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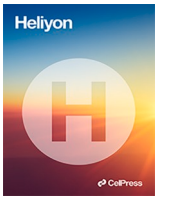

Research article

Anti-proliferation and induction of mitochondria-mediated apoptosis by *Garcinia hanburyi* resin in colorectal cancer cells

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A R T I C L E I N F O

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Colorectal cancer

A B S T R A C T

Introduction: Several parts of *Garcinia hanburyi* are used in traditional medicine for many purposes. In this study, *Garcinia hanburyi* resin (GHR) was explored for possible anti-proliferative effects and the underlying mechanism on colorectal cancer (CRC) cells.
Methods: Gambogic acid (GA) content in GHR was analyzed by HPLC method. The cytotoxicities of GA and GHR were assessed in human CRC cell lines (SW480 and Caco-2) and normal colon cells (CCD841 CoN) using a trypan blue exclusion assay, MTS assay, and cell morphology analysis. Cell cycle and apoptosis at its half maximal inhibitory concentration (IC50) were analyzed using flow cytometry. And, the levels of intrinsic apoptosis-related proteins were measured by Western blot analysis.
Results: GA was the major compound as 71.26% of the GHR. The cell viability of CRC cells was decreased in a time- and dose-dependent manner after exposure to GHR. The selectivity index indicated that GHR had a high degree of selectivity against CRC cells. The same result was obtained for GA treatment. In addition, GHR markedly induced typical apoptotic morphology of CRC cells, but had no obvious effect on normal colon cells. GHR induced apoptosis with the cell cycle arrest at the G2/M phase. An increase in Bax/Bcl-2 ratio and a decrease in procaspase-3 proteins indicated that GHR promoted apoptosis by disrupting the mitochondrial outer membrane permeability and the subsequent activation of caspase-3.
Conclusion: GHR, which contained GA as an active compound, significantly inhibited CRC cell proliferation via the induction of intrinsic apoptosis, while having low toxicity on normal colon cells. Therefore, GHR could be proposed as a potent candidate for the treatment of CRC.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. However, the current treatment regimens for CRC such as surgery, radiation therapy, and chemotherapy have many limitations; e.g., adverse effects, tumor evolution and intra-tumor heterogeneity, drug resistance, and cost [1]. Recently, novel anticancer treatments such as immunotherapy, targeted therapy, and organ transplantation [2], have been drawing attention as an option for CRC therapy, but these too have adverse effects, resulting in drug discontinuation [3], and early mortality risk [4]. Despite all the efforts that have been made to combat cancer, the incidence of CRC is steadily rising [5], so cost-effective therapeutic strategies with minimal adverse effects are urgently required.

Medicinal plants are rich sources of bioactive molecules and have been used in alternative medication for centuries. They contain substances that can be exploited for treatment of various diseases, including CRC [6]. *Garcinia hanburyi* is a perennial plant, small to medium-sized, non-deciduous, with branches. The trunk is quite straight. The bark is thick, grey, smooth, exuding a yellow gum-resin. Leaves are opposite, elliptic or ovate-lanceolate, and the flowers are light yellow, and fragrant. The fruit is round and hard. *Garcinia hanburyi* has a long history of medicinal use in Southeast Asia. Its yellow resin has not only been used as a colorant for arts, but also as a therapeutic drug. It has been used in the treatment of infected wounds and constipation. Previous research has shown that extracts from *Garcinia hanburyi* have various biological activities, e.g., anti-inflammatory, antipyretic, and analgesic effects [7]. The composition of *Garcinia hanburyi* resin (GHR) consists of more than 100 types of xanthone such as betulin, betulinic acid, morellic acid, and isomorellic acid. Gambogic acid (GA, $C_{38}H_{44}O_8$, molecular weight 628.75) is a major active constituent. Previous studies showed that GA can inhibit cell growth in various cancers, e.g., gastric cancer [8], hepatoma [9], breast cancer [10], and oral cancer [11]. Additionally, GA induced cell cycle arrest and apoptosis by increasing nuclear condensation and DNA fragmentation [9,12]. Both the extrinsic and intrinsic pathways were involved in GA-induced apoptosis, as indicated by elevated pro-apoptotic proteins, Bax and Bid, decreased anti-apoptotic proteins, Bcl-2 and Bcl-xL, activated caspase-8, -9 and -3 activity, and reduced mitochondrial transmembrane potential [8,13].

Apoptosis is a form of programmed cell death. As a physiological and pathological mechanism, apoptosis kills cancer cells without imposing damage to normal cells or surrounding tissues. Thus, inducing apoptosis in cancer cells has been an important approach for treatment of CRC. Apoptosis happens in normal cells and plays an important role in the process of developing and maintaining the balance of cells in high-class organisms. Apoptosis-induced cells are characterized by several morphological changes including cytoplasm and organelle condensation, cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA and nuclear fragmentation. Finally, cells are separated into apoptotic bodies [14]. Apoptosis occurs via 2 pathways: 1) the extrinsic pathway, involving cell surface death receptors (Fas/FasL and caspase-8) and 2) the intrinsic pathway, associated with intracellular signals (Bcl-2 family and caspase-9) and mitochondria [15]. Both pathways converge to the activation of the caspase-cascade system, the family of cysteine proteases, subsequently resulting in cell death. Thus, stimulation of apoptosis is a promising target for anticancer therapy.

In this study, GHR was expected to have similar anti-cancer activities to GA, but its underlying mechanism in CRC therapy had not been thoroughly investigated. To investigate novel potential drugs to treat CRC, we determined the role of GHR on cytotoxicity using a trypan blue exclusion assay, MTS assay, and cell morphology analysis, we analyzed cell cycle and apoptosis by flow cytometry, and we evaluated the expression levels of proteins in the intrinsic pathway of apoptosis by Western blot.

2. Materials and methods

2.1. Preparation of GHR

GHR was purchased from the herb shop fully certified and approved by the Thai Government and reconfirmed by Bhanuz Dechayont, holding a license to practice Thai traditional medicine (Thai pharmacy) from the Medical Registration Division, Ministry of Public Health, Thailand. GHR was completely dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 10 mg/ml and further diluted in the following experiments. The final concentration of DMSO in all cell treatments was 1.0% (v/v).

2.2. Cell culture

CRC cell lines (SW480 RRID:CVCL_0546 and Caco-2 RRID:CVCL_0025) and normal colon cell line (CCD841 CoN RRID:CVCL_2871) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and non-essential amino acid under 37 °C, 5% CO_2 atmosphere. When cells were cultured to approximately 80% confluence, they were subcultured or used for the experimental procedure. This study was approved by Thammasat University Institutional Biosafety Committee (TU-IBC) (No. 006/2559).

2.3. High performance liquid chromatography (HPLC) analysis

To investigate the GA content in the GHR, the GHR was dissolved in DMSO and filtered through a nylon membrane of 0.45 μm disc before injection. GHR was analyzed by HPLC (Agilent Technologies 1260, Germany) using an Acclaim™ 3 μm C_{18} column (4.6 × 150 mm) (Thermo Fisher Scientific Co., LTD., Thailand), eluted with acetonitrile: water (45:55 v/v) using a flow rate of 0.6 ml/min at 25 °C and detected with a UV detector at 290 nm. Commercial GA (Sigma-Aldrich Pte Ltd, Singapore) was analyzed as a standard. The percentage of GA content in GHR was calculated according to the method reported in previous literature [16].

