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ORIGINAL ARTICLE

Studies of structure–activity relationship on plant polyphenol-induced suppression of human liver cancer cells

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Abstract
Purpose To study anticancer activities of 68 plant polyphenols with different backbone structures and various substitutions and to analyze the structure–activity relationships.
Methods Antiproliferative activity of 68 plant polyphenols on human liver cancer cells were screened by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide method. Structure–activity relationships were analyzed by comparison of their activities with selected structures. Cell cycle progression was assayed by flow cytometry analysis and apoptosis was analyzed by DNA fragment assay.
Results Based on their backbone structures, 68 polyphenols were sub-classed to flavonoids (chalcones, flavanones, flavones and isoflavones), chromones and coumarins. The order of their potency to suppress the human liver cancer cells is chalcones > flavones > chromones > isoflavones > flavanones > coumarins. Chalcones comprise the most potent group with IC_{50} values ranging from 21.69 to 197 μM. Top nine most potent chalcones in the group have hydroxylation at 2′-carbon position in B-ring. Flavones ranked second in their potencies. Quercetin, 4-hydroxyflavone and luteolin are three hydroxyflavones with highest potencies in this group. Their IC_{50} values are 30.81, 39.29 and 71.17 μM, respectively. Chromones, isoflavones, flavanones and coumarins showed much lower potencies when compared to the first two groups with IC_{50} ranges of 61 to >400, 131 to >400, 138 to >400 and 360.85 to >400 μM, respectively. In mechanistic studies, the most potent chalcone, 2,2′-dihydroxychalcone could induce G2/M arrest and then apoptosis of the cancer cells.
Conclusions An analysis of structure–activity relationship showed that following structures are required for their inhibitory potencies on human liver cancer cells: (1) of the six sub-classes of the polyphenols tested, the unique backbone structure of chalcones with a open C-ring; (2) within the chalcone group, hydroxyl substitution at 2′-carbon of B-ring; (3) hydroxyl substitution at 3′-carbon in B-ring of flavones. However, some other structures were found to decrease their potencies: e.g. substitutions by sugar moieties in flavones. These data are valuable for design and modification of new polyphenols, which could be potential antiproliferative agents of cancer cells.

Keywords Plant polyphenols · Liver cancer · Anticancer activity: apoptosis · G2/M arrest · 2,2′-Dihydroxychalcone

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Introduction

Chemotherapy has still been an important treatment modality for cancers. However, toxicity and poor tolerance to current chemotherapeutic drugs are dose-limiting factors. This has led to a rising interest in developing anticancer drugs from relatively non-toxic sources. Plant polyphenols are one such example. They are relatively non-toxic natural products [1] found in normal diets [2] and herb medicines [3]. They have been shown to have various biological and pharmacological effects including anticancer activity [4]. In vitro, many studies have shown that plant polyphenols suppress various cancer cells [4, 5]. These anticancer effects have also been studied in animal cancer models [6] and clinical cancer patients [7]. Many of the polyphenols with

various structures have been studied. However, our present understanding of the relationships between their chemical structures and anticancer properties is still very limited. A systemic analysis for the structure–activity relationships may be necessary to define more important chemical structures of promising compounds or classes of compounds. They may help in design, modification and development of new polyphenols as potential antiproliferative agents of cancer cells. In this present study, plant polyphenols, which were selected based on their chemical structures, were investigated for their activity to suppress growth of human liver cancer cells in vitro. Backbone structures of selected plant polyphenols include flavonoids (chalcones, flavanones, flavones and isoflavones), chromones and coumarins. Each of the polyphenols in each sub-class was selected according to the their substitution groups and substitution positions.

Materials and methods

Chemicals

The plant polyphenols were obtained from Indofine Chemical Company (Hillsborough, NJ, USA). 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma & Aldrich (St Louis, MO, USA). Media and sera for cell culture were purchased from Life Technologies (Grand Island, NY). Other chemicals were of analytical grade from standard commercial suppliers.

Cell culture

Human liver tumor cells, Hep G2, were obtained from ATCC (Rockville, MD, USA) and grown in MEM medium containing 1 mM sodium pyruvate, 1 mM non-essential amino acid and 10% fetal calf serum (FCS, Life technologies, Grand Island, NY, USA). Cells were maintained in humidified atmosphere of air/CO_2 (19/1) and were subcultured every 2–3 days.

