

*Full Length Research Paper*

# Anticancer activity of guava (*Psidium guajava* L.) branch extracts against HT-29 human colon cancer cells

Sang-Bong Lee<sup>1</sup> and Hae-Ryong Park<sup>2\*</sup>

<sup>1</sup>Gapo High School, Masan 631-320, Korea.

<sup>2</sup>Department of Food Science and Biotechnology, Kyungnam University, Masan 631-701, Korea.

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**Natural products have recently become the focus of increased research interest due to their potential pharmacological activities. Therefore, we established a program to screen natural products for cytotoxic activity using the MTT reduction assay system to test HT-29 human colon cancer cells. During the course of screening, we found that the acetone extracts of guava (*Psidium guajava* L.) branch (GBA) had cytotoxic effects on HT-29 cells. The GBA showed highly cytotoxic effects via the MTT reduction assay, LDH release assay, and colony formation assay. In particular, the GBA of the 250 µg/ml showed 35.5% inhibition against growth of HT-29 cells. As expected, GBA induced characteristic apoptotic effects in HT-29 cells, including chromatin condensation and sharking that occurred 24 h after the cells had been treated at a concentration level of 250 µg/ml. To examine the functions on apoptosis, we used a flow cytometric analysis. The apoptotic cells were distributed according to the cell cycle phase shown by sub-G1 DNA content.**

**Key words:** Natural products, HT-29 cells, cytotoxicity, guava branch, anticancer.

## INTRODUCTION

Cancer is the primary cause of mortality and morbidity in the elderly (Fried, 2003). Elderly persons are at ten times greater risk of developing cancer than persons under 65 years of age (Hayat et al., 2007). Cancer is caused by endogenous and exogenous factors leading to the sequential accumulation of genetic alterations, a scenario known as multi-step oncogenesis (Hanahan and Weinberg, 2000). Cancer can occur as the result of a disruption of this balance, due to either an increase in cell proliferation or a reduction in cell death or both (Kerr et al., 1972). In particular, colon cancer is the most common malignancy of the gastrointestinal tract and the second most common cause of cancer-related death worldwide (Ferlay et al., 2007). Although the prognosis in the majority of patients with early phase diagnosis is good, for patients with diagnoses in the advanced stages the

prognosis is poor (Ansari et al., 2006). Cancer cells are characterized by unregulated growth, as well as insufficient and inappropriate vascular supply (Tomida and Tsuruo, 1999). Moreover, a core of cells was subjected to micro environmental stress conditions, and has decreased apoptotic potential through genetic alterations, thereby resulting in resistance to apoptosis (Kaufman et al., 2002).

Colon cancer is treated by surgery, radiation therapy, chemotherapy, and by other methods. Chemotherapy in general and polychemotherapies in particular, both adjuvant and metastatic settings, have led to significant improvements in patient survival. The use of Irinotecan and/or oxaliplatin in combination with 5-FU and folinic acid is linked with an improvement in median survival and/or progression-free survival (Douillard et al., 2000). However, these combined treatments are intrinsically and potentially cytotoxic against normal cells (Pantazis, 1995). This problem has inspired efforts to develop novel anticancer agents with fewer side effects and lower levels of cytotoxicity against normal tissues and cells

\*Corresponding author. E-mail: parkhy@kyungnam.ac.kr. Tel: 82-55-249-2689. Fax: 82-55-249-2995.

(Park et al., 2004). To overcome these limitations and to improve the effectiveness of cancer chemotherapy, it is necessary to isolate new anticancer drugs that are specifically toxic to cancer cells, and to identify new targets that destroy cancers when exposed to chemotherapy (Vaux and Korsmeyer, 1999). Accordingly, many researchers have tested the use of natural products for cancer treatments. Natural products used in traditional folk medicine have been the source of many medically beneficial drugs, as many medicinal plants have been shown to present interesting biological and pharmacological activity (Battle et al., 2005).

