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Abstract

Solanum nigrum L. (SN) has been used in traditional folk medicine to treat different cancers. It is also used as hepatoprotective and anti-inflammation agent. In this study, we demonstrated that the extract of SN (SNE) induced a strong cytotoxic effect toward HepG2 cells, but much less to Chang liver and WRL-68 cells. The mechanisms of the cytotoxic effect were concentration-dependent. High doses of SNE (2 mg/ml and 5 mg/ml) induced apoptotic cell death in HepG2 cells, as evidenced by increases in the expressions of p-JNK and Bax, mitochodiral release of cytochrome *c* and caspase activation. On the other hand, cells treated with low concentration of SNE (50~1000 μg/ml) revealed morphology and ultrastructural changes of autophagocytic death under electron microscopic observation. Furthermore, these cells showed increased levels of autophagic vacuoles, and LC3-I and LC3-II proteins, specific markers of autophagy. The levels of Bcl-2 and Akt that have been implicated in the down-regulation of autophagy were decreased upon SNE treatment. Taken together, these findings indicate that SNE induced cell death in hepatoma cells via two distinct anti-neoplastic activities of SNE, the ability to induce apoptosis and autophagocytosis, therefore, suggesting that it may provide leverage to treat liver cancer.

Key words: *Solanum Nigrum* L., Apoptosis, Autophagy, LC3, Acidic vesicular organelle

Introduction

Solanum nigrum L. (SN) is a herbal plant indigenous to the Asia, and grows wildly and abundantly in open fields. It has been used in traditional Oriental medicines for treating a various kinds of tumors and is believed to have various biological activities (1). For examples, in the Chinese traditional medicine, SN is used as hepatoprotective and anti-inflammation agent. A freshly prepared extract of the herb is effective in cirrhosis of liver, juice of the leaves alleviates pain in inflammation of the kidney and bladder, and internally for cardialgia (2). More specifically, it has been used to cure hepatic cancer for a long time in Oriental medicine (3). Previous investigations have shown that extracts of SN suppressed the oxidant mediated DNA-sugar damage (3), and the plant exerted cytoprotection against gentamicin-induced toxicity on Vero cells (2) and anti-neoplastic activity against Sarcoma 180 in mice (4). More recent studies revealed that extracts of SN induced apoptosis in MCF-7 cells (1) and inhibited 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced tumor promotion in HCT-116 cells (5). The ethanol extract of dried fruits of SN had a remarkable hepatoprotective effect in CCl_4-induced liver damage (6). These studies suggest that SN possesses a beneficial activity as anti-oxidant and anti-tumor promoting agent, although the mechanism for the activity remains to be elucidated.

SN has been reported to contain many polyphenolic compounds, mainly flavonoids and steroids. The antioxidant and anti-tumor activity of SN may be due to the presence of polyphenolic constituents (7). The presence of steroidal glycosides, steroidal alkaroids, steroidal oligoglycosides, solamargine, and solasonine has also been detected (8, 9).

The purpose of this work was to study the effects of SN on liver cancer cells to evaluate its therapeutic potential in treating liver cancer. In this report, we describe experiments that showed water extracts of SN induced programmed cell death in liver cancer cells. The death mechanism was characterized, revealing that SN not only initiated apoptosis but also caused cell death through autophagocytosis (type II programmed cell death). The results of this investigation provide a scientific evidence for the application of this herbal medicine in liver cancer therapy.

Materials and methods

Preparation of extracts of SN. (SNE) The whole plant of SN was collected from the mountain in Miaoli, Taiwan. The plants were washed, cut into small pieces, shade dried for 3 days, and then dried overnight in an oven. The dried SN (800 g) was mix with water (5000 ml) for 30 minutes, and subjected to continuous hot extraction (100℃, 40 minutes). The resulting water extract was subsequently concentrated with water bath (90℃) until became creamy, and dried in an oven (70℃) that finally gave 185 g (23.125 % of initial amount) of water extract of SN. The concentration used in the experiment was based on the dry weight of the extract.

HPLC conditions. After hydrolysis by boiling in 1 N HCl for 5 min, the components of SNE were determined by HPLC analysis using a Hewlett-Packed Vectra 436/33N system with diode array detector. The HPLC method employed a 5 μm RP-18 column (4.6 × 150 mm i.d.). SNE was filtered through a 0.45 μm filter disc, and 10 μl of which were injected into the column. The chromoatography was monitored at 280 nm, and UV spectra were colleted to confirm peak purity. The mobile phase contained two solvents [A, acetic acid/water (2%, v/v); B, 0.5% acetic acid in water/acetonitrile (50:50, v/v)], and run by a linear gradient method at room temperature as follows: from 10% B to 80%, using a flow rate of 1 ml/min, over 70 min (10).

