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C-PHYCOCYANIN OF *SPIRULINA PLANTESIS* INHIBITS NSP12 REQUIRED FOR REPLICATION OF SARS-COV-2: A NOVEL FINDING *IN-SILICO*

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ABSTRACT: SARS-CoV-2 or COVID-19 is one of the deadly pandemics faced by the world population, which has infected 7 million and claimed the lives of 0.4 million people. In spite of a few drugs available to control the pandemics, a formal vaccine is the least that the world expects under the current scenario. However, the release of a vaccine is expected to come at the cost of its own time. SARS-CoV-2 replicates in the host cells with the aids of the molecular machinery of a complex formed by three non-structural proteins (NSPs) *viz.*, nsp12, nsp8, and nsp7. Recent studies reveal that among the three NSPs, nsp12 is vital for viral replication and is the target for drugs. Several studies have linked the viral infection to a weaker immune system, which is quite likely to be targeted by the virus. In search of such a natural compound that might increase the immunity and block the viral replication within the host, we selected C-Phycocyanin of *Spirulina plantesis* to study its anti-viral property *in-silico*. Spirulina is a free-floating filamentous microalgae growing in alkaline water bodies. It is a well-known source of valuable food supplements, such as proteins, vitamins, amino acids, minerals, *etc*. In the present study, we focused on the possibility of C-Phycocyanin to inhibit the active site of nsp12, which is very much needed for viral replication. Auto Dock, Auto Grid, and Discovery Studios tools reveal that C-Phycocyanin inhibits the active site of nsp12 thereby interfering with the replication of the virus itself.

INTRODUCTION: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) or CoVID-19 as it is commonly called, is one of the recently known viruses which belong to the Coronaviridae family [1]. These viruses possess RNA as their genetic materials, which are known to cause severe diseases in birds as well as mammals.

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The viruses are constituted by enveloped positive single-stranded RNA genome with helically symmetrical capsids [2]. The term was derived from the Latin word *"Corona"* meaning crown [3]. The emergence of SARS-CoV-2 resulted in a pandemic situation throughout the world infecting more than 7 million people and also claimed the lives of almost half a million. Currently, there are no approved drugs for the treatment of SARS-CoV-2.

However, certain drugs used against SARS-CoV and MERS like Remdesivir and Hydroxy-chloroquine are currently being used, of which the former has shown some promising effects [4, 5].

In order to design a drug against the virus, it is equally important to understand the mechanism of action of the pathogen. SARS-CoV-2 has been shown to replicate in the host cells using RNA dependent RNA polymerases (RdRp). Further, the non-structural proteins such as nsp7, nsp8, and nsp12 of which the latter is an RdRp, form an active complex which is ATP dependent. Though this non-structural protein complex is required for the replication of the virus, recent studies have shown that nsp12 plays a major role in replication [6, 7].

With the world expecting a formal vaccine, which is not yet available, treatment for the pandemic is far from reach. It is noticed that spreading happens typically through respiratory droplets created while an infected person coughs or sneezes, similar to how influenza and other respiratory pathogens spread. Under the current circumstances, social distancing, usage of detergents, and self-isolation are the only options available to be free from infection [8]. In the absence of suitable drugs and vaccines for the pandemic, several strategies of using alternative treatment methods are inevitable. Boosting of the immune system could be one of the strategies which might keep the virus away from infecting healthy individuals. One such strategy was initiated by CSIR-CFTRI (Central Food Technological Research Institute), under the Government of India to distribute supplements containing *Spirulina platensis* for the infected people. Though it is not an alternative treatment procedure, it is believed that the algae possess immunomodulatory functions, which might be important to boost the immune system of an individual.

Spirulina platensis is well known blue-green algae that grow in high alkaline conditions and specially used for its high nutritious contents that include amino acids, vitamins, proteins, polysaccharides, and other pigments. Further, cellular assays and animal studies conducted with cold water extracts of *Spirulina platensis* upon the viability and pathogenicity of several influenza A viral strains showed that the extract inhibited viral plaque formation and reduced viral replication in cell cultures, and importantly was shown to be safe and well-tolerated at high doses in cellular and animal toxicity studies.

