Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **34** (10): 1961-7 (2015)

Regular article
Received: May 21, 2015
Revised version: August 4, 2015
Accepted: August 5, 2015

Antitumor Effect of Salidroside on Mice Bearing HepA Hepatocellular Carcinoma

Hanjun SONG [1], Jianjie WANG [1] *, Molin WANG [1], Hang DONG [2], Lijiang LI [2], Tengyuan ZHANG [1] & Shaobo ZHOU [3]

[1] *College of Basic Medicine, Jiamusi University, No. 148 Xuefu Street, Jiamusi 154007, P.R. China*
[2] *The First Affiliated Hospital, Jiamusi University, No. 348 Dexiang Street, Jiamusi 154002, P.R. China*
[3] *Department of Life Science, Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, LU1 3JU, UK*

***SUMMARY*. Salidroside, a phenylpropanoid glycoside extracted from *Rhodiola rosea* L., has antiproliferative effects on tumour cells in mice. However it's antitumor mechanism remains largely unknown. In this study, 4 groups of mice bearing hepatocarcinoma cells were given treatment with vehicle alone, cyclophosphamide (25 mg/kg, *i.p.*) and salidroside, either 100 or 200 mg/kg (*p.o.*) for 14 days. The morphology of tumour specimens was analysed by transmission electron microscopy. Apoptotic cells in sections of mouse tumour tissue were analysed using an *in situ* apoptosis kit. The expression of Bcl-2, Bax and caspase 3 mRNA were examined with RT-PCR. The results showed that the tumour weights in groups 100 or 200 mg/kg/day of salidroside were reduced significantly (45.34 and 52.48% respectively), compared to vehicle groups. Salidroside increased apoptotic cells index, e.g. in 200 mg/kg group, it was four times higher compared to the control group. Even more, treatment with salidroside decreased Bcl-2 mRNA expression and increased Bax and caspase 3 mRNA expressions. These indicated that the antitumor mechanism of salidroside may induce tumour cell apoptosis in mice by triggering the mitochondrial-dependent pathway and activation of caspase 3.**

RESUMEN. Salidrósido, un glucósido fenilpropanoide extraído de *Rhodiola rosea* L., tiene efectos antiproliferativos sobre células tumorales en ratones. Sin embargo, el mecanismo antitumoral sigue siendo en gran parte desconocido. En este estudio, a 4 grupos de ratones portadores de células de hepatocarcinoma se les dio tratamiento con vehículo solo, ciclofosfamida (25 mg/kg, *i.p.*) y salidrósido, ya sea 100 o 200 mg/kg (*p.o.*) durante 14 días. La morfología de las muestras de células tumorales se analizó por microscopía electrónica de transmisión. Las células apoptóticas en secciones de tejido de tumor de ratón se analizaron usando un kit de apoptosis *in situ*. La expresión de mRNA de Bcl-2, Bax y caspasa 3 fueron examinados con RT-PCR. Los resultados mostraron que los pesos de los tumores en los grupos de 100 o 200 mg/kg/día de salidrósido se redujeron significativamente (45,34 y 52,48%, respectivamente), en comparación con los grupos de vehículos. El salidrósido aumentó el índice de células apoptóticas, por ej., en el grupo 200 mg/kg fue cuatro veces mayor en comparación con el grupo control. Aún más, el tratamiento con salidrósido disminuyó la expresión de mRNA de Bcl-2 y aumentó las expresiones de ARNm de Bax y caspasa 3. Estos resultados sugieren que el mecanismo antitumoral de salidrósido puede inducir apoptosis de células tumorales en ratones mediante la activación de la vía mitocondrial dependiente y la activación de la caspasa 3.

