RESEARCH ARTICLE

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Momordica cochinchinensis (Gac) Aril Suppresses Proliferation and Induces Apoptosis of Colorectal Cancer Cells

Ratsada Praphasawat[1,2], Kanathip Singsai[2,3]*, Phuridee Muenkaew[4], Sarita Sirisawat[4], Watanyu Jaewcharoenchai[4], Sinitra Thakaew[5], Supatthra Komkhan[5], Anchalee Rawangkan[5]

Abstract

Background: Gac aril contains high level of carotenoids. This carotenoid possesses several pharmacological properties including antioxidant, anti-inflammatory, and anti-tumor activities. **Objective:** To investigate the anti-cancer activity of Gac aril extract on human colorectal cancer cells and its related mechanisms. **Methods:** Colorectal cancer cell lines HCT116 and HT29 were treated with Gac aril extract and its effects on cytotoxicity and anti-proliferation were analyzed using the MTT/MTS and colony formation assay, respectively. Then, further related mechanisms responsible for anti-proliferation were investigated by cell death detection ELISA and Flow cytometry. **Results:** The results showed that treated cells became rounded up and there was a loss of contact with neighboring cells, leading to a reduction of cell viability. The cytotoxic effects were evaluated IC_{50} for HCT116 and HT29 cells were 2.16 mg/mL and 1.29 mg/mL, respectively but it not toxic to normal HEK293 at the same dose. Moreover, Gac aril extract significantly inhibits proliferative ability with increasing concentrations having a greater effect. Subsequently, the cellular mechanism responsible for suppressive proliferation was validated. It shows apoptosis induction and arrest of cell cycle. **Conclusion:** Our findings demonstrated that Gac aril extract can induce apoptosis and arrest of cell cycle at S and G2/M phases in both HCT116 and HT29 colorectal cancer cells.

Keywords: Gac aril extract- Anti-colony formation- Cell cycle arrest- HCT116- HT29

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Introduction

Colorectal cancer (CRC) is a severe health problem and a leading cause of cancer-related deaths worldwide [1-3]. Many risk factors contribute to colon cancer development. However, a major role, in the pathogenesis of colon cancer, is played by environmental and genetic factors [4]. Most colorectal cancers develop from sequential mutation of tumor suppressor genes and proto-oncogenes, including P53, APC, SMAD4, c-Myc as well as KRAS, that accumulate over time [5]. This has allowed cancer transformation and colon tumor development, which is characterized by increasing cell proliferation and evasion of apoptosis as an important hallmark of cancer [6,7]. As a result, extensive amounts of research have been targeted at the suppression of cell proliferation and induction of apoptosis for developing new anticancer-drug potential [8-10].

Emerging evidence indicates that natural dietary agents have a potential for cancer treatment due to their ability to improve efficacy and diminish toxicity [11]. Several dietary agents possess anti-turmeric potential because they have a targeting proliferative function and triggering apoptosis [12,13]. Currently, *Momordica cochinchinensis* (Lour.) Spreng (*M. cochinchinensis*) in the diet has been extensively studied due to its high carotenoid content [14,15]. The fruit of this plant is abundant in Asia and is found from southern China to northern Austria. *M. cochinchinensis* has long been used as food, and its medicinal aspects have long been known about in South Asia, especially in Vietnam where this fruit is known as Gac. The aril surrounding the seed of Gac fruit is a bright-red membrane, thereby, in Vietnamese tradition, this fruit is used as a colorant for cooking red sticky rice and served at weddings and New Year celebrations. It has been reported that Gac aril has an abundance of bioactive compounds particularly polyphenolic compounds and carotenoids [16,17]. Interestingly, carotenoids from Gac

*[1]Department of Pathology, School of Medicine, University of Phayao, Thailand. [2]Unit of Excellence of Pharmacological Research on Medicinal Plants, University of Phayao, Thailand. [3]Division of Pharmacology, Department of Pharmaceutical Care, School of Pharmaceutical Sciences, University of Phayao, Thailand. [4]Department of Pharmaceutical Care, School of Pharmaceutical Sciences, University of Phayao, Thailand. [5]Department of Microbiology, School of Medical Science, University of Phayao, Thailand. *For Correspondence: kanathip.si@up.ac.th*

fruit are more rich in both lycopene and beta-carotene than in tomatoes and carrots by an amount of 200 and 54 times, respectively [14,17,18].

