Chemical and biological analysis of active free and conjugated bile acids in animal bile using HPLC-ELSD and MTT methods

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Abstract. The aim of the present study was to determine the chemical composition and *in vitro* cytotoxic activity of seven bile samples and bile acids using the high-performance liquid chromatography (HPLC)-evaporative light scattering detector (ELSD) method. Free and conjugated bile acid standards were used to identify and quantify the chemical components of the seven animal bile samples. The MTT assay was used to determine the cytotoxic effect of the animal bile samples and the free and conjugated bile acids on hepatocellular carcinoma MHCC97-L cells. Chemical analysis revealed that the bile samples from the different animals shared little similarity in terms of their composition. A cell viability assay revealed that cattle bile, as well as its major components, DCA, CDCA and TCDCA, exhibited a marked cytotoxic effect on the hepatocellular carcinoma MHCC97-L cells. The bear bile samples that originated from the Asian black bear and the American black bear contained a unique component, TUDCA, which distinguished them from the other animal bile, though their inhibitory action on MHCC97-L cells was not markedly distinct. The present study reveals that cattle bile may be a potential alternative to bear bile for hepatocarcinoma therapy.

Introduction

Liver cancer is one of the most common and prevalent human malignancies in the world. However, prevention and treatment of liver cancer remain inadequate. In the theory and practice of traditional Chinese Medicine, bear bile has been widely used for fighting fever, toxins, inflammation, swelling, pain, liver diseases and cancer (1). However, due to increasing concerns that obtaining bile from bears is cruel and inhuman, bear farming and bile collection has been restricted in China and worldwide by government policies. The use of bear bile is now illegal, as bears are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). A search for alternatives to bear bile is therefore urgently required. Bile from other animal sources is being considered as an alternative to bear bile. Various pharmacological actions between animal bile and bear bile have been compared (2); however, a comprehensive investigation on chemical composition and cytotoxic activity is lacking.

In the present study, a high-performance liquid chromatography (HPLC)-evaporative light scattering detector (ELSD) system was introduced to quantify the conjugated and free bile acids in seven different animal bile samples. Standard chemicals were used to identify and measure the chemical composition of the animal bile samples, and a cell viability assay was used to determine the cytotoxic potential of the animal bile samples as well as the bile acids. The various chemical compositions as well as the *in vitro* cytotoxic activity of the different animal bile samples were determined. The cattle bile contained the active components DCA, CDCA and TCDCA, and was determined to be a potential cytotoxic agent against cancer cell growth.

Materials and methods

Chemicals and sample collection. Sodium tauroursodeoxycholate (TUDCA, T0266), ursodeoxycholic acid (UDCA, U5127), sodium deoxycholate (DCA, D6750), sodium taurochenodeoxycholate (TCDCA, T6260), sodium taurodeoxycholate (TDCA), taurocholic acid (TCA, T4009), sodium chenodeoxycholate (CDCA, C8261), sodium glycodeoxycholate (GDCA, G9910), sodium glycochenodeoxycholate (GCDCA, G0759), sodium glycocholate (GCA, G7132), cholic acid (CA, C1129) and taurine (TR, T0625) were purchased from Sigma-Aldrich (USA). Bile from the American black

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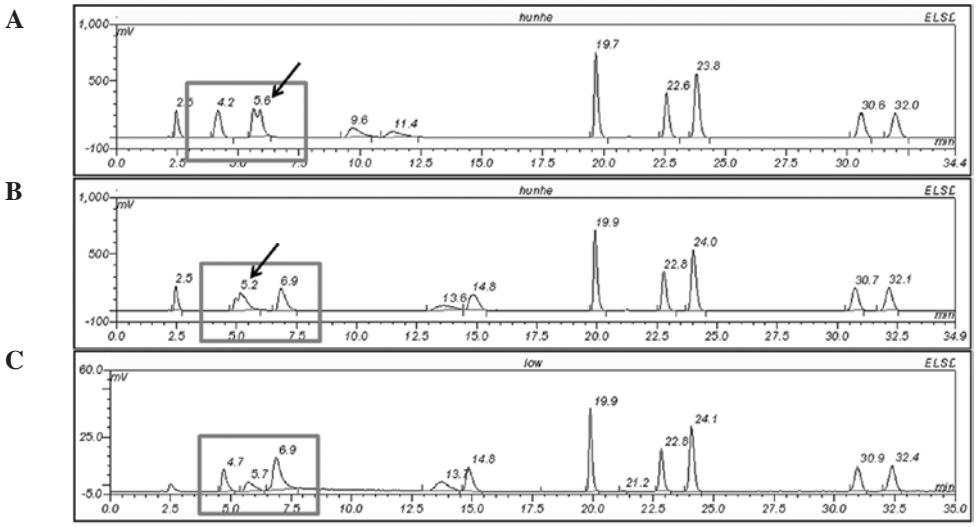

