RESEARCH COMMUNICATION

Induction of Mitochondria-mediated Apoptosis in Human Gastric Adenocarcinoma SGC-7901 Cells by Kuraridin and Nor-kurarinone Isolated from *Sophora Flavescens*

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Abstract

The study was designed as one of a series to find novel anticancer compounds from Chinese herbs. For this purpose, we screened an ethanol extract of 300 herbs against SGC-7901 cells. *Sophora flavescen* was included in those showing potential cytotoxic activity. Target compounds were therefore isolated and analyzed on analytical HPLC. Chromatography showed only one peak with a purity of 97%. The ESI-MS spectrum showed two molecular ions: m/z 424(M+) and 438(M+). Furthermore, combining the data of 1HNMR and 13CNMR, it was deduced that this product was a mixture of two compounds; kuraridin (1) and nor-kurarinone (2). The concentration was [1]:[2]=9:10, the chemical structural formulae are C25H28O6 and C26H30O6. In this study, mechanisms involved by the mixture of compounds 1 and 2-induced growth inhibition including apoptosis and G2/M phase arrest in human gastric adenocarcinoma SGC-7901 cells were examined for the first time. Triggering of the mitochondrial apoptotic pathway was demonstrated by loss of mitochondrial membrane potential, reduction in the Bcl-2/Bax ratio, and significant activation and cleavage of caspase-3. Additionally, the production of reactive oxygen species (ROS) was also increased. Taken together, our results indicated that the cytotoxic efficacy of the mixture of compounds 1 and 2 is mainly due to induction of cell cycle arrest and apoptosis.

Keywords: *Sophora flavescens* - Kuraridin - Nor-kurarinone - apoptosis - HPLC

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Introduction

Gastric cancer is the fourth most frequently diagnosed cancer after lung, breast, and colorectal cancers. It is the second most cause of cancer-related death (738,000 deaths, 9.7%) after lung cancer worldwide (Ferlay et al., 2010). The rate of incidents of gastric cancer is comparatively higher in the Eastern Asia and 65–70% of new cases of deaths were reported from gastric cancer in less-developed countries (Parkin, 2001; Hernandez et al., 2010). In 2005, the incidence rate of gastric cancer (0·3 million deaths and 0·4 million new cases) ranked third among the most common cancers in China (Yang et al., 2005). Currently, surgery is one of the most common treatments of gastric cancer but survival rate of patients with gastric cancer is less than 33%. Thus, there is an immense need to identify novel and promising agents for the cure and treatment of gastric cancer.

In the last few decades, several researchers have identified numerous dietary and botanical natural compounds which possess chemopreventive potential (Amin et al., 2009). Plants are one of the most important source for anticancer agents (Cragg and Newman, 2005). *Sophora flavescens* (Chinese name, Kushen) a medicinal plant, is commonly found in Eastern Asia. Previous studies revealed that *S. flavescens* plant was used as traditional herbal medicine for the treatment of diarrhea, gastrointestinal hemorrhage, and eczema (Zhu, 1998). Chemical and pharmacological studies of *S. flavescens* have illustrated the isolation of alkaloids (Okuda et al., 1965; Sekine et al., 1993; Song et al., 1999), flavonoids (Kyogoku et al., 1973; Kuroyanagi et al., 1999; Kang et al., 2000a; 2000b; Son et al., 2003; Ding et al., 2005; Kim et al., 2006; Shen et al., 2006; Lee et al., 2007; Liu et al., 2010; Oh et al., 2011) and tritepenoid saponins (Ding et al., 2005; Ding et al., 1992).

The present study was designed to find novel and targeted anticancer compounds from Chinese herbs. For this purpose, we screened ethanol extract of 300 Chinese herbs against human gastric cancer using SGC-7901 cells and found that ethanol extract of several Chinese herbs could specifically inhibit cell growth. Then the target compounds were isolated from those Chinese herbs. One of them was kushen, a Chinese herb that has shown

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potential cytotoxic activity. The target compounds isolated from kushen were; kuraridin (1) and nor-kurarinone (2).

