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Exploring the antimicrobial activity of *Origanum majorana L.* against the highly virulent multidrug-resistant *Acinetobacter baumannii AB5075*: UPLC-HRMS profiling with in vitro and in silico studies

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Abstract

Background The infamous multidrug-resistant (MDR) bacterium *Acinetobacter baumannii* is becoming a nightmare in intensive care units across the globe. Since there are now very few effective antimicrobial agents, it is necessary to explore unconventional resources for novel antimicrobials. This study investigated the potential antimicrobial activity of *Origanum majorana* L. against *A. baumannii* employing multiple approaches including antimicrobial susceptibility, fractionation, ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) dereplication, and in silico analysis for target/ligand identification.

Results On the extremely pathogenic MDR strain *A. baumannii* AB5075, *O. majorana* L. has shown a significant growth inhibitory effect (MIC = 0.675 mg/mL). The polar 50% methanol fraction was the most active (MIC = 0.5 mg/mL). The UPLC-HRMS dereplication of the bioactive fraction detected 29 metabolites belonging to different chemical classes. Justicidin B, one of the identified metabolites, was projected by preliminary in silico analysis to be the most highly scoring metabolite for binding with molecular targets in *A. baumannii* with a Fit score = 8.56 for enoyl-ACP reductase (FabI) (PDB ID: 6AHE), suggesting it to be its potential target. Additionally, docking, molecular dynamics simulation, and bioinformatics analysis suggested that this interaction is similar to a well-known FabI inhibitor. The amino acids involved in the interaction are conserved among different MDR *A. baumannii* strains and the effectiveness could extend to Gram-negative pathogens within the ESKAPE group.

Conclusions *Origanum majorana* L. extract exhibits antimicrobial activity against *A. baumannii* using one or more metabolites in its 50% methanol fraction. The characterized active metabolite is hypothesized to be justicidin B which inhibits the growth of *A. baumannii* AB5075 via targeting its fatty acid synthesis.

Keywords *Acinetobacter baumannii*, *Origanum majorana* L., Justicidin B, Virtual screening, FabI inhibitors

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Background

Healthcare practitioners everywhere give careful consideration to *Acinetobacter baumannii* as it is a major cause of nosocomial infections. *A. baumannii* is frequently responsible for outbreaks in intensive care units that show up as urinary tract infections, surgical site infections, pneumonia linked to ventilator use, and sepsis [1]. Based on pooled data from various regions globally, *A. baumannii* was found to produce approximately 25 hospital-acquired infection cases per 1000 patients; in intensive care units, this ratio rose to 54 cases per 1000 patients [2]. Furthermore, the problem has gotten worse due to its resistance to several antimicrobial agents that are currently available, as well as, its acquired resistance to their alternatives.

Multidrug-resistant (MDR) *A. baumannii* are increasingly reported worldwide reaching 45% of *A. baumannii* infections, exceeding the MDR rates reported for all other common nosocomial pathogens [3]. This has placed *A. baumannii* among the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) [4–6]. For multidrug-resistant Gram-negative bacteria, including *A. baumannii*, colistin and carbapenems like doripenem, imipenem, and meropenem, are typically regarded as the last resort treatments [7–9]. Nevertheless, the administration of colistin has been restricted due to its toxicity, including nephrotoxicity and neurotoxicity [10, 11]. The frequency of isolation of carbapenem-resistant *A. baumannii* (CRAB) is increasing with currently few alternatives for treatment. The Centres for Disease Control and Prevention (CDC) has listed CRAB as an "urgent threat" to human health [12, 13]. In a developing country like Egypt, the rate of isolating CRAB from patients reached 80% and higher [14]. After the COVID-19 pandemic, the reported infections by CRAB increased by 78%, which is highly alarming [13].

The development of novel antibiotics is essential due to the high mortality rates associated with CRAB infections [15, 16]. This has warranted WHO to include *A. baumannii,* along with other carbapenem-resistant pathogens, *P. aeruginosa* and Enterobacteriaceae*,* to be critically prioritized in the research and development of new antibiotics [17].

Plant-produced secondary metabolites, sometimes referred to as phytochemicals, may offer chemical resistance against infectious agents. The abundance of these metabolites, some with efficacies comparable to that of synthetic antibiotics, provides a repertoire of chemicals that could be used to successfully create and deploy new antimicrobial agents against MDR-ESKAPE pathogens [18–20].

Origanum majorana L. commonly referred to as Sahtar, Zaatar or sweet marjoram is a photoautotrophic medicinal perennial herb of the *Origanum* genus, which belongs to the Lamiaceae family [21]. This plant is found broadly all around the Mediterranean region, but especially in Morocco, Algeria, Egypt, Spain, and Portugal [22]. Traditional medicine makes extensive use of it, as a possible treatment for a wide range of illnesses including allergies, respiratory infections, hypertension, diabetes, and stomach pain, as well as, an intestinal antispasmodic, [23]. Pharmacological research revealed that this plant's essential oils and extracts possessed a variety of biological qualities, including hepatoprotective, antimutagenic, anticancer, antiparasitic, antibacterial, antifungal, antidiabetic, antioxidant, anti-inflammatory, and analgesic effects [24]. The plant extracts' safety and therapeutic advantages have been verified by a toxicological evaluation [25]. In rats, *O. majorana* L. extracts did not result in any fatalities, which further confirms their safety [26, 27]. *O. majorana* L. has previously been found to contain a wide range of bioactive compounds from various chemical classes, including hydroquinone, sterols, terpenoids, tannins, phenolic acids (arbutin and methyl arbutin), fatty alcohols, and flavonoids (diosmetin, orientin, luteolin, apigenin, vitexin, and thymonin) [28]. Thymol, carvacrol, cis-sabinene hydrate, limonene, terpinene, and camphene are among the plant's abundant volatile oil constituents [28]. It is important to bear in mind that different plant organs, growth stages, and even harvesting techniques can have different effects on the amount and composition of the aforementioned metabolites and volatile oil components within the same species [29]. Antibacterial activity has been demonstrated by the plant against a variety of pathogenic bacteria, including *E. faecalis*, *Bacillus subtilis*, *Escherichia coli*, *K. pneumoniae*, *Serratia* sp. and *Salmonella choleraesuis* [30–32].

In search of prospective treatments for MDR *A. baumannii*, this study aimed to investigate the antibacterial activity of *O. majorana* L. extract and its fractions. UPLC-HRMS was used to analyse the chemical profile of the active 50% methanol fraction (50% MF) in order to establish a relationship between the antibacterial activity and the bioactive secondary plant metabolites. Justicidin B, a secondary metabolite identified in *O. majorana* L. bioactive fraction, has been identified as a potential novel antimicrobial agent against *A. baumannii* and other ESKAPE pathogens, which targets the essential FAS-II fatty acid biosynthesis pathway component FabI.