Figure 1. Optimization of HPLC conditions for bile sample analysis. HPLC chromatogram of the mixed standards under the elution conditions with a starting ratio of A to B of (A) 52 to 48%; (B) 50 to 50% and (C) 51 to 49%. The frame highlights the isolation situation of the monitored peaks.

Table I. HPLC separation conditions.

Time (min)	Methanol (A%)	0.5% acetic acid in water (pH 3.0, B%)
0	51	49
10	51	49
15	75	25
35	75	25

bear (UB), *Ursus Americanus*, was purchased from Pak Shing Tong Ginseng Co. Ltd. (Hong Kong; licence no. APO/PL 1907/06). Bile from the Asiatic black bear (AB) was purchased from Hang Hing Co. (Hong Kong; licence no. APO/PL 2384/07). Snake bile powder (SB), pig bile powder (PB), cattle bile powder (CaB) and chicken bile powder (ChB) were purchased from Yee Po International (China). Bile juice from rabbit (RB) was kindly provided by the Laboratory Animal Unit (The University of Hong Kong).

Sample preparation. Bile samples (2 g) were extracted with a 40-ml methanol-water solution (1:1; v/v) in 50-ml centrifuge tubes for 2 h using an ultrasonic cleaner (Branson, USA) at room temperature and were then centrifuged at 4,000 rpm for 20 min. The supernatant (2 ml) was collected and filtered through a 0.45-μm membrane (Millipore, USA). Pure compounds (Sigma) were dissolved in methanol-water solution (1:1; v/v) for a final concentration of 2 mg/ml and then filtered. For analysis of bioactivity, 30 ml of supernatant was collected, and the solvent was evaporated by a rotary evaporator. The residue was dissolved in water containing 0.1% dimethyl sulphoxide (DMSO) (Sigma) at various concentrations. Pure compounds were dissolved in water containing 0.1% DMSO.

HPLC-ELSD analysis. A Dionex® HPLC system (comprising a quaternary pump 680, an autosampler ASI-100, an injector with a 200 μl loop, a column oven STH 585 and a data system Chromeleon® 6.40) was used in this experiment with an ELSD (2000ES; Alltech, USA). ELSD conditions were as follows: flow rate of purified compressed air as a nebulizing gas, 1.6 l/min; temperature of heated drift tube, 85˚C. A Nova-Pack® C18 column (300 mm x 3.9 mm I.D., particle size, 4 μm; Waters, USA) with a Nova-Pack® C18 Guard column was used as a solid phase. Methanol as organic solvent A and 0.5% acetic acid in Mill-Q water (pH 3.0) as aqueous solvent B were used as mobile phases. A three-step gradient elution was as shown in Table I. The column temperature was 40˚C, and the flow rate was kept constant at 0.9 ml/min.

Cell culture and cell viability assay. The *in vitro* cytotoxic activity of the agents (TCA, GCA, DCA, GCDCA, GDCA, TDCA, taurine, TCDCA, CDCA, UDCA, TUDCA) and the animal bile (PB, SB, CaB, ChB, RB, AB, UB) in the hepatocellular carcinoma cell line MHCC97-L was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells at 80% confluence in a 75 cm^2 flask were trypsinized, and a single-cell suspension was obtained. Cells (10,000) in 200 μl of medium per well were seeded in 96-well plates and incubated for 24 h. Cells were then incubated along with a series of bile samples or pure compounds at various concentrations (2, 4, 8, 16, 32, 64, 128, 256 and 512 μM) for 24, 48 and 72 h. Wells treated with vehicle (0.1% DMSO) served as the controls. After treatment for various time periods, 15 μl of 5 mg/ml MTT (Sigma) was added to each well and incubated for 4 h at 37˚C. The medium was then discarded, and 200 μl of DMSO (Sigma) was added and pipetted up and down to dissolve the crystals within the wells. The absorbance was measured at 570 nm by a Multiskan MS microplate reader (Labsystems, Finland). Each experiment was repeated three times, and the standard deviations were indicated as error bars. Cell viability was calculated as the ratio of the absorbance of cells treated with agents to that of the untreated control multiplied by 100%. A curve was plotted as the percentage of viable cells against the concentration of the agents.

