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CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM *OPISTHOPAPPUS TAIHANGENSIS* (LING) SHIH AND ITS ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXICITY PROPERTIES

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Abstract

This study aims to investigate the chemical composition of essential oil from a Chinese endemic species *Opisthopappus taihangensis* (Ling) Shih and its biological properties. GC/MS analysis of the essential oil identified 36 components, composing 85.3 % of the total oil. The major constituents were germacrene D (13.1 %), caryophyllene (6.6 %), β-eudesmol (6.5 %), caryophyllene oxide (5.1 %), 3-carene, 2-(acetylmethyl)- (4.7 %), eucalyptol (4.7 %) and borneol acetate (3.9 %). The results of *in vitro* antimicrobial activity indicated the essential oil showed a favorable antimicrobial efficiency against *Escherichia colii*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* of which the MIC values were 12.5, 3.125, 1.563 and 3.125 mg/ml, respectively. Moreover, the oil displayed good DPPH scavenging capacity with the IC_{50} of 25.28 mg/ml. Cytotoxicity test showed the oil possessed impressive cytotoxic capacity against H-22 but weak effect against AML-12 with the same dose. Above all, the *O. taihangensis* essential oil maintained significant bio-activity with favorable practical potency.

Introduction

Essential oils (EO) are volatile secondary metabolites derived from aromatic plants. They have been used as drugs, spices and preservatives from ancient times. They were recognized to own antibacterial, antioxidant, hepatoprotective, anti-inflammatory, antiviral and anticancer potency, and even be effective in the prevention and clinical recovery against heavy metal intoxication (Flora *et al*. 2013). The impressive functions of the natural products have captured more interest and have been widely applied in food, cosmetics and pharmaceutical industries.

Compared to synthetic antioxidants, the natural products are more environmentally friendly and have a greater safety with little effects (Ali *et al*. 2014). Though they could be ideal alternatives for food preservation and diseases treatment, research related to EO and other natural products with high quality still needs massive work.

Opisthopappus taihangensis (Ling) Shih belonging to *Opisthopappus* classified in subtribe Anthemideae of the family Asteraceae, is a perennial herb endemic to China and mainly distributed in Taihang Mountains. *O. taihangensis* maintains a good biological property of cold resistance, drought and shade tolerance due to the cliffy and rocky growth condition. Local people used to make tea from the flower of this medicinal plant for liver-clearing and eyesight improving. Now studies about *O. taihangensis* mainly focus on the genetic diversity, intergeneric hybridizations, etc. The biological activities of flavonoid compounds were also detected in previous literature. It demonstrated that *O. taihangensis* extract is richer in quercetin, rutin and chlorogenic acid compared with *Dendranthema indicum*. The high content of bio-active constitutes contribute most to its *in vitro* antioxidant potency by DPPH and ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)) assay, as flavonoids are repeatedly reported to have biological activities. The antibacterial activity of *O. taihangensis* ethanol extract possessed a marked inhibiting effect against most tested microbe strains at previous time.

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Since the research on EO of *O. taihangensis* is still unknown, in the present study the chemical composition and *in vitro* biological activities of EO of *O. taihangensis* were investigated.

Materials and Methods

The aerial parts of *O. taihangensis* were collected in the experimental greenhouse of Zhengzhou University in September-October of 2016. Fresh aerial parts of *O. taihangensis* (100 g) were subjected to sonication in an ultrasonic cleaning instrument for 30 min and hydrodistillion for 4 hrs in a Clevenger-type apparatus. The oil was dried, filtered and preserved in a sealed vial at –20ºC, with the mean yield 0.52% (v/w).