Pharmacological studies have proven that Gac aril possesses antioxidant, anti-inflammatory, neuroprotective, and anticancer effects [14,19]. In cancer research, this compound was tested for in vitro anti-cancer activity using cancer cell lines. Gac aril extract showed the potential to help in the treatment of many types of cancer cells such as liver [20], colon [20], breast cancers [15,18], and melanoma [15]. It was suggested that these effects were associated with antioxidant activities. In addition, a recent study has found that inhibition of cell proliferation might rely on apoptosis induction, as well as the triggering of necrosis in breast and melanoma cancer cells and cell cycle arrest in colon cancer cells. In the present study, we aim to evaluate the cytotoxic effects of Gac aril extract and test its ability to induce anti-proliferation on colorectal cancer cells. Further, we will demonstrate the related mechanisms of this process of apoptosis induction in those cancer cells.

Materials and Methods

Cell culture and in vitro experiments

HCT116 (ATCC-CCL-247TM) and HT29 (ATCC-HTB-38TM) were provided by Associated Prof. Dr. Prasit Suwannalert, Department of Pathobiology, Mahidol University (Thailand). The cells were grown in 60 mm dishes in high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin/streptomycin (Sigma). Both cell lines were maintained at 37◦c in a humidified atmosphere of 5% CO_2.

To assess the anticancer effects of water extract from Gac aril, cell viability was firstly assessed after treatment with 0.5-1.5 mg/mL Gac aril for 24 and 72 h. Then, the IC_{50} concentration (half maximal inhibitory concentration) was determined in both cell lines. To analyze the effect of Gac aril on the anti-proliferation, cells were incubated with Gac aril concentration (0.5-1.5 mg/mL) for 72 h. Apoptosis induction and Cell cycle distribution in both cell lines were evaluated in the presence of 0.3-0.5 mg/mL Gac aril for 72 h.

Gac aril preparation

M. cochinchinensis was collected in Phrae district, Thailand. The Gac aril was separated. The powdered specimens of Gac aril were extracted with distilled water in the ratio of 1:5 and blended. Then filtered and lyophilized at -20 °C, pressure of 250 Pa for 48 h. The dry extracts were kept in -20°c [16]. For treatment, Gac aril extract was dissolved in sterile distilled water and diluted to appropriate experimental concentrations in a culture medium.

Cell viability evaluation

To evaluate the cell viability of HCT116 and HT29, cells at a density of 4.8 x 10^4 cells/well were seeded on a 96-well plate and allowed to attach for 24-48 h. Cell viability was measured using MTT assay of cells treated with different concentrations (0.5-1.5 mg/mL) of Gac aril extracts and incubated for 24 and 72 h. After an incubation period, the medium containing Gac aril extract was removed.

Clonogenic assay or colony forming assay

Cells were prepared at a concentration of 3x102 cells/mL. HCT116 and HT29 cells were cultured in 6 well-plates, incubated in a CO2 incubator at 37 ºC for 24 h. 2 ml of Gac extract was added at concentrations of 500, 1000 and 1500 µg/mL. At the end of 72 h, the colony forming were examined under an inverted microscope. The number of colonies from 9 to 10 fields per concentration was then counted and averaged for each concentration. Then, the relative colony proliferation rate was calculated by the equation,

Relative colony formation rate (%) = 100 x average number of colony Sample/average number of colonies per field Control

Apoptosis determination by ELISA assay

Apoptosis induction was quantified using Cell Death Detection ELISA PLUS kit (Sigma-Aldrich, Mannheim, Germany). This immunoassay allows the specific detection of histone-associated-DNA-fragment in the cytoplasmic fraction after induced cell death. Briefly, HCT116 and HT29 cells were respectively seeded into a 12-well plate and allowed to attach as described above. The media was removed and replaced with Gac aril solution (0, 300, and 500 µg/mL). After 72 h of incubation, cells were harvested in accordance with the manufacturer's instructions. The absorbance was measured at 405 nm using an ELISA reader (synergyH1, Biotek, USA).

Apoptosis determination by Flow cytometry

To determine the effect of *M. cochinchinensis* extract on inducing apoptosis in HT29 and HCT116 cells, the experiment was conducted by Annexin V/PI apoptosis detection Assay according to Khazaei et al. [21] with some modifications. HT29 and HCT116 cells were separately cultured in 6 well plates for 24 h at a density of 1x106 cells/well followed with Gac extract at concentration 0, 300, and 500 µg/mL for 72h. After incubation, cells were tripsinized, washed with PBS and binded with the binding buffer for 200 µl. After that, cells were stained with Annexin V and incubated for 15 min. The stained cells were centrifuged and resuspended in 1% paraformaldehyde. Propidium iodide (PI) was added to each reaction before flow cytometry analysis (FACScan, Becton Dickinson, USA).

