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Antitumor effects of total alkaloids isolated from *Solanum nigrum* *in vitro* and *in vivo*

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Received October 20, 2007, accepted December 12, 2007

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Pharmazie 63: 534–538 (2008) *doi: 10.1691/ph.2008.7789*

This study demonstrated that the total alkaloids isolated from the traditional Chinese medicinal herb *Solanum nigrum* Linne (SNL-A) inhibited the growth of human cervical cancer HeLa cells in culture medium with much lower toxicity to human normal lymphocytes. By means of HE staining and TUNEL assay, our results further revealed that SNL-A induced cell death by apoptosis. An immunohistochemical assay showed down-regulation of the bcl-2 and p53 genes and no obvious change of bax gene in the SNL-A treated cells. Subcutaneous injection of HeLa cells induced tumor formation in nude mice, and SNL-A showed a significant inhibitory effect on tumor formation. These results suggested that SNL-A may be a potential, natural apoptosis-inducing agent for cervical cancer.

1. Introduction

Cancer of the uterine cervix is the second leading cause of death from cancer in women worldwide and also the most prevalent gynecological tumor in China.

Traditional Chinese medicine (TCM) has been used for a long time, with well-documented efficacy, in cancer therapy in China. However, in most cases the underlying mechanism remains unknown. Recent research has revealed that inducing apoptosis is one of the antineoplastic mechanisms of TCM (Zhang et al. 1997; Dong et al. 1997; Zhang et al. 1998; Tanizawa et al. 1994; Ma et al. 2001).

In TCM, *Solanum nigrum* Linne (SNL) has been used for centuries to cure inflammation, edema, mastitis and hepatic cancer (Sultana et al. 1995; Prashanth et al. 2001). Sultana et al. (1995) reported that SNL ethanol extract suppressed the oxidant-mediated degradation of calf thymus DNA. Hu et al. (1999) also showed that SNL exhibited anti-neoplastic effects on several human tumor cell lines. Recently, it has also been demonstrated that an ethanol extract from ripe fruits of SNL inhibited the proliferation of human MCF-7 breast cancer cells and induced cell death by apoptosis (Son et al. 2003). Lee and Lim (2006) reported that a 150KDa glycoprotein isolated from *Solanum nigrum* Linne had a stimulatory effect on caspase-3 activation and PARP cleavage in HCT-116 cells, and blocked nuclear factor-kappa B activation and reduced inducible nitric oxide (iNO) production. However, there has been little systematic study of whether the major pharmacological ingredient, the total alkaloid content of SNL (SNL-A), has anti-cervical cancer effects.

The purpose of this study was to investigate the antitumor activity of SNL-A and to provide scientific evidence identifying SNL-A as a new and effective antitumor herbal medicine.

2. Investigations and results

2.1. Effects of SNL-A on HeLa cells and normal human lymphocyte proliferation

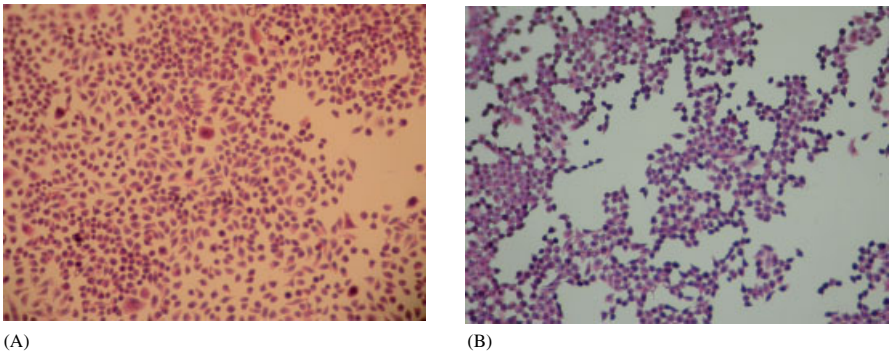
HeLa cells and human lymphocytes were cultured in 10% FCS-containing medium with or without SNL-A for 48 h or 72 h, and cell proliferation was evaluated by the MTT test. The results showed that after 48 h treatment, the concentration of SNL-A causing 50% HeLa cell death (IC_{50}) was approximately 75 μg/mL, while after 72 h treatment, IC_{50} was approximately 50 μg/mL. On the other hand, cytotoxic activities of SNL-A were substantially lower towards normal human lymphocytes. The IC_{50} of SNL-A towards normal human lymphocytes exceeded 500 μg/mL. Based on this experiment, we chose the dose of 75 μg/ml SNL-A and 48 h incubation for the following *in vitro* experiments.