EO of *O. taihangensis* was analyzed using Agilent GC/MS (7890A) instrument under the following conditions. DB-5 MS fused silica capillary column (30 m × 0.25 mm × 0.2 5 µm film thickness); carrier gas: helium at 1.0 ml/min in constant flow, injection volume 1 µl with split ratio 1 : 10, injector temperature 280ºC, column temperature: 50ºC (1 min), to 200ºC at 10ºC/min (2 min) then to 260ºC at 5ºC/min (5 min); Mass spectra: electronic impact at 70 eV and ion source temperature at 230ºC. Mass spectra were set at 35 - 550 a.m.u range. The identification of the constituents was evaluated on the basis of retention index (RI), MS library database (NIST-08), Pubchem, Look Chem database and by comparing with previous literature data.

The antimicrobial activity of the EO of *O. taihangensis* was tested against 5 strains The test microbes were *Escherichia colii* (ATCC25922), *Staphylococcus aureus* (ATCC29213), *Salmonella enterica* (ATCC13076), *Bacillus subtilis* (CMCC(B)63501) and *Candida albicans* (ATCC10231). Microbe suspension was made and adjusted to 10^6 cfu/ml in 0.85 % NaCl by 0.5 McFarland before used.

The disc-diffusion assay was used according to the method of Hussain *et al.* (2008) and Miladi *et al.* (2013) with modification. Kanamycin (1 mg/ml, Genview, America) and Ketoconazole (0.5 mg/ml, Aladdin, China) were used as bacterial and yeast positive control, respectively to determine the sensitivity of each tested microbes. After incubation for 24 hrs, the diameter of inhibition zone was measured using a vernier caliper (0 ~ 150 mm).

The MIC for microbes was determined by modified broth microdilution assay according to the method of Harzallah *et al*. (2011) and Sobrinho *et al.* (2016). The EO was mixed in methanol and diluted to 100 mg/ml as the initial concentration and methanol was adjusted to 1% (w/w). A series of successive two fold dilutions was prepared from 100 mg/ml to 0.1953 mg/ml. Each well of 96-well plate contained 100 µl of oil sample and 100 µl of adjusted suspension of microbes. Sterile water was used as negative control whereas Kanamycin and Ketoconazole were the corresponding positive control. The plates were incubated for 24 hrs and then the growth of microbe was visually detected. The MIC was defined as the lowest EO concentration at which the microbe did not show any visible growth.

The free-radical scavenging activity of *O. taihangensis* EO was measured by DPPH assay referred to Miladi *et al.* (2013) with modification. In a test tube, 0.2 ml of the EO at different concentration ranging from 17.44-87.2 mg/ml, was mixed with 4.8 ml DPPH (Alfa Aesar, America) in methanol (0.04 mg/ml). The mixture was shaken vigorously and stands in the dark for 30 min. The absorbance of the system was measured at 517 nm. DPPH scavenging activity was calculated by the equation below:

$$IP\ (\%) = [(Abs_c - Abs_s)/Abs_c] \times 100 \qquad (1)$$

where *IP* (%) is the inhibition percentage; Abs_c is the absorbance of the control at 30 min and Abs_s is the absorbance of the sample at 30 min. Ascorbic acid (Vitamin C, Aladdin, China) was the positive control. IC_{50} was defined as the sample concentration scavenging 50% of DPPH.

The antioxidant activity of the *O. taihangensis* EO was detected by bleaching of β-carotene/linoleic acid emulsion system described by Kadri *et al*. (2011) and Sobrinho *et al.* (2016) with adoptions. A stock solution of β-carotene/linoleic acid mixture was prepared by dissolving 0.2 ml of 1mg/ml β-carotene (Aladdin, China) in chloroform, 20 µl linoleic acid and 200 µl Tween 80. Chloroform was completely removed under vacuum in rotary evaporator at 50ºC. Then, 50 ml of distilled water was added and the mixture was shaken vigorously for 10 min. A 4.8 ml of this emulsion was dispensed to test tubes containing 0.2 µl of the EO at concentrations of 1 - 20 mg/ml in methanol solution. Sample readings were taken immediately after blended evenly in the spectrophotometer at 470 nm and a second reading was conducted after the samples were put in a 50ºC water bath for 120 min. The negative control was 4.8 ml of the emulsion alone. The blank control was the emulsion without β-carotene. BHT was positive control. The percentage inhibition was calculated as the equation below:

