

Stilbenes contribute to the anticancer effects of *Rheum undulatum* L. through activation of apoptosis

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Abstract. *Rheum undulatum* L. (*R. undulatum*) is a medicinal plant used for the treatment of inflammatory diseases in East Asian countries. Numerous stilbenes isolated from *R. undulatum* have been revealed to possess anticancer effects. The aim of the present study was to evaluate the effect of extracts and compounds isolated from *R. undulatum* on human gastric cancer cell viability and to elucidate their molecular mechanism of action on the apoptosis pathway. The results demonstrated that aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside, isolated from the methanolic extract of dried rhizomes of *R. undulatum*, exhibited anti-proliferative effects on the human gastric carcinoma cell line AGS, with IC₅₀ values of 84.66 \pm 0.44 and 68.28 \pm 0.29 μ M, respectively. The percentage of apoptotic cells increased significantly following treatment with each compound at a concentration of 100 μ M, compared with that in the non-treated group in the image-based cytometry assay. Western blot analysis revealed that these compounds activated the caspase cascade and inhibited B-cell lymphoma-2, an anti-apoptotic protein.

Introduction

Gastric cancer accounts for ~10% of annual cancer-associated mortalities globally in 2011 (1). Gastric cancer is more prevalent in South Korea, Japan, Eastern Europe and South America, compared with other geographical regions (2012) (2). According to cancer statistics in 2013, gastric cancer was the

most frequently diagnosed cancer in males, and the fourth most common cancer among females in South Korea (3). The risk factors for gastric cancer include *Helicobacter pylori* infection, cigarette smoking, alcohol abuse, obesity and a history of gastritis (2). Surgery is considered to be the most effective treatment for gastric cancer, while adjuvant therapies, including chemotherapy and chemoradiation, are additionally used to improve patient survival by up to 15% (4). Furthermore, owing to the diverse resources of medicinal plants, previous researchers have focused on screening natural compounds as molecular targets for cancer prevention, which has resulted in the discovery of numerous anticancer agents, including curcumin, ginsenosides, genistein, and (-)-epigallocatechin-3-gallate (5).

Rheum undulatum L. (*R. undulatum*), also known as rhubarb, is a well-established East-Asian traditional medicine for the treatment of inflammation (6,7), allergies (8), dental diseases (6) and blood stagnation (7,9). Previous studies also revealed that *R. undulatum* has numerous pharmacological effects, including hepatocyte protective (10), anti-obesity and hypolipidemic (7,11), anti-cariogenic (12), anti-allergic (8,9), tyrosinase inhibition (13), anti-diabetic (14), anti-platelet aggregation (15), anti-atherosclerotic (16) and anticancer (6,17-20) effects. Among the natural compounds of *R. undulatum*, stilbenes, a group of non-flavonoid phenolic compounds, are potential candidates for cancer chemoprevention (21). Numerous stilbenes isolated from *R. undulatum*, including deoxyrhapontigenin (18), piceatannol (19) and pterostilbene (20), exhibit the ability to mediate cancer cell death.

Programmed cell death is a key mechanism underlying the pathogenesis of numerous diseases, particularly cancer. Apoptosis, autophagy and programmed necrosis are the three main types of programmed cell death (22). Apoptosis is the principal type of cell death triggered by DNA damage (22). Apoptosis is induced by two independent pathways comprising of the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. In the extrinsic pathway, initiator caspases (caspase-8 and -10) are activated when pro-apoptotic ligands bind to their cognate death receptors, tumor necrosis factor receptor superfamily member 10a (TRAIL) and FAS, via adaptor proteins, such as Fas Associated Via Death Domain (23). Following activation, these initiator caspases cleave and stimulate effector caspases (caspase-3 and -7) to

