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Original Article

Fluorescence Emission and Molecular Docking Studies Identified Novobiocin as a Potent Inhibitor of the Japanese Encephalitis Virus (JEV) Envelope Protein

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ABSTRACT

Introduction: The Japanese Encephalitis Virus (JEV) causes the acute inflammatory disease of the central nervous system known as Japanese encephalitis. JEV is a RNA virus (+strand) that is tiny, enclosed, and from the family Flavivirus. The envelope protein (E), which facilitates JEV entrance into the host cell, has been chosen as a possible molecular aim for therapeutic progress in this work. **Materials and Methods:** The 3D structure of E protein was retrieved from RCSB PDB (id: 3P54). The sdf files of four lead molecules namely etoposide, netropsin, nogalamycin, and novobiocin were downloaded from the PubChem and used for molecular docking against JEV E protein. We then assessed the fluorescence emission intensity of JEV alone and JEV bound to novobiocin at 280–500 nM to confirm the inhibition of JEV by Novobiocin. The secondary structure of JEV was ascertained by measuring its CD spectra. The CD spectra of JEV were examined as a function of temperature to evaluate the protein constancy. **Results:** Our docking and fluorescence emission spectra results showed that JEV E pro has good binding preference for novobiocin among these four test compounds. Moreover, fluorescence emission spectra of JEV E protein with novobiocin also revealed the 5 μM concentration is an effective novobiocin concentration to inhibit the activity of target protein. One top ranked lead molecule namely novobiocin with strong binding affinity (-8.574 Kcal/mol) to JEV E protein was known based on binding energy. Results of the CD Spectra made it quite evident that there is no random coil. Peaks in the negative range (troughs) at 210–230 nm in the CD spectra indicate that JEV has a secondary structure rich in helices. The data unambiguously show that there is no appreciable variation in the JEV curves. **Conclusion:** Conclusion of the current work offer a thorough understanding of how the JEV E protein is inhibited and provide information that will help novobiocin be developed as a therapeutic drug against JEV E protein in certain viral diseases.

Keywords: Envelope protein, Fluorescence emission spectra, Japanese encephalitis virus, Molecular docking, Novobiocin, Therapeutics.

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INTRODUCTION

The most common cause of viral encephalitis globally is the Japanese Encephalitis Virus (JEV), which causes 30000-50000 cases and 10000 fatalities each year in Asia (eastern region).[1] The virus, which is arthropod-borne and naturally circulates amid pigs and wild birds or mosquitoes, can also spread to humans and horses.[1] JEV vaccines are in a variety of forms, but due to cost, licensing challenges, and safety concerns, they are not widely available in Asia.[2-5] JEV, along with the WNV (West Nile virus), TBEV (Tick-borne encephalitis virus) and dengue virus, are all members of the Flavivirus genus.

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RNA viruses (+stranded) called flaviviruses have 9-12 kb genomes that are translated into a single polyprotein before being split up by host and viral proteases into seven nonstructural proteins and the structural proteins Capsid (C), Premembrane (prM), and Envelope (E). When a capsid binds to viral RNA, it creates a nucleocapsid that is encased in a membrane produced from the endoplasmic reticulum that contains the proteins E and prM. E proteins have a hydrophobic coil that facilitates the fusing of the viral and host membranes and are in charge of cellular adhesion.[6-11]

The JEV virion goes through a maturation phase during its life cycle to prevent the fusion peptide as of being inserted mad about the host cell membrane too early. Prior to viral secretion, E in an immature virion forms erratic trimers with fusion loops that are capped by prM. At this point, E is cleaved in the trans-Golgi network.[12-14] The loop is then buried at the interface of an icosahedral network of flat antiparallel homodimers formed by

E.[15,16] Once inside the endosome, where the acidic environment causes an irreparable shift from dimer to trimeric spikes, mature virions bind to cells and are absorbed.[17-20] This results in the release of the nucleocapsid into the cell, which reveals the fusion loops that enter the endosome and connect the host and viral membranes.

