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CHEMICAL COMPOSITION OF ESSENTIAL VOLATILE OILS OF *SELAGINELLA* SPP. AND THEIR ANTIBACTERIAL ACTIVITY

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Abstract

The chemical composition of the volatile oils of three species of *Selaginella viz. S. moellendorfii*, *S. helvetica* and *S. delicatula*, was studied by gas chromatograph-mass spectrometer (GC-MS). The major chemical groups occurring in the essential oils were aliphatic hydrocarbon (19.43 - 42.8%), higher alcohols (4.57 - 11.9%), ketones (3.36 - 20.81%) and sesquiterpene (2.3 - 62.59%). The main constituents of the essential oils were phytone (1.8 - 20.33%), 2-pentylfuran (4.78 - 5.91%) and copaene (53.1%). The antibacterial activity of the volatile oils from three species of *Selaginella* plants was evaluated against three Gram-positive bacteria namely, *Staphylococcus aureus*, *Diplococcus pneumoniae*, *Corynebacterium pyogenes* and three Gram-negative bacteria namely, *Escherichia colii*, *Pseudomonas aeruginosa*, and *Proteusbacillus vulgaris*, by the agar disc diffusion method. The results of antibacterial test indicated that the volatile oils of three species of *Selaginella* showed good antibacterial activity, especially against *Corynebacterium pyogenesi*. Moreover, the essential oils of *S. moellendorfii* have the strongest antibacterial effect against *Corynebacterium pyogenes* (MIC and MBC were both 5 mg/mL). The results clearly demonstrated that the essential volatile oils of three *Selaginella* species can be used as a possible source for antibacterial drug development by the modern pharmaceutical industries.

Introduction

Selaginella moellendorfii, *S. helvetica* and *S. delicatula* have been commonly used in traditional Chinese medicine. They generally belong to the family Selaginellaceae. It is reported that these three species of *Selaginella* can be harvested all year round. Meanwhile, the genus *Selaginella* has a great effect on clearing away heat and toxic materials, invigorating the circulation of blood. The latest researches (Li *et al.* 2017) showed that *Selaginella* plants have antioxidant and antitumor activity. These three species of *Selaginella* distributed in southern China are rich in biflavones, polyoses, volatile oils, alkaloids (Wang *et al.* 2013, 2017).

The total volatile oil of plants has cytotoxic effect, anti-mutagenesis effect and anti-oxidation function (Hsouna *et al.* 2011, Wang *et al.* 2015). There are different types of volatile components in natural plants, including organic acids, monoterpenes, sesquiterpenes, aliphatic compounds, ketone etc. (Wang and Zhao 2010, Jiang *et al.* 2017). These substances have different biological activities. Sesquiterpenes not only have cellular poison activity but also can be used as an anti-inflammatory agent (Wang *et al.* 2016, 2015). Generally, fatty acid has an effect on regulating lipid metabolism and impact on central nervous system (Jump *et al.* 1994, Gravholt *et al.* 1998). Monoterpene, the main active component of volatile oil, has anticholinesterase and pediculicidal activities (Astani *et al.* 2009, Coffi *et al.* 2012).

At present bacteria are becoming more resistant to antibiotics and evaluation of antimicrobial effects of plant secondary metabolites against pathogens are essentially needed. Many substances of volatile oils such as phytone, β-ionone and linolenic acid from *Lippia graveolens* have antibacterial effects (Hernández *et al.* 2 009). Marino *et al.* (2011) studied on the antibacterial

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effects of Lamiaceae and Compositae, indicating that hyssop oils have antibacterial effects. In order to find new and better antibiotics, these are safe and effective for infection control. The studies on the antibacterial aspects of *Selaginella* could be helpful towards the safe and effective antibacterial agent.

So far, there are a few reports on biological activity and chemical analysis of the essential oils from *Selaginella*. Therefore, the aim of this work is to evaluate bioactive feature of the essential oils in three species of *Selaginella* such as *S. moellendorfii*, *S. helvetica* and *S. delicatula*, including composition and antibacterial activities, which will provide scientific basis for further exploring the development and utilization of *Selaginella* plants.

