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Original article

UHPLC–MS/MS Metabolite Fingerprinting and Antimicrobial Mechanisms of *Gymnocarpos sclerocephalus* (syn. *Sclerocephalus arabicus*) from the Saharo–Arabian Desert, Libya

Hamed Bogdadi[1], Nowar Bhari[1], Shamsi Saad Shamsi[2*], Mukhtar Almukashir[3], Ketaam Youness[4]

[1]*Department of Botany, Faculty of Science, Sebha University, Sebha, Libya*
[2]*Department of Medical Laboratory, Faculty of Medical Technology, Sebha University, Sebha, Libya*
[3]*Department of Microbiology, Faculty of Science, Sebha University, Sebha, Libya*
[4]*Department of Chemistry, Faculty of Education, Fezzan University, Traghan, Libya*
Corresponding email. *sha.saad@sebhau.edu.ly*

Abstract
The Saharo-Arabian desert shrub Gymnocarpos sclerocephalus (Decne) Dahlgren & Thulin (Caryophyllaceae) has medicinal uses, but its phytochemistry and bioactivity are understudied. This research defines the metabolite fingerprint of G. sclerocephalus, isolates key constituents, and assesses its antimicrobial properties and preliminary safety. Using UHPLC–MS/MS, we tentatively identified 35 metabolites, primarily flavonoid glycosides and triterpene saponins. Vitexin, daphnoretin, and quercetin were isolated. The crude extract showed antimicrobial activity (MICs 128–512 µg/mL), with the ethyl acetate fraction being most potent (MIC 32-64 µg/mL). Daphnoretin was effective against S. aureus and B. subtilis (MIC 64 µg/mL). The ethyl acetate fraction inhibited S. aureus biofilm formation by ~80% and disrupted bacterial membrane integrity. Synergy was observed between the ethyl acetate fraction and erythromycin against S. aureus and between daphnoretin and gentamicin against E. coli. The extract showed low cytotoxicity in human fibroblasts (CC50 ~350 µg/mL) and low toxicity in zebrafish embryos. These findings provide a chemotaxonomic profile for G. sclerocephalus and demonstrate its potential as a source of antimicrobial agents.
Keywords. Gymnocarpos Sclerocephalus, LC–MS/MS Metabolomics, Chemotaxonomy, Antimicrobial Activity.

Introduction

Desert plants survive in extreme environments by synthesizing unique secondary metabolites that confer adaptive advantages, such as stress tolerance and defense against microbes. The Saharo-Arabian desert region, encompassing North Africa (including southern Libya) and the Arabian Peninsula, harbors diverse flora with underexplored phytochemical properties. These harsh habitats often drive the high accumulation of polyphenols, terpenoids, and osmoprotectants, making desert plants promising sources of bioactive compounds.

Gymnocarpos sclerocephalus (Decne) Dahlgren & Thulin – synonym Sclerocephalus arabicus Boiss. Is a perennial woody herb/shrub in the Caryophyllaceae family, found in arid gravelly plains and wadis of the Sahara and Arabia. Locally known as “aûrmîd” in parts of North Africa [1,2], it has a broad regional distribution and is recognized as a medicinal plant in its native range [3]. Ethnobotanical surveys indicate that some Gymnocarpos species are used in traditional remedies for infections and inflammation, although detailed documentation is limited. The congeneric Gymnocarpos decandrus Forssk., a related desert species in Egypt and Libya, is a well-known forage plant for livestock [4] and has recently attracted scientific interest because of its medicinal properties. G. decandrus extracts have demonstrated antidiabetic, anti-inflammatory, and diuretic activities. Notably, G. decandrus inhibits carbohydrate-hydrolyzing enzymes and the Coxsackie B4 virus in vitro [5,6], validating its use in managing diabetes. These bioactivities are attributed to their high polyphenolic content. HPLC-MS/MS metabolite profiling of G. decandrus identified 19 secondary metabolites, including 13 flavonoids, five saponins, and one phenolic acid, with several compounds (e.g., C-glycosyl flavones) reported for the first time in this genus [6]. These findings underscore the accumulation of diverse phytochemicals (flavonoids, saponins, etc.) in Gymnocarpos spp., which are common in Caryophyllaceae [5].

Phytochemically, Caryophyllaceae family members (e.g., Silene, Herniaria, and Dianthus) are known to produce flavone and flavonol glycosides, triterpenoid saponins, and ecdysteroid compounds [7]. For example, flavonoid C-glycosides (derivatives of apigenin and luteolin) and oleanane-type saponins are abundant in Silene species [7,8]. Until recently, coumarins have rarely been reported in this family. A breakthrough study isolated bis-coumarin daphnoretin and biflavonoids wikstrol A and B from G. decandrus roots [9]. These are the first coumarin and biflavonoid metabolites documented in Caryophyllaceae [9], suggesting that Gymnocarpos may possess a unique chemical profile within its family. This chemotaxonomic insight suggests that G. sclerocephalus could similarly harbor novel or significant compounds, meriting comprehensive metabolomic investigation.

