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## Original Research Article

# Chemical composition of the essential oil of whole plant of *Elsholtzia dense* Benth and its anti-tumor effect on human hepatoma cells

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### Abstract

**Purpose:** To determine the chemical components of the essential oil of *Elsholtzia dense* in Sichuan Province and evaluate the effect of the oil on human hepatoma cells (SMMC-7721) *in vitro*.

**Methods:** The essential oil was extracted using the modified steam-distillation extraction method, and its chemical components were determined by gas chromatography-mass spectrometry (GC-MS). The effect of the essential oil on proliferation of SMMC-7721 cells was studied by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, with L02 and HeLa cells serving as control groups.

**Results:** GC-MS results show that the essential oil of *E. dense* contains 40 components. Thirty seven components were identified and accounted for 98.39 % of the essential oil. The two main components were rosefuran epoxide (53.12 %) and 2-ethyl imidazole (29.8 %). The oil significantly inhibited cell proliferation in a concentration- and time-dependent manner ( $p < 0.05$ ). SMMC-7721 cells were more inhibited than L02 and HeLa cells by the oil, with half maximal inhibitory concentration ( $IC_{50}$ ) values of 26.23 and 25.46  $\mu\text{g/mL}$  after 8-h and 24-h treatments, respectively.

**Conclusion:** Out of the 40 chemical components of the essential oil of *E. dense*, rosefuran epoxide and 2-ethyl imidazole were the most abundant. The oil has a significant anti-tumor effect on SMMC-7721 cells, and thus has a potential to be developed as an anti-liver cancer drug.

**Keywords:** Medicinal herb, *Elsholtzia dense* Benth, Essential oil, Rosefuran epoxide, 2-Ethyl imidazole, Anti-tumor activity

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## INTRODUCTION

*Elsholtzia dense* Benth belonging to Labiatae, is found in the west of China, including Sichuan, Shanxi and Qinghai. *E. dense* is one of the sources of the Tibetan medicine, Qirou [1]. It has anti-inflammatory, anti-pruritic, myogenic, and hemostatic effects [2], and also could remove necrotic tissue and repel mosquito [3]. It has been traditionally applied to treat several diseases such as summer cold, scabies, syphilitic rhinitis, laryngitis, skin itching, furuncle

carbuncle and stomach disease in China [2,3]. The main medicinal components of *E. dense* is its essential oil [2,4-6]. The essential oil of many plants has been reported to have an inhibitory effect on tumor cells [7-13]. However, the anti-tumor effect of the essential oil from *E. dense* in Sichuan Province has been seldom studied.

The chemical composition of the essential oil from *E. dense* has been reported in several studies [2,4-6]. However, the chemical composition of the essential oil differs in different

regions where *E. dense* grows. The chemical components of the essential oil from *E. dense* in Sichuan Province have not been identified until now.

Therefore, the aim of this study was to identify the chemical components of the essential oil from *E. dense* in Sichuan Province and investigate its anti-tumor effect. In this study, the plant was collected from alpine meadow in Hongyuan County of Sichuan Province. The essential oil was isolated from the whole plant using the modified steam-distillation extraction method. The chemical components were identified using gas chromatography-mass spectrometer (GC-MS) analysis. The effect of the essential oil on the proliferation of normal human liver cells (L02), human hepatoma cells (SMMC-7721) and human cervical carcinoma cells (HeLa) was studied by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay.

## EXPERIMENTAL

### Plant materials

The plants of *E. dense* were collected from alpine meadow in Hongyuan County of Sichuan Province, and verified as *E. dense* by Professor Danwei Ma, Sichuan Normal University, China.

### Extraction of the essential oil from *E. dense*

The whole plant of *E. dense* was dried in a shade. According to the modified steam-distillation extraction method previously described [4], the essential oil was obtained from 500 g of the dried whole plants boiled in a required amount of distilled water. Then the essential oil was dried with anhydrous sodium sulfate, and filtered through 0.22  $\mu\text{m}$  membrane. Finally it was stored in a sealed brown reagent bottle at  $-20\text{ }^{\circ}\text{C}$  for the experiments. Using this method the yield of the essential oil from *E. dense* was increased to 0.36 %.