Consequently, over the course of our screening program on the cytotoxic activity of natural products, we found that *Psidium guajava* L. branch exerted cytotoxic effects against HT-29 cells. *P. guajava* L. commonly known as guava is a native plant in tropical America and has long been naturalized in South East Asia. Different parts of the plant are used in the indigenous system of medicine for the treatment of various human ailments. The bark has been employed for treating diarrhea in children. The leaves are used for relief of cough, pulmonary disorders, wounds, and ulcers. The fruit is tonic, cooling, laxative, and antihelmintic (Shen et al., 2008). However, the biological and chemical activity of guava branch has not been investigated. In particular, the anti-proliferative effects of guava branch had not been elucidated until now. In this study, we assessed the cytotoxic effects of acetone extracts of guava branch (GBA) against HT-29 cells and subsequently have a proposed mechanism responsible for its cell death activity.

## MATERIALS AND METHODS

### Materials

Guava (*Psidium guajava* L.) blanches (GB) were collected in early September 2008 at the farm of GuavaKorea Co. (Uiryong, Korea). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT), dimethyl sulfoxide (DMSO), gallic acid, (+) catechin, and vanillin were provided by Sigma Chemical Co. (St. Louis, MO, USA). Lactate dehydrogenase (LDH) release assay kit and Folin-Ciocalteu reagent were purchased by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI 1640 medium, fetal bovine serum and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

### Preparation of GB extracts

The GB was freeze-dried and finely ground using a blender (MC-811C, Novita, Korea) to pass through a 48-mesh sieve. The GB powder (10 g) was extracted with 300 mL of acetone in a shaking incubator (100 rpm) overnight at room temperature and filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). Solvents were then removed by evaporation *in vacuo*, and the dried extracts were obtained. The acetone extract from GB was named as GBA. The GBA was then dissolved in DMSO at a concentration of 25 mg/mL for experiments, and diluted with DMSO when needed.

### Cell culture and treatments

HT-29 human colon cancer cells were obtained from the Korean Cell Line Bank (KCLB). The cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 mg/ml of NaHCO<sub>3</sub> in a 37°C incubator with 5% CO<sub>2</sub>. The GBA was dissolved in DMSO and added to the culture medium so that the final concentration of DMSO was less than 1%.

### MTT reduction assay

Cell viability was measured with blue formazan that was metabolized from MTT by mitochondrial dehydrogenase, which is active only in live cells. HT-29 cells were seeded in 96-well plate at a density of  $1.0 \times 10^5$  cells per well, cultured overnight and pretreated with various concentrations of GBA. After incubation for 24 h, the MTT (5 mg/ml) colorimetric viability test was used to determine the viability of cells. The absorbency of each well was measured at 540 nm using an ELISA reader (BioRad, Model 680, USA), and the percentage viability was calculated.

### LDH release assay

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). The cells were pretreated with various concentrations of GBA for 24 h, and the supernatant was used to assay LDH activity. A colorimetric assay was applied, according to which the amount of formazan salt is proportional to LDH activity in the sample. The intensity of red color formed in the assay and measured at a wavelength of 540 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from vehicle-treated cells and expressed as a percentage of the control.

### Colony formation assay

The cells were seeded at  $1.0 \times 10^5$  cells in a 24-well plate, cultured overnight, and treated with various concentrations of GBA for 24 h. For the colony formation assay, the cells were then diluted in fresh medium lacking GBA, reseeded at  $1.0 \times 10^3$  cells per well in 6-well plates, and cultured under normal growth conditions for 7 or 8 days to form colonies. Formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival was calculated by setting the survival of control cells at 100%. IC<sub>50</sub> values were determined from the dose-response curves of colony formation inhibition.

### Morphological analysis

HT-29 cells were seeded in 6-well plates ( $2.0 \times 10^5$  cells/well) and incubated in a medium at 37°C under a 5% CO<sub>2</sub> for 24 h. The cells were pretreated with various concentrations of GBA. After incubation for 24 h, cellular morphology was observed using a phase-contrast microscope (Nikon, Japan). The photographs were taken at  $\times 100$  magnification.

### Measurement of apoptosis and cell staining

Apoptosis was investigated by staining the cells with Hoechst 33342 (Sigma) and flow cytometric analysis. The cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 h at room temperature. The

fixed cells were washed with PBS, and stained with Hoechst 33342 for 1 h at room temperature. The Hoechst-stained nuclei were excited and emission wavelengths set at 358 and 461 nm, respectively, visualized using a fluorescence microscope. A flow cytometric analysis of the cellular DNA content was performed as described previously. The cells were harvested and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 µg/ml of propidium iodide at room temperature in the dark for 30 min. The apoptotic cells were measured using a FACS caliber flow cytometer (Beckman Coulter).

