Chemical Composition, DPPH Free Radical Scavenging and Antimicrobial Activity of the Essential Oil and Six Compounds Isolated from *Spiraea mongolica* Maxim

ZHANG Wen-huan[1], QIAN Han[1], SONG Ya-jie[2], SHEN Tong[1,3]

(1.College of Chemistry and Bioengineering, Lanzhou Jiaotong University, Lanzhou 730070, China) (2.Marine College, Shandong University, Weihai 264200, China) (3.Lanzhou Shichuang Biotechnology Company, Lanzhou 730070, China)

Abstract: The essential oil was extracted from the dried twigs and leaves of *Spiraea mongolica* Maxim by hydro-distillation and identified by GC-MS. The main compositions of the oil were fatty acids and their derivatives, ethyl palmitate (38.631%), ethyl linolelaidate (23.576%) and ethyl linolenate (14.634%). From the dried twigs and leaves, botulin (1), lupine-3,20-diol (2), 1*β*-hydroxyl-6,9-dien-8-oxoeremophil -11-nor-11-ketone (3), 3-(4-Methoxyphenyl) propanal (4), stigmasterol (5), *β*-Sitosterol (6) were isolated by silica gel column chromatography and identified by NMR data. Their DPPH free radical scavenging and antimicrobial activities were separately evaluated. The essential oil showed good to moderate DPPH free radical scavenging activity (IC_{50} = 900 μg/mL), while had no apparent antimicrobial activity. Compound (4) exhibited reasonably strong DPPH free radical scavenging ability (IC_{50} = 13 μg/mL) and showed diverse antimicrobial competence against *Aspergillus flavus*, *Bacillus subtilis* and *Candida albicans*. Compound (3) showed antimicrobial activity against *Aspergillus flavus, Bacillus subtilis* and weak DPPH free radical scavenging activity. These results of the study had certain significance in the study of traditional Chinese medicine plant and development of new drugs.

Key words: *Spiraea mongolica* Maxim; essential oil; GC-MS; DPPH free radical scavenging activity; antimicrobial activity

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蒙古绣线菊六个化合物及其挥发油化学成分和清除 DPPH 自由基活性、抗菌活性的测定分析

张文环[1]，钱瀚[1]，宋亚洁[2]，沈彤[1,3]

（1.兰州交通大学化学与生物工程学院，甘肃兰州 730070）（2.山东大学（威海）海洋学院，山东威海 264200）（3.兰州世创生物科技有限公司，甘肃兰州 730070）

摘要：通过 GC/MS 对水蒸气蒸馏法提取的蒙古绣线菊枝、叶挥发油化学成分进行分析。挥发油的主要成分为脂肪酸及其衍生物，其中棕榈酸乙酯占挥发油总含量 38.631%、反亚油酸乙酯 23.576%、亚麻酸乙酯 14.634%。通过硅胶柱色谱法分离和核磁鉴定，白桦脂醇(1)、羽扇豆醇-3,20-二醇(2)、1*β*-羟基-6,9-二烯-8-羰基-11-降-11-二酮(3)、3-(4-甲氧基苯基)丙醛(4)、豆甾醇(5)、*β*-谷甾醇(6)六个化合物从蒙古绣线菊枝、叶中分离出来。对它们清除 DPPH 自由基活性、抗菌活性也分别进行了测定。试验结果表明挥发油有良好的清除 DPPH 自由基活性(IC_{50}=900 μg/mL)，对所选菌株没有明显的抗菌活性。化合物(4)显示出较强的清除 DPPH 自由基活性(IC_{50}=13 μg/mL)，对黄曲霉、枯草芽孢杆菌和白色念珠菌均有明显的抑菌活性。化合物(3)清除 DPPH 自由基活性微弱，对黄曲霉和枯草芽孢杆菌显示明显的抑菌活性，这些研究结果对传统中草药的研究和新药物的研制具有一定的意义。

关键词：蒙古绣线菊；挥发油；GC-MS；清除 DPPH 自由基活性；抗菌活性

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作者简介：张文环（1987-），女，硕士研究生，主要从事天然产物的分离、提取及生物活性测定分析

