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Chemical Constituents of *Goniothalamus elegans* Leaves and their Anticancer Activity

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The methanolic extract from *Goniothalamus elegans* leaves showed potent antiproliferative effect on human cancer cells. After purification, goniothalamin (**1**), goniomicin C (**2**), pinocembirn (**3**), stigmasterol (**4**), *trans*-cinnamic acid (**5**) and benzoic acid (**6**) were isolated from the extract. Their structures were determined using spectroscopic analysis data and compared with existing data in the literature. The antiproliferative activity of compounds on cancer cell lines were determined by MMT assay and found that compound **1** displayed strong cytotoxicity towards human colon (HCT116), cervical (HeLa) and breast (MCF-7) cancer cells (IC_{50} 0.35-2.36 µg/mL). The extract and compound **1** showed stronger inhibitory effect on three cancer cells than standard drug cisplatin, while, their antiproliferation on non-cancer cells showed close. Moreover, compound **2** showed cytotoxicity to MCF-7 cells without showing cytotoxicity against non-cancer cells. These results revealed that the extract and compound **2** are potent human cancer cells proliferation and needed further investigations the mechanism as chemotherapeutic agents.

Keywords: *Goniothalamus elegans*, Goniomicin C, Goniothalamin, Anticancer.

INTRODUCTION

Several medicinal plants have been preventing and treating cancer patients for many years [1]. The genus *Goniothalamus* (Annonaceae) is commonly known as winged euonymus in tropical and subtropical Asia and has been widely used in traditional medicine to treatment of heart disease and bloody diarrhea [2,3]. The natural extracted from *Goniothalamus* spp. had been able to induce cytotoxicity in cancer cell lines including colon (HT-29 and Col-2), leukemia (P-388), oralnasopharyngal (KB), breast (MCF-7), lung (Lu-1), alveolar basal epithelial (A549), urinary bladdercancer (T24), rat glioma (ASK) and embryonic kidney (HEK-293) [4-6]. This species have been the isolation and characterization of styryl-lactones, acetogenins, phenanthrene lactams, naphthoquinones, azaanthraquinones, terpenoids, flavonoids and steroids [7-10]. Styryl-lactones bear an α,β-unsaturated δ-lactone is the active compound that able to investigate the cytotoxic properties against several cancer cell lines [11].

Present study with respect to discovery of bioactive compounds with potential pharmaceutical applications from Thai medicinal plants, we have focused on *Goniothalamus elegans*, commonly called "Kao Nang Nee" in Thai [3]. The extracts from the bark of *G. elegans* has been isolated and found 2*H*-tetrahydropyran derivative, styryllactones, aristolactams and also sported to exhibit cytotoxic activities of one or more cancer cell lines including KB, MFC-7 and small cell lung (NCI-H187) [12]. As a continuous interest in this plant, the chemical constituents from the leaves of *G. elegans* and their cytotoxicity were investigated. This work describe the isolation and characterization of six compounds, (+)-goniothalamin (**1**), 4*S*,6*R*-goniomicin C (**2**), (-)-pinocembrin (**3**), stigmasterol (**4**), *trans*-cinnamic acid (**5**) and benzoic acid (**6**) (Fig. 1) from *G. elegans* leaves as well as the methanol extract and compounds **1-3** have been discussed for inhibitory effects on cell proliferation.

