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Article

Coumarins from the Roots of *Angelica archangelica* and Antibacterial Activity Against Methicillin Resistant *Staphylococcus aureus*

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ABSTRACT: *Angelica archangelica* (Fam. Apiaceae) roots were extracted sequentially with *n*-hexane, dichloromethane (DCM) and methanol (MeOH) using a Soxhlet apparatus. The *n*-hexane and DCM extracts were fractionated by Vacuum Liquid Chromatography (VLC) over silica gel followed by Preparative Thin Layer Chromatography (PTLC) to yield two coumarins, osthol (**1**) and osthenol (**2**). However, the methanol extract was fractionated by solid-phase extraction using C18 cartridge followed by analysis of components by analytical High Performance Liquid Chromatography (HPLC) and purification by preparative High Performance Liquid Chromatography (HPLC) to give two coumarins, bergapten (**3**) and heraclenol (**4**) along with sucrose (**5**). Compounds were identified by a series of 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR spectroscopy and mass spectrometric analysis. The crude extracts (*n*-hexane, DCM, and methanol) and compound **1**, were screened for antimicrobial activity against Gram-positive bacteria (Methicillin resistant *Staphylococcus aureus*, MRSA and *Micrococcus luteus*), Gram-negative bacteria, *Pseudomonas aeruginosa*, and a fungus, *Candida albicans*. The antimicrobial assays showed that the crude DCM and *n*-hexane extracts, alongside compound **1**, largely inhibited the growth of the microorganisms at high concentrations. Compound **1,** which was further tested for antibacterial activity against a series of clinical isolates of methicillin resistance *Staphylococcus aureus*, showed moderate level of activity against only one strain.

Key words: *Angelica archangelica*, coumarins, antibiotic resistance, antimicrobial activity, MRSA.

INTRODUCTION

Antibiotic resistance has become a major global public healthcare problem. Although there are several classes of antibiotics currently available for clinical uses, the pathogenic microorganisms have been developing resistance to existing antimicrobial drugs by several mechanisms. The situation of antimicrobial resistance is even worse in developing countries because of the accessibility of antibiotics without a prescription, self-medication and irrational uses of antibitiocs.[1] The global death toll due to antimicrobial resistace has been estimated to rise to 10 million per year by 2050 if no intervention is taken or no novel antibiotic is discovered.[2] This notorious problem of antimicrobial drug resistance has inspired scientists to look for novel antimicrobial compounds, particularly those from natural sources, to act against the clinical isolates of multi-drug resistance microbes.

Angelica archangelica L. (Fam. Apiaceae), commonly known as 'garden angelica' is a species of wild European celery that has been cultivated as a medicinal plant since the 10th century and is predominantly found in Scandinavia and Russia. The roots and rhizomes of this plant have been used as a antimicrobial, antispasmodic, carminative,

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cholagogue, choleretic, diaphoretic, diuretic and a sedative agent.[3,4] The medicinal properties of *A. archangelica* are largely owing to the presence of coumarins and furocoumarins which form their reserve of phytoalexins, a plant's response to parasites and infections. A coumarin known as 6-hydroxy coumarin was found to be a fungicide while other mixtures of phytoalexins were found to be able to eradicate *C. albicans in vitro*, with high potency.[5] *A. archangelica* is also present in the Ayurveda, where it is known as "chanda", and indicated for its use to treat fevers, toothaches and headaches.[6] Previous phytochemical studies on *A. archangelica* have demonstrated the presence of essential oils[7] and coumarins.[8-11] Here we report the isolation and identification of courmarins from the roots of *A. archangelica* using a wide range of chromatographic techniques including HPLC as well as the antimicrobial activities against various microbes including methicillin resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