Eletron Miroscopy. The cells were harvested by trypsinization, washed twice with PBS, and fixed with 2% glutaraldehyde, 4% paraformaldehyde and 1% tannic acid in 0.1 mol/l cacodylate buffer, pH 7.4, for 25 h at 4℃. After washing with PBS, the cells were stained with an osmium-thiocarbohydrazide-osmium (OTO) sequence as described by Willingham and Rutherford (11). This procedure was carried out by incubating cells in 1% OsO_4 in 0.1 mol/l cacodylate buffer, pH 7.4, for one hour followed by 1% thiocarbohydrazide in H_2O for 15 minutes and then 1% OsO_4 for 15 minutes. Extensive washing of cells with distilled water was performed between each step. After staining, the cells were dehydrated in a graded series of EtOH to 100% EtOH and then immersed serially with 1:1 hexamethyldisilazane and absolute ethanol, and pure hexamethyldisilazane for five minutes each. After air drying from hexamethyldisilazane for overnight, the cells were embedded in agarose gel. One-μm thin sections were cut and the gels were coated with 500 Å of gold in a JEOL Vacuum sputter coater and viewed in a JEOL T300 electron microscope with scanning attachment (JEOL, Tokyo, Japan) (12).

Detection of acidic vesicular organelles (AVO) with acridine orange staining. To detect AVOs in SNE-treated cells, the vital staining with acridine orange was performed as described previously (13-15). The treated-tumor cells were stained with acridine orange, adding at a final concentration of 1 μg/ml for 15 minutes. Samples were then examed under a fluorescence microscope. Acridine orange labels acidic vesicular organelles, such as autophagosomes (16). A typical acridine orange-positive cell exhibits granular distribution of acridine orange in the cytoplasm indicative of autophagosome formation.

Results

Composition of SNE. To establish the composition of SNE from *Solanum Nigrum* L., the concentrations of polyphenolic acids were determined by HPLC. The dry weight yield of SNE was 23.125 ± 2.095%, consisting of 20.35 ± 0.967% total polyphenolics using gallic acid as standard. HPLC analysis of the standard polyphenols showed the retention times (RT) of gallic aicd, protocatechuic acid (PCA), catechin, (-)-epigallocatechin galllate (EGCG), caffeic acid, epicatechin, rutin, quercetin and naringenin were 7.94, 15.23, 20.68, 23.47, 25.95, 27.14, 33.31, 51.23 and 55.95 min, respectively (Figure 1). The analysis of SNE revealed the presence of gallic aicd (2.897%), PCA (1.977%), catechin (2.353%), EGCG (1.533%), caffeic acid (1.988%), epicatechin (0.392%), rutin (0.836%) and naringenin (5.106%). The composition of the SNE was summarized in Table 1. The extract was stored at -20℃ and used in the following studies.

Cytotoxic effects of SNE on HepG2 cells. In this study, we first determined the cytotoxicity of SNE by treating HepG2, WRL-68 and Chang liver cells with SNE at various concentrations for 24 h and 48 h followed by MTT assay. The human hepatic cell line WRL-68 has a morphological structure similar to hepatocytes and hepatic primary cultures. Derived from fetal liver, WRL-68 cells secrete α-fetoprotein and albumin, preserve the activity of some characteristic or specific liver enzymes (i.e. alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase and alkaline phosphatase) and exhibit a cytokeratin pattern similar to other hepatic cultures, providing an in vitro model to study the toxic effects of xenobiotics (17).

The addition of SNE exerted a cytotoxic effect in a dose- and time-dependent manner (Figure 2AB). Following a 24 h incubation with 50 μg/ml of SNE in HepG2 cells, the cytotoxicity was around 22.7~27.4% (Figure 2A). However, when 100~5000 μg/ml of the extract was added, a significant increase in cytotoxicity was observed. Furthermore, a time-dependent increase in SNE-induced cytotoxicity was also detected (Figure 2B). The strongest potency of SNE on the cytotoxicity of cells was toward HepG2 liver cancer cells. The concentration of SNE on the inhibition of 50% of HepG2 cells viability (IC_{50}) was 0.625 mg/ml. However, the IC_{50} of SNE to the death of Chang liver (human liver cells; IC_{50}: 3.2 mg/ml SNE) and WRL-68 (human fetal liver cell; IC_{50}: 3.0 mg/ml SNE) were 5.12-fold and 4.8-fold higher than that of HepG2 cells indicating that SNE is less cytotoxic to normal cells.