In-vivo studies on influenza-infected mice given Spirulina extract had higher survival rates compared to vehicle-treated controls. Spirulina extract disrupted the hemagglutination of viral particles to erythrocytes, thus inhibiting the infection process [10].

Some of the compounds in Spirulina like calcium spirulan, which is made of sugar moieties and C-phycocyanin (CP) have been shown to possess anti-viral properties. Earlier *in-vitro* studies revealed that calcium spirulan was effective in treating Human Herpes Simplex Virus and Human AIDS Virus [9]. C-Phycocyanin (CP) is a light-harvesting, pigment-binding protein isolated from algae. Earlier studies demonstrated that CP displays typical apoptotic characteristics, such as nuclear condensation, DNA fragmentation, membrane blebbing, and cell shrinkage. The applications of CP in human tumor cells were found to arrest cell cycle at the G0/G1 phase which blocks the synthesis of DNA, indicating inhibition of tumor cell proliferation [24].

As C-phycocyanin of *Spirulina platensis* has been proved to possess several health benefits, the current study is aimed towards an understanding if C-Phycocyanin, which is an important component of the blue-green algae, can have any antiviral properties against the currently existing SARS-CoV-2 virus through an *in-silico* approach.

MATERIALS AND METHODS:

Protein Structure, Co-factors, and Substrates: X-ray crystal structure of complex nsp12, nsp8, and nsp7 (pdb id: 7btf) [11] were obtained from the Protein Data Bank (PDB) [12]. The substrate used for the docking study is C-phycocyanin, and the 3D structure of C-phycocyanin was generated using ChemSketch tool [13] in mol format. Chimera tool was used to identify the docking of the active site region of nsp12 and the substrate C-Phycocyanin by superimposing [14].

Docking: In order to understand the enzyme-substrate interaction, the substrate was docked into the active site of the protein (with co-factors nsp8-nsp7). Docking was performed by AutoDock 4.0 program[15] using the empirical free energy function and the Lamarckian Genetic algorithm [16]. For the ligand, Gasteiger partial charges were used, and the

non-polar hydrogen molecules are conjoined. The grid map was calculated using AutoGrid, and the grid box dimension was set to 92*94*94 with a spacing of 0.192. After docking, the best conformation of the substrate interaction was analyzed for the interactive residues of substrates by Discovery Studio.

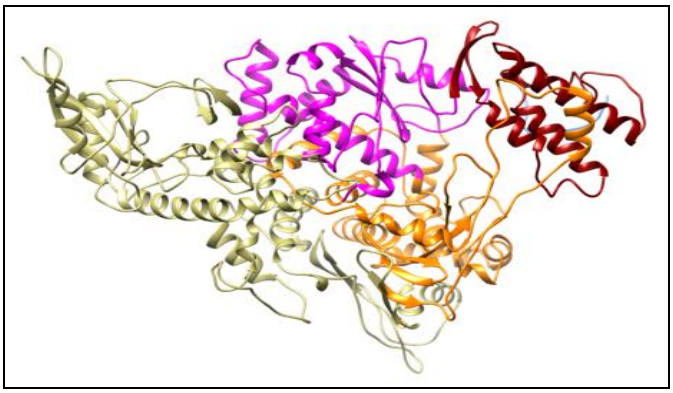

FIG. 1: STRUCTURE OF SARS-Cov-2 nsp12. THE nsp-12 STRUCTURE CONSISTS N-TERMINAL OF NIDOVIRUS WHICH IS HIGHLIGHTED IN TAN, FINGERS DOMAIN IN ORANGE, PALM DOMAIN IN MAGENTA AND A THUMB DOMAIN IN DARK RED

RESULTS:

Structure of SARS-CoV-2 nsp12 RNA-Dependent RNA Polymerase: The SARS-CoV-2 nsp12, which is an RNA-dependent RNA polymerase almost resembles a cupped right hand with fingers, palm and thumb subdomains as shown by earlier studies [17]. The nsp-12 structure consists N-terminal of nidovirus, which is highlighted in tan, fingers domain in orange, palm domain in magenta and a thumb domain in dark red **Fig. 1**.