General. HREIMS was recorded on a Micromass Q-TOF Global Tandem Mass Spectrometer. NMR spectra (both 1D and 2D) were obtained on a Bruker AVANCE 600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C), using the residual solvent peaks as internal standard. Vacuum-liquid chromatography (VLC) for hexane and dichloromethane extracts was carried out using Merck silica gel 60 H. Solid phase extraction for methanol extract was carried out using prepacked column with reversed-phase silica gel (C18). Reversed phase HPLC was performed on a Dionex Ultimate 3000 HPLC system coupled with a photodiode array (PDA) detector, using a Phenomenex Gemini-NX 5 U C_{18} column (150 × 4.6 mm, 5 µm), Phenomenex (Macclesfield, UK), and gradient solvent systems comprising MeOH containing 0.1% TFA (solvent B) (Loughborough, UK) and water containing 0.1% TFA (solvent A) at a flow rate of 1 ml/min. Prepartive HPLC separation was performed on an Agilent prep HPLC system comprising a PDA detector and an autosampler, and using a Hichrom preparative C18 silica column (150 mm x 21.2 mm x 10 µm), and the same solvent system as in the analytical runs, with the only exception of the flow rate, which was 10 ml/min in prep runs. The column temperature was set at 25°C. TLC and PTLC were conducted on normal-phase Merck Silica gel 60 PF_{254} and reverse phase Merck Silica gel RP-18 PF_{254} plates (20 cm × 20 cm). Spots on TLC and PTLC plates were visualised under UV light (254 and 366 nm) and spraying with 1% vanillin-H_2SO_4 followed by heating at 110°C for 5-10 min.

Materials. *A. archangelica* L. root cuts (Batch number: 18388) were purchased from Herbal Apothecary, an authentic supplier of medical plants in the UK. The plant materials were ground into powder using a coffee grinder.

Extraction, fractionation and isolation of compounds. The ground plant materials (386 g) were extracted sequentially with *n*-hexane, DCM and methanol using a Soxhlet apparatus. Each of these three extracts was completely evaporated to dryness using a rotary evaporator. These extracts were analysed by analytical TLC to reveal the presence of compounds of various types.

The *n*-Hexane extract (10 g) was then subjected to VLC over silical gel 60H eluted with a mobile phase of increasing polarity (*n*-hexane-EtOAc and EtOAc-MeOH mixtures of 10-20% increaments). The eluates were combined together on the basis of TLC analysis. VLC fractions eluted with 15-30% EtOAc in *n*-hexane were further subjected to preparative-TLC over Silica gel PF_{254} (Mobile phase 20% EtOAc in *n*-hexane) to yield compound **1** (203 mg).

The DCM extract (8.5 g) was fractionated by VLC over Silica gel 60H using *n*-hexane-EtOAc (from 100% *n*-hexane to 100% EtOAc with 10% increment) and EtOAc-MeOH (from 100% EtOAc to 60% MeOH in EtOAc) mixtures of increasing polarity. The eluates were combined together on the basis of TLC analysis. VLC fractions eluted with 15-25% EtOAc in *n*-hexane were further subjected to

preparative-TLC to yield more of compound **1** (30.5 mg), whilst compound **2** (15.2 mg) was isolated from the VLC fraction eluted with 30-50% EtOAc in *n*-hexane.

The MeOH extract (2.5 g) was fractionated by solid-phase extraction (SPE) on a Strata C_{18} reversed-phase cartridge (20 g, Phenomenex, Macclesfield, UK), eluted with a step-gradient using water-MeOH mixture of decreasing polarity (20-80% MeOH in water), to obtain a total of four SPE fractions. SPE fractions were dried using a rotary evaporator followed by freeze-drying. Reverved-phase preparative HPLC separation of the the SPE fraction eluted 20% MeOH in water gave compound **3** (35.3 mg; retention time 5.5 min) as a crystalline solid. Similarly, reversed-phase preparative HPLC separation of the SPE fractions eluted with 60% MeOH in water yielded compouds **4** (retention time 6.4 min) and **5** (retention time 12.8 min).

Assessment of preliminary antimicrobial activity. The *n*-hexane, DCM and MeOH extracts of *A. archangelica* roots were initially assayed for their antimicrobial activity against three bacterial strains (two Gram-positive- *S. aureus* NCTC 12981 and *Micrococcus luteus* NCTC 7508 and one Gram-negative- *Pseudomonas aeruginosa* NCTC 12903, and a fungal strain, *Candida albicans* ATCC 90028 using 96-well microtitre plate based on the modified resazurin *in vitro* antimicrobial assays.[12]

Assessment of antibacterial activity against MRSA strains. Microdilution titre assay was used for the determination of minimum inhibitory concentration (MIC) of compounds employing 96-well plates. During this experiment, the cation-adjusted Mueller-Hinton broth purchased from Oxoid Microbiology Products, UK was adjusted to have 20 and 10 mg/l of Ca^{2+} and Mg^{2+} ions, respectively. *S. aureus* strains used in this study were ATCC25923 (a standard laboratory strain sensitive to antibiotics like tetracycline), SA1199B, XU212, and EMRSA15.[13] SA1199B overexpresses the NorA MDR efflux pump[14] and XU212 is a Kuwaiti hospital isolate that is a MRSA strain possessing the TetK tetracycline efflux pump,[13] whereas the EMRSA 15 strain[15] was epidemic in the UK. The assay protocol described previously[16] included the uses of a positive control (norfloxacin) and colour indicator (95 mg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT). Bacterial growth was indicated by a color change from yellow to dark blue. MICs were carried out in duplicate.