MTT assay of cell growth

Approximately, 1×10^4 cells were seeded in each well of 96-well tissue culture plates and incubated in a CO_2 incubator for 24 h. The stock solutions of plant polyphenols were prepared freshly in dimethylsulphoxide and aliquots of the stock solutions were added to wells of the plates at desired concentrations. After incubation with the polyphenols for various periods of time, the medium was changed with fresh medium and survival cells in plate wells were determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) assay as reported previously [8]. Twenty-five microlitres of the 5 mg/ml stock solution of MTT was added to each well and incubated at 37°C for 2 h. One hundred microlitres of the extraction buffer (20% SDS w/v, in 50% *N*,*N*-dimethyl formamide v/v, 2.5 of 80% acetic acid and 2.5% of 1 N HCl, pH 4.7) was then added. After an overnight incubation at 37°C, the optical densities at wavelength of 570 nm were measured using a microplate reader (Dynatech Laboratories). The cell survival of each dose of polyphenols was calculated as the ratio of absorbance in wells containing drug treated cells to that in control wells with untreated cells. IC_{50} values were calculated from dose-dependent curves of at least five points.

Cell cycle analysis

HepG2 cells were sub-cultured in each well (2.5×10^5/well) of six well plates and incubated at 37°C for 24 h. Aliquots of a 20-mM stock solution of 2,2′-dihydroxychalcone were then added to the cell cultures to reach final concentrations in the range from 20 to 60 μM and the cells were incubated with the polyphenols for various periods of time. Cell suspensions from either control cultures or 2,2′-dihydroxychalcone-treated cultures were prepared by trypsinization and washed twice with phosphate buffered saline (PBS). The cells were then fixed in 85% ethanol at −20°C for 20 h and rewashed with PBS to remove ethanol. Cells collected by centrifugation were stained in 1 ml propidium iodide (PI)/Triton X-100 staining solution (40 μg PI/ml, 200 μg RNase/ml, 0.1% Triton X-100 in PBS) at 37°C for 1 h. Cell cycle distribution was analyzed by flow cytometry using a BD Flow Cytometer (Coulter Corporation, Miami, FL, USA) and the DNA histograms were analyzed with a Winndi multi-cycle software.

Determination of apoptosis

Detection of hypodiploid (Sub-G1) apoptotic cells was performed by flow cytometry as described above. Further confirmation of apoptosis was accomplished by performing a DNA fragment analysis using an electrophoresis method. HepG 2 cells were treated with 20–60 μM of 2,2′-dihydroxychalcone (2,2′DHC) for 6, 12, 24 and 48 h, respectively, and harvested by trypsinization. DNA from the cells was extracted using a Qiagen DNA mini kit (Qiagen, Hilden, Germany). The DNA samples were then treated with DNAse-free RNAse for digestion of possible contaminated RNAs. Approximately 40 μg of the DNA samples were loaded on a 2% agarose gel and stained with ethidium bromide. Images were taken by the Bio-Rad gel documentation system.

Results and discussions

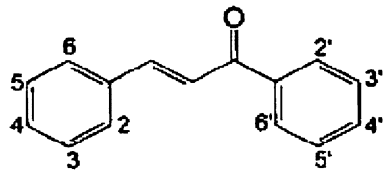
Structure–activity relationships of plant polyphenols in their inhibitory potencies on HepG 2 cells

