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Antioxidants and bioactivities of total flavonoids Extracted from *Kunlun Chrysanthemum* Flowers

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Author Disclosure

The authors declare that there is no competing financial interests exist in this paper.

Abstract

Plant extracts have medicinal and pharmacological values. The aim of the present study was to evaluate the antioxidant activity, antibacterial property, and protective effect on liver injury induced by carbon tetrachloride (CCl_4) in mice as well as the antiproliferative effect on tumor cells of *Kunlun Chrysanthemum* total flavonoids (KCTF). We measured free radical scavenging

activities of KCTF and also evaluated KCTF's antioxidative effects on lipid peroxidation and superoxide dismutase activity. Moreover, KCTF's detoxification effect was assessed on acute liver injury in mouse induced by carbon tetrachloride (CCl_4) and its anti-proliferation property was determined using HeLa cells and esophageal cancer cells. Results indicated that KCTF had significant antioxidant activity. KCTF inhibited the growth of both Gram positive and Gram negative bacteria. However, KCTF had no significant protective effect on acute liver injury induced by CCl_4. Results of the antiproliferative effect of KCTF on HeLa cells and Eca109 cells indicated that KCTF possessed great inhibitory activity with average IC_{50} values of 133.6±0.1885 μg/mL, 192.0±0.07719 μg/mL, respectively. Therefore, the present study indicates that KCTF may have potential application on functional food industry due to its natural antioxidant, bacteriostatic and antiproliferative effects.

Keywords: *Kunlun Chrysanthemum* total flavonoids (KCTF); antioxidant activity; antibacterial property; antiproliferative effect; liver injury

Abbreviations

(KCTF) *Kunlun Chrysanthemum* total flavonoids

(CCl_4) carbon tetrachloride

(UHP) ultra high pressure

(HPLC) high performance liquid chromatography

(DPPH·) 2,2-diphenyl-1-picrylhydrazyl

(OH·) hydroxyl radical

(Vc) Ascorbic acid

(TBHQ) tertiary butylhydroquinone

(SOD) superoxide dismutase

(MDA) malondialdehyde

(MIC) the minimum inhibitory concentration

(ALT) alamine aminotransferase

(AST) aspartate aminotransferase

(HeLa) Human cervical carcinoma cells ,

(Eca109) esophageal cancer cells

(MTT) 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide

(LDH) lactate dehydrogenase

Introduction

Kunlun Chrysanthemum (Coreopsis tinctoria, C. tinctoria) is an annual herbaceous plant which belongs to family Compositae, and commonly grows in the Middle East, Eastern Europe, Western and Central Asia(Liang, HAMulati, Pang, & Sun, 2010). In Xinjiang of China, *C. ticntoria* is commonly known as "*Kunlun Chrysanthemum*" and "snow chrysanthemum", and is cultivated in Karakorum Mountains of 2600 meter above sea level. In traditional Uyghur medicine, *C. tinctoria* has been used by Uyghur people for treatment of various diseases such as hypertension, palpitation and gastrointestinal discomfort(A. S. ZHANG, 2010). In our previous studies, the content of polysaccharides, procyanidins and total flavonoids extracted from *Kunlun chrysanthemun* were 193.3 mg/g, 26.58 mg/g, 281.2 mg/g (unpublished), respectively. In recent years, there are more studies on the bioactive extracts from *Kunlun Chrysanthemum* due to its antioxdation function(Yang, Chen, Yang, & Xin, 2014) and its ability in reducing blood lipid without causing liver damage in hyperlipidemic mouse (R. Fang, Tang, Huang, Chen, & Zhang, 2009; Li et al., 2014). However, most research about biological activity of *C. Tinctoria* has focused on the polysaccharide compounds(Siqun Jing, Chai, et al., 2016) or its other extracts while no comprehensive studies have been performed on its flavonoids extracts.

Flavonoids are one of the major constituents of *Coreopsis* genus(Dias, Bronze, Houghton, Motafilipe, & Paulo, 2010). Flavonoids are important secondary metabolites, and widely distributed in various plants(Meurer-Grimes, 1995). Biological activities of the flavonoids have been reported in many studies, such as antioxidant, anti-tumor, and hypertensive effect (Aron & Kennedy, 2008; Karabin, Hudcova, Jelinek, & Dostalek, 2015). Previous studies using cultured

cells showed that flavonoids could significantly inhibit the growth of various malignant cells(Galijatovic, Walle, & Walle, 2000), such as breast cancer(Bratkov, Shkondrov, Zdraveva, & Krasteva, 2016), liver cancer(Yan & Liu, 2007), cervical cancer(Sheng-Bin & Xie, 2013), and stomach cancer cells (Ji et al., 2004; Zhou, 2006). To our knowledge, there is no study regarding the antibacterial property and protection against liver injury and antiproliferative effects of KCTF on cancer cells.

In the present study, to study the different aspects of bioactivities of KCTF, we evaluated the bioactivities of KCTF by the following studies: (i) the antioxidant activities of KCTF were investigated by experiments in vitro and in vivo; (ii) the antibacterial property of KCTF was identified by the disc diffusion method; (iii) the protective effect on liver injury was studied through the acute liver injury in mice induced by CCl_4 model; (iv) the antiproliferative effect of KCTF was evaluated by MTT assay and optical microscopy. This study may provide a theoretical basis for further development of KCTF in food industry.

Material and Methods

Materials

Dried *Coriopsis tinctoria* flowers were obtained from a local herbalist at Hetian in Xinjiang in August 2012, and were identified by Prof. Abudula Abbas from Xinjiang University, China. The optimum conditions of KCTF by UHP extraction(S. Q. Jing & Zhang, 2013) were as following: extraction temperature 25 ^{o}C, ratio of solid to liquid l:16 (g/mL), pressure 340 MPa, UHP time 3 min, material particles 80 mesh, and under this condition, the yield of KCTF was up to 12.35%. Then, the KCTF was further purified by AB-8 macroporous adsorption resin and with rutin being used as the standard, the content of total flavonoids could reach 52.43% after purification.

