

Anti-methicillin-resistant *Staphylococcus aureus* (Anti-MRSA) flavonoids from Iraqi *Citrus sinensis* (L.) Osbeck

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Abstract

Citrus sinensis (L.) Osbeck (Rutaceae) was investigated as a potential source of anti-MRSA compounds. A bioassay-guided isolation approach afforded fourteen compounds from the peel and leaf extracts of *C. sinensis*, including a new flavanone glycoside, sinensidin (**10**). Structural elucidation was based on spectroscopic analyses, including 1D and 2D NMR and HRMS data. Among the isolated compounds, 3-methoxynobiletin (**4**), sinensetin (**7**), and rutin 3'-methyl ether (**12**) exhibited moderate anti-MRSA activity (MIC = 128–256 µg/mL). The crude extract showed higher apparent activity than the individual compounds, suggesting possible contributions from additional constituents and/or synergistic interactions. This study expands the phytochemical profile of *C. sinensis* and suggests that its polymethoxylated flavonoids and flavonoid glycosides may contribute to anti-MRSA activity.

Keywords *Citrus sinensis* · Rutaceae · MRSA · Polymethoxyflavone · Antimicrobial · Bioassay-guided protocol

Introduction

Antibiotic resistance, also known as antimicrobial drug resistance (AMR), represents one of the most significant global public health challenges today¹. The improper and excessive use of antibiotics has exacerbated this issue, leading to a worsening situation^{2,3}. According to a report published by the World Health Organization (WHO), antimicrobial resistance was responsible for approximately 5 million deaths globally in 2019 alone^{4,5}. Projections suggest that by 2050, the annual death toll due to AMR could rise to 10 million⁶. Given the severity of this public health crisis, the search for novel antimicrobial agents from natural sources has become essential to combat various drug-resistant microbial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA)⁷.

Citrus sinensis (L.) Osbeck, commonly known as the sweet orange, is an evergreen flowering tree belonging to the Rutaceae family. This species typically grows to a height of 7–10 m and accounts for approximately 70% of the global annual production of *Citrus* species⁸. Native to Asia, particularly regions such as China, India, Iraq, and Pakistan, *C. sinensis* is widely distributed in warm and tropical areas. The fruits are widely consumed

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as a rich source of vitamin C, while various parts of the plant are traditionally used in medicinal systems to treat a range of conditions, including angina, anxiety, colds, constipation, coughs, cramps, colic, depression, diarrhoea, hypertension, menstrual disorders, obesity, stress, and microbial infections such as bronchitis, flu, and tuberculosis⁸.

Previous phytochemical investigations of *C. sinensis* have identified several bioactive secondary metabolites, including carotenoids, cinnamic acid derivatives, flavonoids (primarily polymethoxyflavones), alkaloids (mainly acridone types), limonoids, sterols, terpenoids, and vitamins B and C. These compounds exhibit a wide range of reported bioactivities, including anticancer, antihyperlipidemic, anti-inflammatory, antimalarial, antimicrobial, anti-obesity, antioxidant, anxiolytic, and sedative effects⁸. However, the anti-MRSA activity of *C. sinensis* has not been systematically evaluated, and bioassay-guided isolation approaches targeting anti-MRSA constituents remain limited.

Preliminary studies have reported antimicrobial activity of essential oils derived from the peels of *C. sinensis* against multidrug-resistant bacteria⁹. Moreover, hesperetin, coumaric acid, and ferulic acid isolated from Newhall navel orange peel demonstrated activity against a single MRSA strain¹⁰. As part of ongoing research aimed at identifying phytochemicals with anti-MRSA activity^{7,11–13}, this study involves the bioassay-guided isolation of 3-methoxynobiletin (**4**), rutin 3'-methyl ether (**12**), and sinensetin (**7**) as compounds exhibiting anti-MRSA activity. These compounds were isolated from the leaves and peels of *C. sinensis* collected from Iraq, where the plant is traditionally used for the treatment of infections. In addition, ten additional known flavonoids (1–3, 5, 6, 8, 9, 11–14) and a new flavanone glycoside, sinensidin (**10**), were identified. The objective of this study is to isolate and characterise anti-MRSA constituents from *C. sinensis* using a bioassay-guided isolation approach.

Materials and methods

General

Chromatographic solvents were purchased from Fisher Scientific, UK, and were used without further purification. NMR experiments were performed on a Bruker AMX600 NMR spectrometer (600 MHz for ¹H and 150 MHz for ¹³C). Mass spectrometry analyses were conducted using a high-resolution mass spectrometry (HRMS) facility at the National Mass Spectrometry Facility (NMSF) (Swansea, UK), employing Xevo G2-S ASAP or LTQ Orbitrap XL spectrometers. Low- and high-resolution mass spectrometry analyses were also performed at Liverpool John Moores University; HRMS was performed using an Agilent 6200 Series Accurate-Mass Time-of-Flight (TOF) LC/MS system with electrospray ionisation (ESI) in positive ion mode, connected to an Agilent autosampler injection system.

Vacuum liquid chromatographic (VLC) fractions, prepared using silica gel (Sigma-Aldrich, UK), were analysed on a Dionex Ultimate 3000 UHPLC system coupled with a photodiode array (PDA) detector. A Phenomenex Gemini-NX C18 column (150 mm x 4.6 mm, 5 µm, Phenomenex, USA) and gradient solvent systems comprising acetonitrile (ACN, solvent B) and water (solvent A), both containing 0.1% TFA (flow rate 1 mL/min), were employed for method development and preparative-scale separation. Reversed-phase preparative HPLC purification was performed on an Agilent Technologies 1260 Infinity Series prep-HPLC system coupled with a photodiode array detector (Germany), using a Phenomenex LC-18 C₁₈ stainless steel column (150 mm x 21.2 mm, 5 µm, Phenomenex, USA) with the same solvent system at a flow rate of 10 mL/min. The column temperature was maintained at 25 °C.

