Chemotherapy is the main approach for treating advanced and recurrent hepatocellular carcinoma (HCC), but the clinical performance of chemotherapy is limited by a relatively low response rate, drug resistance, and adverse effects that severely affect the quality of life of patients. The aqueous extract of *Solanum nigrum* (AE-SN) is a crucial ingredient in some traditional Chinese medicine (TCM) formulas for treating cancer patients and exhibits antitumor effects in human HCC cells. Therefore, this study examined the tumor-suppression efficiency of AE-SN integrated with a standard chemotherapeutic drug, namely, cisplatin or doxorubicin, in human HCC cells, namely, Hep3B and HepJ5. The results suggested that the integrated treatment with AE-SN-potentiated cisplatin and doxorubicin induced cytotoxicity through the cleavage of caspase-7 and accumulation of microtubule-associated protein-1 light chain-3A/BII (LC-3A/BII), which were associated with apoptotic and autophagic cell death, respectively, in both the Hep3B and HepJ5 cells. In conclusion, AE-SN can potentially be used in novel integrated chemotherapy with cisplatin or doxorubicin to treat HCC patients.

1. Introduction

Liver cancer is one of the most common malignant diseases worldwide, particularly in eastern Asia and sub-Saharan Africa, and hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer [1]. A major challenge in treating HCC is the poor prognosis for advanced and recurrent cases.
Although chemotherapy is the main approach used to treat advanced and recurrent HCC cases, its clinical performance is largely limited by various factors such as a relatively low response rate, drug resistance, and various adverse effects that substantially impact the quality of life (QOL) of HCC patients [2]. The development of complementary and alternative medicines for improving the tumor-suppression efficiency of current chemotherapeutic drugs and managing the QOL of HCC patients has become an accepted optional approach worldwide [3, 4]. Traditional Chinese medicine (TCM) has long been employed in treating various cancers through the use of numerous herb-based formulas; however, most of these formulas lack sufficient, basic clinical medical evidence verifying their antitumor efficacy.

TCM formulas are normally prepared using mixed extracts, with the composition and dose of the ingredients sometimes varying among individual cases. The varying composition and dosage cause difficulty in clarifying the antitumor efficacy of the formulas in clinical trials and experimental studies [2]. An alternative approach is examining the individual ingredients from specific TCM formulas that may contribute to the tumor-suppression efficacy. For instance, recent studies have suggested that certain crude extracts in TCM formulas, such as extracts of *Semen Cocis*, *Scutellaria barbata*, and *Solanum nigrum*, exhibited tumor-suppression efficacy in human HCC cells [5–7]. In recent studies, total flavonoids extracted from *Scutellaria barbata* inhibited cell proliferation and invasion of hepatocarcinoma via mediation of matrix metalloproteinases and metalloproteinases [8], and saikosaponin-D extracted from *Bupleurum chinense* DC also was reported to enhance radio sensitivity on hepatoma cells by adjusting cell cycle or hypoxic conditions [9, 10]. These findings suggested that components and crude extracts of some TCM herbs may inhibit hepatocarcinoma cells via various mechanisms. The crude extracts of *Solanum nigrum* have demonstrated antitumor effects in various types of cancer, including human melanoma and colorectal, endometrial, cervical, and breast cancers [11–15]. Previous studies have indicated that the aqueous extract of *Solanum nigrum* leaves (AE-SN) mainly suppressed tumor cell growth by activating programmed cell death associated with caspase-3-dependent apoptosis [7] and LC-3 A/B-related autophagy [7, 11, 12, 14]. In addition, AE-SN is capable of enhancing the cytotoxicity induced by various chemotherapeutic drugs, including cisplatin, doxorubicin, and docetaxel, in human endometrial and colorectal cancer cells [11, 12], suggesting that AE-SN is a potential ingredient to develop for integrated chemotherapy with standard chemotherapeutic drugs. Because cisplatin and doxorubicin are the standard therapeutic drugs for treating HCC cases, knowing the antitumor effects of AE-SN in combination with either cisplatin or doxorubicin in human HCC cells would be beneficial.

To understand the potential of AE-SN for use in integrated chemotherapy with cisplatin or doxorubicin in human HCC cells, the main aim of the present study was to clarify whether AE-SN enhances the cytotoxicity induced by cisplatin and doxorubicin in human HCC cells. The results showed that a single treatment with AE-SN activated programmed cell death and provides insight into the efficacy of integrating AE-SN with chemotherapeutic drugs in treating HCC cells. The study results provide experimental evidence for supporting further application of AE-SN in HCC therapy.