The polymerase region is comprised of a finger's domain (residues 398–581, 628–687), a palm domain (residues 582–627, 688–815), and a thumb domain (residues 816–919). SARS-CoV-2 nsp12 also contains a nidovirus-unique N-terminal extension (residues 1–397). The finger domains are found to possess index, middle, ring, and pinky loops. The thumb domain is found in the active site to which the finger loops reach to make contact in positive-stranded RNA virus polymerases. In SARS-CoV nsp12, the contacts between the index finger and the thumb domain are particularly extensive with the positioning of an alpha-helix in the index finger loop to pack with the thumb helical bundle. The index finger-thumb interaction site also forms the nsp7-nsp8 heterodimer-binding site, with most of the contacts made between nsp12 and nsp7. The nsp12 RdRp is found to possess minimal activity on its own but the addition of nsp7 and nsp8, which behave as the main co-factors, stimulates the highest polymerase activity [17].

Structure of SARS-CoV-2 nsp8 and nsp7 Cofactors: The nsp8 and nsp7 complex is depicted in **Fig. 2**. While the nsp8 C-terminal head region folds around the helical domains of nsp7, the N-terminal region of nsp8, which spans amino acid residues from 1–81 seems to possess a more extended or disordered conformation.

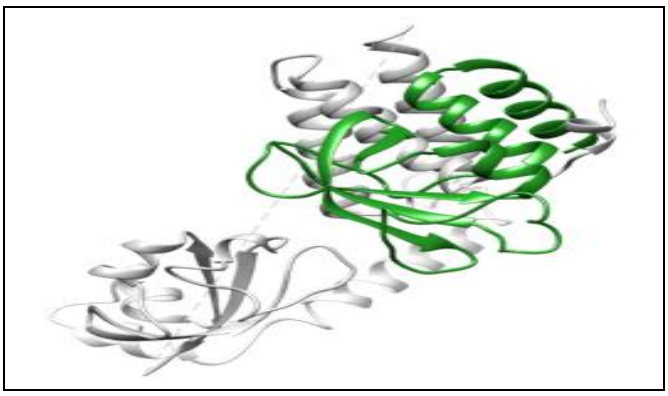

FIG. 2: STRUCTURES OF nsp8 AND nsp7 ARE DEPICTED IN GREY AND GREEN RESPECTIVELY

Interactions of nsp12 with nsp7 and nsp8: A large number of protein-protein interactions govern the SARS-CoV-2 RNA synthesis complex. Both nsp7 and nsp8 have essential roles in the formation and activity of the RNA synthesis machinery. Further, a strong interaction between nsp8 and nsp12 with the other viral nsps suggests that these two proteins form a hub for protein-protein interactions within the viral replication complex.

The nsp12 outer region is a largely negative electrostatic potential, and the nsp7 and nsp8 surfaces contacting nsp12 are also relatively neutral. The second subunit of nsp8 contains some basic residues in the N-terminal region visible in the structure (residues 77–98), contributing to an extension of the positive electrostatics of the template-binding channel. More conserved nsp12 residues contact the nsp8 N-terminal region (residues 77–126), while contacts with the nsp8 C-terminal head domain are mediated primarily by main-chain atoms, which may have less stringent requirements in their amino acid composition to retain binding. The nsp12-nsp8-nsp-7 complex structure is depicted in **Fig. 3**.

