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Computational Screening of Medicinal Plant Phytochemicals to Discover Potent Inhibitors against Hepatitis B Virus

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Hepatitis B virus (HBV) infections are infamous to cause liver damage, hepatocellular carcinoma, and cirrhosis, all of which can be fatal in nature. Nucleotide analogues, which target viral reverse transcriptase, and interferon therapy, which is known to have side effects in recipients, are currently being used to treat such infections. Increasingly, the growing viral resistance towards the first line of drugs has been a concern for the healthcare system worldwide, and therefore the need for new therapeutic interventions has been noted and novel viral targets are being explored. The HBV core protein (HBc), which regulates several viral replication checkpoints in the host cell, is one such possible target for therapeutic development. In this study, we use in silico approach to investigate the potential of various phytochemicals and natural compounds to be developed as antiviral medicines that target HBc protein. For which, the compounds were collected from databases and potential candidates were screened and shortlisted based on their pharmacokinetics and drug-likeness using Lipinski's rule of five. Further, the chosen phytochemicals were subjected to docking analysis, and binding affinities were evaluated to set a cut-off value for selecting the best interactions, which showed better binding energy values compared to standard anti-HBV drugs.

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Further, the two- and three-dimensional interactions of the ligand and target protein complexes were studied to gain insights into the ligand-target bonding patterns, and bioavailability and toxicity profiles were analyzed to understand the safety and efficacy of the selected compounds to be developed as anti-HBV interventions. Upon complete inspection, Ingenol was identified as the best candidate among the chosen phytochemicals, followed by I-asarinin and Withaferin. We hope that the findings from this study will be useful in the development of anti-HBV drug candidates or formulations.

Keywords: Molecular docking; phytochemicals; toxicity testing; bioavailability; binding energy; core protein; hepatocellular carcinoma.

1. INTRODUCTION

Hepatitis (inflammation of the liver) epidemics have been prevalent throughout human history and are mentioned in texts belonging to societies with no social or cultural contact. Although the etiological agent for such epidemics was not known at the time, it was hinted to be of infectious origin, due to its prevalence in populations with appalling hygienic condition [1,2]. With the acceptance of the 'Germ theory of disease' and the discovery of viruses in the 1890s, the research behind this clinical epidemic data started to gain motion and in 1965, the first virus as a causative agent of hepatitis was identified and named as Hepatitis B virus (HBV). Since then, a wide range of viruses have been discovered and identified as pathogens responsible for viral hepatitis, the most infectious of these are members of the Hepadnaviridae family [3].

Currently, there are 18 members in the Hepadnaviridae viral family, among which five are known to infect humans and are named Hepatitis virus A to E. All the five members in the family are RNA viruses except HBV, which is a DNA virus. It is also considered the most infectious among the five, while HAV and HEV are the most controllable ones as they are known to cause only acute infections [3-5]. Viral hepatitis globally results in around 1.4 million deaths each year, of which 90% are the result of HBV and HCV infections. In 2015, HBV alone caused nearly 1.5 million new hepatic infections and 820,000 deaths, a number which is comparable to TB and HIV [6]. Additionally, it is also known to be a leading cause of cirrhosis, hepatocellular carcinoma (HCC), and certain extrahepatic manifestations, the severity of which depends on the age and genetic predisposition of the individual [7,8]. Even though HBV is highly infectious, it is not directly cytopathic, instead, the damage is immune-mediated, due to the expression of viral proteins on the cell surface [9].