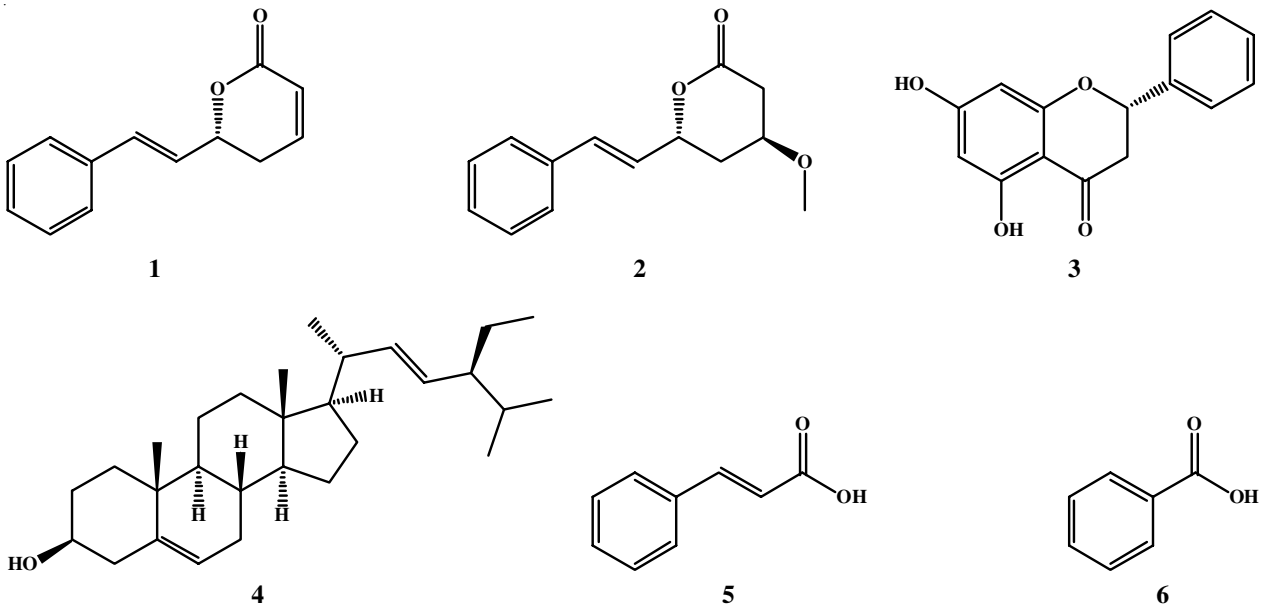

Fig. 1. Structures of the isolated compounds from leaves of *G. elegans*

EXPERIMENTAL

The leaves of G. *elegans* were collected from Phu Pha Shing Forest, Nong Wua So District, Udon Thani Province, Thailand and were identified by Prof. James F. Maxwell, Chiang Mai University. A voucher specimen (SRITUBTIM 18) was deposited at Udon Thani Rajabhat Universtiy Herbarium, Udon Thani, Thailand.

Melting points were determined using an Electrothermal IA9200 digital melting point apparatus. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter and UV spectra were recorded using an Agilent 8453 UV-vis spectrophotometer. IR spectra were obtained using a Bruker Tenser 27 spectrophotometer. NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer using $CDCl_3$ as solvents. The internal standards were referenced from the residue of those solvents. The HR-ESI-TOF-MS were recorded on a Bruker micrOTOF mass spectrometer. The ECD calculations were carried out by Gaussian 09 software with the time-dependent density functional theory (TD-DFT) method using CAM-B3LYP/6–311++G (d,p) and the conductor-like polarizable continuum model (C-PCM) was used for solvent (MeOH) effects. Chromatography was carried out on Merck silica gel 60 (230-400 mesh) and Sephadex LH-20 (40-70 µm; GE Health care). TLC was performed with precoated Merck silica gel 60 PF_{254} and aluminum sheets and the spots were visualized at 254 and 366 nm, sprayed with anisaldehyde reagent and then heated until charred by a heat gun. Commercial grade solvents were distilled at their boiling point ranges prior to use for extraction and chromatographic separations (CC and TLC), whereas AR grade solvents were used for crystallization.

