Antimicrobial potential of the leaves of *Citrus grandis* (L.) Osbeck collected from Iraq: Bioassay-guided isolation of sinensetin as the anti-MRSA compound

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ABSTRACT

Infections caused by antibiotic-drug-resistant microorganisms are a major global health concern, and they result in millions of deaths every year. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of such drug-resistant microbial strains, and new and effective antimicrobial agents are desperately needed to combat infections caused by MRSA. In the search for effective anti-MRSA agents, the leaves of *Citrus grandis* (Rutaceae), also known as *C. maxima*, were investigated. Implementing a bioassay-guided approach, sinensetin (**2**), which is a polymethoxyflavone, was isolated as a promising anti-MRSA compound, showing inhibitory activity against three (EMRSA-15, MRSA340802 and MRSA274819; MIC values 128-256 μg/mL) of five MRSA strains tested in the present study. Five other flavonoids 6,7,8,3',4'-pentamethoxyflavone (**1**)**,** cirsilineol (**3**), nobiletin (**4**), 5-desmethylsinensetin (**5**) and hesperidin (**6**) were isolated from the dichloromethane extract of this plant. They displayed varied levels of antimicrobial activities against the tested microbial strains, *Micrococcus luteus* NCTC 7508, *Escherichia coli* NCTC 12241 and *Pseudomonas aeruginosa* NCTC 12903, and a fungal strain, *Candida albicans* ATCC 90028, but not against *Staphylococcus aureus* NCTC 12981. Sinensetin (**2**) also exhibited strong antimicrobial activity against the fungal strain *C. albicans* with an MIC value of 0.0625 mg/mL. The chemical structures of all isolated compounds were unequivocally elucidated by spectroscopic means (1D and 2D NMR and HR-ESIMS). The present study revealed sinensetin (**2**) as a potential structural template for generating structural analogues and developing anti-MRSA agents and provided scientific evidence supporting the traditional uses of *C. grandis* in the treatment of microbial infections.

Keywords: *Citrus grandis*; MRSA; polymethoxyflavonoids; Rutaceae; sinensetin; *Staphylococcus aureus*

1. Introduction

One of the major global public health concerns is antibiotic resistance or antimicrobial drug resistance (AMR) [1], and the condition has become worse because of the irrational overuse of antibiotics in recent years [2, 3]. The World Health Organization (WHO) estimates that antimicrobial resistance directly caused 1.27 million deaths and contributed to the death of *ca.* 5 million people worldwide in 2019 alone [4, 5]. It is further estimated that by 2050 as many as ten million deaths may occur annually. This dire situation of AMR has necessitated the search for novel compounds, particularly those from natural sources, with potential antimicrobial properties against various drug-resistant microbial strains, e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) [6].

Citrus grandis (L.) Osbeck [*alt. Citrus maxima* (Burm.) Merr.], commonly known as 'pomelo' or 'shaddock', is a perennial tree (5-15 m) from the family Rutaceae [7]. Pomelo, native to Southeast Asia (particularly, Malaysia), is the largest citrus fruit (15-30 cm diameter) and this natural and non-hybrid citrus fruit is also widely grown in Chile, China, Cambodia, Iraq, Japan and Türkiye [7-10]. Various plant parts of *C. grandis* have long been used in traditional medicines for treating fever, oedema, skin infections, pain and anorexia [8, 11]. The fruit is used as an antipyretic, cardiac stimulant, antitoxic and to treat stomachache, and the pulp is used as an appetizer [12]. This plant is a part of Chinese medicine for healing colds and relieving fatigue [9]. In South Korea, leaves are used as a food flavouring agent and used in making tea [13]. In many Asian countries, the leaf oil of *C. grandis* is used to treat skin infections, headaches and abdominal pain, the fruit peel is used for treating cough, swelling, and epilepsy, the flowers are used as a remedy for anxiety and sleep disorders, and the whole fruit is used for the treatment of asthma, leprosy, and mental disorders [8]. Previous phytochemical studies on *C. grandis* revealed the presence of several bioactive plant secondary metabolites including, carotenoids, cinnamic acid derivatives, flavonoids (mainly polymethoxyflavones), alkaloids (mainly acridone type), limonoids, sterols, terpenoids and vitamin B and vitamin C, whereas the bioactivity evaluation established the anticancer, antihyperlipidemic, anti-inflammatory, antimicrobial, antioxidant and cytoprotective (hepatoprotective and nephroprotective) potential of this plant [8]. Most of these studies were about whole fruits, pulps, peels, and flowers, but the least with the leaves of *C. grandis*. Also, the anti-MRSA potential of this plant was not previously evaluated. As a part of our on-

going endeavour to discover phytochemicals with anti-MRSA activity [6, 14-16], we now report on the bioassay-guided isolation of sinensetin (**2**) as a potential anti-MRSA agent against multiple MRSA strains, as well as five other flavonoids, 7,8,3',4'-pentamethoxyflavone (**1**)**,** cirsilineol (**3**), nobiletin (**4**), 5-desmethylsinensetin (**5**) and hesperidin (**6**) from the leaves of *C. grandis* collected from Iraq, where this plant has long been traditionally used to treat infections.

