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Globospiramine Exhibits Inhibitory and Fungicidal Effects against *Candida albicans* via Apoptotic Mechanisms

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Abstract: Candidiasis is considered an emerging public health concern because of the occurrence of drug-resistant *Candida* strains and the lack of available structurally diverse antifungal drug armamentariums. The indole alkaloid globospiramine from the anticandidal Philippine medicinal plant *Voacanga globosa* exhibits a variety of biological activities; however, its antifungal properties remain to be explored. In this study, we report the *in vitro* antifungal activities of globospiramine against *Candida albicans* and *Candida tropicalis* and explore its possible target proteins using *in silico* methods. The colony-forming unit (CFU) viability assay revealed time- and concentration-dependent anticandidal effects of the alkaloid by decreasing almost 50% of the viable CFUs 60 min after treatment. Results of the MIC and MFC assays indicated inhibitory and fungicidal effects of globospiramine against *C. albicans* (MIC = 8 μg/mL; MFC = 4 μg/mL) and potential fungistatic effects against *C. tropicalis* at lower concentrations (MIC = 4 μg/mL; MFC > 64 μg/mL). The FAM-FLICA poly-caspase assay showed metacaspase activation in *C. albicans* cells at concentrations of 16 and 8 μg/mL, which agreed with the MIC and MFC values. Molecular docking and molecular dynamics simulation experiments indicated stable, strong binding of globospiramine with the target proteins 1,3-β-glucan synthase and Als3 adhesin enzymes, which are indirectly involved in apoptotic-driven candidal inhibition.

Keywords: globospiramine; bisindole alkaloid; *Voacanga globosa*; *Candida albicans*; *Candida tropicalis*; molecular docking; molecular dynamics; antifungal; apoptosis

1. Introduction

The increased occurrence and severity of fungal infections have greatly contributed to the escalation of disease-associated morbidity and mortality rates , with approximately 1.5 million deaths annually [1–3]. Treatment failures are mostly attributed to the emergence and re-emergence of resistant strains, which is among the consequences of the irresponsible use of available antifungal

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drugs and innate evolutionary mechanisms of the causative agents against therapeutic pressures [4,5].

Fungal infections are caused by different species, mostly belonging to the genera *Aspergillus*, *Pneumocystis*, *Candida*, and *Cryptococcus*. Among these, *Candida* spp. are the most frequently reported pathogen [6]. *Candida* spp. transition into opportunistic pathogens in immunologically weak and immunocompromised patients, leading to local and systemic infections [7]. However, in the last few decades, there has been an increased incidence of deep fungal infections or chronic candidiasis (long-term infection caused by *Candida* species) even in healthy individuals [8,9]. In addition to *C. albicans*, C. *tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* infections have been reported in clinical settings. These *Candida* cells have developed mechanisms to ensure efficient colonization and infection of their hosts despite therapeutic pressures. Therefore, this necessitates the discovery of new antifungal agents with novel and/or multitargeting mechanisms [10,11].

Current clinically approved antifungals belong to the following structural classes: polyenes, azoles, and echinocandins [12]. Polyenes like amphotericin B target ergosterol, which is selectively found in fungal cell membranes. Azoles inhibit fungal cytochrome P450-dependent enzymes, resulting in impaired ergosterol synthesis. Echinocandins block the 1,3-β-*D*-glucan synthase, disrupting fungal cell wall biosynthesis [13]. The fact that most chemotherapeutic-based treatment armamentarium against candidiasis is limited to these three classes calls for new and diverse drug congeners from different structural classes.

Relevant to our study, indole alkaloids have long been explored for their biological properties. The indole moiety contributes to the efficient binding of compounds to a number of disease targets, thus conferring favorable biological activities. In the context of anticandidal drug discovery, some reported indole alkaloids include kopsifolines A, G, H, I, J, and K from *Kopsia fruticosa* [14], ibogaine [15], and chetomin and chaetoglobinol A from *Chaetomium globosusm* [16]. Moreover, six out of 27 isolated monoterpene indole alkaloids from the plant *Rhazya strictra* demonstrated inhibitory properties against six *Candida* strains [17]. Other anticandidal indole alkaloids derived from marine organisms include indolepyrazines A and B from *Acinetobacter* sp. ZZ1275 [18]. Thus, indole alkaloids and their derivatives have great potential for development as anticandidal agents. Among the prolific producers of biologically active indole alkaloids is the genus *Voacanga* of the family Apocynaceae. In the Philippines, the medicinal plant *Voacanga globosa* (Blanco) Merr. has numerous biological activities [19–21], including *C. albicans* inhibition [22]. As part of our research effort to explore antimicrobial agents from Philippine medicinal plants, we hereby disclose the antifungal activities of the spirobisindole alkaloid globospiramine (Figure 1) previously obtained from *Voacanga globosa* [19] against *C. albicans* and *C. tropicalis* using in vitro and computational approaches (molecular docking and molecular dynamics simulations).

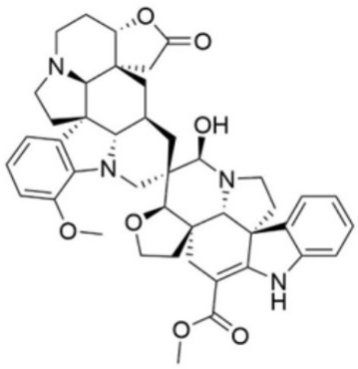

Figure 1. Structure of globospiramine.

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2. Materials and Methods

2.1. Test Compound

Globospiramine was isolated from *Voacanga globosa* as previously reported [19]. The test concentrations of globospiramine were prepared by dilution with dimethyl sulfoxide (DMSO).