### GC-MS analysis of the essential oil

The chemical nature of the essential oil was analyzed by Gas Chromatography-Mass Spectrometer (GC-MS, Agilent 6890-5973, Agilent Technologies Inc. USA). Helium was used as carrier gas. The rate of Agilent DB-Wax capillary column (60 m x 0.25 mm, 0.25  $\mu\text{m}$  film thickness) was set at 1.0 mL/min. During the analysis period the temperature of GC oven was first set at  $35\text{ }^{\circ}\text{C}$  for 10 min and then increased to  $230\text{ }^{\circ}\text{C}$  at the rate of  $3\text{ }^{\circ}\text{C}/\text{min}$ , and finally maintained at  $230\text{ }^{\circ}\text{C}$  for 10 min. Split ratio was

5:1. The injector temperature was set at  $230\text{ }^{\circ}\text{C}$ . Mass spectra were recorded in the mass range of  $m/z$  35–450 at 70 eV.

### Determination of chemical components of the essential oil

The essential oil components was determined by comparison of their relative retention times to standard reference data from the National Institute of Standards and Technology (NIST) version 11 GC-MS libraries (USA). Quantification was determined by percentage peak area calculations using GC-FID.

### Cell culture

SMMC-7721 cells, L02 cells and HeLa cells were grown as monolayers in RPMI-1640 medium supplemented with 10 % fetal bovine serum and 1 % antibiotics (100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin). Cells were maintained at  $37\text{ }^{\circ}\text{C}$  and 5 % of  $\text{CO}_2$ . The cells used in the experiments were in logarithmic growth phase.

### Determination of cell proliferation

SMMC-7721 cells, L02 cells and HeLa cells were seeded at the density of  $7\text{--}10 \times 10^4/\text{well}$  in a 96-well plate (100  $\mu\text{L}/\text{well}$ ). After 12 hour incubation, the cells were adhered to the plate and treated with the essential oil from *E. dense* at 5 different concentrations, 10, 20, 30, 40, 50, 60  $\mu\text{g}/\text{mL}$ . Cells treated with 1 % DMSO were negative control and cells treated with 80  $\mu\text{g}/\text{mL}$  fluorouracil were positive control. After 8 or 24 hour treatment with the essential oil, 20  $\mu\text{L}$  of 5 mg/mL MTT solution was added into the medium. After 4 hour incubation, the medium containing MTT was removed, and the remaining formazan crystals were dissolved by incubation with 150  $\mu\text{L}/\text{well}$  DMSO on the oscillator for 10 min. The absorbance (A) value of each well was measured at the wavelength of 490 nm by microplate reader (MD/Spectra Max M2, USA). All experiments were performed 3 times. The cell proliferation inhibition rate was calculated as:

The cell proliferation inhibition rate (%) =  $[1 - A_{\text{essential oil treated cells}} / A_{\text{DMSO control}}] \times 100\%$ .

### Data statistics and analysis

The data were analyzed using SPSS17.0 (SPSS Inc., USA). Significant differences were analyzed by Least-Significant Difference (LSD) test.  $P < 0.05$  was considered statistically significant. All data are presented as mean  $\pm$  standard deviation (SD). The figures were drawn using Microsoft Excel 2003 (Microsoft, USA).

## RESULTS

### Composition of essential oil

The chemical nature of the essential oil from *E. dense* was studied by GC-MS analysis. The chromatogram of the essential oil from *E. dense* (Figure 1) shows that the essential oil consisted of 40 chemical components. Thirty seven chemical components were identified (Table 1), and they accounted for 98.39 % of the essential oil. Rosefuran epoxide accounted for 53.12 % of the essential oil, followed by 2-ethyl imidazole (29.8 %), durenol (2.87 %), *p*-cymene (2.11 %), 1-cyanoacetyl-3,5-dimethylpyrazole (1.93 %) and 1,5,9,9-tetramethyl-1,4,7,-cycloundecatriene (1.16 %).

### Inhibitory activity of essential oil of *E. dense*

Figure 2 shows a time- and concentration-dependent inhibitory effect of the essential oil on proliferation of 3 cell lines, which is similar to the positive control (treatment with 80  $\mu\text{g}/\text{mL}$  fluorouracil). The essential oil significantly inhibited proliferation of SMMC-7721 and HeLa cells in a concentration dependent manner during 8 hour treatment ( $p < 0.05$ , Figure 2A) and 24 hour treatment ( $p < 0.05$ , Figure 2B). During 8 hour treatment the  $\text{IC}_{50}$  values for SMMC-7721, L02 and HeLa cells were 26.23, 36.64 and 44.46  $\mu\text{g}/\text{mL}$ , respectively. During 24 hour treatment the  $\text{IC}_{50}$  values for SMMC-7721, L02 and HeLa

cells were 25.46, 53.16 and 68.37  $\mu\text{g}/\text{mL}$ , respectively. These results suggest that SMMC-7721 cells are the most sensitive to the inhibitory effect of the essential oil among these three cell lines.