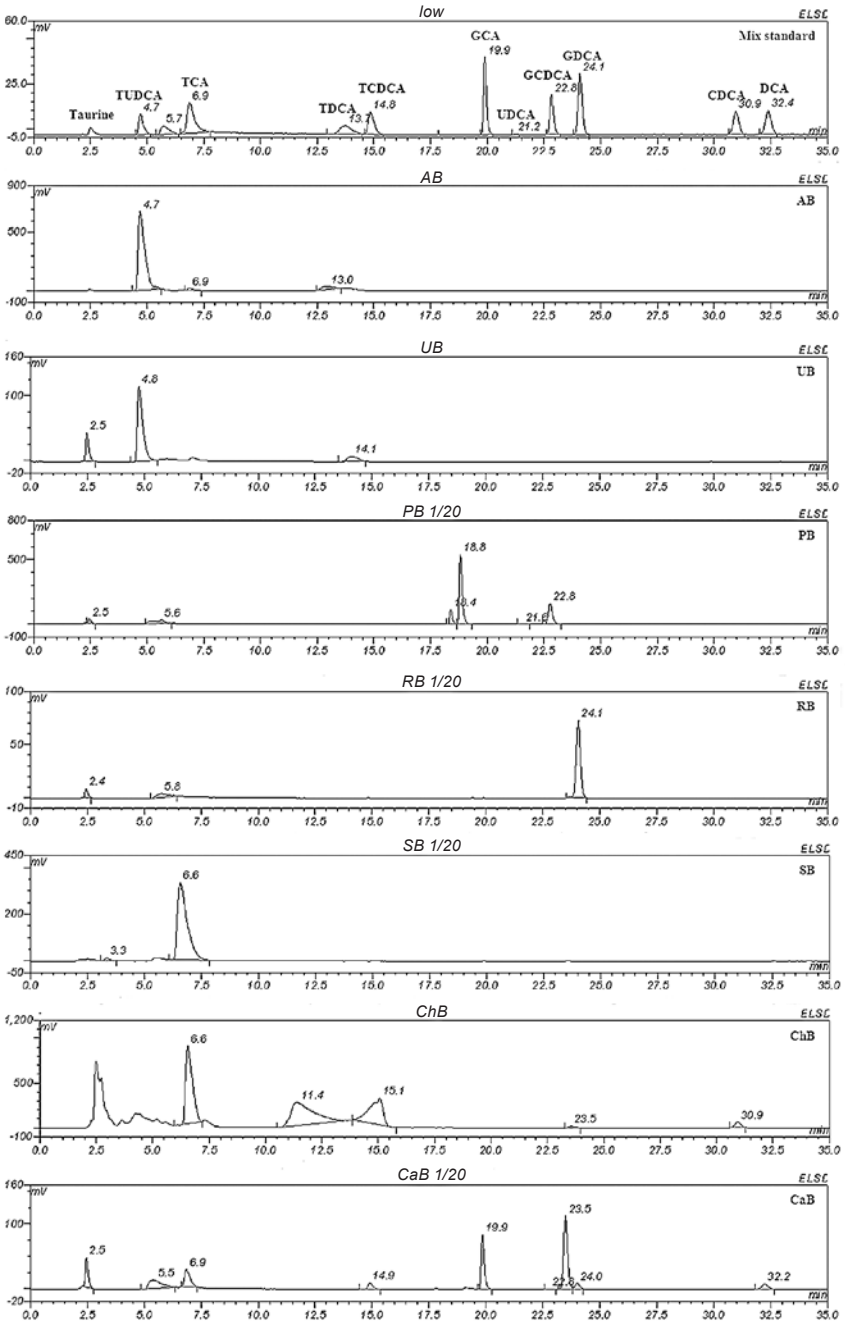

Figure 2. HPLC-ELSD analysis of the seven animal bile samples. Identification of each peak in the mixed standard and bile samples was performed by injecting individual standard samples to compare the retention time.

