Selection of *Staphylococcus aureus* DNA replication inhibitors

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ABSTRACT

Introduction: S. aureus is an important pathogen that causes nosocomial infections, food poisoning by release of enterotoxins and toxic shock syndrome through the injection of super antigens into the bloodstream. The β-clamp protein of S. aureus is a process-promoting factor for most enzymes in prokaryotic DNA replication and its dimerization is essential for the deleterious action of the pathogen.
Objective: In this work, we report the application of the double hybrid system to select β-clamp dimerization inhibitors of S. aureus from Caatinga plant extracts, especially Buchenavia tetraphylla.
Material and Methods: (1) preparation of organic extracts of Buchenavia tetraphylla was prepared as previously reported (2) The β-clamp-β-clamp interaction was evaluated using a hybrid double bacterial system (BTH), based on the reconstitution of the activity of the enzyme adenylate cyclase (cya) from Bordetella pertussis and cloned in Escherichia coli. (3) The antimicrobial activity of active extracts was evaluated by determining the minimum inhibitory concentration (MIC) against S. aureus 8325-4 (4) To identify compounds potentially responsible for the activity, the active extracts were subjected to a non-segmented analysis by LC-MS/Q-TOF (5) The in silico studies were carried out by searching for the ligand structures, construction and validation of the 3D model of the β-clamp and molecular docking between ligands and protein target of S. aureus and the human CYP3A4 hepatic system.
Results and Discussion: There was a high interaction (in the order of 4,800 kcal/mol) between buchenavianine and analogues with β-clamp with no significant difference between them. On the other hand, the interaction between buchenavianine and human CYP3A4 analogues was (of the order of 2,200 kcal/mol) following the same principles of electronegativity of the elements. The purpose of this analysis is to understand how the β-clamp protein interacts with the main human liver metabolization protein. The interaction between buchenavianine and human CYP3A4 analogues was 2 times lower than buchenavianine and S. aureus β-clamp analogues, and this shows that between the two targets studied here (S. aureus β-clamp and human CYP3A4) the ligands will be preferentially attracted to the S. aureus β-clamp, which ratifies them as viable inhibitors of the S. aureus DNA replication process.
Conclusion: The combination of microbiological, chemical and computational resources allowed to indicate molecules present in the plant extract capable of interacting with the β-clamp of S. aureus. The approach presented here promotes a rational biochemical screening for ligand selection with better possibilities for subsequent bench tests, saving financial resources and optimizing results in drug research and development.

Keywords: Teacher Training, Inhibitor Selection, DNA Replication, Staphylococcus aureus.

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LIST OF ABBREVIATIONS/LEGENDS

LC-MS/Q-TOF = Liquid chromatography/time-of-flight/mass spectrometry
BTH = benzothiadiazole
Cya = adenylate cyclase
DnaN = DnaN is the gene that encodes DNA clamp of DNA polymerase III in prokaryotes. The β clamp physically locks Pol III onto a strand of DNA during replication to help increase its processivity. The eukaryotic equivalent of the β clamp is the PCNA.
CFU = In microbiology, the colony forming unit (CFU) is a unit of measurement used to estimate the number of viable bacteria or fungi—that is, capable of multiplying by binary fission under controlled conditions—in a sample.
UHPLC = Ultra high performance liquid chromatography
MIC = minimum inhibitory concentration
RPM = revolutions per minute
NCBI = National Center for Biotechnology Information is a section of the United States National Library of Medicine, a branch of the National Institutes of Health, headquartered in Bethesda, Maryland. The institution was founded in 1988 as a result of legislation proposed by Senator Claude Pepper.
PDB = PDB is a 3D database of proteins and nucleic acids. These data, usually obtained through X-ray diffraction, nuclear magnetic resonance, and cryo-electron microscopy, are submitted by physicists, biologists, and biochemists from all over the world. Available in the public domain, they can be used freely

INTRODUCTION

Staphylococcus aureus It is a notorious pathogenic bacterium that causes a wide range of soft tissue diseases ranging from simple infections (such as boils), to the most serious (pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections), or deep-seated infections (toxic shock syndrome, osteomyelitis, and endocarditis)(Hill & Imai, 2016; Humphreys et al., 2016; Moormeier & Bayles, 2017; T. H. Nguyen, Park, & Otto, 2017; Tong, Davis, Eichenberger, Holland, & Fowler, 2015). In addition *S. aureus* it is considered one of the main causes of healthcare-associated infection (HAI), being mainly related to contamination of surgical wounds and internal medical devices (Humphreys et al., 2016; Moormeier & Bayles, 2017; Tong et al., 2015). This pathogen also causes food poisoning by releasing enterotoxins (Sergelidis & Angelidis, 2017) and toxic shock syndrome through the release of super-antigens into the bloodstream (Bassetti et al., 2017).

Several virulence factors have been identified in isolates of *S. aureus* which are involved in functions such as: (i) promotion of adhesion (Agglutination factor A and protein A) and colonization (hyaluronidase) of host tissues; (ii) evasion of the immune system (carotenoids and coagulases); (iii) host damage (hemolysins, leukotoxins, and leukocidin) (Dunyach-Rémi, NGBA Essebé, The Truth, & Lavigne, 2016; Hill & Imai, 2016; Koymans, Vrieling, Gorham, & van Strijp, 2016; Moormeier & Bayles, 2017; Dryrik & Horswill, 2016; Spaan, van Strijp, & Torres, 2017). Targeting this variety of virulence factors to design drugs is not a trivial task (Kane, Carothers, & Lee, 2016; Kong, Neoh, & Nathan, 2016). This difficulty increases considerably if we take into account that there are clones of S. aureus with decreased susceptibility to antimicrobial drugs for clinical use, high capacity for *S. aureus* resistance to antimicrobial agents.

In the absence of chemotherapeutic resources against *S. aureus* It has become a serious problem worldwide, as an alarming increase in strains resistant to different classes of antibiotics (macrolidia, quinolones, and more recently, vancomycin and oxazolidinone) has also been observed (Das, Dasgupta, & Chopra, 2016; Foster, 2017). Due to this drastic panorama, many researchers have directed their efforts to strengthen and/or develop effective drug discovery programs. In this case, multidisciplinary approaches are used instead of traditional ones based only on conventional methods (such as agar diffusion and minimum inhibitory concentration assays). For example, some strategies are based on genetic platforms such as **Dual Hybrid System** (detailed explanation in the appendices) (Kjelstrup, Hansen, Thomsen, Hansen, & Lobner-Olesen, 2013), phage display (Rentero Rebollo et al., 2016), aiming to select compounds that inhibit specific factors involved in the survival or virulence of bacteria. The application of "Omics" technologies (genomics, transcriptomics, proteomics, metabolomics) has also become popular in the discovery of antimicrobial agents and new therapeutic targets, providing a substantial boost in this field (Dominguez et al., 2017; Dos Santos et al., 2016; MARTINEZ, Agbale, Nomiyama, & Franco, 2016).

