Sergio Rosselli[1]
Antonella Maggio[1]
Gabriella Bellone[1]
Carmen Formisano[2]
Adriana Basile[3]
Carla Cicala[4]
Alessio Alfieri[4]
Nicola Mascolo[4]
Maurizio Bruno[1]

# Antibacterial and Anticoagulant Activities of Coumarins Isolated from the Flowers of *Magydaris tomentosa*



## Abstract

The phytochemical investigation of the acetone and methanol extracts of the flowers of *Magydaris tomentosa* (Desf.) DC afforded six known coumarins as well as (+)-meranzin hydrate (**7**), not previously reported as a natural product. The antibacterial activity of umbelliprenin (**1**), osthol (**2**), imperatorin (**3**), citropten (**4**) and (+)-meranzin hydrate (**7**) was tested against Gram-positive and Gram-negative bacteria. All coumarins (**1–7**) isolated in this study inhibited growth of all bacterial strains tested (MIC between 16 and 256 $\mu$g/mL), the most active being imperatorin (**3**) (MICs between 32 and 128 $\mu$g/mL) and citropten (**4**) (MICs between 16 and 256 $\mu$g/mL). The anticoagulant activity of compounds **1–4** and **7** was also evaluated.

## Key words

*Magydaris tomentosa* · Apiaceae · coumarins · (+)-meranzin hydrate · antibacterial activity · anticoagulant activity

## Introduction

*Magydaris tomentosa* (Desf.) DC. [syn.: *M. pastinacea* (Lam.) Paol.] belongs to the Apiaceae family and is a plant growing in Sicily and Sardinia [1]. The plant, 1 to 2 m in height, is characterized by a striated, sulcate, thick stalk with very large leaves and umbels with 40–50 pedicels with white flowers. The studies on *M. pastinacea* (Lam.) Paol. allowed the isolation of several glucosides from the fresh rhizomes [2], [3] and some known coumarins from the fruits [4]. Phytochemical studies on *Magydarids panacifolia* [5], the only other species of this genus investigated so far, allowed the isolation of magydardienol, a new irregular monocyclic diterpene, whose structure was corrected later [6] and shown to be identical to bonandiol, isolated from *Bonannia graeca* (L.) Halácsy (Apiaceae) [7], and of several coumarins [8].

*M. tomentosa* is a rare species which grows in the mountains of Sicily and causes skin irritations, as we could confirm after collecting the plant material. Here we report the results of studies on the antibacterial and anticoagulant activities of coumarins (**1–4** and **7**; Fig. **1**) isolated from the acetone and methanol extracts of the flowers of *M. tomentosa*.

## Material and Methods

### General

Optical rotations were determined on a JASCO P-1010 digital polarimeter. $^{1}$H- and $^{13}$C-NMR, spectra were recorded in $CDCl_3$ on a Bruker AC 250 E NMR spectrometer (Bruker; Rheinstetten, Germany), using the residual solvent signal ($\delta$ = 7.27 in $^{1}$H and $\delta$ = 77.00 in $^{13}$C) as reference. $^{13}$C-NMR assignments were determined by DEPT spectra. ESI-MS was obtained with an Applied



**Affiliation**
[1] Dipartimento di Chimica Organica, Università degli Studi di Palermo, Palermo, Italy
[2] Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II", Napoli, Italy
[3] Dipartimento di Biologia Vegetale, Università degli Studi di Napoli "Federico II", Napoli, Italy
[4] Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli "Federico II", Napoli, Italy

**Correspondence**
Prof. Dr. Maurizio Bruno · Dipartimento di Chimica Organica · Università degli Studi di Palermo · Viale delle Scienze · Parco d'Orleans II · 90128 Palermo · Italy · Phone/Fax: +39-091-596-905 · E-mail: bruno@dicpm.unipa.it

