Zanthoxylum ailanthoides Sieb and Zucc. Extract Inhibits Growth and Induces Cell Death through G_2/M-phase Arrest and Activation of Apoptotic Signals in Colo 205 Human Colon Adenocarcinoma Cells

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Abstract. *The effects of 50% ethanolic stem extracts of Zanthoxylum ailanthoides Sieb and Zucc. (ZASZ) on the cell viability, cell cycle and apoptosis were investigated in a human colon adenocarcinoma cell line (colo 205). The results demonstrated that ZASZ induced morphological changes and decreased the cell viability. ZASZ promoted Wee1, checkpoint kinase 2 (CHK2), p21 and p53 levels, decreased cyclin B and cdc25c associated with that led to G_2/M phase arrest. ZASZ-triggered apoptosis was confirmed by 4'-6-diamidino-2-phenylindole (DAPI) staining and DNA gel electrophoresis. ZASZ increased the levels of glucose-regulated protein 78 (GRP78) and growth arrest and DNA damage inducible gene 153 (GADD153), and promoted an increase of reactive oxygen species (ROS) and Ca^{2+} release, and loss of mitochondrial membrane potential ($\Delta\Psi_m$) accompanied by cytochrome c release that was due to the decrease of Bcl-2 and increase of Bax levels in the colo 205 cells. ZASZ also induced the protein levels of apoptosis-inducing factor (AIF) and endonuclease G (Endo G), increased the levels of caspase-3, -7 and -9, and stimulated the levels of fatty acid synthase (Fas) and Fas ligand in the colo 205 cells. ZASZ contains phenolic compounds, including flavone, chlorogenic acid and isofraxidin, among which, flavone was found to be the most effective in reducing cell viability and proliferative responses in the colo 205 cells. ZASZ induces cytotoxicity and apoptosis in colo 205 cells which provides the rationale for studies in animal models on the utilization of ZASZ as a potential cancer therapeutic compound.*

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Colon cancer is a common malignancy, and one of the major fatal diseases throughout the world (1). Furthermore, it is the second most frequent cause of death from cancer in Taiwan, where based on the reports from the Department of Health of the Republic of China, 19.6 people per 100 hundred thousand people die each year of colorectal cancer. The therapy for human colorectal cancer in Taiwan includes chemotherapy, radiotherapy and surgery. However, these treatment strategies had limited success. New agents acting on novel targets of carcinoma are needed.

The cyclin-dependent protein kinase (Cdk) family, cyclins and Cdk inhibitors (CdKI) are essential for cellular regulatory processes, particularly in the regulation of transcription and cell cycle progression (G_1 to S or G_2 to M phase) (2, 3). Cdk are regulated by multiple mechanisms which include inhibitory phosphorylation of conserved residues within the active site (4) and binding of additional regulatory molecules (5). It is reasonable to propose that if cyclin and/or Cdk are affected then cell cycle arrest and apoptosis could be induced.

It is well known that apoptosis plays a variety of important roles under normal physiological conditions (6).

Impaired apoptosis is a crucial step in the process of cancer development (7, 8). Cells undergo apoptosis through distinct pathways based on stimulation of caspases, a family of proteases that are important in regulating apoptosis (5). It is well known that compounds that can induce apoptosis can be divided into the fatty acid synthase (Fas) and Fas ligand (FasL) pathways resulting in the activation of the caspase-8, mitochondria-dependent pathway and the caspase-3-dependent pathway. Both pathways trigger the cytoplasmic release of pro-apoptotic mitochondrial proteins and stimulate the endoplasmic reticulum (ER) stress pathway which is attributed to the activation of caspase-12 and eventual apoptosis (9). Therefore, the resistance of cancer cells to treatment might be associated with dysregulation or defects in different steps of the apoptotic pathways.

Numerous naturally occurring compounds are thought to have potential value for cancer prevention and cancer therapy (10, 11) and they were recognized to be a potential inhibitor of tumor cell proliferation, carcinogen-induced carcinogenesis or inducers of apoptosis (12-14). The tender leaves of *Zanthoxylum ailanthoides Sieb and Zucc*. have been used as a dietary supplement in the Taiwanese population for many years (15). In the Chinese population, the plant *Z. ailanthoides* has been used as a herbal medicine for the treatment of heart disease, bone-injury and cold resistance. Our previous study demonstrated that a 50% ethanolic stem extract of *Z. ailanthoides* (ZASZ) was safe from a genotoxic perspective and acted as an antioxidant (16). However, there is no information on effects of ZASZ. Therefore, in the present study, the possible anticancer activity of ZASZ and whether it acts on cell cycle arrest and triggers tumor cell apoptosis were determined.

Materials and Methods

Plant material and preparation of extracts. The ZASZ (50% ethanolic stem extract) was prepared as previously described (16). Briefly, the plant material was obtained from the mountain area of Sinshe Township, Taichung, Taiwan. Each 100 g stem was extracted with 500 ml 50% ethanol at 75˚C for 3 h and decocted three times. The decoctions were filtered and then dried by a vacuum freeze-dryer. The extraction yields were 9.0%. The extracts were sealed in plastic bottles and stored at –70˚C until used. For the present experiments, extracts were dissolved in PBS before adding to cell cultures.

Chemical and reagents. Propidium iodide (PI), 4'-6-diamidino-2-phenyindole (DAPI), ethidium bromide (EtBr), triton X-100, trypan blue and ribonuclease A (RNase A) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). All other chemicals were at least reagent grade.

