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ORIGINAL ARTICLE

An experimental and *in silico* study on the antifungal activity of guaianolides isolated from *Centaurea polypodiifolia* Boiss. against resistant *Candida* strains

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Abstract: The emergence of multidrug-resistant *Candida* strains especially, *Candida auris*, *C. dubliniensis*, and *C. inconspicua* has accelerated the research on antifungal novel molecules. Sesquiterpene lactones, abundant in the Asteraceae family, have drawn attention with their antifungal activity. Therefore, three extracts obtained from the aerial part of Turkish endemic *Centaurea polypodiifolia* were investigated against *Candida albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. dubliniensis* (NCPF 3949a), *C. inconspicua* (NCPF 8523), and *C. auris* (NCPF 8977) and gave MIC values between 6.25 and 12.5 µg/mL. Dichloromethane (DCM-M) extract yielded five known (cynaropicrin, cynarinin A, aguerin B, grosheimin, and dehydrocynaropicrin) and one novel (nourolide) guaianolide-type sesquiterpene lactones. Two semi-synthetic cynaropicrin derivatives were obtained to enhance the molecular diversity of the present study. 1D (^{1}H and ^{13}C APT) and 2D (COSY, HSQC, HMBC, and NOESY) NMR experiments were employed for structure elucidation. MIC values of the tested sesquiterpene lactones against the above-mentioned strains ranged between 3.12 and 50 µg/mL. Molecular dynamics simulations were conducted to investigate the interaction of **4** (aguerin B), which demonstrated the highest antifungal activity, with the exo-β-(1,3)-glucanase and 14-α-demethylase enzymes. These findings revealed stable binding interactions, suggesting that aguerin B has potential as a lead for further antifungal drug development.

Keywords: Antifungal, *Candida*, *Centaurea*, sesquiterpene lactone, fungal resistance

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1 Introduction

Natural compounds isolated from the plants, especially terpenoids (Topçu & Gören, 2007; Gören & Topçu, 2025), provide a great source in drug discovery due to their molecular diversity and good pharmacokinetic properties, despite their disadvantages such as low yield in plants and difficulty in chemical synthesis (Khan et al., 2023; Musso & Biscussi, 2025; Soković & Liaras, 2021; Gurel et al., 2024). Semi-synthesis studies on the natural compounds are employed in order to overcome the disadvantages mentioned. Also, natural compounds and their semi-synthetic derivatives present great potential in discovery of novel molecules with lower toxicity and higher efficacy peculiarly, against the fungal resistance (Geissler et al., 2018; Staniek et al., 2013).

Emergence of antifungal resistance and limitations of current antifungal drug groups have led to a dramatic increase in fungal infections worldwide, especially in immunosuppressed patients. Adverse effects of the current antifungal groups are not easily tolerated by the patients, and toxicity profile of them limits their use in the treatment. Pharmacokinetic problems, such as low tissue penetration, high protein binding, and low bioavailability, hinder achievement and/or maintenance of the required concentration at the infection site, thus a decrease in treatment efficacy. Consequently, the afore-mentioned limitations of the current antifungal agents result in ineffective antifungal treatment, prolonged hospitalization, and financial distress on the healthcare system (Branda et al., 2025; Sanglard & Odds, 2002). In addition to limitations of the current antifungal groups, the rise of fungal resistance and emergence of multidrug-resistant *Candida*

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dubliniensis, C. *inconspicua*, and *C. auris* urged the need for novel antifungal molecules. The threat level of *C. auris*, which has strains resistant to the current antifungal drug groups, has been identified as "urgent" by the Centers for Disease Control and Prevention (Schwartz & Dingle, 2019; Sugita et al., 2004; Sullivan & Coleman, 1998). These occurrences turned research groups' attention to natural compounds for the discovery of novel molecules that offer better pharmacokinetic properties and are capable of breaking the fungal resistance in order to improve the efficacy of antifungal treatment (Villagómez-Guzmán et al., 2025).

Natural compounds, especially sesquiterpene lactones (SLs), have been utilized in antifungal research (Barrero et al., 2000; Sidjui et al., 2014; Soković & Liaras, 2021; Vajs et al., 1999). Molecular diversity of SLs empowers different mechanisms of action required in order to break the antifungal resistance. Antifungal activity mechanisms of the SLs have not been entirely explored yet, but the literature suggests that SLs exhibit antifungal activity by targeting fungal membranes due to their lipophilic nature. Along with disruption of fungal membranes, SLs display antifungal activity by alkylation, thereby inhibiting cellular enzymes (Arif et al., 2009; dos Dantas et al., 2025). Previous reports on the antifungal activity of SLs focus on the presence of α-methylene γ-lactone ring, by reason of its alkylation capacity (Koukoulitsa et al., 2006; Kupchan et al., 1970; Lee et al., 2006). It should also be noted that in terms of antifungal activity of SLs, lower polarity favored over increased alkylation capacity (Ciric et al., 2012; Karioti et al., 2007; Koukoulitsa et al., 2005; Skaltsa et al., 2000). Despite comprehensive antifungal research on the antifungal activity of SLs against different fungal strains, there is limited data on their antifungal activity against *Candida* sp.

SLs might be utilized in novel antifungal molecule research against the multidrug-resistant *Candida* strains, considering their promising antifungal activity reports. Almost 90% of identified SLs are obtained from aerial parts of plants that belong to the Asteraceae family. *Centaurea* L., a member of Asteraceae family, has been utilized in antibacterial, antiviral, antifungal, anti-inflammatory, antiplasmodial, and cytotoxic research due to its SLs content (Khammar & Djeddi, 2012). The genus *Centaurea* is distributed across most of the continents and represented by 794 taxa worldwide (WFO, 2026). Türkiye is one of the main centers of the genus, with 118 of 194 taxa being endemic (Erdoğan et al., 2017; Taşar et al., 2018), which offer a great source for novel antifungal molecule research (Boğa et al., 2016; Popović et al., 2016). Therefore, extracts obtained from the aerial part of endemic *Centaurea polypodiifolia* Boiss. were investigated for their antifungal activity against *Candida albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. dubliniensis* (NCPF 3949a), *C. inconspicua* (NCPF 8523), and *C. auris* (NCPF 8977) in the present study. Natural SLs, guaianolides, isolated from the extract and semi-sytnthetic derivatives of cynaropicrin were also subjected to antifungal tests. Antifungal properties of natural and semi-synthetic SLs were also explored via in silico studies.

2 Materials and Methods

2.1 General Experimental Procedures

Extracts evaporated using a rotary evaporator from Buchi®, Switzerland. Isolation studies were conducted via chromatographic methods, including column and thin layer chromatography (TLC). Supelco Sephadex® LH-20 (Sigma Chem. Co., Germany), Silica gel (Kiesegel 60 0.063–0.200 mm, Merck, Germany), and LiChroprep RP-18 gel (40–63 µM, Merck, Germany) were used as adsorbents during column chromatography. Isolation monitored via Silica gel 60 F_{254} TLC plates (0.25 mm, Merck, Germany). TLC plates were examined under 254 and 366 nM wavelength utilizing a UV lamp (Camag®, Switzerland) and 10% anisaldehyde-sulfuric acid solution, followed by heating. All of the solvents (hexane, dichloromethane, methanol, and acetonitrile) were purchased from Merck, Germany. 1D (^{1}H and ^{13}C APT) and 2D (COSY, HSQC, HMBC, and NOESY) Nuclear Magnetic Resonance (NMR) of SLs were recorded in a Bruker® Ascend NEO® NMR Spectrometer (USA) operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. The relative stereochemistry of the SLs was determined using 2D-NOESY NMR when necessary. Prior to 1D and 2D NMR experiments, all of the SLs were dissolved in deuterated chloroform ($CDCl_3$) except **3,** which was dissolved in deuterated methanol (MeOD) since it was not soluble in $CDCl_3$. ^{1}H and ^{13}C APT 1D NMR and 2D NOESY NMR spectra of stereoisomers **2** and **3** were also recorded in $CDCl_3$ and MeOD (95:5, v/v) for comparison. IR spectrum of novel compound **3** was determined using Bruker® Alpha FT-IR (USA). LC-MS analysis of novel compound **3** was performed with a Thermo Scientific® Q Exactive spectrometer (USA), and optical rotation was measured using a Rudolph Analytical Autopol V Plus® in methanol (USA). Amphotericin B and Resazurin were purchased from Sigma-Aldrich, Germany. During antifungal tests, sterility control of each bottle was performed before it was used. The broth microdilution test was performed by using sterile, disposable microdilution plates (96 U-shaped wells) (LP Italiano SPA, Milano, Italy). Sabouraud dextrose agar was from BBL (Sparks, MD, USA).

