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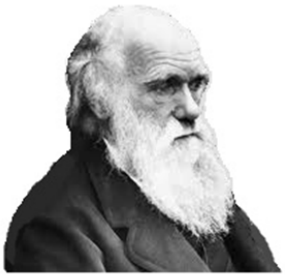

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Preparation, chemical components and biological activities of essential oil from Licorice seeds

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ABSTRACT

The essential oil from licorice seeds was prepared and investigated using gas chromatography-mass spectrometry (GC-MS), resulting in the identification of 47 beneficial components. The oil was found to be abundant in fatty acids: including linoleic acid (43.87%), palmitic acid (10.43%), oleic acid (4.51%), stearic acid (1.83%), and palmitoleic acid (1.25%). The oil's physicochemical properties revealed an acid value of 0.066 mg/g, a peroxide value of 7.267 g/100g, an iodine value of 37.231 g/100g, and a saponification value of 186.823 mg/g, indicating that it can be regarded as a premium edible oil. The antioxidant activity of this oil showed that it has a antioxidant capacity with a free radical scavenging rate of up to 38.53% at a concentration of 15 mg/mL. The *in vitro* anticancer activities against lung cancer A549 cell lines and cervical cancer Hela cell lines showed that it inhibited the cancer cell lines with cell viability of 61% and 56% at a concentration of 100 mg/mL, respectively with a dose-depedent manner. This results indicate that the oil can be considered to be used as edible oil or potential applications in the field of food and medicine.

***Keywords*:** Licorice seeds, essential oil, preparation, chemical components analysis, antioxidant, anticancer

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1. INTRODUCTION

Licorice, a plant belonging to the legume family, is referenced in the Chinese Pharmacopoeia for its medicinal properties, primarily encompassing the roots and underground stems of three species: Glycyrrhiza uralensis Fisch., Glycyrrhiza inflata Batalin, and Glycyrrhiza glabra L. Licorice has been medicinally used in China for over two millennia and is acclaimed as the "King of Medicines." According to classical herbal texts, licorice is sweet, non-toxic, and possesses various effects such as tonifying the spleen and Qi, clearing heat and detoxifying, suppressing coughs, alleviating pain, and mitigating the toxicity of other drugs [1]. Modern pharmacological research has revealed that licorice is rich in many bioactive components: including triterpenoid saponins, flavonoids, polysaccharides, coumarins, and volatile oils [2-4]. It exhibits a wide spectrum of biological activities: such as anti-inflammatory [5], antiviral [6], antimicrobial [7], antitumor [8], antioxidant [9], hypoglycemic [10], testosterone-lowering [11], hepatoprotective [12], and immune-enhancing effects [13]. Due to its rich bioactive components and biological activities, licorice is highly valued for its applications and is widely used in the pharmaceutical industry, food, and other areas: such as tobacco [14].

Traditionally, the medicinal parts of licorice are the roots and rhizomes, leading to a considerable waste of the above-ground portions such as leaves, stems, and seeds. Numerous studies have confirmed that licorice leaves also contain rich chemical constituents and biological activities. The main compounds identified from the leaves are flavonoids and dihydrostilbenes [15, 16], and their extracts exhibit biological activities including antioxidation [17], anti-inflammation [15], anti-prostatitis [18], inhibition of α-glucosidase [19], and maltase. This indicates that further research on the chemical constituents and pharmacological activities of the above-ground parts of licorice is necessary.

However, much work on the chemical constituents and biological activities of licorice and its leaves have been carried out, but the research on licorice seeds is limited. In this study, we mainly studied the preparation of the essential oil from the seeds of licorice, determination its chemical constituents and physicochemical properties. And then, we also investigate its *in vitro* antioxidation and anticancer activities.

2. EXPERIMENTAL SECTION

2. 1. Materials and Reagents

The licorice seeds were purchased from the market in the Inner Mongolia Autonomous Region of China on June 22, 2023. The reagents are available in the market with analytical grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from TCI Chemicals (Shanghai) Development Co., Ltd., and the CCK-8 cell viability assay kit was obtained from Beijing TransGen Biotech Co., Ltd.