Cell lines and culture conditions. Human colon adenocarcinoma cell line (colo 205), human hepatoma cell line (Hep G2), human murine leukemia cell line (WEHI-3), mouse melanoma cell line (B16-F1) and promyelomonocytic cells line (HL60) were obtained from the Food Industry Research and Development Institution (Hsinchu, Taiwan). The colo 205 and WEHI-3 cells were cultured in RPMI 1640 medium, and the Hep G2 and B16-F1 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin- streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) (17). All the cells were maintained in a humidified 5% CO_2 incubator at 37˚C and the cells were sub-cultured every 3-4 days to maintain logarithmic growth and were allowed to grow for 24 h before treatments were applied.

Morphological changes and cell viability analysis. The colo 205, Hep G2, B16-F1 and WEHI-3 cells were plated in 12-well plates at a density of 1×10^5 cells/ml. The cells were treated with different concentrations (31.25, 62.5, 125.0, 250.0 or 500.0 μg/ml) of ZASZ or PBS as a solvent control and grown at 37˚C, 5% CO_2 and 95% air for 24, 48 or 72 h. To determine morphological changes, the cells were photographed by a phase-contrast microscope. Cell viability was determined by a PI exclusion method and by using a flow cytometer (Becton-Dickinson, FACSCalibur, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength as previously described (17).

Cell cycle and apoptosis analysis. The colo 205 cells were incubated with different concentrations of ZASZ (0, 31.25, 62.5, 125.0, 250.0 or 500.0 μg/ml) for 48 h. The cells were then harvested by centrifugation and fixed gently in 70% ethanol (in PBS) at -20˚C overnight. The cells were then re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase A and 0.1% triton X-100 in a dark room for 30 min and analyzed by flow cytometry (17, 18). A FITC annexin V apoptosis detection kit from BD Pharmingen (San Diego, CA, USA) was used to determine and quantify the apoptotic cells by flow cytometry (19).

DAPI staining. The colo 205 cells were placed for 24 h and then incubated with various concentrations of ZASZ (0, 31.25, 62.5, 125.0, 250.0 or 500.0 μg/ml) for 48 h. The cells were washed by PBS, fixed with 4% formaldehyde for 15 min, stained with DAPI (Sigma) at 37˚C for 30 min and photographed using a fluorescence microscope as previously described (17, 20).

DNA fragmentation assay. The colo 205 cells were incubated with different concentrations of ZASZ (0, 31.25, 62.5, 125.0, 250.0 or 500.0 μg/ml) for 48 h. The cells were then harvested by centrifugation, the DNA was isolated and was then gel electrophoresized and stained with EtBr and photographed under UV light as previously described (17, 21).

Measurement of reactive oxygen species (ROS) production, mitochondrial membrane potential ($\Delta\Psi_m$) and Ca^{2+} release. The colo 205 cells were incubated with 250.0 μg/ml of ZASZ for 0, 2, 4, 6 or 24 h. The cells were harvested and washed twice with PBS then re-suspended with specific fluorochromes. For ROS analysis, the cells were re-suspended in 500 μl 10 μM of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen) and incubated at 37˚C for 30 min and then analyzed by flow cytometry as previously described (22, 23). For $\Delta\Psi_m$ analysis, the cells were re-suspended in 500 μl of 3,3'-dihexyloxcarbocyanine ($DiOC_6$, Invitrogen) (1 μmol/l) and incubated at 37˚C for 30 min and

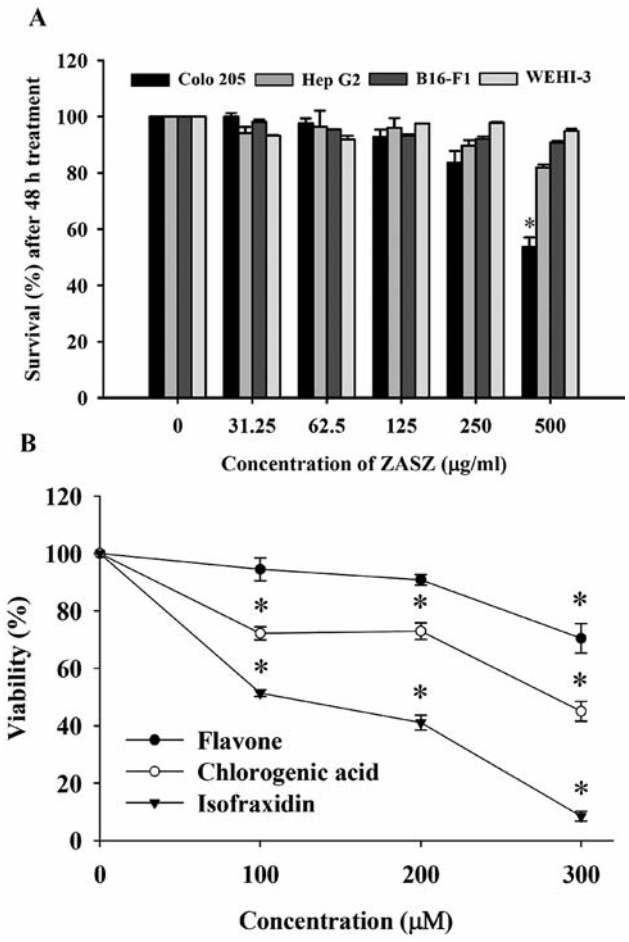

Figure 1. *Effects of the ZASZ on the proliferation of colo 205, B16-F1, Hep G2 and WEHI-3 cells (A) and the active phenolic compounds in ZASZ on the cell viability of colo 205 cells (B). Means±S.D. of three independent experiments. *p<0.05 compared with the control sample.*

analyzed by flow cytometry as previously described (22, 24). To detect $\Delta\Psi_m$ release, the cells were re-suspended in Indo 1/AM (3 μg/ml, Invitrogen) and incubated at 37˚C for 40 min. After incubation, the cells were washed with PBS and analyzed by flow cytometry as previously described (22).