Multidrug-resistant (MDR) microbial infections are a rising global threat, prompting the search for new antimicrobials and antibiotic adjuvants. Plants lacking advanced immune systems have evolved complex chemical defenses against pathogens [10]. Polyphenols, such as flavonoids, can inhibit a broad spectrum of

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microbes through multiple mechanisms, such as disrupting bacterial membranes and cell walls, inhibiting biofilm formation and efflux pumps, and synergizing with conventional antibiotics [10]. In the context of desert plants, these compounds may also protect against environmental microbes in the soil and on plant surfaces. Given reports that Gymnocarpos extracts exhibit antimicrobial effects (e.g., G. decandrus root extract significantly inhibited *Bacillus subtilis* growth) [11], G. sclerocephalus is hypothesized to possess antibacterial and antifungal activities that can be mechanistically elucidated. Indeed, the traditional use of G. sclerocephalus and allied species for treating infections, although anecdotal, aligns with the presence of such bioactive metabolites.

This study aimed to profile the secondary metabolite composition of *Gymnocarpos sclerocephalus* using LC–MS/MS metabolomics, isolate major constituents from its extracts, evaluate the antimicrobial spectrum of the crude extract, fractions, and purified compounds, investigate the modes of antimicrobial action (bactericidal kinetics, antibiofilm and membrane effects, and synergy with antibiotics), and assess safety aspects, including cytotoxicity and toxicity/irritation indicators. By integrating these facets, we sought to provide a comprehensive understanding of the phytochemical fingerprint and biological activities of G. sclerocephalus and to discuss the results in the context of chemotaxonomy and the potential of desert Caryophyllaceae as sources of new antimicrobial agents.

Methods

Plant Material and Identification

Aerial parts of *Gymnocarpos sclerocephalus* (Decne.) Dahlgren and Thulin were collected in spring from arid habitats near Sebha City, southwestern Libya (approximately 27°02′N 14°26′E, altitude ~450 m). The habitat is a Saharan desert scrub with a hyper-arid climate. Plant identification was performed by a botanist, and a Voucher specimen (voucher no. GSC-2025). The accepted taxonomy of this plant is *Gymnocarpos sclerocephalus* (family Caryophyllaceae), with the synonym *Sclerocephalus arabicus* Boiss . (as per the regional flora).

Extraction and Fractionation

The collected plant material was shade-dried (<40°C) and ground into a fine powder. The dried powder (500 g) was exhaustively extracted by maceration in 70% ethanol (3 × 2 L, 48 h each, room temperature). The combined ethanol extracts were filtered and evaporated under reduced pressure at 40°C to yield a crude hydroalcoholic extract (yield: 8.5% w/w). The crude extract (40 g) was resuspended in distilled water (200 mL) and sequentially partitioned with n-hexane (3 × 200 mL), dichloromethane (3 × 200 mL), ethyl acetate (3 × 200 mL), and n-butanol (3 × 200 mL) to fractionate the compounds by polarity. The solvents were removed under vacuum to obtain the following fractions: hexane (F1, 5.2 g), dichloromethane (F2, 2.1 g), ethyl acetate (F3, 7.8 g), n-butanol (F4, 10.4 g), and the remaining aqueous fraction (F5, 12.0 g). Preliminary phytochemical tests indicated that F3 and F4 were rich in polyphenols and saponins (positive ferric chloride and froth tests, respectively), whereas F1 contained mainly non-polar terpenoids (oils and sterols). The fractions were stored at 4°C.

Bioassay-guided isolation was undertaken on the most active fraction (F3, see Results). F3 (7.5 g) was subjected to column chromatography on silica gel, eluted with a gradient of chloroform–methanol (100:0 to 0:100) to yield subfractions F3-A through F3-G. These were monitored by TLC (silica, solvent $CHCl_3$–MeOH 9:1, visualized under UV and by vanillin–sulfuric acid reagent) and tested for antimicrobial activity (agar diffusion assay). Two subfractions (F3-D and F3-E) with strong antibacterial activity were further purified using repeated column chromatography and semi-preparative HPLC. This led to the isolation of compound 1 (15 mg, yellow amorphous powder) and compound 2 (9 mg, colorless needles), along with a smaller amount of compound 3 (4 mg). The compounds were identified by ^1H and ^13C NMR, MS, and by comparison with literature data. Compound 1 was identified as vitexin (apigenin 8-C-β-D-glucoside), Compound 2 as daphnoretin (a biscoumarin), and Compound 3 as quercetin (3,5,7,3′,4′-pentahydroxyflavone).

LC–MS/MS Metabolite Profiling

Untargeted metabolomic analysis of the crude extract was performed using ultra-high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HR-MS/MS). An aliquot of the extract (5 mg/mL in 70% MeOH) was filtered (0.22 µm), and 5 µL was injected into a UHPLC system (Agilent 1290) interfaced with a quadrupole-time-of-flight mass spectrometer (Maxis Q-TOF, Bruker) equipped with an electrospray ionization source. Chromatographic separation was performed using a C18 column (100 mm × 2.1 mm, 1.7 µm) at 40°C, with a gradient mobile phase of 0.1% formic acid in water (A) and acetonitrile (B) from 5% to 95% B over 30 min at 0.3 mL/min. Data were acquired in both positive and negative ion modes (scan range of m/z 100–1500). Automated data-dependent MS/MS fragmentation was enabled (collision energy: 20–35 eV).