Recently it has been reported that, kuraridin, is a naturally occurring flavonoid in the roots of *S. flavescens* and possesses glycosidase inhibition (Kim et al., 2006), antibacterial (Sohn et al., 2004), diacylglycerol acyltransferase (Chung et al., 2004), and tyrosinase inhibition activities (Kim et al., 2003; Son et al., 2003). We, therefore, intended to explore the further biological activities of the mixture of compounds 1 and 2. Thus, in this study, we investigated the cell-growth inhibitory effects of the mixture of kuraridin and Nor-kurarinone and examined their cytotoxic activity and mechanism using SGC-7901 cells.

Materials and Methods

Reagents

Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. DMEM, Methylthiazolyldiphenyl-tetrazolium bromide (MTT), propidium iodide (PI), Hoechst 33258, and dimethyl sulfoxide (DMSO) were purchased from Sigma. Annexin V-FITC apoptosis detection kit and reactive oxygen species assay kit were purchased from Beyotime Institute of Biotechnology. Rabbit polyclonal anti-human Bcl-2, anti-human Bax and cleaved caspase-3 antibodies were purchased from Wuhan Boster Biological Technology Co., Ltd. Mouse anti-β-actin and anti rabbit antibodies were purchased from Santa Cruz Biotechnology. Ponceou and cell lysis buffer for Western and IP were purchased from Bio SS Beijing. Rhodamine123 was purchased from Eugene Co. (Oregon, U.S.A.).

Extraction, isolation and identification of compound 1 and 2

Target compounds were isolated from the plant, *S. flavescens* via fractionation extract as we previously described (Rasul et al., 2011; Shawi et al., 2011)

Cell culture

Human gastric adenocarcinoma SGC-7901 cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum, 100 μ/ml penicillin and 100μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 and 95% air. Cells were seeded in 10 cm dishes and allowed to grow to approximately 70% confluence before experimentation.

Cell proliferation assay

The growth inhibitory effects of the mixture of compounds 1 and 2 on the viability of cells were determined by the MTT assay. Briefly, SGC-7901 cells were plated at 96-well plates for overnight. Cells were treated with different concentrations of the mixture of compounds 1 and 2. After incubation for 24 and 48 hours, cell viability was determined. We added 10 μl MTT (5 mg/ml in phosphate buffered saline) to each well and incubated for 4h. After careful removal of the medium, 150 μl dimethyl sulfoxide (DMSO) was added to each well and shaken carefully. The absorbance was recorded on the Microplate Reader (ELX 800, BIO-TEK Instruments, Inc.) at a wavelength of 570 nm. The effect of the mixture of compounds 1 and 2 was measured on cell growth inhibition and inhibition ratio (I %) was calculated using the following equation:

I% = [A570 (control) - A570 (treated)] / A570 (control) *100

Determination of apoptosis by fluorescence microscopy and flow cytometry

For determination of apoptosis, Briefly, SGC-7901 cells were cultured in 6-well plates overnight. After 20 h, the mixture of compounds 1 and 2 were added to the wells in a volume of 1 mL per well to a final concentration of 40 μg/ml for 24 and 48 h with negative control group. Then, cells were harvested, washed with pre-chilled PBS (4°C), and centrifuged at 1000 rmp for 5 min. After, the supernatant was discarded; the pellet was resuspended gently in 195 μl Annexin V-FITC binding buffer and incubated with 5 μl Annexin V-FITC for 10 min in the dark. Cells were, then, centrifuged at 1000 rpm for 5 min and the pellet was resuspended in 195 μl binding buffer with 10 μl PI in the dark. After filtration (300 apertures), the suspension of each group was analyzed by flow cytometry.