Efforts to find new targets for therapy have indicated that proteins related to DNA replication constitute an attractive antimicrobial target, and one that has been considerably underexplored so far, given the number of clinically available compounds that target this machinery (Sanyal & Doig, 2012; van Eijk, Wittekoek, Kuijper, & Smits, 2017). DNA replication is a process performed by several proteins (Jameson & Wilkinson, 2017). The correct operation of this pathway is decisive for cell growth and proliferation (Jameson & Wilkinson, 2017). The β-clamp protein plays a key role in managing DNA replication (Nanfara, Babu, Ghazy, & Sutton, 2016). In *S. aureus*This protein is encoded by *dnaN* and is made up of a homodimer that surrounds the DNA and slides along the duplex, bringing the polymerase into contact with the DNA to ensure processivity (Johnson & O'Donnell, 2005; Kjelstrup et al., 2013; Nielsen & Lobner-Olesen, 2008). The β-clamp interacts with many different proteins, including DnaE, PolC, δ, PolIV (DinB), PolV (UmuC/D), PolI, MutS, MutL, DNA ligase, and Had (Indiani & O'Donnell, 2006; Johnson & O'Donnell, 2005; Nielsen & Lobner-Olesen, 2008). However, few studies have explored β-clamp as a target for antimicrobial discovery (Kjelstrup et al., 2013; Pandey, Verma, Dhar, & Gaurinath, 2018; Pandey et al., 2017). Based on this, a double hybrid system was built for the selection of compounds with the ability to prevent the dimerization of the replicative protein, resulting in the selection of peptides that inhibited the dimerization of the β-clamp of S. aureus (Kjelstrup et al., 2013).

The β-clamp protein confers processivity for DNAs, polymerases, and other proteins. It acts as a clamp, forming a ring around DNA (a reaction catalyzed by the clamp-loading complex), which diffuses ATP-independent, free, and bidirectionally along the double helix.

In this work, we report the application of the double hybrid system to select inhibitors of the dimerization of the β-clamp of *S. aureus* from Caatinga plant extracts. These compounds may serve as possible candidates for future development in new classes of antibiotics, as well as provide insight into the function of the replication process of *S. aureus*. Read the appendices to observe the systematic order in which the research steps were carried out and detailed explanations of the methods used.

MATERIAL AND METHODS

PLANT EXTRACTS FROM THE CAATINGA

The extracts evaluated in our study were kindly provided by the Natural Products Laboratory of the Department of Biochemistry of the Federal University of Pernambuco. Plant material was collected in the Catimbau National Park (Catimbau National Park, Pernambuco, Brazil) in November 2013. The taxonomic identification was carried out by Dr. Alexandre Gomes da Silva, at the Herbarium of the Agronomic Institute of Pernambuco (IPA), where each specimen of the voucher is deposited.

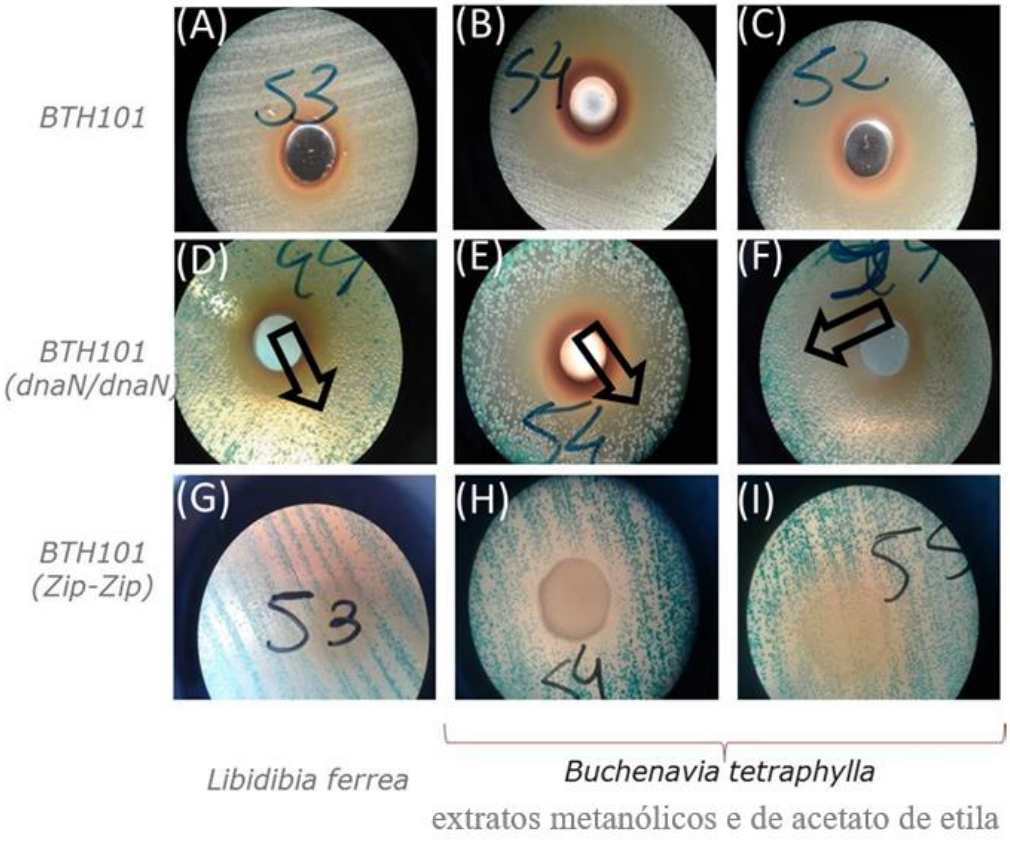
The aqueous extracts were obtained by mixing 25 g of each sample (leaves, branches, etc.) with distilled water in a shaker at 125 rpm for 72 h at 25°C. Then, each extract was filtered and the supernatant was freeze-dried. All extract samples were stored at 4°C prior to use. In addition, the preparation of organic extracts of *Beech avia tetraphylla* has been prepared as reported by (Cavalcanti Filho et al., 2017). See attachments for details of phytochemical screening.

SELECTION OF PLANTS THAT DISRUPT THE B-CLAMP-B-CLAMP INTERACTION

The Interaction β-clamp-β-clamp was evaluated using a hybrid double bacterial system (BTH – see appendices), based on the reconstitution of the activity of the enzyme adenylate cyclase (CYA) of *Bordetella pertussis* and cloned in *Escherichia coli.* In this system, the gene encoding the enzyme cya was divided into two dysfunctional fragments (T18 and T25), which only express the enzyme if reassembled. Each fragment was then fused to a sequence of *dnaN* (encoder of β-clamp) and cloned separately into two recombinant plasmids (p25N-DnaN and pUT18-DnaN). Then a mutant bloodline *E. coli* BTH101 (with deletion in the enzyme gene β-galactosidase) was co-transformed with the two recombinant plasmids (p25N-DnaN and pUT18-DnaN). Given the ability to β-clamp form dimers, the T18 and T25 fragments will be brought together and adenylate cyclase activity will be activated. This would result in the production of the enzyme β-galactosidase, which is evidenced by the formation of blue colonies on plates containing Luria-Bertani (LB) medium supplemented with X-gal (5-bromo-4-chloro-indolyl-p-D-galactoside) (Kjelstrup et al., 2013).

