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Original Article

Antioxidant and antitumor effects of *ferula sinkiangensis* K. M. Shen

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Abstract: Objective: This study is to investigate the antioxidant and antitumor effects of the extract fractions of the *Ferula sinkiangensis* K. M. Shen. Methods: Four different fractions of the *Ferula sinkiangensis* K. M. Shen were obtained by the extraction with petroleum ether, ethyl acetate, n-butanol, and methanol, respectively, which were used to treat the HCT116, Caco-2, HepG2, and MFC cells. Free radical scavenging effects of the ferula fractions were deteced with the DPPH assay. Effects of the ferula fractions on the peroliferatoin of the tumor cells were assessed with the SRB assay. Apoptosis was detected with flow cytometry. Results: The DPPH assay showed that the petroleum ether fraction hardly showed any antioxidant activity, while the ethyl acetate, n-butanol, and methanol fractions exhibited free radical-scavenging capacities, in a dose dependent manner. The SRB assay showed that, the proliferation of the tumor cells could be inhibited by the ferula fractions, in a dose dependent manner. However, differential effects were observed for the different fractions in different model cells. Particularly, the ethyl acetate fraction exerted the most efficient inhibiting effects on the tumor cell proliferation. In addition, the flow cytometry showed that, all the ferula fractions significantly enhanced the apoptotic process in the tumor cells, with differential enhancing capacities in different model cells. Conclusion: Extract fractions of the *Ferula sinkiangensis* K. M. Shen could exert antioxidant, proliferation-inhibiting, and apoptosis-enhancing effects in tumor cells. Particularly, the ethyl acetate fraction exhibits the most potent antioxidant and antitumor effects.

Keywords: *Ferula sinkiangensis* K. M. Shen, extract fractions, antioxidant effects, anti-tumor effects

Introduction

Ferula originates from the roots or resin of the ferulic species, which has long been used in the traditional medicine in China, India, and Iran [1-3]. The antitumor effects of various ferula extracts have been demonstrated in various models [4-6]. The galbanic acid extracted from the *Ferula assafoetida* resin could inhibit the tumor endothelial factors and exert lethal effects on tumors [4]. Moreover, the coumarin extracted from *Ferula diversivittata*, together with sesquiterpene lactones A, D, F, and G, could inhibit the formation of the skin cancer tumors in rats [5]. Furthermore, the umbelliprenin extracted from *Ferula szowitsian*a has been shown to induce the activity of apoptotic proteins, and inhibit the growth of human M4Beu metastatic melanoma cells [7]. *Ferula sinkiangensis* K. M. Shen belongs to the Umbelliferae family, which is the unique species in China, distributed only in Xinjiang [3]. Up to now, few studies have been reported on the antitumor effects of *Ferula sinkiangensis* K. M. Shen.

It has been well accepted that excessive reactive oxygen free radicals would oxidize and damage fats, proteins, and nucleic acids in the body, which might cause cancer, aging, and cardiovascular diseases [8]. On the other hand, the antioxidant defense system, including the SOD enzymes, vitamin C, and other exogenous antioxidants, could protect the body against the oxidative damages induced by these free radicals [9]. Ferula is rich in phenylpropanoid ferulic acid, coumarin, sesquiterpene coumarin, and other terpene lactones, most of which have potent antioxidant activities. The coumarin extracted from the *Ferula diversivittata* root could decline the hydrogen peroxide-induced DNA damages in human lymphocytes [10].

Moreover, the alcohol extract of the *Ferula gummosa* root has been shown to reduce the hemolysis induced by hydrogen peroxide in rats, with similar effects as vitamin C [10-12].

In this study, the antioxidant and antitumor effects of the extract frastions of *Ferula sinkiangensis* K. M. Shen were investigated. Four different extract fractions were first obtained according to the polarity. Antioxidant effects of these fractions were determined with the DPPH method, and their antitumor effects were investigated in various tumor cells.