#### Total phenolic content (TPC)

The TPC of GBA was determined using the method by Gutfinger (Gutfinger, 1981). The GBA (1 ml, 1 mg/ml) was mixed with 0.2 ml 50% Folin-Ciocalteu reagent and 1 ml 2% Na<sub>2</sub>CO<sub>3</sub>, and centrifuged at 12,000×g for 5 min. The absorbance was measured with a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) at 750 nm after 30 min incubation at room temperature. Gallic acid was used as a standard for TPC.

#### Total flavanol content (TFC)

The TFC of GBA was estimated by the vanillin method using catechin as a standard (Price et al., 1978). The GBA (1 ml, 1mg/ml) was mixed with 5.0 ml of 2.0% vanillin (8.0% methanolic HCl). The absorbance was measured with a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) at 500 nm after 20 min incubation in the dark at room temperature. TFC was obtained by using (+)-catechin as a standard.

#### Statistical analysis

All data are the means of ± S.D. of three determinations and data was analyzed using the SPSS package for Windows (Version 11.5). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test. The differences were considered significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Cytotoxic activity of GBA on HT-29 human colon cancer cells

Acetone extracts from guava branch (GBA) preparations were evaluated for their cytotoxic activity in HT-29 cells. As a preliminary test to confirm the cytotoxicity of GBA in HT-29 cells, the cells were pretreated with 50, 100, and 250 µg/ml GBA. HT-29 cells were originally derived from a human colon cancer and were chosen as representative hypovascular tumor. These cells had shown strong tolerance to anticancer agents *in vitro* and *in vivo*. We attempted to determine the cytotoxic activity of GBA and the cytotoxic effect of these extracts as measured by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. As shown in Figure 1A, GBA showed potent cytotoxic activity in HT-29 cells. GBA effected a 30 - 70% reduction in cell viability as compared to the control, in a dose-dependent manner. In order to assess the cytotoxicity occurring in

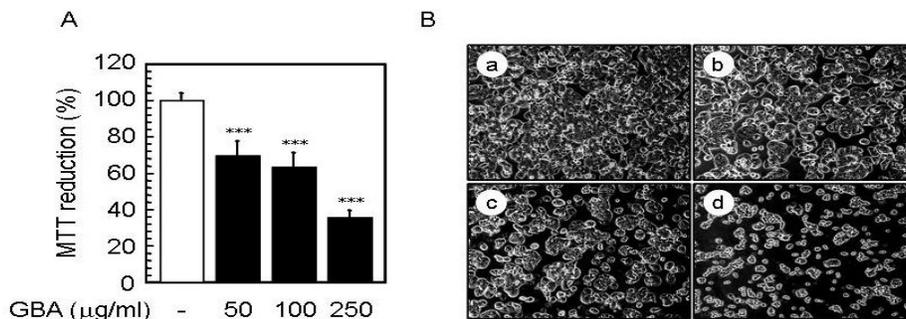
the GBA-treated HT-29 cells, cells were incubated with GBA, and morphological alterations were confirmed via phase-contrast microscopy. As shown in Figure 1B, after 24 h of incubation with various concentrations of GBA, many of the cells showed cytoplasmic shrinkage and loss of normal nuclear architecture, became detached and found floating in the medium. As a result, the number of cytotoxic cells increased with GBA concentration, with the highest having the most pronounced inhibitory effect on cell proliferation than the control.

The cytotoxic effect of GBA was tested via the lactate dehydrogenase (LDH) release assay, based on the extent of LDH leakage into the medium. The augmented release of LDH into the media is reflective of cellular damage. Therefore, we conducted this experiment in order to estimate the release of LDH after treatment with various concentrations of GBA. As expected, GBA caused cytotoxicity in a dose dependent manner by a 35 - 45% increase in LDH-release, as compared to control cells (Figure 2A). In order to further clarify these results, we assessed the effects of GBA on colony formation assay. HT-29 cells were pretreated with 50, 100, and 250 µg/ml of GBA for 24 h. A GBA level of less than 100 µg/ml was required for 50% inhibition of colony formation (IC<sub>50</sub>). The highest concentration of GBA (250 µg/ml) produced a particularly significant reduction in cell viability wherein colony formation was inhibited by 91% (Figure 2B). Consistent with the results of the MTT reduction assay, LDH release assay, and colony formation assay showed that GBA inhibited HT-29 cells proliferation in a dose-dependent manner. These results clearly indicate that GBA can induce cytotoxic activity against HT-29 cells.