The plant polyphenols were grouped as chalcones, flavones, chromones, isoflavones, flavanones and coumarins according to their backbone structures. Cytotoxic effects of 68 plant polyphenols in the six sub-groups on the HepG2 cells were studied dose-dependently. IC_{50} values were obtained from dose-response curves. Tables 1, 2, 3 and 4 show IC_{50} values of each of these sub-classes respectively. Twenty-eight chalcones comprise the largest group of the six sub-groups. As shown in Table 1, 2,2′-dihydroxychalcone tops the group in the cytotoxicity on HepG2 cells with an IC_{50} value of 21.69 μM while 2-hydroxy-2, 4,4′-trimethoxychalcone has the lowest potency with an IC_{50} value of 197 μM. It has been reported that, generally, hydroxyl-derivatives of chalcones have more potent anti-proliferation effects on cancer cells when compared to other chalcone derivatives and other flavonoids [9, 10]. Previous studies have also showed that hydroxylation of chalcones is required for their inhibitory potencies on activity of glutathione S-transferase, export of glutathione conjugates and sensitisation of cancer cells to anticancer agents [11–14]. Therefore, in this study, 28 hydroxychalcones with single or multi hydroxyl substitutions at various positions were chosen to be studied to understand effects of number of hydroxyl substitutions and positions of hydroxyl substitutions on their potencies. The addition of a hydroxyl substitution at the 2′ position generally led to an increase in HepG2 cell growth inhibition (from 2-hydroxylchalcone, IC_{50} = 53.39 μM to 2,2′-dihydroxychalcone, IC_{50} = 21.69). Removal of the 2′-hydroxy substitution also decreased HepG2 cell growth inhibition (from 2′-hydroxychalcone IC_{50} = 33.23 to chalcone IC_{50} = 48.60). As shown in Table 1, all nine potent chalcones have hydroxyl-substitutions at 2′-carbon in Ring B (Table 1, 1–9). Other chalcones with 2′-hydroxylation did not show potent activities. This may be due to their other substitutions which decrease their activity. 2′-Hydroxylation may be an important structure required for the activity of chalcones to suppress the cancer cells. Other reported anticancer chalcones also have hydroxylation at 2′-carbon of ring B [15–18]. These studies further support our point. When compared to other subclasses of the polyphenols studied, the unique structure of open C ring of chalcones may be an important structure for their most potent antiproliferative activity on cancer cells. The hydroxylation at 2′ carbon position in B ring of chalcones may make the unsaturated carbonyl groups in the open C ring more reactive, therefore increase their antiproliferative activity on cancer cells.

Flavones ranked second in their potencies. Quercetin has been studied extensively for its anti-proliferation property on cancer cells [6, 19, 20]. In consistence with the previous studies on other cancer cells [6, 21–23], the present study has also demonstrated that quercetin is the most potent flavone in this group to suppress human liver cancer cells with an IC_{50} value of 30.9 μM. The potency of quercetin may be attributed to hydroxyl substitutions, particularly, its hydroxyl-substitutions at 3′ carbon of ring B and 5 carbon of ring A, since kaempferol (without 3′-hydroxylation) and Fisetin (without hydroxylation at 5 carbon in ring A) have much lower potency with IC_{50} values >400 μM. Other potent inhibitors are also hydroxylflavones (Table 2. 2 and 3, IC_{50}, 39.25 and 71.17 μM, respectively). However, hydroxyl substitutions at other positions may affect their potencies. For example, hydroxylation at 5′-carbon decreased the potency (Table 2. 10, IC_{50} >400 μM compared to Table 2. 1, IC_{50}, 30.9 μM). In addition, lower redox potential of quercetin (0.03 V) when compared to that of kaempferol and Fisetin (0.12 V) may make quercetin more likely to form a semi-quinone radical and then a quinone. These intermediate metabolites may play a role in antiproliferative activity of quercetin [24]. In this study, all flavones with substitutions by sugar moieties showed significantly lower potencies when compared to other flavones without substitutions with sugar moieties (Table 2. 14, 16–18, with IC_{50} values >400 μM). Another report also showed that flavonoids were more effective than their corresponding glycosides in suppressing growth of cancer cells [25].

Tables 3 and 4 shows anti-proliferation effects of chromones, isoflavones, flavanones and coumarins on the human liver cancer cells. When compared to chalcones and flavones, their effects on the cancer cells were much weaker with IC_{50} ranges of 61 to >400, 131 to >400, 138 to >400 and 360.85 to >400 μM, respectively. The order of their potencies is chromones > isoflavones > flavanones > coumarins. Chromones with substitutions by groups such as bromo-, chloro- and formyl groups have moderate potencies (Table 3. Chromones 1–3), while substitutions by a methyl group lowered the potency (Table 3. Chromones, 4). More electrophilic properties of halogens when compared to methyl groups may play a role in their inhibitory effects on cancer cells. Belonging to the group of isoflavones, genistein has been considered as a potential cancer therapeutic agent and studied by many researchers, in vitro, in vivo and in clinical trials [26, 27]. However, in this study, genistein only showed moderate inhibitory effects on the human liver cancer cells when compared to chalcones. IC_{50} values of flavanones ranged from 131.6 to >400 μM. Much weaker effects were observed with coumarins. Four of five coumarins tested have IC_{50} values >400 μM.