Methods and materials

Cell lines and cell culture

Human colon cancer (HCT116) cells, human colon adenocarcinoma (Caco-2) cells, human hepatoma (HepG2) cells, and mouse forestomach cancer (MFC) cells, were purchased from the Cell Institute, Chinese Academy of Sciences, Shanghai, China. These cells were cultured with the RPMI1640 medium (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS; HyClone, Logan, Utah, USA), 100 U/mL penicillin and 100 mg/mL streptomycin, in a 5% CO_2, 37°C incubator.

Extract preparation

Totally 400 g *Ferula sinkiangensis* K. M. Shen resin (as confirmed by the Institute for Food and Drug Control, Yili, Xinjiang; Lot No. 20120004) was subjected to the extraction with 2000 mL petroleum ether (Fuyu Fine Chemical Co., Ltd., Tianjin, China) at 30-60°C for 40 min, followed by another extraction with 2000 mL petroleum ether at below 40°C for 40 min. After filtering, concentration, and drying at 60°C (until no bubbles formed), the petroleum ether fraction was obtained. The residue was then extracted with 2000 mL ethanol (95%), and concentrated under reduced pressure. The concentrated liquid cream was mixed with diatomite (Qingdao Haiyang Chemical Co., Ltd., Qingdao, Shandong, China), and then subjected to the subsequent ultrasonic extraction with ethyl acetate (Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China), n-butanol (Xi'an Chemical Reagent Factory, Xi'an, Shaanxi, China), and methanol (Xi'an Chemical Reagent Factory), respectively (5 min for each extraction). After filtering and drying, the ethyl acetate, n-butanol, and methanol fractions were obtained.

DPPH assay

The free radical scavenging effects were deteced with the 2,2-diphenylpicrylhydrazyl (DPPH) assay [13]. Each ferula fraction and VC were diluted to obtain the dilution series of 400, 200, 100, 50, 25, 12.5, and 6.2 ml/L. 50 μL dilute and 200 μL DPPH solution were added into 96-well plates. After mixing, the plate was incubated in dark for 30 min. Then the absorbance (A) at 515 nm was detected with a microplate reader. The control wells were added with 50 μL methanol and 200 μL DPPH solution, and the blank wells were added with 50 μL test sample and 250 μL methanol. The radical-scavenging rate was calculated according to the following formulation: Radical-scavenging rate (%) = $(A_{control}-A_{sample})/(A_{control}-A_{blank}) \times 100\%$.

SRB assay

The effects of the ferula fractions on the peroliferatoin of the tumor cells were assessed with the sulforhodamine B (SRB) assay [14]. The petroleum ether, ethyl acetate, n-butanol, and methanol fractions of the *Ferula sinkiangensis* K. M. Shen were diluted into 250, 125, 62.5, 31.2, and 15.6 mg/L, respectively. HCT116, Caco-2, HepG2, and MFC cells were planted onto the 96-well plates for 24 h, and then treated with the ferula fractions for another 24 h. The cells were fixed with 50% TCA solution. After washing, 0.4% SRB solution (Sigma-Aldrich, St. Louis, MO, USA) was added into each well. After staining for 30 min, the cells were washed with 1% acetic acid for four times. 150 μL Tris-base lye (10 mmol/L, pH 10.5) was added, followed by 10-min oscillation. The absorbance (A) at 515 nm was recorded. The cells in the control wells were not subjected to the drug administration, and the blank wells were added with medium alone. The tumor cell proliferation-inhibiting rate was calculated according to the following formulation: Proliferation-inhibiting rate (%) = $(A_{control}-A_{treatment})/(A_{control}-A_{blank}) \times 100\%$. The IC_{50} values were obtained with the Graphpad Prism 5 software.