Determination of G_2/M phase arrest and apoptosis-related protein levels. The colo 205 cells were incubated with 250.0 μg/ml of ZASZ for 0, 6, 12, 24 or 48 h. After treatment, the cell lysates were used to determine the protein abundance of cyclin A and B, cell division control 2 (cdc2), cdc25c, checkpoint kinase 2 (CHK2), Wee1, ataxia-telangiectasia mutated (ATM), p21, p53, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X (Bax), cytochrome *c*, apoptosis-inducing factor (AIF) and endonuclease G (Endo G), caspase-9, -3, -7, poly (ADP-ribose) polymerase (PARP), fatty acid synthase (Fas), Fas ligand (FasL), caspase-8, truncated BH3 interacting domain death agonist (tBid), glucose-regulated protein 78 (GRP78), growth arrest and DNA damage inducible gene 153 (GADD153) and β-actin by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (17, 22).

Table I. *Phenolic contents of ZASZ.*

Compound	Content (mg/g extract)
Chlorogenic acid	2.74±0.21
Flavone	22.68±0.90
Isoflaxidin	1.46±0.14

Reported values are the means±SD (n=3).

Characterization of phenolic compounds. Phenolic compounds in the ZASZ extract were analyzed based on the method described by Li *et al*. (25) with a slight modification. Dried ZASZ (0.1 g) was dissolved in 10 ml of 50% ethanol, filtered through a 0.45 μm filter, and analyzed by high-performance liquid chromatography (HPLC). HPLC analysis was performed using a Hewlett- Packard HPLC System (HP 1100 series, Waldron, Germany), consisting of a quaternary pump and a variable wavelength detector (VWD) at 270 nm and equipped with a Li-Chrospher RP-18 cartridge column (250 mm ×4.6 mm, 5 μm, Merck KGaA, Darmstadt, Germany). The mobile phase was a stepwise gradient of water (0.1% v/v phosphoric acid)-acetonitrile (0.01 min, 94: 6; 50 min, 65: 35), and the injection volume was 30 μl. The identification of each compound was based on a combination of retention time and spectral matching by comparison with those of known standards. Peak areas and concentrations were determined using the Hewlett- Packard Chem Station Chromagraphic Management System.

Statistical analysis. Student's *t*-test was used to analyze statistical differences between the ZASZ treated and control groups. A *p*-value less than 0.05 was considered significant. All data are presented as means±S.D. of three independent experiments.

Results

Effects of ZASZ and its active phenolic compounds on cell viability in human cancer cell lines. Each cell line responded differently to the ZASZ treatment with the colo 205 cells most susceptible to ZASZ-induced toxicity (Figure 1A). The 500 μg/ml ZASZ treatment over 48 h decreased cell viability by 46.4%, 18.1%, 9.2% and 5.2% in the colo 205, Hep G2, B16-F1 and WEHI-3 cells, respectively. Therefore, the colo 205 cells were used in the subsequent experiments because of the greater effects of and sensitivity to ZASZ on those cells compared with the other cell lines.

The ten standard polyphenols used for comparison showed the retention times of gallic acid, chlorogenic acid, caffeic acid, flavone, isofraxidin, rutin, genistin, daidzin, quercetin and genistein to be 5.68, 14.78, 16.93, 21.28, 25.89, 26.87, 28.66, 39.43, 43.10 and 49.37 min, respectively, using HPLC analysis (chromatograms not shown). The data in Table I showed that ZASZ contained three major phenolic substances, including chlorogenic acid, flavone and isofraxidin with flavone the most abundant phenol. The effects of chlorogenic acid, flavone and isofraxidin on cell