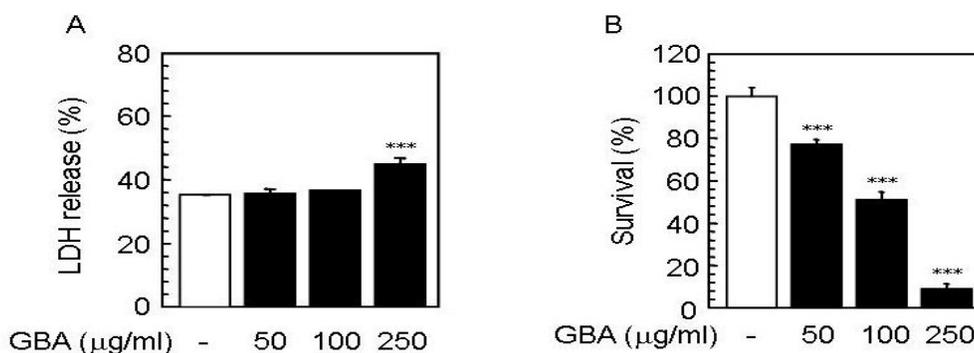
### GBA-induced apoptosis in HT-29 Cells

Apoptosis is a genetically programmed cell death mechanism that can be activated by various stimuli, including chemotherapeutic agents and radiation. The morphological features of apoptosis include chromatin condensation, cell shrinkage, and nuclear fragmentation (Lazebnik, 1998). In order to determine whether the cytotoxic activity of GBA was due to apoptosis, HT-29 cells were treated for 24 h with various concentrations of GBA. The cells, stained with Hoechst 33342 (10 µM) revealed marked chromatin condensation and apoptotic body formation when examined by a fluorescence microscope. In addition, Hoechst 33342 staining revealed that the highest concentration (250 µg/ml) of GBA had the effect of causing highly condensed, fragmented nuclei morphology, and nuclei shrinkage, the typical characteristics of apoptosis (Figure 3A).

To identify the presence of apoptosis, we used flow cytometric analysis after PI staining of cells to evaluate the effects of GBA on HT-29 cells. The apoptotic cells were distributed according to the cell cycle phase by showing sub-G1 DNA content. The amount of apoptotic



**Figure 1.** Cytotoxic effects of acetone extracts of guava (*Psidium guajava* L.) branch (GBA) on HT-29 human colon cancer cells. The cells were exposed to the various concentrations of GBA for 24 h. (A) Cell viability was measured with the MTT reduction assay. After MTT reduction assay, the MTT reduction rate was calculated by setting each of the control survivals equal to 100%. (B) Analysis of morphological changes resulting from GBA treatment of HT-29 cells. The cells were exposed to various concentrations of GBA; a, control; b, 50 µg/ml; c, 100 µg/ml; d, 250 µg/ml and morphological changes were monitored for 24 h. The photographs were taken with a phase-contrast microscope at  $\times 100$  magnification. \*\*\*significant vs. control untreated cells ( $p < 0.001$ ).



**Figure 2.** GBA induced cell death in HR-29 cells. (A) Cell death was measured with the LDH release assay. For the results of the LDH release assay, the data were normalized to the activity of LDH released from vehicle-treated cells, and expressed as percentage of control. (B) Colony formation analysis of HT-29 Cells, the survival rate was calculated by setting each of the control survival rates. After 7 - 8 days, the formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. \*\*\*significant vs. control untreated cells ( $p < 0.001$ ).