Flow cytometry

Cellular apoptosis was detected by the flow cytometry [15-17]. HCT116, Caco-2, and HepG2

Table 1. Characterization of the polar fractions from *Ferula sinkiangensis* K. M. Shen

Polar fraction	Weight (g)	Yield (g/g)	Property
Petroleum ether fraction	51.41	0.12	Yellow oil droplets, strong garlic odor
Ethyl acetate fraction	60.81	0.15	Brown dry extract
N-butanol fraction	10.13	0.03	Brown black dry extract
Methanol fraction	14.14	0.04	Black dry extract

cells were planted onto the 6-well plates for 24 h, and then treated with the petroleum ether, ethyl acetate, n-butanol, and methanol fractions of *Ferula sinkiangensis* K. M. Shen, respectively, at the concentration of 62.5 mg/L, for 24 h. The cells were harvested and washed with PBS, and subjected to centrifugation at 4°C at 500 rpm for 5 min. After washing, the cells were stained with Annexin V-FITC (5 μL) at 4°C for 20 min, and then PI (10 μL) at 4°C for 5 min. The fluorescence was detected with the guava easyCyte 8-HT flow cytometer (Millipore Guava Technologies, Hayward, USA).

Statistical analysis

Data were expressed as mean ± SD. The SAS. JMP 9.0 software was used for statistical analysis. One-way ANOVA was used for the comparison, with the Dunnett and Tukey's HSD tests. $P < 0.05$ was considered statistically significant.

Results

Characterization of extract fractions of ferula sinkiangensis K. M. Shen

The ferula resin was extracted with petroleum ether, ethyl acetate, n-butanol, and methanol, respectively, and the ferula fractions were obtained. The characteristics of these ferula fractions, including the weight, yield, and properties, were shown in **Table 1**. According to these results, the petroleum ether fraction was rich in fats containing sulfur and volatile terpenes; the ethyl acetate and n-butanol fractions were rich in sesquiterpene coumarin with different polarities; and the methanol fraction contained sesquiterpene coumarin and other simple phenylalanine derivatives. The yields of the n-butanol and methanol frations were relatively low, while the petroleum ether and ethyl acetate fractions provided high yields, indicating the value for further development and utilization.

DPPH free radical-scavenging activities of ferula fractions

To investigate the free radical-scavenging effects of the ferula fractions, the DPPH assay was performed. The ferula fraction concentration gradient was set at 400, 200, 100, 50, 25, 12.5, and 6.2 mg/L. Our results showed that the IC_{50} values for the petroleum ether, ethyl acetate, n-butanol, and methanol fractions were 3166, 34.1, 33.8, and 40.3 mg/L, respectively. As shown in **Figure 1**, compared with thhe positive control, the petroleum ether fraction hardly showed any antioxidant activity, while the ethyl acetate, n-butanol, and methanol fractions exhibited potent free radical-scavenging capacities, in a dose dependent manner. These results suggest that the ethyl acetate, n-butanol, and methanol fractions might contribute to the antioxidant acitivity of *Ferula sinkiangensis* K. M. Shen.

Inhibiting effects of ferula fractions on tumor cell proliferation

To investigate the effects of the ferula fractions on tumor cell proliferation, HCT116, Caco-2, HepG2, and MFC cells were treated with the ferula fractions at indicated concentrations (i.e., 250, 125, 62.5, 31.2, and 15.6 mg/L), and the cell proliferation was assessed by the SRB method. Our results showed that, in general, the proliferation of these tumor cells was inhibited by the treatment of the ferula fractions, in a dose dependent manner (all $P < 0.05$) (**Figure 2**). The IC_{50} values for these ferula fractions in these tumor cells were summarized in **Table 2**. In specific, the inhibiting effects of the petroleum ether fraction were relatively weak, and in Caco-2 and HepG2 cells, the treatment at low concentrations even promoted the cell proliferation. The ethyl acetate, n-butanol, and methanol fractions dramatically inhibited the proliferation of these tumor cells. The most potent inhibiting effects were observed for the ethyl acetate fraction, whose IC_{50} values in the HCT116, Caco-2, HepG2, and MFC cells were 43.7, 14.8, 28.7, and 9.0 mg/L, respectively.