2.2. Results of morphological observation of HeLa cells

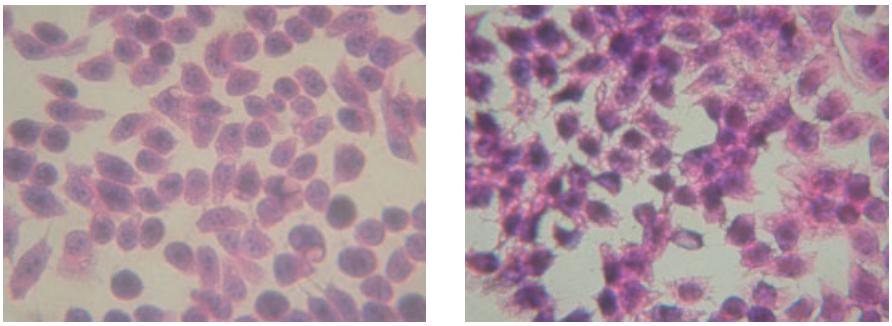
As shown in Fig. 1, the changes of cell morphology after 75 μg/ml of SNL-A treatment were analyzed. In contrast to control HeLa cells, the SNL-A treated cells showed an abnormal metaphase form, in which the chromosomes were abnormally compacted, and the interphase cells were also more rounded and fragmented; this appearance coincided with the description of classical apoptosis.

2.3. Detection of apoptosis of HeLa cells by TUNEL assay

DNA fragmentation is one of the hallmarks of cell apoptosis. In this experiment, terminal deoxynucleotidyl transferase was used to label fluorescein-dUTP to the 3′-end of

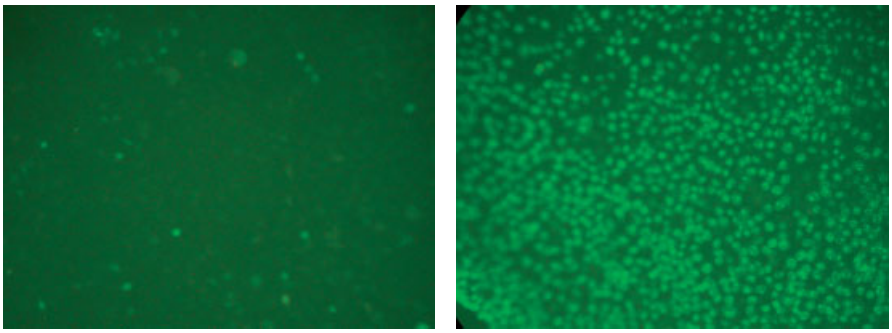


(A) (B)



(C) (D)

Fig. 1:
Effect of 75 μg/mL SNL-A on morphology of the HeLa cells during 48 h of culturing. (A) HeLa cells were incubated with complete culture medium (100×). (B) HeLa cells were incubated with culture medium containing 75 μg/mL SNL-A (100×). (C) HeLa cells were incubated with complete culture medium (400×). (D) HeLa cells were incubated with culture medium containing 75 μg/mL SNL-A (400×)



(A) (B)

Fig. 2:
Determination of DNA fragmentation of HeLa cells by the TUNEL assay. (A) HeLa cells were incubated with complete culture medium without SNL-A for 48 h. (B) HeLa cells were incubated with culture medium containing 75 μg/mL SNL-A for 48 h. The TUNEL assay was performed according to the manufacturer's protocol, and the intensity of fluorescence in the cells was determined by fluorescent microscopy

DNA fragments in individual cells after apoptosis. The fluorescence in HeLa cells intensified significantly after SNL-A treatment (Fig. 2), suggesting that SNL-A induces much more cell death by apoptosis.

