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Article

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**Integrated phytochemical profiling and computational analysis identify
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Abstract

Natural products remain an important source of diverse structures for antiviral drug development, but pinpointing and prioritizing bioactive metabolites from complex plant extracts remains challenging. We developed an integrated approach combining phytochemical analysis and computational methods to identify sesquiterpene lactones from *Artemisia absinthium* L as potential candidates for interaction with the conserved coronavirus main protease (Mpro/3CLpro), a crucial target for viral replication. Using a Box–Behnken design, we optimized ultrasound-assisted extraction, followed by GC-MS and LC-MS profiling to analyze solvent-dependent phytochemical profiles. Ethanol yielded the highest extraction efficiency and enriched dimeric sesquiterpene lactones such as absinthin and anabsinthin, which were purified via preparative HPLC. These compounds were assessed through molecular docking, ADMET and toxicity predictions, and 100 ns molecular dynamics simulations, with X77 and nirmatrelvir used as reference compounds. Both lactones were predicted to fit within the Mpro active site, with anabsinthin showing the most favorable predicted binding profile. Molecular dynamics simulations indicated that the anabsinthin–Mpro complex displayed greater stability, lower backbone deviations, reduced residue fluctuations, and persistent interactions with key residues, including Glu166 and Gln189. In silico pharmacokinetic and toxicity predictions suggested generally favorable early drug-like characteristics without prominent predicted toxicity alerts. This integrated workflow combining extraction optimization, phytochemical profiling, purification, and computational assessment provides a rational strategy for prioritizing natural products for further investigation. Among the compounds examined in this study, anabsinthin emerged as the most promising *A. absinthium*-derived lactone candidate for subsequent experimental evaluation against Mpro. However, these computational predictions require confirmation through biochemical and cellular assays before direct Mpro inhibition or antiviral activity can be established.

Keywords: *Artemisia absinthium*; Sesquiterpene lactones; SARS-CoV-2 Mpro; Molecular docking; Molecular dynamics simulation; ADME/toxicity prediction.

1. Introduction

Although the acute emergency phase of pandemic has ended, coronavirus remains an evolving respiratory pathogen, and the continued emergence of viral variants sustains the need for antiviral preparedness[1–3]. In this context, antiviral discovery is no longer limited to the immediate pandemic response but also aims to diversify chemical scaffolds capable of targeting conserved viral functions. The virus main protease, also known as Mpro, 3CLpro, or nsp5 protease, remains a central target in this strategy because it mediates the proteolytic processing of the viral polyproteins pp1a and pp1ab into functional non-structural proteins required for replication[4,5]. Its essential catalytic role, structural conservation, and absence of closely related human homologues make Mpro a particularly attractive target for structure-based antiviral discovery[6]. The therapeutic relevance of Mpro inhibition has been clinically validated by Nirmatrelvir, the active protease inhibitor component of Paxlovid, which inhibits recombinant SARS-CoV-2 Mpro with reported K_i and IC_{50} values of 3.1 nM and 19.2 nM, respectively[7,8]. Antiviral lead discovery has traditionally relied on both synthetic [9] and natural scaffolds[10]. However, natural products remain valuable sources of antiviral lead scaffolds because they provide chemical diversity that is often underrepresented in synthetic libraries[11,12]. Among plant-derived metabolites, sesquiterpene lactones are of particular interest because they are oxygenated terpenoid compounds[13] with diverse biological activities and structural features capable of supporting protein–ligand recognition[13,14]. Several studies have investigated plant-derived compounds as potential inhibitors of SARS-CoV-2 proteases; however, a substantial part of this literature has focused on broadly distributed phytochemicals, particularly flavonoids, phenolic acids, and other polyphenolic compounds[15–17]. In comparison, bitter sesquiterpene lactones represent a less explored natural-product class for coronavirus protease-directed investigation [18]. *Artemisia absinthium* L. is a medicinal plant known to contain several classes of secondary metabolites, including volatile constituents, flavonoids, phenolic acids, coumarins, and bitter sesquiterpene lactones[19,20]. Among these constituents, Absinthin and Anabsinthin are chemically distinctive representatives of the wormwood bitter-lactone fraction[21]. Unlike broadly distributed compounds such as quercetin, rutin, caffeic acid, or chlorogenic acid, Absinthin and Anabsinthin are more characteristic of the sesquiterpene-lactone profile of wormwood[22]. Their dimeric sesquiterpene lactone architecture makes them especially relevant for exploring under investigated natural scaffolds that may interact with viral protease binding pockets. Previous computational screening has suggested that wormwood-derived phytochemicals may interact with SARS-CoV-2-related

targets[23,24]. However, these studies were generally broad virtual screenings and did not provide a focused Mpro-centred evaluation of Absinthin and Anabsinthin. There remains a lack of integrated analysis. This gap limits the ability to judge whether their predicted binding is structurally meaningful or merely a consequence of broad screening. Accordingly, the present study was designed to evaluate Absinthin and Anabsinthin as underexplored wormwood-derived sesquiterpene lactones against SARS-CoV-2 Mpro using an integrated phytochemical and structure-based computational workflow. The experimental strategy first involved solvent-dependent extraction optimization through a design of experiments approach, followed by GC-MS and LC-MS chemical profiling and targeted purification of the major sesquiterpene lactone fraction. This workflow enabled the selection of absinthin and anabsinthin as chemically distinctive candidates for subsequent in silico evaluation. We hypothesized that these chemically distinctive lactones could fit within the Mpro active site and establish relevant interactions with residues involved in substrate recognition and catalytic-site organization. To test this hypothesis, the study combined molecular docking, interaction analysis, ADME and toxicity prediction, and molecular dynamics simulation.

2. Material and methods

2.1 Plant material collection, identification, and pre-extraction processing

Aerial parts of *Artemisia absinthium* L. were collected in October from cultivated plants maintained within an area dedicated to aromatic and medicinal plants at Mohammed VI Polytechnic University (UM6P), Benguerir, central Morocco (32°13'09.5"N, 7°56'48.5"W). The unique semi-arid soil and climatic conditions of this region are known to enrich plant phytochemical diversity [25]. The plant material was identified by members of the research team based on its characteristic morphological features and their established experience in the handling and study of aromatic and medicinal plants. The material originated exclusively from cultivated plants within the university campus and was not collected from wild populations or protected natural habitats. No voucher specimen was deposited in a public herbarium at the time of collection, and no separate field-collection permit was issued for this material. The plant material was subsequently air-dried for 3 days in a shaded and ventilated environment, ground into a fine powder, and stored in airtight containers protected from light and moisture until extraction.

2.2 Ultrasound-assisted extraction procedure

Ultrasound-assisted extraction (UAE) was performed using hexane and ethanol. Dried plant powder was extracted at room temperature using a probe sonicator (Figure S1). The solid-to-liquid ratio was varied from 0.01 to 0.04 g/mL, and the sonication time varied between 0 and 15 min. After extraction, suspensions were filtered and solvents were removed under reduced pressure to obtain dried extracts. Extraction yield (%) was calculated as the ratio of dried extract mass to initial mass of plant powder. The optimal conditions were selected based on maximum yield and reproducibility.

2.3 Box-Behnken design and response-surface optimization

Extraction optimization was performed using a Box–Behnken design (BBD) with three factors: sonication time (A, min), solid-to-liquid ratio (B, g/mL), and solvent type (C). The design, comprising 28 experimental runs, was generated and analysed using Design-Expert software (Version 13). A quadratic model was fitted to the experimental yield data, and its significance was evaluated by analysis of variance (ANOVA). The complete design matrix and corresponding response values are provided in Table S1 (Supplementary Information).

2.4 GC-MS profiling of volatile and semi-volatile constituents

Hexane and Ethanolic fractions were analysed by GC-MS SCION 8900 GC coupled to a SCION 8900 Triple-Quadrupole mass spectrometer (TQ) (SCION Instruments). Separations were performed on a DB-5ms UI capillary column (30 m × 0.25 mm) with helium as carrier gas at a constant flow of 1.0 mL min⁻¹. The injector was operated in splitless mode (10:1), injector temperature 250 °C, injection volume 1 µL. The oven program was 50 °C (1 min), ramp 10 °C min⁻¹ to 300 °C, hold 10 min. The MS was operated in electron-ionization (EI) mode at 70 eV; ion-source, quadrupole, and transfer-line temperatures were 200 °C, 150 °C, and 280 °C, respectively. Data were acquired in full-scan mode over m/z 40–550 at 5 scans s⁻¹. Identification was based on retention indices (n-alkane series, C8–C30) and library matching (NIST), with manual verification of diagnostic ions.

2.5 LC-MS metabolite profiling of crude extracts

Wormwood extracts were profiled by LC-MS on an Agilent 1260 Infinity II system coupled to an Agilent InfinityLab LC/MSD single-quadrupole, equipped with the multimode (ESI/APCI) source. Chromatographic separation was performed using a binary mobile phase consisting of (A) water and (B) acetonitrile, both acidified with 0.1% formic acid, at a flow rate of 0.5 mL/min. Nitrogen served as the nebulizing gas at 300 °C. Data was acquired in positive and negative ion modes over m/z 100–1000. The conversion of LC/MS files was performed using LC/SQ ChemStation to MassHunter Translator (version 8.04.00), with analyses conducted on

MassHunter Qualitative Analysis Navigator & Workflows (version B.08.00). Tentative identifications were assigned from MS spectra and retention behaviour by comparison with peer-reviewed databases and the primary literature.

2.6 Targeted purification and LC-MS-based identification of absinthin and anabsinthin

The ethanol extract obtained under optimal UAE conditions was filtered and evaporated at 40 °C under reduced pressure. The residue was partitioned with ethyl acetate (50 mL) and washed twice with brine. The organic phase was subjected to a QuEChERS dispersive-SPE cleanup using anhydrous MgSO₄ and a silica-based sorbent (PSA/C18) were added together, the mixture was vortexed for 1 min, centrifuged, the supernatant was filtered and concentrated at 40 °C under reduced pressure. The cleaned extract was then fractionated on a BÜCHI Pure system in preparative HPLC mode with a C18 column (250 × 10 mm, 5 µm). Elution employed a linear water/acetonitrile gradient containing 0.1% formic acid with gradient 0 to 40 % of acetonitrile in 10 min then 40 % to 60 % in 5 min and later 60 % of acetonitrile in 20 min as isocratic mode, Fractions were monitored by UV and ELSD; major peaks were collected automatically, dried, and characterised by LC-MS.

