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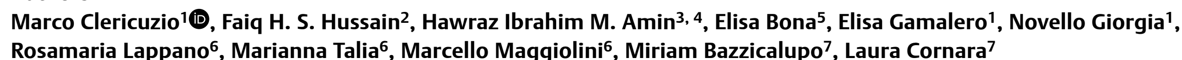
Cytotoxic, Anti-bacterial, and Wound-healing Activity of Prenylated Phenols from the Kurdish Traditional Medicinal Plant Onobrychis Carduchorum (Fabaceae)

Authors



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ABSTRACT

Onobrychis carduchorum (Fabaceae) is a plant widely employed in Kurdish traditional medicine to cure wounds, inflammations, and other skin diseases. We could isolate ten different polyphenols from the acetone extract of this plant: **1**–**4** are isoflavones, having a genistein skeleton; **5**–**7** are flavanones, having a naringenin skeleton; and **8**–**10** are prenylated dihydro-stilbenes. In particular, **8**–**10** have been isolated, so far, only from *Glycyrrhiza glabra* (liquorice). Many of the above prenylated phenols showed significant toxicity on some human breast cancer cell lines, and a relevant growth inhibition of *Staphylococcus aureus* strains. In addition, **9** and **10** had marked wound healing activity. It is suggested that these bioactivities are responsible, at least partly, for the plant's traditional use.

Introduction

The mountain region stretching from extreme Eastern Turkey to North-West Iran, roughly corresponding to historical Kurdistan, is a region where traditional herbal remedies are still largely used by village people. This sector of the Middle East supports a wide number of endemic Fabaceae, probably allied to steppe climates. The genus *Onobrychis* Miller has its main diversity center in SW Asia [1]. *Onobrychis carduchorum* Towns. is an Irano-Turanian perennial herb, diffused in Eastern Turkey, Northern Syria, N Iraq. and NW Iran [1]. The detailed survey by Mükemre et al. [2] on the medicinal plants used in the Çatak region (Turkey) cites two species of *Onobrychis, Onobrychis carduchorum* and *Onobrychis altissima* Grossh. as being traditionally used as a styptic, and to cure wounds and cuts. The plant leaves are simply crushed and spread over the skin [2]. More recently, a second paper has appeared on the traditional medicinal plants of Eastern Turkey (Ağri Province) [3], which reports the same traditional use of *O. carduchorum*, that is, in wound and cut healing, and as a styptic.

The plants of the present investigation were collected by one of the authors (H. M. A.) in the Halgurd Mountain of Iraqi Kurdistan (▶ **Fig. 1**). Local people, interviewed by the collector, answered that the plant is widely employed for several skin diseases, among which are inflammations and wounds. The plant leaves are rubbed on the skin part to be healed, in agreement with the method used in Turkey [2].

No previous investigation exists on the metabolites of *O. carduchorum*. Sainfoin (*Onobrychis viciifolia*), one of the most widely employed legume forages worldwide, also belongs to the genus *Onobrychis*. Several chemical investigations on sainfoin metabolites have appeared in the literature. The aerial parts of *O. vicifolia* yielded various phenolic compounds, including arbutin, rutin, catechin, kaempferol, quercetin, and afzelin [4–6]. The condensed tannins component has also been investigated. It showed antimicrobial activity [6, 7], as well as anthelmintic properties, acting against different parasitic nematodes [8, 9].

In the present work, we followed a bioguided search approach. In particular, we chose an antibacterial test against *Staphylococcus aureus* as the leading assay to discriminate between active and non-active chromatographic fractions. The inhibition halo value observed for vancomycin (used as a positive control, see Material and Methods) was the threshold value we followed to select appropriate fractions.

Starting from the active fractions, we could isolate ten pure phenolic compounds **1**–**10** (▶ **Fig. 2**). They are all known molecules, but some of them have been found, to date, only in one single plant. The *S. aureus* growth inhibition values of compounds **2**–**9** are reported in ▶ **Table 1**.