$$I\ (\%) = [(Abs_{s1} - Abs_{s2}) / (Abs_{c1} - Abs_{c2})] \times 100 \qquad (2)$$

where I (%) is the inhibition percentage; Abs_{s1} is the absorbance of the EO at t = 0 min, Abs_{s2} is the absorbance of the EO at t = 120 min, Abs_{c1} is the absorbance of the control at t = 0 min, and Abs_{c2} is the absorbance of the control at t = 120 min.

Mouse normal liver cells (AML-12) and mouse hepatocellular carcinoma (H-22) were used as test cell lines. AML-12 was grown in DMEM/F-12 with Insulin-Transferrin-Selenium and RPMI 1640 was for H-22. The medium was also mixed with 10% fetal bovine serum 1% (w/v), glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cell lines were cultured in a incubator at 37ºC in 5% CO_2.

The cytotoxic effects of the EO were tested by the MTT assay. A MTT Cell Proliferation and Cytotoxicity Assay Kit (Genview, America) was used. Optical density (OD) of the plate was measured using a Molecular Devices Microplate Reader (CMax Plus) at 490 nm. The values of the blank wells were subtracted from the tested and the control. The percentage viability was calculated following below formula:

$$Viability\ (\%) = [(Abs_s - Abs_b) / (Abs_c - Abs_b)] \times 100 \qquad (3)$$

where viability (%) is the growth viability of cell, Abs_c is the absorbance of the control, Abs_b is the absorbance of the blank and Abs_s is the absorbance of the sample. The test was conducted in quintic for two independent times.

All experiments were performed in triplicate (except cytotoxicity test) and the results were expressed as mean ± standard deviation (SD). Statistics differences were analyzed by one-way ANOVA with SPSS 17.0. Significance of difference was accepted at $p < 0.05$. IC_{50} values were also calculated using SPSS 17.0.

Results and Discussion

Results showed that the yield of EO of *O. taihangensis* was approximately 0.52 % (v/w) on a fresh weight basis. The compositions of the EO, the percentages, calculated RI and RT are listed in Table 1. Thirty six components of the EO were identified under the experimental condition, accounting for 85.3 % of the total. The oil was dominated by Germacrene D (13.1%), followed by caryophyllene (6.6 %), β-eudesmol (6.5 %), caryophyllene oxide (5.1 %), eucalyptol (4.7 %), 3-Carene, 2-(acetylmethyl (4.7 %) and borneol acetate (3.9 %).

Table 1. Chemical composition of the EO obtained from the aerial parts of *O. taihangensis*.

