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Article

The Leaf Essential Oil of *Acorus macrospadiceus* (Yam.) F. N. Wei & Y. K. Li from Vietnam: Chemical Composition and Antimicrobial Activity

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Abstract: In the present communication, the composition and antimicrobial activity of essential oil from the leaves of *Acorus macrospadiceus* (Yam.) F. N. Wei & Y. K. Li from Vietnam were reported. The yield of the essential oil was 0.25 % (w/w). The main constituents of the essential oil were β-caryophyllene (31.1 %), α-selinene (12.6 %), α-asarone (12.0 %) and α-humulene (8.0 %). The essential oil displayed anti-candidal activity against *Candida albicans* ATCC 10231 with a minimum inhibitory concentration (MIC) value of 8.67 µg/mL. In addition, the essential oil exhibited a broad spectrum of antimicrobial activity against the other tested microorganisms with MIC in the range 17.33 µg/mL (*Enterococcus faecalis* ATCC 299212) to 100.33 µg/mL (*Escherichia coli* ATCC 25922). The antimicrobial activities of *A. macrospadiceus* are being reported for the first time.

Keywords: *Acorus macrospadiceu,* essential oil, sesquiterpenes, *Candida albicans, Enterococcus faecalis.*

Introduction

The genus *Acorus*, belonging to the family Acoraceae, is mainly distributed from northern temperate to subtropical regions [1,2]. It was once a member of Araceae and regarded as a relatively primitive group in the family before redesignation as Acoraceae [1]. Species in this genus have multiple values including medicinal, nutritional, ornamental, cultural, economic, and ecological uses. *Acorus macrospadiceus* (Yamam.) F. N. Wei and Y. K. Li were described as a new species in 1985, were regarded as a synonym of *A. gramineus* [3]. Based on multiple approaches including phylogenetics, metabolomics, morphology, ecology, and ethnobotany, *A. macrospadiceus* is now designated as an independent species [4]. *A. macrospadiceus* (local name: Thach xuõng bo) is popular that the local people in ethnic communities use it as a flavoring agent in the preparation of meat and fish. Leaves and rhizomes are both used in cooking [5]. The plant has fennel-like smell [5].

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A previous study on the chemical constituents of essential oils from *A. macrospadiceus* revealed an abundance of estragole (93.56 % of total oil content in leaf and 71.62 % of total oil content in rhizome) [6]. The major constituents found in the oil analysed from Vietnam were *cis*-asarone (45.14 %), *trans*-asarone (15.49 %), β-caryophyllene (12.08 %), and neronidol (6.70 %) [7]. An earlier study on the volatiles from the leaves and rhizomes of *Acorus* spp. found that methyl chavicol (54.019 %) and nootkatone (15.92 %) are the major constituents of *A. macrospadiceus* essential oil [8]. *A. macrospadiceus* essential oil inhibits melanin synthesis in B16F10 melanoma cells (antimelanogenic) and showed antioxidant potential [8].

The main objective of the present paper is to report the chemical composition and antimicrobial activity of essential oils hydro distilled from the leaves of *A. macrospadiceus* growing in Vietnam. This is in continuation of extensive research aimed at the characterization of the volatile constituents and biological potentials of the poorly studied species of Vietnamese flora [9-12].

Material and methods

The leaves of A. macrospadiceus

Quantity (2.2 kg) of the leaves of *A. macrospadiceus* were obtained from plants cultivated in Pù Hoat Nature Reserve (GPS: 19°35'19''N, 104°43'7''E), Vietnam, at an elevation of 870 m, in September 2020. The plant was identified by Dr. Le Thi Huong. A voucher specimen, LTH 907, was deposited in the plant specimen room, Vinh University.