SNE-induced apoptotic death of HepG2 cells. HepG2 cells treated with 2 and 5 mg/ml SNE for 48 h showed typical apoptotic features: cell shrinkage, membrane blebbing, and apoptotic bodies as observed under inverted microscopy. To further confirm the programmed cell death was involved in the cytotoxic effect of SNE on liver cancer cells, SNE-treated HepG2 cells were subjected to apoptosis assays by PI staining of nuclei as described in Materials and methods, and the apoptotic state was quantitated by flow cytometry. The results indicated that after a treatment with high concentration (2 and 5 mg/ml) of SNE for 48 h, an increased proportion of apoptotic cells were observed (Figure 3A). The ratio of cells at the hypodiploid phase were increased to 30.01% and 37.88% when HepG2 cells were exposed to 2.0 mg/ml and 5.0 mg/ml SNE for 48 h, respectively (Figure 3A).

Effect of SNE on JNK phosphorylation and Bax protein expression. JNK plays an important role in apoptotic signaling. In some cell types, JNK regulate the activities of pre-existing Bcl-2 family proteins that mediate mitochondrial release of cytochrome *c*, resulting in caspase activation (18). We investigated whether the SNE-induced apoptosis was modulated by the activation of JNK. The results showed that the cellular level of phospho-JNK was significantly increased to 2.65-fold and 2.81-fold ($P<0.001$) of control level under a treatment of 2 mg/ml and 5 mg/ml of SNE, respectively (Figure 3B). Investigations of the bcl-2 gene family that encodes integral membrane proteins have shown a complex network regulating apoptosis in multiple biological systems (19). We examined the cellular levels of Bax after the treatment of SNE for 48 h in HepG2 cells. The expressions of Bax were significantly induced by the treatment of 2 mg/ml and 5 mg/ml of SNE to 3.45-fold and 3.52-fold ($P<0.001$), respectively (Figure 3B).

Effect of SNL on cytochrome c release and caspase 3 cleavage. Since cytochrome *c* is reported to be involved in the activation of the caspase that executes apoptosis (20, 21), we examined the level of cytochrome *c* in the cytosol by Western blot analysis. The results showed that the amount of cytosolic cytochrome *c* increased 1.88-fold and 2.05-fold ($P<0.001$) in the 2 mg/ml and 5mg/ml SNE-treated HepG2 cells (Figure 3C).

Caspases 3 is a cytosolic protein that exists normally as an inactive precursor with higher molecular weights (32 kDa) that is cleaved proteolytically into low molecular weights (20 kDa) when cell undergoes apoptosis

(22). In this study, there was an increase in the activation of caspase 3 to 1.91-fold, 2.3-fold and 2.45-fold (**P<0.01; ***P<0.001) of control level in response to 1 mg/ml, 2 mg/ml and 5 mg/ml SNE treatment, respectively (Figure 3D).

Low concentration of SNE-induced autophagic cell death in HepG2 cells. The flow cytometric data (Figure 3A) showed that there was only a very small portion (< 4 %) of HepG2 cells displayed a typical morphology of apoptosis when the cells were exposed to low concentrations (50 to 1000 μg/ml) of SNE. At the same circumstances, the survival rates were down to 67.7% and 20% for 50 and 1000 μg/ml, respectively (Figure 2B). These observations indicated that some other death mechanism was initiated in HepG2 cells in response to the treatment of low concentrations (50 to 1000 μg/ml) of SNE.

Electron microscopic characterization, which has been the gold standard for determining the mode of cell death, was next used to distinguish between apoptosis, necrosis and nonapoptotic programmed cell death. Nonapoptotic programmed cell death is principally attributed to autophagy (type II programmed cell death). Autophagy is series of biochemical steps through which eukaryotic cells commit suicide by degrading their own cytoplasm and organelles through a process in which these components are engulfed and then digested in double membrane-bound vacuoles called autophagosomes (23). Transmission electron microscopic analysis of HepG2 cells without SNE treatment revealed normal nuclear and mitochondrial morphology (Figure 4A-a). On the other hand, HepG2 cells treated with 100 μg/ml of SNE for 48 h revealed extensive vacuolization, formation of membranous whorls (also called myelin figures) and depletion of organelles, which are hallmarks of autophagy (Figure 4A-b,c). Cells undergoing autophagic cell death retained an intact nuclear membrane, without chromatin condensation. Initiation of autophagy was associated with an accumulation of lipid droplets in cytoplasm (fatty change of hepatocytes) (Figure 4A-b). This event is due to a decline in protein synthesis (vide infra) which blocks the utilization of lipids for lipid-protein conjugation and is typical of hepatocytes undergoing cellular stress (24).