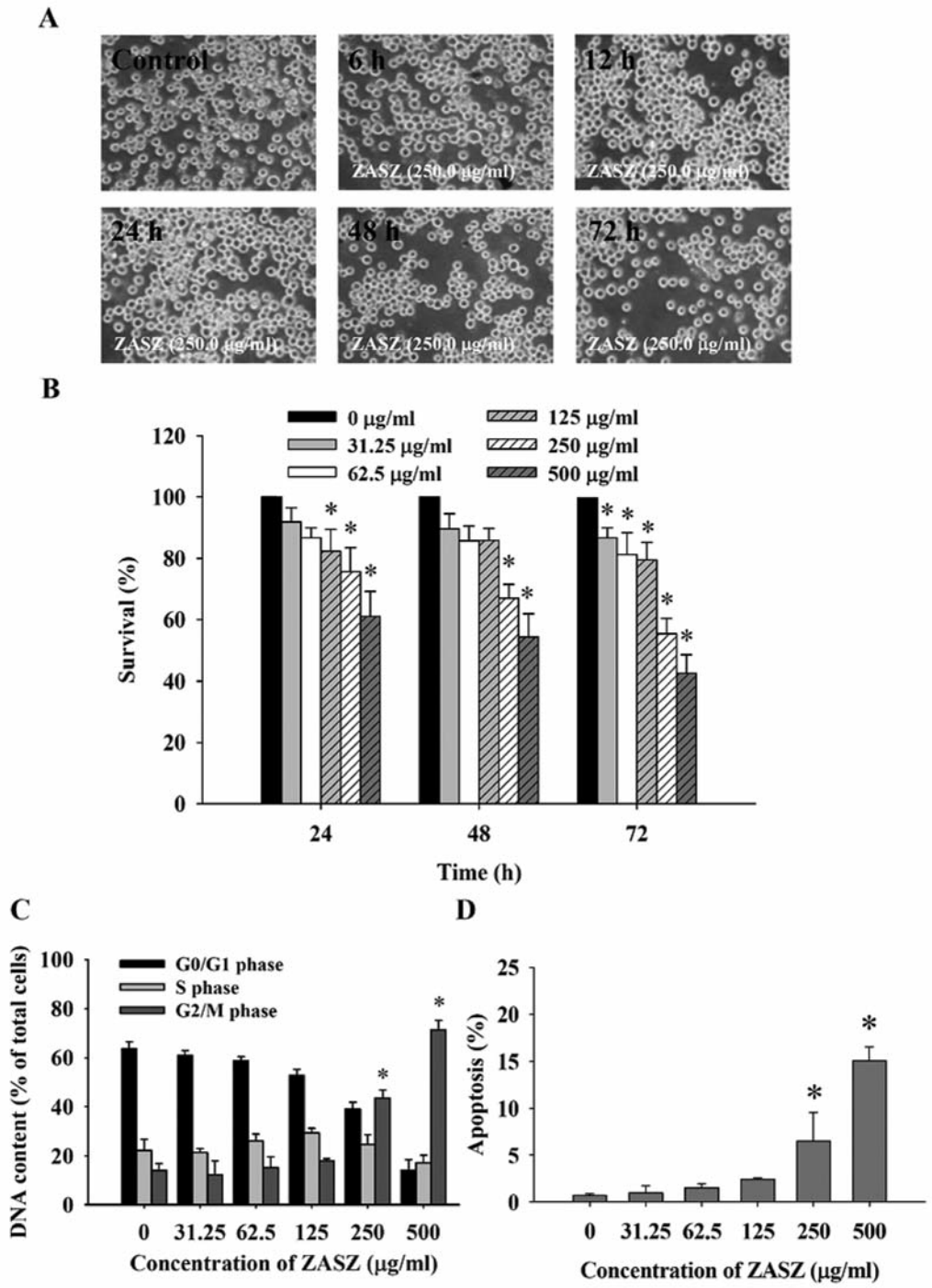

Figure 2. *Effects of ZASZ on cell morphology (A) and cell viability (B) up to 72 h and , DNA content for cell cycle distribution (C) and apoptosis (D) after 48 h treatment in colo 205 cells. Mean±S.D. of three experiments. *p<0.05 compared with the control sample.*

viability were examined, and results from Figure 1B indicated that the induction of toxicity of the three phenolic compounds was dose-dependent. The cell viability followed the order flavone>chlorogenic acid>isofraxidin.

Effects of ZASZ on cell morphology, cell viability, cell cycle distribution and apoptosis. The phase-contrast microscopic examination indicated that ZASZ-induced apoptotic morphological changes such as shrinking and cytoplasmic