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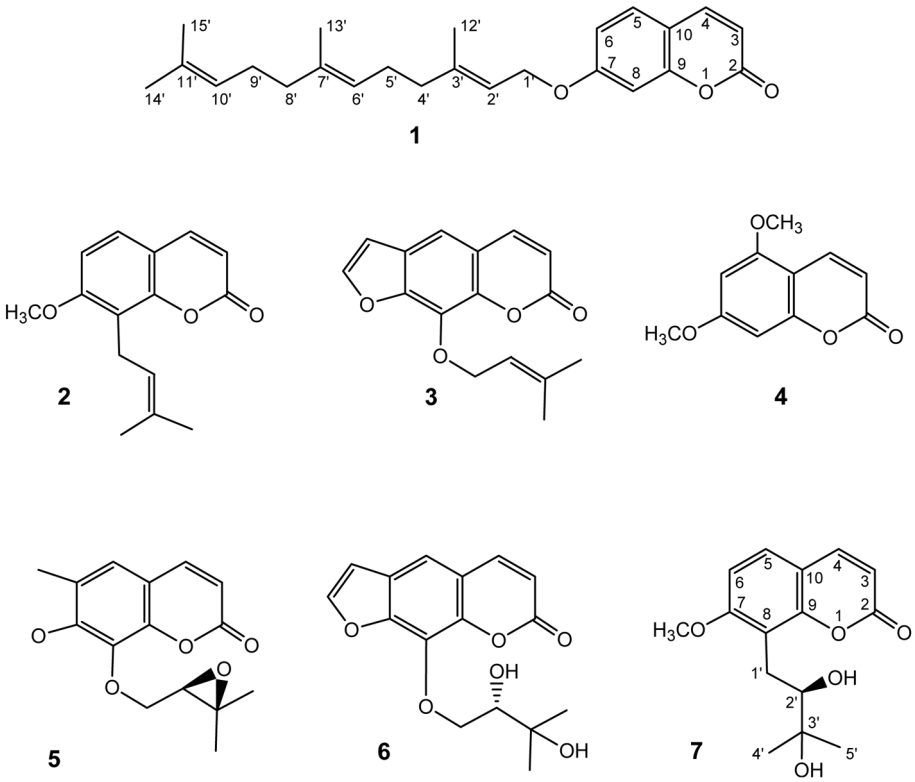


Fig. **1** Chemical structures of compounds **1**–**7**.

Biosystem API-2000 mass spectrometer (Applied Biosystems; Concord, Canada).

**Plant material**

The flowers of *Magydaris tomentosa* (Desf.) DC were collected in June 2005 near Godrano (Palermo) in Sicily, Italy and voucher specimens (PAL 05–608) were deposited in the Herbarium of the Botanical Garden of Palermo, Italy.

**Extraction and isolation**

Dried and powdered flowers (250 g) of *M. tomentosa* were extracted with $Me_2CO$ (5 L) at room temperature for a week. After filtration, the solvent was evaporated to give a gum (21 g) which was chromatographed on a silica gel (Merck No. 7734, deactivated with 15% $H_2O$, 400 g; Merck; Darmstadt, Germany) column eluted with petroleum ether, petroleum ether-EtOAc mixtures and EtOAc. 500 mL fractions were collected as follows: 1–4, petroleum ether; 5–8, petroleum ether-EtOAc (3:2); 9–12, EtOAc. Fractions 3 and 4 were re-chromatographed on a silica gel column. Elution with *n*-hexane-EtOAc (9:1) gave umbelliprenin (**1**). Fractions 5–8 were re-chromatographed on a silica gel column. Elution with *n*-hexane-EtOAc (7:3) gave osthol (**2**), imperatorin (**3**), citropten (**4**) and (+)-heraclenin (**5**).

