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Chemical Composition and Biological Activities of the Essential Oil from *Aristolochia fordiana* Hemsl

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Abstract: The present study investigated the chemical composition of the essential oil obtained from the aerial parts of *Aristolochia fordiana* Hemsl (AF-EO) using GC-FID and GC-MS, and evaluated the *in vitro* biological activities of the essential oil. Forty-nine compounds representing 99.6% of the total oil were characterized. The main constituents were identified as *β*-chamigrene (17.0%), *β*-caryophyllene (11.1%), *α*-bulnesene (11.0%) and *β*-pinene (10.2%). Furthermore, the antibacterial activity of the essential oil of *A. fordiana* was studied using disc diffusion and micro-broth dilution assays. AF-EO exhibited a significant antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* with MIC values below 100 μg/mL. Besides, the results of MTT assays indicated that the essential oil exhibited a moderate cytotoxic activity on HepG2 (liver hepatocellular cells) and MCF-7 (human breast adenocarcinoma cells) cell lines. However, the AF-EO showed a weak antioxidant activity in DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP assays.

Keywords: *Aristolochia fordiana* Hemsl; essential oil; antibacterial activity; cytotoxic activity; antioxidant activity.

1. Introduction

Essential oils are a mixture of numerous compounds formed by aromatic plants as secondary metabolites, and have been known to exhibit a broad range of biological activities including antibacterial, antioxidant, antiviral, antitumor and anti-inflammatory as well as other various activities. In recent years, studies on the pharmacological properties of essential oils have become increasingly significant in the search for natural and safe alternative medicines [1].

The Aristolochiaceae family is distributed throughout tropical and temperate regions of the world [2], and its largest genus is *Aristolochia* which is widespread throughout the North Africa, Europe and Asia [3]. Species of *Aristolochia* have often been reported as important medicinal plants in ethnobotanical studies [4]. *Aristolochia fordiana* Hemsl, a common twining herb belonging to the genus *Aristolochia*, is

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native to Southwestern China [5]. Its rhizome has been used in Traditional Chinese Medicine (TCM) to treat seizures, rheumatism, and abdominal pain [5].

The *Aristolochia* genus is known for its numerous therapeutic properties [6]. According to the literature, several species of *Aristolochia* genus are rich in essential oils, such as *A. chilensis* [7], *A. gigantea*, *A. macroura*, *A.cymbqera*, *A. rodriguesia*, *A. birostris*, *A. papillaris* and *A. triangularis* [8], *A. gibertii* [9, 10], *A. mollissima* [11], *A. delavayi* [12], *A. longa* [13], *A. arcuata*, *A. chamissonis*, *A. elegans*, *A. esperanzae*, *A. galeata*, *A. gigantean*, *A. lagesiana*, *A. malmeana*, *A. melastoma*, *A. pubescens* [14] and *A. odoratissima* [15]. Many biological activities have been described following the use of essential oil from the *Aristolochia* genus, which have demonstrated antimicrobial activity [11-13] and cytotoxic activity [11]. The chemical composition of the essential oils of some *Aristolochia* species have been reported, and the principal components were limonene [9, 10, 14, 16], bicyclogermacrene [9, 10], germacrene D [8, 10, 15], *β*-caryophyllene [8], (*E*)-nerolidol [8, 10], *α*-pinene , *β*-pinene , camphene and *o*-cymene [14].

To the best of our knowledge, the chemical composition and biological activities of the essential oil of *A. fordiana* (AF-EO) have not been investigated. Therefore, we report here the composition of the essential oil obtained from the aerial parts of *A. fordiana* and its *in vitro* antibacterial, cytotoxic and antioxidant activities.

2. Materials and Methods

2.1. Plant Material

The fresh plant material was collected in July 2016 from Guangxi Province of China, and was identified by Associate Prof. Hong Zhao of Marine College, Shandong University. A voucher specimen (NO.10806) was deposited at the Laboratory of Botany of Marine College, Shandong University.

2.2. Isolation of the Essential Oil

The aerial part of the fresh plant material (500g) was subjected to hydrodistillation for four hours，using a modified Clevenger apparatus. The essential oil (0.93g, 0.186% w/w) was obtained using ethyl ether as a collecting solvent and dried over anhydrous sodium sulfate and stored in airtight container in a refrigerator at 4 °C until they were analysed.