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induce cell apoptosis (23). The intrinsic pathway is regulated by the interactions between pro- and anti-apoptotic B-cell lymphoma-2 (BCL-2) family proteins. When DNA damage or metabolic stress occurs, BCL-2-associated X (BAX) and BCL-2 antagonist/killer (BAK) are temporarily stimulated by the activation of BH3-only protein, and BCL-2, BCL-extra large or myeloid cell leukemia 1 are inhibited (23). BAX and BAK are also activated by BH3 interacting domain death agonist (BID), which facilitates a crosstalk between the extrinsic and intrinsic pathways (23). The activation of BAX and BAK stimulates major outer membrane porin, which results in the release of second mitochondria-derived activator of caspase (SMAC) and cytochrome *c* (23). SMAC blocks X-linked inhibitor of apoptosis, an inhibitor of caspase-9, to facilitate the activation of caspase-9 induced by apoptosome formation when cytochrome *c* interacts with apoptotic peptidase activating factor 1 (23). Finally, active caspase-9 activates caspase-3 and -7 to initiate apoptotic cell death (23).

Numerous regulatory factors as mentioned above contribute to the control of apoptotic pathways in multicellular organisms and any abnormal expression of these factors may result in cancer (23). Therefore, an important strategy for cancer chemoprevention is stimulating apoptotic pathways by inhibiting anti-apoptotic BCL-2 family proteins or activating TRAIL death receptors and the caspase cascade (24). The aim of the present study was to evaluate the effects of extracts and compounds isolated from *R. undulatum* on human gastric cancer cell viability and the stimulation of apoptotic cell death, and to elucidate their molecular mechanisms of action on the apoptosis pathway.

Materials and methods

Plant material. The rhizomes of *R. undulatum* were purchased from Kyung-dong Herbal Market (Seoul, South Korea) in 2015. The plant material was authenticated by Dr Rack-Seon Seong, the director of Center of Natural Resources Research, Jeonnam Bioindustry Foundation (Jeonnam, South Korea). The voucher specimen of this plant material (RU201506) was deposited in the Herbarium of College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University (Incheon, South Korea).

Chemicals and reagents. The Ez-Cytox Cell Viability assay kit was acquired from Daeil Lab Service Co., Ltd. (Seoul, South Korea). The primary antibodies against BID (cat. no. 2002; 1:1,000 dilution), BAX (cat. no. 2772; 1:1,000 dilution), BCL-2 (cat. no. 2876; 1:1,000 dilution), cleaved caspase-3 (cat. no. 9661; 1:1,000 dilution), cleaved caspase-8 (cat. no. 9496; 1:1,000 dilution), poly (ADP-ribose) polymerase (PARP) (cat. no. 9542; 1:1,000 dilution), and β -actin (cat. no. 4967; 1:2,000 dilution), the horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (cat. no. 7074; 1:3,000 dilution), and the radioimmunoprecipitation assay (RIPA) buffer were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Pierce™ BCA Protein assay kit was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Sample preparation for cell viability assay. The dried rhizomes of *R. undulatum* were extracted using a Soxhlet extractor with

3 different methods to prepare samples for the cell viability assay. A total of 10 g dried rhizomes of *R. undulatum* was extracted with 100 ml 100% ethanol (EtOH) at 60°C for 24 h to yield the RU1 extract (4.96 g), whereas 10 g dried rhizomes was extracted with 100 ml 50% EtOH at 100°C for 24 h to yield the RU2 extract (5.65 g). For the last sample, 10 g dried rhizomes was extracted with 100 ml of 50% EtOH at 60°C for 13 h to yield the RU3 extract (5.85 g). The 0.5% DMSO-treated group was used as a control.

