Journal of
Proteomics & Bioinformatics

Reddy et al., J Proteomics Bioinform 2016, 9:9
DOI: 10.4172/0974-276X.1000410

Research Article Open Access

A Critical Assessment of *Bombyx mori* Haemolymph Extract on *Staphylococcus aureus* an *In vitro* and *In silico* Approach

Harinatha Reddy A[1]*, Srinivasulu C[2] and Venkatappa B[1]

[1]*Department of Microbiology, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India*
[2]*Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India*

Abstract

The newly reemerging drug-resistant Gram-positive pathogens require the discovery of new drug targets and the development of new therapeutics. We focus on antimicrobial proteins which inhibits growth of *Staphylococcus aureus*. In the present study fifth instar *Bombyx mori* larvae was used and infected with *S. aureus* by intrahaemocoelic injection of bacterial sample. The haemolymph was collected from the healthy and infected larvae after 24 h of infection and stored at -4°C in eppendorf tubes until use. Haemolymph extract was prepared and antimicrobial activity was done by the classical well diffusion method. Haemolymph extract prepared form infected larvae exhibit maximum zone of inhibition on *S. aureus* when compared with healthy haemolymph extract. Bioinformatics tools were used to find out the molecular interaction and binding mode of antimicrobial peptides from *B. mori* such as Moricin and Cecropin on three different drug target proteins Glycerol phosphate lipoteichoic acid synthase (PDB: 2W5Q), ABC transporter (PDB: 1P99) and DNA Gyrase (PDB: 2XCO) from *S. aureus*. Hex Docking software was used for the docking studies. The interaction mode and binding results can be represented in terms of docking energy. The best binding energies were obtained from the docking of DNA gyrase with Moricin and Cecropin (-702.13 Kcal/mol and -639.39 Kcal/mol) respectively.

Keywords: Haemolymph extract; Moricin; Cecropin; Glycerol phosphate lipoteichoic acid synthase; ABC transporter; DNA gyrase

Introduction

The failure of most important drugs on microorganisms, there is an urgent need to develop and other control agents. The objective of study is to find out antimicrobial activity of antimicrobial peptides and their interaction with drug target proteins of *Staphylococcus aureus*. Antimicrobial peptides (AMPs) are natural and synthetic peptides with a broad spectrum activity on fungi, bacteria and fungi [1]. Antimicrobial peptides are oligo peptides with a varying number of amino acids, most of them are cationic, which target bacterial cell membranes and cause disintegration of the lipid bilayer structure [2,3]. The insect antibacterial peptides are very potent in their IC50 even in the sub-micromolar or low micromolar range, majority of peptides interact with bacterial membranes [4]. Insects are one of the major sources of antimicrobial peptides/proteins. Cecropin is the first insect antimicrobial peptide has been purified from *Hyalophora cecropia* pupae. Insect antimicrobial peptide can be classified into four families based on their structures or unique sequences: The α-helical peptides (Cecropin and Moricin), cysteine-rich peptides (insect defensin and drosomycin), proline-rich peptides (apidaecin, drosocin, and lebocin), and glycine-rich peptides/proteins (attacin and gloverin) [5].

Moricin and Cecropin are an antibacterial peptides isolated from haemolymph of domestic silkworm. Moricin is a highly basic antibacterial peptide. It consists of a long alpha-helix with 8 turns from a 42 amino acid sequence over almost the entire protein [6]. Moricin functions as an antibacterial peptide against Gram-positive and Gram-negative bacteria with its main activity being towards Gram-positive bacteria. Cecropins are antimicrobial peptides [7], which are small proteins of about 31-37 amino acid residues active against both Gram-positive and Gram-negative bacteria.