On the otherh hand, in all these tumor cells, the HCT116 cells exhibited the highest resistance

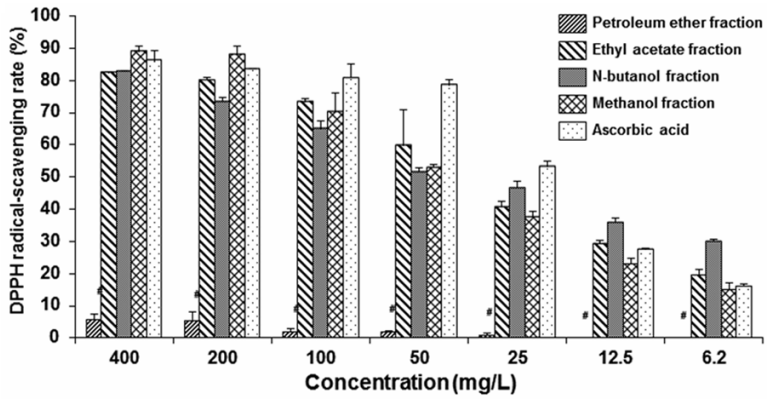

Figure 1. DPPH free radical-scavenging activities of ferula fractions. Free radical scavenging activities of the ferula fractions were assessed with the DPPH assay. Compared with the positive control (ascorbic acid), #P < 0.05.

to the ferula fraction treatments. Taken together, these resutls suggest that ferula fractions could significnatly inhibit tumor cell proliferation. Particularly, the ethyl acetate fraction exerts the most efficient inhibiting effect on tumor cell proliferation, which might contribute to the antitumor acitivity of *Ferula sinkiangensis* K. M. Shen.

Enhancing effects of ferula fractions on tumor cell apoptosis

To investigate the effects of the ferula fractions on tumor cell apoptosis, HCT116, Caco-2, and HepG2 cells were treated with the ferula fractions at 62.5 mg/L, and cellular apoptosis was detected with flow cytometry. Our results showed that, compared with the control HCT-116 cells, the apoptosis rates for the cells treated with all the ferula fractions were significantly elevated (all $P < 0.05$) (**Figure 3**). The most enhancing effect was observed for the petroleum ether fraction. On the other hand, all the ferula fractions significantly increased the apoptosis rates of Caco-2 and HepG2 cells (all $P < 0.05$) (**Figure 3**). Specifically, for Caco-2 cells, the most enhancing effects were observed for the n-butanol and methanol fractions. For HepG2 cells, the most enhancing effects were noted for the petroleum ether and n-butanol fractions. These results suggest that ferula fractions could significantly enhance the tumor cell apoptosis.

Discussion

In the present study, the effects of the exact fractions of *Ferula sinkiangensis* K. M. Shen on proliferation of *in vitro* model cells were first investigated, including HCT116, Caco-2, HepG2, and MFC cells. Our results showed that, for HCT-116 cells, all the fractions could inhibit cell proliferation, in a dose dependent manner. According to the IC_{50} values, the most potent inhibiting effect on HCT116 cell proliferation was observed for the ethyl acetate fraction. For Caco-2 cells, the ethyl acetate, n-butanol, and methanol fractions could significantly inhibit cell proliferation. However, the petroleum ether fraction only exhibited cell proliferation-inhibiting effect at high concentration, while the low concentration slightly promoted cell proliferation. Efficient inhibiting effects were observed for the ethyl acetate and n-butanol fractions, with the IC_{50} values of 14.8 and 15.6 mg/L, respectively. Similar results were observed for HepG2 cells, in which the ethyl acetate, n-butanol, and methanol fractions significantly inhibited cell proliferation, while the petroleum ether fraction had been found to increase the proliferation of HepGg2 cells. This phenomenon might be due to that HepG2 liver cancer cells could absorb and benefit from the aliphatic compounds in the petroleum ether fraction. The most potent proliferation-inhibiting effect was noted for the ethyl acetate fraction, with an IC_{50} value of 28.7 mg/L. All the fractions had been shown to exert significant inhibiting effects on the proliferation of MFC cells. Specifically, the IC_{50} value of the ethyl acetate fraction was 9.0 mg/L. These results suggest that, the ethyl acetate fraction exhibits the most potent inhibiting effect on tumor cell proliferation. This might be associated with the fact that the ethyl acetate fraction is rich in sesquiterpene coumarin and derivatives of 7-hydroxycoumarin, which have be shown to have potent antitumor effects [4-7, 18, 19].

