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Enhanced production of camptothecin by immobilized callus of *Ophiorrhiza mungos* and a bioinformatic insight into its potential antiviral effect against SARS-CoV-2

Rajesh Mamkulathil Devasia [a], Mohammad Altaf [b], Abdulwahed Fahad Alrefaei [c], Salim Manoharadas [d,*]

[a] SCMS Institute of Bioscience and Biotechnology, South Kalamassery, Kochi, Kerala 682033, India
[b] King Saud University, Central Laboratory, Department of Chemistry, College of Science, P.O. Box. 2454, Riyadh 11451, Saudi Arabia
[c] King Saud University, Department of Zoology, College of Science, P.O. Box. 2454, Riyadh 11451, Saudi Arabia
[d] King Saud University, Central Laboratory, Department of Botany and Microbiology, College of Science, P.O. Box. 2454, Riyadh 11451, Saudi Arabia

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ABSTRACT

Camptothetin (CPT) is a quinoline alkaloid originally isolated from the Chinese tree, *Camptotheca acuminata Decne*. CPT was found to have anticancerous and antiviral properties. Derivatives of natural CPT, including topothecan and irinotecan are used clinically to treat a variety of cancers. Apart from *Camptotheca acuminata Decne,* CPT production was also found in the perennial plant *Ophiorrhiza mungos.* In this study we attempted the immobilization of the tissue culture grown callus of *Ophiorrhiza mungos* for the continuous production of a higher concentration of CPT. As evident from previous studies about the antiviral effects of CPT, we wanted to bioinformatically analyze the binding potential of CPT towards two important proteins of SARS-CoV-2, protease (M^{pro}) and RNA dependent RNA polymerase (RdRp). Further docking analysis of the CPT against the exterior spike glycoprotein of SARS-CoV-2 was also done to determine their potential interaction. The immobilized callus of *Ophiorrhiza mungos* produced CPT at a concentration of 420 μg/l by the end of 12 days of growth. The HPLC analysis was done to determine the purity of the CPT synthesized by the immobilization technique. The bioinformatic analysis revealed a higher binding efficiency of CPT and its derivatives, toptecan and irinotecan against M^{pro} and RdRp. The docking analysis of CPT against the spike glycoprotein of SARS-CoV-2 showed hydrogen bonding with the amino acids at K466 with a bond distance of 2.56A° and K355 with a bond distance of 2.40A°. This finding was of particular importance that other compounds including hydroxychloroquine sulphate, lopinavir and ivermectin could bind with the spike protein only by weak Vander wall bonds and no hydrogen bond formation was noticed. Our studies hence evaluate the efficiency of CPT against SARS-CoV-2, by potentially blocking the interaction of the spike glycoprotein with the angiotensin-converting enzyme 2 (ACE2) receptor found on host cells.

1. Introduction

The effectiveness of natural herbal products against human diseases and infections are subject of intense research over several decades. Several studies have pointed out the efficiency of secondary metabolites from medicinal plants in treating infectious diseases in humans (Yuan et al., 2016). One of the well-studied secondary metabolites, that elicit antileukemic and antitumor properties is camptothecin (CPT) (Kessel, 1971; Hutchinson, 1981). CPT is a pyrrolo (3,4-b) quinoline alkaloid originally isolated from the Chinese tree, *Camptotheca acuminata Decne*. (Nyssaceae). Clinical studies have pointed out that CPT is very effective against liver carcinoma, colon cancer, gastro intestinal malignancies and tumors of head and neck (Murphy et al., 2001; Pommier, 2006: Pommier, 2011, 2013). The molecular mechanism by which CPT exerts its anticancerous activity is by inhibiting the enzyme DNA topoisomerase I (topoI). The inhibition of topoI by CPT subsequently leads to apotopsis of the cell (Champoux, 2001).

