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Chemical composition and bioactivity of the essential oil of *Artemisia anomala* from China

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The composition and the bioactivity of the essential oil of *Artemisia anomala*, a traditional medicinal herb in China, were investigated. The composition of the essential oil was analyzed by gas chromatography–mass spectometry (GC–MS), and a total of 39 components representing 98.1% in this plant were identified. The main constituents were camphor (18.3%), 1, 8-cineole (17.3%), *β*-caryophyllene oxide (12.7%), and borneol (9.5%). The antimicrobial activities of the oil were separately evaluated against six micro-organisms, *Bacillus cereus* (CMCCB 63301), *Micrococcus flavus* (ATCC 14698), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus epidermidis* (ATCC 12228), and a clinical-isolated strain *Staphylococcus aureus*. The results showed that the oil possessed inhibitory activity against *B. cereus*, *M. flavus*, *E. coli*, *S. epidermidis*, and *S. aureus*, among which the best inhibition was observed against *B. cereus* with a MIC of 0.313 μL/mL. The antiproliferative activities of the oil were assessed against four human tumor cell lines, A459 (human lung adenocarcinoma), BRO (human melanoma), MCF7 (human breast cancer), and PC3 (human prostate). The oil showed the ability to inhibit proliferation of human cancer cells. And the MCF7 cell line was the most sensitive to the oil with an IC_{50} of 0.09.

Keywords: *Artemisia anomala*; essential oil; GC–MS; antibacterial; antiproliferation

Introduction

Artemisia is a genus of aromatic small herbs or shrubs spreading in northern temperate zones. It is one of the largest and most widely distributed genera of the Asteraceae family and consists of more than 500 species (1). This genus has always been of great botanical and pharmaceutical interest (2–4). Many of *Artemisia* species have long been used in traditional medicines for the treatment of a variety of diseases and complaints as antiseptic, antiatherogenic, antihelmintic, tonic, diuretic, antioxidant, anti-inflammatory, bacteriostatic, or fungistatic agents in countries like China, Japan, South Korea, and Turkey (5–8). Some *Artemisia* species are rich in essential oil (6, 9). There are over 180 species of *Artemisia* in China (10). *Artemisia anomala* S. Moore, a well-known medicinal herb (liu-yue-shuang or liu-ji-nu in Chinese), for example, is distributed in eastern and southern China such as Jiangxi, Anhui, and Zhejiang Province (11). In particular, *A. anomala* is usually processed into beverage in some regions in China to relieve summer heat and dyspepsia (12, 13). It has also shown antioxidative and antibacterial activity of the methanol extract of *A. anomala* (14). This knowledge prompted renewed interest in this plant and served as the basis of interest of this investigation. With reference to the study of *A. anomala*, the general flavonoids, lactones, guaianolides, sesquiterpenes, and sesquiterpenoids in *A. anomala* have been reported (15, 16). However, the compositions and the bioactivity of its essential oil have not been reported yet. With the growing interest in the use of essential oils in both food and pharmaceuticals, a systematic examination of plant extracts for these properties has become more and more important (17).

The goal of this study was to identify the chemical components within the essential oil of *A. anomala* and, in addition, to evaluate the activities of the essential oil against a proliferation of selected bacterial strains and human tumor cell lines.

Materials and methods

Plant material

The aerial parts of *A. anomala* were collected from Mt. Tianmu located in Zhejiang Province in China in July 2004. We chose the flowers and leaves as the sample, which were air-dried in the shade and stored with good ventilation. Voucher specimens were deposited at the MOE Lab for Biodiversity Science and Ecological Engineering, School of Life Sciences, Fudan University, Shanghai, China.

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Essential oil analysis

Extraction of the essential oil

The dried aerial parts of *A. anomala* (400 g) were subjected to water distillation for 3 hours using a Clevenger-type apparatus. Water was removed from the essential oil through an use of anhydrous sodium sulfate, and kept at 4°C prior to chemical and biological analyses.

Chemicals used

All chemicals used were analytical reagent grade. All reagents were purchased from Sinopharm Chemical Reagent Co, Ltd. (SCRC).