Table 1 Effects of chalcone derivatives on the growth of HepG2 cells



OOP	Compound	Hydroxylation pattern	Substitution	IC50 (μM)
1	2,2′-Dihydroxychalcone	2,2′		21.69
2	2,2′-Dihydroxy-3-methoxychalcone	2,2′	3 MO	23.79
3	2′-Hydroxy-4-methylchalcone	2′ 4-Methyl		24.33
4	2′4-Dimethoxychalcone	2′,4′MO		25.26
5	3,2′-Dihydroxychalcone	3,2′		25.98
6	3,4,2′4′-Tetrahydroxychalcone	3,4,2′4′		31.00
7	2′-Hydroxychalcone	2′		33.23
8	2,4,2′,5′-Tetramethoxy-chalcone	2,4,2′,5′ MO		33.28
9	2′-Hydorxy-2,4-methoxy-chalcone	2′	2,4 MO	34.63
10	2′-Hydroxy-2-methoxychalcone	2′	2 MO	40.86
11	2,2′, 4′-Trihydroxychalcone	2,2′,4′		41.11
12	4,2′,5′-Trimethoxychalcone		4,2′,5′	41.29
13	2′-Hydroxy-4,4′,6′-trimethoxy-chalcone	2′	4,4′,6′	42.91
14	2, 4′-Dihydroxychalcone	2, 4′		43.08
15	2′, 5′-Dihydroxychalcone	2′,4′		45.37
16	4’-Chlorochacone		4′ Chloro	46.09
17	Chalcone			48.60
18	2′-Hydroxy-2,4′-methoxy-chalcone	2′	2,4′ MO	48.87
19	2′,4′-Dihydroxy-3,4-dimethoxy-chalcone	2′,4′	3,4 MO	49.33
20	4′-Hydroxychalcone	4′		51.53
21	2′,4′-Dihydroxychalcone	2′, 4′		52.59
22	2-Hydroxychalcone	2		53.39
23	4,2′,5′-Trihydroxychalcone	4,2′,5′		54.14
24	4-Hydroxychalcone	4,		67.77
25	4-Methoxychalcone		4 MO	94.43
26	2′-4′-Dihydroxy-4-methoxychalcone	2′,4′	4 MO	121.71
27	4,2′,4′,5′-Tetrahydroxychalcone	4,2′,4′,6′		166.75
28	2′-Hydroxy-2,4,4′-tri-methoxychalcone	2′	2,4,4′ MO	197.00

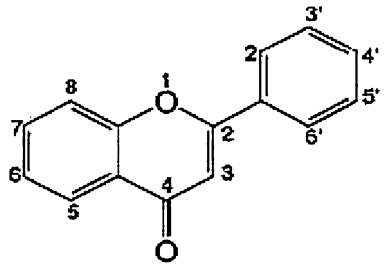
OOP order of potency, *MO* methoxyl

HepG2 cells seeded in 96 well plates were incubated without or with the test compounds (3–300 μM) for 24 h. MTT (25 μl) was then added and incubated for 2 h followed by incubation with lysing buffer for further 24 h. Absorbencies were measured at 570 nm. Cell growth of each concentration of polyphenols was calculated as the ratio of absorbency in treated cells compared with control cells. IC_{50} values were calculated from dose-dependent curves plotted from at least three points. Each point was performed in at four replicates and expressed as mean ± SD (n = or > 4)

Effects of 2,2′-dihydroxychalcone on cell cycle progression

2,2′DHC is the most potent agent of the 68 polyphenols tested. It was further studied to understand its mechanism for cell growth inhibition. Mammalian cell growth and proliferation are mediated via cell cycle progression and cell cycle checkpoints are important targets for regulation of cell growth. Therefore, the effects of 2,2′DHC on cell cycle progression were studied to understand the mechanism through which 2,2′DHC inhibited the cells. As shown in Fig. 1, after exposure to 2,2′DHC at 40 and 60 μM for 6 h, there was a significant increase in the proportion of HepG2 cells in G2/M phase (29.5 and 34.4%, respectively, when