Extraction and isolation: Air-dried leaves of *G. elegans* (1.0 kg) were ground to powder and then extracted at room temperature (3 days each time) with MeOH two times (3 L × 2). Removal of solvent under reduced pressure gave MeOH extract (137 g). The extract was subjected to column chromatography over silica gel eluted with a gradient system of EtOAc-hexane and MeOH-EtOAc to afford nine fractions (M1-M9) on the basis of TLC. Fraction M2 (1.8 g) was subjected to column chromatography, eluted with a gradient system of EtOAc: hexane (1:9-10:0), to give nine subfractions, M2.1-M2.9. The solid of subfraction M2.3 was recrystallized from EtOAc to give compound **1** as colourless crystals (1.8 g). Fraction M3 (959.0 mg) was filtered and crystallized from EtOAc to afford an additional amount of compound **1** (5.5 g). Then, the residue of M3 fraction was separated by column chromatography by using a gradient system of EtOAc:hexane (5:95-100:0) to give 11 subfractions as M3.1-M3.11. Subfraction M3.6 was subjected to column chromatography, eluted with an isocratic system of EtOAc:hexane (1:9) to give compound **6** (88.7 mg) as a white solid. Fraction M4 (6.1 g) was separated over CC, eluted with an isocratic system of EtOAc:hexane (1:4) to give seven subfractions, M4.1-M4.7. Subfraction M4.3 was further purified over Sephadex LH-20 column using MeOH as solvent to afford compound **3** (28.8 mg) as an orange solid. Solid portion of M4.6 subfraction was filtered and recrystallized from EtOAc to give an additional amount of compound **1** (825 mg). Then, the residue of M4.6 was purified by column chromatography, eluted with an isocratic system of EtOAc:hexane (3:7) to yield compound **2** (11.0 mg) as orange viscous liquid. Fraction M6 (1.2 g) was subjected to Sephadex LH-20 column using MeOH as solvent, followed by column chromatography eluting with EtOAc:hexane (3:7) to give compounds **4** (2.7 mg) and **5** (3.8 mg) as white solid. Fraction M8 (2.2 g) was subjected to Sephadex LH-20 column using MeOH:CH_2Cl_2 (7:3) as solvent to give three subfractions as M8.1-M8.3. Subfraction M8.2 was subjected to column chromatography, eluted with an isocratic system of EtOAc:CH_2Cl_2:hexane (3:2:5) to give four subfractions, M8.2.1-M8.2.4. Finally, subfraction M8.2.2 was subjected to column chromatography, eluted with EtOAc:CH_2Cl_2:hexane (3:2:5) to give an additional amount of compound **2** (4.1 mg).

(+)-Goniothalamin (1): Colourless crystals, $[\alpha]_D^{25}$ + 157.0, (*c* 1.0, $CHCl_3$), m.p.: 78-80 ºC, R_f : 0.33 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 1701, 1375, 1244, 1059; ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm), *J*, Hz): 2.52 (2H, m, 5-CH_2), 5.08 (1H, m, 6-CH), 6.07 (1H, dt, *J* = 9.6, 1.6 Hz, 3-CH=), 6.26 (1H, dd, *J* = 16.0, 6.0 Hz, 7-CH), 6.71 (1H, d, *J* = 16.0 Hz, 8-CH), 6.91 (1H, ddd, *J* = 9.6, 4.4, 4.0 Hz, 4-CH=), 7.25 (1H, t, *J* = 8.0 Hz, 4′-CH), 7.34 (2H, t, *J* = 8.0 Hz, 3′-CH, 5′-CH), 7.40 (1H, d, *J* = 8.0 Hz, 2′-CH, 6′-CH); ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm): 29.9 (C-5), 77.9 (C-6), 121.6 (C-3), 125.7 (C-7), 126.7 (C-2′, 6′), 128.3 (C-4′), 128.7 (C-3′,5′), 133.1 (C-8), 135.8 (C-1′), 144.7 (C-4), 163.9 (C=O).