## DISCUSSION

In this study, the essential oil from *E. dense* collected from alpine meadow in Hongyuan County of Sichuan Province consists of 40 chemical components, with 37 components identified. The essential oil from *E. dense* shares some common components with other species of *Elsholtzia*: thymol, 1,8-cineole,  $\beta$ -dehydroelsholizone, elsholtzia ketone, linalool, carvacrol and *p*-cymene [14], however it differs in the chemical composition from other species of *Elsholtzia* [15-17].

In this study the main components of the essential oil from *E. dense* were identified as rosefuran epoxide (53.12 %), 2-ethyl imidazole (29.8 %), durenol (2.87 %), *p*-cymene (2.11 %), 1-cyanoacetyl-3,5-dimethylpyrazole (1.93 %) and 1,5,9,9-tetramethyl-1,4,7,-cycloundecatriene (1.16 %).

The essential oil of *E. dense* in Sichuan Province also differs in the chemical composition from *E. dense* growing in other regions [3-5].

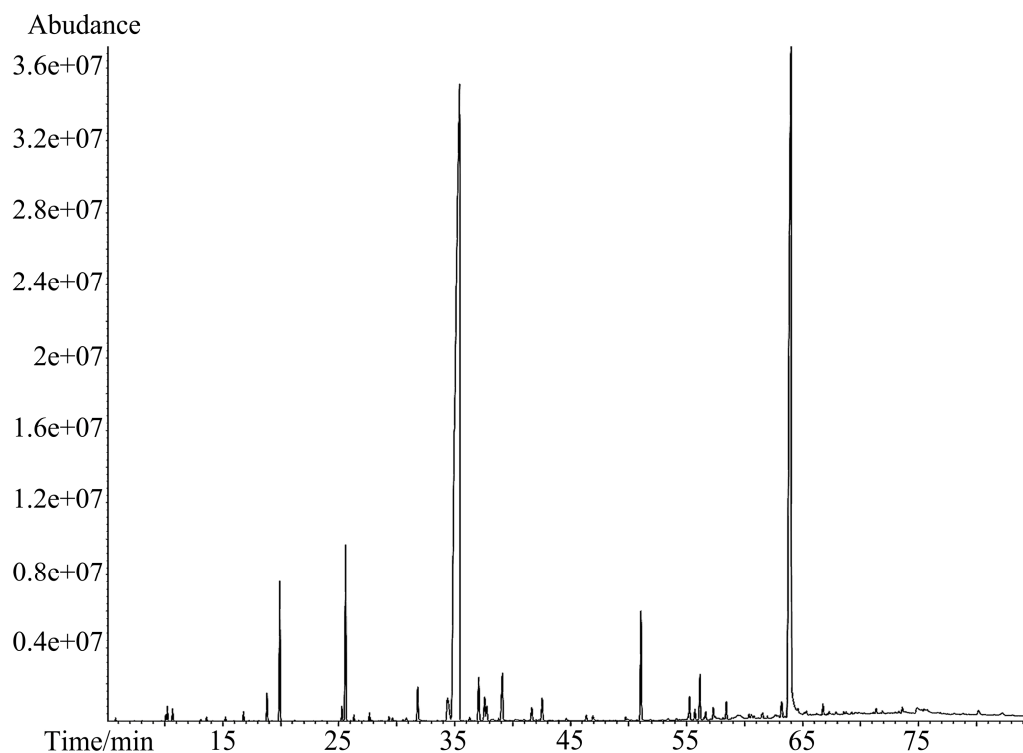


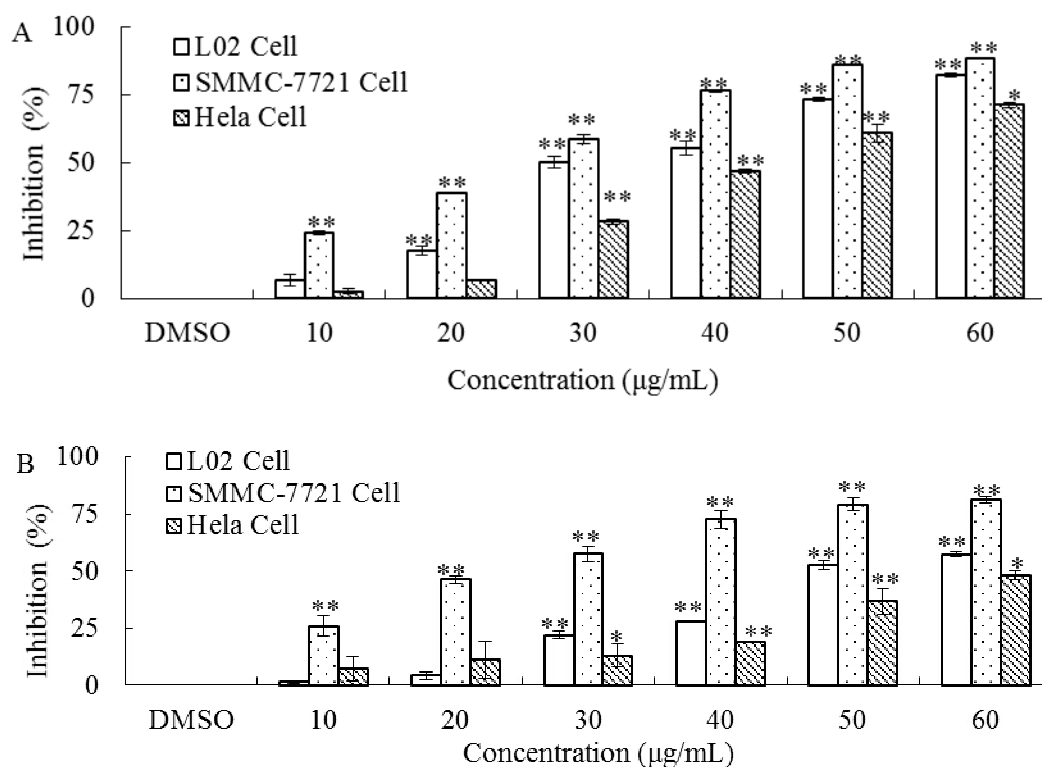
Figure 1: Chromatogram of the essential oil of *E. dense*