Animals

Kunming male mice (20 ± 2 g) 2011-0003/SCXK (Xin) were obtained from Xinjiang Laboratory Medical University Breeding Research Center and basal diet was provided by the Xinjiang Animal Center. Animals were kept under a 12 h/12 h light/dark cycle and allowed free access to food and water. The study protocols were approved by the Ethics Committee on Animal Experiment, Xinjiang University, China.

Microorganisms

Staphylococcu saureus, *Escherichia coli*, *Bacillus subtilis*, *Penicillium citri*, *Aspergillus niger*, *Saccharomyces cerevisiae* were used in this study because they are widely available. All the cultures were obtained from Microorganism Laboratory of College of Life Science and Technology, Xinjiang University (Urumqi, China). The strains were subcultured for further antimicrobial test.

High performance liquid chromatography (HPLC) analysis of KCTF

In order to investigate the main chemical composition of KCTF, HPLC analysis was adopted(Gao et al., 2016) and commercially available Mali glycosides were used as reference substance. The chromatographic separation was performed on a Phenomenex Gemini C18 (250 mm × 4.6 mm, 5μm) with the mobile phase: acetonitrile: 0.5% formic acid solution. The percentage of acetonitrile was changed from 5% to 20% for 60 min. The flow rate, column temperature and detection wavelength were 1.0 mL/min, 35^{o}C and 378 nm, respectively.

In vitro Antioxidant activities of KCTF

Antioxidant activity of KCTF was determined by reducing power, 1,1-diphenyl- 2-picrylhydrazil (DPPH•) radical scavenging, and hydroxyl radical (•OH) scavenging tests. A series concentrations of KCTF (0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml) were prepared. Same concentrations of ascorbic acid (Vc) and Tertiary butylhydroquinone (TBHQ) were used as the reference materials in each experiment. All tests were carried out in triplicate.

Assay of reducing power

The Reducing power of KCTF was determined according to the method of Jing(S. Jing, Ouyang, Ren, Xiang, & Ma, 2013) with slight modifications. Briefly, various concentrations of KCTF (0.1-0.9 mg/mL) in 1.0 mL MeOH were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide in 10 test tubes. The mixtures were incubated at 50°C for 20 min. At the end of the incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixtures and then centrifuged at 5000×g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm with a UV-visible spectrophotometer. Higher absorbance indicates stronger reducing power.

Assay of 1,1-diphenyl-2-picrylhydrazil (DPPH•) radical scavenging activity

Generally, radical scavenging activities of antioxidants in the plant extracts were evaluated using DPPH radicals, which was measured by the method of Xiao *et al* (Xiao et al., 2012). Briefly, 2 mL of 2×10^{-4} mol/L DPPH• ethanol solution was added to 2 mL various concentrations (0.1- 0.9 mg/mL) of KCTF solution. The reaction mixture was incubated for 30 min at room temperature in the dark. The absorbance was measured at 514 nm with a UV/visible spectrophotometer.

Assay of Hydroxyl radical (•OH) scavenging activity

Hydroxyl radical scavenging activity was determined according to reported phenanthroline- Fe^{2+} oxidation method(Zhao, Jian-Ke, Zhao, & Xiao-Xia, 2009) with minor modifications. Briefly, 4.0 mL sodium phosphate buffer (pH 7.4) was mixed with 1.5 mL of 5 mmol/L phenanthroline solution in a test tube. Then, 1.0 mL $FeSO_4$ solution (7.5 mmol/L) and 1.0 mL of the different sample solutions of KCTF (0.1-0.9 mg/mL) were added. Finally, 1.5 mL double distilled water and 1.0 mL 1% H_2O_2 solution were added. The absorbance of the final solutions was measured at 536 nm with a UV-visible spectrophotometer after incubation at 37°C for 60 min.

In vivo antioxidant activities of KCTF

Antioxidant activity in vivo of KCTF was carried out by the method based on Jing *et al.*(Siqun Jing, Zhang, & Yan, 2015) with a few modifications. The concentrations of KCTF used were based on data reported in the literature in conjunction with purity and dosage used in mice. After a week acclimation to the laboratory, 50 mice were randomly divided into five groups: normal control group (NC), low-dose group (LD, 30 mg/kg BW per day, where BW is body weight), middle-dose group (MD, 100 mg/kg BW per day), high-dose group (HD, 300 mg/kg BW per day) and positive control group (NC^+, Vc 800 mg/kg BW per day). Dosages were selected based on previous studies(Siqun Jing et al., 2015). The NC group of mice was given 0.2 ml physiological saline while low, middle and high dose groups were fed KCTF once a day for 28 days, and positive group was fed Vc. The body weight of mice was measured once a week. After the final intragastric administration, the mice were fasted for 12 h and blood were then taken by pricking the eyeball. The mice serum was separated at 3500×g for 15 min and stored at 4^{o}C. The organs of mice were removed out and weighed immediately after they were sacrificed. Then 10% concentration of liver homogenate was made with physiological saline at 4 ℃ and the supernatant was removed and refrigerated after centrifugation at 3500×g for 5 min. The value of

superoxide dismutase (SOD), malondialdehyde (MDA) in serum and liver homogenate was determined by commercial reagent kits according to the instruction manuals.

Antibacterial activity of KCTF

The antibacterial activity of KCTF was measured by the disc diffusion method shown by Wang (Wang, 2014) with a little modification. The activated strain of bacteria, mold spores, and yeast were picked and transferred with loop into 9 mL sterile water respectively, and then shaken well to make spores and cell suspensions which contained $1\sim2\times10^8$ cells/mL of bacteria, $1\sim8\times10^6$ cells/mL of mold spores, and $1\sim7\times10^6$ cells/mL of yeast. Under aseptic conditions, 200 μL of each bacteria suspension was added to the prepared medium plates and coated evenly. Sterile paper discs (9 mm in diameter) were impregnated with 5.00 mg/mL extracts solution, 0.03 mg/g potassium sorbate solution, 95% ethyl alcohol and sterile water saline solution overnight and then placed on the bacteria plate. Bacterial plates were incubateor 18 h - 24 h, while mold plates were incubated at 28±1°C for 48 h and yeast plates were incubated at 36±1°C at 30±1°C for 24 h. The inhibition of bacterial and fungal growth were recorded by measuring the diameter (mm) of the clear zones surrounding the disc which indicate the presence of antimicrobial activity (Siqun Jing, wang, et al., 2016). All data of antimicrobial assays are the average of triplicate analyses.

