Viral Inhibitory potential of Hyoscyamine in Japanese Encephalitis virus infected Embryonated Chick involving multiple signaling pathways

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Abstract

Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis worldwide. Emergence of new genotypes of the virus and increased rate of mutation demands for alternatives that can fight against this deadly pathogen. Although the anti-viral property of *Atropa belladonna* and its active components such as Atropine, Scopolamine has been studied, yet the role of its another important component hyoscyamine against JEV has not yet been established till date. Thus our current study is focussed to investigate the protective effects of hyoscyamine against JEV and its immunomodulatory function in embryonated chick. Pre-treatment with hyoscyamine sulphate indicated significant decrease in viral load in both CAM and brain tissues after 48 hour and 96 hour (post infection). Insilico studies showed stable binding and interaction between hyoscyamine and NS5 (non structural protein 5) making it an effective compound against the virus. Embryonated eggs pre-treated with hyoscyamine sulphate showed upregulation of TLR3, TLR7, TLR8, IL-4, IL-10 as well as interferons and the regulatory factors. Hyoscyamine sulphate caused significant downregulation of TLR4 as compared to other experimental groups as analysed by one-way ANOVA. Hence our study demonstrated the protective function of hyoscyamine in controlling JEV viral replication and its dissemination to brain that may emerge as a promising therapy against JEV in future.

Introduction

Japanese Encephalitis Virus (JEV), a single stranded RNA virus is considered as the major cause of the viral encephalitis resulting in 70000 cases annually among which nearly 25% cases are considered to be fatal [1]. The virus is a member of the Flaviviridae family and is mainly transmitted by *Culex triaeniorhynchus* [2]. The progression, pathogenicity and outcome of the disease is predominantly regulated by the innate immune response of the host [3]. The virus is a neurotrophic virus that enters and binds to the host cell receptors that leads to severe neurological complications and psychological sequelae [4]. Overproduction of different pro-inflammatory mediators may make the situation much more complicated although the generation of anti-viral response mediated by the same is a key factor to control and prevent the disease. In recent times, there is no specific, effective treatment against JEV. Although some vaccines are available against this pathogenic virus yet excessive rate of mutation and emergence of new genotypes of the same has limited their efficacy thus posing serious threat to the animal kingdom [5]. Previously many scientific studies claimed that a strong immune response is highly crucial and also a requisite factor in the control of viral replication, dissemination and consequent pathogenesis [3]. The type I interferons (IFNα/β) are extremely important in mounting a strong innate immunity against different flaviviral infections including JEV [6]. JEV is recognised by numerous type I pattern recognition receptors (PRRs) that includes toll like receptors (TLR), RIG-I (retinoic acid-inducible gene-1) and MDA5 (melanoma differentiation associated gene 5) [7]. During the course of infection, toll like receptors are generally induced by pathogen-associated molecular patterns (PAMPs) that initiates a signaling pathway involving a number of proteins [8]. Studies have reported that the viral genome is recognised by TLR3, TLR7 and TLR8 while the viral glycoprotein mainly attaches to TLR4 [9, 10, 11]. In a

study it has been found that TLR4 deficient mutants has increased resistance against JEV in mice [12]. A knockdown analysis of TLR3 and RIG-I in mice has resulted in inhibition of ERK (extracellular signal regulated kinase), p38MAPK (mitogen activated protein kinase) and AP-1 (activator protein-1) that highlighted their possible role in JEV induced inflammatory response of microglial cells [13]. TLR7 helps in survivality of mice through induction of innate and pro-inflammatory responses [14]. However, TLR7 deficient mice exhibits compensatory regulatory mechanisms through increased expression of TLR8 [11].

As mentioned above, the limited efficacy of drugs and affordable vaccines have led to increased demand for novel therapeutics to control this deadly encephalitis globally. With the help of different cutting edge technologies of bioinformatics approach and molecular biological techniques in hand, the future is waiting for a remarkable breakthrough in JEV drug discovery. Over the past times, the natural compounds have gained enormous importance and also proved to be effective against different microorganisms [15]. The antiviral property of *Atropa belladonna*, a perennial herb of the *Solanaceae* family has already been studied [16]. Not only the plant, but also its active components such as Atropine, Scopolamine has been found to exhibit potential antiviral activity against JEV [17]. However the antiviral effect of another important component of *Atropa belladonna* known as Hyoscyamine has not yet been investigated and thus still remains obscure till date.

Though there was huge increase in zoonotic transmission of viruses, well established animal models for studying infections and pathogenicity are lacking. Various studies have pointed out chick embryonated egg as one of the cheapest model in studying different viral infections including JEV. The chorioallantoic membrane (CAM), an extra embryonic membrane present in chick is accompanied with a highly vascularized system that functions in mimicking the blood brain barrier (BBB) [18]. Although the skin fibroblast grafts could permeate the BBB, however the associated vessels of the purified astrocytes could not do so that clearly indicates that astrocytes plays an important role in the induction of BBB features in the non-neural CAM vessels[19]. Studies have figured out that CAM vessels when exposed to astrocytes containing medium leads to increased expression of the neurothelin, an important marker of the endothelial cells although the same approach does not hold when endothelial cell or glioma-conditioned medium was used [20]. This leads to a conclusive fact that astrocytes secreted certain factors is highly crucial in inducing the BBB characteristics of the endothelial cells. JEV has been found to disrupt the BBB when it enters the centrral nervous system and propagating in the neurons. Although it is highly controversial whether astrocytes forms BBB, still CAM has emerged as a promising model for studying cancer progression, viral pathogenicity and BBB owing to its extensive vascularization with dense capillary network [21]. The presence of these features attributes to consider CAM as a suitable model for studying host-pathogen interactions. In our study, Vero cells have been used for JEV propagation and viral load determination as the same has been reported in various studies for contact inhibition [22, 23]. Considering all the above facts, our present study is focussed to investigate the antiviral effect of hyoscyamine and its role in regulation of different signalling pathways that is involved in disease pathology and also in clinical outcomes.

Materials And Methods

Embryonated Chick Eggs

The embryonated eggs (one day old) have been procured form a poultry farm situated at Arambagh in the district of South 24 Parganas. Embryonated chick eggs from Black Australorp (*Gallus gallus*) has been used in our study. After proper disinfection of the shells using 70% ethanol, the eggs were kept in an incubator by maintaining physiological conditions like 37°C temperature and 60% relative humidity (RH). The eggs were maintained in the laboratory using the candling procedure from the third day onwards and were rotated 3–4 times per day to observe certain changes such as vascularization, viability and also germ development until the 11th day as reported in other studies [24].

Virus Strain

The prototype strain of JEV (SH1 strain; GenBank ID: MH753128.1) was used for our study. The virus was obtained in a lyophilized form from National Institute of Virology (NIV), Pune, India and was cultured in Biosafety Level 2 (BSL2) facility as prescribed by the Department of Biotechnology, Government of India. There is no requirement of explicit ethical approval for the same. The lyophilized form was resuspended in BAPS [phosphate-buffered saline (PBS, pH 7.4) (Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 20% bovine serum albumin (BSA), Thermo Fischer Scientific, Waltham, MA, USA)] and the same was used as a stock which was inoculated into the Vero cell line ((ATCC® CCL-81™) to increase the viral copy number. Standardization of the final copy number of the viral inoculum was done by determining the LD_{50} dose as per standardized protocol described elsewhere [25]. The viral inoculum was prepared by resuspending the isolated virus in BAPS and was subsequently filtered using a 0.22-µm syringe filter before using for treatments as illustrated in the “Experimental Design” section.

Hyoscyamine

Hyoscyamine sulphate was purchased from Sigma-Aldrich (Catalogue No. H1600000), St. Louis, MO, USA. Different concentrations of the same were tested for their ability to reduce the viral load in Vero cells (ATCC® CCL-81™) at 24 h post-infection. On the basis of standardization experiment, 1.48 mM of hyoscyamine sulphate was selected as the optimal dose for further experimental analysis.