RESULTS AND DISCUSSION

Vacuum liquid chromatography followed by PTLC on the *n*-hexane and DCM extracts of *A. archangelica* led to the isolation of compounds **1** and **2**. The LC-MS of compound **1** revealed the molecular ino peak at *m/z* 244 Da. The ^{1}H NMR spectrum (600 MHz, $CDCl_3$, Table 1) showed two sets of doublets for protons resonating at δ_H 6.19 (1H, *d*, *J* = 9.4 Hz, H-3), 7.58 (1H, *d*, *J*=9.4 Hz, H-4), 7.26 (1H, *d*, *J* = 8.6, H-5) and 6.81 (1H, *d*, *J* = 8.6, H-6)- typical of a 7,8 disubstituted coumarin. In addition, the ^{1}H NMR spectrum showed three protons downfield signal at 3.90 for a methoxyl group and two sets of methyl protons at δ_H 1.65 (3H, *s*), 1.82 (3H, s), a set of methylene protons at 3.55 (2H, *d*, *J* = 7.6 Hz, H-1'), an olefinic methine at 5.21 (1H, t, H-2') constuting a prenyl group. The ^{13}C NMR spectrum (150 MHz, $CDCl_3$, Table 1) displayed signals for a total of 15 carbons, including a carbonyl at δ_C 161.6 (C-2), a methoxyl at 56.2, five methine carbons at 113.2 (C-3), 144.0 (C-4), 126.4 (C-5), 107.6 (C-6), 121.3 (C-2'), five quaternary carbons at 160.4 (C-7), 118.2 (C-8), 153.0 (C-9), 113.2 (C-10), 132.8 (C-3'), one methylene carbon at 22.1 (C-1'), and two methyl carbons at 18.1 (C-4') and 26 (C-5'). The positions of the methoxyl and prenyl groups in the molecule were confirmed by the HMBC experiment. In the HMBC experiment, a common ^3J correlation from protons at δ_H 7.26 (H-5), 3.55 (H-1') and 3.90 (OMe) to an oxygen-bearing aromatic quaternary carbon at δ_C 160.4 confirmed the presence of a methoxyl group at C-7. H-4 also showed ^3J correlation to the carbonyl carbon δ_C 161.6 (C-2), also to another oxygenated quaternary carbons at δ_C 153.0 (C-8a) and δ_C 126.4 (C-5), while the H-3 revealed ^3J correlation to a quaternary at δ_C 113.2 (C-10) and ^2J correlation to

the carbonyl carbon δ_C 161.6. The H-6 displayed ^3J correlations to C-8 (δ 118.2) and C-10 (δ 113.2). The proton signal of H-5′ (δ 1.65) had the long-range ^3J correlations with the carbon signals of C-4′ (δ 18.1), C-2′ (δ 121.3) and ^2J correlation to C-3′ (δ 133.8). The HMBC correlations of H-1′ (δ 3.55) with C-8 (δ 118.2; ^2J), C-9 (δ 153.0, ^3J), C-2′ (δ 121.3; ^2J) and to C-3′ (δ 133.8; ^3J) confirmed the attachment of prenyl group through C-8. Therefore, compound **1** was identified as osthol, a common coumarin present in the family Apiaceae. The NMR data of compound **1** was in a good agreement with published data for osthol.[16]

Table 1. ^{1}H and ^{13}C NMR data of compounds 1 and 2.