2.4. Results of IHC

As shown in Fig. 3, after treatment with SNL-A, the expression of bcl-2 and mutant p53 gene product in Hela cells were inhibited significantly (A, B, E, F), but no obvious change was seen for the bax gene product (C, D).

2.5. In vivo antitumor effects of SNL-A

To evaluate the antitumor activity of SNL-P *in vivo*, we created a mouse cervical carcinoma model by s.c. injection of HeLa cells into nude mice. After p.o. administration of 25 mg/kg or 50 mg/kg b.w. of SNL-P daily for 14 days, we found that SNL-A significantly inhibited tumor growth. The tumor weight of the control group was 1.74 ± 0.22 g, while the tumor weight of mice treated with low and high dose SNL-A was reduced to 1.11 ± 0.23 g and 0.77 ± 0.37 g respectively. The tumor inhibition rate was 36.20% and 55.75%, respectively (Table 1). The tumor weight of mice treated with low or

Table: Effect of SNL-A on tumor growth

Groups	Treatment (mg/kg)	Animal number		Body weight (g)		Tumor weight (g)	Inhibition rate (%)
		Beginning	End	Beginning	End		
Control	Vehicle	8	7	20.12 ± 1.69	22.67 ± 3.14	1.74 ± 0.22	
SNL-A	25	8	8	21.01 ± 1.38	23.71 ± 2.27	1.11 ± 0.23^{a}	36.20
	50	8	8	20.57 ± 1.27	22.62 ± 2.01	0.77 ± 0.37^{a}	55.75

Results are expressed as mean ± S.D.; [a] represents significant differences compared with the control, $p < 0.05$

