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CHEMICAL INVESTIGATION AND ANTIPROLIFERATIVE STUDIES OF ISOLATED POLYISOPRENYLATED BENZOPHENONES FROM STEM-BARK OF *Garcinia maingayi*

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Keywords

Garcinia maingayi

Polyisoprenylated Benzophenones

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Flow Cytometry

ABSTRACT

In the current study, sequential solvents extraction from the stem bark of *Garcinia maingayi*, a native plant to Malaysia has led to the isolation of four polyisoprenylated benzophenones: 30-*epi*-cambogin **(GB 1)**, 14-deoxy-30-*epi*-cambogin **(GB 2)**, guttiferone F **(GB 3)**, and 14-deoxy-guttiferone F **(GB 4)**. The structures were elucidated using IR, optical rotation, and NMR spectral data. The compounds were evaluated for antiproliferative effect using MTT assay, apoptosis using Annexin V/7-AAD flow cytometry, cell cycle progression, and activation of caspases 3/7, 8 and 9 and BCL2 mRNA expression in MCF-7, HeLa, and HepG2 cancer cell lines. Compounds **GB 1** to **GB 4** exhibited a remarkable antiproliferative effect on HeLa, MCF-7, and HepG2 cells with IC_{50} values ranging from 5 to 45 µM. Compounds **GB 1** to **GB 4** induced significant cell cycle arrest in the G1 phase corroborated with the decrease in the number of MCF-7 and HepG2 cells in S and G2/M phases ($P<0.05$). Compounds **GB 1** to **GB 4** induced apoptosis at 48 h. Further, among these, compounds **GB 1** and **GB 2** induced significant levels of caspases 3 and 9 in HeLa cells, while **GB 3** induced caspase 9 activities in both MCF-7 and HepG2 cells. No significant induction of caspase 8 was observed suggesting that the apoptotic effects are mainly mediated through the intrinsic pathway. Only compound **GB 1** inhibited the BCL2 mRNA expression significantly in all treated cancer cells. In conclusion, these compounds possess anticancer properties and thus further investigation is crucial on the mechanistic study, structure-activity relationship, and identification of putative molecular targets.

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1 Introduction

Therapeutic uses of medicinal plants in the pharmaceutical drug discovery against cancer have been tested over the past century. World Health Organization (WHO) estimated 21.7 million people will be diagnosed with cancer and 13 million people will die because of cancer by the year 2030 (Ferlay et al., 2015). Cancer incidence rates are increasing rapidly and have become the second leading cause of death globally. In line with the drug development, the *Garcinia* species is one of the important plants studied greatly for medicinal values.

Garcinia is a polygamous dioecious evergreen fruit tree or shrub that belongs to Guttiferae (Clusiaceae) family (Chai, 2000; Joseph et al., 2005; Jabit et al., 2009; Hemshekhar et al., 2011). Approximately 450 species of *Garcinia* are widely distributed in tropical Asia, South Africa, and Polynesia as well as in Australia (Kumar et al., 2013; Patil & Appaiah, 2015). *Garcinia* is widely found in Malaysia specifically in Kedah, Perak, Pahang, Terengganu, Johor (Kochummen, 1998; Jabit et al., 2009) and primarily present in Sabah and Sarawak (Repin et al., 2012; Ling & Julia, 2012). Detailed chemical studies on *Garcinia* species using nuclear magnetic resonance (NMR) spectroscopy revealed the presence of triterpenoids (stigmasterol, sitosterol), benzophenone (isoxanthochymol), xanthone (1,3,7-trihydroxy-2-3-(3-methylbut-2-enyl)xanthone), and benzoic acid derivative (3,4-dihydroxy-methylbenzoate) (Cheng & Cheow, 2008). The presence of phenolic compounds contributed to antioxidant properties (Krishnamoorthy et al., 2014), while xanthone and benzophenone have cytotoxic properties toward various human cancer cell lines (Kumar et al., 2013).

Two newly isolated caged xanthones from *G. wightii* viz., wightiic acid and 16-O-methyl wightiic acid also showed antiproliferative activities. However, the wightiic acid showed exceptional antiproliferative activity with IC_{50} of 4.7 and 5.2 µMin A-375 and MCF-7 cells, respectively (Lekshmi et al., 2020). *Garcinia* xanthone I, a newly isolated xanthone from *G. xanthochymus* induce HepG2 apoptosis through the mitochondrial pathway and proved as a potential drug in the treatment of liver cancer (Jin et al., 2019). There is a lack of study on the Malaysian species of *G. maingayi*. The current study was carried out to reveal the effect of the pure bioactive compounds on various biological activities and their possible mechanism of action. The ability to induce apoptosis is the primary characteristic in considering the efficacy of the compound as a chemopreventive agent is shown in this research.

2 Materials and Methods

2.1 Plant material

Stem-bark of *G.maingayi* was collected from the forest of Semengok, Sarawak, Malaysia in June 2014. The plant was authenticated by Mr. Tinjan Anak Kuda, a botanist from the Forest Department, Sarawak. A voucher specimen of UITM 3017 was deposited at the herbarium of Universiti Teknologi MARA, Sarawak.

2.2 Isolation and purification

The 2.7 kg dried stem-bark of *G.maingayi* was sequentially extracted using hexane, ethyl acetate, dichlomethane, ethanol, and methanol for three days at room temperature. Each filtrate was concentrated using a rotary evaporator at 40 to 60°C. The crude extracts were chromatographed on a silica gel (Merck) column with increasing polarity of organic solvents at various ratios. These steps were repeated successfully using gravity column chromatography and preparative thin-layer chromatography to obtain pure compounds.

2.3 Chemical investigation

The pure compounds were elucidated using Jeol JNM-ECX (Japan), 400 MHz NMR spectrometer with the use of trimethylsilane (TMS) as internal and reference standards for ^{1}H NMR, ^{13}C NMR, HMQC, and HMBC spectra. IR spectra were analyzed using Fourier Transform Infrared (FTIR) (Perkin Elmer). Optical rotations were measured on Jasco Europe P-2000 digital polarimeter.