* Corresponding author.
E-mail address: smanoharadas@ksu.edu.sa (S. Manoharadas).
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Apart from the anticancer activity, CPT is also reported to have antiviral, antiangiogenic and neurotoxic properties (Clements et al., 1999; Uday Bhanu and Kondapi, 2010). In a study by Tai et al. (2019), CPT was used as a chemivirotherapeutic agent along with oncolytic measles virus for specific annihilation of breast cancer cells (Tai et al., 2019) cells. In another study CPT was used to inhibit vaccinia virus deoxyribonucleic acid (DNA) synthesis in HeLa cells (Horwitz et al., 1972). Other studies in the similar context pointed out that CPT functions as a potent inhibitor of replication, transcription and packaging of double-stranded DNA-containing adenoviruses, papovaviruses and herpesviruses, and the single-stranded DNA-containing autonomous parvoviruses in cell free systems (Pantazis et al., 1999). The effectiveness of CPT against retroviruses was also studied in detail. For instance, CPT and its derivatives, 10-hydroxy-CPT and 7-hydoxymethyl-CPT was shown to possess anti-HIV-1 activity (Li et al., 2010). Although the specific mechanism by which CPT exerts its anti-HIV-1 effect was not elucidated in the study, it was speculated that similar to 9-Nitrocamptothecin (9NC), CPT also inhibits the tumor necrosis factor- mediated activation of HIV-1 and selectively initiating apoptosis of HIV infected cells (Hung et al., 2001; Moulton et al., 1998). The structure of CPT is shown in Fig. 1.

The emergence of a betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cause the infectious disease in humans known as COVID-19. SARS-CoV-2 capsid encases a positive-sense, single-stranded RNA with a genome length of 29,881 nucleotide bases (Lau et al., 2015; Khan et al., 2020; Chan et al., 2020; Lu et al., 2020a; Lu et al., 2020b). The phylogenetic analysis state that the SARS-CoV-2 is distantly related to SARS-CoV (approx. 79% similarity) and MERS-CoV (approx. 50% similarity) (Lu et al., 2020; Cherry et al., 2017). The initial attachment of the SARS-CoV-2 to the target cells is facilitated by the exterior spike protein of the virus. There are several antiviral drugs under experimentation which presumably blocks the spike protein rendering inability of the virus to infect the host cells. For instance, the drug ivermectin was shown to bind the spike protein by docking analysis (Lehrer and Rheinstein, 2020). There are also report on other drugs such as Ribavirin, Remdesivir, Sofosbuvir, Galidesivir and Tenofovir, that displays potential activity against SARS-CoV-2, by specifically interacting with the RNA dependent RNA polymerase (RdRp) (Elfiky, 2020).

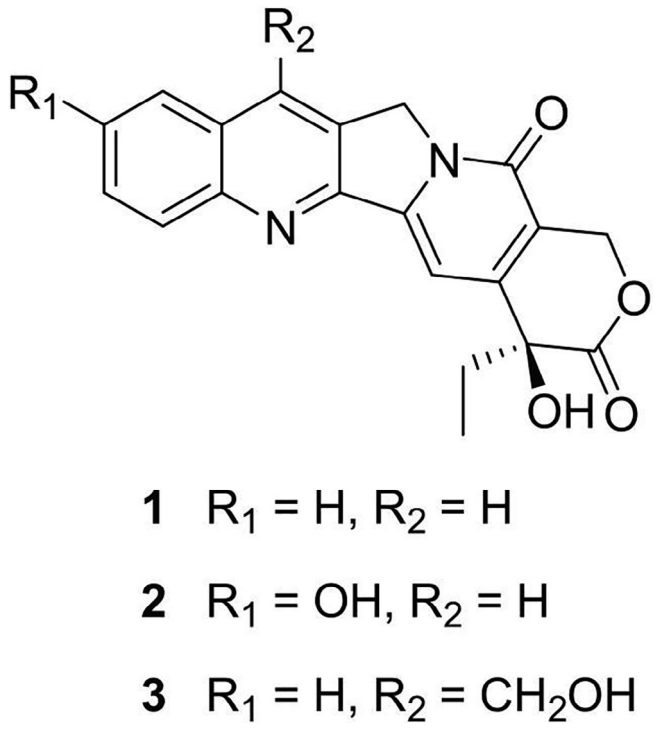

Fig. 1. Structure of Camptothecin and its analogues (1) Camptothecin (2) 10-hydroxy-CPT (3) 7-hydroxymethyl-CPT (Li et al. 2010).