Plant material

The leaves and peels of *Citrus sinensis* (L.) Osbeck were collected from Diyala, Central Iraq (N 33.79684, E 44.623337) in September 2015. The plant materials were air-dried at room temperature and ground into a fine

powder using a laboratory grinder. Dr Shaymaa Al-Majmaie (Diyala University, Iraq) identified the plant by comparison with herbarium specimens. The plant material was obtained from cultivated *Citrus sinensis* plants and did not involve the collection of wild or endangered plant species. The collection and use of the plant material complied with relevant institutional, national, and international guidelines and legislation. A voucher specimen (No. 6534) was deposited at the National Herbarium of Iraq. The plant material was subsequently stored under controlled dry and dark conditions before analysis.

Extraction

The air-dried powdered leaves (194.6 g) and peels (394.4 g) of *C. sinensis* were extracted separately. Sequential extraction was performed using solvents of increasing polarity, *n*-hexane, dichloromethane (DCM), and methanol (MeOH), using a Soxhlet apparatus (900 mL, ten cycles each). The crude extracts were concentrated to dryness using a rotary evaporator and stored at 4 °C for further analysis. The DCM extracts of the leaves and peels, along with the MeOH extract of the peels, showed the highest antimicrobial activity in preliminary in vitro screening using resazurin as an indicator of cell growth¹⁴. These active extracts were subjected to further fractionation to isolate the bioactive compounds.

Antimicrobial assays

Resazurin assay

The modified resazurin assay, as described by Sarker et al.¹⁴, was used to determine the minimum inhibitory concentration (MIC) using a microtitre plate-based method. The assay was conducted under aseptic conditions. Two Gram-positive bacterial strains (*Micrococcus luteus* NCTC 7508 and *Staphylococcus aureus* NCTC 12981), two Gram-negative bacterial strains (*Escherichia coli* NCTC 12241 and *Pseudomonas aeruginosa* NCTC 12903), and a fungal strain (*Candida albicans* ATCC 90028) were included in this study.

Preparation of standard microbial colonies

Microbial strains were cultured in 20 mL of nutrient agar on Petri dishes and incubated for 12–48 h at 35 °C. Single colonies from the incubated plates were transferred to sterilised tubes containing 100 mL of nutrient broth and incubated at 35 °C for 24–48 h. After incubation, the tubes were centrifuged at 4000 rpm for 5 min. The supernatant was discarded, and 20 mL of sterile normal saline was added, followed by centrifugation under the same conditions. This washing process was repeated until the supernatant became clear. The optical density of the bacterial suspension was measured using a spectrophotometer at 500 nm, and the concentration was adjusted to 5×10^6 CFU/mL through dilution and calculation¹⁵.

Preparation of resazurin solution

The resazurin solution was prepared by dissolving 1 mg of resazurin sodium salt (purchased from Aldrich, USA) in 5 mL of sterile distilled water. A vortex mixer ensured complete dissolution and homogeneity. Resazurin served as an indicator of microbial cell growth in the assay¹⁵.

Preparation of tested materials

Stock solutions of the test materials were prepared by dissolving them in 10% (v/v) dimethyl sulfoxide (DMSO) or sterilised water. The concentrations were 10 mg/mL for crude extracts and 1 mg/mL for fractions and pure compounds¹⁵.

Preparation of 96-well plates

All wells of the 96-well plates were filled with 50 μ L of sterile normal saline. The test material (100 μ L) was added to the first row, followed by serial dilution by transferring 50 μ L using multichannel pipettes. Resazurin (10 μ L) was then added to all wells, followed by 10 μ L of microbial suspension (5×10^6 CFU/mL). To prevent dehydration, each plate was loosely wrapped with cling film. Each plate included ciprofloxacin as a positive control for bacterial strains and nystatin for the fungal strain *C. albicans*¹⁵.

Interpretation of results

The normal colour of resazurin is blue. During incubation, if the test materials inhibited microbial growth, the wells retained the blue colour or turned purple or colourless, indicating a positive result. The development of a pink colour indicated no inhibitory effect. The lowest concentration at which no colour change occurred was recorded as the MIC (minimum inhibitory concentration). All assays were performed in triplicate, and mean values were calculated for each sample¹⁵.

Anti-MRSA screening

The anti-MRSA screening was conducted against six methicillin-resistant *Staphylococcus aureus* strains: SA1199B, XU212, MRSA340702, EMRSA-15, MRSA274819, and the standard strain ATCC 25,923. All bacterial strains were obtained from the UCL School of Pharmacy, and the experiments were performed at the University of East London.

Preparation of culture medium

Mueller-Hinton broth (MHB) was prepared according to the supplier's instructions. The medium was adjusted to contain cations at concentrations of 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+} .

Preparation of tested compounds

The compounds and antibiotics were dissolved in predetermined amounts of dimethyl sulfoxide (DMSO), ensuring that the final DMSO concentration did not exceed 1%. The solutions were further diluted with MHB to achieve a starting concentration of 128 μ g/mL.

Preparation of MTT solution

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was prepared by dissolving the required amount of MTT in methanol (MeOH) to obtain a concentration of 5 μ g/mL.