The minimum inhibitory concentration (MIC) values were determined as described by Swenson previously (Swenson et al., 2010). The MIC value was defined as the lowest concentration of a substance preventing visible growth of the test organisms. Sterile water alone was used as control.

Protective effect of KCTF on CCl_4-induced acute liver injury in mice

The protective effect of KCTF on liver injury was observed by establishing a CCl_4-induced acute liver injury model in mice described by Cai (Pintus et al., 2014) with a slight modification. Male Kunming mice (body weight: 18-22 g) were housed in standard cages at a constant temperature of 22±1℃ and relative humidity of 55%±5% with 12 h light-dark cycle (08:00 to 20:00) for at least 1 week before experiment.

For liver protection experiments (X. Yang et al., 2014; Zhong, Gao, Chen, & Zhang, 2015), blank and model groups of mice were intragastrically administrated with 0.2 mL/10 g distilled water as the non-therapeutic control. The positive group was given bifendate (0.4 g/kg BW)

orally for 6 consecutive days. The KCTF groups of mice were orally administered KCTF (0.10 LD, 0.20 MD and 0.40 HD g/kg) for 6 consecutive days. Both bifendate and KCTF were administered at the same time. One hour after the administration of the experimental drugs on day 5, intraperitoneal injection of 0.1% CCl_4 olive oil (0.2 mL/10g) was carried out except the mice in blank group. The mice were fasted overnight for 24 h with CCl_4 injection. Then the mice were sacrificed under anesthesia and the blood was taken by picking the mice's eyeball and centrifuged (3500×g for 10 min) for supernatant analysis. The activities of the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. The liver coefficient was defined as Liver weight / Body weight

Antiproliferative activity against tumor cells

MTT assay

Human cervical carcinoma cells (HeLa), esophageal cancer cells (Eca109) and Vero cells were obtained from Xinjiang University Xinjiang Biological Resources Gene Engineering Key Laboratory (Urumqi, China). In vitro antiproliferative activity of KCTF against two tumor cells (HeLa and Eca109) was used, together with Vero cells (normal cells) as control. The proliferation of cells mentioned above was conducted by 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with some slight modifications (Abe, Ueo, & Akiyoshi, 1994; Mancang et al., 2009). Briefly, logarithmically growing cells were seeded in 96-well culture plates (5×10^4cells/well) for 24 h at 37℃ with 5% CO_2 in the atmosphere. The cultures were washed and treated with a serial concentration of KCTF (12.5, 25, 50, 100, 200, 400, and 800 μg/mL). 20 μl MTT solution (5 mg/mL; Sigma-Aldrich, MO, USA) was then added 12 h, 24 h, 48 h, or 72 h later. After 4 h incubation at 37℃, at the end of the treatment, the incubation medium was discarded, and the formed crystals were dissolved in 100 μl dimethyl sulfoxide. MTT reduction was quantified by measuring the light absorbance of each well at 570 nm using a Universal Microplate Reader (EL800, BIO-TEK Instruments, USA) to evaluate the proliferation of cancer cells. All experiments were performed in triplicate and cell survival was expressed as a percentage of the control, which was considered to be 100%. The IC_{50} value was calculated as the sample concentration that caused a 50% inhibition of cell proliferation.

Determination of morphological changes of cells

Hela cells, Eca-109 cells, and Vero cells (5×10^4 cells/well) were incubated for 24 h in 96 well

plates respectively. After incubation, the cells were untreated or treated with KCTF at different concentrations (12.5, 100 and 800 μg/mL) for 24~72 h(Sang et al., 2013). Then the medium was removed and cells in wells were washed twice with PBS. This was followed by examination under phase contrast inverted microscope (Nikon, Japan) at 200 × magnification.

Fluorescence microscopy observation of Hela cells

The cells, plated onto glass cover slips in 6-well plates and treated with 0 and 10 g/mL of the KCTF for 48 h, were washed twice with PBS and stained with Hoechst 33342 (Sigma, USA) for 15 min at 37℃. After washing with PBS, cover slips were mounted onto microscope slide and nuclear morphology was observed under a fluorescence microscope (Nikon, Tokyo, Japan) at 200 × magnifications.

Lactate dehydrogenase (LDH) activity

According to the reported method (Cheng et al., 2008), Hela cells in logarithmic growth phase were seeded in 24-well culture plate, and each well having 5×10^4 cells/mL cell suspension. After incubation for 12 h, the old medium was gently sucked out. Then 1.0 mL KCTF (0, 800 μg/mL medium) were added in each sample at three replicates and cultured for 48h. Medium was centrifuged at 2000 ×g for 5 min according to literature methods with minor modifications. The amount of LDH in the supernatant was measured as an index of cell necrosis, the amount of LDH in suspended cells was measured as an apoptotic index, the amount of LDH of a culture bottle adherent cell was measured as the level of intracellular lactate dehydrogenase. Apoptosis rate and necrosis rate was calculated as the following: apoptosis rates (%) = LDHa/(LDHa + LDHn + LDHv) × 100%; Necrosis rate (%) = LDHn/(LDHa + LDHn + LDHv) × 100%.

Statically analysis

Data were expressed as means ± standard deviations of three determinations. Statistical analysis was performed using t-test and one-way analysis of variance. A value of $P < 0.05$ was considered significantly different. All computations were made with SPSS 19.0 software.