4*S*,6*R*-Goniomicin C (2): Orange viscous liquid, $[\alpha]_D^{20}$ + 38.8 (*c* 0.1, $CHCl_3$). R_f = 0.35 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 3707, 1721, 1258, 1053; ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm), *J*, Hz): 1.88 (1H, ddd, *J* = 14.4,11.2, 3.2 Hz, 5-α-CH), 2.21 (1H, dddd, *J* = 14.4, 3.2, 3.2, 1.2 Hz, 5-β-CH), 2.72 (1H, dd, *J* = 17.6, 4.4 Hz, 3-α-CH_2), 2.77 (1H, ddd, *J* = 17.6, 4.4, 1.2 Hz, 3-β-CH_2), 3.38 (3H, s, 4-OCH_3), 3.84 (1H, m, 4-$CHOCH_3$), 5.23 (1H, ddd, *J* = 11.2, 6.4, 3.2 Hz, 6-CH), 6.20 (1H, dd, *J* = 15.6, 6.4 Hz, 7-CH=), 6.69 (1H, d, *J* = 15.6 Hz, 8-CH=), 7.28 (1H, m, 4′-CH), 7.33 (2H, m, 3′-CH, 5′-CH), 7.38 (2H, m, 2′-CH, 6′-CH); ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 33.6 (C-5), 35.6 (C-3), 56.2 (C-CH_3O), 71.3 (C-4), 76.1 (C-6), 126.6 (C-7), 126.7 (C-2′, C-6′), 128.2 (C-3′, C-5′), 128.7 (C-4′), 132.4 (C-8), 135.9 (C-1′), 169.6 (C=O).

(–)-Pinocembrin (3): Orange solid, $[\alpha]_D^{20}$ - 26.0 (*c* 0.1, MeOH), m.p.: 196-198 ºC, R_f = 0.55 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 3230, 1637, 1603, 1499, 1461, 1276; ^{1}H NMR (400 MHz, CDC_3, δ (ppm), *J*, Hz): 2.83 (1H, dd, *J* = 16.8, 3.2 Hz, 3-α-CH), 3.09 (1H, dd, *J* = 16.8, 12.4 Hz, 3-β-CH), 5.44 (1H, dd, *J* = 12.4, 2.4 Hz, 2-CHO-), 5.95 (1H, brs, 7-OH), 6.04 (2H, s, 6-CH, 8-CH), 7.38-7.45 (5H, m, 2′-CH, 3′-CH, 4′-CH, 5′-CH, 6′-CH), 12.04 (1H, s, 9-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 43.3 (C-3), 79.2 (C-2), 95.5 (C-6), 96.7 (C-8), 103.2 (C-10), 126.1 (C-2′, C-6′), 128.9 (C-4′), 128.9 (C-3′, C-5′), 138.3 (C-1′), 163.2 (C-5), 164.3 (C-9), 164.6 (C-7), 195.8 (C=O).

Stigmasterol (4): White solid, m.p.: 165-167 ºC, R_f = 0.44 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 3359, 1667, 1461, 1382, 1054; ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm), *J*, Hz): 0.68 (3H, s, 18-CH_3), 0.82 (3H, d, *J* = 6.8 Hz, 27-CH_3), 0.83 (3H, t, *J* = 6.8 Hz, 29-CH_3), 0.84 (3H, d, *J* = 6.8 Hz, 26-CH_3), 0.92 (1H, m, 9-α-CH), 1.01 (1H, s, 14-α-CH), 1.03 (3H, s, 19-CH_3), 1.03 (3H, d, *J* = 7.2 Hz, 21-CH_3), 1.04 and 1.64 (2H, m, 15-αβ-CH_2), 1.08 and 1.86 (2H, m, 1-αβ-CH_2), 1.15 (1H, m, 17-α-CH), 1.20 and 1.42 (2H, m, 28-αβ-CH_2), 1.20 and 2.02 (2H, m, 12-αβ-CH_2), 1.26 and 1.70 (2H, m, 16-αβ-CH_2), 1.42 (1H, m, 8-β-CH), 1.42 (2H, m, 11-αβ-CH_2), 1.48 and 1.98 (2H, m, 7-αβ-CH_2), 1.48 (1H, m, 25-CH), 1.51 (1H, m, 24-CH), 1.53 and 1.82 (2H, m, 2-αβ-CH_2), 2.02 (1H, m, 20-CH), 2.22 and 2.28 (2H, m, 4-αβ-CH_2), 3.52 (1H, m, 3-α-CHOH), 5.02 (1H, dd, *J* = 15.3, 8.3 Hz, 23-CH=), 5.14 (1H, dd, *J* = 15.3, 8.3 Hz, 22-CH=), 5.37 (1H, m, 6-CH=); ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 12.1 (C-29), 12.2 (C-18), 19.6 (C-27), 19.9 (C-26), 21.0 (C-11), 21.1 (C-19), 21.2 (C-21), 24.4 (C-15), 25.4 (C-28), 28.9 (C-16), 31.2 (C-2), 31.9 (C-7), 33.0 (C-25), 34.2 (C-8), 36.5 (C-10), 37.3 (C-1), 39.7 (C-12), 40.5 (C-20), 42.2 (C-13), 42.3 (C-4), 50.2 (C-9), 51.4 (C-24), 56.0 (C-17), 56.9 (C-14), 71.8 (C-3), 121.6 (C-6), 129.3 (C-23), 138.3 (C-22), 140.8 (C-5).