Isolation of essential oil from the leaves of A. macrospadiceus

The plant sample was processed before hydrodistillation by removing debris to obtain 2.0 kg. Thereafter, the leaf samples were pulverized into coarse particles by using a locally made grinder, to obtain 2 kg of sample which was subjected to separate hydrodistillation inside a Clevenger-type apparatus according to established specification as described in the previous studies [9-12]. The plant material was divided into three parts to ensure that hydrodistillation was repeated three times. The sample was carefully introduced into a clean and dry 5 L flask. Distilled water was added into the flask until it covered the surface of the sample completely. The procedure was maintained at normal pressure for 3 h. The essential oils of *A. macrospadiceus* which were distilled over water were collected separately by running through the tap in the receiver arm of the apparatus into clean and previously weighed sample bottles. The oils were kept under refrigeration (4°C) until the moment of analysis. The experiment was conducted in triplicate. The essential oil yield (%) was calculated by the mass (g) of the essential oils divided by the mass (g) of the leaves of the plants.

Gas chromatography (GC) analysis of the essential oil

Gas chromatographic analysis (GC) was performed on Agilent GC 7890A attached to FID detector of Agilent Technologies, USA, and fitted with HP-5MS chromatographic column with a length of 60 m, inner diameter (ID) = 0.25 mm, and film thickness of 0.25 μm. Helium was the carrier gas with a flow rate of 1.0 mL/min. The inlet temperature was 250°C, and the oven temperature was program from 60°C to 240°C at a rate of 4°C/min, with a 2 min hold. The split ratio was 100:1, and the volume of diluted (10 % *n*-hexane solution) essential oils injected into the column was 1.0 μL. The interface temperature was 270°C while the detector temperature was 260°C. Quantification was done using the calibration curves generated from the analyses of representative standard compounds from each class as described previously [9-12].

Gas chromatograph-mass spectrometry (GC/MS) analysis of the essential oil

An Agilent GC 7890A chromatograph equipped with a capillary of molten silica HP-5MS (60 m × 0.25 mm, film thickness of 0.25 μm) and coupled with a mass spectrometer (HP 5973 MSD) was used for GC/MS analysis of the essential oils. Similar conditions as described were used for GC analysis, with Helium (1 mL/min) as the carrier gas as described previously [9-12]. The MS conditions were as follows-ionization voltage

of 70 eV; emission current of 40 mA; acquisition and a scan range of 35-450 amu with a sampling rate of 1.0 scan/s.

The identification of constituents of essential oils from the GC/MS spectral of *A. macrospadiceus* was performed based on a comparison of retention indices (RI Exp.) with reference to a homologous series of *n*-alkanes (C_4-C_{40}), under identical experimental conditions. In some cases, co-injection with known compounds under the same GC conditions was employed. The mass spectral (MS) fragmentation patterns were checked with those of other essential oils of known composition in literature [13] as described recently [9-12].

Antimicrobial activity test

The microorganisms used in the study of the antimicrobial activity of the essential oil of *A. macrospadiceus* were *Enterococcus faecalis* ATCC 299212, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076, and *Candida albicans* ATCC 10231. The strains were obtained from the laboratory stock of the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam. The Mueller-Hinton Agar (MHA) and Sabouraud Agar (SA) were used as testing media respectively for bacteria and fungi.

The Minimum inhibitory concentration (MIC) and median inhibitory concentration (IC_{50}) values were measured by the microdilution broth susceptibility assay as described previously. The choice of investigated concentrations was based on previous reports on similar reports where essential oils are active within specific concentration ranges [9-12]. A 2-fold dilution range was used for the experiment. Stock solutions of the essential oils were prepared in 1 % dimethylsulfoxide. Dilution series (2-fold) were prepared from 16,384 to 2 µg/mL (2^{14}, 2^{13}, 2^{12}, 2^{11}, 2^{10}, 2^9, 2^7, 2^5, $2^{3,}$ and 2^1 µg/mL) in sterile distilled water in micro-test tubes from where they were transferred to 96-well microtiter plates. Bacteria grown in double-strength Mueller-Hinton broth or double-strength tryptic soy broth and fungi grown in double-strength Sabouraud dextrose broth were standardized to 5×10^5 and 1×10^3 CFU/mL, respectively. The last row of the micro-test tubes containing only the serial dilutions of samples without microorganisms was used as a positive (no growth) control. Sterile distilled water and medium served as a negative (no antimicrobial agent) control. Streptomycin was used as the gram-positive antibacterial standard while nystatin and cycloheximide were used as gram-negative antibacterial and antifungal standards, respectively. Streptomycin was used to treat or prevent infections that are proven or strongly suspected to be caused by several gram-positive and gram-negative bacteria including tuberculosis, *Mycobacterium,* endocarditis etc.[14]. Cycloheximide is a naturally occurring fungicide [15]. Nystatin is an antifungal that works by stopping the growth of fungus of the mouth or intestines. It has been used to treat *Candida* infections [16].