2.2. Colony-Forming Unit Viability Assay

A colony-forming unit (CFU) viability assay was performed following previously reported methods [23]. Briefly, *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 750 cells were inoculated in Sabouraud dextrose broth (SDB) and incubated overnight. The resulting culture must reach OD_{600} = 0.3 to 0.8 prior to serial dilution until approximately 500 CFUs/mL were attained. The test compound globospiramine in varying concentrations (2.5, 50, and 100 µg/mL) was added to the diluted suspension and 50 µL were aliquoted from the diluted suspension and spread plated on Sabouraud dextrose agar (SDA) plates after 0, 30, 60, and 90 min. After 48 h of incubation, percentage CFU viability was measured using the following equation:

$$\%\ CFU\ viability = \frac{CFU_x}{CFU_0}\ x\ 100$$

where CFU_x is the number of surviving colonies at each specific time point (x = 30, 60, or 90 minutes) for each test concentration of globospiramine while CFU_0 is the number of colonies at the starting point (time = 0). DMSO (negative/vehicle) and amphotericin B B were utilized as controls. The experiment was performed in triplicate.

2.3. MIC and MFC Determination

The minimum inhibitory concentration (MIC) was assessed based on CLSI M27-A3 2008 guidelines on microdilution for yeast species. Fresh yeast suspension (OD_{600} = 0.3 to 0.8) in SDB was diluted to yield 5.0 x 10^2 to 2.5 x 10^3 cells/mL. Two-fold serial dilutions from 256 µg/mL to 1 µg/mL were prepared in a 96-well microplate for globospiramine. For the positive control amphotericin B, the concentrations used were 0.125 to 16 µg/mL. Each well contained 100 µL of test concentrations, 5 µL standardized yeast suspension in SDB, and 100 µL RPMI 1640 broth medium. Absorbance was recorded using the Glomax Discover Microplate Reader (Promega) after 24 h of incubation. MIC was recorded as the lowest concentration to inhibit increase in OD_{600}. Triplicates were performed. For the minimum fungicidal concentration (MFC) determination assay, 10 µL from the well containing the MIC and two concentrations higher were spread plated on SDA. MFC was recorded as the lowest concentration where no colony growth was observed in the three independent plates post 48 h of incubation.

2.4. FAM-FLICA Poly-Caspase Assay

The FAM-FLICA poly-caspase assay was performed according to the manufacturer's instructions provided in the kit (ImmunoChemistry Technologies, Bloomington, MN) with modifications. The FAM-FLICA reagent has been used in studies to elucidate yeast metacaspase activities [24,25]. For the treatment concentrations, the MIC, 2 x MIC and 4 x MIC of globospiramine against *C. albicans* and *C. tropicalis* were used. In the 96-well microplate, *Candida* cells were exposed to the treatment groups for 24 h. Each wells contained approximately 1 x 10^6 candidal cells/mL. The FAM-FLICA reagent was then added. After 50 min of incubation at 30 °C, fluorescence readouts were obtained using the Glomax Microplate Discover Reader (Promega) (excitation: 490 nm; emission: 530 nm). Three independent experiments were conducted. For statistical analysis, one-way ANOVA was performed followed by pairwise analysis with DMSO (negative/vehicle control) as the reference (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

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2.5. Molecular Docking against C. albicans Proteins

2.5.1. Ligand and Protein Preparation

Globospiramine was considered as the ligand, whereas the *C. albicans* target proteins as receptors and/or molecular targets. The following PDB IDs were used: 1EQP (1,3-β-glucan synthase), 4QUV (δ-14-sterol reductase), 5TZ1 (lanosterol 14-alpha demethylase or CYP51), 5UIV (thymidylate kinase), 4LEB (Als3 adhesin), and 2Y7L (Als9-2). These PDB IDs have already served as key targets in other molecular docking studies and/or are considered pharmaceutical targets of current anticandidal drugs [26–28]. To prepare the ligand, its structure was drawn in ChemDraw (18.1), optimized in Avogadro (1.2.0), and saved as .mol2 file. For the protein preparation, non-standard residues were removed in UCSF Chimera (1.17.3), followed by minimization using the steepest descent and conjugate gradient methods. The output file was saved as .pdb [29].

2.5.2. Molecular Docking and Visualization of Interactions

The prepared ligands and protein targets were combined using UCSF Chimera (1.17.3). Actual docking simulation experiments were carried out using the flexible ligand in a flexible active site protocol based on the BFGS algorithm coupled with AutoDock Vina. Grids were generated to encompass the target binding domains. To visualize the interactions, the output files were processed using BIOVIA Discovery (4.1). [30]

2.6. Molecular Dynamics Simulations

MD simulation experiments were conducted using the Desmond package (Desmond Molecular Dynamics System 6.4 academic version, D. E. Shaw Research ("DESRES"), New York, NY, USA, 2020. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, USA, 2020). The three-dimensional ligand/protein complexes (globospiramine within 1,3-β-glucan synthase (PDB ID: 1EQP) and Als3 adhesin (PDB ID: 4LEB)) obtained by molecular docking studies were prepared using the system builder tool, available in Desmond software, to produce suitable complexes for MD simulation studies. Accordingly, the ligand/protein complexes were placed into an orthorhombic box and solvated by water molecules (TIP3P water model) [31,32]. A physiological concentration of monovalent ions (0.15 M) was used by adding to the biological systems Na^+ and Cl^- ions. MD simulation studies were conducted using the OPLS3 force field [33], and calculations were performed utilizing the CUDA API technology on two NVIDIA graphics processing units (GPUs). A constant number of particles, constant temperature (300 K by Nosé–Hoover thermostat method [34], and pressure (1.01325 bar by Martyna–Tobias–Klein method [35] were considered using the NPT ensemble class. To assess the motion for bonded and non-bonded interactions within the short-range cutoff, the RESPA integrator was adopted (inner time step of 2.0 fs) [36]. To calculate long-range electrostatic interactions (short-range electrostatic interactions were fixed at 9.0 Å), the particle mesh Ewald method (PME) was employed [37]. To equilibrate the biological systems, the default protocol available in Desmond was used. The protocol consists of several constrained minimizations and MD simulations that were applied to each biological system to progressively relax and bring them to equilibrium. The Desmond application's simulation event analysis tools were utilized to examine the MD results produced throughout the MD simulation calculations, as previously reported [38].