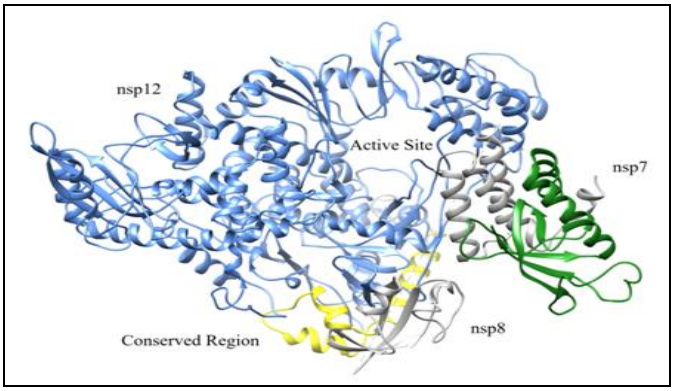

FIG. 3: STRUCTURE OF COMPLEX OF nsp12-nsp8-nsp7 OF SARS-CoV-2. Nsp7 IS SHOWN IN GREEN, nsp8 IN GREY AND nsp12 IN BLUE. THE nsp8 INTERACTS WITH THE CONSERVED nsp-12 RESIDUES (HIGHLIGHTED IN YELLOW), SPANNING AMINO ACID RESIDUES 77-126. THE ACTIVE SITE OF nsp12 IS MARKED TO WHICH THE NATURAL SUBSTRATE ATP BINDS UNDER NORMAL CONDITIONS OF THE VIRAL REPLICATION

The nsp7-nsp8 heterodimer binds to nsp12 on the thumb domain of the polymerase through which ATP enters the channel to reach the active site in the nsp12. The nsp12 index finger loop has been previously identified as necessary for recruitment of nsp12 to replication complexes [17]. The binding of the nsp7-nsp8 heterodimer to this loop suggests that nsp7-nsp8 facilitates the interaction of nsp12 with additional components of the RNA synthesis machinery for incorporation into viral replication complexes.

Structure of C-Phycocyanin of *Spirulina Platensis*: *Spirulina* is a superfood, and C-Phycocyanin is a key ingredient, and it is believed to protect the liver and kidneys during detoxification [18]. Several properties like antioxidation, detoxification, and importantly, inhibition of viral replication are some of the vital functions attributed to C-Phycocyanin present in Spirulina [19, 20]. It consists of four cyclopentane rings with two double-bonded oxygen and two carboxyl groups attached to it **Fig. 4**. 3D structure of C-phycocyanin in relation to its chemical structure [25] was obtained with chem sketch software.

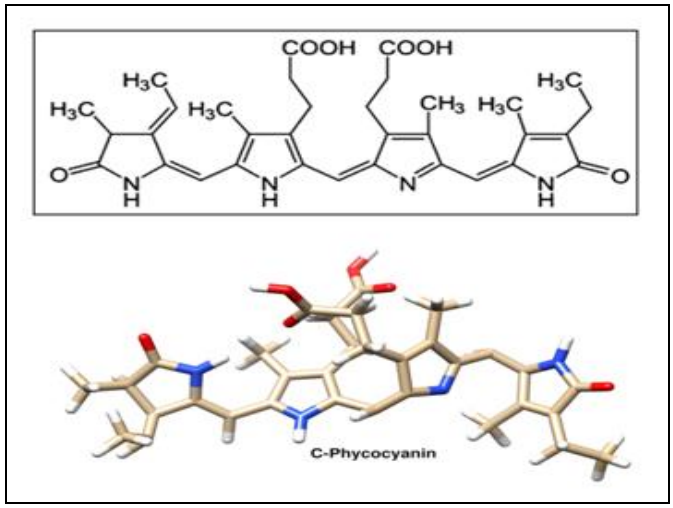

FIG. 4: STRUCTURE OF C-PHYCOCYANIN. THE CHEMICAL STRUCTURE WAS OBTAINED FROM AVAILABLE LITERATURE [25], AND THE 3D STRUCTURE OF C-PHYCOCYANIN OF *SPIRULINA PLATENSIS* WAS PREPARED USING CHEMSKETCH SOFTWARE

Interaction Study of C-Phycocyanin with the nsp Complex of SARS-CoV-2: The natural substrate for the nsp12 RdRp is the adenosine triphosphate (ATP) and the residues that interact with ATP are highly conserved throughout the coronavirus family [21]. ATP-bound crystal structures for SARS-CoV-2 nsp12 are not available in the PDB data bank. To guesstimate the binding mode of ATP in SARS-CoV-2 nsp12, the structure of SARS-CoV-2 nsp12 was superimposed with a poliovirus RdRp structure (PDBID: 2ILY) by using chimera tool **Fig. 5**.