Methods

Bacterial strains and culture conditions

The multidrug-resistant highly virulent *A. baumannii* strain AB5075 [33] was obtained from Prof. Dr. Lory,

Harvard University, USA as a gift, and was used as the test microorganism in all the experiments. The bacterial strain was typically grown in tryptic soy broth (TSB) with shaking at 180 rpm at 37 °C or plated on tryptic soy agar (TSA) and incubated at 37 °C.

Preparation, extraction and fractionation of *O. majorana* L. aerial parts

Origanum majorana L. aerial parts were collected from the Experimental Station of the Faculty of Pharmacy, Cairo University, Giza, Egypt. Mrs Therese Labib, Consultant at Orman Botanic Garden, Dokki, Giza, Egypt, kindly authenticated the plant material. Air-dried powder of *O. majorana* L. aerial parts (0.5 kg) was exhaustively extracted with methanol (5×7 L) and evaporated on a rotary evaporator to yield the total methanolic extract (ME, 75 g). Part of the residue (45 g) was suspended in 350 mL of distilled water and then fractionated using dichloromethane (3×750 mL), which was then evaporated to get DCM-F (25 g). The mother liquor (ML) was applied on the Diaion-HP20 column and eluted firstly with distilled water (1L, discarded), followed by 50% methanol (1 L), and then 100% methanol (1 L). The fractions were evaporated to yield 50% MF (4 g) and 100% MF (6 g), respectively.

Preliminary screening of *O. majorana* L. extract for antimicrobial activity

An overnight culture of *A. baumannii* AB5075 was grown in TSB at 37 °C and 180 rpm. The culture was diluted in TSB to OD600 = 0.1 arbitrary units, then diluted 1:1000 in TSA which was prewarmed to 50 °C. The inoculated TSA (50 mL) was distributed in a 15-cm-wide petri dish. After agar solidification, 10 µL of the extract, or the fraction, were spotted on the agar surface, the spots were dried in a laminar flow cabinet, and the plates were incubated at 37 °C. After 24 h, the plates were inspected visually, and the diameters of the growth inhibition zones were measured using a ruler.

Determination of the minimum inhibitory concentration (MIC)

Following the criteria of the Clinical and Laboratory Standards Institute (CLSI), the MIC was ascertained using the broth microdilution method [34]. The dried extract or fraction was dissolved in DMSO to a final concentration of 100 mg/mL. Then, it was diluted in Muëller-Hinton broth to yield a series of twofold dilutions ranging from 0.001 to 2 mg/mL. One hundred µL aliquots from each concentration were distributed in a 96-well plate. Each well was inoculated with 10 µL (~ 10^5 CFU as determined spectrophotometrically by measuring absorbance at 600 nm and verified by viable count) of a freshly prepared bacterial suspension. The plates were incubated for 24 h at 37 °C. The MIC was the minimum concentration of the tested crude extract or its fractions that inhibited visible bacterial growth. Muëller-Hinton broth containing the equivalent of DMSO served as a negative control and the experiment was performed in triplicate.

UPLC-HRMS analysis of the active fraction

The *O. majorana* L. active subfraction (50% MF) was subjected to UPLC-HRMS analysis followed by dereplication (identification of the secondary metabolites present in the extract) using the Dictionary of Natural Products and Reaxys online databases. The dereplication depended on the HRMS isotope profiles, MS/MS fragmentation, and the databases were filtered to include only the plant genus or family.

A Bruker MAXIS II Q-ToF mass spectrometer connected to an Agilent 1290 UHPLC system was used to analyse the samples. The column used was a Phenomenex Kinetex XB-C18 (2.6 mM, 100×2.1 mm). The following LC gradient profile was used to achieve separation: 5% MeCN + 0.1% formic acid to 100% MeCN + 0.1% formic acid in 15 min at a flow rate of 0.1 mL/min. MS parameters were: mass range *m/z* 100–2000, capillary voltage 4.5 kV, nebulizer gas 4.0 bar, dry gas 9.0 L/min, and dry temperature of 250 °C. MS/MS experiments with a step collision energy of 80–200% were carried out using the Auto MS/MS scan mode.

Virtual target identification

PharmMapper was used to characterize potential target(s) of justicidin B [35]. This platform uses an extracted pharmacophore model and stores it as a library ligand dataset in mol2 format. Then, it rates each molecule in the PDB according to how well it fits the model. This approach yields a pure Fit score that is far more reliable and significant than chance pharmacophore matching. The query structure was submitted to the platform in the pdb format, and the results were sorted based on the Fit scores.

Docking study

Using an AutoDock Vina docking machine, the crystal structures of FabI (PDB ID: 6AHE) were utilized for the docking study [36]. The co-crystallized ligand AFN-1252 was used to determine the binding site and the docking grid-box. The coordinates of the grid-box were set to be x = −36.404, y = −8.512, z = −3.293.

A criterion of 2.0 Å was established for the ligand to binding site shape matching root-mean-square deviation (RMSD). The Charmm force field (v.1.02) with a distance-dependent dielectric and a non-bonded cut-off distance of 10.0 was used to obtain the interaction energies. Then,

an energy grid was set at 5.0 Å from the binding site [36]. Inside the chosen binding pocket, the studied compound, justicidin B, was energy-minimized. Pymol software was used to edit and visualize the generated binding poses [37].

Molecular dynamics simulation (MDS)

NAMD 3.0.0. software was used to perform MDS [38, 39]. Protein systems were constructed with the VMD software's QwikMD toolkit [39, 40], and the protonation states of the amino acid residues were set (pH 7.4). The protein structure was examined for any missing hydrogens, and the co-crystalized water molecules were then removed. Following that, the entire structure was immersed in an orthorhombic box of TIP3P water together with 0.15 M Na^+ and Cl^- ions in a 20 Å solvent buffer. The systems were then energy-minimized and allowed to equilibrate for five nanoseconds. With the help of the VMD plugin Force Field Toolkit (ffTK), the ligand topologies and properties were determined. Afterwards, the produced parameters and topology files were loaded into VMD so that the simulation steps could be carried out and the protein–ligand complexes could be interpreted without errors.

Binding free energy calculations

The molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) method from the AMBER18's MMPBSA.py module was used to calculate the docked complex's binding free energy by applying the following equation complex $\Delta G_{\text{Binding}} = \Delta G_{\text{Complex}} - \Delta G_{\text{Receptor}} - \Delta G_{\text{Inhibitor}}$ [41].