To evaluate the effects of extracts on the dimerization of β-clamp, lineage BTH101 (100 µL) was applied to LB+X-gal plates and relevant antibiotics. Each sample (40 µL to 50 mg/mL) was applied in a 6-mm orifice made in the LB+X-gal (5-bromo-4-chloro-indolyl-p-D-galactoside) plates. The plates were incubated at 37 °C and after 24 h the development of white colonies was indicative of the inhibition of the interactions β-clamp-β-clamp (Kjelstrup et al., 2013). See Figure 1. The specificity of the active extract was confirmed using the empty plasmids (pKT25-zip and pUT18C-zip). Experimental design in the annexes.

Figure 1: Effects of *extracts of Libidibia ferrea* (aqueous extract) and *Buchenavia tetraphylla* (methanolic and ethyl acetate extracts) on the dimerization of β-clamp of *S. aureus*. (A-C): Effects of each extract on the growth of *E. coli* BTH101. (D-F): Effects of each extract on *E. coli* BTH101 co-transformed with p25N-DnaN and pUT18-DnaN plasmids. (G-I): Effects of each extract on *E. coli* BTH101 co-transformed with empty plasmids (pKT25-zip and pUT18C-zip). The arrows indicate the development of white colonies.



DETERMINATION OF ANTIMICROBIAL ACTIVITY OF ACTIVE EXTRACTS.

The antimicrobial activity of active extracts was evaluated by determining the minimum inhibitory concentration (MIC) against *S. aureus* 8325-4. The double serial dilution of each extract was prepared in a 96-well plate using LB broth. Each well received 10 µL of bacterial suspension (approximately 1.5 × 108 CFU/mL). The microplates were incubated at 37 °C. After 24 h, 50 µL of resazurin solution (0.01%) were added to each well. The plates were re-incubated for 2 h at 37 °C, and any change in coloration from blue to pink was recorded as microbial growth. The lowest concentration at which no color change occurred was taken with MIC. See attachments for more details

LC-MS/Q-TOF ANALYSES

To identify compounds potentially responsible for the activity, the active extracts were subjected to a non-segmented analysis by LC-MS/Q-TOF. Chromatography was performed on a Dionex UltiMate® 3000 Quaternary Rapid Separation UHPLC+ system (Thermo Fisher Scientific, Germering, Germany). Separation was achieved in a 1.7u XB-C18 Kinetex column (100 x 2.1 mm, 1.7 µm, 100 Å, Phenomenex, Torrance, CA, USA). Formic acid (0.05%) in water and acetonitrile

(supplied with 0.05% formic acid) were used as mobile phases A and B, respectively. Gradient conditions: 0.0-1.0 min, 5% B; 1.0.-12.0 min, 5-40% B; 12.0-20.0 min 40-100% B, 20.0-22.0 min 100% B, 22.0-22.5 min 100-5% B, and 22.5-25.0 5% B. The mobile phase flow rate was 300 µL $^{min-1}$. The temperature of the column was maintained at 30 °C. Four wavelengths (205 nm, 220 nm, 250 nm, and 390 nm) were monitored by a UV-VIS detector.

Liquid chromatography was coupled to a microOTOF-Q mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ion source (ESI) operated in positive or negative ionization mode. The ionic spray voltage was maintained at +4500 V or -3900 V, respectively. The dry temperature was set to 200 °C and the dry gas flow was set to 8 L min-1. The misting gas was set to 2.5 bar and collision energy to 15 eV. Nitrogen was used as dry gas, nebulizer gas, and collision gas. The m/z range has been set to 50-1400. Auto MSMS mode was used to obtain the MS and MS/MS spectra of the three most abundant ions present at each time point with intelligent exclusion to also include less abundant ions. All files were calibrated based on composite spectra collected from clusters formed in Na+ at the start of each run.

ESTUDOS *IN SILICO*

Structure of compounds

The structure of the selected compounds was obtained from the PubChem Project (https://pubchem.ncbi.nlm.nih.gov/) database. Each composite was converted into 3-dimensional (3D) files using Easy convert software.

Construction and validation of the β-clamp structure of *S.aureus*

The 3D structure of the β-clamp of *S. aureus* is not available in the PDB and was modeled by the *Swiss-Model Automated Protein Modeling Server* with default settings (http://swissmodel.expasy.org/). The amino acid sequence of S. *aureus β-clamp* (composed of 377 residues) used in this study was obtained from the NCBI (WP_000969811.1). The server selected the 3D structure (obtained experimentally) of the B. subtilis *β-clamp* (PDB ID: 4TR6) as a model. The quality of the selected S. aureus *β-clamp* model was verified with Z-score (PROVE: http://services.mbi.ucla.edu/SAVES/) and Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

Docking molecular

Molecular docking simulations to investigate the binding of the active compound and its derivatives to the predicted β-clamp structure of *S. aureus*. Molecular coupling was performed on the server *Swissdock* (http://www.swissdock.ch/docking), a web service based on EADock DSS software

(Grosdidier, Zoete, & Michielin, 2011 — Brazil). The interaction of all compounds with the human CYP3A4 enzyme was also analyzed. For this, the structure of human CYP3A4 was obtained from PDB (PDB ID: 5VDC).

See appendices for a detailed understanding of the systematic order of the methods used in this work.

RESULTS AND DISCUSSION

EXTRACTS OF CAATINGA PLANTS CAPABLE OF AFFECTING B-CLAMP DIMERIZATION IN *S. AUREUS*

The first step of this work was to select extracts of Caatinga plants capable of inhibiting the formation of dimers by the β-clamp. This protein is the process-promoting factor for most enzymes in prokaryotic DNA replication and its dimerization is essential for correct action (30). Since only a few compounds are reported to inhibit β-clamp (16, 27, 28), we tried to analyze whether caatinga plants could be a source of this type of compounds.

The plants of the caatinga were chosen because they are cultivated in a unique biome, exclusive to Brazil, marked by long periods of drought and high levels of solar radiation (Rodrigues, Silva, Eisenlohr, & Schaefer, 2015). The Caatinga is located in the Northeast of Brazil and covers about 12% of the country's territory, extending over large areas of the states of Ceará, Piauí, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia and also a part of the north of Minas Gerais. Extreme environmental conditions have an influence on the metabolism of caatinga plants (Oliveira et al., 2017) making them capable of producing new compounds that are interesting targets for drug research. Several examples demonstrate the antimicrobial potential of natural products derived from Caatinga plants (da Silva et al., 2013; Malafaia et al., 2017; Silva et al., 2015).