Cell cycle analysis

HCT116 and HT29 cells were grown in 6-well plate (2×10^5 cells/well) for 24 h and incubated with or without various concentrations of extract (0-500 µg/mL) for 72 h. The cell suspension was then pelleted, centrifuged, washed twice with cold PBS, and fixed with 70% ethanol at -20°C for 24 h. Following, the cells were stained with a nuclear staining dye 50 µg/mL of propidium iodide (Biolegend, California) containing RNaseA (Geneaid, Taiwan) solution (20 µg/mL) for 30 min in the dark. The

DNA contents at different phases of the cell cycle were measured by flow cytometry using a FACScan apparatus (FACScan, Becton Dickinson, USA).

Statistical analysis

Results are expressed as mean ± standard derivation (SD) of n experiments. To determine statistical significance, independence t-test was used for comparing a single treatment mean with a control. One-way ANOVA with post-hoc Tukey test was used for comparing multiple treatments, including flow cytometry apoptosis test and cell cycle arrest test. All statistical analysis were performed using SigmaPlot 12.0 software. Differences were considered statistically significant at P-value ≤ 0.05.

Results

Gac aril inhibits the viability of colorectal cancer cells

To study the effect of Gac aril extract on colorectal cancer in vitro, we treated colorectal cancer HCT116 and HT29 cells with increasing concentrations of Gac aril extract for 96 h. The viability of treated cells was evaluated using the trypan blue exclusion method. After incubation, we observed the morphology of treated cells under microscopy. We found that the morphology changed with Gac aril treatment, when compared to the untreated control group. Cells became a round shape and lost their adhesiveness, leading to decreased cell viability in the Gac aril treatment groups, as shown in Figure 1. We further assessed the cell viability and found that both types of colorectal cancer cells were also affected in a concentration-dependent manner. Percentage of cell viability of HCT116 and HT29 cells showed in Table 1. In HCT116 cells, we found a significant decrease in cell survival rates with a 0.1 mg/mL amount of Gac aril treatment, and the IC_{50} value for HCT116 was approximately 2.16 mg/mL. We also confirmed that there was a reduction in cell viability in the other colorectal cancer cells. In the case of HT29, the viability of the cells showed similarly a significant reduction with increasing concentrations of Gac aril. The number of living cells observed in the highest concentration (1.5 mg/mL) was 21.54%, which considering the IC_{50} value, was 1.29 mg/mL. Taken together, Gac aril treatment led to the inhibition of cell viability in colorectal cancer cells. Therefore, cell viability greater than 70% was chosen to negate cell death during the subsequent experiment.

Table 1. Percentage of Cell Viability of HCT116 and HT29 Cells, Results were Repeated at Least Three Independent Experiments.

Concentration (μg/mL)	Cell viability (%)	
	HCT116	HT29
Control	100	100
GAC 500	104.81 ± 0.25	62.55 ± 1.11
GAC 1000	90.11 ± 0.29	54.22 ± 0.98
GAC 1500	59.41 ± 0.14	50.69 ± 0.09

Gac aril inhibits the colony formation of colorectal cancer cells

The effect of Gac aril extract on HCT116 colony formation by clonogenic assay showed that Gac aril extract was able to inhibit colony growth of HCT116 cells at concentrations of 500, 1000 and 1500 μg/mL. The percentage of relative colony formation rate were 88.46 ± 1.16, 46.15 ± 0.79, and 26.92 ± 0.48, respectively. There was a statistically significant difference ($p < 0.001$) when compared to the control group. Similar to the findings with regards to HCT116, Gac aril treatment in HT29 cells showed a decrease in colony forming, but apparently, a weaker signal was observed in the HT29 cells, compared to the HCT116 cells. The inhibition of colony growth of HT29 cells was found when treated with Gac aril extract at concentrations of 500, 1000, and 1500 μg/mL. The percentage of relative colony formation rate were 66.67 ± 1.03, 48.48 ± 1.07, and 45.45 ± 0.71, respectively. All concentrations were a statistically significant difference ($p < 0.001$) when compared to the control group (Figure 2).

Gac aril induced apoptosis in colorectal cancer cells

To identify the possible mechanism responsible for the suppression of cell proliferation by Gac aril, we investigated whether Gac aril-induced programmed cell