Autophagy is characterized by AVO formation, which is detected and measured by vital staining of acridine orange. Acridine orange moves freely to cross biological membranes and accumulates in acidic compartment, where it is seen as fluorescence bright red (13, 25, 26). As shown in Figure 4B-b~f, vital staining of HepG2 cells with acridine orange showed the accumulation of AVO in the cytoplasm of cells exposed to 10~1000 μg/ml of SNE. In contrast, there were relatively few AVOs in the cytoplasm of control cells (Figure 4B-a).

Involvement of LC3 in SNE-induced autophagy. LC3 is localized in autophagosome membranes during amino acid starvation-induced autophagy (27, 28). Recent investigation showed that there are two forms of LC3 proteins in cells: LC3-I and LC3-II (26, 27). LC3-I is the cytoplasmic form of LC3 and is processed into LC3-II, which is associated with the autophagosome membrane. Therefore, the amount of LC3-II is correlated with the extent of autophagosome formation. Using the immunoblotting analysis with anti-LC3 antibody, we examined the expressions of LC3-I (18 kDa) and LC3-II (16 kDa) in HepG2 cells treated with SNE. As shown in Figure 5A, the level of total LC3 (LC3-I and LC3-II proteins) increased in HepG2 cells 24 h after exposure to SNE. Moreover, a marked increase in LC3-II protein was also detected in these cells. These results indicate that SNE stimulated not only the accumulation of LC3 protein, but also the conversion of a fraction of LC3-I into LC3-II.

Effect of SNE on Bcl-2 expression and Akt phosphorylation. Autophagy is a multi-step process, and various signaling pathways have been implicated in its up- or down-regulation (29-32). Bcl-2 has also been shown to

regulate autophagy in cancer cells. Down-regulation of Bcl-2 using antisense technology triggered autophagy, but not apoptosis, in HL60 human leukaemic cells (31, 33). We investigated whether the SNE-induced autophagy was modulated by Bcl-2. The results showed that the cellular level of Bcl-2 was significantly decreased to 80%, 25% and 18% of that of control in the cells exposed to 100 μg/ml, 500 μg/ml and 1000μg/ml of SNE treatment, respectively (Figure 5B). Furthermore, Akt is a serine-threonine kinase, located downstream of class I PI3K, that activates the kinase mTOR, leading to suppression of autophagy. We examined the level of phospho-Akt in the cytosol by Western blot analysis. The results showed that the amount of phospho-Akt decreased to 45%, 40% and 25% of control in the cells exposed to 100 μg/ml, 500 μg/ml and 1000 μg/ml of SNE treatment, respectively ($P<0.001$) (Figure 5C). These results indicate that Bcl-2 and Akt were involved in the SNE-induced autophagy.

Discussion

Solanum nigrum L. (SN) is a common herb that grows wildly and abundantly in open field. It has been used in traditional folk medicine because of its diuretic and antipyretic effects. More specifically, it has been used to cure inflammation, edema, mastitis and hepatic cancer for a long time in oriental medicine (34). The phytochemical studies revealed the presence of an alkaloid called solamargine, nigrum I, nigrum II and a glycoside named solasodine (6). It has also been shown that SN contains many polyphenolic compounds, mainly flavonoids and steroids. Our results showed that SNE is consisting of 20.35 % total polyphenols, such as gallic aicd (2.897%), PCA (1.977%), catechin (2.353%), EGCG (1.533%), caffeic acid (1.988%), epicatechin (0.392%), rutin (0.836%), naringenin (5.106%) and unknow polyphenols (Figure 1 and Table 1). The antioxidant and anti-tumor activity of these extracts has been suggested to be due to the presence of polyphenolic constituents (7). Nevertheless, there is little conclusive evidence demonstrating the effectiveness of SN on treating already existed 50 to 1000 μg/ml tumors or malignancies animals.

In Chinese traditional medicine, SN is believed to have anti-tumor properties, including liver cancer, breast cancer, lung cancer, stomach cancer, colon cancer and bladder cancer, and is also used as hepatoprotective and anti-inflammation agent, although the mechanism for the activity remains to be elucidated (2, 3). The latest report revealed that the ethanol extract of dried fruits of *Solanum nigrum* has a remarkable hepatoprotective effect against the CCl_4-induced liver damage (6). This study developed some understanding of the effects of SN on liver cancer cells to evaluate its therapeutic application in treating liver cancer.