Raw data were processed for feature detection, alignment, and dereplication. Putative identification of metabolites was achieved by comparing accurate masses, isotopic patterns, and MS^2 spectra against databases (METLIN and MassBank). Fragmentation patterns were interpreted to elucidate the aglycone-sugar relationships for flavonoids (loss of 162 amu for hexose, etc.). Tentative identifications were assigned

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confidence levels according to the Metabolomics Standards Initiative guidelines (level 2: probable structure match by library or literature).

Antimicrobial Assays

Microorganisms: Test strains were obtained from clinical isolates and included Gram-positive bacteria (Staphylococcus aureus 25923, *Bacillus subtilis* 6633), Gram-negative bacteria (Escherichia coli 25922, Pseudomonas aeruginosa 27853), and a yeast (Candida albicans 10231). Cultures were grown in Mueller–Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth for C. albicans.

Minimum Inhibitory Concentration (MIC): MICs of the crude extract, fractions, and isolated compounds were determined using the broth microdilution method (CLSI guidelines). Two-fold serial dilutions of each sample (initial concentration 1024 µg/mL for extracts/fractions and 512 µg/mL for pure compounds) were prepared in 96-well plates (in MHB or SDB). Microbial suspensions (0.5 McFarland, ~10^6 CFU/mL) were added, and the plates were incubated at 37°C (24 h for bacteria and 48 h for Candida). The MIC was defined as the lowest concentration with no visible growth. Minimum bactericidal/fungicidal concentrations (MBC/MFC) were determined by subculturing 10 µL of the clear wells onto agar plates. All assays were performed in triplicate. Gentamicin (for bacteria) and nystatin (for Candida) served as positive controls, and 1% DMSO served as a solvent control (showing no inhibition) [12].

Time–Kill Kinetics: Time–kill studies were conducted for selected treatments against S. aureus (as a representative sensitive pathogen) following standard methods. Briefly, exponentially growing S. aureus (~10^6 CFU/mL) in MHB was exposed to compound 2 (daphnoretin) at concentrations of 1× and 4× its MIC. At 0, 2, 4, 8, and 24 h intervals, samples were taken, diluted in PBS, and spread on nutrient agar for colony counting after 24 h of incubation. Time–kill curves (log_10 CFU/mL vs time) were plotted. Bactericidal activity was defined as a ≥3 log_10 CFU/mL reduction from the initial inoculum. Control growth (no drug) was performed in parallel [13].

Antibiofilm Activity: The ability of G. sclerocephalus extracts to prevent biofilm formation was evaluated using S. aureus and P. aeruginosa. Overnight cultures were diluted in tryptic soy broth (supplemented with 1% glucose for S. aureus) to a concentration of approximately 10^6 CFU/mL. Aliquots were added to 96-well polystyrene plates with or without sub-MIC concentrations of the test samples (e.g., 1/4× and 1/2× MIC of the ethyl acetate fraction and isolated flavonoids). After incubation (24 h, 37°C), planktonic cells were removed, and the biofilms were gently washed with PBS. Biofilm biomass was quantified using crystal violet staining. Briefly, 0.1% crystal violet was added for 15 min, excess stain was rinsed off, and the bound dye was solubilized in 30% acetic acid. The absorbance was measured at 590 nm. The percentage inhibition of biofilm formation was calculated relative to the untreated control biofilm. Additionally, pre-formed 24 h biofilms were treated with the samples for another 24 h to assess biofilm eradication by measuring residual viable biofilm cells using the XTT metabolic assay. All biofilm assays were performed in triplicate [14].Membrane Integrity Assay: To probe membrane-disruptive action, a propidium iodide (PI) uptake assay was used on S. aureus. PI is a DNA-binding dye that penetrates only cells with compromised cell membranes. S. aureus mid-log cultures (10^7 CFU/mL in PBS) were treated with the Gymnocarpos extract, fractions, or compounds at 1× MIC for 1 hour. Syto9 (green-fluorescent nucleic acid stain for live cells) and PI (red-fluorescent for dead cells) were added (from the Live/Dead BacLight kit, Invitrogen). After 15 min in the dark, the samples were analyzed using fluorescence microscopy and measured using a fluorescence plate reader (ex/em 480/620 nm for PI). The percentage of PI-positive cells was calculated. Untreated bacteria (negative control, low PI uptake) and bacteria heated at 70°C for 10 min (positive control for complete membrane damage) were included for baseline comparison. In addition, the leakage of intracellular contents was assessed by measuring the release of 260 nm-absorbing materials (nucleic acids) into the supernatant of E. coli treated with and without the extract (using a spectrophotometer).

