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Identification of a New Benzophenone from *Psidium guajava* L. Leaves and its Antineoplastic Effects on Human Colon Cancer Cells

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ABSTRACT: *Psidium guajava* L. leaves have a long history of being consumed as herbal teas in many countries. The aim of this study was to identify compounds with anticancer potentials from *Psidium guajava* L. leaves. Utilizing various extraction and chromatographical techniques, we have isolated one new (**2**) and two known compounds (**1, 3**). Structural analyses by the spectroscopic methods of TOF-MS, ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC identified these three compounds as guavinoside E (**1**), 3,5-dihydroxy-2,4-dimethyl-1-*O*-(6′-*O*-galloyl-*β*-D-glucopyranosyl) -benzophenone (**2**), and guavinoside B (**3**). Cell viability assays showed that compounds **2** and **3** inhibited the growth of HCT116 human colon cancer cells in a dose-dependent manner, where compound **2** was more potent than compound **3**. Based on flow cytometry analysis, compound **2** showed stronger activity in inducing cellular apoptosis in cancer cells than compound **3**. Furthermore, compounds **2** and **3** modulated expression levels of key proteins involved in cell proliferation and apoptotic signaling. Specifically, compound **2** increased the levels of p53, *p*-ERK1/2, *p*-JNK, and cleaved caspases 8 and 9, and compound **3** increased the levels of p53 and cleaved caspase 8. Overall, this study provided identities of three bioactive compounds from *P. guajava* L. leaves and their anti-cancer effects against human colon cancer cells, which could facilitate the utilization of these compounds and *P. guajava* L. leaves as potential chemoprevention agents against colon carcinogenesis.

KEYWORDS: *Psidium guajava* L. *Leaves, Benzophenone, colon cancer, colony formation, apoptosis*

1. Introduction

Colorectal cancer is one of the leading cause of cancer associated death worldwide and its incidence and mortality is closely associated with diet and lifestyle.[1,2] Diet-based cancer chemoprevention is defined as using dietary compounds to reverse, suppress, or prevent carcinogenesis.[3] Due to their potential efficiency and infrequency of side effects, natural products and their bioactive components have potential applications in the prevention of colon cancer.[4] Many bioactive phytochemicals have been reported to affect one or more processes involved in carcinogenesis, including carcinogen metabolism, DNA repair, cellular proliferation, apoptosis, cell cycle, angiogenesis, and metastasis.[5-7] Therefore, increasing attention is being paid to exploring new bioactive compounds derived from fruits, vegetables, and natural plants for their anti-cancer properties.

P. guajava L., belonging to the Myrtaceae family, has been found in tropical and subtropical areas worldwide, particularly in Mexico, South America, Europe, Africa, and Asia.[8] The fruits of *P. guajava* L. are a good source of nutrition, as well as medicine.[9-11] In addition to the fruits, other parts of the plant, such as the leaves, have also been widely utilized. In Japan, a guava leaf tea, which contains aqueous guava leaf extract, is now commercially available and approved as one of the Foods for Specified Health Uses.[12] Studies have demonstrated the leaves had various bioactivities, including anti-diarrheal, anti-cancer, anti-hypertensive, anti-oxidant, anti-microbial, hepatoprotective, and hypoglycemic properties.[12-14] These health-promoting activities have been attributed to the abundant bioactive compounds

present in the leaves, including polyphenols, triterpenes, flavonoids, essential oils, saponins, tannins, and alkaloids.[15-17] Methanol extracts of *P. guajava* L. leaves have shown anti-tumor activities against human breast carcinoma benign cells (MCF-7) and murine fibrosarcoma (L929sA) cell lines.[18] The aqueous extract of budding guava leaves showed anti-tumor effects against both human prostate epithelial (PZ-HPV-7) and carcinoma (DU-145) cell lines, as well as anti-angiogenesis and anti-migration activities, respectively.[19, 20] Additionally, essential oils derived from *P. guajava L.* have shown anti-cancer activities when tested against human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines.[21] These studies were performed using concentrated crude extracts of *P. guajava L.* leaves; however, the main active compounds in these extracts were rarely determined and the underlying molecular mechanisms remain to be thoroughly characterized. In this study, we investigated *P. guajava* L. leaves, and isolated one new compound, 3,5-dihydroxy-2,4-dimethyl-1-*O*-(6′-*O*-galloyl-β-D-glucopyranosyl)-benzophenone (**2**), and two previously identified compounds, guavinoside E (**1**), and guavinoside B (**3**). Furthermore, the structures of these compounds were verified through analysis of spectroscopic data (TOF-MS, ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC), and their inhibitory effects against human colon cancer cells and underlying mode of action were characterized.

2. Materials and methods

2.1. Chemicals.

RPMI 1640 media, fetal bovine serum (FBS), and trypsin-EDTA were purchased from Mediatech Inc. (Herndon, VA, USA). DMSO, penicillin, streptomycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for p53 and phosphorylated c-JUN N-terminal kinase (*p*-JNK) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for cleaved caspase 8, cleaved caspase 9 and phosphorylated extracellular signal-regulated kinase p44/42 (*p*-ERK1/2) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibody specific for β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material.

P. guajava L. leaves were collected prior to fruiting in July 2013 in Maoming, Guangdong province, P.R. China, and were identified by associate professor Boru Liao. The collected leaves were air-dried under the shade for one week to keep the moisture content below 5% and stored at 4 °C at the College of Food Science, South China Agricultural University (Guangzhou, China).