HBV is an enveloped virus, with a 3.2 Kb partially double-stranded DNA as its genetic material and is divided into 8 sub-types based on the variation in genetic composition and geographical location. All of these sub-types have a similar genomic design where 4 overlapping Open Reading frames (ORF) code for 7 different proteins, which are as follows Hepatitis B surface (HBs) protein (3 subtypes), Hepatitis B core (HBc) protein, Hepatitis B envelope (HBe) protein, X protein, and HBV DNA polymerase; all of which play major roles in the viral replication cycle from attachment and entry into the cell to completion of viral assembly and formation of Dane particles [10]. Another unique property of HBV is that it is the only known virus to use covalently closed circular DNA (cccDNA) and maintain it inside the hepatocyte nucleus as its replicative center. In certain cases, cccDNA is found in an intact form even after the seroclearence of the virus and is considered to be the reason for the re-emergence of the infection [9,10].

Current treatment for HBV focuses on viral suppression therapy using broad-spectrum antivirals, which include nucleot(s)ide analogs and PEG-interferon. The nucleot(s)ide analogs suppress viral replication by inhibiting the viral polymerase and the interferon therapy enhances the host immune response. Though the antivirals work they lack in providing a complete cure for the disease, also the first line regime includes entecavir, tenofovir fumarate, or tenofovir alafenamide, which have poor availability in Sub-Saharan African and West Pacific countries. Similarly, the PEG-interferon regime is not perfect as its efficacy is limited due to poor patient tolerance, but its seroclearence rate is high compared to other medications [9,11,12]. Also, HBV and HAV vaccines are available and have promising results, but they need to be given in a specific time frame to obtain the best results, e.g., the first dose has to be given within 24 hours of pregnancy, which is not a practical solution to consider on a global scale and only

34% new-borns receive this dose [5,6]. Thus, there is a need to discover and bring in new therapeutic interventions to aid in hepatitis treatment.

Among all HBV proteins, Hbc being the core unit forms the structural basis of the virus and is also known to play other major roles in the replication cycle and hence has recently been considered as a plausible target for drug discovery [12]. HBc protein in its active form is present as a dimer of identical 183-residue polypeptide chains. Several of such dimers bind together to form an icosahedral lattice structure called a Nucleocapsid. This is the main core of the virus where the relaxed circular DNA (rcDNA) and Viral DNA polymerase, which is a reverse transcriptase, are placed. Apart from being the structural component, HBc also plays other regulatory roles. One of which is the encapsulation of pre-genomic RNA (pgRNA) into the premature nucleocapsid; C-terminal domain (CTD) is found to be responsible for this process, the exact mechanism for which is yet to be discovered but is hinted to be based on the arginine-rich CTD amino acid sequence [12,13]. The arginine-rich sequence is also vital for the activity of HBV DNA polymerase (reverse transcriptase), which forms rcDNA from pgRNA, hence converting premature to mature nucleocapsid. The mature nucleocapsid further can complete the viral assembly and go on to infect other hepatocytes or it can enter the nucleus to replenish the cccDNA pool and help in the prevalence of infection. Additionally, HBc as an antigen has been shown to delay the immune response towards the infected cells and aid in the carcinogenic activity of X protein to give rise to HCC [12-15]. Thus, the importance of HBc in the viral cycle is quite clear and can be targeted for potential drug screening.

Drug discovery has come a long way since random trials and testing out toxins on animals, rather its primary goal lies in discovering and developing novel molecular scaffolds with high binding affinity and selectivity for the target while shortlisting candidates with a good pharmacokinetic profile [16]. Virtual screening procedures are being used commonly to uncover the abilities of available compounds to work as novel inhibitors, where in silico techniques like docking, pharmacophore mapping, and shape-based screening (SBS) can be used to screen candidate compounds having the potential to act against HBc from a huge dataset, and these potential molecules can further be subjected to pharmacokinetics and toxicity screening strategies to know their probable safety and efficacy [15,17]. Molecular docking lies at the baseline of such work and its purpose is to find realistic binding geometries for a suggested ligand with known target site(s) and precisely align the ligand at the binding site and evaluate the strength of the interaction. Based on the accuracy of results and the time required to obtain them, the in-silico strategies are gaining ground and being extensively used as a base strategy for novel drug discovery processes [18,19,20].