Synergy Testing: The combined effect of G. sclerocephalus extract with standard antibiotics was examined using the checkerboard microdilution method. Two combinations were tested based on preliminary indications of mechanism: (1) the ethyl acetate fraction with erythromycin against S. aureus (to investigate efflux pump inhibition or membrane permeability effects that might re-sensitize Gram-positive bacteria), and (2) daphnoretin with gentamicin against E. coli (to test if compounds facilitate aminoglycoside uptake in Gram-negatives). Serial two-fold dilutions of the plant sample and antibiotic were prepared across wells in a 96-well grid such that each row had a fixed fraction of the antibiotic MIC and each column had a fixed fraction of the plant extract MIC. After adding the bacterial inoculum and incubation, the fractional inhibitory concentration index (FIC) was calculated as FIC_A + FIC_B, where FIC_A = MIC_A in combination / MIC_A alone, and similarly for B. An FIC_I ≤ 0.5 was interpreted as synergistic, >0.5–1 as additive, >1–4 indifferent, and >4 antagonistic. All tests were performed in duplicate.

Cytotoxicity Assay

The cytotoxicity of the crude extract and major isolated compounds was evaluated using a mammalian cell line to gauge selective toxicity. Human skin fibroblasts (HFF-1) were cultured in DMEM with 10% fetal bovine serum. Cells were seeded into 96-well plates (~1×10^4 cells/well) and allowed to adhere for 24 h. Extracts and compounds were added (seven concentrations from 1 to 500 µg/mL, in triplicate) and incubated for

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48 h. Cell viability was determined using the MTT assay [7]. Briefly, 0.5 mg/mL MTT reagent was added for 2 h, the medium was removed, and the purple formazan product was dissolved in DMSO. The absorbance at 570 nm (reference 630 nm) was measured using a microplate reader. The percentage viability relative to the untreated control was calculated. The concentration that reduced viability by 50% (CC_50) was estimated from dose–response curves. Additionally, lactate dehydrogenase (LDH) release was measured in the culture supernatants (CytoTox 96 kit) to detect membrane damage at the highest concentrations.

Zebrafish Embryo Toxicity

Embryonic zebrafish (Danio rerio) assays were performed as an in vivo toxicity model. Zebrafish eggs (wild-type strain) were collected and synchronized at the 1–4 cell stage. Healthy embryos (n=10 per group) were transferred to 24-well plates (one embryo per well in 2 mL embryo medium). *G. sclerocephalus* extract was added at concentrations of 0 (control), 10, 50, 100, and 200 µg/mL (0.5% DMSO as carrier). The embryos were incubated at 28°C in a 14:10 h light: dark cycle. Outcomes were monitored at 24, 48, 72, and 96 h post-fertilization (hpf): embryo coagulation (mortality), hatching success, and morphological abnormalities (edema, spinal deformities, etc.) were recorded under a stereomicroscope. Heart rate was measured at 48 hpf as a sublethal toxicity indicator. The maximum non-lethal concentration (MNLC) was determined. All experiments adhered to the ethical guidelines for animal use.

Data Analysis

Unless otherwise noted, all experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD). For quantitative assays (biofilm inhibition, viability, etc.), statistical significance between treated and control groups was determined by one-way ANOVA with Tukey's post hoc test (using GraphPad Prism v8), with $p<0.05$ considered significant. The synergy checkerboard was interpreted as described above, without statistical testing. All graphs were plotted using Prism.

Results

Phytochemical Profile by LC–MS/MS

Untargeted LC–MS/MS analysis of *Gymnocarpos sclerocephalus* extract detected ≥57 peaks in the total ion chromatograms within a retention time of 5–25 min. A set of 35 major metabolites was tentatively annotated in the LC–MS/MS dataset. The detected metabolites included flavonoid glycosides, phenolic acids/other phenolics, triterpene saponin–like features, and coumarin/biflavonoid constituents (Table 1).

Flavonoids were the most abundant class, with approximately 18 assigned peaks, comprising flavone and flavonol glycosides. Vitexin (apigenin 8-C-glucoside; $[M–H]^-$ m/z 431.097) was detected as a major constituent. C-glycosyl flavone features consistent with isovitexin and orientin were annotated using diagnostic fragment losses of 120 and 90 amu, respectively. A prominent peak at RT 7.1 min with m/z 609.146 ($[M+H]^+$) was identified as rutin, producing fragment ions at m/z 303. Isoquercitrin and cynaroside were annotated using a characteristic glucose loss of 162 amu.

A metabolite detected at RT of 22.5 min with $[M–H]^-$ m/z 367.066 was assigned to daphnoretin; its MS/MS spectrum included a fragment at m/z 183. Two peaks at RT ~19–20 min with $[M–H]^-$ m/z ~537 were annotated as wikstrol A, and wikstrol B. Protocatechuic acid ($[M–H]^-$ m/z 153.019) was detected with an MS/MS fragment at m/z = 109. The minor phenolic acids included caffeic acid (m/z 179) and ferulic acid (m/z 193). Primary metabolite ions, including sucrose (m/z 341), were observed.

Multiple high-mass features in the negative mode (approximately m/z 1200–1500) showed sequential sugar-loss patterns (162 amu) and aglycone-related ions. One abundant ion at $[M–H]^-$ m/z 1255 fragmented to m/z 1089 and 925 and produced an aglycone-related fragment at m/z 503. Approximately six saponin-like features were detected.