Methicillin-resistant *S. aureus* identified over 4 decades ago, has undergone rapid evolutionary changes becoming a dominant pathogen [8]. *S. aureus* is an opportunistic pathogen cause severe infections in humans. *S. aureus* isolates have been associated with nosocomial infections and rapidly developed resistance to multiple drug classes [9]. The core resistance phenotype that seems to be most associated with the persistence of *S. aureus* in the hospital is methicillin resistance [10]. MRSA is a worldwide problem, the number of hospitalized cases increases form last ten years by MRSA. Vancomycin remains the first line intravenous drug for severe MRSA infections [11,12]. *S. aureus* has a remarkable ability to develop antibiotic resistance, Vancomycin resistance has resulted in a steady decline in the efficacy of these valuable antibiotics [13]. The resistance to antibiotics is mainly due to the slow growth rate and low metabolic activity of bacteria. The use of natural Antimicrobial Peptides (AMPs) has been increasing in the place of antibiotics to kill pathogenic bacteria is an attractive therapeutic approach [14]. The antimicrobial peptides are an alternative for the antibiotics, the advantages of antimicrobial peptides are selectivity, fast killing, broad antimicrobial spectra and no resistance development [15,16]. Bioinformatics tools were used to find out the molecular interaction and binding mode of antimicrobial peptides from *Bombyx mori* such as Moricin and Cecropin on three different drug target proteins of *S. aureus* (Glycerol phosphate lipoteichoic acid synthase, ABC transporter and DNA Gyrase).

The membrane embedded enzyme lipoteichoic acid synthase enzyme responsible for lipoteichoic acid synthesis in *S. aureus* [17]. Lipoteichoic Acid (LTA) is an important cell wall component of *S. aureus*. Therefore glycerol phosphate lipoteichoic acid synthase

***Corresponding author:** Harinatha Reddy A, Department of Microbiology, Sri Krishnadevaraya University, Anantapur-515003, Andhra Pradesh, India, Tel: +919000616285; E-mail: biohari14@gmail.com

Received August 10, 2016; **Accepted** September 12, 2016; **Published** September 19, 2016

Citation: Reddy HA, Srinivasulu C, Venkatappa B (2016) A Critical Assessment of *Bombyx mori* Haemolymph Extract on *Staphylococcus aureus* an *In vitro* and *In silico* Approach. J Proteomics Bioinform 9: 226-231. doi: 10.4172/0974-276X.1000410

essential for bacterial growth and serve as a target for antibiotic development [18]. ATP-Binding Cassette (ABC) transporters are widespread among microorganisms and comprise one of the largest protein families and targets for the development of antibacterial vaccines and therapies. ABC transporters hydrolyzes ATP to provide energy for the import and export of a wide variety of substrates in to the cell [19]. DNA gyrase also known as topoisomerase II is an essential bacterial enzyme that catalyzes the ATP-dependent negative super coiling of double-stranded closed circular DNA. DNA gyrase as the intracellular target of a number of antibiotics as a paradigm for other DNA topoisomerases [20].

Materials and Methods

Rearing of *Bombyx mori* larvae

Fifth instar larvae of CSR2 verities were obtained from Regional Sericulture Research Station, Rapthadu, Anantapur (District) and reared in the laboratory by the improved method of rearing technique [21]. The silkworms are maintained on mulberry leaves at a temperature of 27°C and relative humidity of 75%. The life span of the silkworm under these conditions was 30-32 days.

Preparation and inoculation of bacterial sample in Luria broth (LB)

Mannitol salt agar (MSA) medium was prepared a loopful sample of *S. aureus* bacterial sample was streaked on MSA and incubated at 37°C for 24 h. After incubation *S. aureus* formed golden yellow color colonies on MSA medium. A loopful of bacteria was taken with the help of a loop and inoculated in to the Luria broth (LB 1000 ml) and incubated at 37°C overnight. The inoculated LB sample was centrifuged for 15 min at 4000 rpm. By discarding the supernatant, pellet sediment at the bottom of the tube was dissolved in 100 ml of distilled water. The number of bacteria cells in the bacterial culture suspension was calculated by colony-forming unit (CFU).

Infection of silkworm larvae with bacterial strain

Fifth instar silkworm larvae divided in to two groups, each group consisting of 20-30 larvae. One group was infected with the bacterium (2×10^9 cfu/ml) by intrahaemocoelic injection of 0.1 ml bacterial sample. A similar number of larvae injected with distilled water and considered as a control. Both control and infected larvae reared under room temperature. The time of infection was recorded, the haemolymph was collected from the infected and control group larvae by cut were made on the proleg cuticle at 24 h post infection. The collected haemolymph was centrifuged at 2000 rpm for 15 min at 4°C. The resulting supernatant was stored at -4°C in eppendorf tubes until use. The protein concentration in supernatant was determined with Folin Ciocalteau's reagent according to the Lowry method [22].

Antibacterial activity evaluation by Agar well diffusion assay

In vitro antibacterial activity was evaluated by Agar well diffusion method using Mueller Hinton Agar (MHA). Antimicrobial activity of *B. mori* haemolymph was determined against *S. aureus* obtained from the department of microbiology Sri Krishnadevaraya University, Andhra Pradesh.