通讯作者：沈彤（1971-），男，教授，主要从事天然产物化学及有机化学合成的研究与开发

The genus *Spiraea* comprises about one hundred species, mainly distributed in the northern temperate zone. The current distribution and differentiation center of this genus in China possess seventy species[1,2]. In traditional medicine, some of the genus *Spiraea* leaves, fruits, and roots are used to treat inflammation, cough, headache, toothache, malaria and fever, and also as diuretic, narcotic, analgesic and emetic[3,4]. The hetisine-type C_{20}-diterpene alkaloids isolated from *S. fritschiana var. parvifolia* and its derivatives showed a certain effect on antiplatelet aggregation activity[5]. The spiramine C from *S. japonica L.f. var. acuta Yu* and *S. japonica L. f. var. ovalifolia Franch* and its derivatives spiramine C1 could inhibit PAF-induced platelet aggregation and had concentration-dependent property[6]. Water extracts of *S. pubescens* could reduce alcohol-induced glutathione depletion, decrease the content of MDA (methane dicarboxylic aldehyde) and reduce the lipid peroxidation of liver damage caused by the alcohol[7]. Three caffeoyl hemiterpene glycosides from *S. prunifolia* have been determined to have potent anti-oxidative and anti-inflammatory activities[8].

S. mongolia Maxim with relatively small production is mainly distributed over Inner Mongolia, Jilin and Ningxia provinces[9]. This plant is often used as folk Tibetan medicine and Mongolia medicine to treat swelling and ulcer on the body surface, trauma, ascites, pulmonary congestion, uterine bleeding and other diseases[10]. Xie H. H. obtained betulin, betulinic acid, betulinin acid-3,5dihydroxyl-cinnamate and daucosterol from the isolation of *S. mongolia* Maxim for the first time[11]. Learned from literatures, betulin and its derivatives showed great potential in anti-HIV and cancer treatment as biological agents, particularly betulinic acid with a wide range of biological and pharmacological activities, it had anti-HSV-1, anti-ulcer and analgesic effect, could inhibit plasmodium falciparum and deworm[12~16]. Hence the essentiall oil and the extraction of *S. mongolia* Maxim were worthy of study.

1 Experimental

1.1 Plant material and extraction of the essential oil

The twigs and leaves of *S. mongolia* Maxim were collected from Hezuo Country Gansu Province P. R. China in August 2014, and identified by Prof. Yongqiang Tian from College of Chemistry and Bioengineering, Lanzhou Jiaotong University. The voucher specimen (NO. LP14004) was deposited in the Laboratory Natural Products Research, College of Chemistry and Bioengineering, Lanzhou Jiaotong University.

The essential oil of the air-dried twigs and leaves (500 g) from *S. mongolia* Maxim was obtained by hydro-distillation for 5 hours at 180 ℃. The essential oil was dried over anhydrous sodium sulfate, and then it was stored in refrigerator at 4 ℃ until GC-MS analysis. It was extracted in yield of 0.03%.

1.2 GC/MS analysis

Analytical GC of the essentia oil was carried out using a Hewlett Packard 6890 equipped with flame ionization detector (FID) and a HP-5 MS capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm). Oven temperature program: 60~280 ℃ (60~200 ℃ at 15 ℃/minute; 200~280 ℃ at 5 ℃/minute), 200 ℃ (5 minutes), 280 ℃ (20 minutes); injector temperature: 280 ℃; carrier gas: helium, adjusted to a linear velocity of 40.118 cm/second; injection volume of 0.2 μL splitting ratio 1:40. GC/MS analysis of the essential oil was performed on a Hewlett Packard 6890 GC coupled with a Hewlett Packard mass selective detector 5973 using HP-5 MS fused silica column (30 m × 0.25 mm). The essential oil compounds were identified from their GC retention indices (RI), comparison of their mass spectra to those recorded in NIST and fragmentation patterns reported in literature. These identified essential oil compositions and their percentage were presented in Table 1.

1.3 Extraction and isolation from *S. mongolia* Maxim

The air-dried twigs and leaves of *S. mongolia* Maxim (5.5 kg) was powdered and then extracted three times with methanol (7 d each time) at room temperature. The extract was concentrated under decompressed distillation to get a residue of 500 g. Then