2.3. Essential Oil Analysis and Identification

The AF-EO sample was analysed by an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID). Column: capillary column HP-5MS (30 m × 0.25 mm i.d.; 0.25 μm film thickness). The operational conditions were as follows: temperature program from 60 °C (1 min) to 200 °C (5 min) at 6 °C/min and then from 200 °C to 280 °C (2 min) at 5 °C/min; the injector temperature was set at 250 °C; the flow rate of Helium as the carrier gas was set at 1.2 mL/min; the injection volume was 0.2 μL.

Analyses of the essential oil were carried out on a Hewlett Packard 6890 gas chromatograph (Agilent) fitted with a HP-5MS fused silica column, coupled with a Hewlett Packard 5975C mass selective detector operated by HP Enhanced ChemStation software, version A.03.00. GC parameters were the same as those mentioned for GC-FID. The injection volume was 0.2 μL of 1% solution prepared in *n*-hexane with split ratio 1:50. Mass spectra were acquired in EI mode at 70 eV. The mass range was from *m/z* 50 to 550.

Essential oil compounds identification was based on retention indices (relative to C_7-C_{30} *n*-alkanes, under the same experimental conditions), and computer matching with NIST 14 MS Search 2.2 Mass Spectral Database for GC-MS as well as by comparisons of their mass spectra with data already available in the literature [17, 18].

2.4. Antibacterial Activity Assay

The Gram positive bacteria, *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633) and the Gram negative bacteria, *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were selected for antibacterial test.

The antibacterial activities were evaluated using disc agar diffusion method recommended by CLSI (Clinical and Laboratory Standards Institute) [19]. Filter paper discs (6 mm in diameter) were impregnated with 10 µL of the samples (10 mg/mL) or chloramphenicol (1 mg/mL) as a positive control and incubated at 37 °C for 24 h. Antibacterial effect was assessed by measuring the diameter of the inhibition zone (DIZ), which visibly presents the absence of bacterial growth, including the 6 mm disk.

The MIC values were performed in the 96 well-microplates using the microdilution assay according to the literature previously described by Ellof (1998) with slight modifications [20] The essential oil was diluted and transferred into each well (100 µL per well). Chloramphenicol was used as the reference antibiotic control. The inoculum was added to all wells (100 µL per well). The 12th well was considered as growth control (it contained only the culture medium and strain). The plates were incubated at 36 °C for 18 h. 20 µL of 1% TTC (2,3,5-triphenyl tetrazolium chloride) aqueous solution was used as an indicator of microbial growth [21]. For the determination of the MBC, a sample of 100 µL from each well (without any colour alteration) was subcultured on the MHA (Mueller Hinton agar) plates and incubated at 37 °C for 18-24 h (overnight). The MBC is defined as the lowest concentration without any bacterial growth. Experiments were carried out in triplicates to minimise the experimental error. The result of antibacterial activity test is given in Table 2.

2.5. Cytotoxic Activity Assay

2.5.1. Cell Culture

MCF-7 (human breast adenocarcinoma cell line) and HepG2 (liver hepatocellular cells) cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (200 U/mL of penicillin and 50 µg/mL of streptomycin). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

2.5.2. MTT Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine the cell viability as described in a previous report [22]. The cells (5×10^3 cells per well) were seeded in 96-well cell culture plates in 200 µL of culture medium RPMI 1640 and grown for 24 h to allow cell attachment.

The essential oil was solubilised in DMSO, and afterwards diluted with culture medium for use. Doxorubicin was used as a positive control. The dilutions of the AF-EO (0.016-2 mg/mL) were added to the wells, except the negative control wells where only culture medium was added. All samples were done in triplicate. The microplates were incubated for 24, 48, and 72 h. After incubation, 20 µL of MTT (5 mg/mL in PBS) were added to each well and incubated for 4 hours under the same culture conditions. Formazan crystals were dissolved in 100 µL DMSO. The optical density was measured at 570 nm using an enzyme linked immunosorbent assay (ELISA) reader.

The cytotoxic activity was expressed as the concentration of the AF-EO producing 50% inhibition of cell growth (IC_{50}). The percentages of cell growth were calculated as follows:

$$\text{Cell growth (\%)} = [\text{A (sample)} / \text{A (control)}] \times 100\% \quad (1)$$

The results of the cytotoxic activity tests are given in Table 3.