Our results demonstrated a significant cytotoxic effect of SNE on HepG2 cells that was mediated via two mechanisms depending on the exposed concentrations. When HepG2 cells were treated with high concentration (2 mg/ml and 5 mg/ml) of SNE, the cells underwent apoptotic cell death as evidenced by increases in sub-G1 cells, and cellular levels of phospho-JNK, Bax, cytosolic cytochrome *c* and cleaved-caspase 3. Exposure of low concentration (50~1000 μg/ml) of SNE did not result in apoptosis, but rather our results point to autophagocytosis (autophagy of type II programmed cell death) as the main mode of death under such condition.

Autophagy is a physiological mechanism that involves the sequestration of cytoplasm and intracellular organelles into membrane vacuoles and results in their eventual enzymatic degradation (23). In response to appropriate stimulation, depolarized mitochondria are known to move into autophagic vacuoles. Thus, mitochondrial dysfunction may be a point of overlap between apoptotic and autophagocytic processes (35). The fusion of the edges of the membrane sac forms a closed double-membrane structure, the so-called

autophagosome. Finally, the autophagosome fuses with a lysosome to become the autolysosome within which lysosomal hydrolases degrade the sequestered cellular constituents. HepG2 cells treated with 50~1000 μg/ml of SNE demonstrated an ultrastructural appearance consistent with the characteristics of autophagy under electron microscopic observation. Furthermore, SNE-treated cells were able to be stained with acridine orange, a specific marker for autophagic vacuoles (36). Confirmatory experiments were performed with anti-LC3 antibody showing that SNE stimulated not only the expression of LC3 protein, but also the conversion of a fraction of LC3-I into LC3-II. Furthermore, the levels of Bcl-2 and Akt, that have been implicated in down-regulation of autophagy were decreased upon SNE treatment, confirming the involvement of Bcl-2 and Akt in the SNE-induced autophagy (32).

Nonapoptotic cell death is mainly attributed to autophagy that is considered to be an alternative way to kill tumor cells when the cells are chemoresistant on the basis of ineffective apoptosis (37). A number of studies have reported that autophagy is activated in cancer cells in response to various anticancer therapies, such as tamoxifen in breast cancer cells (38), temozolomide (TMZ, a DNA alkylating agent) and arsenic trioxide in malignant glioma cells (24). As for natural products, resveratrol, a phytoalexin that is present in grape nuts and red wine, induced autophagy in ovarian cancer cells (39), and soybeen B-group triterpenoid saponins caused autophagy in colon cancer cells (40). The results of present study add SNE to the list of natural products that possess autophagic effect in addition to its apoptotic effect. We have demonstrated that the cytotoxic effects after expose to high concentration (2 mg/ml and 5 mg/ml) SNE is caused by apoptosis and low concentration SNE is caused by autophagy. However, the molecular basis for the differentiate effect responding to high and low concentrations of SNE needs further investigation. There might be a cross-talk between the pathways of apoptosis and autophagy.

In summary, we have identified two distinct anti-neoplastic activities of SNE in liver cancer cells, the ability to induce apoptosis and autophagocytosis. This response and its ability to kill these cells hint at the possibility that this agent may be useful as an adjuvant therapy to treat liver tumors. These findings pave the way for additional experiments to consider the molecular basis for this response and studies to determine whether adequate concentrations of bioactive SNE can be attained to treat liver tumors.

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Table and Figures

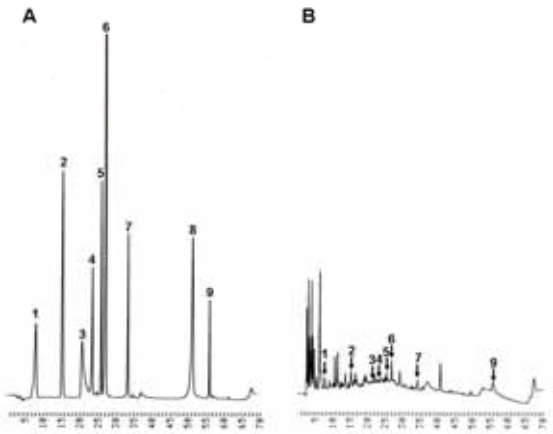

Figure 1. (A) HPLC chromatograms of SNE (10 mg/ml; 10 μl). polyphenolic compounds correspond to peaks 1-9 as in (B). (B) HPLC chromatogram of nine kinds of standard (10 mg/ml; 10 μl). Peaks: 1, gallic acid; 2, protocatechuic acid (PCA); 3, catechin; 4, (-)-epigallocatechin gallate (EGCG); 5, caffeic acid; 6, epicatechin; 7, rutin; 8, quercetin; 9, naringenin. Detector was set at 280 nm. The arrow indicated the retention time (RT) of the merge of SNE and the different standards.