INTRODUCTION

Hepatocellular carcinoma is the second and third most common cause of cancer death in males and females, respectively [1,2]. The prognosis of these patients is poor because there is no effective treatment [3,4]. Although the therapeutic methods for hepatocellular carcinoma have made great progress, more than 80% of cases at advanced stage are inoperable. Moreover, despite patients having had surgery at an early stage, the incidence of recurrence is approximately 20-25% in year one after operation [5-7]. Therefore, it is important to invest new drugs for its effective treatment.

Natural herbal plants have been demonstrated to be reliable and are potential sources used to treat cancers. Many Chinese herbal medicines have shown to be effective in cancer chemopre-

KEY WORDS: apoptosis, hepatocellular carcinoma, salidroside.

* Author to whom correspondence should be addressed. *E-mail:* jmsdxjianjie@163.com

ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

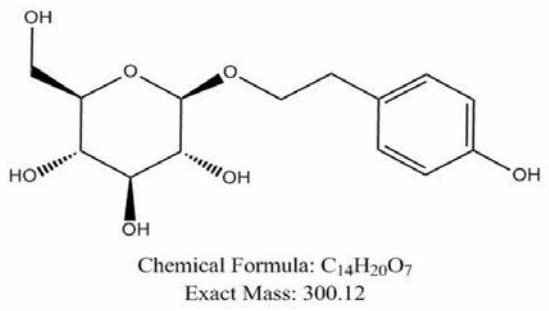

Figure 1. The structure of salidroside.

vention and chemotherapy, with none or few side effects [8]. Salidroside [2-(4-hydroxyphenyl) ethyl-β-D-glucopyranoside], is a phenylpropanoid glycoside and it is extracted from the plant Rhodiola *rosea* L. (Fig 1). Some reports have shown that salidroside has various pharmacological effects including antioxidant, antifatigue, anti-inflammation and immune enhancement [9-11], as well as antitumor effects, e.g. inhibiting the proliferation of cervical cancer, leukaemia cells K562, melanoma cloudman S91, lung cancer cells A549, breast cancer, stomach and liver cancer [12-16]. Our previous study showed that salidroside (100 and 200 mg/kg) inhibited the proliferation of tumour cells in hepatocellular (HepA) mice and promoted the activities of cytotoxic T lymphocyte and natural killer cells with no side effects [17]. However, the antitumor mechanism of salidroside remains largely unknown. Therefore, this present study investigates this by using a HepA bearing mouse model.

MATERIALS AND METHODS

Chemicals and cell line

Salidroside was obtained from Shenyang Pharmaceutical University (Shenyang, China). Cyclophosphamide (CTX) was purchased from HengRui pharmacy Inc. (Jiangsu province, China). The terminal deoxyribonucleotide transferase-mediated nick-end labeling assays (TUNEL) kit was purchased from KeyGEN Bio (Nanjing, China). Trizol was purchased from Gibco (Gaithersburg, MD, USA). Reverse transcriptase cDNA synthesis kit was purchased from Takara Bio (Dalian, China). The HepA Hepatocellular cell line was obtained from Cancer Institute of Chinese Academy of Medical Sciences (Beijing, China). All other chemicals used were analytical reagent grade.

Experimental animals

Forty female Kunming mice aging 6-8 weeks old (20.0 ± 2.0 g), were provided by the Experimental Animal Center of Dalian Medical University. They were maintained under standard environment conditions and fed with a standard pellet diet and free access to water. All animal experiments were conducted in accordance with the NIH Guide for the care and Use of Laboratory Animals (NIH Publication No.80-23; revised 1978 and the number approved by Administrated-Committee of Laboratory Animals was 062310).