Conservation of the FabI sequences

The amino acid sequence of the FabI (PDB ID: 6AHE) enzyme, which showed the best-Fit score with justicidin B, was retrieved from the NCBI database. To confirm the conservation of FabI in different strains of *A. baumannii* and different strains of ESKAPE pathogens, the protein sequence of FabI was analysed by the BlastP tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) [42], using a nonredundant protein sequences database against each organism (*A. baumannii*, *E. faecium*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *Enterobacter* spp.) with an expect threshold of 0.001. Proteins were considered conserved if they had a max alignment score of more than 200.

The conservation of amino acids in the predicted binding site for justicidin B was further confirmed by the alignment of the sequences of representative proteins from MDR *A. baumannii* strains (including those from *A. baumannii* ATCC 19606 (PDB ID:6AHE) and *A. baumannii* AB5075) and from representative strains of different ESKAPE pathogens. The sequences of the target proteins were retrieved from the NCBI database. The protein sequences were aligned using the multiple sequence alignment Clustal Omega tool with default settings (https://www.ebi.ac.uk/Tools/msa/clustalo/) [43].

Results

O. majorana L. total methanolic extract and its fractions have potent growth inhibitory effects against *A. baumannii*

The *O. majorana* L. total methanolic extract was spotted on the trypticase soy (TS) agar seed inoculated with *A. baumannii* AB5075. It showed a 14-mm zone of growth inhibition after 24-h incubation (Fig. 1A). Using the broth microdilution method, the minimum inhibitory concentration (MIC) of the total methanolic extract of *O. majorana* L. was determined at 0.675 mg/mL. Chromatographic techniques were used to prepare subfractions by partitioning with solvents of different polarities and applying them on a Diaion column. The 50% methanol fraction showed considerable antimicrobial activity (Fig. 1B). Upon measuring the MIC for this fraction, it showed a value of 0.5 mg/mL. On the other hand, the non-polar dichloromethane fraction had a higher MIC value of 0.875 mg/mL.

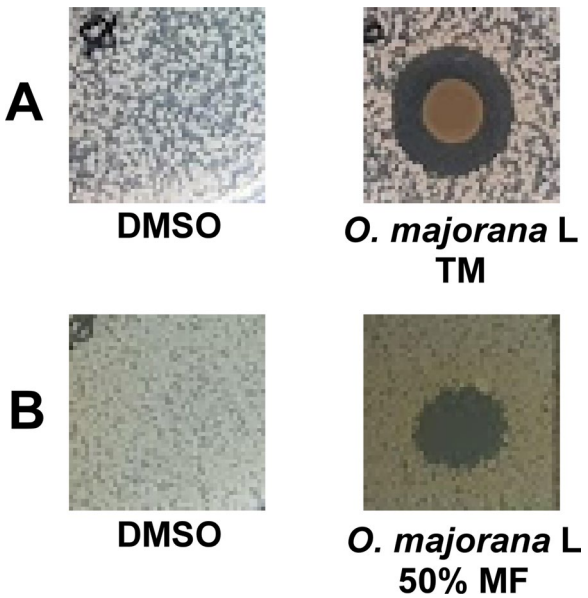


Fig. 1 Growth-inhibitory effects of *O. majorana* L and its fractions against *A. baumannii*. Ten µl of the total methanolic (TM) extract (**A**) of *O. majorana* L or the 50% methanol fraction from Diaion-HP20 (50% MF) (**B**) were spotted on TSA plates inoculated with *A. baumannii* AB5075 and the plates were incubated overnight at 37 °C. In both cases, equivalent amounts of the solvent (DMSO) were spotted on the same plates. The experiment was repeated three times, and representative images were presented

UPLC-HRMS analysis of *O. majorana* L. active fraction reveals multiple metabolites.

To unlock the chemical diversity of the active fraction of the *O. majorana* L., UPLC-HRMS profiling revealed the presence of 29 metabolites belonging to different structural classes, comprising two terpenes, two alkaloids, one lignan, nine flavonoid aglycones, four flavonoid glycosides, seven phenolic acid derivatives, and two alicyclic derivatives. Moreover, three hits were not identified, indicating new compounds or compounds that were not reported before in the family Lamiaceae (Table 1).

Virtual screening-based target characterization

PharmMapper was employed to suggest a suitable protein target for the identified metabolites. The retrieved results were arranged according to their Fit scores. Only bacterial targets related to the *Acinetobacter* genus were considered. A threshold Fit score of 7 was set to select the best-scoring hits. As a result, *A. baumannii*-derived enoyl-ACP reductase (FabI) (PDB ID: 6AHE) was found to be among the top-scoring hits for justicidin B (Fit score = 8.56), where all the remaining bacterial hits were below the cut-off score of 7. Hence, this target (i.e. FabI) was proposed as its potential target, and justicidin B was the only compound among the 29 LC-HRMS-dereplicated metabolites that scored above the threshold Fit score.

Re-docking justicidin B modelled structure into the FabI active site achieved binding mode and docking score comparable to those of the co-crystalized inhibitor AFN-1252 (docking scores = −10.67 and −11.75 kcal/mol, respectively). Justicidin B was able to establish two H-bonds with TYR-149 and TYR-159. In addition, it was involved in multiple hydrophobic interactions with PHE-96, LEU-102, TYR-149, and ILE-203. Similarly, the co-crystallized inhibitor was involved in the same hydrophobic interactions and formed H-bonds with both TYR-159, as seen in justicidin B, and with ALA-97 (Fig. 2A, B).

According to subsequent molecular dynamic simulation (MSD) experiments (100 ns-long), the justicidin B structure was able to achieve stable binding mode throughout the simulation, with an average Root Mean Square Deviation (RMSD) of 1.96 Å, which was similar to that of the co-crystallized inhibitor AFN-1252 (average RMSD = 1.65 Å; Fig. 2C). Such stable bindings of both justicidin B and AFN-1252 were translated into stable and significant interaction energies (electrostatic and van der Waals) inside the enzyme's binding site, where both compounds showed total interaction energies averaged around − 26.16 and − 44.27 kcal/mol, respectively (Fig. 3A, B). Hence, their calculated absolute binding free energies were comparable as well ($\Delta G_{Binding}$ = -8.11 and -9.52 kcal/mol, respectively).

According to the previous modelling and MD simulation findings, it could be hypothesized that *O. majorana* L. extract was able to inhibit the growth of *A. baumannii* via targeting its fatty acid synthesis by one of its metabolites (i.e. justicidin B).