Working stock was prepared as 1 ml of bacterial strain was initially inoculated in 100 ml of sterile Mueller Hinton broth and incubated for 37°C ± 1°C for 24 h respectively. Then 0.2 ml of the test organism from the working stock were seeded into 100 ml sterile MHA and allowed to stay at 37°C for 3 h. When the MHA solidifies, six holes of uniform diameter (7 mm) were made using sterile aluminum borer. Then, 70 µl of haemolymph extract standard solution (sets of five dilutions 10, 20, 30, 40, 50 mg/ml prepared in double-distilled water) respectively and control (Ciprofloxacin 25 mg/ml) were placed in each hole separately under aseptic condition. The plates were then maintained at room temperature for 2 h to allow the diffusion of the solution into the medium. All the bacterial plates were then incubated at 37°C ± 1°C for 24 h and the zone of inhibition was measured (mm, including the diameter of the bore (7 mm)) and the results were recorded.

Ligand preparation

The 3D Structure of the Moricin (PDB: 1KV4), Cecropin (PDB: 2LA2) peptides was retrieved from PDB (Protein data bank) and prior to initiating the docking simulations, all non-protein molecules were removed from the PDB.

Protein preparation

The crystal structures of Glycerol phosphate lipoteichoic acid synthase (PDB: 2W5Q), Dipeptide ABC transporter-PG110 (PDB: 1P99) and DNA Gyrase (PDB: 2XCO) from *S. aureus* were retrieved from the Protein Data Bank (http://www.rcsb.org). Prior to initiating the docking simulations, all non-protein molecules were removed, for any alternative atoms locations only the required location was retained. The downloaded proteins are energy minimized for docking studies.

In silico modeling

Docking studies: Hex is an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein and DNA molecules. Hex can also calculate protein-ligand docking, assuming the ligand is rigid, and it can superpose pairs of molecules using only knowledge of their 3D shapes. In our present work, we have used Hex docking software for protein-protein docking studies. It requires both ligand PDB files and receptors PDB files.

The Moricin and Cecropins are taken as ligands and the bacterial proteins were taken as receptors in this study. The ligands were docked against bacterial proteins.

Result and Discussion

Antimicrobial activity

The haemolymph extract prepared form infected larvae exhibited good activity on *S. aureus* when compared with healthy haemolymph (Figure 1). The maximum zone of inhibition (26 mm) was exhibited by highest concentration (50 mg/ml) of haemolymph extract prepared form infected larvae (Figure 2). Zone of inhibition suggested that peptides are synthesized during the infection with exhibits antimicrobial activity on *S. aureus*. Antimicrobial peptides are synthesized in response to microbial infection or septic body injury mainly in insect fat body and in certain blood cells and then rapidly released into haemolymph where they act synergistically against microorganisms [23-26]. Antimicrobial proteins appear to be ubiquitous and multicomponents of the innate immune mechanisms existing in *B. mori* [27].

The recognition of the pathogens and parasites by the invertebrate immune system may involve soluble proteins present in the haemolymph as well as proteins localized at the surface of the haemocytes or other cells [28]. Antibacterial peptides can also be induced in epidermal cells in response to wounding or infection in the cuticles [29]. The process of synthesizing antibacterial proteins may take few minutes or hours after the infection and these are secreted into the haemolymph [30]. Injection of bacteria into

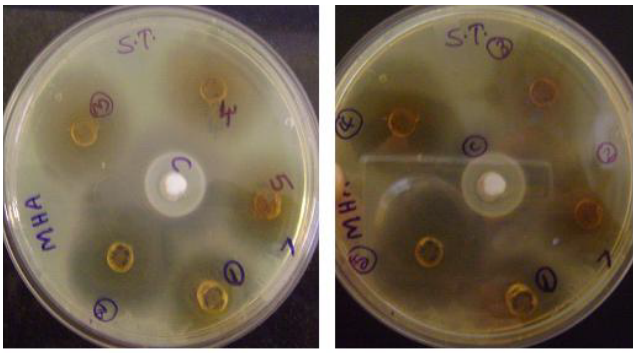

Figure 1: Antimicrobial activity of *Bombyx mori* haemolymph (A). Haemolymph form control group (B). Haemolymph form infected group.