The test was based on the assessment of growth through turbidimetry (use of optical density as a measure of growth). The cultures of tested microorganisms grown overnight are diluted and read on a spectrophotometer at 600 nm in comparison with McFarland reagents (Barium Sulphate) to obtain the microbial load as standardized culture. After incubation at 37°C for 24 h, the MIC values were determined to be the lowest concentration of essential oils which completely inhibited the growth of micro-organisms.

The IC_{50} values were determined by the percentage of microorganisms that inhibited growth based on the turbidity measurement data of EPOCH2C spectrophotometer (BioTeK Instruments, Inc Highland Park Winooski, VT, USA) and Rawdata computer software (Brussels, Belgium) according to the following equations:

$$5\ \text{Inhibitin} = \frac{OD_{control(+)} - OD_{test\ agent}}{OD_{control(+)} - OD_{control(-)}} \times 100$$

$$IC_{50} = High_{conc} - \frac{(High_{inh}\ \% - 50\ \%) \times (High_{conc} - Low_{conc})}{(High_{inh}\ \% - Low_{inh}\ \%)}$$

where OD is the optical density, control (+) is the cells in medium without the antimicrobial

agent, test agent corresponds to a known concentration of the antimicrobial agent, control (-) is the culture medium without essential oils, High Conc/Low Conc is the concentration of test agent at high concentration/low concentration, and High Inh%/Low Inh% is the % inhibition at high concentration/% inhibition at low concentration.

Statistical analysis

All results of chemical composition and antimicrobial experiments were repeated three times and are expressed as mean ± standard deviation (SD).

Results and discussion

Chemical constituents of A. macrospadiceus

The hydrodistillation of the leaves of *A. macrospadiceus* produced light-yellow coloured essential oil. The average yield of essential oil was 1.13 g ± 0.01 (w/w) or 0.25 % (w/w). Table 1 presents the compounds as identified by GC/MS. Thirty-seven compounds accounting for 99.6 % of the essential oil contents were identified in the leaves of *A. macrospadiceus*. The monoterpene classes of compounds are less common in the essential oil (ca. 3.4 %). The composition of the essential oil was dominated by sesquiterpene hydrocarbons (70.8 %) and phenylpropanoids (18.2 %). The main constituents of the essential oil were β-caryophyllene (31.1 %), α-selinene (12.6 %), α-asarone (12.0 %), α-humulene (8.0 %), β-cubebene (6.0 %) and β-asarone (5.7 %). Other compounds occurring in significant amounts include caryophyllene oxide (3.2 %), β-selinene (3.1 %), and methyl chavicol (2.4 %).

Table 1. Percentage composition of essential oil from the leaves of *A. macrospadiceus*

No.	RT (min)	Compounds[a]	RI (Exp.)	RI (Lit.)	Concentration (%)[b]
1	10.02	α-Pinene	938	932	0.5
2	11.20	Sabinene	978	968	0.2
3	11.37	β-Pinene	984	978	0.3
4	15.28	Linalool	1101	1100	2.4
5	18.86	Methyl chavicol	1203	1198	0.3
6	24.14	α-Cubebene	1359	1354	1.0
7	24.33	α-Longipinene	1365	1364	0.3
8	25.08	α-Copaene	1388	1387	0.4
9	25.44	β-Bourbonene	1399	1398	1.4
10	25.51	β-Cubebene	1401	1400	6.0
11	26.64	β-Caryophyllene	1437	1433	31.1
12	26.85	β-Gurjunene	1444	1445	0.2
13	27.35	(*Z*)-β-Farnesene	1460	1452	0.4
14	27.53	Prezizaene	1466	1470	0.3
15	27.68	α-Humulene	1470	1472	8.0
16	28.27	β-Chamigrene	1489	1490	0.6
17	28.30	ar-Curcumene	1490	1492	0.2
18	28.50	Germacrene D	1497	1495	1.1
19	28.70	β-Selinene	1503	1503	3.1
20	28.86	*trans*-Muurola-4(14),5-diene	1509	1510	1.7
21	28.97	α-Selinene	1512	1513	12.6
22	29.10	β-Bisabolene	1516	1515	1.0
23	29.51	γ-Cadinene	1530	1528	1.0
24	29.66	δ-Cadinene	1535	1532	0.4
25	30.42	*trans*-Cadinene ether	1561	1559	0.4

table 1. (continued).