2.4 Antiproliferative analysis

2.4.1 Cancer cell lines

Human cervical epithelial carcinoma cell line (HeLa)(ATCC®CCL-2™), human breast adenocarcinoma cell line (MCF-7)(ATCC®HTB-22™), and human hepatocellular carcinoma cell line (HepG2)(ATCC®HB-8065™) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin and 100 UI/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All the cell lines were maintained at 37ºC in a 5% CO_2 incubator.

2.4.2 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Leong et al. (2007) and Bradshaw et al. (2005) with some modifications was used. Cancer cell lines were seeded into 96-well plates (Nunc, Denmark) at concentrations ranging from $1x10^4$ to 10^5 cells. Compounds, DMSO, and doxorubicin hydrochloride (positive control) (Nacalai Tesque, Japan) were added into respective wells and incubated for 48 h at 37ºC in 5% CO_2 incubator. MTT reagent (5 mg/ml) (Sigma, UK) was added and incubated further for 4 h, followed by the addition

of 100 µl of DMSO. The absorbance was measured using a microplate reader (Omega, Germany) at 570 nm. The dose-response curves and 50% growth inhibition concentrations (IC_{50}) were determined from GraphPad Prism (version 8.0.2).

2.4.3 Flow cytometry

Propidium iodide staining coupled with flow cytometry was used in the evaluation of cell cycle progression. The population of apoptotic cells was measured using the PE Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Both floating and attached cells were collected, stained, and analyzed using a FACS Calibur flow cytometer and the CellQuest Pro software (version 5.1.1; BD Biosciences, USA) (BD Biosciences, 2008).

2.4.4 Caspases activation

The catalytic activity of caspases 3/7, 8, and 9 was measured at 48 h after treatment using Caspase-Glo 3/7, Caspase-Glo 8, and Caspase-Glo 9 assay kits (Promega, Madison, WI, USA) based on manufacturer's guidelines (Promega, 2018).

2.4.5 BCL2 mRNA expression

Treated cancer cell lines were further preceded to RNA extraction and the cDNA was synthesized using high capacity RNA-to-cDNA master mix based on manufacturers' protocol(Qiagen, Valencia, CA, USA; Applied Biosystems, Carlsbad, California, USA) (Qiagen, 2013). FastStart Universal SYBR green master reagent (Roche, Indianapolis, USA) and Bio-Rad iQ5 real-time PCR detector system (Bio-Rad, Richmond, CA, USA) (Pabla & Pabla, 2008) were used. Bio-Rad iQ5 Optical System Software V1.0 was used for data analysis. Table 1 shows primer sequences used in the expression. The 40 cycle's conditions were 3 min at 94°C followed by 40 s at 94°C, 40 s at 60°C, and 25 s at 72°C. The housekeeping gene, GAPDH was used in the normalization (Reboucas et al., 2013).

2.5 Data analysis

Data were reported as mean±standard deviation from a minimum of three independent experiments. Levels of significance were determined using student t-test, whereas treatments were compared to the control. $P < 0.05$ was considered statistically significant unless otherwise specified.

3 Results and Discussion

3.1 Structural elucidation

The 30-*epi*-cambogin (GB 1), 14-deoxy-30-*epi*-cambogin (GB 2), guttiferone F (GB 3) and 14-deoxy-guttiferone F (GB 4) were isolated from *G. maingayi* (Figure 1).Table 2 summarizes the detail of compounds, while Table 3 shows the ^{1}HNMR and ^{13}CNMR spectra of the compounds GB 1 to GB 4. The molecular formula of GB 1 was $C_{38}H_{50}O_6$ with the mass of 602 and optical rotation ([α]D) of -129º. The molecular formula of GB 2 was $C_{38}H_{50}O_6$ differing from GB 1 by an oxygen atom. Compounds GB 1 and GB2 were different on the aromatic ring in which GB 1 has two OH groups at C13 and C14, while GB 2 has only one OH group at C13. The molecular formula of GB 3 is similar to that of GB 1 with $C_{38}H_{50}O_6$, while the molecular formula of GB 4 is similar to that of GB 2.

The NMR spectra of GB 4 were generally similar to that of GB 3 except for the absence of an OH group at C14 which was replaced by an aromatic proton. Compounds GB 1 and GB 2 were obtained as a yellow powder, while compounds GB 3 and GB 4 as yellow and honey consistency.

Compounds GB 1 and GB 3 were also reported obtained from the root wood of *Allanblackia stuhlmannii* (Engl.) in the early phase of research (Fuller et al., 1999) with the same structural properties. However, compounds GB 2 and GB 4 obtained in this study are new compounds and reported isolated for the first time from Malaysian species of *G. maingayi*.

3.2 Antiproliferative effect

Figure 2 shows the dose-response curves of cancer cell lines treated with various concentrations of compounds **GB 1** to **GB 4** for 48 h. These compounds exhibited cytotoxicity with IC_{50} values in the range of 5 to 45 µM as shown in Table 4. The number of cells reduced drastically upon treatment as compared to vehicle control (0.1% DMSO) indicating cytotoxic effect (Figure 3).

The monolayer cells detached and form spherical shapes revealing death mode upon treatment. The original anchorage-dependent cancer cells losses their adhesiveness or lose their substrate attachment and becomes rounded, further shrinks due to cell death (Freshney, 2005; Kleinsmith, 2006). These morphological changes confirm the presence of anticancer properties in the isolated compounds that act as mitogen factors.

Visible cell death suspensions upon treatment were seen in HeLa cells as compared to other cells. Kenji et al. (2003) reported garcinol, isogarcinol and xanthochymol from *G. purpurea* displayed a strong apoptosis-inducing effect against human leukemia cell lines namely NB4, HL60, U937, and K-562 via nuclear fragmentation and DNA ladder formation. Kai-Wei et al. (2012) reported garcinielliptones S and phloroglucinol from *G. subelliptica* remarkably increased the cell death of human bladder carcinoma cells (NTUB1) at 72 h with $\mathbf{IC_{50}}$ values of 45.1 ± 7.8, 13.5 ± 2.3 and 3.3 ± 0.1 µM, respectively. These studies were in agreement with the current study supporting that the compounds isolated from *G. Maingayi* are potential antiproliferative agents.