The mainstream of flavivirus-neutralizing antibodies bind E and can prevent attachment and fusion as well as other entrance process steps.[21-26] Widely cross-reactive antibodies are produced following a flavivirus infection; however the polysera from a particular infection will first kill a portion of other viruses. This occurrence serves as the foundation for the serocomplex classification arrangement, which divides flaviviruses into groups based on tests for cross-neutralization using polysera from heterologous infections.[27] Within a certain serocomplex, clinical symptoms of infection can include hemorrhagic fever and febrile sickness. The St. Louis Encephalitis Virus (SLEV), the West Nile Virus (WNV), and the archetypal member JEV all belong to the JEV serocomplex and are known to cause acute or deadly encephalitis as well as flu-like symptoms.[27,28] The most famous serocomplexes are those represented by TBEV, yellow fever virus, and DV, which also exhibit particular tropisms and pathogeneses.

Because the virus is enzootic, effective antiviral therapy is critical for JEV. This feature of the virus allows it to survive in the environment and persist to the point where it is never completely eliminated.[29] Modern technology has led to an increase in research towards the creation of antiviral drugs for JE over the past few decades. The main goal of JEV antiviral research is to quickly and affordably make a therapeutic treatment available to JEV risk groups.[29,30]

In attendance is currently no explicit treatment available for JEV strains. Although there are a few vaccines, they cannot treat all JEV strains, thus further research is necessary before they can be properly evaluated and used to treat JEV infections. The structural data that are now available provide a new method for locating antiviral medicines that are effective against JEV infection's initial stages. A method for therapeutic intervention is to stop encapsulated viruses at the point of viral entrance. The future offers optimism for a development in JEV drug discovery thanks to cutting edge computational approaches and biomedical science. The recent resurgence of encephalitis illnesses has alarmed the scientific community worldwide because it has now become a major issue. The majority of JEV outbreaks take place in tropical nations like India, Pakistan, Indonesia, and Brazil, mid others, where sizable populations are living in poverty.

Although the thing is astonishing that so rare effective antivirals have been created contrary to the JEV, the difficulties still lie forward. New structural insights into the JEV life cycle and viral interactions with cellular molecules and antibodies greatly aid in the discovery of novel inhibitor classes. Strong structure-based approaches may quickly concentrate the expansion of extremely effective compounds given the computational approach's capacity to attain high-resolution structures of viral components and inhibitory compounds.[31-36]

The goal of the current investigation was to ascertain how the JEV protein interacts with the test substances namely etoposide, netropsin, nogalamycin, and novobiocin. By using molecular docking and investigations of fluorescence emission spectra, binding affinity was identified. Our fluorescence emission spectra and docking results showed that JEV has a strong preference for novobiocin among these four test chemicals for binding. The current study's findings offered a thorough understanding of JEV inhibition and contributed knowledge toward the creation of novobiocin as a medicinal molecule against the JEV in specific disorders.

MATERIALS AND METHODS

Molecular Docking Investigation

Molecular docking learning work was performed to found interactions of test compound with the goal protein. The study was carried out on the structure (3D) (Figure 1) of target protein namely Japanese Encephalitis Virus Envelope Protein (JEV) retrieved from the provided link (as JEV_model_01.pdb; PDB ID: 3P54) using Maestro 10.1 program (Schrodinger Inc. USA). The target protein for the contemporary study was first arranged using protein preparation wizard tool. Water molecules and all additional disagreeable residues were detached after preprocessing. It was at that time imperiled to hydrogen bond optimization and energy minimization. Using 'Sitemap' tool of Maestro, most probable binding sites were identified and the best binding site identified for the target protein was selected for further processing. The binding site was defined as grid box consuming receptor grid generation tool in Glide.