2.2 Plant Material

Aerial part of *Centaurea polypodiifolia* (CPvP) were collected from Sivas/Türkiye in 2017 and identified by Prof. Dr. Emine Akalın. Herbarium sample is stored in the Istanbul University Faculty of Pharmacy Herbarium (ISTE) with the voucher number ISTE 116469. Plant material was cut into 3.5–4 cm pieces and air-dried in the shade at room temperature.

2.3 Extraction and Isolation

1350 g of plant material were used for extraction. Coarsely chopped dry plant material was macerated with dichloromethane (DCM) at room temperature to obtain an SLs-rich extract. Plant material was subjected to maceration with dichloromethane (DCM) for 20 minutes twice, and a 1:5 plant material: solvent ratio was used (DCM-M). After maceration, dried plant material was pulverized and extracted again with DCM in a Soxhlet apparatus (DCM-S).

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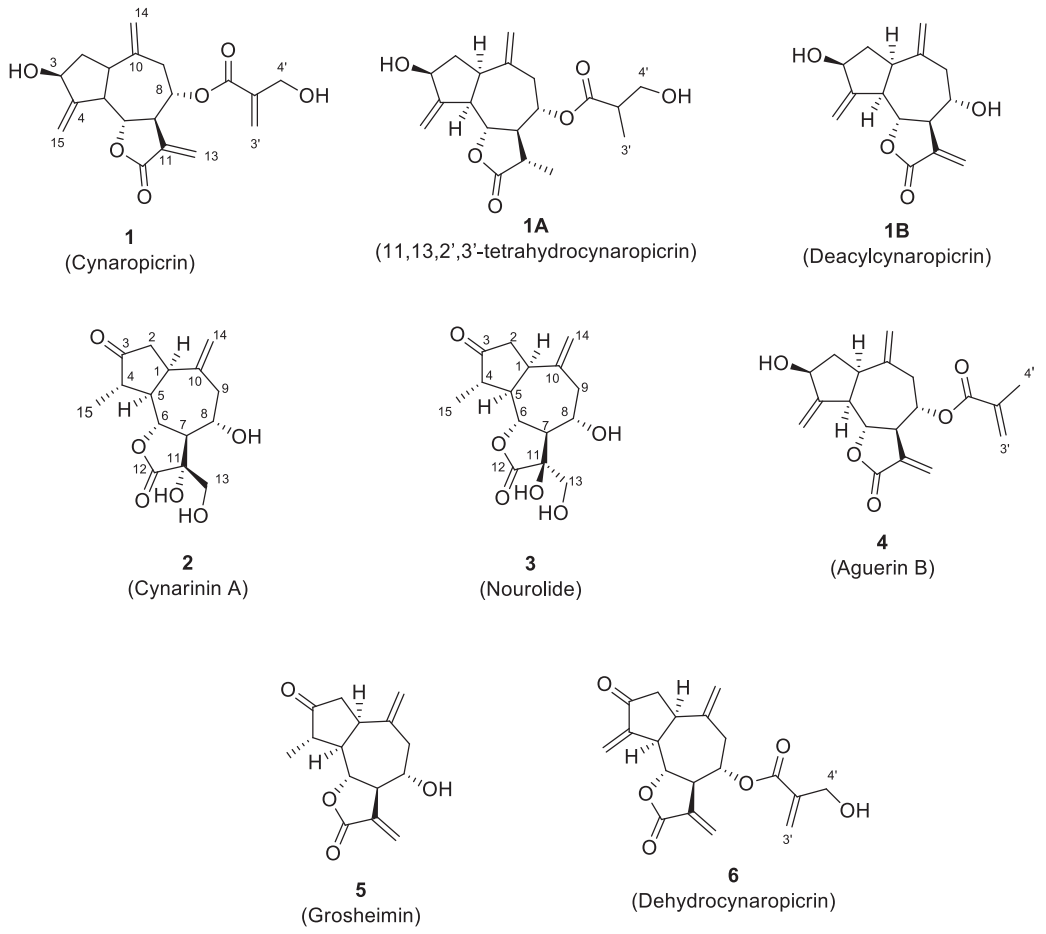

Figure 1. Chemical structures of natural and semi-synthetic sesquiterpene lactones

Once the Soxhlet extraction with DCM was over, plant material was dried again and extracted with methanol (MeOH) in a Soxhlet apparatus (MeOH-S). All of the extracts were dried under vacuum using a rotary evaporator at 45°C. All dried crude extracts were stored at −20°C.

Isolation studies were conducted on DCM-M extract due to its SLs-rich nature and afforded five known guaianolides; **1** (1800 mg), **2** (4.8 mg), **4** (10.6 mg), **5** (15.8 mg), and **6** (1.5 mg) (Figure 1). The isolation schema is given in the supplementary data. DCM-M extract fractioned in Sephadex LH-20 column and dichloromethane: methanol (3:1, v/v) was used as mobile phase. According to TLC examinations, fractions were combined. Fr. 48–76 was subjected to Sephadex LH-20 column chromatography with hexane: dichloromethane: methanol (7:4:1, v/v/v) mobile phase and afforded subfraction Fr. 171. Sephadex LH-20 methanol column chromatography was performed on subfraction Fr. 171. Fr. 6–8, which is a subfraction of Fr. 171, subjected to Sephadex LH-20 column chromatography with hexane: dichloromethane:methanol (7:4:1 v/v/v) of Fr. 6–8 and afforded novel compound **3** (10 mg).

2.4 Semi-synthesis Studies

Semi-synthesis studies were conducted on cynaropicrin (**1**), the major compound in DCM-M extract, and afforded compounds **1A** and **1B** (Figure 1).

50 mg (0.14 mmol) cynaropicrin dissolved in 50 mL methanol, dried with sodium borohydride. Small portions of sodium borohydride (75 mg, 1.98 mmol) were added to the solution under constant stirring at room temperature for 25 minutes. The endpoint determination of the reaction was evaluated using TLC (toluene:dichloromethane:ethyl acetate:acetonitrile, 2:2:2:1, v/v/v/v). At the end of the reaction, a saturated $NaHCO_3$ solution (100 mL) was added to neutralize excess sodium borohydride. Liquid-liquid extraction with dichloromethane yielded the reaction mixture. Sephadex LH-20 column chromatography of the reaction mixture with hexane:dichloromethane:methanol (7:4:1, v/v/v) mobile phase afforded Fr. 46–49, semi-synthetic **1A** (5.9 mg).