The plant material was further extracted with MeOH (5 L) for a week at room temperature. After filtration and evaporation of the solvent, a gum (8 g) was obtained, which was chromatographed on a silica gel (Merck No. 7734, deactivated with 15% $H_2O$, 200 g) column and eluted with petroleum ether-EtOAc 3:7 (2 L) and $CHCl_3$-MeOH 4:1 (2 L). The fractions (250 mL each) eluted with $CHCl_3$-MeOH were re-chromatographed on a silica gel column and then by preparative TLC (Merck, 2 mm, EtOAc) to give heraclenol (**6**) and (+)-meranzin hydrate (**7**). The spectroscopic data of these substance were in complete agreement with those reported in the literature.

*Umbelliprenin* (**1**): 50 mg; m.p. 63–64 °C (petroleum ether-EtOAc); $^1$H-NMR and $^{13}$C-NMR: see Table **1**.

*Osthol* (**2**): 500 mg; m.p. 83–84 °C (petroleum ether-EtOAc).

*Imperatorin* (**3**): 1.55 g; m.p. 102 °C (needles from petroleum ether-$Me_2CO$).

*Citropten* (**4**): 1.8 g; m.p. 146 °C (petroleum ether-$Me_2CO$).

*(+)-Heraclenin* (**5**): 5 mg; m.p. 108–109 °C (MeOH); $[\alpha]_D^{22}$: +21.1° (*c* 0.10, pyridine).

*Heraclenol* (**6**): 5 mg; m.p. 110–113 °C (MeOH); $[\alpha]_D^{22}$: +11.1° (*c* 0.10, pyridine).

*(+)-Meranzin hydrate* (**7**): 40 mg; m.p. 124–125 °C (*n*-hexane-EtOAc); $[\alpha]_D^{22}$: +40.9° (*c* 0.34, $CHCl_3$); $^1$H-NMR ($CDCl_3$, 250 MHz): $\delta$ = 7.62 (1H, d, *J* = 9.5 Hz, H-4), 7.35 (1H, d, *J* = 8.7 Hz, H-5), 6.88 (1H, d, *J* = 8.7 Hz, H-6), 6.55 (1H, d, *J* = 9.5 Hz, H-3), 3.94 (3H, s, $OCH_3$), 3.64 (1H, br dd, *J* = 10.5, 2.4 Hz, H-2′), 3.10 (1H, dd, *J* = 13.8, 2.4 Hz, $H_a$-1′), 3.00 (1H, dd, *J* = 13.8, 10.5 Hz, $H_b$-1′), 1. 34 (6H, s, $CH_3$–4′, $CH_3$–5′); $^{13}$C NMR ($CDCl_3$, 62.7 MHz): $\delta$ = 163.8 (C, C-2), 160.5 (C, C-7), 153.3 (C, C-9), 143.9 (CH, C-4), 126.8 (CH, C-5), 115.8 (C, C-8), 113.0 (C, C-10), 112.7 (CH, C-3), 107.4 (CH, C-6), 78.0 (CH, C-2′), 72.8 (C, C-3′), 56.1 ($CH_3$, $OCH_3$), 25.9 ($CH_2$, C-1′), 25.4 ($CH_3$, $CH_3$–4′), 24.0 ($CH_3$, $CH_3$–5′).