Currently, the most widely studied agents targeting HBc under trial are Core protein assembly modulators (CpAMs), which focus on inhibiting capsid assembly and pre-genomic RNA (pgRNA) encapsulation. The cost factor of CpAMs is the major drawback of this strategy. Also, they focus on a single step of the replicative cycle, instead of targeting multiple steps at a time [12]. Hence, in this study, we focus on evaluationg the potential of phytochemicals as anti-HBc compounds using molecular docking techniques and aim that the information generated from the study would be helpful to generate a list of candidates for refining and lead optimization process to develop an effective analog or cocktail of therapeutic interventions against HBV.

2. MATERIALS AND METHODS

2.1 Protein Retrieval

The HBc protein of Hepatitis B virus acts as a protective barrier for the nucleocapsid and has various functions which are essential for viral infection, thus the respective protein was targeted. A 3.5 Å-resolution structure of a recombinant core assembled from full-length Hepatitis B capsid/core virus protein (HBc) by cryo electron microscopy (cryoEM) (PDB ID: 32JV)(https://www.rcsb.org/structure/3J2V) [21] was retrieved from RCSB Protein Data Bank [22]. The 3-D structure of the same is shown in Fig. 1.

2.2 Ligand Retrieval

Using Dr. Duke's Drug Bank Database [23] and Indian Medicinal Plants, Phytochemistry And Therapeutics (IMPPAT) database [24], a total of 1855 phytochemicals were retrieved.

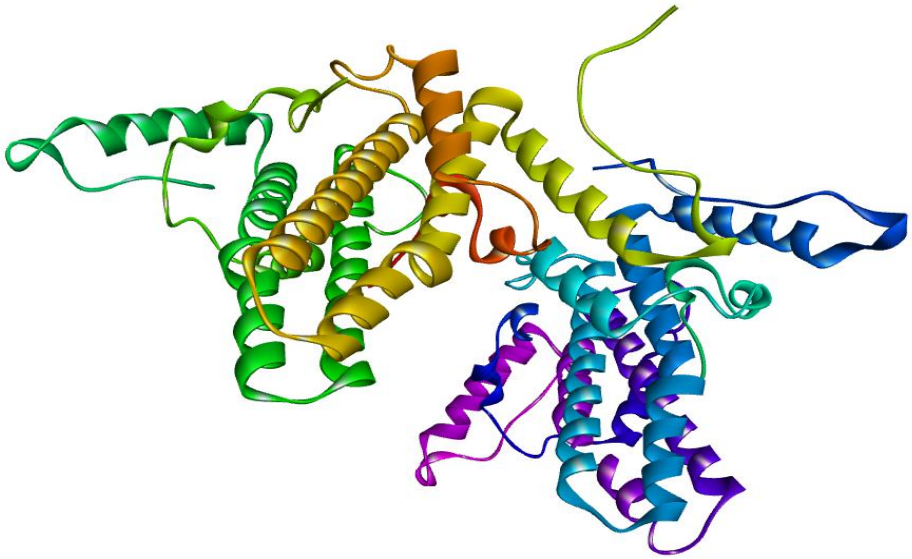

Fig. 1. 3-dimensional structure of Core protein of Hepatitis B virus

2.3 ADME Analysis

Drug-likeness and pharmacokinetics properties of a bioactive compound must be known to consider it as an eligible and viable candidate for drug discovery studies. An online web tool SWISS-ADME [25] was used to determine the above mentioned properties for the retrieved phytochemicals on the basis of Lipinski's rule of five. The canonical simplified molecular input line entry system (SMILE) of each of the phytochemical compound was submitted onto the webserver for determining its fulfilment of lipinski's rule of five. The compound must have a molecular mass less than 500 dalton, not more than 5 hydrogen bond donors (the total number of nitrogen-hydrogen and oxygen-hydrogen bonds), not more than 10 hydrogen bond acceptors and an octanol-water partition coefficient (XLog P) less than 5. [26].