Suspension of the bacterial subculture

All bacterial strains were subcultured one day before the experiment. The strains were streaked onto nutrient agar slopes using a sterile loop and incubated at 37 °C for 12–18 h.

Anti-MRSA assay

The assay was performed using 96-well plates. Initially, 100 μ L of MHB was added to all wells except those in column 12. In the first row, 100 μ L of the test compounds or antibiotics was added. Using a multichannel pipette, the contents of the first well were mixed thoroughly, and 100 μ L was transferred to the wells of the second column.

This serial dilution process was continued up to column 10. Finally, 100 μL from column 10 was transferred to column 12. An inoculum density of 5×10^5 CFU/mL of each test organism was prepared in normal saline (9 g/L) by comparison with a 0.5 McFarland standard. MHB (125 μL) was dispensed into 10 wells of a 96-well microtitre plate (Nunc, 0.3 mL volume per well). The plates were incubated at 37 °C for 18 h. To determine the minimum inhibitory concentrations (MICs), 20 μL of MTT solution was added, followed by incubation for 20 min.

Interpretation of results

This colourimetric method indicated bacterial growth by a colour change from yellow to purple/blue. The MIC was recorded as the lowest concentration at which no visible growth was observed. All assays were performed in triplicate, and mean values were calculated. Norfloxacin was used as a positive control. The method was adapted from the broth microdilution technique according to the National Committee for Clinical Laboratory Standards, with minor modifications involving the use of Mueller-Hinton broth^{13,16–20}.

Isolation of compounds

The active DCM extracts from the leaves and peels of *Citrus sinensis* were subjected to vacuum liquid chromatography (VLC) separation²¹ on silica gel. A stepwise mobile phase of increasing polarity was employed, consisting of chloroform (CHCl_3) and MeOH as follows: 100% CHCl_3 , 2% MeOH in CHCl_3 , 4% MeOH in CHCl_3 , 6% MeOH in CHCl_3 , 8% MeOH in CHCl_3 , 10% MeOH in CHCl_3 , 20% MeOH in CHCl_3 , and 30% MeOH in CHCl_3 (each fraction, 200 mL). The active MeOH extract of the peels was further fractionated by solid-phase extraction (SPE) on a Strata C_{18} (20 g) cartridge²¹ using 30%, 50%, 80%, and 100% MeOH in water as eluents (200 mL each).

The VLC and SPE fractions were assessed for antimicrobial activity. The active VLC fraction F7 (20% MeOH in CHCl_3) from the DCM extract of the leaves, and VLC fractions F6 (10% MeOH in CHCl_3) and F7 (20% MeOH in CHCl_3) from the peel DCM extract, were selected for further analysis. Additionally, the active SPE fractions SPE2 (50% MeOH in water) and SPE3 (80% MeOH in water) of the MeOH extract of the peels were analysed by reversed-phase analytical HPLC to develop optimal separation conditions for preparative HPLC.

Reversed-phase preparative HPLC using various gradient elution methods, as described in the Results section, yielded fourteen flavonoids (1–14). The structures of all compounds were elucidated based on extensive 1D and 2D NMR data analysis, MS data interpretation, and comparison with literature data for previously reported compounds (1–9, 11–14)¹⁵.

Results and discussion

The Soxhlet extraction of the dried, ground leaves of *Citrus sinensis* (194.6 g) yielded three extracts, as *n*-hexane (6.2 g, 3.19%), DCM (10.2 g, 5.24%), and MeOH (14.3 g, 7.35%). Similarly, extraction of the peels (394.4 g) produced three extracts, as *n*-hexane (7.3 g, 1.9%), DCM (12.7 g, 3.2%), and MeOH (25.2 g, 6.39%). Preliminary antimicrobial screening indicated that the DCM extracts showed the highest activity among the extracts tested, followed by the MeOH extracts of the leaves and peels (Table 1). These extracts showed activity against all tested microorganisms, with minimum inhibitory concentration (MIC) values ranging from 4.8 to 625 $\mu\text{g/mL}$.

The *n*-hexane extract of the leaves showed the lowest level of activity among the six extracts, with effects against *Escherichia coli*, *Micrococcus luteus*, and *Staphylococcus aureus* observed only at higher concentrations (MIC = 2500–5000 $\mu\text{g/mL}$). The *n*-hexane extract of the peels also showed activity at higher concentrations against all tested organisms except *S. aureus*, with the lowest MIC observed against *M. luteus* (MIC = 156 $\mu\text{g/mL}$). The DCM extracts of both the leaves and peels showed activity against all strains, with the peel extract

Table 1 Antimicrobial activity of the extracts of the leaves and peels of *C. sinensis*, the VLC fractions of the DCM extracts, the SPE fraction of the MeOH extract of peels, and the isolated compounds (1–14).

