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Original Research Article

In vitro antioxidant, antibacterial and anti-tumor activities of total flavonoids from *Elsholtzia densa* Benth

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Abstract

Purpose: *To investigate the in vitro antioxidant, antibacterial and anti-tumor activities of total flavonoids from Elsholtzia densa Benth of Sichuan Province, China.*
Methods: *The total flavonoids of Elsholtzia densa Bent were extracted utilizing the ultrasonic extraction method, and purified by D101 macroporous adsorption resin. An in vitro antioxidant test, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay and iCELLigence system were used to evaluate their antioxidant, antibacterial and anti-tumor activities.*
Results: *The results showed that the total flavonoids exhibited good scavenging ability in hydroxyl radical (•OH), DPPH free radical (DPPH•), and super oxide anion radical (O_2^-•). Antioxidant activity was higher than for control (ascorbic acid). Their antibacterial activity was good with minimum inhibitory concentration (MIC) of 2, 4 and 14 µg/mL against Escherichia coli, Staphylococcus aureus and Bacillus subtilis, respectively. Anti-proliferation data from the iCELLigence system studies showed that the total flavonoids significantly inhibited the growth of five types of cells, including a normal human hepatocytes cell line (L02), two human hepatocellular carcinoma cell line (SMMC-7721 and HepG-2), a human cervical cancer cell line (Hela) and a Baby Hamster Syrian Kidney cell line (BHK-21) ($p < 0.05$). AO/EB staining indicate that the total flavonoids might cause apoptosis of Hela cells.*
Conclusion: *The results suggest that the total flavonoids from Elsholtzia densa Bent are potential natural antioxidants and antimicrobial agent, with anti-cancer properties.*

Keywords: *Elsholtzia densa Benth., Total flavonoids, Antioxidant activity, Antibacterial activity, Anti-tumor activity*

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INTRODUCTION

Elsholtiza densa Benth. is a Labiatae herb of the genus *Elsholtzia* [1], also known as cough grass, wild basil, and smelly *Elsholtzia* [2,3]. It is a very important nectar plant that produces a very high volume of nectar [4]. It is widely distributed in the provinces of Shanxi, Sichuan, Qinghai, Tibet, Gansu and other places in China, found at 1800 ~ 4100 m high on the edges of forest, on mountains, in meadows, in rivers, on hillsides, and in wastelands [5,6]. It has been used as a

therapeutic agent for the treatment of tuberculosis-injury paralysis, hematemesis, influenza, epidemic toxin, summer cold, fever, heat stroke, acute gastritis, and other kind of diseases [7,8], as will as used as spices and in tea.

Flavonoids are known for their antimicrobial, antioxidant, analgesic, antiseptic, and anti-inflammatory activities [9,10]. It has potential benefits in anti-tumor therapy, due to its availability and low toxicity. Investigating potential effective treatment of various cancers via systematic screening of a variety of flavonoids-based natural products has attracted much attention over the past few decades [11].

Elsholtiza densa Benth. from the Sichuan-Tibetan Plateau was used in this study. Total flavonoids from *Elsholtiza densa* Benth. were extracted using an ultrasonic extraction method.

An in *vitro* antioxidant test and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay were used to evaluate its antioxidant and antibacterial effects. The iCELLigence system was used to evaluate *Elsholtiza densa* Benth.'s anti-tumor activity on five different cells.

EXPERIMENTAL

Materials

Elsholtiza densa Benth. whole plant was gathered at an altitude of 3504 m in a Hongyuan County of Sichuan Province, China alpine meadow in July of 2015 and identified by Professor Danwei Ma (Sichuan Normal University, China). A voucher specimen (no. *E.dense*-2015-7) was deposited in the herbarium of College of Life Science, Sichuan Normal University, Chengdu, China. *Elsholtiza densa* Benth. was dried in a drying mechanism and then ground into a powder.

Isolation of flavone extract

Total flavonoids from *Elsholtiza densa* Benth. whole plants were extracted using an ultrasonic extraction method which was repeated three times. Thirty-gram of *Elsholtiza densa* Benth. dry powder was mixed with 50 % ethanol at the ratio of 1 : 30 (w/v) extracted via an ultrasonics process for 30 min. The residue was collected after filtration. The filtrate residue (total flavonoids) was collected via a rotary evaporator (Rong Rong biochemical instrument equipment Co., Ltd., Shanghai, China).