2.3. Equipment.

^{1}H NMR, ^{13}C NMR, HSQC, and HMBC spectra (CD_3OD) was recorded with AV 600 NMR spectrometer (Bruker Biospin AG, Switzerland): at 600 (^{1}H) and 150 (^{13}C) MHz; residual solvent peaks as internal standard; Chemical shifts (δ) in ppm and coupling constant (*J*) in Hz. Mass spectra were generated using a Synapt G2-Si

Q-TOF-mass spectrometer (Waters, MA, USA) equipped with an electrospray ionization (ESI) source. The instrument was operated in the negative mode with capillary voltage 2.5 kV, sampling cone 40 V, source temperature 100 °C, desolvation temperature 350 °C, cone gas 50 L/h, desolvation gas 600 L/h, nebulizer 5.5 Bar. All reagents and chemicals were purchased from commercial sources.

2.4. Preparation of Plant Extracts.

The air-dried *P. guajava* L. leaves (118.0 kg) were crushed and extracted three times with 10 equivalent volumes of 70% ethanol. These extracts were filtrated and concentrated in a vacuum evaporator in a water bath, and then further extracted with ethyl acetate. The extracts were once again concentrated and then separated by column chromatography on a 17 L column containing 6.00 kg of silica gel (80-100 mesh) and eluted with a mixture of ethyl acetate and petroleum ether (3:1 v/v). For each elution, 4 L per bottle of the separated solutions were collected, resulting in a total of 75 bottles. Differences between each separation were determined using the thin-layer chromatography (TLC) method with iodine vapor as the detection reagent, and fractions with similar TLC profiles were incorporated. In total, 15 crude fractions (Fr. A-O) were collected. Fraction H (31.6 g) was processed by reverse phase silicone column chromatography with a gradient elution of acetonitrile: H_2O (25:75 to 100:0). Based on TLC analysis, fractions containing similar components were pooled and re-crystallized in methanol to obtain compounds **1** (0.50 g), **2** (0.51 g), and **3** (2.12 g). There is no methanol left for next experiments.

2.5. Cell Culture and Treatments.

Human colon cancer HCT116 and HT29 cells (ATCC, Manassas, VA, USA) were cultured with RPMI 1640 media supplemented with 5% heat inactivated FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37 °C with 5% CO_2 and 95% air. Cells were kept sub-confluent and sub-cultured every 3-4 days. Normal human colon diploid fibroblasts CCD-18Co cells (ATCC, Manassas, VA, USA) were cultured with DMEM media supplemented with 10% heat inactivated FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37 °C with 5% CO_2 and 95% air. DMSO was used as the vehicle for treatment delivery to the cells, and the final concentration of DMSO in the culture media was 0.1% v/v for all experiments.

2.6. Cell Viability Assay.

HCT116 (2000 cell/well), HT29 (2000 cell/well) and CCD-18Co (1.5×10^4 cells/well) cells were seeded into 96-well plates. After incubating for 24 h to allow for cell attachment, the cells were treated with serial dilutions of four compounds of interest (purity ≥ 98%) in serum complete medium. The cell viability was then determined using the MTT assay as previous reported.[22, 23]

2.7. Colony Formation Assay.

Assays assessing colony formation were conducted as previously described.[24] HCT116 cells (500 cells/well) were seeded into 6-well plates. After incubating for 24 h to allow for cell attachment, the cells were treated with several different concentrations of compounds **2** and **3** in 2 mL of serum complete medium. The medium was changed every 4 days. After incubating for 12 days, the HCT116 cell colonies were rinsed with PBS and then stained with 0.2% crystal violet for 15 min.

After rinsing with ddH_2O to remove residual dye, the plates were imaged with high resolution scanner (HP Inc. Palo Alto, CA, USA). Next, 2 mL of 1% SDS were added to each well to solubilize the stain. The plates were agitated on an orbital shaker until the color was uniform with no areas of dense coloration in the bottom of wells. The absorbance of each well was determined at a wavelength of 570 nm.

2.8. Analysis of Apoptosis by Flow Cytometry.

HCT116 cells (5×10^4 cells/mL) were seeded into 6-well plates. After 24 h, the cells were treated with several different concentrations of compounds **2** and **3** in 2 mL of serum complete medium. After another 72 h, any floating cells were harvested, and adherent cells were detached using 0.25% trypsin-EDTA. After washing with 1 mL of ice-cold PBS, the number of apoptotic cells was analyzed as previously described.[25] Apoptosis was quantified using annexin V/PI double staining with an apoptosis detection kit (Biovision, Mountain View, CA, USA). Cells in early apoptosis and late apoptosis/necrosis were quantified with a BD LSR II cell analyzer (BD Biosciences, San Jose, CA, USA).[26]

2.9. Immunoblotting.

HCT116 cells (5×10^4 cells/mL) were seeded into 15 cm cell culture dishes. After incubating for 24 h to allow for cell attachment, the cells were treated with compounds **2** and **3** (80 μM) for 72 h. The cells were then washed with ice-cold PBS and extracted with RIPA lysis buffer containing 25 mM of pH 7.2 Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA; Boston Bioproducts, Ashland, MA, USA)

supplemented with 1% protease inhibitor cocktail. Whole cell lysates were then analyzed by Western blot as previously reported.[27-29] Antibodies specific for p53, *p*-ERK1/2, *p*-JNK, cleaved caspase 8 and cleaved caspase 9 were used for immunoblotting. β-Actin (Sigma-Aldrich) was used as a loading control.

2.10. Statistical Analysis.

All results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Statistically significant differences between groups were identified using Student's two-tailed t-test (two groups) or analysis of variance (ANOVA) when comparing more than two groups. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Identification of three compounds from *P. guajava* L. Leaves.

Utilizing different extraction solvents, silica-gel column chromatography, and crystallization, one new (**2**) and two known compounds (**1, 3**) were successfully isolated from the leaves of *P. guajava* L. and identified. These compounds were guavinoside E (**1**), 3,5-dihydroxy-2,4-dimethyl-1-*O*-(6′-*O*-galloyl-β-D-glucopyranosyl)-benzophenone (**2**), and guavinoside B (**3**).

