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Anti-bacterial effect of phytoconstituents isolated from *Alimatis rhizoma*

Chengfu Li[1], Wei Yan[1,2], Enji Cui[1*] and Changji Zheng[1*]

Abstract

Five compounds including three triterpenoids and two sesquiterpenes were isolated from *Alimatis rhizoma*. Their chemical structures were determined to be alisol B 23-acetate (**1**), alisol C 23-acetate (**2**), alisol B (**3**), alismol (**4**) and alismoxide (**5**) by various spectroscopic analysis, including ^{1}H-NMR, ^{13}C-NMR, HMBC and MS spectra. Compounds **1**–**5** were evaluated for their antibacterial potential against 6 strains of bacteria including three drug-resistant bacteria (one methicillin-resistant *Staphylococcus aureus* strain CCARM 3506, two quinolone-resistant *Staphylococcus aureus* strains CCARM 3505 and CCARM 3519), two G^+ bacteria (*Streptococcus mutans* KCTC 3289 and *Staphylococcus aureus* KCTC 209) and one G^- bacterium (*Escherichia coli* KCTC 1924). Compounds **1**–**5** showed strong antibacterial effect against *S. mutans* KCTC 3289, their MIC values were 2, 64, 16, 32 and 32 μg/mL, respectively. The antibacterial activity results of compounds **1**–**5** against these bacteria were reported for the first time. The results indicate that *Alimatis rhizoma* are potential sources of new antibacterial material.

Keywords *Alimatis rhizoma*, Phytoconstituents, Antibacterial activity, Structure–active relationship

Introduction

Since the advent of antibiotics, it has cured countless infected patients around the world, but with the passage of time and people's abuse of antibiotics, it has led to the emergence of drug-resistant strains. The original strong antibacterial effect is getting worse, even some antibiotics have been loss of function which seriously threatens human life [1]. Today the drug resistance crisis is becoming more and more serious, and has became one of the biggest threats facing the global society. If the trend of bacterial resistance is not effectively controlled, human will fall into the crisis of no drugs available, so it is urgent to separate active compounds with good antibacterial activity from natural medicines.

*Correspondence:
Enji Cui
ejcui@ybu.edu.cn
Changji Zheng
zhengcj@ybu.edu.cn
[1] College of Pharmacy, Yanbian University, Yanji 133000, People's Republic of China
[2] Changchun Food and Drug Inspection Center, Changchun 130012, People's Republic of China

Alimatis rhizoma, the dried tuber of *Alisma orientalis*, is a widely used traditional herbal medicine in China, and broadly distributed in Fujian, Sichuan, Jiangxi, Yunnan province and other areas of China. Triterpenes and sesquiterpenes [2] were typical metabolites of *A. rhizoma*, also it contained diterpenes, volatile oils and alkaloids. *A. rhizoma* had various pharmacological effects including lowering blood lipids [3], anti-urolithiatic effect [4], anti-inflammatory effect [5, 6], anti-fatty liver effect [7, 8], diuretic effect [9], protecting cardiovascular system [10] and anti-cancer effects [11, 12].

In this research, we demonstrated the isolation and structural characterization of three protostane-type triterpenoids (**1**–**3**) and two guaiane-type sesquiterpenes (**4**, **5**) from the methanolic extract of *A. rhizoma*. All compounds were explored for antibacterial activity test.

Materials and methods

Plant materials

Alimatis rhizoma were purchased from YanBian Wei Ye pharmacy in YanJi and Prof. Hui-Zi Lv of College of Pharmacy, YanBian University, YanJi, China identified.

A voucher specimen (YBU-R012) was stored at the Medicinal Chemistry of Natural Products Laboratory, YanBian University, YanJi, China.

Instruments and reagents

NMR spectra were recorded on a Bruker 300 MHz (AV 300, Germany). Mass spectra were measured on a time of flight-mass spectrometry (TOF-MS, AXIMA-QIT, Shimadzu, Tokyo, Japan). Column chromatography was performed using silica gel (200–300 mesh, Qingdao Haiyang, Qingdao, China) and octadecylsilyl (ODS) silica gel (Kieselgel 60, Art 7734, Merck, Germany). Thin-layer chromatography (TLC) on pre-coated silica gel GF_{254} (200×200 mm, Branch of Qingdao Haiyang Chemical, Qingdao, China) and ODS (Kieselgel 60 F254, Art 5714, Merck, Germany) were carried out [13].