Purification of total flavonoids

D101 macroporous adsorption resin (Source leaf Biotechnology Co., Ltd., Shanghai, China) was washed three times with distilled water, and then washed with 1:3 (ethanol : water, v/v) 95 % ethanol solution several times until no white turbidity remained. The resin was soaked in 2 % hydrochloric acid for 3 h and then rinsed with distilled water. It was soaked in 5 % NaOH solution for 3 h and then rinsed with distilled water [12].

Five grams of flavone extract was dissolved in 500 mL of distilled water in a chromatography column and mixed with 50 % ethanol solution to collect the elution solution. It was extracted again via rotary evaporator. The extract was placed in a conical flask, sealed with gauze, and vacuum-dried for 1 h to collect the powder. The extract yield was calculated as a percentage of the whole grass powder.

Evaluation of scavenging activity of hydroxyl free radical (•OH)

The reaction mixture contained 200 µL of 1.8 mol/mL $FeSO_4$, 150 µL of 1.8 mol/mL salicylic acid-ethanol solution, 10 µL of 0.03 % H_2O_2, and 140 µL of total flavonoids solution. The reaction was started by adding H_2O_2. After incubation at 37 ℃ for 30 min, the absorbance value (A) was measured at 510 nm. Distilled water was used as the blank control (A_0), and ascorbic acid was used as the positive control in the sample solution. Scavenging activity (S) of •OH was calculated using Eq 1.

$$S\ (\%) = \{(A_0\text{-}A)/A_0\}100 \quad \text{..... (1)}$$

Determination of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity

The gradient solution of total flavonoids (0.01 mg/mL, 0.02 mg.mL, 0.03 mg/mL, 0.04 mg/mL, 0.05 mg/mL and 0.06 mg/mL) were prepared in a 25 % ethanol solution and mixed with 400 µL of 1 mol/L DPPH solution (Magnolia Town Industrial Development Zone, Xindu, Chengdu, China). The reaction mixture was incubated at 37 ℃ for 30 min with the absorbance value (A) at 517 nm. 95 % ethanol solution was used as the blank control (A_0), and ascorbic acid was used as the positive control. Scavenging activity of DPPH was calculated using Eq 2.

$$S\ (\%) = \{(A_0\text{-}A)/A_0\}100 \quad \text{..... (2)}$$

Determination of superoxide anion radical (O_2^{-}•) scavenging activity

A series of 150 µL total flavonoid solutions of different concentrations (ranging from 0.18 mg/mL to 0.26 mg/mL) mixed with 500 µL of a 0.1 mol/L Tris - HCl buffer solution (pH = 8.2) was heated at 25 ℃ for 20 min, and reacted with 300 µL of 3 mmol/L pyrogallol solution (Magnolia Town Industrial Development Zone, Xindu, Chengdu, China) which was dissolved in 10 mmol/L hydrochloric acid for 9 min. The reaction was terminated with 1 mL concentrated hydrochloric acid solution with the absorbance value (A) at 320 nm. The absorbance value of 3 mmol/mL pyrogallol solution was measured as A_x. Double distilled water was used as the blank control (A_0), and ascorbic acid was used as the positive control in the sample solution. Scavenging activity of O_2^{-}• was as in Eq 3.

$$S (\%) = \{(A_0-A-A_x)/A_0\}100b \quad (3)$$

Assessment of antibacterial activity

In vitro antibacterial activities of total flavonoids from *Elsholtiza densa* Benth. were qualitatively and quantitatively assessed using the minimum inhibitory concentration (MIC) values. *Escherichia coli (E. coli), Staphylococcus aureus (S. Aureus)* and *Bacillus subtilis* were grown in LB medium at 37 ℃. Antibacterial activity was determined by MTT (Sigma, U.S.) assay using the 96-well plate method [13]. The positive control was treatment with 1 mg/mL of Ampicillin solution.