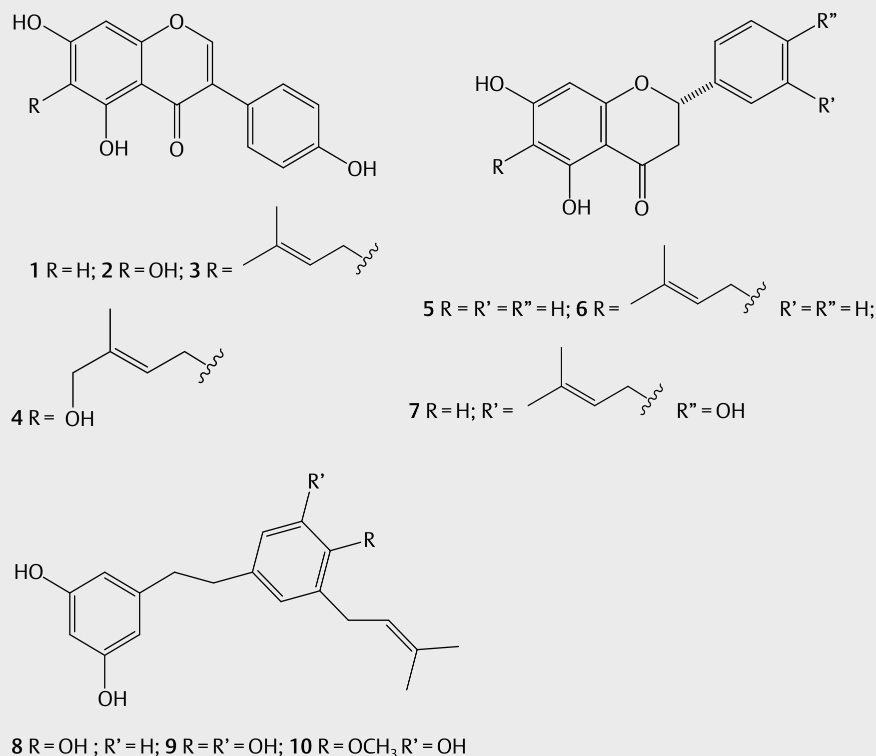
The second bioactivity we tested on pure compounds (when provided with enough material) was the cytotoxicity against different breast tumor cell lines, in particular, MCF-7, SkBr3, and MDA-MB-231 (data reported in ▶ **Table 2**). Breast cancer is the most common type of cancer among women worldwide, and plants belonging to the Fabaceae family have often yielded metabolites showing cytotoxicity to breast tumor cells. For instance, the bark methanol extract from *Guibourtia tessmannii* showed antiproliferative activity in tumor cells, including the MCF-7 breast cancer cell line [10]. Similarly, two stilbenes isolated from the Nigerian plant *Cajanus cajan* proved the capability to interfere with the viability of different cancer cell lines, including MCF-7 [11]. Finally, five isoliquiritigenin chalcones isolated from *Spatholobus suberectus*, and some synthetic analogues, were evaluated for their cytotoxic potential against the MCF-7 and MDA-MB-231 breast cancer cells [12].

Results and Discussion

The acetone crude extract of leaves and flowers of *O. carduchorum* was found to be rich in phenolics. Ten of them (compounds **1**–**10**) (▶ **Fig. 2**) could be isolated in pure form and were identified by 1D and 2D ^{1}H and ^{13}C NMR spectra analysis (the chemical shift values are reported in ▶ **Tables 1S–3S**, Supporting Information) and mass spectrometry. Our spectral data were coherent with those of the literature.

Genistein (**1**), a flavonoid particularly common in Fabaceae, was inactive in the *S. aureus* inhibition test. For this reason, it was not subjected by us to further bioactivity assays. Moreover, the gen-

▶ **Fig. 1** **a** Geographical position of M. Halgurd in Iraqi Kurdistan. **b** Position of region **a** in the Middle East. **c** *Onobrychis carduchorum* (specimens of our collection).



▶ **Fig. 2** Structure of compounds **1**–**10**.

▶ **Table 1** *S. aureus* strains response to the isolated and control compounds. Values are reported as inhibition halos in mm.

