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Molecular Structure and Anti-proliferative Effect of Galangin in HCT-116 Cells: *In vitro* Study

Ghassan Mohammad Sulaiman*

Biotechnology Division, Applied Science Department, University of Technology, Baghdad, Iraq

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*Corresponding Author
Tel/Fax: +964 (0) 7902781890
E-mail: gmsbiotech@hotmail.com

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Abstract Galangin is a naturally occurring plant flavonoid with potential anticancer activity. In present work, the Becke three-parameter hybrid exchange functional method and the Lee-Yang-Parr correction functional methods were used to investigate the structural properties of galangin. The structure-activity relationship analysis has been performed to determine its antioxidant pharmacophore by using density functional theory method and quantum chemical calculations. The free radical scavenging activities of galangin were analyzed with the use of 2, 2-diphenyl-1-picrylhydrazyl and compared with Vitamin C as a control. Galangin decreased the cell proliferation rate in HCT-116 cells and showed concentration- and time-dependent response. Galangin significantly increase the inhibitory effect on HCT-116 clonogenicity and promotes cell cycle arrest at the G2/M or G1 phase, as confirmed by flow cytometry analysis.

Keywords: galangin, molecular structure, clonogenicity, cell cycle, HCT-116

Introduction

Flavonoids are naturally occurring polyphenolic compounds that are ubiquitously in plants, that possess a variety of beneficial health effects. Flavonoids have been characterized, and therapeutically significant levels of dietary flavonoids are used by humans living in Western countries because of the unique properties of such compounds. *In vitro* studies suggest that flavonoids can protect the human body from reactive oxygen species (ROS), such as hydroxyl, alkoxyl, or peroxyl radicals; galangin may have powerful reactivity that can protect the body from various life-threatening diseases (1-3). Flavonoids may also have cancer prevention effects. The antitumor effects of flavonoids may be attributed to suppress multiple signaling pathways in the process of tumor promotion, including tumor cell transformation, invasion, metastasis and angiogenesis, through inhibiting kinases, reducing transcription factors, modulating cell cycle, and activating cell death process (4-6).

Galangin (3,5,7-trihydroxyflavone), belongs to a class of flavonoids known as flavonols. Galangin is present in *Alpinia officinarum*, a plant used for many years in Asia as herbal therapeutics and honey; compounds from this plant are major components of propolis, which is a natural composite balsam produced by honeybees from the gum of various plants (7-9). Galangin showed various pharmacological activities, such as anti-mutagenic, anti-clastogenic, anti-oxidative, radical scavenging, and metabolic enzyme modulating activities (10-12). Furthermore, galangin showed cancer preventive properties in several cancer cell lines (13-15). Based on previous reports, galangin could inhibit the cell proliferation and induce apoptosis of cancer cells. Therefore, galangin may induce apoptosis and cell cycle arrest of human colon carcinoma cells. The mechanism by which galangin suppress human colon carcinoma cells (HCT-116) through molecular features is not well known. To understand this phenomena, the relationship between the structure-activity and the antioxidant pharmacophore of galangin against a strong oxidative agent (DPPH) were studied by using density functional theory method and quantum chemical calculations.

Materials and Methods

Materials DMEM medium, fetal bovine serum (FBS), and galangin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin and streptomycin were used to prevent microbial contamination (Biosource International, Nivelles, Belgium). RNase A and propidium iodide (PI) were purchased from Sigma-Aldrich. Moreover, ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) were used for the spectrophotometric determination of galangin-free radical scavenging activities. All reagents used were of analytical grade.

Free radical scavenging activity The free radical scavenging activity of the extracts was measured using DPPH (16). The DPPH radical is

deep violet in color because of its unpaired electron. The radical scavenging capability of the galangin can be determined spectrophotometrically based on absorbance loss at 517 nm when the pale yellow non-radical form is produced. In the assay, equal volumes (0.5 mL) of DPPH (60 μM) and galangin (50 or 100 μg mL^{-1}) were mixed in a cuvette and left to stand for 30 min at room temperature. Ascorbic acid (vitamin C) was used as standard. The absorbance was read at 517 nm in a UV/VIS spectrophotometer (Lambda 19; Perkin-Elmer, Inc., Shelton, CT. USA).