The chemical structures of these compounds are presented in Figure 1.

Compound **1** was a yellow amorphous powder and had a positive $FeCl_3$ reaction. In its ^{1}H-NMR (CD_3OD, 600 MHz), the peaks were at 2.10 (3 H, s), 2.14 (3 H, s), 2.49 (1 H, t, *J*=8.4 Hz), 3.00 (2 H, m), 3.18 (1 H,t, *J*=8.4 Hz), 3.60 (1 H, dd, *J*=5.8, 11.8 Hz), 3.72 (1 H, dd, *J*=2.4, 11.8 Hz), 4.20 (1 H, d, *J*=7.7 Hz), 7.37 (2 H, t, *J*=7.6 Hz), 7.47 (1 H, t, *J*=7.6 Hz), and 7.61 (2 H, d, *J*=7.6 Hz). In its ^{13}C-NMR (CD_3OD, 150 MHz), the peaks were at 8.5, 9.1, 63.2, 71.7, 74.9, 77.4, 77.6, 105.0, 109.6, 111.2, 113.8, 128.5 (×2), 130.6 (×2), 132.5, 142.8, 153.0, 157.7, 160.7, and 202.5. Compound **1** was determined to be guavinoside E by comparing its ^{1}H and ^{13}C NMR data with the literature.[30]

Compound **2** was a yellow amorphous powder and had a positive $FeCl_3$ reaction. Its molecular weight was 572 amu according to its quasi-molecular ion peak at 571.1471 (M-H) in the ESI-MS. The ^{1}H NMR spectrum (Table 1) of Compound **2** revealed two methyl groups at δ_H 2.08 and 2.14 (each 3 H, s) with one typical galloyl proton signal at δ_H 7.13 (2 H, s). The signals at δ_H 7.2-7.6 (5 H, m) were assigned as benzoyl protons and the anomeric proton signal at δ_H 4.18 (1 H, d, *J*=7.8Hz) suggested a sugar moiety was present. The ^{13}C NMR spectrum (Table 1) of compound **2** contained twenty-three signals, including seven galloyl carbon signals (δ_C 110.40×2, 121.55, 139.83, 146.50×2, and 168.38), six glucose carbon signals (δ_C 64.41, 70.5, 74.85, 75.18, 77.42, and 105.26), two methyl carbon signals (δ_C 8.41 and 9.22), seven

benzoyl carbon signals (δ_C 201.63, 143.20, 131.93, 130.55×2, and 128.28×2), signals for one full substituted benzene carbon based on the m-trihydroxy model (δ_C 160.95, 158.99, 153.40, 112.98, 111.15, and 109.98).

The carbons were assigned based on the HMBC experiments. By carefully comparing compound **2** with the NMR data for the known compound **1**, it is clear that compound **2** contained one additional O-galloyl group. Moreover, in the HMBC spectrum (Figure 2, Supplementary Figure S1), the cross-peaks between δ_H 4.3 (2 H, m, H-6′) and δ_C 168.38 (C-7″) suggested the connection of a galloyl at C-6′ of the sugar unit. Thus, the chemical structure was deduced to be **3**, 3,5-dihydroxy -2,4-dimethyl-1-*O*-(6′-*O*-galloyl-*β*-D-glucopyranosyl)-benzophenone.

Compound **3** was a yellow amorphous powder and had positive $FeCl_3$ reaction. In its ^{1}H-NMR (CD_3OD, 600 MHz), the peaks were at 2.11 (6 H, s), 3.4-3.6 (4 H, m), 4.40 (1 H, dd, *J*=4.2, 12.0 Hz), 4.45 (1 H, dd, *J*=1.8, 12.0 Hz), 4.74 (1 H, d, *J*=7.8 Hz), 7.05 (2 H, s)， 7.37 (2 H, m), 7.49 (1 H, m), 7.61 (2 H, m). In its ^{13}C-NMR (CD_3OD, 150 MHz), the peaks were at 10.0 (×2), 64.3, 71.5, 75.4, 75.8, 77.7, 105.4, 110.2 (×2), 111.6, 112.6 (×2), 121.4, 129.0 (×2), 129.9 (×2), 132.9, 139.8, 141.6, 146.4 (×2), 156.2 (×2), 159.6, and 168.3. Compound **3** was identified as guavinoside B by comparing its NMR data with work by Matsuzaki et al.[31]

3.2. Inhibitory Effects of the Isolated Compounds on human colon cancer cells.

To evaluate the potential anti-cancer properties of the isolated compounds, the ability of these compounds to inhibit the growth of two human colon cancer cell lines was tested by MTT assay. The HCT116 and HT29 cells were treated with serials of concentrations of the three compounds ranging from 20 to 100 μM for 24 h, 48 h and 72 h. As shown in Figure 3, compound **1** showed marginal effects on the growth of both HCT116 and HT29 cells at concentrations up to 100 μM, which concurred with a previous report.[30] Compounds **2** showed growth inhibition on the HCT116 and HT29

cells in a dose-dependent and time-dependent manner. Particularly, after treating 72 h, compound **2** inhibited HCT116 cells growth up to 81.4% at dose of 100 μM, with an IC50 of 60 μM. Compound **2** showed stronger effect on HCT116 cells than on HT29 cells, probably because it is more sensitive to HCT116 cells than HT29. Similar trends were also obtained in HCT116 and HT29 cells after compound **3** treatment. The growth rate of HCT116 cell was inhibited by compound **3** by 66.2% at dose of 100 μM for 72 h, with an IC50 of 80.3 μM. Compound **2** had a significantly stronger inhibition than compound **3** on the two colon cancer cell lines at matching doses of 80, and 100 μM, despite their similar chemical structures. 5-FU, current chemotherapeutics used in colon cancer patients, exhibited significantly stronger inhibition than other three natural compounds on both HCT116 and HT29 cells after 48 h and 72 h treatment. While for HT29 cells, 5-FU showed no inhibition after 24 h treatment as other three compounds.