The corona viral genome encodes four major structural proteins named spike (S) protein, nucleocapsid (N) protein, membrane (M) protein and the envelop (E) protein. The entry of SARS-CoV-2 to the target cell is facilitated by the spike protein. The spike protein consists of three major units, a short intracellular tail, a trans membrane anchor and a large ectodomain which consist of receptor binding spike S1 subunit and a membrane fusing spike S2 subunit (Fan, 2016). SARS-CoV-2 recognizes the host cell receptor, Angiotensin Converting Enzyme 2 (ACE2), by the S1 subunit of the spike glycoprotein. The viral entry to the lung cells predominantly causes mild to moderate level upper respiratory tract infections (Yang et al., 2020). In humans, the ACE2 is an enzyme found on cell surface of varying cell types, including lung cells, arteries, heart, kidney and intestine (Hamming et al., 2004). The ACE2 enzyme on the surface of the host cells catalyze the cleavage of angiotensin II (a vasoconstrictor peptide) into angiotensin 1–7 (a vasodilator), hence lowering the blood pressure (Keidar et al., 2007). An increased level of angiotensin II can increase inflammation and death of cells in the alveolar cells, which is critical for the oxygen transport in the body. The death by SARS-CoV-2 usually occurs due to respiratory failure as a result of alveolar damage. Upon binding with SARS-COV-2 spike protein, the normal function of ACE2 receptor is inhibited and the regulation of angiotensin II is challenged, thus increasing the levels of angiotensin II which damage tissues especially in the lungs and heart.

Here we attempted to analyze the effective of CPT against SARS-CoV-2 by molecular docking analysis against the spike glycoprotein. In addition, we also attempted to map the binding affinity of CPT and its derivatives against two critical proteins of the virus such as RNA dependent RNA polymerase (RdRp), protease (Mpro). In addition, we evaluated the efficiency of immobilized laboratory grown callus of the plant *Ophiorrhiza mungos* in production of camptothecin. Earlier, *O. mungos* and *O. pumila* have been reported as the sources of camptothecin and 9-methoxycamptothecin (Roja, 2006). However, no immobilization techniques have been ever tested for its production.

2. Materials and methods

2.1. Media preparation and callus development

Murashige and Skoog medium (MS) supplemented with benzyl amino purine (BAP) (1 mg/l; Hi media, India) and 2, 4-Dichloro phenoxy acetic acid (2 mg/l; Hi media, India) was used for the initiation and development of callus cultures. Sucrose (3%; Hi media, India) was added to the medium as a carbon source. The pH of the medium was adjusted for 5.7 using 0.1 N hydrochloric acid (HCl) and 0.1 N sodium hydroxide (NaOH). 0.8% agar (w/v) was used for solidifying the media.

Stem explants of *Ophiorrhiza mugos* were used for the initiation of callus development. The explants were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) and washed with sterile distilled water for 4–5 times. The explants were inoculated to the prepared medium and incubated at 16 h of light/8 h dark cycle at 25 ± 2 °C in plant tissue culture chamber. The cultures were kept under observation for 20 days for the initiation of callus. The developed calli were sub cultured every 15 days on the same medium for two months so as to obtain healthy growth.

2.2. Encapsulation of callus

Friable callus cells developed on the specific medium was encapsulated under in vitro conditions. Briefly, 2.5% of sodium algi-

nate solution and 100 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) was prepared and autoclaved. The sodium alginate solution containing approximately 2 g of callus was added drop wise to the chilled calcium chloride solution initiating the formation of callus entrapped alginate beads. These beads were washed with MS salts solution and was transferred to sterile culture medium. The cultures were maintained on an orbital shaker with 60 rpm shaking conditions at room temperature of 25 ± 2 °C with 50 to 75% humidity and 16 h of photoperiod.

2.3. Extraction of secondary metabolite

Every eight days the cultures from the flasks were harvested by filtration through a sieve filter. The pH of the filtered medium taken for analysis and was basified with concentrated NaOH to pH 12. The compound was extracted by solvent extraction process by using chloroform (Jones and Kinghorn, 2012). Briefly, equal volume of chloroform was added to the filtered culture media and was mixed thoroughly. The bottom phase with the produced CPT was extracted out and the chloroform layer was washed with water and it was completely evaporated by suction and was air dried. The CPT was dissolved in HPLC grade water (Sigma, USA). The extracted product was further quantified by HPLC analysis.