Table 1. LC–MS/MS metabolite fingerprint of G. sclerocephalus extract: Key classes and representative marker features.

Class	Representative marker / annotated feature	RT (min)	Precursor ion (mode)	MS/MS diagnostic information
Flavonoid C-glycoside	Vitexin (apigenin 8-C-glucoside)	—	431.097 ($[M–H]^-$)	C-glycoside fragmentation; major constituent
Flavonoid C-glycoside	Isovitexin (tentative)	—	—	Diagnostic neutral losses 120 and 90 amu
Flavonoid C-glycoside	Orientin (tentative)	—	—	Diagnostic neutral losses 120 and 90 amu
Flavonol O-glycoside	Rutin (quercetin-3-O-rutinoside)	7.1	609.146 ($[M+H]^+$)	Fragment ion 303 (aglycone)
Flavonol O-glycoside	Isoquercitrin (tentative)	—	—	Neutral loss 162 amu (glucose)
Flavone O-glycoside	Cynaroside (tentative)	—	—	Neutral loss 162 amu (glucose)

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Bis-coumarin	Daphnoretin	22.5	367.066 ([M–H]⁻)	Fragment ion 183
Biflavonoids	Wikstrol A / B (tentative)	~19–20	~537 ([M–H]⁻)	Assigned as biflavonoids (two flavone units)
Phenolic acid	Protocatechuic acid	—	153.019 ([M–H]⁻)	Fragment ion 109 (loss of CO_2)
Phenolic acid	Caffeic acid	—	179 ([M–H]⁻)	Minor feature
Phenolic acid	Ferulic acid	—	193 ([M–H]⁻)	Minor feature
Saponin-like feature	Example high-mass saponin feature	—	1255 ([M–H]⁻)	1089 (−Glc), 925 (−2Glc), aglycone-related 503
Primary metabolite ion	Sucrose ion	—	341	Observed (not emphasized)

Antimicrobial Activity of Extracts and Fractions

The antimicrobial activity of the crude extract and solvent fractions against the bacterial test organisms is shown in Table 2. The crude hydroalcoholic extract showed MICs of 256 µg/mL against S. aureus and 128 µg/mL against B. subtilis, respectively. The MICs against E. coli and P. aeruginosa were 512 µg/mL. Fractionation yielded differential activities across the fractions. The ethyl acetate fraction (F3) showed MICs of 64 µg/mL against S. aureus, 32 µg/mL against B. subtilis, and 256 µg/mL against both E. coli and P. aeruginosa. The n-butanol fraction (F4) showed MICs of 128–256 µg/mL against S. aureus and B. subtilis, and 512 µg/mL against E. coli and P. aeruginosa. The hexane (F1) and DCM (F2) fractions exhibited MICs >512 µg/mL against the bacterial panel. The aqueous fraction (F5) showed no measurable inhibitory activity against the bacterial panel within the tested range.

Table 2. MICs (µg/mL) of G. sclerocephalus crude extract and solvent fractions against bacterial test organisms.

Sample	S. aureus	B. subtilis	E. coli	P. aeruginosa
Crude extract (70% EtOH)	256	128	512	512
F1 (hexane)	>512	>512	>512	>512
F2 (DCM)	>512	>512	>512	>512
F3 (EtOAc)	64	32	256	256
F4 (n-BuOH)	128–256	128–256	512	512
F5 (aqueous)	No activity detected	No activity detected	No activity detected	No activity detected

Antimicrobial Activity of Isolated Compounds

The MICs of vitexin and daphnoretin against the bacterial panel are presented in (Table 3). Vitexin showed MICs of 128 µg/mL against S. aureus and 256 µg/mL against B. subtilis, and >512 µg/mL against E. coli. Daphnoretin showed MICs of 64 µg/mL against S. aureus and B. subtilis and 256 µg/mL against E. coli. Quercetin had an MIC of 256 µg/mL against S. aureus.

Table 3. MICs (µg/mL) of vitexin and daphnoretin against the bacterial test organisms.

Compound	S. aureus	B. subtilis	E. coli
Vitexin (Compound 1)	128	256	>512
Daphnoretin (Compound 2)	64	64	256

Yeast Susceptibility and Positive Control

The crude extract inhibited Candida albicans with an MIC of 256 µg/mL. Daphnoretin inhibited C. albicans with a MIC of 128 µg/mL. Nystatin exhibited an MIC of 4 µg/mL and MFC of 8 µg/mL (Table 4).

Table 4. Antifungal susceptibility of C. albicans and nystatin control.

Agent	MIC (µg/mL)	MFC (µg/mL)
Crude extract (70% EtOH)	256	Not fungicidal at the highest tested concentration
Daphnoretin (Compound 2)	128	Not fungicidal at the highest tested concentration
Nystatin (control)	4	8

Bactericidal Activity (MBC)

For S. aureus, the crude extract showed an MBC of 512 µg/mL. The ethyl acetate fraction (F3) exhibited an MBC of 128 µg/mL. Daphnoretin exhibited an MBC of 128 µg/mL. Vitexin showed bactericidal activity at 4× MIC (Table 5).