2. 2. Instruments

Gas chromatography-mass spectrometry (GCMS-QP2010 Ultra, Shimadzu); Fourier transform infrared spectrometry (IRAFFINITY-1, Shimadzu); Ultraviolet-visible spectrophotometer (U-3310, Hitachi); Full-wavelength multifunctional microplate reader (Multiskan GO, Thermo Fisher); Rotary evaporator (RE-52AA, Shanghai Yarong); burettes

(10 mL, 25 mL, 50 mL, Sinopharm); pipettes (25 mL, Sinopharm); conical flasks (250 mL, 500 mL, Sinopharm).

2. 3. Extraction of licorice seeds oil

Fifty kilograms of licorice seeds were air-dried naturally, and the seeds were ground into a powder. The powder was added into a 50L container and with 40L soaked with 40 Lpetroleum ether for two weeks. Then, the mixture was filtered and the filtrate was concentrated under reduced pressure to obtain the residue, which was filtered over neutral alumina to obtain the orange-yellow color oil (the oil is shown in **Figure 1**).

Figure 1. The essential oil obtained from the seeds of licorice

2. 4. Determination of Physicochemical

Properties of licorice seed oil sample preparation were conducted according to GB/T 15687-2008, and the acid value, saponification value, peroxide value, and iodine value of the oil were analyzed and determined according to GB/T 5009.229-2016, GB/T 5534-2008, GB/T 5538-2005, and GB/T 5532-2008, respectively.

2. 4. 1. GC-MS analysis

1g of licorice seed oil was diluted with dichloromethane, and 1 mL of the licorice seed oil-dichloromethane solution was filtered through a 0.22 μm filter membrane for analysis [20].

The gas chromatography conditions were as follows: column, RXi-5Sil MS (30 m × 0.25 mm × 0.25 μm) capillary column; inlet temperature, 250 °C; temperature program, initial

column temperature of 60 °C, increased to 200 °C at 10 °C/min, then to 300°C at 5 °C/min and held for 10 minutes; carrier gas, high-purity helium (99.999%); injection volume, 1 μL; split ratio, 20:1. The mass spectrometry conditions were: EI source; ionization voltage, 70 eV; ion source temperature, 230 °C; full scan mode; scanning mass range, m/z 35-500.

2. 4. 2. FT-IR analysis

A suitable quantity of licorice seed oil, which was dried using anhydrous sodium sulphate, was administered onto a potassium bromide plate for further examination. The spectral range was defined from 400 to 4000 cm^{-1}, with a resolution of 4.0, and the scanning procedure was repeated 32 times. The experiment was carried out under a consistent ambient temperature of 25°C.

2. 4. 3. Antioxidant activity

The DPPH free radical method is a fast, straightforward, and cost-effective way to evaluate the ability of substances to remove free radicals. The antioxidant activity of licorice seed oil was measured using the DPPH free radical method[20], with the following experimental procedure: (1) Five milligrams of DPPH were weighed out and dissolved in ethyl acetate to a final volume of 50 mL to prepare the DPPH stock solution; (2) Five milliliters of the DPPH stock solution and 45 mL of ethyl acetate were combined in a volumetric flask, mixed thoroughly, and stored in the dark as the DPPH working solution (prepared immediately before use); (3) One milliliter of the DPPH working solution was placed in a cuvette, and its absorbance at 519 nm (A_0) was measured; (4) Licorice seed oil was diluted with ethyl acetate to prepare solutions of varying concentrations (1 mg/mL, 2 mg/mL, 4 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL), and 1 mL of each concentration was added to 5 mL of freshly prepared DPPH working solution. The mixture was allowed to react in the dark for 30 minutes, and the absorbance at 519 nm (A) was measured. The DPPH scavenging rate (%) was calculated using the formula: DPPH scavenging rate (%) = $(A_0 - A) / A_0 \times 100\%$.

2. 4. 4. *In vitro* anticancer evaluation

The anticancer activity of this licorice seed oil against lung cancer (A549) and Hela cells (Hela) was evaluated using the CCK-8 method. The licorice seed oil was solubilized in DMEM at a concentration of 100 mg/mL and subsequently diluted with DMEM to generate five distinct concentrations (100 mg/mL, 50 mg/mL,25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL) as stock solutions for below experiment.

The experiment will be divided into three groups: A (blank group) which will be treated with DMEM and CCK-8; B (control group) which will be treated with DMEM, CCK-8, and cells; and C (experimental group) which will be treated with DMEM, CCK-8, cells, and varying amounts of licorice seed oil solution.