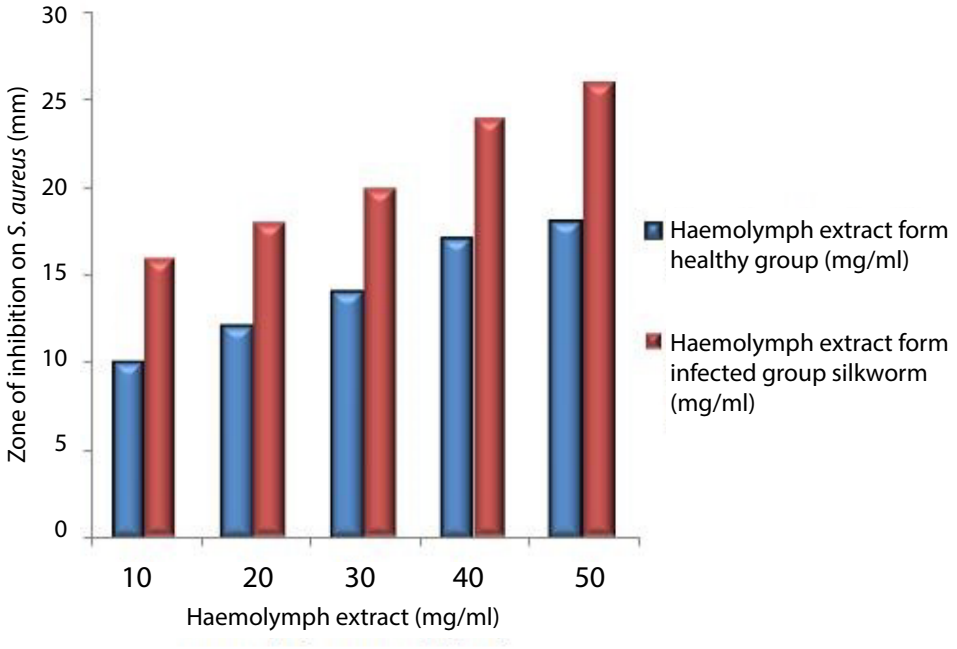

Figure 2: Graphical representation of antimicrobial activity of *Bombyx mori* haemolymph.

the haemocoel elicits the synthesis of a number of antimicrobial peptides and proteins [31]. The haemolymph showed antibacterial activity, it offers to suggest that a broad spectrum of antibacterial peptides was secreted in response to immunization [29].

In silico molecular docking

Moricin and Cecropin are antibacterial peptides isolated from haemolymph of domestic silkworm. Moricin and Cecropin functions as antibacterial peptides against Gram-positive and Gram-negative bacteria, with its main activity being towards Gram-positive bacteria. The 3D structures are retrieved from the protein data bank.

The docking results of Moricin and Cecropin with *S. aureus* target proteins are summarized in Table 1. As noted before good convergence was achieved, with the best docked conformations also found to have the highest binding energy and the greatest number of conformations per cluster. The binding energy was represented in terms of Kcal/mol. Conformations appear a strong hydrogen bond interaction of Moricin and Cecropin with target proteins. The best binding energies were obtained from the docking of DNA gyrase with Moricin and Cecropin are -702.13 Kcal/mol and -639.39 Kcal/mol respectively (Table 1).

DNA gyrases are an important target of antibacterial agents. DNA gyrase are enzymes that catalyse changes in the topology of DNA [32,33], i.e., they can interconvert relaxed and supercoiled forms. These enzymes are found in all cell types and are essential for survival. Due to their essential nature and their mechanisms of action, topoisomerases have become key drug targets both for antibacterial and anti-cancer chemotherapy [34-36]. In the present hypothesis Moricin and Cecropin bind to DNA gyrase enzyme with high binding energy and inhibits DNA gyrase activity when compared to other drug target proteins such as Glycerol phosphate lipoteichoic acid synthase and Dipeptide ABC transporter-PG110. The amino acid residues that participated in hydrogen bonding are summarized in Table 1.