2.4. HPLC analysis of the synthesized CPT

High performance liquid chromatography analysis was carried out for determining the alkaloid present in the culture during the different weeks of interval. It was performed in JASCO HPLC using C-18 column (30X3.9 mm) using UV detector set at λ 254 nm. The flow rate was 0.8 ml/minute with the solvent system set at a ratio of 42:58 of Acetonitrile: Water, respectively. Camptothecin present in the extracts were identified by comparison of their retention time with that of the authentic samples. A standard solution of 1 mg of alkaloid in 2 ml diluted with MeOH was used in different concentrations and injected on HPLC. The retention time and the area under the peak were noted down and a graph of concentration against area was plotted. The concentration of the separated alkaloid was determined from the standard calibration curves.

The performance and reproducibility of the instrument within the specified limits was measured by comparison with those produced by a reference or standard over an appropriate range of measurements. The calibration of the system was followed after every maintenance job. The studies using the same HPLC instrument has been previously reported (Roja, 2006).

2.5. Phytochemicals and protein selections

From previous reports on SARS-CoV-2 treatment the drugs were selected and used for the comparison with camptothecin. The SDF files of the drugs and camptothecin were retrieved from PUBCHEM compound database (https://pubchem.ncbi.nlm.nih). All structures are converted to Mol2 and PDB format using Open babel software (O'Boyle et al., 2011). Viral protease (M^{pro}), RNA dependent RNA polymerase (RdRp) and the viral spike glycoprotein was used for docking studies. All the structures were retrieved from PDB Database as 6LU7 for Mpro, 7BTF for RdRP and 6VSB for SARS-COV-2 spike glycoprotein (www.rcsb.org).

2.6. Docking analysis

The selected drugs and camptothecin were screened one by one by studying their interaction with protein targets by means of AutoDock Vina tool (Trott and Olson, 2011). The docked structures were retrieved in PDB format and binding affinities were calculated by means of PRODIGY software. The PDB structures were evaluated and possible hydrogen bonds and the bond distances were analyzed by means of chimera software version.1.13.1 (Pettersen et al., 2004).

3. Results and discussion

3.1. Enhanced synthesis of camptothecin by immobilized callus of Ophiorrhiza mungos

In order to counter the problem of the low synthesis of camptothecin in the culture medium under laboratory conditions, we used immobilized callus of *O. mungos* (Fig. 2a) and determined the rate of camptothecin production over a period of 14 days. The rate of camptothecin synthesis by the immobilized cells peaks at 12 days after initial inoculation. The production of camptothecin was comparatively low in the first week (90 μg/l) with the amount of camptothecin increasing to 420 μg/l until the end of second week of analysis (Fig. 2b). The purity of the synthesized camptothecin was analyzed by HPLC analysis. In concurrence with the standard, the synthesized camptothecin was pure with a single peak (Fig. 2c).

Plant cell immobilization was earlier described for entrapping the cells of *Catharanthus roseus*, *Morinda citrifolia, Thalictrum* and *Digitalis lanata* (Brodelius and Pedersen, 1993; Yokoyama, 1996; Suzuki et al., 1988; Alfermann and Petersen, 1995). Immobilization of plant cell plays an important role in the production of high value chemicals and secondary products (Smetanska, 2008). The effective advantage of plant cell immobilization is the production of desired compounds continuously without cell wash out. In addition, the immobilization enables a group of cells to function at the same time thereby, increasing the productivity of the cells. The immobilization technique routinely uses hydro colloidal gels such as alginate and carrageenan to entrap the cells inside the matrix (Kim and Chang, 1990).