Computational study Gaussian (Gaussian 09; Revision A.02, Gaussian Inc., Wallingford, CT, USA) was used to calculate the ground-state geometry of galangin and was fully optimized with hybrid density functional theory (DFT) by using basis set 6-31G. The Becke three-parameter hybrid exchange functional in combination with the Lee-Yang-Parr correction functional was used to optimize the geometries functions. The highest occupied molecular orbital (HOMO) distribution and lowest unoccupied molecular orbital (LUMO) distribution of galangin radical molecules were also discussed in this study (17-19).

Cell line and growth inhibition The HCT-116 (ATCC CCL-247) cell line was cultured in DMEM medium supplemented with 10% FBS, 20 mM Hepes, and 2 mM L-glutamine under a humidified atmosphere of 5% CO_2 at 37°C in tissue culture flasks (T 25 cm^2, Iwaki; Bibby Sterilin, Staffordshire, UK). For growth inhibition assays, HCT-116 cells were seeded in 24-well flat-bottom culture plates (Falcon™; Fisher Scientific, Pittsburgh, PA, USA) at a density of 2×10^4 cells mL^{-1}. After 48 h in the exponentially growing phase, the cells were treated with galangin at concentrations of 5, 10, 15, and 20 μg mL^{-1} for 24, 48, and 72 h. The number of cells was counted every 24 h by using an automated cell counter (TC 20™; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Clonogenic survival assay To investigate the inhibition of colony formation, HCT-116 cells were seeded at a density of 1,000 cells mL^{-1} in 6-well flat-bottom culture plates (Falcon™; Fisher Scientific) and incubated for 48 h in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C. Cells were treated with galangin at concentrations of 5, 10, 20 and 40 μg mL^{-1}, and incubated for additional 120 h (in three replicates). When the non-treated cells were readily visible under the microscope as a confluent monolayer, the medium was removed and the wells were washed with 2 mL of filtered PBS. Then, the colonies were stained with 200 μL per well of crystal violet for 2 min. After removing the stain, extensive washing was performed in tap water for 10 min, after which washing in distilled water was performed twice for 10 min each time. Then, the plates were tapped on towel paper, dried, and photographed.

Cell cycle analysis Cell cycle analysis was performed according to the method of Darzynkiewicz and Huang (20) with slight modifications. HCT-116 cells were seeded in 6 multi-well plates at a density of 2×10^4 cells mL^{-1} and cultured for 48 h. The cells were treated with different concentrations of galangin for 24 h. Cells were harvested at a density of 2×10^6 and then transferred to a polypropylene conical tube and centrifuged at 400×*g* for 10 min, after which the supernatant was discarded. The pellet was fixed with 96% ethanol drop-wise with continuous vortexing and was incubated in an ice bath for 30 min. The tube was stored overnight at 4°C. Thereafter, the cell pellet was collected by centrifugation at 400×*g* for 10 min before resuspending in 1 mL of washing buffer. Further centrifugation at 400×*g* for 10 min was performed to collect the cell pellet, after which 2 mL of propidium iodide (PI) solution and 25 μL of RNase A were added. The mixture was left to stand overnight in the dark at 4°C. Fluorescence emitted from the PI-DNA complex was quantified after excitation of the fluorescent dye by FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 cells were examined for each sample, and fluorescence emission was set at 610 nm (FL3). The distribution of DNA content was expressed as the G1, S, and G2/M phases.

Statistical analysis The grouped data were statistically evaluated using ANOVA with SPSS program (SPSS/14.0; SPSS Inc., Chicago, IL, USA). Values were presented as the mean±SD of the three replicates of each experiment.

Results and Discussion

Many flavonoids exhibit antioxidant activity and anti-proliferative effects against tumor derived cell lines, including cell lines of melanoma, leukemia, lung, prostate, and breast carcinoma origin and human colon carcinoma cells (21-24). A series of experiments were performed on galangin to assess the possible protective action of this compound against oxidative reaction and to evaluate its antitumor activity in HCT-116 cells.

Free radical scavenging activity of galangin To evaluate the free radical-scavenging activity of galangin, the DPPH reduction-based method was used and reported in Fig. 1. The anti-antioxidant activity was significantly reduced and was concentration dependent. This activity reduction was more prominent at 100 μg mL^{-1} with 59.72% compared with vitamin C. The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds. DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (25). The inhibitory action of galangin may serve as a significant indicator of its free radical scavenging activity. Antioxidants can donate electrons to reactive radicals, converting them into more stable and unreactive species (26,27).