To determine the toxicity of the isolated compounds on normal colon cells, we evaluated their growth inhibitory effects on CCD-18Co cells by MTT assay. As shown in Figure 3C, the three compounds we isolated (up to 100 μM) did not cause significant inhibitory effect on the growth of normal colon cells, which means these three natural compounds have no toxicity on normal colon cells. These compounds would also need evaluation of any potential side effects when tested at therapeutic doses in vivo. Unfortunately, like most chemotherapeutic agents, 5-FU has numerous toxic effects, such as diarrhea, mucositis, myelosuppression, and thrombophlebitis of peripheral veins.[32] Therefore, it's urgent to explore new bioactive compounds derived

from natural plants for their anti-cancer properties.

Taken together, compound **2** and **3** exhibited the most potent inhibitory effect on HCT116 cells and no toxicity on normal colon cells, so these two compounds were subjected for further investigation as described below.

3.3. The Compounds 2 and 3 Significantly Suppressed Colony Formation of HCT116 Cells.

To further verify the inhibitory effects of compounds **2** and **3** on HCT116 cell growth, colony formation assays were conducted following treatment of HCT116 cells with different concentrations (10, 20, and 30 μM) of the two compounds. As shown in Figure 4, compounds **2** and **3** significantly inhibited the formation of colonies at all concentrations tested. Colony formation was reduced by compound **2** to 70.14, 33.05, and 14.26% of the control at 10, 20 and 30 μM and by compound **3** to 94.08, 42.45, 28.32% of the control at 10, 20, and 30 μM, respectively. At all three concentrations tested, compound **2** showed stronger inhibition on the colony formation of cancer cells than compound **3**. These results were consistent with the inhibitory effects of these two compounds observed in the MTT assays. Overall, these results demonstrated that compounds **2** and **3** significantly inhibited proliferation of HCT116 cells, where inhibition by compound **2** was significantly stronger than by compound **3**.

3.4. Compounds 2 and 3 induced cellular apoptosis in human cancer cells.

Cellular apoptosis is a form of programmed cell death carried out through a specific process. Induction of apoptosis can destroy the balance between cellular

proliferation and death, and abnormality in apoptosis could lead to diseases such as cancer.[33, 34] Consequently, promoting apoptosis in cancer cells has been regarded as an effective approach for cancer chemoprevention.[35] To delineate the mechanisms related to inhibition of cancer cell growth by compounds **2** and **3**, cellular apoptosis of HCT116 cells was evaluated. After treating cells for 72 h with compounds **2** and **3**, flow cytometry was used with Annexin V/Propidium Iodine (PI) double staining to detect and quantify cells in early and late apoptosis. As shown in Figure 5, 40, 60, and 80 μM of compound **2** increased the size of the apoptotic cell population by 1.50-fold (3.65%), 2.33-fold (5.67%), and 10.08-fold (24.53%), respectively, as compared to the control cells (2.43%) in a dose-dependent manner. While compound **3** did not significantly induce apoptosis in HCT116 cells at 40 and 60 μM, while at 80 μM compound **3** increased the apoptotic cell population by 3.64-fold (8.87%) compared to the control (2.43%). Compound **2** exhibited significantly stronger effects in the induction of cellular apoptosis in cancer cells than compound **3**, which is similar to the results obtained from the growth inhibition and colony formation assays. Therefore, our results demonstrated that compounds **2** and **3** inhibited HCT116 cell growth at least partially by increasing the programmed cell death in cancer cells, with compound **2** being much stronger in inducing cellular apoptosis than compound **3**. These findings were in accordance with previous reports characterizing the anti-tumor properties of *P. guajava* L. extracts, which were closely associated with the induction of apoptosis.[36]

3.5. Modulation of Key Proteins Related to Cell Proliferation and Apoptosis by

Isolated Compounds.

To further determine the molecular mechanisms underlying inhibition of HCT116 cells by compounds **2** and **3**, the effects of these compounds on the expression of key proteins associated with cell proliferation and apoptosis were evaluated. As a tumor suppressor protein, p53 plays important roles in inhibiting carcinogenesis and controlling the cellular response to DNA-damaging agents, such as cancer chemotherapeutics and ionizing radiation.[37, 38] Mutation of the p53 leading to the loss of its function is one of the most commonly detected abnormalities in human cancers. In this study, it was found treatment with compounds **2** and **3** led to significantly increased expression levels of p53 by 38% and 45%, respectively, compared to the control, which could lead to subsequent cellular events including apoptosis. These results support a previously published study on the association of upregulation of p53 by β-caryophyllene oxide isolated from *P. guajava* L. and the resulting apoptotic effects.[39]

Mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases that play important roles in the regulation of cell growth, proliferation, differentiation, and apoptosis.[40] MAPKs include growth factor-regulated extracellular signal-related kinases (ERKs), stress-activated MAPKs, and c-Jun NH2-terminal kinases (JNKs).[41] ERK and JNK have been proved to promote cell apoptosis[42, 43]. Following treatment with compounds **2**, levels of *p*-ERK1/2 and *p*-JNK proteins were significantly elevated by 5.45- and 1.64-fold, respectively, compared to the control. While the total-protein of ERK and JNK did not show any difference between control and

compound **2** treatment group. Meanwhile, compound **3** had no significant effect on the expression of *p*-ERK1/2 and *p*-JNK at the concentrations tested, suggesting that inhibition of cell growth by compound **3** is not through MAPK signaling pathway.