2.7 In silico Analysis

2.7.1 Mpro structure selection and receptor preparation

The crystal structure of SARS-CoV-2 main protease, Mpro, in complex with the non-covalent inhibitor X77 was retrieved from the Protein Data Bank using the accession code 6W63 [26]. This structure was selected because it provides an experimentally resolved ligand-bound active site and has an X-ray resolution of 2.10 Å, allowing reliable structure-based docking analysis. Prior to docking, the co-crystallized ligand X77 was extracted and saved separately for redocking validation and comparative analysis. The protein structure was then prepared using AutoDockTools [27] by removing the co-crystallized ligand, crystallographic water molecules, and non-essential heteroatoms. Polar hydrogens were added, appropriate atomic charges were assigned, and the prepared receptor was saved in PDBQT format for molecular docking.

2.7.2 Ligand structure retrieval and preparation

Absinthin and Anabsinthin were obtained from PubChem [28] in 3D SDF format, while X77 was extracted directly from the 6W63 crystal structure. All ligands were converted from SDF or PDB format into PDB format using Open Babel [29]. The resulting structures were then

prepared using AutoDockTools, where hydrogens and charges were assigned, rotatable bonds were defined, and each ligand was saved in PDBQT format for docking calculations.

2.7.3 Molecular docking

Molecular docking was performed using AutoDock Vina [30]. The docking grid was centered on the binding pose of the co-crystallized ligand X77 to reproduce the experimentally defined active site. The grid box encompassed the main catalytic and substrate-binding residues of Mpro, including His41, Thr45, Met49, Gly143, Ser144, Cys145, His163, Met165, Glu166, Gln189, and Gln192. The grid center was set at $x = -23.154$, $y = 15.059$, and $z = -30.384$, with grid dimensions of $22 \times 22 \times 30$ Å. This grid covered the predicted binding cavity with an estimated volume of 525 Å³. Docking calculations were performed using an exhaustiveness value of 32, and the best-ranked poses were selected according to the predicted binding affinity and their orientation within the active site.

2.7.4 Redocking validation and post-docking interaction analysis

To validate the docking protocol, X77 was redocked into the prepared 6W63 binding site using the same docking parameters applied to the studied ligands. The docked pose of X77 was then superimposed onto the original co-crystallized pose using PyMOL[31], and the RMSD was calculated to assess the ability of the protocol to reproduce the experimental binding orientation. Post-docking interaction analysis was performed on the selected binding poses. Two-dimensional protein–ligand interaction diagrams were generated using BIOVIA Discovery Studio Visualizer, while three-dimensional binding orientations and pose representations were prepared using PyMOL.

2.7.5 Drug-likeness and ADME prediction

The pharmacokinetic properties of Absinthin, Anabsinthin, and X77 were predicted using SwissADME [32]. The evaluated descriptors included topological polar surface area, TPSA, lipophilicity, LogP, aqueous solubility, LogS, gastrointestinal absorption, blood-brain barrier permeation, P-glycoprotein substrate status, CYP2D6/ CYP3A4 inhibition, Lipinski rule compliance [33], and PAINS alerts. The SwissADME bioavailability radar was used to visualize the drug-likeness profile of each compound, while the BOILED-Egg model [34] was used to assess the predicted relationship between gastrointestinal absorption, brain permeation, and P-glycoprotein-related efflux.

2.7.6 Toxicity and safety-liability prediction

The toxicity profiles of the compounds studied were evaluated using ProTox-III [35] and pkCSM [36]. ProTox-III was used to predict the median lethal dose, LD50, expressed in mg/kg, and the corresponding toxicity class. Molecular weight and dose-value distribution outputs were also retrieved from ProTox-III to support comparative toxicity interpretation. Complementary toxicity-related endpoints were predicted using pkCSM, including AMES toxicity, maximum tolerated human dose, hERG I inhibition, hERG II inhibition, oral rat acute toxicity, oral rat chronic toxicity, hepatotoxicity, skin sensitization, *Tetrahymena pyriformis* toxicity, and minnow toxicity. These predictions were used as preliminary in silico indicators of safety liabilities and were interpreted comparatively across the studied compounds.

2.7.7 Molecular dynamics simulation setup and trajectory analysis

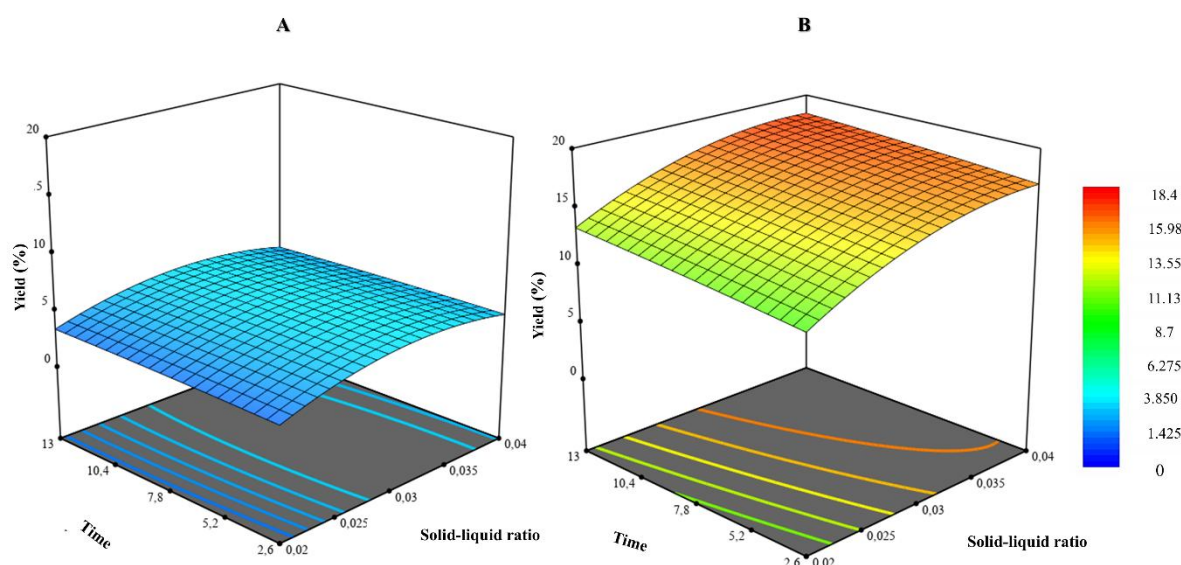
Molecular dynamics simulations were performed using the Desmond module of the Schrödinger suite [37]. The selected protein-ligand complexes were prepared and subjected to system generation using the System Builder tool. The systems were parameterized using the OPLS4 force field [38] and solvated in an orthorhombic simulation box using the SPC water model. The box dimensions were generated using the buffer-based calculation method, and the systems were neutralized with ions at a salt concentration of 0.15 M. Before production simulation, each system was relaxed using the standard Desmond relaxation protocol. Production molecular dynamics simulations were performed for 100 ns under the NPT ensemble at a temperature of 300 K and a pressure of 1.01325 bar. Trajectory frames were recorded at intervals of 100 ps, generating approximately 1000 frames per simulation. The energy recording interval was set to 1.2 ps. post-simulation analyses, including RMSD, RMSF, and protein-ligand contact profiling, were performed to assess the dynamic stability of the complexes, as commonly applied in structure-based drug-discovery workflows [39].

3. Results and discussion

3.1 Box-Behnken optimization of ultrasound-assisted extraction yield

In recent years, ultrasound-assisted extraction has emerged as an efficient and sustainable alternative to conventional solid–liquid extraction methods, mainly due to acoustic cavitation phenomena that enhance solvent penetration, disrupt plant cell walls, and improve mass transfer while reducing extraction time and solvent consumption [40]. In this context, recent works from our laboratory have further highlighted the relevance of ultrasound-assisted approaches for the recovery and processing of natural compounds under mild and greener conditions [41]

258 The ultrasound-assisted extraction of *Artemisia absinthium* L. was refined using a Box–
 259 Behnken design (Supplementary Table S1). A statistically robust quadratic model was
 260 established, explaining 93.3% of the yield variability ($R^2 = 0.9328$, $p < 0.0001$) and showing
 261 strong predictive capability (Adjusted $R^2 = 0.9030$; Predicted $R^2 = 0.7962$). Based on ANOVA
 262 (Supplementary Table S2) and the coded model equations (Supplementary Table S3), ethanol
 263 showed a strong positive contribution ($C = +5.56$), whereas the solid-to-liquid ratio presented
 264 a significant negative quadratic effect ($B^2 = -1.51$), indicating an optimum beyond which the
 265 yield plateaus. Accordingly, the predicted optimal conditions (time = 5.26055 min, solvent =
 266 EtOH, solid-to-liquid ratio = 0.036 g/mL) identified solvent type as the most influential factor
 267 ($p < 0.0001$), followed by the solid-to-liquid ratio ($p = 0.0036$), while sonication time was not
 268 significant ($p = 0.4452$). Response surface plots (Fig. 1) further indicated that ethanol produced
 269 markedly higher yields (up to 19.4%) than hexane ($\leq 9.0\%$) across the experimental domain.



270
 271 **Fig. 1** Response surface plots showing the effect of sonication time and solid–liquid ratio on
 272 the extraction yield (%) of *Artemisia absinthium* L by UAE using (A) hexane and (B) ethanol
 273 as solvents

274 3.2 GC-MS profiles of volatile and semi-volatile constituents

275 GC–MS analysis revealed distinct volatile profiles in the ethanolic and hexane extracts of *A.*
 276 *absinthium* (Table 1). Both extracts contained characteristic oxygenated monoterpenes,
 277 including thujone and camphor, which are widely reported chemotaxonomic markers in

278 wormwood-derived volatile fractions [42–44]. Their presence in both extracts is consistent with
 279 their moderate lipophilicity, allowing partitioning into solvents of different polarity.
 280 The hexane extracts additionally contained higher molecular weight, non-polar constituents. A
 281 sesquiterpene hydrocarbon (C₁₅H₂₄) was detected at 20.8 min, alongside polycyclic aromatic
 282 compounds, including dehydrochamazulene, were also observed; this azulene pigment often
 283 formed via thermal rearrangement of guaianolide-type sesquiterpene lactones during GC
 284 analysis. A series of isomeric C₁₄H₁₄ compounds (match scores 735–761) was also detected and
 285 is tentatively attributed to aromatic plant metabolites and/or thermal degradation products.
 286

287 **Table 1.** Key volatile compounds identified by GC–MS in *A. absinthium* extracts.