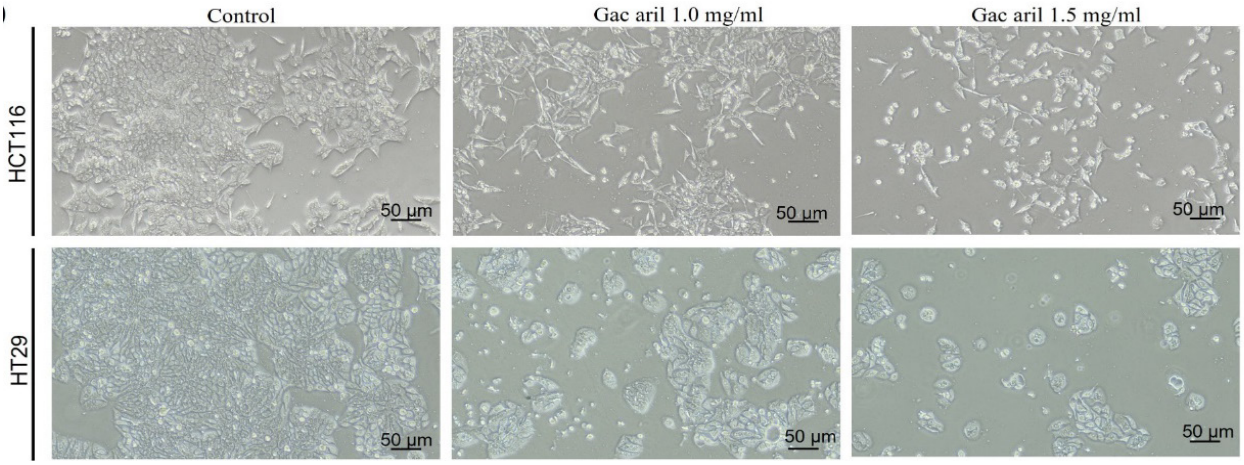

Figure 1. Effect of Gac Aril Extract on the Viability of Human Colorectal Cancer Cells. Representative images showing HCT116 and HT29 cancer cells treated with Gac aril at 1.0 and 1.5 mg/mL, respectively for 96 h.

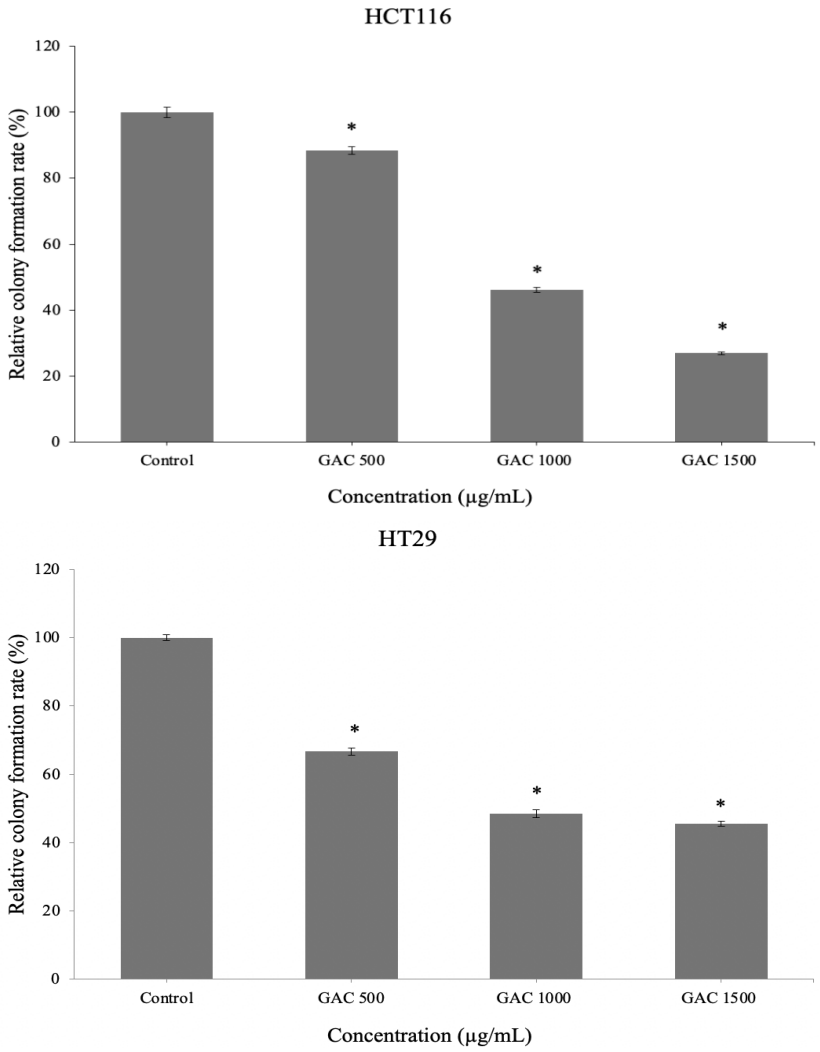

Figure 2. Anti-Proliferative Effect of Gac Aril on Two Colorectal Cancer Cells. HCT116 and HT29 were treated with Gac aril at 0.5-1.5 mg/mL for 72 h. Proliferation rate was assessed using MTS assay. Results represent mean ± SD of four wells per experiment. Three independent experiments were repeated. *p < 0.001 compared to the control.

death or apoptosis in colorectal cancer cells. Therefore, we quantitated a DNA fragment that is a biochemical hallmark of apoptosis by using Cell death detection ELISA PLUS. As expected, we found a significant increase in apoptosis induction in HCT116 and HT29 following Gac aril treatment at 500 µg/mL for 72 h when compared with the control group (Figure 3a-b). To confirm this finding, we investigated this effect in another measurement, flow cytometry, the result showed in Figure 4a-c. Interestingly, the apoptosis induction was significantly increased in HCT116 and HT29 cells after treatment with Gac aril at 300 and 500 µg/mL, respectively (Figure 4c). These results indicated that Gac aril effectively induced apoptosis in HCT116 and HT29 colorectal cancer cells, correlating with its inhibitory effect on cell proliferation.