Animal model and treatment with drugs

The HepA hepatocellular cell line (10^7 cell/mL) was injected into the mouse peritoneal in 10 mice (0.2 mL/mouse). After 7-10 days, ascites produced were drawn from HepA mice under aseptic conditions, and they were diluted to the concentration of 10^7 cells/mL in saline. HepA cells (0.2 mL) were subcutaneously injected into the left limb axilla. After 24 h of inoculation, mice were weighed and randomly divided into four groups (n = 10). The group was administered with vehicle alone (normal saline, *p.o.*), and is the control group. The other group was treated with cyclophosphamide (CTX, 25mg/kg, *i.p.*, CTX group), which is considered as the standard antitumor reference drug. The other two groups were administered with salidroside (either 100 or 200 mg/kg, *p.o.*, respectively). After 14 days [6,17], all mice were weighed and killed, and the transplanted tumours in four groups were removed and weighed. According to the mean weight of the tumour, the rate of tumour inhibition was calculated in the following method: *the rate of inhibition (%) = (mean tumour weight of control group - mean tumour weight of treated group) /mean tumour weight of control group × 100 %.*

Histology analysis

Tumour specimens were fixed in 10% (v/v) neutral formalin solution for 24 h and processed routinely by embedding in paraffin. Tissue serial sections (4 μm) were stained with haematoxylin and eosin (H&E staining) and examined under a light microscope.

Morphology analysis by transmission electron microscopy

Tumour specimens (1 mm^3) were pre-fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer at 4 °C for 2 h, and then rinsed thoroughly in phosphate buffer and post fixed in 1%

OsO_4 at 4 °C for 30 min. After being fixed, specimens were dehydrated through a graded ethanol series, and embedded in Epon (Epoxy resin, Spi supplies, West chester, USA). The ultrastructure of cells were analysed in ultrathin sections (70 nm) in a TEM (Hitachi H-800, Tokyo, Japan). After, the sections were stained with uranyl acetate and lead citrate [6].

TUNEL assay

Apoptotic cells in sections of mouse tumour tissue from the four groups were detected by using an *in situ* apoptosis detection kit according to the manufacturer's instructions. The distinctly brown staining of cells represents apoptotic cells while the blue staining of cells shows non-apoptotic cells under a light microscope. The average number of positive cells was counted in five randomly selected optical fields (200 tumour cells/per field). The *apoptotic index (%)* was calculated as: *(number of apoptotic cells / total number of cells)* × *100%.*

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA in tumour tissues was extracted using Trizol reagent (Invitrogen). RT-PCR was performed using reverse transcriptase cDNA synthesis kit according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA and then followed by PCR amplification using specific primers: Bcl-2, forward: (5'-CCTGGCACCTGGCGG ATAGC-3') and reverse: (5'-CGACTGAAGAGTGAGC-CCAGCAGAAC-3'); Bax, forward: (5'-GCTCT-GAACAGATCATGAAGACAG-3') and reverse: (5'-CAATCCA AAGTGGACCTGAGG-3'); caspase-3, forward: (5'-TTTGTTTGTGTGCTTCTG AGCC-3') and reverse: (5'-GATGTTCTGGAGA GCCCCG-3'); β-actin, forward (5'-AATGGGTCA-GAAGGACTCCTATGTGG-3') and reverse: (5'-CGCCTA GAAGCACTTGCGGTG-3'). Bcl-2 or Bax was amplified by 30 cycles at 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 40 sec in order. Caspase 3 was amplified by 30 cycles at 94 °C for 45 sec, 52 °C for 45 sec, and 72 °C 1 min. All procedures were followed by a 7 min extension at 72 °C. PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide and visualized by ultraviolet-induced fluorescence. The intensity of bands was quantified by LabWork 3.0 UVP software (UVP, USA).

Statistical analysis

All results were presented as mean ± standard deviation (S.D.). One-way analysis of variance and Duncan's multiple range tests were used to compare differences among the groups, and $P < 0.05$ indicated a statistically significant result.

RESULTS

Effects of Salidroside on tumour weight *in vivo*

After daily administration of CTX (25 mg/kg, *i.p.*) and salidroside (100 or 200 mg/kg, *p.o.*), for two weeks, tumour weight was significantly lower compared to the control group. The mean tumour weight was 3.22 ± 0.67 g in the control group, whereas it was 1.76 ± 0.45 and 1.53 ± 0.43 g in salidroside group (100 or 200 mg/kg respectively), and was 1.47 ± 0.31 g in the CTX group. The tumour inhibition rate was 45.34, 52.48, and 54.35 % in salidroside (100 or 200 mg/kg) and CTX group, respectively (Table 1).