Cell culture

Normal human hepatocyte cell line L02, human hepatocellular carcinoma cell line SMMC-7721 and HepG-2, human cervical cancer cell line Hela and baby hamster syrian kidney cell line BHK-21 (State Key Laboratory of Biotherapy, Sichuan University West China Hospital, Chengdu, China) were grown as monolayers in a RPMI-1640 medium (Harry Bioengineering Co., Ltd, Chengdu, China) supplemented with 10 % fetal bovine serum (Harry Bioengineering Co., Ltd, Chengdu, China) and 1 % antibiotics containing 100 µg/mL of streptomycin and 100 U/mL of penicillin. The cells were incubated at 37 ℃ with 5 % CO_2 during the log growth phase.

Anti-proliferation assay using iCELLigence system

After cells were seeded at the determined concentration in 250 µL of the medium in E-plate 8, proliferation was monitored every 1 h by the iCELLigence system (Essen biological Co. Ltd, Hangzhou, China). Approximately 24 h after seeding, while the five kinds of cells (L02 cells, SMMC-7721 cells, HepG-2 cells, Hela cells and BHK-21 cells) were in the log growth phase, the cells were exposed to 400 µL of medium containing six different concentrations of total flavonoids (0, 0.5, 1, 1.5, 2 and 2.5 mg/mL). Cells grown in either medium or medium+25% ethanol solution were negative controls, and cells grown in 5-fluorouracil (5-FU) were positive controls. All experiments lasted for 96 h.

Examination of cell morphology by inverted microscopy

Hela cells were seeded in a 6-well plate at the density of 3×10^5 /mL. After 24 h incubation (37 ℃ with 5 % CO_2), cells were cultured in 2 mL of RPMI-1640 medium containing total flavonoids from *Elsholtiza densa* Benth. at concentrations of 0.5, 1.5 and 2.5 mg/mL. The negative control used 25 % ethanol solution. After 48 h incubation, cell morphology was observed and photographed under inverted phase contrast microscopy (Leica, Germany). The cells were then suspended for the next experiment.

Assessment of apoptosis by fluorescence microscopy

A 50 µL cell suspension was mixed with 2 µL of AO/EB solution (100 µg/ml AO and 100 µg/mL EB). Twelve µL was taken and placed on a clean glass slide to examine and image the progress of cell apoptosis under inverted phase contrast fluorescence microscopy (Leica, Germany). After AO/EB double staining, the living cells (green), viable apoptotic cells (green), non-viable apoptotic cells (red) and non-viable non-apoptotic cells (red) were observed via fluorescence microscopy.

Statistical analysis

The data were analyzed using SPSS17.0 (SPSS Inc., USA). Significant differences were analyzed using Least-Significant Difference (*LSD*) test. $P < 0.05$ was considered statistically significant. Relevance were analyzed using the double variable method. All data are presented as mean ± standard deviation (SD).

RESULTS

The yield of the total flavonoids

A total of 0.826 g of flavonoids were isolated from 30 g of *Elsholtzia densa* Benth. powder with a yield of 2.75 %.