Effect of Gac aril on cell cycle

To identify the possible mechanism responsible of Gac aril on cell cycle in colorectal cancer cells. Therefore, we quantitated a DNA content at different phases of the cell cycle were measured by flow cytometry. We found that Gac aril significantly suppressed cell cycle at S and G2/M phases in concentrations of 300 and 500 µg/mL on HCT116, while it was significant at only 500 µg/mL on HT29 cells (Figure 5a-b).

Discussion

Colon cancer is a major cause of morbidity and mortality worldwide [22]. Developing new drugs to treat it is a remaining global challenge. As a result, plant-derived medicine has been increasing looked at as a means of doing this [8,10]. *Momordica cochinchinensis* has long been used as a food and for its medicinal properties. Importantly though, water extract from Gac fruit (*M. cochinchinensis*) has been shown to kill melanoma and breast cancer cell lines through multiple mechanisms (apoptosis and necrosis) and to inhibit the growth of the colon 26-20 adenocarcinoma and HepG2 carcinoma cell lines [20]. In this study, we have shown its anticancer capacity in relation to colorectal cancer cells. We have also shown that Gac aril has beneficial effects on HCT116 and HT29 colorectal cancer cells. Gac aril treatment is shown to produce a morphological change, which was observed in both HCT116 and HT29 cells. Gac aril-treated cells showed signs of cellular damage including round