Statistical analysis. The data were analyzed using the Student's t-test and are expressed as the mean ± SD.

Results

Optimization of HPLC conditions. Various separation conditions were tested to obtain the optimal resolution for the free and conjugated bile acids. A mixed standard solution (10 μl) was eluted with different starting ratios of mobile phases. The separation was monitored to determine the optimal elution conditions. The optimal condition with a starting ratio of A to B of 51 to 49% was selected for sample analysis based on good baseline resolution and stable duration. The optimization of HPLC conditions is presented in Fig. 1.

Identification and quantification of free and conjugated bile acids in animal bile samples, including bile crystals from Asian and American bears. Isolation of the mixed standardized chemicals and the seven animal bile samples was performed under the optimized conditions (Fig. 2). An individual standard sample was analyzed under the same conditions to identify the peaks in the bile samples. Notably, the chemical composition of the seven animal bile samples varied, showing extensive differences. Both AB and UB samples contained TUDCA, a particular component only found in bear bile juice, but not in any of the other animal bile samples. UB also contained a small amount of TCDCA, while AB did not. PB contained a large amount of UDCA, whose taurine conjugated form is TUDCA, whereas CaB was

Table II. Relative content (%) of the conjugated and free bile acids in the seven animal bile samples.

	UDCA	DCA	TCA	GCA	TDCA	CDCA	GDCA	GCDCA	TUDCA	TCDCA
PB	16.112							34.691		
SB			94.857							
RB							18.356			
CaB		8.615	15.058	18.020				1.777		9.881
ChB		6.940			11.446	1.242				7.182
AB									6.522	
UB									2.344	0.685

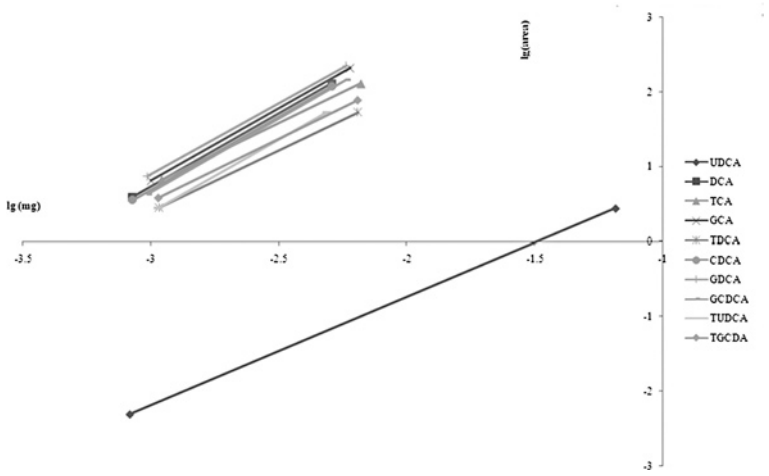

Figure 3. Standard curves of the ten studied free and conjugated bile acids.

Table III. IC_{50} of animal bile in hepatocellular carcinoma MHCC97-L cells.

IC_{50}	AB (μM)	UB (μM)	PB (μM)	CaB (μM)
24 h	487.97	512.02	NA	106.41
48 h	374.14	202.58	330.86	72.74
72 h	378.92	263.52	58.29	45.47

composed of a series of DCA-based chemicals, including DCA, CDCA, TDCA and TCDCA. SB was mainly comprised of TCA, while RB contained large amounts of GDCA. The standard curve is presented in Fig. 3, and the chemical composition of the seven animal bile samples is shown in Table II.