the extract (500 g) was isolated by a silica gel column chromatography (CC, 200-300 mesh, 3000 g) eluting with n-hexane-acetone (15:1, 10:1, 5:1 and 2:1) to obtain four fractions (Fr.1 - Fr.4). Fr.2 (n-hexane-acetone = 10:1, 80 g) was isolated by CC (300-400 mesh, 2000 g) eluting with $CHCl_3$-Acetone (30:1, 20:1, 15:1, 10:1), four small fractions (Fr.2-1, Fr.2-2, Fr.2-3, Fr.2-4) were correspondingly got. After repeatedly purified by CC, compound 5 (30 mg), 6 (15 mg) and 1 (500 mg) were respectively obtained from the fraction of $CHCl_3$-Acetone elution (Fr.2-1, Fr.2-1, Fr.2-2). Fr.3 (n-hexane-acetone = 5:1, 100g) was isolated by CC (300-400 mesh, 2000g) eluting with $CHCl_3$-Acetone (20:1, 15:1, 10:1, 5:1), four small fractions (Fr.3-1, Fr.3-2, Fr.3-3, Fr.3-4) were correspondingly got. After repeatedly purified,-compound 3 (30 mg), 2 (12 mg) and 4 (18.6 mg) were respectively obtained from the fraction of $CHCl_3$-Acetone elution (Fr.3-1, Fr.3-1, Fr.3-3). Their chemical structures were showed in Fig.1.

1.4 DPPH free radical scavenging activity

To assess the antioxidant potential of the essential oil and compounds, different concentration gradients (ranging from 2.5 μg/mL to 10 mg/mL) were adopted to evaluate its radical scavenging rate against 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) with the standard antioxidants butylated hydroxyl toluene (BHT) as the positive control. The detailed procedure was performed by following reported method earlier[17, 18]. Inhibition of free radical DPPH in percent (I%) was calculated using the equation: $I(\%) = [(A_0 - A_t) / A_0] \times 100\%$, where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of the blank sample. The sample concentration providing 50% inhibition (IC_{50}) was roughly read out from the inhibition percentages trend graph. All the experiments were repeated three times and all measurement were performed in triplicate. The DPPH free radical scavenging activity of the essential oil and four compounds was presented in Fig.2.

1.5 Antimicrobial activity

The essential oil and compounds were tested against 8 micro-organisms. Reference strains were as follows: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus subtilis ATCC 6633*, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Aspergillus flavus* ATCC 9643, *Mucor mucedo* ATCC 9643, *Phytophthora parasitica* ATCC 62653. The disc agar diffusion method was employed for the estimation of antimicrobial activity. The positive controls were chloramphenicol and nystatin. Briefly, a suspension liquid of standard micro-organism (2×10^8 CFU/mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were individually impregnated with 10 μl of the diluted essential oil liquor (10 mg/mL), compound liquor (10 mg/mL), chloramphenicol (1 mg/mL) and nystatin (1 mg/mL), and then placed on the incubated plates one night at 37 ℃ for bacteria and 72 hours at 28 ℃ for the fungus. The DDs were measured and expressed in millimetre. All the experiments were repeated three times, all measurements were performed in triplicate and were expressed as average of three analyses ± standard deviation. The results of antimicrobial activity are presented in Table 2.

2 Results and discussion

2.1 Chemical constituent of the essential oil

In Table 1, about 27 compounds were identified from essential oil. The primary components were fatty acids and their derivatives. The lipid compounds accounted for most of the proportion of the essential oil. A high content of ethyl palmitate (38.631%) was the major constituents, followed by ethyl linolelaidate (23.576%), ethyl linolenate (14.634%) and ethyl myristate (6.47%). The presence of ethyl 9-hexadecenoate (2.099%), hexahydrofarnesyl acetone (2.093%), ethyl cinnamate (1.972%) and ethyl stearate (1.592%) was also significant for the oil profile. Due to a large number of literature reports, the genus *Spiraea* plants contain a great deal of terpenoids and some alkaloids[5~7], so this result is unexpected, and it just verifies the reason why few people go to further study the essential oil of *S. mongolia* Maxim. While these substances are important materials for fine chemistry and organic intermediate, also widely used in medicines, cosmetic industry and food additive field, especially palmitate (38.631%) has been heavily used in

industrialized production as food additives.

2.2 Six compounds isolated from *S. mongolica* Maxim

From the air-dried twigs and leaves of *S. mongolia* Maxim, six compounds were obtained by the silica gel column chromatography, thereinto five compounds are terpenoids[5~7]. At the same time, betulin (1) was extracted in quantity to 500 mg. As all known, betulin (1) and betulinin acid have very high bioactivity, so the plant *S. mongolica* Maxim is worthy of attention. The following are the spectral data of six compounds.

