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Investigation of bioactive components responsible for the antibacterial and anti-biofilm activities of *Caroxylon volkensii* by LC-QTOF-MS/MS analysis and molecular docking†

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Caroxylon volkensii is a wild desert plant of the family Amaranthaceae. This study represents the first report of the metabolomic profiling of *C. volkensii* by liquid chromatography quadrupole-time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS). The dereplication study of its secondary metabolites led to the characterization of 66 known compounds. These compounds include catecholamines, tyramine derivatives, phenolic acids, triterpenoids, flavonoids, and others. A new tyramine derivative, alongside other known compounds, was reported for the first time in the Amaranthaceae family. The new derivative and the first-reported compounds were putatively identified through MS/MS fragmentation data. Given the notorious taxonomical challenges within the genus *Salsola*, to which *C. volkensii* previously belonged, our study could offer a valuable insight into its chemical fingerprint and phylogenetic relationship to different *Salsola* species. The antibacterial potential of *C. volkensii* methanolic extract (CVM) against *Pseudomonas aeruginosa* was screened. The minimum inhibitory concentration (MIC) of CVM ranged from 32 to 256 $\mu\text{g mL}^{-1}$. The anti-quorum sensing potential of CVM resulted in a decrease in the percentage of strong and moderate biofilm-forming isolates from 47.83% to 17.39%. It revealed a concentration-dependent inhibitory activity on violacein formation by *Chromobacterium violaceum*. Moreover, CVM exhibited an *in vivo* protective potential against the killing capacity of *P. aeruginosa* isolates. A molecular docking study revealed that the quorum-sensing inhibitory effect of CVM can be attributed to the binding of tyramine conjugates, ethyl-*p*-digallate, and isorhamnetin to the transcriptional global activator *LasR*.

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1. Introduction

Since the first use of antibiotics, they have revolutionized the treatment of bacterial infections.¹ Nevertheless, this important achievement is increasingly failing, owing to the dissemination of bacterial resistance to almost all classes of antibiotics,

constituting a great worldwide health challenge. The number of patients with untreatable bacterial infections is increasing and consequently, the mortality rate of these infections is unfortunately high.² Deaths brought on by bacterial infections are estimated to be the second most common cause of death worldwide.³ This most serious situation dictates the necessity of finding novel approaches to combat the bacterial resistance.¹

Quorum sensing (QS) is a communication mechanism employed by bacterial cells to control the expression of virulence genes based on changes in their population density.⁴ It is one of the major mechanisms used by bacteria for biofilm formation, adaptive antibiotic resistance, increasing virulence, and overcoming immune responses.^{5–7} Quorum sensing inhibition is among the novel therapeutic approaches used for controlling antibiotic-resistant bacteria.^{6,8,9}

Plants have been employed since ancient times in traditional medicine for addressing infectious diseases before the era of antibiotics.^{10,11} In recent times, there has been a renewed interest in exploring plants for the discovery of antimicrobials. This is attributed to their capacity to generate numerous

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bioactive secondary metabolites that possess the potential to combat bacterial infections and boost the human immune system.^{12–14}

Caroxylon volkensii (Schweinf. & Asch.) Akhani & Roalson is an annual herb belonging to the family Amaranthaceae. According to the International Plant Names Index (IPNI), the plant was mentioned for the first time under the name of *Salsola volkensii* (Schweinf. & Asch.) in Mémoires de l'Institut Égyptien Collation 2: 130 (1887). It had previously been classified as a member of the Old-World *Salsola* (family Chenopodiaceae), which encompassed a large number of desert plants.¹⁵ The taxonomic classification of this plant was revised by Akhani *et al.* (2007), who transferred it to the genus *Caroxylon*.¹⁶ The plant was also suggested by Feodorova (2015) to be segregated into the genus *Nitrosalsola*, based on its anatomical and molecular phylogenetic data.¹⁷ Hence it has two synonyms, *C. volkensii* (Schweinf. & Asch.) Akhani & Roalson and *Nitrosalsola volkensii* (Schweinf. & Asch.) Theodorova and the basionym of *Salsola volkensii* Asch. & Schweinf. It was described in the flora of Egypt as shrubs or subshrubs having small, alternate, sessile leaves with solitary flowers forming dense or loose spikes at the leaf axils and showing 5-segmented perianth.¹⁸ It is also recognized by its characteristic odor of decaying fish when bruised.¹⁸ It grows in the desert and sandy plains of Egypt, Saudi Arabia, Palestine, and Iraq.¹⁸ The limited native land of the plant and its occurrence in desert and arid environments have so far restricted the research studies on its biological activities and phytochemical components. Only one research study has described the hepatoprotective effects of its alcoholic extract against CCl₄-induced hepatotoxicity in Sprague Dawley rats.¹⁹ Various plant species within the Old-World *Salsola* genus have been documented for their ability to exhibit antimicrobial effects against diverse pathogens.¹⁵ Hence, in the present research, we investigated the antibacterial properties of *C. volkensii* and clarified its capabilities in inhibiting biofilm formation and disrupting quorum sensing in clinical isolates of *Pseudomonas aeruginosa*.

Dereplication by Liquid Chromatography coupled to Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS/MS) is a powerful analytical approach used in natural product research and metabolomics. It combines the separation capabilities of liquid chromatography with the high-resolution and accurate-mass measurement capabilities of quadrupole time-of-flight mass spectrometry. It involves rapid identification of previously characterized compounds, even from complex mixtures of natural products. Employing dereplication strategies minimizes redundant efforts of purification and re-identification of known compounds and prioritizes the isolation of novel chemical entities.²⁰ The dereplication strategy using LC-QTOF-MS/MS has been extensively used for rapid identification of natural products in plants, bacteria, and fungi.^{20–22}

In the current study, we investigated the phytochemical content of *C. volkensii* for the first time, using a dereplication process by LC-QTOF-MS/MS analysis. From a taxonomical perspective, it is worth mentioning that the genus *Salsola*, to which *C. volkensii* was belonging, is among the most contentious plant genera. The taxonomy of *Salsola* species is

characterized by an ongoing debate and confusion within the scientific community.¹⁵ This underscores the importance of our study in understanding the taxonomic classification of *C. volkensii* and its taxonomic relationship to other *Salsola* species and to Amaranthaceae plants through the analysis of its metabolomic profile. We also performed a docking study of the major identified compounds against the *LasR* enzyme, which plays a role in controlling the expression of numerous genes associated with biofilm formation, pathogenicity, and virulence.

2. Materials and methods

2.1. Plant material

Aerial parts of *Caroxylon volkensii* (Schweinf. & Asch.) Akhani & Roalson (Fig. 1) were collected from Aswan in March 2017 and were identified by Dr Yassin Al-Sodany, professor of Plant Ecology, in the Botany Department within the Faculty of Science at Kafrelsheikh University.