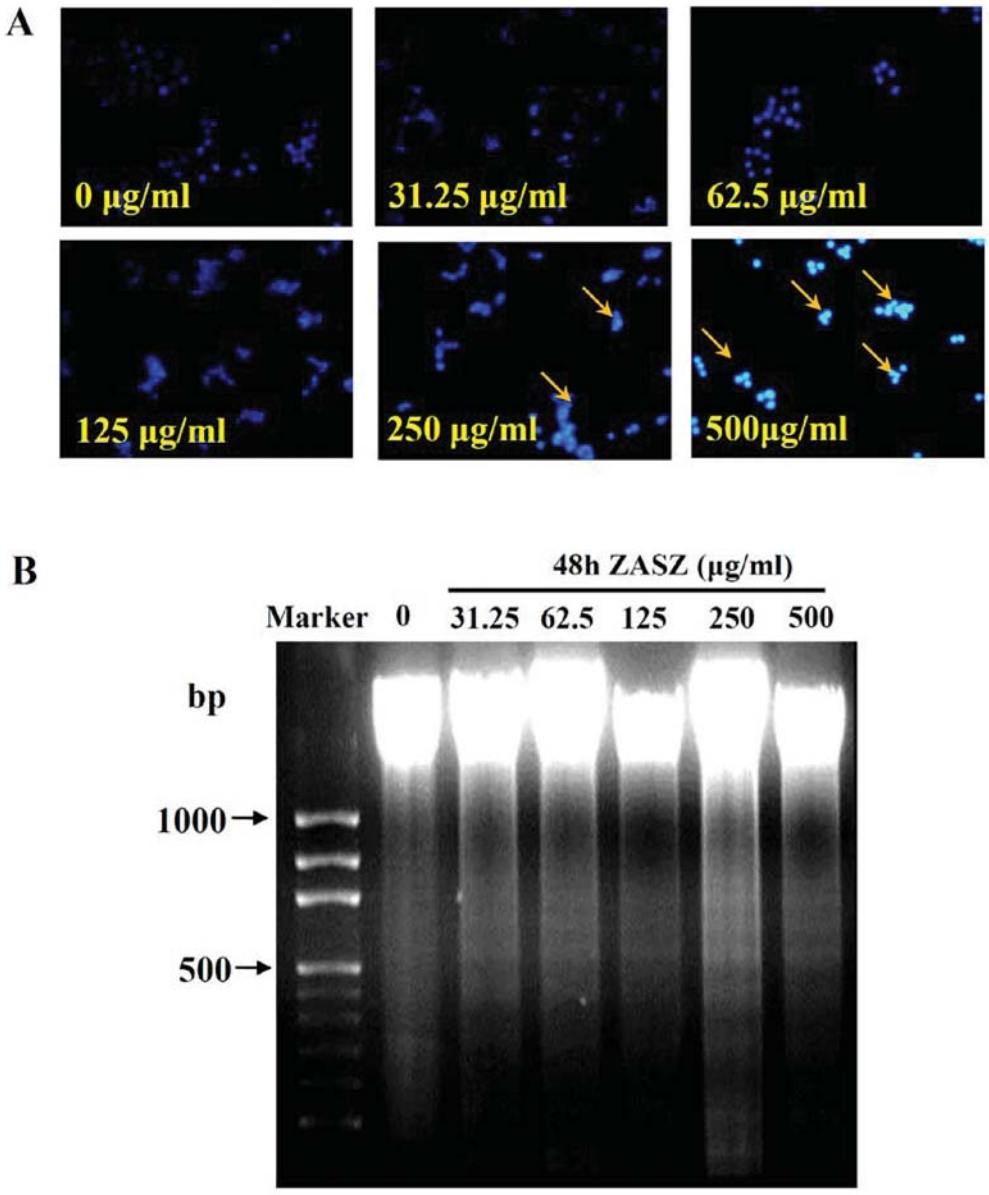

Figure 3. *The induction of apoptosis by ZASZ in colo 205 cells was determined by DAPI staining and fluorescence microscopy (A) and DNA gel electrophoresis and UV light (B).*

blebbing were found (Figure 2A) and a PI stain analysis by flow cytometry showed that the percentage of viable cells was significantly different between the ZASZ-treated and the control groups (Figure 2B). These effects were dose- and time-dependent. ZASZ induced G_2/M-phase arrest in the colo 205 cells (Figure 2C) and the effect was dose-dependent. It can be seen in Figure 2D that ZASZ also induced apoptosis in the colo 205 cells. To further confirm that ZASZ induced apoptosis, the results determined by DAPI staining. Figure 3A shows that apoptotic cell death occurred after ZASZ treatment and Figure 3B demonstrates evidence of DNA fragmentation in the ZASZ -treated colo 205 cells.

Effects of ZASZ on ROS and Ca^{2+} levels and $\Delta\Psi_m$. The ROS levels were stimulated when the colo 205 cells were incubated with 250 µg/ml of ZASZ and peaked after a 2 h exposure, then decreasing slightly up to 24 h. However, the ROS levels were still higher in the ZASZ-treated cells than in the control cells for at least 24 h (Figure 4A). Flow cytometric analysis indicated that the $\Delta\Psi_m$ decreased in the ZASZ-treated cells as

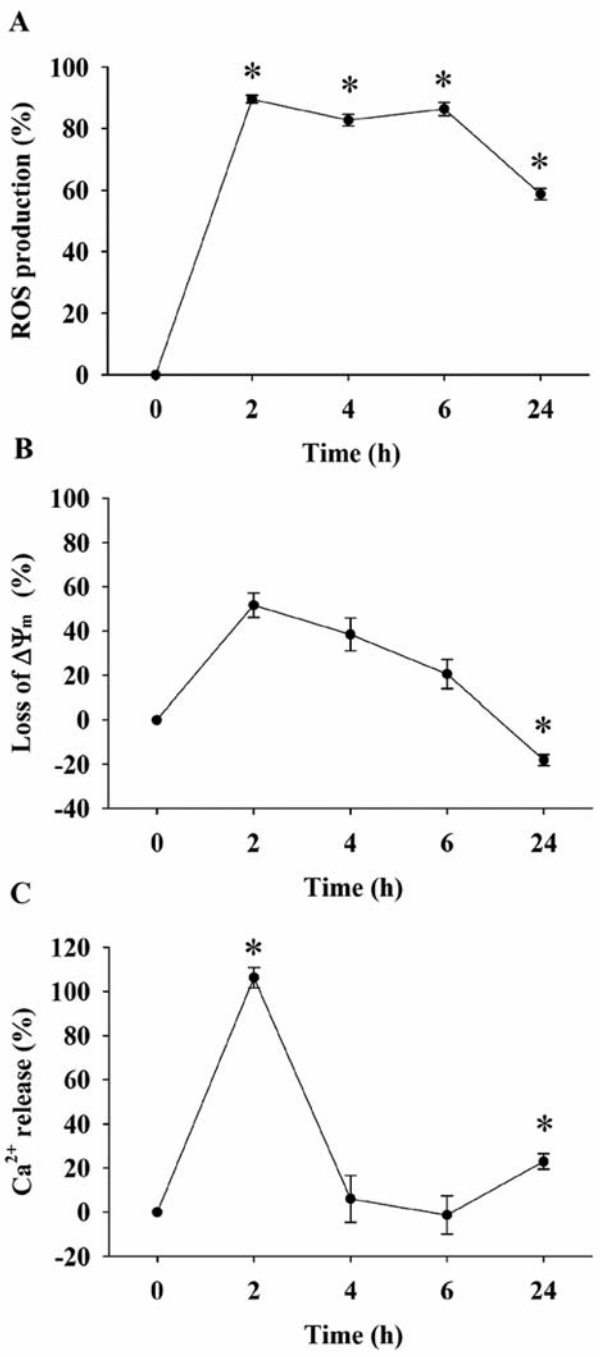

Figure 4. *Effects of ZASZ (250 μg/ml) on the levels of reactive oxygen species (ROS) (A), mitochondria membrane potential ($\Delta\Psi_m$) (B) and Ca^{2+} release (C) in colo 205 cells. Mean±S.D. of triplicated experiments. *p<0.05 compared to control sample.*

compared with the control cells after 24 h treatment (Figure 4B). Also, it can be seen in Figure 4C that the cytoplasmic Ca^{2+} level peaked at 2 h and then began to decrease but were higher than the control cells at 24 h ($p<0.05$).