The antimicrobial activities of these extracts were confirmed by MIC determinations (Table 1). Among them, the aqueous extract of *L. ferrea* showed the lowest value (MIC: 97.65 µg/mL), followed by methanolic extract (MIC: 195.31 µg/mL) and ethyl acetate extract (MIC: 390.62 µg/mL) of *B. tetraphylla*. The antimicrobial activity of extracts and compounds of *B. tetraphylla* and *L. ferrea* (including those obtained from specimens grown in the Caatinga) has been reported in other studies against *S. aureus* (da Silva et al., 2013; de Oliveira et al., 2012) and other microorganisms (Cavalcanti Filho et al., 2017; de Oliveira Marreiro et al., 2014). However, to the best of our knowledge, this is the first time that the presence of β-clamp inhibitors has been reported for these plants. In addition, evidence on interference with the cell division of *S. aureus* and *C. albicans* were suggested by electron microscopy for the hydroalcoholic extract of *L. ferrea* (da Silva et al., 2013) and methanolic from *B. tetraphylla* (Cavalcanti Filho et al., 2017)respectively. See Figure 1 after the references.

Table 1: Antimicrobial activity of extracts of *Libidibia ferrea* (aqueous extract) and *Buchenavia tetraphylla* (methanolic extracts and ethyl acetate) against *S. aureus*.

Plant	Extract	MIC (µg/mL)
Beech avia tetraphylla	Methanolic	195,31
	Ethyl Acetate	390,62
Iron Libya	Aqueous	97,65

DETECTION OF BUCHENAVIANINE AND ITS DERIVATIVES IN THE ACTIVE EXTRACT OF *B. TETRAPHYLLA*

The chemical characterization of the methanolic extract of the leaves of *B. tetraphylla* revealed the presence of buchenavianine and its derived compounds (Figure 2). Buchenavianin and derivatives [N-desmethylbuchenavianine, O-desmethylbuchenavianine, N,O-bis-(desmethyl)-buchenavianine] are classified as flavoalkaloids with a piperidine moiety at carbon 8. Flavoalkaloid is a term used for compounds that contain both flavonoids and alkaloid components (Blair, Calvert, & Sperry, 2017). The convergence of two distinct biosynthetic pathways results in natural products that exhibit a wide range of interesting biological activities that would not be expected for flavonoids or alkaloids alone (Blair et al., 2017). Buchenavianin and N-desmethylbuchenavian were first isolated from the leaves of *Beech avia macrophylla*, while O-desmethylbuchenavian and N,O-bis-(desmethyl)-buchenavianine were first found in the fruit of the same plant (Blair et al., 2017). There are few reports on biomedical applications of buchenalianin and its derivatives that include the cytoprotective effect against human immunodeficiency virus (HIV) (Beutler, Cardellina, McMahon, Boyd, & Cragg, 1992) and inhibition of kinases with cyclin CDK1/cyclin B and CDK5/p25 by ATP competition (T. B. Nguyen et al., 2012). Buchenavianine and its derivatives have also been detected in the extract of *Buchenavia tomentosa* with activity against species of *Candida* (Teodoro et al., 2015).

Based on this, we decided to select buchenavianin and its derivatives for the *in silico evaluations*. The structures of Buchenavinine (C22H23NO4), N-desmethylbuchenavinine (C21H21NO4), O-desmethylbuchenavinine (C21H21NO4), N,O-bis-(desmethyl)-buchenavianine (C21H21NO4) and the analogous compounds CHEMBL2029614 (C21H21NO4) and CHEMBL2029615 (C21H21NO4) were obtained from the PubChem Project database and are shown in Figure 3.

Figure 2: Detection of buchenamelanin and derivatives in the methanolic extract of *B. tetraphylla* by LC-MS/Q-TOF in positive mode.

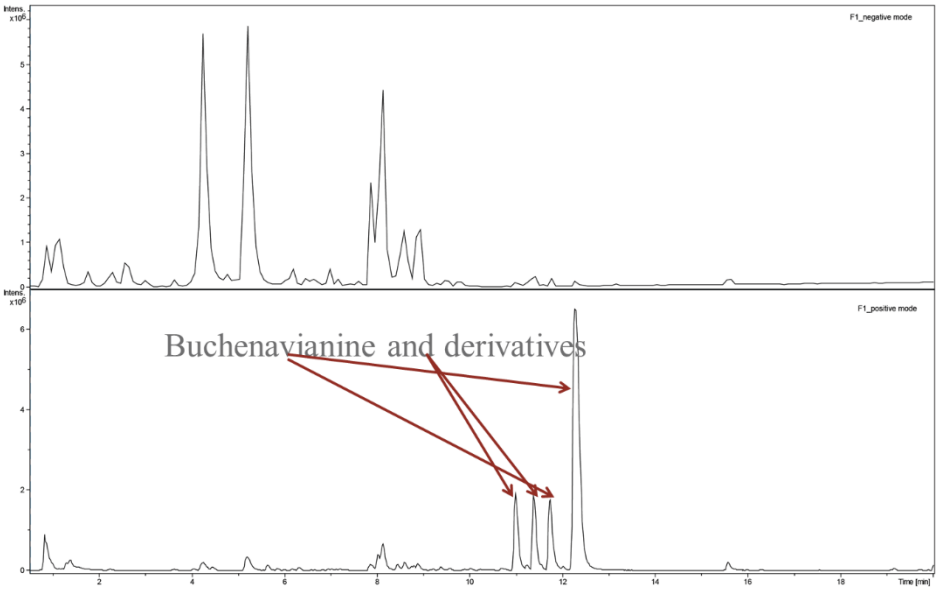
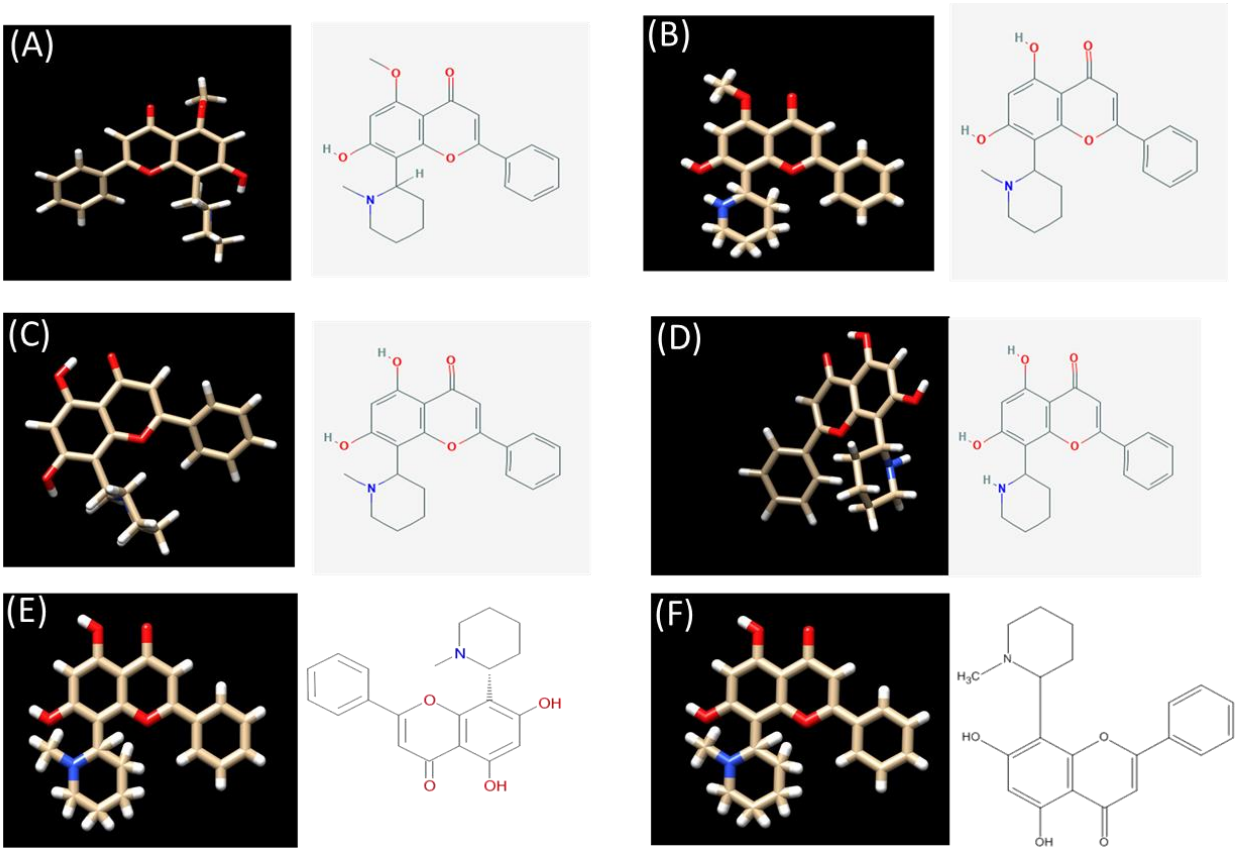