3.3 Cell cycle progression

The effects of compounds **GB 1** to **GB 4** in MCF-7 and Hep G2 on cell cycle progression is shown in Figure 4. These compounds induced significant cell cycle arrest in **G1** phase corroborated with the decrease in the number of cells in S and G2/M phases ($P<0.05$). Due to excessive cell death, cell cycle analysis was not able to perform on HeLa cells. The link between apoptosis and cell cycle progression in cancer cells is considered a possibly effective approach to control tumour growth (Roger & Mike, 2006). A classic cell cycle progression is represented by early-G1, late-G1, S, and G2/M phases. Higher G1 populations were seen in both MCF-7 and HepG2 treated cells, and this causes a concomitant decrease in the population of cells in the G2/M phase as compared to control. The results showed the G1 cell cycle arrest induces cell death in the cancer cell lines. The current study agrees with Zhang et al. (2016) that a novel compound nujiangexathone A from *G. nujiangensis* suppressed HeLa by arresting the cells at G0/G1 phases from 61.65 to 73.65% and decreased the cells in the G2/M phases from 23.55 to 12.60% after 48 hours at 20 µM.

3.4 Induction of apoptosis

Compounds **GB 1** to **GB 4** induced apoptosis in all the cancer cell lines by a significant percentage of apoptosis following treatment for 48 h using Annexin V/7-AAD staining. Compounds **GB 1** and **GB 2** exhibited the highest percentage of apoptosis in HeLa cells, while the lowest was shown by compound **GB 2** in HepG2 cells. However, 0.1% of DMSO as vehicle control showed a lower apoptotic rate indication the cells are viable. Figure 5 shows the scatter plots of the treated cancer cells entering to first early apoptotic stage and finally to the late apoptotic stage. It is seen that lesser treated cells enter the necrotic stage (upper left).

3.5 Activation of Caspases

Caspases 3/7, 8, and 9 activities in HeLa, MCF-7 and HepG2 activation following treatment exhibited a remarkable apoptotic effect (Figure 6).

Table 1 Primer sequences for qPCR

Gene	Forward primer	Reverse primer
BCL2	5'-ATCGCCCTGTGGATGACTGAGT-3'	5'-GCCAGGAGAAATCAAACAGAGGC-'3
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA-3'

Table 2 Summarised details of the isolated polyisoprenylated benzophenones from the stem-bark of *G. maingayi*

Features	GB 1	GB 2	GB 3	GB 4
Findings	Known compound	New compound	Known compound	New compound
Isolated from Crude extracts	Methanol	Dichloromethane	The mixture of ethanolic and hexane	Ethyl acetate
Name	30-*epi*-cambogin	14-deoxy-30-*epi*-cambogin	Guttiferone F	14-deoxy-guttiferone F
Form	Yellow powder	Yellow powder	Yellow and honey consistency	Yellow, honey consistency
Molecular formula	$C_{38}H_{50}O_6$	$C_{38}H_{50}O_5$	$C_{38}H_{50}O_6$	$C_{38}H_{50}O_5$
Molecular mass	602	586	602	586
Optical rotation	-129º	-143º	-298º	-255º

Table 3 ^{1}H NMR and ^{13}C NMR spectra of compounds GB 1 to GB 4

Position	GB 1	GB 2	GB 3	GB 4	GB 1	GB 2	**GB 3**	**GB 4**
		δC,ppm				δH, ppm (type)		
1	171.7	171.9	199.0	198.5	-	-	-	-
2	125.2	125.2	116.0	116.5	-	-	-	-
3	194.6	193.9	193.9	193.6	-	-	-	-
4	68.2	68.3	69.8	69.9	-	-	-	-
5	46.1	46.4	49.7	49.8	-	-	-	-
6	46.2	46.4	46.9	46.8	1.33, m	1.45, m	1.45, m	1.41, m
7	39.3	39.7	42.7	42.7	1.86, dd (14.6, 7.3) 2.14, d (14.6)	1.98, dd (14.2, 7.3) 2.28, d (14.2)	2.03, m 2.35, d (14.2)	2.04, m 2.35, d (13.8)
8	51.2	51.3	58.0	57.9	-	-	-	-
9	207.3	207.4	209.3	209.3	-	-	-	-
10	193.2	193.7	195.0	195.4	-	-	-	-
11	129.9	133.2	127.8	132.1	-	-	-	-
12	114.3	114.8	116.5	115.8	7.21, d (1.8)	7.29, t (1.4)	6.91, m	6.87, m
13	144.7	156.2	143.8	156.1	-	-	-	-
14	150.6	121.7	149.9	121.2	-	7.14, dt (7.8, 1.4)	-	6.90, m
15	114.4	129.7	114.4	128.9	6.61, d (7.9)	7.18, t (7.8)	6.58, d (8.0)	7.10, t (7.8)
16	123.8	120.6	124.2	120.1	6.87, dd (7.9, 1.8)	6.96, dt (7.8, 1.4)	6.93, m	6.85, m
17	25.5	25.7	27.1	26.3	2.30, dd (13.7, 5.2) 2.54, dd (13.7, 5.5)	2.43, dd (13.7, 5.5 2.64, dd (13.7, 8.2	2.72, m 2.58, d (12.4)	2.71, m 2.52, d (13.7)
18	119.6	119.9	120.2	120.0	4.77, m	4.90, t (5.5)	5.08, m	-
19	134.6	139.0	135.4	137.7	-	-	-	5.08, m
20	25.9	26.2	26.2	26.2	1.47, s	1.58, s	1.79, s	1.78, s
21	17.9	18.1	18.3	18.1	1.45, s	1.57, s	1.73, s	1.68, s
22	22.3	22.6	22.8	22.7	1.03, s	1.15, s	1.15, s	1.11, s
23	26.6	26.9	26.5	27.1	0.85, s	0.97, s	1.00, s	0.99, s
24	29.2	29.4	29.0	29.1	2.03, m 2.49, m	2.15, m 2.59, m	2.05, m 2.10, m	2.02, m 2.09, m
25	124.8	125.0	123.9	123.9	4.77, m	4.90, t (5.5)	4.92, t 7.3)	4.90, m
26	133.0	133.8	133.1	133.0	-	-	-	-
27	25.8	26.0	25.8	25.9	1.54, s	1.66, s	1.65, s	1.67, s
28	17.9	18.0	18.1	18.0	1.56, s	1.65, s	1.52, s	1.51, s
29	28.3	28.4	36.3	36.2	0.84, m 2.91, dd (14.0, 3.7)	0.93, m 3.03, dd (14.2, 3.7)	1.98, m 1.88, dd (14.0, 4.2)	1.90, m 1.93, m
30	42.8	42.9	43.7	43.6	1.28, m	1.39, m	2.67, m	2.74, m
31	86.7	86.7	148.1	148.2	-	-	-	-
32	28.5	28.6	112.8	17.8	0.78, s	0.88, s	4.36, s 4.40, s	1.60, s
33	21.1	21.3	18.0	112.7	1.12, s	1.23, s	1.59, s	4.40, s 4.43, s
34	29.6	29.7	32.7	32.7	1.67, m 1.91, m	1.79, m 2.02, m	2.03, m	2.00, m 2.12, m
35	121.3	121.5	122.7	122.7	5.07, t (7.3)	5.15, td 6.6,1.4)	5.03, t 6.8)	5.04, m
36	133.7	134.7	132.1	135.1	-	-	-	-
37	25.6	25.9	25.9	25.8	1.65, s	1.74, s	1.69, s	1.67, s
38	18.0	18.2	18.1	18.2	1.48, s	1.60, s	1.54, s	1.54, s