Compound	Halo
2	6.0 ± 0.0
3	13.7 ± 1.2
4	6.0 ± 0.0
5	12.0 ± 0.0
6	6.0 ± 0.0
7	12.3 ± 0.6
8	15.7 ± 0.6
9	17.3 ± 0.6
10	18.3 ± 0.6
Vancomycin[1]	16.0 ± 0.6
Negative control[2]	6.3 ± 0.3

[1]Vancomycin was used as a positive control for the *S. aureus* assay.
[2]Linseed oil was used as a negative control for all organisms.

▶ **Table 2** Antiproliferative activity elicited upon 48 exposure of tested compounds in MCF-7, SkBr3, and MDA-MB-231 breast cancer cells, as determined by the MTT assay.

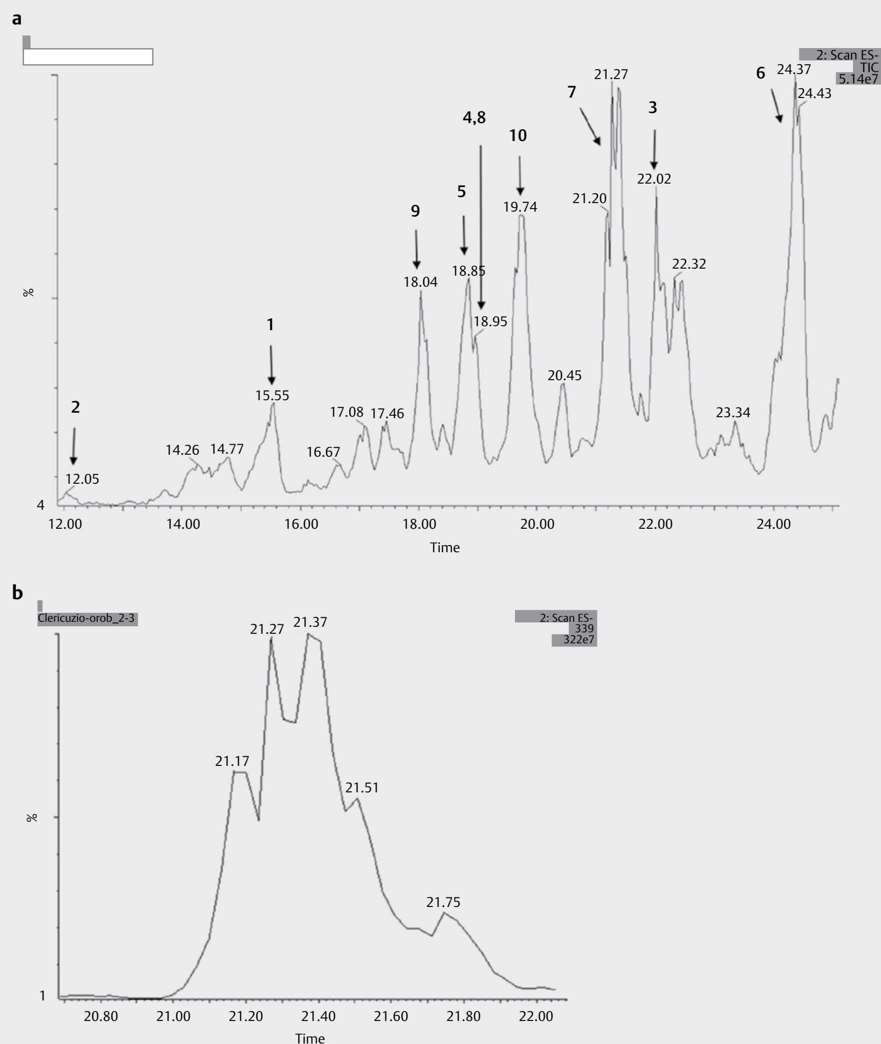
Compound	MCF-7	SkBr3	MDA-MB-231
2	35 (± 3)	35 (± 4)	> 50
3	4 (± 1)	5 (± 1)	5 (± 1)
5	10 (± 2)	26 (± 2)	25 (± 3)
7	9 (± 1)	7 (± 1)	8 (± 2)
8	7 (± 1)	6 (± 1)	17 (± 3)
9	7 (± 1)	7 (± 1)	7 (± 1)
10	30 (± 5)	5 (± 1)	12 (± 1)
Cisplatin	16 (± 3)	7 (± 2)	14 (± 1)

IC_{50} (± SD) values are expressed in μM.

istein effect on breast cancer cells has been already documented by one of us (M. M.) [13].