3. Results

3.1. Effects of Globospiramine on C. albicans and C. tropicalis CFU Viability

Screening for inhibitory activities of globospiramine against the pathogenic yeast species *C. albicans* and *C. tropicalis*, CFUs, were investigated in vitro using a CFU viability assay. Globospiramine exhibited time- and concentration-dependent activities against *C. albicans* and *C. tropicalis* by significantly decreasing the percentage of viable CFUs. After 60 min exposure to

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globospiramine at 2.5 µg/mL, the CFU count for both *Candida* species was reduced to approximately 50% of the original count (Figure 2).

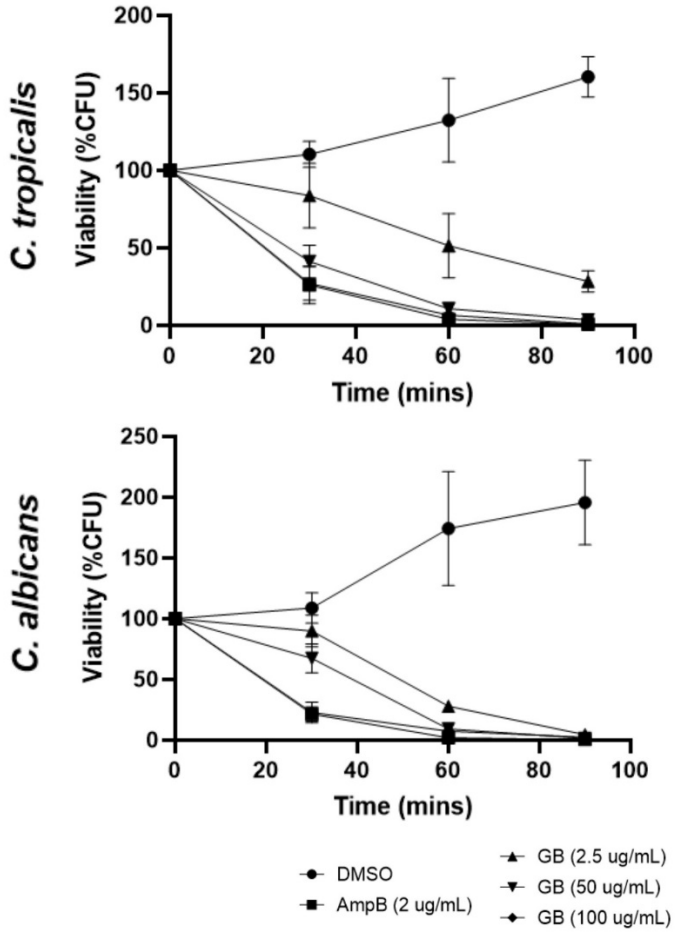

Figure 2. Globospiramine demonstrated time- and concentration-dependent effects on the CFU viability of *C. albicans* and *C. tropicalis*.

3.2. MIC and MFC of Globospiramine versus C. albicans and C. tropicalis

The MIC and MFC were determined for both *Candida* species to support the CFU viability data using microdilution and spread plate techniques. The lowest concentration that significantly prevented increased OD_{600} was considered as MIC, whereas MFC was the lowest concentration that completely inhibited the growth of yeast colonies. In agreement with the results of the CFU viability assay, globospiramine showed moderately strong MIC (8 ug/mL) and MFC (8 µg/mL) values against *C. albicans.* (Table 1) compared with the positive standard drug control, amphotericin B. Interestingly, a lower concentration of globospiramine was effective against *C. tropicalis* (MIC = 4 µg/mL); however, such activity might be limited to growth inhibition, or a much higher concentration (MFC > 64 µg/mL) is necessary to promote fungicidal effects (Table 1).

Table 1. MIC and MFC of globospiramine and positive control amphotericin B against *C. albicans* and *C. tropicalis*.

	Globospiramine	Amphotericin B
MIC (µg/mL)		
C. albicans	8.0	0.50
C. tropicalis	4.0	0.50
MFC (µg/mL)		
C. albicans	10.67	0.83
C. tropicalis	>64.0	1.67

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3.3. Apoptosis-Inducing Activities of Globospiramine vs C. albicans and C. tropicalis

The metacaspase-activating activity of globospiramine vs *C. albicans* and *C. tropicalis* cells was also investigated to determine its possible mechanism of action. Globospiramine induced a significant increase in relative fluorescence units (RFUs) compared with the vehicle control DMSO – triggering metacaspase activation responses in *C. albicans* cells at 16 ($p < 0.01$) and 8 μg/mL ($p < 0.05$) test concentrations (Figure 3). These concentrations corroborated to the MIC and MFC values shown in Table 1. Meanwhile, globospiramine did not induce apoptosis in *C. tropicalis* cells. This is to be expected since the MFC value against *C. tropicalis* was noted > 64 μg/mL.