Extraction and isolation of *A. rhizoma*

The air-dried powdered *A. rhizoma* (5 kg) was soaked in methanol (MeOH), then obtained 298 g of methanol extract by filtration and concentration. The extract was dissolved with 0.5 L of distilled water, and successively fractionated with petroleum ether (PE, 0.5 L×3) and ethyl acetate (EtOAc, 0.5 L×3). The concentrated EtOAc extract (50.89 g) was applied to normal phase silica gel (SiO_2) column chromatography (CC), then fully eluted with a gradient of dichloromethane (CH_2Cl_2)-MeOH (1:0–0:1) to obtain 13 fractions (EA–EM). Fr.EF (11.22 g) was subjected to SiO_2 column, and gradient elution was performed using PE-EtOAc (100:1–0:1) to get 5 fractions (Fr.EF1–EF5). Compound **1** (100 mg) was obtained from Fr.EF3. Fr.EF4 (5.54 g) was further subjected to SiO_2 CC with CH_2Cl_2–MeOH (20:1–0:1) to yield 4 fractions (Fr.EF4-1–EF4-4) and give a compound **2** (18.9 mg). Fr.EF4-2-3 (169 mg) was purified by SiO_2 column and eluted by PE-EtOAc (20:1–0:1) to produce a compound **5** (28.8 mg). Normal phase silica gel column chromatography was performed on Fr.EH. After using PE-EtOAc (15:1–0:1) to perform gradient elution, and then Fr.EH2 was eluted with PE-EtOAc (25:1–0:1) to obtain a compound **3** (166.7 mg). The PE extract (38.56 g) was used on SiO_2 CC and fully eluted with PE-EtOAc (100:1–0:1) to obtain 6 fractions (Fr.PA–PF). Fr.PC (11 g) was purified by SiO_2 column using PE-EtOAc (100:1–0:1) to yield 3 fractions (Fr.PC1–PC3). Fr.PC2 was purified through SiO_2 column and eluted with PE-CH_2Cl_2 (100:1–0:1) to give a compound **4** (30 mg).

Alisol B 23-acetate (**1**): White amorphous powder; TOF-MS m/z 537 [M+Na], $C_{32}H_{50}O_5$; ^{1}H-NMR (300 MHz, $CDCl_3$, δ_H): 4.59 (1H, m, H-23), 3.80 (1H, m, H-11), 2.73 (1H, d, J=8.5 Hz, H-24), 2.55 (1H, dd, J=13.2, 5.7 Hz, H-12), 2.07 (3H, s, OAc), 1.71 (1H, d, J=10.7 Hz, H-9), 1.33, 1.31, 1.14, 1.07, 1.05, 1.03, 0.96 (3H, each, s), 1.02 (3H, d, J=7.1 Hz, H-21); ^{13}C-NMR (75 MHz, $CDCl_3$, δc): in Table 1.

Alisol C 23-acetate (**2**): White amorphous powder; TOF-MS m/z 551 [M+Na], $C_{32}H_{48}O_6$; ^{1}H-NMR (300 MHz, $CDCl_3$, δ_H): 4.55 (1H, m, H-23), 3.98 (1H, m, H-11), 2.72 (1H, d, J=8.5 Hz, H-24), 2.42 (1H, m, H-15β), 2.07 (3H, s, OAc), 1.88 (1H, m, H-15α), 1.75 (1H, m, H -22α), 1.19 (3H, d, J=7.0 Hz, H-21), 1.32, 1.29, 1.22, 1.09, 1.08, 1.07, 0.89 (3H, each, s); ^{13}C-NMR (75 MHz, $CDCl_3$, δ_c): in Table 1.