### 2. Materials and Methods

#### 2.1. Cell Lines and Regents

Two human HCC cell lines, namely, Hep3B and Hep15, and one normal human pulmonary fibroblast, namely, WI-38, were purchased from the Biorepository Collection and Research Center (Hsinchu, Taiwan). Hep3B is an HCC cell line commonly used to examine antitumor components, and, by comparison, Hep15 is a more malignant and resistant cell line that exhibits high expression of survivin [16]. This study used these two HCC cell lines to examine the antitumor effects of AE-SN and used WI-38 cells to examine the cytotoxicity of AE-SN in normal human cells. All of the cell lines were maintained in Dulbecco’s modified Eagle’s medium ( Gibco, Grand Island, NY, USA) in addition to 100 U/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO₂ humidified incubator. Cisplatin and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell lysis buffer was prepared using a solution of 150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/mL of leupeptin, and 100 μg/mL of aprotinin. The primary antibodies used to detect protein expression and activation were LC-3 A/B, caspase-3, and caspase-7 (Cell Signaling Technology, Danvers, MA, USA) as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH, AbFrontier, Seoul, South Korea). A secondary antibody, donkey anti-rabbit horseradish peroxidase conjugate, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HPLC-grade acetoniitrile (ACN) and methanol were purchased from JT Baker (Phillipsburg, NJ, USA). The purified solamargin was purchased from Tauto Biotech (Shanghai, China).

AE-SN was prepared using the TCM processing method to simulate the AE-SN administered to patients in TCM clinical practice. In brief, 50 g of dried *Solanum nigrum* leaves was immersed in 750 mL of distilled water, gradually heated to 100°C, and maintained at 100°C for 1 h. The AE-SN solution was further condensed to a final concentration of 1 g of raw material/mL through heating at 100°C. The final AE-SN stock solution (1 g/mL) was filtered using a 0.22 μm filter before the experiment.

#### 2.2. Quantitative Analysis of Solamargin in AE-SN

Solamargin is considered as a crucial antitumor component that existed in *Solanum nigrum* and can be a marker component for AE-SN [17]. The concentration of solamargin was therefore determined in the AE-SN stock solution by using liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Before analysis, 100 μL AE-SN stock solution was mixed with 900 μL mixture of water : methanol (30 : 70, v/v). LC-MS/MS analysis was performed in the mobile phase of HPLC-grade acetoniitrile (ACN) and deionized water with a Syncronis C18 column (Thermo Scientific, MA, USA). The flow rate is 0.5 mL/min with splitted 0.25 mL/min to mass. The ionization mode of the mass spectrometry condition was
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Figure 1: Extracted ion chromatography of solamargine. (a) The parent ion of purified solamargine was fragmented into two daughter ions (blue and red peaks). (b) Fragmented ions presented in AE-SN.

2.3. Cell Viability Assay and Morphological Observation. Hep3B and HepJ5 cells were plated onto 96-well plates, with $5 \times 10^3$ cells per well, and cultured overnight before treatment. To evaluate the antitumor effects of AE-SN, the cells were treated with 0 to 2.0 mg/mL of AE-SN for 48 h. To evaluate the antitumor effects of integrated treatment with the chemotherapeutic drugs and AE-SN, the cells were treated with 0 to 20 μM cisplatin or 0 to 10 μM and 0.5, or 1.0 mg/mL of AE-SN for 48 h. In this study, cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 1(b)). The concentration of solamargine in AE-SN stock solution was 77 μg/mL by using MRM analysis.

Table 1: IC50 values in HCC cells treated with AE-SN alone or in combination with AE-SN and either chemotherapeutic drug. The IC50 values were analyzed using cell viability data determined after 48 h treatment with AE-SN (Figure 2) and with AE-SN integrated with either chemotherapeutic drug (Figure 3).

<table>
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<tr>
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<th>Hep3B</th>
<th>HepJ5</th>
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<tr>
<td><strong>AE-SN (mg/mL)</strong></td>
<td>0.96</td>
<td>0.97</td>
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<tr>
<td><strong>Combination of AE-SN (mg/mL)</strong></td>
<td>0</td>
<td>0.5</td>
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<tr>
<td>Cisplatin (μM)</td>
<td>6.75</td>
<td>2.74</td>
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<tr>
<td>Doxorubicin (μM)</td>
<td>4.65</td>
<td>1.31</td>
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2.4. Western Blotting Analysis. Hep3B and HepJ5 cells were planted into 6 cm dishes with $5 \times 10^5$ cells per dish and cultured overnight. The cells were treated with a control medium, namely, 5 μM of cisplatin or 2 μM of doxorubicin, and 0 or 1.0 mg/mL of AE-SN for 48 h. After 48 h of incubation, the cells were collected using a cell lysis buffer. The protein concentrations of the cell lysates were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and equalized before western blotting analysis. The cell lysates were separated using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred into a polyvinylidene fluoride membrane (Pall Corp, Port Washington, NY, USA).