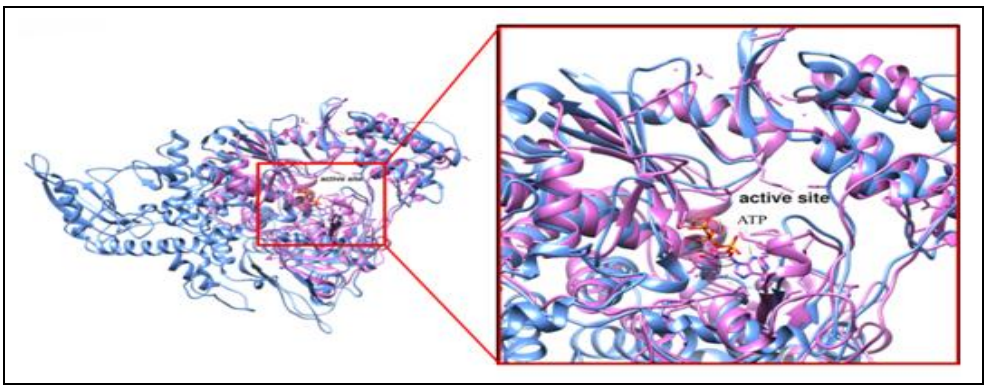

FIG. 5: THE SUPERIMPOSITION OF SARS-CoV-2 nsp12 WITH POLIOVIRUS RdRp FOR IDENTIFYING THE ACTIVE SITE REGION. THE POLIO RdRp SEEMED TO CLEARLY SUPERIMPOSE SARS-CoV-2. THE ENLARGED IMAGE SHOWS ATP, THE NATURAL SUBSTRATE BINDING TO THE ACTIVE SITE

After superimposition, the receptor residues such as Asp 760, Asp 761, Asp 618 Arg 553, and Arg 555 are identified as the active site residues, and then the substrate was docked. The following steps were generally applied: The nsp12-nsp8-nsp7 complex for docking studies was prepared by adding hydrogen atoms and gastiger charges to the system with their standard geometry. The mol2 file of the substrate C-Phycocyanin was loaded, and then grid box was set around the natural substrate active site region. Docking was performed for 1000 dock conformations of the substrate. The obtained poses were studied, and the poses that showed the best binding energy were selected and stored for substrate–protein interactions **Fig. 6**. Interestingly, it was observed that C-Phycocyanin interacts perfectly with the active site of the nsp12 RdRp similar to that of the natural substrate, ATP. Out of 1000 poses, 840 docked poses showed the lowest binding energy of -8.63 kcal/mol, and the average binding energy of all 1000 was -4 kcal/mol.