Alisol B (**3**): White amorphous powder; TOF-MS m/z 472, $C_{30}H_{48}O_4$; ^{1}H-NMR (300 MHz, $CDCl_3$, δ_H): 3.86 (1H, m, H-11), 3.19 (1H, m, H-23), 2.92 (2H, m, H-20 and

Table 1 ^{13}C-NMR spectroscopic data of compounds **1–5** (δ in ppm)

	1	2	3	4	5
C-1	30.9	30.8	31.0	55.0	50.6
C-2	36.7	33.6	33.8	24.8	22.5
C-3	220.1	219.4	220.1	40.2	40.4
C-4	47.0	47.0	47.0	80.7	80.2
C-5	48.4	48.7	48.5	47.3	50.3
C-6	20.1	20.0	20.1	121.3	121.3
C-7	34.2	34.8	34.3	149.8	149.7
C-8	40.7	40.0	40.6	30.0	25.1
C-9	49.9	49.8	49.6	37.1	42.5
C-10	36.9	36.9	36.9	153.9	75.3
C-11	70.1	69.8	69.8	37.4	37.3
C-12	34.4	35.5	34.3	21.3	21.2
C-13	138.2	177.2	138.1	21.5	21.4
C-14	57.0	49.8	57.0	24.1	21.5
C-15	30.6	45.7	30.6	106.5	21.3
C-16	29.1	208.1	29.1		
C-17	134.1	134.4	134.8		
C-18	23.2	23.1	23.3		
C-19	25.7	25.5	25.5		
C-20	27.8	26.7	27.7		
C-21	20.1	20.1	20.2		
C-22	33.7	35.0	38.8		
C-23	71.5	71.9	69.1		
C-24	65.1	64.9	67.8		
C-25	58.5	58.7	59.2		
C-26	19.4	19.7	19.2		
C-27	24.7	24.7	24.9		
C-28	29.6	29.6	29.6		
C-29	20.0	19.3	20.1		
C-30	23.8	23.1	24.0		
–O–$\underline{C}OCH_3$	170.1	170.2			
–O–$CO\underline{C}H_3$	21.2	21.2			

1–5 in $CDCl_3$, 75 MHz

22β), 2.80 (1H, dd, J=13.4, 5.7 Hz, H-12α), 2.69 (1H, d, J=8.1 Hz, H-24), 1.73 (1H, d, J=10.7 Hz, H-9), 1.50 (1H, m, H-22α), 1.02 (3H, d, J=7.0 Hz, H-21), 1.30, 1.22, 1.11, 1.07, 1.05, 1.04, 0.98 (3H, each, s); ^{13}C-NMR (75 MHz, $CDCl_3$, δ_c): in Table 1.

Alismol (**4**): Colorless oil; TOF-MS m/z 220, $C_{15}H_{24}O$; ^{1}H-NMR (300 MHz, $CDCl_3$, δ_H): 5.57 (1H, s, H-6), 4.72, 4.78 (1H. each, s, H_2-15), 1.26 (3H, s, H-14), 1.01 (3H, d, J=6.8 Hz, H-13), 0.99 (3H, d, J=6.8 Hz, H-12); ^{13}C-NMR (75 MHz, $CDCl_3$, δ_c): in Table 1.

Alismoxide (**5**): White amorphous powder; EI-MS m/z 220 $[M\text{-}H_2O]^+$, $C_{15}H_{26}O_2$; ^{1}H-NMR (300 MHz, $CDCl_3$, δ_H): 5.50 (1H, d, J=2.6 Hz, H-6), 1.27 (3H, s, H-15), 1.21 (3H, s, H-14), 1.01 (3H, d, J=6.7 Hz, H-13), 0.98 (3H, d, J=6.7 Hz, H-12); ^{13}C-NMR (75 MHz, $CDCl_3$, δ_c): in Table 1.