The annual production of CPT is five times less than that of its requirement (Kai et al., 2014). Camptothecin and its derivatives, named irinotecan and topotecan are extensively used to treat various cancers and also to treat certain viral diseases. All the clinically relevant CPT derivatives used are synthesized from natural CPT, which is obtained by the extraction of stem and fruit of the plant *Camptotheca acuminata* (Wall et al., 1966). The natural growth of *C. acuminata* takes around 9–10 years to become a mature plant from the seed germination level. In order to isolate natural CPT, 1000–1500 tons of wood chip are utilized every year to meet the increasing market demand of the drug (Watase et al., 2004). Plant cell and tissue culture is an efficient method for producing important secondary metabolites and other plant derived compounds. Apart from *C. acuminata,* a high-level accumulation of CPT in in vitro grown plants of *O. mungos* compared to that of naturally growing plants has been reported (Namdeo, 2007). The whole plant contains 0.2 to 5 mg/g (dry weight) of CPT, depending on the type of tissue analyzed (Lopez-Meyer et al., 1994). The amount of CPT content was 0.08% in leaves and 0.14% in the stem. The strategy to produce CPT in vitro by monitoring growth parameters with the objective to its scale up its production has been reported by other workers (Murthy et al., 2014; Kai et al., 2014). However, low concentration of the synthesized CPT in the culture medium was a major concern.

In our study we exploited the immobilization technique to entrap the tissue culture grown cells of *O. mungos*. The growth of stem explants of *O. mungos* was cultured on MS medium as described in materials and methods. Successive subcultures increased the growth rate of the callus, which were initially growing slowly. Further growth of the callus was initiated by transferring it to liquid medium of similar composition. Based on our