2.2. Chemicals

Methanol and acetonitrile, as organic solvents of HPLC grade, were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (HPLC-grade) was acquired from Sigma Chemical Co. (Saint-Louis, MO, USA). Deionized water was obtained using a GenPure purification system (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Preparation of the plant extract

50 g of the dried powdered plant material was extracted with methanol (250 mL) assisted by sonication for 30 min at room temperature. The extraction process was repeated three times. The supernatant was filtered and dried using a rotary evaporator at 45 °C, giving 6.5 g of total dry crude extract. A portion of 1 mg of the extracted sample was dissolved in 1 mL of methanol and subsequently filtered using 0.45 µm polyvinylidene difluoride (PVDF) syringe filters for utilization in LC-MS/MS analysis.

2.4. LC-MS/MS analysis

The methanol extract of *C. volkensii* (CVM) was analyzed on an HPLC-MS system consisting of a 1260 Infinity II Flexible HPLC



Fig. 1 *Caroxylon volkensii* (Schweinf. & Asch.) Akhani & Roalson plant photographs.

coupled to a 6530 Q-TOF-MS (Agilent Technologies, USA). An injection of 8 μL from the processed crude extract was introduced into a Zorbax RP-18 column provided by Agilent Technologies (150 mm \times 3 mm, 2.7 μm), maintained at a temperature of 40 $^{\circ}\text{C}$, with a flow rate set at 0.5 mL min^{-1} . The mobile phase system comprised a combination of solvent A (0.1% formic acid) and a gradual gradient increase from 0 to 100% of solvent B (acetonitrile + 0.1% formic acid) over a duration of 120 min. Mass spectra were acquired in both positive and negative electrospray ionization (ESI) modes, employing a capillary voltage of 4000 V. The recorded spectra encompassed the m/z values range from 100 to 1500. Parameters such as the capillary temperature and drying gas flow were adjusted to 320 $^{\circ}\text{C}$ and 10 L min^{-1} , respectively. The collision energy and the nebulization pressure were adjusted to 18–45 eV and 40 psi respectively. The LC-MS/MS data acquired was transformed using Proteowizard msconvert, followed by processing and analysis through MZmine.

2.5. Biological study

2.5.1. *In vitro* antibacterial activity and susceptibility to *Caroxylon volkensis*. The utilized culture media and chemicals employed were sourced from Oxoid in the UK and Merck in the USA. A collection of 23 clinical isolates of *P. aeruginosa* was acquired from the culture repository at the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University, Egypt. The reference isolate utilized was *P. aeruginosa* ATCC 27853.

In vitro testing of antibacterial susceptibility to CVM was conducted using the agar well diffusion method.²³ Following the inoculation of Muller-Hinton agar (MHA) plates with bacterial suspensions, wells were created and loaded with CVM, ciprofloxacin (used as a positive control), and dimethyl sulfoxide (DMSO) at a concentration of 10% (employed as a negative control). Subsequently, the plates underwent an overnight incubation at 37 $^{\circ}\text{C}$. CVM demonstrated antibacterial activity by generating zones of inhibition around the wells. The determination of minimum inhibitory concentrations (MICs) was carried out using the broth microdilution method as previously outlined.²⁴

2.5.2. Anti-biofilm and anti-quorum sensing activities of *Caroxylon volkensis*. Initially, the ability of the examined isolates to form biofilms was assessed through the crystal violet assay as previously reported.²⁵ The optical density (OD) at 490 nm was measured using an ELISA reader (Sunrise Tecan, Austria). The tested isolates were categorized into four groups based on their OD values: Non-biofilm producer (NBP) for OD values between the cut-off (OD_c) and twice OD_c, Weak biofilm producer (WBP) for OD values between twice OD_c and four times OD_c, Moderate biofilm producer (MBP) for OD values between four times OD_c and six times OD_c, Strong biofilm producer (SBP) for OD values greater than six times OD_c. The cut-off OD (OD_c) was determined as the mean OD plus three times the standard deviation (SD) of the negative control. After grouping the bacterial isolates into four groups (strong, moderate, weak, and non-biofilm forming), they were treated with CVM (0.5 MIC). Subsequently, the assessment of biofilm-

forming ability was repeated to evaluate the impact of CVM. The quorum quenching potential (quorum inhibitory action) of CVM was elucidated as previously described.²⁶ In brief, various concentrations of CVM were created through a serial two-fold dilution method, commencing from 1 $\mu\text{g mL}^{-1}$ and progressing up to 1024 $\mu\text{g mL}^{-1}$. The prepared concentrations were then added to *Chromobacterium violaceum* (ATCC 12472) suspension and left at 30 $^{\circ}\text{C}$. After overnight incubation, the mixtures were centrifuged and DMSO (500 μL) was added to the formed pellets. Vigorous vortexing was carried out to extract the violacein pigment (purple), subsequently quantified by measuring the optical density (OD) at 585 nm using an ELISA reader (Sunrise Tecan, Austria). The control group involved a *C. violaceum* suspension without the presence of CVM.

2.5.3. *In vivo* protection assay. Thirty male albino mice were provided by the animal facility of Cairo University, Egypt. The mice, weighing between 22 g and 26 g, were provided with standard pellet meals and access to filtered water. Furthermore, the mice were housed in a controlled environment with a temperature set at 25 $^{\circ}\text{C}$ and subjected to a 12 hour light and dark cycle.²⁷ Following a 7 day acclimation period for the mice, the *in vivo* experiment was conducted in accordance with the established guidelines for laboratory animal care and services. The study received ethical approval with the designated number TP/RE/06-22P-014 from the Research Ethical Committee of the Faculty of Pharmacy at Tanta University, Egypt.

A survival assay was utilized to evaluate the protective efficacy of CVM against *P. aeruginosa* infection.¹ Briefly, *P. aeruginosa* bacteria in phosphate-buffered saline (1×10^8 CFU mL^{-1}) with and without treatment with CVM (0.5 MIC) were injected *via* an intraperitoneal route into the mice. The mice were randomly divided into three groups, each consisting of 10 mice: group I (non-infected control group), group II (infected with *P. aeruginosa* but untreated with CVM), and group III (infected with *P. aeruginosa* and treated with CVM). The survival of the mice was observed over a period of 14 days.

The assays were carried out in triplicate and the results are presented as mean \pm standard deviation (SD). Statistical significance was evaluated using two-way ANOVA with a significance level set at $p < 0.05$. To assess the survival of mice, Kaplan–Meier survival curves were employed. The analysis was conducted using Graph Pad Prism software (Version 8, GraphPad Software Inc., USA).