Lung cancer A549 and cervical cancer Hela cells (1×10^6 cells/mL) were inoculated into 96-well plates at 100 μL per well and incubated in an incubator (95% air, 5% carbon dioxide) at 37 °C for 12 hours. Once the cell lines reached 90% growth in the logarithmic phase, the culture medium was removed from each well, and every well in groups A and B received 100 μL of new DEME. Then, 100 μL of various concentrations of essential oil solution (five repetitions of each concentration) were added to each well in group C, and the plates were incubated for another 48h at 37 ℃. In addition to the medium, 10 μL of CCK-8 was introduced

into every well of groups A, B, and C, and cultured for an additional hour at a temperature of 37 °C, while being shielded from light. The absorbance (OD value) related to the sample was measured at 450 nm using an enzyme marker. The OD value detected in each group was used to calculate the cell survival rate = $[(A_{experimental} - A_{blank}) / (A_{control} - A_{blank})] \times 100\%$.

3. RESLUTS AND DISCUSSION

3. 1. Physicochemical properties of licorice seed oil

It can be seen from **Table 1** that the acid value of licorice seed oil was less than 3 mg/g. A lower acid value indicates a lower content of free fatty acids in the oil, suggesting the better oil quality. The higher peroxide value of the oil may have been related to the high-temperature treatment during seed oil extraction and the storage methods employed. The iodine value, which reflects the content of unsaturated fatty acids in the oil, is less than 100 g/100g for licorice seed oil, classifying it is a non-drying oil and suitable for industries: such as soap, pharmaceutical, and lubricant production. The saponification value of the oil is also one of the indicators for evaluating oil quality; a higher saponification value indicates a smaller molecular weight of the fatty acids, which can be more easily absorbed by the human body.

Table 1. Physicochemical properties of licorice seeds oil

Item	Indicator
Acid value (KOH)/(mg/g)	0.066±0.003
Peroxide value (g/100g)	7.267±0.023
Iodine value (g/100g)	37.231±0.016
Saponification value (KOH)/(mg/g)	186.823±1.818

3. 2. The chemical compositions of licorice seed oil

The chemical compositions of licorice seed oil was investigated using GC-MS (**Figure 2**). After searching the NIST11.lib spectral library and referencing relevant literature, a total of 47 compounds were identified and the results were summarized in **Table 2**.

It was observed from **Table 2** that the major constituents in licorice seed oil were linoleic acid (43.87%), palmitic acid (10.43%), oleic acid (4.51%), stearic acid (1.83%), and palmitoleic acid (1.25%).

Linoleic acid [22], which is abundant in licorice seed oil, has been reported in the literature to be an essential fatty acid for humans, offering benefits such as reducing cholesterol levels, preventing atherosclerosis, and decreasing the incidence of coronary heart disease and cancer. The presence of a high content of linoleic acid in licorice seed oil suggests its potential to be a premium edible oil with health benefits for humans.