50 mg (0.14 mmol) cynaropicrin dissolved in 10 mL methanol. NaOMe (2.5%) solution was added dropwise to the mixture (2 mL) and under constant stirring for 5 minutes. The determination of the endpoint of the reaction was evaluated with TLC (dichloromethane: methanol, 95:5, v/v). The reaction mixture was subjected to PTLC with dichloromethane:methanol (95:5, v/v) and afforded semi-synthetic **1B** (2 mg) (Araki et al., 2024; Cravotto et al., 2005).

2.5 Structure Elucidation

All of the 1D-NMR (^{1}H and ^{13}C APT) and 2D-NMR (HSQC, HMBC, and NOESY) spectra and structure elucidation of

Table 1. ^{1}H (500 MHz) and ^{13}C APT (125 MHz) 1D-NMR shifts and NOESY correlations of **2** and **3** in $CDCl_3$:MeOD (95:5, v/v) (δ in ppm)

	Compound 2			Compound 3		
	δ_H	δ_C	NOESY	δ_H	δ_C	NOESY
1	3.10 (td, *J* = 8.2, 4.0 Hz, 1H)	39.7	H7	3.10 (td, *J* = 8.4, 4.3 Hz, 1H)	39.4	H5, H7
2α	2.49 (m, 2H)	43.4	H14b	2.50 (m, 1H)	43.4	–
2β						–
3	–	218.9	–	–	219.8	–
4	2.27 (tt, *J* = 7.7, 3.6 Hz, 1H)	47.4	H6	2.26 (m, 1H)	51.6	H6
5	2.27 (tt, *J* = 7.7, 3.6 Hz, 1H)	51.4	H7	2.26 (m, 1H)	47.3	H15, H1
6	4.02 (dd, *J* = 10.1, 8.5 Hz, 1H)	81.7	H4, H8, H13a	4.03 (m, 1H)	81.8	H4
7	2.64 (t, *J* = 10.4 Hz, 1H)	55.9	H5, H1	2.63 (t, *J* = 10.3 Hz, 1H)	56.0	H1
8	3.80 (td, *J* = 10.3, 5.6 Hz, 1H)	69.9	H6, H8, H13b, H13a	4.03 (m, 1H)	70.2	H9β
9α	2.17 (dd, *J* = 12.3, 10.0 Hz, 1H)	48.3	–	2.83 (dd, 12.5, 5.5 Hz, 1H)	47.7	H14a
9β	2.81 (dd, *J* = 12.4, 5.6 Hz, 1H)		–	2.19 (dd, 12.5, 10.1 Hz, 1H)		H8
10	–	143.8	–	–	143.9	–
11	–	76.3	–	–	78.4	–
12	–	173.1	–	–	177.3	–
13a	4.57 (d, *J* = 11.1 Hz, 1H)	64.4	H6, H8	3.83 (m, 2H)	62.6	–
13b	4.32 (dd, *J* = 11.2, 2.0 Hz, 1H)		H8			–
14a	5.02 (s, 1H)	114.9	–	5.04 (s, 1H)	114.9	–
14b	4.74 (s, 1H)		H2	4.73 (s, 1H)		H9α
15a	-	14.6	–	1.19 (d, *J* = 6.7 Hz, 3H)	14.5	H5
15b			–			

all compounds are given in supplementary data along with COSY, HSQC, HMBC, and NOESY correlation tables. The relative stereochemistry of the compounds was determined using 2D-NOESY NMR when necessary. Compound **3** was dissolved in MeOD prior to NMR experiments since it was not soluble in $CDCl_3$. NMR spectra of novel **3** and its stereoisomer **2** were also recorded in $CDCl_3$:MeOD (95:5, v/v) for comparison (Table 1, Figure 2).

Compound 3: white amorphous powder; $[\alpha]^{19.8}{}_{D}$: 120° (*c*, +0.06, MeOH); UV (MeOH) λ_{max} (log ε): 206 and 287 nm; ^{1}H and ^{13}C APT in MeOD (supplamentary data) in CDCl3:MeOD (95:5, v/v in Table 1); (-)-HRESIMS: *m/z* 296.1250 $[M]^-$ (calcd *m/z* 296.12504); IR V_{max} (NaCl) cm^{-1}: 3384, 2933, 2360, 1772, 1734, 1643, 1463, 1374, 1350, 1280, 1221, 1175, 1133, 1066, 979, 910.

2.6 Antifungal Activity and SAR Studies

Antifungal activity of extracts, natural SLs, and semi-synthetic cynaropicrin derivatives was tested against *Candida albicans* ATCC 90028 (American Type Culture Collection, USA), *C. krusei* ATCC 6258, *C. auris* NCPF 8977 (National Collection of Pathogenic Fungi, UK Health Security Agency), *C. dubliniensis* NCPF 3949a, and *C. inconspicua* NCPF 8523. Microdilution method according to the standard protocol by the Clinical and Laboratory Standard Institute (CLSI) was employed. Amphotericin B, extracts, and SLs were dissolved in 100% dimethyl sulfoxide as recommended by CLSI guidelines. RPMI 1640 broth with L-glutamine without sodium bicarbonate and 0.165 M MOPS buffer (34.54 g/lt) was employed. pH of the medium was adjusted to 7.0 at 25°C (Clinical and Laboratory Standards Institute, 2008b, 2008a, 2020). Resazurin solution (0.02%) was prepared in sterile distilled water, filtered, and stored at 4°C (Sanchotene et al., 2008).

Inoculum suspension of yeasts preparation was also performed according to CLSI guidelines as well. The yeast colonies were incubated at 35°C for 48 H on Sabouraud dextrose agar. (Clinical and Laboratory Standards Institute, 2008b, 2008a, 2020). The colonies were subcultured to 5 mL of 0.85% sterile saline. Their turbidity was adjusted to 0.5 McFarland Standard spectrophotometrically at 530 nM. It was diluted to 1:50 and then 1:20 with RPMI 1640, final concentration was 0.5×10^3 to 2.5×10^3 CFU/mL.

Serial dilution of samples and amphotericin B was done in a microplate with RPMI 1640 medium. DMSO control (<2% DMSO/RPMI), positive control (without sample), and negative control (broth only) wells were employed along with the sample wells (100 µL). All of the wells, other than the negative control wells, were inoculated with 100 µL respective inoculum. The final concentrations of all extracts and isolated compounds were 50 to 0.002 µg/mL, and amphotericin B was 16 to 0.007 µg/mL.

The microplates were incubated for 24–48 h at 35°C. 30 µL resazurin solution was added to each well, and microplates were incubated for another 24 h. The purple color was defined as negative, while the pink color was defined as positive for fungal growth. The minimum concentration (MIC) at which no growth was observed was taken as the MIC value (Clinical and Laboratory Standards Institute, 2008b, 2008a, 2020; Sanchotene et al., 2008).