The expression and activation of the selected protein markers, namely, LC-3 A/B, caspase-3, and caspase-7, as well as the internal control, GAPDH, were then determined using corresponding primary and secondary antibodies. The immunoreactivity was detected using an electrochemiluminescence western blotting detection kit (Western Lightning Plus-ECL, PerkinElmer Inc., Waltham, MA, USA).

2.5. Statistical Analysis. Analyses of the half maximum inhibitory concentration (IC50) were performed using CalcuSyn software (Biosoft, Cambridge, UK), whereas Student’s t test and one-way ANOVA were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cytotoxicity of AE-SN Alone and with the Chemotherapeutic Drugs in Hep3B and HepJ5 Cells. In this study,
48 h treatment with 0 to 2 mg/mL of AE-SN gradually inhibited the growth of Hep3B and HepJ5 cells in a concentration-dependent manner (one-way ANOVA, $P < 0.01$, Figure 2(a)). The IC$_{50}$ values of AE-SN for the Hep3B and HepJ5 cells were 0.96 and 0.97 mg/mL, respectively. AE-SN treatment also resulted in the production of cell fragments and debris, which were less than 12 $\mu$m in diameter in comparison with cells treated with the control medium in both Hep3B and HepJ5 cells (Figure 2(b)). Collectively, these results suggested that AE-SN inhibited cell growth and demonstrated cytotoxicity in Hep3B and HepJ5 cells.

To evaluate cytotoxicity on the integrated treatment with chemotherapeutic drugs and AE-SN, cisplatin (1 to 20 $\mu$M) or doxorubicin (1 to 10 $\mu$M) was used to treat the Hep3B and HepJ5 cells, respectively, with 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h (Figure 3). The IC$_{50}$ values of the cisplatin integrated with 0.5 mg/mL of AE-SN in the Hep3B and HepJ5 cells were reduced to 2.74 $\mu$M and 2.84 $\mu$M, respectively, whereas those of doxorubicin were reduced to 1.31 $\mu$M and 1.42 $\mu$M. In other words, the IC$_{50}$ values for cisplatin and doxorubicin integrated with 0.5 mg/mL of AE-SN were reduced to 40% and 30% of the values of the drugs used alone in the Hep3B and J5 cells, respectively. Human pulmonary fibroblast cells, WI-38, were also treated with 0, 0.5, or 1.0 mg/mL of AE-SN and cisplatin or doxorubicin to identify the cytotoxicity of the combined treatment (Figures 3(c) and 3(f)). AE-SN was not likely to enhance cisplatin- or doxorubicin-induced cytotoxicity in the WI-38 cells in comparison with that in the

Figure 2: AE-SN treatment inhibited Hep3B and HepJ5 cell growth. (a) Hep3B and HepJ5 cells were treated with 0.1 to 2 mg/mL of AE-SN for 48 h, and the cell viability was determined using an MTT assay. The data are presented as the mean ± standard deviation. (b) Hep3B and HepJ5 cells were treated with 1.0 mg/mL of AE-SN for 48 h, and the cell size distribution was determined according to the cell diameter by using a Scepter cell counter. Arrows indicate a cell diameter of 12 $\mu$m.
Figure 3: AE-SN-potentiated cisplatin and doxorubicin induced cytotoxicity in Hep3B and HepJ5 cells but had no effect on normal human pulmonary fibroblasts (WI-38 cells). (a–c) Cells were treated with 0 to 20 μM cisplatin and 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h. (d–f) Cells were treated with 0 to 10 μM doxorubicin and 0, 0.5, or 1.0 mg/mL of AE-SN for 48 h. The cell viability was determined using an MTT assay; the data are presented as the mean ± standard deviation.
3.2. Activation of Programmed-Cell-Death-Related Protein in AE-SN and Chemotherapeutic-Drug-Treated Cancer Cells. Hep3B and HepJ5 cells treated with AE-SN demonstrated a clear morphological change similar to the formation of phagolysosome-like vacuoles (Figures 4(a) to 4(d)). This morphological change was also observed in AE-SN-treated human endometrial and colorectal carcinoma cells [11] and related to the activation of autophagy.