Cytotoxic effect of animal bile samples on hepatocellular carcinoma MHCC97-L cells. Since bear bile is regarded as a therapeutic agent for liver diseases according to classic Chinese Medical theory, and has been used for the treatment of liver cancer by ancient and modern Chinese Medical practitioners (3), the cytotoxic effect of bile from two bear species and other animals on the hepatocellular carcinoma cell line MHCC97-L was examined using the MTT assay. Our results revealed moderate cytotoxic effects for both types of bear bile. Consistent with the difference in their chemical composition, UB (IC_{50}= ~200 μM) exhibited more significant cytotoxic activity than AB (IC_{50}= ~400 μM). Bile from pig or cattle is usually used as an alternative to bear bile due to concerns for protecting endangered species. Our results revealed that both CaB and PB exhibited potent cytotoxic activity in MHCC97-L cells after a 72-h treatment (Table III). In contrast, both SB and RB revealed pro-proliferative activity in MHCC97-L cells; SB had a more extensive promotional effect on cell proliferation than RB (Fig. 4).

Cytotoxic effect of free and conjugated acids on the growth of hepatocellular carcinoma MHCC97-L cells. The cytotoxic activity of free and conjugated bile acids on human carcinoma has been well documented in previous *in vitro* and *in vivo* studies (3,4). In order to provide a systematic report on the cytotoxic activity of bile acids from animal bile in the liver cancer cell line MHCC97-L, we conducted experiments to examine the *in vitro* cytotoxicity of ten free and conjugated bile acids, which were originally isolated from animal bile. The results are presented in Fig. 5. DCA, CDCA and TCDCA demonstrated a significant cytotoxic activity in MHCC97-L cells, while TDCA, GDCA and GCDCA exhibited lower cytotoxic activity, even though they share similar chemical structure. UDCA and its taurine conjugated form, TUDCA, revealed no cytotoxic activity in MHCC97-L cells, whereas