2. Materials and Methods

2.1. General

Chromatographic solvents were from Fisher Scientific, UK, and used without further purification. The NMR experiments were performed on a Bruker AMX600 NMR spectrometer (600 MHz for ^{1}H, and 150 MHz for ^{13}C). MS was conducted using high-resolution mass spectroscopy facility (HR-MS) at the National Mass Spectrometry Facility (NMSF) (Swansea, UK) on Xevo G2-S ASAP or LTQ Orbitrap XL1 spectrometers. Low and high-resolution MS analyses were also obtained at Liverpool John Moores University; HR-MS using Agilent 6200 Series Accurate-Mass Time-of-Flight (TOF) LC/MS using electro-spray ionisation (ESI) in positive ion mode connected to an Agilent auto-sampler injection system. The vacuum liquid chromatographic (VLC, silica gel, Sigma-Aldrich, UK) fractions were analyzed on a Dionex Ultimate 3000 UHPLC coupled with a photodiode array (PDA) detector. A Phenomenex Gemini-NX 5 U C18 column (150 × 4.6 mm, 5 μm, Phenomenex, USA), and gradient solvent systems comprising acetonitrile (ACN, solvent B) and water (solvent A) (both contained 0.1% TFA, flow rate: 1 mL/min) were employed for method developments for preparative-scale separation and isolation of compounds. The reversed-phase preparative HPLC purification was performed on an Agilent Technologies, 1260 Infinity Series prep-HPLC coupled with a photo-diode-array detector, (Germany) using a Phenomenex LC-18 C_{18} stainless steel column (150 × 21.2 mm, 5 μm, Phenomenex, USA) with the same solvent system as mentioned above, but with a 10 mL/min flow rate. The column temperature was set at 25°C.

2.2. *Plant material*

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

powder using a coffee grinder. A voucher specimen (No. 15324) for this collection was deposited at the National Herbarium of Iraq.

2.3. Extraction

The air-dried ground leaves (351 g) of *C. grandis* were extracted sequentially with solvents of increasing polarity, i.e., *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 4oC for further work. The active DCM extract with the highest level of antimicrobial activity in the initial *in vitro* antimicrobial screening using resazurin as an indicator of cell growth [17] was subjected to further fractionation leading to the isolation of active compounds.

2.4. Antimicrobial assay (resazurin assay)

The modified resazurin test described by Sarker et al. [17] was used to determine the minimum inhibitory concentration (MIC) using a microtitre plate. The key feature of this assay is the use of a standard concentration of bacterial suspension. This assay was performed under aseptic conditions. Two Gram-positive (*Micrococcus luteus* NCTC 7508 and *Staphylococcus aureus* NCTC 12981) and two Gram-negative (*Escherichia coli* NCTC 12241 and *Pseudomonas aeruginosa* NCTC 12903) bacterial strains and a fungal strain, *Candida albicans* ATCC 90028 were used in this study. All these microorganisms were obtained from the properly identified and appropriately maintained cultures at the Microbiology Laboratory of the School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, UK.

Preparation of standard microbial colonies: Microbial strains were cultured on 20 mL nutrient agar in Petri dishes and incubated for 12-48 h at 35 °C. Single colonies from incubated plates were transferred to sterilized tubes containing 100 mL nutrient broth and incubated at 35 °C for 24 -48 h. After the incubation, the tubes were centrifuged at 4000 rpm for 5 min. The supernatant was discarded, and 20 mL sterile normal saline was added to the tubes and again centrifuged at the same conditions. Centrifugation was repeated until the supernatant became clear. A spectrophotometer was used at 500 nm to determine the optical density of the bacterial suspension with dilution factor and calculations to obtain a concentration of 5×10^6 cfu/mL.

Preparation of resazurin solution: The resazurin solution was prepared by dissolving 1 mg of resazurin (resazurin sodium salt purchased from Aldrich, USA) in 5 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogeneous solution. Resazurin was used in this assay as an indicator of cell growth.

Preparation of tested materials: The stock concentration was prepared by dissolving the tested materials in 10% (v/v) DMSO or sterilized water. The stock concentration was 10 mg/mL for crude extracts, while 1 mg/mL for the fractions and pure compounds.

Preparation of 96-well plates: All wells on 96-well plates were filled with 50 µL sterilized normal saline. The test material (100 µL) was added to the first row of the plate and serial dilutions were made using multichannel pipettes by transferring 50 µL. Resazurin (10 µL) was added to all wells and finally, 10 µL of bacterial suspension (5×10^6 cfu/mL) was added to each well. To prevent bacterial dehydration, each plate was wrapped loosely with cling film. Every plate contained the antibiotic ciprofloxacin as a positive control for the bacterial strains and nystatin for the fungal strain *C. albicans*.

Interpretation of results: The normal resazurin colour is blue. During incubation, if the test materials inhibited the microorganisms, they acquire the resazurin's blue colour, or become purple or colourless, which is considered a positive result, whereas the development of pink colour represents no effect of the test materials on the microbes. The lowest concentration at which the colour change occurs is considered an MIC (minimum inhibitory concentration) value. The mean of three values was calculated.

2.5. Anti-MRSA screening

The anti-MRSA screening was performed against five methicillin-resistant *S. aureus* strains, SA1199B, XU212, MRSA340702, EMRSA-15, MRSA274819, and against the standard strain ATCC25923. All the 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 instruction given by the supplier. The MHB was adjusted to contain cations- 20 mg/L Ca^{2+} and 10 mg/L of Mg^{2+}.

Preparation of tested compounds: Compounds and antibiotics were dissolved in predetermined amounts of DMSO (less than 1% concentration in the final well), which was further diluted with MHB to obtain the targeted starting concentration (128 µg/mL).

Preparation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution: It was achieved by dissolving the required amount of MTT in MeOH to obtain a concentration of 5 µg/mL.

Suspension of subculture of the bacteria: All bacterial strains were subcultured one day before the experiment. Bacterial strains were subcultured in nutrient agar slopes by streaking the bacteria with a loop followed by incubation at 37^{o} for 12-18 h.