2.4 Protein and Ligand Preparation

Protein: The 3-dimensional structure of the core protein of Hepatitis B virus was eradicated of the water molecules and was added with Kollman charges along with polar hydrogen atoms using UCSF Chimera tool 1.15 [27]. This protein molecule was then saved in PDB format.

Ligand: The bioactive compounds fulfilling the Lipinski's rule of five were chosen as the appropriate ligands. The 3-D SDF format files of the same compounds were obtained from the PubChem database. These formats were then used for structure variation generation, optimizing and minimizing the energy from the ligands via PyRx virtual screening tool [28].

2.5 Molecular Docking

The bioactive compounds fulfilling the Lipinski's rule were subjected to molecular docking with the Hepatitis core protein with Auto Dock Vina using PyRx virtual tool [28]. The free energy formed between the ligands and the core protein was determined. The Auto Dock Vina software's method of determining bound conformation of ligand-protein complex is based on free binding energies which are calculated using the empirical force field. The docking method aims to bind the ligand onto the active sites of the target protein and gives rise to the best docked conformation with minimal energy as an output. A non-rigid approach for docking was applied, in which the protein and ligand dimensions were kept flexible.

2.6 Analyzing and Output Visualization

The docking conformation of the ligand-protein complex with the lowest free binding energy was analyzed via Biovia Discovery Studio Visualiser [29]. The complexes formed between the respective ligand and protein with free binding energy greater than 8 Kcal.mol^{-1}were chosen and analyzed via their 2 and 3 dimensional structures. The analysis was based on monitoring and differentiating the intermolecular

interactions including conventional hydrogen bonds, hydrophobic interactions, Van der Waal forces, Pi- sigma bonds, Pi-Pi T shaped bonds, Alkyl bonds, Pi- Alkyl bonds, Unfavorable acceptor- acceptor bonds, Unfavorable donor-donor bonds, Carbon hydrogen bonds, Pi- donor hydrogen bonds and Amide Pi- stacked bonds.

2.7 Bioavailability Radar analysis

The compounds fulfilling the Lipinski's rule of five were subjected to the bioavailability radar i.e ADME analysis using physicochemical parameters [25] such as LIPO, Lipophilicity: -0.7 < XLOGP3 < +5; SIZE, Molecular size:150 g/mol < mol. wt. < 500 g/mol; POLAR, Polarity: 20 Å2 < TPSA <130 Å2; INSOLU, Insolubility: 0 < Log S (ESOL) < 6; INSATU, Instauration: 0.25 < Fraction Csp3 < 1; FLEX, Flexibility: 0 < Number of rotatable bonds < 9 were the positive indicatives of suitable oral consumption. These physicochemical parameters covered each axis within which the ideal radar parameters were represented as a pink region. So, for a phytochemical to be considered as a drug and suitable for oral consumption their corresponding radar should fall within the pink region. Any violation seen in the above-mentioned parameters directly rejects the chosen ligand for further analysis as it does not qualify the bioavailability radar requirements [30].

2.8 Toxicity Prediction

Computational toxicity predictions are rather faster and safer than the identification of toxic doses in animals, and also contribute in reducing the amount of animal experiments being conducted. The oral assessment for a bioactive compound involves absorption, distribution, metabolism, excretion and toxicological properties (ADMET) analysis. The above-mentioned parameters were calculated for the bioactive compounds with zero violations of Lipinski's rule of five and bioavailability radar to evaluate their oral consumption properties. Protox-II [31] and ADMET 2.0 [32] tools were used for the analysis. These tools are virtual webservers differing from each other on the basis of the properties measured. Protox II tool was used for determination of the Toxicity profile, Toxicity class and the median lethal dose-50 of the bioactive compounds showing no violations for lipinski's rule of five as well as bioavailability radar. The prediction system is associated with the chemical structure and the canonical simplified molecular input line entry system (SMILE) of the respective compound. The LD-50 value (in mg/kg weight) of the respective ligand (bioactive compound) places them on a toxicity class scale gradually increasing the class and safety of the compound. Class I being extremely toxic (LD-50 ≤ 5) and Class VI being non- toxic and safe to consume (LD50 > 5000) [33]. Furthermore, The ADMET lab 2.0 server was used for determination of toxicity parameters such as Herg Blockers, H-ht, DiliAmes toxicity, Eye irritation, Rat oral acute toxicity, Fdamdd, Skin sensitization, Carcinogenicity, Eye corrosion, Respiratory toxicity and Environmental toxicity [34].