Extract	RT (min)	Identified Compound	Molecular Formula	Match Factor	Notes
Ethanol	11.6	Thujone	C ₁₀ H ₁₆ O	862	Characteristic monoterpene of wormwood
Ethanol	12.5	(+)-2-Bornanone (Camphor)	C ₁₀ H ₁₆ O	860	Common in <i>Artemisia</i>
Hexane	11.6	Thujone	C ₁₀ H ₁₆ O	863	Characteristic monoterpene of wormwood
Hexane	12.5	(+)-2-Bornanone (Camphor)	C ₁₀ H ₁₆ O	874	Common in <i>Artemisia</i>
Hexane	12.5	1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one (Camphor)	C ₁₀ H ₁₆ O	850	Camphor isomer (library match)
Hexane	20.8	(S,1Z,6Z)-8-Isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene	C ₁₅ H ₂₄	836	Germacrene-type sesquiterpene hydrocarbon
Hexane	27.2	Dehydrochamazulene	C ₁₄ H ₁₄	717	Azulene sesquiterpenoid; thermal degradation

				product	
Hexane	27.2	C ₁₄ H ₁₄ isomers	C ₁₄ H ₁₄	735– 761	Aromatic derivatives

The detected thujone isomers situate these extracts within the historic *A. absinthium* chemotype, though their concentrations are pharmacologically insignificant [42]. Consequently, our focus shifts to the major ethanolic fraction constituents found via LC-MS: the sesquiterpene lactone isomers, absinthin and anabsinthin.

3.3 LC-MS metabolite profiles and solvent selectivity of crude extracts

LC-MS analysis revealed distinct and solvent-dependent metabolite profiles in the ethanolic and hexane extracts of *Artemisia absinthium* L. The spectral data was reported and provided in the Supplementary Information. Table 2 summarizes selected LC-MS features tentatively classified based on observed m/z values, retention times, reported ESI polarity, fragmentation behaviour, and consistency with previously described *Artemisia* metabolites [45,46]

The ethanolic extract exhibited a broad chromatographic profile spanning approximately 1.47–12.19 min, consistent with extraction of mid-polar to polar secondary metabolites. Several compounds (CPDs) detected in the ethanolic extract showed recurrent high-mass ions in the m/z 460–520 range, frequently accompanied by dehydration-type and lower-mass fragment ions. Such ion patterns are consistent with dimeric sesquiterpene lactones reported in *A. absinthium*, including absinthin/anabsinthin-type scaffolds. At the crude-extract level, these features are interpreted conservatively as absinthin/anabsinthin-type dimeric lactone-related ions, reflecting a combination of molecular, dehydrated, and fragment species rather than isolated molecular ions. Lower-mass ions at $m/z \approx 248.9$ detected in the ethanolic extract co-eluted with the dimer-related CPDs and are therefore attributed to in-source fragmentation of dimeric sesquiterpene lactones, rather than to free monomeric lactones in ethanol.

In contrast, the hexane extract displayed a more selective enrichment of low- to mid-polarity constituents. LC-MS spectra were dominated by CPDs with m/z values in the ~230–290 range, consistent with monomeric guaianolide-type sesquiterpene lactones. Recurrent ions at $m/z \approx 248.9$ and $m/z \approx 280$ –282 were therefore tentatively assigned to santonin- and artabsin-type guaianolides, which are preferentially extracted by non-polar solvents. Additional hexane-specific CPDs at m/z 325.0 (RT 4.238 min) and m/z 388.7 (RT 6.277 min) are consistent with oxidized guaianolide-type sesquiterpene derivatives reported in *Artemisia* species [47–50] Although high-mass ions in the m/z ~460–520 range were occasionally observed in hexane

extracts, these signals were weak and occurred alongside extensive background and cluster-type ions, precluding assignment to dimeric lactones.

Table 2. Key metabolites tentatively identified by LC–MS in *A. absinthium* extracts.

Extract	CPD No.	RT (min)	Observed <i>m/z</i>	ESI mode (as reported)	Tentative classification
Ethanol	CPD 3	1.588	520.9, 588.8, 452.8	–ESI Scan:2 / ±ESI TIC	Absinthin/Anabsinthin-type dimeric lactone-related ions
Ethanol	CPD 4	1.588	498.4	+ESI Scan:1	Protonated dimer-related ion
Ethanol	CPD 16	5.723	248.9	+ESI Scan:1	Fragment ion of dimeric lactones
Hexane	CPD 6	1.995	248.9	+ESI Scan:1	Santonin/Artabsin-type monomeric guaianolide
Hexane	CPD 38	9.742	282.0	±ESI TIC	Artabsin-related monomeric lactone
Hexane	CPD 9	4.238	325.0	–ESI Scan:2	Oxidized guaianolide derivative
Hexane	CPD 22	6.277	388.7	+ESI Scan:1	Oxidized sesquiterpene lactone

The full reports of detected ions for both extracts are provided in Supplementary Information.

Overall, the LC–MS data demonstrate clear solvent selectivity, with ethanol preferentially enriching dimeric sesquiterpene lactones (absinthin/anabsinthin-type), whereas hexane enriches monomeric guaianolides (santonin/artabsin-type) and oxidized lactone derivatives. On this basis, absinthin/anabsinthin-type features detected in the ethanolic extract were selected for profiling-guided purification and subsequent *in silico* prioritization against SARS-CoV-2 Mpro.

3.4 Profiling-guided purification of absinthin/Anabsinthin isomers

A sequential purification strategy was employed to enrich and isolate absinthin/anabsinthin isomers from the ethanol extract. Initial matrix cleanup of the crude extract (Fig. 2A) using a modified QuEChERS protocol, followed by preparative HPLC, yielded an enriched fraction (Fig. 2B). Analytical HPLC of this fraction showed two partially resolved peaks at 8.275 min (Peak A, 33.0%) and 8.458 min (Peak B, 67.0%), with a chromatographic resolution (*R_s*) of 1.62, indicating effective removal of interfering constituents. The predominant isomer (Peak B) was subsequently isolated to chromatographic purity by preparative HPLC (Figure 2C), as evidenced by a single symmetrical peak.

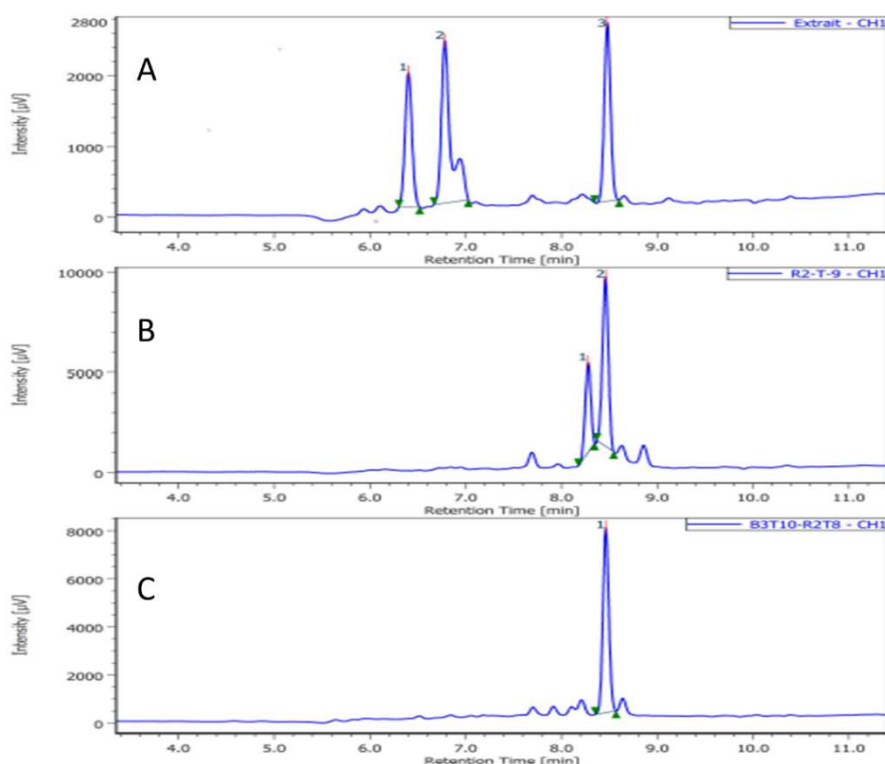


Fig. 2 The tentative purification of absinthin/anabsinthin isomers from *Artemisia absinthium* ethanolic extract; (A) Crude extract showing co-elution of target isomers; (B) Enriched fraction after QuEChERS cleanup and preparative HPLC, showing partial separation of isomers (Peak A: 8.275 min, 33.0%; Peak B: 8.458 min, 67.0%; $R_s = 1.62$); (C) Purified isomer B (absinthin/anabsinthin) following second preparative HPLC purification, displaying a single peak at 8.458 min.

Structural verification of the purified isomers was carried out using LC–MS, that revealed a prominent ion tentatively assigned to a dehydrated protonated species of absinthin/anabsinthin, consistent with in-source loss of water from the parent dimeric sesquiterpene lactone ($C_{30}H_{40}O_6$)[51–55]. Such in-source dehydration and fragmentation behavior is well documented for guaianolide-type dimers under electrospray ionization conditions. In addition, characteristic fragment ions detected in the m/z ~248–310 range, including ions at m/z 309.9, 230.8, and 228.8, are consistent with reported guaianolide dimer fragmentation pathways[56–58], thereby supporting the tentative structural assignment of the purified compounds as absinthin/anabsinthin isomers. The absence of significant background ions in the mass spectrum indicates a high degree of chromatographic purity. With the compound’s identity and purity tentatively established, it was subsequently subjected to in-silico screening against Mpro a target that remains largely unexplored for these sesquiterpene lactones.

3.5 Structure-based computational evaluation of absinthin and anabsinthin against Mpro

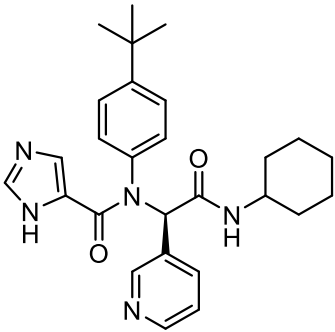
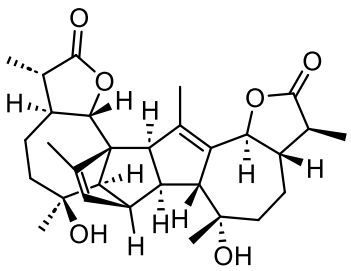
Mpro was selected because it is essential for viral polyprotein processing and replication [5], and its inhibition is clinically validated by Nirmatrelvir [7], the active Mpro inhibitor in Paxlovid. The crystal structure 6W63 was chosen because it contains Mpro complexed with X77, a potent non-covalent inhibitor, thereby providing an experimentally resolved active-site reference for docking and interaction analysis. Its 2.10 Å X-ray resolution and absence of reported mutations support its suitability for structure-based evaluation. Regarding ligand selection, Absinthin and Anabsinthin were selected because they are characteristic bitter sesquiterpene lactones of *Artemisia absinthium* [19,20], unlike common plant polyphenols that are widely distributed and extensively screened [10,59]. Their selection is supported by the phytochemical profile of wormwood, where the two compounds are reported among the bitter sesquiterpene-lactone constituents, and by previous docking studies that suggests COVID-target-related binding potential for both compounds [23,24]. Together, these considerations support the present work as a focused evaluation of underexplored wormwood-derived scaffolds. In this study, the compounds were evaluated not only based on docking score, but also by binding-mode quality, interaction similarity to references, predicted ADMET behavior, and dynamic stability using molecular dynamics.