***trans*-Cinnamic acid (5):** White solid, m.p.: 132-134 ºC, R_f = 0.76 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 3440, 1675, 1632, 1466, 1450; ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm), *J*, Hz): 6.43 (1H, d, *J* = 16.0 Hz, 2-CH=), 7.41 (3H, m, 6-CH, 7-CH, 8-CH), 7.58 (2H, m, 5-CH, 9-CH), 7.80 (1H, d, *J* = 16.0 Hz, 3-CH=); ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 117.2 (C-2), 128.3 (C-5, C-9), 129.0 (C-6, C-8), 130.7 (C-7), 134.0 (C-4), 147.0 (C-3), 171.7 (C=O).

Benzoic acid (6): White solid, m.p.: 120-123 ºC, R_f = 0.77 (40% EtOAc in hexane), IR (KBr, ν_{max}, cm^{-1}): 3107, 1715; ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm), *J*, Hz): 7.48 (2H, t, *J* = 6.4 Hz, 3-CH, 5-CH), 7.62 (1H, t, *J* = 6.4 Hz, 4-CH), 8.17 (2H, d, 6.8 Hz, 2-CH, 6-CH), ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 128.5 (C-3, C-5), 129.3 (C-1), 130.2 (C-2, C-6), 133.8 (C-4), 171.7 (C=O).

Cell proliferation assessment: Cell proliferation assays were performed using a MTT colorimetric method [13,14]. Antiproliferative activities of the extract and the selected compounds were determined in three cancer cell lines as human colon cancer (HCT116), human cervical cancer (HeLa) and human breast adenocarcinoma (MCF-7) cell lines and non-cancer cells as African green monkey kidney (Vero) cells. Briefly, the cells (8×10^3 cells) were seeded into 96-well plates, after incubation for 24 h, the compounds at varied concentrations (µg/mL) were added to the cell culture, which was then incubated for 24, 48 and 72 h. After each period of exposure, 10 µL of MTT [3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide] (5 µg/mL) was added per well and incubated for 2 h at 37 ºC. After that the formazan dye was dissolved with 100 µL of DMSO and the absorbance at 550 nm was measured on a microplate reader (Bio-Rad laboratories, Herculus, CA, USA). The reference substance was cisplatin.

RESULTS AND DISCUSSION

Structural elucidation of compounds 1-6: Chromatographic separation of the methanolic extract from the leaves of *G. elegans* let to isolation and identification of six compounds, two styryllactones (**1**, **2**), one flavonoid (**3**), one steroid (**4**) and two carboxylic acid (**5**, **6**). From the NMR experiments, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, NOESY, HSQC, HMBC spectra analyses and comparison with literature data, the complete assignment of chemical structures were established. The ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** showed the presence of mono-substituted phenyl moiety, lactone and two vinyllic protons/carbons. The coupling constants of Compound **2** providing evidences for 2 to be 4*R*,6*S* or 4*S*,6*R*-stereoisomer (Fig. 2: $J_{6ax/5ax}$ = 14.4; $J_{6ax/5eq}$ = 3.2; $J_{5ax/4eq}$, $J_{5eq/4eq}$ = 3.2; $J_{4eq/3ax}$, $J_{4eq/3aq}$ = 4.4 Hz). The absolute configuration was determined on the basis of the CD spectral analyses. The CD curves of both compound **2** showed negative Cotton effect around 203 nm and the positive Cotton effect around 210 nm in Fig. 3, indicating the same absolute configuration of 4*S*,6*R* isomer. The ^{1}H & ^{13}C NMR spectra of compound **3** showed