2.5.4. Docking study. The procedure was carried out utilizing AutoDock Vina 1.2.3 (ref. 28 and 29) following the details outlined in the ESI.[†]

3. Results and discussion

3.1. LC-MS/MS analysis

Liquid chromatography quadrupole-time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS) analysis was used for the identification of the secondary metabolites present in the methanolic extract of *C. volkensis* (CVM). The plant sample was analyzed in both negative and positive ESI modes to identify the highest-possible number of secondary metabolites. The total ion chromatograms (TIC) obtained from both modes are presented in Fig. S1–S3.[†]



The dereplication study of the plant secondary metabolites against the Dictionary of Natural Products (DNP) and METLIN databases led to the identification of 67 compounds belonging to diverse chemical classes. The identification of these compounds relied on the concordance of their ions in positive and/or negative ESI modes with literature data and reviewing the biosynthetic abilities of other *Salsola* species. Furthermore, the structures of some of these compounds were putatively verified through MS/MS fragmentation data. The molecular formula, mode of ionization, retention time, mass error (≤ 10 ppm), peak area, MS/MS fragmentation data, as well as bibliographic references of each phytochemical are presented in Table S1.† The identified compounds are categorized into six chemical classes, which will be discussed in detail.

3.2. Alkaloids and nitrogenous compounds

The dereplication study led to the characterization of 30 nitrogenous compounds of various classes (Table S1† and Fig. 2). Salsoline (1.2), an isoquinoline alkaloid previously found in different *Salsola* species,^{30–34} was identified in our study. Additionally, we report here the presence of its *O*-demethylated analog, salsolinol (1.1), in the family Amaranthaceae for the first time. Its identification was supported by the MS/MS fragmentation data (Fig. S4†). Another methylated isoquinoline derivative was tentatively identified based on the correspondence of its molecular ion peak in the positive mode to salsolidine (1.3.1) or *N*-methylsalsolidine (1.3.2) that were reported previously in other *Salsola* species.³⁴ Compound 1.4 with $[M - H]^-$ at m/z 218.0816 was tentatively characterized as a pyrrolo[2,1-*a*]isoquinoline derivative that could be either salsoline A (1.4.1)^{35–38} or salsoline B (1.4.2).³⁹ Compound 1.5 was

identified as a terrestrial acid that had previously been isolated from *S. collina*.³⁷

CVM was found to be rich in *N*-methyltyramine (1.7), which showed the highest peak intensity among the metabolites detected in the positive ESI mode (Fig. S1†). Its identification was putatively verified through MS/MS fragmentation data (Fig. S6†) which agree with the human metabolome MS/MS spectra with the ID number HMDB0003633.⁴⁰ Other tyramine derivatives include catecholamines, such as dopamine/octopamine, synephrine, norepinephrine, and their glucosylated analogs (1.6–1.18) were also detected (Table S1† and Fig. 2). The MS/MS spectra of some of these compounds are presented in the ESI file (Fig. S5–S10†).

In this study, catecholamine derivatives were reported in *Salsola* species for the first time. While tyramine and other catecholamines were previously reported in *Spinacia oleracea* belonging to the Amaranthaceae plant family.^{41,42} It is worth noting that catecholamines were previously reported to be synthesized in many plants as a response to stress conditions.^{42,43} They were suggested to be involved in growth regulation, carbohydrate metabolism, nitrogen detoxification, and protection against pathogens.⁴² The presence of catecholamines in *C. volkensis* may be attributed to its growth in a stressful environment under arid conditions. We are suggesting that catecholamines could be the constituents responsible for the traditional use of other *Salsola* species as antihypertensive agents.¹⁵

Another group of nitrogenous compounds that are formed by the conjugation of tyramine analogs with phenolic acids was also detected. This group is represented by moupinamide (*N*-*trans*-feruloyltyramine, 1.22), which was reported in several *Salsola* species.^{15,44} Other derivatives such as cinnamoyl (1.19),

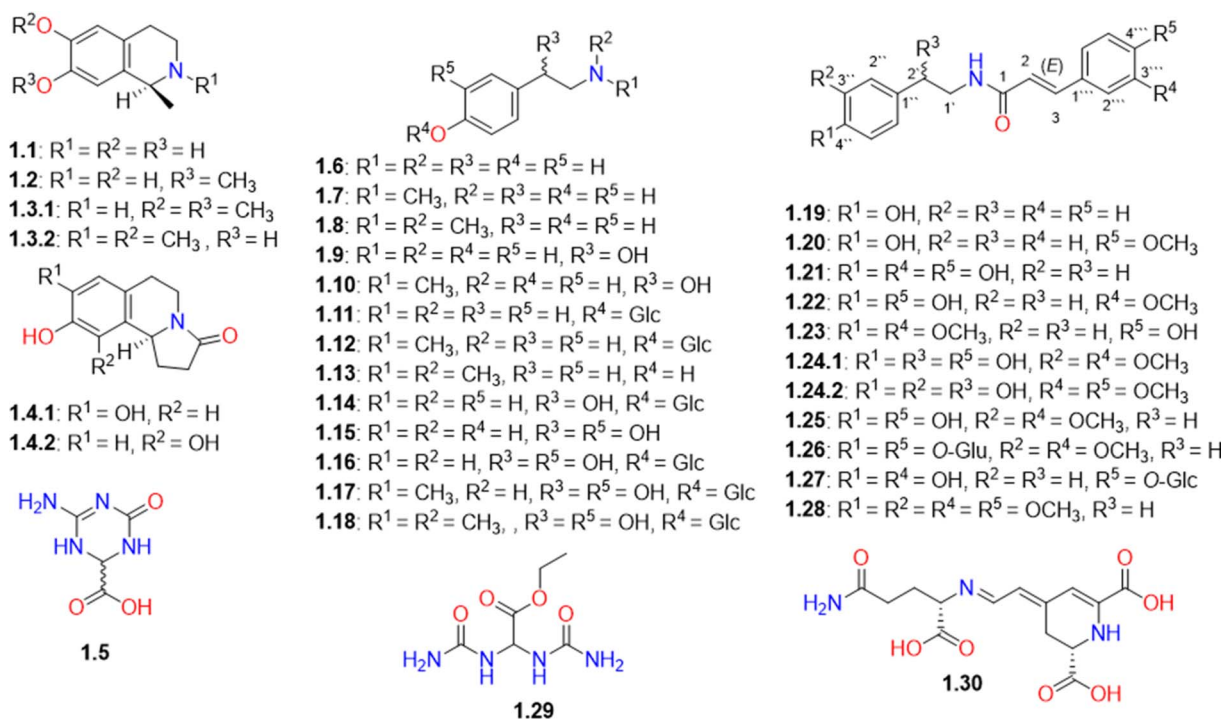


Fig. 2 Structures of alkaloids and other nitrogen-containing compounds (1.1–1.30) identified in *Caroxylon volkensis*.