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Table 5. Bactericidal endpoints (MBC) for S. aureus.

Treatment	MIC (µg/mL)	MBC endpoint (µg/mL)
Crude extract	256	512
F3 (EtOAc)	64	128
Daphnoretin (Compound 2)	64	128
Vitexin (Compound 1)	128	4× MIC

Kinetics of Bacterial Killing

The time–kill analysis of S. aureus treated with daphnoretin is summarized in (Table 6). At 1× MIC (64 µg/mL), viable counts decreased by approximately ~1 log10 at 4 h and returned to near the baseline by 24 h. At 4× MIC (256 µg/mL), viable counts decreased by 3.5 log10 within 4 h, with no detectable viable bacteria after 8 h and no growth at 24 h (detection limit: 10 CFU/mL). The crude extract at 4× MIC killed approximately 99.9% of cells within 24 h. Vitexin showed a plateau of approximately 1–2 log10 kill at 4× MIC.

Table 6. Time–kill kinetics against S. aureus

Agent	Exposure	4 h outcome	8 h outcome	24 h outcome
Daphnoretin	1× MIC (64 µg/mL)	~1 log10 decrease	—	Regrowth to near baseline
Daphnoretin	4× MIC (256 µg/mL)	3.5 log10 decrease	No detectable CFU	No detectable CFU (DL 10 CFU/mL)
Crude extract	4× MIC	—	—	~99.9% kill
Vitexin	4× MIC	1–2 log10 decrease	—	Plateau (1–2 log10 kill)

Inhibition of Biofilm Formation

Biofilm formation assays showed that the ethyl acetate fraction (F3) reduced S. aureus biofilm biomass by approximately ~80% at 1/2× MIC (32 µg/mL) and by ~50% at 1/4× MIC (16 µg/mL) relative to untreated controls (crystal violet quantification). For P. aeruginosa, F3 at 1/2× MIC (128 µg/mL) reduced biofilm biomass by approximately 60%.

For isolated compounds, daphnoretin at 1/2× MIC (32 µg/mL) reduced S. aureus biofilm formation by approximately ~70%, whereas vitexin produced approximately ~40% inhibition at an equivalent relative exposure level. For pre-formed (24 h) S. aureus biofilms, exposure to the Gymnocarpos fraction or daphnoretin at 4× MIC for 24 h reduced biofilm viability by approximately ~1 log.

Effects on Bacterial Cell Membranes

The membrane integrity assays are summarized in (Table 7). Live/Dead staining of untreated S. aureus showed <5% PI-positive cells. Following exposure to F3 at MIC (64 µg/mL) for 1 h, approximately ~50% of the cells were PI-positive; at 2× MIC, approximately ~85% of the cells were PI-positive. Daphnoretin at 64 µg/mL produced approximately ~70% PI-positive cells after 1 h, while vitexin at 64 µg/mL produced approximately ~20% PI-positive cells after 1 h (Table 7). Nucleic acid leakage from E. coli increased after exposure to the extract at 2× MIC for 2 h, with supernatant absorbance at 260 nm increasing by ~ ~3-fold relative to the control (Table 7).

Antibiotic Synergy

The checkerboard assay results are summarized in Table 7. For S. aureus, the combination of F3 with erythromycin produced an FIC index (FIC_I) of 0.5 at a condition where F3 was applied at 1/4× MIC and erythromycin at 1/4× MIC. Under these conditions, the erythromycin MIC decreased from 0.5 µg/mL to 0.125 µg/mL (Table 7). For E. coli, gentamicin alone showed an MIC of 1 µg/mL, and daphnoretin alone showed an MIC of 256 µg/mL. In combination, gentamicin MIC decreased to 0.25 µg/mL when daphnoretin was applied at 64 µg/mL; the calculated FIC components were FIC_G = 0.25 and FIC_D = 0.25, resulting in FIC_I = 0.50 (Table 7).

Cytotoxicity and Zebrafish Toxicity

The safety endpoints are summarized in (Table 7). In HFF-1 fibroblasts, crude extract viability remained >90% at concentrations of up to 100 µg/mL. The crude-extract CC_50 was approximately 350 µg/mL. At 500 µg/mL, viability was approximately ~60%, and lactate dehydrogenase (LDH) release increased. No morphological changes were observed at concentrations ≤200 µg/mL (Table 7). The compound-level cytotoxicity endpoints included daphnoretin CC_50 ~200 µM (~80 µg/mL) and vitexin CC_50 >300 µM (>150 µg/mL). The selectivity indices reported in the Results section were ~5.5 for crude extract and ~2.5 for daphnoretin (Table 7).

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In zebrafish embryos, exposure to up to 100 µg/mL produced mortality of 0–10% at 96 hpf, compared with 0% in controls (not statistically significant). At 200 µg/mL, 20% of embryos failed to hatch by 72 hpf, and some larvae showed pericardial edema and a curved tail axis; the heart rate was approximately ~10% lower than that of the control at 48 hpf. Mortality did not reach 50% at 200 µg/mL, and the LC_50 was extrapolated to be >200 µg/mL. No neurobehavioral changes (touch response) were observed at the sublethal doses (Table 7).