Molecular Docking Analysis

The crystal structure of the non-structural protein of JEV (PDB ID: 4K6M) and the ligand (Hyoscyamine: Pubchem ID: 154417) was taken from RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) and PubChem servers respectively. The structure of the ligand and the protein were optimized using Chimera and Avogadro respectively. The PDB files of the protein and the ligand were converted into PDBQT [Protein Data Bank, Partial Charge (Q), and Atom type (T)] using Autodock MGL Tools [26]. A three-dimensional grid box has been generated to define binding pocket and the active site was chosen on the basis of the internal ligand position found in the crystal structure of the protein. The

ligand and the receptor were kept flexible and rigid respectively and molecular docking was performed using the Autodock command line tool on the basis of gradient optimization algorithm. The best fit docked complex was visualised using Pymol and the 2D interactions were analysed using Biovia Discovery Studio [27, 28].

MD Simulation

Interaction of the ligand and the receptor was further analysed by molecular dynamics (MD) simulation using Schrodinger Maestro version 11.9.011 by applying thermodynamic conditions such as pressure, temperature, density and volume. The OPLS2005 force field was used to prepare the protein-ligand complex [29]. Furthermore, the system was built using the pre-defined SPC water model applying orthorhombic periodic boundary conditions at a distance of 10 Å units. Importantly, prior to MD simulation, the charge of the complexes was electrically neutralized by balancing with 0.015M of K^+/Cl^- ions, and the energy minimization was done through heating and equilibrium processes. The system's ultimate production phase lasted 100 ns at 300 K and 1 Atm pressure, employing Nose-Hoover method [30, 31] with NPT ensemble. Based on the interactions and dynamical properties of the complexes, the optimal conformations were selected. To assess the structural changes, the trajectories of each protein-ligand complex were analysed using specific parameters like RMSD (root mean square deviation), RMSF (root mean square fluctuation), intermolecular interactions.

Treatment of embryonated eggs with JEV

The experiments were designed using different volumes of JEV that were inoculated into embryonated eggs and LD50 dose was determined according to the protocol of Reed and Muench [25]. The LD_{50} was found to be 30µl (4.6×10^7 copy number). The eggs were divided into four groups containing 4 eggs in each group for our experimental study. At first, the egg shell surfaces were sterilized with 70% ethanol and were then carefully punctured by applying moderate pressure. A depression was created on the lateral surface of the egg by applying slight suction that was applied over the hole at the air space [32]. The details of treatment in different experimental groups has been clearly summarised in Table 1. Following inoculation, the holes over the egg surface were sealed with molten wax and were kept horizontally in an incubator at a temperature of 37□C for 48 hours. The eggs were subjected to candling after every 12 hours to check their viability and the eggs that were dead within 24 hours post inoculation were sorted out and were discarded. After 48 hours, the eggs were sacrificed by chilling on ice and after proper disinfection, CAM, Brain and Amniotic fluid were harvested and collected.

Table 1
Different experimental groups along with the details of the treatment

Sl no.	Experimental groups	Treatment details
1.	Group I (Control)	40 µl of BAPS was used
2.	Group II (Infection group)	30 µl of viral inoculum along with 10 µl of BAPS was used.
3.	Group III (Pre-treatment group)	10 µl of hyoscyamine sulphate was used followed by 30 µl of viral inoculum.
4.	Group IV (Medicine control group)	10 µl of hyoscyamine sulphate along with 30 µl of BAPS.
**30 µl of viral stock with 4.6×10^7/ml copy number was used.		
**1.48mM of hyoscyamine sulphate stock was used in our study.		

Determination of viral load using by One-Step qRT-PCR

The viral load in CAM, Brain and Amniotic fluid among the experimental sets was determined using Japanese Encephalitis Virus Kit genesig® Standard Kit (Primedesign Ltd., UK) as per instructions of the manufacturer. 20 µl of the reaction mix was prepared which contains 14 µl of Oasig One Step 2X qRT-PCR Mastermix and 1 µl of primer/probe mix. 6 µl of Nuclease free water was used in case of negative control. The reaction was carried out using CFX96 Real time instrument (Bio-Rad, USA) where the cyclical conditions are as follows: Reverse transcription at 55°C for 10 mins, Enzyme inactivation at 95°C for 2 mins, Denaturation at 95°C for 10 second and data collection at 60°C for 60 second. The viral copy number was determined on the basis of linear regression analysis of the standard curve from the positive control RNA and the efficiency of the PCR reaction was determined.

RNA Isolation

Total RNA was isolated from Brain, CAM and Amniotic fluid from all the experimental sets using Trizol reagent (Thermo fisher, USA) as per standardized protocol as described elsewhere [33]. In our process, one hundred milligrams per one hundred microliter of the sample was used for extraction of RNA using Trizol. DNase I (Thermo Scientific, USA) was used to avoid any genomic DNA contamination in the reaction. The RNA pellet was finally eluted in 40 µl DEPC water. The purity was checked using a Nanodrop Spectrophotometer (Eppendorf, Germany) and those with a A_{260}/A_{280} ratio ranging between 2.0 to 2.2 were used for further experimental analysis [34]. The RNA samples were stored at -80°C until further use.

Gene Expression Study

The expression levels of different genes such as TLR-3, TLR-4, TLR-7, TLR-8, IFNα, IFNβ, IRF-3 and IRF-7 were studied to assess the pathogenicity of viral infection and the role of Hyoscyamine in reducing the viral load in different experimental groups. RNA was isolated as described above and cDNA was

synthesized from 1µg of the total RNA using One Step cDNA Synthesis Kit (Takara, Japan) as per standardised protocol. The expression levels were checked through Semiquantative Real time PCR using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in CFX96 Real-Time instrument (Bio-Rad, USA) under amplification conditions as described elsewhere. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as a housekeeping gene for data normalization. $2^{-\Delta\Delta Ct}$ method [35] was used to estimate the relative fold change in gene expression levels. The primer sequences for the above mentioned genes for chick has been provided elsewhere [36–39].

Statistical Analysis

The experiments were performed in triplicates (n = 3), and the data are represented as + SD. To check and analyse the differences between different experimental groups One-way and two-way ANOVA (analysis of variance) and t-test were used using GraphPad Prism (Version 5) [40]. Pearson's correlation (Pearson's Coefficient) was used for correlation study and a $p < 0.001$ was considered as statistically significant in our study.

Results

Optimization of Hyoscyamine Sulphate dose

Different concentrations of the compound were tested in order to check and validate their efficacy in viral load reduction. The viral load has reduced to a certain extent with increase in concentration of hyoscyamine with the highest reduction at 1.48 mM. Higher concentrations indicated negligible effect on the viral load. Thus 1.48 mM was selected as the concentration in further experiments.

Molecular docking of Hyoscyamine against NS5 Protein of JEV

Molecular docking studies indicated that the ligand (hyoscyamine) binds with an affinity of -6.4kcal/mol to the active site of the receptor (NS5 protein of JEV) (Fig. 2a). As revealed from pour docking study, GLY 85 participated in H bond interactions, SER 56, GLY 58, TRP 87, CYS 82, GLY 86, GLY 81, GLY 83, HIS 110 and ASP 146 have Pi-Anion interactions with hyoscyamine (Fig. 2b) signifying the role of the same in regulating the protein activity.

MD Simulations

Molecular dynamics simulation was used to investigate the stability, intermolecular interaction and the confirmation between hyoscyamine and the non structural protein 5 (NS5) of JEV. To reveal the structural changes of the protein-ligand complex, the final production step was executed.