GC–MS conditions

Chemical analysis was performed using a Hewlett-Packard Series 6890 gas chromatograph (GC; Finnigan Voyager, San Jose, CA, USA). It was equipped with FID using a fused silica HP-5 capillary column (30 m long, 0.25 mm in diameter, and 0.25 μm film thick) with a mixture of 5% diphenyl-polysiloxane and 95% dimethyl-polysiloxane as the stationary phase. The analysis was performed on a combined GC–MS instrument (Finnigan Voyager, San Jose, CA, USA) using the same HP-5 capillary column. An aliquot (1 μL) of the essential oil was injected into the column using a 10:1 split injection. The injection port temperature was set at 250°C. The GC program included an initial column temperature of 60°C for 2 minutes, which was then increased to 250°C at a rate of 10°C/minutes, and held isothermal for 10 minutes. Helium was used as the carrier gas (1.0 mL/min). The mass spectrometer was operated in the 70 eV EI mode with scanning from 41 to 450 amu at 0.5 seconds; and the ion source temperature was set at 200°C. The linear retention indices (RIs) of the components were determined relative to C_8–C_{24} *n*-alkanes standards (Sigma Chemical Company, St. Louis, MO, USA).

The compounds within the volatile oil were identified by matching their mass spectral fragmentation patterns with those stored in the spectrometer database using the NIST data spectral library (NIST, 2000) and comparison of their RI. The RIs were calculated by the fused silica HP-5 capillary column using an *n*-alkane series (C_6–C_{22}) under the same GC conditions as for samples. The GC chromatogram is showed in Figure 1.

