive ion mode) *m/z* 401.0629 [M + Na]$^+$ (calcd for $C_{21}H_{14}NaO_7$, 401.0637).

Cleistonkinin D (**4**): white microcrystalline; mp 328–330 ^{o}C; $[\alpha]_D^{30}$ +88.1 (*c* 0.042, $CHCl_3$); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 226 (-16.5), 257 (+0.5), 278 (-6.6), 346 (+15.9) nm; IR (KBr) ν_{max} 3547, 2912, 1745, 1656, 1602, 1489, 1440, 1396, 1346, 1240, 1195, 1114, 1051, 1004, 929, 854, 821 cm^{-1}; UV (MeOH) λ_{max} nm (logε) 204 (4.30), 230 (4.22), 257 (4.07), 332 (3.67), 344 (3.62); NMR data see Table 2; HRESIMS (positive mode) *m/z* 419.0749 [M + Na]$^+$ (calcd for $C_{21}H_{16}NaO_8$, 419.0743).

Cleistonkinin E (**5**): white microcrystalline; mp 270–271 ^{o}C; $[\alpha]_D^{30}$ +234.6 (*c* 0.108, $CHCl_3$); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 203 (-20.1), 225 (-5.5), 239 (-6.3), 261 (+1.9), 278 (-3.2), 357 (+4.3) nm; IR (KBr) ν_{max} 3466, 2899, 1726, 1629, 1589, 1475, 1446, 1338, 1269, 1238, 1188, 1099, 1029, 1012, 927, 885 cm^{-1}; UV (MeOH) λ_{max} nm (logε) 204 (4.30), 230 (4.22), 257 (4.07), 332 (3.67),

344 (3.62); NMR data see Table 2; HRESIMS (positive mode) *m/z* 405.0583 $[M + Na]^+$ (calcd for $C_{20}H_{14}NaO_8$, 405.0586).

Cleistonkiside A (**6**): white microcrystalline; mp 174–176 ^oC; $[\alpha]_D^{30}$ -218.18 (*c* 0.44, $CHCl_3$); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 201 (-39.4), 216 (-19.7), 236 (-5.0), 251 (+0.6), 279 (-3.5), 295 (+2.9) nm; IR (KBr), ν_{max} 3523, 2900, 1747, 1618, 1504, 1475, 1440, 1338, 1232, 1070, 1033, 997, 925 cm^{-1}; UV (MeOH) λ_{max} nm (logε): 204 (4.30), 230 (4.22), 257 (4.07), 332 (3.67), 344 (3.62); NMR data see Table 3; HRESIMS (negative mode) *m/z* 659.1601 $[M - H]^-$ (calcd for $C_{31}H_{31}O_{16}$, 659.1612).

Cleistonkiside B (**7**): white microcrystalline; mp 184–185 ^oC; $[\alpha]_D^{30}$ -101.2 (*c* 0.083, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 204 (-13.7), 217 (+0.2), 234 (-3.0), 252 (+0.6), 285 (-1.0), 306 (+0.02) nm; IR (KBr), ν_{max} 3329, 2895, 1755, 1643, 1490, 1440, 1382, 1309, 1242, 1165, 1139, 1070, 1012, 933, 869, 806 cm^{-1}; UV (MeOH) λ_{max} nm (logε) 204 (4.30), 230 (4.22), 257 (4.07), 332 (3.67), 344 (3.62); NMR data see Table 4; HRESIMS (negative mode) *m/z* 663.1930 $[M - H]^-$ (calcd for $C_{31}H_{35}O_{16}$, 663.1925).

2.4. Cytotoxic activity assay

Hep3B (Human hepatoma), Hela (human cervical carcinoma), MCF7 (human breast cancer), Pan C1 (pancreas epithelioid acarcinoma) and A549 (human lung adenocarcinoma) were kindly provided by Prof. Jeong-Hyung Lee, Department of Biochemistry, College of Natural Sciences, Kangwon National University, Korea. The cells were cultured at 37 ^oC in RMPI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a 5% CO_2 incubator. Cells between 5 and 20 passages were used for the assays. The cytotoxic activity was measured by using a modified MTT assay [13]. Viable cells were seeded in 96-well plates at a density of 1.0×105 cells/mL. Cells were treated with various concentrations of essential oils (0.3 1, 3, 10, and 30 µg/mL) and then incubated at 37^{o}C for 24 h in fresh RMPI1640 medium. Cells were subsequently incubated at 37 °C with MTT (0.5 mg/mL) for 4 h. After removal of supernatant, formazan crystals were dissolved in isopropanol and the optical density was measured at 570 nm. Camptothecin (Andrich, purity >95%) was used as a positive control.

*2.5. TD-DFT electronic CD (ECD) calculation of **4**–**7***

A conformational search was carried out with Spartan'14 software (Wavefunction Inc., Irvine, CA) for the proposed absolute configuration as shown at the molecular mechanics level (MMFF) [14]. All MMFF minima were re-optimized using DFT calculations at the B3LYP/6-31G(d) level with the Gaussian 09 program. The geometry was optimized starting from various initial conformations with vibrational frequency calculations confirming the presence of minima. The DFT calculations (B3LYP/LanL2DZ) were performed on the lowest-energy conformations using 30 excited states and a polarizable continuum model (PCM) in methanol. The ECD spectra of the conformers were combined using Boltzmann weighting with program SpecDis 1.71 [15]. After applying a UV-shift correction, the computed CD spectra were compared with the CD curves experimentally obtained.