Morphological changes of cell apoptosis

The morphological changes of cell apoptosis were further studied in order to understand anti-tumor mechanisms. Results in Fig. 2 showed that in the control group, tumour cells of different sizes and shapes were arranged closely. The cells displayed a small cytoblastema and a larger thickly stained nucleus and obvious heteromorphism and hyperplasia as well. However the

Groups	n	Treatment (mg/kg)	Body weight (g)		Mean weight of tumour (g)	Inhibition rate (%)
			Start	End		
Control	10	Vehicle	20.23 ± 2.02	21.79 ± 2.23	3.22 ± 0.67	
CTX	10	25	21.48 ± 1.89	19.85 ± 1.34	1.47 ± 0.31*	54.35%
salidroside	10	100	20.69 ± 2.04	22.26 ± 2.85	1.76 ± 0.45*	45.34%
	10	200	21.38 ± 1.91	23.17 ± 2.34	1.53 ± 0.43*	52.48%

Table 1. The inhibitory effect of salidroside on HepA solid tumours * $p < 0.05$ as compared with control group, values are mean ± S.D.

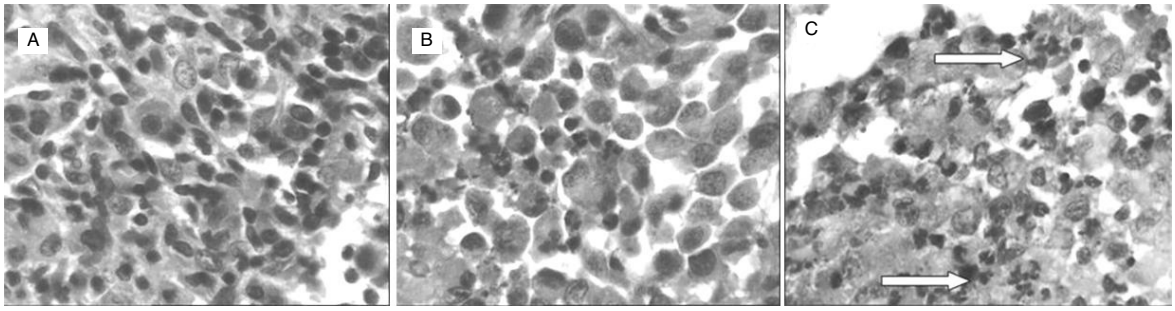

Figure 2. Morphological changes of tumours in the HepA model treated with salidroside (H&E staining ×400).(**A**) The control group. (**B**) CTX group. (**C**) Salidroside group (200 mg/kg). Arrows indicates the apoptotic cells.

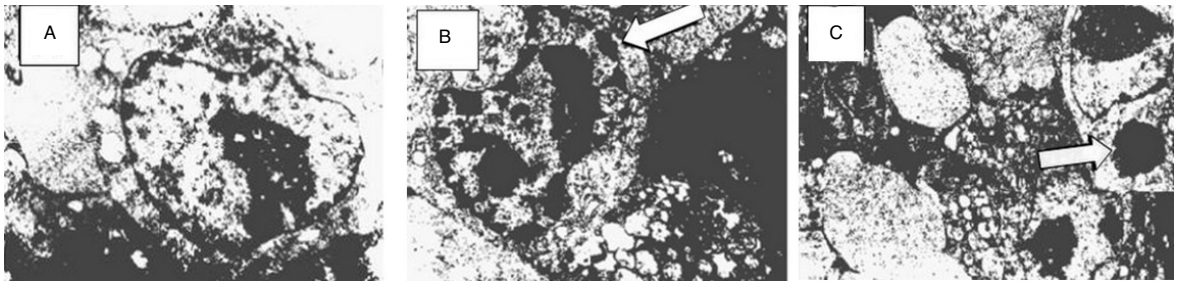

Figure 3. Morphological changes of tumour cells in the HepA model treated with salidroside by electron microscopy. (**A**) The control group; (**B**) CTX group; (**C**) Salidroside group (200 mg/kg). Arrows reveal cell shrinkage, chromatin condensation, irregularly shaped crescents at the nuclear edges, and fragmentation of cells into membrane-wrapped apoptotic bodies.