Tested samples	MIC values in µg/mL				
	Gram-positive bacteria		Gram-negative bacteria		Fungus
	<i>Micrococcus luteus</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>
Leaves					
n-Hexane extract	5000	5000	2500	NA	NA
DCM extract	312.5	625	625	5000	312.5
MeOH extract	1250	2500	2500	5000	5000
Peels					
n-Hexane extract	156	NA	5000	5000	2500
DCM extract	4.8	625	312.5	625	156
MeOH extract	312.5	5000	1250	1250	625
VLC fractions of the DCM extract of the leaves					
F1	NA	NA	NA	NA	NA
F2	NA	NA	NA	NA	NA
F3	NA	NA	NA	NA	NA
F4	NA	NA	NA	NA	NA
F5	NA	NA	NA	NA	NA
F6	NA	NA	NA	1000	NA
F7	625	625	NA	1000	625
F8	NA	1000	NA	NA	1000
VLC fractions of the DCM extract of the peels					
F1	NA	NA	NA	NA	NA
F2	NA	NA	NA	NA	NA
F3	NA	NA	NA	NA	NA
F4	500	NA	NA	NA	500
F5	500	NA	NA	1000	500
F6	7.81	1000	500	500	7.81
F7	15.6	500	NA	1000	15.6
F8	NA	NA	NA	NA	NA
SPE fractions of the MeOH extract of the peels					
SPE1	500	NA	NA	NA	500
SPE2	625	1000	1000	NA	500
SPE3	31.2	125	250	500	15.6
SPE4	500	NA	NA	NA	500
Isolated compounds (1–14)					
1	500	NA	NA	NA	NA
2	250	NA	NA	500	250
3	250	NA	NA	500	500
4	500	250	NA	500	250
5	500	NA	NA	250	250
6	250	NA	NA	NA	NA
7	250	250	500	250	62.5
8	NA	NA	NA	NA	NA
9	NA	NA	NA	NA	NA
10	125	NA	NA	NA	250
11	NA	NA	NA	NA	NA
12	250	250	250	250	250
13	125	NA	NA	500	250
14	500	NA	NA	NA	500
Positive controls					
Ciprofloxacin	97.6	97.6	15.5	19.5	Not applicable
Nystatin	Not applicable	Not applicable	Not applicable	Not applicable	97.6

NA = No activity at the tested concentration.

exhibiting relatively higher apparent activity than the leaf extract. The DCM extract of the peels showed activity against *M. luteus* with an MIC value of 4.8 µg/mL.

A similar trend was observed for the MeOH extracts of the leaves and peels, with the peel extract showing relatively higher apparent activity (Table 1). The peel extracts showed relatively greater apparent antimicrobial activity than the leaf extracts (e.g., MIC=4.8 µg/mL vs. 625 µg/mL against *M. luteus*), which may reflect the combined presence of polymethoxyflavones and other unidentified constituents rather than the effect of polymethoxyflavones in isolation.

The lowest MIC value against *Candida albicans* was observed for the DCM extract of the peels (MIC=156 µg/mL), while the MeOH extract of the leaves showed the lowest level of activity (MIC 5000 µg/mL). The MeOH extracts displayed activity against all five microbial strains, although their MIC values (312.5–5000 µg/mL) were higher than those of the DCM extracts (Table 1). Consequently, the active DCM extracts of the leaves and peels were subjected to VLC-assisted fractionation, resulting in eight fractions for each extract. Since the MeOH extract of the peels showed relatively improved antimicrobial activity compared with the MeOH extract of the leaves, it was further fractionated using solid-phase extraction (SPE) on a Strata C₁₈ cartridge (20 g, Phenomenex, UK). This process yielded four SPE fractions (SPE1–SPE4) eluted with 30%, 50%, 80%, and 100% MeOH in water. All VLC and SPE fractions were evaluated for antimicrobial activity to identify fractions for subsequent isolation of bioactive compounds.

The first five VLC fractions (F1–F5: 100% CHCl₃, 2% MeOH in CHCl₃, 4% MeOH in CHCl₃, 6% MeOH in CHCl₃, and 8% MeOH in CHCl₃) of the DCM extract of the leaves did not show detectable antimicrobial activity under the conditions tested. VLC fraction 6 (F6, 10% MeOH in CHCl₃) exhibited activity only against *Pseudomonas aeruginosa* (MIC 1000 µg/mL), while fraction 8 (F8, 30% MeOH in CHCl₃) displayed activity against *Staphylococcus aureus* and *Candida albicans* (MIC=1000 µg/mL). VLC fraction 7 (F7, 20% MeOH in CHCl₃) of the DCM extract of the leaves was comparatively more active, showing activity against all tested organisms (MIC=625–1000 µg/mL) (Table 1).

The first three VLC fractions (F1–F3: 100% CHCl₃, 2% MeOH in CHCl₃, and 4% MeOH in CHCl₃) and fraction 8 (F8, 30% MeOH in CHCl₃) of the DCM extract of the peels did not show detectable antimicrobial activity under the conditions tested. VLC fractions 4 (F4, 6% MeOH in CHCl₃) and 5 (F5, 8% MeOH in CHCl₃) exhibited activity against *Micrococcus luteus* (MIC=500 µg/mL) and *Candida albicans* (MIC=500 µg/mL), with F5 also displaying activity against *Pseudomonas aeruginosa* (MIC 1000 µg/mL). VLC fraction 6 (F6, 10% MeOH in CHCl₃) showed activity against all tested organisms (MIC=7.81–1000 µg/mL), while fraction 7 (F7, 20% MeOH in CHCl₃) exhibited activity against all microorganisms (MIC=156–1000 µg/mL) except *Escherichia coli* (Table 1).