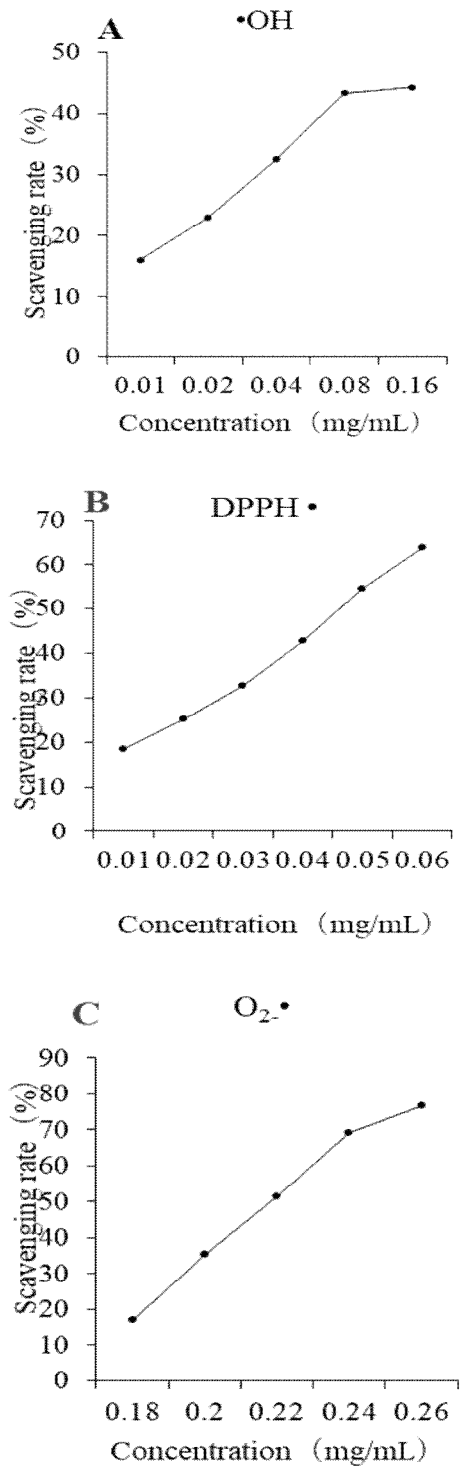


Figure 1: Scavenging rates of total flavonoids from *Elsholtzia densa* Benth. on hydroxyl free radical (A), DPPH radical (B), superoxide anion radical (C)

Antioxidant activity of total flavonoids

Figure 1 presents the scavenging rates of the total flavonoids from *Elsholtzia densa* Benth. on •OH (Figure 1A), DPPH • (Figure 1B) and O_2-• (Figure 1C). A total flavonoids had concentration-dependent scavenging ability on •OH, DPPH• and O_2-•. The IC_{50} value on •OH was 0.183 mg/mL, p = 0.061, r = 0.861. The IC_{50} value on DPPH• was 0.046 mg/mL, P = 0.000, r = 0.995. The IC_{50} value on O_2-• was 0.218 mg/mL, P = 0.001, r = 0.992. The IC_{50} values of ascorbic acid on the three kinds of free radicals were 0.072 mg/mL, 0.008 mg/mL and 1.166 mg/mL, respectively. Therefore, the scavenging rate of total flavonoids on •OH and DPPH• was lower than that of ascorbic acid, and the scavenging rate of O_2-• was much higher than that of ascorbic acid.

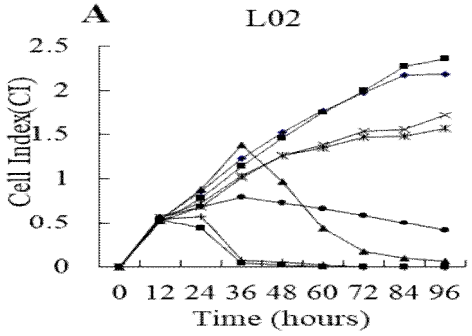
Antibacterial activity of total flavonoids

Total flavonoids from *Elsholtzia densa* Benth. were found to have antibacterial activity. It demonstrated significant inhibition against *E. coli* and *S. Aureus* with MIC values of 2 and 4 μg/mL, which was much higher than that of Ampicillin (*P < 0.05)*. The MIC value of total flavonoids on *Bacillus subtilis* was 14 μg/mL.

Effect of total flavonoids on cell proliferation

In this study, the iCELLigence system are used to evaluate the effect of total flavonoids from *Elsholtzia densa* Benth. on the proliferation of tumor cells. Figure 2 shows that the total flavonoids significantly inhibited the growth of five kinds of cells depending on its concentration level. Positive control (five different cells treated with 80 μg/mL of 5-FU) showed growth inhibition.

The inhibitory effect of total flavonoids was stronger than the positive control in all five kinds of cells in certain concentrations of total flavonoids (greater than or equal to 1.5 mg/mL). When the concentration was lower than 1.5 mg/mL, the inhibitory effect of total flavonoids was still higher than the positive control in HepG-2 cells, Hela cells and BHK-21 cells, whereas it was lower in L02 cells and SMMC-7721 cells.