number of tumour cells in the salidroside treatment groups, especially in the high-dose group, decreased markedly and tumour cell chromatin accumulated at the side of the nucleic membranes. The nucleic shape was irregular and the surface of the nucleic membrane was rough. The nucleus was broken but was encapsulated by an intact membrane containing intact organelles and apoptotic bodies. TUNEL staining results showed that the apoptotic index reached 23.32% in the salidroside group (Fig 4).

Tumour cell apoptosis in salidroside treated groups was further confirmed by electron microscopy. Results in Fig. 3 show representative pictures of apoptotic tumour cells in salidroside treated groups that revealed cell shrinkage, chromatin condensation, irregularly shaped crescents at the nuclear edges, and fragmentation of cells into membrane-wrapped apoptotic bodies.

Expression of apoptosis-related genes in tumour tissues

To further determine the mechanisms of apoptosis induced by salidroside, the expression of Bcl-2, Bax and caspase 3 mRNA were examined with RT-PCR. The results in Fig. 5 shows that compared to the control group, the expression of Bcl-2 mRNA decreased, while the expression of Bax and caspase 3 mRNA increased in both salidroside treated groups. The down-regulation of Bcl-2 mRNA and up-regulation of Bax mRNA led to a decrease in the ratio of Bcl-2/Bax. However, the expression of the above apoptosis-related gene did not significantly change in the CTX group.

DISCUSSION

This study further investigated into the effects and mechanisms of salidroside on mice harbouring HepA cells. The results demonstrated that salidroside plays an important role in antitumor activity. Compared with the control group, salidroside administration can significantly decrease tumour weight, increase tumour inhibition rates, induce morphological changes indicative of apoptosis, increase tumour cell apoptosis, regulate Bcl-2 and Bax mRNA expressions, reducing Bcl-2/Bax ratio, and increase caspase 3 activity. These findings are consistent with previous studies using salidroside, which induced cell apoptosis and cell-cycle arrest in human breast cancer cells and A549 lung cancer cells [15,16].