Results and discussion

HPLC chromatograms of KCTF

The result of HPLC analysis of KCTF showed that under such chromatographic conditions, KCTF mainly contained 2 characteristic components and one of the higher content was found to be Mali glycosides (Kunlun chrysanthemum chalcone 4′-O-β-glucoside) after comparing with the spectrum of Mali glycosides standard. Nonetheless, the area of another peak was too small to be analyzed (Fig. 1).

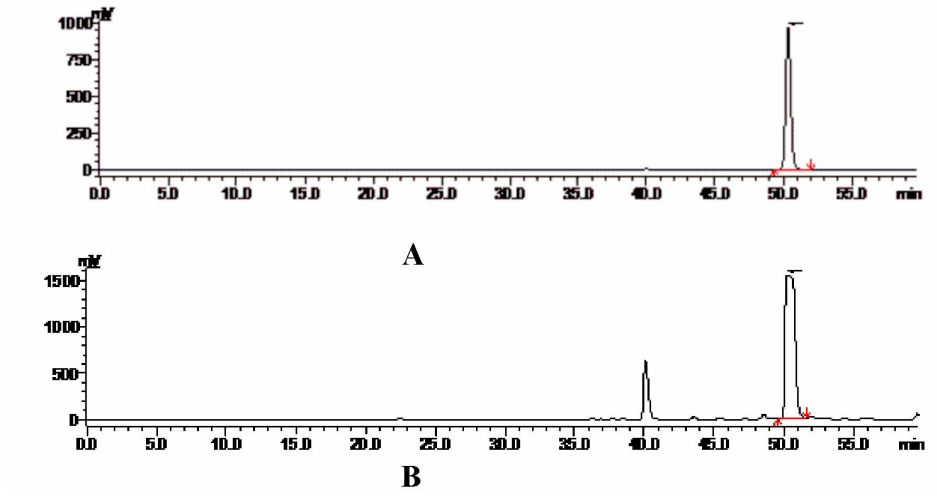


Fig. 1 HPLC chromatogram of KCTF at 378 nm. A, control (Mali glycosides); B sample (KCTF).

Antioxidant activity in vitro

Reducing power

Reducing power may serve as an important indicator of a compound's potential antioxidant activity. The antioxidants like Vc could interrupt or inhibit the chain reaction by capturing and removing free radicals, and TBHQ terminate the chain reaction by providing a hydrogen atom (Huang & Zheng, 2006; X. Y. Zhang, Li, Wu, Bai, & Liu, 2005). As known in Fig. 2A, the reducing power of samples increased with the increasing concentration of KCTF. However, the growing trend becoming slower at the concentration of 0.7 mg/mL. The range of reducing power of the three tested samples from strong to weak was as following: TBHQ > Vc > KCTF. This result indicated that KCTF has antioxidant capacity.

DPPH radical scavenging activity

DPPH• is a stable free radical, which has been widely used as a substrate to estimate antioxidative activity of antioxidants. The results showed (shown in Fig. 2B) the concentration-dependent curves of KCTF on DPPH radical scavenging ability. The scavenging

effect of KCTF on DPPH radicals was 32.24% at the concentration of 0.9 mg/mL, and weaker than that of Vc and TBHQ (68.12% and 97.23%). In general, the KCTF has antioxidant activity (IC_{50} = 1.369±0.05184 mg/mL) but was lower than that of Vc (IC_{50} = 0.3358±0.08868 mg/mL) and TBHQ (IC_{50} = 0.07399±0.2326 mg/mL). The DPPH• scavenging activities could be attributed to their hydrogen donating abilities. Hence, the mechanism may be due to the supply of hydrogen by KCTF, which combines with radicals and forms a stable radical to terminate the radical chain reaction (Lai, Lai, Zhao, & Chen, 2010).

OH radical scavenging activity

Among the oxygen radicals, ·OH is the most active and toxic free radical, and induces severe damage to adjacent biomolecules. Therefore, ·OH scavenging ability can be accepted as an illustrator of antioxidant activity. Fig. 2C shows that the scavenging activity of KCTF on ·OH was in a concentration-dependent manner and inhibition rate on ·OH is 44.28% at the concentration of 0.9 mg/mL while that of Vc and TBHQ at the same concentration (0.9 mg/ml) were 57.62% and 69.43%, respectively. Moreover, the IC_{50} of KCTF, Vc and TBHQ for scavenging ·OH were 1.126±0.3148 mg/mL, 0.7362±0.08024 mg/mL, and 0.4411±0.1040 mg/mL, respectively.

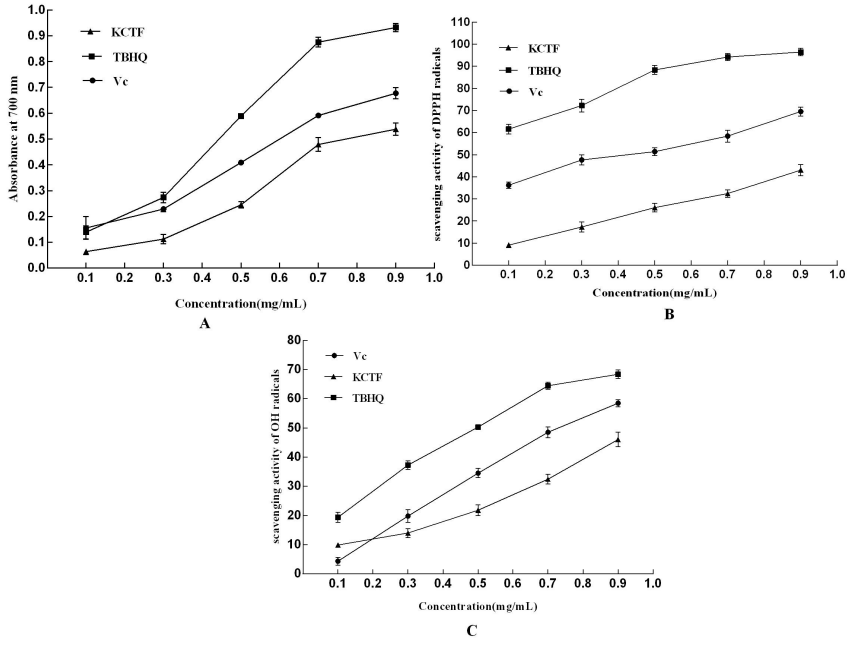


Fig. 2. Antioxidant activity of KCTF in vitro. A, Reducing power of KCTF; B, DPPH• radical scavenging activity of KCTF; C, Hydroxyl radical (•OH) scavenging activity of KCTF