Cancers are induced by endless proliferation of tumor cells due to abnormalities in cell structure, function, and metabolism. Therefore, apoptotic process has been intensively studied to prevent and fight against the occurrence and development of tumors [20]. In the present study, effects of the ferula fractions on the

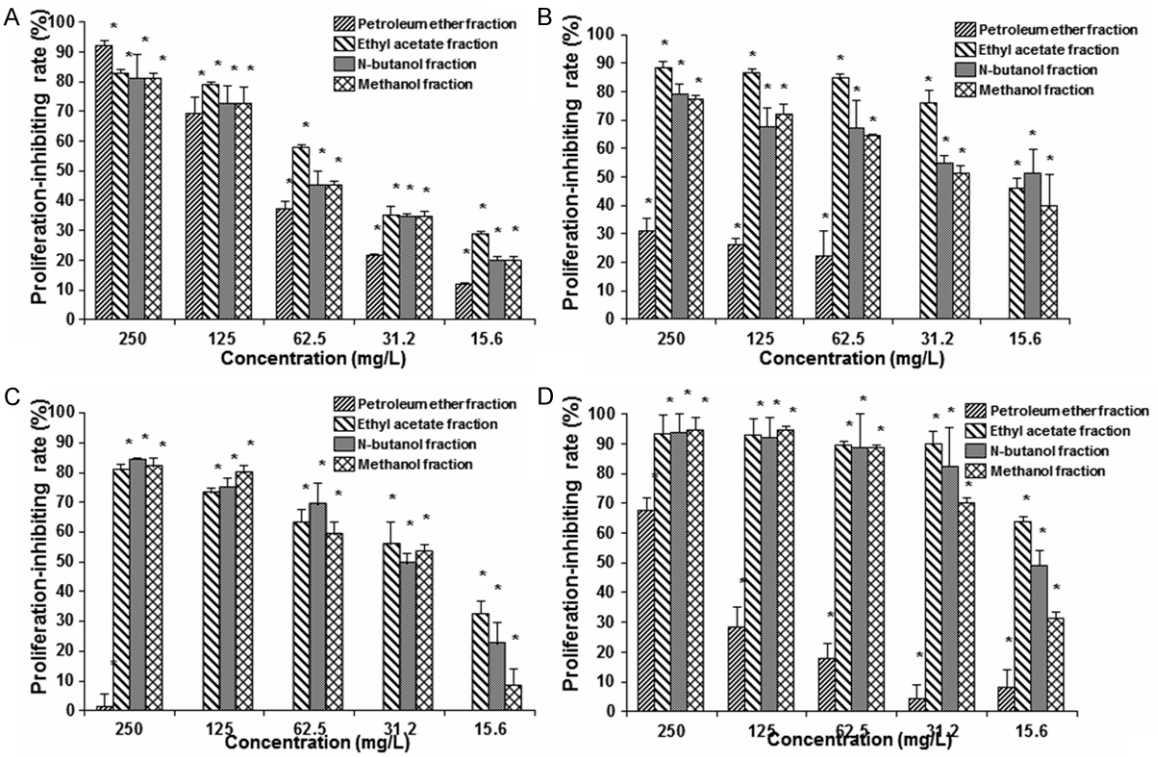

Figure 2. Inhibiting effects of ferula fractions on tumor cell proliferation. Ferula fractions at indicated concentrations (i.e., 250, 125, 62.5, 31.2, and 15.6 mg/L, respectively) were used to incubate the HCT116 (A), Caco-2 (B), HepG2 (C), and MFC (D) cells, respectively. Cell proliferation was assessed by the SRB method, and the proliferation-inhibiting rates were caculated. Proliferation-inhibitin rates for the petroleum ether fraction at 31.2 and 15.6 mg/L in the HCT116 cells were -0.5±5.9 and -47.7±13.1, respectively; and the proliferation-inhibitin rates for the petroleum ether fraction at 125, 62.5, 31.2, and 15.6 mg/L in the HepG2 cells were -24.2±12.4, -30.3±15.6, -39.1±7.6, and -27.2±12.7, respectively. These data were not shown in the figure. Compared with the control group (where the proliferation-inhibiting rate was zero), $^{*}P < 0.05$.