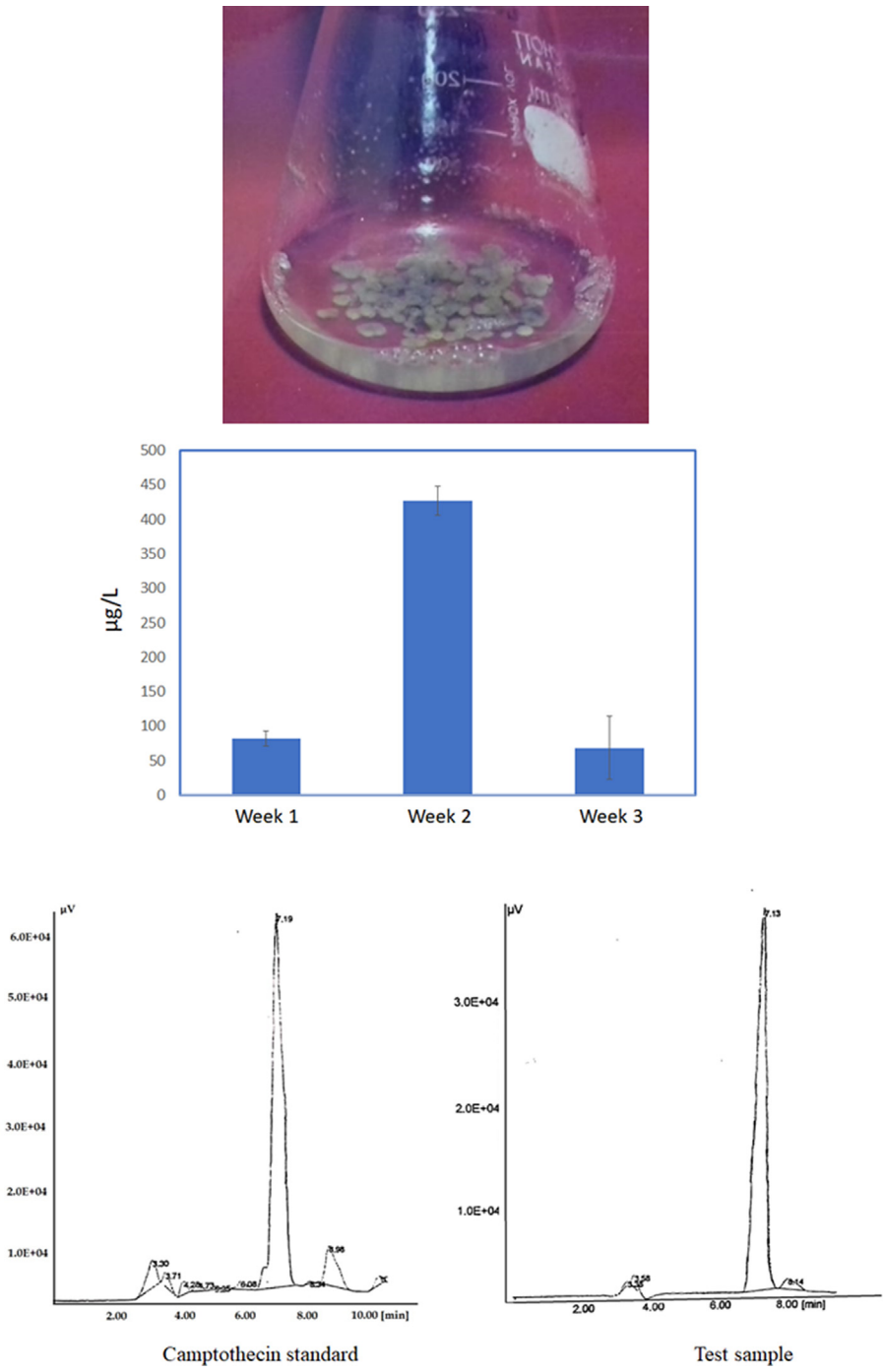

Fig. 2. Immobilization of the callus of *Ophiorrhiza mungos* and the analysis of the synthesized camptothecin. (a) The immobilized callus suspended in liquid growth culture (b) Concentration of the synthesized camptothecin at first week (1–7 days), second week (8–14 days) and third week (15–21) days of growth. (c) Analysis of the synthesized camptothecin by HPLC in comparison with the standard.

preliminary investigations of these callus for its ability to produce CPT, immobilization of the callus cells was done as described in materials and methods. HPLC analysis of the culture medium with the immobilized callus after one week of the culture revealed the presence of the CPT alkaloid with a reasonable detection concentration of 420 µg/l by 12 days (Fig. 2b). The alkaloid was detected as a single peak with a retention time of 7–8 min (Fig. 2c). This retention time was comparable with the standard sample of CPT (Fig. 2c).

The level of synthesized CPT, however reduced from its maximum yield after the end of 12 days of incubation. At the end of third week of growth, there was a significant reduction of the level of the synthesized CPT. The percentage reduction in the level of CPT was calculated to be approximately six times lower than the level of CPT at the end of second week. We speculate that this could be either because of the degradation of CPT or modifications of CPT could have led to the formation of other unidentified products. Further studies are warranted to experimentally prove our speculation. By suitably manipulating the culture medium and by genetic transformation methods, it may be possible to synthesize a higher level of CPT.

3.2. Bioinformatic approach shows strong binding affinity of CPT towards M^{pro} and RdRp proteins from SARS-CoV-2 virus

Camptothecin was earlier shown to possess antiviral activity. In this context, we wanted to bioinformatically determine the binding affinity of CPT towards two critical proteins of SARS-CoV-2 virus, the protease (M^{pro}) and the RNA dependent RNA polymerase (RdRp). The infective and replicative cycle of SARS-CoV-2 consists of virus entry, genome replication, assembly and germination of virions. Three vital proteins M^{pro}, RdRp and spike glycoproteins are responsible for the spreading of the virus (Guo et al., 2020; Walls et al., 2020). Once inside the host cell, the positive stranded RNA of SARS-CoV-2 is translated to a polyprotein, that is cleaved by the protease (M^{pro}). This cleavage accelerates the formation of replication-transcriptase complex. The RdRp protein on the other hand helps in the replication of the whole genome of the virus.

We analyzed the binding affinity of CPT and its derivatives, Toptecan and Irinotecan towards Mpro and RdRp proteins by PRODIGY software. The binding affinities of all the compounds tested is shown in Table. 1. The affinity of binding lies within a range of 3.7 to 8.9 Kcal/mol, indicating that all these drugs have comparable potential as active compounds for these receptor molecules. Ivermectin, Lopinavir and Hydroxychloroquine sulphate (HCQS) were also analyzed for its binding affinity towards Mpro and RdRp. As evident from the data (Table 1), Irinotecan had the highest binding affinity towards M^{pro} and the affinity was comparable with Lopinavir. The weakest binding out of the tested compounds against M^{pro} was observed with HCQS, with the value as low as −3.70 Kcal/mol. The binding affinity of CPT and its two derivatives towards RdRP protein was also higher in the range of −7.0 to −8.2 Kcal/mol. Interestingly, the binding affinity of HCQS with RdRp was the lowest out of the compounds tested with −4.30 Kcal/mol (Table 1). This data foresees the potentiality of CPT and its derivatives as a prominent antiviral agent against SARS-CoV-2 by inhibiting M^{pro} and RdRp.