We could isolate a second isoflavone from *O. carduchorum*, viz. 6-hydroxy-genistein (**2**). It resulted in being inactive in both our cytotoxic (▶ **Table 2**) and antibacterial assays (▶ **Table 1**). This flavonoid was first isolated from *Baptisia hirsuta* (Fabaceae), where it was present along with 7-*O*-disaccaride [14].

6-Prenylgenistein (**3**), also known as wighteon (in the past sometimes referred to as erythrinin B), is the first of many prenylated phenolics found in the present work. These prenylated phenols gave a typical deep purple spot on TLC, when revealed with SV (sulpho-vanilline) at 100 °C. Moreover, they were easily identified by NMR spectroscopy, owing to their high-field aliphatic resonances, both at ^{1}H and at ^{13}C. Wighteon (**3**) was the most active molecule in our cytotoxic assays, showing an IC_{50} value of 4 to 5 μM against MCF-7, SkBr3, and MDA-MB-231 breast cancer cells (▶ **Table 2**). It was also significantly active against *S. aureus* (▶ **Table 1**). In the lit-



▶ **Fig. 3** **a** HPLC-MS spectrum (TIC, ESI$^-$) of the *O. carduchorum* leaves subextract (chlorophyll free) investigated in the present work. **b** SIM (single-ion monitoring) of m/z = 339 ([M - H]$^-$ of **7** and its isomers.

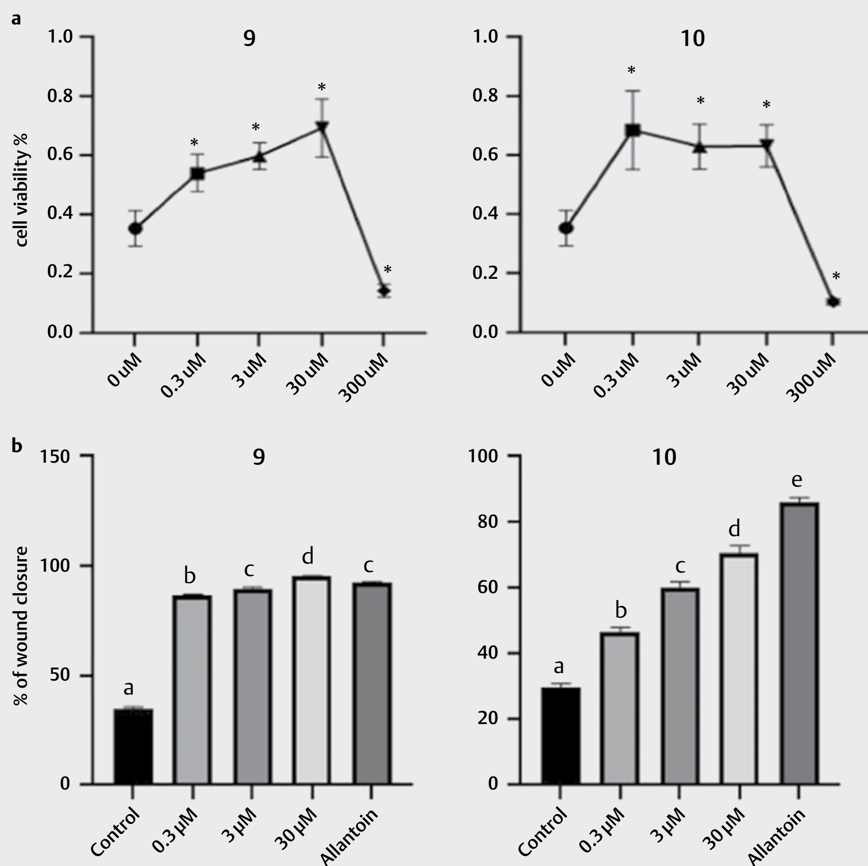
erature, compound **3** has been isolated from various other plant sources, mainly belonging to the legume family, as, for instance, *Sophora* [15], *Lupinus* [16], *Bolusanthus* [17], and also from *Maclura* (Moraceae) [18]. Compound **3** was active in various bioassays [16–21].