Anti-MRSA assay: This experiment was performed using 96-well plates. The first step in this assay was to add (100 µL) of MHB to all wells except those in column 12. To the first row of the plate, 100 µL of test compounds or antibiotics were added. Using a multi-channel pipette, the materials of the first well were mixed properly, followed by the transfer of 100 µL of the well contents to the wells of the second column and so on until column 10. Finally, 100 µL content from the wells of column 10 was transferred to the wells of column 12.

An inoculum density of 5×10^5 cfu of each of the test organisms was prepared in normal saline (9 g/L) by comparison with a 0.5 MacFarland standard. MHB (125 µL) was dispensed into 10 wells of a 96-well microtitre plate (Nunc, 0.3 mL volume per well). Then microtitre plates were incubated at 37°C for 18 h. To determine the minimum inhibitory concentrations (MICs), 20 µL of MTT was added to the microtitre plate and incubated for 20 min. It is a colourimetric method, bacterial growth was indicated by the colour changing from yellow to dark blue and the MIC was recorded as the lowest concentration at which no growth was observed. As a positive control norfloxacin, a well-known antibiotic was used.

The method used to determine the MIC is considered a broth microdilution method according to National Committee for Clinical Laboratory Standards with modification using nutrient broth as the medium [15, 19-22].

2.6. Isolation of compounds

The most active DCM extract of *C. grandis* was subjected to vacuum liquid chromatographic (VLC) separation [23] on silica gel using a step-wise mobile phase of increasing polarity using the solvent mixture comprising chloroform ($CHCl_3$) and MeOH as follows 100% $CHCl_3$, 2% MeOH in 100% $CHCl_3$, 4% MeOH in 100% $CHCl_3$, 6% MeOH in 100% $CHCl_3$, 8% MeOH in 100% $CHCl_3$, 10% MeOH in 100% $CHCl_3$, 20% MeOH in 100% $CHCl_3$ and 30% MeOH in 100% $CHCl_3$ (each fraction was 200 mL). The VLC fractions were assessed for antimicrobial activity and the most active fraction F7 (20% MeOH in 100% $CHCl_3$) was analysed by reversed-phase analytical HPLC to develop the optimum separation conditions for preparative HPLC separation. Reversed-phase preparative HPLC analysis [linear gradient 20-80% acetonitrile (ACN) in water over 30 min, isocratic 80% ACN for the next 5 min, linear gradient 80-20% ACN in water for 5 min, and isocratic 20% ACN in water for 5 min] of the VLC fraction F7 afforded six compounds (**1**-**6**), the structures of which were unambiguously confirmed by extensive 1D and 2D NMR data analyses and MS spectroscopic data interpretation as well as comparison with the respective literature data.

3. Results and Discussion

The Soxhlet extraction of the dried ground leaves of *C. grandis* afforded three extracts, *n*-hexane (5.9 g, 1.68%), DCM (13.5 g, 3.84%) and MeOH (15.9 g, 4.53%). Initial antimicrobial screening of these extracts revealed the most activity in the dichloromethane (DCM) extract (Table 1), which was active against all the tested microorganisms (MIC = 0.3125-0.625 mg/mL). The *n*-hexane extract was only active against *Micrococcus luteus* (MIC = 0.625 mg/mL), *Escherichia coli* (MIC = 5 mg/mL) and *Candida albicans* (MIC = 5 mg/mL), while the MeOH extract was active against all five microbial strains. However, the MIC values of the MeOH extract against the tested microorganisms (0.625 - 5 mg/mL) were much higher than those of the DCM extract (Table 1). The most active DCM extract was subjected to VLC fractionation affording eight fractions. Among the VLC fractions, fraction F7 (20% MeOH in $CHCl_3$ fraction) displayed the highest level of antimicrobial activity, and it was active against all five microbial strains tested in this study with the MIC values ranging between 0.0156 and 0.625 mg/mL (Table 1). While fractions F1-F5 and F-8 were not at all active against any of the tested microbial strains at the highest tested concentration, fraction F-6 (10% MeOH in 100% $CHCl_3$) was active against all the tested microbial strains (MIC = 0.5-1 mg/mL), but the activity was much less potent than that of F7 (Table 1).

Reversed-phase preparative HPLC analysis of the most active VLC fraction F7 of the DCM extract [linear gradient 20-80% acetonitrile (ACN) in water over 30 min, isocratic 80% ACN for the next 5 min, linear gradient 80-20% ACN in water for 5 min, and isocratic 20% ACN in water for 5 min] afforded six compounds (**1**-**6**) (Figure 1) with the retention times (t_R) 14.58, 19.00, 20.27, 21.19, 23.93 and 28.45 min, respectively, which were identified unequivocally as 6,7,8,3',4'-pentamethoxyflavone (**1**; 2.5 mg) [24]**,** sinensetin (**2**; 3.5 mg) [24], cirsilineol (**3**; 2.9 mg) [25], nobiletin (**4**; 4.3 mg) [26], 5-desmethylsinensetin (**5**; 2.7 mg) [27] and hesperidin (**6**; 3 mg) [28] by 1D and 2D NMR as well as MS data analyses. While the MS data (Table 2) confirmed the molecular mass of all these compounds, the experimental ^{1}H and ^{13}C NMR data (Table 3) were comparable with the published data available in the literature for respective compounds. Five (**1**-**5**) of the six isolated flavonoids are polymethoxyflavones, which are typical of the genus *Citrus* and known to have various bioactivities [29].