Position	δ_H in $CDCl_3$ (600 MHz)		δ_C in $CDCl_3$ (150 MHz)	
	1	2	1	2
2	-	-	161.6	162.0
3	6.19, *d*, *J*= 9.4 Hz	6.24, *d*, *J*= 9.2 Hz	113.2	114.5
4	7.58, *d*, *J*= 9.4 Hz	7.36, *d*, *J*= 9.2 Hz	144.0	144.6
5	7.26, *d*, *J*= 8.6 Hz	7.22, *d*, *J*= 8.4 Hz	126.4	126.8
6	6.81, *d*, *J*= 8.6 Hz	6.78, *d*, *J*= 8.4 Hz	107.6	107.3
7	-	-	160.4	160.8
8	-	-	118.2	118.0
9	-	-	153.0	153.5
10	-	-	118.3	118.2
1’	3.55, *d*, *J*= 7.6 Hz	3.63, *d*, *J*= 7.4 Hz	22.1	22.7
2’	5.21, *t*, *J*= 7.6 Hz	5.27, *t*, *J*= 7.4 Hz	121.3	121.8
3’	-	-	133.8	134.2
4’	1.82, *s*	1.86, *s*	18.1	18.7
5’	1.65, *s*	1.77, *s*	26.0	26.3
7-OMe	3.90, *s*	-	56.2	-

The ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of compound **2** showed almost an identical pattern of peaks to those for compound **1**. The only difference was that this compound did not show any peak for the methoxyl protons which was replaced by a hydroxyl group instead. Accordingly, the compound **2** was identified as osthenol by direct comparism of its spectrum with those of osthol and replacing the MeOH with the hydroxyl group at position 7.

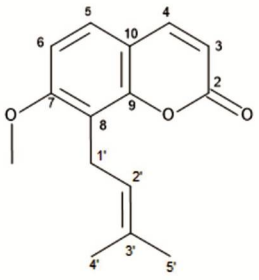
Solid-phase extraction followed by preparative HPLC separation of the MeOH extract of *A. archangelica* afforded compounds **3-5**. The ^{1}H NMR spectrum (600 MHz, $CDCl_3$, Table 2) of compound **3** displayed two doublets (J = 9.8 Hz) for protons at δ_H 6.26 (H-2) and 8.42 (H-3), one aromatic proton as singlet at δ_H 7.25 (H-9), and three proton singlet for a methoxyl group at δ_H 4.31 (4-OMe). Furthermore, two aromatic protons exhibited at δ_H 7.77 (1H, *d*, J = 2.4 Hz, H-2) and δ_H = 7.14 (1H, d, J = 2.4 Hz, H-3) indicated the presence of a furan ring in the molecule. Therefore, on the basis of the spectral data, compound **3** was identified as bergapten, a common furocoumarin in the various plant families including Rutaceae and Apiaceae, and was previously reported from *A. archangelia*.[8]. LC-MS showed the molecular ion *m/z* = 216 Da. The ^{1}H NMR data of the compound **3** were in good agreement with those published in the literature.[17]

The LC-MS of compound **4** revealed the molecular into peak *m/z* at 304 Da. The ^{1}H NMR spectrum (600 MHz, CD_3OD, Table 2) of compound **4** displayed peaks for a furocoumarin, which were similar to those of compound **3**. In addition, the ^{1}H NMR spectrum revealed signals for oxymethylene protons at δ_H 4.79 (H-1’), an oxymethine proton as a triplet at δ_H = 4.38 (H-2’), two methyl groups as singlets at δ_H 1.23 (H-4’) and δ_H 1.29 (H-5’). The ^{13}C NMR spectrum (150 MHz, CD_3OD, Table 2) displayed a total of 16 carbons, including a carbonyl at δ_C 162.8 (C-2), five aromatic methine carbons at

114.0 (C-3), 141.8 (C-4), 94.8 (C-8), 146.9 (C-2'), 103.4 (C-2'), five quaternary carbons at 152.0 (C-5), 118.1 (C-6), 160.2 (C-7), 154.1 (C-9), 108.2 (C-10), one oxymethylene carbon at 76.0 (C-1'), one oxymethine 78.2 (C-2'), one oxygenated aliphatic quaternary 72.8 (C-3') and two methyl carbons at 24.9, (C-4') and 27.3 (C-5'). Accordingly, compound 4 was identified as heraclenol, another furocoumarin. Both ^{1}H and ^{13}C NMR data of the compound 4 were in good agreement with published data for heraclenol.[18,19] Heraclenol is a furocoumarin that is known to exist in the genus *Angelica* and in the family Apiaceae.[20] It is also found in *Ducrosia anethifolia*,[21] *Heracleum candicans* [22] and *Prangos pabularia*. [23]

Table 2. ^{1}H data of compounds 3 and 4 and ^{13}C NMR data 4.