2.4. Trypan blue exclusion assay

SW480, Caco-2, and CCD841 CoN cells were seeded into 12-well plates at 2×10^5 cells in each well overnight and then treated with varied concentrations of GHR, ranging from 0 to 2.5 μg/ml in CRC cells and 0–10 μg/ml in normal colon cells. Cells were incubated at 37 °C for 24, 48, and 72 h. After that, cells were trypsinized, washed, and resuspended in PBS. We mixed 1 part of 0.4% trypan blue and 1 part of cell suspension and allowed the mixture to incubate for approximately 3 min at room temperature. Cells should be counted within 3–5 min because longer incubation periods may cause cell death and reduce viability. The unstained (viable) and stained (nonviable) cells were counted separately in the hemocytometer. The percentages of viable cells were calculated as follows (1):

$$\text{The percentages of viable cells} = \left[\frac{\textit{total number of viable cells per ml of aliquot}}{\textit{total number of viable cells} + \textit{nonviable cells per ml of aliquot}}\right] \times 100 \quad (1)$$

2.5. MTS assay

SW480 and Caco-2 cells were seeded into 96-well plates at 1×10^4 cells in each well, then treated with 1% DMSO as a control, and varied concentrations of GA (0, 0.25, 0.5, and 1 μM; equivalent to 0, 0.16, 0.31, 0.63 μg/ml). Similarly, SW480, Caco-2, and CCD841 CoN cells were incubated in media including 1% DMSO and different concentrations of GHR, as in the trypan blue staining assay. The incubation was carried out at 37 °C for 48 and 72 h. MTS assay was performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, USA). Cells in each well were added with 20 μl of MTS solution and incubated for 1 h. The absorbance was determined at 490 nm using a microplate reader and calculated using the following equation (2). The half maximal inhibitory concentrations (IC50) were calculated using Prism software.

$$\text{The percentages of viable cells} = \left[\frac{A_{490}\ \textit{of treated sample}}{A_{490}\ \textit{of control}}\right] \times 100 \quad (2)$$

2.6. Calculation of selectivity index

The degree of selectivity of GHR was expressed by selectivity index (SI). The SI values were calculated as the IC50 for the CCD841 CoN normal colon cell line divided by the IC50 for either SW480 or Caco-2 CRC cell lines. A compound with SI value greater than 2 is considered as high selectivity against cancer cells, whereas one with SI value less than 2 demonstrates general toxicity to normal cells [17].

2.7. Observation of morphological changes using an inverted microscope

In morphology studies, SW480, Caco-2, and CCD841 CoN cells were seeded at 1×10^4 cells/well in 96-well plates overnight and then incubated at 37 °C for 24, 48, and 72 h with or without GHR at the corresponding concentrations. The medium was discarded, and cells were washed twice with PBS. The morphological changes of apoptotic cells were observed and photographed using an inverted microscope (Nikon, USA) at 200× magnifications.

2.8. Cell cycle analysis

SW480 and Caco-2 cells were seeded into 12-well plates at 2×10^5 cells in each well and then treated with GHR and 1% DMSO as a control. Caco-2 cells were treated with IC50 of GHR at 48 and 72 h. Differently, since in SW480 the IC50 of GHR at 72 h was comparable to the IC50 at 48 h, the maximum dose was used instead to observe more distinct results. The incubation was carried out at 37 °C, 5% CO_2 atmosphere for 48 and 72 h. Cells were trypsinized with 0.05% trypsin and washed twice with PBS. After that, cells were fixed with cold 70% ethanol overnight. The treated cells were stained for 1 h using Muse™ Cell Cycle Assay kit (Merck, USA). Finally, the DNA content and cell cycle distribution were analyzed by BD FACSverse™ flow cytometer (BD Biosciences, USA).

2.9. Apoptosis detection by flow cytometry

SW480 and Caco-2 cells were seeded into 12-well plates at 2×10^5 cells in each well overnight. After incubation with GHR (0.55 and 0.80 μg/ml for SW480, and 0.91 and 1.07 μg/ml for Caco-2) for 24, 48, and 72 h, cells were trypsinized, washed with PBS, and stained with Annexin V-FITC and propidium iodide (PI) working solutions, following the manufacturer's protocol of the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, USA). Cells were incubated at room temperature for 15 min and then analyzed by a flow cytometer (DxFLEX, Beckman Coulter, USA). Using the software provided by the manufacturer, quantification was conducted from scatter plots, where early apoptotic cells stained with Annexin V-FITC were presented in the lower right quadrant and late apoptotic cells stained with both Annexin V-FITC and PI were presented in the upper right quadrant. The lower left quadrant (Annexin V-/PI-) represented the live cells and the upper left quadrant (Annexin V-/PI+) was determined as the population of necrotic cells.

2.10. Western blotting

Following the treatment of GHR at IC50 into SW480 and Caco-2 cells for 72 h, cell pellets were collected, lysed with RIPA buffer supplied with protease inhibitor (Ameresco, USA), and homogenized by sonication. The cell lysates were centrifuged at 12,000 rpm at 4 °C for 15 min. Protein levels in supernatants were measured using Pierce BCA assays kit (Thermo Scientific, USA). Equal proteins (50 μg) were separated using SDS-polyacrylamide gel (12% resolving gel and 4% stacking gel) and transferred onto polyvinylidene fluoride membranes by electrical blotting. The membranes were incubated with Odyssey blocking buffer for 1 h at room temperature and incubated with appropriate primary antibodies (dilution 1:1000) at room temperature overnight with shaking. All antibodies were purchased from Cell Signaling Technology, USA; #9662 Caspase-3, #4223 Bcl-2, #2772 Bax, and #2144 α-Tubulin (internal control). After that, the membranes were incubated with an accordant secondary antibody at room temperature for 1 h. Band visualization and quantitation were carried out using a Li-COR Odyssey Imager (Li-COR, USA).

2.11. Statistical analysis

All experiments were conducted in triplicate, repeated at least three times, and calculated as mean ± SD. The analyzes were performed with commercially available software (SPSS v13.0). The significant differences were determined by one-way analysis of variance (ANOVA) followed by Turkey test. P value ≤ 0.05 was considered statistically significant. The results of statistical analysis, including P values were provided in the supplementary data.

3. Results

3.1. HPLC analysis of GA content in GHR

In Fig. 1A, a GA standard solution showed a distinct and sharp individual peak at the retention time (Rt) 24.26 min, while the GHR compound showed five remarkably separated peaks at Rt 19.78, 21.71, 23.79, 24.23, and 24.92 min (Fig. 1B). The Rt 24.23 min was found to match the GA standard solution, assuming that GHR contained mainly GA. The percentage of GA was calculated to be 71.26% based on area normalization.