4-methoxy-cinnamoyl (**1.20**), and caffeoyl (**1.21**) tyramine conjugates were also detected (Table S1† and Fig. 2). Another related derivative with a molecular ion peak $[M + H]^+$ at m/z 328.1534 was characterized as (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-(4-methoxyphenethyl)acrylamide (**1.23**), which was reported to be naturally occurring in *Sinomenium acutum* and *Zanthoxylum piperitum*.^{45,46} However, this is the first report of identification of this compound in the family Amaranthaceae, which is supported by MS/MS fragmentation data (Fig. S11†).

Compound **1.24** with $[M - H]^-$ at m/z 358.1287 was tentatively characterized as 2'-hydroxy- 3''-methoxymoupinamide (**1.24.1**) or *N*-(3',4'-dimethoxy-cinnamoyl)-norepinephrine (**1.24.2**) that were previously detected in different *Salsola* species.^{37,38,47} *N*-*Trans*-feruloyl-3-*O*-methyldopamine (**1.25**) and rubemamine (*E*)-*N*-(3,4-dimethoxyphenethyl)-3-(3,4-dimethoxyphenyl)acrylamide (**1.28**) previously identified in family Amaranthaceae^{37,48} were also detected. While aristomanoside (**1.26**), a di-glucoside derivative of **1.25** previously reported in *Aristolochia manshuriensis*,⁴⁹ was detected herein for the first time from the Amaranthaceae family.

Furthermore, we detected the m/z values for several new moupinamide analogs that could be biosynthetically accessible through hydroxylation, methylation, or glycosylation of the identified analogs. However, we couldn't find the MS/MS fragmentation data that support their identification. Fortunately, we got enough MS/MS fragmentation spectra (Fig. S13 and S14†) that could support the identification of only one new analog identified as 4'''-*O*-β-D-glucopyranosyl caffeoyltyramine (**1.27**).

In this research, a urea derivative, namely ethyl 2,2-diureidoacetate (syn. allantoinic acid ethyl ester, **1.29**) was detected from a natural source for the first time. Its identification in CVM was supported by the MS/MS fragmentation spectrum presented in Fig. S15.† In addition, compound **1.30**, with a molecular ion peak $[M + H]^+$ at m/z 340.1131897, was tentatively identified as vulgaxanthin I that had been previously identified in *Beta vulgaris*, a plant belonging to the family Amaranthaceae.⁵⁰

3.3. Phenolic acids and simple phenols

The current LC-MS study revealed the presence of several phenolic compounds in the methanolic extract of *C. volkensii* (Table S1† and Fig. 3). These compounds included cinnamic acid (**2.1**), ferulic acid (**2.2**), rosmarinic acid (**2.3**), vanillic acid glucoside (**2.5**), and resorcinol (**2.6**), which had been identified previously in different plants belonging to family Amaranthaceae. Two phenolic compounds (**2.4** and **2.7**) were observed in relatively high concentrations in CVM as indicated by their high peak intensities in the TIC (negative mode, Fig. S2†). Compound **2.7** was identified as ethyl-*p*-digallate previously isolated from *Mangifera indica*.⁵¹ This study represents the first instance of identifying this compound within the plant family Amaranthaceae. Its identification was supported by the MS/MS fragmentation data presented in Fig. S17,† while compound **2.4** was identified as the sulfonic acid derivative, tichocarpol A, which had previously been isolated from *Tichocarpus crinitus* algae.⁵² This study represents the first report on the occurrence of this compound in higher plants. For this

reason, it is important to highlight that the identification process of this compound extends beyond mere concordance of its ion in negative ESI mode with existing literature and the close correspondence of its experimental molecular weight with the theoretical value, showcasing a remarkably low mass error of just 2.56 ppm. Our identification is further bolstered by the ESI-MS/MS spectrum (Fig. S16†), which reveals molecular ion fragments that support our identification. It is noteworthy that *Caroxylon volkensii* belongs to the Amaranthaceae family, which encompasses former Chenopodiaceae members, including halophytes and *Salsola* species recognized for their abundant mineral content, including sulfur.^{53,54} Other sulfur-containing compounds had been previously reported in *Salsola* species.^{44,55} Additionally, halophytes share a high salt tolerance similar to algae,^{56,57} whereas members of the Chenopodiaceae family are recognized as the most salt-tolerant higher plants.⁵⁸

3.4. Triterpenoids and their derivatives

CVM was found to be rich in triterpenes (Table S1† and Fig. 4). Only one triterpene of the ursane nucleus, named 2α,3β,23,24-tetrahydroxurs-12-en-28-oic acid (**3.1**), was detected, while triterpenes of the oleanane skeleton were found to be more predominant. They included free oleanolic acid (**3.2**) as well as its combination derivatives with different sugar units of variable length. Among these derivatives, salsolide C (**3.4**) and salsolide E (**3.5**) were present in high concentrations in CVM, as indicated by their high peak intensities in the TIC (negative mode, Fig. S3†). They had previously been isolated from different *Salsola* species.^{59–62} Compound **3.7**, with a molecular ion peak $[M - H]^-$ at m/z 955.492, also appeared in a high concentration. It was tentatively identified as β-D-glucopyranosiduronic acid, (3β)-28-(β-D-glucopyranosyloxy)-28-oxoolean-12-en-3-yl 3-*O*-β-D-galactopyranosyl (**3.7.1**) or β-D-glucopyranosiduronic acid, (2β,3β)-28-(β-D-glucopyranosyloxy)-2-hydroxy-28-oxoolean-12-en-3-yl 3-*O*-(6-deoxy-α-L-mannopyranosyl) (**3.7.2**), previously reported in the plant family Amaranthaceae.^{63,64}

3.5. Flavonoids

Several types of flavonoids were detected in CVM. They can be subcategorized into flavones, isoflavones, flavanols, and isoflavanes. The identified flavones were isorhamnetin (**4.1**) and its glycosidic analogues, including isorhamnetin-3-*O*-β-D-glucopyranoside (**4.2**), narcissoside (**4.3**), isorhamnetin-3-*O*-β-D-apiofuranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1→6)]-β-D-glucopyranoside (**4.4**), and chrysoeriol-7-*O*-β-D-glucopyranoside (**4.5**). Few isoflavones, compounds **4.6–4.8**, were tentatively identified (Table S1† and Fig. 5), based on the consistency of their ion peaks to known isoflavones isolated from *Salsola somalensis*.^{65,66} However, one flavanol, catechin (**4.9**), and one isoflavane, salisoflavan (**4.10**), which had previously been reported in *S. imbricata*,^{67,68} were also detected.