Cell Cycle Analysis

SGC-7901 cells were seeded in 12-well plates and were treated with 40 μg/ml concentration of the mixture of compounds 1 and 2 for 24 and 48 h. After treatments, the percentages of cells in the different phases of the cell cycle were evaluated by determining the DNA content after propidium iodide staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1000 rpm at 4°C for 5 min. Pellets were fixed in 70% ice-cold ethanol overnight. After fixation the cells were centrifuged again, were washed twice with PBS, and incubated in PBS containing 1 mg/ml RNase for 10 min at room temperature. Finally, samples were stained with 1mg/ml propidium iodide for 30 min at 4°C. Data acquisition was done on Flow Cytometry and analyzed by Cell Quest software.

Western Blotting

SGC-7901 cells were cultured and treated with the mixture of compounds 1 and 2(40 μg/ml) for different time periods and western blot analysis was carried out. Briefly, the cells were trypsinised, collected in 1.5ml centrifuge tube and were washed twice with PBS, centrifuged at 1500g for 5 min and then the cell pellets were resuspended in lysis buffer and lysed on ice for 30 min with vertex after every 5 min. After centrifugation at 13000g for 15 min, the supernatant fluids were collected and the protein content of the supernatant was quantified by a NanoDrop 1000 spectrophotometer (Thermo scientific, USA). The protein lysates were separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ). The membranes were soaked in blocking buffer (5% skimmed milk) for 2 h. Proteins were detected using polyclonal antibodies. Rabbit anti-human Bcl-2 antibody (1:200) or Rabbit anti-human Bax antibody (1:200) or Rabbit anti-human cleaved caspase-3 antibody (1:500) overnight at

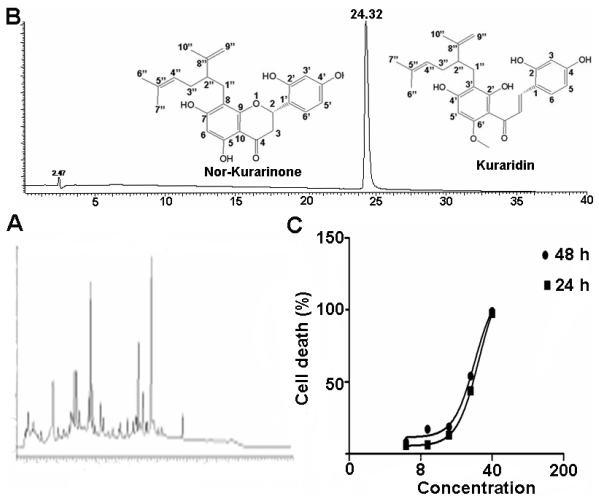

Figure 1. Isolation of Compounds and their Cytotoxic Effects. (A) Analytical HPLC chromatogram of raw ethanol extract of *Sophora flavescens* plant; (B) structures of (1) Kuraridin and (2) Nor-Kurarinone; (D) SGC-7901 cells were treated with various concentrations of the mixture of compounds 1 and 2 for 24 and 48 h. Cell viability was determined by MTT assay. Data shown are means ± SDs (n = 3)

4 °C and visualized using anti-rabbit or anti-mouse IgG conjugated with HRP and DAB as the HRP substrate.

Statistical analysis of Data

To evaluate the significance of difference of represented data and results, statistical analysis was carried out using the Student's t-test. $p < 0.05$ was regarded as statistically significant. All experiments were repeated at least three times. Data are expressed as mean ± S.D.

Results and Discussion

We began our investigation with the screening of ethanol extract of *S. flavescens* against SGC 7901 cells to evaluate its inhibitory effects on cell growth and led to the isolation of the target compound using HPLC. The analytical HPLC chromatograph of ethanol extract has been shown in the Figure 1A. When the target compound was analyzed on analytical HPLC, the chromatograph showed that there was only one peak and the purity was 97%. The ESI-MS spectrum showed two molecular ions: m/z 424(M+) and 438(M+). Furthermore, combining the data of ^{1}HNMR and ^{13}CNMR, it was deduced that the product was a mixture of compound 1 and 2, the concentration was [1]:[2]=9:10, the chemical structural formulae are $C_{25}H_{28}O_6$ and $C_{26}H_{30}O_6$, structures are shown in Figure 1B.