The conservation of the predicted interaction sites of justicidin B and FabI in *A. baumannii* and among members of the ESKAPE group

To check if the predicted interaction of justicidin B and FabI could be extended to other strains of *A. baumannii*, we performed a blast analysis for the enoyl-ACP reductase (FabI) amino acid sequence against *A. baumannii* strains in the National Centre for Biotechnology Information (NCBI) database. The alignment score was > 200, indicating a high degree of conservation of the overall amino acid sequence of this protein. Upon performing a multiple sequence alignment of the amino acid sequences of the FabI protein in fifteen MDR and CRAB strains, all five amino acids involved in the interaction were conserved (TYR-149, TYR-159, PHE-96, LEU-102, and ILE-203) (Fig. 4A). Moreover, to determine if this interaction could be extended to other members of the ESKAPE pathogens, the FabI amino acid sequence of representative MDR strains from the members of this group was retrieved when another multiple sequence alignment was performed (Fig. 4B). The results of this alignment indicated that all the five amino acids involved in the interaction between justicidin B and FabI are conserved (TYR-149, TYR-159, PHE-96, LEU-102, and ILE-203) in the Gram-negative members: *K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp. On the other hand, for the Gram-positive members *S. aureus* and *E. faecium*, all the interaction sites were conserved except the non-polar amino acid ILE-203 which was replaced with another non-polar amino acid, valine, and TYR replaced PHE-96 in *E. faecium*. This change in one amino acid in these two strains could have a minimal impact on justicidin B binding.

Discussion

The rise of antibiotic-resistant pathogens and the difficulties in developing antimicrobials have aroused concerns among medical professionals for the well-being of humanity. Plants, a rich source of undiscovered metabolites, offer a solution as a natural source of antimicrobial compounds because they are attainable and generally safe to use. *O. majorana* L. or sweet marjoram is an aromatic, herbaceous, perennial plant in the family Lamiaceae. The plant has been used as a flavouring and herbal spice and is widely reported for its antibacterial activity against

Table 1 LC-HRMS analysis of the 50% methanol fraction (50% MF) of *Origanum majorana* L

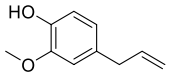
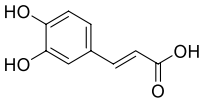
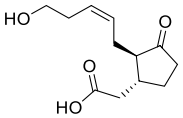
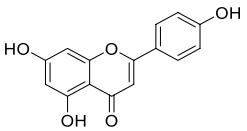
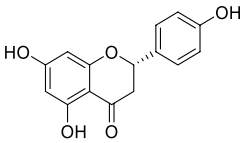
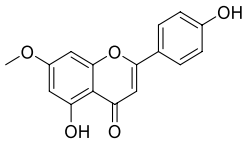
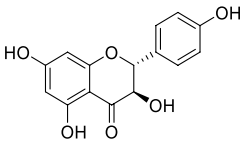
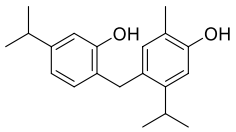
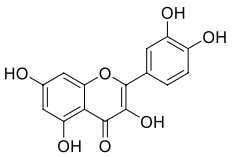
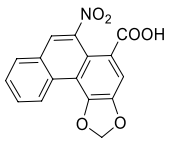
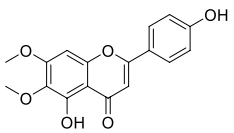
HRMS m/z	Mol. formula	Tentative identification	Compound structure
165.0911	$C_{10}H_{12}O_2$	Eugenol	
181.04954	$C_9H_8O_4$	Caffeic acid	
227.1279	$C_{12}H_{18}O_4$	12-hydroxyjasmonic acid	
271.0603	$C_{15}H_{10}O_5$	Apigenin	
273.0758	$C_{15}H_{12}O_5$	Naringenin	
283.0812	$C_{13}H_{14}O_7$	No hit from the family	
285.0757	$C_{16}H_{12}O_5$	Genkwanin	
289.0706	$C_{15}H_{12}O_6$	Aromadendrin; dihydrokaempferol	
299.2005	$C_{20}H_{26}O_2$	Majoradiol	
303.0499	$C_{15}H_{10}O_7$	Quercitrin; sophoretin; flavin	
312.0502	$C_{16}H_9NO_6$	Aristolochic acid II	
315.0865	$C_{17}H_{14}O_6$	Salvigenin; cirsimaritin	

Table 1 (continued)

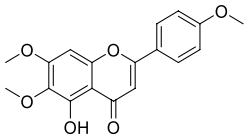
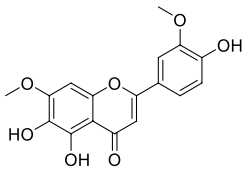
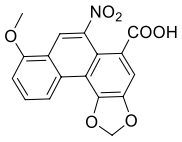
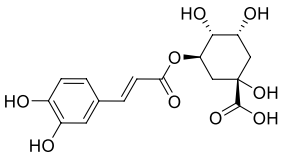
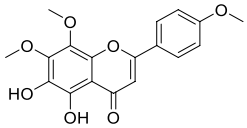
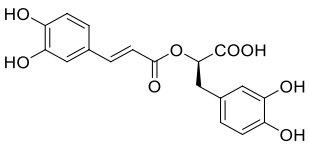
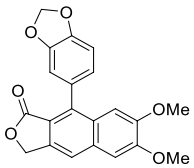
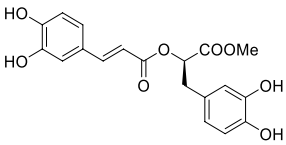
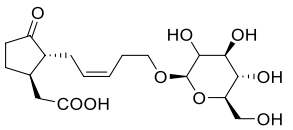
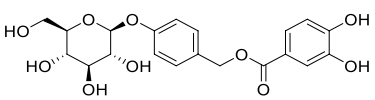
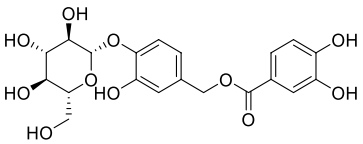
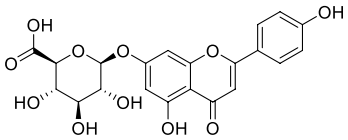
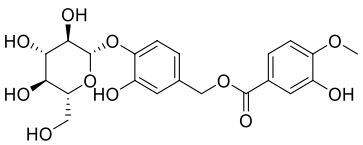
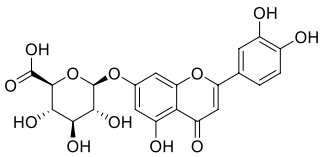
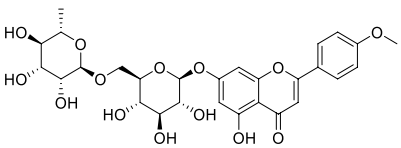
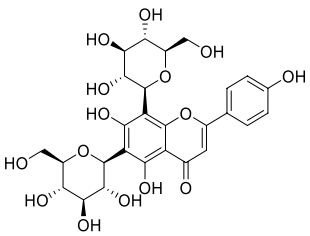
HRMS m/z	Mol. formula	Tentative identification	Compound structure
329.1019	$C_{18}H_{16}O_6$	5-Hydroxy-4′,6,7-trimethoxyflavone	
331.0812	$C_{17}H_{14}O_7$	5,6,4′-Trihydroxy-7,3′-dimethoxyflavone	
342.0608	$C_{17}H_{11}NO_7$	Aristolochic acid I; aristolochic acid	
355.1023	$C_{16}H_{18}O_9$	Chlorogenic acid; 5-*O*-caffeoylquinic acid	
359.1125	$C_{19}H_{18}O_7$	Gardenin B	
361.0917	$C_{18}H_{16}O_8$	(R)-(+)-rosmarinic acid	
365.1032	$C_{21}H_{16}O_6$	Justicidin B	
375.1074	$C_{19}H_{18}O_8$	Rosmarinic acid methyl ester	
379.1023	$C_{18}H_{18}O_9$	No hits from the family	
389.1806	$C_{18}H_{28}O_9$	12-Hydroxyjasmonic acid 12-*O*-hexoside	
407.0972	$C_{19}H_{18}O_{10}$	No hits from the family	
423.1285	$C_{20}H_{22}O_{10}$	Amburoside A	