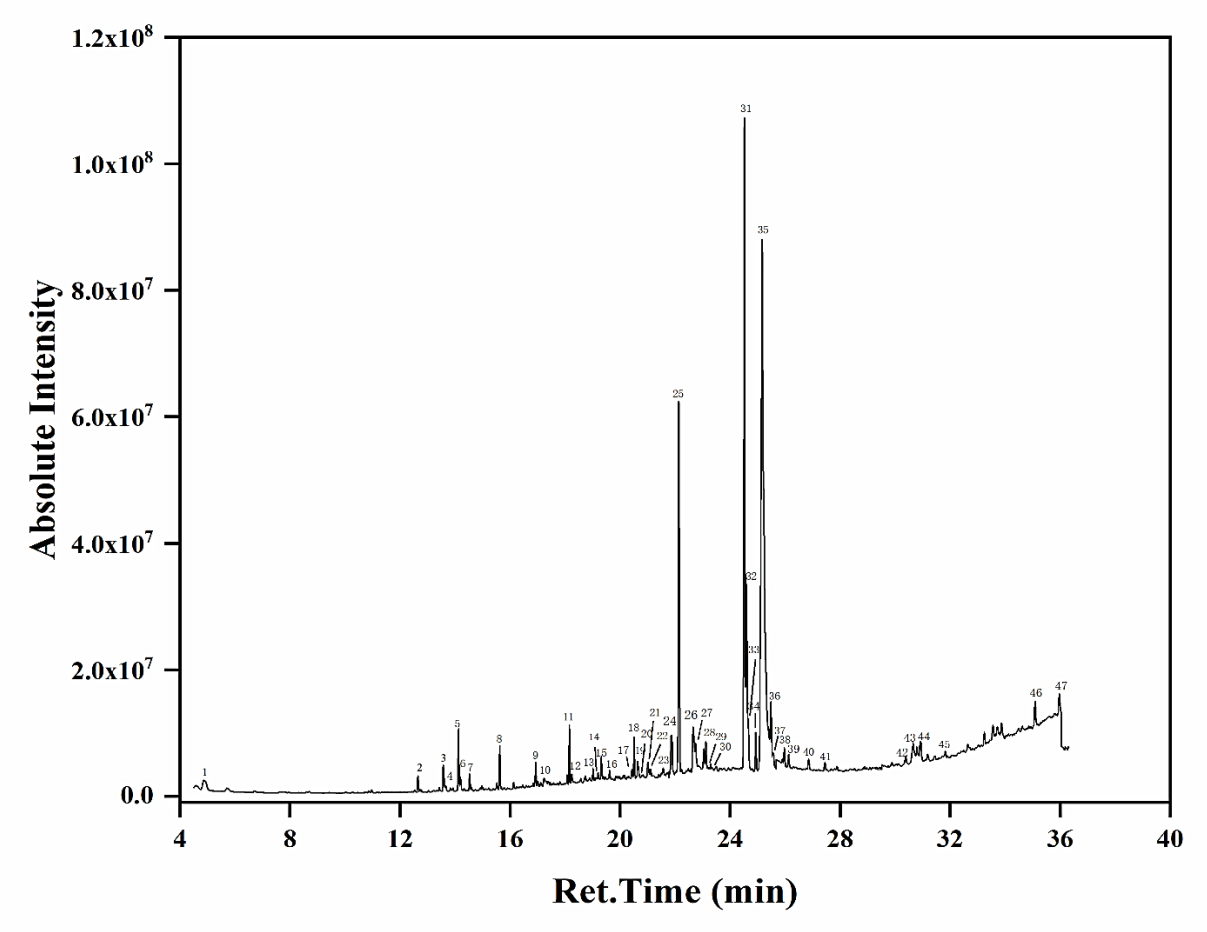

Fig. 2. The GC-MS chromatogram of the seeds oil of licorice.

Table 2. The chemical compositions of the seeds oil of licorice.

No.	Names	Formula	RT/mins	% Peak Area
1	p-Xylene	C_8H_{10}	4.870	0.34
2	Dodecane	$C_{12}H_{26}$	12.655	0.24
3	Caprolactam	$C_6H_{11}NO$	13.575	0.55
4	4-Methoxybenzaldehyde	$C_8H_8O_2$	13.650	0.13
5	Anethole	$C_{10}H_{12}O$	14.125	1.02
6	2,4-Decadienal	$C_{10}H_{16}O$	14.195	0.27
7	(2E,4E)-Deca-2,4-dienal	$C_{10}H_{16}O$	14.530	0.30

8	Tetradecane	$C_{14}H_{30}$	15.625	0.58
9	Hexadecane	$C_{16}H_{34}$	16.935	0.36
10	3,5-Di-tert-butylphenol	$C_{14}H_{22}O$	17.235	0.26
11	Heptadecane	$C_{17}H_{36}$	18.160	0.80
12	Hexanoic acid, 2-ethyl-, 2-ethylhexyl ester	$C_{16}H_{32}O_2$	18.245	0.16
13	7-Tetradecyne	$C_{14}H_{26}$	19.015	0.18
14	Indan-5-ol	$C_9H_{10}O$	19.200	0.15
15	Octadecane	$C_{18}H_{38}$	19.315	0.38
16	Methyl tetradecanoate	$C_{15}H_{30}O_2$	19.620	0.17
17	1-Hexadecanol	$C_{16}H_{34}O$	20.435	0.18
18	Nonadecane	$C_{19}H_{40}$	20.510	0.63
19	Dioctyl ether	$C_{16}H_{34}O$	20.640	0.45
20	Methyl heneicosanoate	$C_{22}H_{44}O_2$	20.835	0.13
21	Oleic alcohol	$C_{18}H_{36}O$	21.015	0.41
22	Perhydrofarnesyl acetone	$C_{18}H_{36}O$	21.115	0.14
23	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	$C_{20}H_{40}O$	21.570	0.22
24	Methyl Z-9-hexadecenoate	$C_{17}H_{32}O_2$	21.870	1.25
25	Methyl palmitate	$C_{17}H_{34}O_2$	22.135	7.19
26	Palmitic acid	$C_{16}H_{32}O_2$	22.650	2.85
27	Stearic acid	$C_{18}H_{36}O_2$	22.660	1.08
28	Ethyl palmitate	$C_{18}H_{36}O_2$	23.045	0.39
29	n-Eicosane	$C_{20}H_{42}$	23.120	0.51
30	13-Tetradecynoic acid methyl ester	$C_{15}H_{26}O_2$	23.500	0.13
31	Methyl linoleate	$C_{19}H_{34}O_2$	24.520	13.78