Figure 3: Structures of buchenavianine and its derivatives used for *in silico studies*. (A) Buchenavianine; (B) N-desmethylbuchenavianine; (C) O-desmethylbuchenavianine; (D) N,O-bis-(desmethyl)-buchenavianine; (E) CHEMBL2029614; (F) CHEMBL2029615.



VALIDATION OF THE 3D STRUCTURE MODEL FOR B-CLAMP OF *S.AUREUS*

The β-clamp model of *S. aureus* was predicted by homology modeling using the β-clamp structure of *B. subtilis* (PDB ID: 4TR6). The two proteins exhibit 50% similarity in their amino acid sequences (Figure 4). The modeled structure is shown in Figure 5. The stereochemical quality of the

predicted model was determined by the Z-score and Ramachandram plot. The Z-score indicated that only 4.5% of atoms did not follow the Gaussian distribution, which indicated that the predicted structure has a high level of atoms in the correct configuration (95.5%) (Figure 6A). In addition, the Ramachandram plot (Figure 6) showed that 96.8% and 2.6% of amino acid residues are in favorable and permissible regions, respectively (Figure 6B). All these results confirmed that the model obtained for β-clamp of *S. aureus* has stereochemical quality and can be used for modeling studies.

Figure 4: Alignment of amino acid sequences from S. *aureus β-clamp and* B. subtilis *β-clamp.*

```
β_clamp_S_aureus        -MMEFTIKRDYFITQLNDTLKAISPRTTLPILTGIKIDAKEHEVILTGSDSEISIEITIP
β_clamp_B_subtilis      SHMKFTIQKDRLVESVQDVLKAVSSRTTIPILTGIKIVASDDGVSFTGSDSDISIESFIP
                          *:***::* :: .::*.***:* ***:******** *.:. * :*****:****  **

β_clamp_S_aureus        KTVDGEDIVNISETGSVVLPGRFFVDIIKKLPGKDVKLSTNEQFQTLITSGHSEFNLSGL
β_clamp_B_subtilis      KEEGDKEIVTIEQPGSIVLQARFFSEIVKKLPMATVEIEVQNQYLTIIRSGKAEFNLNGL
                        *  ..::**.*.: **:** .*** :*:****   *::.::*: *:* **::****.**

β_clamp_S_aureus        DPDQYPLLPQVSRDDAIQLSVKVLKNVIAQTNFAVSTSETRPVLTGVNWLIQENELICTA
β_clamp_B_subtilis      DADEYPHLPQIEEHHAIQIPTDLLKNLIRQTVFAVSTSETRPILTGVNWKVEQSELLCTA
                        * *:** ***:....***: ..:***:* ** **********:****** :::.**:***

β_clamp_S_aureus        TDSHRLAVRKLQLEDVS-ENKNVIIPGKALAELNKIMSDNEEDIDIFFASNQVLFKVGNV
β_clamp_B_subtilis      TDSHRLALRKAKLDIPEDRSYNVVIPGKSLTELSKILDDNQELVDIVITETQVLFKAKNV
                        *******:** :*:  . .. **:****:*:**.**:.**:* :**.::..*****. **

β_clamp_S_aureus        NFISRLLEGHYPDTTRLFPENYEIKLSIDNGEFYHAIDRASLLAREGGNNVIKLSTG-DD
β_clamp_B_subtilis      LFFSRLLDGNYPDTTSLIPQDSKTEIIVNTKEFLQAIDRASLLAREGRNNVVKLSAKPAE
                         *:****:*:***** *:*:: : :: ::. ** :************ ***:***:   :

β_clamp_S_aureus        VVELSSTSPEIGTVKEEVDANDVEGGSLKISFNSKYMMDALKAIDNDEVEVEFFGTMKPF
β_clamp_B_subtilis      SIEISSNSPEIGKVVEAIVADQIEGEELNISFSPKYMLDALKVLEGAEIRVSFTGAMRPF
                         :*:**.*****.* * : *:::** .*:***. ***:****.::. *:.*.* *:*:**

β_clamp_S_aureus        ILKPKGDDSVTQLILPIRTY
β_clamp_B_subtilis      LIRTPNDETIVQLILPVRTY