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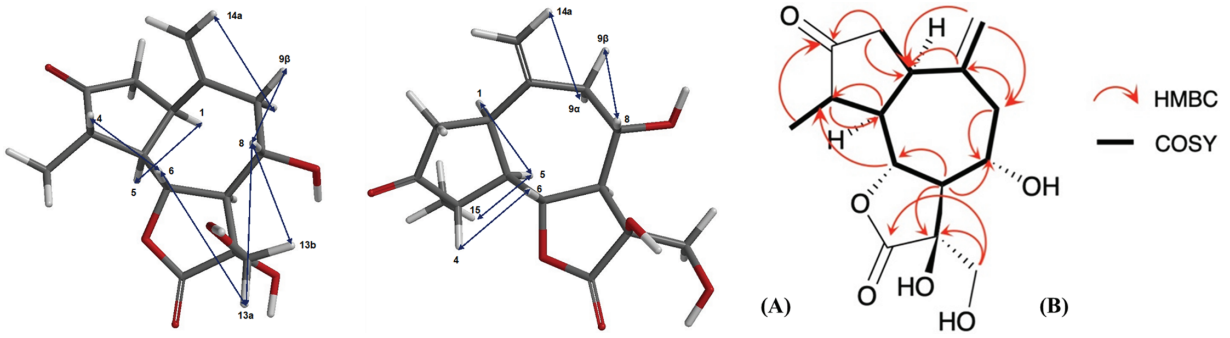

Figure 2. (A) ^{1}H-^{1}H NOESY correlations of compounds **2** and **3**, respectively; (B) Key ^{1}H-^{1}H COSY and HMBC correlation of **3**

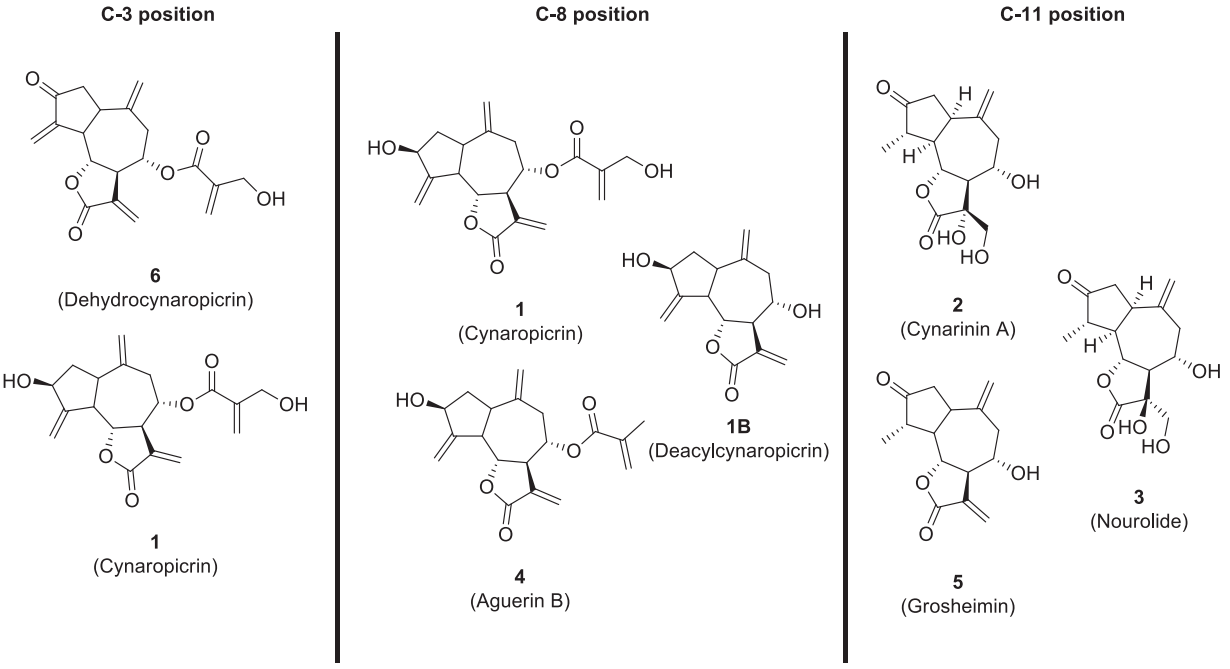

Figure 3. Variation points of sesquiterpene lactones obtained in the present study

Molecular diversity of the SLs obtained in the present study enabled SAR (structure-activity relationship) studies on the antifungal activity. SLs that only show variation at one position (namely C-3, C-8, and C-11) were employed during SAR studies (Figure 3). Variation between **1** and **6,** qualified comparison of hydroxyl and carbonyl at C-3 position. **1**, **1B**, and **4** were utilized to evaluate influence of esterification at C-8 position on the antifungal activity. Also, guaianolides that only differentiate by their lactone rings, **2**, **3**, and **5**, provided an opportunity to investigate the antifungal potential of SLs without α-methylene-γ-lactone.

2.7 In Silico Evaluation of Physicochemical and ADME Properties

The physicochemical properties of the compounds such as molecular weights, hydrogen bond acceptor and donor numbers, topological polar surface area, rotatable hydrogen bond number; lipophilic properties such as Log P; water solubility properties such as Log S; pharmacokinetic properties such as GI and blood-brain barrier passage; drug-like properties such as Lipinski and Veber rule were evaluated using Ligand-Based ADME/Tox prediction module in Schrödinger (Tok et al., 2024).

2.8 Computational Methods

All proteins—exo-β-(1,3)-glucanase (PDB ID: 1EQP), 14-α-demethylase (PDB ID: 6AYB), sterol reductase (PDB ID: 4QUV), and thymidylate synthase (PDB ID: 5UIV)—were retrieved from the Protein Data Bank (PDB) and used for molecular docking simulations using Schrödinger's 2022-4 small molecule drug discovery suite (Schrödinger, 2022). Following protein preparation, binding sites were identified by generating a grid centered on the native ligands—KKK, NDP, and ADP—for all proteins except exo-β-(1,3)-glucanase. Since the probable binding site of exo-β-(1,3)-glucanase could not be determined based on a native ligand, the SiteMap module (Halgren, 2009) was used, and the identified SiteMap-1 site (da Alves et al., 2020; Vieira Melo et al., 2024)

was selected to create the grid box. Furthermore, Glide re-docking was performed using the Standard Precision (SP) method for all proteins, except for sterol reductase, which was docked using the Extra Precision (XP) mode. All other parameters were kept at their default values.

2.9 Molecular Dynamics Simulations

All Molecular dynamics (MD) simulations for exo-β-(1,3)-glucanase (PDB ID: 1EQP) and 14-α-demethylase (PDB ID: 6AYB) were conducted using Flare™ v9.0.0 software (Cresset, UK), which utilizes the OpenMM engine. For each protein–ligand complex, an orthogonal water box was generated using the three-site extended simple point charge (SPC/E) model to solvate the system. To simulate physiological conditions, the ionic concentration was adjusted to 0.15 M with NaCl. A small-molecule force field was set to AMBER for 14-α-demethylase. Periodic boundary conditions were implemented, and long-range electrostatic interactions were calculated via the Particle Mesh Ewald (PME) method. Simulation data were recorded every 20,000 time steps. Each system was subjected to a 500-nanosecond MD run under NPT conditions, ensuring constant pressure and temperature during the production phase.

3 Results and Discussion

3.1 Structure Elucidation of Natural and Semi-Synthetic SLs

Isolation studies yielded five known and one novel guaianolide-type sesquiterpene lactones with different functional groups. Also, two different cynaropicrin semi-synthetic derivatives (**1A** and **1B**) were obtained (Figure 1).

Compound **1** was the major compound in DCM-M extract. According to ^{1}H NMR spectra taken in $CDCl_3$, **1** was identified as cynaropicrin (Sang et al., 2005), which was previously isolated from *Cynara scolymus* (Corbella et al., 1972; Zhao et al., 2005) and from various *Centaurea* species such as *Centaurea solstitialis* (Wang et al., 1991), *C. behen* (Rustaiyan et al., 1981), *C. helenioides* (Yaylı et al., 2006), and Turkish endemic *C. drabifolia* subsp. *detonsa*. Compound **2** was identified as cynarinin A, which was previously obtained from *Cynara scolymus* (Zhao et al., 2005), *Centaurea kotschyi* subsp. *persica* (Serino et al., 2022), and Turkish endemic *Centaurea drabifolia* subsp. *detonsa* (Formisano et al., 2017). Compound **4** was designated as aguerin B based on 1D and 2D NMR spectra (Rustaiyan et al., 1981). Previously, aguerin B was isolated from *Centaurea behen* (Rustaiyan et al., 1981), *C. canariensis* (González et al., 1978), *C. solstitialis* (Wang et al., 1991), and recently from *Centaurothamnus maximus* (Al-Saleem et al., 2023). NMR spectra analysis revealed the structure of compound **5** as grosheimin (Rustaiyan et al., 1981). Grosheimin was previously isolated from *Centaurea behen* (Rustaiyan et al., 1981), *Centaurea helenioides* (Yaylı et al., 2006), and *Cynara scolymus* (Zhao et al., 2005). Compound **6** was identified as dehydrocynaropicrin, which was previously isolated from *Cynara scolymus* (Zhao et al., 2005). Cynaropicrin derivative compound **1A** was established as 11,13,2′,3′-tetrahydrocynaropicrin (Cravotto et al., 2005). Compound **1B** was defined as deacylcynarpicrin, which is naturally found in *Centaurothamnus maximus* C (Al-Saleem et al., 2023).