3.6. Steroids

Two steroids (Table S1† and Fig. 6) were detected in CVM. The first steroid is the common campesterol (**5.1**), which had



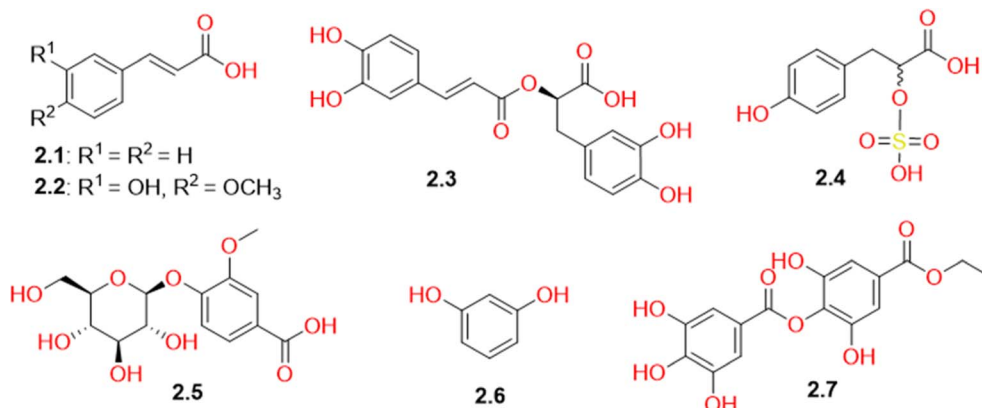


Fig. 3 Structures of phenolic acids and simple phenols (2.1–2.7) identified in *Caroxylon volkensii*.

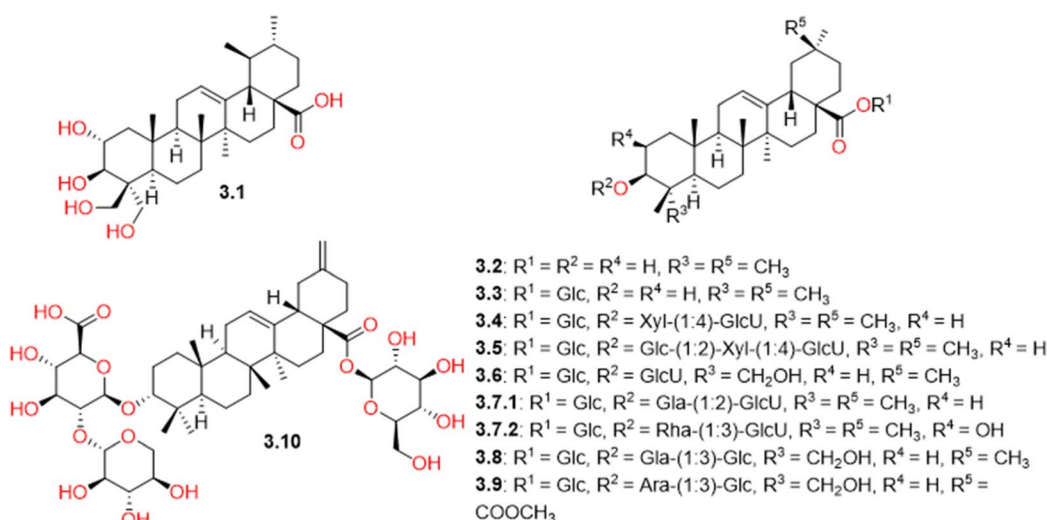


Fig. 4 Structures of triterpenoids (3.1–3.10) identified in *Caroxylon volkensii*.

previously been isolated from *S. collina*.⁶⁹ The second is amasterol (5.2), an ecdysteroid isolated before from the roots of *Amaranthus viridis*, was reported to exhibit a growth inhibitory activity against lettuce seedlings and to possess antifungal activity against the pathogen *Helminthosporium oryzi*.⁷⁰

3.7. Miscellaneous compounds

Other secondary metabolites biosynthesized from mixed pathways, including the malonate, isoprenoid, and shikimate pathways, were detected in CVM and categorized as miscellaneous compounds (Table S1† and Fig. 7). They included the fatty acid component, 9,12,13-trihydroxyoctadeca-10(*E*),15(*Z*)-dienoic acid (6.1), which had previously been isolated from *Salsola tetrandra*.⁷¹ Plastoquinone 3 (6.2), a chloroplast component involved in photosynthesis, was also detected. It had previously been isolated from the plant *Spinacia oleracea*, belonging to the family Amaranthaceae.⁷² Also, *N*-cyclohexanecarbonylpentadecylamine (6.3), previously detected in *Senna siamea* pods⁷³ and *Polyporus umbellatus*,⁷⁴ was identified for the first time in the family Amaranthaceae. Its identification

was supported by the MS/MS fragmentation spectrum (Fig. S18†) as evident from the pseudo molecular ion peak $[M + H]^+$ corresponding to palmitamide at m/z 256.26349. This compound showed high peak intensity in the TIC (positive mode) of the CVM (Fig. S1†). Compound 6.3 had previously shown a selective inhibition of the acidamidase hydrolyzing *N*-acylethanolamines.⁷⁵ Several isoprenoid derivatives (6.4–6.8) were also identified in CVM. They included the sesquiterpene glucoside, amarantholidoside IV (6.4), which had previously been isolated from *Amaranthus retroflexus*,⁷⁶ in addition to two monoterpenes, *viz.* carvone (6.5) and chenopnone (6.6), and two sesquiterpenes, namely chenopdiol (6.7) and 6,11-dihydroxy-3-eudesmen-2-one (6.8).

3.8. Biological activity

Pseudomonas aeruginosa plays a significant role in causing severe and life-threatening infections, particularly in individuals with compromised immune systems, such as those with cancer or AIDS. Numerous isolates of *P. aeruginosa* exhibit the ability to form biofilms, rendering them more resistant to