No.	RT (min)	Compounds[a]	RI (Exp.)	RI (Lit.)	Concentration (%)[b]
26	30.65	(*E*)-Nerolidol	1568	1561	0.8
27	31.67	Caryophyllene oxide	1603	1601	3.2
28	32.28	β-Asarone	1625	1627	5.7
29	32.42	Humulene oxide II	1629	1630	0.6
30	32.86	1-*epi*-Cubenol	1645	1644	0.2
31	33.47	Ageratochromene	1667	1669	0.2
32	33.68	*neo*-Intermedeol	1674	1672	0.7
33	34.00	α-Asarone	1685	1688	12.0
34	34.85	Pentadecanal	1716	1716	0.5
35	35.09	Asaronaladehyde	1725	1727	0.2
36	36.98	*cis*-5-Hydroxycalamenene	1795	1782	0.2
37	44.78	Phytol	2116	2119	0.4
		Total			99.6
		Monoterpene hydrocarbons (Sr. No. 1-3)			1.0
		Oxygenated monoterpene (Sr. No. 4)			2.4
		Sesquiterpene hydrocarbons (Sr. No. 6-24)			70.8
		Oxygenated sesquiterpenes (Sr. No. 25-27, 29, 30, 32, 36)			6.1
		Diterpenes (Sr. No. 37)			0.4
		Phenylpropanoids (Sr. No. 5, 28, 33, 35)			18.2
		Chromenes (Sr. No. 31)			0.2
		Aliphatic aldehyde (Sr. No. 34)			0.5

[a]Elution order on HP-5MS column
RI (Exp.) Retention indices on HP-5MS column
RI (Lit.) Literature retention indices (NIST, 2018);
[b]Standard deviation were insignificant and excluded from the Table to avoid congestion
RT, retention times (min)

A comparative analysis of the present result with previous data on the essential oils of *A. macrospadiceus* revealed some variation in their compositional pattern. Estragole and nootkatone, the major compounds of the leaf essential oils reported from China [7,8], were not identified in the present studied oil sample. In addition, the content of methyl chavicol, a characteristic compound of *A. macrospadiceus* was identified in a much lower quantity (2.4 % vs. 54.09 %) in the present investigated oil sample when compared with a previous study [8]. However, the content β-caryophyllene (31.1 %) competes favorably with value (12.08 %) obtained from a previously analysed oil sample from Vietnam [7], although the contents of α-asarone and β-asarone are much lower. The differences in the compositional pattern of *A. macrospadiceus* essential oils grown in Vietnam may be due to locality of harvest, phonological stage of the plant, etc. Based on the chemotaxonomic analysis, essential oils from *A. macrospadiceus* may be thought to exist in three chemical forms namely:

i. Essential oil with high content of estragole [6].
ii. Essential oil dominated by methyl chavicol and nootkatone [8].
iii. Essential oil containing a significant amount of asarone and β-caryophyllene [7] and this study.

Results of antimicrobial test

Essential oil from the leaf of *A. macrospadiceus* displayed antimicrobial activity towards five of