Structure of the four test compounds namely etoposide, netropsin, nogalamycin, and novobiocin (Figure 2AD) were retrieved from Pubchem database as sdf files and their energy was abated using LigPrep module of Maestro. At pH 7.0 2.0, all possible ionization states were generated and minimized. Glide was used to dock the generated ligand molecules into the active binding site in standard/extra precision mode (SP/XP). Different compounds interactions (hydrogen bonds, pi-pi interactions, and hydrophobic contacts) were detected when test compounds were docked into the active region of the target protein, and these interactions may be thought to be the cause of the compounds' observed activity.

Fluorescence Emission Spectra based inhibition study of JEV by Novobiocin

In a JASCO 6300 spectrofluorometer, the fluorescence spectra of the target natural protein "JEV" and the JEV-novobiocin complex were measured. For both excitation and emission, a slit with a

width of 10 nm was employed. For the intrinsic tryptophan fluorescence assay, spectra of the human recombinant protein JEV (BioVision, Cat # P1112-50; source *E. coli*, purity > 90%) were obtained. Using fluorescence emission spectra, the binding of JEV to novobiocin (Sigma, CAS No.: 56677-21-5) was studied. Emission spectra were captured from 300 to 500 nm after samples were stimulated at 280 nm.

Circular dichroism (CD) spectra

To ascertain the structure (secondary) of the JEV protein, Circular Dichroism (CD) spectra were taken. Using bovine serum albumin as the reference, the concentration of protein was calculated using the Bradford method. 0.2 mg/mL of protein was the final concentration. 2 mm band width, 1 second of be an average of time, and four scans covering the wavelength (205-250 nm) range of at room temperature were the requirements for recording CD spectra. Using a quartz cuvette with a 0.1 cm path length, the CD spectra were captured using a Jasco J-715 spectropolarimeter.

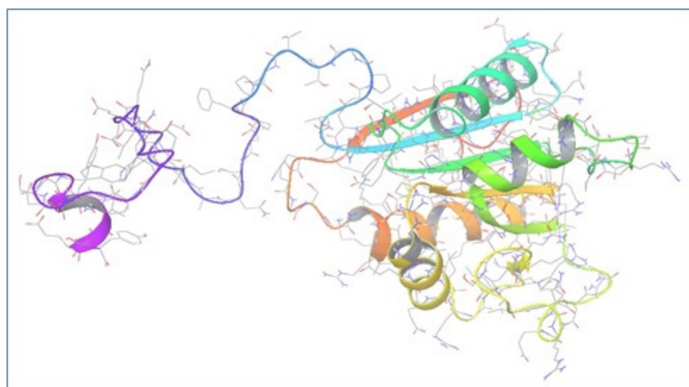


Figure 1: JEV Protein (Helicase ATP binding domain).

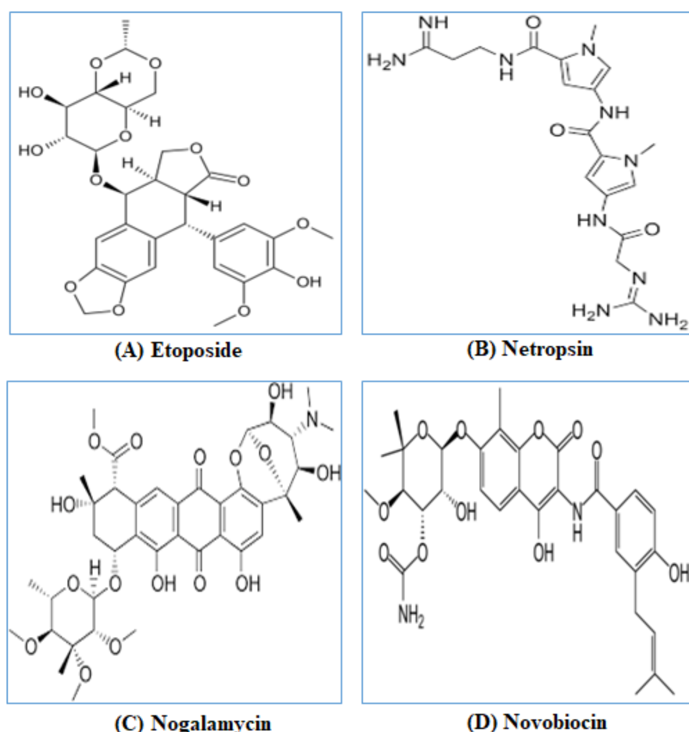


Figure 2: Structure of test compounds.