All of the guaianolides obtained in the present study were screened and reviewed in SciFinder®. Dehydrocynaropicrin (**6**) was isolated from *Centaurea* species for the first time. Compound **3** was identified as a novel compound and was named nourolide.

Molecular formula of **3** was established as $C_{15}H_{20}O_6$ according to (–)-HRESIMS: *m/z* 296.1250 $[M]^-$ (calcd *m/z* 296.12504) and ^{13}C APT NMR spectrum. The IR spectra displayed absorption bands for hydroxyl (3384 cm^{-1}) and γ-lactone (1772 cm^{-1}) functionalities. The ^{13}C NMR spectrum of **3** showed 14 carbon signals, including resonances at δ_C 177.3 (C-12) and 62.7 (C-13), as supported by IR spectra. Prominent HMBC correlations of H_3-15 (δ_H 1.19, d, J = 6.68 Hz) and H_2-2 (δ_H 2.50, m) with δ_C 219.2 evidenced the presence of a carbonyl function at C-3, which did not show resonance in the ^{13}C APT NMR spectrum. Remaining carbon signals classified as one methyl (C-15, δC 14.50), two quaternary at δ_C 143.9 and 78.4 (for C-10 and oxygenated C-11, respectively), one exocyclic methylene at δ_C 114.9, two oxygenated methine at δ_C 82.0 and 70.2 (for C-6 and C-8, respectively), four methynes and two methylenes (Table 1). The ^{1}H NMR spectrum displayed resonances for a typical guaianolide frame with a triplet at δ_H 2.63 (J = 10.27 Hz, H-7) and two singlets at δ_H 5.04 and 4.73 for H_2-14. The ^{1}H and ^{13}C APT NMR data given above resembled those of cynarinin A (**2**) (Yıldırım et al., 2025). In contradistinction to **2**, the hydroxyl moiety substituted at C-11 is β-oriented in **3**. The β-orientation of the hydroxyl moiety resulted in an upfield shift of H_2-13 AB signals to δ_H 3.83 (δ_H 4.32 and 4.02 for **2**) along with a downfield shift of H-8 to δ_H 4.03 (δ_H 3.80, td, J = 10.50, 5.56 Hz for **2**). Also, the NOESY spectrum of **3** did not display any correlation between H_2-13 protons and H-6/H-8 protons, as distinct from **2**.

3.2 Antifungal Activity

The rise of antifungal resistance, emergence of multidrug-resistant *Candida* sp. such as; *Candida auris, C. inconspicua*, and *C. dubliniensis*, and limitations of the current antifungal agents have accelerated the research on novel antifungal compounds. Natural compounds, SLs in particular, have been utilized in antifungal research (Barrero et al., 2000; Schwartz & Dingle, 2019; Sugita et al., 2004; Sullivan & Coleman, 1998). MIC values of the extracts and the SLs in the present study are given in Table 2.

The extracts gave the same MIC value against *Candida albicans*, *C. auris*, *C. dublinienesis*, and *C. inconspicua* (MIC = 12.5 μg/mL), but the best antifungal activity was achieved against *C. krusei* with MIC values at 6.25 μg/mL concentration. SLs showed equal or lower antifungal activity than DCM-M extract against *C. albicans*, *C. auris*, and *C. inconspicua*. Only **4** (Aguerin B) exhibited two-fold higher activity than the DCM-M extract against *C. dubliniensis* and *C. krusei*, with MIC values 6.25 μg/mL and

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Table 2. MIC values of extracts and sesquiterpene lactones (μg/mL)

Samples	*C. albicans* ATCC 90028	*C. auris* NCPF 8971	*C. dubliniensis* NCPF 3949a	*C. krusei* ATCC 6258	*C. inconspicua* NCPF 8523
DCM-M	12.5	12.5	12.5	**6.25**	12.5
DCM-S	12.5	12.5	12.5	**6.25**	12.5
MeOH-S	12.5	12.5	12.5	**6.25**	12.5
1	12.5	25	12.5	50	12.5
2	12.5	12.5	12.5	12.5	12.5
3	12.5	25	12.5	12.5	12.5
4	12.5	12.5	**6.25**	**3.12**	12.5
5	12.5	25	12.5	12.5	12.5
6	12.5	12.5	12.5	12.5	12.5
1A	12.5	25	12.5	12.5	12.5
1B	12.5	12.5	12.5	12.5	12.5
Amph. B	≤0.007	0.014	≤0.007	2	≤0.007
DMSO control	+	+	+	+	+
Positive control	+	+	+	+	+
Negative control	–	–	–	–	–
Sample MIC Range: 50 – 0.002 μg/mL (12 dilutions)					

3.12 μg/mL, respectively. Higher or equal antifungal activity of the extracts might be explained by their natural compound richness, which enables act on different mechanisms, hence the synergistic effect (Tenório et al., 2024).

All of the tested SLs gave the same MIC value against *C. albicans* and *C. inconspicua* (MIC = 12.5 μg/mL). **1** exhibited two-fold lower antifungal activity than **6** against *C. auris*, which was attributed to the hydroxyl moiety at the C-3 position. Likewise, two-fold higher antifungal activity of **1B** and **4** than **1** marked the effect of esterification and the acid at the C-8 position on the activity. According to the results of the present study, esterification with methacrylic acid (**4**), which has an alkylation function, increases the antifungal activity against *C. auris* compared to esterification with 2-(hydroxymethyl) acrylic acid (**1**). On the other hand, the equal antifungal activity of **4** and **1B** might be explained by the increased polarity of **4** due to esterification. Interestingly, **2** exhibited two-fold higher antifungal activity than its stereoisomer **3** and **5**, a guaianolide with α-methylene γ-lactone ring. In terms of antifungal activity against *C. dubliniensis*, only **4** showed two-fold higher antifungal activity (MIC = 6.25 μg/mL) than the other SLs. Dissimilarly from antifungal activity against *C. auris*, **1** and **1B** gave the same MIC value (MIC = 12.5 μg/mL) against *C. dubliniensis*, which emphasized the influence of esterification with methacrylic acid. **1** displayed the worst antifungal activity against *C. krusei* (MIC = 50 μg/mL) while **6** showed MIC value at 12.5 μg/mL concentration. Four-fold lower antifungal activity of **1** was attributed to the carbonyl moiety at the C-3 position, parallel with antifungal activity against *C. auris*. **4**, a guaianolide with methacrylic acid esterification at the C-8 position, exhibited the best antifungal activity with 3.21 μg/mL MIC value. Four-fold higher antifungal activity of **4** than **1B** revealed the positive effect of esterification with an acid that has an alkylation function. Also, **1B** showed four-fold higher antifungal activity against *C. krusei* than **1**, this might be explained by the influence of esterification on polarity, mirroring the antifungal activity against *C. auris*.