To evaluate the cytotoxic effects of the mixture of compounds 1 and 2 on the growth of human gastric adenocarcinoma SGC-7901 cells, we treated SGC-7901 cells with various doses of the mixture of compounds 1 and 2 for 24 and 48 h. Cell proliferation was measured by MTT assay. Figure 1C demonstrates that the mixture of compounds 1 and 2 inhibited cellular proliferation in dose and time dependent manner. Based on these results, we selected a moderate concentration (40 μg/mL) for the molecular mechanistic study of the mixture of compounds

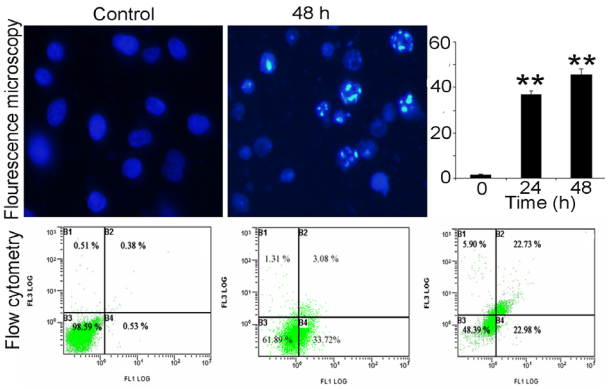

Figure 2. Apoptosis Induced by the Mixture of Compounds 1 and 2 in SGC-7901 Cells. (A) SGC-7901 cells stained with Hoechst 33258 after treating with 40 µg of the mixture of compounds 1 and 2 for 24 and 48 h - DNA morphological changes were examined under the fluorescence microscopy; (B) Cells were stained with Annexin V-FITC/PI and were analyzed by flow cytometry - Cell populations shown in the lower right (Annexin V+/PI-) represent apoptotic cells, upper right (Annexin V+/PI+) represent necrotic cells

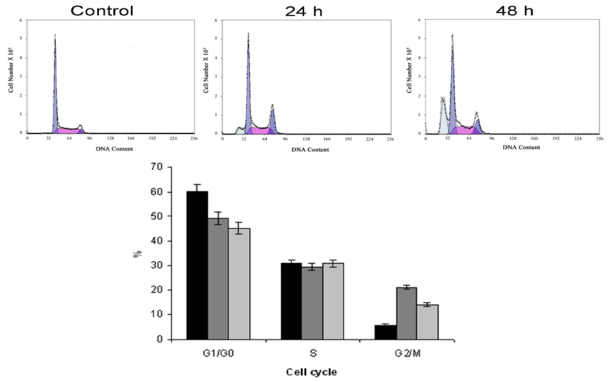

Figure 3. Cell Cycle Arrest of SGC-7901 Cells by the Mixture of Compounds 1 and 2. Cell cycle distribution in SGC-7901 cells treated with the mixture of compounds 1 and 2 for 24 and 48h was analyzed by flow cytometry. Apoptotic nuclei with hypodiploid DNA content corresponding to sub G1 peak

1 and 2 in time dependent manner.

To determine whether treatment of SGC-7901 cells with the mixture of compounds 1 and 2 resulted in the apoptotic or necrotic cell death, it was observed by using the Hoechst 33258 staining. The marked morphological changes, such as nuclear fragmentation, condensation of chromatin and apoptotic bodies of cells were observed in treated cells with the mixture of compounds 1 and 2 for 24 and 48 h under fluorescence microscopy as compared to control group. In order to get further confirmation, the apoptosis induced by the mixture of compounds 1 and 2 was evaluated by Annexin V-FITC and PI staining. Annexin V- FITC and PI staining was rendered to distinguish early from late apoptosis and necrosis. The percentage of apoptotic cells induced by 40 µg/ml of the mixture of compounds 1 and 2 were 36.80% and 45.71% after 24 and 48 h respectively (Figure 2).