3.2. Effect of GA on CRC cell proliferation

Anti-proliferative effect of GA on CRC cells was investigated by treating the SW480 and Caco-2 cells with GA at various concentrations. GA was dose-dependent cytotoxic to both cells, compared to 1% DMSO, the control. The IC50 of GA on SW480 was 0.46 μM at 48 h and 0.38 μM at 72 h (Fig. 2A). On the other hand, the IC50 of GA on Caco-2 was 0.63 μM at 48 h and 0.57 μM at 72 h (Fig. 2B). The results showed that increasing incubation time reduced the IC50 of GA in both cell lines, indicating that longer exposure to GA made cells more susceptible. Moreover, GA can potentially inhibit the proliferation of SW480 cells more than Caco-2 cells.

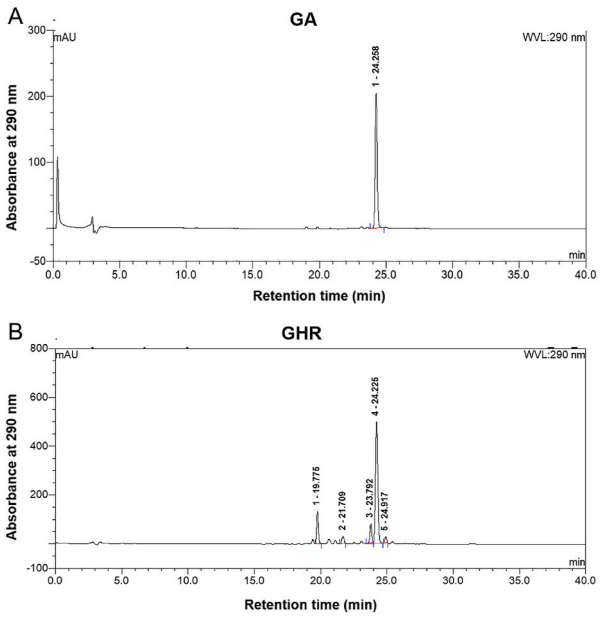

Fig. 1. Identification of gambogic acid (GA) in the *Garcinia hanburyi* resin (GHR). The chromatograms represent the HPLC analysis of standard GA (A) and GHR (B).

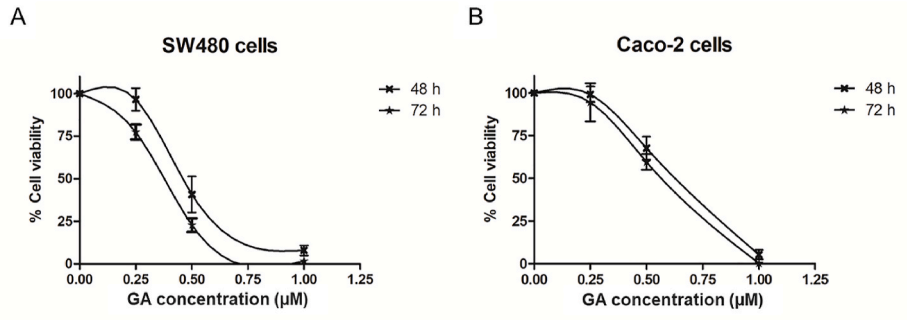

Fig. 2. Cytotoxic effect of GA on CRC cells. SW480 (A) and Caco-2 (B) cells were incubated with varied concentrations of GA for 48 and 72 h. Data represent the mean ± SD of triplicate experiments and were considered significant differences when **P* value ≤ 0.05.

3.3. Effect of GHR on the proliferation of CRC cells and normal colon cells

To study the effect of GHR on the proliferation of CRC and normal colon cells, SW480, Caco-2, and CCD841 CoN cells were treated with 1% DMSO as a control and designated concentrations of GHR for 24, 48, and 72 h. Using trypan blue exclusion assay, GHR inhibited all cell lines in a dose- and time-dependent manner (Fig. 3A–C). Since the percentages of cell viability were dramatically decreased at 48 and 72 h, the IC50 of GHR at these time points was further investigated using MTS assay. Similarly, the results showed that GHR exhibited cytotoxic activity against both CRC cell lines. IC50 of GHR on SW480 was 0.55 μg/ml at 48 h and 0.52 μg/ml at 72 h (Fig. 3D). The IC50 of GHR on Caco-2 was 1.07 μg/ml at 48 h and 0.91 μg/ml at 72 h (Fig. 3E). However, SW480 was likely more sensitive to the GHR than the Caco-2 was. This effect was consistent with the result of the GA on CRC cell proliferation. The response to GHR was almost not different between 48 and 72 h in both CRC cell lines. The results indicated that the toxicity of GHR was not dependent on the exposure time.

On the other hand, the IC50 of GHR in CCD841 CoN cells was 7.00 μg/ml at 48 h and 4.46 μg/ml at 72 h (Fig. 3F), which was much higher than the IC50 levels in CRC cells. The ideal drug should have a high therapeutic index giving maximum anti-cancer activity with minimal cytotoxicity. As shown in Table 1, all SI values were higher than 2, suggesting that GHR was more cytotoxic against the CRC cells than against the normal colon cells.

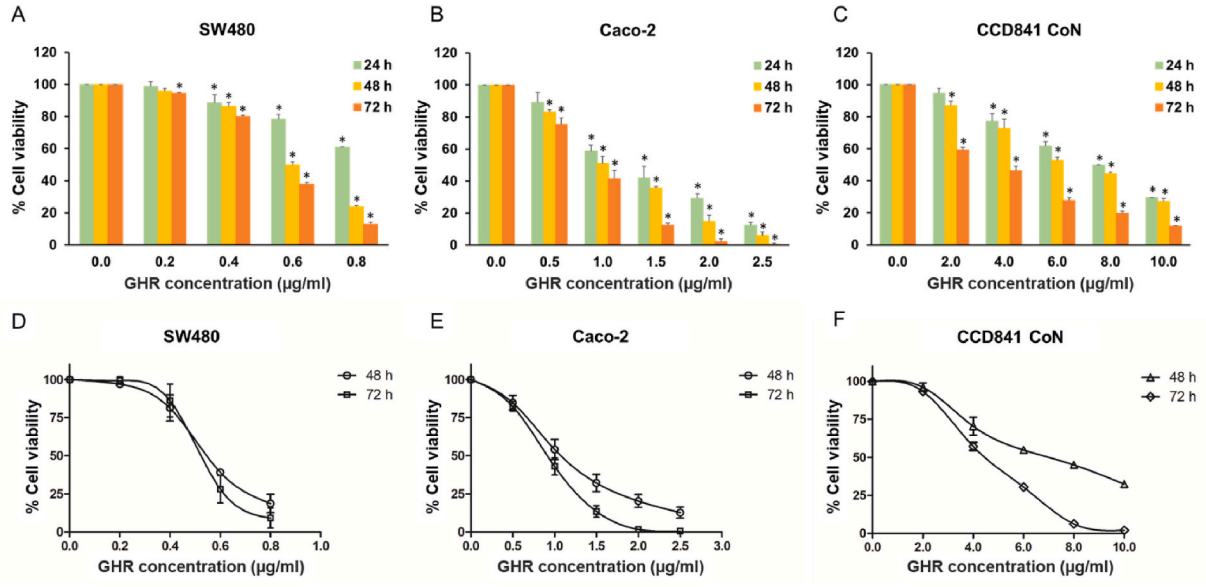

Fig. 3. GHR-induced cytotoxicity in CRC cells, but not in normal colon cells. SW480 (A), Caco-2 (B), and CCD841 CoN (C) cells were incubated for 24, 48, and 72 h with varied concentrations of GHR, depending on the cell line. The %cell viability was measured by trypan blue exclusion assay. Similarly, SW480 (D), Caco-2 (E), and CCD841 CoN (F) cells treated with various concentrations of GHR for 48 and 72 h were analyzed using MTS assay. Data represent the mean ± SD of triplicate experiments and were considered significant differences when **P* value ≤ 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Selectivity index (SI) of GHR on CRC cells (SW480 and Caco-2) versus normal colon cells (CCD841 CoN).