Materials and Methods

Three different species of *Selaginella* viz. *S. moellendorfii*, *S. helvetica* and *S. delicatula* were collected from different places of China, in January, 2018 (Table 1). Collected plant materials were air dried in the laboratory at 25°C for 15 days in a light-proof environment, certified as genuine by Yang Jianwen, Director of Zunyi Medical University.

Table 1. Collection sites of different species of *Selaginella*.

No.	Abbreviation	Collection site	Latitude	Longitude	Altitude (m a.s.l)
1	Sm	Zunyi, Guizhou Province, China	N38°25′20.75″	E115°19′47.31″	25
2	Sh	Meizhou, Guangdong Province, China	N24°08′40.08″	E115°43′2.28″	86
3	Sd	Hekou, Yunnan Province, China	N35°20′5.03″	E116°26′50.96″	110

Sm = *Selaginellamoellendorfii*, Sh = *Selaginellahelvetica*, Sd = *Selaginelladelicatula*.

Three Gram-positive strains viz. *Staphylococcus aureus* (ATCC 25623), *Diplococcus pneumonia* (ATCC 8789) and *Corynebacterium pyogenes* (ATCC 6051) and three Gram-negative bacteria viz. *Escherichia coli* (ATCC 26922), *Pseudomonas aeruginosa* (ATCC 19420), and *Proteusbacillus vulgaris* (CMCC (B) 51352) were used as test organisms. All test organisms were provided by Chengdu Jinsheng Technology Co., LTD (Chengdu, China), and cultured at 37°C on nutrient agar and nutrient broth medium. N-hexane, a homologous series of straight-chain n-alkanes (C_8 - C_{33}, 40 mL) and anhydrous sodium sulfate were purchased from Ruizhx Technology Co., Ltd. (Beijing, China). All other reagents were used as analytical grade and purchased from Chang Zheng Chemical Company (Chengdu, China).

The ground sample (100g) was subjected to hydrodistillation for 6 hrs, using 5000 ml round-bottom flask. Distillation liquid was extracted three times with 30 ml n-hexane, and mixed together. The volatile oils obtained dried by anhydrous Na_2SO_4 and, after filtration, kept up 4°C for further use.

The essential oils were determined by GC-MS analyses. The test data were achieved on an Agilent Technologies 6890N GC equipped with mass spectrometer detectors using a HP-5MS capillary column (30.00 m × 0.25 mm, 0.25 μm film thicknesses). Initial column temperature conditions were 50°C for 5 min, 10°C/min ramp until 200 °C, held for 5 min, 10°C/min ramp 250°C, and then held isothermally at 250 °C for 5 min using helium 5.0 ultrapure carrier gases at 1.0 ml/min. The runtime for a single chromatographic analyzed was 35 min. The injector

temperature was set at 250 °C. The acquisition range was 50 - 550 m/z in electron-impact (EI) mode using an ionization voltage of 70 eV. The essential oils (0.1 µL) were injected into the GC systems.

The essential oils were identified by comparison of their retention indices and mass spectra with those recorded in the Nist (Nist 05, 2005) and WileyNist (WileyNist07 2007). Then, Kovat's retention indices values (RI) calculated according to the following formula:

$$RI = 100 - (A\text{-}C(n)/C(n+1) - C(n)) + 100n$$

where A is the retention time of analyte; C(n) is the retention time of nalkane with n carbon atoms eluting before A; C(n + 1) is the retention time of the next nalkane with (n + 1) carbon atoms eluting after A; and n is the number of carbon atoms in the n-alkanes.

The antibacterial activities of different species of *Selaginella* were determined against *Staphylococcus aureus*, *Diplococcus pneumoniae*, *Corynebacterium pyogenes*, *Escherichia colii*, *Pseudomonas aeruginosa* and *Proteusbacillus vulgaris* by the disc diffusion susceptibility method (Ruiz-Bustos *et al.* 2009). Bacteria were grown in Müller Hilton broth at 35°C for 18 hrs and then each cell density was adjusted according to 0.5 McFarland standard. After that, 100 µl of bacterial suspension was punched into Müller Hilton Agar. 100 µl of each sample prepared in dimethyl sulfoxide (DMSO) was placed on inoculated Petri dishes and incubation at 37°C for 24 hrs and pure DMSO was used as negative control. Antimicrobial activity was evaluated by determining minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of samples according to the method as previously described (Behrooz *et al.* 2014). The study was performed in triplicates of each sample.