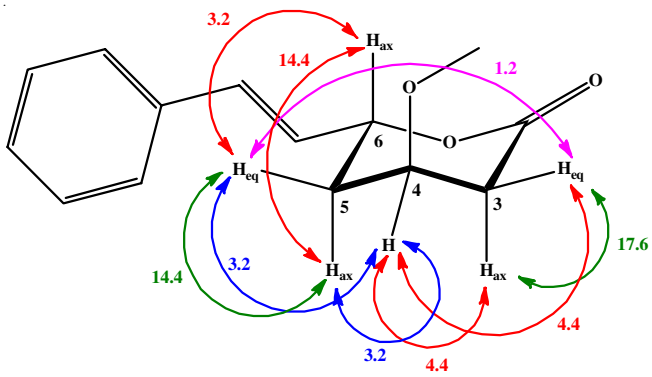

Fig. 2. Coupling constant (*J* values) in Hz of compound **2**

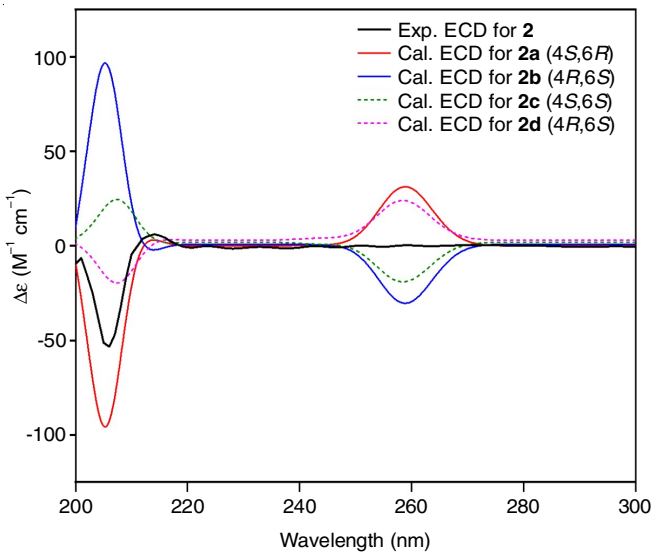

Fig. 3. Experimental and calculated ECD spectra of compound **2**

typical pattern of flavanone revealed the presence of an ABX system. All the isolated compounds were identified as (+)-goniothalamin (**1**) [15], 4*S*,6*R*-goniomicin C (**2**) [6], (-)-pinocembrin (**3**) [16], stigmasterol (**4**) [17], *trans*-cinnamic acid (**5**) [18] and benzoic acid (**6**) [19,20] as depicted in Fig. 1.

Cell proliferation assessment: Inhibitory activity of methanolic extract and compounds **1-3** were investigated on three cancer cells (HCT116, HeLa and MCF-7) and non-cancer cells (Vero cells) for exposure times of 24, 48 and 72 h. The results are summarized in Fig. 4 and indicated that they inhibited cancer cell proliferation in a time- and dose dependent manner. The methanol extract showed potent cytotoxic activity against all three cancer cells with IC_{50} values in the range of 0.85-1.55 µg/mL at 72 h. While compound **1** showed strong antiproliferative activity in HCT116 (IC_{50} 1.27 ± 0.15 µg/mL), HeLa (IC_{50} 0.35 ± 0.02 µg/mL) and MCF-7 cells (IC_{50} 2.36 ± 0.11 µg/mL). Compound **2** showed moderate cytotoxicity against MCF-7 cells (IC_{50} = 9.04 ± 0.66 µg/mL) and showed no cytotoxicity against Vero cells (IC_{50} > 50 µg/mL). Moreover, the methanolic extract and compound **1** showed more cytotoxic activity on three cancer cells than those of cisplatin. Furthermore, the IC_{50} values of antiproliferative activity on vero cells of three of them are close.