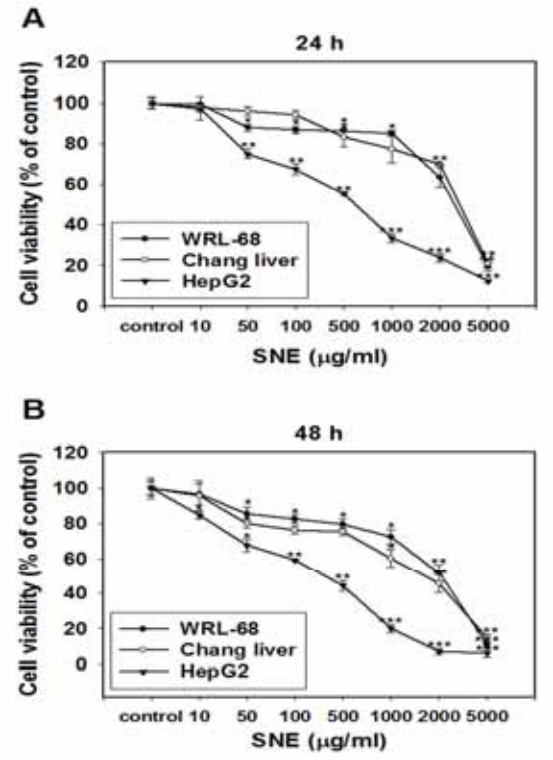

Figure 2. Effects of SNE on the viability of HepG2, WRL-68 and Chang liver cells. Cells were treated with 0, 10, 50, 100, 500, 1000, 2000 or 5000 μg/ml of SNE for 24 h and 48h before subjected to a MTT assay for cell viability. The data were expressed as a percentage of the control (dose 0), and presented as mean ± SD from three independent experiments. Results were statistically analyzed with Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

Table 1. Polyphenolic compounds of the SNE

Polyphenolic compound	Peak No.	%
Gallic acid	1	2.897 ± 1.10
PCA	2	1.977 ± 0.95
Catechin	3	2.353 ± 1.05
EGCG	4	1.533 ± 0.63
Caffeic acid	5	1.988 ± 0.49
Epicatechin	6	0.392 ± 0.11
Rutin	7	0.836 ± 0.32
Naringenin	9	5.106 ± 2.01