Table 1
Binding affinity of different antiviral drugs towards M^{pro}, RdRP protein from SARS-CoV2.

Sl No.	Name of the compound	Binding affinity with (M^{pro}) in Kcal/mol	Binding affinity with (RdRp) in Kcal/mol
1	Ivermecitin	−7.50	−8.30
2	Lopinavir	−8.00	−8.40
3	Hydroxychloroquine sulphate	−3.70	−4.30
4	Camptothecin	−7.50	−7.30
5	Topothecan	−7.50	−7.00
6	Irinotecan	−8.90	−8.20

3.3. Molecular docking analysis shows hydrogen bond formation by CPT against spike protein of SARS-CoV-2

In order to get more insights to the binding of camptothecin, hydroxychloroquine sulphate, lopinavir and ivermectin towards the external spike protein of SARS-CoV-2, molecular docking analysis of all the four compounds with spike glycoprotein of SARS-CoV-2 was done. Among the docked compounds, lopinavir, ivermectin and camptothecin was found to bind to the S1 region of spike glycoprotein, which is the site of interaction with the host cells ACE2 receptor. In contrast, the binding of hydroxychloroquine sulphate occurred in the S2 region of the spike glycoprotein. Interestingly, it was found that CPT forms hydrogen bonds with spike glycoprotein at two key positions with amino acids, K466 with a bond distance of 2.56A° and K355 with a bond distance of 2.40A° (Fig. 3a). All the other compounds including HCQS, ivermectin and lopinavir formed weak Vander Waals bonds with the spike glycoprotein of SARS-CoV-2 (Fig. 3b, c, d).

It was earlier shown that amino acids L-455, F-486, Q-493, S-494, N-501 and Y-505 are responsible for the binding of spike protein with the Angiotensin-converting enzyme 2 (ACE2) receptor (Andersen et al., 2020; Qiu et al., 2020). ACE2 is the receptor molecule present in human cells, to which SARS-CoV-2 attaches with the spike glycoprotein receptor. It can be inferred that the region from 355 to 530 of the spike glycoprotein is critical for the receptor binding on the host cells in SARS-CoV-2. Interestingly, CPT bind at positions 355 and 466. An educated guess can therefore point out that the binding of CPT with the critical spike protein of the virus may block the ACE2 receptor binding and further processing of the virus.