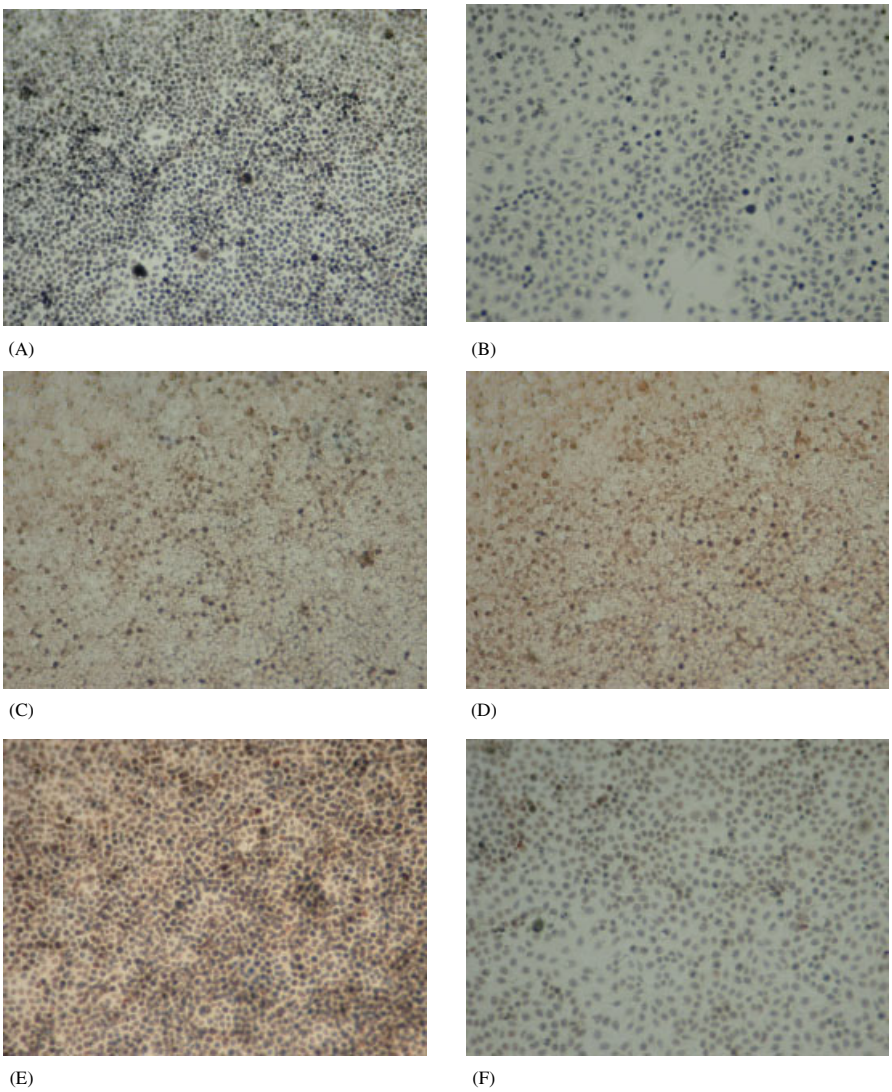


Fig. 3:
Immunohistochemistry (IHC) analysis of bcl-2, bax and p53 gene expression. Panels A, C, and E indicate, respectively, bcl-2, bax and p53 gene products in the control cells. Panels B, D and F indicate, respectively, bcl-2, bax and p53 gene products in cells treated with 75 μg/mL SNL-A for 48 h

high dose SNL-P was significantly lower than that of the control group ($p < 0.05$).

3. Discussion

The MTT assay showed that SNL-A can inhibit the growth and cause the death of HeLa cells, and the IC_{50} values showed that the HeLa cell line was much more sensitive to SNL-A than normal human lymphocytes, so a relative low toxicity might be expected for SNL-A in further clinical research.

Programmed cell death (apoptosis), the deletion of certain cells in tissues without concomitant inflammation, is advantageous in tissue homeostasis. Its morphological features (e.g. apoptotic bodies, chromatin condensation, DNA fragmentation, and sub-G1 peak in the DNA histogram) are generally employed as hallmarks of cell death by apoptosis (Arends and Wyllie 1991; Dive et al. 1992; Darzynkiewicz et al. 1992). The action of total alkaloid isolated from *Solanum nigrum* Linne (SNL-A) satisfied the criteria of apoptosis in HeLa cells, indicating that SNL-A induced cell death by apoptosis. The study presented here demonstrated that SNL-A inhibited proliferation and induced apoptosis in human cervical cancer HeLa cells.

To evaluate the mechanism of apoptosis induced by SNL-A further, the production of apoptosis-associated genes was monitored by IHC. Genetic alterations resulting loss of apoptosis or disturbance of apoptosis-signaling pathways are likely to be critical components of carcinogenesis (Schulte-Hermann et al. 1994). Apoptosis is modulated by anti-apoptotic and pro-apoptotic effectors, involving a large number of proteins. The pro-apoptotic and anti-apoptotic members of the bcl-2 family act as a rheostat in regulating programmed cell death and are considered to be targets of anticancer therapy (Baell and Huang 2002; Goodsell 2002). The bcl-2 family of genes serves as both positive and negative regulators of cell death (Antonsson and Martinou 2000). The intracellular level and the ratio of Bcl-2 and Bcl-2-related proteins dictate whether a cell should engage in the apoptotic program (Reed 1997). SNL-A-induced apoptosis showed a significant down-regulation of the Bcl-2 protein, while Bax levels remained unchanged. This result indicated that SNL-A might trigger apoptosis via deregulation of bax and bcl-2 gene expression in HeLa cells. p53 is known to induce apoptosis through inhibition of bcl-2, amplification of death signals and activation of caspases (Haupt et al. 2003). Mutations in p53 have been reported to occur in 40% of all human tumors. While wild type p53 functions as a tumor suppressor gene, mutant p53 functions as an oncogene. Overexpression of mutant p53 may enhance genetic instability by facilitating cell proliferation and inhibiting DNA repair and apoptosis. In particular, p53 mutations have been reported to be associated with overexpression