Cell line	Selectivity index (SI)	
	48 h	72 h
SW480	12.73	8.58
Caco-2	6.54	4.90

3.4. Effect of GHR on CRC and normal colon cell morphology

In the present study, we examined the effect of GHR on SW480, Caco-2, and CCD841 CoN cell morphology. Untreated cells were demonstrated in Fig. 4/5/6 A, E, and I. All cell lines treated with 1% DMSO maintained their original morphology form and were adherent to the tissue culture flasks (Fig. 4/5/6 B, F, J). However, exposure of SW480 (Fig. 4) and Caco-2 (Fig. 5) cells to different concentrations of GHR for 24 (Fig. 4C–D, 5C-D), 48 (Fig. 4G–H, 5G-H), and 72 h (Fig. 4K-L, 5K-L) markedly induced changes in cell morphology. A significant proportion of cells revealed typical apoptotic features such as rounding, shrinkage, membrane blebbing, and detachment. The number of cells undergoing apoptosis increased proportionally to the GHR concentration and incubation time. In contrast, the CCD841 CoN cells were less affected by GHR treatment. Even when the concentration of GHR was increased from 0.55 (Fig. 6C, G, K) to 0.80 μg/ml (Fig. 6D, H, L), the normal colon cells were still alive and intact, being sharply demarcated and elongated. Taken together, these data indicated that GHR triggered morphological changes of apoptosis in CRC cells but had much less effect on CCD841 CoN cell morphology.

3.5. Study of cell cycle after GHR treatment

To clarify the mechanism for the GHR-induced cytotoxicity in CRC cells, the cell cycle distribution at 48 and 72 h was examined by flow cytometric analysis. Representative histograms from one of at least three independent experiments are shown. The percentages of cells in sub-G1, G1, S and G2/M phases were analyzed and demonstrated as bar graphs. Statistical significance ($P < 0.05$) was tested versus control at the same incubation periods. Both SW480 (Fig. 7) and Caco-2 (Fig. 8) cells displayed an increase in the percentage of cells in the sub-G1 and G2/M phase and a decrease in the G1 phase at both 48 (Fig. 7A–B and Fig. 8A–B) and 72 h (Fig. 7C–D and Fig. 8C–D). The results exhibited that exposure of the SW480 and Caco-2 cells to GHR led to a dose- and time-dependent accumulation of cells in sub-G1 population, indicating an increase of cells with fractional DNA content, a situation occurring during apoptosis, and the significant increase of arrested cells at G2/M phase indicated that the cell cycle arrest was related to the induction of apoptosis.

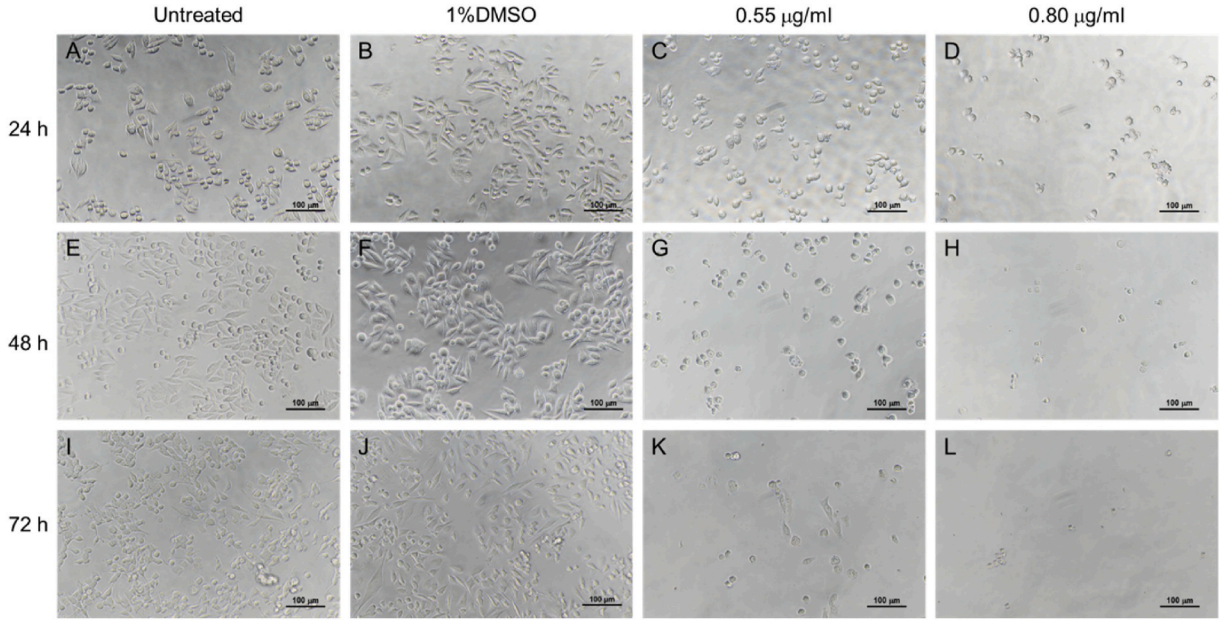

Fig. 4. Effects of GHR on SW480 cell morphology. SW480 cells were treated with 1% DMSO and GHR at concentrations of 0.55 and 0.80 μg/ml for 24 h (A–D), 48 h (E–H), and 72 h (I–L). The representative images are shown from three independent experiments (magnification, ×200).