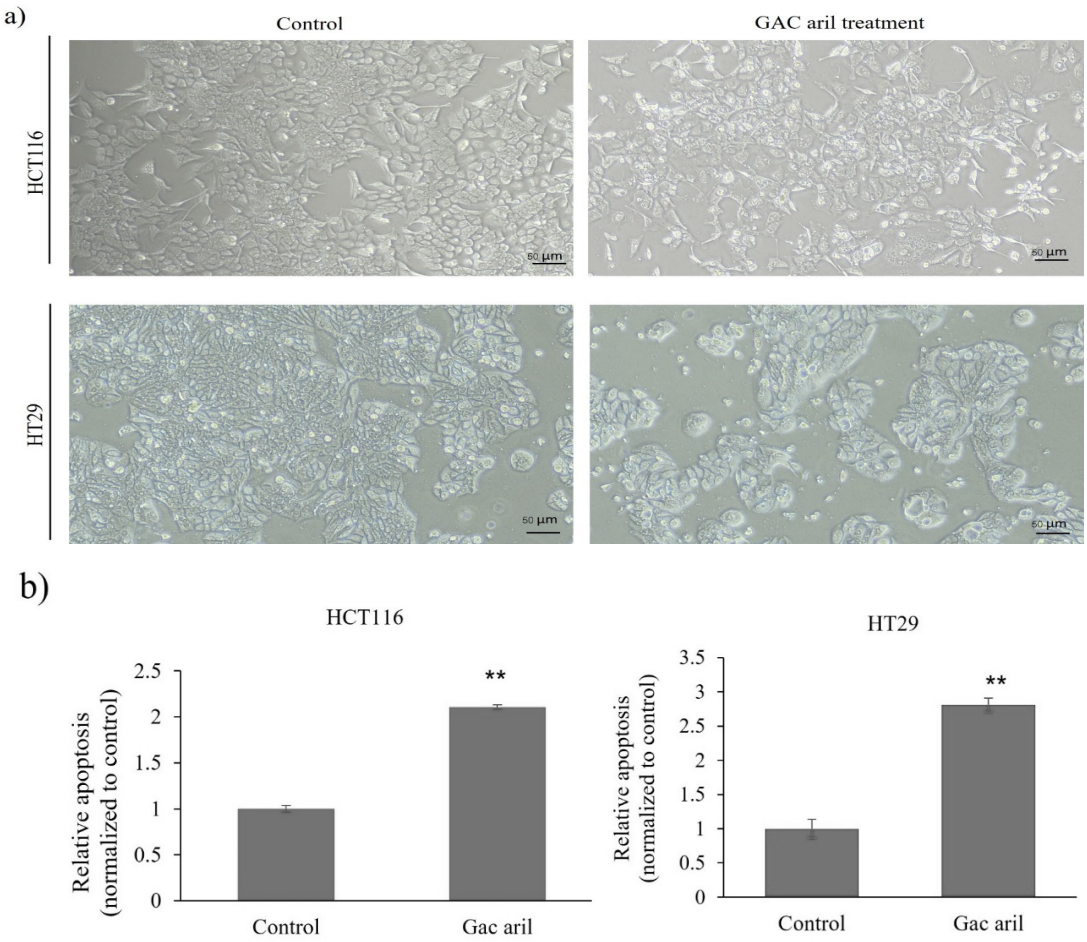

Figure 3. Apoptosis Induction of Colorectal Cancer Cells by Gac Aril Measured by ELISA. a) Representative image showing HCT116 and HT29 cells treated with Gac aril 500 µg/mL for 72 h. b) Graph showing apoptosis induction in HCT116 and HT29 cells treated Gac aril 500 µg/mL. Results were an average of four wells per experiment, repeating 2 experiments. **p < 0.001 relative to the control.

up and detachment from surface culture. The results of cytotoxicity testing demonstrated that the IC_{50} value of Gac aril was at 2.16 and 1.29 mg/mL for HCT116 and HT29, respectively. The level of cytotoxicity found in this study was greater than in an earlier study done on colon 26-20 cells, which caused approximately 30-35% loss of cell viability at 1.24 mg/mL. It was consistent with HepG2 cells where an IC_{50} value of 1.24 mg/mL was examined after water-soluble extract treatment [20]. In addition, it has been previously reported that an examination of cell viability between Gac aril extracts from water and HAE (Hexane:acetone:ethanol), the water extract from Gac aril showed dramatically higher cytotoxicity against both breast cancer and melanoma cancer cells than did the corresponding HAE [15]. However, our study demonstrated that the total cell viability was similar for the two cell lines tested. Thus, we further assessed whether Gac aril extract might inhibit the cell proliferative function in those cancer cells.

Excessive cell proliferation is an important physiologic quality of cancer cells, leading to cancer development [7]. Anticancer therapies can be used to interrupt cancer cell proliferation [23-25]. In the current study, we found that Gac aril treatment can reduce the cell proliferation activity of colorectal cancer cells. There was a significant decrease in cell proliferation in HCT116 and HT29 cells with Gac treatment, as observed in other cancers. It has also been reported to inhibit the growth of colon 26-20 adenocarcinoma cells in transplanted Balb/c mice, as well as to suppress cell proliferation in both colon 26-20 and HepG2 cells [20]. The observed inhibition is in agreement with previous reports that aril extract of Gac fruit showed cytotoxicity and antiproliferation of MCF-7 cells [15]. Their results suggested that the extract contained relatively high amounts of carotenoids and reflects the inhibition of cancer cell proliferation.

To elucidate which the mechanism is responsible for the suppression of cell proliferation, we firstly determined the apoptosis induction in colorectal cancer cells. Apoptosis is a physical pathway leading to cell death upon programmed genetic regulation. Apoptotic cells appeared to round-up and lost contact with neighboring cells. The nucleus of apoptotic cells is condensation and cleavage to DNA fragments [7,26]. However, cancer cells have an effort to evasion this property. Therefore, targeting apoptosis is possible for anticancer drug development [12,13,27]. Our findings demonstrate that treating of Gac aril exhibits cellular change as seen in HCT116 as well as HT29 cancer cells. We found that Gac aril treatment dramatically induced apoptosis of HCT116 colorectal