Betulin (1): white needles; mp 252-253℃; 1H-NMR (500MHz, CDCl3): δH (ppm) 0.670 (m, 1H, -CH); 0.751 (s, 3H, -CH3); 0.812 (s, 3H, -CH_3); 0.960(s, 3H, -CH_3); 0.970 (s, 3H, - CH_3); 1.587 (s, 3H, - CH_3); 1.665 (s, 3H, - CH_3); 1.850 (m, 1H, -CH); 2.380 (m, 1H, -CH); 3.180 (m, 1H, -CH); 3.790 (m, 1H, -CH); 4.578 (d, J= 1.4, 1H, =CH); 4.583 (d, J= 1.4, 1H, =CH). ^{13}C-NMR (125MHz, $CDCl_3$): δ 38.72(C1); 27.48 (C2); 78.99 (C3); 34.00 (C4); 55.31 (C5); 18.31 (C6); 34.25 (C7); 40.94 (C8); 50.42 (C9); 37.18 (C10); 20.85 (C11); 25.23 (C12); 37.32 (C13); 42.73 (C14); 27.06 (C15); 29.19 (C16); 42.74 (C17); 48.78 (C18); 47.81 (C19); 150.48 (C20); 29.77 (C21); 33.98 (C22); 27.99 (C23); 27.99 (C24); 16.11 (C25); 18.32 (C26); 15.36 (C27); 60.57 (C28); 109.7 (C29); 20.85 (C30).

lupine-3,20-diol (2): microcrystals; mp 162-163℃; ^{1}H-NMR (500MHz, $CDCl_3$): δH (ppm) 0.84 (s, 3H, -CH_3); 0.81 (s, 3H, -CH_3); 0.96 (s, 3H, -CH_3); 1.12 (s, 3H, -CH_3); 0.97 (s, 3H, -CH_3); 0.76 (s, 3H, -CH_3); 1.06 (s, 3H, -CH_3); 1.22 (s, 3H, -CH_3). ^{13}C-NMR (125MHz, $CDCl_3$): δ 79.23 (C3); 73.72 (C20); 55.45 (C5); 50.54 (C19); 50.21 (C18); 48.56 (C9); 44.87 (C17); 43.77 (C14); 41.60 (C8); 40.44 (C22); 39.07 (C4); 38.95 (C1); 37.72 (C13); 37.33 (C10); 35.82 (C7); 34.81 (C16); 31.78 (C30); 29.28 (C21); 28.95 (C15); 28.22 (C23); 27.82 (C2); 27.64 (C12); 25.02 (C29); 21.64 (C11); 19.44 (C28); 18.57 (C6); 16.39 (C24); 16.38 (C26); 15.61 (C25); 15.08 (C27).

1β-hydroxy-6,9-dien-8-oxoeremophil-11-nor-11-ket one (3): colorless gum, 1H-NMR (500MHz, CDCl3): δH (ppm) 4.56 (t, 1H, -CH); 2.07 (dddd, J= 13.0, 3.5, 3.5, 3.2, 1H, -CH2); 1.98 (dddd, J= 13.0, 13.0, 13.0, 3.2, 1H, -CH2); 1.52 (m, 1H,-CH2); 1.61 (m, 1H, - CH2); 1.68 (m, 1H, - CH); 7.67 (s, 1H, =CH); 6.18 (s, 1H, =CH); 2.54 (s, 3H, -CH3); 1.38 (s, 3H, -CH3); 1.15 (d, J= 6.6, 3H, -CH3). ^{13}C-NMR (125MHz, CDCl3): δ 73.34 (C1); 34.48 (C2); 25.06 (C3); 41.06 (C4); 44.35(C5); 161.97 (C6); 136.06 (C7); 184.59 (C8); 126.84 (C9); 165.62 (C10); 198.86 (C11); 31.02 (C12); 18.75 (C13); 16.13 (C14).

3-(4-Methoxyphenyl) propanal (4): yellow powder. ^{1}H-NMR (500MHz, CDCl3): δ 9.649 (d, J= 7.75, 1H, =CHO); 7.154 (m, 4H, -PhH); 6.596 (dd, J= 7.75, 7.70, 1H, =CH); 6.145 (s, 1H, =CH); 3.944 (s, 3H, -CH3).

Stigmasterol (5): white crystal; mp:165-167℃. ^{1}H-NMR (500MHz, $CDCl_3$): δ_H (ppm) 3.51 (m, 1H, -CH); 7.23 (s, 1H, -OH); 0.67 (s, 3H, -CH_3); 0.98 (s, 3H, - CH_3); 5.43 (m, 1H, =CH); 5.32 (dd, 1H, =CH); 5.17 (dd, 1H, =CH). ^{13}C-NMR (125MHz, $CDCl_3$): δ 37.1 (C1); 31.8 (C2); 71.9 (C3); 39.8 (C4); 140.9 (C5); 121.7 (C6); 33.8 (C7); 33.9 (C8); 50.3 (C9); 36.6 (C10); 21.2 (C11); 42.2 (C12); 42.2 (C13); 56.8 (C14); 24.4 (C15); 28.7 (C16); 56.2 (C17); 11.8 (C18); 19.50 (C19); 6.2 (C20); 18.7 (C21); 138.2 (C22); 129.4 (C23); 45.8 (C24); 29.2 (C25); 19.9 (C26); 19.1 (C27); 23.1 (C28); 11.9 (C29).