*Recorded at 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR in δ ppm: assignments based on DEPT, HMCQ and HMBC [s singlet, m multiplet, d doublet, dd doublet of doublet, t triplet]

Table 4 *In vitro* cytotoxic effect of compounds GB 1 to GB 4 as expressed in IC_{50} values at 48 h

Sample	IC_{50} values (µM)		
	HeLa	**MCF-7**	**HepG2**
GB 1	13.24 ± 1.53	5.48 ± 0.20	8.02 ± 1.53
GB 2	15.49 ± 1.39	5.30 ± 0.32	9.25 ± 1.57
GB 3	33.71 ± 2.33	12.46 ± 0.57	18.03 ± 1.99
GB 4	45.10 ± 1.27	22.97 ± 0.97	24.97 ± 1.04
Doxorubicin	0.29 ± 0.01	1.45 ± 0.69	0.45 ± 0.01

Compounds **GB 1** and **GB 2** induced significant (>two-fold induction and P<0.01 by student t-test) levels of caspases 3/7 and 9 activities in HeLa cells, while compound **GB 3** induces caspase 9 activities in both MCF-7 and HepG2 cells. Compound **GB 4** is the least potent among all the compounds tested, as apoptosis was only detected in MCF-7 and HepG2 cells. Compound **GB 4** induces significant caspase 9 activation suggesting activation of the intrinsic pathway.

Interestingly, compound **GB 4** did not induce caspases 3/7, 8, or 9 activities in HepG2 cells despite a significant induction of apoptosis suggesting that the compound might induce cell death through a caspase-independent mechanism. No induction of caspase 8 was observed suggesting that the apoptotic effects induced by compounds **GB 1** to **GB 4** are mainly mediated through the intrinsic apoptotic pathway. Activation of the Bcl2 protein family usually occurs in response to DNA damage via the mitochondrial pathway (Damagoj & Wayne, 2007).

Similarly, Ahmed et al. (2012) and Sethi et al. (2014) reported garcinol from the fruit rind of *G. indica* killed prostate (LNCaP, C4-2B and PC3) and pancreatic ca(BxPC-3) cancer cells by downregulating NF-kB and phosphoinositide 3- kinase (PI3K)/serine/threonine-specific protein kinase (Akt) signaling pathways. Besides, Xiong et al. (2014) cytochrome c (Crompton, 2000). This process will further induce cell death selectively and eliminates tumour cells.

3.6 BCL2 mRNA expression

Results given in figure 7 shows compound **GB 1** inhibited BCL2 mRNA expression significantly (P< 0.01) in HeLa, MCF-7, and HepG2 cells, while no such inhibitory effect was observed in other compounds treated cells.

The levels of BCL expression was reduced by 3.5, 3.2, and 1.8-fold in HeLa, MCF-7, and HepG2 cells, respectively. Bcl2 family of proteins is an anti-apoptotic marker and central regulators of mitochondrial cell-intrinsic apoptotic which is directly related to apoptosis induction. The results clearly shown that the BCL2 are down-regulated in all the cancer cells treated with compound **GB 1**. The BCL2 itself can bind to pro-apoptotic members such as Bax and thus releases cytochrome c (Crompton, 2000). This process will further induce cell death selectively and eliminates tumour cells.