Among the four SPE fractions, fraction 3 (SPE3, 80% MeOH in water) was comparatively more active, showing activity against all microbial strains assessed in this study (MIC=156–500 µg/mL). Fraction 2 (SPE2, 50% MeOH in water) also showed activity against all organisms (MIC=500–625 µg/mL) except *Pseudomonas aeruginosa* (Table 1). The first fraction (SPE1, 30% MeOH in water) exhibited activity only against *Micrococcus luteus* and *Candida albicans* (MIC=500 µg/mL). Considering the overall antimicrobial activity profile, particularly against *Staphylococcus aureus*, VLC fraction 7 (F7) of the DCM extract of the leaves, VLC fractions F6 and F7 of the DCM extract of the peels, and SPE fractions SPE2 and SPE3 were selected for further HPLC analysis to purify the active compounds. The relatively higher apparent activity of fractions F6 and F7 compared with individual isolated flavonoids may reflect possible synergistic interactions among multiple constituents, a phenomenon frequently observed in phytochemical studies.

Reversed-phase preparative HPLC analysis of VLC fraction F7 of the DCM extract of the leaves of *C. sinensis* (linear gradient 30–100% acetonitrile (ACN) in water over 30 min, isocratic 100% ACN for 5 min, linear gradient 100–30% ACN in water for 5 min, and isocratic 30% ACN in water for 5 min, flow rate 10 mL/min) afforded five compounds (1, 3, 5, 6, and 8) (Fig. 1) with retention times (*t_R*) of 20.39, 21.36, 22.32, 31.61, and 33.45 min, respectively.

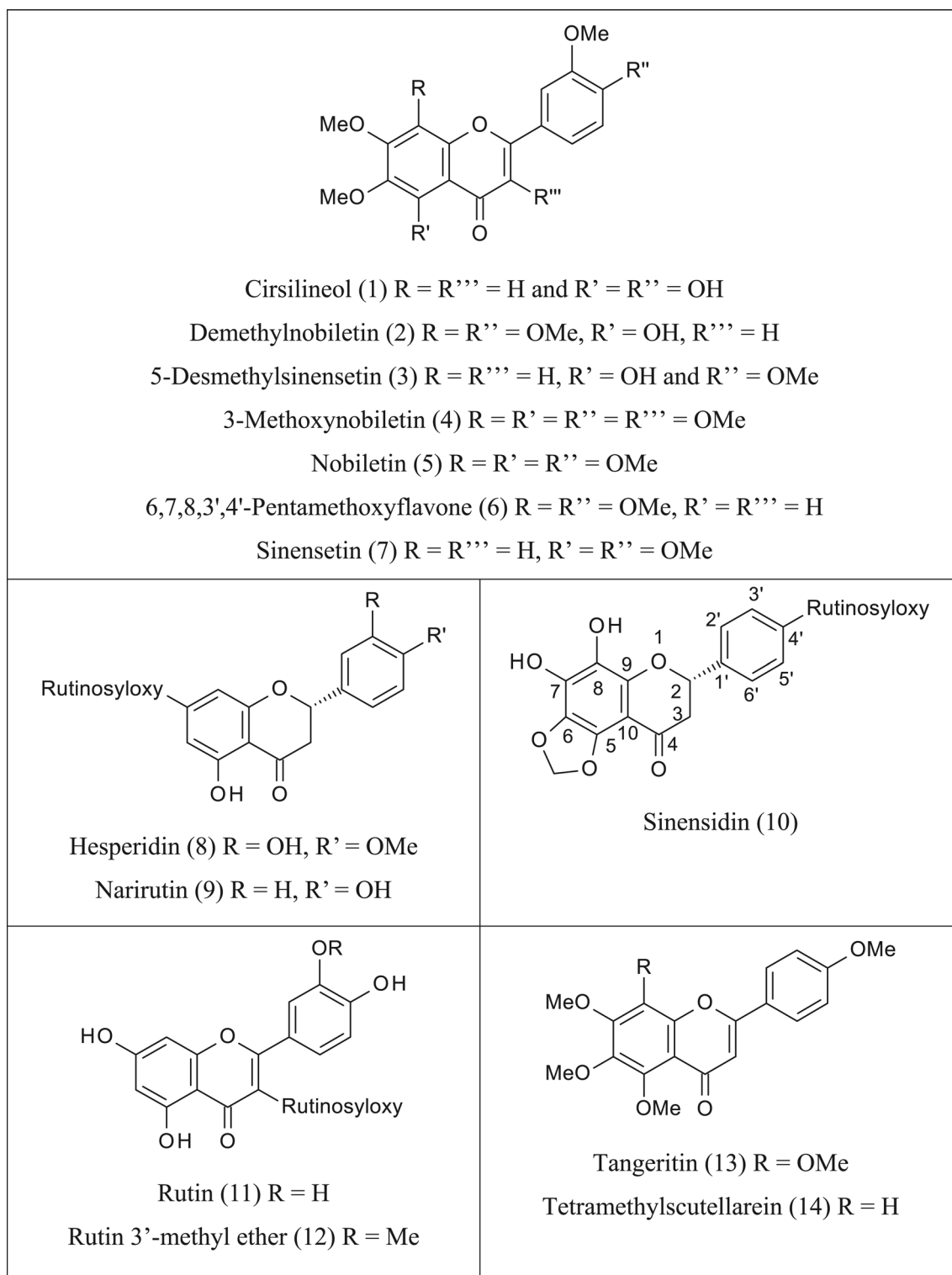


Fig. 1 Structures of flavonoids isolated from the active fractions of the extracts of the leaves and peels of *C. sinensis*.