Table 2 Effects of flavone derivatives on growth of HepG2 cells



OOP	Compound	Common name	Hydroxylation pattern	Substitutions	IC_{50} (μM)
1	3,5,7,3′,4′-Penta-hydroxyflavone	Quercetin	3,5,7,3′,4′	30.87	
2	4-Hydroxyflavone		4	39.25	
3	3,5,3′,4′-Tetrahydroxy-flavone	Luteolin	3,5,3′,4′	71.17	
4	6-Hydroxyflavone		6	73.46	
5	3,5,7-Trihydroxyflavone Galangin		3,5,7	118.90	
6	5,7,3′-Trihydroxy-4′-glucosideflavone		5,7,3′	4′-Glucoside	172.29
7	5,7-Dihydroxy-4′-methoxyflavone	Acacetin	5,7	4 MO	221.67
8	3′,4′-Dihydroxyflavone		3′,4′		331.04
9	3,5,7,2′-Tetra-hydroxyflavone		3,5,7,2′		364.66
10	3,5,7,3′,4′,5′-Hexa-hydroxyflavone	Myricetin	3,5,7,3′,4′,5′		>400
11	3,5,7,4′-Tetrahydroxy-flavone	Kaempferol	3,5,7,4′		>400
12	3,5,7,4′-Tetrahydroxy-5′-methoxychalcone	Isorhamnetin	3,5,7,4′	5’ MO	>400
13	3,7,3’,4’-Tetrahydroxy-flavone	Fisetin	3,7,3′,4′		>400
14	5,7,3′,4′-Tetrahydroxy-flavone	Quercitrin	5,7,3′,4′	3-*O*-Rhamnose	>400
15	5,7,4′-Trihydroxyflavone	Apigenin	5,7,4′		>400
16	5,7,4′,5′-Tetrahydroxy-flavone	Isoquercitrin	5,7,4′,5′	3-Glucoside	>400
17	5,3′-Dihydroxy-7-rutinose-4-methoxyflavone	Diosmin	5,3′	7 Rutinose; 4′ MO	>400
18	5,4′-Dihydroxy-7-glucoside-apioseflavone	Apiin	5,4′	7-Glucoside-apiose	>400

OOP order of potency, *MO* methoxyl

HepG2 cells seeded in 96 well plates were incubated without or with the test compounds (3–400 μM) for 24 h. MTT (25 μl) was then added and incubated for 2 h followed by incubation with lysing buffer for further 24 h. Absorbencies were measured at 570 nm. Cell growth of each concentration of polyphenols was calculated as the ratio of absorbency in treated cells compared with control cells. IC_{50} values were calculated from dose-dependent curves plotted from at least three points. Each point was performed in at four replicates and expressed as mean ± SD (n = or >4). IC_{50} >400, less than 50% of cells were killed by 400 μM of the test compound

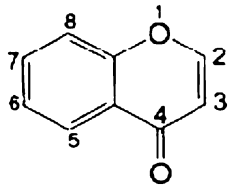
compared to 23.0% of control, $P < 0.01$). At 12th hour of treatment, the proportion of G2 M cells increased dose-dependently (23.7, 27.5 and 39.13% of total cell number at the concentration of 20, 40 and 60 μM, respectively, when compared to 20.6% of control, $P < 0.05$, $P < 0.01$ and $P < 0.01$). However, G2/M cells decreased progressively from 24 to 48 h of treatment with 60 μM 2,2′-dihydroxychalcone (from 39.13 at 12th hour to 21.54% at 24th hour, 11.99 at 36th hour and 1.31% at 48th hour) with a corresponding increase of cells in sub-G1 phase (apoptotic cells) (from 10.34 at 12th hour to 43.98% at 24th hour, 56.60 at 36th hour and 92.97% at 48th hour). These data suggest that G2/M arrested cells underwent apoptosis subsequently. This time- and dose-dependent analysis of flow cytometry showed that 2,2′-dihydroxychalcone induces G2/M arrest of the human liver cancer cells. This G2/M arrest proceeding apoptosis may be a mechanism for its inhibitory effects on growth of the cancer cells.

Determination of apoptosis in 2,2′-dihydroxychalcone-treated cells

In the analysis of flow cytometry, the control cells showed no sub-G1 peak (sub-G1 cell population less than 5%) within 48 h. 2,2′-dihydroxychalcone-treated cells revealed an apparent sub-G1 peak at the time point of 24 h (Fig. 1) and dose-and time-dependent responses of the peak to 2,2′DHC treatment were observed. When compared to

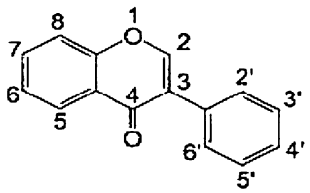
Table 3 Effects of chromones and isoflavones on growth of HepG 2 cells