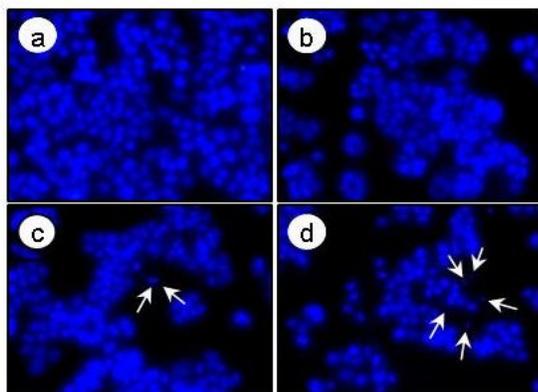
cells measured in the sub-G1 phase were approximately 30-fold greater for the GBA-treated HT-29 cells than for the control (Figure 3B). These results clearly indicate that the anti-proliferative effect of GAE on HT-29 cells was attributable to apoptosis and suggest that guava branch may be an important natural anticancer chemotherapeutic agent.

#### Total phenolic content (TPC) and total flavanol content (TFC) of GBA

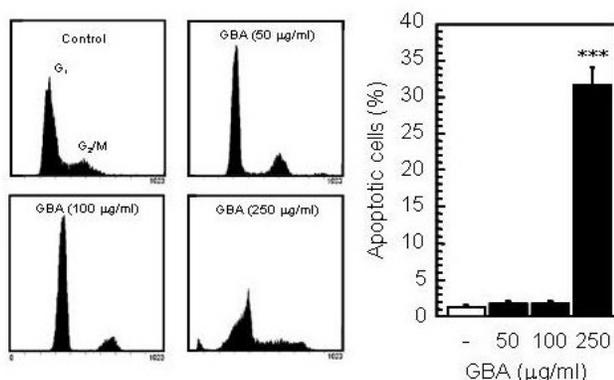
Many plants contain bioactive phytochemicals which may play important roles in disease prevention as well as health promotion. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing

compounds, and organosulfur compounds (Liu, 2004), among which phenolic substances often play a primary or a synergistic role. Chen et al. reported that aqueous extract of guava leaf inhibited brain derived metastatic prostate cancer DU-145 cells in a dose- and time-dependent manner (Chen et al., 2007), and they ascribed the anticancer activity of guava extract to its extraordinarily high polyphenolic ( $165.61 \pm 10.39$  mg/g) and flavonoid ( $82.85 \pm 0.22$  mg/g) contents. As shown in Table 1, total phenolic content (TPC) and total flavanol content (TFC) of GBA were  $575.94 \pm 1.25$  mg/g and  $35.99 \pm 1.62$  mg/g, respectively. TPC of acetone extracts of Korean guava branch in this study was 3.47 fold higher than that of aqueous extract of Taiwan guava leaf (Tomida and Tsuruo, 1999). Lower TFC value of GBA compared to the flavonoid content of Taiwan guava leaf

A



B



**Figure 3.** GBA-induced apoptosis in HT-29 cells. (A) The cells were treated with various concentrations (a; control, b; 50 µg/ml, c; 100 µg/ml, and d; 250 µg/ml) of GBA for 24 h. Fixed cells were stained with Hoechst 33342 (10 µM) and examined by fluorescence microscopy at a magnification of  $\times 400$ . The arrows indicate apoptotic cells. (B) A flow cytometric analysis of HT-29 cells at the indicated concentration (a; control, b; 50 µg/ml, c; 100 µg/ml, and d; 250 µg/ml) of GBA was conducted after the cells had been incubated for 24 h. The cell cycle distribution was determined by a flow cytometric analysis of the DNA content after staining with propidium iodide. \*\*\*significant vs. control untreated cells ( $p < 0.001$ ).

might be because flavanol is only a part of the large family of flavonoid compounds. On the other hand, lupeol, a triterpene, of guava has been reported as an anticancer compound (Saleem, 2009). Though it's difficult to identify which compounds of GBE play a critical role in anticancer activity against HT-29 cell line, we paid attention to the exorbitant amount of TPC and TFC of GBA.

In conclusion, our results clearly demonstrate that GBA significantly induces cytotoxicity and an increase in the sub-G1 phase of HT-29 cells. These results show that GBA may exert anticancer activity by the induction of apoptosis via inhibition of cell cycle. Therefore, our

**Table 1.** Total phenolic content (TPC) and Total flavanol content (TFC) of GBA (unit: mg/g).

TPC	TFC
575.94 $\pm$ 1.3	35.99 $\pm$ 1.6

All measurements were done in triplicate.

results suggest that GBA may be a potential candidate for a novel therapeutic agent in the field of anticancer drug discovery.

## ACKNOWLEDGMENT

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