Table 2. Inhibiting effects of ferula fractions on tumor cell proliferation

	IC_{50} (mg/L)			
	Petroleum ether fraction	Ethyl acetate fraction	N-butanol fraction	Methanol fraction
HCT116 cells	68.7	43.7	60.3	59.6
Caco-2 cells	373.6	14.8	15.6	28.3
HepG2 cells	-	28.7	36.6	43.5
MFC cells	179.2	9.0	14.9	21.8

Note: No IC_{50} value was obtained for the petroleum ether fraction in HepG2 cells.

apoptosis of tumor cells were next detected with flow cytometry. Our results showed that, all the ferula fractions could enhance the apoptosis of HCT116, Caco-2, and HepG2 cells. For HCT116 cells, the most efficient apoptosis-promoting effect was observed for the ethyl acetate fraction. For Caco-2 cells, the most potent effects were noted for the n-butanol and methanol fractions. For HepG2 cells, the most effective apoptosis-enhancing effects were observed for the ethyl acetate and n-butanol fractions. The promoting effects of ferula fractions on tumor cell apoptosis might be associated with the enhancing expression of histone deacetylase (HDAC) [6], which may also be responsible for the proliferation-inhibiting effects of these ferula fractions. Further in-depth studies are still needed to confirm these findings.

Antioxidant effects of the fractions were also investigated. Our results showed that, the ethyl acetate, n-butanol, and methanol fractions could significantly scavenge DPPH free radicals, with the most efficient effect for the ethyl acetate fraction. However, the petroleum ether fraction did not show any free radical-scaveng-ing effects. Based on these results, the ethyl