of bcl-2 (Lucken-Ardjomande and Martinou 2005). Our results showed that the expression of mutant p53 in SNL-A treated cancer cells decreased significantly compared with that of control cells, which may lead to apoptosis together with bcl-2 by some route. However, the precise mode of progression of the p53 dependent pathway needs further analysis.

SNL-A belongs to the class of steroidal glycoalkaloids and the steroid molecules can bind to intracellular receptor proteins by direct diffusion across the plasma membrane of target cells (Tropp 1997). This binding might further activate the receptors, which then regulate the transcription of apoptosis-relative genes. Then, finally, changes in the products of apoptosis-associated genes result in the apoptosis of HeLa cells.

In our experiments, an *in vivo* study eventually provided direct evidence to support the antitumor effect of SNL-A. SNL-A, at doses of 25 mg/kg and 50 mg/kg. b.w., significantly reduced tumor growth in nude mice induced by the subcutaneous injection of HeLa cells, accompanied by no significant cytotoxic effects on the mice. This result further confirmed the potential application of SNL-A in the treatment of cervical cancer.

Further investigation is being carried out in our laboratory to separate the total alkaloids of SNL and to determine in more detail the molecular mechanism of apoptosis in HeLa cells induced by SNL-A, e.g. the pathway of this apoptotic process, the signal transduction pathway(s) involved, and the possible changes in expression of other apoptosis-related genes. However, this study does suggest that SNL-A could be a candidate for developing a low-toxicity antitumor agent.

4. Experimental

4.1. Sample preparation and extraction

Air-dried *Solanum nigrum* L. (682.6 g) was cut in small pieces, further percolated with 4500 mL hot distilled water under ultrasonic vibration for 90 min, and then concentrated under reduced pressure. The aqueous extract was adjusted with sulfuric acid to pH 2–3, then centrifuged at 3000 rpm for 10 min, and the supernatant was collected and concentrated under reduced pressure at 65 °C. The resulting extract was further extracted with n-butanol under ultrasonic vibration for separation the water layer was collected, and this step was repeated 25 times. It was adjusted to pH 9–10 with sodium hydroxide, and centrifuged under 3000 rpm for 10 min, and the supernatant was collected and concentrated under reduced pressure at 65 °C. It was again, extracted with n-butanol under ultrasonic vibration for separation, the n-butanol layer was collected and the alkaloid portion extracted from *n*-butanol with sulfuric acid solution of pH 2–3, this step being repeated 25 times. After that, the acid solution was adjusted to pH 7.2–7.5, and concentrated under reduced pressure at 65 °C, to give the crude alkaloids from *Solanium nigrum* L. Methanol was added to dissolve the crude alkaloid, filtered through paper (Whatman No. 3) to remove the sulfate. Finally, the collected filtrate was concentrated with a rotary evaporator and further frozen and dried to give 1.7513 g (0.256% of initial amount), identified as total alkaloid of *Solanium nigrum* L (SNL-A).

The qualitative analysis of SNL-A was carried out with Mayer, Bertrand and Sonnenschein precipitation reagent and the color of the precipitate showed light yellow, brown red and brown yellow.