Table 7. Membrane integrity, antibiotic synergy, and safety endpoints of G. sclerocephalus extract/fraction/compounds.

Result Domain	Assay/Endpoint	Organism/Model	Treatment (Dose; Time)	Outcome
Membrane Integrity	Live/Dead (PI uptake)	S. aureus	Untreated (Control)	<5%
	Live/Dead (PI uptake)	S. aureus	F3 (EtOAc) (at 1×MIC)	~50%
	Live/Dead (PI uptake)	S. aureus	F3 (EtOAc) (at 2×MIC)	>85%
	Live/Dead (PI uptake)	S. aureus	Daphnoretin (at 2×MIC)	~70%
	Live/Dead (PI uptake)	S. aureus	Vitexin (at 2×MIC)	~20%
	Nucleic-acid leakage (A260)	E. coli	Crude Extract (at X µg/mL)	~3-fold increase
Antibiotic Synergy	Checkerboard (FIC_I)	S. aureus	F3 + Erythromycin	FIC_I = 0.50
	MIC shift (erythromycin)	S. aureus	With subinhibitory F3	0.5 → 0.125 µg/mL
	Checkerboard (FIC_I)	E. coli	Gentamicin + Daphnoretin	FIC_I = 0.50
	MIC shift (gentamicin)	E. coli	With Daphnoretin	1 → 0.25 µg/mL
Cytotoxicity	Fibroblast viability	HFF-1	Crude Extract (≤ 100 µg/mL)	>90%
	CC_50	HFF-1	Crude Extract	~350 µg/mL
	High-dose viability + LDH	HFF-1	Crude Extract (at 200 µg/mL)	~60% viability
	Morphology observation	HFF-1	Crude Extract (≤ 100 µg/mL)	No changes observed
	CC_50	HFF-1	Daphnoretin	~200 µM (~80 µg/mL)
	CC_50	HFF-1	Vitexin	>300 µM (>150 µg/mL)
Selectivity Index	SI (CC50 / MIC)	—	Crude Extract; Daphnoretin	SI ≈ 5.5 (dimensionless)
Zebrafish Toxicity	Mortality at 96 hpf	Zebrafish embryos	Crude Extract (≤ 100 µg/mL)	0–10%
	Hatch failure	Zebrafish embryos	Crude Extract (at 200 µg/mL)	~20%
	Developmental findings	Zebrafish embryos	Crude Extract (at 200 µg/mL)	Pericardial edema; curved tail
	Heart rate change	Zebrafish embryos	Crude Extract (at 100 µg/mL)	~10% reduction
	LC_50	Zebrafish embryos	Crude Extract	> 200 µg/mL
	Touch response	Zebrafish embryos	Crude Extract (≤ 100 µg/mL)	No neurobehavioral changes

Discussion

Our study provides an integrated chemical–biological profile of *Gymnocarpos sclerocephalus* by aligning LC–MS/MS metabolite fingerprinting with bioassay-guided fractionation and mechanism-oriented microbiology readouts. The LC–MS/MS data showed a metabolite space dominated by flavonoid glycosides, which is consistent with the broader chemotaxonomic signal reported for Caryophyllaceae, where C- and O-glycosylated flavones and flavonols are recurrent and informative at the genus/family level [15]. In parallel, the detection of multiple high-mass saponin-like features agrees with the established occurrence of triterpenoid saponins in Caryophyllaceae and related lineages, supporting the interpretation that amphiphilic glycosides contribute to the chemical defense profile of desert-adapted taxa [16]. Within

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Gymnocarpos, prior metabolomics/isolation work has indicated that phenolics (including flavonoids and small phenolic acids) are prominent constituents [4], and the present dataset extends this pattern for G. sclerocephalus collected near Sebha by providing a coherent "class-level" fingerprint (flavonoids, phenolic acids, saponin-like features) anchored by diagnostic ions and MS/MS behavior.

A key outcome was the recovery of coumarin/biflavonoid–type signals alongside abundant flavonoid glycosides. The annotation of daphnoretin and wikstrol-like biflavonoids positions G. sclerocephalus closer to the specialized polyphenolic chemistry previously reported from Gymnocarpos congeners, where daphnoretin and wikstrols were isolated/characterized and proposed as distinctive constituents at the genus level [17]. In this context, the simultaneous presence of common Caryophyllaceae-type flavonoid glycosides and relatively uncommon coumarin/biflavonoid signatures supports the use of combined metabolite-class fingerprints and marker features as practical chemotaxonomic evidence when evaluating population-level variation across Saharo-Arabian ranges.

The antimicrobial outcomes align with the fractionation logic and the known structure–activity tendencies of plant phenolics. The enrichment of activity in the ethyl acetate fraction is consistent with the concentration of mid-polar polyphenols and related constituents typically responsible for growth inhibition in bacterial assays [18]. The compound-level activity pattern—daphnoretin showing stronger antibacterial performance than vitexin against the Gram-positive panel—fits the broader observation that flavonoids often act as moderate inhibitors alone but can meaningfully contribute to multi-component effects when concentrated, combined, or paired with other membrane-active constituents [5]. The antibiofilm findings further strengthen the functional relevance of the phenolic space, since both vitexin and quercetin-class flavonoids have been reported to interfere with staphylococcal surface properties and biofilm-associated phenotypes in controlled studies, supporting the plausibility of the measured reductions in biomass under sub-inhibitory exposures [7,8]. Given that microtiter crystal-violet workflows are widely used to quantify early biofilm biomass across diverse organisms, the assay choice is also methodologically aligned with standard biofilm quantification practice [6].