Extraction and isolation of stilbene compounds. The dried rhizomes of *R. undulatum* (6.0 kg) were sonicated thrice for 4 h at 65°C with 5.0 l of 100% methanol. The solvent was removed *in vacuo* to yield 700.0 g of crude extract. Following dispersion in 2 l of water, the crude extract was solvent-partitioned sequentially with 2 l of chloroform (CHCl₃) and 2 l of ethyl acetate (EtOAc) to give CHCl₃ (7.7 g), EtOAc (118.0 g), and H₂O (335.0 g) fractions. The EtOAc fraction (118.0 g) was separated by silica gel open column chromatography (4.5 cm diameter x 60 cm long; Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck, NJ, USA) with a CHCl₃-MeOH (5:1 to 1:1, v/v, SK Chemicals, South Korea) gradient solvent system to yield E1, E2 and E3 fractions. Compounds C1 (17 mg) and C2 (14 mg) were acquired from the E1 (5.4 g) fraction using silica gel open column chromatography (3.5 cm diameter * 30 cm long) with an *n*-hexane-EtOAc (2.5:1, v/v, SK Chemicals, Gyeonggi-do, South Korea) isocratic solvent system. The E2 fraction (10.6 g) was separated on a silica open gel column (3.5 cm diameter x 35 cm long) eluted with a CHCl₃-MeOH-H₂O (3.5:1:0.15, v/v/v) isocratic solvent system to obtain E2A (2.3 g), E2B (1.8 g), and E2C (2.0 g) subfractions. Compounds C3 (45 mg) and C4 (100 mg) were isolated from the E2C subfraction using YMC RP-18 open column chromatography (150 μ m, Fuji Silysia Chemical Ltd., Japan; 2.5 cm diameter * 30 cm long) with a MeOH-H₂O (1:1.2, v/v) isocratic solvent system. All the open column chromatography was performed at the room temperature at a flow rate of 3 ml/min. The isolated compounds were identified as aloe-emodin (C1), chrysophanol 1-O- β -D-glucopyranoside (C2), rhapontigenin (C3) and rhaponticin (C4), by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), 1D and 2D NMR spectroscopic analysis. The process of isolation is summarized in Fig. 2.

Cell culture. The human gastric cancer cell line AGS was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured with RPMI-1640 medium (Cellgro; Corning Incorporated, Corning, NY, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), and supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 1% sodium pyruvate. The cultured cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability assay. AGS cells were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h at 37°C, and then treated with 25, 50, 100 and 200 μ g/ml of the *R. undulatum* extracts. Following 24 h of treatment, the cultured cells were incubated with an Ez-Cytox Cell Viability assay kit for 1 h at 37°C, according to the manufacturer's protocol. Cell viability

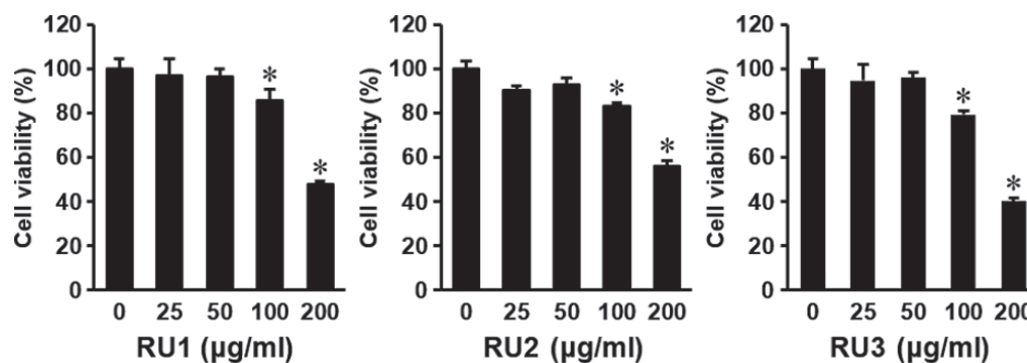


Figure 1. Effect of *Rheum undulatum* dried rhizome extracts on the proliferation of the human gastric carcinoma cell line AGS. The dried rhizomes of *Rheum undulatum* were extracted with Soxhlet extractor by different methods to prepare samples for the cell viability assay. RU1: with 100% EtOH at 60°C for 24 h; RU2: with 50% EtOH at 100°C for 24 h; and RU3: with 50% EtOH at 60°C for 13 h. AGS cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were treated with test samples at concentrations ranging from 25 to 200 µg/ml to evaluate cytotoxicity. Cell viability was determined using an EZ-Cytox Cell Viability assay kit for 24 h following treatment with *Rheum undulatum*. *P<0.05 vs. the 0.5% DMSO-treated (control) group.

was evaluated by measuring the optical density change at 450 nm using a microplate reader (PowerWave XS; BioTek Instruments, Inc., Winooski, VT, USA).