Antioxidant activity in vivo

The influence of KCTF on mice body weight was shown in Table 1. There was no significant difference ($p > 0.05$) of mice final body weight among all the groups. The results implied that the concentration of KCTF had little effect on the weight of mice and its weight gain. Superoxide dismutase (SOD) is the main antioxidant enzyme of removing free radicals that are generated during metabolic processes. Malondialdehyde (MDA) is metabolic parameters of lipid peroxidation, which leads to destruction of cell function. As shown in Tables 2 and 3, the serum SOD content of MD and HD groups was significantly ($P < 0.05$) or extremely significantly ($P < 0.01$) higher than that of NC group while LD group of KCTF had no significant difference. However, all three different doses could significantly improve liver homogenate SOD activities ($P < 0.01$ and $P < 0.05$) and the effect of positive group (NC^+) were equivalent to that of the HD group. Thus, our results showed that KCTF could improve SOD activity in mice and reflected an obvious dose-dependent effect. Various dose groups of KCTF all significantly decreased MDA content both in serum and in liver homogenate. Particularly, both MD group and HD group significantly decreased MDA content compared with NC group ($P < 0.01$). The observations indicated that KCTF could decrease the MDA content in mice in a dose-dependent manner.

Table 1 Body weight change of mice before and after gastric perfusion

Groups	Number of samples	Gavage in a dosage (mg/(kg BW day))	Weight before gastric perfusion (g)	Weight after gastric perfusion (g)	Added value of weight (g)
NC	10	–	19.36±0.45	30.98±0.53	11.62±0.09
NC^+	10	150	20.70±0.27	32.82±0.64	12.12±0.37
KCTF/LD	10	100	18.98±0.35	30.43±0.76	11.45±0.41
KCTF/MD	10	300	19.61±0.76	31.36±0.56	11.74±0.80
KCTF/HD	10	500	18.73±0.33	30.99±0.47	12.33±0.14

Table 2 Effects of KCTF on MDA and SOD of mouse serum

Groups	Number of samples	Gavage in a dosage (mg/(kg BW day))	Superoxide dismutase (SOD) U/mL	Malondialdehyde(MDA) nmol/mL
NC	10	–	129.67±10.23	11.47±2.67
NC^+	10	150	142.42±14.09**	9.05±1.07*
KCTF/LD	10	100	134.31±12.47	9.52±1.24*
KCTF/MD	10	300	140.21±8.76*	6.18±1.45**
KCTF/HD	10	500	149.43±11.98**	5.06±2.22**

Note: compared with control group: * $p < 0.05$, ** $p < 0.01$.

Table 3 Effects of KCTF on MDA and SOD of mouse liver homogenate

Groups	Number of samples	Gavage in a dosage (mg/(kg BW day))	Superoxide dismutase (SOD) U/mL	Malondialdehyde(MDA) nmol/mL
NC	10	–	141.62±22.30	6.41±0.67
NC^+	10	150	158.02±18.34**	4.05±0.43**
KCTF/LD	10	100	152.78±19.45*	5.79±0.25
KCTF/MD	10	300	156.21±8.76*	4.18±0.65**
KCTF/HD	10	500	165.14±12.32**	3.06±0.58**

Note: compared with control group: * $p < 0.05$, ** $p < 0.01$.

In summary, the above results of experiments in vitro and in vivo proved that KCTF had antioxidant activity. Overall, KCTF could help to strengthen the free radical scavenging ability in mice and could be used as a potential antioxidant agent.

Antibacterial activity of KCTF

Table 4 shows that the KCTF had good inhibitory effect on bacteria, especially on the Gram positive bacteria, which is the same as a previous study carried on *Artemisia rupestris L* flavonoids by Fang *et al.* (M. Z. Fang et al., 2010). Results showed that the diameters of inhibition zone of KCTF (5.00 mg/mL) against *Bacillus subtilis* and *Staphylococ*cus aureus were

16.40±0.02 mm and 15.52±0.09 mm, respectively, while the MIC value of them was at the same value of 0.31 mg/mL. Furthermore, yeasts and molds are less susceptible than bacteria to treatment by KCTF. The above results clearly show that KCTF is a potential antibacterial agent. Therefore, the mechanisms involved in antibacterial activity of KCTF are worthy of further investigation.

Table 4 The diameter(mm) of the inhibition zones of different samples and the MIC value of KCTF

	Escherichia coli.	*Staphylococcus aureus*	Bacillus subtilis	Penicillium	*Aspergillus niger*	*Saccharomyces cerevisiae*
KCTF (5.00 mg/mL)	14.74± 0.54	15.52± 0.09	16.40± 0.02	10.57± 0.15	11.08± 0.10	11.27± 0.02
Ethyl alcohol (95%)	9.80±0.09	10.00±0.12	10.50±0.09	9.52±0.08	10.21±0.05	9.53±0.07
Aseptic saline	0	0	0	0	0	0
0.03% potassium sorbate	10.17±0.09	10.12±0.11	10.03±0.11	9.57±0.04	10.25±0.11	9.89±0.05
MIC value* **KCTF(mg/mL)**	0.63	0.31	0.31	5	2.5	1.25

Note：The diameter of the inhibition zones are mean±SD (mm) from the experiments in triplicate. The MIC value* of KCTF is expressed in mg/mL.

Effect of KCTF on CCl_4-induced acute liver injury in mice

The effect of KCTF on weight of mice and the effect of KCTF on the acute liver injury induced by CCl_4 in mice was given in Tables 5 and 6, respectively.