32	Methyl oleate	$C_{19}H_{36}O_2$	24.590	4.51
33	Methyl 11-octadecenoate	$C_{19}H_{36}O_2$	24.665	0.88
34	Methyl stearate	$C_{19}H_{38}O_2$	24.930	0.75
35	Linoleic acid	$C_{18}H_{32}O_2$	25.165	30.09
36	Docosa-13,16-dienoic acid	$C_{22}H_{40}O_2$	25.485	2.60
37	All-cis-9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	25.570	0.88
38	Heneicosane	$C_{21}H_{44}$	25.975	0.33
39	Eicosyl acetate	$C_{22}H_{44}O_2$	26.130	0.26
40	N,N-Dimethyldodecanamide	$C_{14}H_{29}NO$	26.855	0.22
41	Tetracosane	$C_{24}H_{50}$	27.440	0.18
42	Trilinolein	$C_{57}H_{98}O_6$	29.340	0.25
43	Pentacosane	$C_{25}H_{52}$	30.385	0.17
44	2-Octanoylfuran	$C_{12}H_{18}O_2$	30.925	0.51
45	Heptacosane	$C_{27}H_{56}$	31.825	0.15
46	Squalene	$C_{30}H_{50}$	35.090	0.65
47	Tetracontane	$C_{40}H_{82}$	35.970	0.53

3. 3. FT-IR Analysis of licorice seed oil

Fourier transform infrared (FT-IR) spectroscopy, a technique based on the chemical composition and structural bonds of substances, is widely utilized in the development and application of natural products as well as in chemical research.

FT-IR analysis of licorice seed oil was conducted [23, 24], with the spectrum presented in **Figure 3**. The absorption peak at 3008 cm^{-1} was attributed to C-H stretching vibrations. The most intense absorption peaks were observed in the region between 2854 and 2926 cm^{-1}, which were attributed to the asymmetric and symmetric stretching vibrations of CH_2 groups. A strong stretching peak at 1745 cm^{-1} was confirmed to be associated with C=O and -CHO groups. Absorption peaks in the range of 1363 to 1458 cm^{-1} corresponded to C-H bending vibrations, while peaks between 1000 and 1300 cm^{-1} were indicative of C-O stretching vibrations. The absorption at 723 cm^{-1} was attributed to -CH=CH- bending vibrations. The above spectral features indicate that licorice seed oil contains mostly aliphatic compounds, which is consistent with the compositions analyzed in **Table 2**.