Image-based cytometry assay. AGS cells were seeded on 6-well plates at a density of 4×10^5 cells/well for 24 h at 37°C in order to allow the cells to adhere to the bottom of the well, and were then treated with the isolated compounds of *R. undulatum* at concentrations of 100 µM. Following a 12 h treatment, the cells were collected and stained in Annexin Binding Buffer (Thermo Fisher Scientific, Inc.) with Annexin V Alexa Fluor 488 (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min in dark at the room temperature. Annexin V-positive cells were determined with a Tali image-based cytometer (Invitrogen; Thermo Fisher Scientific, Inc.) and analyzed by TaliPCApp version 1.0 software (Invitrogen; Thermo Fisher Scientific, Inc.).

Western blot analysis. AGS cells were seeded on 6-well plates at a density of 4×10^5 cells/well and treated with aloe-emodin and chrysophanol 1-O-β-D-glucopyranoside at a concentration of 100 µM. At 24 h following treatment, the cells were collected and lysed in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride to obtain whole-cell extracts. Proteins in the whole-cell extracts of each test sample were quantified by using the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of proteins (20 µg/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature. The separated proteins were identified by incubation at 20°C with epitope-specific primary and secondary antibodies (as described previously) prior to visualization with ECL Advance Western Blotting Detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK) and a FUSION Solo Chemiluminescence system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Statistical analysis. The data are presented as the mean ± standard deviation. Statistical significance was determined by one-way analysis of variance with a Bonferroni correction for multiple comparisons using SigmaStat

version 4.0 software (Systat Software, Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with *R. undulatum* extracts reduces cell viability. The viability of AGS cells was determined using cell viability assays to evaluate the cytotoxic effects of the three extracts from the dried rhizomes of *R. undulatum*. The extracts, designated RU1, RU2 and RU3, were prepared using different extraction methods. AGS cells were seeded on 96-well plates at a density of 1×10^4 cells/well and then treated with the three extracts at concentrations (25, 50, 100 and 200 µg/ml). Following 24 h of treatment, cell viability was evaluated using the Exactor Cell Viability assay kit. As depicted in Fig. 1, all extracts exhibited antiproliferative effects on AGS cells in a dose-dependent manner at concentrations of 100 and 200 µg/ml. The IC₅₀ values of RU1, RU2, and RU3 extracts were 208.02 ± 2.99 , 222.67 ± 2.10 , and 175.89 ± 1.40 µg/ml, respectively (Fig. 1). The RU3 extract, which was extracted using 50% EtOH as a solvent and heated at 60°C for 13 h, had the strongest antiproliferative effect on AGS cells. A number of previous studies focused on the anticancer effects of the methanolic extract of *R. undulatum* (6,18,20). In the present study, the ethanolic extract of this medicinal plant also exhibited inhibitory effects on cancer cell proliferation. Collectively, these results demonstrate that the differences in ethanol concentration, and temperature and time of extraction process, affected the pharmacological effect of extracts.

Compounds isolated from *R. undulatum* reduce cell viability in a dose-dependent manner. The isolated compounds were also evaluated for cytotoxic effects on the human gastric carcinoma cell line AGS. The results are depicted in Fig. 3. Among the four isolated compounds, aloe-emodin and chrysophanol 1-O-β-D-glucopyranoside exhibited an increased potential for anti-proliferative effects on AGS cells. The IC₅₀ values of aloe-emodin and chrysophanol 1-O-β-D-glucopyranoside were 84.66 ± 0.44 and 68.28 ± 0.29 µM, respectively (Fig. 3). To confirm whether the anticancer effects of aloe-emodin and chrysophanol 1-O-β-D-glucopyranoside on AGS cells were