*2.6. Acid Hydrolysis of **6** and **7***

Each solution of **6** or **7** (each 5.0 mg) in 1N HCl (dioxane/H_2O: 1/1, 2.5 mL) was heated at 95 ^oC for 5 h. The filtrates from hydrolysate were neutralized with DOWEX HCR-S ion-exchange resin and filtered. The filtrates were concentrated under reduced pressure and examined for carbohydrates by silica gel TLC [Kieselgel 60 (Merck Art 5554), *i*-PrOH/Me_2CO/H_2O (5/3/1)] comparing with authentic samples. The R_f values of each sugar were as follows: glucose, 0.43; and apiose, 0.54. The remaining residue was purified by preparative TLC. The sugars were identified as D-glucose {$[\alpha]_D^{30}$ +53 (*c* 0.18, H_2O)}, and D-apiose {$[\alpha]_D^{30}$ +16 (*c* 0.12, H_2O)}.

3. Results and discussion

Dried and ground fruits of *C. tonkinensis* (5 kg) were extracted with EtOH at room temperature. The solvent was removed under reduced pressure, and the residue was suspended in water and extracted with *n*-hexane. The water layer was freeze-dried and the residue (168 g) was purified by repeated open column chromatography to give compounds **1**–**12** (Figure 1).

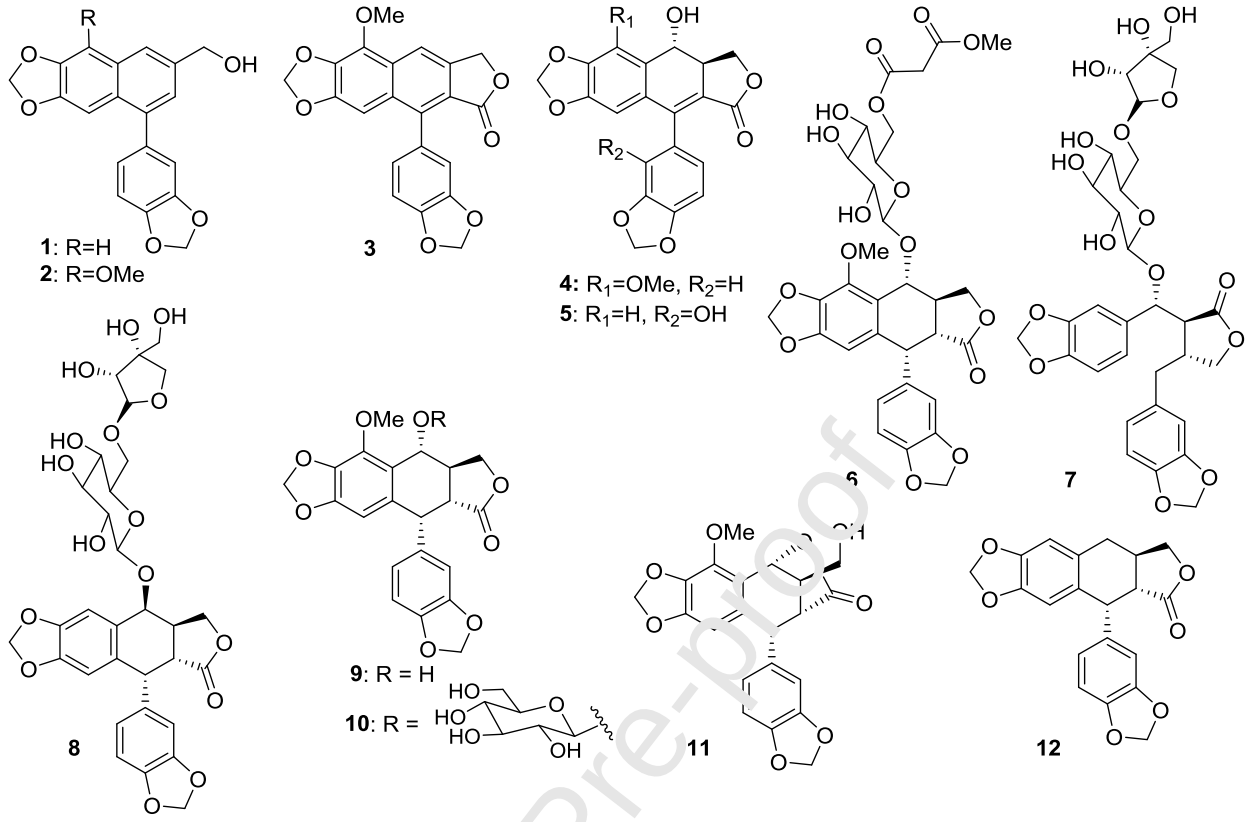

Fig. 1. Structure of compounds **1**–**12**.