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Figure 5: Model for the β-clamp structure of *S. aureus* predicted by homology modeling.

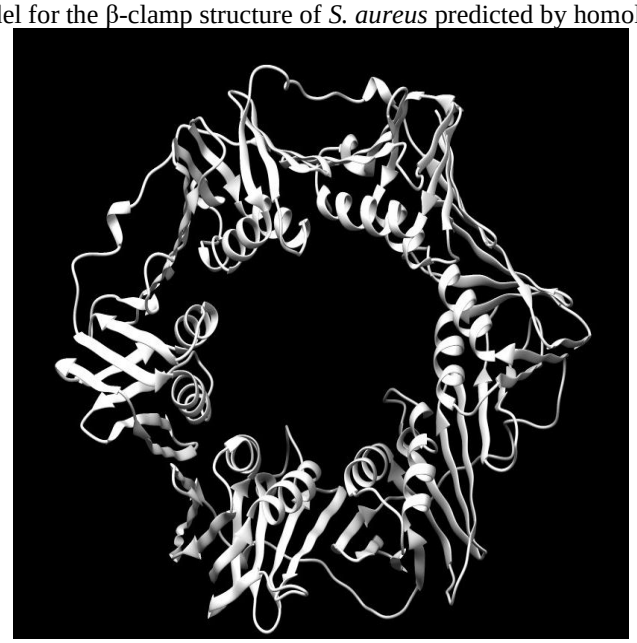
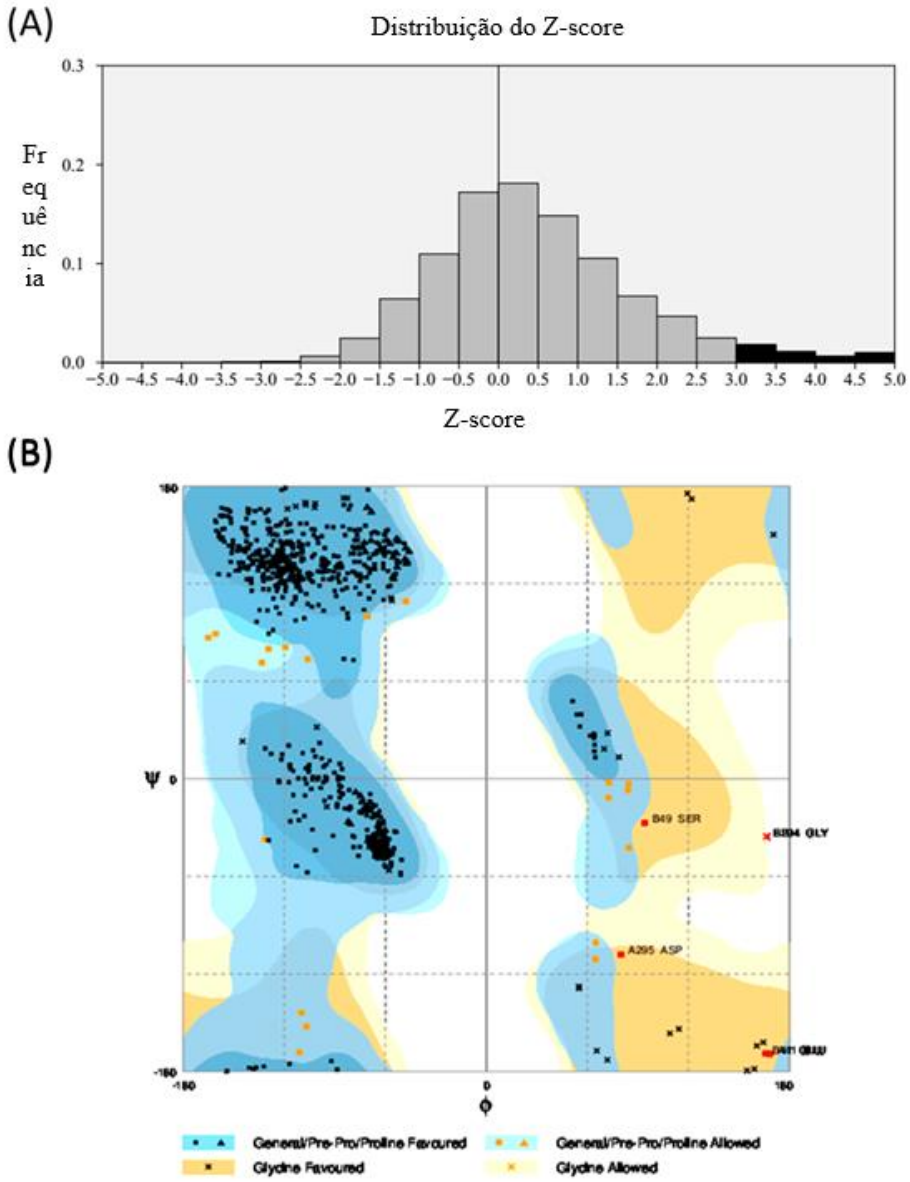


Figure 6: Validation of the 3D structure model for β-clamp of *S. aureus* determined by Z-score (A) and Ramachandram plot (B). In (A) Only 4.5% (122) atoms are outside the Gaussian distribution, indicating that the structure has 95.5% correction. In (B) we have the number of residues in the favored region is 720 (96.8%), in the allowed region it is 19 (2.6%).



B-CLAMP ANCHORAGE STUDIES OF *S.AUREUS* AND SELECTED COMPOUNDS

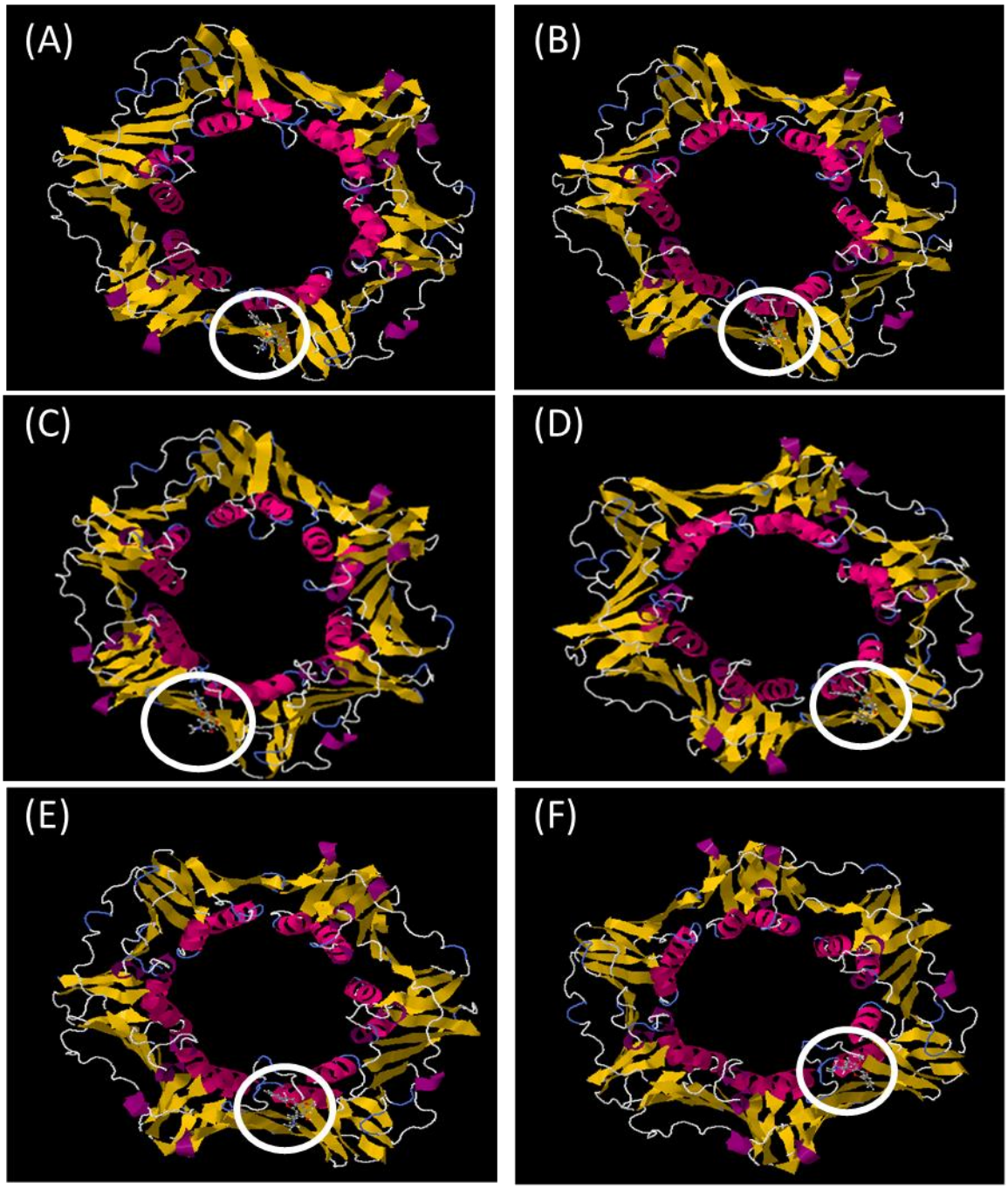
The interaction of buchenavianine and related compounds with β-clamp of *S. aureus* It was predicted by molecular coupling. Swissdock presents the results in terms of FullFitness and Gibbs free energy (ΔG) (Table 2). Favorable binding modes were obtained on the basis of FullFitness and cluster formation. FullFitness values ranged from -4894.34 kcal/mol to -4879.57 kcal/mol. Regarding the estimated ΔG, the values ranged from -6.64 kcal/mol to -7.50 kcal/mol. The ranking of the compounds was performed using the value of FullFitness as previously adopted in other studies (Saha, Islam, Artist, & Hasan, 2013). Figure 7 shows the visualization of the most energetically favorable bond of each compound in β-clamp of *S. aureus*.

The best FullFitness was found for N,O-bis-(desmethyl)-buchenavianin with a value of -4894.34 kcal/mol and estimated ΔG of -7.26 kcal/mol, for the most favorable interaction. The analogue CHEMBL2029615 (FullFitness = -4892.24 kcal/mol, estimated ΔG = -7.43 kcal/mol) and CHEMBL2029614 (FullFitness = -4891.09 kcal/mol, estimated ΔG = -7.31 kcal/mol) exhibited results similar to those observed for N,O-bis-(desmethyl)-buchenavianine. O-desmethylbuchenavianin (FullFitness = -4888.70 kcal/mol, estimated ΔG = -6.64 kcal/mol), N-desmethylbuchenavianin (FullFitness = -4880.36 kcal/mol, estimated ΔG = -7.50 kcal/mol) and buchenavianin (FullFitness = - 4879.57 kcal/mol, estimated ΔG = -7.40 kcal/mol) were ranked fourth, fifth, and sixth in relation to their ability to bind to β-clamp of *S. aureus*. As we know, exothermic reactions release heat and are therefore able to stabilize a biochemical system, in accordance with the principles of chemistry (thermodynamics) and physics (Hamiltonian Lagrangian mechanics)

Table 2: Interaction between S. *aureus* β-clamp and selected compounds.

Classification	**Compound**	**FullFitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**
1	**N,O-bis-(desmethyl)-buchenavianine**	-4894,34	-7,26
2	**CHEMBL2029615**	-4892,24	-7,43
3	**CHEMBL2029614**	-4891,09	-7,31
4	**O-desmethylbuchenavianina**	-4888,70	-6,64
5	**N-desmetilbuchenavianina**	-4880,36	-7,50
6	**Buchenavianina**	-4879,57	-7,40