No.	Compounds	%	RI_{cal}[A]	RT[B]
1	α-Pinene	2.3	929	5.401
2	β-Pinene	0.4	979	6.114
3	β-Myrcene	0.9	991	6.224
4	Eucalyptol	4.7	1032	6.970
5	Pincarveol	0.6	1139	8.688
6	Camphor	2.0	1143	8.802
7	Pinocarvone	0.5	1164	9.005
8	Borneol	2.4	1167	9.158
9	Terpinen-4-ol	1.4	1182	9.272
10	α-Terpineol	1.3	1189	9.475
11	Borneol acetate	3.9	1284	10.759
12	Terpinyl acetate	1.6	1350	11.597
13	Caryophyllene	6.6	1419	12.674
14	β-Copaene	0.7	1432	12.777
15	Humulene	1.3	1454	13.125
16	γ-Muurolene	1.9	1477	13.332
17	Germacrene D	13.1	1481	13.463
18	Aromadendrene	0.6	1486	13.557
19	Bicyclogermacrene	1.3	1495	13.623
20	δ -Cadinene	0.4	1518	13.848
21	β-elemene	3.2	1549	14.224
22	Nerolidol	0.6	1564	14.309
23	Spathulenol	1.5	1577	14.637
24	Caryophyllene oxide	5.1	1581	14.724
25	Cineole	2.4	1631	15.26
26	Cyclosativene	2.0	1727	15.362
27	β-Eudesmol	6.5	1649	15.565
28	Isolongifolene, 9,10-dehydro-	0.8	1622	15.681
29	α-Bisabolol	2.5	1684	15.812
30	cis-Z-α-Bisabolene epoxide	0.8	1695	15.87
31	3-Carene, 2-(acetylmethyl)-	4.7	1951	19.482
32	n-Hexadecanoic acid	1.9	1968	19.584
33	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	0.9	2106	20.019
34	Phytol	2.8	2114	22.079
35	Linolenic acid ethyl ester	0.4	2171	23.036
36	Heptacosane	0.5	2700	31.683
Major groups		%		
Monoterpene hydrocarbons		3.6		
Oxygenated monoterpenes		17.5		
Sesquiterpene hydrocarbons		26.7		
Oxygenated sesquiterpenes		27.0		
Diterpene		7.6		
Others		2.8		
Total identified		85.3		

[A] RI_{cal} is Calculated Retention Index on the HP-5MS column. [B] RT is Retention Time.

In the chemical composition analysis by GC-MS, sesquiterpenes and oxygenated sesquiterpenes were found to be the predominant fraction comprising 53.7 % of all, and oxygenated monoterpenes also attained a proportion of 17.5 %. No previous literature reported the composition analysis of *O. taihangensis* EO, but many of these identified constituents were widely found in other genus of the Asteraceae family, namely *Anthemis odontostephana* (Sajjadi *et al*. 2014), *Eupatorium intermedium* (Czaikoski *et al*. 2016) and *Wedelia prostrate* (Dai *et al*. 2013). Many compounds such as caryophyllene, caryophyllene oxide, terpinen-4-ol, the main constitutes of *O. taihangensis* EO with biological activities, were detected. Previously a wide spectrum of antimicrobial activity was reported (Carson and Riley 1995, Matasyoh *et al*. 2009).

The antimicrobial activity results of EO of *O. taihangensis* presented in Table 2 and Fig. 1 showed that the EO had a broad spectrum of antibacterial property against the most test microbes. The most significant effect of the oil was against *B. subtilis*, with the largest inhibition zone diameter (18.94 ± 1.18 mm) and smallest MIC (1.563 mg/ml). The inhibiting potency of EO against *B. subtilis* was as effective as the antibiotic Kanamycin of 1mg/ml. *S. aureus* and *C. albicans* were after that which shared the similar diameter of inhibition zones and the same MIC value (3.125 mg/ml). There was no significant difference ($p < 0.05$) between the two. The effect against *E. colii*, of which the diameter was 7.32 ± 0.23 mm with the MIC value (12.5 mg/ml). *S. enterica* was the least susceptible strain in the test, showing no visible inhibition zone. It is evident that the EO performed better inhibitory against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and yeast (*C. albicans*) compared to Gram-negative bacteria (*E. colii* and *S. enterica*).

Table 2. Antimicrobial activity of the *O. taihangensis* EO expressed as inhibition zone diameter and minimum inhibitory concentration (MIC).

Strain	Types	IZ[A] (mm±SD)			MIC values (mg/mL)		
		Essential oil	Kanamycin	Ketoconazole	Essential oil	Kanamycin	Ketoconazole
E.coli ATCC25922	Gram-negative	7.32 ± 0.23c	18.60±1.23	NT[B]	12.5	0.391	NT
S.aureus ATCC29213	Gram-positive	11.09 ± 1.03b	24.77±0.60	NT	3.125	0.391	NT
S. enterica ATCC13076	Gram-negative	-[C]	14.91±0.58	NT	NT	NT	NT
B.subtilis CMCC(B)63501	Gram-positive	18.94 ± 1.18a	18.92±1.54	NT	1.563	0.002	NT
C.albicans ATCC10231	Yeast	10.38 ± 0.64b	NT	13.96 ± 0.71	3.125	NT	0.008

A = IZ is Inhibition Zone in diameter (mm ± SD) around the discs impregnated with 10 µl of the EO. B = NT is Not Tested. C = is no visible IZ.