2.6. Antioxidant Activity Assay

The scavenging activities of the essential oil on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulphonate) cation radical were determined according to the method as previously described [23,24,25]. The scavenging activity was expressed by IC_{50} value that is the effective concentration at which free radicals are scavenged by 50%.

The reducing ability was determined by using Ferric reducing antioxidant power (FRAP) assay as previously described with slight modifications. The FRAP reagent was prepared by mixing sodium acetate buffer (300 mmol/L, pH 3.6), a solution of TPTZ (10 mmol/L) in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ using the proportion 10:1:1 (v/v/v). A 40 µL of appropriately diluted sample extract and 160 µL of FRAP reagent were mixed in a 96-well plate and incubated at 37 °C for 40 min in the dark. In the case of the blank, 40 µL methanol was added to 160 µL FRAP reagent. The absorbance of the resulting solution was measured at 593 nm using a plate reader. An analytical curve with different concentrations of Trolox (linearity: 0.1-40 µg/mL; $R^2 = 0.998$) was plotted to quantify the ferric reducing antioxidant power of the essential oil. The potential antioxidant activity was expressed as Trolox equivalent antioxidant capacity in µmol Trolox × g^{-1}. The outcome of scavenging activity test is given in Table 4.

3. Results and Discussion

3.1. Chemical Composition of the Essential Oil

The hydrodistillation of the aerial parts of *A. fordiana* (500g) provided an essential oil characterized by a typical odor, in a yield of 0.186% (w/w) of the fresh weight. Identification of the essential oil constituents was performed by comparing GC-MS retention data with retention indices obtained by the combination of the essential oil with C_7-C_{30} *n*-alkanes as internal standards. A total of forty-nine compounds were identified, which represent 99.6% of the total composition of the essential oil (Table 1). The oil composition is dominated by the presence of sesquiterpenes comprising 50.6% of the total composition, followed by oxygenated sesquiterpenes (24.2%), monoterpenes (18.5%) and oxygenated monoterpenes (6.3%). The principal chemical constituents were found to be *β*-chamigrene (17.0%), *β*-caryophyllene (11.1%), *α*-bulnesene (11.0%), *β*-pinene (10.2%), *β*-eudesmene (6.7%), limonene (5.6%), linalool (4.2%), bicyclogermacrene (4.2%) and longifolene (4.1%). Previous studies on essential oils of the aerial parts of some other *Aristolochia* species have been reported and similar data were found in their chemical compositions, as all of them were dominated by a higher proportion of sesquiterpenes [8-10, 14-16, 26]. However, the presence of *β*-chamigrene and *α*-bulnesene, mentioned in this work as major constituents, had never been previously reported in principal components for the *Aristolochia* species.

3.2. Antibacterial Activity

The *in vitro* antibacterial activities of the AF-EO were assessed by the disc agar diffusion and micro-broth dilution methods against two Gram-positive and two Gram-negative bacterial strains. The antibacterial activities were expressed as the diameters of inhibition zone (DIZ), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The results are shown in Table 2.

The AF-EO exhibited a significant antibacterial activity against only the tested gram-positive bacteria *S. aureus* (MIC=0.078 mg/mL, MBC=0.078 mg/mL) and *B. subtilis* (MIC=0.078 mg/mL, MBC=0.078 mg/mL), while among Gram-negative bacteria only *P. aeruginosa* (MIC=2.5 mg/mL) was slightly affected by the AF-EO, particularly *E. coli* was completely resistant. The probable cause of the relative tolerance of Gram-negative bacteria to essential oils has been correlated with the presence of a hydrophilic outer layer [27]. The outer membrane of Gram-negative bacteria is rich in hydrophilic lipopolysaccharides (LPS) which act as a barrier against penetration of hydrophobic compounds. Hence, Gram-negative bacteria show a higher degree of resistance against EOs [28]. In addition, antibacterial effects of the most abundant compounds in the AF-EO, viz., *β*-caryophyllene, *β*-pinene, limonene and linalool have been previously reported [29-31].