RMSD

MD simulation focuses on the behaviour of protein and ligand at a particular time. In our study, the whole simulation was subjected for 100ns where certain properties of the complexes were investigated as the

backbone RMSDs during the course of simulation. The X-axis of the RMSD plot indicates the simulation time (ns) whereas the Y-axis indicates RMSD fluctuation of the protein-ligand complex. It has been observed that the NS5-Hyoscyamine complex was initially stable upto 40 ns. After 40 ns, the complex fluctuated upto 3.5 Å. Again the complex was found to be in a stable conformation upto 70ns. Although the some fluctuations were observed upto 42 Å after 75 ns, the complex is found to be stable during the remaining course of simulation.

RMSF

To equate the flexibility of individual residue in the protein-ligand complex, the root-mean-square fluctuations (RMSF) were assessed and plotted. The RMSF of the NS5 protein fluctuated highly up to ~ 5.5Å at the N-terminal and up to ~ 2.5Å the C terminal). The RMSF of the protein–ligand complex denoted the minor fluctuation for our complex. There were some deviations at some time points during the simulation period of 100 ns.

Role of Hyoscyamine in viral load reduction

Hyoscyamine sulphate pre-treatment has resulted in reduction of viral load in both brain and CAM tissues (Fig. 5a and 5b). Viral load reduction has been observed in 24, 48 and 72 and 96 hour post infection, where the lowest load being observed in both CAM and brain after 72 hour and 96 hour. The result indicates that Hyoscyamine sulphate has remarkable antiviral property ($p < 0.0001$) as analysed by two-way ANOVA. However, no significant change in viral load has been observed in case of amniotic fluid in any of the time points (data not shown).

Regulation of TLR Gene Expression by Hyoscyamine in JEV infected CAM and Brain

It has been observed that JEV infection increases the expression of TLR-3 mRNA in CAM and brain. Pre-treatment with Hyoscyamine sulphate along with JEV caused upregulation of TLR-3 as compared to other groups. On the other hand, TLR-4 expression is significantly increased in virus infected group as compared to hyoscyamine sulphate pre-treated group. Also, the level of TLR-8 mRNA got significantly increased and the expression level of TLR-7 got reduced upon Hyoscyamine Sulphate pre-treatment.

Modulation of Type I Interferon's (IFNs) and Interferon Regulatory Factors (IRF) in CAM and Brain by Hyoscyamine pre-treated embryonated eggs

Our study highlighted the fact that viral infection has been found to downregulate the expression levels of Interferon's (IFNα and IFNβ) and Interferon Regulatory Factors (IRF3 and IRF7). Interestingly, the situation is exactly reversed when pre-treatment with Hyoscyamine Sulphate has been found to upregulate their expression levels ($p < 0.0001$). Similar changes have been observed in both CAM and brain tissues among different experimental groups as analysed statistically.

Alteration in the levels of Interleukins (IL-4 and IL-10) by Hyoscyamine sulphate

Virus infected CAM and brain tissues showed downregulation of IL-4 and IL-10. However, treatment with Hyoscyamine sulphate prior to inoculation of virus demonstrated upregulation of these two interleukins ($p < 0.0001$) as analysed by one-way ANOVA in comparison with the JEV infected group.

Discussion

The first line innate immune receptors in battling against different pathogenic microbial infections especially virus are the Toll-like receptors [41] as established by the fact that these receptors has been shown to exert innate immune responses during the course of infection by flavivirus [42]. Studies have revealed that interaction of the viral RNA with TLR3 (a crucial pattern recognition receptors i.e PRRs) induces a signalling cascade that activates signalling pathways like MAPK and ERK [43]. Our study have figured out that TLR3 expression was higher in Hyoscyamine sulphate pre-treated embryonated chick eggs while the virus infected group having higher viral load also showed less TLR3 expression (Fig. 6a and 6b). Increased TLR expression (Hyoscyamine sulphate treated embryonated chick) has been found to be suppressed when they were pre-treated with Hyoscyamine sulphate. Similar observations were observed when Scopolamine hydrobromide was used against JEV in the same infection model in our previous studies [17]. Also studies with other viruses such as novel Bunya virus that corresponded with our findings [44]. Thus it may be speculated that JEV would possibly lower the expression of TLR3 which is correlated with our findings. Alternatively, it can be said that Hyoscyamine sulphate has an antiviral property that is mediated through induction of TLR3 expression in embryonated chick although reports with other Flaviviruses showed conflicting results. Induction of TLR3 causes activation of innate and adaptive immune cells and recognises dsRNA ultimately leading to activation of adaptor protein TRIF (TIR-domain containing adaptor-inducing interferon-β) [45]. This complex mediates translocation of NF-κB (nuclear factor kappa light chain enhancer of activated B cells) and the interferon regulatory factors into the nucleus that leads to type I interferon's production [45]. The role of Hyoscyamine sulphate in modulating TLR4 expression was also investigated. Previous studies pointed the negative role of TLR4 that facilitated replication of JEV in mice [46]. However, our findings indicated significant upregulation of TLR4 gene expression in embryonated chick eggs infected with virus (Fig. 6c and 6d) reflecting the fact that death of the chicks mediated by the virus permits successful viral replication evading the antiviral response of the host. Hyoscyamine pre-treated chick embryo in both CAM and brain showed lowest viral load that depicts a strong correlation between viral load and expression of TLR4 (Fig. 6c and 6d). In influenza virus infections, various knockout studies with TLR4 gene in mice showed less viral load and proinflammatory responses [47, 48]. Mice without TLR4 gene has been found to have enhanced expression of type I interferon's as well as JEV specific IgG and IgM antibodies that are important host antiviral innate and adaptive immune factors [49]. Thus it may be said that modulation in TLR4 gene expression and immune signalling has resulted in low viral load in the hyoscyamine pre-treated embryonated eggs. TLR7 and TLR8 are highly crucial in activating a signalling cascade that ultimately leads to the generation of pro-inflammatory cytokines and type I interferon's through TRIF and MyD88 [11, 50, 51]. In our study, TLR7 is upregulated in both the tissues in virus infected group. On the other hand, eggs pre-treated with hyoscyamine showed much increased expression of the same as compared to virus

infected group (Fig. 6e and 6f). Interestingly, no significant difference in expression of TLR8 has been observed between medicine treated group (only hyoscyamine sulphate) and hyoscyamine sulphate pre-treated chick embryo (Fig. 6g and 6h). Although various studies have investigated that deficiency in TLR7 gene is not related to survivality in JEV infections, there has been increased viral load in CAM and brain tissues of mice that highlights the pivotal role of TLR7 in regulation of viral dissemination and propagation of JEV. In another study, elevated expression of TLR7 and TLR8 has been found in virus infected group that was found to be further enhanced in embryo pre-treated with Scopolamine hydrobromide that correlates with our findings to a great extent [17]. It may be anticipated that variation in TLR8 gene expression is due to use of different experimental models with alterations in pathogenesis and experimental conditions. Although JEV down regulates TLR gene expression as reported in other studies, hyoscyamine sulphate treatment leads to induction in TLR gene levels that mounts a strong immune response. Owing to suppression of TLR gene (low TLR levels) by JEV, it may be speculated that pre-treatment with hyoscyamine sulphate causes inactivation of the viral particles. However some viral particles may remain active and will continue to lower down expression of TLR gene while at the same time hyoscyamine sulphate will try to elevate it. This tug-of-war situation (dynamic interaction of Hyoscyamine sulphate and JEV) accounts for increased expression of TLR gene in the presence of hyoscyamine sulphate alone as compared with medicine pre-treated group.

Elevated levels of the type I interferon's (IFNα and IFNβ) was found in CAM and brain tissues infected with virus. Pre-treatment with Hyoscyamine sulphate followed by virus infection caused further upregulation in both the type I interferon levels (Fig. 7a-7d).