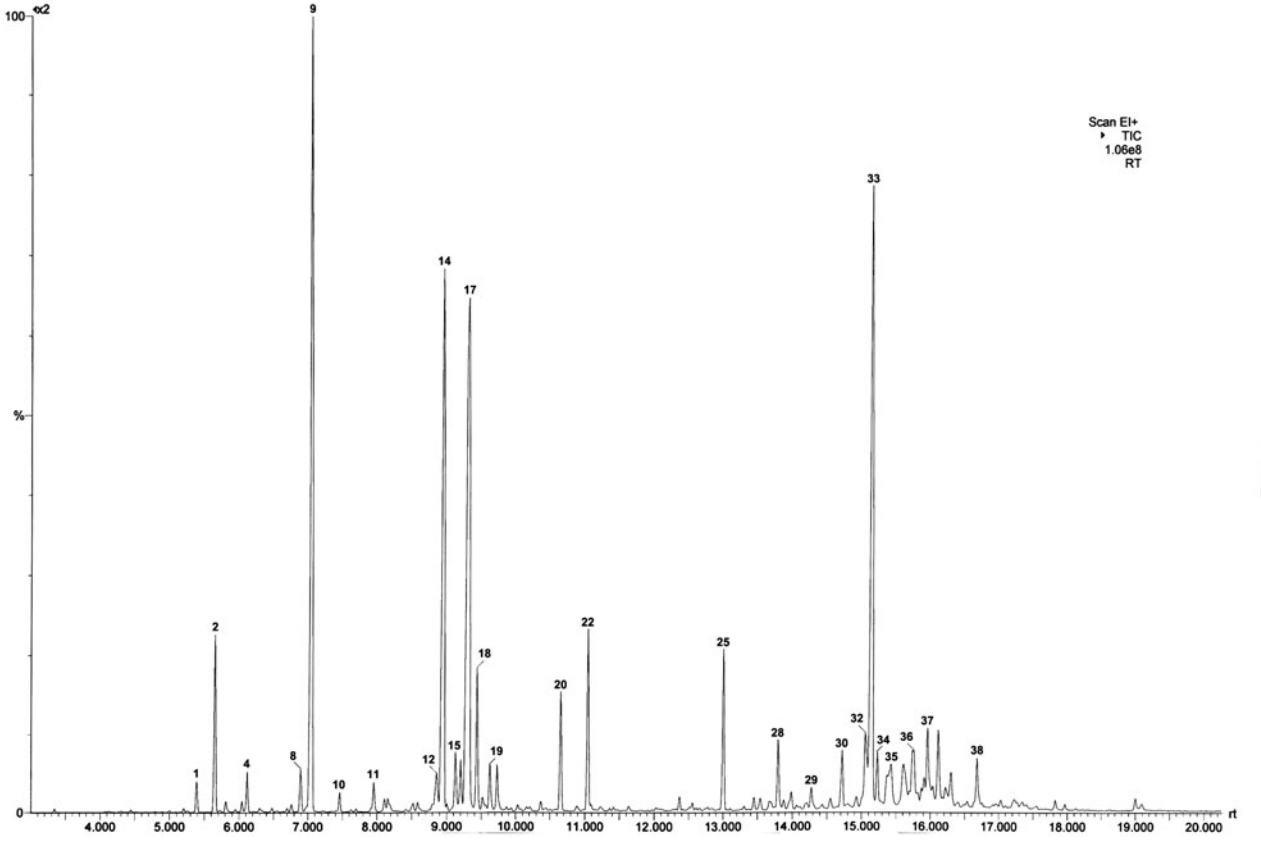

Figure 1. GC chromatogram of *Artemisia anomala* oil on HP-5 capillary column.

Antibacterial analysis

Bacteria strains tested

The essential oil was individually tested against the following six micro-organisms: *Bacillus cereus* (CMCCB 63301), *Micrococcus flavus* (ATCC 14698), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus epidermidis* (ATCC 12228), and the clinical-isolated strain *Staphylococcus aureus*. All the strains were purchased by Institute of Microbiology, Chinese Academy of Sciences.

Antibacterial testing

Antibacterial activity was measured using a broth microdilution method (18). All test samples were prepared in Mueller Hinton Broth (MHB, Oxoid) supplemented with a Tween 80 detergent (final concentration of 1% v/v). The bacteria were cultured in MHB for 12–16 hours at 37°C. The number of bacterial cells was adjusted to an approximate density of 1×10^7 (CFU)/mL in MHB. For each well of a 96-well plate, a mixture of 20 μL of bacterial suspension and 80 μL of the MHB containing a test agent was added and mixed. The test samples of the essential oil were prepared in the concentrations of 20.000, 10.000, 5.000, 2.500, 1.250, 0.625, 0.313, and 0.156 μL/mL, including one growth control (MHB + Tween 80), one sterility control (MHB + Tween 80 + test oil), one positive control (MHB + Tween 80 + bacterial), and one negative control (MHB + Tween 80 + ampicillin). Ampicillin was prepared in the concentrations of 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, and 3.9 ng/mL in parallel experiments in order to control the sensitivity of the test micro-organism. All tests were performed in triplicate except the one of ampicillin that was carried out in duplicate.

Plates were incubated under ambient atmospheric conditions for 24 hours at 37°C. After the incubation, all plates were evaluated for relative growth inhibition using optical density (OD) values. Growth inhibition was expressed as the ratio of OD values of bacterial growth incubated with test compounds to those of control wells. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the essential oil required to completely inhibit macroscopic growth of bacteria.

Cytotoxicity testing

Cell culture and cancer cell lines: Four human cancer cell lines were used to test the cytotoxicity of the isolated essential oil. These include: A459 (human lung adenocarcinoma), BRO (human melanoma), MCF7 (human breast cancer), and PC3 (human prostate cancer), provided by Pharmaceutical Development Center, University of Texas M. D. Anderson Cancer Center, Houston, USA. A549 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium): F12 (1:1) medium with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin, and 15 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) buffer. BRO cells as well as MCF7 cells were grown in DMEM (low glucose) medium with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL of streptomycin, 1 mM Sodium pyruvate, and 1 mM MEM nonessential amino acids. PC3 cells were grown in RPMI1640 medium with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL of streptomycin. All cell cultures were grown in a temperature-controlled incubator maintained at 37°C and with an atmosphere of 5% CO_2.

Cancer cells were plated (3–5,000 per well) in 96-well plates and incubated overnight at 37°C. Dilutions of the essential oil were added and allowed to incubate continuously with cells for up to 72 hours. The viability of cells treated with essential oil samples of various dilutions was determined by a standard MTT reduction assay which is based on the relative ability of mitochondria to convert the yellow MTT dye to a blue formazan product. After the required treatment time, aliquots (100 μL) of MTT solution (1 mg/mL) were added to each well and incubated for 2 hours at 37°C. The supernatant was then removed and 100 μL of isopropanol was added to dissolve the blue formazan crystals that had formed as a result of mitochondrial dehydrogenase activity in live cells. The relative color intensity was measured by a microplate spectrophotometer at 595 nm. Linearity of the color intensity relative to cell number within the range expected in the study was determined at the outset of the experiment. Relative ability of the essential oil dilutions to inhibit growth of cancer cells was determined by comparing growth of oil-treated cells relative to that of untreated cell proliferation. The concentration of essential oil required to inhibit cell growth by 50% (IC_{50}) over the 72 hours experimental period was then calculated.