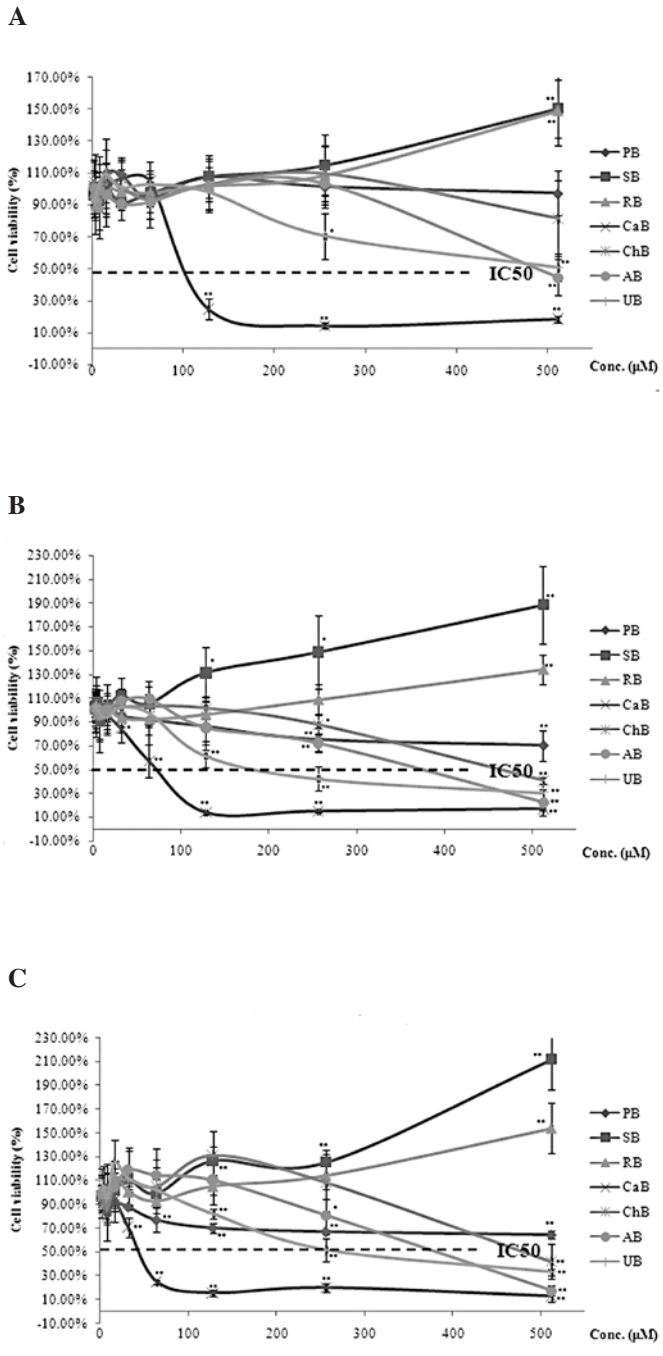

Figure 4 Cytotoxic activity of the seven animal bile samples on hepatocellular carcinoma MHCC97-L cells. MHCC97-L viability upon treatment with the animal bile samples for (A) 24 (B) 48 and (C) 72 h. The curves revealed a parallel shift at 24, 48 and 72 h in a dose-dependent manner, apart from RB and SB. The order of cytotoxic strength was CaB, UB, AB, PB and ChB; whereas RB and SB had no cytotoxic effect. All experiments were conducted in triplicate, and the results were analyzed for statistical significance ($^{*}p<0.05$, $^{**}p<0.01$ compared to the control).

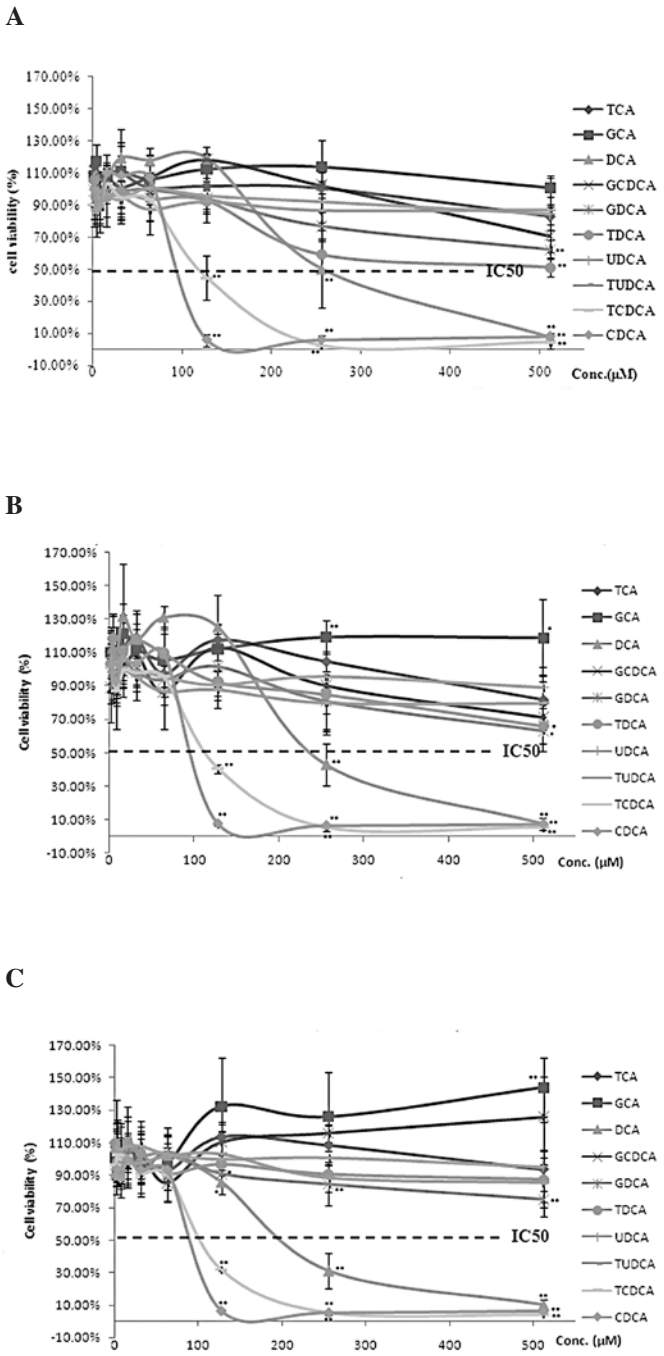

Figure 5. Cytotoxic effect of free and conjugated bile acids on hepatocellular carcinoma MHCC97-L cells. MHCC97-L viability upon treatment with free and conjugated bile acids for (A) 24 (B) 48 h and (C) 72 h. DCA, CDCA and TCDCA exhibited a significant cytotoxic effect in a dose-dependent manner at 24, 48 and 72 h. Other free acids had a weak cytotoxic effect, whereas GCA and GCDCA induced cancer cell proliferation and growth. All experiments were conducted in triplicate, and the results were analyzed for statistical significance ($^{*}p<0.05$, $^{**}p<0.01$ compared to the control).