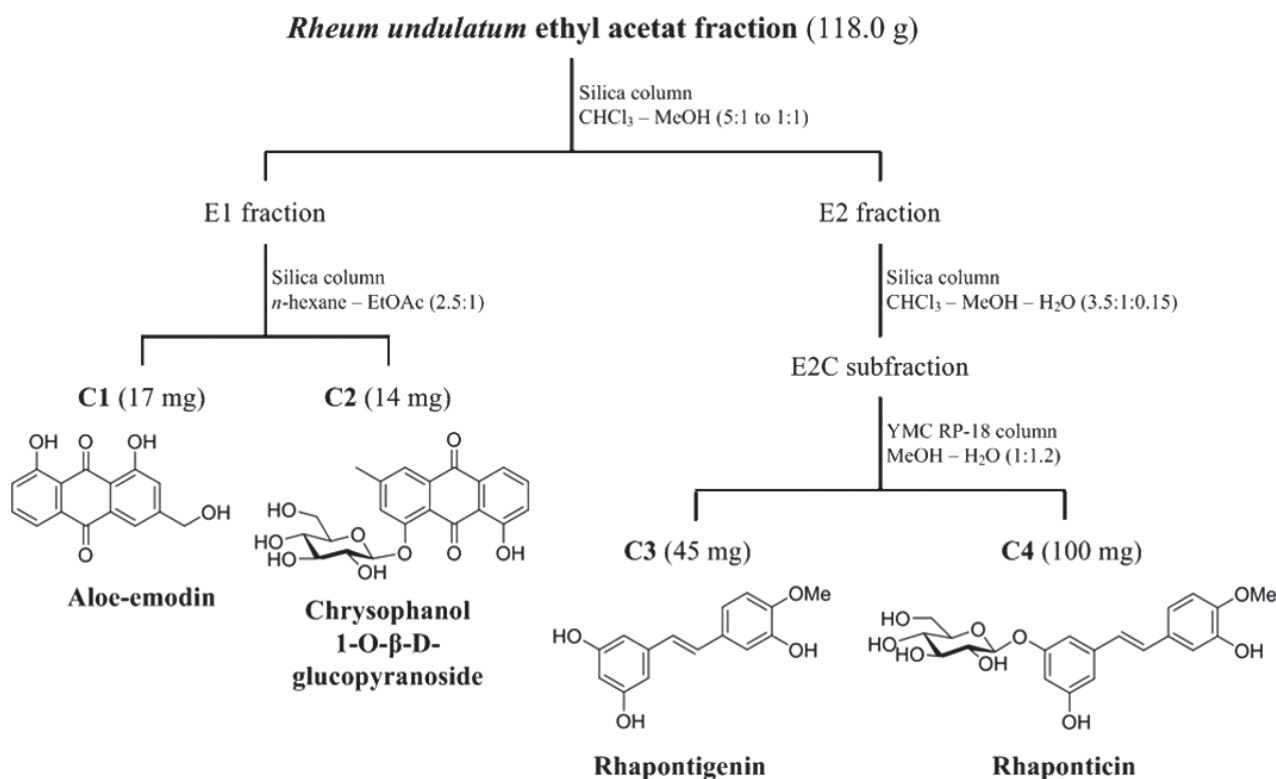


Figure 2. Isolation scheme of compounds from the ethyl acetate fraction of *R. undulatum*. C1: aloe-emodin; C2: chrysophanol 1-O-β-D-glucopyranoside; C3: rhapontigenin; and C4: rhaponticin.