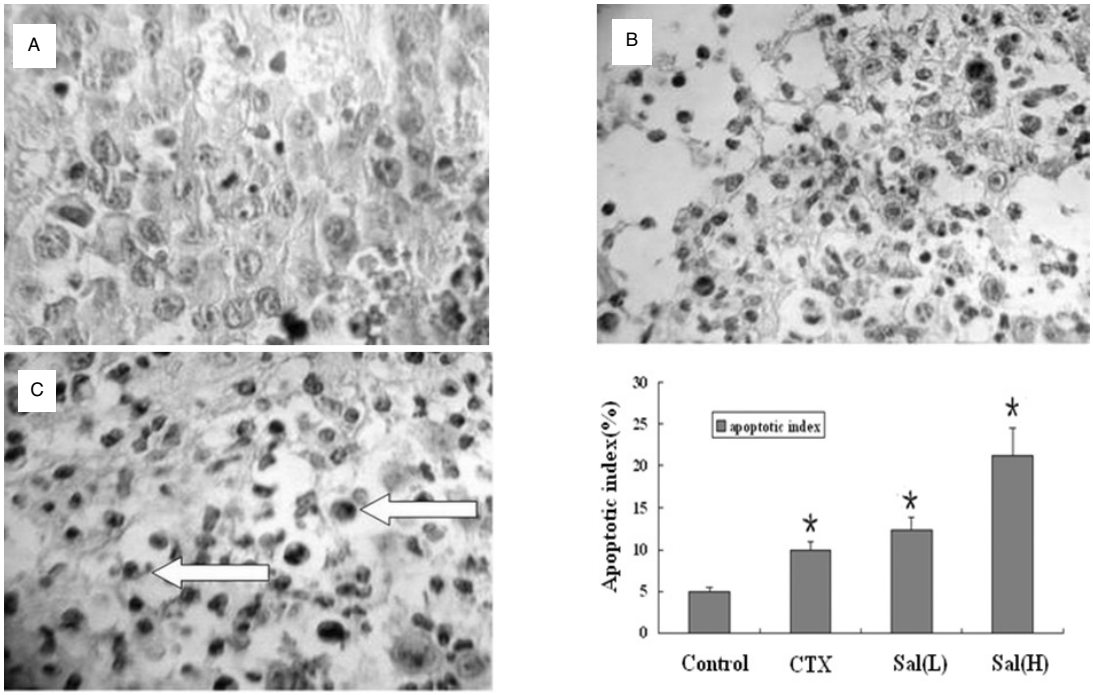

Figure 4. Morphological and apoptotic index changes of tumour cells in the HepA model treated with salidroside (TUNEL staining ×200). (**A**) The control group. (**B**) CTX group (**C**) salidroside group (200 mg/kg). (**D**) The changes of apoptotic index in the HepA cancer model *in vivo* treated with CTX and salidroside (100 and 200 mg/kg) for 14 days. * P < 0.05 *vs*. Control group.