Compound **1** was isolated as white powder. Its negative HRESIMS showed the deprotonated molecular ion [M - H]$^-$ at *m/z* 321.0753 which together with ^{13}C NMR data are consistent with the molecular formula of $C_{19}H_{14}O_5$. The ^{1}H NMR spectra of **1** showed the resonances of an ABX aromatic ring system at δ_H 6.92 (H-2′), 6.91 (H-5′) and 6.89 (H-6′), two singlet aromatic protons at δ_H 7.19 (H-3) and 7.10 (H-6), and two *ortho*-coupled spin system at δ_H 7.25 (H-8′) and 7.64 (H-7) in the aromatic region (Table 1). Furthermore, three broad singlets, each of which integrates to two protons was also observed at δ_H 4.82 (CH_2-9), 6.04 (CH_2-10) and 6.02 (CH_2-9′). ^{13}C NMR data analysis with the aid of HSQC experiment revealed the resonances of 19 carbons, including two methylenedioxy groups, seven aromatic methines, one oxygenated methylene group and nine sp^2 quaternary carbons (Table 2). Analyses of 2D NMR spectra of **1**, especially HMBC spectrum indicated a naphthalene system in the structure of **1** (Figure 2). The location of the oxygenated methylene at C-8 was established by correlation of protons at δ_H 4.82 (CH_2-9) with carbons at δ_C 124.5 (C-7), C-8 (δ_C 136.4) and C-8′ (δ_C 124.9). Similarly, the ABX ring was

linked to C-7′ as demonstrated by cross-peaks of C-7′ at δ_C 139.6 with protons at δ_C 6.92 (H-2′) and 6.89 (H-6′). Finally, the two methylenedioxy groups at C-4/C-5 and C3′/C4′ were determined by cross-peaks of CH_2-10 protons (δ_H 6.04) with C-4 (δ_C 148.0) and C-5 (δ_C 147.7), and CH_2-9′ protons (δ_H 6.02) with C-3′ (δ_C 147.6) and C-4′ (δ_C 147.0). Compound **1** was reported here for the first time and named cleistonkinin A.

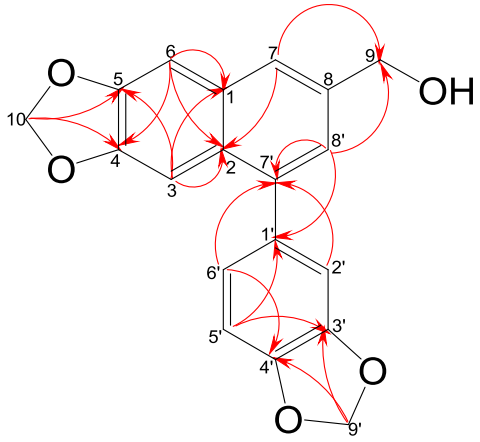

Fig. 2. Selected HMBC correlations for **1**.

Table 1 ^{1}H NMR data for **1**–**7** (mult. *J* in Hz).

Cn°	**1***	**2***	**3***	**4****	**5****	**6***	**7****
2							6.94 (1H, s)
3	7.19 (1H, s)	6.93 (1H, s)	6.8[illegible] (1H, s)	6.07 (1H, s)	6.29 (1H, s)	6.34 (1H, s)	
5							6.79 (1H, d, 8.0)
6	7.16 (1H, s)						6.76 (1H, d, 8.0)
7	7.64 (1H, br. s)	8.06 (1H, d, 1.0)	8.14 (1H, s)	5.12 (1H, d, 13.5)	4.71 (1H, dd, 6.2, 14.5)	5.27 (1H, d, 10.0)	5.21 (1H, d, 3.5)
8				3.38 (1H, m)	3.28 (1H, m)	3.18 (1H, m)	2.52 (1H, m)
9	4.82 (2H, s)	4.83 (2H, s)	5.37 (2H, s)	4.12 (1H, t, 9.0)	4.19 (1H, dd, 8.7, 8.5)	4.06 (1H, dd, 8.0, 9.0)	
				4.64 (1H, t, 9.0)	4.62 (1H, dd, 9.0, 8.7)	4.61 (1H, dd, 8.0, 8.0)	
10	6.04 (2H, br.	6.03 (2H,	6.05 (2H, br.	5.96 (2H,	6.01 (1H, br.	5.98 (1H, br.	5.92 (2H, br.