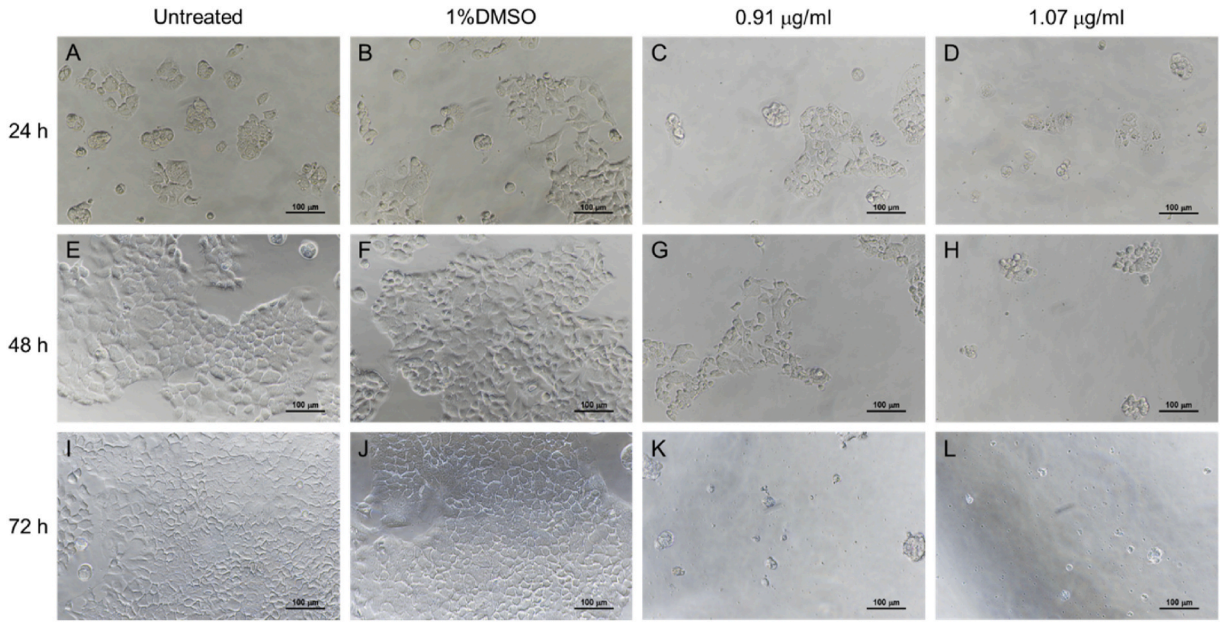

Fig. 5. Effects of GHR on Caco-2 cell morphology. Caco-2 cells were treated with 1% DMSO and GHR at concentrations of 0.91 and 1.07 μg/ml for 24 h (A–D), 48 h (E–H), and 72 h (I–L). The representative images are shown from three independent experiments (magnification, ×200).

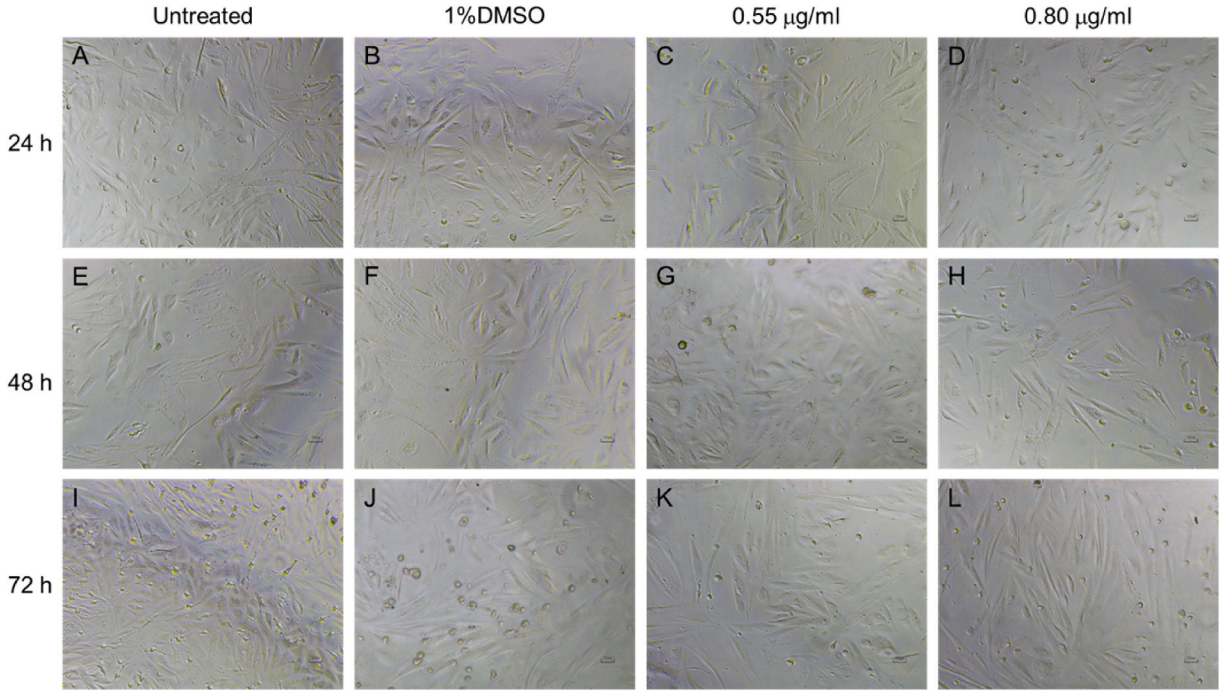

Fig. 6. Effects of GHR on the CCD841 CoN cell morphology. CCD841 CoN cells were treated with 1% DMSO and GHR at concentrations of 0.55 and 0.80 μg/ml for 24 h (A–D), 48 h (E–H), and 72 h (I–L). The representative images are shown from three independent experiments (magnification, ×100).

3.6. Detection of apoptosis after GHR treatment

To further explore the apoptosis-inducing capability of GHR, SW480 (Fig. 9A) and Caco-2 (Fig. 9B) cells were treated with GHR at 24, 48 and 72 h. The collected cells were analyzed by a flow cytometer using Annexin V-FITC/PI double staining. In both CRC cell lines,