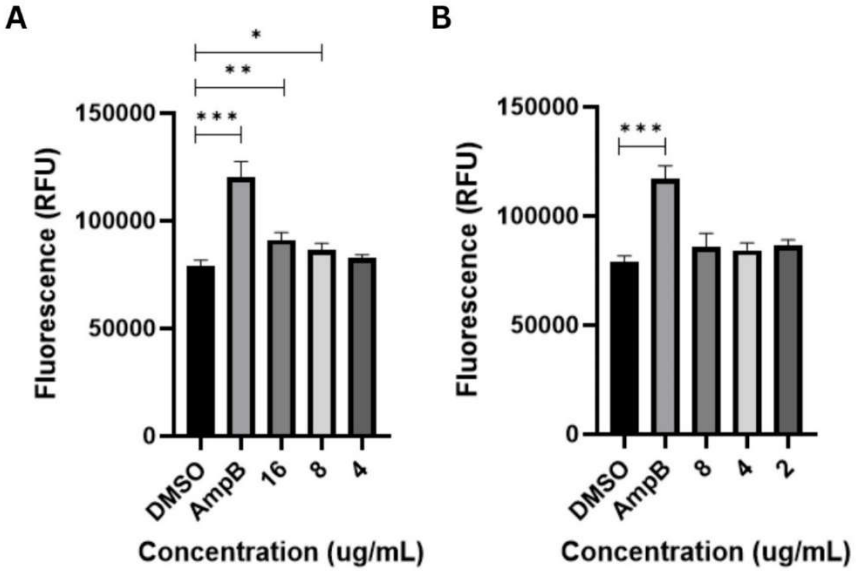

Figure 3. The spirobisindole alkaloid globospiramine significantly induced apoptosis in (**A**) *C. albicans* cells at 16 and 8 μg/mL concentrations, which agreed with its MIC and MFC. However, these effects were not observed in (**B**) *C. tropicalis* cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). DMSO was used as a negative control and amphotericin B (AmpB) (0.5 μg/mL) as a positive control.

3.3. Molecular Docking against C. albicans Targets

Probing the putative molecular targets of globospiramine is the next step after demonstrating its in vitro anti-*C. albicans* properties. Six protein targets previously reported to play significant roles in the pathogenesis of candidiasis caused by *C. albicans* were selected for molecular docking experiments, namely 1EQP (1,3-β-glucan synthase), 4QUV (δ-14-sterol reductase), 5TZ1 (lanosterol 14-alpha demethylase or CYP51), 5UIV (thymidylate kinase), 4LEB (Als3 adhesin), and 2Y7L (Als9-2). Globospiramine showed the best binding affinities to 1,3-β-glucan synthase and Als3 adhesin (Table 2, Figure 4A-4B). Compared with the positive controls caspofungin and amphotericin B, our compound showed much better binding energy against all these targets.

Table 2. Binding energies and interactions of globospiramine against *C. albicans* protein targets.

PDB IDs	Globospiramine		Positive Controls					
			Caspofungin		Amphotericin B		Co-crystallized ligand / inhibitor	
	BE (kcal/mol)	Interactions	BE (kcal/mol)	Interactions	BE (kcal/mol)	Interactions	BE (kcal/mol)	Interactions
1EQP (1,3-β-glucan synthase)	-10.5	His253 (H-bond), Phe258, Phe229 (*pi-pi*	-8.0	Trp277, Gln230 (H-bond), Asp227 (salt	-8.8	Asn305, Asp151 (H-bond), Phe258,	-	-

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		stacked), Trp277, Val231, Tyr255, Phe229, His253 (*pi*-alkyl), Glu192, His253, Gln230, Glu262 (C-H bond)		bridge), His254, Glu262 (C-H bond), Val273, Phe258, Phe144, Tyr255, Trp373 (alkyl, *pi*-alkyl), His253, Arg265 (unfavorable interaction)		Phe144 (*pi*-alkyl), Tyr 153 (unfavorable donor-donor, C-H bond)		
4QUV (δ-14-sterol reductase)	-9.5	Arg324, His320 (H-bond), Val96, His320 (*pi-sigma*), Leu253, Met99 (alkyl), Arg106, Arg323 (unfavorable positive-positive)	-7.2	Arg106, Arg323, Arg324, Lys406 (H-bond), Tyr414, Trp352, Leu346, Cys403, Trp411, Lys319, Val96 (alkyl, *pi*-alkyl), His320 (*pi-pi* stacked), Gln97 (C-H bond), Arg324 (unfavorable positive-positive)	-7.5	Gln97, Glu250, Arg323, Arg324, Gly343 (H-bond), Met99, Leu253 (alkyl), Arg106 (unfavorable positive-positive)	-9.5	His248, Arg313, Thr254, Lys259, Lys319, Trp256, Arg395, Asn316, Thr255 (H-bond), Asp244, Asp399, Arg395 (attractive charge, *pi*-cation), Glu201 (C-H bond), Lys319 (unfavorable positive-positive), Tyr245 (*pi-pi* T-shaped), Arg398, Val252 (*pi*-alkyl, alkyl)
5TZ1 (lanosterol 14-alpha demethylase or CYP51)	-7.4	Arg469 (H-bond), Glu444 (attractive charge), Val452, Val454 (alkyl), Ser453, Lys451 (C-H bond)	-5.7	Met508, Pro462, His468, Leu439, Leu471, Gly303 (H-bond), His468 (C-H bond), Ile304 (*pi-sigma*), Leu87, Phe233, Tyr64, Phe380, Phe228, Val509, Leu150, Ile304, Ile131, His377, Pro230, Leu88, Lys90	-3.3	Phe463 (H-bond), His468 (C-H bond), Tyr118 (*pi*-lone pair), Leu376, Ile379, Ala146, Ile304, Leu204, Phe475 (alkyl, *pi*-alkyl), Cys470, Ile379, Gly464, Arg381, Thr311, Phe475, Leu150, Ile471, Tyr132	-10.6	Gly303, Ile304 (C-H bond), Ser507, His377 (halogen), Tyr118, Tyr132 (*pi-pi*), Leu121, Phe233, Leu376, Pro230, Ile304, Ile131, Lys143 (alkyl, *pi*-alkyl)