Table **1** Spectroscopic data of umbelliprenin (**1**) in $CDCl_3$

| | *H (J Hz)* | *C* | *HMBC* |
|---|---|---|---|
| 2 | | 161.30 | H-4, H-3 |
| 3 | 6.26 d (9.6) | 112.94 | |
| 4 | 7.63 d (9.6) | 143.43 | H-5 |
| 5 | 7.37 d (8.1) | 128.66 | H-4 |
| 6 | 6.85 dd (8.1, 2.4) | 113.21 | |
| 7 | | 162.15 | H-5, H-8, H-6, H-1′ |
| 8 | 6.82 d (2.4) | 101.57 | H-6 |
| 9 | | 155.86 | H-4, H-5, H-8 |
| 10 | | 112.41 | H-4, H-6, H-8, H-3 |
| 1′ | 4.61 d (6.6) | 65.47 | |
| 2′ | 5.48 brt (6.6) | 118.41 | H-1′, H-4′, Me-12′ |
| 3′ | | 142.39 | H-1′, H-5′, Me-12′ |
| 4′ | 2.13 m | 39.51 | H-2′, H-10′, H-5′, Me-12′ |
| 5′ | 2.15 m | 26.12 | H-4′ |
| 6′ | 5.08 t (6.6) | 123.47 | H-5, H-8′, Me-13′ |
| 7′ | | 135.58 | H-8′, Me-12′ |
| 8′ | 1.97 m | 39.65 | H-10′, H-9′, Me-13′ |
| 9′ | 2.05 m | 26.63 | H-8′ |
| 10′ | 5.10 t (6.6) | 124.28 | Me-14′, Me-15′ |
| 11′ | | 131.43 | H-9′, Me-14′, Me-15′ |
| 12′ | 1.78 s | 16.77 | H-2′, H-4′ |
| 13′ | 1.60 s | 16.03 | H-6′, H-8′ |
| 14′ | 1.69 s | 25.69 | Me-15′ |
| 15′ | 1.59 s | 17.68 | Me-14′ |

### Antimicrobial activity

The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth dilution method [9]. The following twelve bacterial strains, selected as representative of the classes of Gram-positive and Gram-negative, were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA): *Bacillus subtilis* (ATCC 10774), *Enterobacter aerogens* (ATCC 13048), *Enterobacter cloacae* (ATCC 10699), *Enterococcus faecalis* (ATCC 14428), *Escherichia coli* (ATCC 11229), *Klebsiella pneumoniae* (ATCC 10031), *Proteus mirabilis* (ATCC 7002), *Proteus vulgaris* (ATCC 12454), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 10699), *Staphylococcus aureus* (ATCC 13709) and *Staphylococcus epidermidis* (ATCC10875).

### Preparation of the compounds

The samples were prepared by dissolving 10 mg of the dry residue in 10 mL of sterile physiological Tris buffer, pH 7.4, 0.05 M [10].

### Antimicrobial assay

Bacterial strains were grown on MH agar plates (DIFCO; Detroit, MI, USA) and suspended in MH broth (DIFCO). The MIC values against bacterial strains were measured using the MH broth-dilution method [9]. The inoculum suspensions were prepared from 6 h broth cultures and adjusted to a 0.5 McFarland standard turbidity. The compounds, sterilized by 0.45 m Millipore filters, were added to MH broth medium. Serial 10-fold dilutions were made that furnished a concentration range from 1000 to 0.01 $\mu$g/mL for the compounds. The lowest concentrations of the solutions with activity underwent 2-fold dilutions for a more accurate measurement of the MIC [11]. The bacterial suspensions were aerobically incubated for 24 h at 37 °C. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. Control cultures, containing only sterile physiological Tris buffer were also prepared. In addition, MIC values for tetracycline hydrochloride (Pharmacia; Milano, Italy), benzylpenicillin sodium (Cynamid; Catania, Italy) and cefotaxime sodium (Roussel Pharmacia; Milano, Italy) were also determined in MH broth, using the standard method.

### Anticoagulant activity

*Ex vivo studies:* All animal experiments complied with the Italian D.L. n° 116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC). Compounds **1**–**4** and **7** were dissolved in ethanol and $H_2O$ (ratio 1 : 1) and both compounds and the vehicle control were intraperitoneally administered to male Wistar rats (200–230 g; Harlan Nossan; San Pietro al Natisone, Italy), at the doses of 1, 3 and 10 mg/kg once a day for 2 consecutive days. 20 hours following the second administration, rats were anesthetized with enflurane and blood was collected by cardiac puncture and anticoagulated with trisodium citrate (3.8 % w/v; 1 : 10). Platelet-poor plasma (PPP) was immediately prepared by centrifugation at 2500 rpm for 15 minutes at 4 °C. Anticoagulant activity was measured by a coagulameter (KoaguLab MJ Ortho Diagnostic Systems; Raritan, NJ, USA). To measure prothrombin time (PT), samples of 100 $\mu$L of PPP were incubated at 37 °C for 3 min; coagulation was then induced by adding 200 $\mu$L of prothrombin reagent (Ortho Diagnostic Systems).