Effects of ZASZ on G_2/M-phase arrest and apoptosis-associated specific protein levels. The Western blotting analysis is shown in Figures 5 and 6. Protein levels of CHK2, Wee1, ATM, p21, p53 (Figure 5B), Bax (Figure 5C), cytochrome *c*, AIF, Endo G (Figure 5D), caspases-9, -3, and -7 (Figure 6A), Fas, FasL, caspase-8, tBid (Figure 6B), GRP78 and GADD153 (Figure 6C) increased and cyclins A and B , cdc2, cdc25c (Figure 5A), Bcl-2 (Figure 5C) and PARP (Figure 6A) reduced following ZASZ treatment in the colo 205 cells.

Discussion

Until now, a cure for colon cancer is not satisfying and numerous studies focused on finding new agents for colon cancer therapy. In this study, we showed that ZASZ induced G_2/M arrest and Western blot analysis showed that it was mediated through the decreased protein expressions of cdc25c, Wee1 and CKI proteins ($p21^{CIP1/WAF1}$), a simultaneous decrease in cyclins A and B and enhanced binding of CKI-Cdk. The concentration of 250.0 μg/ml of ZASZ was selected for all the Western blotting analysis based on the viability studies (Figure 2) and was close to the IC_{50} for 72 h exposure. $p21^{CIP1/WAF1}$ is a universal inhibitor of Cdk(s) and $p27^{KIP1}$ is commonly upregulated in response to antiproliferative signals (26). The present data also showed that ZASZ induced the expression of p53 in the colo 205 (p53 wild-type) cells (Figure 5B). The tumor suppressor gene p53 is activated in response to various genotoxic stresses, resulting in cell cycle arrest or apoptosis. Cell cycle arrest that is dependent on p53 requires transactivation of $p21^{WAF1/CIP1}$, GADD45 and cyclin G through the binding of the cyclin-cdk complex (27). The blockade of cell cycle progression by ZASZ was through activation of $p21^{WAF1/CIP1}$ involving a p53 dependent pathway (Figure 5A and B). It has been reported that p53 is responsible for the up-regulation of Fas, Bax, Bad and Bcl-xl resulting in apoptotic cell death (28). Therefore, ZASZ not only increased the cell cycle-related protein ($p21^{WAF1/CIP1}$), but also enhanced the proapoptotic expression of Bax, Fas and reduced levels of the anti-apopototic protein Bcl-2 (Figure 5 and 6).

Normal cells maintain a balance of pro-apoptotic and anti-apoptotic Bcl-2 family proteins which integrate the diverse death- and survival-signals to control the fate of the cell (8, 29) including both Bcl-2 and Bax. The present data demonstrated that the ZASZ-induced apoptosis was associated with an increase of Bax and a reduction of Bcl-2 protein levels, and both Bcl-2 family proteins were in agreement with the notion that an imbalance between pro- and anti-apoptotic proteins of the Bcl-2 family results in apoptosis (8, 29). Anti-apoptotic Bcl-2 family proteins are also known to prevent the release of caspase-9 (30), and regulate the release of cytochrome *c* from the mitochondria