Chromones



OOP	Compound	Common name	Hydroxylation pattern	Substitutions	IC_{50} (μM)
1	3,6-Dibromochromone			3,6 Bromo	61.57
2	6,8-Dichlorochromone			6,8 Chloro	94.98
3	3-Formylchromone			3 Formyl	100.20
4	6-Methylchromone			6 Methyl	>400

Isoflavones



OOP	Compound	Common name	Hydroxylation pattern	Substitutions	IC_{50} (μM)
1	5,7-Dihydroxy-4′-methoxyisoflavone	Biochanin A	5,7	4′ MO	131.59
2	5,7,4′-Trihydroxy-isoflavone	Genistein	5,7,4′		189.92
3	7,4′-Dihydroxy-isoflavone	Daidzein	7,4′		>400

OOP order of potency, *MO* methoxyl

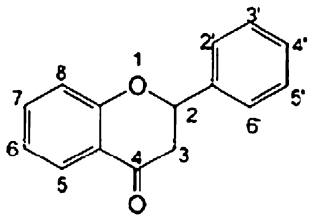
HepG2 cells seeded in 96 well plates were incubated without or with the test compounds (3–400 μM) for 24 h. MTT (25 μl) was then added and incubated for 2 h followed by incubation with lysing buffer for further 24 h. Absorbencies were measured at 570 nm. Cell growth of each concentration of polyphenols was calculated as the ratio of absorbency in treated cells compared with control cells. IC_{50} values were calculated from dose-dependent curves plotted from at least three points. Each point was performed in at four replicates and expressed as mean ± SD (n = or >4). IC_{50} >400, less than 50% of cells were killed by 400 μM of the test compound

control sub-G1 population of 4.57%, at 24th hour of treatment, 20.11, 29.47 and 43.98% cell populations were detected in sub-G1 peak with treatment by 20, 40 and 60 of 2,2′DHC, respectively ($P < 0.05$, $P < 0.05$ and $P < 0.01$). At 36th and 48th hour of treatment, more obvious accumulations of cells in sub-G1 peaks was observed (Fig. 1, 36 and 48 h). Apoptotic cells with fragmented DNA should appear in the sub-G1 peak and this has been used as a marker for detection of apoptotic cells [28]. However, the accumulation of cells in sub-G1 peak does not necessarily indicate DNA oligonucleosomal fragmentation, cells with less DNA content and some cellular debris may also accumulate in the sub-G1 region [29]. Therefore, gel electrophoresis was used to detect DNA oligonuleosomal fragments, which are a typical feature of apoptotic cells. As shown in Fig. 2, DNA laddering was observed in 2,2′-dihydroxychalcone-treated cells at the time points of 12 and 24 h. DNA ladders reflect DNA fragments in certain stage of apoptotic cells. With progression of apoptosis, the DNA fragments may further degrade to cause weakness or disappearances of DNA ladders in a gel analysis. In this study, when higher concentrations of 2,2′-dihydroxychalcone were used, further degradation of DNA fragments may cause DNA laddering bands at 60 μM to become weak when compared to that at 40 μM. These results further confirm induction of apoptosis in the cancer cells by 2,2′-dihydroxychalcone. 2,2′-dihydroxychalcone-induced apoptosis proceeded with G2 arrest was implicated to be involved in the growth inhibition of the cancer cells. However, mechanisms for the induction of G2 arrest and apoptosis still need to be studied.

In summary, potencies of the plant polyphenols to suppress the cancer cells varied among the various groups with different backbone structures and within each group with

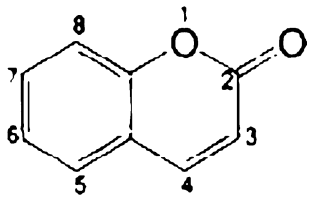
Table 4 Effects of flavanones and coumarins on growth of HepG2 cells

Flavonones



OOP	Compound	Common name	Hydroxylation pattern	Substitutions	IC_{50} (μM)
1	2′-Hydroxyflavanone		2′		138.24
2	4′-Hydroxyflavanone		4′		145.15
3	Flavanone				149.41
4	3,7,3′,4′,5′-Penta-methoxyflavanone			3,7,3′,4′,5′ MO	261.15
5	6-Hydroxyflavanone		6		322.04
6	6,2′,3′-Trimethoxyflavanone			6,2′,3′	334.50
7	5,7-Dihydroxyflavanone	Pinocembrin		5,7	>400
8	3,5,7,3′,4′-Penta-hydroxyflavanone	Taxifolin		3,5,7,3′4′	>400
9	3,7,3′,4′-Tetrahydroxy-flavanone	Fustin		3,7,3′,4′	>400
10	5,4′-Dihydroxy-7-neo-hesperidosideflavanone	Naringin			>400