RESULTS

Study of Molecular Docking

To determine the test compounds' ability to link to the target protein, molecular docking was used. The docking scores of test compounds have been enlisted in Table 1 and presented in Figure 3A and 3B. Novobiocin (Pubchem Id 54675769) was found to show maximum binding capacity with JEV target protein (_model_01.pdb) and having docking score of-8.574 Kcal/mol compared to other test compounds (enlisted in Table 1). Novobiocin exhibited hydrogen bond interactions with target protein residues (Thr 151, Glu 64 and Gly 32) and other Van der Waal interactions with target protein residues (Leu 27, Pro 29, Val 61, Ala 150, Pro 158 and Pro 160). Docking scores of other test compounds with the target protein have been enlisted in Table 2.

Fluorescence Emission Spectra of novobiocin and JEV

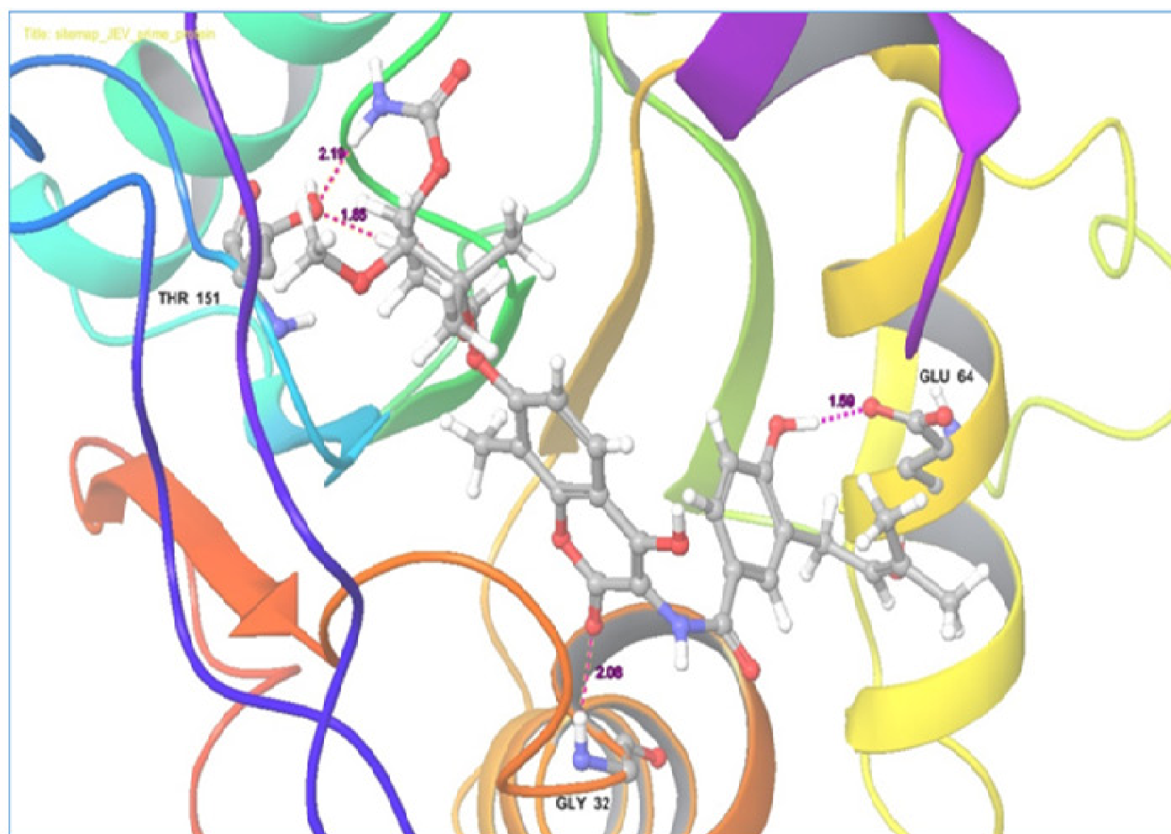
We then assessed the fluorescence emission intensity of JEV alone and JEV bound to novobiocin at 280-500 nM to comprehend the change in tertiary structure by evaluating the intrinsic fluorescence of aromatic amino acid (tryptophan) residues contained in JEV. When JEV was combined with novobiocin, its fluorescence emission intensity was noticeably lower than when JEV was used alone (Figure 4). These results support novobiocin's ability to bind to and inhibit JEV. Results also indicated that a concentration of 5 µM is sufficient to stifle JEV activity.