In order to verify the statistical significance, all analyses were carried out in triplicate and results were expressed as mean ± SD.

Results and Discussion

A total of 52 volatile metabolites, 27 in Sd (*Selaginella delicatula)*, 32 in Sm (*Selaginella moellendorfii)* and 27 in Sh (*Selaginella helvetica)*, were identified based on mass spectra with the reference database and calculated retention indices (RI_{calc}) of HP-5MS capillary column (Table 2). The retention indices of the experimental data were in good agreement with those reported before in the literature. Up to about 50 min, a linear correlation between retention index and retention time was obtained. The average relative standard deviations (RSD, %) for the retention indices ranged from 0.7 to 4.1%. Total ion chromatogram (TIC) of volatile metabolites from three *Selaginella* species by HS-SPME-GC-MS is shown in Fig. 1.

The metabolites identified were of different chemical classes, including ketones (5), sesquiterpene (7), monoterpenes (2), aliphatic esters (4), higher alcohols (6), aldehydes (2), furan compounds (1), fatty acids (6) and aliphatic hydrocarbon (18). The amounts of sesquiterpene in *S. helvetica* (expressed as GC peak areas) were significantly higher than those found in the other *Selaginella* species. Obvious differences in the average peak areas of almost all of the metabolites identified were found among these *Selaginella* plants.

A total of 27 metabolites were identified by GC-MS from *S. delicatula* and aliphatic hydrocarbon (42.8%) was the major component. Ketones (20.81%) were the second most abundant metabolite followed by higher alcohol (11.9%), furan compounds (4.78%), aliphatic esters (1.55%), phenolics (0.94%), fatty acids (3.41%), monoterpenes (0.53%) and aldehydes (0.64%). A typical total ion chromatogram (TIC) of volatile metabolites from *S. delicatula* using GC-MS is shown in Fig.1. The distribution of volatile metabolites chemical classes is presented in Fig. 2. In the individual metabolites, phytone (20.33%) was found to be the main component followed by phytol(7.03%), 3-ethyl-tetracentane (6.44%)(Table 2).

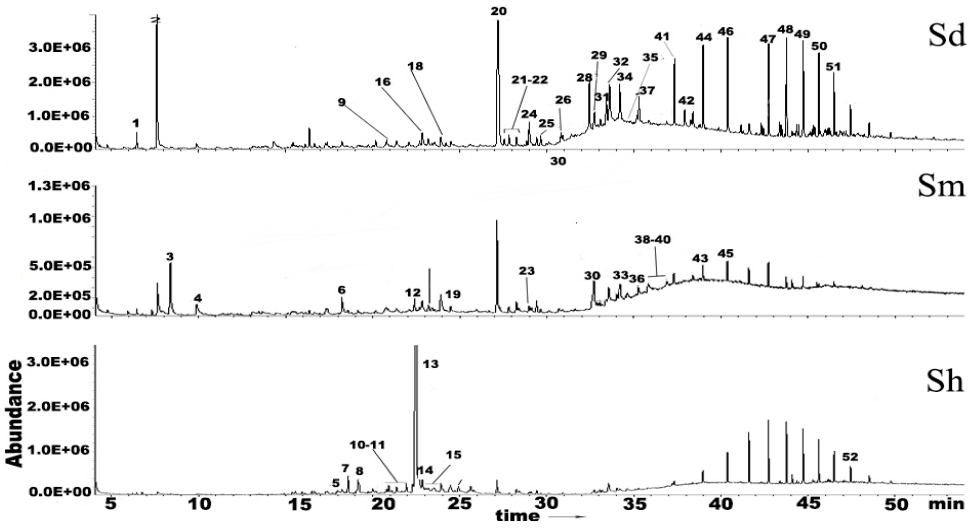

Fig. 1. Analysis diagram of the volatile oil of three species of *Selaginella* by GC-MS.