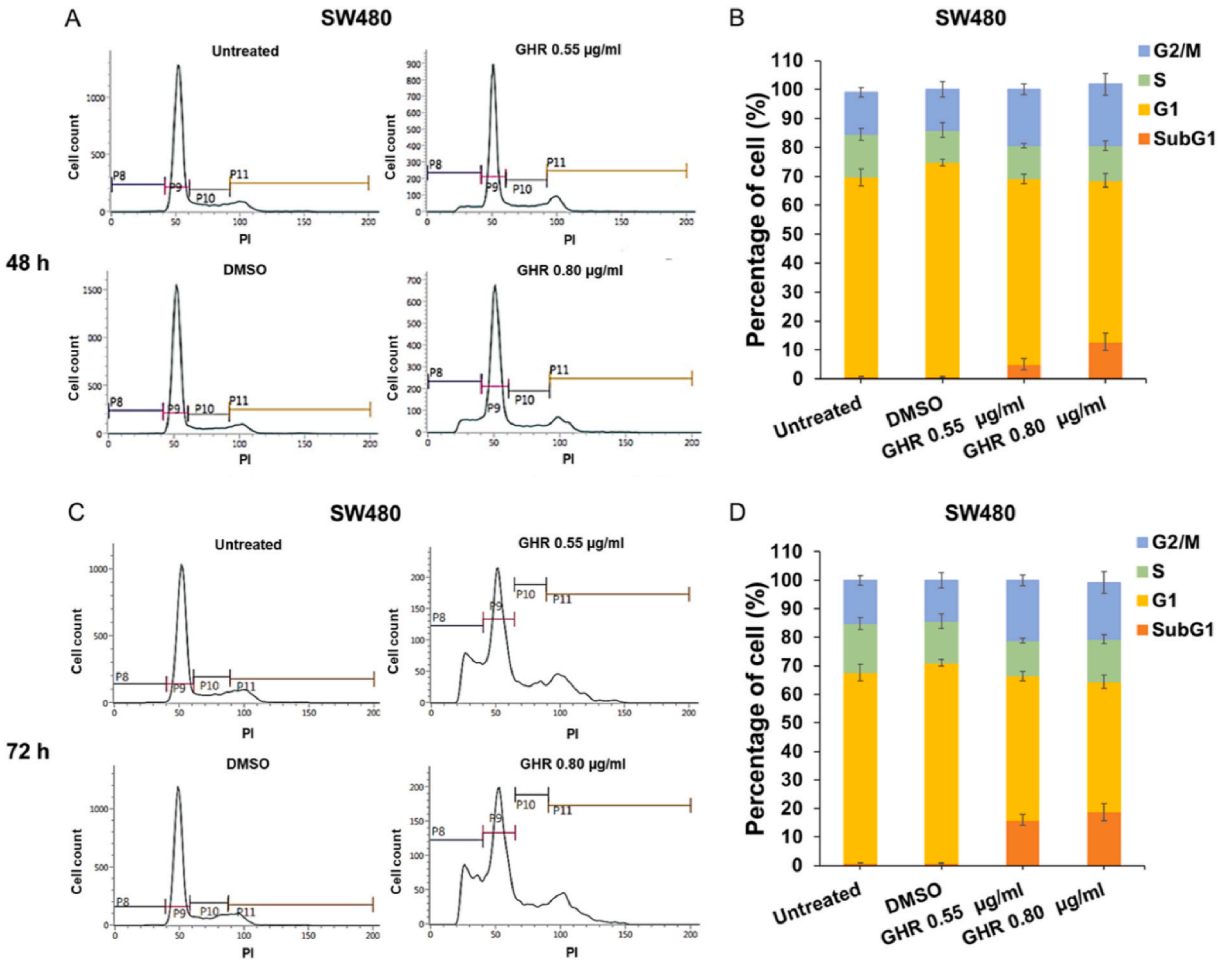

Fig. 7. Accumulation of SW480 cells in sub-G1 and G2/M phase after GHR treatment. Cells were treated with 0.55 and 0.80 µg/ml GHR for 48 h (A) and 72 h (C). Representative histograms showed the mean percentages of cells in each phase of the cell cycle at 48 h (B) and 72 h (D). Means and SD bars were based on triplicate experiments.

the percentages of live cells significantly decreased, while early apoptotic cells started to emerge at 24 h after GHR treatment. At 48 h, the population of cells at both early and late apoptotic stages had significantly increased compared to the control, and most of the cells had entered into late apoptosis at 72 h after exposure to a high concentration of GHR. The flow cytometric data confirmed that GHR induced cell death through apoptosis, as evidenced by an explicit shift from the viable cell to early and late apoptotic cell populations in a dose- and time-dependent manner.

3.7. Western blot analysis of apoptosis-related proteins

To investigate the mitochondria-dependent apoptosis pathway, the changes in the protein levels of Bax, Bcl-2 and procaspase-3 were evaluated in GHR-treated SW480 (Fig. 10A) and Caco-2 (Fig. 10B) cells. Western blot analysis showed that GHR induced up-regulation of Bax but down-regulation of Bcl-2 in a concentration-dependent manner, resulting in an increased ratio of pro-apoptotic (Bax)/anti-apoptotic (Bcl-2) members, which is involved in changes of the mitochondrial membrane permeability and structure.

A downstream step is the caspase pathway, a cascade of cysteine proteases. Particularly, caspase-3 plays a central role in the execution of apoptosis. Activated caspase-3 cleaves a wide variety of cellular proteins that cause typical morphological changes in apoptotic cells [18]. The involvement of caspase-3 in apoptosis induction was determined in GHR-treated SW480 and Caco-2 cells. As a result, a significant reduction of procaspase-3 was observed in a concentration-dependent manner in both CRC cell lines.

4. Discussion

It is now well-accepted that medicinal plants are a valuable source of bioactive molecules for the treatment of various human

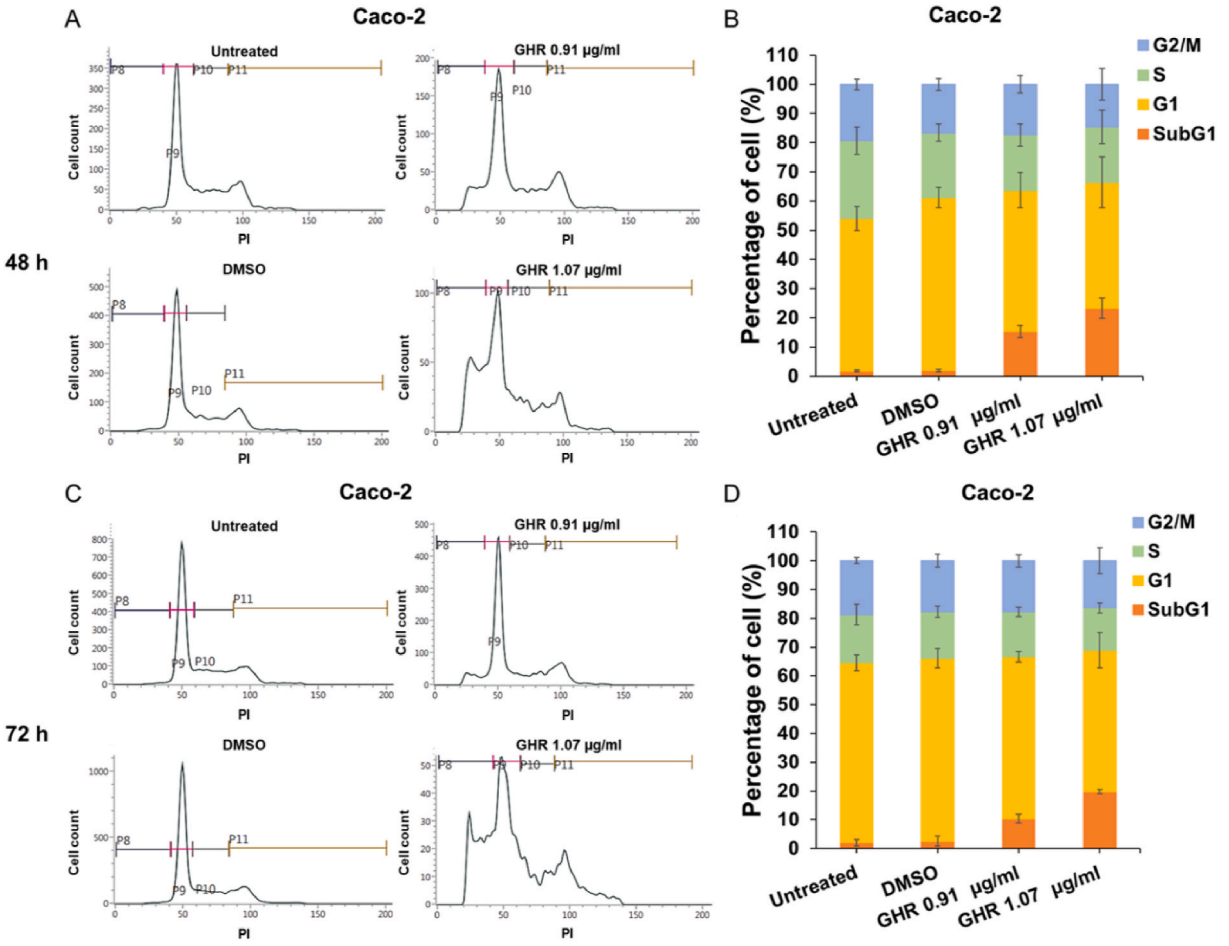

Fig. 8. Induction of sub-G1 accumulation and G2/M arrest in GHR-treated Caco-2 cells. Cells were treated with 0.91 and 1.07 µg/ml GHR for 48 h (A) and 72 h (C). The mean percentages for different phases of cell cycle were illustrated in the histograms; 48 h (B) and 72 h (D). Means and SD bars of three independent experiments were reported.