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				(*pi*-alkyl, alkyl), Arg381, Tyr132, Lys143 (unfavorable interactions)		(unfavorable bonds)		
5UIV (thymidylate kinase)	-9.4	Gly155, Asp91, Arg39 (H-bond), Asp13, Arg39, Glu159 (*pi*-cation / *pi*-anion / salt bridge), Glu159, Ser18 (C-H bond), Lys17 (*pi*-alkyl)	-8.2	Ser18, Asp13, Asp91, Arg92, Lys17, Lys35, Arg39, Gly157 Gly155 (H-bond), Glu162, Glu159 (salt bridge, attractive charge), Asp13, Asp91, Lys35, Gly155 (C-H bond), Ile196, Arg153, Lys17, Arg39, Val199 (alkyl, *pi*-alkyl)	-7.7	Arg92, Lys35, SerA (H-bond), Glu162, Gln159 (salt bridge, attractive charge), Pro37 (alkyl), Asp13 (C-H bond), Ser18 (unfavorable donor-donor)	-8.9	Arg92, Lys17, Arg14, Ser18, Gly16 (H-bond), Glu159, Asp91, Asp13 (attractive charge, *pi*-anion), Lys35 (unfavorable donor-donor), Tyr100 (*pi*-*pi*), Leu51 (*pi*-alkyl)
4LEB (Als3 adhesin)	-10.6	Thr168 (H-bond), Asp169 (attractive charge), Asp169, Tyr166 (C-H bond), Val161 (*pi*-*sigma*), Val161, Leu167 (alkyl)	-6.5	Thr168, Tyr226, Thr20, Asn22 (H-bond), Trp295 (*pi*-cation), Pro29, Arg171, Tyr21 (alkyl, *pi*-alkyl), Asn22 (*pi*-donor H-bond)	-7.7	Asn22 (H-bond), Tyr226 (*pi*-alkyl), Arg294 (unfavorable positive-positive)	-	-
2Y7L (Als9-2)	-8.1	Thr293 (C-H bond), Trp294 (*pi*-cation), Tyr21, Pro160, Val161 (*pi*-alkyl)	-6.4	Thr168 (H-bond), Arg171, Val22, Pro160, Val161, Ile167, Tyr23, Phe225, Pro29 (alkyl, *pi*-alkyl)	-7.1	Glu86, Ser210, Asn213 (H-bond), Asn211 (C-H bond), Tyr261 (*pi*-alkyl)	-	-

(-) not identified / no co-crystallized ligand attached.

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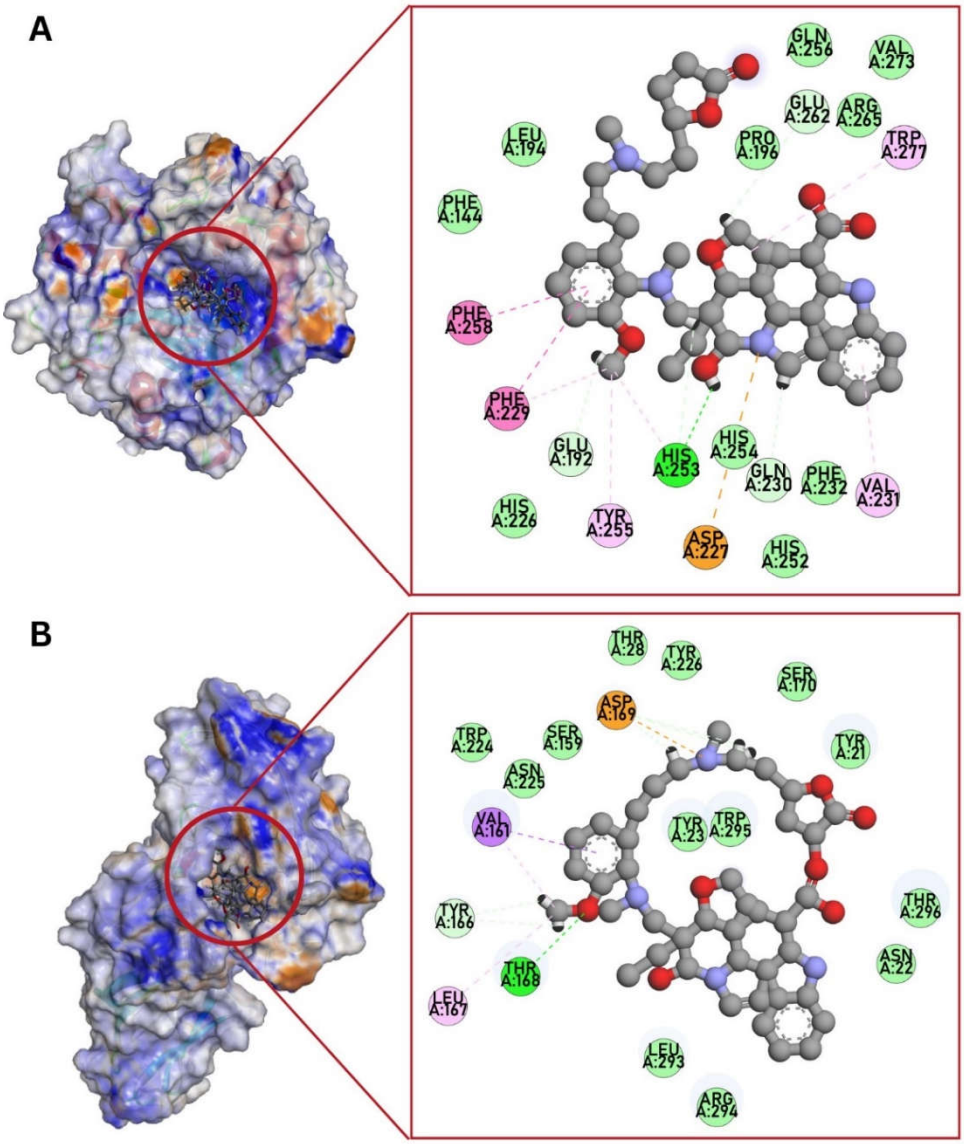

Figure 4. Dock poses of globospiramine to (**A**) 1,3-β-glucan synthase and (**B**) Als3 adhesin.