Besides, the oil compositions, such as α-pinene, β-pinene, (-)-Germacrene D, etc. have been reported to exhibit significant antimicrobial activity (Da *et al*. 2012). The interactions between compounds and diffusion of various hydrophobic agents across the cell membrane might also affect the antimicrobial effect (Rashid *et al*. 2013). In the test, Gram-negative bacteria were less susceptible to the oil. It might be due to the lipopolysaccharide in the outside membrane resisting the antimicrobial agents (Cosentino *et al*. 1999).

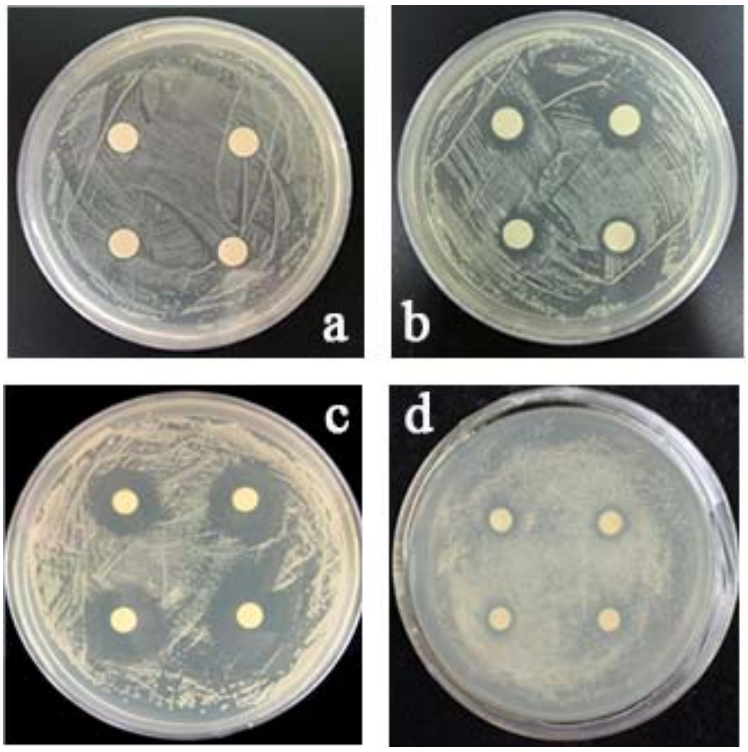

Fig. 1. The inhibition effect of *O.taihangensis* EO against *E.colii*-a, *S.aureus*-b, *B.subtilis*-c and *C.albicans*-d.