Mechanism-oriented readouts in this study converge on membrane compromise as an important proximal event, particularly for the most active fraction/compound. Propidium iodide (PI) uptake is a standard indicator of permeability changes in Gram-positive bacteria and is frequently applied to distinguish membrane permeabilization kinetics under antimicrobial exposure [9]. The observed increases in PI-positive populations, together with leakage endpoints, are therefore consistent with a model in which concentrated fraction constituents and daphnoretin-rich conditions accelerate envelope disruption, whereas flavonoid-only conditions are comparatively weaker or require higher exposures to produce similar permeability shifts. These mechanistic data provide a direct bridge between "which chemical space is enriched by fractionation" and "which cellular target class is perturbed," which is essential when positioning desert plant metabolites as leads, rather than descriptive bioactivity signals.

The synergy results were also consistent with the mechanistic direction of the dataset. Interpretation of checkerboard interactions is commonly grounded in fractional inhibitory concentration (FIC) indexing, with FIC thresholds frequently used to operationalize synergy in antimicrobial combination testing[110]. The obsrvation of F3 with erythromycin against S. aureus is consistent with the known ability of plant secondary metabolites to modulate bacterial susceptibility, including permeability shifts or interference with resistance-enabling processes [18]. More broadly, medicinal-plant synergy frameworks have precedent in well-characterized systems where a "non-antibacterial" metabolite potentiates an antibacterial alkaloid by inhibiting efflux, demonstrating that plant chemistry can yield resistance-modifying functionality rather than only direct killing [19]. The daphnoretin–gentamicin interaction against E. coli is consistent with a permeability-enabled uptake interpretation, as aminoglycoside efficacy is strongly influenced by barrier and transport constraints, and membrane perturbation can reduce the effective antibiotic requirement.

Finally, the safety endpoints of this study provide an initial translational frame by placing antimicrobial-effective concentrations alongside mammalian cell viability and zebrafish embryo tolerance windows. While these models do not substitute for in vivo pharmacology, the combined selectivity and acute developmental screens help define whether the observed antimicrobial and antibiofilm actions occur at concentrations plausibly separable from overt cytotoxicity during early stage assessment. Taken together, the study positions G. sclerocephalus as a chemotaxonomically informative Saharo-Arabian Caryophyllaceae species whose metabolite fingerprint (flavonoid glycosides + saponin-like features + genus-linked coumarin/biflavonoid signals) can be linked to measurable antimicrobial, antibiofilm, membrane-permeabilizing, and antibiotic-potentiating behaviors, providing a rational basis for subsequent targeted isolation and mode-of-action refinement in desert populations.

Conclusion

This study demonstrates that *G.sclerocephalus* from the Saharo-Arabian desert possesses a diverse array of secondary metabolites and notable antimicrobial activities. LC–MS/MS metabolomic fingerprinting and compound isolation revealed that the plant's chemistry is dominated by flavonoid glycosides, triterpenoid saponins, and coumarin derivatives, reflecting both common Caryophyllaceae traits and unique genus-specific metabolites. These compounds collectively confer antibacterial and antifungal effects, which are

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most pronounced against gram-positive bacteria. The crude extract, especially its polyphenol-rich fraction, showed bacteriostatic to bactericidal action, which is partially attributable to membrane-disrupting mechanisms. We provided evidence that G. sclerocephalus metabolites not only inhibit planktonic microbes but also prevent biofilm formation and can synergize with conventional antibiotics, features that are highly advantageous in addressing persistent and drug-resistant infections.

In terms of safety, *G. sclerocephalus* extract exhibited a reasonable therapeutic margin, with low cytotoxicity to mammalian cells and minimal toxicity in a zebrafish model. Although plants concentrate calcium oxalate crystals as a physical defense, proper extract preparation can mitigate irritation risks. The findings of this study support the traditional medicinal use of G. sclerocephalus and encourage further research on its bioactive principles. G. sclerocephalus emerges as a promising source of natural antimicrobial agents, potentially useful as standalone therapeutics or as adjuvants to existing antibiotics. Moreover, the chemotaxonomic knowledge gained enriches our understanding of the distribution of secondary metabolites in desert plant lineages in the family.

Overall, this study underscores the value of desert plants, such as *Gymnocarpos sclerocephalus*, in drug discovery and provides a scientific basis for their safe medicinal application. Future in vivo efficacy studies and compound development are warranted to fully harness the observed antimicrobial potential. By integrating metabolomic profiling with pharmacological assays, we established a foundation for G. sclerocephalus as a notable addition to the pharmacopeia of arid-zone medicinal flora.

Conflict of interest. Nil

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