	s)	br. s)	s)	br. s)	s) 6.04 (1H, br. s)	s) 5.99 (1H, br. s)	s)
2′	6.92 (1H, d, 1.0)	6.91 (1H, br. s)	6.78 (1H, d, 1.5)	6.64 (1H, s)		6.64 (1H, s)	6.44 (1H, d, 1.0)
5′	6.91 (1H, d, 8.0)	6.90 (1H, d, 8.0)	6.94 (1H, d, 8.0)	6.87 (1H, d (8.0)	6.95 (1H, d, 8.0)	6.73 (1H, d, 8.0)	6.66 (1H, d, 8.0)
6′	6.89 (1H, dd, 1.5, 8.0)	6.88 (1H, br. d, 8.0)	6.76 (1H, dd, 1.5, 8.0)	6.59 (1H, d, 8.0)	6.67 (1H, d, 8.0)	6.74 (1H, d, 8.0)	6.41 (1H, dd, 1.0, 8.0)
7′						4.53 (1H, d, 4.5)	2.24 (1H, dd, 7.5, 13.5) 2.48 (1H, m)
8′	7.25 (1H, d, 1.5)	7.28 (1H, d, 1.0)				2.68 (1H, dd, 4.5, 14.5)	2.88 (1H, m)
9′	6.02 (2H, br. s)	5.99 (2H, br. s)					3.93 dd (5.0, 8.5) 4.28 dd (8.5, 8.5)
10′			6.05 (1H, d, 0.5) 6.07 (1H, d, 0.5)	5.95 (2H, br. s)	6.06 (1H, br. s) 6.07 (1H, br. s)	5.91 (1H, br. s) 5.92 (1H, br. s)	5.98 (1H, br. s) 6.00 (1H, br. s)
1″						4.38 (1H, d, 7.5)	3.98 (1H, d, 7.0)
2″						3.39 (1H, m)	3.04 (1H, m)
3″						3.53 (1H, dd, 9.0, 9.0)	3.03 (1H, m)
4″						3.37 (1H, dd, 9.0, 9.0)	3.01 (1H, m)
5″						3.39 (1H, m)	3.07 (1H, m)
6″						4.28 (2H, m)	3.35 (1H, m) 3.77 (1H, br. d, 10.5)
1‴							4.90 (1H, d, 3.0)
2‴						2.87 (1H, d, 15.5)	3.73 (1H, d, 3.0)

						3.10 (1H, d, 15.5)	
4‴							3.54 (1H, d, 9.0) 3.81 (1H, d, 9.0)
5‴							3.32 (2H, m)
6-OMe	4.19 (3H, s)	4.21 (3H, s)	3.91 (3H, s)				
7-OH			5.29 (1H, br. s)	5.98 (1H, d, 6.2)			
2′-OH				6.79 (1H, br. s)			
2″-OH							5.26 (1H, d, 4.0)
3″-OH							4.94 (1H, d, 5.0)
1‴-OMe						4.06 (3H, s)	
3‴-OH							4.46 (1H, s)
5‴-OH							4.74 (1H, t, 5.5)

*Recorded in $CDCl_3$; **Recorded in DMSO-d_6

Signals of 1D NMR spectra of cleistonkinin B (**2**) were close to those of **1** except for the presence of a methoxy group and a sp^2 quaternary carbon instead of the aromatic methine group which suggested **2** had the same carbon skeleton as **1** with an additional methoxy group. This observation was confirmed by analyses of 2D NMR spectra. The methoxy group at C-6 was defined by cross-peaks of C-6 at δ_C 139.4 with the methoxy protons at δ_H 4.19 in the HMBC spectrum.

Compound **3** was obtained as a white, microcrystalline solid. Its positive HRESI mass spectrum showed a sodium adduct ion $[M + Na]^+$ at *m/z* 401.0629. 1D NMR data of **3** suggested its structural similarity to **2**. However, resonance of a sp^2 quaternary carbon in place of an aromatic methine, and an additional carbonyl group at δ_C 169.9 (C-9′) were revealed for **3**.

Analysis of 2D NMR spectra assigned the structure cleistonkinin C (**3**) which is an aryl naphthalene lignan lactone. The lactone ring was defined by HMBC cross-peaks of the carbonyl carbon C-9′ at δ_C 169.9 with CH_2-9 protons at δ_H 5.37.

Compound **4** was isolated as a white microcrystalline solid and optically active $[\alpha]_D^{30}$ +88.1 (*c* 0.042, $CHCl_3$). Its positive HRESI mass spectrum exhibited the sodium adduct molecular ion $[M + Na]^+$ at *m*/*z* 419.0749, suggesting the molecular formula of $C_{21}H_{16}O_8$. In comparison of 1D NMR data with **3**, the resonances of two sp^3 methines were observed for **4**, instead of a double bond in the structure of **3**. This was confirmed by correlations of a spin-spin coupling system H-7 (δ_H 5.12)/H-8 (δ_H 3.38)/CH_2-9 (δ_H 4.12 and 4.64) in the COSY spectrum of **4**. The chemical shifts of CH-7 suggested its linkage to oxygen (Tables 1 and 2). The relative configuration of **4** was established by proton coupling constant analysis of H-7 which had *anti*-coupling constant (*J* = 13.5 Hz) indicating a *trans*-pseudodiaxial relationship between H-7 and H-8. In order to resolve the absolute configuration of **4**, its circular dichroism (CD) spectrum was compared with calculated electronic circular dichroism (ECD) spectra of the two possible enantiomers, (7*S*,8*R*)-**4** and (7*R*,8*S*)-**4** [16,17]. The ECD quantum chemical calculations were performed using the Gaussian 09 software [18]. To obtain minimum energy conformers, geometry optimization of each possible enantiomer of **4** was conducted. The calculated ECD spectra of (7*S*,8*R*)-**4** and (7*R*,8*S*)-**4** were generated using the time-dependent density functional method at the B3LYP/LanL2DZ level. The calculated ECD spectrum of (7*S*,8*R*)-**4** displayed Cotton effects in good agreement with those of the experimental CD spectrum of **4** (Figure 3). The *S*- and *R*-configurations were thus assigned for the chiral carbons C-7 and C-8 of cleistonkinin D (**4**), respectively.