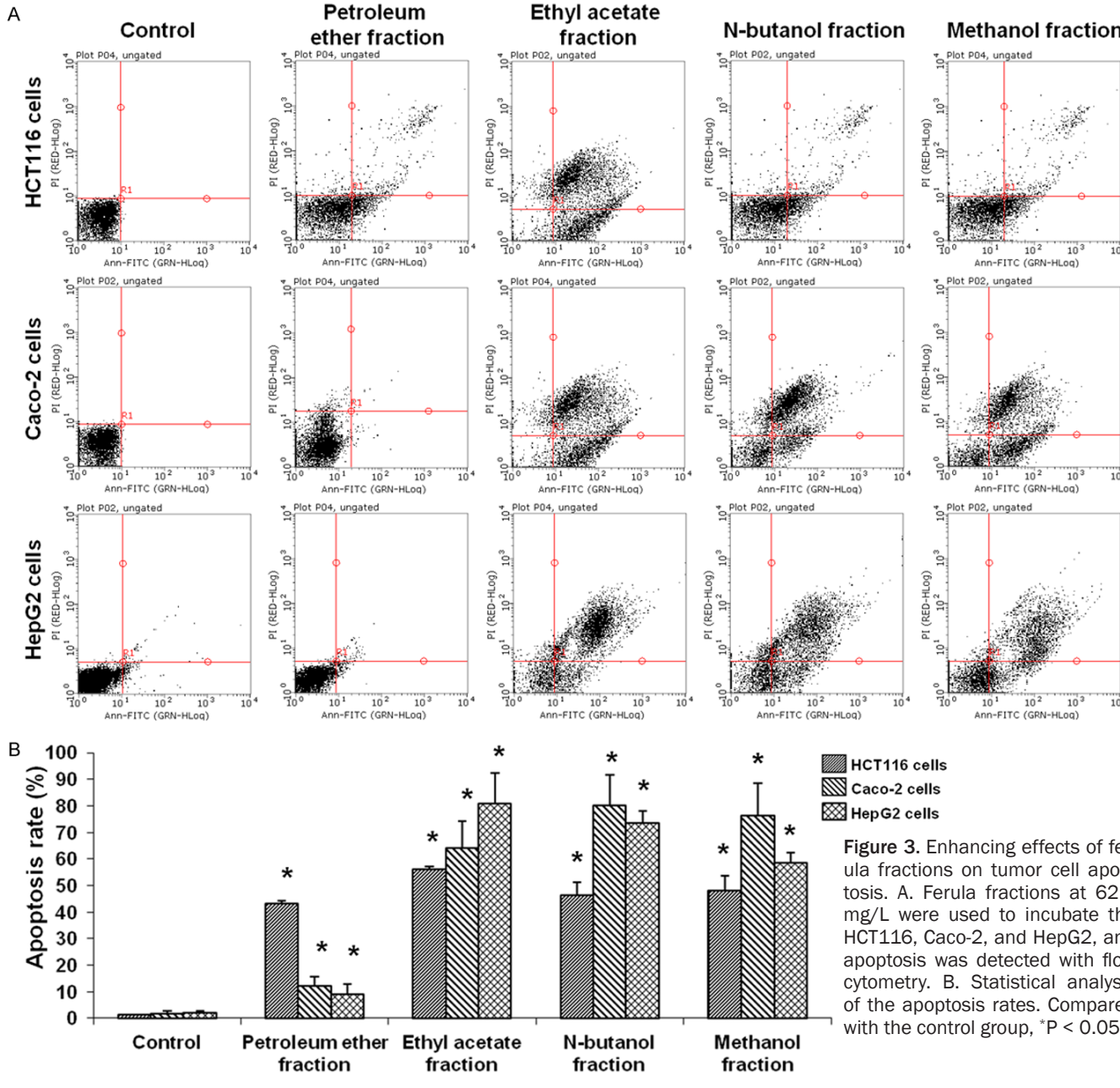

Figure 3. Enhancing effects of ferula fractions on tumor cell apoptosis. A. Ferula fractions at 62.5 mg/L were used to incubate the HCT116, Caco-2, and HepG2, and apoptosis was detected with flow cytometry. B. Statistical analysis of the apoptosis rates. Compared with the control group, $^{*}P < 0.05$.

acetate fraction of *Ferula sinkiangensis* K. M. Shen was associated with the most potent proliferation-inhibiting and apoptosis-promoting effects in tumor cells. Therefore, we suppose that the ethyl acetate fraction greatly contribute to the antioxidant and antitumor activities of *Ferula sinkiangensis* K. M. Shen.

According to the National Cancer Institute, the antitumor effects of natural drugs could be primarily confirmed when the extract exhibits an IC_{50} value < 30 mg/L or the inhibitory rate achieves 80% at the concentration of 100 mg/L [21]. Our results showed that, the IC_{50} values for the ethyl acetate fraction of *Ferula sinkiangensis* K. M. Shen on Caco-2, HepG2, and MFC cells were all lower than 30 mg/L. Particularly, for MFC cells, the IC_{50} value of the ethyl acetate fraction was only 9.0 mg/L. Therefore, it is of great significance to investigate the antitumor effects of the ferula ethyl acetate fraction in further *in vivo* researches and even clinical studies, especially regarding the treatment of gastric cancers.

In conclusion, our results showed that the ferula petroleum ether fraction of *Ferula sinkiangensis* K. M. Shen hardly showed any antioxidant activity, while the ethyl acetate, n-butanol, and methanol fractions exhibited free radical-scavenging capacities, in a dose dependent manner. Moreover, the proliferation of tumor cells could be inhibited by the treatments of ferula fractions. Furthermore, all the ferula fractions significantly enhanced the tumor cell apoptosis. However, differential effects on the cell proliferation and apoptosis were observed for the different fractions in different model cells. Particularly, the ferula ethyl acetate fraction exhibits the most potent antioxidant and antitumor effects. These findings would provide evidence for the application of *Ferula sinkiangensis* K. M. Shen and its fractions in the treatment of cancers.

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Disclosure of conflict of interest

None.

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