It was indicated in Table 5 that the final body weight of mice in all the other groups had no significant differences compared with normal group ($p > 0.05$). The results indicated that KCTF had small impact on the weight of mice and their weight gain. As is shown in Table 6 that ALT and AST activities in model group showed extremely significantly different ($p<0.01$) when compared with blank control group, indicating that the model was successfully made. However, the results showed that there was no obvious protective effect of different doses of KCTF on

acute liver injury induced by CCl_4. Furthermore, after comparison of the effect on liver protection of total flavonoids, Chrysanthemum and polysaccharides extracted from *Kunlun Chrysanthemum*, we find that only high does group of *Kunlun Chrysanthemum* Chrysanthemum revealed significant protective effect on acute liver injury induced by CCl_4 (unpublished).

Table 5 The effects of KCTF on body weight of mice that had acute liver damage (x ± s, n=10)

Groups	Dose (g/kg)	Initial Weight (g)	Final weight (g)
Blank		9.7±1.5	25.8±3.3
Model		20.0±1.4	25.4±2.0
Positive	0.40	20.0±1.6	23.2±1.7
KCTF/LD	0.10	20.0±1.6	23.2±2.0
KCTF/MD	0.20	19.6±1.1	23.6±1.4
KCTF/HD	0.40	20.2±1.9	23.7±3.0

Table 6 Effects of KCTF on AST and ALT activities in mouse serum with liver injury induced by CCl4 (x ± s, n=10)

Groups	Dose (g/kg)	AST (u/L)	ALT (u/L)	Liver coefficient (%)
Blank		112.63±22.20	62.52±14.46	4.89±0.40
Model		279.16±181.57**	293.66±212.73**	5.07±0.27
Positive	0.4	188.08±60.98	235.12±193.03	5.20±0.70
KCTF/LD	0.1	343.17±22.63	464.46±269.24	4.73±0.41#
KCTF/MD	0.2	348.27±227.94	357.27±159.51	5.02±0.29
KCTF/HD	0.4	412.20±209.57	445.49±169.80	5.05±0.35

Note: compared with blank group: ** $p < 0.01$, compare with model group; # $p < 0.05$.

Antiproliferative activity of KCTF

Effect of KCTF on proliferation of Hela cells, Eca-109, and Vero cells

HeLa cells, Eca-109 cells and Vero cells were incubated with different concentrations (12.5, 25, 50, 100, 200, 400 and 800 μg/mL) of KCTF for a certain time (12, 24, 48, and 72 h) and then were measured by MTT assay (Fig. 3). KCTF could inhibit the proliferation of HeLa cells and Eca-109 cells with average IC_{50} values of 133.6±0.1885 μg/mL, 192.0±0.07719 μg/mL, respectively. The inhibitory effect of KCTF on Hela cells and Eca-109 cells showed a significant increasing trend with the increase of the concentration and time while the inhibitory effect on Vero cells was weak. The inhibitory rate on Vero cells was only 21.68% after incubation for 72 h at a concentration of 400 μg/mL. Compared with untreated control cells, KCTF had no significant immediate effect on cell viability when applied at low concentrations (12.5-100 μg/mL) to Hela cells. KCTF could specifically inhibit the growth of Hela cells from 100 to 800 μg/mL ($P < 0.01$) and the inhibitory rate of 800 μg/mL dosage was 91.27% at 48 h similar to 92.16% at 72 h. At the concentration of 800 μg/mL at 72 h, the inhibitory rate of Eca-109 cells was 66.38%. KCTF exhibited significant proliferation inhibition effect on HeLa cells and Eca-109 cells..

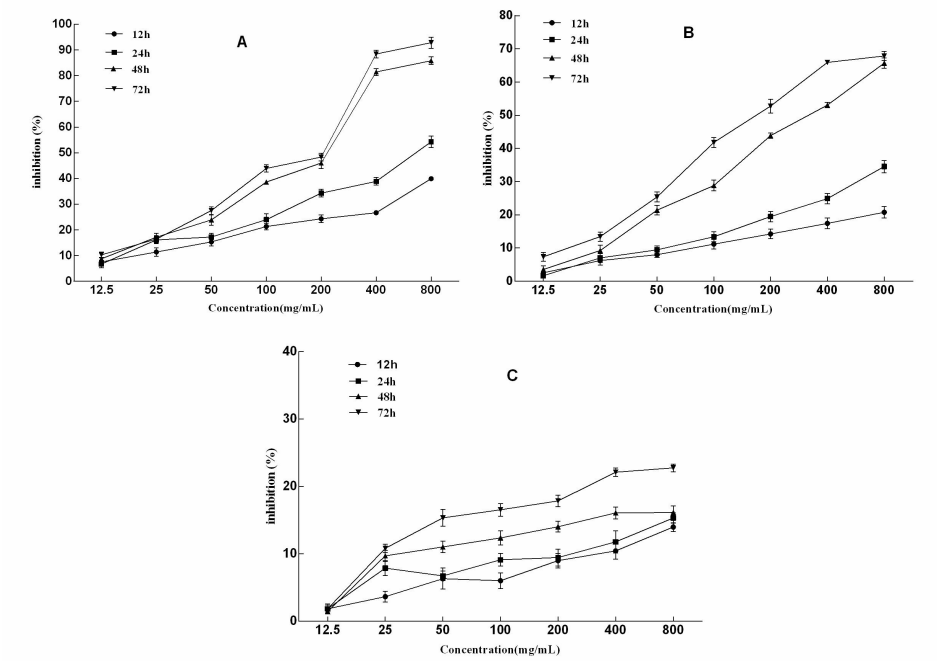
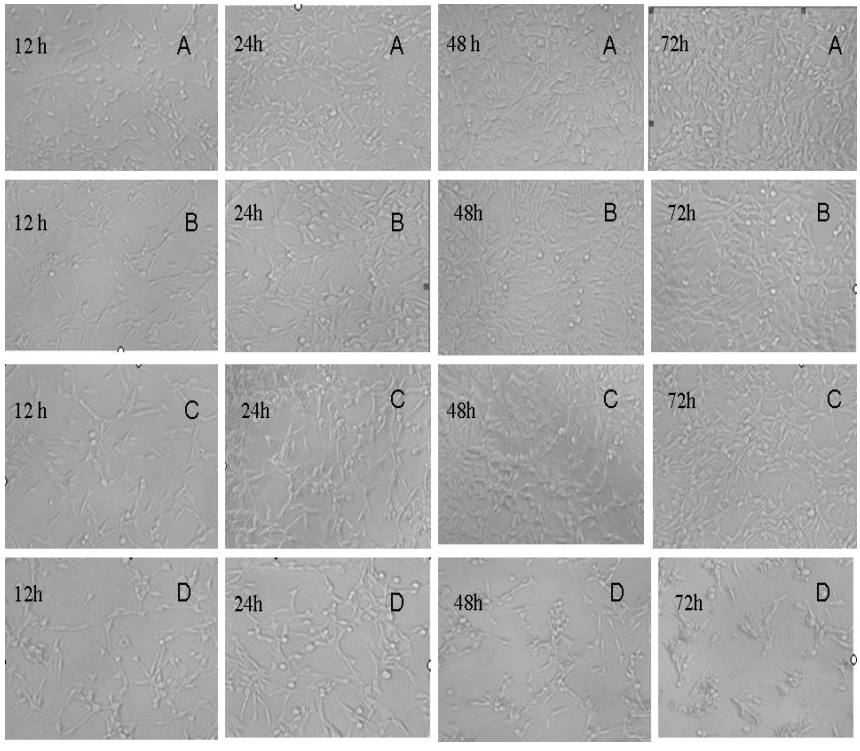