Activation of the initiator caspase 8 by death receptors or caspase 9 by mitochondria can, in turn, activate the effector caspases 3, 6, and 7. These activated caspases then cleave structural proteins, which ultimately leads to apoptosis.[44-46] Therefore, caspases 8 and 9 are key regulators of apoptosis. Compound **2** induced a significant increase of 2.02- and 1.41-fold in the levels of cleaved caspases 8 and 9, respectively, compared to the control. However, compound **3** only upregulated the expression of cleaved caspase 8 by 1.76-fold and had no effect on the expression of cleaved caspase 9 compared to the control.

In summary, compound **2** (80 μM) significantly increased expression levels of p53, *p*-ERK1/2, *p*-JNK, and cleaved caspases 8 and 9 in HCT116 cells. Compound **3** (80 μM) significantly increased the expression levels of p53 and cleaved caspase 8, but it did not significantly change the expression levels of *p*-ERK1/2 and cleaved caspase 9 in HCT116 cells. Overall, compound 2 showed stronger effect on modulation of the key proteins related to cell proliferation and apoptosis on HCT116 cells than compound **3**, despite their similar chemical structures. This finding was in consistent with previous results we obtained in cell proliferation, colony formation and apoptosis. The different effects of the two compounds might be associated with the different position of the glycoside on the hydroxyl moiety. However, our data are yet too preliminary to predict an exact structure-activity relationship, we need further

experiments to verify our hypothesis.

4. Conclusion

In conclusion, we identified three compounds, including a new benzophenone glucoside, from *P. guajava* L. leaves. In addition, this study demonstrated that compounds **2** and **3** effectively inhibited the growth and colony formation of the human colon cancer line HCT116 by inducing apoptosis and modulating multiple signaling proteins involved in regulation of proliferation and programmed cell death. Furthermore, compound **2** was found to not only be a stronger inhibitor of growth and colony formation than compound **3**, but it was also more effective at inducing apoptosis and influencing related signaling proteins. These differences between the two compounds may due to differences in glycoside position, which could influence the bioactivity of these two compounds. Overall, the results of this study provide insight into the anti-tumor components of *P. guajava* L. leaves and demonstrated that compound **2** has promising anti-cancer properties. This suggests that *P. guaijava* L. or its preparations containing the compounds **2** and **3** have the potential to be used as new agents for colon cancer prevention. These results warrant future investigations on the *in vivo* cancer chemoprevention effects of *P. guaijava* L. or these compounds.

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Notes

The authors have no competing financial interests to declare.

Abbreviations

TLC: thin-layer chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; DMSO, dimethyl sulfoxide; IR, inhibitory rate; PI, Propidium iodide; ERK1/2, extra cellular signal-regulated kinase p44/42; JNK, phosphorylated c-JUN N-terminal kinase.

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Figures Captions

Figure 1. Chemical structures of the three compounds, guavinoside E (**1**), 3,5-dihydroxy-2,4-dimethyl-1-*O*-(6′-*O*-galloyl-β-D-glucopyranosyl)-benzophenone (**2**), and guavinoside B (**3**), isolated from the leaves of *P. guajava* L.

Figure 2. Key HMBC correlations for compound **2.**

Figure 3. Inhibitory effects of compounds on human colon cancer cells HCT116 (A) and HT29 (B), and normal human colon cells CCD-18Co (C). Cells were treated with different concentrations of the test compounds for 24 h, 48 h, and 72 h. Growth inhibition was measured by MTT assay as described in the Materials and Methods. Data are expressed as mean ± SD (n = 6, from three independent experiments), and the different notations indicate statistically significant difference ($p< 0.01$) based on ANOVA analysis followed by Duncan's multiple range test. "#" indicates significant difference between the effects of compounds **2** and **3**, as well as at different concentrations ($p<0.01$). "*" indicates a significant difference between the effects of compounds **1** and **2** ($p< 0.01$). "**" indicates a significant difference between the effects of compounds **2** and 5-FU ($p< 0.01$).

Figure 4. Compounds **2** and **3** inhibited colony formation of HCT116 cells. Cells were seeded into 6-well plates. After incubating for 24 h, cells were treated with serial dilutions of compounds **2** and **3**. After incubating for 12 days, the colonies formed were photographed and measured as described in the Materials and Methods. Data are expressed as mean ± SD (n=3), and the different notations in the bar charts indicate statistically significant difference ($p< 0.01$) based on Student's two-tailed t-test.

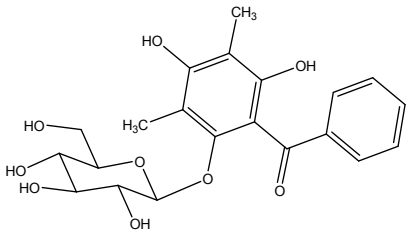
Figure 5. Effects of compounds **2** and **3** on apoptosis of HCT116 cells. Cells were seeded in 6-well plates for 24 h, and then treated with compounds **2** and **3**. After treatment for 72 h, cells were collected and the frequency of apoptosis was measured as described in the Materials and Methods. Data are expressed as mean ± SD (n = 3). Statistical analysis was conducted separately for all treatments for early and late apoptosis. The different notations in the bar graphs indicate statistically significant differences ($p<0.01$) based on ANOVA analysis followed by Duncan's multiple range test.

Figure 6. Effects of compounds **2** and **3** on key proteins related to cellular proliferation and apoptosis in HCT116 cells. Cells were seeded in 15 cm culture dishes for 24 h, and then treated with 80 μM compounds **2** and **3**. After incubating for 72 h, the cells were collected and analyzed by Western blot as described in the Materials and Methods. The numbers underneath the blots represent band intensity (normalized to β-actin, means are from three independent experiments) as measured by ImageJ software. The standard deviations (all within ±15% of the means) are not shown. The experiments were repeated three times. β-actin served as a loading control. *indicates statistical significance in comparison with the control ($p< 0.05$, n = 3).