Antibacterial susceptibility test

The antibacterial test was performed using six strains of bacteria. The Culture Collection of Antimicrobial Resistant Microbes of Korea (CCARM) supplied three drug-resistant bacteria strains of methicillin-resistant *Staphylococcus aureus* (MRSA CCARM 3506) and quinolone-resistant *Staphylococcus aureus* (QRSA CCARM 3505 and CCARM 3519). The Korean Collection for Type Cultures (KCTC) supplied the remaining bacteria strains including two G^+ bacteria of *Streptococcus mutans* (KCTC 3289) and *Staphylococcus aureus* (KCTC 209), one G^- bacteria of *Escherichia coli* (KCTC 1924). The bacteria were cultured in Mueller–Hinton Broth (MHB) and grown to mid-log phase, and then diluted 1000 times in the medium under the same environment. Under aseptic conditions, the cells were inoculated into MHB broth, and then evenly distributed into 96-well microtiter plates for further cultivation. The antibacterial activity of the compounds was tested by serial dilution to determine the minimum inhibitory concentration (MICs). A microtiter ELISA reader was used to measure the absorbance at 650 nm to determine bacterial growth.

Results and discussion

Three tetracyclic triterpenoids (**1**–**3**) and two sesquiterpenes (**4**, **5**) were isolated from the methanolic extract of *A. rhizoma*. Their chemical structures were determined as alisol B 23-acetate (**1**), alisol C 23-acetate (**2**), alisol B (**3**), alismol (**4**), alismoxide (**5**) (Fig. 1) using spectroscopic methods including 1D, 2D NMR (HMBC, HMQC) and TOF-MS spectra.

Compound **1**, white amorphous powder. Its molecular weight was determined to be 514 from the molecular ion peak at *m/z* 537 [M+Na] in TOF-MS. The ^{1}H-NMR spectrum showed three oxygenated methine proton signals at δ_H 4.59 (m, H-23), 3.80 (m, H-11) and 2.73 (d, J=8.5 Hz, H-24), one acetyl group at δ 2.07 (3H, s), seven tertiary methyl groups at δ 0.96, 1.03, 1.05, 1.07, 1.14, 1.31, 1.33 (each 3H, s) and one secondary methyl proton signal at δ_H 1.02 (d, J=7.1 Hz). The ^{13}C-NMR spectral data revealed 32 carbons, including one ketone carbon (δ_c 220.1), one carbonyl carbon (δ_c 170.1), two quaternary olefinic carbons (δ_c 138.2 and 134.1), three oxygenated methine carbons (δ_c 65.1, 70.1, 71.5), one oxygenated quaternary carbon (δ_c 58.5), seven tertiary methyl carbons (δ_c 19.4, 20.0, 23.2, 23.8, 24.7, 25.7, 29.6), one secondary methyl carbon (δ_c 20.1) and one acetyl methyl carbon (δ_c 21.2). Thus, compound **1** was identified to be alisol B 23-acetate by comparison with those found in the literature [14].

Compound **2** showed very similar ^{13}C-NMR spectrum with that of **1**, except for one carbonyl carbon at δ_c 208.1 (C-16) in place of one methylene carbon. In comparison with chemical shifts of C-13, C-14 and C-15, downfield shift at C-13 (δ_c 177.2) and C-15 (δ_c 45.7), upfield shift at C-14 (δ_c 49.8) were observed in **2**. Compound **2** was determined to be alisol C 23-acetate by comparison with previously reported data [14].

Compound **3** showed very similar NMR signals with that of **1**, except that acetyl group of C-23 was converted to hydroxyl group in **3**. By comparison with previously reported data, **3** was identified as alisol B [14].

Compound **4**, colorless oil. The molecular formula was determined to be $C_{15}H_{24}O$ on the basis of a TOF-MS peak at m/z 220 [M]. By analyzing the carbon spectrum data, compound **4** was indicated that it was a sesquiterpene skeleton. The ^{1}H-NMR spectrum showed one olefinic methine proton signal at δ_H 5.57 (1H, s, H-6), a couple of exomethylene proton signals at δ_H 4.78 and 4.72 (1H, each, s, H_2-15), and three methyl proton signals at δ_H 1.26 (3H, s, H-14), δ 1.01 (3H, d, J=6.8 Hz, H-13) and 0.99 (3H, d, J=6.8 Hz, H-12). The ^{13}C-NMR spectrum confirmed the presence of 15 carbon signals, including four olefinic carbons at δ_c 153.9 (C-10), 149.8 (C-7), 121.3 (C-6) and 106.5 (C-15), one oxygenated carbon at δ_c 80.7 (C-4), three methine carbons at δ_c 55.0 (C-1), 47.3 (C-15) and 37.4 (C-11), along with three methyls at δ_c 24.1 (C-14), 21.5 (C-13) and 21.3 (C-12). From the NMR spectroscopic data, suggested that **4** is bicyclic sesquiterpenes, which is derivative of pseudoguaiane-type sesquiterpene. Thus, the structure of **4** was identified as alismol [15].