Table 1. Chemical composition of the essential oil of *A. fordiana*

Peak No.	Compound [a]	RI [b]	RI [c]	% Area	Ref.
1	*β*-Pinene	979	979	10.2	[17, 18]
2	Pseudolimonene	1006	1006	2.3	[17]
3	Limonene	1032	1032	5.6	[17, 18]
4	*γ*-Terpinene	1060	1060	0.1	[17, 18]
5	Terpinolene	1093	1093	0.2	[17, 18]
6	Linalool	1100	1100	4.2	[17, 18]
7	*allo*-Ocimene	1131	1131	0.1	[17, 18]
8	(*E*)-2-Menthenol	1145	1144	0.1	[17]
9	Camphor	1155	1152	0.1	[17, 18]
10	Terpinen-4-ol	1185	1184	0.2	[17, 18]
11	*α*-Terpineol	1198	1198	1.2	[17, 18]
12	Piperitone	1263	1263	0.1	[17, 18]
13	Bornyl acetate	1293	1293	0.4	[17, 18]
14	*δ*-Elemene	1338	1338	0.1	[17, 18]
15	*α*-Longipinene	1348	1348	0.6	[17, 18]
16	*α*-Cubebene	1359	1360	0.1	[17, 18]
17	*β*-Patchoulene	1378	1377	0.1	[17]
18	*α*-Copaene	1389	1390	0.3	[17, 18]
19	*β*-Elemene	1396	1396	0.2	[17, 18]
20	Longifolene	1404	1404	4.1	[17, 18]
21	*α*-Cedrene	1412	1411	0.1	[17, 18]
22	*β*-Gurjunene	1426	1426	0.9	[17, 18]
23	*β*-Caryophyllene	1441	1439	11.1	[17, 18]
24	*β*-Farnesene	1461	1461	1.0	[17, 18]
25	*β*-Chamigrene	1478	1478	17.0	[17, 18]
26	Bicyclogermacrene	1489	1489	4.2	[17, 18]
27	*β*-Eudesmene	1508	1509	6.7	[17]
28	*γ*-Cadinene	1531	1528	0.5	[17, 18]
29	*δ*-Cadinene	1538	1539	3.1	[17, 18]
30	Cubenene	1549	1552	0.2	[17]
31	3,7(11)-Selinadiene	1554	1551	0.3	[17]
32	*α*-Bulnesene	1517	1515	11.0	[17, 18]
33	*α*-Cedrene epoxide	1564	1570	1.3	[17]
34	Epiglobulol	1582	1582	0.1	[17]
35	Globulol	1590	1590	0.2	[17, 18]
36	Viridiflorol	1601	1601	0.1	[17, 18]
37	Guaiol	1607	1605	0.2	[17, 18]
38	*τ*-Cadinol	1615	1615	0.2	[17, 18]
39	Cedrol	1623	1619	0.3	[17, 18]
40	Neointermedeol	1633	1631	0.4	[17]
41	Cubenol	1645	1644	0.3	[17, 18]
42	Widdrol	1651	1651	0.1	[17]
43	*α*-Cadinol	1657	1657	1.9	[17, 18]
44	Intermedeol	1673	1675	3.8	[17, 18]
45	*α*-Bisabolol	1693	1693	0.3	[17, 18]
46	Aristol-1(10)-en-9-ol	1706	1704	0.2	[17]
47	*β*-Nootkatol	1723	1722	3.4	[17]
48	*β*-Costol	1769	1774	0.3	[17]
49	*α*-Muurolene-14-ol	1785	1782	0.1	[17]
	Total identified			**99.6**	

[a] Compounds are listed in order of their elution from a HP-5MS column; [b] (retention index): RI-non-isothermal Kovats retention indices on a HP-5MS column relative to C_7–C_{30} n-alkanes; [c] linear retention indices according to NIST Chemistry WebBook and the literature on a HP-5MS column.

Table 2. Antibacterial activity of essential oil of *A. fordiana*

Microorganism	Diameter of the inhibition zones (mm) [a]		MIC (mg/mL) [b]		MBC (mg/mL) [c]	
	AF-EO	Ch	AF-EO	Ch	AF-EO	Ch
Gram positive						
Staphylococcus aureus ATCC 6538	22.9 ± 0.4	25.7 ± 0.8	0.078	0.039	0.078	0.156
Bacillus subtilis ATCC 6633	23.6 ± 0.6	28.3 ± 1.0	0.078	0.020	0.078	0.078
Gram negative						
Escherichia coli ATCC 25922	6.4 ± 0.2	25.9 ± 0.6	>5	0.039	N.T.	0.156
Pseudomonas aeruginosa ATCC 27853	6.7 ± 0.3	28.8 ± 0.4	2.500	0.020	N.T.	0.040

The diameter of the inhibition zones (mm), including the disc diameter (6 mm), are given as the mean ± SD of triplicate experiments. Diameter of the inhibition zones [a] of AF-EO (1 mg/mL); positive control: Ch, chloramphenicol (0.01 mg/mL); MIC [b]: Minimal inhibitory concentration; MBC [c]**:** Minimal bactericidal concentration; N.T.: not tested.