All six compounds (**1**-**6**) were subjected to resazurin assay as the extracts and fractions mentioned earlier, and sinensetin (**2**) was found to be active against all tested microorganisms with the minimum inhibitory concentration (MIC) values ranging from 0.0625 to 0.50 mg/mL (Table 1), and among the tested compounds, it was the only compound active against *Staphylococcus aureus* (MIC = 0.25 mg/mL). Sinensetin (**2**) was highly active against the fungal strain *Candida albicans* (MIC = 0.0625 mg/mL), which is line with a recent finding where a combination of this compound (**2**) and lysozyme, phytoalexin, chitosan oligosaccharide, 18 beta/20 alpha-glycyrrhizin and betaine has been observed to be effective in the treatment of vulvovaginal candidiasis [30]. Hesperidin (**6**) did not show any antimicrobial activity against the tested microbial strains, but all polymethoxyflavones (**1**-**5**) were active against *Micrococcus luteus* (MIC = 0.25 – 0.50 mg/mL). Furthermore, 6,7,8,3',4'-pentamethoxyflavone (**1**)**,** sinensetin (**2**), nobiletin (**4**) and 5-desmethylsinensetin (**5**) exhibited antimicrobial activity against *Pseudomonas aeruginosa* and *Candida albicans* (MIC = 0.0625 – 0.50 mg/mL) (Table 1).

Because of the observed antimicrobial activity of sinensetin (**2**) against *S. aureus* (MIC = 0.25 mg/mL) and none other compounds being active against *S. aureus*, sinensetin (**2**) was tested for its anti-MRSA potential against the MRSA strains SA1199B, XU212, MRSA340702, EMRSA-15MRSA274819, and against the standard strain ATCC25923 (Table 4). Except for the MRSA strains XU212 and SA119B, this methoxylated flavone (**2**) showed significant anti-MRSA

activity against the other three MRSA strains, EMRSA-15, MRSA340702 and MRSA274819 (MIC = 128-256 μg/mL) as well as the standard *S. aureus* strain ATCC25923. The anti-MRSA activity of sinensetin (**2**) against MRSA340702 was just two-fold less active than the positive control norfloxacin (MIC = 64 μg/mL).

Sinensetin (**2**), which is well distributed in the *Citrus* genus, e.g., *C. aurantium, C. reticulata* and *C. sinensis* [31, 32], has been shown to possess antibacterial activity [33-35], particularly against *Staphylococcus aureus* and this activity is mediated by targeting staphylocoagulase and preventing biofilm formation [36]. This polymethoxyflavone (**2**) together with lysozyme, phytoalexin, chitosan oligosaccharide, 18 beta/20 alpha-glycyrrhizin and betaine was effective (bacteriostatic) against aerobic vaginitis and bacterial vaginosis [30]. However, to the best of our knowledge, this is the first report on the potential anti-MRSA activity of sinensetin (**2**) against five MRSA strains. In addition to the antimicrobial property, sinensetin (**2**) has been shown to have various other bioactivities with therapeutic potentials [30, 33, 34], e.g., anti-adipogenic [37], anticancer [38, 39], anti-inflammatory [40], antioxidant [34, 40], immunomodulatory [41], nephroprotective [42] and neuroprotective [41] properties. Compounds **1**-**5** are polymethoxyflavones, and the only structural differences among them are in the number of methoxylations and the position of the methoxyl functionality on the flavone skeleton (Figure 1). Sinensetin (**2**), a pentamethoxyflavone, which showed the best antimicrobial activity against the tested microbial strains, has the methoxyl functionality at C-5, C-6, C-7, C-3’ and C-4’, whereas another pentamethoxyflavone, 6,7,8,3',4'-pentamethoxyflavone (**1**) has the methoxyl functionality at C-6, C-7, C-8, C-3’ and C-4’, but was not active against *S. aureus*. The only structural difference between sinensetin (**2**) and 6,7,8,3',4'-pentamethoxyflavone (**1**) is the presence of the methoxyl group at C-5 in **2**, but at C-8 in **1**, and this slight difference in the structure might have contributed to better antimicrobial profile, particularly the activity against *S. aureus*, of sinensetin (**2**) than 6,7,8,3',4'-pentamethoxyflavone (**1**). It also appears that having fewer methoxyl functionality in cirsilineol (**3**), which is a trimethoxyflavone, has made this compound among the polymethoxyflavones isolated in this study, the least active against the tested microorganisms. Therefore, it may be inferred that the position and the number of methoxyl functionality on the flavone skeleton contribute to the overall antimicrobial profile of polymethoxyflavones (**1**-**5**).

4. Conclusions

The present work afforded the antimicrobial assay-guided isolation of five methoxylated flavones, 6,7,8,3',4'-pentamethoxyflavone (**1**)**,** sinensetin (**2**), cirsilineol (**3**), nobiletin (**4**), 5-desmethylsinensetin (**5**) and a well-known flavanone glycosides hesperidin (**6**) from the leaves of *Citrus grandis* of Iraqi origin, for the first time. Among the flavonoids, sinensetin (**2**) was found to be the most active antibacterial compound against all test organisms and displayed considerable anti-MRSA potential against three (EMRSA-15, MRSA340802 and MRSA274819) of five MRSA strains assessed in this study. The anti-MRSA potential of sinensetin (**2**) is also presented here for the first time. Based on the current findings, it can be assumed that sinensetin (**2**) could be an ideal structural template for generating structural analogues and developing potential anti-MRSA therapeutic agents. The current findings provide some scientific evidence in support of the traditional uses of *C. grandis* in the treatment of microbial infections.