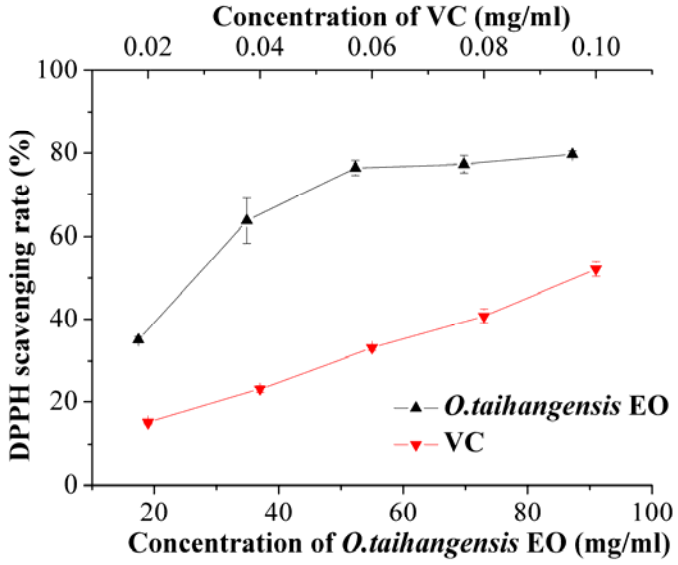

Fig. 2. Antioxidant activity of the EO of *O. taihangensis* by DPPH scavenging assay.

The results of DPPH test are shown in Fig. 2. At the EO concentration of 17.44, 34.88, 52.32, 69.76 and 87.2 mg/ml, the percentage of inhibition ranged from 35.01 to 79.65%. The EO performed its antioxidant activity with an IC_{50} = 25.28 mg/ml (Table 3). The reference standard, ascorbic acid, exhibited an IC_{50} = 0.105 mg/ml, which showed much higher DPPH radical scavenging activity compared to the *O.taihangensis* EO. According to relevant lectures, the antioxidant potential of EOs might be attributed to the chemical compositions (Derwich *et al.* 2011).

In the β-carotene/linoleic acid system, however, the oil showed weak inhibiting capacity in the tested concentration range (Fig. 3). The major reason was the presence of some turbidity in the system when using high concentrations of EO, which affected the detection of the absorbance. Some small organic molecular substances insoluble in water was found to precipitate out from the water system, forming the milky white deposit. Thus, low-dose of the EO range was used with low inhibiting rate, and no IC_{50} was estimated by SPSS. The reference standard BHT showed favourable bleaching inhibiting effect with the IC_{50} of 7.38 μg/ml.

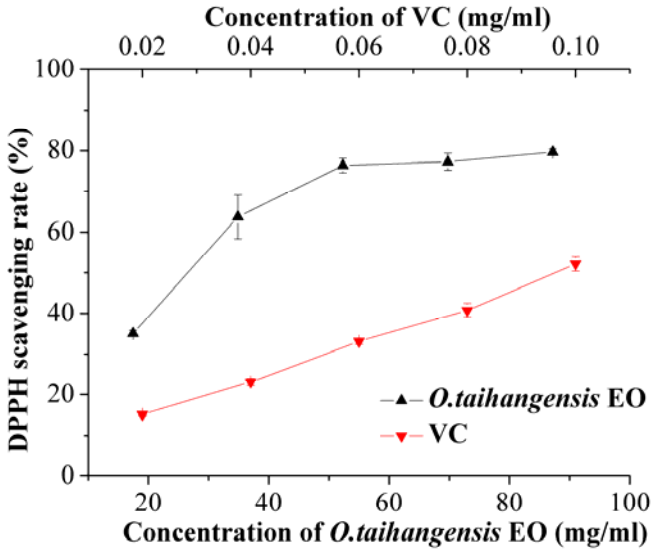

Fig. 3. Antioxidant activity of the EO of *O. taihangensis* by β-carotene/linoleic acid assay.

Table 3. Total antioxidant capacity of the *O. taihangensis* EO according to the DPPH and β-carotene/linoleic acid assays.

Methods	DPPH	β-carotene/linoleic acid
	IC_{50}[A]	IC_{50}
EO	25.28 mg/ml	NC[B]
Ascorbic acid	0.11 mg/ml	-[C]
BHT	-[C]	7.38 μg/ml

[A] IC_{50} is defined as the sample oil concentration of 50 % inhibiting effect estimated in 100 %.
[B] NC is not calculated. [C] - is not test.