Table 1 (continued)

HRMS m/z	Mol. formula	Tentative identification	Compound structure
439.1235	$C_{20}H_{22}O_{11}$	Oreganol-A; protocatechuoyl calleryanin	
447.0921	$C_{21}H_{18}O_{11}$	Apigenin-7-*O*- glucuronide	
453.1391	$C_{21}H_{24}O_{11}$	Oreganol-B	
463.0873	$C_{21}H_{18}O_{12}$	Luteolin-7-*O*- glucuronide	
593.1864	$C_{28}H_{32}O_{14}$	Acacetin-7-*O*-rutinoside	
595.1656	$C_{27}H_{30}O_{15}$	Vicenin-2; apigenin 6,8-di-C-hexoside	

multiple pathogens, including *E. coli*, *B. subtilis*, *B. megaterium*, *S. aureus, P. aeruginosa*, and *Proteus vulgaris* [44, 45].

In the current study, the methanolic extract of *O. majorana* L. demonstrated remarkable antimicrobial activity against *A. baumannii* with a MIC of 0.675 mg/mL. The antimicrobial activity of the methanolic and ethanolic extracts and the decoction of *O. majorana* L. was reported previously against many Gram-positive and Gram-negative pathogens and *Candida* spp. [46, 47]. Only one study evaluated and proved its antimicrobial activity against *A. baumannii,* using the disc diffusion method [48]. The MIC of the methanolic extract was reported previously to be > 100 µg/mL against *S. aureus* and *E. coli* [49]. A higher MIC (1.56 mg/mL) was determined for the decoction of *O. majorana* L. against *S. aureus* and *K. pneumoniae* [47]. The ethanolic fraction has MIC between 40 and 80 µg/mL, against *E. coli* and *K. pneumoniae* [46]. Additionally, the essential oil fraction of the plant was found to be highly active against different bacteria, fungi and protozoa [31, 49, 50]. However, it was not quantitatively tested against *A. baumannii*.

To pinpoint the active fraction responsible for the antimicrobial activity against *A. baumannii* in this study, the plant was fractionated, where the polar 50% methanol fraction was found to be the most active subfraction (MIC = 0.5 mg/mL). Chemical profiling of the bioactive fraction by UPLC-HRMS analysis revealed 29 metabolites belonging to diverse chemical classes. The dominant metabolites were phenolic acids, flavonoids, and lignans [51]. Phenolic acids such as rosmarinic acid, caffeic acid, and chlorogenic acid were previously reported in *O. majorana* L. [29]. They are beneficial against several chronic diseases such as degenerative diseases, cancer,