**Table 1:** Chemical components of the essential oil of *E. dense*

S/no.	Compound	Retention time (min)	Relative content (%)	Molecular formula
1	Acetone	5.754	0.05	
2	(1R)-(+)- $\alpha$ -pinene	10.095	0.09	C <sub>10</sub> H <sub>16</sub>
3	3-thujene	10.223	0.21	C <sub>10</sub> H <sub>16</sub>
4	2-methyl-3-buten-2-ol,	10.671	0.18	C <sub>5</sub> H <sub>10</sub> O
5	$\beta$ -phellandrene	13.605	0.06	C <sub>10</sub> H <sub>16</sub>
6	$\beta$ -myrcene	15.233	0.06	C <sub>10</sub> H <sub>16</sub>
7	<i>D</i> -limonene	16.788	0.14	C <sub>10</sub> H <sub>16</sub>
8	$\gamma$ -terpinene	18.816	0.40	C <sub>10</sub> H <sub>16</sub>
9	<i>o</i> -cymene	19.923	2.11	C <sub>10</sub> H <sub>14</sub>
10	3-octanol	25.281	0.19	C <sub>8</sub> H <sub>18</sub> O
11	Durenol	25.593	2.87	C <sub>10</sub> H <sub>14</sub> O
12	Perillen	26.31	0.08	C <sub>10</sub> H <sub>14</sub> O
13	mushroom alcohol	27.651	0.12	C <sub>8</sub> H <sub>16</sub> O
14	5-methyl-2-furancarboxaldehyde	29.342	0.07	C <sub>6</sub> H <sub>6</sub> O <sub>2</sub>
15	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	29.641	0.05	C <sub>18</sub> H <sub>52</sub> O <sub>7</sub> S <sub>7</sub>
16	(-)- $\beta$ -bourbonene	30.83	0.08	C <sub>15</sub> H <sub>24</sub>
18	unidentified	34.389	1.00	
19	rosefuranepoxide	35.405	53.12	C <sub>10</sub> H <sub>14</sub> O <sub>2</sub>
20	1-ethyl-3-(1-methylethyl)- Benzene	36.295	0.08	C <sub>11</sub> H <sub>16</sub>
21	4-pyridazinamine	37.073	0.94	C <sub>4</sub> H <sub>5</sub> N <sub>3</sub>
22	longifolene	37.587	0.55	
23	acetophenone	37.764	0.34	C <sub>8</sub> H <sub>8</sub> O
24	1,3,5,7-tetraethyl-1-ethylbutoxysiloxy cyclotetrasiloxane	38.799	0.04	
25	1,5,9,9-tetramethyl-1,4,7,- cycloundecatriene	39.105	1.16	C <sub>15</sub> H <sub>24</sub>
26	2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	41.652	0.32	C <sub>15</sub> H <sub>24</sub>
27	unidentified	42.547	0.51	
28	<i>d</i> -cadinene	44.629	0.04	C <sub>15</sub> H <sub>24</sub>
29	perillaldehyde	46.35	0.13	C <sub>10</sub> H <sub>14</sub> O
30	6,7,8,9-tetrahydro-6-methyl-4H-pyrido[1,2-a]pyrimidin-4-one	46.925	0.13	C <sub>9</sub> H <sub>12</sub> N <sub>2</sub> O
31	1-cyanoacetyl-3,5-dimethylpyrazole	51.063	1.93	C <sub>8</sub> H <sub>9</sub> N <sub>3</sub> O
32	3-(4-methylpent-3-enyl)thiophene	55.255	0.52	
33	caryophyllene oxide	56.147	0.82	C <sub>15</sub> H <sub>24</sub> O
34	<i>cis</i> -decalin	58.426	0.33	C <sub>10</sub> H <sub>18</sub>
35	1,4-dihydroxy- <i>p</i> -menth-2-ene	60.382	0.13	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>
36	espatulenol	61.555	0.16	C <sub>15</sub> H <sub>24</sub> O
37	2-ethyl-1H-imidazole	63.991	29.80	C <sub>5</sub> H <sub>8</sub> N <sub>2</sub>