Table Captions

Table 1. NMR data of compound **2** (CD_3OD, 600 MHz for ^1H-NMR, and 150 MHz for ^{13}C-NMR)

Figure 1.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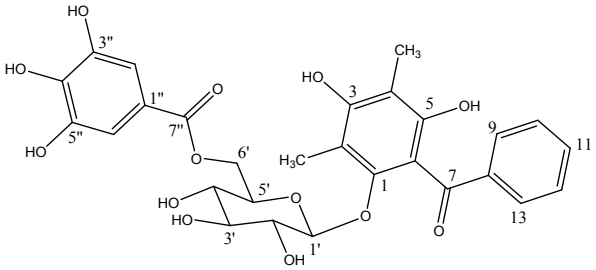
H3C
HO
HO
HO
O
O
OH
O

1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HO
1"
7"
O
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5"
HO
6'
CH3
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H3C
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5'
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13
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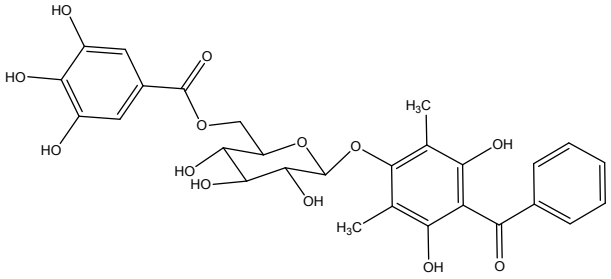
OH

2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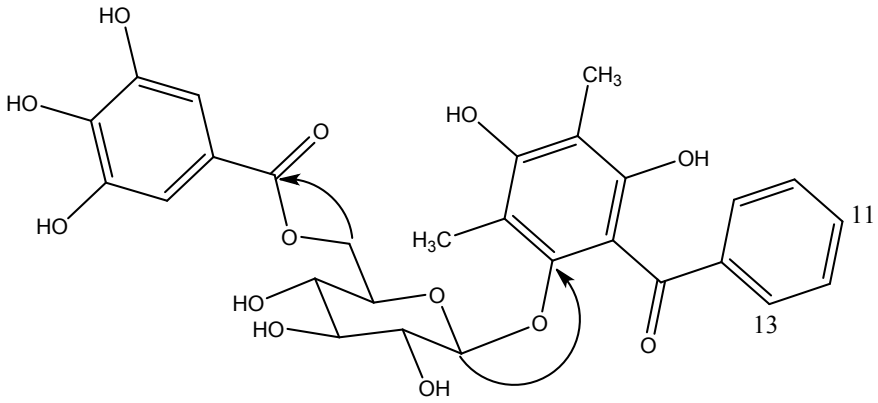
O

3

547 **Figure 2.**



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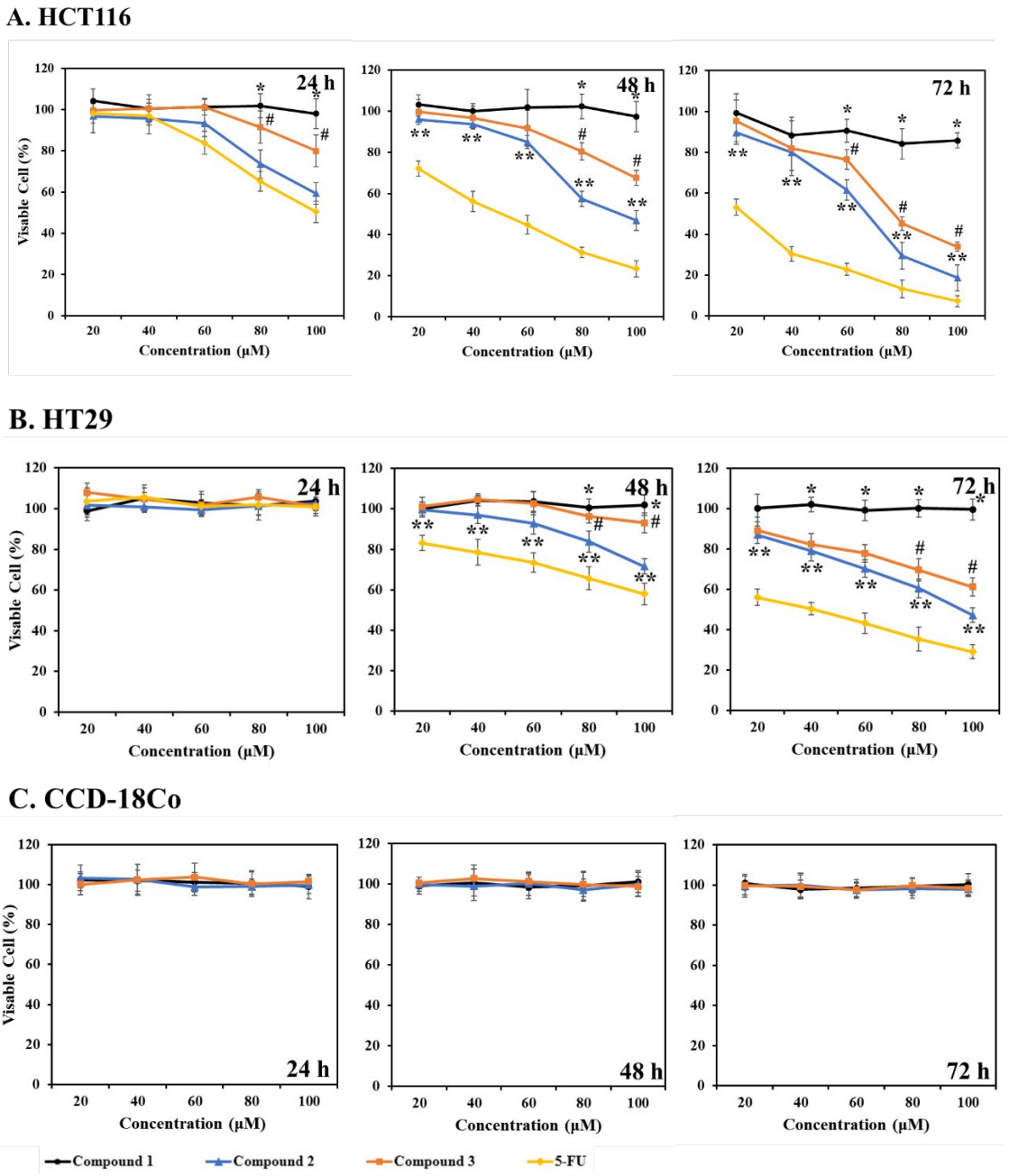
549 **Figure 3.**