3. RESULT AND DISCUSSION

Even though vaccines and antivirals exist, finding a perfect cure against HBV has been difficult. The antivirals which have been discovered and are currently in use have a broad spectrum of action and focus on a selective step to eliminate the replication cycle, which in turn affects the efficacy of viral elimination [11,6].The vaccines have proved to be effective against the spread, but cannot be considered as a preventive measure globally, due to the specific scheduled dosage requirements and low availability in developing and underdeveloped nations [5,4].The concept of using known natural compounds like phytochemicals or their analogs, individually or in combination as therapeutic interventions for a disease is fascinating and could be the necessary solution required to manage the global HBV healthcare burden [35,36,37,18].

HBV genome encodes for 7 proteins in total, of which HBc protein has recently been recognized as the most plausible target for the drug designing process, as it forms the structural basis of the virus and plays regulatory roles in other steps of the replication [38]. The Hepatitis B Virus Core gene (C gene), which is divided into the core and the pre-core regions, encodes for the HBc and HBe proteins respectively. The HBc forms the nuclear structure of the virus, while the HBe is a secretory protein responsible for the immune-modulatory effects in the host [14,39].

The 3D structure of the HBc protein, required to form the nucleocapsid and regulate the action of viral reverse transcriptase, was retrieved in PDB format from the RCSB PDB data repository. In this study, we have focused to investigate the binding of different phytochemicals to the HBc protein and hence to check their potential to work

as inhibitors of viral assembly and the replication cycle, using an in-silico molecular docking approach. 1855 ligands were selected for the same, the ligands chosen were of a diverse chemical nature and majorly belonged to flavonoids, organic compounds, alkaloids, polyphenols, terpenoids, carboxylic acids, steroids, quinones, carbohydrates, benzene and derivatives, and lipids and fatty acids groups (Fig. 2).

Computational in-silico molecular docking techniques were used in this investigation. A total of 1855 phytochemicals were chosen as ligands from the IMPPAT and Dr. Duke's Drug Bank Database.The selected phytochemicals were screened on the basis of Lipinski's rule of five. Compounds exhibiting zero violations were then docked with the HBc core protein as the target protein. The binding energies generated were documented. The toxicity levels of compounds with the lowest binding energy were examined using the PROTOX-II and ADMET tools. The binding energies were measured up to -8.5 Kcal/mol, and ligands with binding energies greater than -8.0 Kcal/mol were chosen. The complexes generated between these compounds and the target protein were then analyzed by applying 2 Dimensional and 3 Dimensional analysis using Discovery studio, which revealed the number of hydrogen bonds in each interaction. To examine the safety and efficacy of these compounds as antiviral medicines against Hepatitis B infection, the top 10 complexes were chosen based on drug likeliness, toxicity class, median lethal dose (LD 50), and number of hydrogen bonds formed.