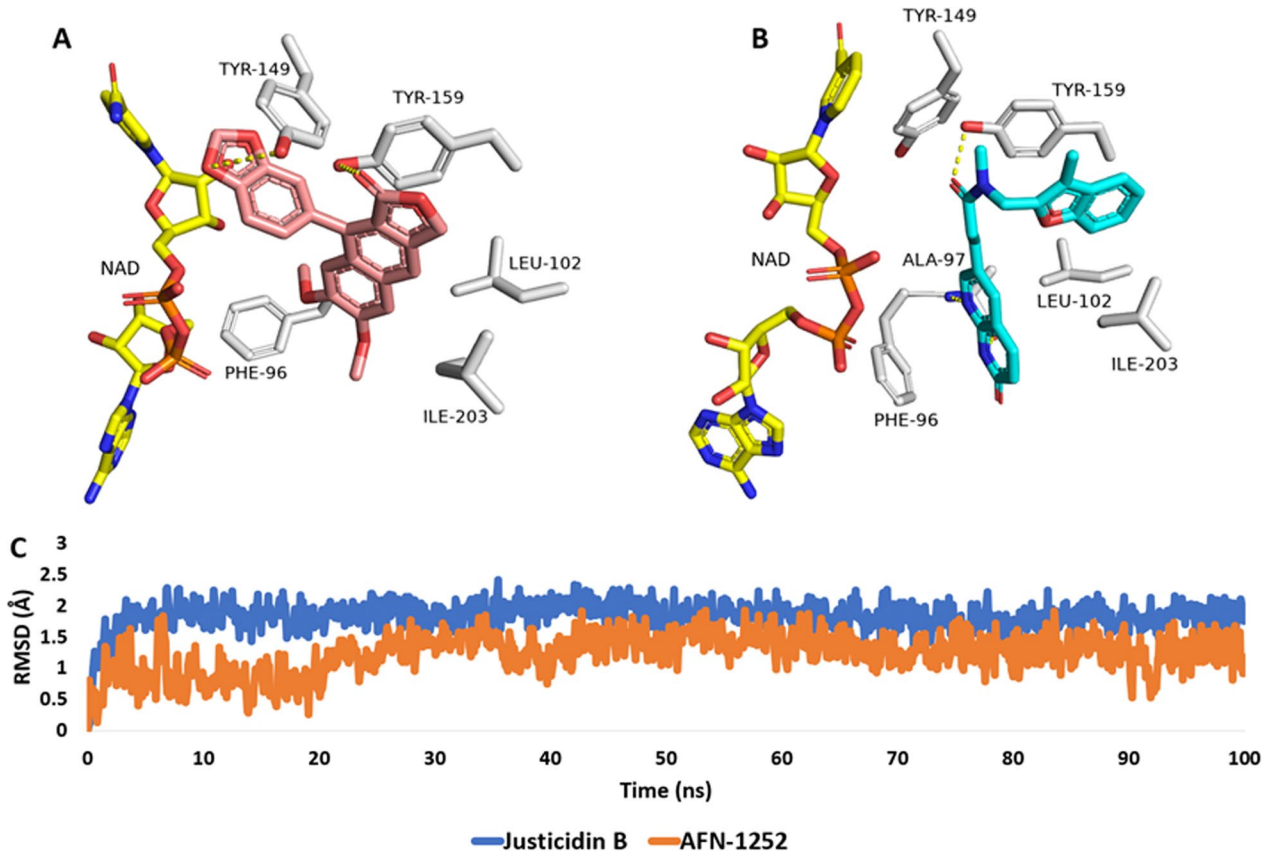


Fig. 2 Binding modes of justicidin B (brick red-coloured structure) (**A**) and the co-crystalized inhibitor AFN-1252 (Cyan-coloured structure) (**B**) inside the binding site of FabI (PDB ID: 6AHE). **C** RMSDs of both structures (i.e. Justicidin B and AFN-1252) inside the binding site of FabI throughout 100 ns-long MD simulation. The yellow-coloured structure is the co-factor Nicotinamide Adenine Dinucleotide (NAD)

diabetes, and ageing [52]. However, to the best of our knowledge, no reports are available about the antimicrobial activity of these metabolites from *O. majorana* L. against *A. baumannii*. The antimicrobial activity of many of these metabolites against Gram-positive and Gram-negative pathogens, such as naringenin [53], quercetin [54], and caffeic acid [55], was previously reported.

Two alicyclic derivatives, 12-hydroxy jasmonic acid and its 12-*O*-glucoside, were previously isolated for the first time from *Origanum* species, i.e. polar extracts of *O. vulgare* L [56]. Later, they were isolated from *O. dictamnus* L. Only 12-hydroxy jasmonic acid and not its glucoside exhibited a strong antibacterial effect against *A. hemoliticus* [57]. When screened for its phytotoxic potential against different phytopathogens, bioguided fractionation of the most active *O. majorana* L. fraction led to the isolation of different molecules, including a new compound named majoradiol, a carvacrol dimer, which was characterized but was not tested for its bioactivity due to its scarcity [58]. Aristolochic acid I and aristolochic acid II were isolated from *O. vulgare* and showed potent activity against leukaemia. Additionally, this is the first report to find that aristolochic acid I and aristolochic acid II demonstrated potent antithrombin activity [59]. Justicidin B, an arylnaphthalene lactone, is a plant-derived subclass of lignans which is distributed in many plants and different families. It is the main active component of *Phyllanthus piscatorum*, exhibiting strong antifungal, antiprotozoal, and anti-proliferative properties [60].

Caffeic acid was isolated from *Origanum dictamnus* L. and demonstrated a good antibacterial effect against *A. hemoliticus* and *P. aeruginosa* [57]. Chlorogenic acid, rosmarinic acid, and rosmarinic acid methyl ester were isolated from the methanol extract of *O. dictamnus* L. and exhibited weak antibacterial activity against *S. aureus* [61]. Amburoside A was previously isolated from *O. micranthum* and exhibited a weak carbonic anhydrase inhibitory effect [62]. Oreganol A and B were isolated from the extract of dried leaves *O. vulgare* and exhibited strong 1,1-dipehnyl-2-picrylhydrazyl (DPPH) radical scavenging activity [63].

Naringenin was previously reported in *O. dictamnus* L. and showed a strong antibacterial effect against *Acinetobacter hemoliticus* [57]. Despite the structural similarity