The non-structural protein 5 (NS5) is the largest flaviviral protein that is required for viral replication in the host [51] and it has also been investigated that this protein functions in blocking the antiviral responses mediated by the type I interferon's. Mutation studies with this protein confirmed prevention of viral replication [52]. To study the interaction of hyoscyamine and NS5 protein, in silico analysis has been performed. Molecular docking studies indicated that hyoscyamine binds to the active site of the non-structural protein 5 (NS5) with a moderate binding affinity that is stabilized by different types of interactions (Fig. 2a and 2b). However to analyse the stability of the complex, molecular dynamics simulation has been performed for 100 ns. MD simulation studies indicated stability of the complex though some minor fluctuations has been observed as indicated in the RMSD and RMSF plots (Figs. 3 and 4).

In a study by Chang et al.;(2013) it has been found that phosphorylation of the interferon regulatory factors (especially IRF3) was blocked by subgenomic flaviviral RNA that resulted in translocational inhibition of the same to the nucleus [53, 54]. Our studies indicated that there was downregulation of the IRF3 gene in the infection group (virus treated group) that was significantly enhanced in hyoscyamine sulphate pre-treated group. Similar findings have been observed for IRF7 also (Fig. 8a-8d).

Induction of antiviral interferon's by interleukins like IL-4 and IL-10 helps to fight against pathogenic viral infections [55, 56]. Various studies have indicated their protective function in case of JEV infections [57].

Scientists have investigated that during the progression of JE in mice, these interleukins has been found to be downregulated. Similar findings has been observed in our study where infection with the virus caused downregulation of these interleukin levels (Fig. 9a-9d). On the contrary, embryonated eggs pre-treated with hyoscyamine sulphate resulted in elevated expression of the same. Taken together, it can be said that elevation in IL-4 and IL-10 levels caused induction of TLR and type I interferons in hyoscyamine sulphate pre-treated embryonated eggs. Alternatively, these interleukins play a significant role in the attenuation of viral infection and the action of hyoscyamine sulphate against JEV is mediated by TLR, IFNs, and Interleukins. Our results established parallel findings in both CAM and brain tissues that establishes CAM as an ideal blood-brain-barrier (BBB) model. Although there was huge controversies regarding the same, hyoscyamine sulphate traversed across the barrier mediating its action in both the tissues that was used in our study.

Thus it can be concluded that the mechanism of action of hyoscyamine sulphate is mainly through the JAK-STAT pathway involving TLR, IFNs and interleukins that leads to generation of anti-inflammatory response and suppression of pro-inflammatory cytokines. Our study has provided clear insights regarding the prophylactic action and immunomodulatory role of the compound (hyoscyamine sulphate) against JEV in embryonated chick as a model system.

Conclusions

The lower viral load in hyoscyamine sulphate pre-treated CAM tissues of embryonated chick indicated significant upregulation of TLR3, TLR7 and TLR8 with a consequent downregulation of TLR4 that modulates the expression of the interferons. Similar results have been indicated in case of brain tissues of the same experimental model. Thus it is anticipated that the alteration of TLRs, interferon's as well as their regulatory factors and interleukins by hyoscyamine sulphate demonstrated the putative potential of this compound as a promising anti-JEV compound though further scientific investigations and validations needs to be performed in the coming days in order to investigate its comprehensive mechanism of action behind its application as a novel therapeutics against JEV.

Declarations

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Author Contributions

Study conception and design: AB, SR. Acquisition of data: AB, RN, MS, LC, SR, Analysis and interpretation of data: AB, RN. Drafting of manuscript: AB Critical revision: AB, MS, SR. Supervision and research resources supply: RN, MS, SR. All authors read and approved the final version of the manuscript.

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Figures

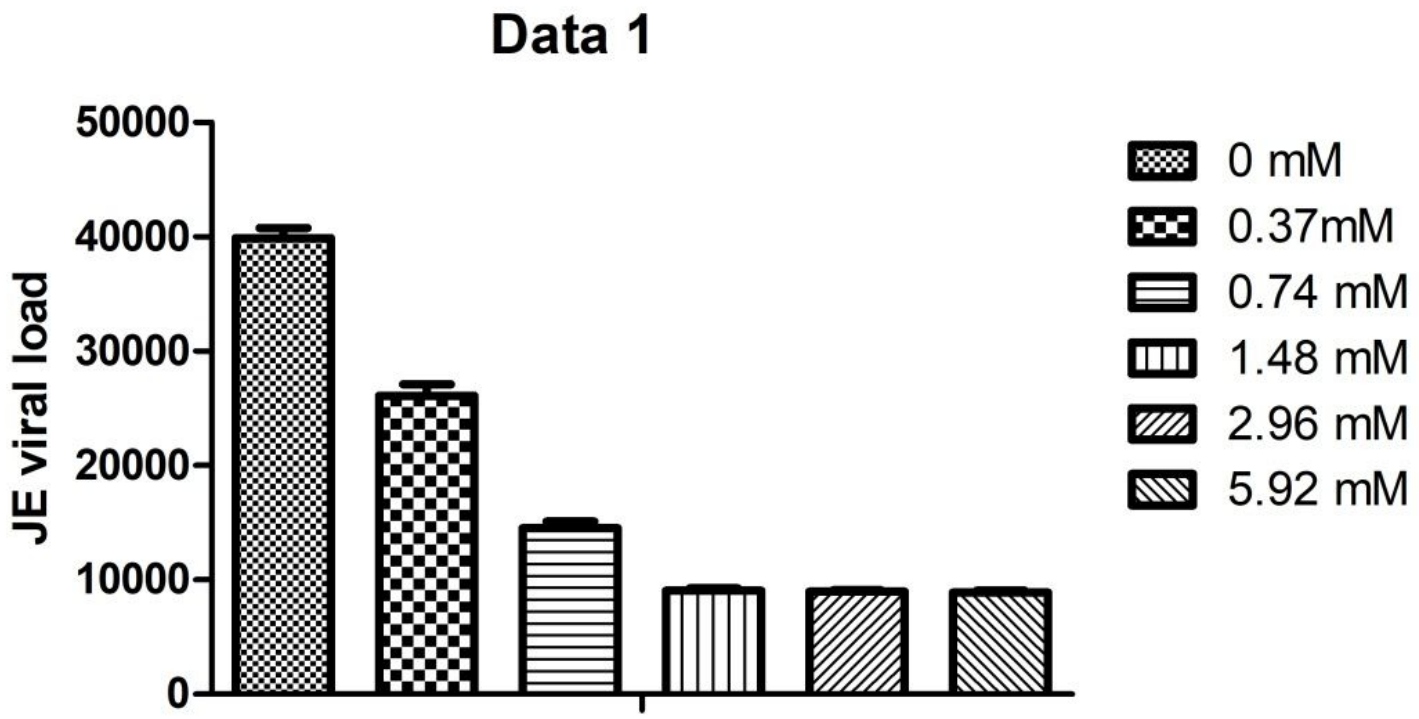

Figure 1

Standardization of hyoscyamine sulphate concentration. The viral load in Vero cells treated with different concentration of hyoscyamine sulphate was determined. Data in the bar diagram indicates ±SD.