3.1 Evaluation of Pharmacokinetic and Pharmacological Properties

Lipinski's rule of five helps us to check the bioavailability of ligands, that is the absorption, distribution, metabolism, and excretion (ADME) of the drug candidates concerning the host system. The rule gives us predefined values for the molecular and physicochemical properties in the form of the number of Hydrogen bond donors/acceptors, molecular weight, and lipophilicity; which have to be satisfied by the compound. To satisfy this rule the compound should have less than 10 H-bond acceptors, lees than 5 H-bond donors, Molecular weight below 500dalton and Log P, which indicates the lipophilic character, should be less than 5 [16,40]. Such prerequisites, which are given by the rule help us predict the nature of the drug, and in turn determine the likeliness of the compound to be successful as a drug candidate e.g., smaller lipophilic compounds would have higher permeability, and compounds with positive charge would have a better chance to be taken up by the cell using passive diffusion [16,42].

The compounds chosen for the study belong to several chemical families as shown in Fig. 2 and Lipinski's rule was used to determine their likeness to work as a drug. Out of 1855, 36.93% that is 685 compounds didn't show any violations of Lipinski's rule, while 63.07% compounds showed at least one form of violation of the rule. 24.85, 16.98, 13.15, and 8.09% compounds violated one, two, three, and four rules respectively (Fig. 3). Hence, these compounds were excluded from the study, while the compounds which didn't violate any rule were assigned for further investigation.

3.2 Molecular Docking

Molecular docking is an in-silico technique used to predict the interaction between ligands and their potential molecular targets using a structure-based scoring strategy. The workflow starts by predicting the possible molecular orientations of a ligand within a receptor and then scoring such orientations to estimate the best complementarity possible. This scoring compares the range of interactions based on their binding energies, which assists the drug development process by shortlisting the best ligand-target pairs to move forward in selecting the right drug candidate [15,43,19]. 685 bio available compounds chosen using Lipinski's rule were used to perform molecular docking with HBc protein. Table 1, shows the binding energy (BE) values of the top 10 ligands, among which Cladospironebisepoxide and 5-Dehydrouzarigenin had the best BE value of -8.5 kcal/mol, indicating a strong interacting potential with the target protein, HBc, while, Ingenol had the lowest BE value of -8.0 kcal/mol, and all others stayed in between. Table 2, shows BE values of currently used drugs for the treatment of HBV, of which Entecavir has the best BE of -6 kcal/mol; followed by Tenofovir, Lamivudine, Adefovir, and Telbivudine which have the BE values as -5.6, -5.4, -5.2, and -5.2 kcal/mol respectively. Fig. 4 shows a graphical representation of the standard and test drugs binding energies. According to which it was seen that, the chosen phytochemicals had better BE values than the drugs currently in the market.

These higher BE values indicate a strong interacting potential with the target protein due to the possible ligand-target bonding patterns and the strength of such bonds. The presence of alkyl and pi-alkyl bonds promotes the hydrophobic interaction of ligands into specific binding pockets of the target, while the presence of hydroxyl groups promotes the formation of hydrogen bonds to stabilize the interaction. Many such bonds exist that aid in stabilizing the ligand-target binding and free energies of the system, which are used to score the strength and efficiency of the interaction in the form of BE values. Considering the BE values of chosen phytochemicals with correlation to the present drugs, the phytochemicals seem to own untapped potential and can be considered for further processing.