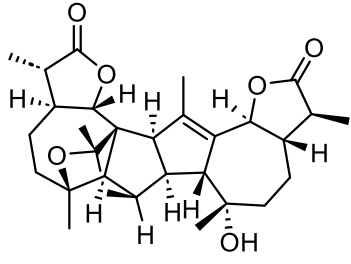
3.5.1 Molecular docking and comparative Binding-Mode Analysis

To assess the reliability of the docking protocol, the co-crystallized ligand X77 was first redocked into the active site of SARS-CoV-2 Mpro using the 6W63 crystal structure. The superposition between the crystallographic and re-docked X77 poses gave an RMSD of 0.72 Å (Fig. 3A), which indicates that the docking protocol was able to reproduce the experimentally observed binding orientation with high accuracy, and supports the use of the same docking parameters for subsequent evaluation of Absinthin and Anabsinthin within the X77-defined binding pocket. This redocking accuracy is consistent with previous Mpro docking validation studies. Sharma et al. reported an RMSD of 0.62 Å for the superimposed crystallographic and re-docked X77 structures [60]. After validation, X77, Absinthin, and Anabsinthin were docked into the same active-site region (Table 3). X77 showed a docking score of -8.3 kcal/mol, which is highly consistent with the study by Sharma et al., who reported a docking energy of -8.4 kcal/mol for the reference ligand X77 against SARS-CoV-2 Mpro [60]. Absinthin and Anabsinthin displayed scores of -8.8 and -9.1 kcal/mol, respectively. These values suggest that both natural compounds can be accommodated within the Mpro binding cavity. However, the

390 difference between the three scores remains moderate and should not be interpreted as evidence
 391 of stronger inhibitory activity. Instead, the results suggest that the wormwood selected lactones
 392 show docking behavior compatible with the catalytic pocket and merit deep evaluation by
 393 molecular dynamics simulations. In the post-docking interaction analysis, the interaction profile
 394 of X77 provided a useful reference for interpreting the binding behavior of the two
 395 sesquiterpene lactones. The reference (Fig. 3B) occupied the central active-site cleft and
 396 established a broad interaction network involving residues from the catalytic and substrate-
 397 recognition regions [5]. It formed hydrogen bonds with Glu166 and Gly143, π -related contacts
 398 with His41, π -sulfur interactions with Met49 and Cys145, and additional contacts with Leu141,
 399 Asn142, Met165, and nearby residues. This pattern is structurally meaningful because His41
 400 and Cys145 form the catalytic dyad [61], while Gly143, Ser144, and Cys145 contribute to the
 401 oxyanion-hole region involved in stabilizing the catalytic intermediate[61–63]. Within this
 402 context, Glu166 deserves particular attention. It is not simply a peripheral contact residue[64];
 403 it contributes to the architecture of the S1 subsite and is involved in maintaining the correct
 404 shape of the substrate-binding region[65]. The S1 subsite is especially important for Mpro
 405 specificity because the enzyme preferentially cleaves viral polyprotein sequences after a
 406 conserved P1 glutamine[62].

407 **Table 3.** Molecular docking scores of X77, Absinthin and Anabsinthin

Ligands	Structure	Docking Score (Kcal/mol)
X77		-8.3
Absinthin		-8.8

Anabsinthin		-9.1
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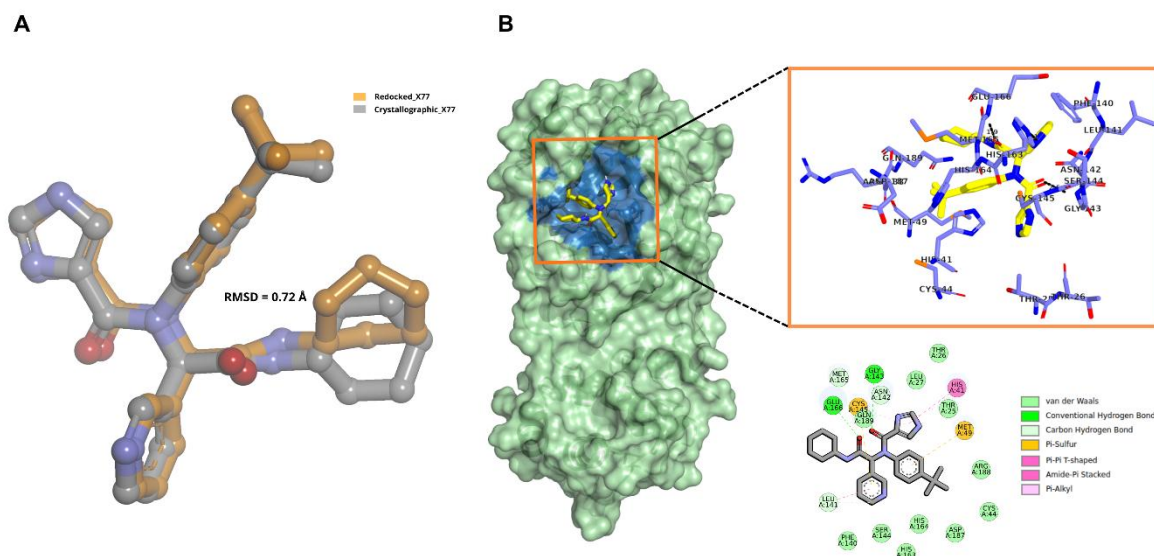


Fig. 3 (A) Validation of the molecular docking protocol by redocking of the co-crystallized X77 inhibitor into SARS-CoV-2 Mpro; (B) Binding-site localization and interaction profile of the reference inhibitor X77 within the SARS-CoV-2 Mpro active site

Structural studies also show that the active conformation of Mpro depends on the proper organization of the substrate-binding site, particularly around the S1 region[62–65]. Therefore, ligands positioned near Glu166 may influence a key recognition zone rather than only occupying a nonspecific hydrophobic cavity. In Figure 4, Absinthin adopted a pose within the same general binding pocket (Fig. 4A) but showed a more compact interaction pattern than X77. Its predicted stabilization mainly involved hydrogen bonding with His41, a carbon hydrogen bond with Gly143, and van der Waals contacts with Met49, Met165, and Glu166 (Fig. B and C). Direct interaction with His41 is favorable because this residue belongs to the catalytic dyad. At the same time, the absence of a clear direct interaction with Cys145 means that Absinthin should not be described as directly targeting the full catalytic dyad. A more

appropriate interpretation is that Absinthin may interfere with the catalytic-pocket environment by occupying the X77-binding region and contacting residues involved in catalysis and substrate positioning. Anabsinthin showed the most favorable docking score among the three ligands, with a predicted binding energy of -9.1 kcal/mol (table1). Its pose was mainly anchored by a predicted hydrogen bond with Gln189 (Fig. D and E), together with van der Waals contacts involving His41, Met49, Met165, and Glu166 (Fig. 4F). This interaction pattern is different from that of Absinthin and X77, but it remains relevant because Gln189 is in the flexible region bordering the substrate-binding cleft[5]. The region containing the S2 and S4 subsites is known to show conformational flexibility upon ligand binding, and Gln189 is frequently involved in adapting the pocket around bound inhibitors[66,67]. Therefore, the Gln189 interaction may be important for explaining how Anabsinthin is accommodated in the active site despite lacking the extended polar network observed for X77. Comparison of the three ligands suggests distinct binding behaviors. X77 displayed the richest interaction network, involving both catalytic and substrate-recognition residues (Fig. 3B). By contrast, Absinthin showed more direct interactions with His41 and Gly143, which are associated with the catalytic and oxyanion-hole regions. Anabsinthin appeared to rely more on pocket adaptation and anchoring through Gln189, while maintaining contacts with residues surrounding the S1/S2 region, including Glu166, Met165, Met49, and His41. This distinction is important, but it remains preliminary because this docking prediction remains static [68] and the later MD analysis can clarify whether these docking contacts are transient or persist during simulation.

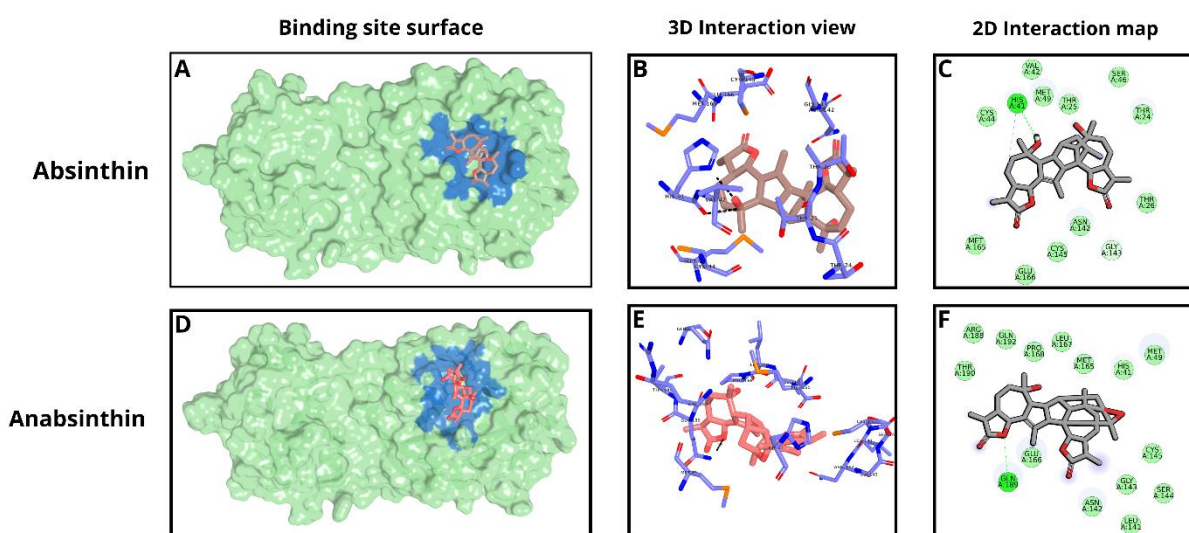


Fig. 4 Comparative docking poses and interaction maps of absinthin and anabsinthin within the SARS-CoV-2 Mpro active site

3.5.2 Predicted Drug-Likeness and ADME Profiles

The drug-likeness and pharmacokinetic profiles of the two sesquiterpene lactones were evaluated using SwissADME and compared with the co-crystallized Mpro ligand X77. This step was included to determine whether the favorable docking results were accompanied by acceptable physicochemical and early ADME properties. As shown in Table 4, the two phytochemicals have identical molecular weights of 496.64 g/mol, placing them close to the upper limit accepted by Lipinski's rule of five [69,70]. These compounds are relatively large natural products, but remain within the classical molecular weight threshold of 500 g/mol. Their MLogP values were 3.95 for Absinthin and 4.04 for Anabsinthin, compared with 2.19 for X77. This suggests that both compounds are more lipophilic than X77, which may be consistent with their hydrophobic contacts within the Mpro binding pocket observed in the post-docking analysis. However, this higher lipophilicity should also be interpreted carefully because excessive hydrophobicity may affect solubility and pharmacokinetic behavior[71,72]. Both natural compounds respected the hydrogen-bonding criteria of Lipinski's rule, with 6 hydrogen bond acceptors and 2 or 1 hydrogen bond donors for Absinthin and Anabsinthin, respectively. Lipinski analysis suggests that both compounds show acceptable rule-of-five compliance, although their molecular weight and lipophilicity are close to the upper favorable range.