Cell viability was determined by MTT kit assay. Fig. 4 showed the cytotoxic activity of the oil against the two tested cell lines, mouse hepatocellular carcinoma H-22 and mouse liver cell AML-12. The cell viability of H-22 decreased remarkably from 50 to 250 μg/ml concentrations. The estimated IC_{50} of the oil against H-22 by SPSS was 91.307 μg/ml. However, AML-12 still performed high cellular viability when the concentration increased to the range of 400 to 600 μg/ml. AML-12 even exhibited better cell proliferation viability over 100% compared to the blank control when using low dose of EO (below 400 μg/ml).

The results of MTT assay demonstrated the impressive cytotoxic activity of *O. taihangensis* EO against the liver cancer cell. The cytotoxicity was likely due to the comprehensive effect of the majority fractions of the oil, including oxygenated monoterpenes and sesquiterpenes that made up over 75 % of the total. Components with high content included caryophyllene and caryophyllene oxide, germacrene D and β-eudesmol, and were proved to have anticancer capacity in other reports and they should contribute greatly to inhibit the growth of cancer cell. *O. taihangensis* EO exhibited high cytotoxicity against the mouse liver cancer cell, which coincides with the traditional function of Chinese traditional chrysanthemum, and it may have a potential therapeutic significance in cancer treatment. More investigations about the concrete components making function and their mechanism need to be conducted in near future.

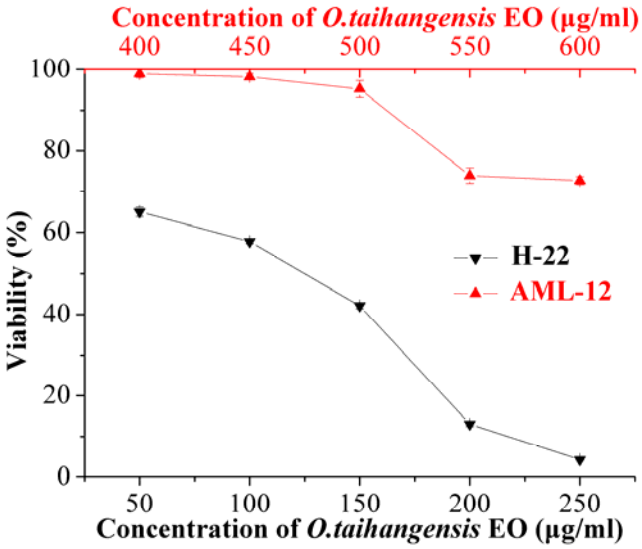

Fig. 4. Cytotoxic activity of the *O. taihangensis* EO against two mouse cell lines.

In the present study, the basic components of *O. taihangensis* EO were identified and proved its potential to resist the growth of microbes, especially against Gram-positive bacteria. It was presumed to correlate to sesquiterpenes and oxygenated monoterpenes. Those constitutes were recorded to have strong biological activities. On the other hand, about 15% of the oil was unidentified substances, requiring more precise analysis assay to make it clear and detect the function. Moreover, the oil possessed free-radical scavenging capacity. According to previous research findings, the antioxidant potential of EOs could be attributed to the chemical compositions (Derwich *et al.* 2011) and the polarities of their chemicals (Ebrahimabadi *et al.* 2010). The most interesting observation was that low dose of EO performed strong lethality against H-22 cancer cell

but little effect on the normal liver cell lines with the same dose applied. Thus, the EO needs further concern and study to detect its ant-cancer ability, and which compound function. It is also inferred that interaction between those substances make difference. Thus, aiming to the effective single component, systematic chemical identification and biological activity detection assays are needed at cell or molecular level.

This is the first comprehensive study on the EO of *O. taihangensis*, providing new insights into the biological function of the medicinal plant. The *O. taihangensis* EO was proved to maintain antimicrobial, antioxidant and cytotoxic activities. It is expected to be favourable in pharmaceutical and food industry field. The oil could be a natural alternative to certain synthetic antibacterial agents or antioxidants. Also, certain effective constitutes with high content could be extracted, purified and used in food preservation, cosmetics or drug agents for further exploitation.

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