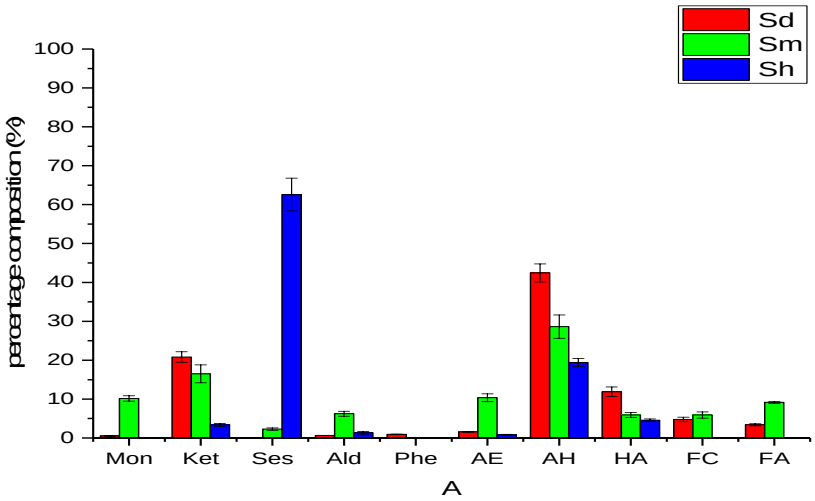

Fig. 2. Distribution of volatile metabolites chemical classes of three species of *Selaginella*.

Chemical class code: Ald: aldehyde;Ket: ketone; Mon: monoterpenes;Ses: sesquiterpenes: FA: fatty acids; FC: furan compounds; AE: aliphatic esters; HA: higher alcohols; AE: Aliphatic esters; AH: Aliphatic hydrocarbon;Phe: phenolic

A total of 32 volatile metabolites from *Selaginella moellendorfii* belonging to distinct chemical groups *viz.* aliphatic (28.63%), ketones (17.66%), aliphatic esters (10.34%), monoterpenes (10.17%), fatty acids (9.16%), aldehydes (6.24%), and in lower amounts furan compounds (5.91%), higher alcohols (4.79%), sesquiterpenes (2.3%). The distribution of different volatile metabolites chemical is presented in Fig. 2. According to the experimental results, phytone (10.6%) was the major component. Limonene (9.51%) was the second most abundant metabolite followed by palmitic acid ethyl ester 7.72%.

Table 2. Essential oil component of three species of *Selaginella*.

No.	RI	Rt/ min	English name	Formula	Family	Volatileoil compounds content from three habitats (%)		
						Sd	Sm	Sh
1	917	6.47	Alpha pinene	$C_{10}H_{16}$	Monoterpenes	0.53 ± 0.02	0.66 ± 0.01	
2	996	7.64	2-pentylfuran	$C_9H_{14}O$	Furan compounds	4.78 ± 0.06	5.91 ± 0.08	
3	1030	8.39	Limonene	$C_{10}H_{16}$	Monoterpenes		9.51 ± 0.11	
4	1102	9.91	Nonyl aldehyde	$C_9H_{18}O$	Aldehydes		2.54 ± 0.05	
5	1428	18	Isoeugenene	$C_{15}H_{24}$	Sesquiterpene			1.27 ± 0.03
6	1436	18.26	β-ionone	$C_{13}H_{20}O$	Ketones		3.07 ± 0.03	0.76 ± 0.02
7	1439	18.6	Aromadendrene	$C_{15}H_{24}$	Sesquiterpene			1.01 ± 0.04
8	1491	19.16	Vanilla ethyl ketone	$C_9H_{10}O_3$	Ketones			0.43 ± 0.01
9	1508	20.92	Elemicin	$C_{12}H_{16}O_3$	Aldehydes	0.64 ± 0.02	3.7 ± 0.05	1.35 ± 0.01
10	1527	21.39	α-muurolene	$C_{15}H_{24}$	Sesquiterpene			1.77 ± 0.05
11	1549	21.94	Cadinene	$C_{15}H_{24}$	Sesquiterpene			2.31 ± 0.03
12	1565	22.31	Pentadecene	$C_{15}H_{30}$	Aliphatic hydrocarbon		0.97 ± 0.05	1.35 ± 0.03
13	1576	22.52	Copaene	$C_{15}H_{24}$	Sesquiterpene			53.1 ± 0.45
14	1605	22.6	Cedar alcohol	$C_{15}H_{26}O$	Higher alcohols			1 ± 0.02
15	1608	22.68	Cedrol	$C_{15}H_{26}O$	Higher alcohols			1.46 ± 0.01
16	1621	22.86	n-pentadecane	C15H32	Aliphatic hydrocarbon	0.92 ± 0.02	2.66 ± 0.05	
17	1631	22.87	Ylangene	$C_{15}H_{24}$	Sesquiterpene			2.3 ± 0.03
18	1635	23.92	Farnesol	$C_{15}H_{26}O$	Higher alcohols	0.26 ± 0.01	0.74 ± 0.03	0.79 ± 0.04
19	1677	24.48	y-muurolene	$C_{15}H_{24}$	Sesquiterpene		2.3 ± 0.03	0.83 ± 0.02
20	1738	27.15	Phytone	$C_{18}H_{36}O$	Ketones	20.33 ± 0.41	10.6 ± 0.23	1.8 ± 0.07
21	1765	27.83	Myristic Acid	$C_{14}H_{28}O_2$	Fatty acids	0.41 ± 0.02	1.72 ± 0.01	1.8 ± 0.07
22	1807	28.25	Methyl Myristate	C15H30O2	Aliphatic esters	0.54 ± 0.03	1.57 ± 0.05	0.29 ± 0.03
23	1816	28.98	2-hexadecanone	C16H32O	Ketones		1.07 ± 0.04	
24	1834	28.99	n-heptadecane	C17H36	Aliphatic hydrocarbon	1.41 ± 0.05		
25	1852	29.41	2-Heptadecanone	C17H34O	Ketones	0.48 ± 0.06	1.76 ± 0.04	0.37 ± 0.06