## Results

Examination of the acetone and methanol extracts of the flowers of *Magydaris tomentosa* yielded six known coumarins: umbelliprenin (**1**) [12], [13], osthol (**2**) [14], imperatorin (**3**) [15], citropten (**4**) [15], (+)-heraclenin (**5**) [16] and (–)-heraclenol (**6**) [4]. Another coumarin, (+)-meranzin hydrate (**7**), was also found, which has never previously been isolated, only the enantiomer being known as natural product [17]. The (+)-meranzin hydrate (**7**) has been synthesized starting from the coumarin turtuoside [18]. Herein, we present the results of an extensive 2D spectroscopic study (HSQC, HMBC, COSY) on umbelliprenin (**1**) that allowed us to give full $^1$H- and $^{13}$C-NMR assignments (Table **1**) not previously reported; therefore the published data [13] are amended. Coumarins isolated in this study inhibited all bacterial strains (MIC between 16 and 256 $\mu$g/mL). In the antibacterial test, the most active were imperatorin (**3**) (MICs between 32 and 128 $\mu$g/mL) and citropten (**4**) (MICs between 16 and 256 $\mu$g/mL). Except for *Bacillus subtilis*, both Gram-positive and Gram-negative bacteria displayed the same sensitivity to tested coumarins, with MICs between 32 and 64 $\mu$g/mL. *Bacillus subtilis* was the least sensitive strain (MIC between 128 and 256 $\mu$g/mL) (Table **2**).

Compounds **1**–**4** at the dose of 3 and 10 mg/kg *i.p.* significantly ($P < 0.01$) prolonged the PT compared to control samples. Conversely, compound **7**, at the same doses, significantly ($P < 0.01$) reduced the PT value (Fig. **2**). Therefore, none of the compounds tested showed anticoagulant activity at the doses of 1 mg /kg *i.p.*

Table **2** Antibacterial activity of compounds **1–4** and **7**

| *Bacteria* | *1* | *2* | *3* | *4* | *7* | *Cefotax.* | *Penicil.* | *Tetrac.* |
|---|---|---|---|---|---|---|---|---|
| *Enterococcus faecalis* (ATCC 14428) | 64 | 64 | 32 | 32 | 64 | R | 2 | 2 |
| *Staphylococcus epidermidis* (ATCC10875) | 64 | 32 | 32 | 32 | 64 | 0.1 | 0.03 | 0.1 |
| *Staphylococcus aureus* (ATCC 13709) | 128 | 64 | 32 | 16 | 128 | 2 | 0.03 | 2 |
| Bacillus subtilis (ATCC 10774) | 256 | 256 | 128 | 256 | 256 | 32 | 32 | 2 |
| *Enterobacter aerogenes* (ATCC13048) | 128 | 128 | 32 | 32 | 128 | R | R | 4 |
| *Enterobacter cloacae* (ATCC10699) | 128 | 64 | 32 | 32 | 128 | R | R | 4 |
| *Escherichia coli* (ATCC11229) | 128 | 256 | 32 | 32 | 128 | 0.1 | 64 | 4 |
| *Klebsiella pneumoniae* (ATCC277736) | 128 | 64 | 32 | 64 | 128 | 0.1 | R | 16 |
| *Proteus mirabilis* (ATCC 7002) | 128 | 32 | 32 | 32 | 256 | 0.03 | 4 | 32 |
| *Proteus vulgaris* (ATCC12454) | 64 | 64 | 64 | 64 | 128 | 0.03 | R | 4 |
| *Pseudomonas aeruginosa* (ATCC 27853) | 128 | 128 | 64 | 64 | 256 | 1.6 | R | 32 |
| *Salmonella typhi* (ATCC 19430) | 64 | 128 | 64 | 64 | 128 | 0.5 | 4 | 1 |

[a] Values represent MIC values expressed in $\mu$g/mL. A comparison is shown between the antibiotic activity of the compounds with reference antibiotics: (cefotax.) Na cefotaxime, (penicil.) benzylpenicillin sodium, (tetrac.) tetracycline.
R = absence of inhibition even at the highest concentration used (1000 $\mu$g/mL).