Target protein	Binding energies of Moricin and Cecropin with target proteins		Interacting amino acids in target protein	Amino acids involved in H-bonding	
	Moricin	Cecropin		Moricin	Cecropin
Glycerol phosphate lipoteichoic acid synthase	-406.69 kcal/mol	-576.67 kcal/mol	Lys 597, Gln 231, Glu 522 and Leu 322, Tyr 417, Asn 482	Asn 33, Lys 36, Lys 38	Arg 16, Glu 9, Gln 31
Dipeptide ABC transporters	-538.54 kcal/mol	-634.57 kcal/mol	Gly 172, Asn 144, Gln 193 and Val 210	Arg 20, Asn 23, Asp 30	Lys 6
DNA gyrase	-702.13 kcal/mol	-639.39 kcal/mol	Lys 581, Arg 1048, Glu 1156, Gln 1368 and Ala 509, Met 1029, Gly 1178, Thr 1181	Val 15, Arg 20, Asn 23, Ala 42	Glu 9, Arg 16, Ala 33, Lys 37

Table 1: HEX docking energy values (kcal/mol) for Moricin and Cecropin peptides with target proteins in *Staphylococcus aureus* and amino acids involved in H-bonding between target protein and ligands.

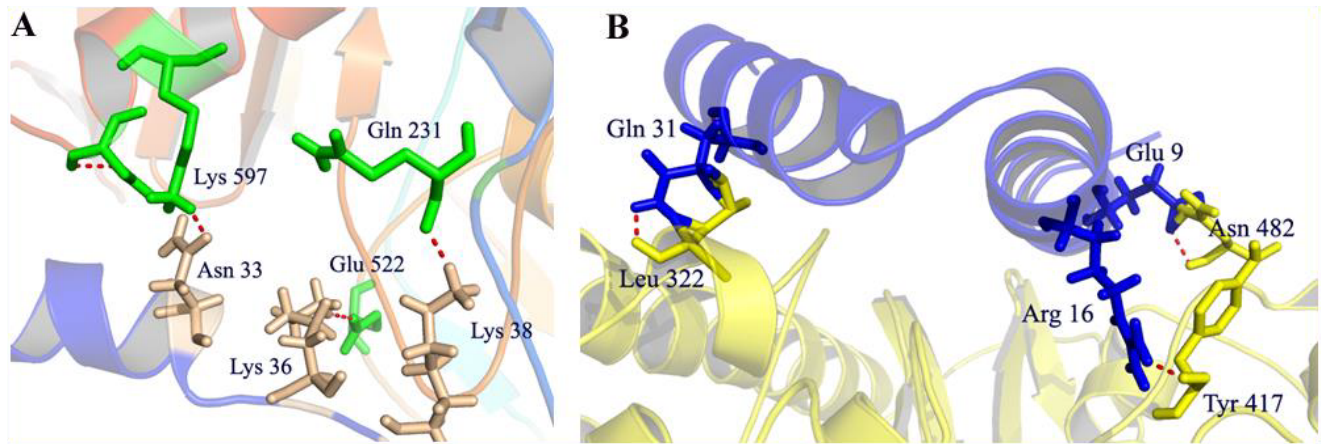

Figure 3: Docking mode of glycerol phosphate lipoteichoic acid synthase with (A) Moricin and (B) Cecropin. The interacting amino acids are represented in sticks and the protein is displayed in cartoon conformation. Moricin and Cecropin cartoons represented in blue colour.