In order to evaluate the relationship between galangin's structure and antioxidant activities, the proposed reaction mechanisms based on the experiment of DPPH radicals and the molecule of galangin

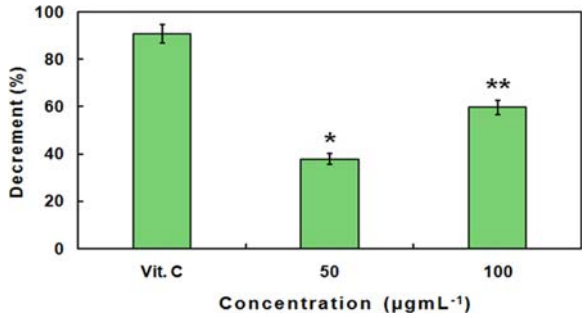

Fig. 1. DPPH free radical scavenging activity of galangin. Results are expressed as percentage decrement of absorbance at 517 nm with respect to control. Each value represents the mean±SD of three experiments.

was calculated by using a model system. Five resonance limit structures were visualized in scheme of galangin, as shown in Fig. 2. This proposed mechanism may give an example to assess the capacity for other radical quenching methods. However, phenolic groups are known to stabilize a radical formed on phenolic carbon with their resonance structure and are positively correlated with the number of hydroxyl groups (28). Galangin possesses the structures essential for flavonoid antioxidant activity, as follows: 3,5,7 hydroxylation, the presence of a double bond between carbons 2 and 3, conjugated with the B ring. However, the antioxidant activities of galangin are due to the presence of hydroxyl group at carbon 3 in association with the double bond C2-C3 conjugated with the B aromatic ring. The presence of this double bond conjugated with the aromatic ring at galangin molecule, the hydroxyl group linked to the C3 can easily undergo one- electron oxidation to give hydroxyl radical (OH·) which allow electron delocalization in position 2 and in B ring. The present results are in accordance with the work of Gregoris and Stevanato (29). Interestingly, the opposed behavior of galangin was observed in apigenin (another flavone), which differ for the position of the hydroxyl group. Both of them delocalize the unpaired electron in different positions of the B aromatic ring, but galangin, with hydroxyl group at C3, shows potent and high antioxidant capacity, unlike apigenin, characterized by a hydroxyl group in C4'. Furthermore, it reported that naringenin and apigenin show low antioxidant capacity because they do not have the double bond C2-C3, while high antioxidant capacity is observed in galangin which presents the hydroxyl group at C3 and the double bond C2-C3 (29,30). The antioxidants can show their protective roles to the free radical through removal of a hydrogen atom from an aromatic hydroxyle group that is donated to free radicals. As an aromatic compound, galangin can support unpaired electrons through delocalization around the electron system. Moreover, galangin can show direct evidence for antioxidants to scavenge a reactive oxygen species in the body cause oxidative damage to cellular components (31,32).

HOMO and LUMO distribution of galangin To further gain an electronic molecular insight into the antioxidant capacity of galangin, The HOMO and the LUMO states were performed by using DFT-based quantum chemical descriptors. The calculated HOMO and LUMO energies showed that charge transfer occurred within the molecules related to the ability of the molecule to donate or accept electrons, respectively. Furthermore, distribution of the frontier