Compound **4** is the wighteon derivative, with the prenyl chain oxidized to primary alcohol at one of the geminal methyls (C-4"). It was obtained by us in very small amounts, so that it was not possible to perform cytotoxic activities on it. In our assay of inhibition of *S. aureus* growth, **4** resulted in being much less active than **3** (▶ **Table 1**). In the literature, **4** has been found in the roots of *Bolusanthus speciosus* (along with **3**) and was given the trivial name of isogacaonin C [17]. This compound resulted in being active against four selected bacteria, including *S. aureus*. Its activity was comparable or slightly inferior to the non-oxidized parent compound **3**[17], in contrast with our findings.

Metabolites **5**–**7** are flavanones of the naringenin series. Pinocembrin **5** was active only against MCF-7 cancer cells (▶ **Table 2**) and showed little inhibition against *S. aureus* (▶ **Table 1**). It is a widespread flavonoid in plants [22] and has also been found in propolis [23].

6-Prenylpinocembrin (**6**), also known as isoglabranin, was isolated by us in too small amounts to be tested in our cytotoxicity assays. In our antibacterial assays, it showed not to be active against *S. aureus* (▶ **Table 2**). Isoglabranin has been isolated for the first time from *Derris rariflora*, a Brazilian member of Leguminosae [24], and then reported from *Dalea elegans* (Fabaceae) [22] and *Helichrysum teretifolium* (Compositae) [25].

3'-Prenylnaringenin (**7**) showed moderate to good cytotoxicity against MCF-7, SkBr3, and MDA-MB-231 cells, with IC_{50} values of 9, 7, and 8 μM, respectively (▶ **Table 2**), but was weakly active against *S. aureus* (▶ **Table 1**). It was the only prenylnaringenin we could isolate from *O. carduchorum* in pure form, even if HPLC-MS analysis (▶ **Fig. 3**) clearly indicated that more isomers of **7** were present in the extract. They are very likely prenylnaringenins bearing the prenyl group in different positions. In fact, both 6- and



▶ **Fig. 4** **a** Effect of incubation with 0.3–300 μM of compounds **9** and **10** on cell viability, evaluated by the MTT assay on HaCaT keratinocytes. Data are means ± SD (n = 9) of formazan absorbance at 570 nm, standardized as percent of control. $^*P < 0.0001$ according to Dunnett's test. **b** Increased wound closure induced by **9** and **10** at 0.3, 3, and 30 μM in a scratch wound healing assay conducted on HaCaT *keratinocytes?*. Data are means ± SEM (n = 60–420) of wound closure, measured at 24 h since wounding and expressed as percentage of total closure. Significant differences among treatments are evidenced with different letters ($p < 0.0001$ according to Bonferroni's test).

8-prenylnaringenin have been reported in the literature, for example, from hop (*Humulus lupulus*) [26]. Compound **7** was first reported from *Erythrina eriotriocha* (Fabaceae) [20] and was then found in *Garcinia dulcis* (Guttiferae) [27], *Azadirachta indica* (Meliaceae) [28], and *Schizolaena hystrix* (Sarcolaenaceae) [29]. It showed weak cytotoxicity against A2780 human ovarian cells [29].