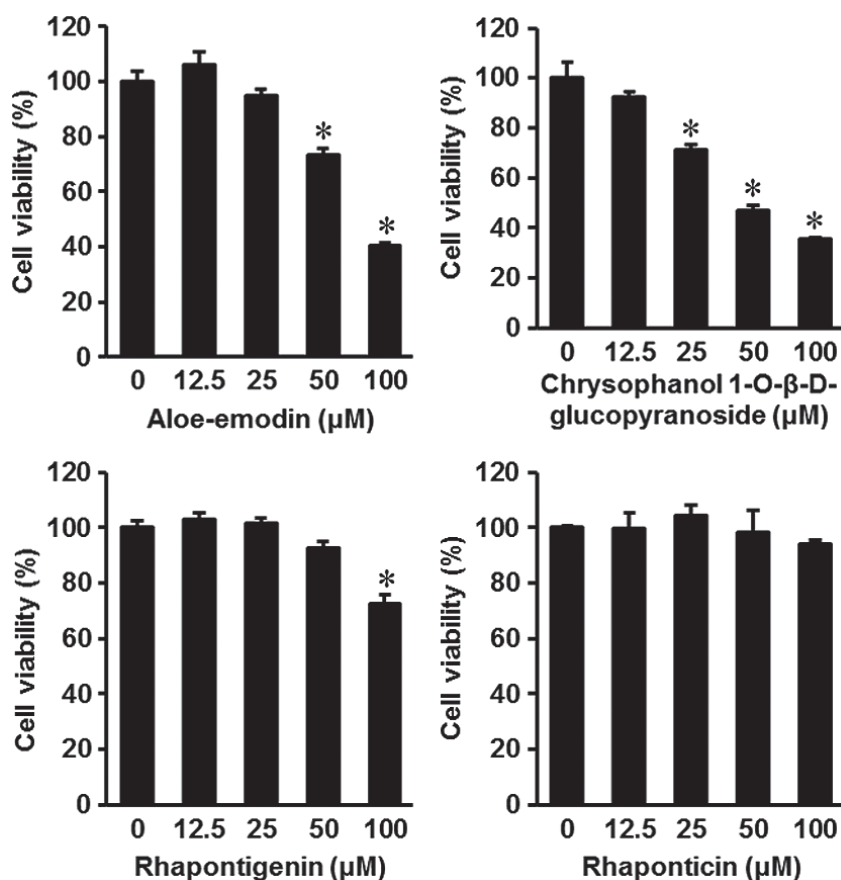


Figure 3. Effect of compounds isolated from *Rheum undulatum* dried rhizomes on the proliferation of the human gastric carcinoma cell line AGS. The AGS cells were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h. The cells were treated with aloe-emodin and chrysophanol 1-O-β-D-glucopyranoside at concentrations of 12.5, 25, 50 and 100 μM to evaluate cytotoxicity. Cell viability was determined using an EZ-Cytox Cell Viability assay kit for 24 h after treatment. *P<0.05 vs. 0.5% DMSO-treated (control) group.