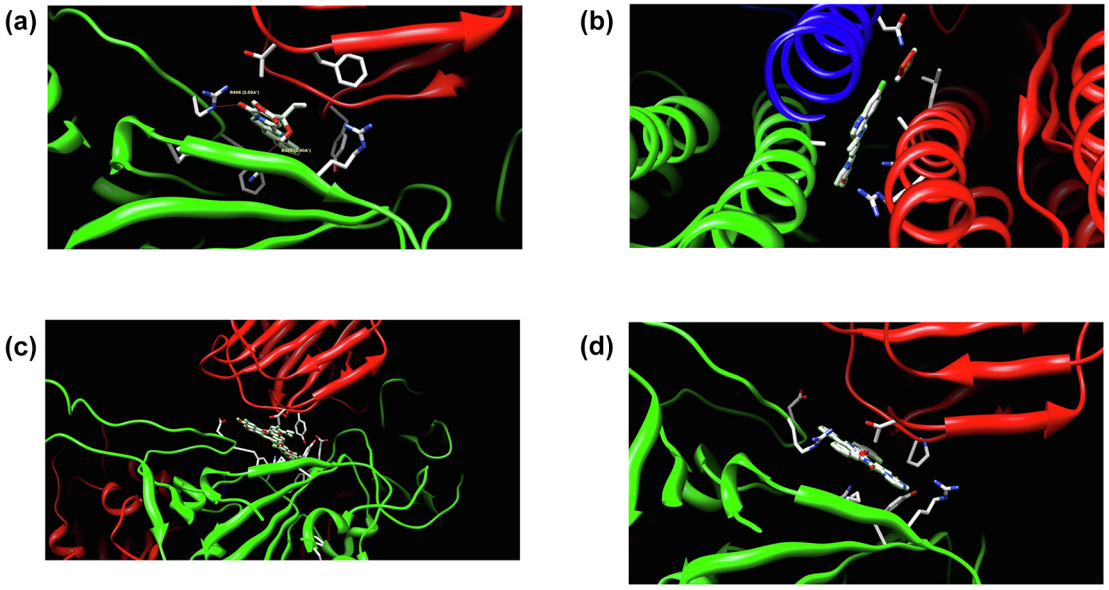

Fig. 3. Docking analysis of camptothecin and other antiviral drugs with spike glycoprotein of SARS-CoV2 (a) Docked molecular structure of camptothecin with spike glycoprotein of SARS-CoV2. Hydrogen bond formation with specific amino acids is shown as red dotted lines (b) Hydroxychloroquine sulphate docked with the spike glycoprotein (c) The docked molecular structure of ivermecitin with the spike glycoprotein (d) Lopinavir docked with the spike glycoprotein.

Beta coronaviruses, including SARS-CoV-2, MERS-CoV and SARS-CoV are single stranded RNA viruses encased in a membrane envelope are pathogenic for humans (Su et al., 2016). The homotrimeric transmembrane spike glycoprotein of SARS-CoV and SARS-CoV-2 shares structural homology and conserved ectodomains. This observation is significant as earlier strategies employed in blocking of SARS-CoV entry to the host cells by interfering with the interaction with the ACE2 receptor may be applicable also to SARS-CoV-2 (Hoffmann et al., 2020; Walls et al., 2020). The proven antimalarial drugs, chloroquine and hydroxychloroquine are being tested as potential candidate drugs against SARS-CoV-2, primarily due to their ability to inhibit the terminal phosphorylation of ACE2 and to elevate the pH in endosomes, respectively (McKee et al., 2020). The spike protein of SARS-CoV-2 was shown to be highly immunogenic to the host immune system, thus inhibiting the SARS-CoV-2 S-mediated cell entry (Walls et al., 2020). The S protein-ACE2 receptor interaction disruption strategies are of remarkable therapeutic value, owing to the higher binding affinity (10–20 fold) of SARS-CoV-2 S protein to ACE2 in comparison to SARS-CoV (Tang et al., 2020). Apart from the S protein, other highly conserved proteins such as RdRp and Mpro shares 95% similarity between SARS-CoV and SARS CoV-2. RdRp is a critical protein that is required for the replication of the viral genome within the host cell. Mpro protein is a protease that cleaves the viral polyprotein into functional units within host cells. The Mpro protein shares a homology of 96% between SARS-CoV and SARS-CoV2 (Morse et al., 2020). Drugs such remdesivir and lopinavir has been shown to be effective against MERS-CoV in mice (Sheahan et al., 2020). In addition, they also potentially inhibit the functionality of RdRp in Ebola virus and proteases of SARS-CoV-2 in humans. These drugs are tested as potential candidate drugs against SARS-CoV-2 in two international clinical trials (SOLIDARITY Trial and DisCoVeRy Trial) (McKee et al., 2020). Potential antiviral agents comprising of natural products and phytochemicals should be assessed for treatment against SARS-CoV-2 infection (McKee et al., 2020).

4. Conclusion

This study, for the first time evaluates the potentiality of camptothecin production by the immobilized callus of *Ophiorrhiza mungos*. The immobilized callus produced camptothecin at a significantly higher concentration of 420 μg/l as compared to the extraction of camptothecin from the whole plant. A strong binding efficiency of CPT and its derivatives, topothecan and irinotecan against the protease (M^{pro}) and RNA dependent RNA polymerase (RdRp) from SARS-CoV-2 was observed from the bioinformatic analysis. Apart from these proteins, camptothecin could also be successfully docked with the spike glycoprotein of SARS-CoV-2. Interestingly, a strong hydrogen bond formation was seen between camptothecin and critical amino acids K466 and K355 of the spike glycoprotein. These results foresee the potentiality of camptothecin and its derivatives as antiviral agents against SARS-CoV-2

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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