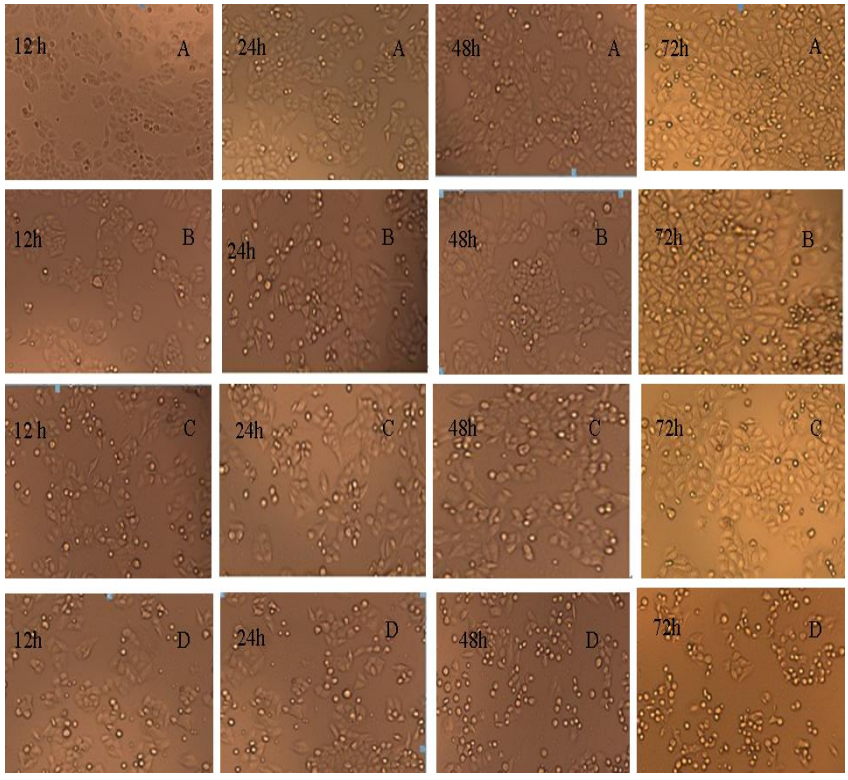
Fig.3. Antiproliferative effect of KCTF on tumor cells and Vero cells: (A) time curve of different concentrations of KCTF against HeLa cells proliferation, (B) time curve of different concentrations of KCTF against Eca-10 cells proliferation, (C) time curve of different concentrations of KCTF against Vero cells proliferation

Influence of KCTF on Hela cells, Eca-109 cells and Vero Cells morphology

The morphology changes of Hela cells, Eca-109 cells and Vero Cells treated with KCTF were observed by phase contrast inverted microscope (Fig. 4). The control groups (untreated with KCTF) of Hela cells and Eca-109 cells grew against the wall of flask and formed a monolayer with regular polygons morphology. But after incubation with KCTF at different concentration, the number of the cells significantly decreased and morphology changed. Cells became shrinkage and turned round, arranged loosely and with gradual ill adherence. However, KCTF has little effect on the cell morphology and the number of Vero cells after treatment with KCTF for 72 h. The Vero cells yielded a slight change, and small portion of which became round cells. Results illustrated that KCTF had antiproliferative effect against Hela cells and Eca-109 cells with little cytotoxic effect on normal Vero cells.



(1)



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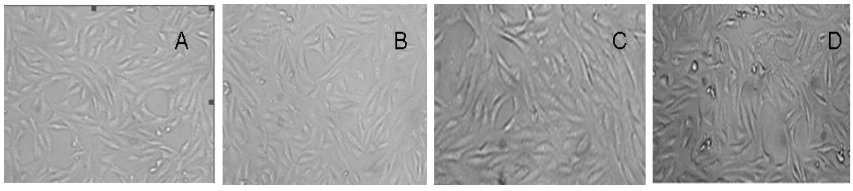

(3)

Fig. 4 Effect of KCTF on cells morphology at different concentrations and treat time (× 200). (1) Hela calls; (2) Eca-109 calls; (3) Vero cells (72 h). A, control; B, 12.5 μg/mL; C, 100 μg/mL; D, 800 μg/mL.