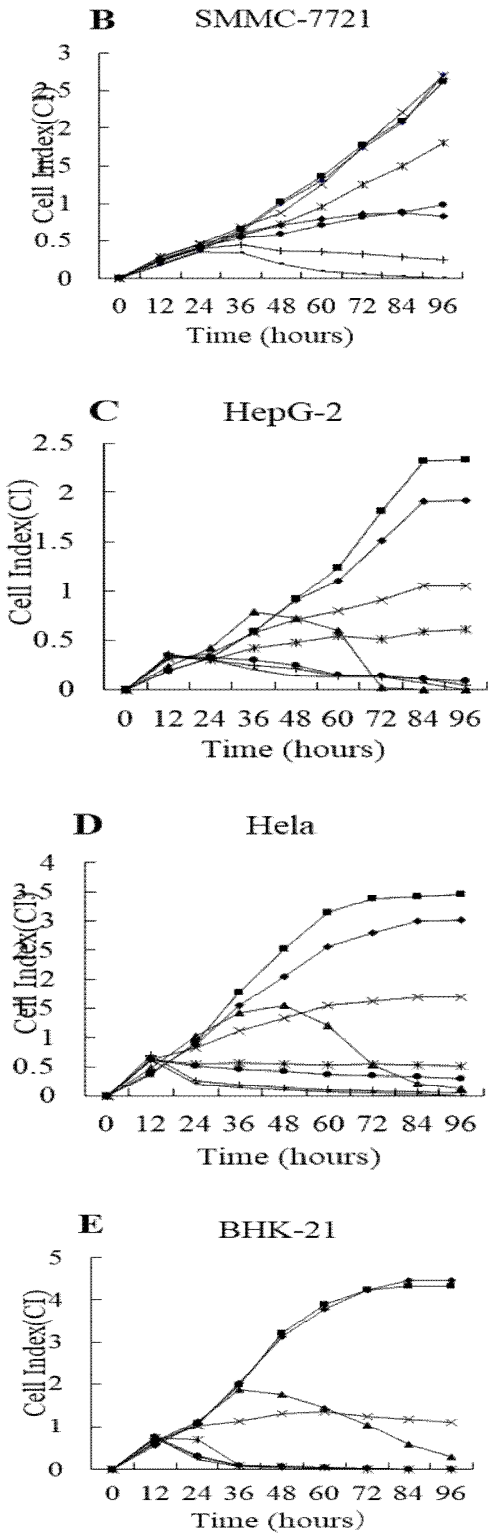


Figure 2: Effect of total flavonoids from *Elsholtzia densa* Benth. on the proliferation of L02 cells (A), SMMC-7721 cells (B), HepG-2 cells (C), Hela cells (D) and BHK-21 cells (E) during 0-96 h treatment in the iCELLigence assay. Time and dose dependent effect of total flavonoids on the proliferation of L02 cells, SMMC-7721 cells, HepG-2 cells, Hela cells, and BHK-21 cells was observed.

Effect of total flavonoids on the morphology Hela cells

Figure 3 shows the morphological changes in Hela cells after 48 h of treatment with total flavonoids from *Elsholtzia densa* Benth. The control group of Hela cells had square aggregate growth, cell adhesion, high refractive index, and apparent split phase (Figure 3 a). During the total flavonoids low-concentration treatment (0.5 mg/mL) procedure, a small number of cells had a circular shape and cell adhesion was decreased (Figure 3 b). When the total flavonoids concentration was 1.5 mg/mL, most cells became round and wrinkled (Figure 3 c). At the highest concentration (2.5 mg/mL), all the cells became round, and the shrinkage phenomenon was obvious. The total flavonoids demonstrated significant cytotoxicity and caused cell apoptosis at the highest concentration (Figure 3 d).

Effect of total flavonoids on *a*poptosis of Hela cells

Figure 4 shows that the apoptosis of Hela cells by 48 h treatment with the total flavonoids from *Elsholtzia densa* Benth. at concentrations of 0.5, 1.5 and 2.5 mg/mL. Compared with the negative control group, the living cells (VNA), viable apoptotic cells (VA), non-viable apoptotic cells (NVA), and non-viable non-apoptotic cells (NVNA) were found in Hela cells treated with the total flavonoids from *Elsholtzia densa* Benth. at concentrations of 0.5, 1.5 and 2.5 mg/mL. The number of non-viable apoptotic cells (NVA) increased as the total flavonoids concentration increased.