3.4. Molecular Dynamics Simulations

To improve the reliability of target identification and validate the docking results, we conducted MD simulation experiments on the most promising targets. In particular, the complexes 1,3-β-glucan synthase/globospiramine and Als3 adhesin/globospiramine were considered for MD simulation studies. Figure 5 shows the MD simulation output for the complex 1,3-β-glucan synthase/globospiramine. Based on 100 ns of MD simulation, we observed a general stability of the selected biological system, highlighted by the low RMSD values of the protein and the ligand and by the low RMSF value, indicating small fluctuations in the biological system. Considering the main interactions found by molecular docking studies, we observed that the H-bond established with residue His253 was maintained, although it became water-mediated. In addition, a strong H-bond network was detected with residue Asp227. Other polar contacts, mainly water-mediated with His254, Glu262, Arg265, and Asp280, were observed. The hydrophobic interactions with Phe229, Tyr255, Phe258, and Trp277 were well maintained during the simulation, with additional hydrophobic contacts with Phe232 and His252.

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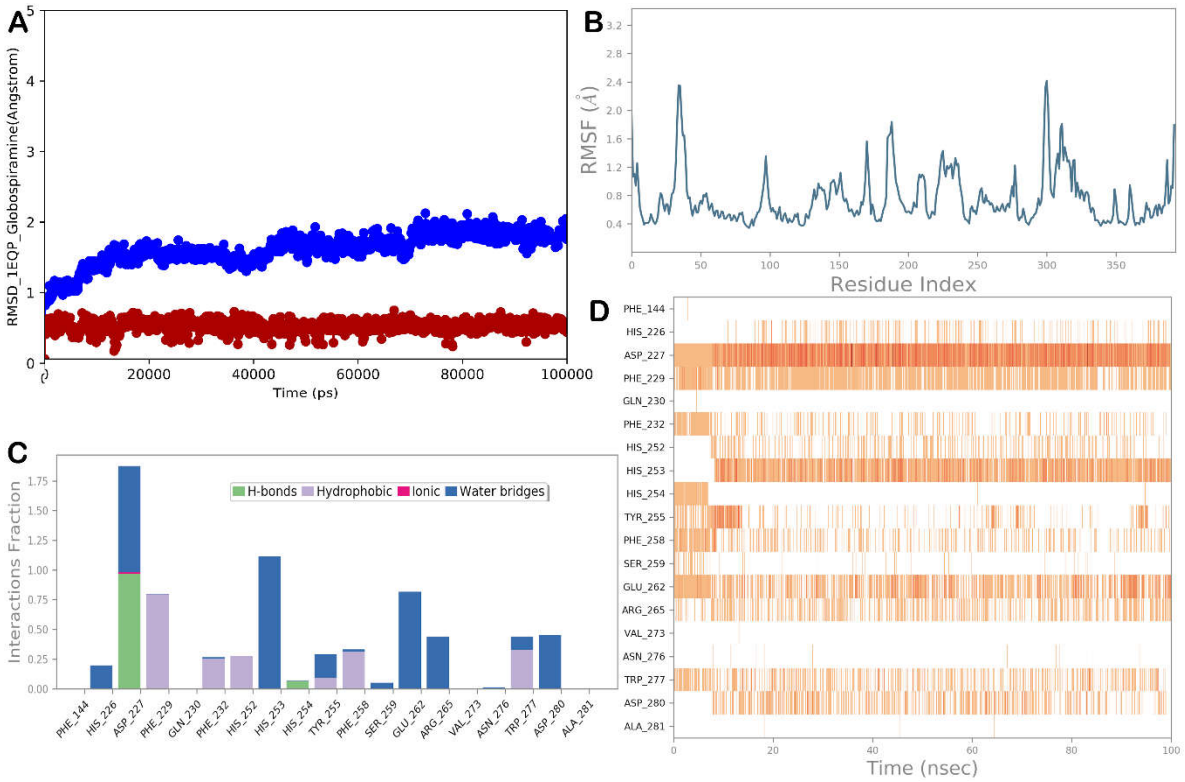

Figure 5. (A) RMSD evaluation (protein: blue line; and ligand: red line). (B) RMSF assessment for the complex 1,3-β-glucan synthase (1EQP)/globospiramine, obtained by docking studies, following a 100 ns MD simulation. (C, D) globospiramine observed throughout the MD run. Four types of interactions can be distinguished: water bridges (blue), ionic (magenta), hydrophobic (grey), and H-bonds (green). Over the trajectory, the stacked bar charts are normalized. For instance, a value of 0.7 indicates that a particular contact is maintained 70% of the time during simulation. Values greater than 1.0 could occur because a protein residue could interact with the ligand more than once using the same subtype. A timeline explanation of the primary interactions is shown in the following diagram in the figure. Those residues that interact with the ligand in each trajectory frame are displayed in the output. A darker orange hue denotes several contacts that some residues have with the ligand. Maestro and Desmond software tools were utilized to generate the pictures (Maestro, Schrödinger LLC, release 2020-3).