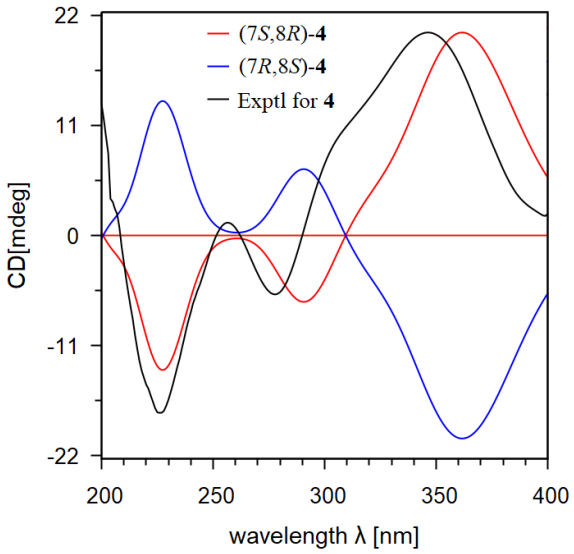

Fig. 3. Calculated ECD and experimental CD spectra of **4**.

^{1}H and ^{13}C NMR spectra of **5** exhibited characteristic features of an aryl-dihydronaphthalene lactone type as **4**. The ^{1}H NMR spectrum of **5** presented two singlet protons at δ_H 6.29 (H-3) and 7.14 (H-6), and two *ortho*-coupled spin systems at δ_H 6.95 (d, J = 8.0 Hz, H-5′) and 6.67 (d, J = 8.0 Hz, H-6′) in the aromatic region which were consistent with the presence of a 1,2,4,5-tetrasubstitued and a 1,2,3,4-tetrasubstituted benzene rings. The spin-spin coupling system H-7 (δ_H 4.71)/H-8 (δ_H 3.28)/CH_2-9 (δ_H 4.19 and 4.62) was observed in the COSY spectrum of **5** as in the structure of **4**. The chemical shifts of C-4, C-5, C-2′, C-3′, C-4′, CH-7 and CH_2-9 (Tables 1 and 2) suggested their connection to oxygen. Intensive analysis of 2D NMR data confirmed the structure of **5**. The relative configuration of **5** was similar to that of **4** as indicated by *anti*-coupling constant (J = 14.5 Hz) between H-7 and H-8. The absolute configurations of the **5** was established by comparison of its experimental and calculated electronic circular dichroism (ECD) spectra (Figure 4). The ECD spectrum of (7*S*,8*R*)-**5** was in agreement with the experimental CD spectrum of **5** indicating 7*S*- and 8*R*-configurations for cleistonkinin E (**5**).

Table 2 ^{13}C NMR data for **1**–**7**.

Cn^{o}	**1***	**2***	**3***	**4****	**5****	**6***	**7****
1	131.1	125.4	129.0	126.3	137.3	121.5	132.1
2	128.4	128.7	130.7	130.4	128.9	135.9	107.1
3	102.4	97.1	98.4	104.7	108.3	105.4	147.2

4	148.0	149.0	149.6	148.5	149.1	150.4	146.6
5	147.7	133.5	136.1	139.9	145.4	137.0	107.9
6	104.2	139.4	135.7	142.5	105.1	141.9	120.2
7	124.5	119.0	113.9	73.4	71.7	76.4	75.6
8	136.4	134.9	139.1	43.1	42.5	37.6	51.7
9	65.5	65.8	68.3	70.8	70.0	71.9	176.4
10	101.1	101.1	101.7	101.6	101.8	101.8	101.0
1′	134.8	134.0	128.5	128.4	127.9	132.9	132.2
2′	110.4	110.4	110.6	110.5	146.8	110.7	108.7
3′	147.6	146.9	147.6	147.2	147.5	147.5	147.1
4′	147.0	147.5	147.5	147.7	146.4	146.9	145.5
5′	108.3	108.2	108.2	108.3	107.9	107.9	107.9
6′	123.2	123.2	123.5	123.8	123.4	124.0	121.6
7′	139.6	139.4	139.8	145.6	144.7	44.3	38.5
8′	124.9	125.7	119.5	113.6	119.7	45.5	36.2
9′	101.1	101.0	169.9	168.4	167.9	173.9	71.9
10′			101.2	102.5	101.3	101.3	101.6
1″						98.1	99.1
2″						73.3	73.6
3″						76.8	75.8
4″						69.8	70.2
5″						73.8	76.5
6″						64.2	67.4
1‴						166.6	109.4
2‴						40.4	76.0
3‴						166.8	78.8
4‴							73.3
5‴							63.4
6-OMe		60.0	60.1	60.9		52.6	
1‴-OMe						60.2	