4.2. Cell line and culture

Human cervical cancer line, HeLa, was supplied by the Shanghai Institute of Biochemistry. Normal human lymphocytes were separated by the Ficol Hypaque method. Both Hela cells and normal lymphocytes were maintained in RPMI 1640 (GIBICO BRL) supplemented with 10% FCS, 10 U/mL penicillin, and 10 mg/mL streptomycin. The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

4.3. Experimental animals

Female BALB/c nu/nu mice aged 6–8 weeks weighing 18–22 g were obtained from the Experimental Animal Center of Xiehe Medical University (Beijing, China) and allowed free access to food and water. The animals were treated according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and their experimental use was approved by the Animal Ethics Committee of the university.

4.4. Cell proliferation assay

Cells were placed in 96-well culture plates (1.0×10^4 cells per well). After 24 h incubation, the cells were treated with different concentrations of SNL-A (0, 25, 50, 100, 200, 500 μg/ml). All cultures were kept for 48 h or 72 h at 37 °C in a humidified incubator. Cell concentration was checked by MTT assay (Cheng 2000). Growth inhibition rate was calculated by the following equation:

$$\text{Inhibition rate} = [(\text{OD control well-OD treated well})/\text{OD control well}] \times 100\% \quad (1)$$

4.5. Preparation for H.E. staining

After 48 h 75 μg/mL SNL-A treatment, the adherent cells were fixed in 4% formalin, and stained by the H.E. staining method, then examined for tumor cell morphology under a light microscope.

4.6. Terminal deoxynucleotidyl transferase (TdT) end-labeling assay

Specific 3′-hydroxyl ends of DNA fragments generated by endonuclease-mediated apoptosis are preferentially repaired by terminal deoxynucleotidyl transferase (TdT) (Gavrieli et al., 1992). The oped to label these strand-breaks with streptovidin-horseradish peroxidase conjugated nucleotides followed by the addition of a substrate.

After SNL-A treatment, the adherent cells were treated according to the instructions of the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH Mannheim, Germany), and finally observed under a fluorescence microscope. The cells whose nucleus turned a distinct green were considered as positive cells.

4.7. Immunohistochemical (IHC) assay

The IHC assay was carried out to detect the protein products of some apoptosis-associated genes. The cells, cultured on glass cover-slips, were treated or not with a dose of 75 μg/mL SNL-A for 48 h, fixed in 10% neutral buffered formaldehyde for 10 min, and then transferred to PBS. Then the adherent cells were stained by the standard immunohistochemical SP (streptavidin peroxidase conjunction) method and observed by light microscopy.

4.8. In vivo antitumor effect of SNL-A

The doses chosen to administer orally to the tumor-bearing mice were based on our preliminary tests. Animals were randomly divided into test groups consisting of 8 nude mice per group. Under sterile conditions, HeLa cells (5×10^6) in 0.1 ml of sterile physiological saline were subcutaneously injected into the left axilla s.c of each mouse (day 0). Twenty-four hours after inoculation, SNL-A was given orally at a dose of 25 mg/kg b.w. and 50 mg/kg b.w. once a day for two weeks. At the end of the experiment, the tumors were excised and weighed. Control mice were administered physiological saline orally following the corresponding SNL-A schedule. The tumor inhibition rate was expressed according to the following formula:

$$\text{mean tumor weight of control group} - \frac{\text{mean tumor weight of treated group}}{\text{mean tumor weight of control group}} \times 100 \quad (2)$$

4.9. Statistical analysis

Results are expressed as mean ± standard deviation. The difference between control and SNL-A treated cells was evaluated using Student's t test. A p value less than 0.05 was considered statistically significant.

Acknowledgements: This study was supported by the Science and Technology Development Fund (D08) of Science and Technology Investing Company, Qinhuangdao, China. The authors would like to thank Prof. Zhao Jian-cheng, a plant taxonomy expert (College of Life Science, Hebei Normal University, Shijiazhuang, China) who examined and authenticated the *Solanum nigrum* Linne sample used.

References

Antonsson B, Martinou J (2000) The Bcl-2 protein family. Exp Cell Res 56: 50–57.