CRediT authorship contribution statement

Shaymaa Al-Majmaie: Investigation, Methodology, Conceptualisation, Formal analysis. Data curation. **Lutfun Nahar:** Conceptualisation, Co-supervision, Visualization, Data analysis, Writing (original draft, reviewing and final editing), Submission. **M Mukhlesur Rahman:** Methodology and anti-MRSA study, Reviewing the first draft. **George P. Sharples:** Conceptualisation, Co-supervision, Visualization, Data analysis, Writing (original draft, reviewing and final editing). **Satyajit D. Sarker:** Conceptualisation, Principal supervision, Visualization, Data analysis, Structure elucidation, Writing (original draft, reviewing and final editing), Submission.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

Data can be made available on request.

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References

1. D. M. Livermore, Bacterial resistance: Origins, epidemiology, and impact, Clinical Infectious Diseases 36 (2003) S11–S23.

2. W. M. Sweileh, Global research publications on irrational use of antimicrobials: call for more research to contain antimicrobial resistance. Globalization and Health 17 (2021) 94.

3. M. J. Hossain, N. Jabin, F. Ahmmed, A. Sultana, S. M. A. Rahman, M. Islam, Irrational use of antibiotics and factors associated with antibiotic resistance: Findings from a cross-sectional study in Bangladesh, Health Science Reports 6 (2023) e1465.

4. Antimicrobial Resistance Collaborators, Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis, The Lancet 399 (2022) 629-655.

5. WHO (The World Health Organization) 2023. Antimicrobial resistance. Available online: https://www.who.int/news-room/fact-sheets/detail/antimicrobialresistance (accessed on 20 September 2024).

6. S. Al-Majmaie, L. Nahar, M. M. Rahman, S. Nath, P. Saha, A. D. Talukdar, G. P. Sharples, S. D. Sarker, Anti-MRSA constituents from *Ruta chalepensis* (Rutaceae) grown in Iraq, and *in silico* studies on two of most active compounds, chalepensin and 6-hydroxy-rutin 3', 7-dimethyld ether, Molecules 26 (2021) 1114.

7. J. O. Kokwaro, Flora of Tropical East Africa, Rutaceae, Balkema, Rotterdam, The Netherlands (1982) pp. 1-52.

8. R. J. Anmol, S. Marium, F. T. Hiew, W. C. Han, L. K. Kwan, A. K. Y. Wong, F. Khan, M. M. R., Sarker, S. Y. Chan, N. Kifli, L. C. M., Ming, Phytochemical and therapeutic potential of *Citrus frandis* (L.) Osbeck: A review, Journal of Evidence-Based Integrative Medicine 26 (2021) 1-20.

9. Z. Taiping, P. Shaolin, P., Introduction to the origin and evolution of Pomelo and its distribution in China, Chinese Journal of Ecology 5 (2000) 010.

10. D. J. Mabberley, A classification for edible citrus: an update, with a note on *Murraya* (Rutaceae), Telopea 25 (2022) 271-284.

11. M. Rahmatullah, T. Ishika, M. Rahman, A. Swarna, T. Khan, M. N. Monalisa, S. Seraj, S. M. Mou, M. J. Mahal, K. R. Biswas, Plants prescribed for both preventive 235 and therapeutic purposes by the traditional healers of the Bede community residing by the Turag River, Dhaka district, American Eurasian Journal of Sustainable Agriculture 5 (2011) 325-331.

12. B. A. Arias, L. Ramón-Laca, L., Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region, Journal of Ethnopharmacology 97 (2005) 89-95.

13. H. Kim, J. Y. Moon, A. Mosaddik, S. K. Cho, Induction of apoptosis in human cervical carcinoma HeLa cells by polymethoxylated flavone-rich *Citrus grandis* Osbeck (Dangyuja) leaf extract, Food and Chemical Toxicology 48 (2010) 2435-2442.

14. B. K. Datta, L. Nahar, M. M. Rahman, A. I. Gray, A. A. Auzi, S. D. Sarker, Polygosumic acid, a new cadinane sesquiterpene, from *Polygonum viscosum* inhibits the growth of drug-resistant *Escherichia coli* and *Staphylococcus aureus* (MRSA) *in vitro*, Journal of Natural Medicines 61 (2007) 391-396.

15. T. R. Nurunnabi, L. Nahar, S. Al-Majmaie, S. M. Rahman, M. H. Sohrab, M. M. Billah, F. M. Ismail, M. M. Rahman, G. P. Sharples, S. D. Anti-MRSA activity of oxysporone and xylitol from the endophytic fungus *Pestalotia* sp. growing on the Sundarbans mangrove plant *Heritiera fomes*, Phytotherapy Research 32 (2018) 348-354.

16. M. Alloush, C. Mallion, S. D. Sarker, M. M. Rahman, Coumarins from the roots of *Angelica archangelica* and their antibacterial activity against methicillin-resistant

Staphylococcus aureus, Dhaka University Journal of Pharmaceutical Sciences 20 (2022) 275-281.

17. S. D. Sarker, L. Nahar, Y. Kumarasamy, Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods 42 (2007) 321-324.

18. M. M. Rahman, M. Garvey, L. J. Piddock, S. Gibbons, Antibacterial terpenes from the oleoresin of *Commiphora molmol* (Engl.), Phytotherapy Research 22 (2008) 1356-1360.

19. M. M. Rahman, W. K. Shiu, S. Gibbons, J. P. Malkinson, J. P., Total synthesis of acylphloroglucinols and their antibacterial activities against clinical isolates of multidrug resistant (MDR) and methicillin-resistant strains of *Staphylococcus aureus*, European Journal of Medicinal Chemistry 155 (2018) 255-262.