To examine the characteristics of the mixture of compounds 1 and 2-induced cell growth inhibition, flow cytometric analysis was performed (Figure 3). Incubation of fixed and permeabilized cells with propidium iodide (PI) resulted in the quantitative PI binding with total cellular DNA, and the fluorescence intensity of PI-labeled

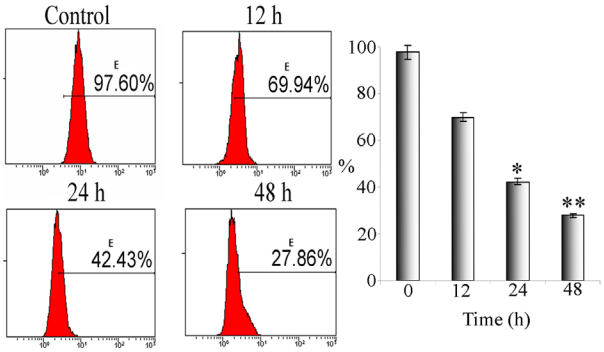

Figure 4. Effects of the Mixture of Compounds 1 and 2 on Mitochondrial Transmembrane Potential of SGC-7901 Cells. The number represents the percentage of mitochondrial transmembrane potential in each condition

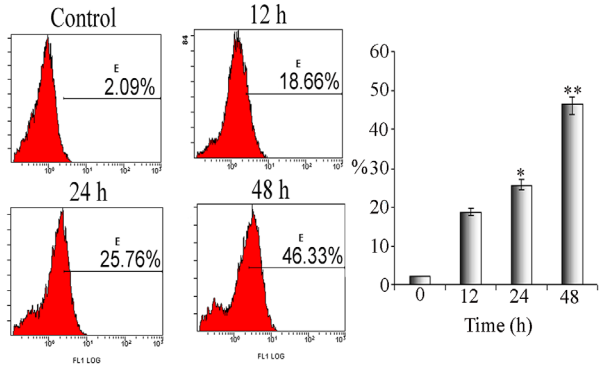

Figure 5. Effects of the Mixture of Compounds 1 and 2 on Intracellular ROS Levels of SGC-7901 Cells. The number represents the percentage of ROS level in each condition.

was relative to the DNA content. Apoptotic nuclei with hypodiploid DNA content harmonized to sub G1 peak (Figure 3). These results suggested that the mixture of compounds 1 and 2 could induce apoptotic cell death in SGC-7901 cells.

It has been reported that cell cycle progression is tightly controlled by various checkpoints in normal cells, while regulation of cell cycle is altered due to abnormal cell growth in cancerous cells. These checkpoints become activated due to certain factors such as DNA damage, exogenous stress signals, and defects during the replication of DNA or failure of chromosomes in attaching themselves to the mitotic spindle. The failure of this regulation is the hallmark of cancer. Several studies reveal that to maintain the proper cell cycle progression in cancer cells is a potential and effective strategy to halt tumor growth (Grana and Reddy, 1995; Kastan et al., 1995; Pavletich, 1999).

To determine the effect of the mixture of compounds 1 and 2 on cell cycle distribution, SGC-7901 cells were treated with the mixture of compounds 1 and 2 for 24 and 48 h, and were subjected to cell cycle analysis as described in material and method. It resulted in the increased accumulation of cells in the G2/M phase. The percentage of cells was increased from 5.98% in untreated cells to 21.21%, and 14.15% in cells treated with the mixture of compounds 1 and 2 for 24 and 48 h, respectively. It was observed that, maximum accumulation of cells in G2/M phase was observed at 24 h, which was decreased after an exposure for 48 h. Interestingly, the reduction in the percentage of cells in the G2/M phase, after 48 h of treatment with the mixture of compound 1 and 2, was linked with a concomitant increase in the apoptosis (Fig. 3). These findings suggest that G2/M phase arrest is one of the mechanisms through which the mixture of compounds 1 and 2 induce cytotoxicity in SGC-7901 cells. Several studies demonstrated that numerous chemotherapeutic and chemopreventive agents had potential antiproliferative effects via arresting the cell division at certain checkpoints in the cell cycle (Mantena et al., 2006; Srivastava and Gupta, 2006).