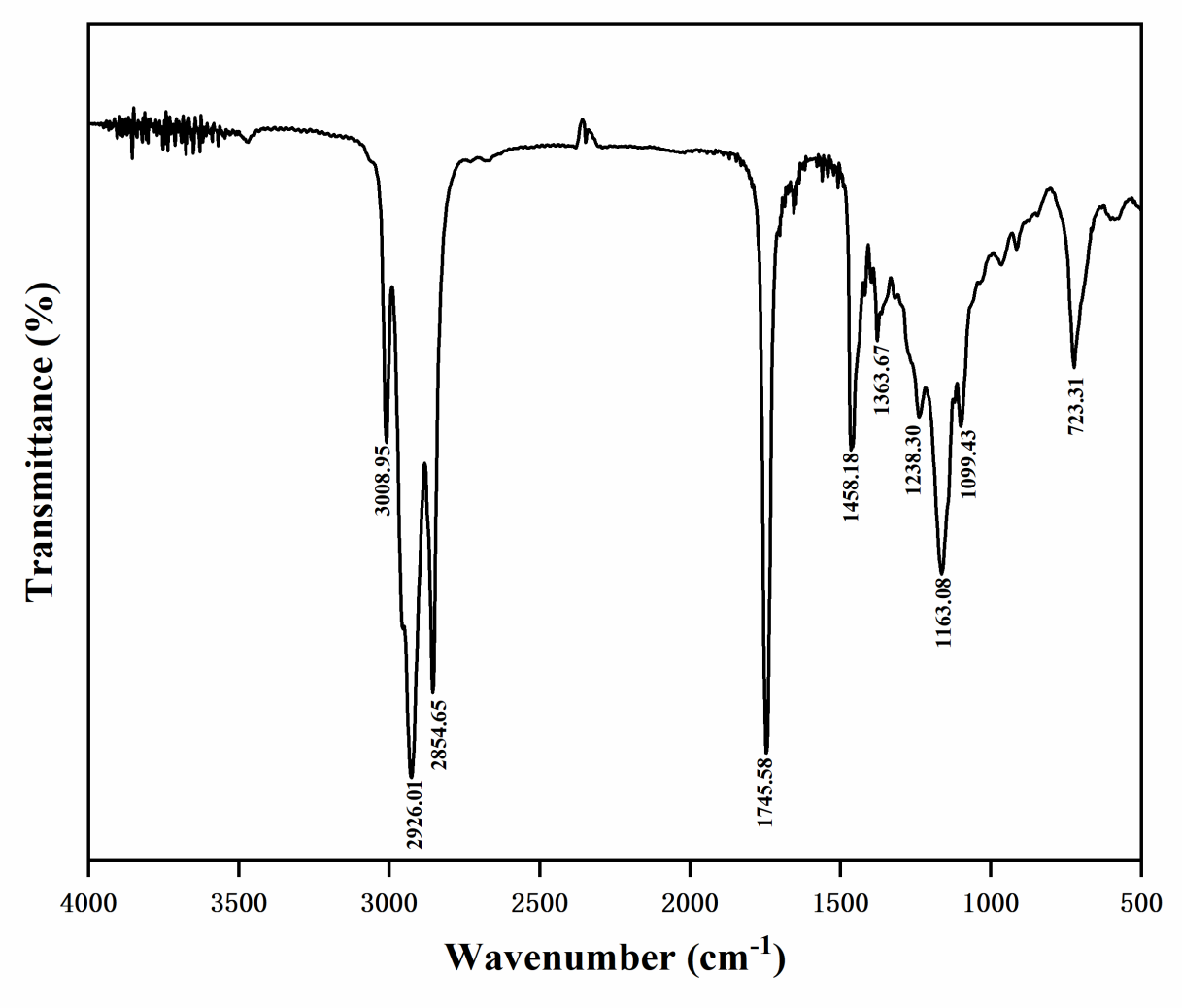

Fig. 3. FT-IR spectra of licorice seeds oil in the frequency range 4000-500 cm^{-1}

3. 4. Antioxidant effect of licorice seed oil

The scavenging rate of DPPH was observed to continuously increase with the escalating concentration of licorice seed oil, as indicated in **Figure 4**. At a concentration of 15 mg/mL, the radical scavenging rate reached 38.53%, indicating that the essential oil possessed a certain level of antioxidant capacity. However, further research could be explored for enhancing the antioxidant activity of licorice seed oil by combining it with other substances, aiming to maximize its development and utilization.