A similar preparative HPLC procedure (linear gradient 20–50% ACN in water over 30 min, isocratic 50% ACN for 3 min, linear gradient 50–20% ACN in water for 5 min, and isocratic 20% ACN in water for 5 min, flow rate 10 mL/min) applied to VLC fraction F6 of the DCM extract of the peels of *C. sinensis* afforded six compounds, 7 ($t_R = 28.28$ min), 14 ($t_R = 33.21$ min), 4 ($t_R = 34.35$ min), 13 ($t_R = 37.03$ min), 2 ($t_R = 39.84$ min), and additional quantities of 5 ($t_R = 32.00$ min). Preparative HPLC analysis of VLC fraction F7 of the peels (linear gradient 20–80% ACN in water over 30 min, isocratic 80% ACN for 5 min, linear gradient 80–20% ACN in water for 5 min, and isocratic 20% ACN in water for 5 min, flow rate 10 mL/min) resulted in the isolation of seven additional compounds: 11 ($t_R = 14.01$ min), 9 ($t_R = 17.70$ min), 14 ($t_R = 22.42$ min), and additional quantities of 7 ($t_R = 19.74$ min), 5 ($t_R = 21.75$ min), 4 ($t_R = 23.03$ min), and 13 ($t_R = 24.33$ min).

Reversed-phase preparative HPLC analysis of the SPE fractions SPE2 and SPE3 of the MeOH extract of the peels (linear gradient 20–80% MeOH in water over 30 min, isocratic 80% MeOH for 2 min, linear gradient 80–20% MeOH in water for 2 min, and isocratic 100% MeOH for 2 min, followed by isocratic 20% MeOH in water for 5 min) afforded compounds 11 ($t_R = 13.78$ min), 10 ($t_R = 15.03$ min), 12 ($t_R = 15.50$ min), 9 ($t_R = 17.70$ min), 8 ($t_R = 19.18$ min), 13 ($t_R = 26.13$ min), and 5 ($t_R = 28.82$ min).

Among the isolated compounds, compounds 1–9 and 11–14 were identified as known natural products: cirsiineol (1, 3.3 mg)²², demethylnobiletin (2, 3.1 mg)²³, 5-desmethylinensetin (3, 2.8 mg)²⁴, 3-methoxynobiletin (4, 4.4 mg)²⁵, nobiletin (5, 2.9 mg)²⁶, 6,7,8,3',4'-pentamethoxyflavone (6, 3.6 mg)²⁷, sinensetin (7, 4.8 mg)²⁷, hesperidin (8, 6.1 mg)²⁸, narirutin (9, 1.9 mg)²⁹, rutin (11, 9.1 mg)³⁰, rutin 3'-methyl ether (12, 4.1 mg)⁷, tangeretin (13, 6.7 mg)³¹, and tetramethylscutellarein (14, 3.7 mg)²⁶. The structures of these compounds were elucidated based on 1D and 2D NMR data, HRMS data, and comparison with published literature (Tables S1–S3). The experimental ¹H and ¹³C NMR data were consistent with reported values for the respective compounds. Most of these flavonoids are polymethoxyflavones, which are characteristic of the *Citrus* genus and are associated with a range of reported bioactivities³².

Compound 10, isolated as a golden-yellow oily substance (2.1 mg), was identified as a new flavanone glycoside. Its structure was proposed based on spectroscopic data. The HRMS data indicated a molecular formula of C₂₈H₃₂O₁₆, with a pseudomolecular ion [M + H]⁺ at m/z 625.1761 (calculated 625.1768). The ¹H NMR spectrum (Table 2) showed aromatic proton signals at δ_H 7.47 (2 H, d, $J = 8.8$ Hz) and 7.16 (2 H, d, $J = 8.8$ Hz), consistent with a *para*-disubstituted phenyl ring at C-2 of the flavanone skeleton, similar to reported substitution patterns in related flavonoids. A singlet at δ_H 6.22 (2 H) was consistent with a methylenedioxy group. Signals at δ_H 5.53 (1 H, dd, $J = 3.1, 12.4$ Hz), 2.83 (1 H, dd, $J = 3.1, 17.1$ Hz), and 3.29 (1 H, dd, $J = 17.1, 12.4$ Hz) corresponded to H-2 and H₂–3 of a flavanone skeleton.

Two anomeric proton signals at δ_H 4.96 (1 H, d, $J = 7.0$ Hz) and 4.71 (1 H, d, $J = 1.4$ Hz) were consistent with β -D-glucopyranosyl and α -L-rhamnopyranosyl residues, indicating the presence of a rutinose disaccharide. The coupling constants ($J = 7.0$ Hz for glucose and $J = 1.4$ Hz for rhamnose) and the rhamnose methyl doublet at δ_H 1.21 ($J = 6.2$ Hz) support the proposed sugar assignments. Minor splitting of the glucose anomeric signal at δ_H 4.96 (integrating to one proton) may reflect conformational effects in CD₃OD rather than indicating the definitive presence of an epimeric mixture, although this possibility cannot be excluded. Overlapping signals in the δ_H 3.33–4.20 region were attributed to the remaining sugar protons.

The absence of additional aromatic proton signals suggested full substitution of the A ring, which limited unambiguous assignment of the positions of oxygenated carbon atoms based on available HMBC correlations. Accordingly, aspects of the structural assignment, particularly with respect to substitution pattern and stereochemical detail, should be regarded as tentative within the limits of the available spectroscopic data. The ¹H–¹H COSY spectrum (Table 2) supported scalar couplings, facilitating the assignment of sugar proton resonances. The ¹³C NMR spectrum (Table 2) showed 28 carbon signals, including a carbonyl carbon at δ_C 203.6 (C-4 of the flavanone nucleus), 12 carbons attributable to the rutinose unit (anomeric carbons at δ_C 102.3 and 103.4, a rhamnose methyl signal at δ_C 18.1, and oxymethine carbons at δ_C 71.6–78.5), a methylenedioxy carbon at δ_C 101.3, and 12 aromatic carbons (four methine, six oxygenated quaternary, and two quaternary carbons) (Table 2). The ¹H–¹³C HSQC spectrum supported the assignment of protonated carbons.