Antifungal activity of SLs has been researched extensively by various research groups. In spite of comprehensive reports, the antifungal activity mechanisms of SLs have not been completely explored yet. Previous studies indicate that SLs display antifungal activity by disrupting fungal membrane integrity due to their lipophilic character and alkylation of cellular enzymes. In addition, their lipophilicity is related to penetration into the fungal cell wall, which enables the inhibition of cellular enzymes (Arif et al., 2009; dos Dantas et al., 2025). Various reports on antifungal activity of SLs favor the presence of α-methylene γ-lactone ring due to its alkylation capacity (Koukoulitsa et al., 2006; Kupchan et al., 1970; Lee et al., 2006). Equal MIC values of **2**, **3**, and **5** in the present study against the tested strains other than *C. auris* revealed that SLs with geminal methyleneoxy-hydroxyl moeity at the C-11 position exhibited similar antifungal activity as a SL with α-methylene γ-lactone ring. Notably, the two-fold antifungal activity of **2** than **5** against multidrug-resistant *C. auris* indicates SLs without α-methylene γ-lactone ring might be utilized as potent antifungal compounds. Additionally, **3** displayed two-fold lower antifungal activity than **2** against the afore-mentioned strain, which highlighted the influence of polarity in antifungal activity (Arif et al., 2009; dos Dantas et al., 2025). Influence of the variations at C-3, C-8, and C-11 positions was summarized as comparision of two functional groups in Table 3.

Data on the antifungal activity of SLs against *Candida* strains are limited in contradiction to comprehensive antifungal investigations conducted on the other fungal species. Despite various reports on antifungal activity of SLs against different fungal strains, among the SLs in the present

Table 3. Functional groups that have a positive effect on antifungal activity at C-3, C-8, and C-11 positions[a]

		C. auris	*C. dubliniensis*	*C. krusei*
C-3	-OH =O	=O	-	=O
C-8	-OH -OCOC(CH_2OH)=CH_2	-OH	-	-OH
	-OH -OCOC(CH_3)=CH_2	-	-OCOC(CH_3)=CH_2	-OCOC(CH_3)=CH_2
	-OCOC(CH_2OH)=CH_2 -OCOC(CH_3)=CH_2	-OCOC(CH_3)=CH_2	-OCOC(CH_3)=CH_2	-OCOC(CH_3)=CH_2
C-11[b]	α-CH_2OH/β-OH β-CH_2OH/α-OH	β-CH_2OH/α-OH	-	-

Note: [a] *C. albicans* and *C. inconspicua* were excluded from the table since all of the tested guaianolides gave the same MIC values.

[b] =CH2 at C-11 position was not included since it gave the same MIC value with α-CH_2OH/β-OH at C-11 position.

study, only cynaropicrin (**1**) and aguerin B (**4**) were tested against *Candida* strains (Schinor et al., 2004; Shakeri et al., 2018). Schinor et al. (2004) reported that cynaropicrin did not show antifungal activity against the standard *Candida albicans* (ATCC 1023) strain and two field strains; *C. albicans* and *C. tropicalis*, at 2.5 mg/mL concentration, while cynaropicrin exhibited MIC values ranging between 12.5 µg/mL and 50 µg/mL against the tested strains in the present study. Antifungal assessment of aguerin B and cynaropicrin against *C. albicans* (ATCC 10231) gave 250 µg/mL and 62.5 µg/mL MIC values, respectively (Shakeri et al., 2018). This is not in correlation with our results since aguerin B (**4**), which displayed the best antifungal activity among the tested SLs, exhibited equal antifungal activity with cynaropicrin (**1**) against *C. albicans*.

3.3 In silico Evaluation of Physicochemical and ADME Properties

We assessed the drug-likeness of the natural and semi-synthetic guaianolides, as this characteristic plays a crucial role in both the physicochemical properties and pharmacokinetics of potential drugs (Başoğlu-Ünal et al., 2022; Tok et al., 2024). Additionally, many new drug candidates may be eliminated in animal or clinical trials because of their poor pharmacokinetic properties, despite their good activity in preclinical studies. The result is a high budget outlay and a significant loss of time and energy in the drug discovery process. Thus, evaluating the pharmacokinetics and physicochemical properties of new small molecules is significant. Specifically, for orally administered drugs, compliance with Lipinski's Rule of Five and Veber's criteria is often used as a benchmark for evaluating drug-likeness.

All guaianolide compounds in our series met Lipinski's Rule of Five criteria, displaying LogP (lipophilicity) values ranging from 0 to 2.5. This indicates that the compounds possess an appropriate level of lipophilicity, a key factor in absorption and oral bioavailability. These results suggest that the compounds possess promising characteristics for further drug development in terms of their potential for oral administration. Selected physicochemical and pharmacokinetic parameters are summarized in Table 4.

3.4 In-silico Studies: Molecular Docking and Molecular Dynamics

It is well known that several enzymes play crucial roles in the manifestation of antifungal activity, including exo-β-(1,3)-glucanase, 14-α-demethylase, sterol reductase, and thymidylate synthase. Therefore, to gain insight into the potential mechanism of action of the isolated compounds, molecular docking studies were carried out against all four targets. However, the compounds did not exhibit any significant binding affinity toward thymidylate synthase or Δ14-sterol reductase. This was evident from the docking score values obtained after mm/GBSA refinement, where even the active compounds—such as **4** and **1A**—showed positive docking scores, given in Table 4, and the moderately active compounds had considerably high (i.e., less favorable) values. Moreover, as reported by Li et al. in a previous study published in *Nature* (Li et al., 2019), a clear correlation exists between docking energy scores and binding affinity, further supporting our observations. All in all, we believe that the antifungal activity of these compounds is likely mediated through the inhibition of exo-β-(1,3)-glucanase or 14-α-demethylase, rather than via interaction with sterol reductase or thymidylate synthase.

SiteMap analysis was utilized for the compound **4**–1EQP complex to identify possible binding pockets, as the selected PDB structure lacked a native ligand complex. Five potential binding sites were identified, and the literature, along with our analysis, guided the selection of SiteMap-1 (da Alves et al., 2020; Vieira Melo et al., 2024) as the most probable binding region. The docked pose was used for MD simulation. The RMSD (root mean square deviation) value of the protein heavy atoms was found to be quite low and consistent during

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Table 4. Some physicochemical and ADME properties of isolated sesquiterpene lactones and their MM-GBSA docking energy scores

Comp.	MW[a]	HBA[b]	HBD[c]	LogPo/w[d]	PSA[e]	HOA%[f]	DS[g]			
							1EQP	5JLC	5UIV	4QUV
1	346.38	7.40	1	2.342	107.86	84.72	−48.31	−45.55	3.12	−34.79
2	296.32	8.15	2	0.149	118.56	64.67	−31.03	−49.38	−20.07	−27.31
3	296.32	8.15	2	0.149	118.56	64.67	−31.03	−49.38	−20.07	−27.31
4	330.38	6.70	1	2.363	86.14	90.63	−46.57	−50.83	8.87	−26.32
5	262.31	6.70	1	0.929	82.32	79.29	−37.87	−48.29	−24.01	−22.48
6	344.36	7.70	0	1.280	113.75	74.95	−39.15	−36.43	−15.57	−22.66
1A	350.41	7.40	1	2.012	93.80	89.03	−44.71	−50.17	1.48	−22.54
1B	262.31	6.40	2	1.098	76.16	84.24	−31.12	−43.35	−13.20	−19.07

Note: [a]Molecular weight (g/mol) (recommended value <500).
[b]Hydrogen bond acceptors (recommended <10).
[c]Hydrogen bond donors (recommended <5).
[d]Logarithm of the octanol/water ratio coefficient of compound (recommended value <5).
[e]Polar surface area (Å) (recommended value ≤140 Å).
[f]Percentage oral absorption (<25% weak and >85% strong).
[g]MM/GBSA dG Bind Energy (kcal/mol).