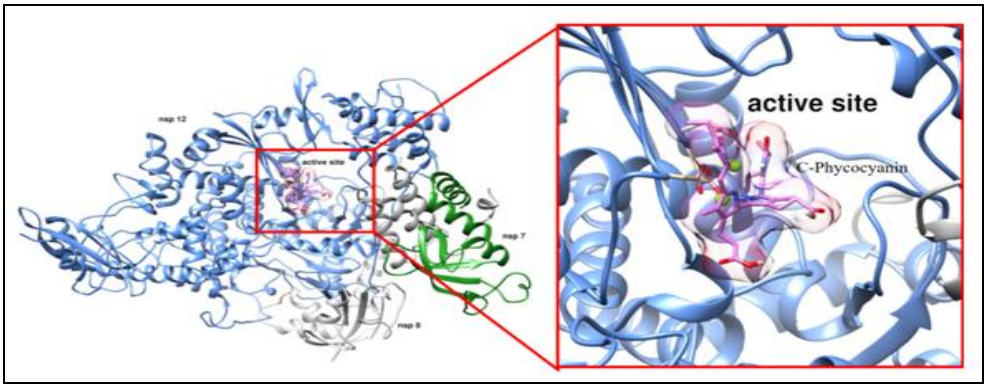

FIG. 6: THE NSP12-NSP8-NSP-7 COMPLEX (PDB ID: 7BTF) STRUCTURE WHERE GREEN INDICATES COFACTOR NSP7, LIGHT GREY INDICATES COFACTOR NSP8 AND BLUE INDICATES NSP12. THE ENLARGED IMAGE SHOWS THAT C-PHYCOCYANIN BINDS IN THE ACTIVE SITE OF THE NSP12, WHERE ATP MOLECULES NORMALLY BIND WHICH IN TURN ACTIVATES THE REPLICATION MECHANISM OF SARS-COV-2

TABLE 1: BINDING ENERGIES OF DIFFERENT DOCKING POSES OF C-PHYCOCYANIN TO SARS-CoV-2 nsp12

S. no.	Docked Pose	Binding Energy (kcal/mol)
1	840	-8.63
2	974	-8.57
3	347	-8.44
4	890	-8.32
5	869	-8.13
6	732	-7.9
7	60	-7.8
8	49	-7.79
9	211	-7.76
10	172	-7.48

A lowest binding energy for C-Phycocyanin indicates that the docked substrate might favour its interaction with the active site region and nearby residues. The top 10 lowest binding energies between the active site of nsp12 and C-Phycocyanin are represented in **Table 1**. Typically, the goal of docking here is to identify if C-Phycocyanin substrate can easily bind to the receptor nsp12-nsp8-nsp7 is energetically most favorable binding pose. The negative values of the docking energies for the substrate suggest that the latter (C-phycocyanin in this case) binds spontaneously without consuming energy. Based on the negative binding energy, we can predict that C-Phycocyanin is a potential molecule that can inhibit the replication mechanism by blocking the binding of ATP molecule, which is a natural substrate to nsp12, which is the key step in SARS-CoV-2 replication mechanism within the host.

From previous studies on poliovirus [22], it is found that the positively charged residues such as Lys159, Arg174, Arg163, Lys167, Lys172 and Lys359 interact with the triphosphate part of ATP and the diverse residues such as Lys61, Ile176, Glu177, Asp238 and Ser288, interact with nucleoside part of ATP. The negatively charged residue Asp323 interacts with the Mg^{2+} ion (here residues numbers are with respect to PDBID: 2ILY). The current in-silico observation is in line with earlier studies.

Based on binding energy, 840 conformations of C-Phycocyanin were considered to analyze if key residues of nsp12 does interact with the substrate, using the Discovery Studio tool. As expected, positively charged residues Lys 551, Arg 555, Arg 553, and Lys 798 interact with C-Phycocyanin, and the negatively charged residues Asp 760 interacts

with the Mg2+ ion **Fig. 7**. The Lys 551, Asn 691, Ser 759, Cys 799, Trp 617, Glu 811, Tyr 619, Pro 620 are residues which interact with the substrate through Van der Waals forces. Arg 555, Arg 553, Asp 618 and Asp 760 makes a conventional bond with C-Phycocyanin and Leu 758, Trp 800 and Lys 798 link through an Alkyl interaction (pi-alkyl interactions there is the interaction of pi-electron cloud over an aromatic group and electron group of any alkyl group) with the substrates. These are probable active site residues required for the replication to occur. As C-Phycocyanin interacts with these amino acids, it is highly expected to inhibit the replication mechanism of viruses.