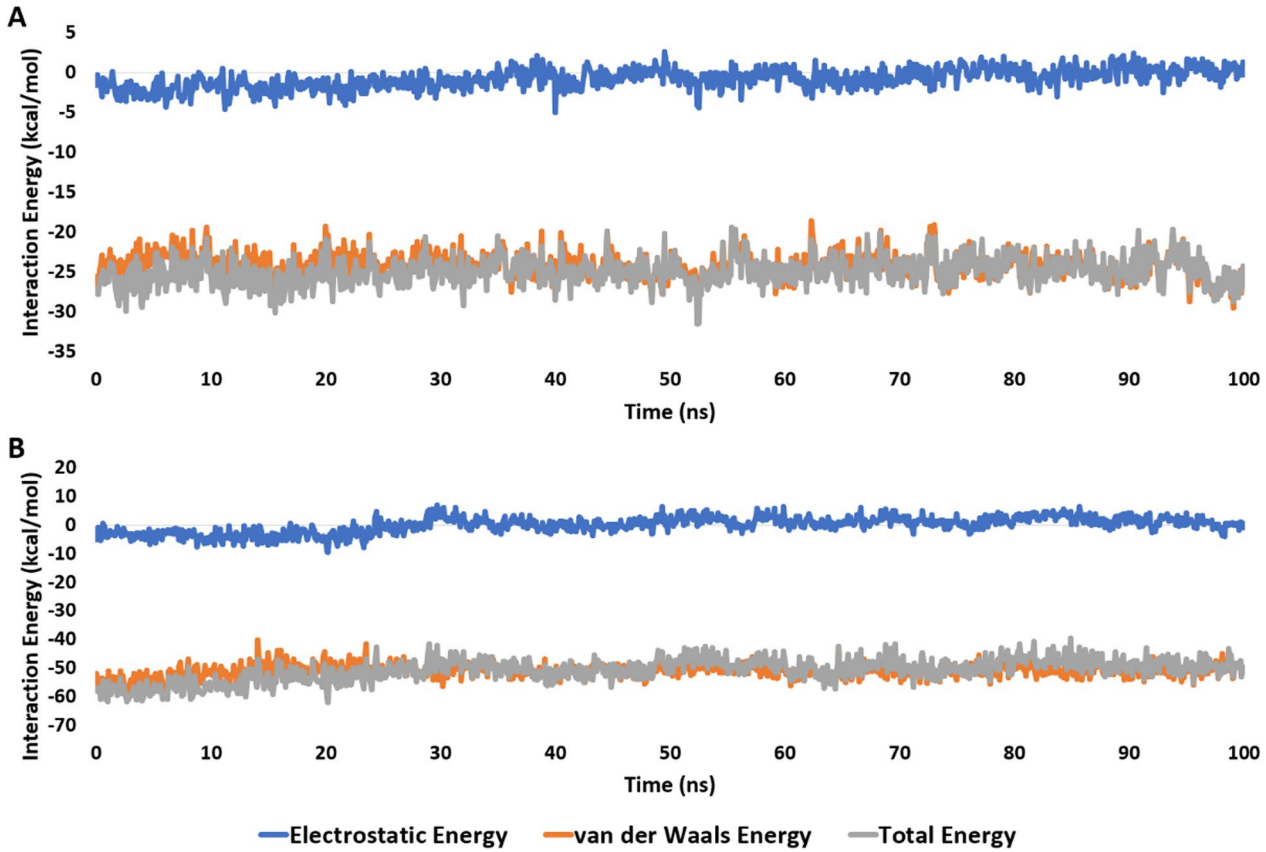


Fig. 3 Interaction energies (i.e. electrostatic and van der Waals interaction energies) of justicidin B (**A**) and AFN-1252 (**B**), respectively inside the binding site of FabI over the course of 100 ns-long MD simulation

with naringenin, apigenin was also isolated from the same species in the same study by Chatzopoulou and co-workers but did not demonstrate any antibacterial activity [57]. Genkwanin, salvigenin, and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone were isolated from the chloroform extract of glandular hairs of *O. x intercedens* [64]. Genkwanin is known for its potent antiviral activity against African swine fever viral infection in Vero cells in a dose-dependent manner [65]. Gardenin B, quercitrin, 5-hydroxy-4',6,7-trimethoxyflavone, and aromadendrin were isolated from *O. majorana* L. with other flavonoid aglycones [58]. Aromadendrin demonstrated moderate anti-inflammatory activity by the inhibition of COX-1 [66]. Apigenin-7-*O*-glucuronide, luteolin-7-*O*-glucuronide, acacetin-7-*O*-rutinoside, and vicenin-2 were isolated from the methanol extract of *O. dictamnus*. None of them demonstrated antibacterial effects against the Gram-positive or Gram-negative strains tested [61].

It can be challenging to characterize a given molecule's biological target. The success rate of identifying accurate molecular targets has greatly increased due to the ongoing development of in silico methods like virtual screening and molecular modelling. Nowadays, a plethora of ligand-based or structural search methods are employed in various online target identification platforms. PharmMapper is a dependable platform that makes use of its pharmacophore model to screen and propose the most probable protein targets for a query chemical [35]. Pharmacophore-based screening works on the basic premise that pharmacophore maps, which delineate the spatial arrangement of structural features, are the primary determinants of the binding of certain compounds to their protein targets. Therefore, molecules whose shapes match these pharmacophore maps have the best chance of binding to the same protein target.

PharmMapper software was employed to screen the 29 metabolites detected in the 50% methanol fraction for binding with putative protein targets in *A. baumannii*. The metabolite justicidin B recorded the highest Fit score (8.56) with the *A. baumannii*-derived enoyl-ACP reductase (FabI). Justicidin B is an aryl naphthalene lignan previously identified in the Lamiaceae family [67], but it is identified here for the first time in *O. majorana* L. extract. The antimicrobial activity of justicidin B was previously

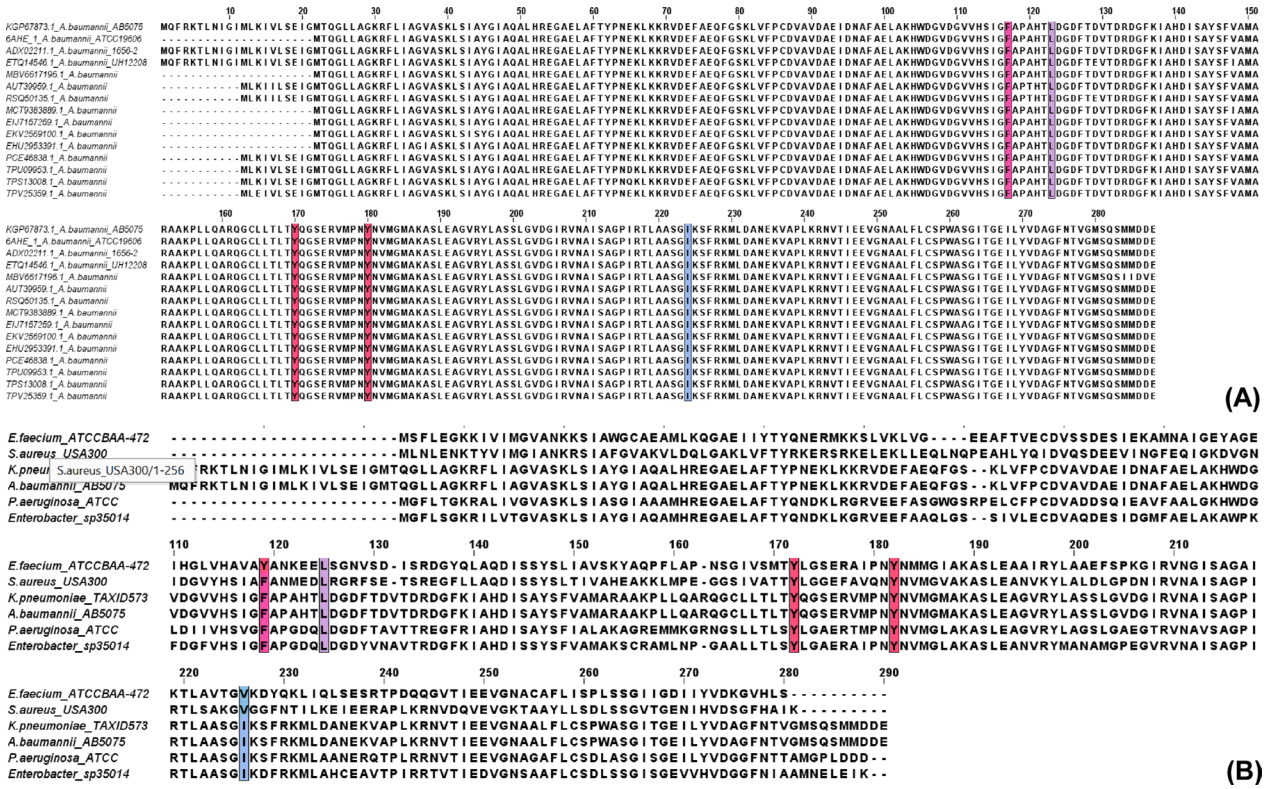


Fig. 4 Alignment of the protein sequences of the enoyl-ACP reductase (FabI) in (**A**) MDR and CRAB *A. baumannii* strains and (**B**) among ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens. The amino acid sequence of each protein was retrieved from the NCBI database and aligned using Clustal Omega software. The alignment visualization was done using Jalview 2.11.3.1. The amino acids involved in justicidin B binding are highlighted