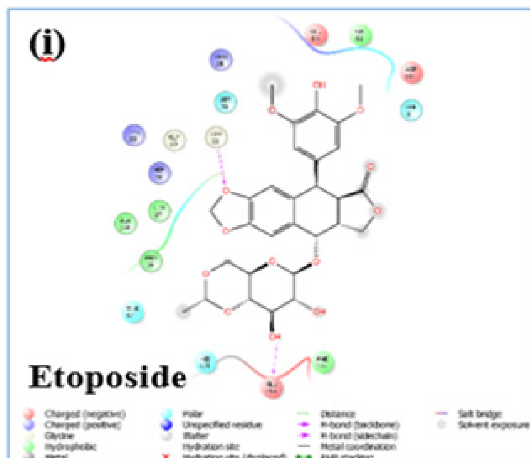
Table 1: Results of compounds with JEV-Protein.

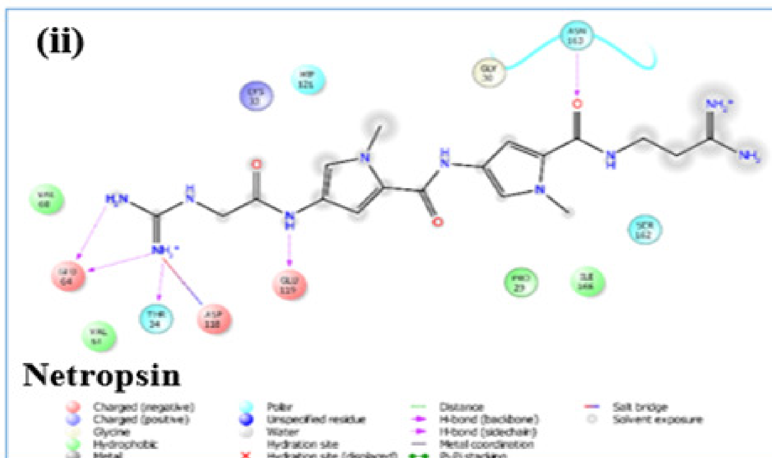
Title Compounds Ids	Compound Name	Docking score (KCal/mol)
54675769	Novobiocin	-8.574
4461	Netropsin	-3.452
5289019	Nogalamycin	-2.902
36462	Etoposide	-3.463

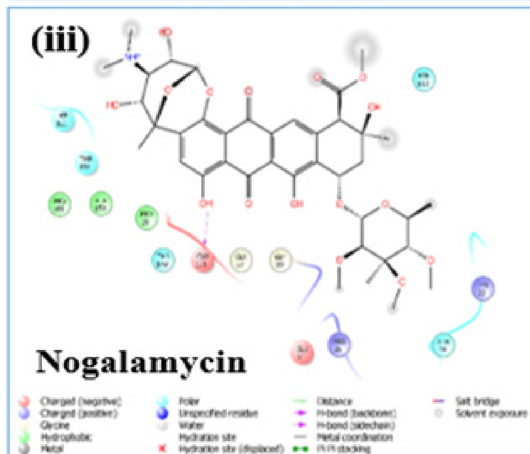
Table 2: Results of compounds with JEV Protein.