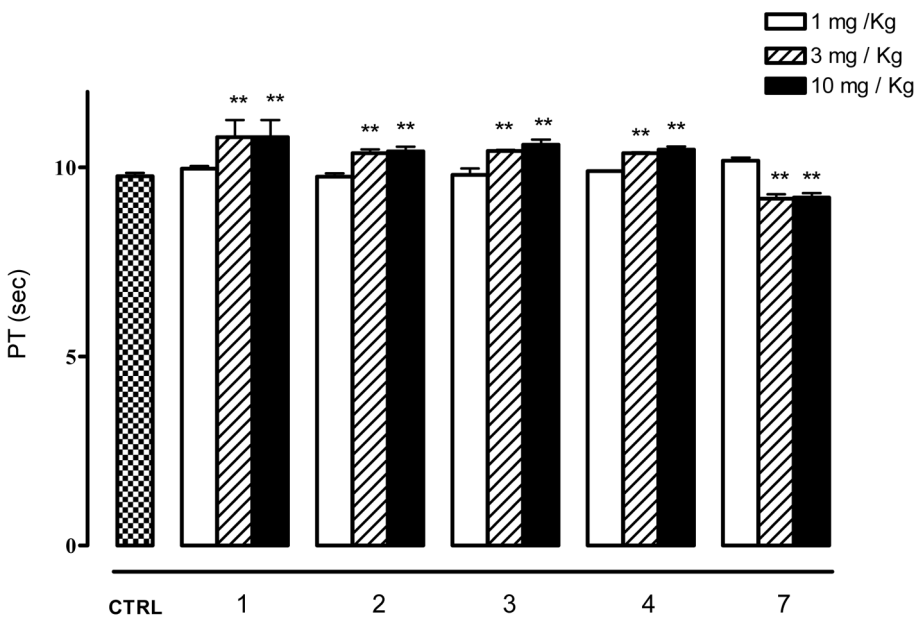


Fig. **2** Anticoagulant activity of compounds **1–4** and **7**. PT value measured on rat plasma following 2 days treatment with compounds **1–4** and **7** (1–10 mg/kg *i.p.*). $^{**}p < 0.01$ vs. control (CTRL; Dunnett's test).

## Discussion

As for antibacterial activity, the tested coumarins were active both against Gram-negative and Gram-positive bacteria. This finding is interesting because conventional antibiotics are often more active against Gram-positive than Gram-negative bacteria. Even the particularly resistant *Pseudomonas aeruginosa* is inhibited by the substances. The pattern of chemical selectivity towards Gram-positive bacteria is not restricted to compounds from plants, but is a general phenomenon observed among most antibiotics. The antibacterial activity of plant extracts against Gram-negative bacteria has been shown in others studies. The extracts from some bryophytes, pteridophytes and edible fruits are active mainly against Gram-negative bacteria that are antibiotic-resistant such as *Pseudomonas aeruginosa* [19], [20], [21], [22]. Contrary to the literature, the antibacterial activity of the tested coumarins is directed both against Gram-positive and Gram-negative bacteria. Among the tested coumarins, imperatorin (**3**) and citropten (**4**) are the most active. The substances' activities show that the antibacterial activity is mainly due to the coumarin ring and that substituents can modulate the potency.

*Ex vivo* studies show that compounds **1–4** exert anticoagulant activity in a test generally and widely used to estimate anticoagulant agents [23]. It is worth noting that compound **7**, carrying two hydroxy groups on the side chain, exhibits a pro-coagulant activity as demonstrated by the shortening of the PT value.


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