Table 2 ^1H (600 MHz) and ^{13}C (150 MHz) NMR data, and ^1H - ^1H COSY, ^1H - ^{13}C HSQC and ^{13}C - ^{13}C HMBC key correlations of sinensidin (10).

Number	Chemical shift δ in ppm		^1H - ^1H COSY	^1H - ^{13}C HSQC	^{13}C HMBC	
	δ_{H} , coupling constant J in Hz	δ_{C}			$^2 J$	$^3 J$
Flavanone skeleton						
2	5.53, dd, 1 H, $J = 3.1, 12.4$	81.5	H-3	C-2	C-1'	C-4
3	2.83, dd, 1 H, $J = 3.1, 17.1$ 3.29, dd, 1 H, $J = 17.1, 12.4$	43.1	H-2	C-3	C-2, C-4	C-1'
4	-	203.6	-	-	-	-
5	-	147.7**	-	-	-	-
6	-	135.5	-	-	-	-
7	-	151.6	-	-	-	-
8	-	129.4	-	-	-	-
9	-	147.6**	-	-	-	-
10	-	111.6	-	-	-	-
1'	-	132.7	-	-	-	-
2', 6'	7.47, d, 2 H, $J = 8.8$	129.1	H-3', H-5'	C-3', C-5'	C-1'	C-2, C-4'
3', 5'	7.16, d, 2 H, $J = 8.8$	118.0	H-2', H-6'	C-2', C-6'	C-4'	C-1'
4'	-	160.4	-	-	-	-
5,6-O-CH ₂ -O-	6.22, s, 2 H	101.3	-	-O-CH ₂ -O-	-	C-5, C-6
Glucose unit						
1''	4.96, d, 1 H, $J = 7.0$	102.3	H-2''	C-1''	-	C-4'
2''	3.88*	75.5	H-1'', H-3''	C-2''	-	C-4''
3''	3.48*	78.5	H-2'', H-4''	C-3''	-	C-5''
4''	3.39*	71.6	H-3'', H-5''	C-4''	-	C-2'', C-6''
5''	3.34*	78.4	H-4'', H-6''	C-5''	-	C-3''
6''	3.92, 3.70*	69.9	H-5''	C-6''	-	C-1''
Rhamnose unit						
1'''	4.71, d, 1 H, $J = 1.4$	103.4	H-2'''	C-1'''	-	C-6''
2'''	3.48*	74.2	H-1''', H-3'''	C-2'''	-	C-4'''
3'''	3.29*	71.8	H-2''', H-4'''	C-3'''	-	C-5'''
4'''	3.69*	72.5	H-3''', H-5'''	C-4'''	-	C-2''', C-6'''
5'''	3.60*	77.3	H-4''', H-6'''	C-5'''	-	C-3'''
6'''	1.21, d, 3 H, $J = 6.2$	18.1	H-5'''	C-6'''	C-5'''	C-4'''

Spectra obtained in CD₃OD.

*Unresolved overlapped peaks, determined by 2D COSY, HSQC, and HMBC experiments.

**Interchangeable; could be either carbon.

The key HMBC correlations (Table 2) included H-1'' (glucose anomeric proton) to C-4', H-6'' to C-1' (rhamnose anomeric carbon), and methylenedioxy protons to C-5 (δ_{C} 147.7) and C-6 (δ_{C} 135.5) of the flavanone A ring. These correlations are consistent with a 4'-*O*-rutinosyl substitution. In addition, the H-6'' to C-1' correlation supports the proposed 6 to 1 linkage between glucose and rhamnose. The observed methylenedioxy correlations are consistent with attachment between adjacent aromatic carbons. Due to full substitution of ring A and the absence of aromatic ^1H signals, unambiguous assignment of all oxygenated ^{13}C signals based solely on HMBC data was not feasible. However, the observed correlations, together with comparative analysis with compound 4 (Table S3) and previously reported data, are consistent with oxygenation at C-5, C-6, C-7, and C-8. A C-5/C-6 methylenedioxy linkage is tentatively proposed for compound 10 based on the available evidence. Annotated HSQC and HMBC spectra supporting these assignments are provided in the Supplementary Information (Figures S1-S6). Accordingly, the structure of compound 10 is tentatively proposed as a new flavanone glycoside, named sinensidin (10), with structural features assigned within the limits of the available spectroscopic data.

All fourteen compounds (1–14) were subjected to the resazurin assay as described for the extracts and fractions. Compounds 3-methoxynobiletin (4), sinensetin (7), and rutin 3'-methyl ether (12) showed activity against

Table 3 Anti-MRSA activity of 3-methoxynobiletin (4), sinensetin (7), and rutin 3'-methyl ether (12).

Tested compounds	MIC values in µg/mL					Standard <i>S. aureus</i> strain ATCC25923
	MRSA strains					
	XU212	SA1199B	EMRSA15	MRSA340702	MRSA274819	
3-Methoxynobiletin (4)	NA	NA	256	NA	NA	256
Sinensetin (7)	NA	NA	256	128	256	128
Rutin 3'-methyl ether (12)	256	NA	NA	256	256	128
Norfloxacin	16	32	1	64	64	2

NA = No activity at the tested concentrations.

all tested microorganisms, with MIC values ranging from 128 to 256 µg/mL (Table 1). These compounds were among those exhibiting activity against *Staphylococcus aureus* (MIC = 250 µg/mL). Sinensetin (7) showed antifungal activity against *Candida albicans* (MIC = 62.5 µg/mL), which is consistent with recent reports describing its activity in combination with other compounds in antimicrobial applications³³.