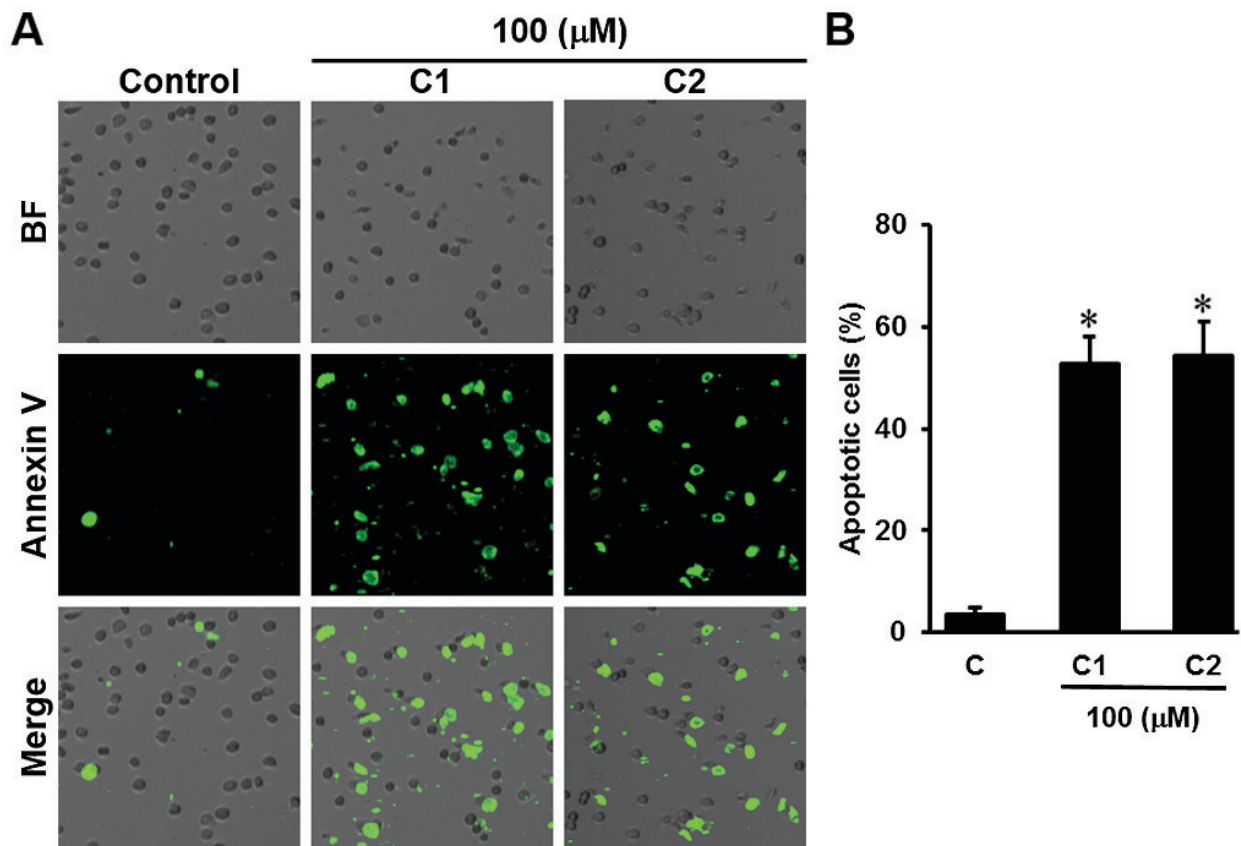


Figure 4. Effect of *Rheum undulatum* compounds on the induction of apoptotic cell death. (A) Microscopic pictures from image-based cytometry assay. Cells were treated with aloe-emodin or chrysophanol 1-O- β -D-glucopyranoside at a concentration of 100 μ M. Following treatment with these compounds for 12 h, the cells were stained with Annexin V-Alexa Fluor 488 to identify the apoptotic cells. The magnification cells: from left to right, AGS cells were treated with 0.5% DMSO (Control), aloe-emodin (C1), or chrysophanol 1-O- β -D-glucopyranoside (C2); from top to bottom, the same magnification cells were taken pictures under bright field (BF), fluorescence to detect Annexin V-stained cells (Annexin V), or the merge form of bright field and fluorescence (Merge). (B) The comparative graph illustrates the percentage of apoptotic cells in each group. * $P < 0.05$ vs. the 0.5% DMSO-treated (control) group. C: 0.5% DMSO-treated (control) group; C1, aloe-emodin; C2, chrysophanol 1-O- β -D-glucopyranoside; and BF, bright field.

mediated by apoptotic pathway activation, image-based cytometry was performed. Following treatment with aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside at a concentration of 100 μ M, the percentage of apoptotic cells labeled with Annexin V significantly increased ($P < 0.05$) to 52.66 ± 5.50 and $54.33 \pm 6.65\%$, respectively, compared with $3.33 \pm 1.52\%$ in the control group (Fig. 4).

Aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside increase the expression of apoptosis-associated proteins in AGS cells. Western blot analysis was performed to identify the mechanisms by which aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside induced apoptosis. Western blot analysis results demonstrated that aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside stimulated the expression of apoptosis-associated proteins in AGS cells. As depicted in Fig. 5, full-length and cleaved caspase-8, and full-length caspase-3 expression markedly increased following treatment with aloe-emodin or chrysophanol 1-O- β -D-glucopyranoside at a concentration of 100 μ M. Treatment with these compounds also increased PARP cleavage in AGS cells. Additionally, aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside induced the down-regulation of the anti-apoptotic protein, BCL-2. The pro-apoptotic BCL-2 family proteins, BID and BAX, were slightly induced when exposed to chrysophanol 1-O- β -D-glucopyranoside.