Compound Name	H-bonds forming residues	Other Interactions
Novobiocin	Gly 32; Glu 64; and Thr 151	Leu 27; Pro 29; Val 61; Ala 150; Pro 158 and Pro 160
Netropsin	Thr 34; Glu 64; Glu 119; and Asn 163	Pro 29; Val 60; Val 61; and Ile 166
Nogalamycin	Glu 119	Pro 29; Ala 150 and Pro 160
Etoposide	Gly 32 and Glu 119	Leu 27; Pro 29; Val 61; Phe 122; and Ala 150









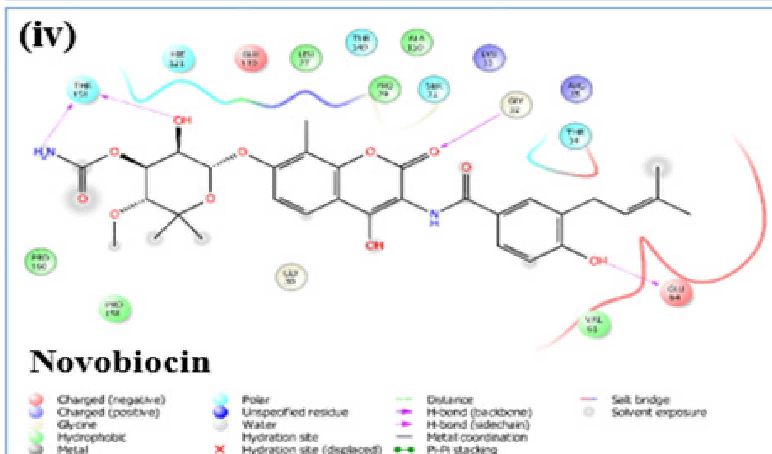


Figure 3: Docking of JEV Protein (Helicase ATP binding domain) with four test compounds.

Circular dichroism spectra

CD spectra are a great way to Figure out a protein's secondary structure. The shape and strength of the CD spectrum differ for the-helix, sheet and random coil configurations. This makes it possible to estimate the proportion of respectively structure (secondary) kind that exists in a protein by looking at its far-UV CD-spectra. The secondary structure of JEV was ascertained by measuring its CD spectra. Results made it quite evident that there is no random coil. Peaks in the negative range (troughs) at 210-230 nm in the CD spectra indicate that JEV has a secondary structure rich in-helix (Figure 5).

It is common knowledge that CD spectra can be used to calculate a transition change's slope or melting temperature (Tm). Protein stability is measured by the metric Tm. The CD spectra of JEV were examined as a role of temperature to evaluate the protein stability. The data unambiguously show that there is no appreciable variation in the JEV curves (Figure 6).

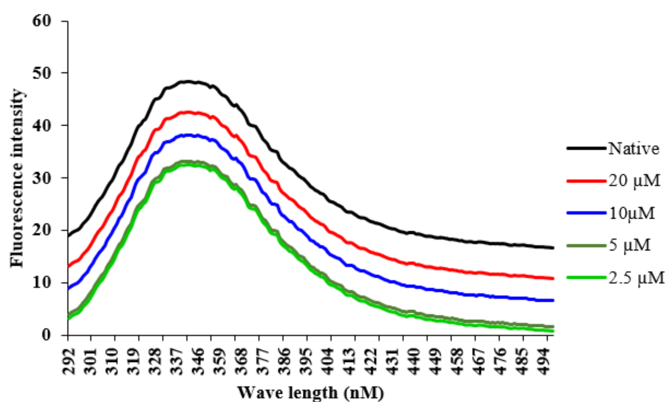


Figure 4: Fluorescence emission spectra of native JEV Protein and in presence of novobiocin.