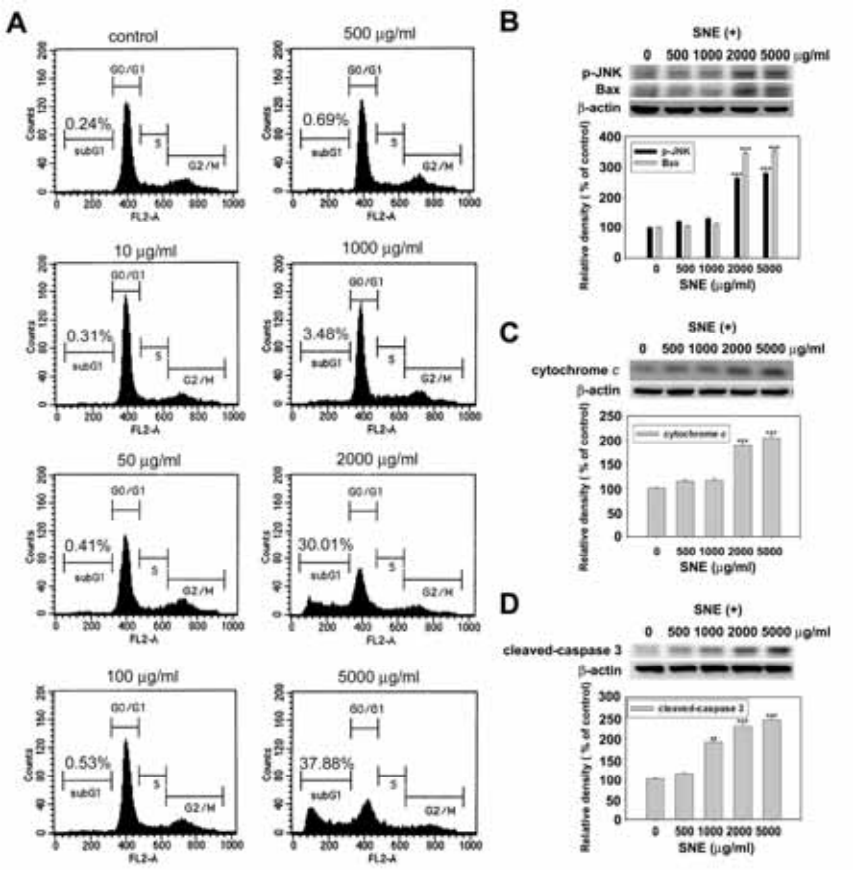

Figure 3. Apoptosis effects of SNE on HepG2 cells. (A) HepG2 cells were treated with 0, 10, 50, 100, 500, 1000, 2000 or 5000 μg/ml of SNE for 48h and subjected to flow cytometric analysis after PI staining. The figure shows a representative staining profile for 8,000 cells per experiment. Sub G1 was defined as apoptotic cells. The figure is a representative of three independent experiments. (B~D) Effects of SNE on the expression of apoptotic protein. HepG2 cells were treated with SNE at a concentration of 0, 500, 1000, 2000 and 5000 μg/ml for 48 h, then subjected to Western blotting to analyze (B) p-JNK and Bax, (C) cytochrome *c* and (D) cleaved-caspase 3 expression as described in Materials and Methods. The levels of these proteins were subsequently quantitated by densitometric analysis with that of control being 100%. Data were presented as mean ± SD from three independent experiments. Results were statistically evaluated by using one-way ANOVA with *post-hock* Dunnett's test. (**P < 0.01; ***P < 0.001).

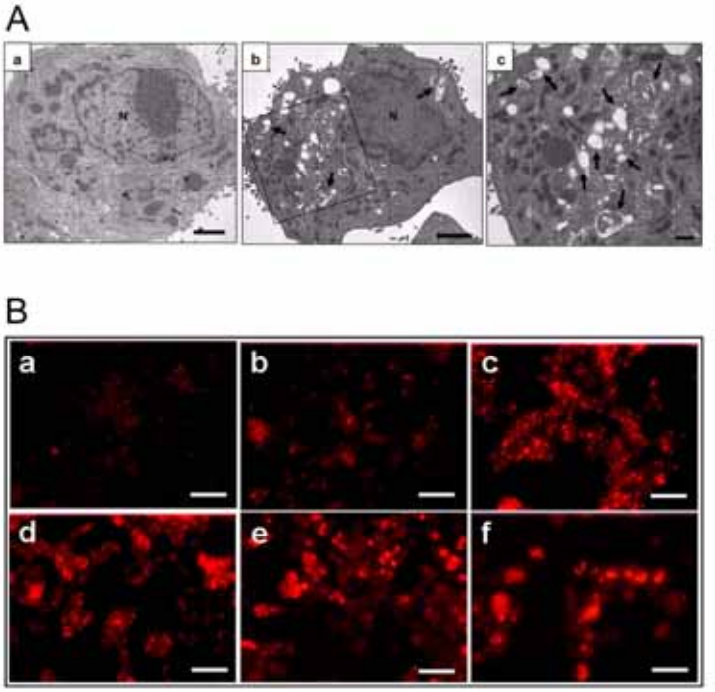

Figure 4. SNE-induced autophagic death in HepG2 cells. (A) Electron micrographs showing the ultrastructure of HepG2 cells treated with SNE (100 μg/ml) for 48 h. *a*, control; very few autophagic vacuoles were observed in un-treated HepG2 cells. *b*, SNE-treated HepG2 cells. Numerous autophagic vacuoles (arrows) were observed. *Bars* (a-b), 2 μm. *c*, an ultrastructure of SNE-treated HepG2 cells at high magnification containing numerous autophagic vacuoles (arrows). *Bar* (c), 3 μm. (B) After addition of SNE for 48 h, acridine orange stain of HepG2 cells showed AVOs formation in a dose-dependent manner. *a*, 0; *b*, 10 μg/ml; *c*, 50 μg/ml; *d*, 100 μg/ml ; *e*, 500 μg/ml; *f*, 1000 μg/ml. *Bars*, 50 μm.