Regarding the Als3 adhesin/globospiramine complex, the MD simulation results are illustrated in Figure 6. In addition, in this case, we observed a general stability of the biological system with a small fluctuation of the protein, as indicated by the RMSD and RMSF values. Considering the main contacts governing the binding mode of globospiramine within the selected binding site of the Asl3 adhesin, we observed that the H-bond with Thr168 was well maintained as well as the ionic interactions with the reside Asp169. Additional polar contacts that could contribute to stabilizing the binding mode were detected with residues Ala19, Asn22, and Arg294. Hydrophobic interactions with residues Val161, Tyr166, and Leu167 were still evident at low frequencies. More favorable hydrophobic contacts were established from globospiramine with Tyr226 and Trp295.

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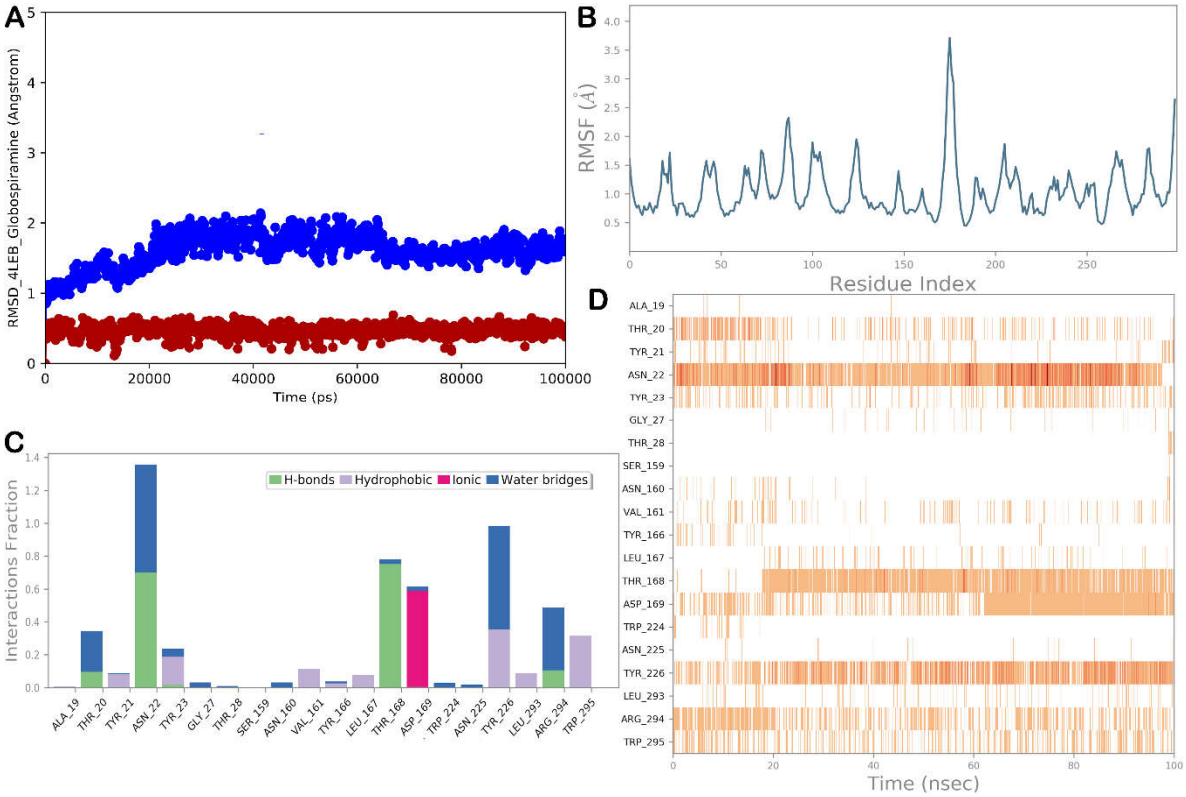

Figure 6. (A) RMSD evaluation (protein: blue line; and ligand: red line). (B) RMSF assessment for the complex Als3 adhesin (4LEB)/globospiramine, obtained by docking studies, following a 100 ns MD simulation. (C, D) globospiramine observed throughout the MD run. Four types of interactions can be distinguished: water bridges (blue), ionic (magenta), hydrophobic (grey), and H-bonds (green). Over the trajectory, the stacked bar charts are normalized. For instance, a value of 0.7 indicates that a particular contact is maintained 70% of the time during simulation. Values greater than 1.0 could occur because a protein residue could interact with the ligand more than once using the same subtype. A timeline explanation of the primary interactions is shown in the following diagram in the figure. Those residues that interact with the ligand in each trajectory frame are displayed in the output. A darker orange hue denotes several contacts that some residues have with the ligand. Maestro and Desmond software tools were utilized to generate the pictures (Maestro, Schrödinger LLC, release 2020-3).

4. Discussion

Globospiramine is a spirobisindole alkaloid from the Philippine endemic medicinal plant *Voacanga globosa*. It has been reported to exhibit biological activities including cholinesterase inhibitory, antiviral, and antimycobacterial properties [19,20]. Meanwhile, *V. globosa* extracts demonstrated anticancer and antifungal activities [21,22]. Generally, indole alkaloids are known to exert antifungal activities [14,39]. For example, decreased viability of fluconazole-resistant *C. albicans* was observed upon treatment with *Tabernaemontana divaricata* indole alkaloids [40]**.** Other studies have reported potential targets of these indole alkaloids, such as isocitrate lyase and other extracellular enzymes implicated in the lipolytic and proteinase activities of *C. albicans* [15,41].