It is well known that the apoptosis involves a dysfunction of mitochondrial membrane integrity, which leads to cell death (Kang et al., 2010). Disintegration of the mitochondrial membrane potential and the redistribution of cytochrome c are critical events in the apoptotic cascade (Kluck et al., 1997; Wang, 2001). A sudden fall down of $\Delta\Psi$m was observed in some anticancer compounds-induced apoptosis in cancer cells (Chen et al., 2007). In this study, we investigated the effects of the mixture of compounds 1 and 2 on the $\Delta\Psi$ m. The rhodamine 123 was used to quantify the mitochondrial membrane potential. The decrease of rhodamine 123 fluorescence is directly proportional to the loss of mitochondrial membrane potential. The fluorescence intensity of rhodamine 123 was observed by flow cytometry which was 97.60%, 69.94%, 42.43%, and 27.86% at 0, 12, 24, and 48 h respectively (Figure 4).

The biological effects of flavonoids are mainly mediated by the formation of reactive oxygen species through the redox activation (Strathmann et al., 2010). ROS are famous mediators for intracellular signaling of cascades. Excessive generation of ROS can induce oxidative stress, loss of cell functioning, and apoptosis (Slater et al., 1995). ROS can also be involved in the process of lipid peroxidation and/or the cross linking of thiol groups in proteins; both of these processes can induce the opening of the mitochondrial permeability transition pore (PTP) (Vercesi et al., 1997; Lee et al., 2007). We assumed that the mixture of compounds 1 and 2 might arouse ROS level, which could be involved in apoptosis induced by the mixture of compounds 1 and 2. Therefore, the intracellular ROS level was measured using the ROS- detecting fluorescence dye 2, 7–dichlorofluorescein diacetate (DCF-DA) because the DCF assay is highly sensitive, linear, and precise for measuring oxidative stress in irradiated cells (Wan et al., 2003). The level of ROS was significantly increased in a time-dependent manner after treating the cells with the mixture of compounds 1 and 2. The ratio of DCF-positive cells was 2.09%, 18.7%, 25.8% and 46.3% at 0, 12, 24, and 48 h respectively (Fig. 5). The findings evidenced that the mixture of compounds 1 and 2 had enhanced the generation of ROS in SGC-7901 cells. The chemotherapeutic agents causing enhancement in oxidative stress are likely to be toxic to the cancer cells because they are found to be involved in the biological processes like cell cycle arrest, DNA repair, and apoptosis (Kang et al., 2010).

The main proteins which stimulate apoptotic process are caspases. These belong to the family of cysteine proteases. Caspase-3 is one of the inducer of apoptosis that is responsible either totally or partially for the

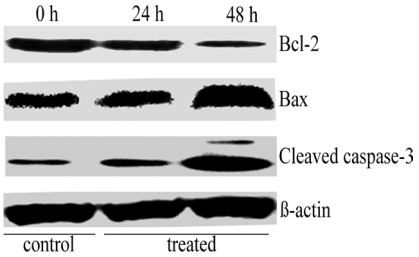

Figure 6. Effects of the Mixture of Compounds 1 and 2 on the Expression Levels of Apoptosis-related Proteins in SGC-7901 Cells