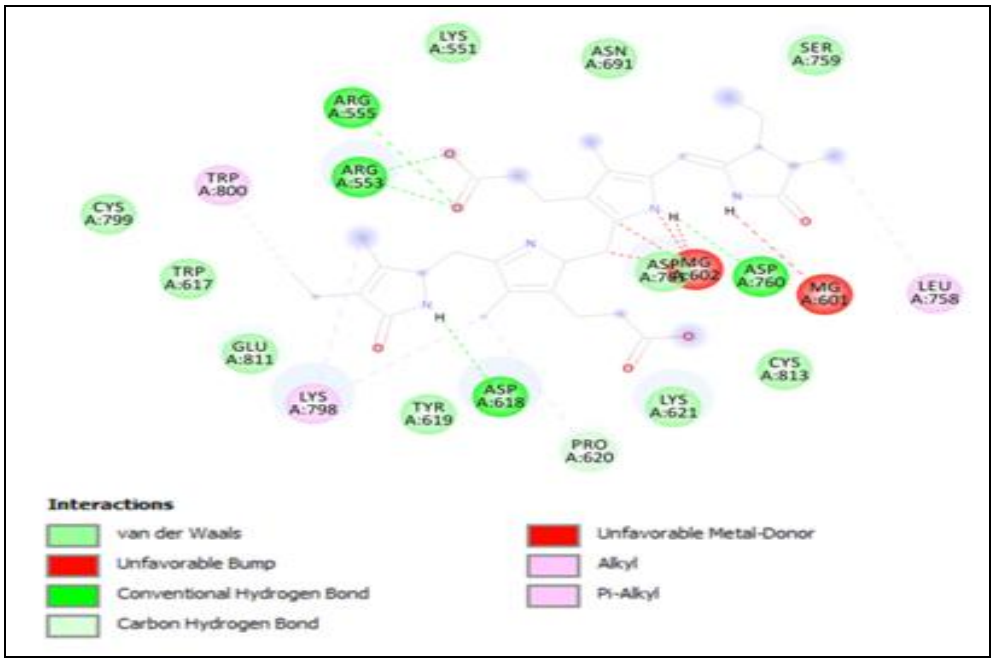

FIG. 7: 2D REPRESENTATION OF THE CHEMICAL INTERACTIONS OF C-PHYCOCYANIN WITH RECEPTOR nsp12-nsp8-nsp7

The image represents the interaction of C-Phycocyanin with aminoacid residues of the active site of SARS-CoV-2 nsp12. The interactions are of different types like van der Waals, Conventional Hydrogen Bonds, Carbon Hydrogen Bonds, Alkyl and Pi-Alkyl Bonds.

DISCUSSION: The emergence of SARS-CoV-2 resulted in a pandemic situation throughout the world, and there were no specific drugs or vaccines currently available for the treatment of the pandemic. However, broad-spectrum antiviral compounds that demonstrated activity against the earlier SARS-CoV or MERS-CoV are now being considered for the treatment of infection caused by the novel coronavirus SARS-CoV-2. For instance, the drug Remdesivir (RDV) which was used during SARS-CoV and MERS outbreaks are now being used for patients. RDV was demonstrated to compete with the ATP binding site of the nsp12 rdrp [5]. *In-vitro* studies suggest that once the ATP analogue binds to the active site of nsp12, RNA synthesis of the virus is arrested. Therefore, targeting the active site of nsp12 might be one of the ways to curb the infection of SARS-CoV-2.

Search for Natural Ways of Treatment – Blue-Green Algae as Key Sources: Several studies are being conducted in search of treatment of SARS-CoV-2, which includes naturally existing organisms or compounds with antiviral properties. Several studies have been conducted on the use of blue-green algae against viral diseases such as HIV-1, HSV-2, RSV, *etc*. [23] 694 Cyanophyta (Blue green algae) members were screened for antiviral activity against HIV-1 and 529 taxa against HSV-2 and RSV. These studies showed high potentials of Cyanophyta members as antiviral agents. These studies revealed the immense potentiality of blue-green algae against viruses. The most commonly consumed and cultivated alga is *Spirulina* (also known as *Arthrospira platensis*), which also shows high antiviral activities and is one such organism that is quite well known to provide immuno-modulatory functions [23].