Compounds **8**–**10** are prenylated dihydrostilbenes, a class of metabolites mainly found in Leguminosae, and often provide important bioactivities. Interestingly, all three were found, so far, only in liquorice (*Glycyrrhiza glabra*), a well-known and widely used medicinal plant [30]. The present paper is therefore the first report of their occurrence in other plants. Here, we could find that they have a noteworthy capacity to inhibit *S. aureus* growth. In particular, the methoxy derivative **10** was the most potent one, and had an activity higher than that of vancomycin, used as a positive reference compound (▶ **Table 1**). In addition, **8** was active on MCF-7 and SkBr3 cells, **10** only on SkBr3 cells, and **9** was equally active on all three tumor cell lines (▶ **Table 2**).

Dihydrostilbenes **8**–**10** were reported to have high antioxidant activity [30]. The EtOAc extract of *G. glabra* leaves, containing, among others, compounds **8**–**10** together with pinocembrin **5** and iso-glabranin **6**, was found to have important anti-genotoxic and anti-inflammatory activities [31].

Wound healing activity

As the main ethnic use of *O. carduchorum* consists in the topic application on the skin to heal wounds and inflammations, we thought it would be of the maximum importance to test the molecules isolated from the plant in *in vitro* wound healing activity. We found that compounds **9** and **10** were significantly active on keratinocytes, as both displayed a biphasic effect on keratinocyte viability, showing a significant stimulation of cell growth in the range of 0.3–30 μM and a strong decrease in cell viability at the 300 μM dose (▶ **Fig. 4a**). The scratch wound assay conducted using subtoxic concentrations showed a significant increase in the wound closure rate compared to both the control and allantoin, induced by all doses tested of compounds **9** and **10** (▶ **Fig. 4b**).

This marked wound healing activity is in agreement with the previously quoted ethnobotanical studies [2, 3]. Sainfoin, one of the few European members of *Onobrychis* (*O. viciifolia*), has been

reported as a traditional medical plant in Northern Italy (Ligurian Apennines) for the treatment of wound healing [32]. The presence of the prenylated dihydrostilbenes **8**–**10** in *G. glabra* might as well be responsible for its wound healing benefits. The plant is used in Ayurveda and folk medicine for this purpose [33], and its skin regeneration activity on rats has been documented by Oloumi et al. [34].

As a conclusive remark, the ethnical use of *O. carduchorum* for skin diseases and inflammations seems to be well supported by the abundant presence of prenylated polyphenols (isoflavones, flavanones, and dihydrostilbenes), having antibacterial, cytotoxic, and wound healing activities, along with the more common radical scavenging ability. In addition, various non-prenylated flavonoids were present in the plant extract, but they were generally much less active than their prenylated counterparts. It has already been reported that prenylation increases the lipophilicity of flavonoids, and hence membrane permeability, enhancing the binding affinity of flavonoids to target biomolecules [35].

Materials and Methods

Plant material

O. carduchorum was collected in Halgurd Mountain, Rawanduz, Kurdistan region (Iraq) during spring 2017. The plant was identified by botanist Prof. Abdulhussain Al-Xayat from the University of Salahaddin-Erbil, Iraq. A voucher specimen was deposited at (ESUH) Education Salahaddin University Herbarium, Kurdistan (registry number ESUH-OC2017). Freshly cut flowers, leaves, and rhizomes were dried in a drying room with active ventilation at room temperature (about 20–22 °C) until constant weight. Subsequently, each part was separately finely minced in a grinding mill. In the end, we obtained 500 g of dry material from leaves, 100 g from flowers, and 750 g from roots. Only the aerial parts (leaves + flowers) were investigated in the present work.

Extraction

The dry powder obtained from the leaves (100 g) and flowers (40 g) were separately suspended in acetone (1 L, 2 times) and stirred at 30 °C for 4 h. The solutions were filtered and then dried under vacuum at rotavapor at 38 °C. The crude extract obtained from the leaves amounted to 6.0 g, and that from flowers to 0.95 g. Chlorophyll (and other very lipophilic material) was removed from the leaf extract by an RP-18 SPE (solid-phase extraction) cartridge, eluted with 95 % aq. MeOH. The eluted solution was again dried at rotavapor, yielding 2.6 g of dry material. This subextract from leaves and the flowers crude extract were compared by TLC (normal and reversed phase) and by HPLC-MS and were found to be almost identical. Both extracts were separately subjected to column chromatography.