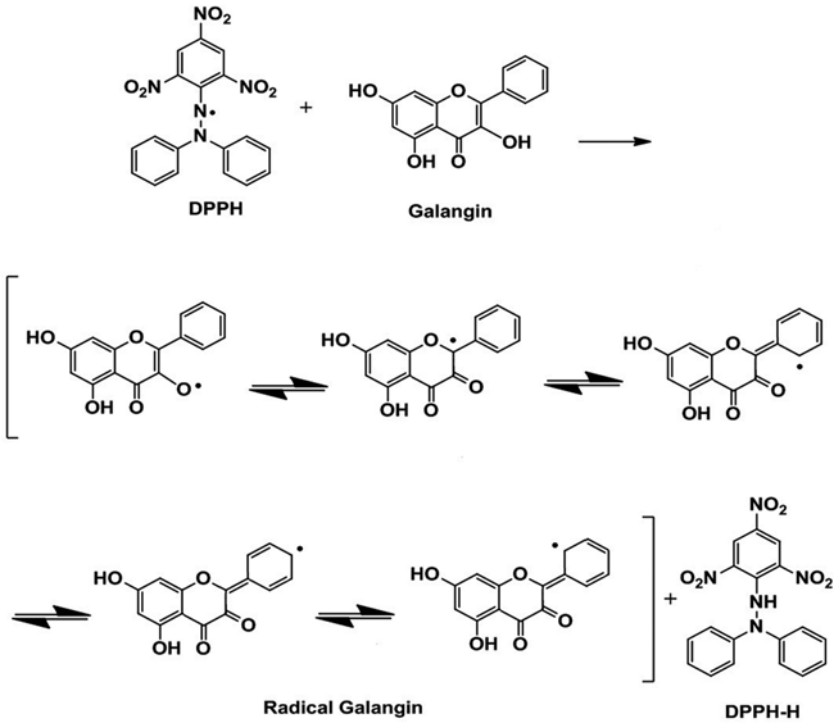

Fig. 2. The reaction scheme between DPPH free radicals and galangin.

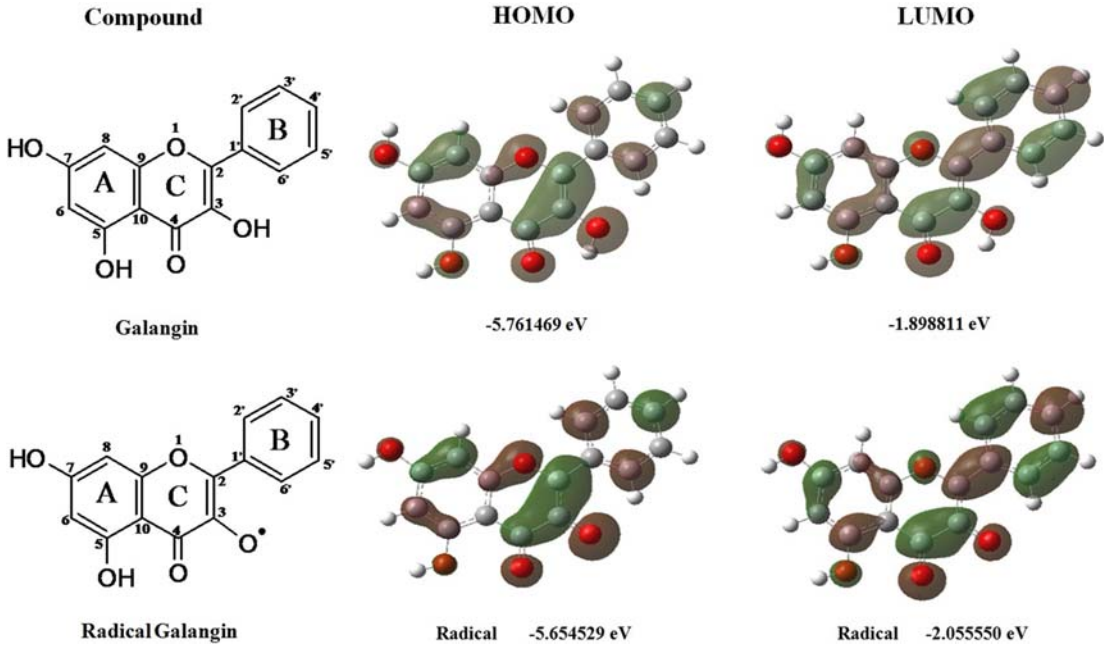

Fig. 3. The highest occupied molecular orbital (HOMO) distribution and lowest unoccupied molecular orbital (LUMO) distribution of galangin radical molecules.

molecular orbital is extremely important in the analysis of antioxidant capacity and can indicate the active site exposed to attack of free radicals. However, the HOMO and LUMO distributions for galangin molecule, along with the frontier molecular orbital energy gap (LUMO-HOMO energy difference, Eg) and the electron volt values, are given in Fig. 3. According to the energy plots, the present experiment suggested that the antioxidant potential of galangin was verified by the high number of hydroxyl groups in the molecule and by the alleviation of formation of phenoxyl radicals generated from these groups. The antioxidant potential is strongly related to the presence of the hydroxyl groups which gives high antioxidant potential to the flavonoid molecule and also to the stability of the created phenoxyl radical. However, the LUMO energy plots clearly indicated that high electron density contributed between B and C rings and between C-2 and C-3, which clearly assured the stability of free radicals between rings.