(Contd.)

No.	RI	Rt/ min	English name	Formula	Family	Volatileoil compounds content from three habitats (%)		
						Sd	Sm	Sh
26	1883	30.81	Methyl hexadecanoate	C17H34O2	Aliphatic esters	0.5 ± 0.03		
27	1913	32.46	1-heptadecanol	C17H36O	Higher alcohols	4.61 ± 0.54		
28	1936	32.72	Palmitic acid ethyl ester	$C_{18}H_{36}O_2$	Aliphatic esters		7.72 ± 0.32	0.54 ± 0.02
29	1949	32.75	Margaric Acid(P)	C17H34O2	Fatty acids	1.88 ± 0.02		
30	1952	32.91	Linoleic acid	C18H32O2	Fatty acids		0.84 ± 0.01	
31	1964	33.08	Oleic Acid	$C_{18}H_{34}O_2$	Fatty acids	1.12 ± 0.05		
32	1983	33.56	Phytol	$C_{20}H_{40}O$	Higher alcohols	7.03 ± 0.06	4.05 ± 0.02	1.32 ± 0.01
33	2022	34.02	Fitone	C18H36O	Ketones		1.16 ± 0.02	
34	2038	34.2	Octadecane	C18H38	Aliphatic hydrocarbon	3.15 ± 0.31	4.52 ± 0.21	
35	2059	34.64	Butyl phthalate	$C_{20}H_{30}O_4$	Aliphatic esters	0.51 ± 0.03	1.05 ± 0.05	
36	2065	35.27	Stearic acid	C18H36O2	Fatty acids		1.5 ± 0.04	
37	2096	35.3	1-chlorooctadecane	$C_{18}H_{37}Cl$	Aliphatic hydrocarbon	1.32 ± 0.04		
38	2109	35.83	n-nonadecane	C19H40	Aliphatic hydrocarbon		4.8 ± 0.22	
39	2129	36.9	2-Methyloctadecane	$C_{17}H_{36}$	Aliphatic hydrocarbon		4.08 ± 0.31	
40	2137	37.3	Nonadecanoic Acid	C19H38O2	Fatty acid		5.1 ± 0.01	
41	2147	37.34	n-eicosane	$C_{20}H_{42}$	Aliphatic hydrocarbon	2.55 ± 0.01		0.57 ± 0.02
42	2153	37.92	Totarol	$C_{20}H_{30}O$	phenols	0.94 ± 0.03		
43	2171	38.39	n-heneicosane	$C_{21}H_{44}$	Aliphatic hydrocarbon		1.28 ± 0.01	
44	2192	38.96	Docosanoic	$C_{22}H_{46}$	Aliphatic hydrocarbon	4.7 ± 0.11	1.85 ± 0.13	0.81 ± 0.06
45	2205	40.37	n-tricosane	$C_{23}H_{48}$	Aliphatic hydrocarbon		2.01 ± 0.02	1.98 ± 0.31
46	2219	40.41	3-Methyltricosane	$C_{24}H_{50}$	Aliphatic hydrocarbon	6.44 ± 0.33		
47	2235	42.74	n-tetracosane	$C_{24}H_{50}$	Aliphatic hydrocarbon	6.22 ± 0.01	1.58 ± 0.08	2.96 ± 0.21
48	2246	43.76	n-pentacosane	C25H52	Aliphatic hydrocarbon	5.7 ± 0.03	2.21 ± 0.01	3.56 ± 0.26
49	2267	44.72	2-Methyltetracosane	C25H52	Aliphatic hydrocarbon	4.43 ± 0.11	1.55 ± 0.10	3.38 ± 0.07
50	2289	45.5	n-hexacosane	C26H54	Aliphatic hydrocarbon	3.79 ± 0.02	1.12 ± 0.64	2.99 ± 0.31
51	2312	46.5	n-heptacosane	C27H56	Aliphatic hydrocarbon	1.81 ± 0.21		
52	2331	47.45	n-Octacosane	C28H58	Aliphatic hydrocarbon			1.83 ± 0.15