β-sitosterol (6): white crystal; mp: 136-140℃; ^{1}H-NMR (500MHz, $CDCl_3$): δ_H (ppm) 3.51 (m, 1H, -CH); 7.23 (s, 1H, -OH); 0.67 (s, 3H, -CH_3); 0.98 (s, 3H, -CH_3); 5.46 (m, 1H, =CH). ^{13}C-NMR (125MHz, $CDCl_3$): δ 37.1 (C1); 31.8 (C2); 71.9 (C3); 39.8 (C4); 140.9 (C5); 121.7 (C6); 33.8 (C7); 33.9 (C8); 50.3 (C9); 36.6 (C10); 21.2 (C11); 42.2 (C12); 42.2 (C13); 56.8 (C14); 24.4 (C15); 28.7 (C16); 56.2 (C17); 11.8 (C18); 19.50 (C19).

2.3 DPPH free radical scavenging activity

Given that the compound Stigmasterol (5) and β-sitosterol (6) are common plant components, activity research has been studied thoroughly, so the following antioxidant and antibacterial activity experiments were no longer continued. In Fig.2, the essential oil presented a good trend of antioxidant activity and give the value of IC_{50} about 900 μg/mL, compound (4) showed a considerable antioxidant activity with IC_{50} about 13 μg/mL, other compounds give too weak antioxidant activity compared with positive control BHT (30 μg/mL). So compound (4) was worth to research deeply or give it organic modifications to make them safe, environmental antioxidants in health care industry and cosmetic

industry.

2.4 Antimicrobial activity

It was obvious that the essential oil had no apparent antimicrobial activity against all above pathomycete and pathogenic bacteria from Table 2. The compound (4) had obvious antimicrobial activity against *A. flavus* ATCC 9643, *C. albicans* ATCC 10231 and *B. subtilis* ATCC 6633. The compound (3) had antimicrobial activity against *A. flavus* ATCC 9643 and *B. subtilis* ATCC 6633. Other two compounds had no apparent antimicrobial activity against the above 8 pathogenic bacteria. According to the above results, further investigations needed to be conducted in the future, especially the antimicrobial effect mechanism of compound (4) and (3) needed to be studied more deeply and completely, the range of pathogens should be extended to more microbial species such as more plant pathogens.