|ΔR|	**|R6-R1|**	**|ΔFullFitness|=14.77 Order 4,000**	**Compare the sort order with Table 3**

Figure 7: *Molecular docking* between β-clamp of *S. aureus* and selected compounds. Compounds are classified by their FullFitness values. (A) N,O-bis-(desmethyl)-buchenavianine; (B) CHEMBL2029615; (C) CHEMBL2029614; (D) O-desmethylbuchenavianine; (E) N-desmethylbuchenavianine; (F) Buchenavianine. The white circles indicate the position of each ligand.



ANCHORING STUDIES WITH HUMAN CYP3A4 AND SELECTED COMPOUNDS

Cytochrome P450 comprises several enzymes that play an essential role during the metabolism of exogenous compounds such as drugs and alcohol. There are at least four isoforms for CYP (cytochrome P450), but 3A4 is the most abundant in the liver and the one that contributes most to bile acid detoxification, the termination of the action of steroid hormones, and the elimination of phytochemicals in food and most medications (Nebert, Wikvall, & Miller, 2013; Olsen, Oostenbrink,

& Jorgensen, 2015). In this regard, it is important to evaluate the effect of a new drug candidate with CYP3A4.

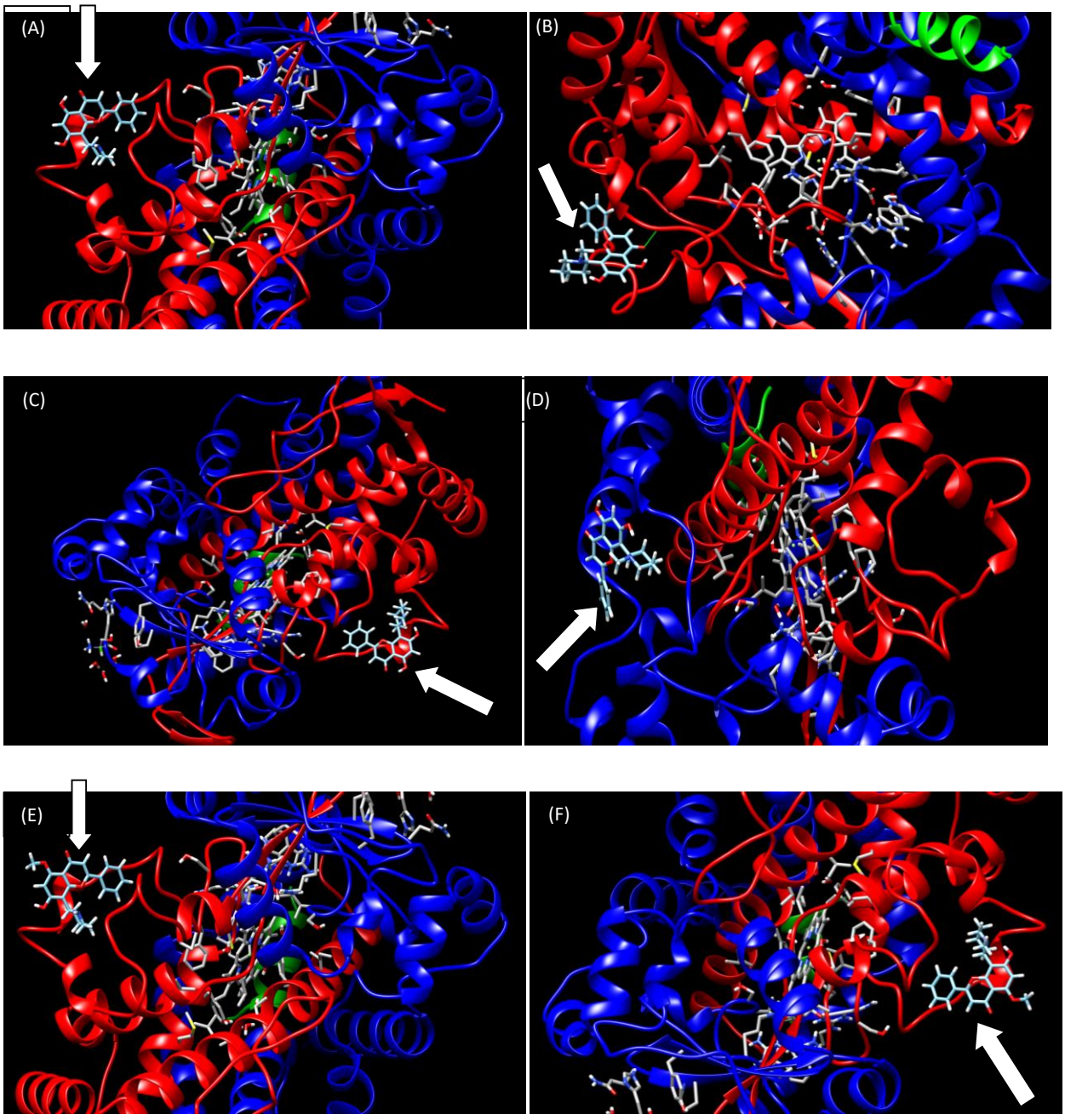
The interaction of buchenaviannin and human CYP3A4-related compounds was predicted by molecular coupling (Table 3). FullFitness values ranged from -2217.75 kcal/mol to -2201.27 kcal/mol. Regarding the estimated ΔG, the values ranged from -6.82 kcal/mol to -7.09 kcal/mol. Figure 8 shows visualization of the most energetically favorable binding of each compound in human CYP3A4.

The best FullFitness was found for N,O-bis-(desmethyl)-buchenavianin with a value of -2217.75 kcal/mol and estimated ΔG of -6.86 kcal/mol, for the most favorable interaction. The analogue CHEMBL2029615 (FullFitness = -2215.91 kcal/mol, estimated ΔG = -7.09 kcal/mol) and CHEMBL2029614 (FullFitness = -2213.84 kcal/mol, estimated ΔG = -6.98 kcal/mol) showed results similar to those observed in N,O-bis-(desmethyl)-buchenavianine. O-desmethylbuchenavianin (FullFitness = -2214.25 kcal/mol, estimated ΔG -7.01 kcal/mol), N-desmethylbuchenavianin (FullFitness = -2203.70 kcal/mol, estimated ΔG = -6.82 kcal/mol) and buchenavianin (FullFitness = -2201.26 kcal/mol, estimated ΔG = -7.04 kcal/mol) were ranked third, fifth, and sixth with respect to their ability to bind to human CYP3A4. The interaction between buchenavianine and human CYP3A4 analogues was 2 times lower than buchenavianine and S. *aureus* β-clamp analogues and this shows that between the two targets analyzed here (S. aureus β-clamp and human CYP3A4) the ligands will be preferentially attracted to *the S. aureus* β-clamp, which ratifies them as viable inhibitors of the S. *aureus DNA replication process*.

Table 3: Interaction between human CYP3A4 and selected compounds.

Classification	**Compound**	**FullFitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**
1	**N,O-bis-(desmethyl)-buchenavianine**	-2217,7524	-6,8654685