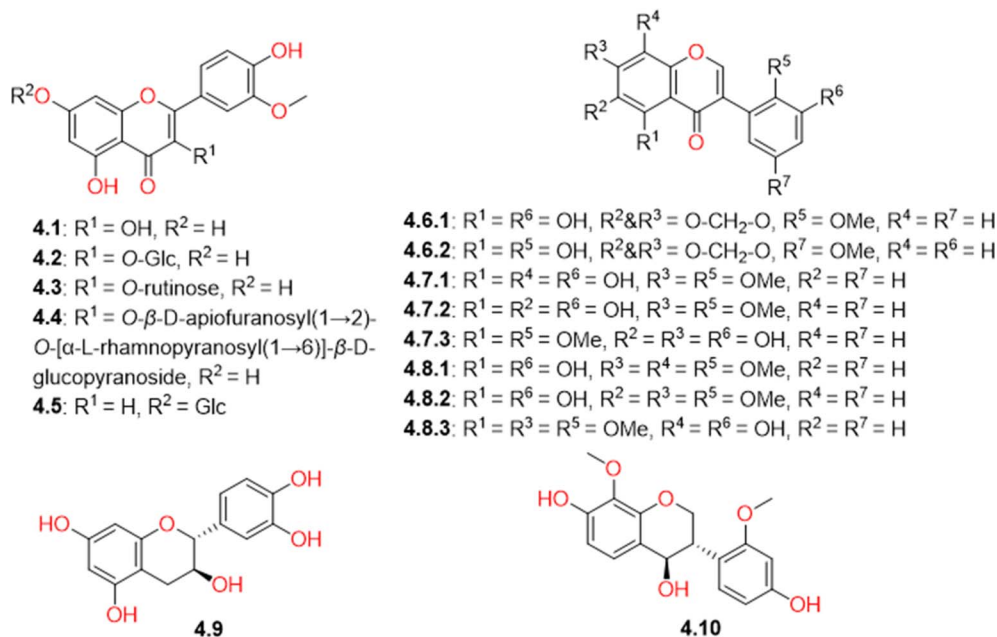


Fig. 5 Structures of flavonoids (4.1-4.10) identified in *Caroxylon volkensii*.

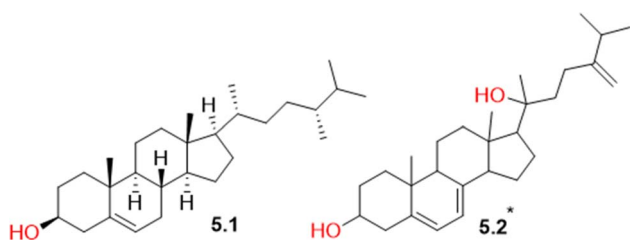


Fig. 6 Structures of steroids (5.1 and 5.2) identified in *Caroxylon volkensii*. *Stereochemistry is undefined.

existing antibiotics.⁷⁷ Thus, it is crucial to explore alternative approaches to the conventional use of antibiotics. Plants serve as a natural reservoir of numerous bioactive compounds with

a wide range of biological activities. Researchers worldwide have extensively explored the antibacterial properties of plant extracts against isolates of *P. aeruginosa*.^{26,77-80} Herein, we present the antibacterial, anti-biofilm, and anti-quorum sensing potentials of CVM against *P. aeruginosa* isolates.

3.9. *In vitro* antibacterial activity

The preliminary screening of the antibacterial activity of CVM was conducted using the agar well diffusion method. Remarkably, it demonstrated the presence of an inhibition zone around the wells, signifying its antibacterial potential against the tested isolates. MIC values were established through the broth microdilution method, revealing that the MIC values of CVM against *P. aeruginosa* isolates varied from 32 to 256 $\mu\text{g mL}^{-1}$, as detailed in Table 1.

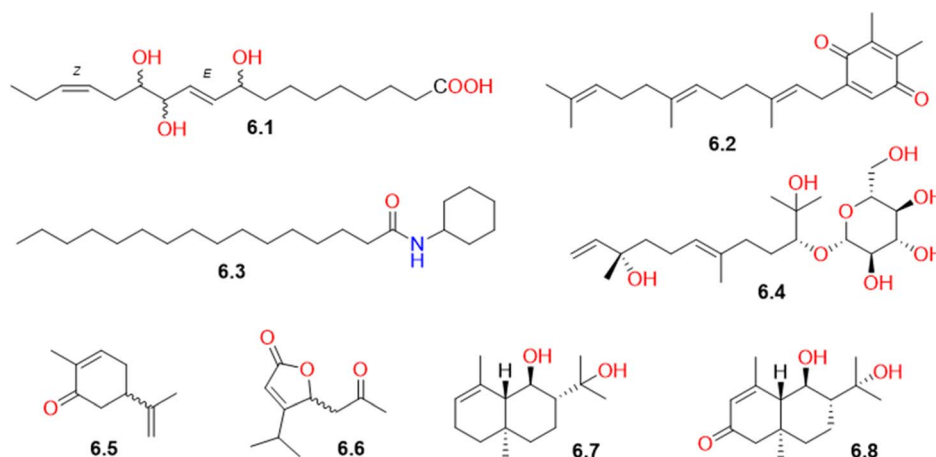


Fig. 7 Structures of biosynthetically miscellaneous compounds (6.1-6.8) identified in *Caroxylon volkensii*.



Table 1 Values of the minimum inhibitory concentrations (MICs) of *Caroxylon volkensii* against the tested isolates

Isolate code	MIC ($\mu\text{g mL}^{-1}$)	Isolate code	MIC ($\mu\text{g mL}^{-1}$)
P1	32	P13	128
P2	32	P14	256
P3	256	P15	128
P4	64	P16	32
P5	128	P17	32
P6	32	P18	64
P7	32	P19	256
P8	128	P20	128
P9	256	P21	128
P10	32	P22	64
P11	64	P23	32
P12	64		

3.10. Anti-biofilm and anti-quorum sensing activities

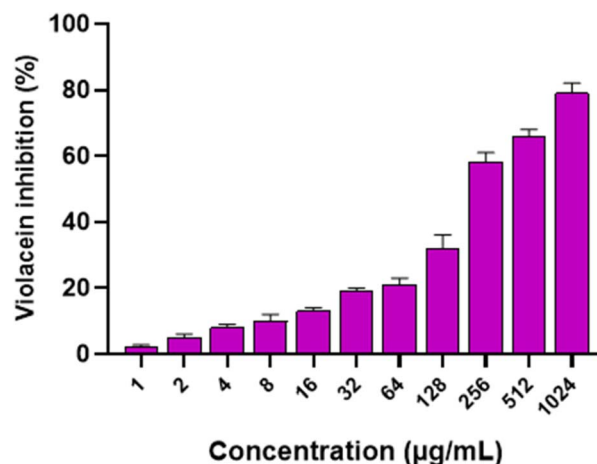
Bacterial virulence quenching is a crucial tactic that has proven to be highly effective in reducing the dissemination of bacterial resistance.⁸¹ This strategy is able to reduce the pathogenesis of the pathogenic bacteria, thus helping the host immune system to eradicate bacteria, and avoid the development of resistance. Besides, there is an important advantage to such an approach as it does not affect the normal flora of the host.^{82,83} Biofilm is a pivotal target for many antivirulence compounds as biofilm eradication can affect disease progression.⁸⁰ Thus, we investigated the impact of CVM on the biofilm forming ability of the tested isolates using the crystal violet assay as shown in Table 2. CVM demonstrated the ability to hinder biofilm formation in the tested isolates, leading to a reduction in the percentage of isolates classified as strong and moderate biofilm formers from 47.83% to 17.39%.