DISCUSSION

Most current JEV infection treatment regimens put more emphasis on supportive care than they do on trying to make the virus weaker. A study looking at the use of ribavirin (a broad-spectrum antiviral) in controlled clinical trials against the virus found that it has a less significant impact in treating JEV. 37 Rosmarinic acid, a naturally occurring compound found in many Labiatae herbs and touted to have antiviral activity against Flaviviruses, was used in preclinical research. This drug reduced JEV (GP78 strain) viral replication in mouse brains. 38 Another chemical, curcumin, has been shown to have antiviral effects against JEV in an *in-vitro* study. This chemical reduced the amount of reactive oxygen species in the cell while maintaining the integrity of the cell membrane, preventing cell. Additionally, curcumin was found to decrease stress-related proteins and compounds that indicate apoptosis.[39] To test for JEV action, several authorized antivirals have been repurposed. These studies used high-throughput screening, computational techniques, and other ways to investigate specific pharmacological targets. Manidipine, cilnidipine, and benidipine hydrochloride are calcium inhibitors that prevent virus infection at any stage, including entrance, replication, and even budding.[40] Despite showing great promise and having high efficacy in *in vitro* or *in vivo* systems, these drugs moreover unsuccessful to exhibit the same efficacy in human studies or were deemed unsuitable for use in a clinical investigation. The only study to date effective against JEV was a placebo-controlled clinical probationary through interferonalalpha-2a in confirmed JE cases in children. Sadly, a survey conducted 3 months after its release demonstrated the ineffectiveness of interferon-alpha-2a non treating JE.[41] Standard treatment drugs go against the receptors or enzymes involved in vital viral processes. Focusing on host cell components like proteases as targets, however, is a further, alternate approach to halt the spread of the virus. Such an alternate strategy can simultaneously target several viruses and decreases the risk of antiviral drug resistance emerging. Despite this benefit, targeting

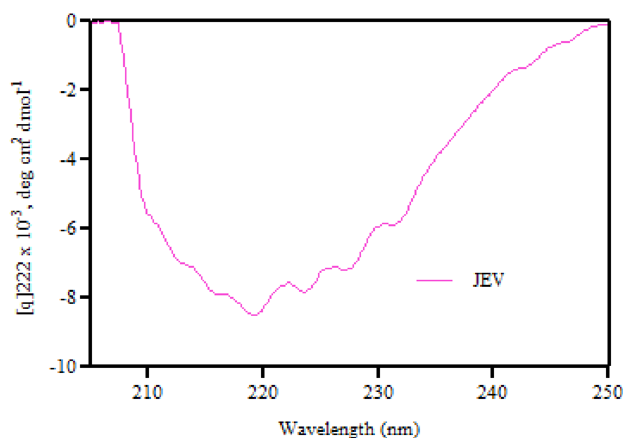


Figure 5: Circular dichroism (CD) spectra of JEV.

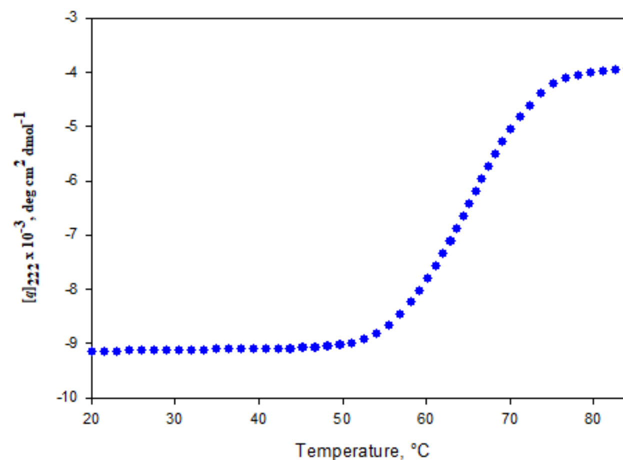


Figure 6: CD spectra of JEV at different temperature.