DISCUSSION

As reported by other researchers, human cardiovascular diseases, cerebrovascular diseases, cancer, Alzheimer's disease, and paralysis might be related to the presence of oxygen free radicals (ROS) [15]. Natural flavonoids extracted from many plants species have obvious antioxidant and antibacterial activities. The methanol extract from *Teucrium polium* L. yielded rutin and apigenin, which were found to be the most active fractions as radical-scavengers [16]. Scavenging activity of •OH and O_2-•, total antioxidant activity, iron chelating activity, and reducing power of the total flavonoids from *Diospyros kaki* L. leaves were significantly higher than that of rutin [17]. The flavones from chestnut flower exhibited antibacterial activity against *E.coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* [18]. *E. coli*, *Saccharomycetes* and *Staphylococcus*

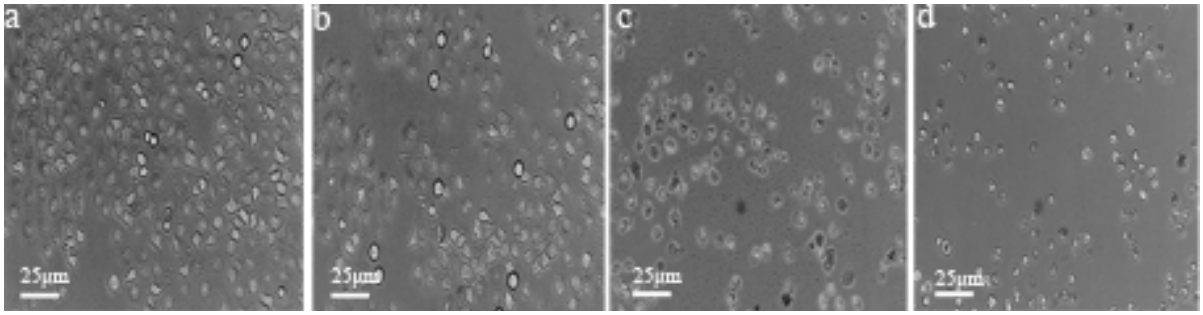


Figure 3: Effect of total flavonoids from *Elsholtzia densa* Benth. on the morphology of Hela cells. Morphological changes in Hela cells at 48 h treatment with 25 % ethanol solution (a), 0.5 mg/mL (b), 1.5 mg/mL (c) and 2.5 mg/mL (d) total flavonoids.

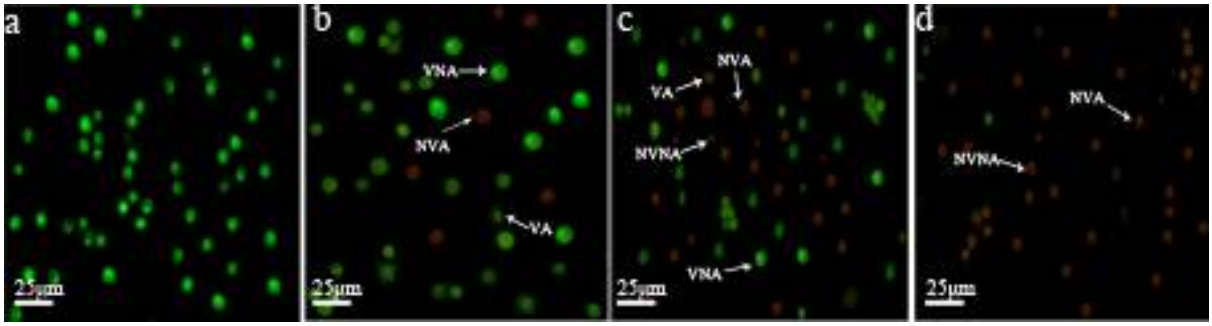


Figure 4: Morphological analysis of total flavonoids from *Elsholtzia densa* Benth. treated Hela cells by AO/EB dual staining. Induced apoptosis in Hela cells at 48 h treatment with 25 % ethanol solution (a), 0.5 mg/mL (b), 1.5 mg/mL (c) and 2.5 mg/mL (d) total flavonoids.

aureus were inhibited by the flavonoids from purslane [19].

The results of this study showed that total flavonoids from *Elsholtzia densa* Benth. had scavenging ability on •OH, DPPH•and O_2-• with the IC_{50} values were 0.183 mg/mL, 0.046 mg/mL, 0.218 mg/mL, respectively. Compared with ascorbic acid, the scavenging rate of total flavonoids on •OH and DPPH • was lower compared to the scavenging rate of ascorbic acid, but the scavenging rate of O_2-• was higher than ascorbic acid with an IC_{50} value of only 18.9 %. The total flavonoids from *Elsholtzia densa* Benth. was found to have antibacterial activity on *E. coli*, *S. Aureus* and *Bacillus subtilis*. It showed obvious inhibition on *E. coli* and *S. aureus* with MIC values that were 2 and 4 μg/mL. The MIC values on *Bacillus subtilis* was 14 μg/mL. The ranked inhibitory effect of total flavonoids on three kinds of bacteria was *E. coli* > *S. aureus* > *Bacillus subtilis*. According to the above results, the total flavonoids from *Elsholtzia densa* Benth. have great antioxidant and antibacterial activities and can be used as a natural antioxidant and antimicrobial drug.