Table 4. Lipinski rule compliance of the compounds along with X77

Compound	MW	MW	MLogP	HBA	HBD	Lipinski Violations
Absinthin	496.64	496.64	3.95	6	2	No
Anabsinthin	496.64	496.64	4.04	6	1	No
X77	459.58	459.58	2.19	4	2	No

The SwissADME bioavailability radar shown in Fig. 5A supports this interpretation. Both natural compounds display physicochemical profiles that are broadly comparable to X77, especially in terms of size, polarity, lipophilicity, solubility, and flexibility. Their radar profiles indicate that the compounds are not outside the general range expected for orally relevant small molecules, but they remain near the limits for size and lipophilicity. The more detailed ADME prediction is presented in Table 5. Absinthin and Anabsinthin showed TPSA values of 93.06 Å² and 82.06 Å², respectively, close to X77 (90.98 Å²). These values are consistent with the predicted high gastrointestinal absorption reported for all three compounds. The predicted logS

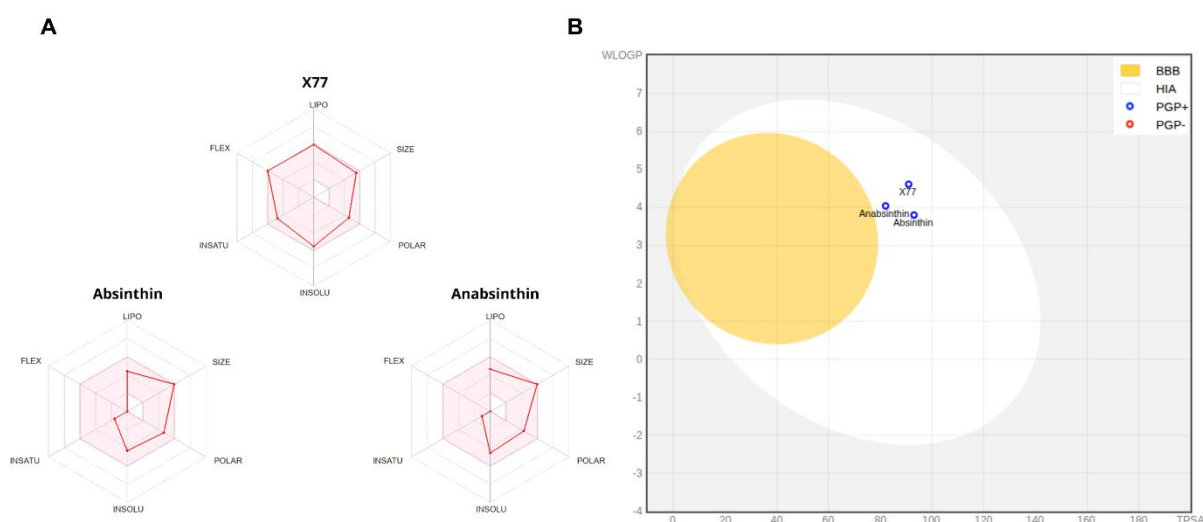
values of Absinthin (−4.33) and Anabsinthin (−4.59) classified both compounds as moderately soluble, similarly to X77 that showed the lowest predicted solubility value (−5.53). The BOILED-Egg model in Fig. 5B is consistent with the tabulated SwissADME predictions. All ligands are positioned in the white region related to predicted gastrointestinal absorption, but outside the yellow region is associated with blood-brain barrier permeation[34]. This profile is favorable in the present context because Mpro is not a central nervous system target, and the lack of predicted BBB permeation may reduce the probability of unnecessary CNS exposure. However, all three compounds were predicted to be P-glycoprotein substrates, as indicated by their blue markers in the BOILED-Egg plot. This point should be considered a potential limitation, since P-gp-mediated efflux may reduce intracellular accumulation and could influence oral exposure[73]. A notable difference between the natural compounds and X77 was observed in the predicted CYP inhibition profile. X77 was predicted to inhibit both CYP2D6 and CYP3A4, whereas the two sesquiterpene lactones were not predicted to inhibit either enzyme. At least computationally, this suggests that both compounds may present a lower risk of CYP-mediated drug-drug interactions than X77[74]. In addition, none of the three compounds showed PAINS alerts, reducing the likelihood that their predicted activity is associated with common assay-interfering substructures [75]. Taken together, the predicted ADME analysis shows that the two wormwood-derived constituents combine acceptable drug-likeness with favorable predicted gastrointestinal absorption, moderate solubility, absence of BBB permeation, no PAINS alerts, and no predicted CYP2D6/CYP3A4 inhibition. Their main limitations are their relatively high molecular weight, elevated lipophilicity, and predicted P-gp substrate status. Therefore, these compounds should not be presented as optimized drug candidates, but rather as promising natural scaffolds with acceptable early ADME features that support further computational and experimental investigation.

Table 5. ADME Profile of the compounds along with X77

	Absinthin	Anabsinthin	X77
TPSA (Å ²)	93.06	82.06	90.98
logP	3.95	4.04	2.19
logS	-4.33	-4.59	-5.53
Solubility Class	Moderately soluble	Moderately soluble	Moderately soluble
GI Absorption	High	High	High
BBB Permeant	No	No	No

P-gp Substrate	Yes	Yes	Yes
CYP2D6 Inhibitor	No	No	Yes
CYP3A4 Inhibitor	No	No	Yes
PAINS alert	0	0	0

501



502

503 **Fig. 5** (A) SwissADME bioavailability radar profiles of X77, absinthin, and anabsinthin; (B)
504 BOILED-Egg prediction of gastrointestinal absorption

505 3.5.3 Predicted toxicity and safety-related liabilities

506 The preliminary toxicity profile of X77, Absinthin, and Anabsinthin were evaluated using
507 ProTox-III and pkCSM. These predictions were used as an early safety filter rather than as
508 definitive toxicological evidence, since computational toxicity models remain dependent on
509 training data, chemical similarity, and endpoint-specific assumptions. In the ProTox-III
510 prediction, Absinthin showed the highest predicted oral LD50 value (1330 mg/kg), followed by
511 X77 (650 mg/kg) and Anabsinthin (452 mg/kg). Since higher LD50 values generally indicate
512 lower predicted acute oral toxicity, this result suggests that Absinthin may present a more
513 favorable predicted acute toxicity profile than both X77 and Anabsinthin. Although the latter
514 showed the most favorable docking score, it had the lowest predicted LD50 among the three
515 compounds. However, all three compounds remained within the same ProTox toxicity class,
516 Class IV, which corresponds to compounds predicted as harmful if swallowed rather than highly
517 or extremely toxic. The distribution plots shown in Fig. 6 provide additional context for these

predictions. In the molecular weight distribution panel (Fig. 6A), Absinthin and Anabsinthin are positioned above the mean molecular weight of the ProTox dataset. X77 is also above the dataset mean but slightly lower than the two natural compounds. In the dose-value distribution panel (Fig. 6B), the predicted LD50 of Absinthin is closer to the dataset mean than those of X77 and Anabsinthin, providing visual context for its comparatively less concerning predicted acute toxicity profile. By contrast, Anabsinthin is positioned further toward the lower-dose region, consistent with its lower LD50 value. The pkCSM toxicity predictions partly supported the favorable safety of the natural compounds but also highlighted endpoint-specific differences [36] (Table 6). All three compounds were predicted to be AMES-negative. In addition, neither Absinthin nor Anabsinthin was predicted to inhibit hERG I or hERG II channels, whereas X77 was predicted as a hERG II inhibitor. This difference is relevant because hERG channel inhibition is associated with cardiac safety concerns, particularly QT prolongation and arrhythmogenic risk [76,77]. The hepatotoxicity prediction also favored the two natural compounds. X77 was predicted to be hepatotoxic, whereas the selected phytochemicals were not. Similarly, none of the three compounds was predicted to cause skin sensitization. The pkCSM rat acute toxicity values showed the same general range for the three ligands, with Absinthin having the highest predicted value, followed by Anabsinthin and X77. This trend is broadly consistent with the ProTox-III result for Absinthin, although the exact ranking of X77 and Anabsinthin differed between the two tools. Therefore, these results should be interpreted as complementary screening outputs rather than absolute toxicity rankings. For chronic oral toxicity, Absinthin showed the highest predicted LOAEL value (1.507 log mg/kg_bw/day), followed by Anabsinthin (1.108) and X77 (0.408). Since LOAEL reflects the lowest dose associated with adverse effects in repeated exposure settings, the higher predicted values for the two natural compounds suggest a potentially better chronic toxicity margin than X77. However, both the selected candidates showed low predicted maximum tolerated dose values in humans, indicating that dose-related safety may still require careful evaluation in later experimental studies.