3.3. Cytotoxic Activity

To investigate the cytotoxic activities, two human tumour cell lines, HepG2 (liver hepatocellular carcinoma cells) and MCF-7 (human breast adenocarcinoma cells), were exposed to increasing concentrations of the essential oil. Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. As shown in Table 3, the IC_{50} values for the cytotoxic effects of the AF-EO on HepG2 and MCF-7 cells were calculated to be 0.69 mg/mL and 0.22 mg/mL for 72 h, respectively. Additionally, the essential oil showed a dose-related cytotoxic activity on the corresponding cell lines in the tested range of concentrations. Likewise, it exhibited an increasing cytotoxicity over longer exposure times, which indicated a time-dependent effect of the AF-EO. The major components present in the AF-EO, such as *β*-caryophyllene, limonene and linalool, have been reported to possess significant cytotoxic effects [29, 32, 33]. However, as compared to the positive control doxorubicin, the essential oil exhibited a moderate cytotoxicity.

Table 3. Cytotoxic activity of the essential oil of *A. fordiana* against HepG2 Cells and MCF-7

	HepG2		MCF-7	
	AF-EO IC_{50} (mg/mL)	Doxorubicin IC_{50} (μg/mL)	AF-EO IC_{50} (mg/mL)	Doxorubicin IC_{50} (μg/mL)
24h	＞2	2.64 ± 0.14	1.31 ± 0.04	1.12 ± 0.04
48h	1.19 ± 0.10	0.88 ± 0.02	0.43 ± 0.02	0.34 ± 0.03
72h	0.69 ± 0.05	0.49 ± 0.04	0.22 ± 0.01	0.13 ± 0.02

IC_{50}: the concentration of compound that affords a 50% reduction in cell growth (after 24, 48, and 72 h of incubation); Doxorubicin was tested as a reference; Expressed as the mean ± SD of triplicate experiments.

3.4. Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulphonate) radical cation scavenging activity and FRAP (ferric reducing antioxidant potential) assays were used to measure the antioxidant capacities of the essential oil. The results

are presented in Table 4. It was observed that the AF-EO exhibited a weak DPPH radical scavenging activity with an IC_{50} value of 2.448 mg/mL compared with the standard, BHT (IC_{50} value of 0.043 mg/mL) and Trolox (IC_{50} value of 0.018 mg/mL). However, the essential oil showed a poor antioxidant activity in the ABTS assay (IC_{50} value > 5 mg/mL). In view of the results of FRAP assay, the essential oil showed a moderate ferric ion reducing activity with a TEAC (Trolox equivalent antioxidant concentration) value of 19.11 μmol Trolox × g^{-1}.

Table 4. Results of antioxidant activity *in vitro* (DPPH, ABTS and FRAP) of the essential oil of *A. fordiana*

Test Sample	DPPH IC_{50} (mg/mL) [a]	ABTS IC_{50} (mg/mL) [a]	FRAP (μmol Trolox × g^{-1})
AF-EO	2.448 ± 0.153	>5	19.11 ± 1.26
BHT [b]	0.043 ± 0.002	0.016 ± 0.001	
Trolox [b]	0.018 ± 0.001	0.013 ± 0.001	

[a] IC_{50} = The concentration of compound that affords a 50% reduction in the assay; [b] Positive control used.

As a conclusion, this study reported, for the first time, the chemical composition and biological activities of the essential oil from the aerial parts of *A. fordiana*. Apart from its weak antioxidant activity, the AF-EO possessed a potent antibacterial activity against referenced gram-positive strains and also showed a moderate cytotoxic activity on HepG2 and MCF-7 cells. The presence of high concentration of *β*-caryophyllene, *β*-pinene, limonene and linalool could be responsible for the observed antibacterial and cytotoxic activities of the essential oil.

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