Proliferation and clonogenic survival assay of HCT-116 cells To visualize the relationships between antioxidant and structural properties described in Fig. 1, 2 and 3, we evaluated the cytotoxicity of galangin on HCT-116 cells *in vitro* using short-term and long-term systems. To assess the short-term growth inhibitory effect of galangin, four concentrations (5, 10, 15, and 20 μg mL^{-1}) were applied on HCT-116 cells for 24, 48, and 72 h. After treatment, the cell number was measured using an automated cell counter, as shown in Fig. 4. The number of cells was markedly inhibited by galangin in a concentration- and time-dependent manner ($p<0.05$). The number of tumor cells after treatment with 20 μg mL^{-1} at 72 h was significantly reduced, and galangin at a concentration of 20 μg mL^{-1} was more effective

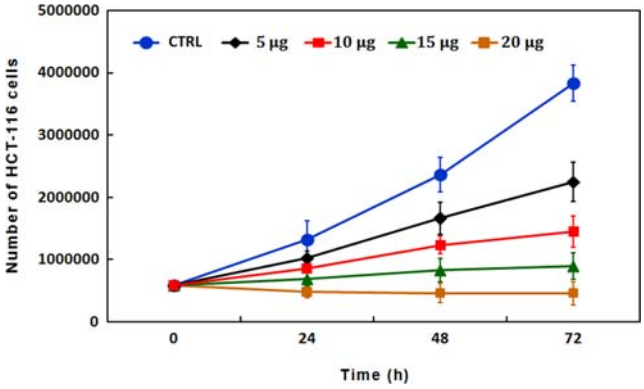

Fig. 4. Growth inhibition of HCT-116 cells after treatment with different concentrations of galangin for 24, 48 and 72 h. The values were mean±SD from three experiments.

than the other three concentrations at the same period of incubation compared with non-treated cells (Fig. 4). A clonogenic survival assay was conducted to measure the long-term cytotoxicity of galangin at four different concentrations. Results of the galangin clonogenic survival assay are shown in Fig. 5. The mortality data obtained in present experiment enabled the prediction of potential cytotoxic and tumor reduction effects of galangin.

Cell cycle distribution of HCT-116 cells To assess whether inhibition of cell growth by galangin was due to the induction of cell cycle arrest or cell death, flow cytometry was performed to detect cellular DNA contents. The cell-cycle distributions were analyzed for 24 h, and the results are shown in Fig. 6. A slight change was

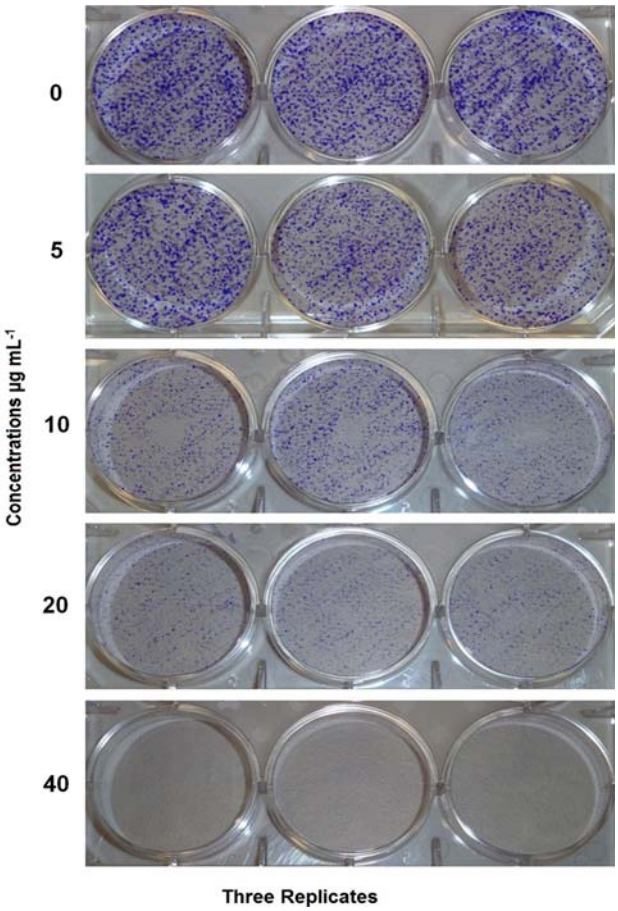

Fig. 5. Clonogenic survival assay of HCT-116 cells after treatment with different concentrations of galangin. Three experiments were indicated.