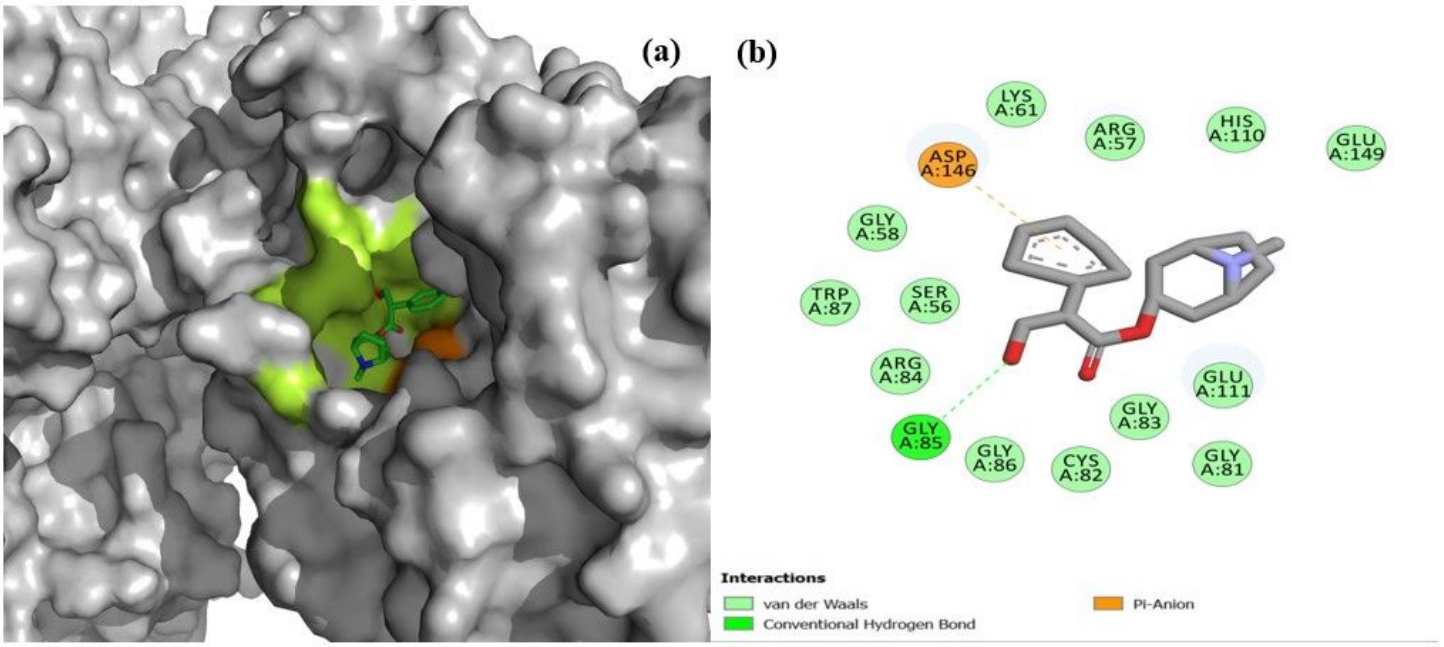

Figure 2

Representation of different types of interactions between the amino acids at the active site of the protein (NS5 of JEV) with hyoscyamine. Fig. 2(a) showing that hyoscyamine is bound at the active pocket of NS5 (non structural protein 5 showing in grey surface) along with multiple interactions in different colours. Conventional hydrogen bonding is showing in red, vanderwaals interaction is showing in lime green and pi-anion interaction is showing in orange. Fig 2(b) showing 2D various interactions between hyoscyamine and the amino acids present in active site. The ligand is shown in grey stick model and interacting residues are shown in multiple colours.

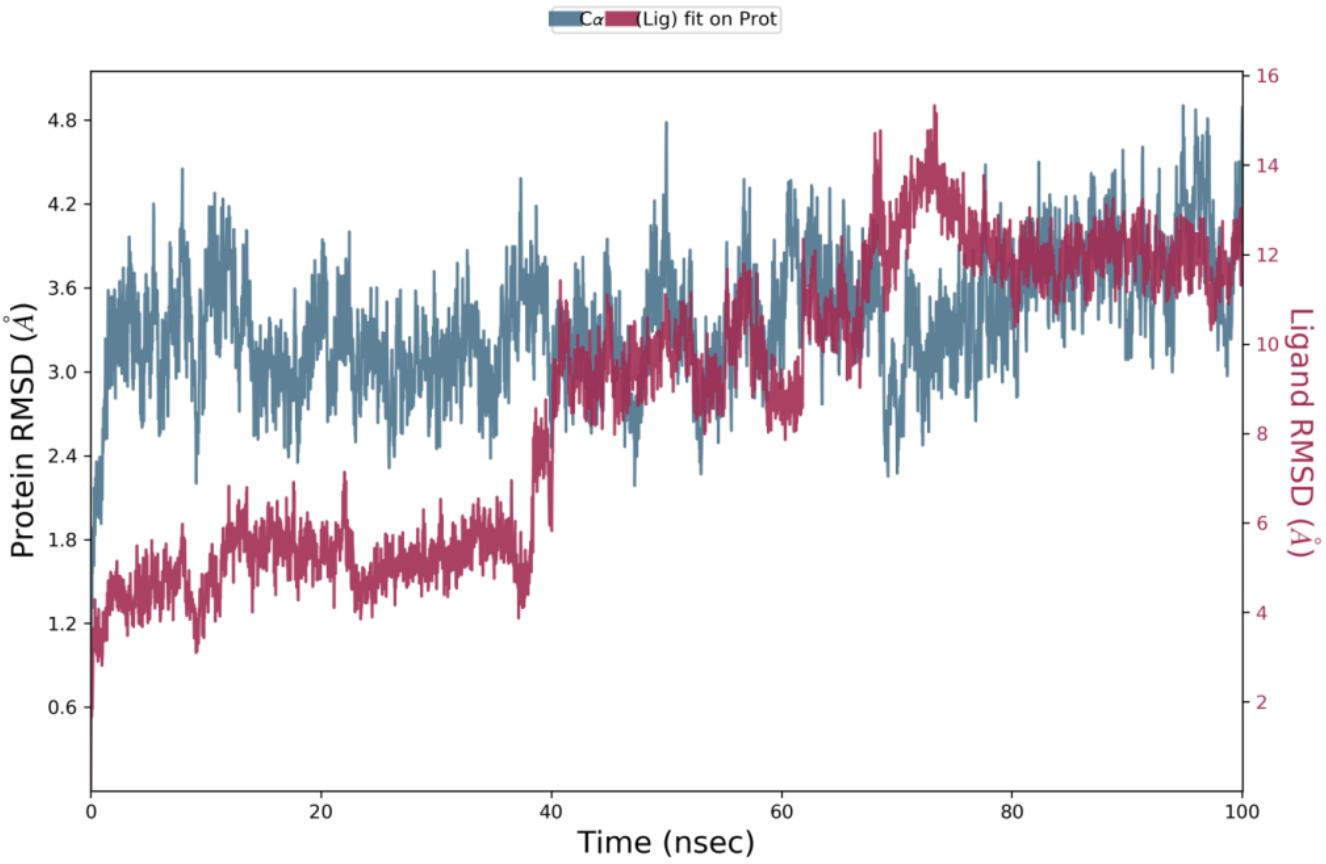

Figure 3

Root mean square deviation plot of hyoscyamine docked with non structural protein 5 (NS5) of JEV throughout the MD simulation. The RMSD was measured as the average distance between the backbone atoms of the protein–ligand structures.