Position	δ_H in $CDCl_3$ (600 MHz)		δ_C in $CDCl_3$ (150 MHz)
	3	4	4
2	-	-	162.8
3	6.26, *d*, *J*= 9.8 Hz	6.29, *d*, *J*= 9.8 Hz	114.0
4	8.42, *d*, *J*= 9.8 Hz	8.43, *d*, *J*= 9.8 Hz	141.8
5	-	-	152.0
6	-	-	118.1
7	-	-	160.2
8	7.25, *s*	7.21, *s*	94.8
9	-	-	154.1
10	-	-	108.2
2'	7.77, *d*, *J*= 2.4 Hz	7.79, *d*, *J*= 2.4 Hz	146.9
3'	7.14, *d*, *J*= 2.4 Hz	7.23, *d*, *J*= 2.4 Hz	103.4
1'	-	4.79, *d*, *J*= 7.2 Hz	76.0
2'	-	4.38, *t*, *J*= 7.2 Hz	78.2
3'	-	-	72.8
4'	-	1.23, s	24.9
5'	-	1.29, s	27.3
7-OMe	4.31, *s*	-	-



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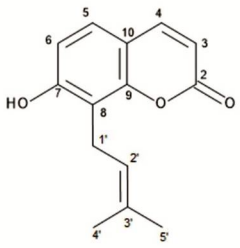

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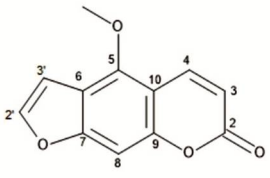

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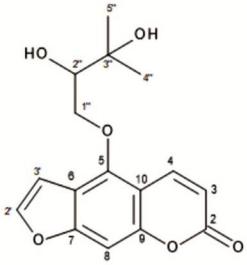

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Table 3. Antibacterial activity (MICs in μg/ml) of extracts and compound 1 isolated from *A. archangelica.*

Extract/ Compound/Antibiotic	*S. aureus* NCTC 12981	*P. aeruginosa* NCTC 12903	*M. luteus* NCTC 7508	*C. albicans* ATCC 90028
Hexane	5	2.5	2.5	0.625
DCM	5	N/A	5	$5x10^{-1}$
Methanol	N/A	N/A	N/A	$5x10^{-1}$
Compound **1**	0.25	0.5	0.5	0.5
Ciprofloxacin	1.95×10^{-3}	9.76×10^{-4}	1.95×10^{-3}	N/A
Nystatin	N/A	N/A	N/A	4.88×10^{-4}

Table 4. Anti-MRSA activity (MICs in μg/ml) of compound 1 isolated from *A. archangelica* against methicillin-resistant *S. aureus*.

Compound/ Antibiotic	XU212	ATCC25923	SA1199B	EMRSA-15
1	512	-	256	-
Norfloxacin	16	2	32	1

The ^{1}H NMR spectrum of compound **5** (600 MHz, CD_3OD) showed anomeric proton at δ_H 5.40 (1H, d, J = 3.28 Hz, H-1), a doublet at δ_H 4.11(1H, d, J = 8.28), a triplet for methine proton at 4.03 (1H, t, H-4') and a number of signals resonating between 3.2-3.9 ppm, typical proton signals for sugar molecule. The DEPTQ spectrum NMR spectrum displayed a total of 12 carbons, including a quaternary carbon at δc 105.2 (C-2') and eight oxygenated methine carbon at δc 93.5 (C-1), 73(C-2), 74.4 (C-3), 71.2 (C-4), 74.2 (C-5), 79.2 (C-3'), 75.7 (C-4'), 83.4 (C-5'), and three oxymethylene carbons at δC 62.1 (C-6), 63.8 (C-1') and 63.5 (C-6'). Based on the ^{1}H and DEPTQ data, compound **5** was identified as sucrose.[24]

The antimicrobial activities of *n*-hexane, DCM and MeOH extracts and compound **1** were tested against three bacteria (two Gram-positive and one Gram-negative) and one fungus (Table 3). The *n*-hexane and DCM extrats as well as compound **1** showed low level of activities against the test organisms whilst the methanol extract did not exhibit any activity. Compound **1** also revealed low to moderate antibacterial activities (Table 4) against two strains (SA1199B; MIC= 256 μg/ml and XU212; MIC= 512 μg/ml) of methicillin resistant *S. aures*.

CONCLUSION

The structure eludation of the compounds reported here was confirmed by a series of both 1D and 2D NMR spectroscopy and mass spectrometry. The outcomes of this phytochemical research followed by antibacterial study are encouraging to look for new antibacterial compounds with potential activity against clinical isolates of multi-drug resistant microorganisms.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this research.

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