host cells still has significant downsides due to cytotoxicity and cellular side effects.[42,43]

High-resolution pictures of viral proteins have been made possible by recent developments in structural virology. They are the capsid protein,[44] E protein,[45] NS5,[46] NS3 C-terminal (NTPase/helicase catalytic domain),[47] and NS2B-NS3 (JEV protease).[48] Because of their crucial roles in how the infection develops, the majority of antiviral medication candidates have focused on the NS3, NS5, and E proteins among these proteins.[49] The best techniques for determining biologically active hits against molecular targets have been bioinformatics tools.[50] The use of these methodologies has broadened the research field and opened up new avenues for disease-related medication discovery. To assess the binding affinity of JEV with four test compounds namely etoposide, netropsin, nogalamycin, and novobiocin, we used molecular docking technique. It is a popular structure-based bioinformatics method for identifying medicines against target molecules.[51] Finding new medicinal compounds and foreseeing ligand-target interactions at the molecular level are made possible by molecular docking.[52-55] Novobiocin demonstrated the highest binding affinity to the target protein JEV among the four test substances. The molecule with the highest potency, novobiocin, has a docking score of-8.574 Kcal/mol. Along with other weak interactions, Novobiocin displayed four hydrogen bonds with the target protein's Thr 151, Glu 64, and Gly 32 residues (JEV model 01.pdb). In the target protein's receptor binding region, the test chemical Novobiocin showed favourable orientation and a variety of weak interactions that may be the basis for its activity. Van der Waals, covalent, and Pi-Pi interactions are thought to be the main driving force behind Novobiocin's and other test compounds' binding to the target protein, which results in its biological activity, according to a molecular docking study of the compounds.[56] Furthermore, the novobiocin-induced inhibition of JEV activity was validated by the *in-vitro* fluorescence emission spectra of native JEV and JEV in the presence of novobiocin. Additionally, we discovered that a 5 M concentration of novobiocin is sufficient to block JEV activity. Together, the results of docking and fluorescence emission spectra showed that JEV has a strong affinity for novobiocin and that its activity can be inhibited at a concentration of 5 M.

Understanding a protein's secondary structure can be done very well using CD spectra. For the configurations of helix, sheet, and random coils, the CD spectrum's strength and shape are different. As a result, it is now possible to determine from a protein's far-UV CD spectra what percentage of each secondary structure type it has. By analyzing its CD spectra, it was possible to determine JEV's secondary structure. The findings made it quite clear that there is no random coil. The CD spectra's negative peaks (troughs) in the 210-230 nm region show that JEV contains a secondary structure with several-helices. It is well known that the melting temperature or grade of a transition change can be determined from CD spectra (Tm). The metric Tm is used to assess protein stability. To evaluate the protein stability, the CD spectra of JEV were studied as a function of temperature. The findings conclusively demonstrate that the JEV curves do not exhibit any discernible change.

CONCLUSION

The results of the present investigation, in conclusion, provided a full understanding of how JEV is suppressed and offered knowledge to the development of novobiocin as a therapeutic molecule against the JEV in several viral diseases. Our analysis of the fluorescence emission spectra and docking data revealed that, among the four test compounds, JEV strongly prefers novobiocin for binding. The current study's findings provided a full understanding of JEV inhibition and provided knowledge for the development of novobiocin as a therapeutic molecule against the JEV in particular diseases.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

JEV: Japanese encephalitis; **C:** Capsid; **prM:** Premembrane; **E:** Envelope; **CD:** Circular dichroism; **Tm:** Melting temperature. **WNV:** West Nile virus, **TBEV:** Tick-borne encephalitis virus.

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