diseases, including cancer. In the last few decades, numerous traditional knowledge-based drugs have been extracted and utilized. *Garcinia hanburyi* is a plant which is widely used in folk medicine as an anti-infective and laxative drug. However, the detailed mechanism of the cytotoxic effect of GHR on CRC cells has not been elucidated yet. Our study demonstrates that GA is the main active ingredient in GHR. Individual immortalized cell lines typically do not represent all subtypes of cancers in a particular tissue of origin, so to produce more reliable results, we tested the anti-proliferative effect of GHR on CRC cells in two types of CRC cell lines; SW480 and Caco-2.

By means of a trypan blue exclusion assay and an MTS metabolic assay, we found that GHR markedly inhibited the proliferation of SW480 and Caco-2 cells in a dose- and time-dependent manner. However, SW480 was more sensitive to the GHR than the Caco-2. Interestingly, the same phenomenon was observed in GA-treated cells; SW480 was more susceptible to GA than Caco-2, confirming that GA is an essential active compound in GHR. The different susceptibility is probably due to different cell characteristics. Caco-2 cells, which are enterocytes with a polarized epithelium with a homogenous distribution of microvilli polarized epithelial cells, can form a differentiated monolayer that resembles the human intestinal epithelium [19], whereas SW480 cells correspond to non-differentiated cells, and their surface protein expression differs from the Caco-2 cells with irregular microvilli and with high losses of the tight junction, indicative of highly disorganized epithelium [20]. Moreover, the difference in genetic alteration profiles between these cell lines might be affected by the presence of germline mutations. SW480 contains only *APC* gene mutation, while Caco-2 has both *APC* and *β-catenin* gene mutations [21]. Thus, the magnitude of cell response might differ depending on their genetic abnormalities.

We also tested the cytotoxicity of GHR in normal colon cells (CCD841 CoN) and calculated the SI, a key parameter that provides a measurement of its potential toxicity. The SI of GHR was notably high, ranging from 4.90 to 12.7, meaning that CRC cells were destroyed even at low concentrations of GHR, while the normal colon cells were unaffected. This finding is consistent with a previous study reporting that GA had a low toxicity on normal cells and tissues [22]. The results were further confirmed by determining the cellular morphological changes to define cell death using an inverted microscope. GHR-treated SW480 and Caco-2 cells lost cellular

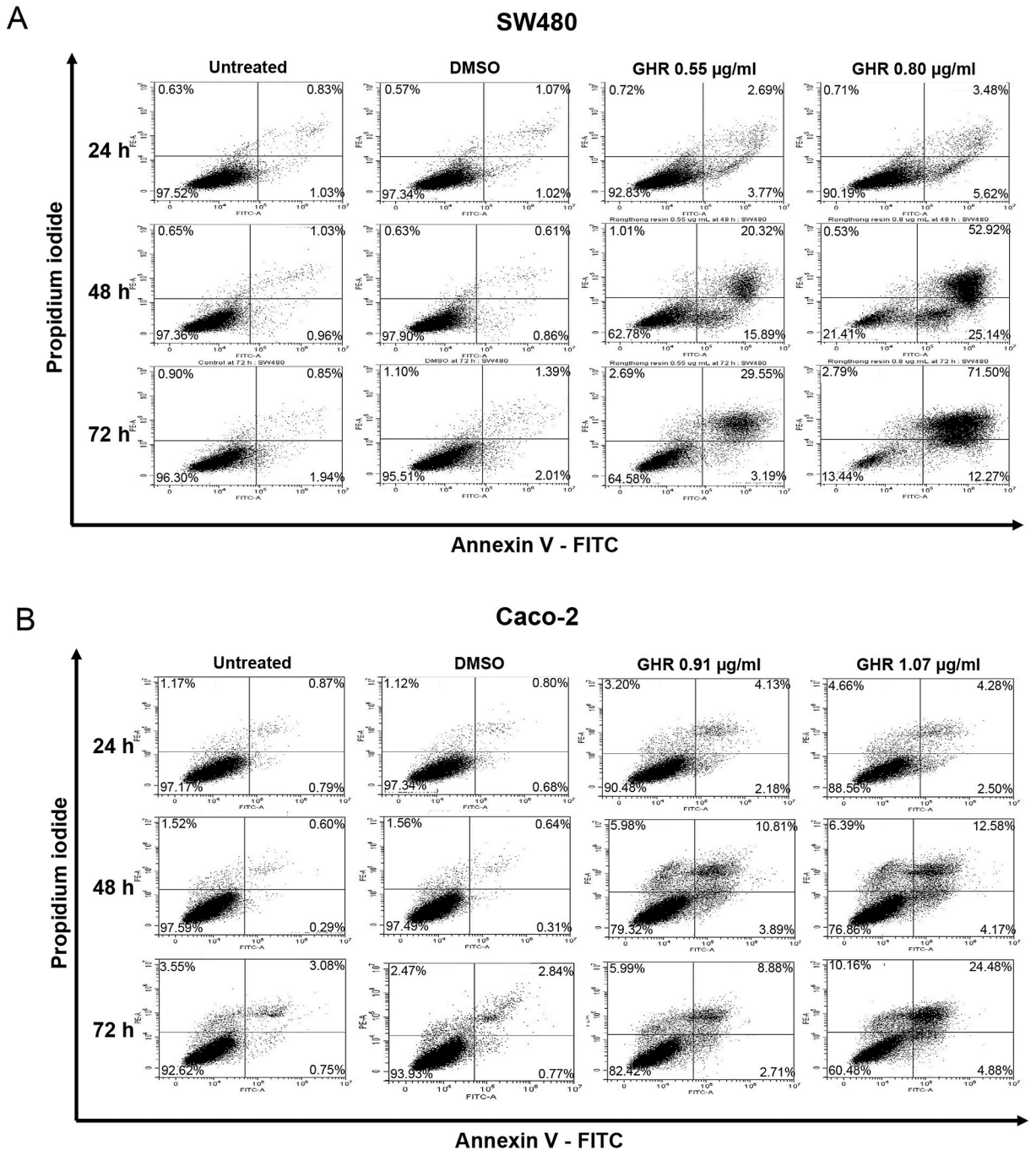

Fig. 9. Induction of apoptosis by GHR in dose- and time-dependent manner. SW480 (A) and Caco-2 (B) cells were cultured in media containing different concentrations of GHR, and then subjected to time-series analysis using flow cytometer. The percentages of cells in each stage were presented in the quadrant of representative scatter plots.

adhesion from the surrounding matrix and neighboring cells, and appeared floating in the culture medium. Detachment from their basal membrane is a characteristic of apoptosis of monolayer adherent cells, a process known as anoikis [23]. Anoikis is a crucial mechanism for the elimination of misplaced or detached cells, which decreases the metastasis of cancer cells to other sites. However, GHR had no such extensive effect on CCD841 CoN cells. This study, for the first time, demonstrates the high selectivity of GHR towards CRC cells, so it can be proposed as a candidate drug. However, further, *in vivo*, study is required to investigate possible side effects.