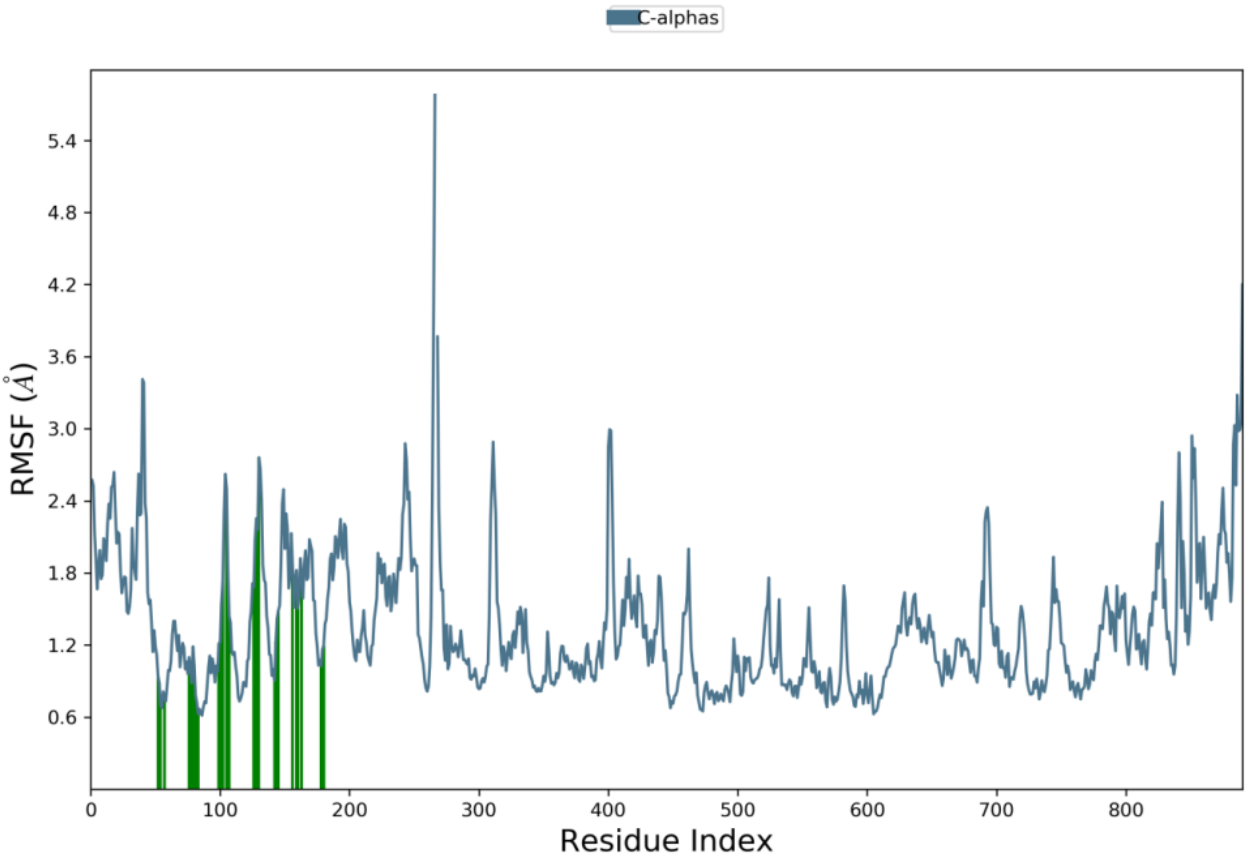

Figure 4

RMSF plot for Non Structural Protein 5 (NS5) of JEV. The green coloured vertical bars represent the protein residues that interact with the ligand.

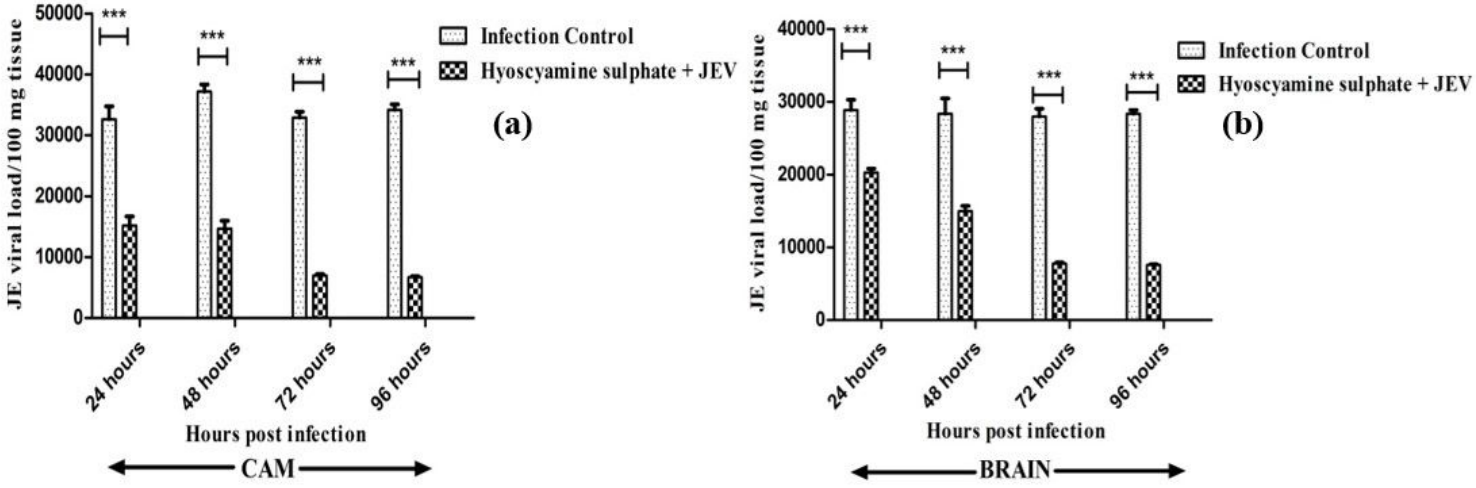

Figure 5

Graphical representation showing the change in viral load in CAM and brain (5a and 5b) across different time points as determined by one-step qRT-PCR in different groups used for our experiments. The embryonated chick eggs were inoculated with the viral inoculum (Infection control) and also pre-treated with hyoscyamine sulphate followed by infection with JEV. The data in the bar diagram represents ±SD. *** $p < 0.0001$ considered as statistical significant. BAPS indicates phosphate buffered saline containing 20% bovine serum albumin, JEV indicates Japanese encephalitis virus, CAM indicates chorioallantoic membrane and SD indicates standard deviation.