observed in cells in the G2 phase after treatment with 5 μg mL^{-1} of galangin (23%). With increasing concentration up to 10 and 15 μg mL^{-1}, the percentage of cells in the G2 phase markedly increased (by 48% and 60%, respectively). Treatment with galangin at 20 μg mL^{-1} induced the growth arrest of G1 cells at approximately 55% while concomitantly decreasing the percentage of S and G2/M cells compared with control cells. These behaviors of galangin were similar to those effects of luteolin (another falvone) which recorded by Tsai *et al*. (33) in human glioblastoma cells. They found two different regulations of cell cycle in a concentration-dependent manner by inducing arrest either in S phase or G2M phase. However, the results of present work clearly confirmed the function of galangin in the induction of cell cycle arrest in colon cancer cells. These results corroborate the findings of many published studies on several other types of human cancer cells. Galangin blocks transition from G0/G1 phase to S phase of Hs578T cells (13) and chronic myelogenous leukemia cell lines (14). Moreover, galangin arrested the development of human gastric cancer SGC-7901 cells at the G2 phase of the cell cycle after 24 h treatment time (34). Therefore, galangin may show different effects in various cancer cell lines. In addition to its effect causing cell cycle blockage, galangin has been demonstrated to promote apoptosis in tumor cells (35). On the basis of the above mentioned results, this study supports the therapeutic potential of galangin and its use as a possible antitumor agent. In conclusion, the present results indicate that galangin display a potent inhibition to HCT-116 cells and induced both G1 and G2/M cell cycle perturbations. The molecular structure calculations of galangin and its antioxidant potentials revealed a significant association found between the DPPH radical scavenging capacity and suppression of cell growth, making the galangin a promising candidate for tumor treatment. The theoretical approach for the molecular structure of galangin and its antioxidant activities showed that a good correlation existed between the scavenging potentials of galangin and reduced proliferation of HCT-116 cells. In addition, galangin showed multiple activities in the cell, and galangin may also show antigenotoxic activity against alkylating agents (10) or inhibition of the arylhydrocarbon receptor activation (36). Galangin may have antiproliferative potential and may be used as an efficient chemotherapeutic drug and/or as a food additive for treating human colon cancer.

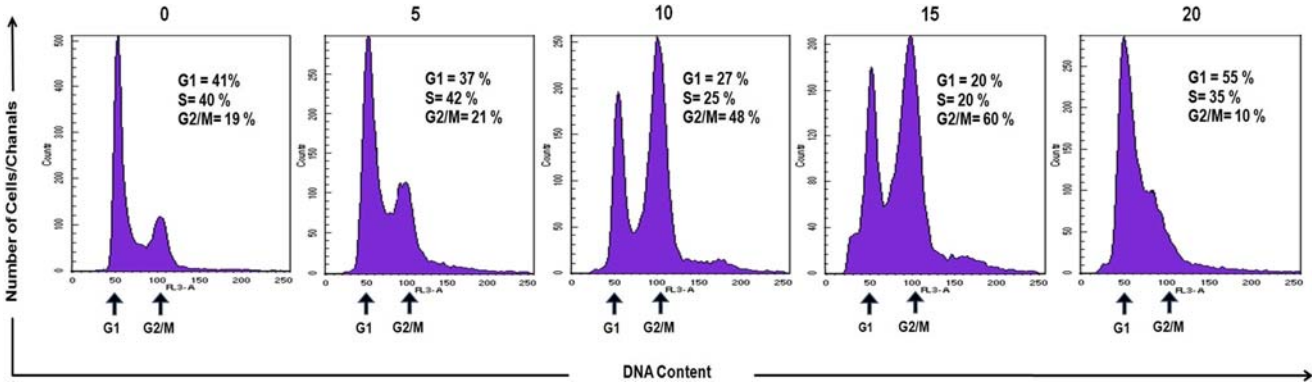

Fig. 6. Effect of galangin on cell cycle distribution in HCT-116 cells involved arrest in the G2/M and G1 phase. Cells were harvested 24 h later and assayed for DNA content by propidium iodide (PI) staining and flow cytometry was then performed for cell cycle distribution. Flow cytometry histograms from one representative experiment in which 5, 10, 15 and 20 μg mL^{-1} of galangin were used are presented. Percentage of cell distribution data for each treatment is indicated.

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Disclosure The authors declare no conflict of interest.

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