*Recorded in $CDCl_3$; **Recorded in DMSO-d_6

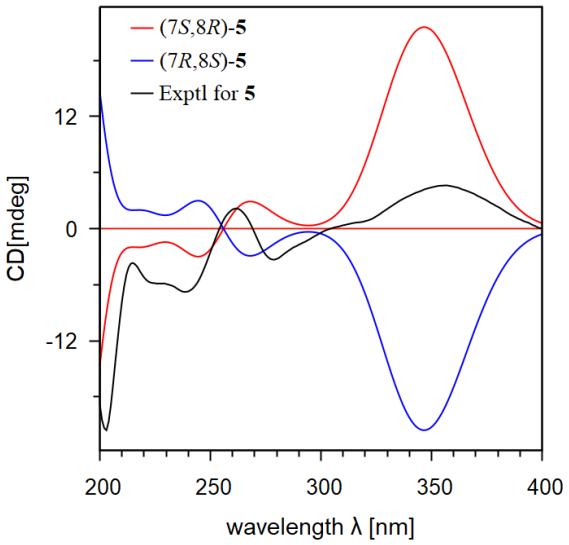

Fig. 4. Calculated ECD and experimental CD spectra of **5**.

Compound **6** was isolated as a white solid and optically active $[\alpha]_D^{30}$ -218.1 (*c* 0.44, $CHCl_3$). Its HRESIMS in negative mode presented the pseudo molecular ion $[M - H]^-$ at *m/z* 659.1601 which together with the ^{13}C NMR data are consistent with a molecular formula of $C_{31}H_{32}O_{16}$. The ^1H NMR spectrum of **6** indicated a singlet proton at δ_H 6.34 (H-3), an ABX system characterized from three protons at δ_H 6.64 (H-2′), 6.73 (H-5′) and 6.74 (H-6′) in the aromatic region. Analysis of the ^{13}C NMR with the aid of the HSQC spectrum of **6**, and the comparison of its NMR data with those of related compounds [10,11], revealed the presence of an aryltetralin lignan glycoside skeleton. This was confirmed by analysis of 2D NMR spectra of **6**, where the methine, methylene, and carboxylate carbon resonances of the aryltetralin lignan framework at δ_C 37.6 (C-8), 45.5 (C-8′), 71.9 (C-9), 76.4 (C-7) and 173.9 (C-9′) were assigned. The presence of a sugar moiety at C-7 was revealed by the cross-peaks of C-7 with the anomeric proton H-1″ at δ_H 4.38 in the HMBC spectrum. The malonate element was connected to C-6″ via ester bridge as shown by the HMBC correlations of C-1‴ at δ_C 166.6 with the protons CH_2-6″ at δ_H 4.28 (Figure 5). Proton coupling constants of the sugar protons indicated the presence of *β*-glucopyranose (Table 1). The proton H-7 had an *anti* coupling constant (*J* = 10.0 Hz), suggesting a *trans*-pseudodiaxial relationship between H-7 and H-8. Whereas, proton H-8′ had a *gauche* (*J* = 4.5 Hz) and an *anti* (*J* = 14.5 Hz) coupling constants. This indicated a pseudoequatorial and a

pseudoaxial orientations for H-7′ and H-8′, respectively. This observation was clearly confirmed by interactions of H-7 (δ_H 5.27) with H-8′ (δ_H 2.68), and those of H-7′ (δ_H 4.53) with H-8′ and one proton of CH_2-9 at δ_H 4.06 in the NOESY spectrum. A positive Cotton effect at 295 nm (Δε +2.9) was observed in the CD spectrum of **6** revealing the *R*-configuration for C-7′ [10,19,20]. Base on the relative configuration established above, the *R*-configuration was thus assigned for the remaining chiral centers, C-7, C-8 and C-8′. The hydrolyzed sugar had a positive optical rotation activity and an identical R_f value with the standard D-(+)-glucose, which suggested the D-configuration for the glucose moiety of cleistonkiside A (**6**).

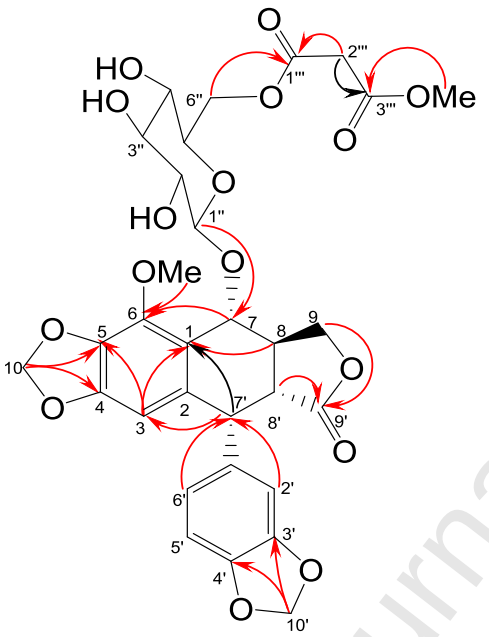

Fig. 5. Key HMBC correlations for **6**.