Hesperidin (8), narirutin (9), and rutin (11) did not show detectable antimicrobial activity against the tested strains. However, other flavonoids (1–3, 5, 6, 10, and 12–14) showed activity against *Micrococcus luteus* (MIC = 125–500 µg/mL). Cirsilioneol (1) and 6,7,8,3',4'-pentamethoxyflavone (6) showed limited activity, with no detectable effects against other organisms at the tested concentrations. Demethylnobiletin (2), 5-desmethylnobiletin (3), nobiletin (5), and tangeretin (13) exhibited similar antimicrobial profiles, showing activity against *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Candida albicans* (MIC = 250–500 µg/mL). Sinensidin (10) and tetramethylscutellarein (14) also showed antifungal activity against *Candida albicans* (MIC = 250 and 500 µg/mL, respectively) (Table 1).

The activity of 3-methoxynobiletin (4), sinensetin (7), and rutin 3'-methyl ether (12) against *Staphylococcus aureus* (MIC = 250 µg/mL) led to their further evaluation against five clinical MRSA strains: XU212, SA1199B, EMRSA-15, MRSA340702, and MRSA274819 (Table 3). 3-Methoxynobiletin (4) showed activity only against EMRSA-15 (MIC = 256 µg/mL), while sinensetin (7) and rutin 3'-methyl ether (12) showed activity against three MRSA strains. Sinensetin (7) showed the lowest MIC against MRSA340702 (MIC = 128 µg/mL), while rutin 3'-methyl ether (12) showed activity against XU212, MRSA340702, and MRSA274819 (MIC = 256 µg/mL).

3-Methoxynobiletin (4), sinensetin (7), and rutin 3'-methyl ether (12) also showed antibacterial activity against the standard strain *Staphylococcus aureus* ATCC 25,923, with MIC values of 256, 128, and 128 µg/mL, respectively. These MIC values (128–256 µg/mL) indicate weak to moderate antimicrobial activity for isolated natural products. To our knowledge, this study reports anti-MRSA activity for 3-methoxynobiletin for the first time and further supports the selective activity of sinensetin (7) and rutin 3'-methyl ether (12). The anti-MRSA activity of sinensetin (7) was lower than that of the positive control norfloxacin (MIC = 64 µg/mL) against MRSA340702 and MRSA274819.

3-Methoxynobiletin (4) has been previously reported from the peels of *Citrus aurantium* var. *sinensis*³⁴ and *C. unshiu* Markovich³⁵. Although its antimicrobial potential has been described, activity against MRSA strains has not been previously reported. This polymethoxyflavone has also been isolated from *Ageratum conyzoides*³⁶ and *Ageratina coelestina*³⁷ (Asteraceae), where antifungal properties have been reported³⁷. Sinensetin (7), widely found in the *Citrus* genus (e.g., *C. aurantium*, *C. grandis*, *C. reticulata*, and *C. sinensis*)^{15,38,39}, has been reported to exhibit antibacterial activity, including against *Staphylococcus aureus*^{15,40–42}. This activity has been associated with potential interference with staphylocoagulase activity and inhibition of biofilm formation⁴³. Rutin 3'-methyl ether (12), also known as isorhamnetin 3-O-rutinoside, has been reported as an antimicrobial compound with anti-MRSA activity from *Ruta chalepensis* (Rutaceae)⁷. This compound has also been identified in *Campanula rapunculoides* (Campanulaceae)⁴⁴, *Caragana sinica* (Fabaceae)⁴⁵, and *Coriandrum sativum* (Apiaceae)⁴⁶.

Conclusions

A bioassay-guided isolation study led to the identification of two polymethoxyflavones, 3-methoxynobiletin (4) and sinensetin (7), along with rutin 3'-methyl ether (12), from the leaves and peels of *Citrus sinensis* of Iraqi origin. These three flavonoids exhibited a moderate level of activity against various strains of methicillin-resistant *Staphylococcus aureus* (MRSA), with minimum inhibitory concentration (MIC) values ranging from 128 to 256 µg/mL. In addition to these moderately active compounds, ten other known flavonoids (1–3, 5, 6, 8, 9, 11–14) and a new flavanone glycoside, named sinensidin (10), were isolated from the active fractions. However, these compounds were inactive at the tested concentrations (MIC ≥ 1000 µg/mL). Among the isolated flavonoids, 3-methoxynobiletin (4), sinensetin (7), and rutin 3'-methyl ether (12) demonstrated the highest activity among the isolates, though still only moderate in potency. The anti-MRSA potential of 3-methoxynobiletin (4) is reported here for the first time. Based on the findings of this study, these flavonoids (4, 7, and 12) may serve as structural templates for the design of more active analogues. Notably, the crude extracts displayed substantially higher potency (MIC as low as 4.8 µg/mL) than the isolated compounds, suggesting the presence of additional active principles and/or synergistic interactions. Further LC-MS/MS profiling and bioassay-guided fractionation are warranted to identify these constituents. While the isolated polymethoxyflavones show only moderate activity, they may still provide useful scaffolds for analogue development. Future work should also include physico-chemical, preclinical, and clinical studies to evaluate their therapeutic potential.

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Data availability The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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