Conclusion

In a nutshell, compounds **GB 1** to **GB 4** induced not only cell cycle arrest but also apoptosis in cancer cell lines in a dosage-dependent manner. However, only compound **GB 1** inhibited the BCL2 mRNA expression. Further investigation on the mechanistic study, chemical modification, structure-activity relationship, and identification putative molecular target of compounds **GB 1** to **GB 4** through *in silico* molecular docking can be considered.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

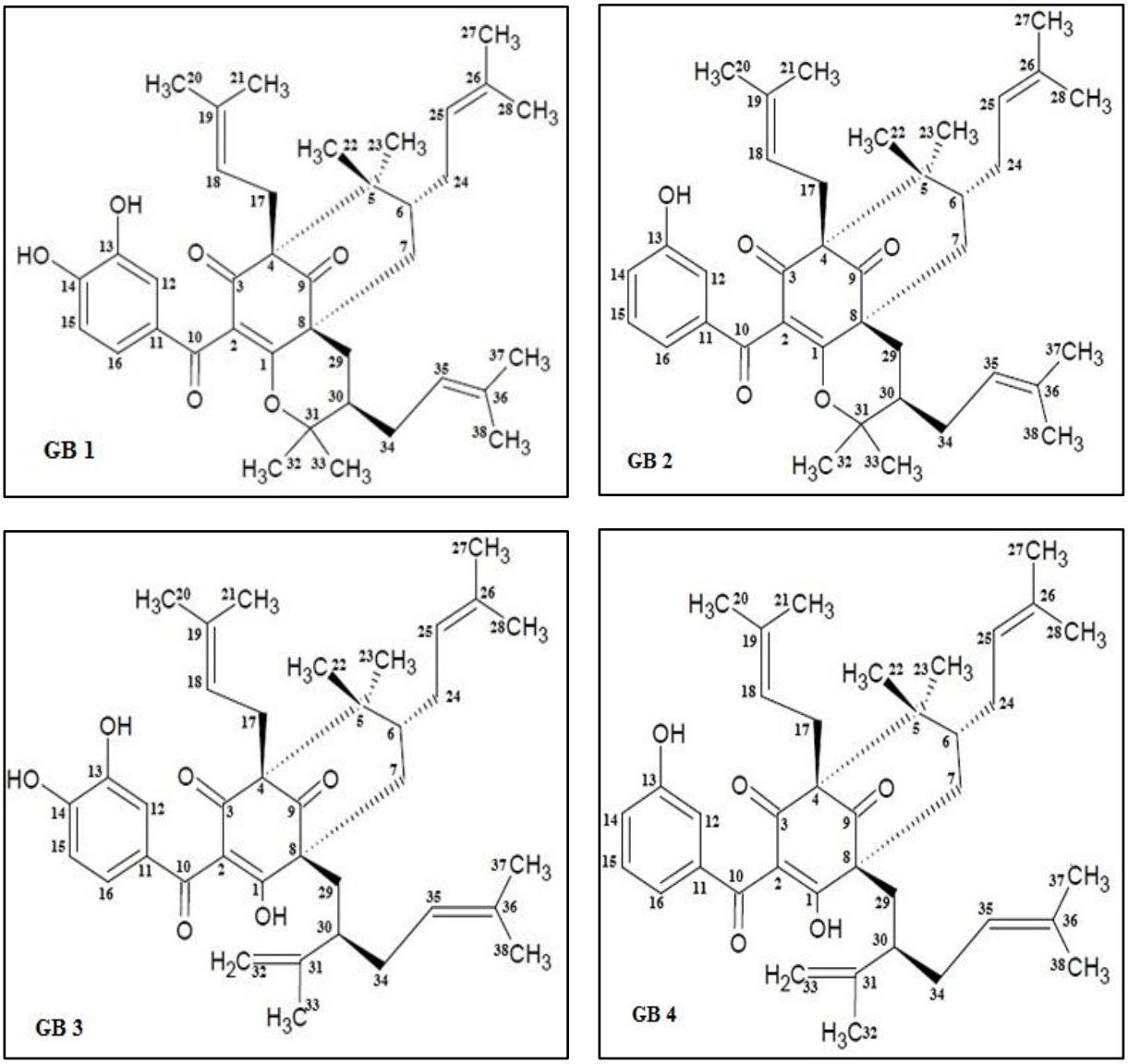


Figure 1 Chemical structures of 30-*epi*-cambogin (**GB 1**), 14-deoxy-30-*epi*-cambogin (**GB 2**), guttiferone F (**GB 3**) and 14-deoxy-guttiferone F (**GB 4**)