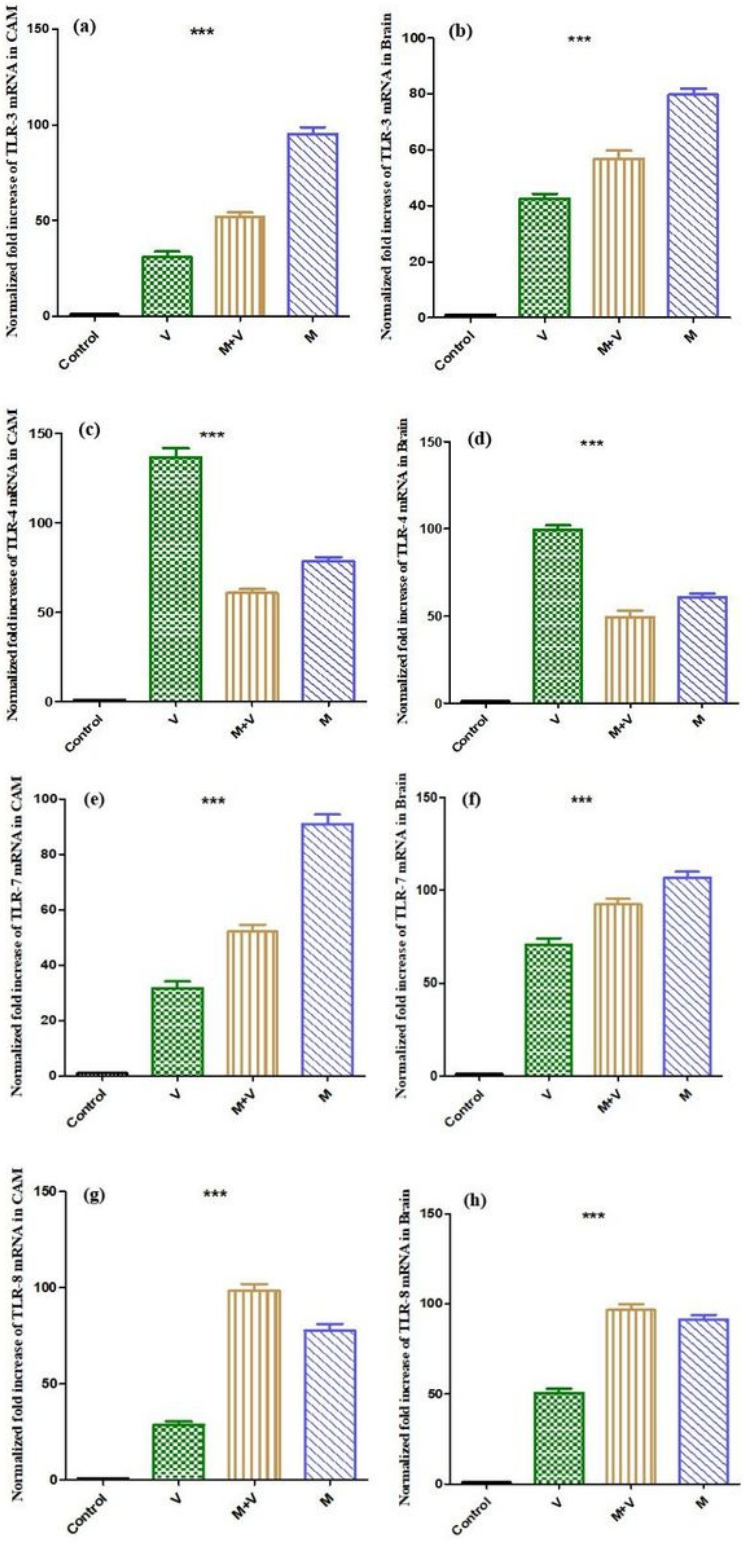

Figure 6

Graphical representation showing the change in gene expression levels of different toll like receptors (TLRs) in CAM and Brain (6a-6h). The bar diagram is showing the relative in the expression level of the same in different experimental groups. GAPDH mRNA was used for normalization. The bars are represented as ±SD. ***$p < 0.0001$ as analysed by one-way ANOVA. “V” denotes CAM and brain tissues infected with virus, “M+V” denotes CAM and brain tissues that has been pre-treated with hyoscyamine

sulphate followed by infection with the virus, “M” denotes pre-treatment only with hyoscyamine sulphate. IL stands for interleukins and SD stands for standard deviation.

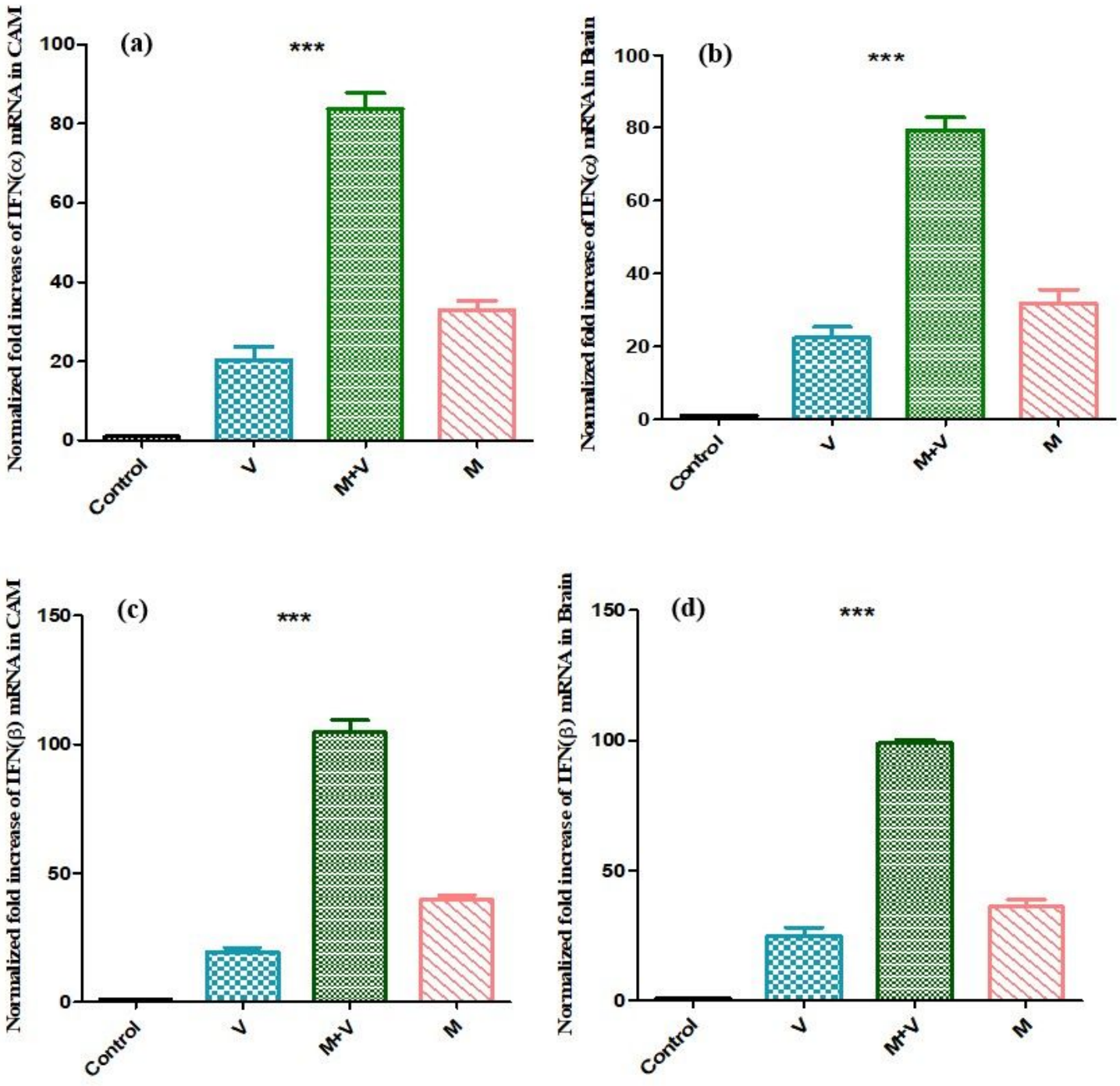

Figure 7

Graphical representation showing the change in gene expression levels of IFN (α) and IFN (β) in CAM and Brain (7a-7d). The bar diagram is showing the relative in the expression level of the same in different

experimental groups. GAPDH mRNA was used for normalization. The bars are represented as ±SD. ***p < 0.0001 as analysed by one-way ANOVA. “V” denotes CAM and brain tissues infected with virus, “M+V” denotes CAM and brain tissues that has been pre-treated with hyoscyamine sulphate followed by infection with the virus, “M” denotes pre-treatment only with hyoscyamine sulphate. IL stands for interleukins and SD stands for standard deviation