proteolytic cleavage of many key proteins such as poly (ADP-ribose) polymerase PARP (Cohen, 1997; Wang, 2001). Cytochrome c is released as a result of changes in mitochondrial membrane potential and mitochondrial permeability transition pores, which are induced by some signals during the mitochondrial mediated apoptosis. Cytochrome c release, being an important factor in mitochondrial mediated apoptosis, may induce the activation of caspase-3 which in turn results in the division of Pro-caspase-3 into two large units which leads to the automatic division of larger subunit into an active small subunit (Kluck et al., 1997). The activities of caspase-3 were examined in SGC-7901 cells treated with the mixture of compounds 1 and 2 for 24 or 48 h. We found that, the mixture of compounds 1 and 2 induced the cleavage of pro-caspase-3 into lager subunit after 24 h, which subsequently led to the automatic division of larger subunit into an active small subunit on the exposure of the mixture of compounds 1 and 2 till 48 h in SGC-7901 cells. The study concluded that the mixture of compounds 1 and 2 could lead to activation and cleavage of caspase-3 in SGC-7901 cells (Figure 6). The mixture of compounds 1 and 2 increased the activation of caspase-3 resulting in the death of SGC-7901 cells which affirmed the caspase dependent mitochondrial mediated apoptosis.

It was also reported that, change in the mitochondrial membrane potential and the release of cytochrome c from the mitochondria is also associated with ratio of Bax/Bcl-2 proteins (Kluck et al., 1997). Bcl-2 family proteins are mostly involved in the mitochondrial apoptotic pathway (Reed, 1998). Anti-apoptotic proteins like Bcl2 and pro-apoptotic like Bax are important members of this family. Bcl-2 and Bax work in an antagonistic manner. Bcl-2 suppresses apoptosis by stabilizing the mitochondrial membrane while Bax provoke apoptosis by increasing mitochondrial membrane permeability; which lead to the release of cytochrome c from mitochondria (Shimizu et al., 1999). The balance between the ratios of Bax/Bcl-2 is the measure of cell death under the influence of apoptotic stimulus such as the excessive generation of ROS (Chan, 2007). Thus, the expression of Bcl-2 and Bax was measured in cells treated with the mixture of compounds 1 and 2 by western blot analysis. An increase in expression of Bax and a decrease in expression of Bcl-2 in SGC-7901 cells were observed in a time-dependent manner after being exposed to the mixture of compounds 1 and 2 (Figure 6). Therefore, we concluded that the mixture of compounds 1 and 2 could promote opening of the mitochondrial permeability transition pore by the down

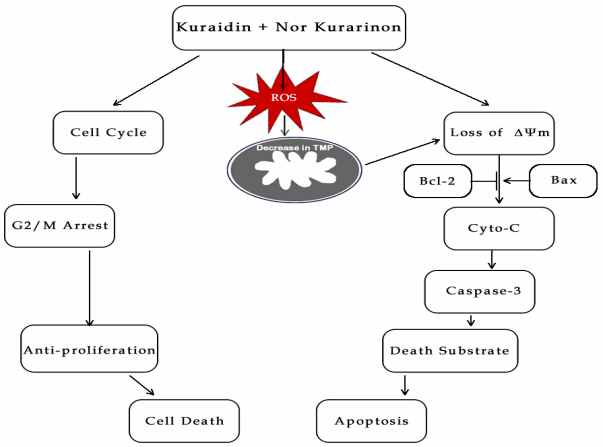

Figure 7. Hypothetical Model for Mechanism of Cytotoxic Activity of the Mixture of Kuraridin and Nor-Kurarinone

regulation of Bcl-2 and up-regulation of Bax.

Our results show that the mixture of compounds 1 and 2 can significantly inhibit the proliferation and induce apoptosis in SGC-7901 cells via mitochondrial apoptotic pathway. Apoptosis induced by the mixture of compounds 1 and 2 was associated with the dysfunction of ΔΨ m, reduction of Bcl-2/Bax ratio, followed by the activation and cleavage of caspase-3 (Figure 7). This study also discloses that mixture of compounds 1 and 2 are involved in the stimulation of ROS production. Our results indicate that the cytotoxic efficacy of the mixture of compounds 1 and 2 was mainly due to induction of G2/M phase cell cycle arrest as well as apoptosis. The mixture of compounds 1 and 2 may prove useful for the development of a novel agent for the treatment of gastric adenocarcinoma cells. Further studies of this possiblility are clearly warranted.

Acknowledgements

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