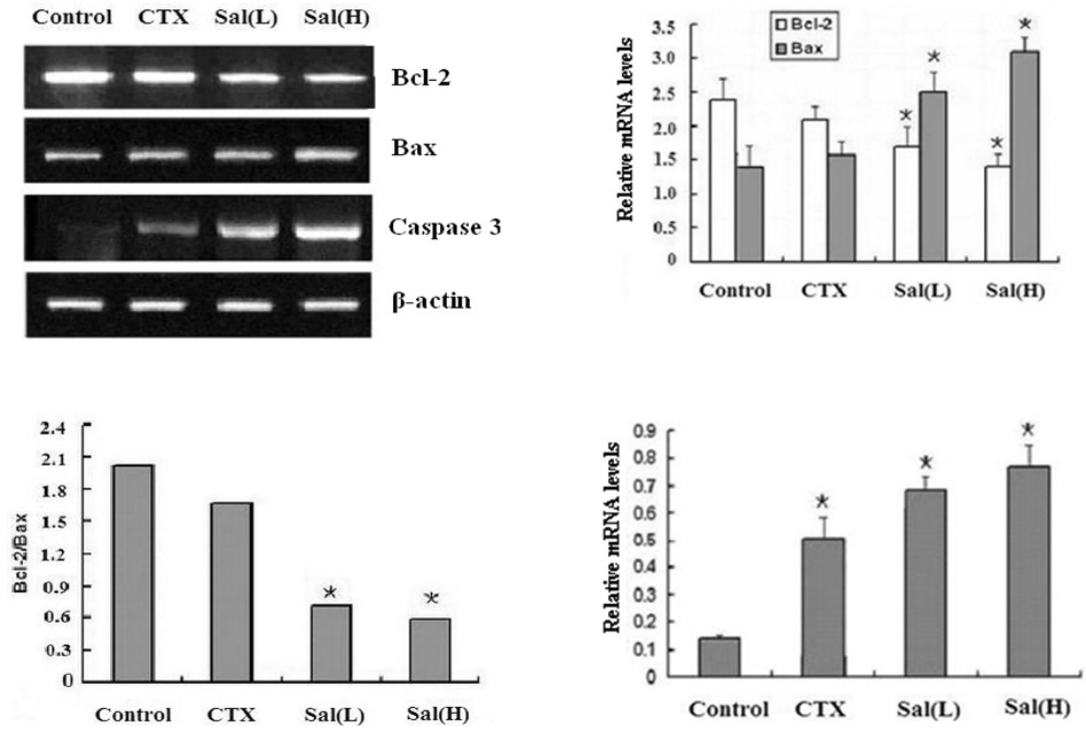

Figure 5. Expression of apoptosis-related genes in tumour tissues. RT-PCR was performed to determine apoptosis-related mRNA expression. β-actin was used as a control. (**B**) The intensities of the Bcl-2 and Bax bands were quantified and are shown as relative expression level after being normalized using β-actin. *P < 0.05, vs. control group. (**C**) The ratio of Bcl-2 and Bax. (**D**) The intensity of caspase 3 was quantified and is shown as the relative expression level after being normalized by β-actin. *P < 0.05, *vs.* control group.

Apoptosis, a physiological process in controlling cell number and proliferation, helps maintain homeostasis of multicellular organisms. It is well recognized that an alteration in cellular homeostasis occurs in cancer, disrupting the balance between cellular proliferation and cell death (apoptosis) [18,19]. The imbalance of cell proliferation and cell death might induce tumour generation. Many anticancer drugs induce tumour cell apoptosis, which is an obvious strategy for cancer therapy [6,20]. Traditional chemotherapeutic drugs often kill tumour cells by preventing mitosis and proliferation [8]. Our data suggest that salidroside can induce apoptosis of HepA cells *in vivo,* demonstrated by observations of cell shrinkage, chromatin condensation, irregular nucleic shape, and the presence of apoptotic bodies, under light microscopy and electron microscopy.

The mechanisms of apoptosis induced by drugs are complex due to the differences in cell types and drugs. The process is controlled by a number of proteins. Among them, mitochondrial and cell-surface death receptor-mediated apoptosis are the two principal pathways leading to programmed cell death. The mitochondrial pathway is thought to play a major role in response to cancer treatment and is mediated by the Bcl-2 family proteins, which are consistently over-expressed in many tumour cells [21-23] and they act as repressors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members, *e.g.,* Bax, act as promoters [24]. Their effects are dependent on the balance between antiapoptotic Bcl-2 and proapoptotic Bax [25,26]. Results in this present study showed that in the salidroside group, the expression of Bcl-2 decreased and the expression of Bax increased. The up-regulation of Bax expression and the reduction of Bcl-2 expression led to a decrease in the ratio of Bcl-2/Bax, which may be responsible for the drug-induced apoptotic process.

Caspase 3 is an executioner caspase of the apoptosis pathway [27]. In the present study, after administration with salidroside there was an increase in caspase 3 mRNA, which indicated that salidroside also promotes caspase 3 gene expression.

Therefore, the most likely explanation for the antitumor mechanisms of salidroside is that salidroside results in DNA damage of tumour cells and that in response, a positive ratio between Bcl-2 and Bax leads to cytochrome c release from the mitochondria, which triggers the mitochondrial-dependent pathway, finally leading to the activation of the caspase 3 and eventually to apoptosis.

CONCLUSION

In summary, salidroside results in the growth inhibition of tumour cells by inducing apoptosis in HepA tumour-bearing mice. The mechanism is likely to occur through the triggering of the mitochondrial-dependent pathway and caspase 3 activation.

Funding. This research was funded by the Natural Scientific Foundation of HeiLongJiang province (D201165) and supported by the Major Project of Jiamusi University (Sz2014-001).

Acknowledgement. Thanks for the suggestions and proof reading by Ms Julie Fernando.