20. W. K. Shiu, M. M. Rahman, J. Curry, P. Stapleton, M. Zloh, J. P. Malkinson, S. Gibbons, Antibacterial acylphloroglucinols from *Hypericum olympicum*, Journal of Natural Products 75 (2011) 336-343.

21. E. Ioannou, A. Quesada, M. M. Rahman, S. Gibbons, C. Vagias, V. Roussis, Structures and antibacterial activities of minor dolabellanes from the brown alga *Dilophus spiralis*, European Journal of Organic Chemistry 2012 (2012), 5177-5186.

22. F. S. Tareq, C. M. Hasan, M. M. Rahman, M. M. M. Hanafi, L. Colombi Ciacchi, M. Michaelis, T. Harder, J. Tebben, M. T. Islam, P. Spiteller, Anti-*Staphylococcal* calopins from fruiting bodies of *Caloboletus radicans*, Journal of Natural Products 81 (2018) 400-404.

23. R. Reid, R., S. Sarker, Isolation of natural products by low-pressure column chromatography, in Natural Products Isolation, 3rd edition (2012), Humana Press – Springer-Verlag, USA.

24. S. Han, H. M. Kim, J. M. Lee, S. Y. Mok, S. Lee, Isolation and identification of polymethoxyflavones from the hybrid *Citrus*, Hallabong, Journal of Agricultural and Food Chemistry 58 (2010), 9488-9491.

25. L. Hammoud, R. Seghiri, S. Benayache, P. Mosset, A. Lobstein, M. Chaabi, F. León, I. Brouard, J. Bermejo, F. Benayache, A new flavonoid and other constituents from *Centaurea nicaeensis* All. var. *walliana* M., Natural Product Research 26 (2012) 203-208.

26. Z. Li, Z. Zhao, Z. Zhou, Simultaneous Separation and Purification of Five polymethoxylated flavones from "Dahongpao" Tangerine (*Citrus tangerina* Tanaka) using macroporous adsorptive resins combined with prep-HPLC. Molecules 23 (2018) 2660.

27. W. M. Alarif, A. Abdel-Lateff, A. M. Al-Abd, S. A. Basaif, F. A. Badria, M. Shams, S.-E. N. Ayyad, Selective cytotoxic effects on human breast carcinoma of new methoxylated flavonoids from *Euryops arabicus* grown in Saudi Arabia, *European* Journal of Medicinal Chemistry 66 (2013) 204.

28. J. Nieto, A. Gutierrez, 1H NMR spectra at 360 MHz of diosmin and hesperidin in DMSO solution, Spectroscopy Letters 19 (1986) 427-434.

29. R. Toledo, M. Tomas-Navaro, J. E. Yuste, P. Crupi, F. Vallejo, An update on citrus polymethoxyflavones: chemistry, metabolic fate and relevant bioactivities, European Food Research and Technology 250 (2024) 2179-2192.

30. Z. Zeng, P. Li, J. Y. Lu, X. Q. Li, M. Li, Y. F. Wu, M. Z. Zheng, Y. Cao, Q. P., Z. J. Ge, L. Zhang, A non-antibiotic antimicrobial drug, a biological bacteriostatic agent, is useful for treating aerobic vaginitis, bacterial vaginosis and vulvovaginal candidiasis, Frontierts in Microbiology 15 (2024) 1341878.

31. W. Cai, S. Zhang, Y. Wang, C. Liu, R. Luo, Differential distribution of characteristic constituents in peel and pulp of Auruntium Fructus Immaturus (*Citrus aurantium* L.) using MALDI mass spectrometry imaging, Fitoterapia 177 (2024) 106067.

32. J. J. Shi, L. H. Peng, W. X. Chen, W. L. Qiao, K. Wang, Y. Y. Xu, J. L. Cheng, Evaluation of chemical components and quality in Xinhui Chenpi (*Citrus reticulata* 'Chach') with two different storage times by GC-MS, Food Science and Nutrition 12 (2024) 5036-5051.

33. L. H. Jie. I. Jantan, S. D. Yusoff, J. Jalil, K. Husain, Sinensetin: An insight on its pharmacological activities, mechanisms of action and toxicity, Frontiers in Pharmacology 11 (2021), 553404.

34. K. Patel, D. K. Patel, Therapeutic effectiveness of sinensetin against cancer and other human complications: A review of biological potential and pharmacological activities, Cardiovascular and Henatological Disorders Drug Targets 22 (2022) 144-154.

35. N. Permadi, M. Nurzaman, F. Doni, E. Julaeha, Elucidation of the composition, antioxidant and antimicrobial properties of essential oil and extract from *Citrus aurantifolia* (Christm.) Swingle peel, Saudi Journal of Biological Sciences 31 (2024) 103987.

36. B. Ge, C. Hu, Y. Qian, Y. Tang, Q. Zhang, S. Jiang, Z. Mu, M. Zhang, Sinensetin interferes with *Staphylococcus aureus* infections by targeting staphylocoagulase and improves infections survival rates in mouse model pneumonia, Journal of Applied Microbiology 135 (2024) lxae235.

37. P. Rajan, P. Natraj, S. S. Ranaweera, L. A. Dayarathne, Y. J. Lee, C. H. Han, Antiadipogenic effect of the flavonoids through the activation of AMPK in palmitate (PA treated HepG2 cells, Journal of Veterinary Science 23 (2022) 21256.