Chromatography and HPLC-MS

LC separations were carried out by a medium pressure liquid chromatography system, consisting of an Alltech 426 HPLC pump equipped with a VWR LaPrep 3101 UV detector. Only RP-18 columns were used, eluted with water-methanol-acetonitrile mixtures. Typically, a first coarse separation was made with a 160 × 50 mm column (irregular 25–40 µm silica), with a flow rate set at 23 mL/min. The employed gradient started from 80 % H_2O:20 % MeOH and arrived to 10 % H_2O 90 % ACN. This allowed us to obtain eight fractions, ranging from 200 to 600 mL each. A bioassay-guided search (see above) allowed us to know that the active fractions were those from 3 to 6. Eventually, a finer separation, with a 250 × 22 mm column (spherical 20 µm silica) at a flow rate of 15 mL/min, was employed for fractions 3–6. In both cases, the elution was monitored by absorbance at 245 nm. The use of normal phase elution on unmodified silica gel was also attempted, but it led to worse separation and extensive degradation of the metabolites.

HPLC-MS analyses were performed on a Waters modular system equipped with a 1525 binary pump, a 2487 UV/Vis detector, and an SQD 3100 ESI MS detector. All mass spectra were acquired in the negative ionization mode. The column employed was a Sepachrome Robusta 150 × 2.1 mm, RP-18 5 µm (Sepachrome). The gradient was set as follows: 0–4 min 70 % H_2O 30 % CH_3OH; 12 min 45 % H_2O 55 % CH_3OH 23–26 min 10 % H_2O 90 % CH_3OH. Water was added with HCOOH (0.1 %). The flow rate was set at 0.5 mL/min.

NMR

NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer, operating at 499.802 MHz (^1H) and 125.687 MHz (^{13}C). All spectra were recorded in acetone-d_6; reference peaks were set at 2.05 (^1H), and at 29.8 and 206.3 (^{13}C).

Cytotoxicity

Cell culture

MCF-7 and MDA-MB-231 breast cancer cells were maintained in DMEM/F-12 supplemented with 5 % FBS, 100 mg/mL penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen, Gibco). SkBr3 breast cancer cells were cultured in RPMI − 1640 medium supplemented with 5 % FBS, 100 mg/mL penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen, Gibco). Cells were switched to medium without serum the day before experiments and thereafter treated in medium supplemented with 2.5 % FBS. HaCat human keratinocytes, purchased from DKFZ, Deutsches Krebsforschungszentrum, Heidelberg, Germany [36], were maintained in DMEM enriched with 10 % (v/v) FBS, 1 % glutamine, and 1 % antibiotic (EuroClone) at 37 °C in a 5 % CO_2 humidified atmosphere.

MTT assay

The effects of each compound on cell viability were determined with the MTT assay, which is based on the conversion of MTT to MTT formazan by mitochondrial enzymes. The positive control cisplatin (purity ≥ 98 %) was purchased from Calbiochem. Cells were seeded in quadruplicate in 96-well plates in regular growth medium and grown until 70 % confluence. Cells were washed once they had attached and then were treated with increasing concentrations (1–100 µM) of each compound for 48 h in regular medium supplemented with 2.5 % FBS. Relative cell viability was determined by the MTT assay according to the manufacturer's protocol (Sigma-Aldrich). Mean absorbance for each sample dose was expressed as percentage of the cells treated with vehicle absorbance and plotted versus sample concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50 % of those in the untreated control wells.