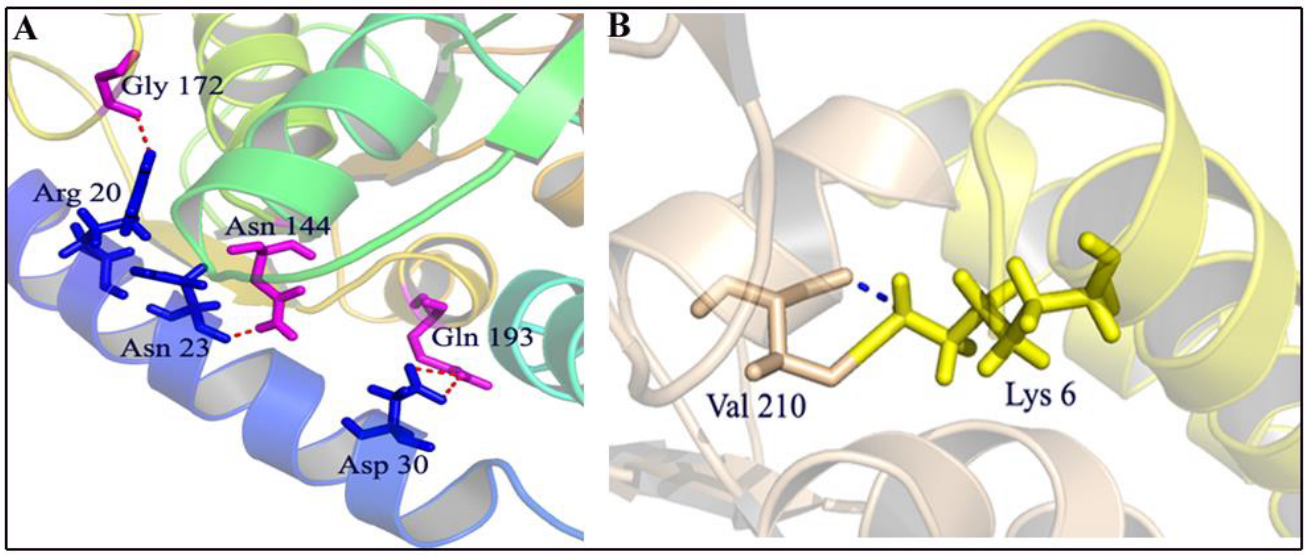

Figure 4: Docking conformation of dipeptide ABC transporter with (A) Moricin and (B) Cecropin. The interacting amino acids are represented in sticks and the protein is displayed in cartoon conformation. Moricin and Cecropin cartoons represented in blue colour and yellow color respectively.

The docking conformation of Moricin and Cecropin with *S. aureus* target proteins such as Glycerol phosphate lipoteichoic acid synthase, Dipeptide ABC transporter-PG110 and DNA gyrase are displayed in Figures 3-5 respectively.

Conclusion

The immune response induced against *Staphylococcus aureus* in response to infection is the synthesis of antimicrobial peptides in

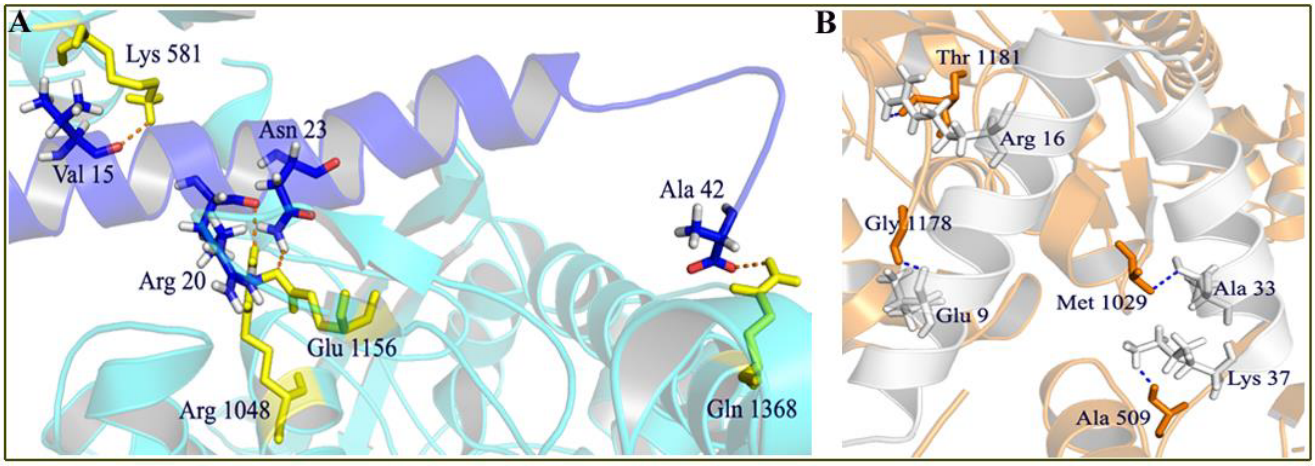

Figure 5: Docking conformation of DNA gyrase with (A) Moricin and (B) Cecropin. The interacting amino acids are represented in sticks and the protein is displayed in cartoon conformation. Moricin and Cecropin cartoons represented in blue and grey white color respectively.

silkworm haemolymph. The isolated haemolymph from infected silkworm larvae consists of peptides which exhibits antimicrobial activity on *S. aureus*. In the present hypothesis Moricin and Cecropin bind to DNA gyrase enzyme with high binding energy and inhibits DNA gyrase activity when compared to other drug target proteins. It is essential that research should continue to isolate and purify the antimicrobial peptides form insects haemolymph to control the opportunistic infections caused by *S. aureus*.

Acknowledgement

The authors express their sincere thanks to DST INSPIRE Fellowship, New Delhi for financial support to carry out this research work.

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