The molecular weight of **5** was determined to be 238 from the molecular ion peak at *m/z* 220 $[M\text{-}H_2O]^+$ in EI-MS, indicating that the structure of **5** had one more hydroxyl group than **4**. NMR signals of **5** were similar to those of **4**, with the exception of the signals from exomethylene double bond of **4** converted into methyl group (δ_H 1.27, δ_c 21.3) and tertiary alcohol group (δ_c 75.3) in **5**. Consequently, compound **5** was determined as alismoxide [15].

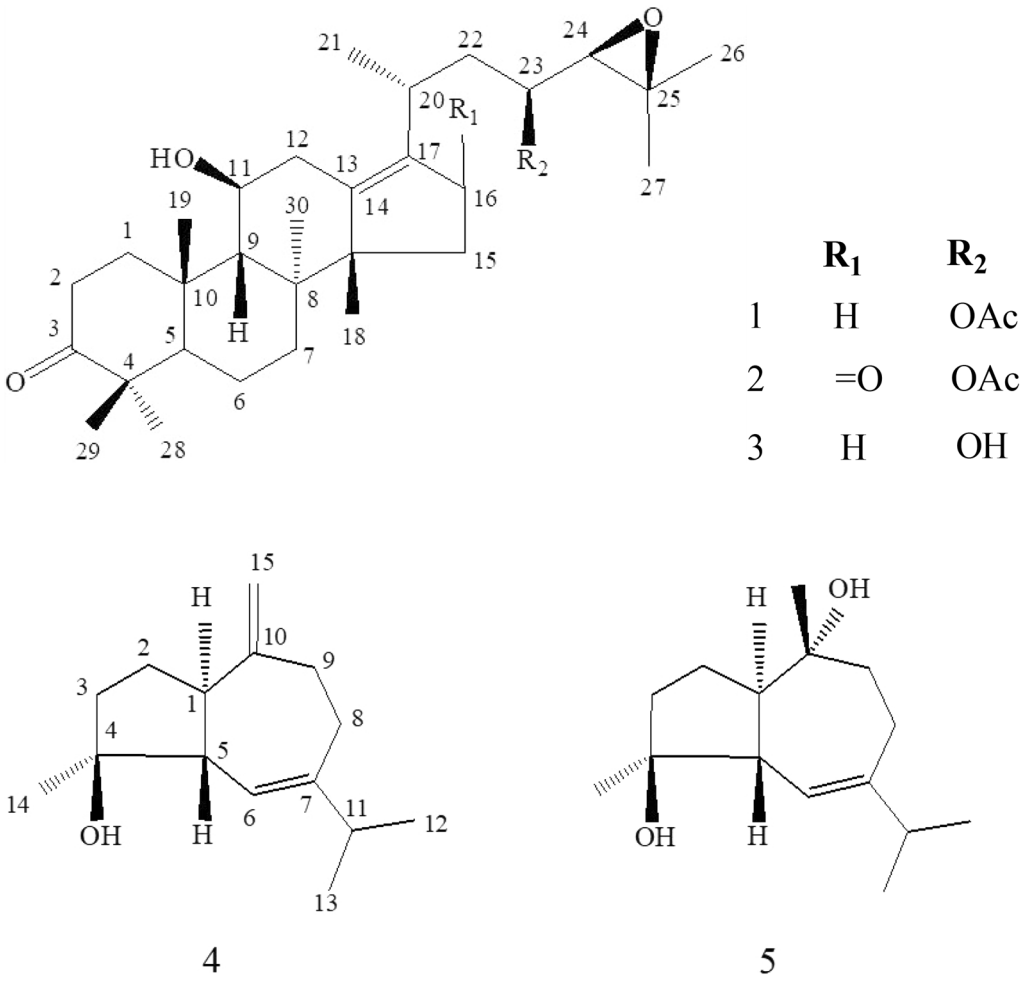

Fig. 1 Chemical structures of isolated compounds **1–5** from *A. rhizoma*

Antibacterial activity

All isolated compounds were evaluated the antibacterial potential against six strains of bacteria including three drug-resistant bacteria (MRSA CCARM 3506, QRSA CCARM 3505 and CCARM 3519), two G^+ bacteria (*S. mutans* KCTC 3289 and *S. mutans* KCTC 209) and one G^- bacteria (*E.coli* KCTC 1924). All compounds displayed strong antibacterial effect against *S. mutans* 3289 with the MIC values of 2–64 μg/mL (Table 2). Among them, compound **1** had the strongest antibacterial activity, even more than streptomycin (MIC: 8 μg/mL), positive control. In particular, when acetyl group of compound **1** was converted to hydroxyl group in **3**, the MIC value has increased from 2 to 16 μg/mL; methylene group at C-16 of **1** was converted to ketone group in **2**, the MIC value has also increased from 2 to 64 μg/

Table 2 The MICs of five compounds isolated from *A. rhizoma* (μg/mL)

Bacteria	1	2	3	4	5	Chloramphenicol	Streptomycin
S. aureus CCARM 3519	> 256	> 256	> 256	128	> 256	8	16
S. aureus CCARM 3506	> 256	> 256	128	128	> 256	8	8
S. aureus CCARM 3505	> 256	> 256	> 256	128	> 256	8	16
S. mutans KCTC 3289	2	64	16	32	32	1	8
S. aureus KCTC 209	> 256	> 256	> 256	128	> 256	8	> 64
E. coli KCTC 1924	> 256	> 256	> 256	128	> 256	4	> 64

Table 3 The MBCs of five compounds isolated from *A. rhizoma* (μg/mL)

Bacteria	1	2	3	4	5	Chloramphenicol	Streptomycin
S. aureus CCARM 3519	> 256	> 256	> 256	256	> 256	8	16