Results and discussion

Chemical composition of the essential oil

The percent yield of the essential oil obtained from the water-distilled aerial parts of *A. anomala* in relation to dry weight of the plant was 0.2% (v/w). The extracted essential oil was light yellow in color. The chemical constituents in the essential oil of *A. anomala* were identified by GC–MS and their RI values are listed in Table 1. Totally, 39 compounds, representing 98.1% (based on peak areas) of the essential oil, were identified. Most of those compounds can be classified

Table 1. Constituents of the essential oil extracted from *A. anomala*.

No[a]	RT[b]	Component[c]	RI[d]	FID (%)
1	5.38	α-pinene	940	0.8
2	5.65	Camphene	956	3.3
3	6.05	Sabinene	979	0.2
4	6.12	β-pinene	982	1.2
5	6.25	Dimethyl octanol	999	0.2
6	6.48	α-phellandrene	1008	0.1
7	6.78	4-carene	1021	0.3
8	6.89	m-cymene	1029	1.4
9	7.05	1,8-cineole	1038	17.3
10	7.46	γ-terpinene	1064	0.6
11	7.95	Linalool	1100	0.7
12	8.86	3-thujone	1110	2.0
13	8.88	Pinocarveol	1147	1.2
14	8.95	Camphor	1153	18.3
15	9.15	Isoborneol	1165	0.8
16	9.20	Verbanol	1168	2.4
17	9.32	Borneol	1174	9.5
18	9.44	p-menth-1-en-4-ol	1184	2.0
19	9.62	α-terpineol	1195	0.7
20	10.65	Thujenal	1202	1.3
21	10.90	Verbanyl acetate	1267	0.4
22	11.04	Bornyl acetate	1292	2.5
23	12.38	α-copaene	1388	0.4
24	12.55	2,3-bornediol	1413	0.8
25	13.00	γ-caryophyllene	1436	3.8
26	13.48	β-farnesene	1461	0.5
27	13.55	β-caryophyllene	1470	0.3
28	13.79	α-humulene	1477	1.0
29	14.28	2-methyl-6-tolyl-2-heptene	1490	1.1
30	14.72	Germacrene D	1496	1.1
31	14.90	Longifolene	1511	0.6
32	15.05	Cadina-1,4-diene	1536	1.1
33	15.15	β-caryophyllene oxide	1571	12.7
34	15.26	Spathulenol	1595	0.8
35	15.41	Ledol	1611	3.8
36	15.76	Cadinol	1658	0.6
37	15.96	Globulol	1674	1.5
38	16.68	Aromadendrene oxide	1687	0.9
39	17.24	Bisabolol	1694	0.3
Identified compounds				98.1
Monoterpene hydrocarbons			8	7.8
Oxygenated monoterpenes			14	59.7
Sesquiterpene hydrocarbons			8	8.7
Oxygenated sesquiterpenes			7	20.6
Others			2	1.3

Notes: [a]Compounds are listed in order of their elution from an HP-5 column. [b]RT – retention times values as determined on HP-5 column. [c]All the compounds are identified by comparison of the mass spectrum with those of the computer mass libraries and by comparison of RI with those from the literatures. [d]RI – retention indices as determined on HP-5 column using the homologous series of $C_{8\text{-}24}$ *n*-alkanes, and compared to the RI values listed in the website of *Flavornet and Human Odor Space*, which are collected from articles published since 1984 using Gas chromatography-olfactometry (GCO) to detect odorants in natural products (http://www.flavornet.org/flavornet.html).

into four classes: eight monoterpene hydrocarbons (7.8%), 14 oxygenated monoterpenes (59.7%), eight sesquiterpene hydrocarbons (8.7%), seven oxygenated sesquiterpenes (20.6%). The major constituents of the essential oil were found to be camphor (18.3%), 1, 8-cineole (17.3%), β-caryophyllene oxide (12.7%), and borneol (9.5%).