Compound **7** was obtained as optically active white solids {$[\alpha]_D^{30}$ -101.2 (*c* 0.083, MeOH)}, and showed a pseudo molecular ion $[M - H]^-$ at *m/z* 663.1930 in the negative HRESIMS which was in agreement with a molecular formula of $C_{31}H_{36}O_{16}$. Its ^{1}H NMR exhibited the resonances of two aromatic ABX systems which were confirmed by analysis of ^{13}C NMR with the aid of HSQC experiment. The ^{1}H-^{1}H COSY data indicated the correlations of H-7 (δ_H 5.21)/H-8 (δ_H 2.52)/H-8′ (δ_H 2.88)/CH_2-7′ (δ_H 2.24 and 2.48), and those of H-8′/CH_2-9′ (δ_H 3.93 and 4.28). In the HMBC spectrum of **7**, cross-peaks of the carbonyl carbon C-9 (δ_C 176.4) with H-7 and CH_2-9′ indicated the formation of the lactone ring. The lignan skeleton was revealed by correlations of C-7 (δ_C 75.6) with H-2 (δ_H 6.94) and H-6 (δ_H 6.76), and those of C-7′ (δ_C 38.5) with H-2′ (δ_H

6.44) and H-6′ (δ_H 6.41). Additionally, the positions of the two methylendioxy groups at C-3/C-4 and C-3′/C-4′ were defined by HMBC correlations of C-3 (δ_C 147.2) and C-4 (δ_C 146.6) with methylene protons CH_2-10 (δ_H 5.92), and C-3′ (δ_C 147.1) and C-4′ (δ_C 145.5) with protons CH_2-10′ (δ_H 5.98 and 6.00). The presence of two sugar units was also observed which were assigned to a glucopyranose and an apiofuranose by analysis of 2D NMR data and proton coupling constants (Table 1). The bonding of the glucopyranose substructure to C-7 was established from the HMBC cross-peak of the anomeric carbon at δ_C 99.1 (C-1″) to the proton at δ_H 5.21 (H-7), and the linkage of the apiofuranose to C-6″ of the glucopyranose moiety by the correlation of the carbon at δ_C 67.4 (C-6″) with the anomeric proton at δ_H 4.90 (H-1‴) (Figure 6). The anomeric proton H-1″ had a coupling constant J = 7.0 Hz, assigning a β-form for the glucopyranose unit while, the downfield chemical shift of the anomeric carbon C-1‴ (δ_C 109.4) suggested a β-apiofuranose conformation in the structure of **7** [11,21]. NOE correlation of H-7 (δ_H 5.21) with H-8′ (δ_H 2.88) indicated that CH-7 and H-8′ were co-facial. This observation demonstrated a *trans* relationship for H-8/H-8′ which was supported by the $\Delta\delta_{Ha\text{-}9'\text{-}Hb\text{-}9'}$ value of 0.35 (this value ≥ 0.2 for *trans*, and ≈ 0 for *cis*) [22,23]. The hydrolyzed glucose and apinose had positive optical rotation values, assigning the D-configuration for both glucose and apiose moieties of **7**. Since, D-glucose, D-apiose, and *trans* configuration for H-8/H-8′ were determined, four possible stereoisomers, 7*R*,8*S*,8′*R*, 7*R*,8*R*,8′*S*, 7*S*,8*R*,8′*S* and 7*S*,8*S*,8′*R* should be attributed for the aglycone of **7**. The ECD spectra of four diastereomers were generated and compared with the experimental CD spectrum of **7** (Figure 7). The ECD spectrum of the diastereomer 7*R*,8*S*,8′*R*-**7** was in accordance with the experimental CD spectrum of **7**. Thus, the 7*R*, 8*S* and 8′*R*-configurations were assigned for **7**. This suggestion was in agreement with the negative Cotton effect at 234 nm in the CD spectrum of **7**, indicating the *R*-configuration for C-8′ [24].

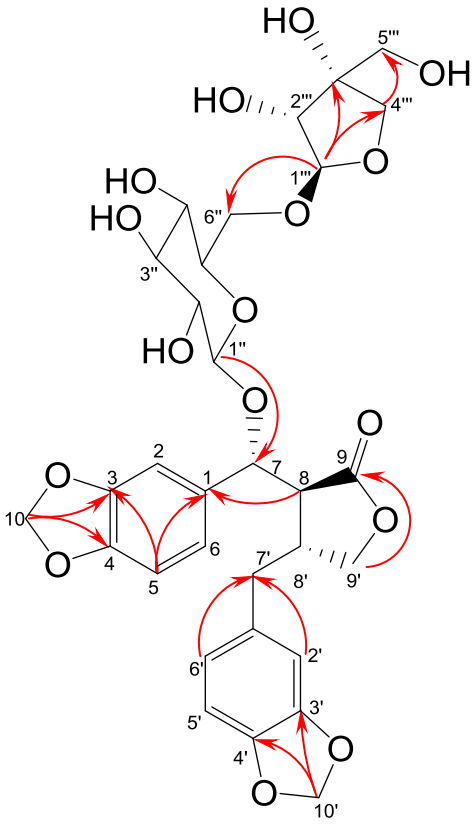

Fig. 6. Key HMBC correlations for **7**.