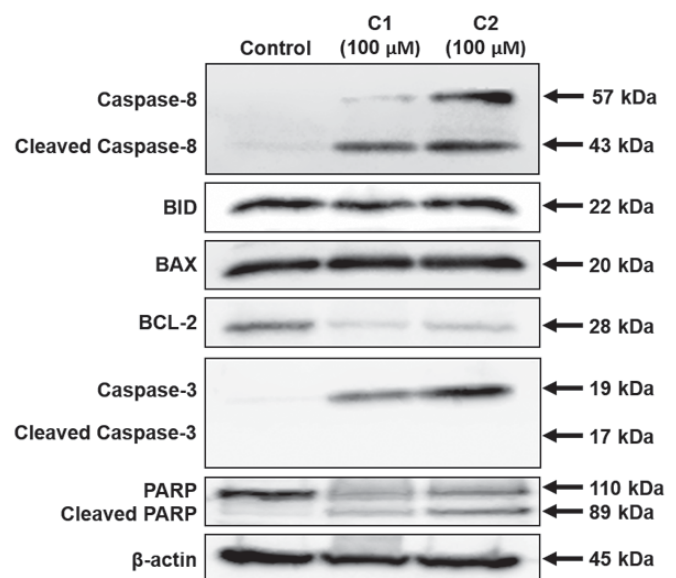


Figure 5. Effect of *Rheum undulatum* compounds on apoptosis-associated protein expression in the human gastric carcinoma cell line AGS. Western blot analysis was performed to determine the mechanisms by which these compounds stimulated apoptosis pathway. β -actin was used as a loading control. C1, aloe-emodin; C2, chrysophanol 1-O- β -D-glucopyranoside; BID, BH3 interacting domain death agonist; BCL-2, B-cell lymphoma-2; BAX, BCL-2-associated X; PARP, poly (ADP-ribose) polymerase.

Discussion

The present study is not the first to report the anticancer effects of *R. undulatum* on gastric cancer cell lines. According to the study by Hong *et al* (6), the methanolic extract of *R. undulatum* induced apoptotic cell death through activation of caspase cascade and inhibition of BCL-2 protein expression. The present study focused on screening the anti-apoptotic effects of the major phytochemical components in *R. undulatum* methanol extracts and the results demonstrated that aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside significantly induced apoptotic cell death, as observed by image-based cytometry. Aloe-emodin is a well established drug candidate for cancer therapy (25). Chen *et al* (25) reported that aloe-emodin at a concentration of 150 μ M induced apoptotic cell death in human gastric carcinoma cells by stimulating the release of apoptosis-inducing factor and cytochrome *c*, as well as by activating caspase-3. In the present study, aloe-emodin at a concentration of 100 μ M also induced apoptosis in AGS cells via the mitochondrial pathway and death receptor pathway, with the activation of caspase-8. Additionally, aloe-emodin was observed to regulate the intrinsic apoptotic pathway by downregulating the expression of BCL-2. Furthermore, chrysophanol 1-O- β -D-glucopyranoside exhibited similar anticancer effects to aloe-emodin, and therefore could be suitable for chemotherapeutic application.

In conclusion, the initial phytochemical analysis of the methanolic extract of dried rhizomes from *R. undulatum* resulted in the isolation of four stilbenes. Among these compounds, aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside effectively suppressed the viability of AGS cells, with IC₅₀ values of 84.66 \pm 0.44 and 68.28 \pm 0.29 μ M, respectively. In a previous study, various stilbenes, including resveratrol, pterostilbene, piceatannol and pinosylvin, have been observed to increase cancer cell death (21). The results of western blot analysis in the present study demonstrated that aloe-emodin and chrysophanol 1-O- β -D-glucopyranoside activated the caspase cascade in apoptosis pathway and downregulated the anti-apoptotic protein, BCL-2.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JHK and KSK conceived and designed the experiments; TAT, DL, SP, SHK, and JGP performed the experiments; TAT, DL and SHK analyzed the data; JHK and KSK contributed the reagents/materials/analysis tools; TAT, JHK and KSK wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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