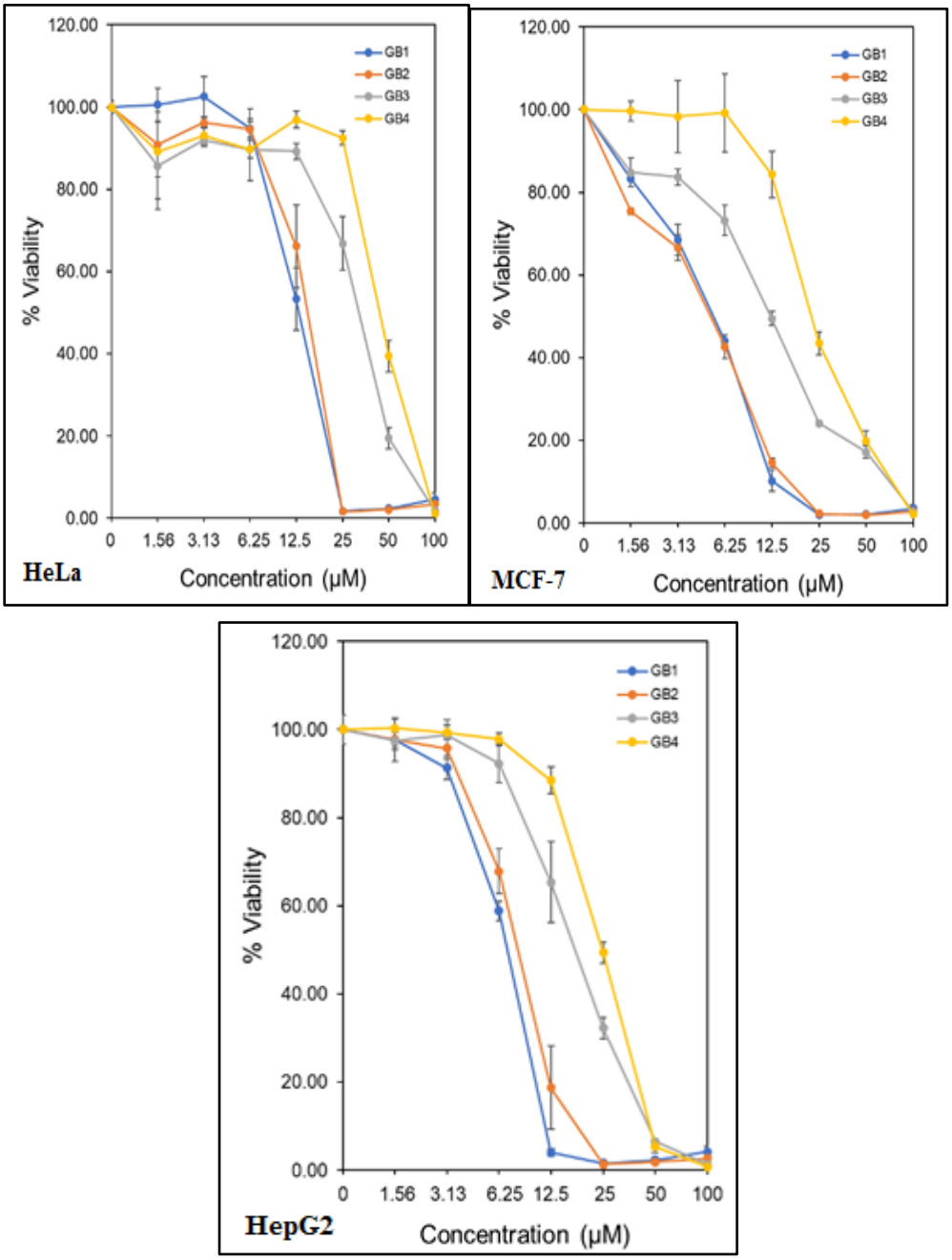


Figure 2 The antiproliferative effect in HeLa, MCF-7 and HepG2 cancer cell lines treated with compounds **GB 1** to **GB 4** at 48 h. The points represent means ± standard deviation (n=3)

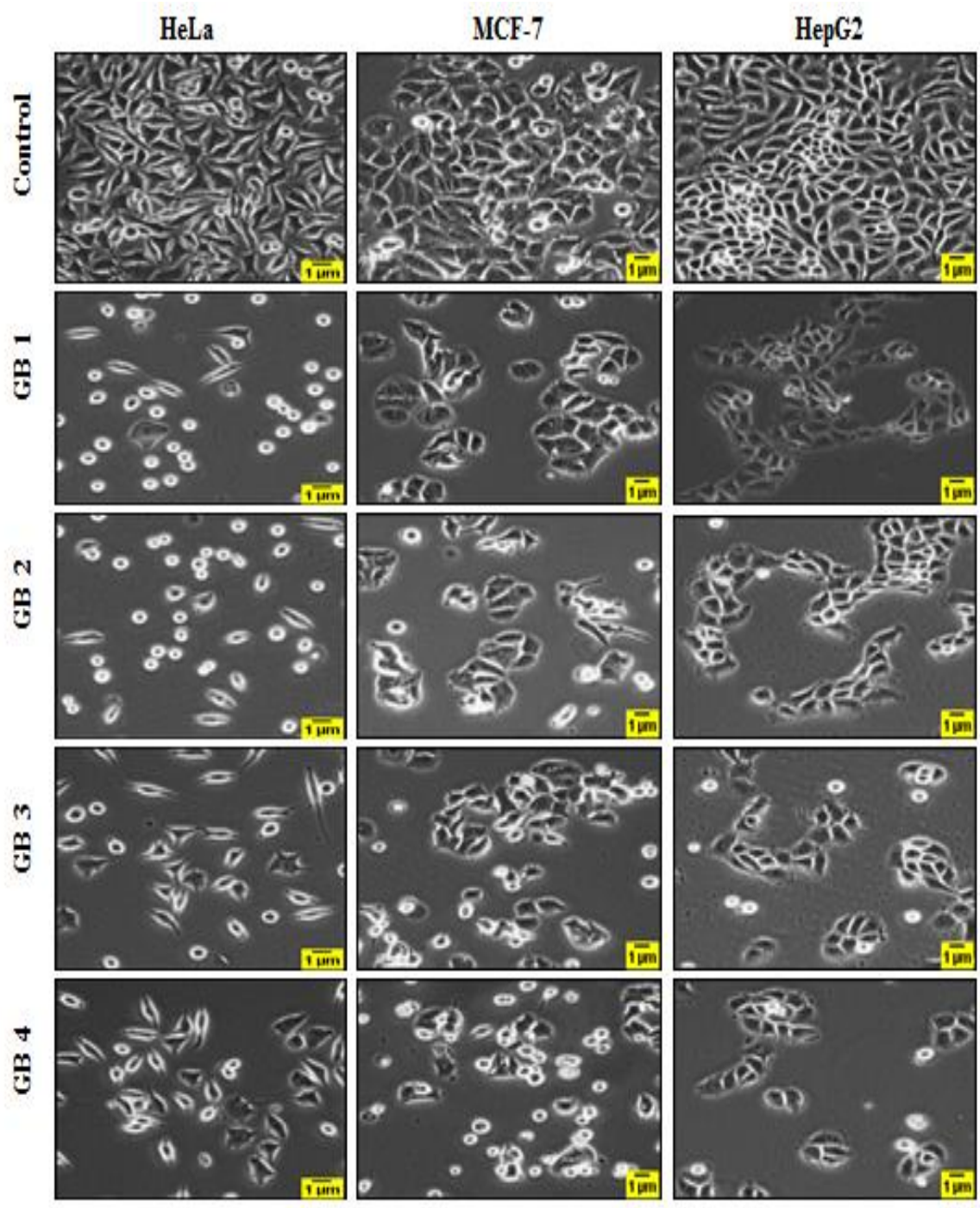
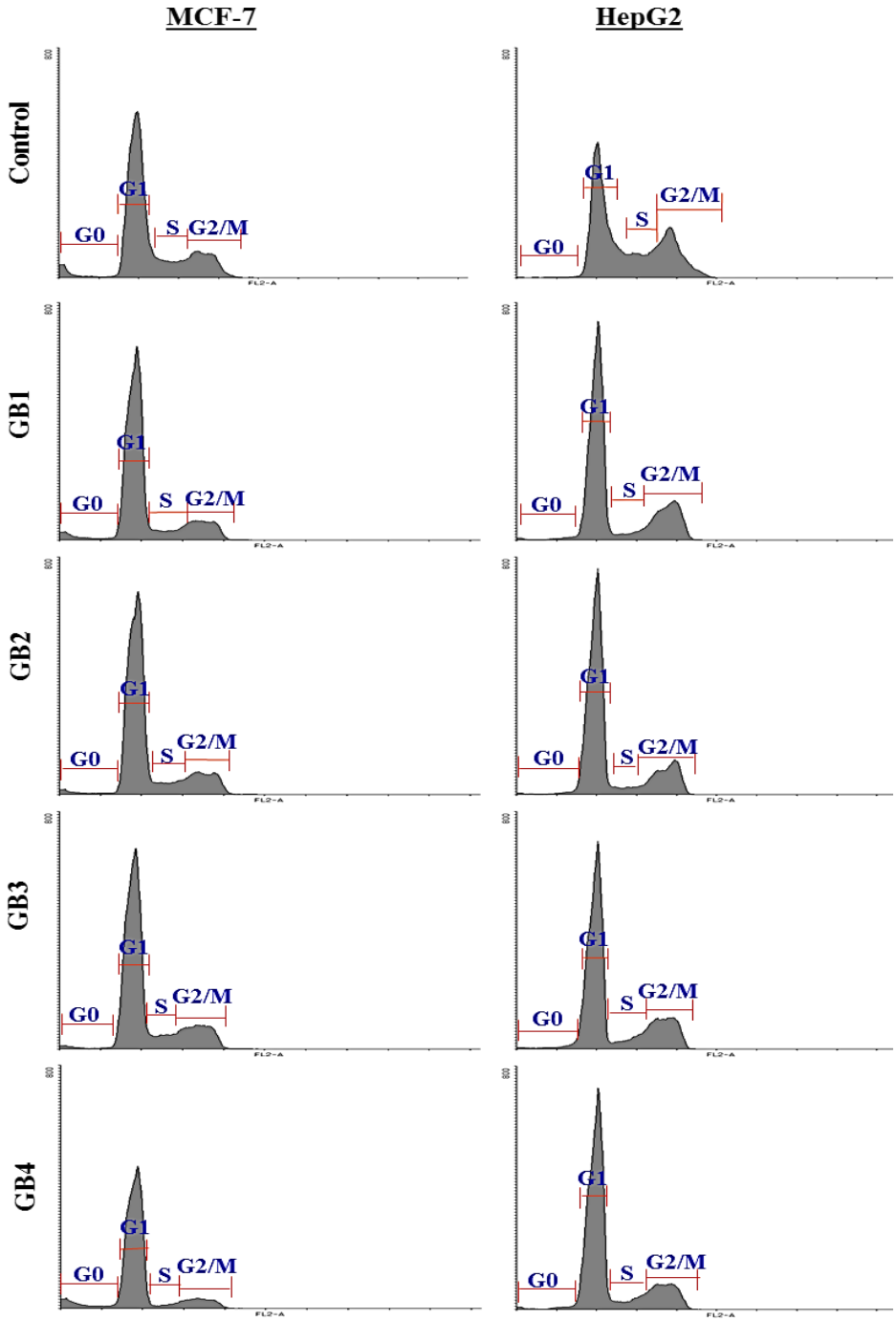