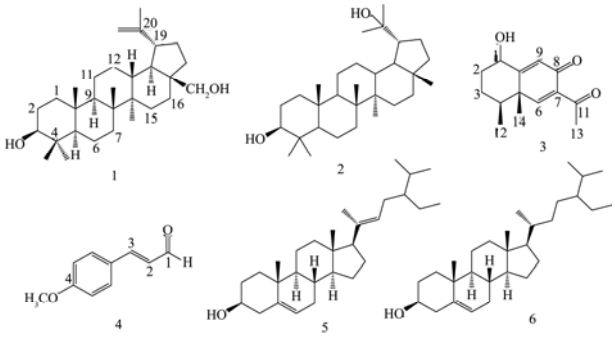

Fig.1 Six compounds isolated from *Spiraea mongolica* Maxim

Table 1 Chemical composition of the essential oil from *Spiraea mongolica* Maxim

Component	RI[t]	RI[a]	Percentage/%
Ethyl cinnamate	9.286	1325	1.97
Ethyl 9-oxononanoate	9.486	1346	0.05
Phosphoric acid, diethyl octyl ester	9.753	1375	0.13
Ethyl dodecanoate	10.186	1420	0.66
Dimethyl sebacate	10.931	1497	0.51
Solaboost SPF	11.786	1567	0.29
Z-7-Tetradecenoic acid	11.919	1578	1.19
Ethyl myristate	12.097	1593	6.47
Hexadecanal	12.375	1611	0.13
Hexahydrofarnesyl acetone	12.697	1631	2.09
Ethyl pentadecanoate	12.819	1638	0.05
Diisobutyl phthalate	12.997	1649	0.39
Ethyl elaidate	13.119	1656	0.16
Ethyl pentadecanoate	13.364	1671	0.54
(E)-Trogodermal	13.830	1699	0.86
Ethyl 9-hexadecenoate	14.786	1741	2.10
Ethyl palmitate	15.608	1778	38.63
Octadecanal	15.941	1793	0.13
Methyl 8-[2-((2-[(2-ethylcyclopropyl) methyl]cyclopropyl)methyl)cyclopropyl]octanoate	16.241	1806	0.07
2-Hydroxy-1,1,10-trimethyl-6,9-epidioxydecalin	17.108	1842	0.11
Ethyl heptadecanoate	17.352	1853	1.26
Geranyl isovalerate	17.496	1859	0.40
Ethyl linolelaidate	19.096	1928	23.58
Ethyl linolenate	19.241	1935	15.73
Ethyl stearate	19.530	1948	1.59
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	19.818	1961	0.16
Octadecanal	20.085	1973	0.06
Total			99.31

Note: RI[a] (retention index): measured relative to n-alkanes (C_6-C_{24}) on DB-5 column.

Table 2 The antimicrobial activity of essential oil and compounds from *Spiraea mongolica* Maxim

Micro-organism	Essent-ial oil	1	2	3	4	Positivecontrol[a]	Positivecontrol[b]	Negativecontrol[c]
	DD[1]	DD[1]	DD[1]	DD[1]	DD[1]	DD[2]	DD[2]	DD[2]
Staphylococcus aureus	-	-	-	-	-	18.6±0.6		6±0
Escherichia coli	-	-	-	-	-	19.2±1.0		6±0
Bacillus subtilis	-	-	-	7.8±0.1	9.2±0.1	20.1±0.2		6±0
Pseudomonas aeruginosa	-	-	-	-	-	15.4±0.4		6±0
Candida albicans	-	-	-	-	9.0±0.1		10.7±0.3	6±0
Aspergillus flavus	-	-	-	8.6±0.2	8.5±0.1	-	11.1±0.4	6±0
Mucor mucedo	-	-	-	-	-		12.0±1.1	6±0
Phytophthora parasitica	-	-	-	-	-		11.5±0.4	6±0

Note: 1. DD, diameter of zone of inhibition (mm) including disc diameter of 6 mm. 2. [1] Tested at a concentration of 10 mg/mL; [2] Tested at a concentration of 1 mg/mL. 3. [a] = chloramphenicol; [b] = nystain; [c] = acetone.

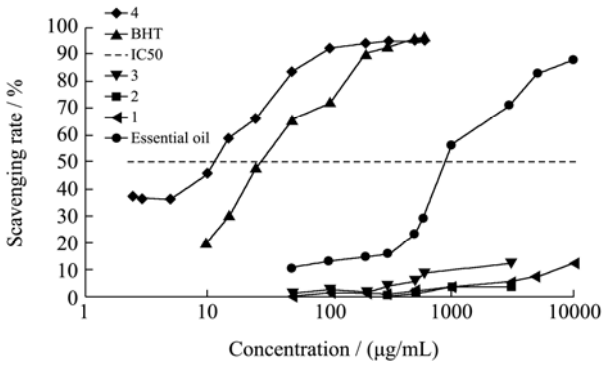

Fig.2 The scavenging rate and IC_{50} of the essential oil and four compounds from *Spiraea mongolica Maxim* in antioxidant activity

Note: Adopting the logarithmic coordinate denotes the abscissa.

3 Conclusion

The essential oil and the compound (4) may play key roles in antioxidant activity of *Spiraea mongolica* Maxim, further research needed to be done to study their other antioxidant activity, their antioxidative mechanism, or safety as antioxidants, to carry out organic modification. The compound (1) made a great contribution in the diverse biological activites of *S. mongolica* Maxim. The compound (2) showed antimalarial activity against Plasmodium falciparum in vitro, it consisted with the insecticidal activity of *S. mongolica* Maxim. The compound (3) and (4) may have synergistic effect on antifungal infection in the efficacy of *S. mongolica* Maxim as Chinese herbal medicine. The results in present study let us know the substances that play a crucial role in a certain activity of *S. mongolica* Maxim, at the same time, the rusults provided material basis for deeper research of *S. mongolica* Maxim and proved the curative treatment of traditional Chinese medicine plant. These compounds with high and various biological activity provided the basis for the development of new, effective drugs, particularly in the current era of resistanc increased, natural products as anti-tumor, anti-cancer drugs combined with other medications should be able to achieve good treatment results and overcome resistanc.

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