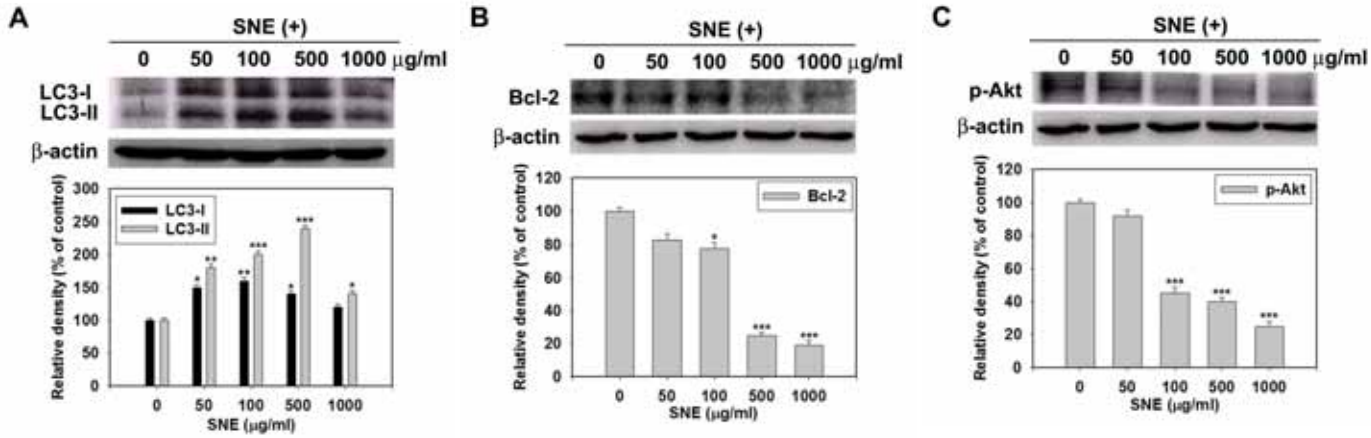

Figure 5. Involvement of LC3, Bcl-2 and p-Akt in SNE-induced autophagy in HepG2 cells. HepG2 cells were treated with SNE at a concentration of 0, 50, 100, 500 or 1000 μg/ml for 12 h, then subjected to Western blotting to analyze (A) LC3, (B) Bcl-2 and (C) p-Akt expression as described in Materials and Methods. The levels of these proteins were subsequently quantitated by densitometric analysis with that of control being 100%. Data were presented as mean ± SD from three independent experiments. Results were statistically evaluated by using one-way ANOVA with *post-hock* Dunnett's test. (*P < 0.05, **P < 0.01 and ***P < 0.001).

計畫成果自評

此研究計畫執行期間，本實驗室完成以下工作：

一、分析龍葵水萃取物中多酚化合物之含量，並以 HPLC 分析其多酚成份及含量。
二、發現以龍葵水萃取物處理人類肝癌細胞 (HepG2)及正常肝細胞 (WRL-68)，其對肝癌細胞較具毒性。
三、龍葵水萃取物致肝癌細胞死亡的機轉：高濃度處理之下，促使細胞經由 apoptosis 機制死亡；低濃度處理之下，則促使細胞經由 autophagy 路徑走向死亡，兩者皆為程式性凋亡之機制。
四、證實龍葵水萃取物致肝癌細胞 HepG2 apoptosis 之分子機轉：高濃度的龍葵萃取物處理之下，可藉由活化細胞內 p-JNK, Bax, Cytochrome *c* release 及 cleavage-Caspase 3，而促進細胞死亡。
五、證實龍葵水萃取物致肝癌細胞 HepG2 autophagy 之分子機轉：低濃度的龍葵萃取物處理之下，以電子顯微鏡觀察發現其可促進肝癌細胞產生 autophagosome，以 AVO 染色也發現同樣的結果，同時細胞內的 autophagy marker: LC3-I 及 LC3-II 表現量明顯增加，此外，可抑制 autophagy 的 Bcl-2 及 p-Akt 在細胞內的表現也明顯減少許多。

此計劃執行期間證實龍葵水萃取物具有促進肝癌細胞死亡之功能，在相同濃度下對正常細胞反而毒性較小，此一發現對肝癌之治療也許提供了一個參考的方向。未來本實驗室將針對以下項目，對龍葵之功能做更深入之探討。

一、以其他肝癌細胞株及其他癌細胞進行實驗，探討龍葵抗癌之功能及詳細分子機轉。
二、以動物實驗證實龍葵抗癌之能力。
三、萃取龍葵多酚化合物進行細胞及動物實驗，探討龍葵抗癌之能力及其有效成分。