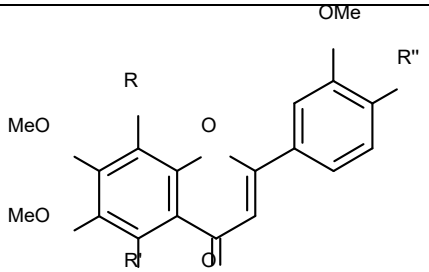
38. T. Ji, L. Ye, E. P. Xi, Y. Liu, X. M. Wang, S. Wang, Sinensetin inhibits angiogenesis in lung adenocarcinoma via the miR-374c-5p/VEGF-A/VEGFR-2/AKT axis. Cell Biochemistry and Biophysics published online (2024): DOI 10.1007/s12013-024-01352-3.

39. J. I. Seo, J. S. Yu, Y. Zhang, H. H. Yoo, Evaluating flavonoids as potential aromatase inhibitors for breast cancer treatment: *In vitro* studies and *in silico* predictions, Chemico-Biological Interactions 392 (2024) 110927.

40. Y. Desmiaty, N. M. D. Sandhiutami, E. Mulatsari, F. A. Maziyah, K. Rahmadhani, H. O. Z. Algifari, F. A. Jantuna, Antioxidant and anti-inflammatory activity through inhibition of NF-kB and sEH of some citrus peel and phytoconstituent characteristics, Saudi Pharmaceutical Journal 32 (2024) 101959.

41. R. Y. Gan, Y. Liu, H. Li, Y. Xia, H. Guo, F. Geng, Q. G. Zhuang, H. B. Li, D. T. Wu, Natural sources, refined extraction, biosynthesis, metabolism and bioactivities of dietary polymethoxyflavones (PMFs), Food Science and Human Wellness 13 (2024) 27-49.

42. Z. L. Kong, W. S. Lv, Y. Y. Wang, Y. J. Huang, K. Che, H. Q. Nan, Y. Xin, J. X. Wang, J. T. Chen, Y. G. Wang, J. W. Chi, Sinensetin ameliorates high glucose-induced diabetic nephropathy via enhancing autophagy *in vitro* and *in vivo*, Journal of Biochemical and Molecular Toxicology 37 (2023) 23445.



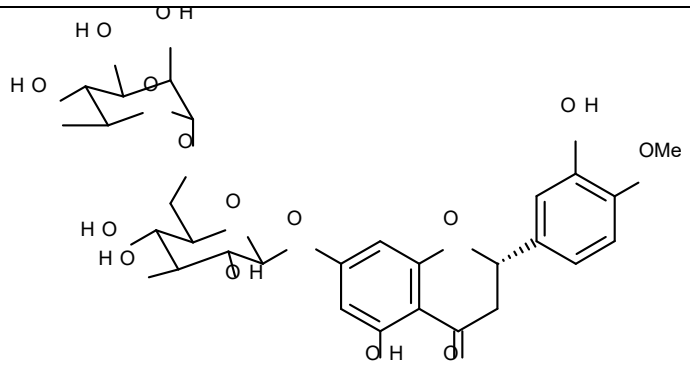
6,7,8,3',4'-Pentamethoxyl-flavone (**1**) R = R'' = OMe, R' = H

Sinensetin (**2**) R = H, R' = R'' = OMe

Cirsilineol (**3**) R = H and R' = R'' = OH

Nobiletin (**4**) R = R' = R'' = OMe

5-Desmethylsinensetin (**5**) R = H, R' = OH and R'' = OMe



Hesperidin (**6**)

Figure 1. Structures of flavonoids isolated from the most active VLC fraction (F7) of the active DCM extract of the leaves of *C. grandis*

Table 1.

Antimicrobial activity of the extracts of the leaves of *C. grandis*, the VLC fractions of the DCM extract, and the isolated compounds (**1**-**6**) from the active VLC fraction

Tested samples	MIC values in mg/mL				
	Gram-positive bacteria		Gram-negative bacteria		Fungus
	Micrococcus luteus	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
n-Hexane extract	0.625	Not active	5.00	Not active	5.00
DCM extract	0.3125	0.625	0.3125	0.3125	0.625
MeOH extract	0.625	5.00	1.25	5.00	2.5
VLC fractions of the DCM extract					
F1	Not active	Not active	Not active	Not active	Not active
F2	Not active	Not active	Not active	Not active	Not active
F3	Not active	Not active	Not active	Not active	Not active
F4	Not active	Not active	Not active	Not active	Not active
F5	Not active	Not active	Not active	Not active	Not active
F6	0.5	1.0	1.0	0.5	0.5
F7	0.0156	0.25	0.625	0.125	0.0312
F8	Not active	Not active	Not active	Not active	Not active
Isolated compounds (**1**-**6**)					
1	0.25	Not active	Not active	0.50	0.25
2	0.25	0.25	0.50	0.25	0.0625
3	0.50	Not active	Not active	Not active	Not active
4	0.50	Not active	Not active	0.25	0.25
5	0.25	Not active	Not active	0.50	0.50

6	Not active	Not active	Not active	Not active	Not active
Positive controls					
Ciprofloxacin	9.76×10^{-4}	9.76×10^{-4}	1.55×10^{-2}	1.95×10^{-4}	Not applicable
Nystatin	Not applicable	Not applicable	Not applicable	Not applicable	9.76×10^{-4}

Table 2.