It can be depicted from the chromatographic profile, there were 27 volatile metabolites from *S. helvetica viz.* sesquiterpenes (62.59%), aliphatic (19.43%), higher alcohols (4.57%), ketones (3.36%), and in lower amounts of aldehydes (1.35%), and aliphatic esters (0.83%). In the individual metabolites, copaene (53.1%) was found to be the main component followed by n-pentacosane (3.56%) and 2-Methyltetracosane (3.38%).

As illustrated in Table 3, the volatile oils of three *Selaginella* species showed better antimicrobial activities against all bacterial strains tested. In addition, all the samples exhibited higher antimicrobial effect against *Corynebacterium pyogenes*, among which the volatile oils of *S. moellendorfii* had the best antibacterial activity (MIC and MBC were both 5 mg/mL). It is worth mentioning that equal amounts of the volatile oils in three plants had different antimicrobial activities on other different bacterial strains. The MIC and MBC values of the essential oils for tested bacterial strains were both in the range of 5-40 mg/ml. Unfortunately, the MBC values of the essential oils for *Staphylococcus aureus* had not been gained when the concentration of essential oils of *S. delicatula* and *S.helvetica* reached the maximum. On the whole, *Escherichia colii*, *Pseudomonas aeruginosa* and *Corynebacterium pyogenes* were sensitive to the essential oils of *S. delicatula*; *Diplococcus pneumoniae* was sensitive to the essential oils of *S. helvetica*.

Table 3. Antibacterial activity of three different species of *Selaginella* (mg/ml).

Samples	*Staphylococcus aureus*		*Diplococcus pneumoniae*		*Corynebacterium pyogenes*		*Escherichia coli*		*Pseudomonas aeruginosa*		*Proteusbacillus vulgaris*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Sm	40	40	>40	NT	5	5	>40	NT	40	40	>40	NT
Sd	>40	NT	40	40	10	20	20	20	20	20	40	40
Sh	>40	NT	20	20	10	10	40	40	40	40	>40	NT

NT: Not tested.

In this study, chemical composition and antibacterial activities of the essential oils of three species from *Selaginella* have been described in detail. The results indicated that the essential oils have better antimicrobial effect against tested Gram-positive and Gram-negative bacteria. The essential oil of three *Selaginella* plants could be effective for controlling of bacteria strains, especially *Corynebacterium pyogenesi*, and the essential oils of *S. moellendorfii* have the best antibacterial effect against *Corynebacterium pyogenes* (MIC and MBC were both 5 mg/ml). Chemical constituents of the essential oils from three herbs were firstly studied by GC-MS, which were composed of aldehydes (0.64 - 6.24%), ketone (3.36 - 20.81%), monoterpenes (5.31 - 6.57%), sesquiterpenes (2.3 - 62.59%), fatty acids (1.8 – 9.16%), furan compounds (4.78 - 5.91%), aliphatic esters (0.83 - 10.34%), aliphatic (19.43 - 42.8%) and higher alcohols (4.57 – 11.9%). From the study it can be concluded that three species of *Selaginella* may be used as a potential natural antimicrobial agent in the future of the pharmaceutical industry.

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