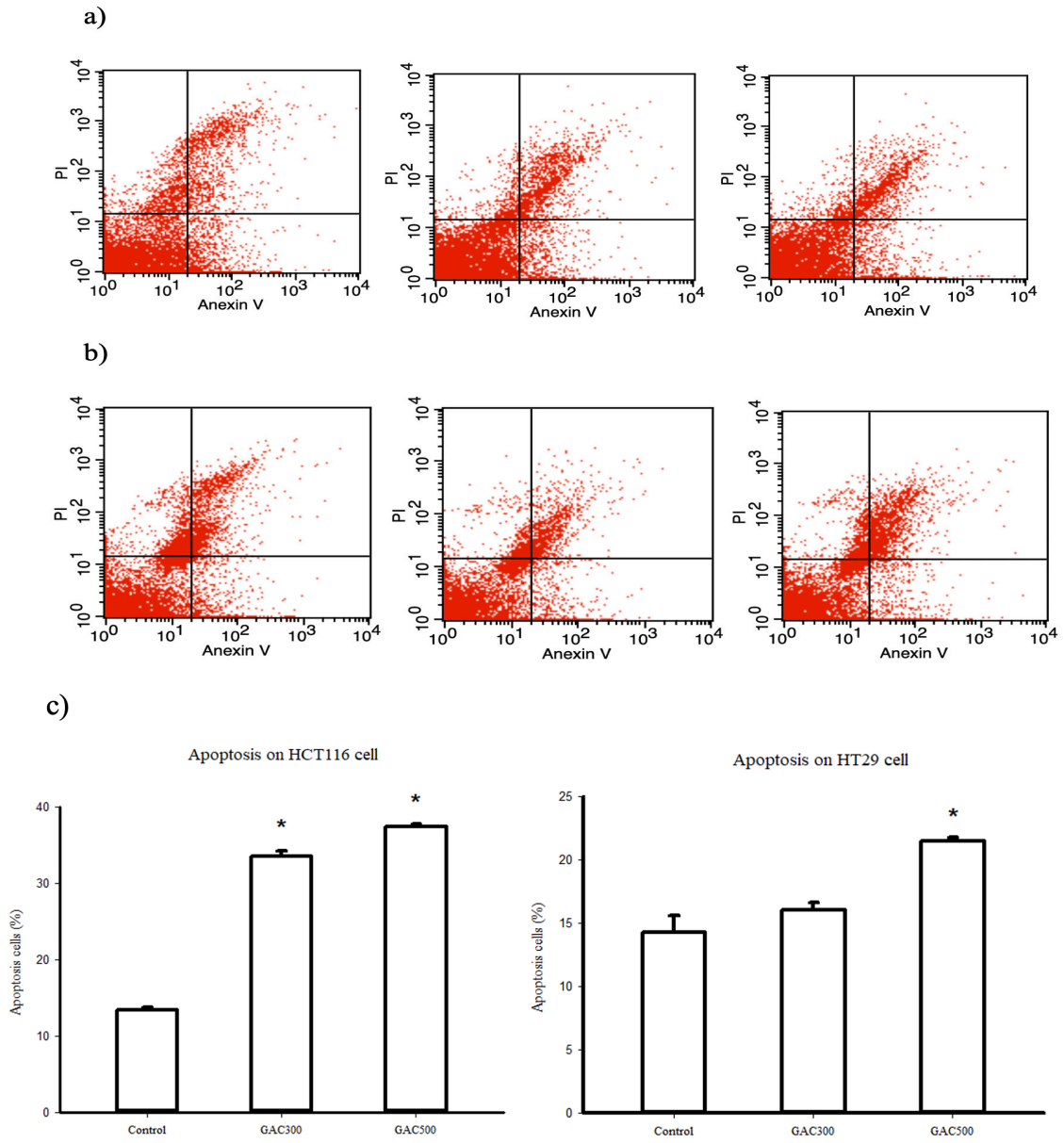

Figure 4. Effect of Gac Aril on Apoptosis Measured by Flow Cytometry. a) representative of the effect of Gac on HCT116 cells stained with PI and Anexin V at control, Gac 300, and Gac 500 µg/mL, respectively. b) representative of the effect of Gac on HT29 cells apoptosis at 0, 300, and 500 µg/mL of Gac extract, respectively. and c) percentage of apoptosis on HCT116 and HT29 cells. The experiments were done in triplicate at each concentration, and the results were expressed as the mean ± SD. *p < 0.001 compared to the control.

cancer cells. These effects have already been confirmed in HT29. Thus, apoptosis induction was clearly enhanced with Gac aril treatment of both cancer cells and led to cell death. With regards to previous research, it is reported that Gac aril induced apoptosis in MCF-7 breast cancer cells as evidenced by an increasing percentage of cells in an apoptosis stage. Interestingly, this study provided a pathway associated with apoptosis induction in MCF-7, through the upregulation of pro-apoptotic bax gene expression and activation of caspase 6, 8, and 9 activities, which corresponds to the high carotenoid content of the Gac aril extract [18,28]. Moreover, Gac aril water extract has also been found to induce an increase of apoptosis/necrosis cell death in breast cancer and melanoma cell lines as detected by flow cytometry.

However, our study did not show which compound was associated with the anti-cancer effects of Gac aril water extract. We searched for reports of active components involved that might have influenced cancer. It has been reported that the antitumor component of the water extract of Gac aril was confirmed as a protein with a molecular weight of 35 kDa, which showed suppression of tumor growth both in vivo and in vitro experiments [20]. In addition, we noticed that the cytotoxicity testing of Gac aril on noncancerous cells should be employed concurrently. Indeed, previous research has demonstrated that Gac aril water extract showed selective cytotoxicity towards neoplastic cells but was not toxic to normal fibroblasts (NHDF), except at high concentrations [15]. To conclude, we have established the anticancer effects of Gac aril extract on two types of human colorectal cancer cell lines, HCT116 and HT29. Gac aril was found to inhibit