Table IV. IC_{50} of bile acids in hepatocellular carcinoma MHCC97-L cells.

IC_{50}	DCA (μM)	CDCA (μM)	TCDCA (μM)
24 h	258.68	100.01	122.43
48 h	244.29	101.57	119.22
72 h	212.20	95.17	109.61

TCA and GCA even exhibited a weak stimulative activity on MHCC97-L cell proliferation. The IC_{50} values of DCA, CDCA and TCDCA are presented in Table IV.

Discussion

Bear bile and bile extractions belong to the category of animal drugs in Traditional Chinese Medicine. The use of bear bile in

Chinese Medical practice has a long history in attenuating fever, toxification, inflammation, swelling, pain, liver diseases and cancer (1). The use of bear bile is now illegal since bears are classified as endangered animal species in CITES. The identification of alternatives to bear bile is therefore necessary. Bile from other animals is considered an alternative due to its similar origin (2,5,6). However, a comprehensive study on the chemical composition and bioactivity of animal bile is necessary.

Studies have revealed that PB solution has similar pharmacological action as bear bile in regards to its anti-inflmmatory, anticonvulsive and analgesic activities (1). It has been reported that PB is used as an alternative to bear bile in specific Chinese Medicinal formulas (2). In the present study, we found that PB contains a large amount of UDCA, the unconjugated form of TUDCA, which is only produced in bears. Both UDCA and TUDCA have been previously found to have anti-inflammatory, anti-apoptotic, cell protective and anticholestatic properties (7-11). In the present study, no significant cytotoxic activity of PB, as well as UDCA and TUDCA, was observed.

The chemical composition of CaB, another type of bile that is usually used as an alternative to bear bile, was found to differ from that of the bear bile in our study. CaB, which mainly contains DCA, CDCA and TCDCA, had excellent cytotoxicity against hepatocellular carcinoma MHCC97-L cells. DCA, CDCA and TCDCA have been reported to inhibit growth, induce apoptosis and suppress metastasis in breast, esophageal, and colon cancers (12-14). Chinese Medicine literature has reported that bile may attenuate liver diseases and cancer (1). Our study on the cytotoxic activity of CaB, as well as its active components, DCA, CDCA and TCDCA, reveals for the first time that these three bile acid derivates and CaB are potential agents for liver tumor treatment. Similar results were also found for ChB.

RB was previously found to be another alternative source to bear bile (6). However, in the present study, we found that RB had a totally distinct chemical composition to bear bile. RB exhibited no cytotoxic activity and even weakly promoted MHCC97-L cell proliferation, which is consistent with the activity of GDCA (main active compound in RB). Notably, we observed a strong and constant stimulation of MHCC97-L cell proliferation by SB, in which TCA is the major and only component identified by the HPLC analysis. TCA was found to promote the occurrence of cholangiocarcinoma induced by diisopropanolnitrosamine in hamsters (15), although the exact mechanism needs further investigation.

In conclusion, the use of bile from other animal sources as an alternative to bear bile has been considered based on their similar chemical or pharmacological profiles. The chemical composition and *in vitro* cytotoxic activity of seven animal bile samples, PB, SB, RB, CaB, ChB, AB and UB, were evaluated in this study. Both free and conjugated bile acids in the animal bile samples were evaluated. HPLC-ELSD analysis revealed the distinct chemical composition of the different animal bile samples. A cell viability assay revealed that bile from cattle exhibits more marked inhibitory activity on hepatocellular carcinoma cell growth and proliferation than bear bile. DCA, CDCA and TCDCA are the major active compounds in cattle bile. Our results support the potential of cattle bile as an alternative to bear bile in liver cancer prevention and therapy.

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