Figure 3 Morphological changes in MCF-7, HeLa and HepG2 cells induced by compounds **GB 1** to **GB 4** and 0.1% DMSO (vehicle control) using microscopy analysis (100x)



|Figure 4 Cell cycle arrests induced by compounds **GB 1** to **GB 4** in MCF-7 and HepG2 cellsat 48 h, followed by propidium staining and flow cytometry

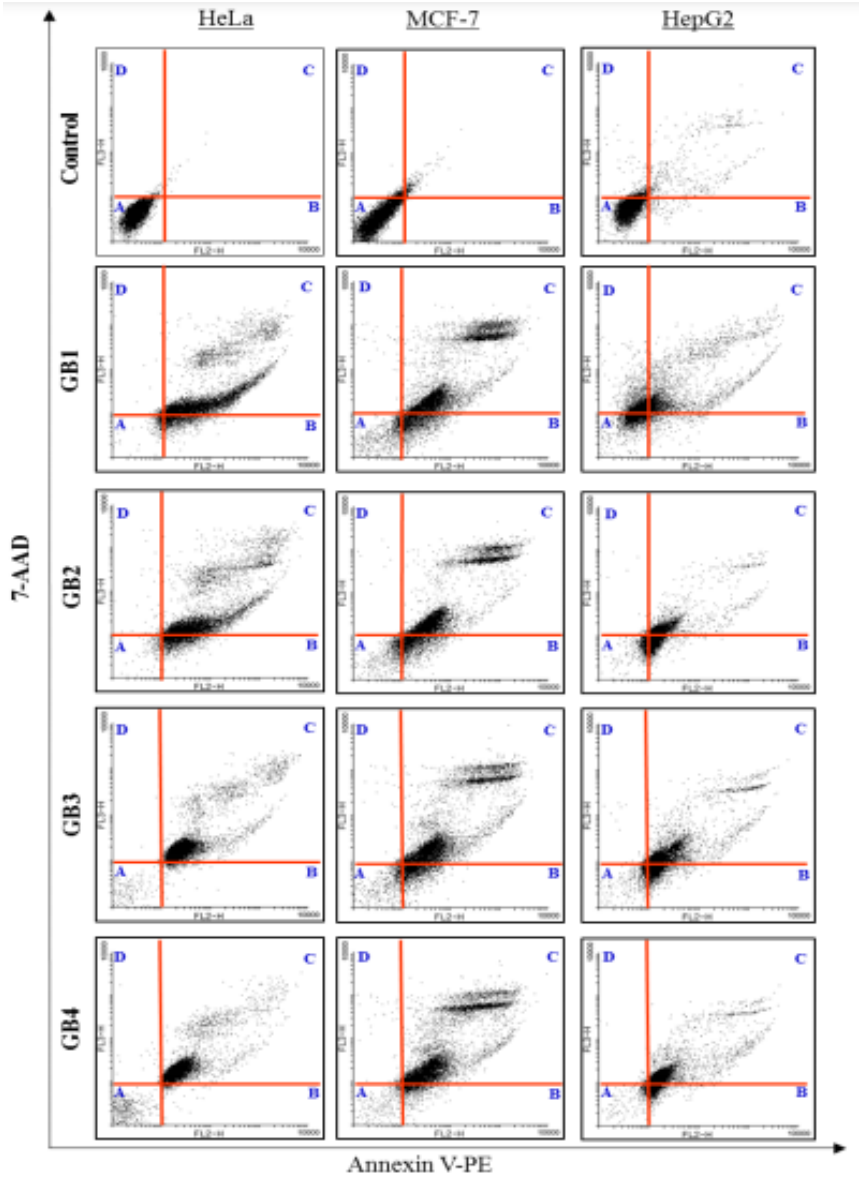


Figure 5 Flow cytometric PE Annexin-V/7-ADD binding profiles of MCF-7, HeLa and HepG2 cancer cell lines treated with 0.1% DMSO (vehicle control) and compounds **GB 1** to **GB 4** at 48 h. Regions labelled A indicating viable cells, B is early apoptotic cells, C is late apoptotic cells and D is necrotic cells

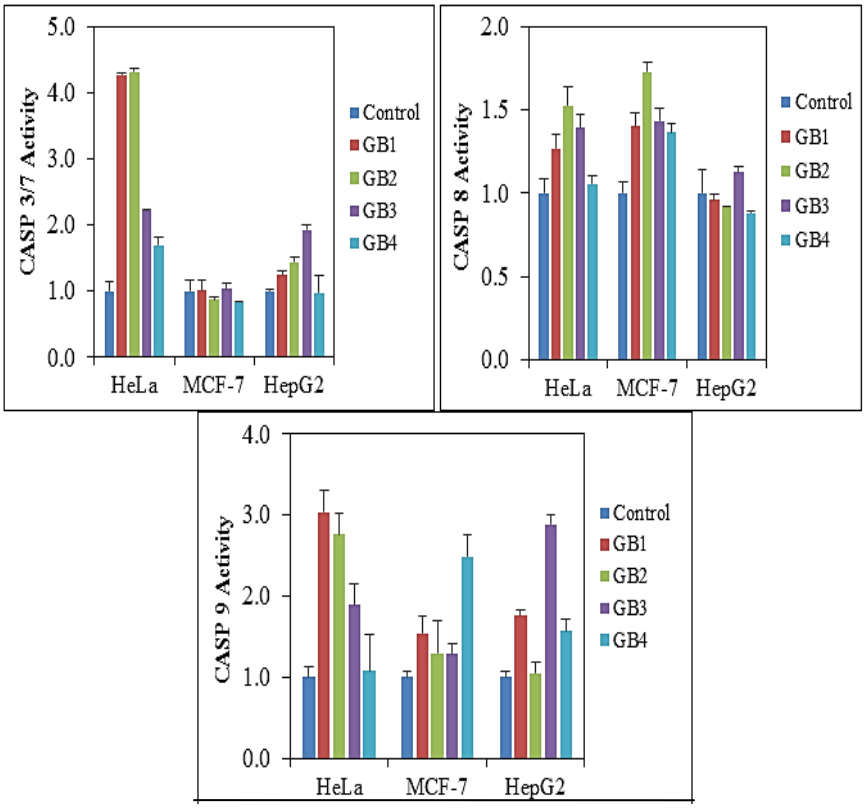


Figure 6 Effects of compounds **GB 1** to **GB 4** on caspases 3/7, 8 and 9 activities in HeLa, MCF-7 and HepG2 cells. The bars represent the mean ±standard deviation (n=3) and * indicates fold change > 2 and P < 0.05 (Student t-test)

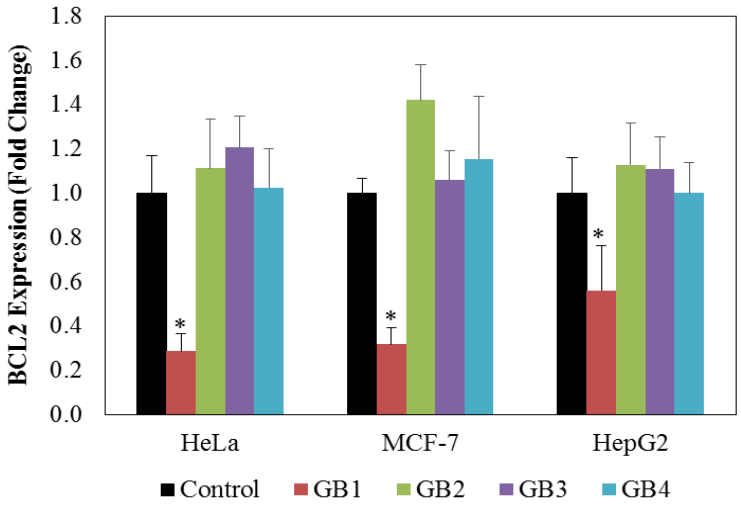


Figure 7 BCL2 mRNA expression profile in MCF-7, HeLa and HepG2 cancer cell lines treated with compounds **GB 1** to **GB 4** at 48 h. Bars indicate mean ± standard deviation (n = 3). * indicates P < 0.01, Student t-test

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