Table 6. Predicted toxicity profile of X77, absinthin, and anabsinthin using ProTox-III and pkCSM

Parameter	X77	Absinthin	Anabsinthin
ProTox-III LD50 (mg/kg)	650	1330	452
ProTox toxicity class	Class IV	Class IV	Class IV

AMES toxicity	No	No	No
Max. tolerated dose, human (log mg/kg/day)	1.019	-0.482	-0.332
hERG I inhibitor	No	No	No
hERG II inhibitor	Yes	No	No
Oral rat acute toxicity, LD50 (mol/kg)	2.627	2.853	2.759
Oral rat chronic toxicity, LOAEL (log mg/kg_bw/day)	0.408	1.507	1.108
Hepatotoxicity	Yes	No	No
Skin sensitisation	No	No	No
T. pyriformis toxicity (log µg/L)	0.285	0.287	0.285
Minnow toxicity (log mM)	2.843	1.656	2.281

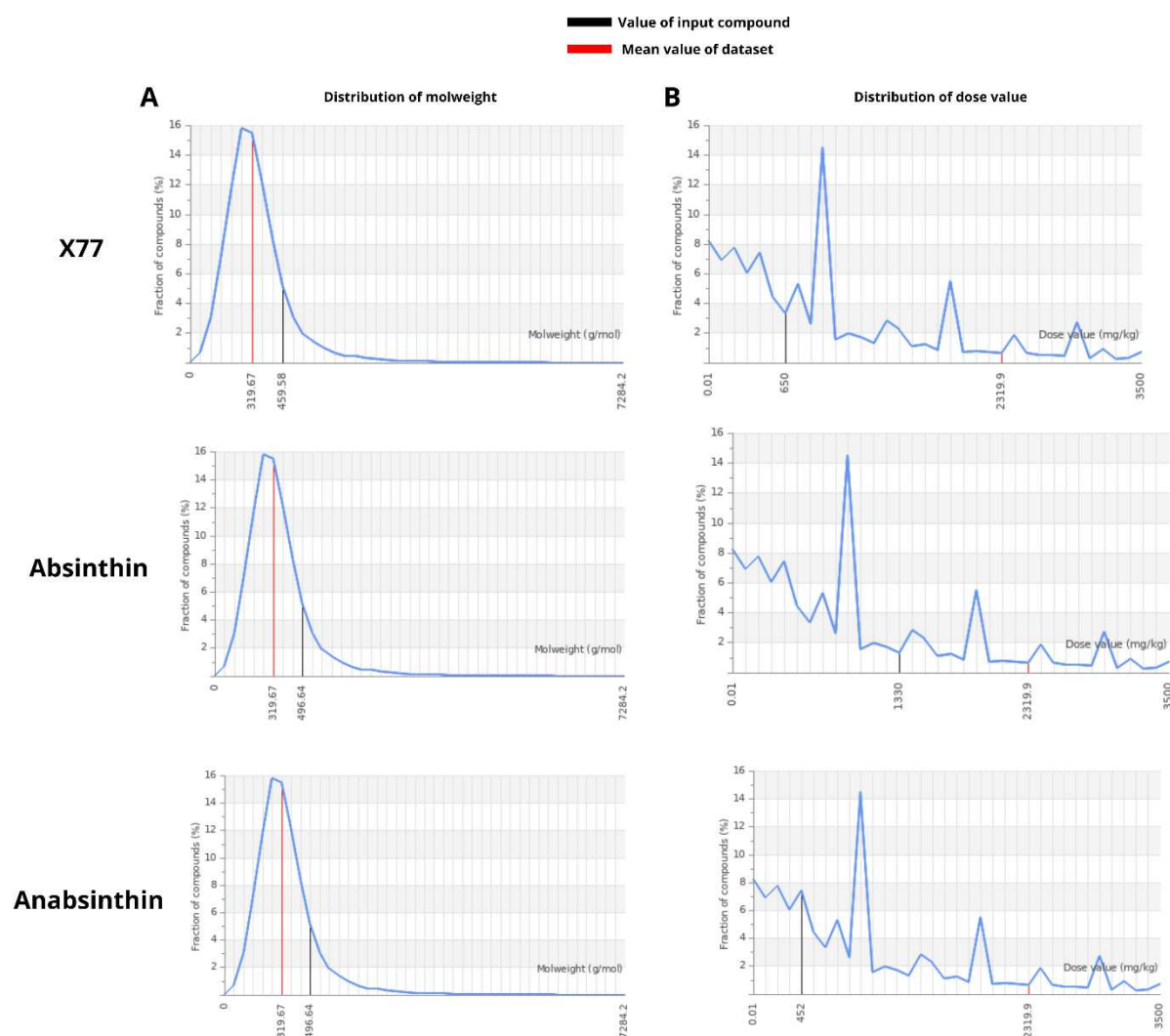


Fig. 6 ProTox-III toxicity-related molecular weight and predicted acute toxicity distribution profiles

3.5.4 Molecular dynamic-based stability assessment

The molecular dynamics results provided an additional dynamic assessment beyond docking and ADMET screening by showing how each Mpro-ligand complex behaved under time-dependent conditions[78]. In this stage, the apo enzyme was used as a baseline to distinguish ligand-induced effects from the intrinsic flexibility of Mpro, while X77 served as the crystallographic reference ligand of the selected structure. Importantly, Nirmatrelvir was introduced specifically in the MD analysis as an additional pharmacological reference, although it was not included in the previous docking and ADMET sections. This addition was justified because Nirmatrelvir is a clinically validated SARS-CoV-2 Mpro inhibitor and therefore provides a pharmacologically relevant benchmark for interpreting the dynamic behavior [78]of

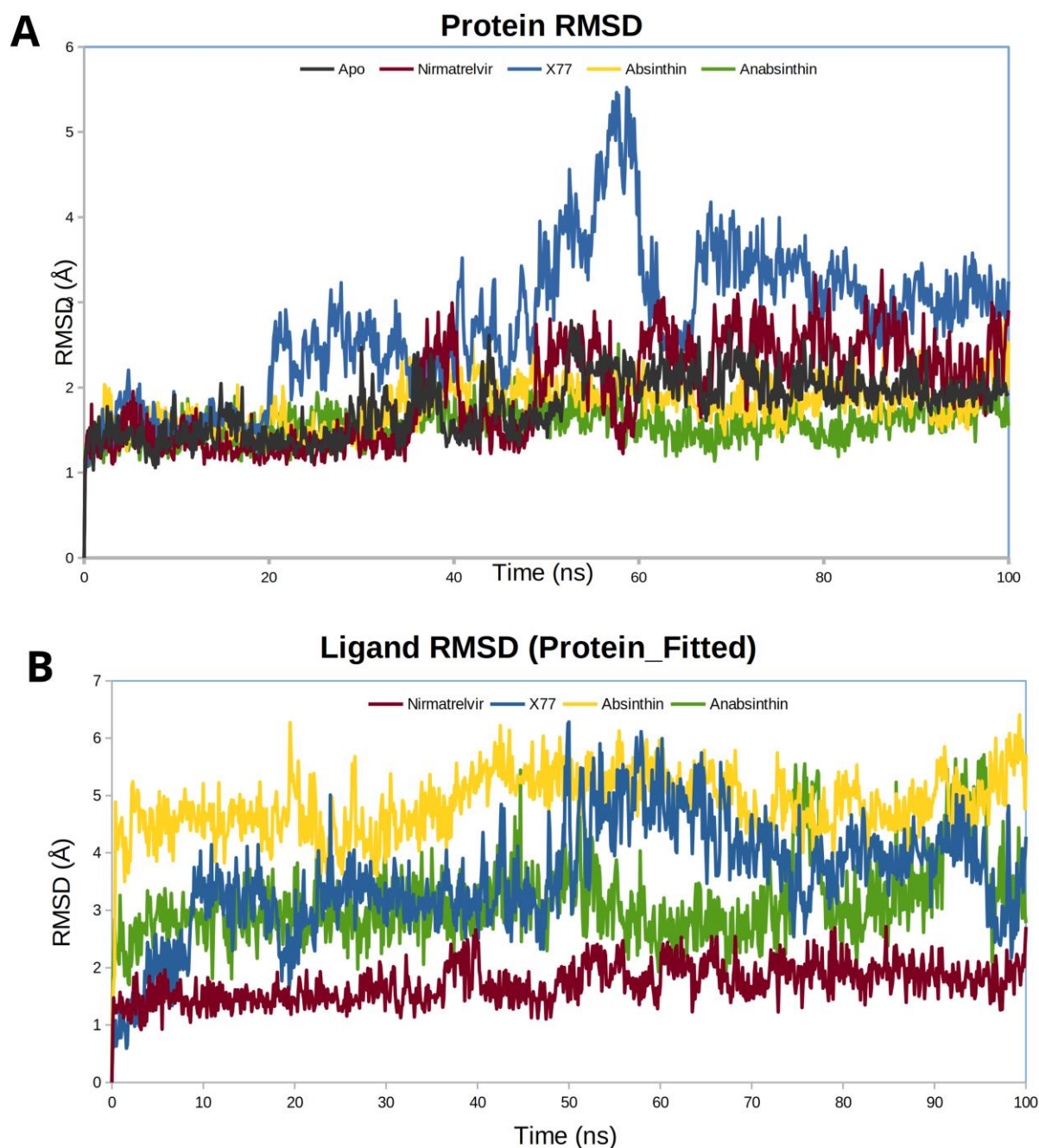
the wormwood selected lactones. Accordingly, the MD results were interpreted by comparing global protein stability, ligand positional stability, residue-level flexibility, and interaction persistence across the apo, X77-, Nirmatrelvir-, Absinthin-, and Anabsinthin-bound systems. This comparative framework allowed the natural compounds to be assessed not only against the co-crystallized ligand X77, but also against a clinically established Mpro inhibitor, giving stronger biological context to the RMSD (Fig. 7), RMSF (Fig. 8), and protein-ligand (Fig. 9) contact analyses.

3.5.5 Protein and ligand RMSD analysis

The dynamic stability of SARS-CoV-2 Mpro in its apo form and in complex with X77, Nirmatrelvir, Absinthin, and Anabsinthin was evaluated through protein backbone RMSD (Fig. 7A) and protein-fitted ligand RMSD (Fig. 7B) over 100 ns MD simulations. The RMSD analysis provides two complementary levels of interpretation: protein RMSD reflects the global structural deviation of Mpro during simulation [79], whereas ligand RMSD describes the positional stability of each ligand within the binding site after fitting the trajectory on the protein framework [80]. For the protein RMSD shown in Fig. 7A, the apo form displayed a mean RMSD of 1.795 ± 0.348 Å, with a terminal mean of 1.964 Å during the last 20 ns, which reveals that the unbound Mpro structure remained globally stable after initial relaxation. This apo RMSD profile applies to previous Mpro MD studies reporting stable backbone behavior around the 2 Å range. For example, Ramadhan et al., using GROMACS and the 6M0K structure, reported an average Mpro backbone RMSD of 2.16 Å over 100 ns and interpreted it as evidence of low fluctuation and stable protein behavior. Despite differences in PDB structure and simulation workflow, this agreement supports the reliability of the apo system used here as a dynamic baseline [81]. Among the ligand-bound systems, Anabsinthin produced the lowest protein RMSD, with a mean value of 1.576 ± 0.196 Å and a terminal mean of 1.655 Å. This suggests that Anabsinthin did not disturb the Mpro backbone and may even contribute to maintaining a compact and stable protein conformation. This is an important observation because a promising inhibitor should ideally bind without inducing excessive structural instability in the target enzyme. Absinthin also maintained an acceptable protein RMSD profile, with a mean RMSD of 1.797 ± 0.280 Å, very close to the apo system. Its terminal RMSD increased to 1.950 Å due to moderate late-stage conformational adjustment but without evidence of major backbone destabilization. Therefore, from the protein perspective, both wormwood-derived compounds, especially Anabsinthin, preserved the structural integrity of Mpro throughout the simulation. In contrast, the X77-bound complex showed the highest