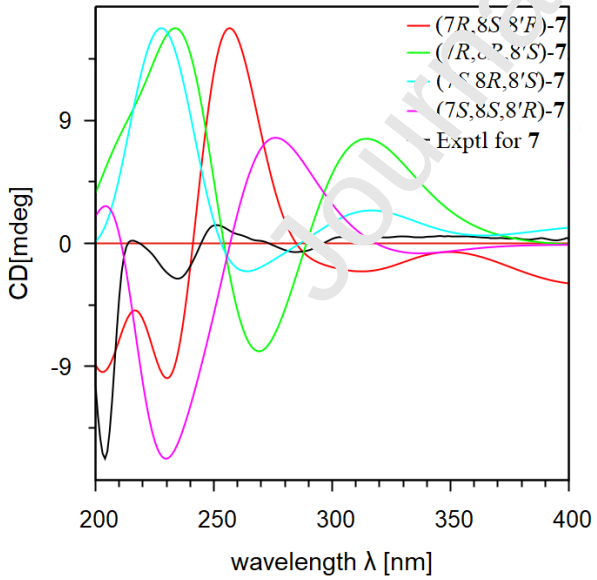

Fig. 7. Calculated ECD and experimental CD spectra of **7**.

Five known compounds, cleisindoside B (**8**) [11], cleistantoxin (**9**) [10], cleisindoside D (**10**) [11], neocleistantoxin (**11**) [10] and polygamain (**12**) [25], were also isolated from this plant. Their structures were determined from NMR data and comparison with those previously reported.

Compounds **1**–**9** were evaluated for their cytotoxicity against a panel of cancer cell lines, Hep3B (Human hepatoma cell lines), HeLa (human cervical carcinoma cells), MCF7 (human breast cancer cells), Pan C1 (pancreas epithelioid carcinoma cell lines) and A549 (human lung adenocarcinoma cells). The most active compound was cleistantoxin (**9**) which seems to be less cytotoxic against HeLa in comparison with A549, Hep3B, Pan C1 and MCF7 (Table 3). Whereas, neocleistantoxin (**11**) was more active toward A549, HeLa, Pan C1 and MCF7 in comparison with Hep3B cells. This is the first report of cytotoxicity of **9** and **11** against A549, Hep3B and Pan C1 cell lines. Compound **2** and **6** had selective inhibition with moderate cytotoxicity against Pan C1 and A549 cell lines, respectively. Compound **5** had weak inhibition against MCF7, Hep3B and Pan C1 cells, and was inactive against A549 and HeLa cell lines event at concentration of 20 μM. The moderate cytotoxicity of compounds **1**–**7** was in agreement with the SAR study for podophyllotoxin and its derivatives. Accordingly, modification of the *trans*-fused γ-lactone ring [26] led to the loss of cytotoxicity. Additionally, compounds with β-substituents at C-7 exhibited greater inhibitory against DNA topo II than those with α-configuration [27].

Table 3 *In vitro* cytotoxic activity of **1**–**7**, **9** and **11** (IC_{50} values are expressed in μM)

Compounds	A549	HeLa	MCF7	Hep3B	Pan C1
1	>20	>20	>20	>20	>20
2	>20	>20	>20	>20	15.3
3	>20	>20	>20	>20	>20
4	>20	>20	>20	>20	>20
5	>20	>20	17.7	13.3	18.5
6	18.1	>20	>20	>20	>20
7	>20	>20	>20	>20	>20
9	0.5	7.2	0.03*	0.8	0.7

11	2.1	1.2	2.2	>20	5.1
Camptothecin	1.6	2.9	0.2	0.1	0.9

*Value taken from ref. 10

Declaration of Competing Interest

The authors declare no conflict of interest.

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Author's statement

We, the undersigned author(s) of the manuscript entitled **"Cytotoxic Lignans from Fruits of Cleistanthus tonkinensis"** hereby declare that the above manuscript which is submitted for publication in the Fitoterapia is NOT under consideration elsewhere. The manuscript is NOT published already in part or whole in any journal or magazine for private or public circulation. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content. The order of authorships on the byline is a joint decision of all the coauthors.

Supplementary data

Supplementary material

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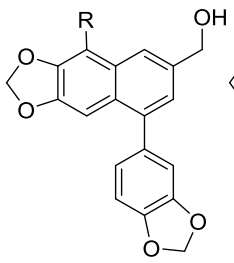
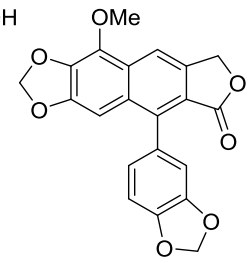
Graphical abstract



1: R=H
2: R=OMe



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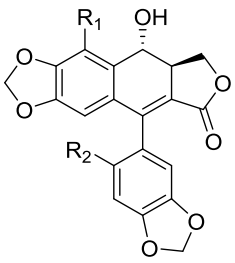
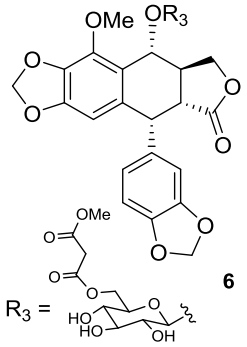
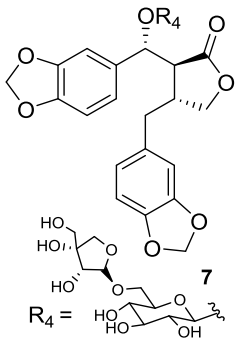


4: R_1=OMe, R_2=H
5: R_1=H, R_2=OH



R_3 =

6



R_4 =

7