To further understand whether AE-SN increases the activation of cell death protein when combined with either cisplatin or doxorubicin in the two HCC cell lines, the activation of LC-3 A/B and caspase-7 was observed in HCC cells treated with 1 mg/mL of AE-SN and 5 μM cisplatin or 2 μM doxorubicin for 48 h. As shown in Figure 5, the results suggested that, in both Hep3B and HepJ5 cells, AE-SN induced LC-3 A/B II accumulation in all of the treatment groups. The cleavage of caspase-7 was also enhanced by AE-SN cotreatment in cisplatin-treated Hep3B and HepJ5 cells and in doxorubicin-treated HepJ5 cells, but not in doxorubicin-treated Hep3B cells. Doxorubicin cotreatment seemed to eliminate the AE-SN-induced caspase-7 cleavage in the Hep3B cells.

4. Discussion

In this study, the impact of AE-SN on tumor-suppression efficiency was evaluated by employing the preparation of AE-SN that is used in TCM clinical practice. The IC$_{50}$ values of AE-SN for Hep3B and HepJ5 cells indicated a moderate anti-tumor effect through direct exposure. Our previous studies showed that administering AE-SN at approximately 0.5 to 1 mg/mL for 48 h yielded similar IC$_{50}$ values in human endometrial and colorectal cancer cells [11,12]. In comparison with the tumor-suppression efficiency observed in previous studies using AE-SN extracts prepared from dried powder through water extraction [7,13,14,19,20], the tumor-suppression efficiency of AE-SN in this study was similar. The results of these experimental studies collectively suggest that cancer treatment may be a reasonable application for the preparation of AE-SN used in TCM practice. In addition, integrated treatment of AE-SN with cisplatin and doxorubicin may substantially reduce the required dose of cisplatin and doxorubicin required to achieve the same tumor-suppression efficiency, thereby improving the QOL of HCC patients during chemotherapy.

AE-SN was suggested to activate both autophagic and apoptotic cell death in many human cancer cell lines by inducing the cleavage of caspase-3 and accumulation of LC-3 A/B II [7]. By contrast, this study indicated that AE-SN
Figure 5: Activation of selected protein markers in HCC cells treated with AE-SN and either cisplatin or doxorubicin. (a) Cells were treated with a control medium, namely, 5 \( \mu \)M cisplatin or 2 \( \mu \)M doxorubicin with 0 or 1.0 mg/mL of AE-SN for 48 h. The activation of LC-3 A/B and caspase-7 was determined using western blotting analysis. GAPDH served as an internal control. (b) Semiquantitation of the LC-3 A/B II and cleaved caspase-7 in the Hep3B and HepJ5 cells. The data are presented as the mean ± standard deviation. * indicates statistical significance in comparison with the previous group, namely, Con versus AESN (vehicle control versus AE-SN), Cs versus Cs + AESN, and Dx versus Dx + AESN (two-tailed Student's t-test, \( P < 0.05 \)).
unable to resist AE-SN-induced cytotoxicity and tended to be more vulnerable to cisplatin- and doxorubicin-induced cytotoxicity in combined treatment with AE-SN. The molecular mechanisms involved in this AE-SN-mediated HCC cytotoxicity require further investigation.

Some steroidal alkaloid glycosides and glycoproteins are considered the major active substances responsible for AE-SN-induced programmed cell death. Solamargine is one of the major compounds present in AE-SN that can activate caspase-3-related apoptosis and LC-3-A/B-II-related autophagy in human leukemia cells [22], and a 150 kDa glycoprotein isolated from AE-SN was reported to be another effective antigenic compound for activating caspase-3-related apoptosis in human colorectal, cervical, and HCC cells [23–25]. However, these isolated compounds do not seem to completely explain the tumor-suppression mechanism of AE-SN because the specific compound that activates the cleavage of caspase-7 remains unknown. Further investigation is therefore required to identify the exact composition of the AE-SN substance that contributes to the antitumor effects.

This study clarified the antitumor effects of AE-SN and the potential of AE-SN for enhancing cytotoxicity induced by cisplatin and doxorubicin in human HCC cells in vitro. In consideration of the absorption rate of AE-SN through the gastrointestinal tracts and its metabolism in vivo, the potential of AE-SN for enhancing cytotoxicity induced by combining cisplatin and doxorubicin in HCC cells needs to be further explored. Our results suggest that AE-SN can be used in novel integrated chemotherapy with cisplatin and doxorubicin to improve tumor-suppression efficiency in HCC treatment.

**Conflict of Interests**

The authors declare that there is no conflict of interests.

**Authors’ Contribution**

Chien-Kai Wang and Yi-Feng Lin contributed equally to this study.

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**References**