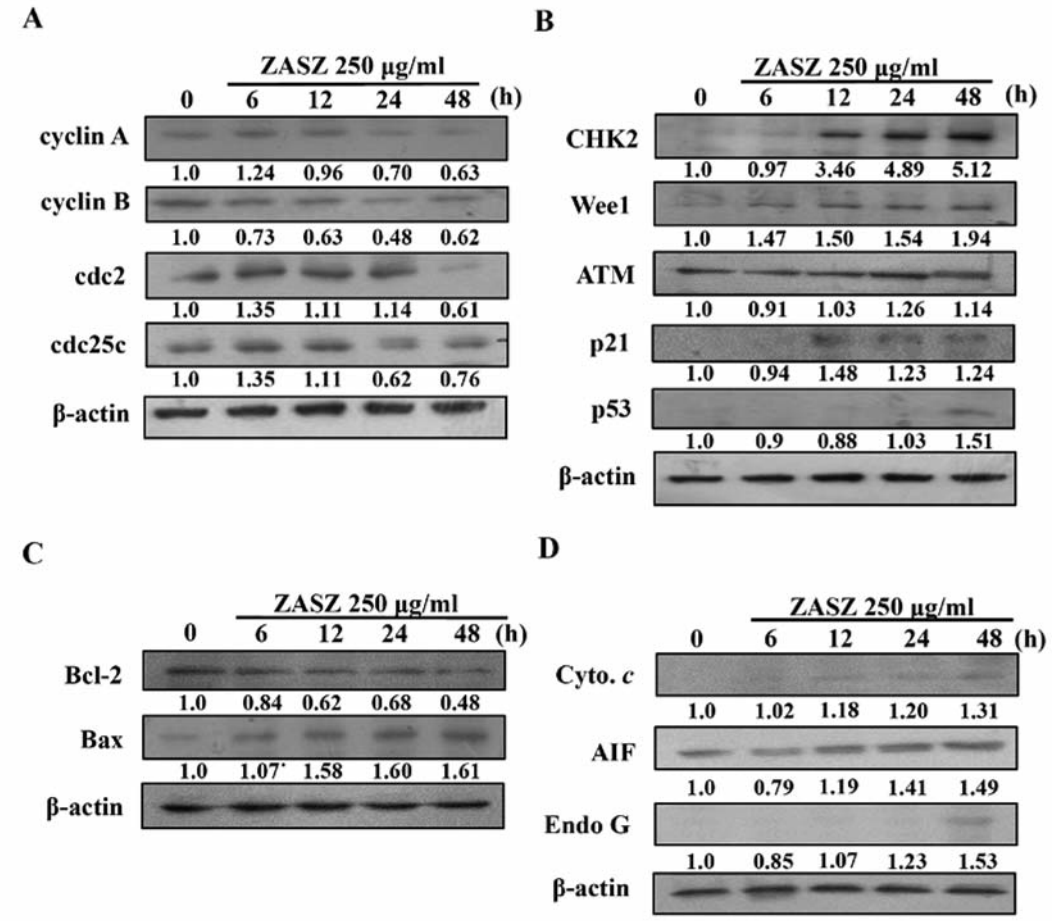

Figure 5. *Effects of ZASZ on the protein levels of A: cyclin A and B, cdc2 and cdc25c; B: CHK2, Wee1, ATM, p21 and p53; C: Bcl-2 and Bax; D: cytochrome c, AIF and Endo G in colo 205 cells, shown by Western blotting. β-Actin, internal control.*

into the cytosol (31, 32). The over-expression of Bcl-xl protein (anti-apoptotic) can block the release of cytochrome c in response to a variety of apoptotis signals (32). However, the Bax protein (pro-apoptotic) promotes cytochrome *c* release from the mitochondria (31, 32). Western blotting demonstrated that ZASZ decreased the $\Delta\Psi_m$ and promoted the levels of cytochrome *c* release from the mitochondria (Figure 5D). The induction of the execution protease, caspase-3 was also demonstrated. This was in agreement with many reports demonstrating that released cytochrome c from mitochondria activates initiator caspase-9 to activate a sequential cascade of caspases, especially caspase-3 resulting in the proteolysis of death substrates and subsequent DNA degradation and apoptotic cell death (8, 29, 33, 34).

ZASZ also increased ROS and Ca^{2+} levels in the colo 205 cells (Figure 4A and C). ROS can stimulate ER stress resulting in Ca^{2+} release and plays an important role in the induction of apoptosis (35, 36). ZASZ increased the levels of GADD153 and GRP78 (Figure 6C) also associated with ER stress because GADD153 is a modulator and GRP78 is a sensor of ER stress. Thus, ZASZ induced cytotoxicity in the colo 205 cells which was mediated by several different pathways associated with apoptosis as depicted in Figure 7.

ZASZ was found to contain approximately 3% phenolic compounds mostly flavone, chlorogenic acid and isofraxidin. Flavone was highest in abundance and was the least effective in reducing cell viability in the colo 205 cells. Flavones are polyphenolic compounds that occur naturally in many plants used for food. *In vitro* studies on the potential anticancer activity of flavones have been reported in different cell types (37-39). Flavone may contribute to the antiproliferative and apoptotic activities of the ZASZ extracts used in the present study.

In conclusion, ZASZ induces morphological changes, decreases the percentage of viable cells, and induces G_2/M phase arrest of cell cycle and apoptosis involving ROS production, ER stress, caspase-3, AIF and Endo G signaling pathways. Further studies are needed on the effectiveness of ZASZ in cancer cells particularly studies in animal models.