2	**CHEMBL2029615**	-2215,9163	-7,0911956
3	**O-desmethylbuchenavianina**	-2214,2532	-7,006671
4	**CHEMBL2029614**	-2213,8435	-6,977535
5	**N-desmetilbuchenavianina**	-2203,7002	-6,822664
6	**Buchenavianina**	-2201,2568	-7,044045
|ΔR|	**|R6-R1|**	**|ΔFullFitness|=16.4956 Order 2,000**	**Same order as in table 2, changes only in classification 3**

Figure 8: *Molecular docking* between CYP3A4 of *S. aureus* and selected compounds. Compounds are classified by their FullFitness values. (A) N,O-bis-(desmethyl)-buchenavianine; (B) CHEMBL2029615; (C) O-desmethylbuchenavianine; (D) CHEMBL2029614; (E) N-desmethylbuchenavianine; (F) Buchenavianine. The arrows indicate the position of each binder.



CONCLUSION

The objective of this study was to evaluate the degree of interaction between potential inhibitors and the β-clamp protein of *S. aureus* by means of microbiological and chemical assays and computer simulations. The comparative modeling of the β-clamp and the validation of its 3D structure were satisfactory (Figures 4, 5 and 6). From the results of molecular docking (Figures 9 and 10) it is concluded that buchenavianine alone confers stability when compared to its derivatives

and/or analogues. The latter have electronegative elements (oxygen, nitrogen,...) in their structure, which is why there is a decrease in binding energy due to the stabilization of the electronic cloud. According to Hamilton Lagrange's principle, a stable system will always be a system with maximum negative entropy (lowest numerical value), for a reason that we can intuitively perceive: phenomena with high internal vibration cannot exist in nature because the energy of movement of their particles disaggregates the structure. We also concluded, from Tables 2 and 3, that there is a high interaction (order of 4,800 kcal/mol) between buchenavianine and β-clamp analogues. On the other hand, the interaction between buchenavianine and analogues with human CYPE3A4 was of the order of 2,200 following the same principles of electronegativity of the elements. Therefore, it is concluded that the approach presented here promotes a rational biochemical screening for the selection of ligands with better possibilities for subsequent bench tests, saving financial resources and optimizing results in research and development of medicines. The combination of microbiological, chemical and computational resources made it possible to indicate molecules present in the plant extract capable of interacting with the β-clamp. Buchenavinine was shown to be satisfactory by the laboratory and *in silico* studies carried out here, with potential for technological development as an inhibitor of the dimerization of the β-clamp protein of S. aureus and thus interrupt the bacterial DNA replication machinery during infections caused by this pathogen.

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