38	1-(1,1-dimethylethyl)-2-methylene-cyclopropane	66.765	0.23	
39	unidentified	71.351	0.10	
40	propanediamide	73.612	0.22	C <sub>3</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub>

In this study it is the first time to study the anti-tumor effect of the essential oil from *E. dense* in Sichuan Province, although the essential oil of many plants has been reported to be anti-tumor [7-13]. The essential oil from *E. dense* had a significant inhibitory effect on the proliferation of L02, SMMC-7721 and HeLa cells in a concentration dependent manner. The IC<sub>50</sub> values show that SMMC-7721 cells were the most sensitive cells to the inhibitory effect of the essential oil. The inhibitory effect on proliferation of SMMC-7721 cells was time-dependent,

however the inhibitory effect on proliferation of L02 and HeLa cells was alleviated when extending its treatment time from 8 hours to 24 hours. For 24 hour treatment with the essential oil, the effect was alleviated possibly because stress cell signaling in L02 and HeLa cells was stimulated and responded during this period. These results reveal that the essential oil from *E. dense* is cytotoxic, exerting stronger inhibitory effect on human hepatoma cells than normal liver cells. Therefore, it has great potential for development as an anti-liver cancer drug.



**Figure 2:** Inhibitory effects of the essential oil from *E. dense* on proliferation of L02, SMMC-7721 and HeLa cells. (A): 8 hour treatment with the essential oil; (B): 24 hour treatment with the essential oil. DMSO: cells were treated with 1 % DMSO; \*  $p < 0.05$  relative to DMSO treated cells, \*\*  $p < 0.01$  relative to DMSO treated cells

## CONCLUSION

The yield of the essential oil from *E. dense* increased to 0.36 % with the use of the modified steam-distillation extraction method. The main chemical components of the essential oil from *E. dense* are rosefuran epoxide, 2-ethyl imidazole, durenol, *p*-cymene, and 1-cyanoacetyl-3,5-dimethylpyrazole. The essential oil of *E. dense* is cytotoxic, exerting stronger inhibitory effect on human hepatoma cells than normal liver cells. Therefore, the essential oil has great potential for development as an anti-liver cancer drug. Further studies are required to identify the active components responsible for its anti-tumor activity.

## DECLARATIONS

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### Conflict of Interest

No conflict of interest 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 pertaining to claims relating to the content of this article will be borne by them.

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