The distribution of cell cycle phases was examined to figure out which cell cycle phase was affected by GHR. The results revealed that GHR induced G2/M phase cell cycle arrest and subsequently apoptosis. G2/M phase arrest of cell cycle progression provides a

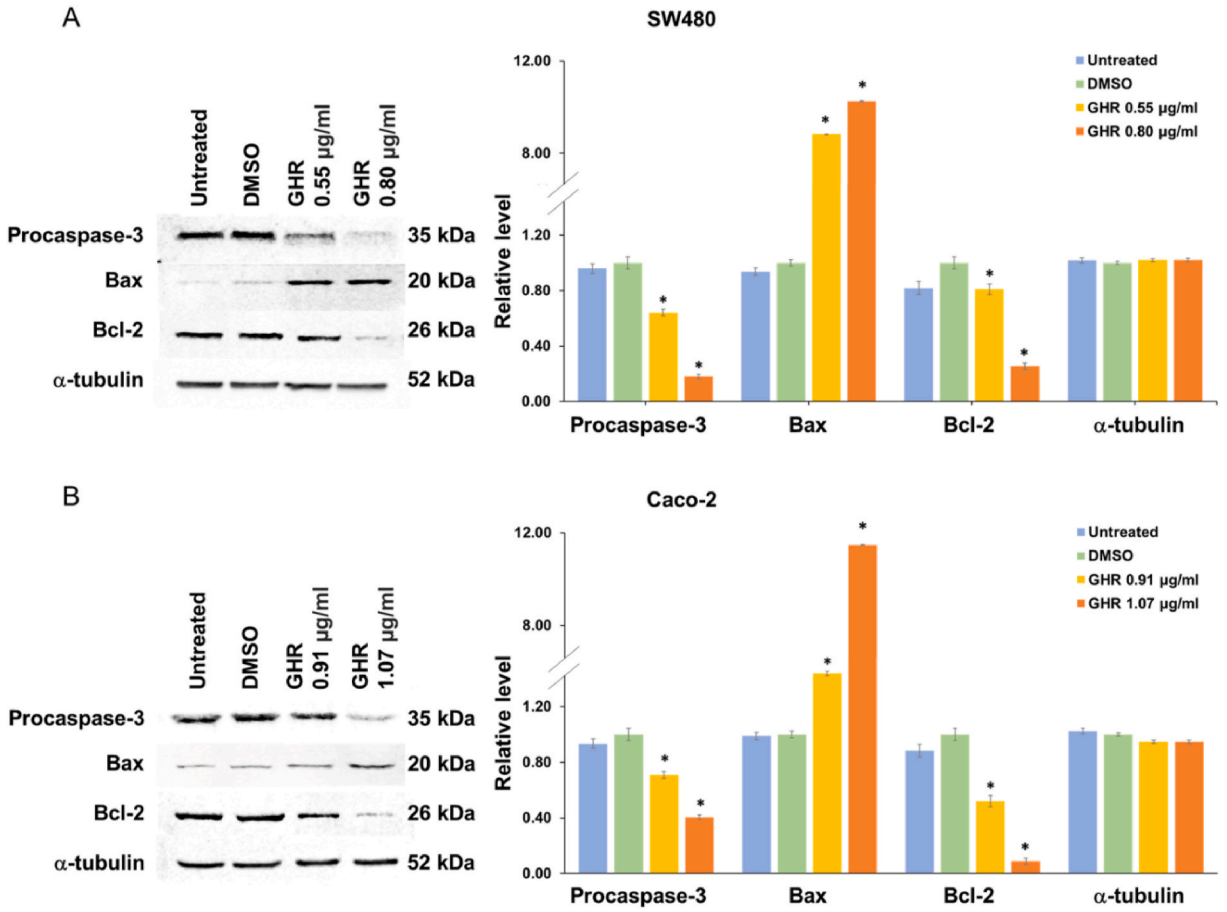

Fig. 10. Alteration of apoptosis-related proteins in CRC cells after GHR treatment. After SW480 (A) and Caco-2 (B) cells were exposed to GHR for 72 h, cell lysates were collected and Western blot analysis was performed with antibodies against Bcl-2, Bax and procaspase-3. Histograms represent a quantitative analysis of band intensities normalized as to α-tubulin. Data were expressed as mean ± SD from triplicate experiments. $^{*}P \leq 0.05$ indicates a significant difference. Images of original uncropped western blots were provided in the supplementary material.

chance for DNA-damaged cells to either repair the damage, particularly to DNA, or alternatively undergo apoptosis pathways [24]. Our results are concordant with previous studies, showing that GA can induce G2/M phase arrest and apoptosis in several malignant cells, including osteosarcoma MG63 cells [25], gastric carcinoma BGC-823 cells [26], nasopharyngeal carcinoma CNE-2 cells [27] and breast carcinoma MCF-7 cells [28]. The results showed that the proportions of viable, early apoptotic, and late apoptotic cells changed significantly according to the GHR treatment with different concentrations and incubation periods. A clear shift from the live cell population to early and late apoptotic cell populations was observed during the incubation time from 24 to 72 h. The total apoptotic ratios were considerably increased compared to the control.

Bax and Bcl-2 family proteins play crucial roles in apoptosis induction by regulating mitochondrial membrane permeability. Activation of Bax results in pore formation in the outer mitochondrial membrane and release of cytochrome *c* into the cytoplasm [29], where protease activating factor (Apaf-1), ATP and procaspase-9 are recruited to form the apoptosome. Then, caspase-9 is activated, and in turn activates caspase-3 and other effector molecules, which eventually leads to cell death [30]. In contrast, anti-apoptotic Bcl-2 binds to and inactivates Bax and other pro-apoptotic proteins, thereby inhibiting apoptosis [31]. Our results demonstrated that Bax was up-regulated, while Bcl-2 and procaspase-3 were decreased in both SW480 and Caco-2 following increasing concentrations of GHR. An elevation of the Bax/Bcl-2 ratio and markedly increased proteolytic cleavage of procaspase-3 indicated that GHR promoted cell apoptosis via a mitochondria-mediated pathway. This finding supports previous results that GA induced apoptosis in Hep3B and Huh7, hepatocellular carcinoma cell lines, through caspases-3 activation [9], and also elevated Bax but reduced Bcl-2 levels in gastric cancer cell BGC-823 [8]. In addition, the anti-cancer effect of GHR might be attributed to its activity in down-regulation of the Wnt/β-catenin signaling pathway, which contributes to the initiation and progression of various types of cancers, including CRC. It was recently reported that ethanolic extract of GHR inhibited tumor growth through the Wnt/β-catenin signaling pathway in an orthotopic mouse model [32].

5. Conclusion

GHR, containing mainly GA, suppressed the proliferation of CRC cells by promoting G2/M cell cycle arrest and mitochondria-mediated apoptosis. GHR might be a promising source for new cancer-selective drugs for CRC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16411.

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