More advantages like anti-diabetes, anti-cholesterol, anti-oxidation *etc*, have been attributed to this blue-green algae for long, in addition to its high nutritive values. Being aware of its properties, CSIR-CFTRI (Council of Scientific & Industrial Research Centre for Food Technological Research Institute), Mysore, under the Government of India had recently announced the use of Spirulina supplemented food items to keep the immune system active to at least prevent the infection of SARS-CoV-2.

C-Phycocyanin Targets the nsp12 Active Site of SARS-CoV-2: *Spirulina platensis* is a superfood with many minerals and proteins in it and because it also contains anti-viral ingredients, it prompted us to look for compounds which might inhibit the replication of SARS-CoV-2. C-Phycocyanin and calcium spirulan present in *Spirulina platensis* are key compounds that are known to possess these anti-viral properties. We selected C-Phycocyanin for the current study. The basic interaction study between viral nsp12 and C-Phycocyanin hints that the compound clearly competes with the ATP that binds to the active site of nsp12. C-Phycocyanin binds to the active site of the SARS-CoV-2 as shown in our *in-silico* study and might inhibit the viral RNA dependent RNA polymerase (RdRp) protein, thereby high jacking the replication mechanism of the virus, which, otherwise multiplies, synthesizes multiple viral proteins within the host cells and ultimately increases the toxicity in the infected people. However, the binding of C-Phycocyanin to the active site of nsp12 in spite of not being an analog of ATP deserves further immediate research.

C-Phycocyanin has antiviral properties, and it doesn't only inhibit the viral replication but also detoxifies the body by protecting the liver and kidneys by activating the immune systems. The initial study on the interaction of substrate, C-Phycocyanin in this case, with viral proteins seems promising. However, further advances in silico analysis, including dynamics and quantum studies along with *in-vitro* experiments, are inevitable to validate the effects of C-Phycocyanin against SARS-CoV-2. Though the current scenario, as predicted by recent studies, shows that strict preventive measures such as lockdown and social distancing might have proved to be effective [26], the post-lockdown era might be highly susceptible to the spread of the pandemic. The above finding might definitely lead to a breakthrough in identifying environmentally safe compounds against the deadly pandemic and C-Phycocyanin, being one such compound that exists naturally in Spirulina platensis can be one of the immediate reliefs from the infection, provided sufficient proven *in-vitro* data can be generated at the earliest.

CONCLUSION: SARS-CoVID-19 belongs to one of the large family of viruses (coronaviridae), which induces disorders including diarrhea, fever, cough, sneezing, difficulty in breathing, upper and lower respiratory tract infections and death if untreated. With the world looking for immediate relief either through drugs or vaccines, we took into account the blue-green algae, *Spirulina platensis* which is well known to possess anti-viral properties, in addition to its strong nutritional values. Specifically, we targeted one of the important ingredients, C-Phycocyanin, for its anti-viral property, particularly against SARS-CoV-2 through *in-silico* approaches. The first results clearly depict that C-Phycocyanin interacts with the nsp12 RdRp of SARS-CoV-2, which is the target for several drugs. To the best of our knowledge, this is the first report of C-Phycocyanin specifically targeting the active site of the main protein responsible for viral replication.

The report demands immediate dynamics and quantum studies along with *in-vitro* experiments. In the meanwhile, consuming Spirulina supplemented food ingredients might be one of the strategies to combat the deadly pandemic.

Author Contribution: T. Kiran Raj and T. S. Gopenath conceptualized the study. T. Kiran Raj conducted the experiments. T. S. Gopenath drafted the Manuscript. R. Ranjithkumar and B.M. Kanthesh helped with the Manuscript and Discussion.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest.

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