Cancer is one of the most common diseases with high mortality rates. Prevention and treatment of cancer have attracted much attention over the past few decades [20]. In recent years, many studies have showed that flavonoids extracted from many plants exhibit anti-tumor effect. Flavonoids have low toxicity, great antioxidant activity, and may induce apoptosis of tumor cells. Therefore, the study of natural flavonoids as anti-tumor drugs has become a popular topic [11]. Casticin, penduletin, and 5-hydroxy-7,4′-dimethoxyflavone isolated from *Croton betulaster* Müll Arg. can inhibit the growth and viability of the human glioblastoma cell line GL-15 [21]. The flavonoids from *Dryopteris erythrosora* have shown obvious cytotoxic effects on the lung cancer cell line A549 [22], and flavonoids from Korean *Citrus aurantium* L. can induced apoptosis in cell line A549 [23]. In this study, the total flavonoids from *Elsholtzia densa* Benth. decreased CI values of L02 cells, SMMC-7721 cells, HepG-2 cells, Hela cells and BHK-21 cells in a concentration dependent manner ($p < 0.05$), and had a great inhibitory effect on Hela cells and BHK-21 cells. The inhibitory effect of 1.0 mg/mL total flavonoids was lower on normal cells (L02 cells) than on tumor cells (SMMC-7721 cells). Real-Time Cellular Analysis (RTCA) technology was used to obtain cellular physiological data by capturing image data throughout the entire time course. Total flavonoids induced morphological alterations, cell shrinkage, and chromatin condensation of Hela cells. Hela cells stained with AO/EB dual stain

showed that the number of apoptotic cells gradually increased as the concentration of total flavonoids increased. The above results show that total flavonoids from *Elsholtzia densa* Benth. have obvious cytotoxicity which can induce tumor cell apoptosis.

Studies have indicated that certain chemotherapeutic drugs can inhibit the proliferation of cancer cells and induce apoptosis by inducing oxidative stress as well as in normal cells. Cisplatin may change the redox status of cancer cells and normal cells, resulting in increased levels of ROS in the cell, which limits the clinical application of cisplatin [24]. It was found that the inhibitory effect on proliferation of osteosarcoma MG-63 cells was enhanced when a combination of cisplatin and emodin was applied, due to emodin offsetting the cisplatin-induced oxidative stress [25]. The flavonoids from *Dryopteris erythrosora* showed obvious cytotoxic effects on a lung cancer cell line (A549), and the anti-tumor activity was slightly increased with improving antioxidant potential of fern flavonoids [22]. Plant derived drugs are safer than synthetic medications, thus resulting in their wider utility. Therefore, drug therapy combined with medication in development of tumor, which has antioxidant activity and anti-tumor activity of *Elsholtzia densa* Benth., may exert stronger therapeutic properties.

CONCLUSION

A yield of 2.75 % of flavonoids were isolated from *Elsholtzia densa* Benth. using ultrasonic extraction. These flavonoids showed great scavenging ability on O_2-•, as well as significant inhibition of *E. coli* and *S. aureus.* It also induces apoptosis and demonstrates significant toxicity effects against tumor cells. Therefore, it is a potential anti-tumor and antibacterial drug.

The gastrointestinal polyps and associated symptoms disappeared after approximately 1 year of TCHM therapy without any complications during the follow-up. This case suggests that TCHM could play an important role in the treatment of gastrointestinal polyps. It may be a better choice for the patients who refuse surgery or cannot be surgically operated on, because the Chinese traditional medicine treatment of chronic gastritis and gastrointestinal polyps method is simple and less painful. Relevant data are however limited, and randomized controlled trials are still needed to confirm its efficacy in a larger population.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities regarding claims related to the content of this article will be borne by them.

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