reported against *S. aureus*, *E. coli,* and *P. aeruginosa.* In addition, justicidin B has been proven to have antiviral, antifungal, and antiparasitic properties. It also exhibits anti-inflammatory, antioxidant and anticancer properties [68]. It was originally isolated through the bioactivity-guided fractionation of the dichloromethane extract of *P. piscatorum* and demonstrated non-specific cytotoxicity, in addition to potent in vitro fungicidal and antiprotozoal effects [60]. It recently demonstrated strong antiviral potential against Zika virus [69], an effective inhibitor of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [70], and strong cytotoxic activity against HeLa cervical cancer cells through targeting key proteins involved in apoptosis regulation [71]. It was even isolated from a marine-derived actinomycete, *Nocardia* sp. ALAA 2000 and exhibited a broad-spectrum antimicrobial effect against a panel of Gram-positive and Gram-negative strains as well as different fungi [72].

FabI was predicted as a possible binding target for justicidin B. FabI is a bacterial catalytic enzyme that reduces a carbon–carbon double bond in an enoyl moiety covalently linked to an acyl carrier protein. This is the rate-determining step in the elongation cycle of fatty acids used in lipid metabolism and biotin biosynthesis in bacteria [73, 74]. Being a unique bacterial enzyme, FabI has been considered a potential target for developing new antibacterial therapeutics [75, 76]. It has been discovered that FabI is necessary for bacterial viability. Recently, a wide range of compounds has been recognized as FabI inhibitors, such as triclosan, imidazoles, indole naphthyridinones, thiopyridine, and 4-pyridone [77].

Lately, there has been a growing recognition of promising antimicrobial targets involved in fatty acids synthesis pathways [78]. Novel FabI inhibitors are considered promising antimicrobial agents against MDR bacteria which could be identified by using molecular docking and virtual screening with the FabI X-ray crystal structures that are currently accessible [79]. Fabimycin is a previously discovered FabI inhibitor and an antibiotic candidate with in vivo activity against Gram-negative pathogens [80]. Our docking studies and MDS suggested justicidin B as a novel inhibitor against *A. baumannii* FabI; it binds the enzyme in a pattern similar to its co-crystalized ligand.

The sequence of FabI enzyme that bound to justicidin B was found to be conserved in *A. baumannii* and ESKAPE

pathogens strains available in the NCBI database with alignment scores > 200. The conservation of FabI enzyme among different bacterial species was confirmed previously [81]. The conservation of the amino acids involved in justicidin B binding was confirmed. Only in *S. aureus* and *E. faecium*, the amino acid ILE-203 was replaced with valine, and PHE-96 by tyrosine, respectively, which should have minimal impact on justicidin B binding. This predicts justicidin B as a promising antimicrobial agent through FabI inhibition with potential broad-spectrum activity. However, the impact of changes in the amino acid sequence in these two Gram-positive pathogens is yet to be determined.

Finally, the predicted activity of justicidin B (an aryl naphthalene lignan) as a broad-spectrum antimicrobial against *A. baumannii* and other ESKAPE pathogens through FabI inhibition still needs to be confirmed by different phenotypic and genotypic studies in a step towards the introduction of new antimicrobial compounds. By tracing the aryl naphthalene lignans containing metabolites, we could take a substantial step towards discovering plant-based chemicals as prospective inhibitors of conserved protein targets in MDR-ESKAPE pathogens.

Conclusions

O. majorana L. extract offers a promising source of natural antimicrobial extracts that could be successfully used against the troublesome *A. baumannii* infections. With further experimental confirmations, justicidin B can be employed as a lead molecule for the development of novel inhibitors against MDR *A. baumannii* and other ESKAPE pathogens through interaction with its potential target, the FabI, and the subsequent inhibition of lipid metabolism and biotin biosynthesis in bacteria.

Abbreviations

DPPH	1,1-Dipehnyl-2-picrylhydrazyl
100% MF	100% Methanol fraction
50% MF	50% Methanol fraction
ACP	Acyl carrier protein
CRAB	Carbapenem-resistant *A. baumannii*
FabI	Enoyl-ACP reductase
ESKAPE pathogens	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
ffTK	Force field tool kit
MF	Methanol fraction
MIC	Minimum inhibitory concentration
MMPBSA	Molecular mechanics Poisson–Boltzmann surface area
ML	Mother liquor
MDR	Multidrug-resistant
NCBI	National centre for biotechnology information
NAD	Nicotinamide adenine dinucleotide
RMSD	Root-mean-square deviation
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
MSD	Subsequent molecular dynamic simulation
CDC	The centres for disease control and prevention
CLSI	The Clinical and Laboratory Standards Institute
DCM-F	Dichloromethane fraction
ME	Total methanolic extract
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UPLC-HRMS	Ultra-performance liquid chromatography–high-resolution mass spectrometry

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Not applicable.

Statement regarding plants

Origanum majorana L. aerial parts were collected from the Experimental Station of the Faculty of Pharmacy, Cairo University, Giza, Egypt. Mrs Therese Labib, Consultant at Orman Botanic Garden, Dokki, Giza, Egypt, kindly authenticated the plant material. Voucher specimen No. (PHARM-16.12.2020) was kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Author contributions

Conceptualization, A.S.A., A.M.E. and M.E.R.; methodology, N.H.M., A.E.M.S., A.M.S., R.A.E., N.M.E., M.Y, W.M., M.T.K.; software, A.E.M.S., A.M.S., R.A.E., M.E.R.; validation, A.S.A., A.M.E., A.S.A. and M.E.R.; formal analysis, N.H.M., A.E.M.S., R.A.E., M.Y., W.M., N.M.E., A.M.S.; investigation, N.H.M., A.E.M.S., R.A.E.; resources, A.S.A. and M.E.R.; data curation, A.S.A., A.M.E. and M.E.R.; writing—original draft preparation, N.H.M., A.E.M.S., R.A.E., A.M.S., N.M.E., M.T.K., W.M., M.Y.; writing—review and editing, A.S.A., A.M.E. and M.E.R.; supervision, A.S.A., A.M.E. and M.E.R.; project administration, A.S.A., A.M.E. and M.E.R.; funding acquisition, A.S.A. and M.E.R.. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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