the tested microorganisms, and anti-candidal activity, with the minimum inhibitory concentration (MIC) values in most cases < 50 µg/mL. (Table 2). The essential oil exhibited anti-candidal action towards *C. albican*s ATCC 10231 with a MIC value of 8.67 µg/mL, with IC_{50} value of 16.0 µg/mL. The order of antibacterial activity was *Enterococcus faecalis* ATCC 299212 (MIC, 17.33 µg/mL) > *Staphylococcus aureus* ATCC 25923 (MIC, 20.45 µg/mL) > *Pseudomonas aeruginosa* ATCC 27853 (MIC, 21.23 µg/mL) > *Bacillus cereus* ATCC 14579 (MIC, 25.67 µg/mL) > *Escherichia coli* ATCC 25922 (MIC, 100.33 µg/mL). The MIC and IC_{50} values provided evidence that the leaf essential oil of *A. macrospadiceus* displayed potent antimicrobial and anti-candidal activities against the tested microorganisms except for *Salmonella enterica* ATCC 13076. Recent findings indicated that substances with MIC values ≤ 100 µg/mL were considered to be of good antimicrobial activity [14]. Thus, *A. macrospadiceus* should be considered a promising antimicrobial agent because the essential oil displayed antibacterial activity with most MIC < 50 µg/mL. Streptomycin, the standard antimicrobial agent for gram-positive bacteria displayed antimicrobial activity with MIC values in the range 0.28 µg/mL to 3.20 µg/mL. In addition, nystatine used as a standard antimicrobial agent for gram-negative bacteria had MIC value of 8.0 µg/mL, with cycloheximide, an anti-candidal agent, showing activity at MIC of 3.20 µg/mL. This is the first report on the antimicrobial activity of the essential oil of *A. macrospadiceus*.

The antimicrobial activities of the essential oil of *A. macrospadiceus* can be related to its main compounds or some synergy between the major and minor compounds. These compounds exhibit activity by firstly destroying the microbial cytoplasmic wall to enhance the permeability and passage of large protons and ions [16]. Nevertheless, the antibacterial effect can be sum up as cumulative actions of several compounds and not to a specific compound [9-12]. Further, due to the complexity of the composition of the essential oils, it is also difficult to explain the mechanism of action of these blends but is important to underline that the wide variety of composition is

Table 2. Antimicrobial activity of the leaf essential of *A. macrospadiceus*

Sample	Gram (+)			Gram (-)			Yeast
	Enterococcus faecalis ATCC 299212	*Staphylococcus aureus* ATCC 25923	*Bacillus cereus* ATCC 14579	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Salmonella enterica* ATCC 13076	*Candida albicans* ATCC 10231
				MIC (µg/mL) [a]			
A. macrospadiceus	17.33± 0.11	20.45 ± 0.50	25.67 ± 0.50	100.33±0.67	21.23 ± 0.11	-	8.67 ± 0.00
Streptomycin	0.28	1.29	3. 20	Nt	Nt	-	Nt
Nystatine	Nt	Nt	Nt	8.0	-8.0	-	Nt
Cycloheximide	Nt	Nt	Nt	Nt	Nt	-	3.20
				IC_{50} (µg/mL) [a]			
A. macrospadiceus	32.0 ± 0.42	64.0 ± 0.59	64.0 ± 0.55	256.0 ±1.00	64.0 ± 0.55	-	16.0 ± 0.10

[a] Means of three replicates analysis; - No activity; Nt Not tested

a positive factor that may limit the development of resistance which is otherwise very common for synthetic drug [16]. The major constituents of essential oil *A. macrospadiceus* e.g. β-caryophyllene [17] and μ-asarone [18] have shown anti-microbial activities against a host of microorganisms. The antimicrobial activities of some other compounds present in the essential oil have been reported [9-12], and are likely to account for the observed antimicrobial activity.

Conclusions

This study found out that β-caryophyllene (31.1 %), α-selinene (12.6 %), α-asarone (12.0 %), and α-humulene (8.0%) were the main constituents of the leaf essential oil of *A. macrospadiceus*. The essential oil displayed anti-candidal activity against *C. albicans* ATCC 10231 with MIC value of 8.67 μg/mL. In addition, the essential oil also exhibited good antimicrobial activity towards *E. faecalis* ATCC 299212 (MIC 17.33 μg/mL), *S. aureus* ATCC 25923 (MIC 20.45 μg/mL), *B. cereus* ATCC 14579 (MIC 25.67 μg/mL), and *P. aeruginosa* ATCC 27853 (MIC 21.23 μg/mL). The oil sample did not show any activity against *S. enterica* ATCC 13076. Therefore, the essential oil of *A. macrospadiceus* may have potential applications as antimicrobial agents.

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Competing interests

The authors declare that they have no competing interests.

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