Moreover, the quorum quenching potential of CVM was elucidated on *Chromobacterium violaceum* standard isolate. *C. violaceum* is frequently used in microbiology labs as a bacterial model possessing a QS system. The impact of potential quorum quenching compounds is examined by monitoring the production of its violacein pigment. This pigment has a violet color and its production is regulated by the QS system of *C. violaceum*.⁸⁴ Interestingly, CVM revealed a concentration-dependent inhibitory activity on violacein production as shown in Fig. 8, and this finding can be correlated with its anti-biofilm activity.

Table 2 Impact of *Caroxylon volkensii* on the biofilm forming ability of *P. aeruginosa* isolates

Biofilm formation ^a	Before treatment	After treatment
Non-biofilm producer (NBP)	8	11
Weak biofilm producer (WBP)	4	8
Moderate biofilm producer (MBP)	6	3
Strong biofilm producer (SBP)	5	1

^a NBP: non-biofilm producer ($\text{ODc} < \text{OD} < 2 \text{ ODc}$), WBP: weak biofilm producer ($2 \text{ ODc} < \text{OD} < 4 \text{ ODc}$), MBP: moderate biofilm producer ($4 \text{ ODc} < \text{OD} < 6 \text{ ODc}$), SBP: strong biofilm producer ($6 \text{ ODc} < \text{OD}$).

**Fig. 8** Bar chart illustrating the concentration-dependent inhibitory impact of *Caroxylon volkensii* on the production of violacein pigment by *C. violaceum* (ATCC 12472).

3.11. In vivo antibacterial activity

We elucidated the *in vivo* protective effect of CVM to assess its antibacterial activity *in vivo*. Interestingly, CVM significantly ($p < 0.05$) diminished the killing ability of the treated isolates in comparison with the non-treated ones. The survival curve for the three examined groups was graphed, as depicted in Fig. 9. In group I, all mice survived throughout the entire experiment. In group II, three mice perished after 2 days, another three after 5 days, and the remainder after 8 days. Group III experienced the demise of only one mouse after 6 days, with the remaining mice in group III surviving until the 14th day.

3.12. Docking study

Molecular docking is a popular computational tool in drug discovery. It is most helpful for estimating the interaction of the ligands with target proteins.^{85,86} *Las* is a well-studied quorum system in *P. aeruginosa*. This system orchestrates the biofilm formation in a sophisticated way to ensure the infection establishment in the host.⁸⁷ *LasR* is a transcriptional global activator that regulates the expression of several genes associated with biofilm formation, pathogenicity, and virulence.^{88–90} *LasR* must interact with its inducer, the 3-oxo-C12 derivative of acylhomoserine lactone (AHL), synthesized by Acyl-homoserine-lactone synthase (*LasI*) in order to be activated.⁹¹ In the present investigation, we conducted a molecular docking experiment using AutoDock Vina software to investigate the potential secondary metabolites responsible for the anti-quorum sensing activity of CVM through binding to the *LasR* enzyme. For the docking study, 18 compounds, identified as the main components in CVM by exhibiting the highest peak intensities in the TIC (Fig. S1–S3†) and the highest peak areas as presented in Table S1,† were selected. The peak areas of all the identified compounds, presented in Table S1,† were arranged in descending order, and the first 18 compounds with the highest peak areas were selected for the docking study. Among the tested compounds, tyramine derivatives showed great binding



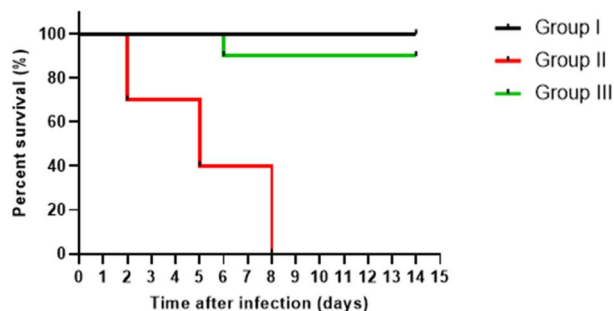


Fig. 9 Kaplan–Meier survival curve of the three groups; group I (non-infected control group, black), group II (infected with *P. aeruginosa* but untreated with CVM, red), and group III (infected with *P. aeruginosa* and treated with CVM, green).

affinity towards *LasR* (Table 3). Moupinamide (**1.22**) showed the highest docking score ($-11.1 \text{ kcal mol}^{-1}$), with a better binding affinity than the co-crystallized ligand, AHL ($-8.723 \text{ kcal mol}^{-1}$). Visualization of the molecular model of binding of moupinamide (**1.22**) and other tyramine derivatives, compounds **1.23** and **1.25**, showed H-bonding with Arg-61, which is important for *LasR* interaction with its auto-inducer.^{92,93} They also exhibited H-bonding with Tyr-93 and Leu-125 and π - π stacking with the Trp-88 amino acid residue in the ligand-binding domain (LBD) of *LasR* (Fig. 10 and S20†). Our predicted anti-quorum sensing activity for the identified tyramine derivatives was in accordance with the findings reported by David *et al.* (2018), which demonstrated tyramine derivatives as quorum sensing inhibitors against *Agrobacterium tumefaciens*.⁹⁴ Ethyl-*p*-digallate (**2.7**) and isorhamnetin (**4.1**) also showed remarkable docking scores of -9.75 and $-9.9 \text{ kcal mol}^{-1}$, respectively, towards *LasR* (Table 3). Ethyl-*p*-digallate (**2.7**) revealed H-bonding with Arg-61, Tyr-93, Thr-75,

and π - π stacking with Trp-88 (Fig. S20†), while isorhamnetin (**4.1**) showed H-bonding with Arg-61, Trp-60, Thr-115, and π - π stacking with Tyr-64 (Fig. S20†). This result is supported by our previous finding of the anti-quorum sensing activity of gallic acid and the flavonoid compound, catechin.⁹⁵

It is important to highlight that tyramine conjugates, which are predicted to be responsible for the anti-quorum sensing potential of CVM, are reported to be present in numerous plant genera.⁹⁶ They are documented to contribute to the growth, development, and defence mechanisms of several plants.⁹⁶ The activity of the enzyme responsible for the biosynthesis of moupinamide (feruloyltyramine, **1.22**) was reported to be increased by 5–8 fold after the infection of *Nicotiana tabacum* with the tobacco mosaic virus.⁹⁷ In recent years, there has been growing interest in tyramine conjugates with phenolic acids, extending from their role in plants to their biological activities and applications in the food industry.^{46,96,98} They have been documented to exhibit antioxidant, anti-cancer, anti-inflammatory, anti-diabetic, and neuroprotective activities.⁹⁶ Moreover, *N-trans*-feruloyltyramine has been reported to exert anti-bacterial activity against *Staphylococcus aureus* with a MIC value of 7.8 mg mL^{-1} , in comparison to the positive control, tetracycline ($125 \text{ } \mu\text{g mL}^{-1}$),⁹⁹ which supports the findings of our docking study. However, additional, in-depth studies are necessary to validate this finding. Many researchers have studied the mass production of tyramine conjugates using both chemical synthesis and bioengineering approaches.⁹⁶ The maximum yield (96.3%) for producing *N-trans*-feruloyltyramine (**1.22**) from ferulic acid and tyramine hydrochloride was attained through a green one-step lipase-catalyzed synthesis.^{96,98} The high production yield of tyramine conjugates encourages further exploration of their antimicrobial activity to be used as potential novel alternatives to traditionally used antibiotics.