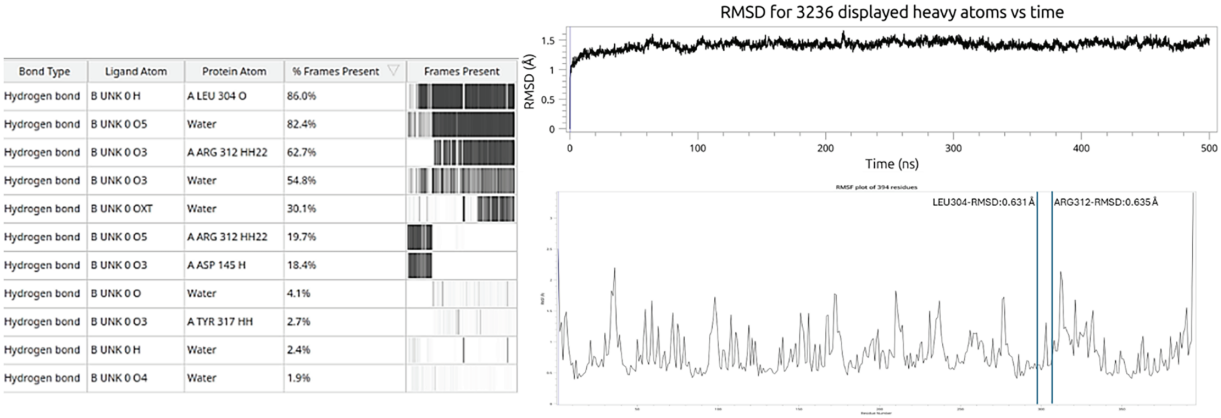

Figure 4. Key molecular interactions and dynamic stability of the compound 4 in the exo-β-(1,3)-glucanase active site (PDB ID: 1EQP). Clustering table illustrating the interactions, RMSD plot showing complex stability over 500 ns., and RMSF profile indicating localized flexibility near the binding site

500 ns of the simulation (Figure 4). The stability illustrates that both protein-ligand complexes have reached equilibrium and are structurally stable.

In particular, aminoacid residues exhibited low RMSF values less than 2.5 Å. These findings suggested that the protein-ligand complex is stable and interaction of the ligand likely favors the overall stability of the complex. Based on these observations, it was possible to conclude that in the case of exo-β-(1,3)-glucanase, the protein-ligand complexes were energetically favorable which is evident by the strong binding illustrated by low energy values and relatively consistent interactions among residues seen in **4** (Figure 4).

Upon analyzing the ligand-protein interaction diagram, significant amino acid residues involved in the interaction with **4** was identified for exo-β-(1,3)-glucanase. In the binding site of exo-β-(1,3)-glucanase, key interactions between the ligand and the enzyme were identified. As shown in Figure Z, the hydrogen bonds with LEU304, and ARG312 lasted for 86%, and 63% of the simulation time. Additionally, the ligand formed a hydrogen bond with some water molecules in the binding pocket for ~30–80% of the simulation time. The -OH group of the ligand interacted with LEU304, while the C=O of the lactone ring was involved in interactions with ARG312, as can be seen in Figure 5. The critical involvement of LEU304 and ARG312 in ligand binding was also previously reported in the literature which underscores their significance in maintaining the ligand's activity at the binding site (da Alves et al., 2020; Vieira Melo et al., 2024).

These findings suggest that the identified interactions, particularly with LEU304, play a pivotal role in the antifungal activity of the compound. The nucleophilic characteristics of these residues may enhance the binding efficiency, as previously discussed.

The second part of the analysis focuses on **4** in complex with 14-α-demethylase (6AYB), based on the results obtained from the MD simulations.

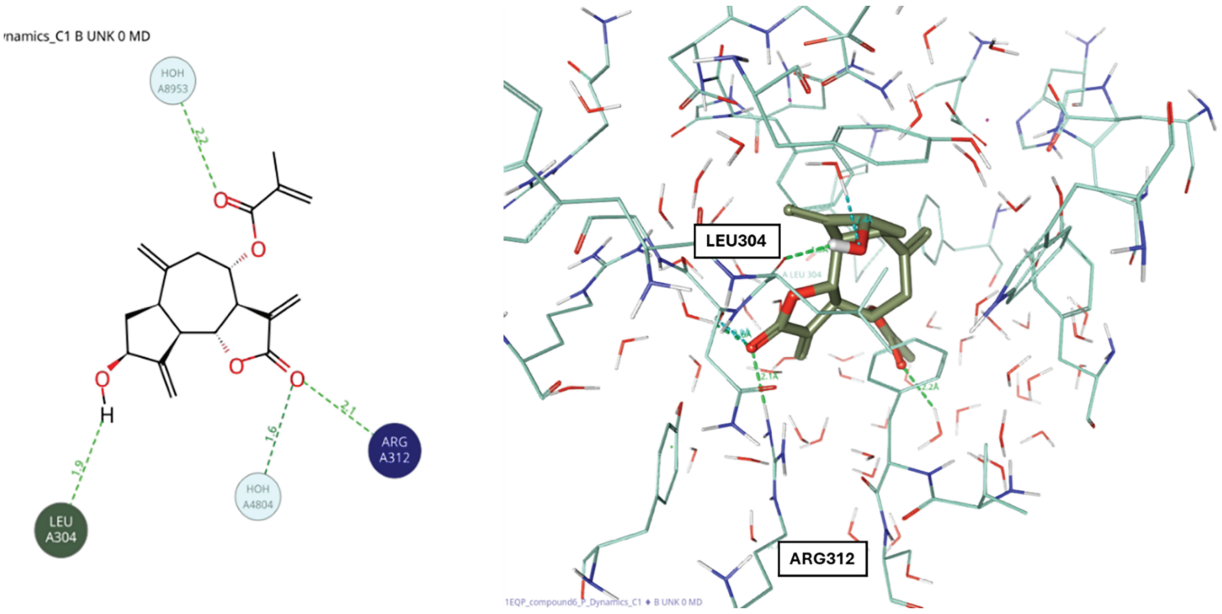

Figure 5. 2D and 3D frequency of ligand contacts for the compound 4-exo-β-(1,3)-glucanase and dynamic stability of the compound 4 in the exo-β-(1,3)-glucanase

Bond Type	Ligand Atom	Protein Atom	% Frames Present	Frames Present
Hydrogen bond	B UNK 0 O3	Water	99.3%	
Hydrogen bond	B UNK 0 O5	A THR 297 HG1	51.9%	
Hydrogen bond	B UNK 0 O	Water	26.0%	
Hydrogen bond	B UNK 0 O5	Water	24.8%	
Hydrogen bond	B UNK 0 OXT	Water	22.5%	
Hydrogen bond	B UNK 0 H	Water	10.3%	
Hydrogen bond	B UNK 0 H	A MET 360 O	6.8%	
Hydrogen bond	B UNK 0 OXT	A TYR 107 HH	6.7%	
Hydrogen bond	B UNK 0 O5	A TYR 120 HH	6.1%	
Hydrogen bond	B UNK 0 O4	Water	3.8%	
Hydrogen bond	B UNK 0 H	A MET 110 SD	3.1%	
Hydrogen bond	B UNK 0 H	A TYR 107 OH	2.4%	
Hydrogen bond	B UNK 0 O5	A GLY 432 H	2.3%	
Sulfur-lone Pair	B UNK 0 O4	A CYS 430 SG	1.8%	