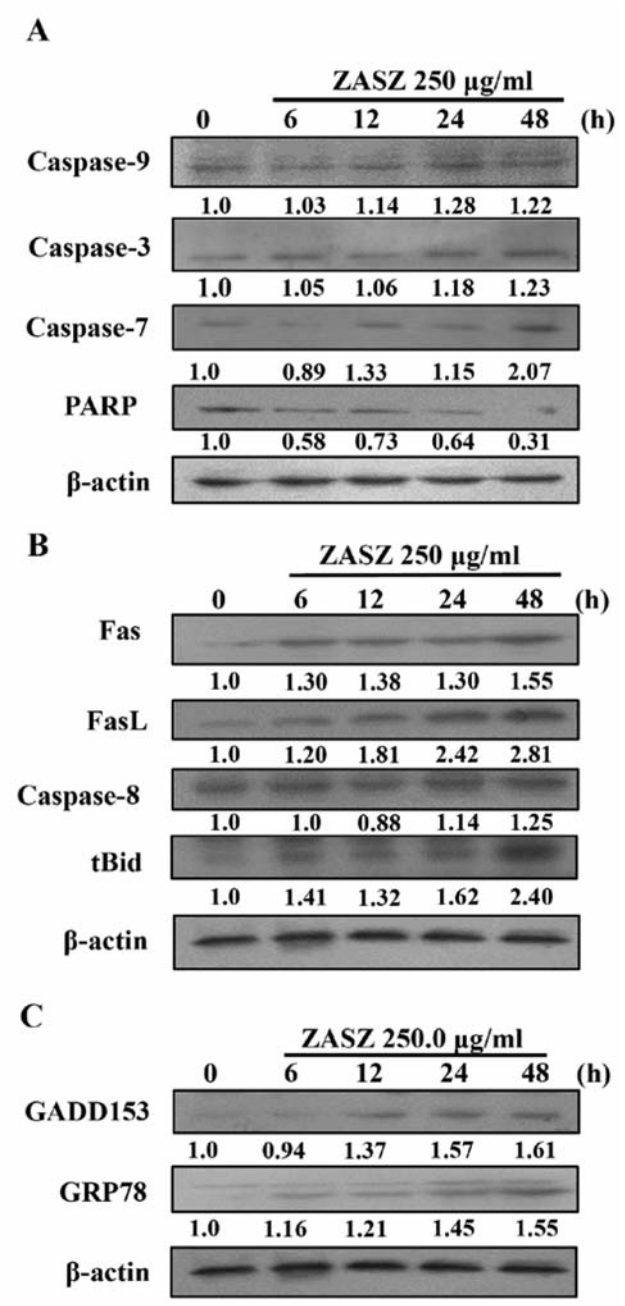

Figure 6. *Effects of ZASZ on the protein levels of A: caspase-9, -3, -7 and PARP; B: Fas, FasL, caspase-8 and tBid; C: GADD153 and GRP78 in colo 205 cells, shown by Western blotting. β-Actin, internal control.*

Acknowledgements

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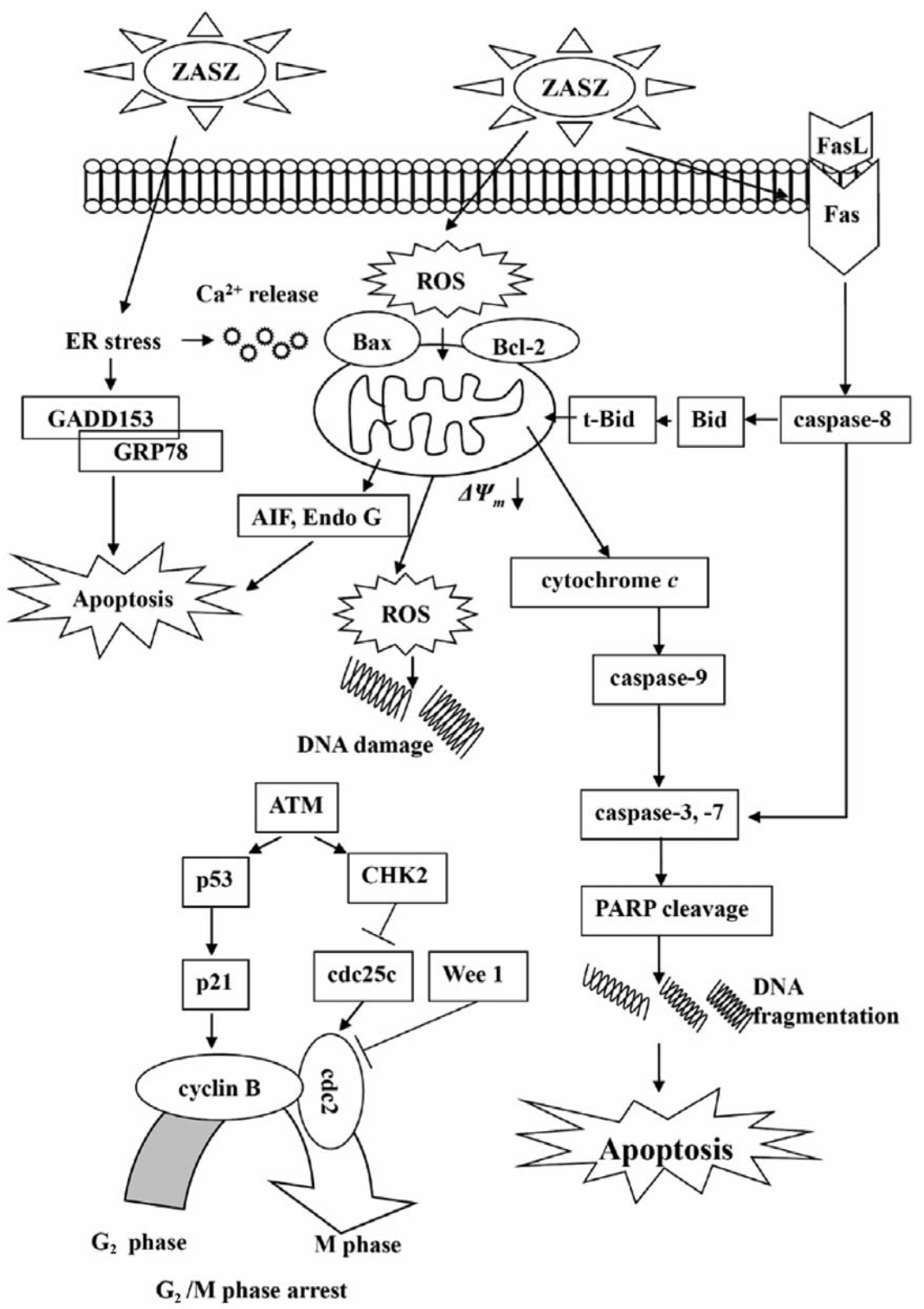

Figure 7. *Proposed possible signaling pathways included by ZASZ treatment. ZASZ induces ROS, ER stress, GRP78 and GADD153 expression leading to* Ca^{2+} *release, promotes Bax, but inhibits Bcl-2 before decreasing the* $\Delta\Psi_m$ *leading to cytochrome c release, caspase-9 activation and caspase-3 activity causing apoptosis.*

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