The essential oil compounds of many *Artemisia* species have been reported. Previous research, for example, showed that bornane derivatives and 1,8-cineole are the major characteristic components of the

Artemisia genus. Camphor (a bornane derivative) and 1,8-cineole, which are widely used in the liqueur-making industry in many countries of the world, were reported to be major constituents of the essential oils of *A. asatica* (19), *A. austriaca* (9), *A. afra* (20), *A. diffusa* (9, 21), *A. annua* (22), *A. scoparia* (6), *A. santonicum*, and *A. spicigera* (2), respectively. In our study, camphor and 1, 8-cineole were also found to be the major constituents of the essential oil. However, the presence of other major components such as *β*-caryophyllene oxide and borneol varied with the particular *Artemisia* species (9).

Antibacterial activity

Antibacterial activities of the essential oil of *A. anomala* were detected with the broth microdilution method against six strains of bacteria. The results of the MICs revealed that the essential oil possessed strong antibacterial activity (Table 2). In the presence of oil, the best inhibition was observed against *B. cereus* with a MIC of 0.313 μL oil/mL of incubation broth, followed by *M. flavus*, *S. epidermidis, S. aureus*, and *E. coli* with MICs at 0.625, 1.25, 5, and 10 μL/mL, respectively. The essential oil was not active against *P. aeruginosa* in the maximum dilution (20 μL/mL) in this experiment.

In general, there was a correlation between the antimicrobial activity and the percentage of some of the major components. Both camphor and 1, 8-cineole are well-known chemicals for their pronounced antimicrobial properties (23, 24), and the antimicrobial activity of *β*-caryophyllene oxide and borneol has been previously reported (25, 26). Kordali et al. compared antimicrobial activity of the essential oil consisting of a high proportion of oxygenated monoterpenes and sesquiterpenes with that of a relatively lower proportion of oxygenated monoterpenes and sesquiterpenes in many *Artemisia* species, and concluded that the latter had weaker antimicrobial activity than the former (27). This study provides new evidence to support the conclusion that the oil of oxygenated monoterpenes and sesquiterpenes is a stronger antimicrobial agent.

Table 2. The MICs of *A. anomala* essential oil.

Microorganism	MIC[a] of the essential oil (μL/mL)	MIC of the ampicillin (ng/mL)
Staphylococcus epidermidis ATCC 12228	1.250	15.6
Staphylococcus aureus (clinical isolated strain)	5.000	250.0
Micrococcus flavus ATCC 14698	0.625	62.5
Escherichia coli ATCC 11775	10.000	+[b]
Bacillus cereus CMCCB 63301	0.313	125.0
Pseudomonas aeruginosa ATCC 10145	+	+

Notes: [a]MIC values are reported as the mean of three experiments. [b]'+': not active within the testing concentrations.

Table 3. The IC_{50} values of *A. anomala* essential oil.

Cell line	A549	BRO	MCF7	PC 3
IC_{50}[a]	0. 7	0. 2	0. 09	0. 31

Notes: [a]IC_{50} values are reported as the mean of three experiments. Data are presented as μL of oil/mL of tissue culture medium.

It has been noted that some essential oils, containing complex mixtures of many compounds, exhibit stronger antimicrobial activities than do single pure compounds isolated from the essential oil (28). The essential oil consists of complex mixtures of numerous components as shown in this study and more than one active compound may have contributed to a collective antimicrobial activity. It will be of great interest to determine individual contributions of each compound, and possible additive and synergistic effects among individual oil components.

Cytotoxicity against four human cancer cell lines

The antiproliferative properties of the *A. anomala* essential oil were assessed against A459, BRO, MCF7, and PC3 cell lines. The IC_{50} values were calculated and are shown in Table 3.

Several studies have reported that some essential oils of plant can inhibit the growth of cancer cells (23, 29). Our data showed that the relative ability of the essential oil to inhibit proliferation of human cancer cells indicated a differential sensitivity. That is, MCF7 cell line was almost eight times as sensitive to the essential oil as A549 cell line. The dominate sensitivity of MCF7 to the essential oil invites further exploration of the relative activity of the oil against other human breast cancer cell lines and perhaps *in vivo* evaluation in animals as well.

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