S. aureus CCARM 3506	> 256	> 256	256	256	> 256	8	8
S. aureus CCARM 3505	> 256	> 256	> 256	256	> 256	8	16
S. mutans KCTC 3289	2	64	16	32	32	1	8
S. aureus KCTC 209	> 256	> 256	> 256	128	> 256	8	> 64
E. coli KCTC 1924	> 256	> 256	> 256	128	> 256	4	> 64

mL. This observation suggested that the absence of acetyl group at C-23 or the presence of ketone group at C-16 in protostane-type resulted in the loss of antibacterial activity against *S. mutans* 3289. In addition, compounds **4** and **5** exhibited the same antibacterial activity with MICs of 32 μg/mL, indicating that the presence of exomethylene group at C-10 of guaiane-type sesquiterpene had not effect on antibacterial activity. Moreover, the minimum bacterial concentration (MBC) values of five compounds were lower than 4 × MIC (Table 3).

In conclusion, five compounds including three triterpenoids and two sesquiterpenes were isolated from *A. rhizoma*. All compounds showed strong antibacterial activities against *S. mutans* 3289 with MIC values of 2–64 μg/mL. Among them, compound **1** had a good potential for use antibacterial agents. In addition, from the structure effective relationship, it found that acetyl group at C-23 and/or ketone group at C-16 of protostane-type is probably active group. It is the first report on antibacterial activity of all isolated compounds against six bacteria strains. Taken together, these results could provide potential sources of antibacterial compounds for *A. rhizoma*.

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Author's contributions
CF Li analyzed data and wrote the original draft. W Yan analyzed the antibacterial activity. EJ Cui reviewed and edited the manuscript. CJ Zheng administrated this study. All authors read and approved the final manuscript.

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Availability of data and materials
All data analyzed during this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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