REFERENCES

1. Parkin, D.M (2001) *Lancet Oncol*. **2**: 533-43.
2. Koda, M., Y. Murawaki, A. Mitsuda, K.Ohyama, Y. Horie, T. Suou, *et al*. (2000) *Cancer* **88**: 529-37.
3. Llovet, J.M (2005) *J. Gastroenterol*. **40**: 225-35.
4. Teo, E.K & K.M. Fock (2001) *Dig. Dis*. **19**: 263-8.
5. Xu, S.P., G.P. Sun, H.Wang & W. Wei (2006) *Anhui Med. Pharm. J*. **1**: 63-5.
6. Sun, G.P., H. Wang, S.P. Xu, Q. Wu, Z.D. Chen & W. Wei (2008) *Eur. J. Pharmacol*. **584**: 246-52.
7. Chuinhui, Z., H. Siuiyu, C. Meiqun, X. Guilin & L. Yunhui (2008) *Anticancer Drugs* **19**: 401-9.
8. Wang, P.J., Q.W. Li, K.Li, X.B. Zhang, Z.S. Han, J.J.Wang, *et al*. (2012) *Pharmazie* **67**: 733-9.
9. Majewska, A., G.Hoser, M. Furmanowa, N.Urbanska, A. Pietrosiuk, A.Zobel, *et al*. (2006) *J. Ethnopharmacol*. **103**: 43-52.
10. Calcabrini, C., R.De Bellis, U.Mancini, L.Cucchiarini, L. Potenza, R. De Sanctis, *et al*. (2010) *Arch. Dermatol. Res*. **302**: 191-200.
11. Pooja, A.S. Bawa & F. Khanum (2009) *Phytother Res*. **23**: 1099-102.
12. Ma, N., C.L. Yuan, H. Sheng & R.S.Wang (2006) *Jiangxi J. Tradit. Chin. Med*. **37**: 55-6.
13. Luo, M., H. Sheng & Y.L.Wang (2005) *JiLin Med. J*. **26**: 1285-6.
14. Qi, W.L., Y. Li, H.F. Lu, H.M. Shi & W.H. Fan (2006) *China Oncology* **16**: 710-3.
15. Hu, X., X. Zhang, S. Qiu, D.Yu & S. Lin (2010) *Biochem. Biophys. Res. Commun*. **398**: 62-7.

16. Wang, J., J.Z. Li, A.X. Lu, K.F. Zhang & B.J. Li (2014) *Oncol. Lett.* **7**: 1159-64.
17. Song, H.J., S.C. Lv, L.J. Li, D.P. Liu, X.Z.Yin & X. Han (2011) *Chin. J. Gerontol.* **31**: 3991-2.
18. Schimnich, A.A., B.C. Barnhart & M.E. Peter (2002) *Curr. Opin. Cell Biol.* **14**: 721-6.
19. Kirkin, V., S.Joos, & M. Zornig (2004) *Biochim. Biophys. Acta* **1644**: 229-49.
20. Scorrano, L & S.J. Korsmeyer (2003) *Biochem. Biophys. Res. Commun.* **304**: 437-44.
21. Ohtsuka, T., D. Buchsbaum, P. Oliver, S. Makhija, R. Kimberly & T. Zhou (2003) *Oncogene* **22**: 2034-44.
22. Xin, M & X. Deng (2005) *Biol. Chem.* **280**: 10781-9.
23. Li, K., Q.W. Li, T. Zhang, Z.S. Han, J.Li, Z.W. Liu, *et al.* (2011) *Afr. J. Biotechnol.* **40**: 7766-71.
24. Wang, J.J., Q.W.Li, Y.T. Ou, C.J.Li, X.X. Sun & Y.Z. Tian (2010) *Lat. Am. J. Pharm.* **29**: 369-75.
25. Cheung, T.H., T.K.Chung, K.W. Lo, M.Y. Yu, S. Krajewski, J.C. Reed & Y.F. Wong (2002) *Gynecol. Oncol.* **86**: 14-8.
26. Mlejnek, P. (2001) *J. Cell. Biochem.* **83**: 678-89.
27. Reed, J.C. (2000) *Am. J. Pathol.* **157**: 1415-30.