protein RMSD, with a mean of 2.751 ± 0.850 Å, a terminal mean of 3.037 Å, and a maximum value reaching 5.523 Å. This deviation suggests a slight conformational drift of the protein-ligand complex during the simulation. Since X77 is a co-crystallized ligand, this result should be interpreted carefully because the high RMSD does not necessarily mean that X77 is biologically inactive, but it indicates that under the present simulation conditions, the X77-bound system underwent larger structural rearrangements than the other complexes. Nirmatrelvir showed an intermediate protein RMSD profile, with a mean of 1.936 ± 0.574 Å and a terminal mean of 2.392 Å. The stability of the nirmatrelvir-bound system agrees with previous computational reports. Ramadhan et al. used Nirmatrelvir as a positive Mpro control and reported an average protein backbone RMSD of 1.98 Å, which is highly comparable to the 1.936 Å observed in the present study[81]. Moreover, Mizuno et al. discussed that, during MD simulations of the Mpro–Nirmatrelvir complex, both the protein main-chain RMSD and ligand RMSD converged, and the ligand remained positioned within the active site [82]. The ligand RMSD analysis (Fig. 7B) provided a more direct view of ligand positional stability inside the Mpro binding pocket. Nirmatrelvir displayed the most stable ligand RMSD profile, with a mean value of 1.723 ± 0.325 Å and a terminal mean of 1.881 Å. This low and relatively consistent ligand RMSD indicates that Nirmatrelvir maintained a conserved binding pose throughout the trajectory, which is consistent with its known role as a clinically validated Mpro inhibitor. By comparison, Anabsinthin showed an intermediate ligand RMSD, with a mean of 3.154 ± 0.712 Å and a terminal mean of 3.652 Å. This indicates that although Anabsinthin strongly stabilized the protein backbone, the ligand itself underwent slight positional or conformational rearrangement within the binding site. This behavior may reflect adaptation of the ligand inside the Mpro pocket rather than complete instability, especially because the associated protein RMSD remained low. Therefore, Anabsinthin appears to be well tolerated by the Mpro backbone, but its binding pose may be more dynamic than that of Nirmatrelvir. Absinthin displayed the highest ligand RMSD among the tested compounds, with a mean value of 4.859 ± 0.532 Å and a terminal mean of 4.977 Å, which indicate poor conservation of its initial docking pose during the simulation. This persistently elevated ligand RMSD suggests substantial displacement or reorientation relative to the starting docking pose. Interestingly, this ligand mobility was not accompanied by major protein destabilization, since the protein RMSD remained close to the apo system. This indicates that Absinthin may remain within or near the binding region while adopting alternative orientations, but its initial docking pose appears less conserved during MD simulation. X77 also exhibited substantial ligand movement, with a mean ligand RMSD of 3.552 ± 0.970 Å and a maximum of 6.281 Å, consistent with the strong protein

629 RMSD drift observed for the X77-bound complex. This combined protein-ligand behavior
630 suggests that the X77 system underwent significant structural rearrangement during simulation,
631 making it less stable under the applied MD conditions than Nirmatrelvir and Anabsinthin.
632 Overall, the RMSD results indicate that Nirmatrelvir provides the most stable ligand-binding
633 pose, while Anabsinthin provides the most stable protein backbone profile. This distinction is
634 important because low protein RMSD suggests that the ligand does not destabilize Mpro,
635 whereas low ligand RMSD suggests that the ligand remains locked in a conserved binding
636 orientation. In this context, Anabsinthin emerges as the more promising wormwood-derived
637 candidate than Absinthin because it combines the lowest protein RMSD with a moderate,
638 although not minimal, ligand RMSD. Absinthin, despite maintaining acceptable protein
639 stability, shows high ligand RMSD, suggesting greater pose flexibility or weaker anchoring of
640 its initial docking orientation. Therefore, the RMSD analysis supports the hypothesis that
641 Anabsinthin forms a dynamically more compatible complex with Mpro than Absinthin, while
642 Nirmatrelvir remains the strongest reference in terms of ligand positional stability.



644 **Fig. 7** Molecular dynamics analysis of protein and ligand RMSD during 100 ns simulations
 645

646 3.5.6 Residue-Level flexibility analysis by RMSF

647 The RMSF analysis was performed to evaluate the residue-level flexibility of SARS-CoV-2
 648 Mpro in its apo form and in complex with X77, Nirmatrelvir, Absinthin, and Anabsinthin (Fig.
 649 8). In contrast to RMSD, which describes the global structural deviation of the protein over
 650 time, RMSF provides a residue-by-residue view of local mobility and highlights flexible loops,

651 termini, and ligand-induced stabilization or destabilization. Overall, the Anabsinthin-bound
652 complex displayed the lowest RMSF profile, with a mean RMSF of 0.814 ± 0.312 Å, clearly
653 below the apo enzyme (1.081 ± 0.582 Å) and all other ligand-bound systems. This indicates
654 that Anabsinthin reduced local residue fluctuations across the Mpro structure. Importantly, only
655 2 residues exceeded 2.0 Å in the Anabsinthin complex, compared with 18 residues in apo Mpro,
656 15 residues in the Absinthin complex, 13 residues in the Nirmatrelvir complex, and 49 residues
657 in the X77 complex. This strongly supports the previous protein RMSD result, where
658 Anabsinthin also showed the lowest backbone RMSD and the most stable global protein
659 behavior. After excluding terminal residues, which are naturally more mobile and can
660 artificially inflate RMSF values, Anabsinthin still showed the lowest core RMSF, with a core
661 mean of 0.781 Å and no core residue above 2.0 Å. This is important because it confirms that
662 the stabilizing effect is not only due to reduced terminal movement but is also observed across
663 the structured protein core. Region-based analysis further supported this observation because
664 Anabsinthin showed the lowest average RMSF in all approximate Mpro structural regions,
665 including Domain I, Domain II, the linker region, and Domain III. In comparison, X77 showed
666 the highest residue-level flexibility, with a mean RMSF of 1.510 ± 0.742 Å and a maximum
667 value of 6.536 Å. The high number of residues above 2.0 Å suggests broader structural mobility
668 in the X77-bound trajectory. This agrees with the previous protein RMSD analysis, where the
669 X77 complex showed the highest RMSD drift. Since X77 is a co-crystallized ligand, this does
670 not mean it is an invalid reference, but it suggests that under the current MD conditions, the
671 X77-bound system experienced stronger conformational rearrangement than the other
672 complexes. Nirmatrelvir showed a moderate RMSF profile, with a mean RMSF of $1.146 \pm$
673 0.466 Å. Although its global RMSF was higher than Anabsinthin, its selected pocket-associated
674 residue RMSF was very low, with a mean of 0.860 Å, which supports strong local stabilization
675 of the binding site. This fits well with the ligand RMSD result, where Nirmatrelvir showed the
676 most stable ligand pose. Therefore, nirmatrelvir behaves as expected for a validated Mpro
677 inhibitor. Absinthin showed a global RMSF profile close to apo Mpro, with a mean RMSF of
678 1.132 ± 0.468 Å. However, unlike Anabsinthin, it did not produce broad residue-level
679 stabilization. Its selected pocket-associated RMSF was 1.303 Å, higher than Nirmatrelvir and
680 Anabsinthin, suggesting more local flexibility around key binding-site regions. This is
681 consistent with the ligand RMSD analysis, where Absinthin showed high ligand RMSD, which
682 reflects substantial reorientation or displacement from its starting docking pose during MD. For
683 selected pocket-associated residues, the mean RMSF followed the order, Anabsinthin $\approx$
684 Nirmatrelvir < X77 < Absinthin < apo. This is a very relevant observation for the Mpro

inhibition story. It suggests that Anabsinthin does not merely stabilize distant regions of the protein but also maintains low flexibility around residues associated with the catalytic/binding pocket. Therefore, among the two wormwood-derived sesquiterpene lactones, Anabsinthin appears dynamically more favourable than Absinthin, combining low protein RMSD with the lowest RMSF profile.

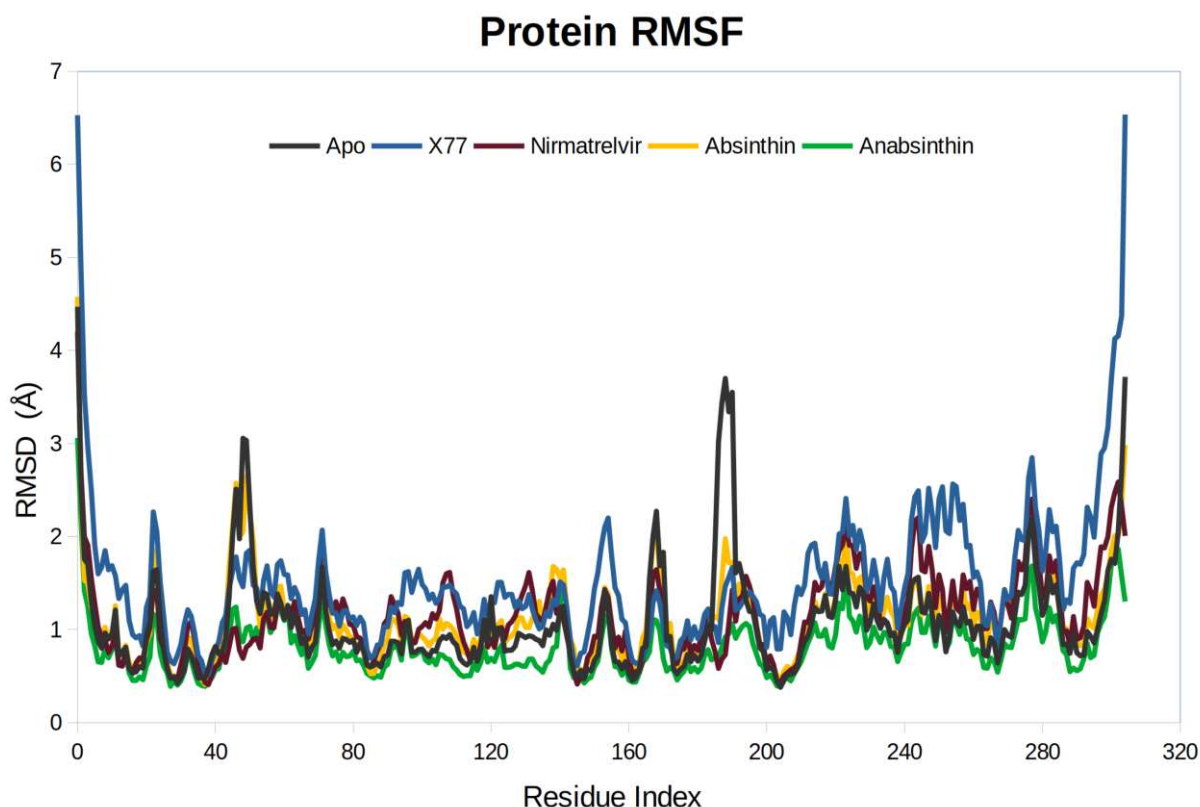


Fig. 8 Residue-level flexibility analysis of SARS-CoV-2 Mpro complexes by RMSF

3.5.7 Persistence of protein-Ligand interaction during MD simulation

Protein-ligand contact analysis was performed to identify residues involved in ligand contacts within the SARS-CoV-2 Mpro binding site during the 100 ns MD simulations (Fig. 9). The interaction fractions reflect the percentage of simulation time during which each residue-maintained contact with the ligand. Therefore, highly persistent contacts indicate residues that actively contribute to ligand anchoring, while weak or transient contacts suggest a more dynamic binding mode. For the X77-bound complex, the interaction profile was dominated by a strong and persistent contact with Glu166 (Fig. 9a), exceeding a normalized interaction fraction of 1.0, indicating multiple contacts involving the same residue during the trajectory. Additional interactions were observed with residues located around the Mpro catalytic pocket,