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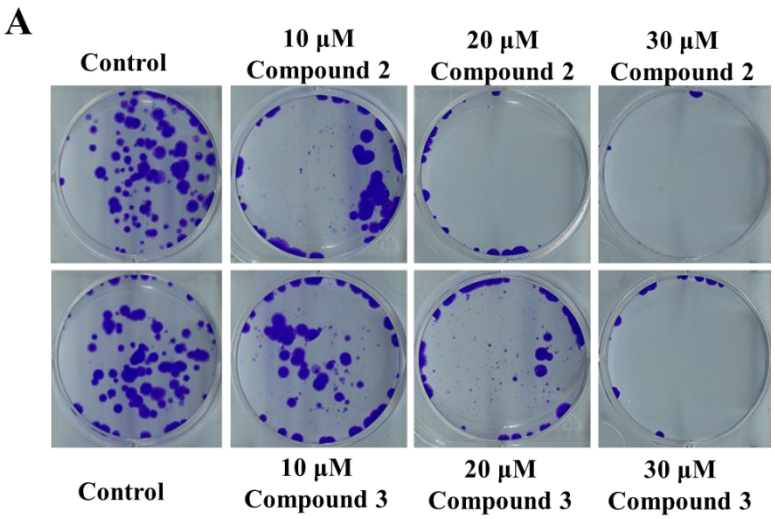
553 **Figure 4.**



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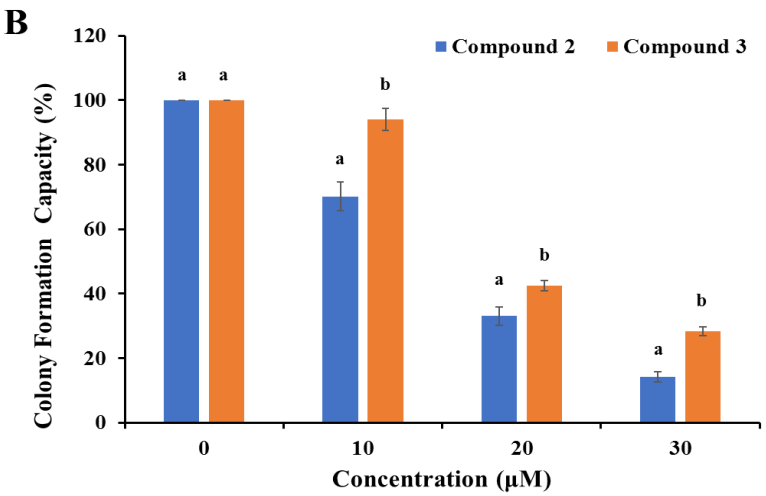