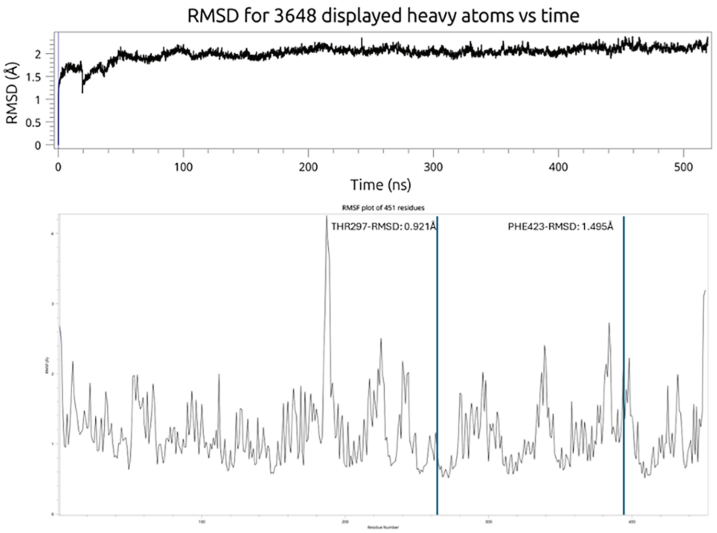

Figure 6. Key molecular interactions and dynamic stability of the compound 4 in the 14-α-demethylase active site (PDB ID: 6AYB). Clustering table illustrating the interactions, RMSD plot showing complex stability over 500 ns., and RMSF profile indicating localized flexibility near the binding site

In contrast to exo-β-(1,3)-glucanase, where SiteMap was used to predict the binding pocket, the binding site for 6AYB was defined based on the co-crystallized native ligand (ligand ID: KKK), which was considered a reliable reference point for docking and subsequent simulations (Debnath et al., 2017).

Throughout the 500 ns molecular dynamics simulation, the RMSD plot indicated that the protein-ligand complex achieved a stable conformation following an initial equilibration phase, suggesting a favorable binding profile (Figure 6). In particular, **4** formed a persistent hydrogen bond with THR297 for 51.9% of the total simulation time. Notably, a water-mediated hydrogen bond was observed between the ligand and **PHE423** via **HOH2238**, which remained stable for 99.3% of the simulation time. This strong and consistent interaction underscores the importance of structured water molecules in stabilizing the ligand at the active site.

Additionally, the RMSF values around the active site residues remained below 2.5 Å, reflecting minimal local flexibility and further confirming the structural stability of the protein-ligand complex (Figure 6). The ligand-protein contact map and clustering results demonstrated that the interactions were not only stable but also frequent, involving residues that are critical for enzymatic activity, as previously reported (Debnath et al., 2017). A more detailed visualization

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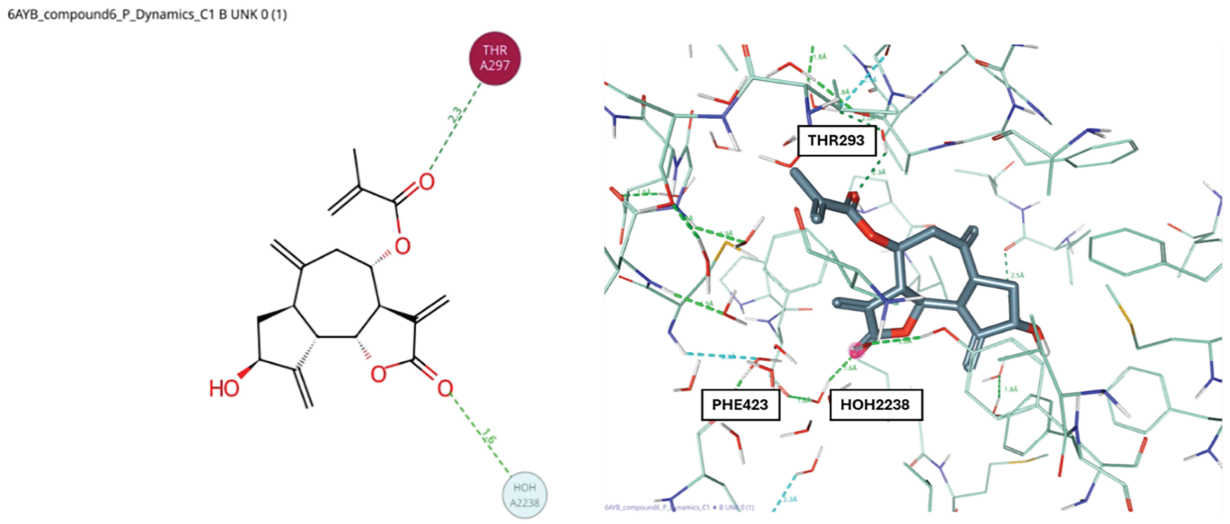

Figure 7. 2D and 3D frequency of ligand contacts for the compound 4-14-α-demethylase (PDB ID: 6AYB)

of these interactions is presented in Figure 7, which includes both 2D and 3D frequency maps of ligand contacts.

Altogether, these findings support the hypothesis that **4** exhibits antifungal effects, most probably through stable and energetically favorable interactions with 14-α-demethylase, with key contributions from hydrogen bonding and water-bridged contacts, particularly with THR297 and PHE423. The dynamic stability and interaction profile are consistent with a strong binding mechanism that may contribute to the observed biological activity.

4 Conclusion

Natural compounds have always presented great potential in drug discovery research. Emergence of antifungal resistance and limitations of the current antifungal agent groups expedited research on novel antifungal compounds, especially against multidrug-resistant *Candida auris*, *C. dubliniensis* and *C. inconspicua*. SLs in particular have been utilized in novel antifungal compound research extensively. *Centaurea* L. genus is rich in SLs therefore, natural compounds of Turkish endemic *C. polypodiifolia* were investigated herein for the first time. Introduction of novel guaianolide, nourolide (**3**), highlighted the potential of Turkish endemic *Centaurea* sp. as a natural compound source. Also, all of the tested SLs showed high antifungal activity against the tested five strains, especially aguerin B (**4**) exhibited the best antifungal activity against *C. krusei* and multidrug-resistant *C. dubliniensis*. Molecular docking studies on 1,3-β-glucan synthase, 14-α-demethylase, sterol reductase, and thymidylate synthase also designated aguerin B as the most potent compound. Molecular dynamics (MD) simulations provided valuable insight into the stability and interaction profiles of the most active SL (**4**, aguerin B) within the binding pockets of 1,3-β-glucan synthase and 14-α-demethylase. The persistent hydrogen bonds and stable RMSD values observed throughout the 500 ns simulations strongly support the potential of aguerin B as a promising antifungal lead molecule. These findings validate the in vitro results and highlight the importance of integrating MD simulations into the early stages of antifungal drug discovery for structure-based optimization of guaianolides. SLs investigated in the present study demonstrated promising antifungal activity against the tested *Candida* strains; however, further studies on their cytotoxicity are required to assess their suitability as lead compounds.

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Author Contributions

Selin Tufan: Writing of the original draft, data curation, extraction and isolation of natural SLs, semi-synthesis studies, structure elucidation, and evaluation of results. Faika Başoğlu: Writing of the original draft and in silico studies. Abdulilah Ece: Supervision of in silico studies. Dilek Şatana: Antifungal activity studies. Mahmut Miski: Supervision of structure elucidation and semi-synthesis studies. Nur Tan: Reviewing and editing of the original draft, supervision of data curation and result evaluation, and project administration.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they have no competing financial interests.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products.

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