Table 3 Docking scores of *Caroxylon volkensii* secondary metabolites against the transcriptional activator receptor (*LasR*) using AutoDock Vina 1.2.3

Compound no.	Peak area	Name	Docking score
1.1	2.69×10^7	Salsolinol	−7.439
1.2	3.06×10^7	Salsoline	−7.605
1.7	1.46×10^9	<i>N</i> -Methyltyramine	−7.467
1.8	4.47×10^8	Hordenine	−7.548
1.11	1.92×10^7	Tyramine- <i>O</i> - β -D-glucoside	−8.204
1.22	3.33×10^7	Moupinamide (<i>N-trans</i> -feruloyltyramine)	−11.100
1.23	2.27×10^7	(<i>E</i>)-3-(4-Hydroxy-3-methoxyphenyl)- <i>N</i> -(4-methoxyphenethyl)acrylamide	−9.565
1.25	1.39×10^7	<i>N-trans</i> -feruloyl 3- <i>O</i> -methyldopamine	−9.762
1.29	5.23×10^7	Allantoic acid ethyl ester	−6.673
2.4	6.46×10^7	Tichocarpol A	−8.437
2.7	2.36×10^7	Ethyl- <i>p</i> -digallate	−9.755
3.4	4.78×10^7	Salsolide C	−8.450
3.5	2.32×10^7	Salsolide E	−8.052
4.1	1.72×10^7	Isorhamnetin	−9.923
4.3	4.71×10^7	Isorhamnetin-3- <i>O</i> -rutinoside (narcissin)	−7.169
6.1	1.38×10^7	9,12,13-Trihydroxyoctadeca-10(<i>E</i>),15(<i>Z</i>)-dienoic acid	−8.101
6.2	2.19×10^8	Plastoquinone 3	−7.180
6.3	1.17×10^9	<i>N</i> -Cyclohexanecarbonylpentadecylamine	−6.845
Co-crystallized ligand	—	3-Oxo-C12 acylhomoserine lactone (AHL)	−8.723



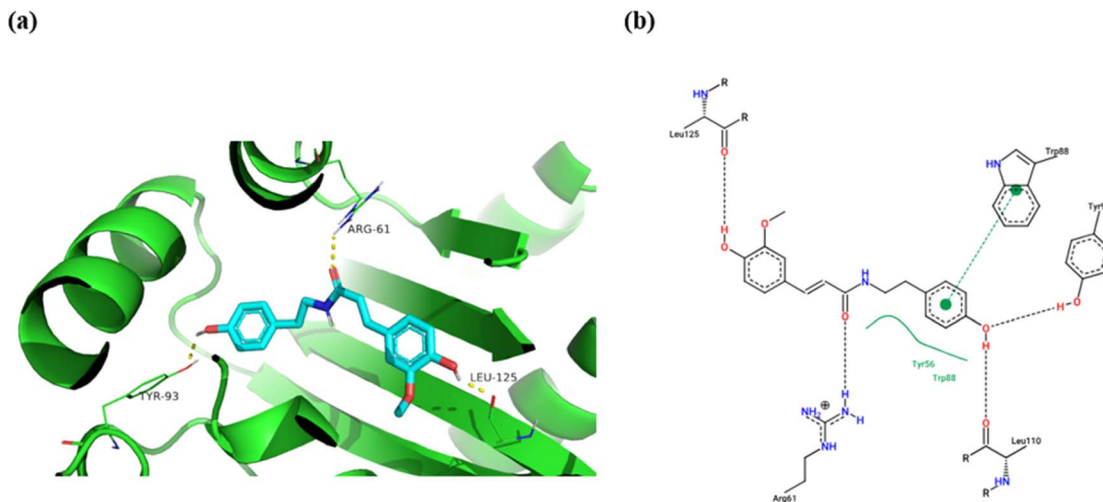


Fig. 10 Molecular binding of moupinamide (1.22) to the transcriptional activator receptor (LasR, PDB code: 2UV0); (a) 3D molecular binding model, (b) 2D molecular binding model demonstrating LasR amino acid residues involved in the interaction.

4. Conclusion

In the current study, the bioactive components of *C. volkensii* have been investigated for the first time, using LC-QTOF-MS/MS analysis. This study has revealed the great chemo-diversity of the plant. It was found to be rich in catecholamines, tyramine derivatives, phenolic acids, triterpenoids, and flavonoids. The obtained results can be most helpful for understanding the potential medicinal applications of *C. volkensii* and its phylogenetic relationship to other plant species in the Old-World *Salsola* and the family Amaranthaceae. Our study has shown that *C. volkensii* could be a potential therapeutic candidate for *P. aeruginosa* infections owing to its anti-biofilm and quorum-quenching potential. Through a molecular docking study, tyramine conjugates, ethyl-*p*-digallate (2.7), and isorhamnetin (4.1) were predicted to be responsible for the anti-quorum sensing inhibitory activity of CVM through binding to the transcriptional global activator, LasR. They can be considered as novel alternatives or adjuncts to the traditionally utilized antibiotics, which suffer from the spread of resistance. Therefore, additional *in vitro* and *in vivo* in-depth studies are necessary to validate these findings. Moreover, further future studies are suggested to elucidate their anti-quorum sensing activity against other bacterial species.

Author contributions

Conceptualization, M. H. E., F. M. A. B., E. E.; methodology, M. H. E., U. R. A., A. A. K., E. E.; investigation, M. H. E., U. R. A., F. M. A. B., G. B., A. A. K., E. E.; data curation, M. H. E., U. R. A.; writing original draft, M. H. E., E. E.; revising and editing the manuscript, M. H. E., U. R. A., F. M. A. B., G. B., A. A. K., E. E.

Conflicts of interest

There are no conflicts to declare.

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