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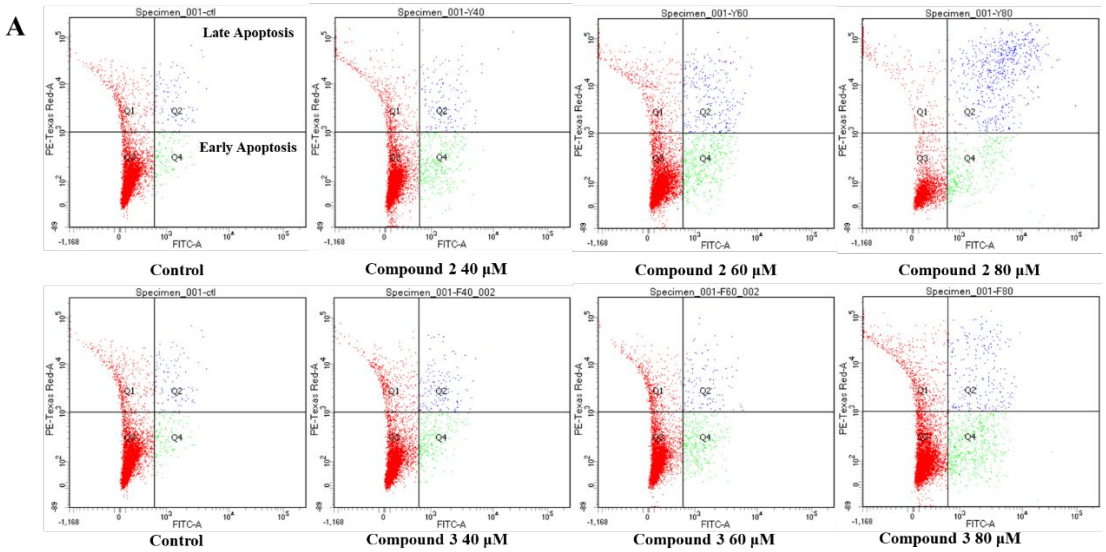
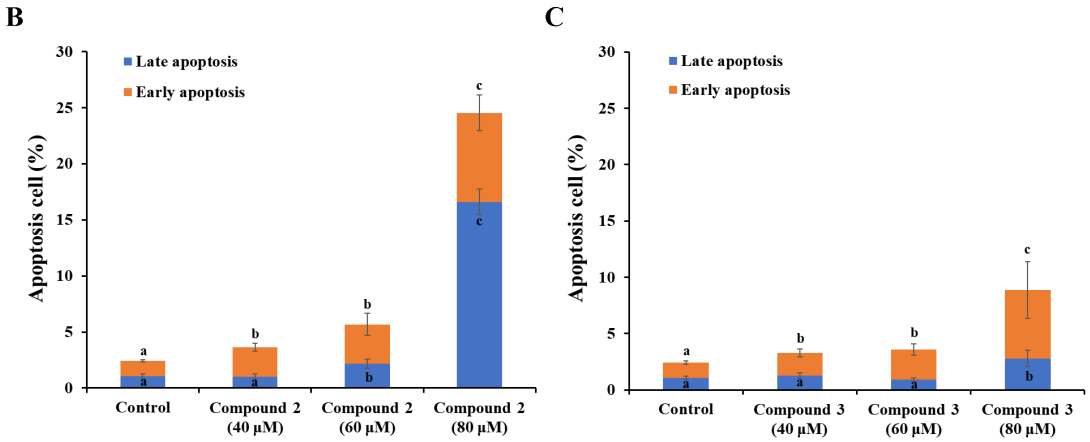
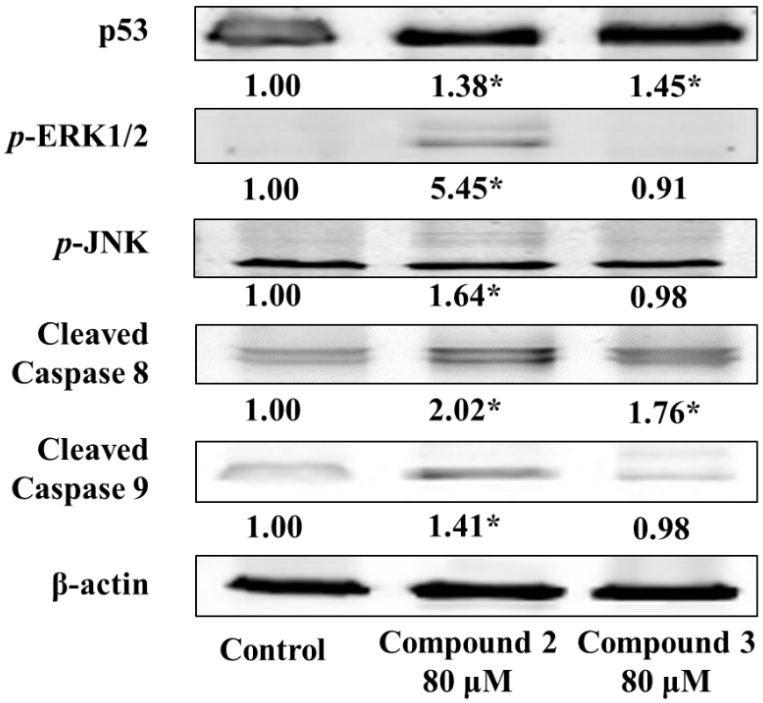
Figure 5.


A
Late Apoptosis
Early Apoptosis
Control
Compound 2 40 μM
Compound 2 60 μM
Compound 2 80 μM
Control
Compound 3 40 μM
Compound 3 60 μM
Compound 3 80 μM


B
C
Late apoptosis
Early apoptosis
Apoptosis cell (%)
Control
Compound 2 (40 μM)
Compound 2 (60 μM)
Compound 2 (80 μM)
Compound 3 (40 μM)
Compound 3 (60 μM)
Compound 3 (80 μM)

559 **Figure 6.**



560

561 **Table 1.**

Proton		Carbon	
		C-1	153.40
		C-2	111.15
2-CH_3	2.14 (3H, s)	2-CH_3	9.22
		C-3	160.95
		C-4	109.98
4-CH_3	2.08 (3H, s)	4-CH_3	8.41
		C-5	158.99
		C-6	112.98
		C-7	201.63
		C-8	131.93
H-9	7.45 (1H, m)	C-9	130.55
H-10	7.27 (1H, m)	C-10	128.28
H-11	7.39 (1H, m)	C-11	143.20
H-12	7.27 (1H, m)	C-12	128.28
H-13	7.45 (1H, m)	C-13	130.55
H-1′	4.18 (1H, d, *J*=7.8Hz)	C-1′	105.26
H-2′		C-2′	74.85
H-3′	2.55 (1H, t, *J*=8.4Hz)	C-3′	77.42
H-4′		C-4′	70.5
H-5′		C-5′	75.18
H-6′	4.3 (2H, m)	C-6′	64.41
		C-1″	121.55
H-2″	7.13 (1H, s)	C-2″	110.40
		C-3″	146.50
		C-4″	139.83
		C-5″	146.50
H-6″	7.13 (1H, s)	C-6″	110.40
		C-7″	168.38

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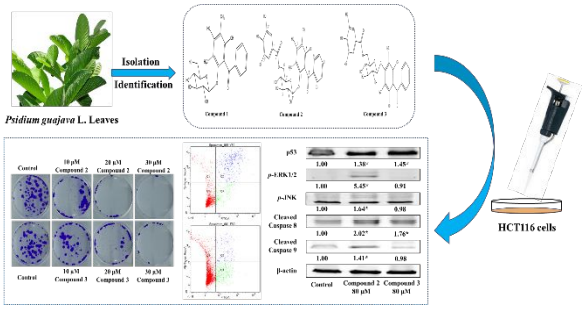
Graphical abstract:



We identified a new benzophenone from *Psidium guajava* L. Leaves, explored its antineoplastic effects and molecular mechanism on HCT116 cells.