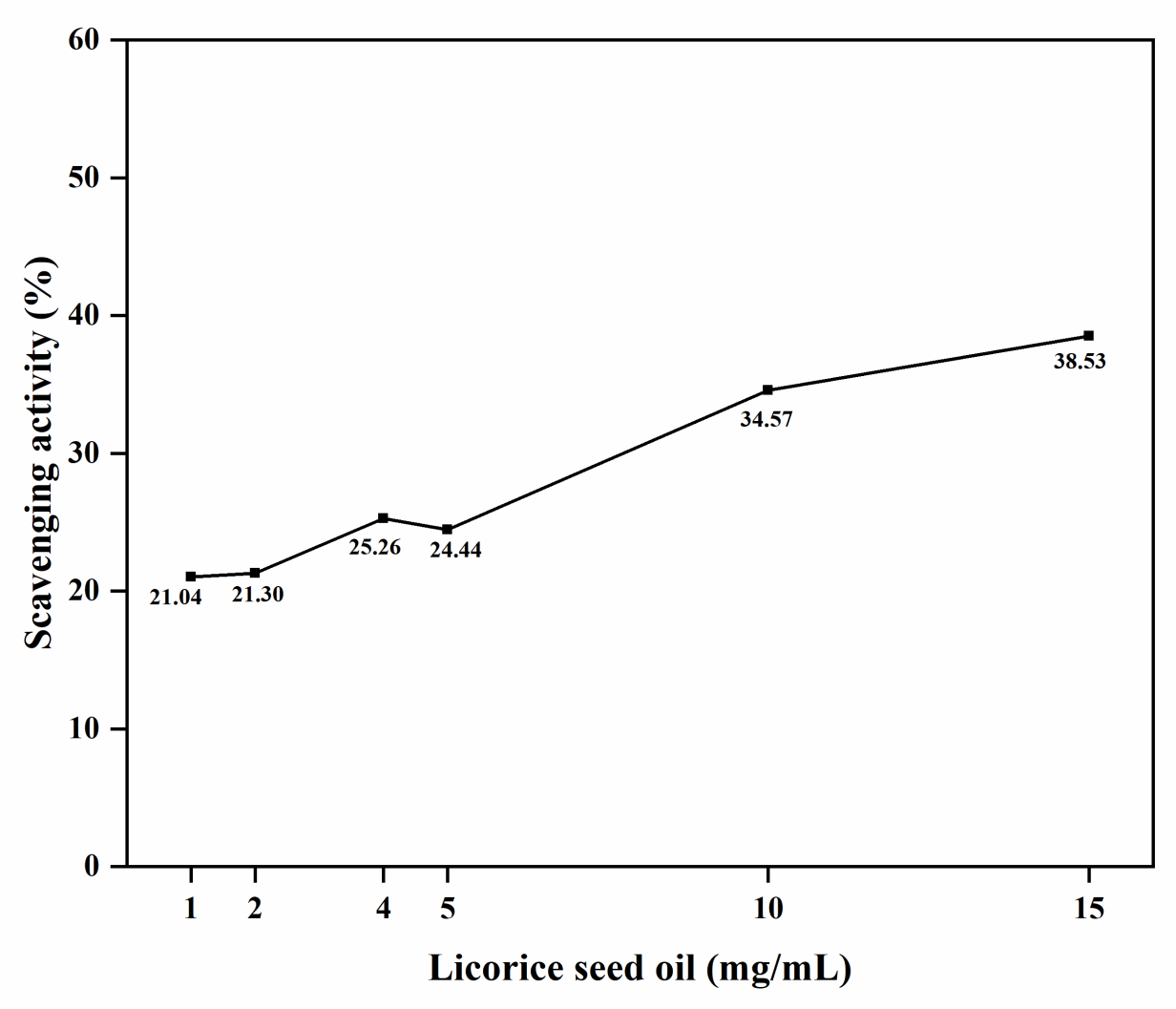

Fig. 4. The scavenging ability of different amounts of licorice seeds oil at 5mL DPPH stocking solutions

3. 5. Anticancer evaluation

In the recent past, there has been a steady rise in the incidence and mortality rates associated with lung and cervical cancers, which pose substantial threats to human health. An *in vitro* assessment of the antitumor potential of licorice seed oil was conducted against lung cancer (A549) and Hela cells using CCK-8 assay.

The viability of A549 cells and Hela cells was found to be 61% and 56%, respectively, when treated with a concentration of 100 mg/mL of licorice seed oil. The inhibitory impact of licorice seed oil on the proliferation of A549 and Hela cells was in a dose-dependent manner (**Figure 5**). With the progressive increasing in the concentration of licorice seed oil, there was a continual decline in the viability of cancer cells, suggesting the oil's potential utility as an anticancer therapeutic.