Among the emerging targets in anticandidal drug discovery is the apoptosis pathway. Herein, we report the apoptosis-inducing potential of globospiramine against *C. albicans* and *C. tropicalis* cells using the FAM-FLICA poly-caspase assay. Yeast and mammalian apoptosis share markers such as DNA fragmentation, metacaspase activation, and reactive oxygen species (ROS) accumulation. FAM-FLICA has been reported to bind to the yeast metacaspase, thus being used to assess yeast apoptosis [24,25,42,43]. The antifungal activities of natural products have been investigated in the context of apoptosis induction as a mechanism of their fungicidal action [44–46]. Our results indicate that the fungicidal effect of globospiramine against *C. albicans* occurs through metacaspase activation leading

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to apoptosis, which might also explain the correlation between MIC, MFC, and metacaspase-inducing activity at the same concentration. Meanwhile, against *C. tropicalis*, globospiramine may be fungistatic and not fungicidal, although other possible modes of cell death like necrosis may be investigated. Therefore, further mechanistic investigations on the effect of globospiramine in promoting the growth inhibition of *C. tropicalis* cells are warranted. Thus, our study indicates for the first time that the *V. globosa* phytoconstituent globospiramine has anticandidal activity and could be responsible for the purported antifungal activity of the medicinal plant.

Based on the molecular docking and MD simulation experimental data, globospiramine potentially exerts its antifungal activity by targeting 1,3-β-glucan synthase and Als3 adhesin. The first putative target, 1,3-β-glucan synthase, is an enzyme important for fungal cell wall synthesis. This enzyme facilitates the creation of β(1→3) glycosidic bonds within 1,3-β-glucan molecules, utilizing uridine diphosphate–activated glucose (UDP-Glc) as the source of sugar and transporting the resulting glucan across the membrane [47]. In general, echinocandins like amphotericin B are known to elicit inhibitory effects on this enzyme in the plasma membrane. Unstable and impaired fungal cell walls then result in morphogenic and intracellular changes that cause cell death [48,49]. In addition, the fact that fungal cell walls and the enzyme itself are absent in human cells make it more ideal as a therapeutic target. Recently, this enzyme has served as a key target among newly discovered compounds and other antifungals [50,51]. Drug-induced damage to the *C. albicans* cell membrane and cell wall upon inhibition of this enzyme was also reported to result in cellular stress, subsequent apoptosis, and G0/G1 cell cycle arrest [52]. However, there are a myriad of reports on phenotypic changes in the enzyme caused by gene mutations in the coding region of FKS1, a gene involved in biosynthesis of 1,3-β-glucan synthase. As a result, such mutations promoted echinocandin resistance [53,54]. Therefore, the discovery of multitargeting agents against 1,3-β-glucan synthase and other virulence factors and/or molecular entities is deemed a logical strategy.

The second putative target is Als3 adhesin. The ALS gene family encodes cell surface proteins in *C. albicans*. These proteins function in adhesion to host cells and various surfaces and are thus implicated in biofilm formation. Biofilms are important determinants in yeast infections and are directly associated with drug failure and resistance. Interestingly, ALS proteins, including Als3 adhesin, are not expressed in human cells [55–57]. Therefore, Als3 is considered an emerging, pathogenetically relevant target for new generation antifungals against *Candida* species [58]. It is noticeable that globospiramine showed almost similar binding propensity and stability to this protein target compared with that of 1,3-β-glucan synthase. This strengthens the multitargeting nature of our compound against these two proteins. The fact that few studies on Als3 as a therapeutic target have been reported provides another opportunity for globospiramine to be considered as a promising alkaloidal template against novel molecular targets in *C. albicans*.

This study reports the possible multitargeting antagonistic effects of globospiramine on *C. albicans*. Two scenarios are possible: (1) these effects are independent from each other, such as the case of the anticandidal drug caspofungin, which exhibits concentration-dependent mechanisms against *C. albicans*, including inhibition of 1,3-β-glucan synthesis, promotion of apoptosis, and induction of necrosis [59], or (2) the inhibition of these proteins may directly or indirectly result in apoptosis and/or necrosis. It is important to note that previous studies have indicated the role of these enzymes in apoptosis. For example, 1,3-β-glucan synthase is involved in cell wall synthesis. Globospiramine effectively bound to 1,3-β-glucan synthase may potentially interrupt the normal biosynthetic pathway to polymerize the candidal cell wall leading to ROS hyperaccumulation – a hallmark of apoptosis [60]. Rapid killing of *C. albicans* by certain set of antimicrobial peptides may be due to apoptosis preceded by cell wall disruption and ROS accumulation [61]. A lipopeptide has also been reported to inhibit *C. albicans* growth by directly impairing the fungal cell wall, leading to an increase in ROS levels and mitochondrial dysfunction, which can activate apoptotic pathways [62]. Inhibition of Als3 on the other hand triggers stress signals and responses due to loss of adhesion-dependent survival signals in *C. albicans*. These stress signals can activate the apoptotic machinery via oxidative stress, DNA damage, or mitochondrial impairment. Additionally, biofilm formation

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may be disrupted upon inhibition of this protein. In fact, apoptosis has been reported in *Candida* cells under the therapeutic pressure of amphotericin B [46,63].

5. Conclusions

Overall, our study reports the fungicidal potential of the spirobisindole alkaloid globospiramine from the Philippine medicinal plant *Voacanga globosa*, particularly against *Candida albicans* via apoptotic-aided mechanisms, along with potential dual-inhibitory activity against the disease-implicated targets 1,3-β-glucan synthase and Als3 adhesin. Our in vitro findings also demonstrated the fungistatic effects of globospiramine against *C. tropicalis*. Accordingly, globospiramine could represent a good biomolecular candidate for exploring and discovering anticandidal drugs with improved therapeutic effects.

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