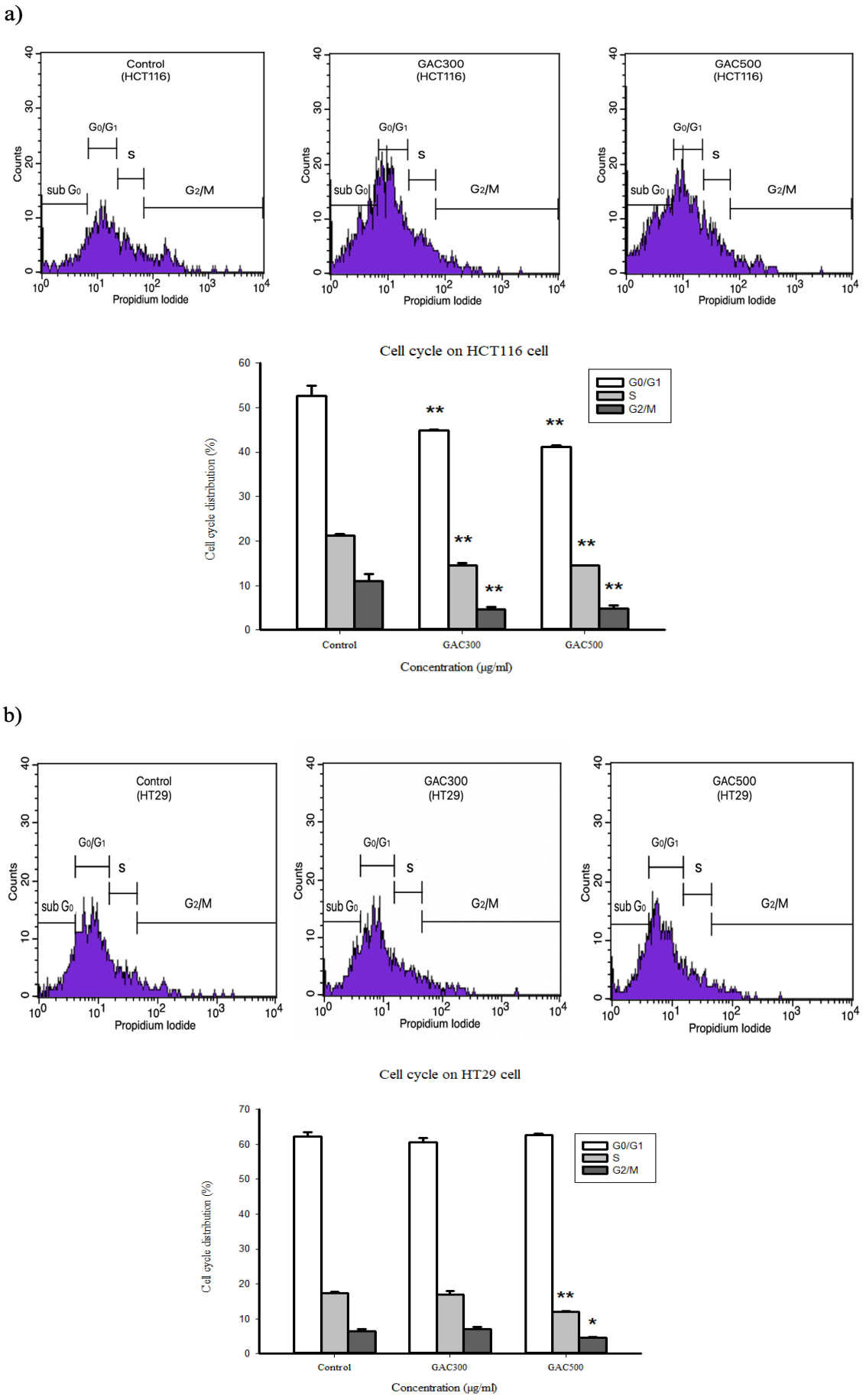

Figure 5. Effect of Gac Aril on Cell Cycle in a) HCT116 and b) HT29 cells measured by flow cytometry. The experiments were done in triplicate at each concentration, and the results were expressed as the mean ± SD. *p < 0.05, **p < 0.001 compared to the control.

cell proliferation in both cell lines. We have provided apoptosis induction and cell cycle arrest at S and G2/M phases as a mechanism responsible for the suppression of cell proliferation and on those colorectal cancer cells.

Author Contribution Statement

Conceptualization and design: Praphasawat R, Singsai K, Rawangkan A. Project administration and Funding acquisition: Praphasawat R, Singsai K. Investigation: Muenkaew P, Sirisawat S, Jaewcharoenchai W, Thakaew S, Komkhan S. Writing-original draft: Praphasawat R. Writing-review and editing: Singsai K.

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Conflict of interest

The authors declare no conflict of interests.

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