Effect of KCTF on Hela cell apoptosis

Apoptosis, also known as programmed cell death, is a vital physiological process that removes cells at the appropriate time in order to better control the number of cells in development throughout the life of an organism (Yao et al., 2010). It is a strict regulatory pathway responsible for the order to remove the superfluous, elderly, and damaged cells(Goldar, Khaniani, Derakhshan, & Baradaran, 2015). The relationship between apoptosis and cancer has been stressed, and more evidence indicate that the relative processes of tumor transformation,

progression and metastasis involve the changes in normal apoptosis pathways (C. L. Zhang, Li-Jun, Shin-Ichi, Satoshi, & Takashi, 2003). It was found that many tumor chemotherapeutic drugs play an anticancer effect on malignant cells by inducing apoptosis (Sun, Luo, & Zhang, 2011). The apoptosis inducing activities of KCTF on Hela cells were investigated through Hoechst 33258 staining assay. The nuclei of live cells treated with Hoechst 33258 expressed uniformly light blue under the observation of fluorescence microscope. Apoptotic cells had bright blue nuclei because of karyopyknosis and chromatin condensation while the nuclei of dead cells could not be stained. Hela cells treated with KCTF at 256.28 ± 1.08 μg/mL for 48 h were stained with Hoechst 33258. Compared with the normal blue of control group, the nuclei of Hela cells appeared to be highly condensed and the granular fluorescence intensity was high, indicating that KCTF could induce apoptosis in Hela cells (Fig. 5).

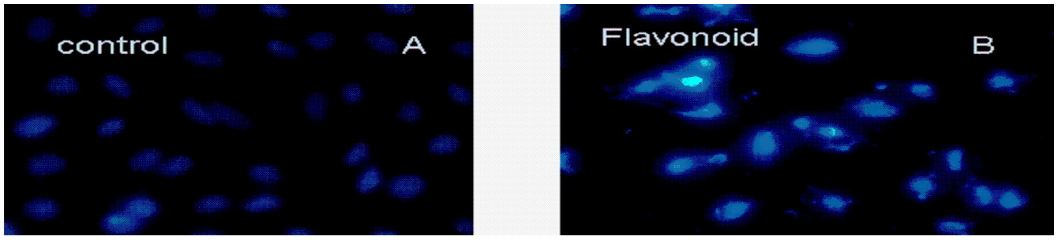

Fig.5 KCTF induced HeLa cell apoptosis was examined by Hoechst 33258 staining. Morphological change of Hela cells observed under an inverted phase contrast microscope (200 ×). A, control; B, after treatment with 256.28 ± 1.08 μg/mL KCTF for 48 h according to IC_{50} Value.

Effect of KCTF on LDH activity

Lactate dehydrogenase (LDH) can catalyze lactate acid to pyruvic acid which can be detected by kit. As seen from Fig. 6, under the dosage of 800 μg/mL KCTF, the number of necrotic cells is more than those of apoptotic cells in Hela cells at 0~12 h, and apoptotic cells significantly increased at 24 h. The result showed that KCTF could induce apoptosis on Hela cells in a suitable time.

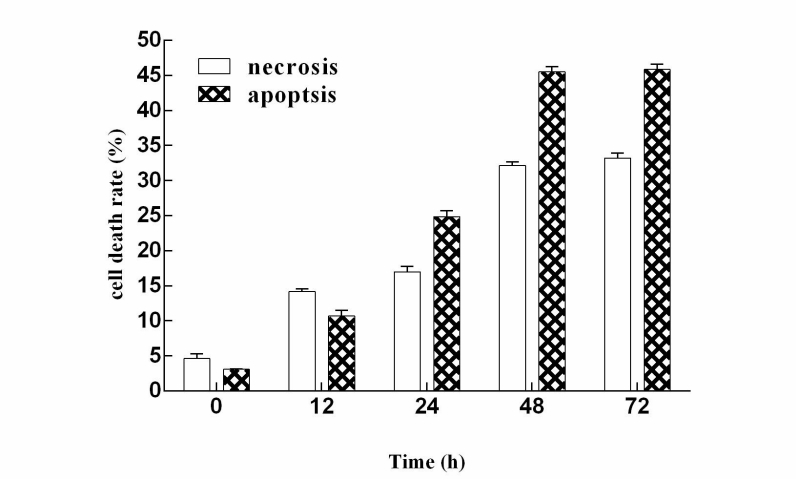

Fig. 6. Cell death assessed by LDH activity was expressed as percentage of apoptosis

Thus, the results showed that KCTF had antiproliferative activity, which could inhibit the growth of HeLa cells and Eca-109 cells but had little cytotoxic effect on Vero cells.

Conclusion

In this study, KCTF was extracted by UHP method and the content of total flavonoids reached 52.43% after purification. The bioactivities results indicated that KCTF had remarkable antioxidant activity in vitro and in vivo. In vitro antioxidant experiments showed that the reducing power and the scavenging capacity of ·OH and DPPH· increased in a concentration-dependent manner and in vivo KCTF inhibited MDA formation while it enhanced the activities of SOD in mice. In addition, KCTF inhibited the growth of both Gram positive and Gram negative bacteria by the disc diffusion method while there was no significant protective effect on acute liver injury induced by CCl_4 in mice. Furthermore, the results of MTT assay exhibited that KCTF had pronounced antiproliferative activity in a dose- and time-dependent manner, which inhibited the growth of HeLa cells and Eca-109 cells at low concentrations with average IC_{50} values of 133.6±0.1885 μg/mL, 192.0±0.07719 μg/mL, respectively. It was observed that there was little effect on the morphology and proliferation of normal Vero cells while the morphology of HeLa cells and Eca-109 cells changed and the apoptotic cells displayed condensed nuclei via Hoechst 33258 staining. LDH activity indicated that KCTF could induce apoptosis on Hela cells in a suitable time. In conclusion, the present study suggests that KCTF has a potent antioxidant activity, antibacterial prosperity and antiproliferative effect against

tumor cells and can be utilized as a novel natural health-promoting bioactive constituent in functional food industry. Further investigations in terms of structure of KCTF and mechanisms of action are in progress.

Acknowledgements

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