Coumarin



OOP	Compound	Common name	Hydroxylation pattern	Substitutions	IC_{50} (μM)
1	2-Hydroxycoumarin		2	360.85	
2	4-Hydroxycoumarin		4		>400
3	6,7-Dihydroxy-6-glucosidecourmarin	Esculin	6,7	6 Glucoside	>400
		Umbelliferone	7		>400
4	7-Hydroxycoumarin				
5	7,8-Dihydroxycoumarin	Dapnetin	7,8		>400

OOP order of potency, *MO* methoxyl

HepG2 cells seeded in 96 well plates were incubated without or with the test compounds (3–300 μM) for 24 h. MTT (25 μl) was then added and incubated for 2 h followed by incubation with lysing buffer for further 24 h. Absorbencies were measured at 570 nm. Cell growth of each concentration of polyphenols was calculated as the ratio of absorbency in treated cells compared with control cells. IC_{50} values were calculated from dose-dependent curves plotted from at least three points. Each point was performed in at four replicates and expressed as mean ± SD (n = or >4). IC_{50} >400, less than 50% of cells were killed by 400 μM of the test compound

different substitutions and different substitution positions. Of the six sub-classes of plant polyphenols screened, chalcones are the group with highest potency to suppress the cancer cells and followed in the order of potencies are: flavones, chromones, isoflavones, flavanones and coumarins. By an analysis of the structure–activity relationships, following structures were found to be required for their inhibitory potencies: (1) of the six sub-classes of the polyphenols tested, the unique backbone structure of chalcones with a open C-ring, (2) within the chalcone group, hydroxyl substitution at 2′-carbon of B-ring, (3) hydroxyl substitution at 3′-carbon in B-ring of flavones. However, some other structures were found to lower their potencies: e.g. substitutions by sugar moieties in flavones. These data are valuable for development of plant polyphenols as possible anti-proliferation agents of cancer cells and for design and