including, Gly143, Ser144 and Cys145 (Fig. 9b). This supports that X77 remained associated with the canonical Mpro substrate-binding region. However, despite this dense contact network, the previous RMSD and RMSF analyses showed that the X77 complex displayed the highest protein RMSD and the largest residue fluctuations. This suggests that X77 maintained several local contacts but did not prevent broader conformational rearrangements of the protein during the simulation. For Nirmatrelvir, the contact profile was more consistent with a stable inhibitor-binding mode. The ligand maintained highly persistent interactions with Glu166 and Cys145, two key residues of the Mpro active site region [83]. As shown in Fig. 9c, the interaction fraction of Glu166 reached approximately 2.0, which does not represent a simple single-contact occupancy of 200%, but rather reflects the formation of multiple simultaneous hydrogen-bond interactions involving the same residue during the trajectory. This interpretation is supported by the 2D interaction diagram in Fig. 9d, where Glu166 forms two persistent hydrogen bonds with two distinct polar motifs of Nirmatrelvir. Therefore, Glu166 acted as a major anchoring residue for Nirmatrelvir within the Mpro binding site. This interaction is particularly important because this residue contributes to the stabilization of ligands within the S1 pocket and helps maintain the active site architecture [64]. Nirmatrelvir also formed additional contacts with His41, Gly143, Ser144, and Thr190, creating a broad and stable interaction network. This agrees very well with the ligand RMSD result, where Nirmatrelvir showed the lowest ligand RMSD among all systems, confirming that its binding pose remained the most stable throughout the simulation. For Absinthin (Fig. 9 e and f), the interaction profile was different. Instead of forming a strong canonical interaction network with Glu166/Cys145, Absinthin showed more distributed and weaker contacts, mainly involving Met49, His164, Glu166, Gln189, Thr190, and Gln192, with several water-mediated interactions. The presence of contacts with Met49 and Gln189 indicates that Absinthin remained within the broader Mpro binding groove, especially around the S2/S4 regions [84]. However, the absence of highly persistent interactions with the catalytic dyad or the oxyanion-hole region explains its high ligand RMSD. In other words, Absinthin was not associated with major protein backbone destabilization, as shown by its acceptable protein RMSD and RMSF, but its binding pose was not strongly locked inside the active site. For Anabsinthin, the interaction pattern was more focused and more favorable than Absinthin. Anabsinthin maintained persistent contacts with Glu166 and Gln189 as shown in Fig. 9g and h; together with additional weaker contributions from residues such as Met49, Asn142, and Leu167. The involvement of Glu166 is especially relevant because it links Anabsinthin to a key anchoring residue of the Mpro binding site. This helps explain why Anabsinthin showed the lowest protein RMSD and the lowest RMSF profile in the previous

analyses. Although its ligand RMSD was higher than Nirmatrelvir, the contact analysis suggests that Anabsinthin did not simply dissociate from the pocket; rather, it likely underwent positional adaptation while maintaining stabilizing interactions with important binding-site residues.

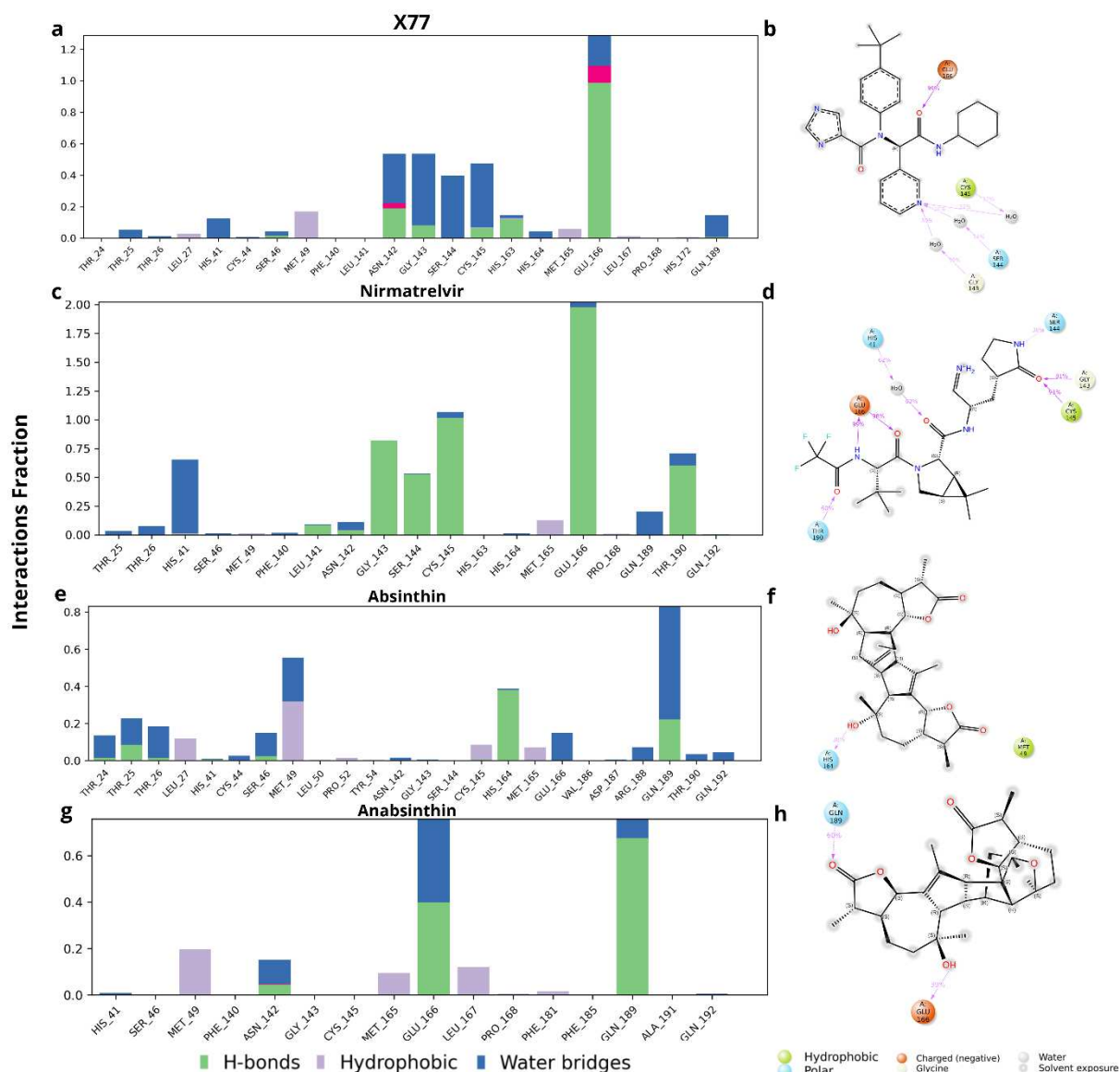


Fig. 9 Dynamic protein–ligand interaction fingerprints during molecular dynamics simulations

Overall, the contact analysis supports the interpretation obtained from RMSD and RMSF. Nirmatrelvir remains the best reference ligand in terms of pose stability, due to its persistent and well-distributed active-site interactions. Anabsinthin appears to be the most promising natural compound, because it combines strong protein stabilization with meaningful contacts involving Glu166 and Gln189. Absinthin, although not strongly destabilizing the protein, shows

a less anchored interaction profile, consistent with its high ligand RMSD. Therefore, among the two wormwood-derived sesquiterpene lactones, Anabsinthin presents the more favorable dynamic behavior against Mpro.

4. Conclusion

This study established an integrated plant-to-computational workflow combining solvent-dependent phytochemical profiling, targeted purification, and in silico evaluation of *Artemisia absinthium* sesquiterpene lactones. Ethanol-based extraction, GC-MS/LC-MS profiling, QuEChERS cleanup, and preparative HPLC enabled the enrichment of absinthin/anabsinthin-type isomers, whose tentative assignment was supported by LC-MS analysis. Molecular docking indicated that both absinthin and anabsinthin could be accommodated within the Mpro catalytic pocket, with anabsinthin showing the most favourable predicted binding score. ADME/toxicity predictions suggested acceptable early drug-like properties, while 100 ns molecular dynamics simulations further highlighted anabsinthin through improved complex stability, reduced residue-level fluctuations, and persistent interactions with key binding-site residues, particularly Glu166 and Gln189. Overall, these computational findings identify anabsinthin as the most promising *A. absinthium*-derived lactone scaffold investigated in this study and support its prioritization for further experimental evaluation as a potential Mpro-oriented natural product candidate. However, these results remain predictive and require confirmation through biochemical enzyme-inhibition assays and relevant cellular models before any direct inhibitory or antiviral activity can be established.

Authors Contributions

Conceptualization and methodology: SL, AT, REL and RB; Extraction, Optimization, Purification, Data collection and analysis: SL, AT and FM; In silico study: SL, SEL and RD; Investigation, Formal analysis: HT, RBN, NB; Writing – original draft: SL, SEL and AT; Writing – review & editing, Supervision: AT, RB and REL; Supervision, Resources, Project administration, Conceptualization: RB and REL.

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Data availability

All data generated or analysed during this study are included in this article and its Supplementary Information files.

Declarations

Consent for publication: Not applicable.

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