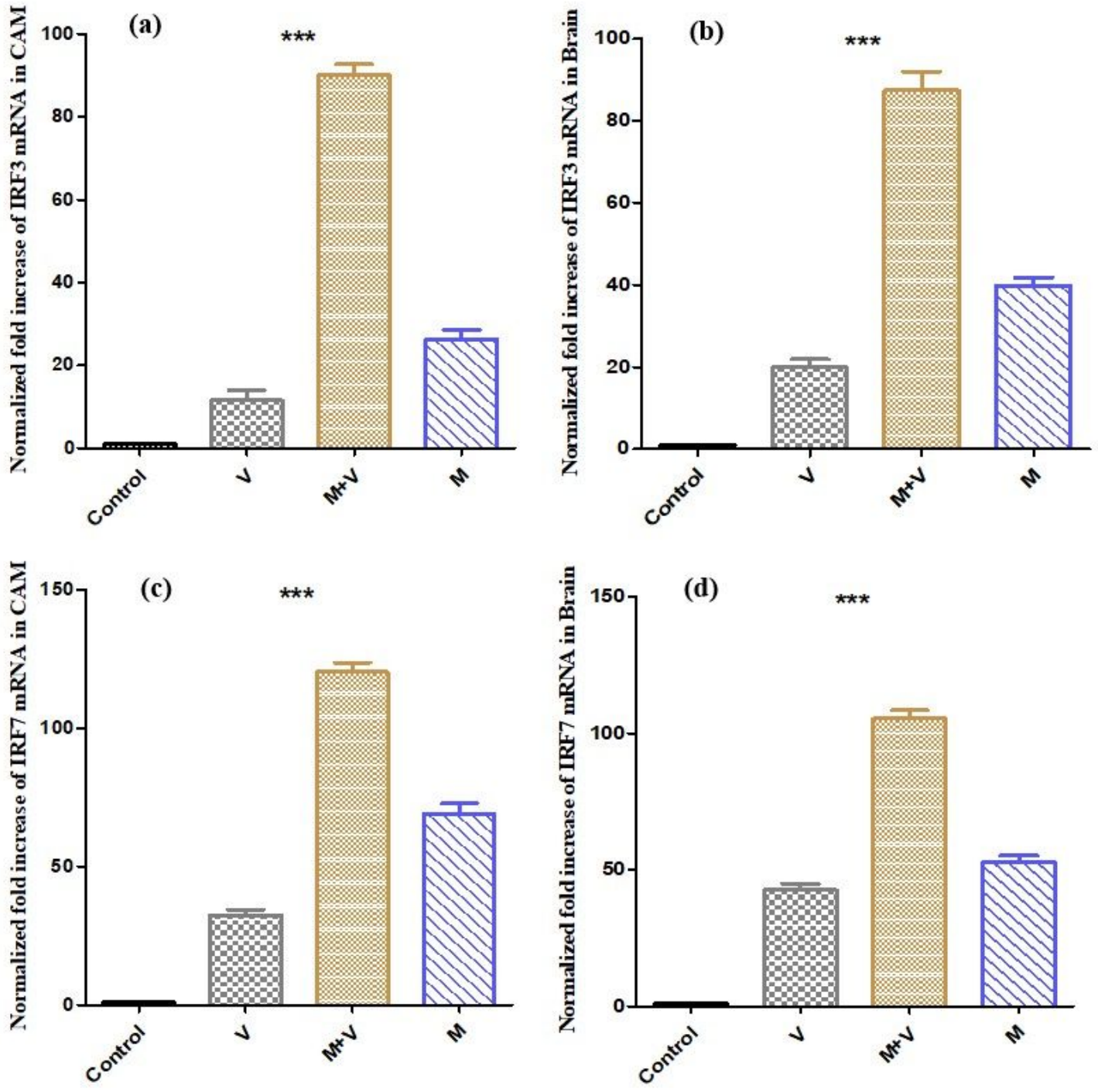

Figure 8

Graphical representation showing the change in gene expression levels of Interferon Regulatory Factors (IRFs) in CAM and Brain (8a-8d). The bar diagram is showing the relative in the expression level of the same in different experimental groups. GAPDH mRNA was used for normalization. The bars are represented as ±SD. ***$p < 0.0001$ as analysed by one-way ANOVA. “V” denotes CAM and brain tissues infected with virus, “M+V” denotes CAM and brain tissues that has been pre-treated with hyoscyamine sulphate followed by infection with the virus, “M” denotes pre-treatment only with hyoscyamine sulphate. IL stands for interleukins and SD stands for standard deviation.

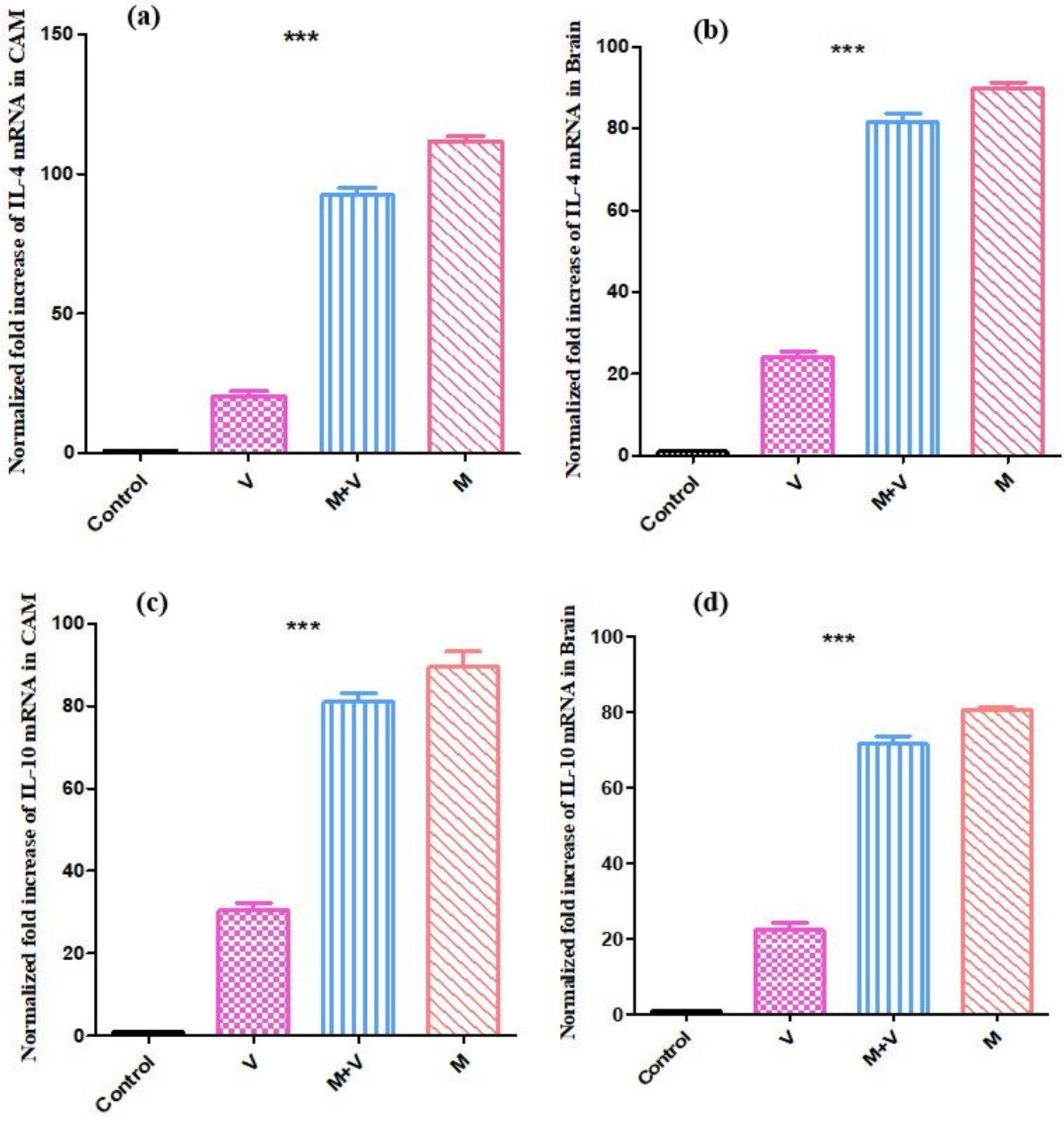

Figure 9

Graphical representation showing the change in gene expression levels of IL4 and IL-10 in CAM and Brain (9a-9d). The bar diagram is showing the relative in the expression levels of IL-10 in different experimental groups. GAPDH mRNA was used for normalization. The bars are represented as ±SD. ***$p < 0.0001$ as analysed by one-way ANOVA. “V” denotes CAM and brain tissues infected with virus, “M+V” denotes CAM and brain tissues that has been pre-treated with hyoscyamine sulphate followed by infection with the

virus, “M” denotes pre-treatment only with hyoscyamine sulphate. IL stands for interleukins and SD stands for standard deviation.