Antibacterial activity

Microorganisms

S. aureus strains were collected by the Microbiology Departments of "SS. Antonio and Biagio and Cesare Arrigo" Hospital (Alessandria, Italy) of the "Città di Alessandria" Clinic (Alessandria, Italy) and "Cardinal Massaia" Hospital (Asti, Italy). Forty-four clinical strains of *S. aureus*, including 17 methicillin-resistant, were isolated from bronchial aspirate (4 strains), cutaneous swabs (13 strains), throat swabs (3 strains), blood cultures (2 strains), eye swabs (1 strain), heel swabs (1 strain), nasal swabs (4 strains), pacemaker pocket swabs (1 strain), pus swabs (1 strain), urine cultures (1 strain), vaginal swabs (1 strain), wound swabs (10 strains), ulcer swabs (1 strain), and tracheal aspirate (1 strain). All strains were identified using the VITEK® 2 automated system (BioMerieux). A detailed description of the *S. aureus* strains here employed can be found in ▶ **Table 4S**, Supporting Information.

Minimal inhibitory concentration of antibacterial agents

The minimal inhibitory concentration (MIC) of the 15 antibacterial drugs [Benzylpenicillin (BPC), Oxacillin (OXA), Gentamicin (GEN), Levofloxacin (LVX), Erythromycin (ERY), Clindamycin (CLI), Linezolid (LZD), Daptomycin (DAP), Teicoplanin (TEC),Vancomycin (VAN), Tetracycline (TET), Tigecycline (TGC), Fusidic acid (FUS), Rifampicin (RIF), Trimethoprim-sulphamethoxazole (SXT)] were measured by a VITEK 2 AST card using a VITEK 2 automated system (BioMerieux). *S. aureus* NCTC 6571 strain was used as a reference strain. Briefly, strain suspensions obtained in physiological solution were adjusted to 0.5 McFarland by measuring absorbance at 600 nm. These suspensions were then loaded into the instrument in VITEK 2 AST cards that provided a series of antibiograms and tests for the detection of resistance (ESBL, cefoxitin screen, high level aminoglycoside resistance, inducible clindamycin resistance).

Disk diffusion assay

The following assays were carried out with 44 *S. aureus* strains and the reference strain *S. aureus* NCTC 6571: Vancomycin antibacterial effects were evaluated according to the EUCAST Disk Diffusion Method for Antimicrobial Susceptibility" v. 7.0 (January 2019). Vancomycin disk (30 µg) was purchased from Biolife, Italia. The sensitivity to the isolated compounds was assessed using an agar disk diffusion method. Strain suspensions (0.5 McFarland) obtained in physiological solution were swabbed on Mueller Hinton Agar (Biolife Italiana S.r.l.) plates. Filter paper disc (6.0 mm diameter) were placed on the agar surface and 10 µL of resuspended new molecules in DMSO were added. Pure DMSO (D-8418; Sigma-Aldrich) (10 µL) and organic linseed oil (10 µL) disks were used as negative controls, while vancomycin was considered a positive control. Plates were incubated at 37 °C for 24 h. All experiments were performed in triplicate. The halos were measured in mm using calipers. In order to attribute antibacterial activity, the halos of the different compounds were compared to the Vancomycin halo, which was used as the threshold value.

In vitro scratch wound assay

An *in vitro* wound healing assay was conducted to determine the migration rate of HaCat cells exposed to **9** and **10**. Aliquots of 2×10^5 cells per well were seeded on a 12-well plate and incubated in complete culture medium. Confluent cell layers were mechanically scratch wounded using a sterile 100 µL pipette tip and exposed for 24 h to extract fractions, complete medium as a control, and 50 µg/mL of Allantoin (≥ 98 %; Sigma-Aldrich) employed as a positive control [37]. Three wells for each treatment were used in this experiment. After incubation, cells were fixed for 15 min in FINEfix working solution (Milestone s.r.l.) with 70 % ethanol and then stained with 0.1 % toluidine blue. The migration rate was estimated by measuring the distance between wound edges at 0 and 24 h using ImageJ software (https://imagej.nih.gove/ij/). Cell migration was expressed as percentage of wound closure. Data were analyzed with GraphPad Prism, version 8.0.1, by ordinary one-way ANOVA with Bonferroni's correction for multiple comparison.

Supporting Information

NMR data for compounds **2–10** are reported in **Tables 1S–3S**. The complete list of the *S. aureus* strains employed in the present work is available in **Table 4S**.

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Conflict of Interest

The authors declare that there are no known conflicts of interest associated with this publication.

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