Arends MJ, Wyllie AH (1991) Apoptosis: Mechanisms and roles in pathology. Int Rev Exp Pathol 32: 223–254.

Baell JB, Huang DC (2002) Prospects for targeting the Bcl-2 family of proteins to develop novel cytotoxic drugs. Biochem Pharmacol 64: 851–863.

Cheng BL (2000) The culture technology of animal cells. South China University of Technology Press, Guangzhou, p. 131–132.

Darzynkiewicz Z, Bruno S, del Bino G, Garczyca W, Holz MA, Lassota P, Traganos F (1992) Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795–808.

Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wyllie AH (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochim Biophys Acta 1133: 275–285.

Dong Y, Yang MM, Kwan CY (1997) In vitro inhibition of proliferation of HL-60 cells by tetrandrine and coriolus versicolor peptide derived from Chinese medicinal herbs. Life Sci 60: PL135–140.

Goodsell DS (2002) The molecular perspective: Bcl-2 and apoptosis. Stem Cells 20: 355–356.

Haupt S, Louria-Hayon I, Haupt Y (2003) p53 licensed to kill? Operating the assassin. J Cell Biochem 88: 76–82.

Hu K, Kobayashi H, Dong A, Jing Y, Iwasaki S, Yao X (1999) Antineoplastic agents: steroidal glycosides from *Solanum nigrum*. Planta Med 65: 35–38.

Lee SJ, Lim KT (2006) 150KDa glycoprotein isolated from *Solanum nigrum* Linne stimulates caspase-3 activation and reduces inducible nitric oxide production in HCT-116 cells. Toxicol in vitro 20: 1088–1097.

Lucken-Ardjomande S, Matinou JC (2005) Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane. CR Biology 328: 616–631.

Ma J, Fu NY, Peng WL, Wu WY, Xu AL (2001) Apoptosis induced by isoliquiritigenin in human gastric cancer MGC-803 cells. Planta Med 67: 754–757.

Prashanth KV, Shashidhara S, Kumar MM (2001) Cytoprotective role of *Solanum nigrum* against gentamicin-induced kidney cell (Vero cells) damage *in vitro*. Fitoterapia 72: 481–486.

Reed JC (1997) Double identity for the proteins of the Bcl-2 family. Nature 387: 773–776.

Schulte-Hermann R, Grasl-Kraupp B, Bursch W (1994) Tumor development and apoptosis. Int Arch Allergy Immunol 105: 363–367.

Son YO, Kim J, Lim JC, Chung Y, Chung GH, Lee JC (2003) Ripe fruits of *Solanum nigrum* inhibit cell growth and induce apoptosis in MCF-7 cells. Food Chem Toxicol 41: 1421–1428.

Sultana S, Perwaiz S, Iqbal M, Athar M (1995) Crude extracts of hepatoprotective plants, *Solanum nigrum* and *Cichorium intybus* inhibit free radical-mediated DNA damage. J Ethnopharmacol 45: 189–192.

Tanizawa A, Fujimori A, Fujimori Y, Pommier Y (1994) Comparison of topoisomerase I inhibition, DNA damage and cytotoxicity of camptothecin derivatives presently in clinical trials. J Natl Cancer Inst 86: 836–842.

Tropp BE (1997) Steroid hormones. In: Biochemistry – Concepts and Applications (Ed. Tropp BE) Brooks/Cole, Pacific Grove p. 492–494.

Zhang GQ, Ren RG, Kong QY et al., (1997) Effects of radix salviae miltiorrhizae on proliferation, apoptosis and c-myc protein expression of fibroblast in culture of kidney with lupus nephritis. Chin J Integrated Traditional Western Med 17: 473–475.

Zhang X, Yang XP, Pan QC (1998) Studies on the anticancer effect and apoptosis in human liver cancer cell line BEL-7402 by sodium artesunate. Chin J Herbal Drugs 29: 467.