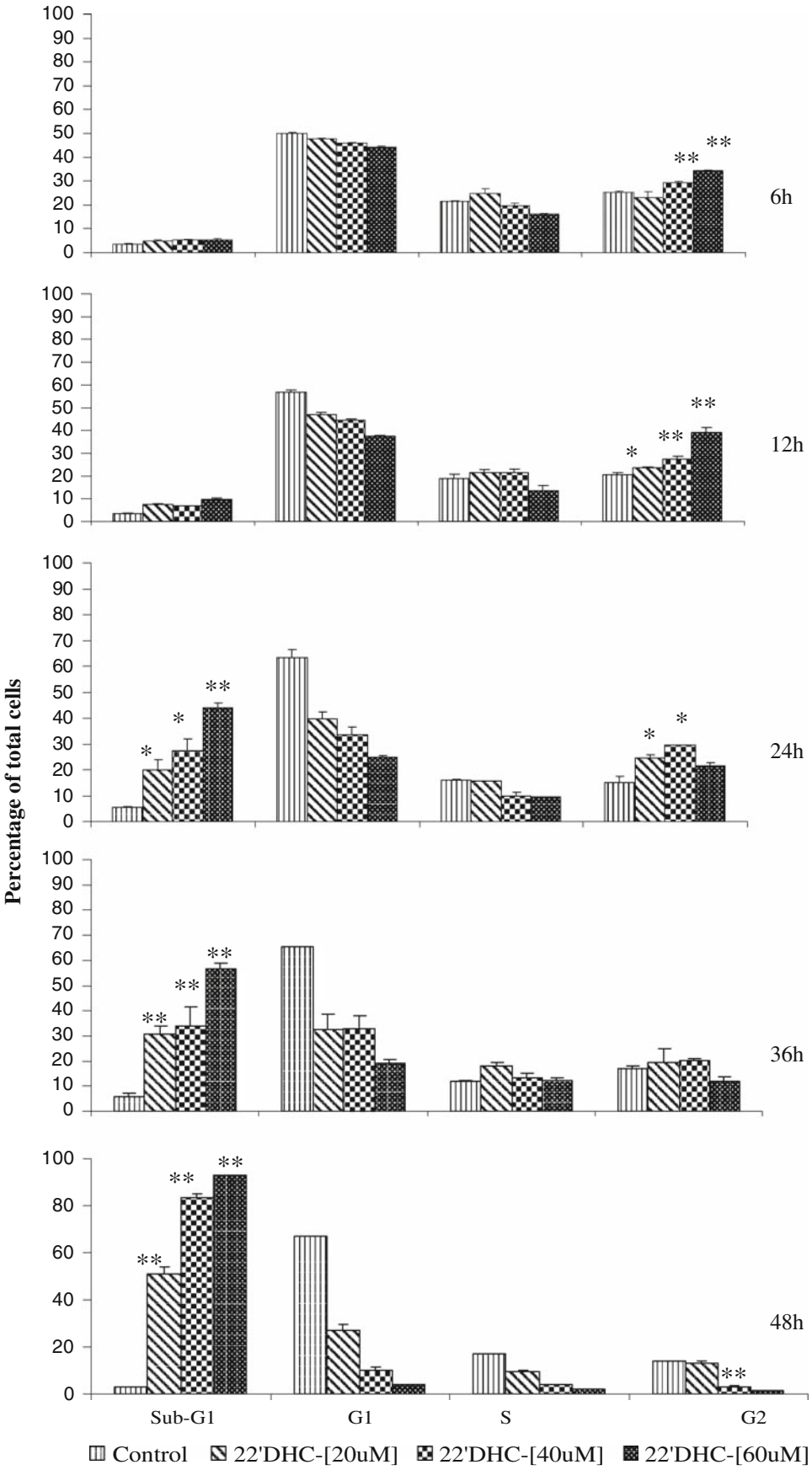


Fig. 1 Flow cytometry analysis of HepG2 cells treated with 2,2′-dihydroxychalcone. HepG 2 cells were incubated with 2,2′-dihydroxychalcone and harvested at varying time-points. Cells were fixed in 85% cold ethanol for 20 h at −20°C, washed with PBS and then incubated in PI/triton X-100 staining solution for 1 h. The cell samples were analyzed by a BD flow cytometer. Results are from three independent experiments and differences were analyzed by student's *t*-test. $^{**}P < 0.01$, $^{*}P < 0.05$, when compared to control. 22′DHC, 2,2′-dihydroxychalcone

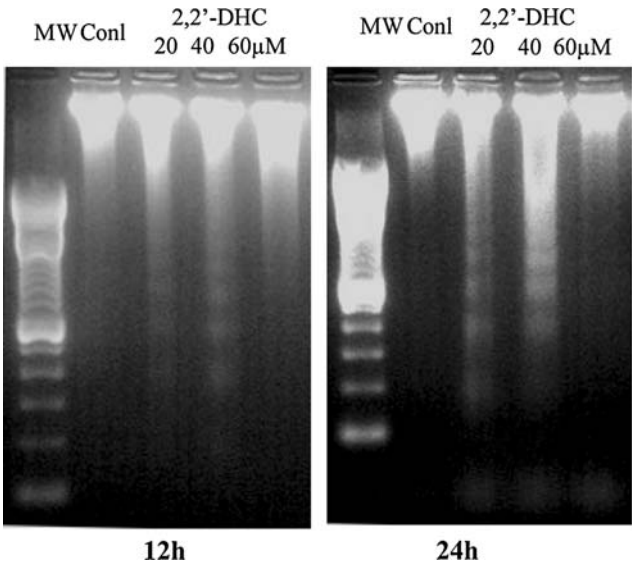


Fig. 2 Determination of apoptosis of HepG2 cells treated with 2,2′-dihydroxychalcone. HepG2 cells were treated with 2,2′-dihydroxychalcone at the concentration of 20, 40, and 60 μM for 12 and 24 h. The cells were then collected by tripsinization and DNA was extracted using a Qiagen kit. DNA samples were incubated at 56°C with 200 μg/ml of DNA free RNAse for 1 h. Approximately 40 μg of DNA in 30 μl of loading buffer was loaded onto a 2% agarose gel and run in TAE buffer at 5 V/cm for 2 h

modification for new anticancer agents as well. In mechanistic studies, the most potent chalcone, 2,2′DHC was found to induce G2/M arrest and apoptosis of the cancer cells. These processes may be involved in their inhibitory effects on the cancer cells.

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