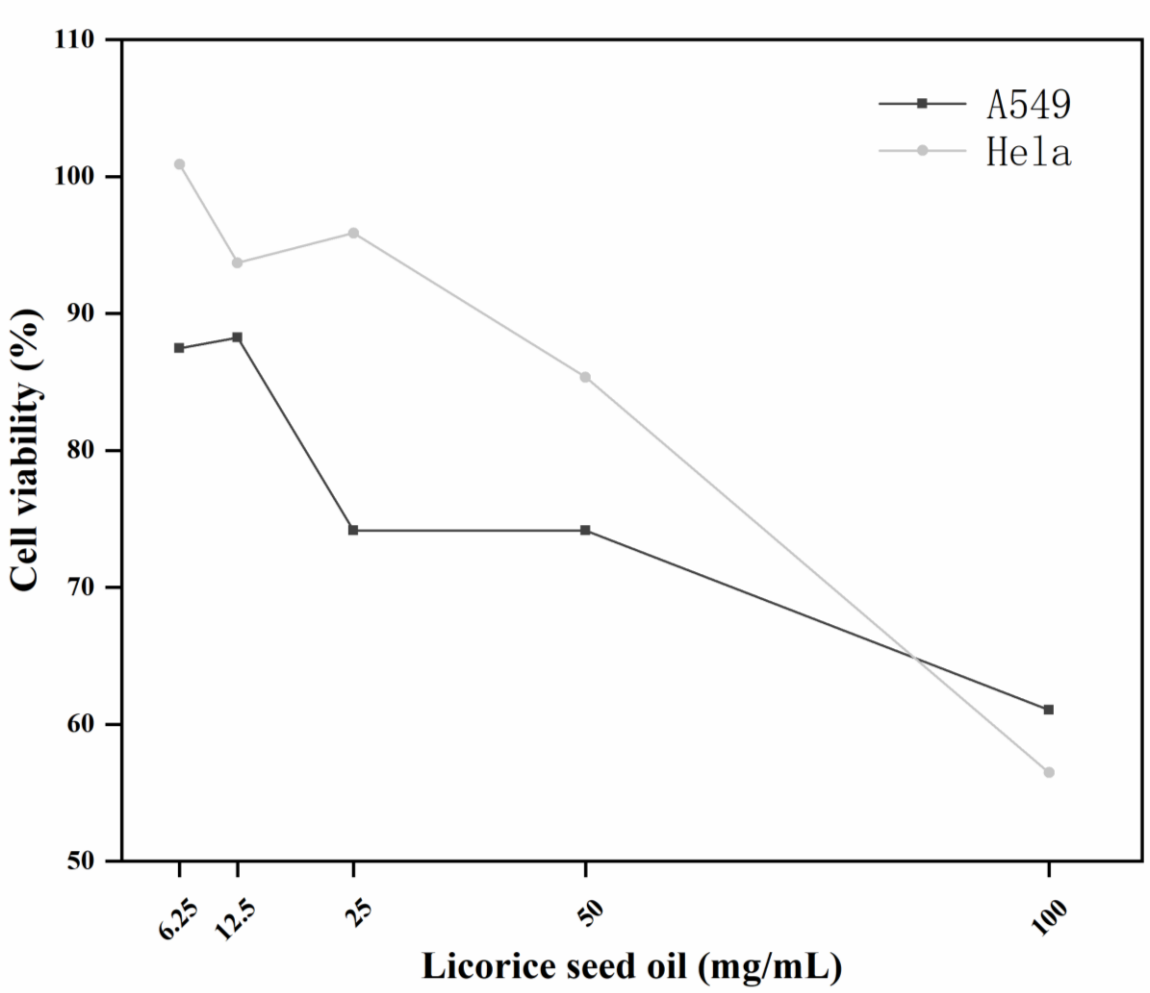

Fig. 5. The licorice seeds oil effects on A549 and Hela tumoral cells' viability

4. CONCLUSION

In this work, we obtained the essential oil from the seeds of licorice and its physicochemical properties were also confirmed as the acid value of 0.066 mg/g, a peroxide value of 7.267 g/100g, an iodine value of 37.231 g/100g, and a saponification value of 186.823 mg/g, respectively. GC-MS and FT-IR analyses were employed to determine its chemical constituents, revealing 47 organic compounds, which are rich in unsaturated and saturated fatty acids. The predominant components included linoleic acid (43.87%), palmitic acid (10.43%), oleic acid (4.51%), stearic acid (1.83%), and palmitoleic acid (1.25%). The oil exhibited higher antioxidant and anticancer activities, it can be used for development of antioxidant and anticacer agents.

Licorice seed oil has potential applications not only as a premium edible oil but also for its antioxidant and anticancer properties. Additionally, as a non-drying oil, it is suitable for use in industries such as soap manufacturing, pharmaceuticals, and lubrication.

Further research on this oil is being carried out now.

Acknowledgments

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