HR-ESIMS data of the isolated flavonoids (**1**-**6**)

*Pseudo*molecular ion and molecular formula	Flavonoids					
	1	**2**	**3**	**4**	**5**	**6**
$[M+H]^+$ ion	*m/z* 373.1317 (calculated 373.1287)	*m/z* 373.1284 (calculated 373.1287)	*m/z* 345.0974 (calculated 345.0974)	*m/z* 403.1388 (calculated 403.1392)	*m/z* 359.1141 (calculated 359.1130)	*m/z* 611.1953 (calculated 611.1976)
Molecular formula	$C_{20}H_{20}O_7$	$C_{20}H_{20}O_7$	$C_{18}H_{16}O_7$	$C_{21}H_{22}O_8$	$C_{19}H_{18}O_7$	$C_{28}H_{34}O_{15}$

Table 3.

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for the isolated flavonoids (**1**-**6**)

Carbon number	^{1}H NMR chemical shift δ_H in ppm (coupling constant *J* in Hz)						^{13}C NMR chemical shift δ_C in ppm					
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
2	-	-	-	-	-	5.50 *dd* (12.0, 3.1)	161.2	161.7	164.4	161.4	164.4	78.8
3	6.60 *s*	6.60 *s*	6.58 *s*	6.64 *s*	6.60 *s*	2.78 *dd* (3.1, 17.2)	107.7	107.6	104.7	107.1	104.9	42.5
4	-	-	-	-	-	-	177.6	177.7	183.3	177.7	183.0	197.5
5	6.97 *s*	-	-	-	-	-	96.9	152.3	153.6	149.6	153.6	163.5
6	-	-	-	-	-	6.14 *d* (2.5)	158.0	144.8	133.1	144.4	133.1	96.8
7	-	-	-	-	-	-	146.0	158.1	159.1	152.2	159.1	165.6
8	-	6.97 *s*	6.55 *s*	-	6.55 *s*	6.12 *d* (2.5)	140.7	96.6	91.1	138.1	91.0	96.0
9	-	-	-	-	-	--	143.0	154.9	153.5	148.7	153.4	163.0
10	-	-	-	-	-		113.2	113.1	106.5	115.1	106.5	103.8
1’	-	-	-	-	-	-	124.5	124.4	123.8	124.2	124.2	131.4
2’	7.33 *d* (2.1)	7.33 *d* (2.1)	7.33 *d* (2.1)	7.41 *d* (2.1)	7.34 *d* (2.1)	6.93 d (2.1)	109.1	109.1	108.7	111.3	109.2	114.6
3’	-	-		-	-	-	149.7	149.7	147.2	149.6	149.7	146.9
4’	-	-		-	-	-	153.0	152.9	149.6	152.9	152.7	148.4
5’	6.96 *d* (8.4)	6.96 *d* (8.4)	7.04 *d* (8.3)	7.00 *d* (8.5)	6.98 *d* (8.0)	6.95 *d* (8.4)	111.5	111.5	115.4	111.5	111.6	112.5
6’	7.51 *dd* (8.4, 2.1)	7.51 *dd* (8.4, 2.1)	7.50 *dd* (8.3, 2.1)	7.57 *dd* (8.5, 2.1)	7.51 *dd* (8.0, 2.1)	6.91 *dd* (8.4, 2.1)	120.0	120.0	121.1	119.9	120.4	118.4
5-OMe	-	3.92 *s*	-	3.95 *s*	-	-	-	61.9	56.7	62.5	-	-
6-OMe	3.99 *s*	3.99 *s*	3.93 *s*	3.95 *s*	3.98 *s*	-	56.7	62.6	61.2	62.2	56.7	-
7-OMe	3.99 *s*	3.96 *s*	3.97 *s*	4.10 *s*	3.93 *s*	-	61.9	56.5	-	61.9	61.2	-
8-OMe	3.92 *s*	-	-	4.02 *s*	-	-	62.5	-	56.5	62.1	-	-
3’-OMe	3.96 *s*	3.96 *s*	3.97 *s*	3.96 *s*	3.99 *s*	3.78 *s*	56.5	56.4	-	56.3	56.5	56.2

4'-OMe	3.98 *s*	3.99 *s*	-	3.97 *s*	3.97 *s*	-	56.4	56.7	-	56.2	56.5	-
Glucose 1	-	-	-	-	-	4.98 *d* (8.5)	-	-	-	-	-	101.1
Glucose 2	-	-	-	-	-	3.63*	-	-	-	-	-	76.0
Glucose 3	-	-	-	-	-	3.45*	-	-	-	-	-	78.8
Glucose 4	-	-	-	-	-	3.40*	-	-	-	-	-	70.7
Glucose 5	-	-	-	-	-	3.54*	-	-	-	-		76.7
Glucose 6						3.42 m						66.8
Rhamnose 1	-	-		-	-	4.52 *d* (1.9)	-	-		-		103.8
Rhamnose 2	-	-		-	-	3.27*	-	-		-		71.7
Rhamnose 3	-	-		-	-	3.28*	-	-		-		72.5
Rhamnose 4	-	-		-	-	3.18*	-	-		-		73.4
Rhamnose 5	-	-		-	-	3.38*	-	-		-		68.8
Rhamnose 6	-	-		-	-	1.09 *d* (6.2)	-	-		-		18.3

Spectra obtained in $CDCl_3$ for compounds **1**-**5**, and in CD_3OD for compound **6**

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

Table 4.

Anti-MRSA activity of sinensetin (**2**)

Tested compounds	MIC values in μg/mL					
	MRSA strains					Standard *S. aureus* strain
	XU212	SA1199B	EMRSA-15	MRSA340702	MRSA274819	ATCC25923
Sinensetin (**2**)	Not active	Not active	256	128	256	128
Norfloxacin	16	32	1	64	64	2