Conclusion

Two styryllactones as (+)-goniothalamin (**1**) and 4*S*,6*R*-goniomicin C (**2**), (−)-pinocembrin (**3**), stigmasterol (**4**), *trans*-cinnamic acid (**5**) and benzoic acid (**6**) were isolated from the methanolic extract from the leaves of *G. elegans*. The methanolic extract and compound **1** showed potent inhibitory on the proliferation of all three cancer cells with IC_{50} values in the range of 0.85-2.36 µg/mL, that were stronger than the standard drug, cisplatin. Especially, compound **2** showed moderate cytotoxicity against breast cancer (MCF-7), but, it showed no cytotoxicity to vero cells. It is interesting to observe that both methanolic extract and compound **2** should be used to develop as the treatment of cancer.

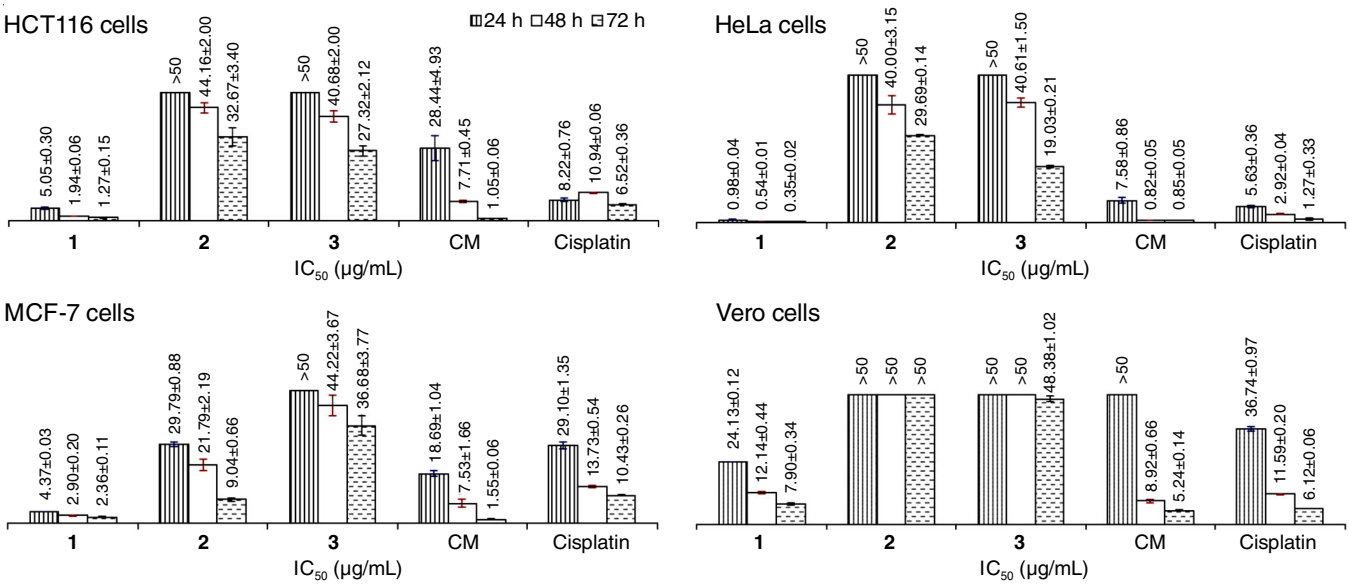

Fig. 4. IC_{50} values of antiproliferative activity on the HCT116, HeLa, MCF-7, and Vero cells of compounds **1-3**, methanol extract (CM) and standard drug (cisplatin)

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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