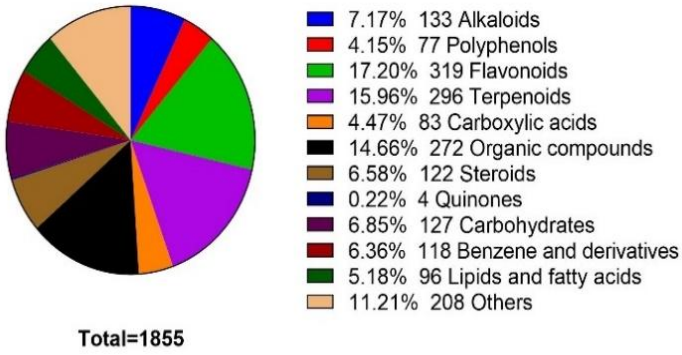

Fig. 2. Classification of phytochemicals chosen in the study

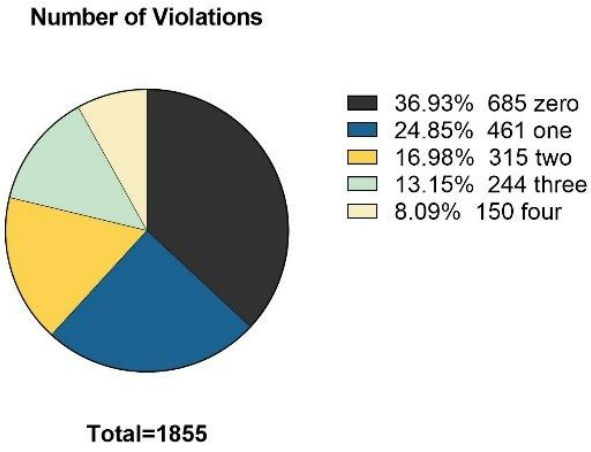

Fig. 3. Distribution of drugs based on violation of Lipinski's rule of five

Table 1. Table representing binding energy of top ten phytochemicals

Sr. No	Ligand	Binding Energy n(ΔG) (kcal/mol)
1	Cladospironebisepoxide	-8.5
2	5-Dehydrouzarigenin	-8.5
3	Gummadiol	-8.4
4	4-Hydroxysesamin	-8.3
5	Sesamolin	-8.2
6	l-asarinin	-8.1
7	Hetisinone	-8.1
8	Shinjulactone	-8.1
9	Withaferin	-8.0
10	Ingenol	-8.0

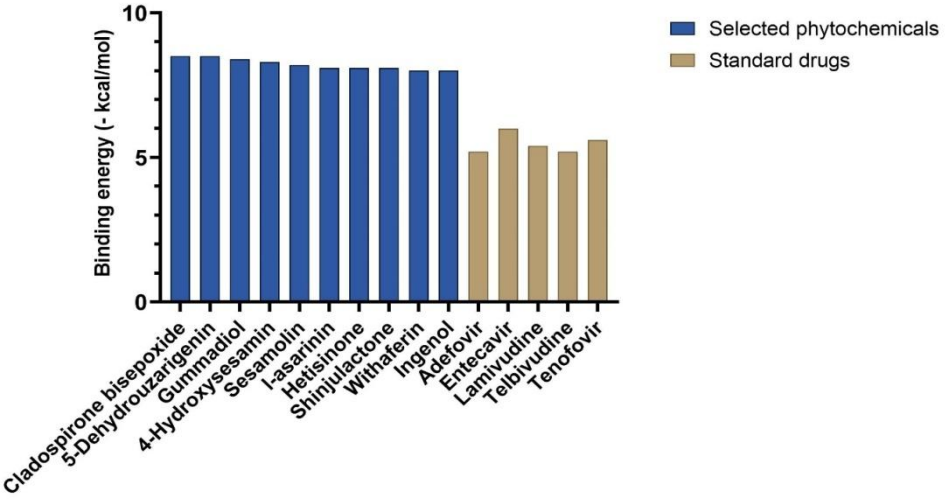

Fig. 4. Comparative binding energy of the best ligands and the drugs currently employed for the treatment of Hepatitis B virus

Table 2. Table representing binding energy of currently used drugs for the treatment Hepatitis B virus

Sr. No	Ligand	Binding Energy n(ΔG) (kcal/mol)
1	Adefovir	-5.2
2	Entecavir	-6
3	Lamivudine	-5.4
4	Telbivudine	-5.2
5	Tenofovir	-5.6

The Stability and bonding patterns of the top ten ligand-HBc protein complexes were analysed, among all the bonds observed during molecular docking, hydrogen bonds are considered most important in determining the specificity of the ligand and drug designing as they play a crucial role in drug absorption and metabolism.

Cladospironebisepoxide had a -8.5 kcal/mol BE value and formed 3 types of bonds with HBc. VAL 120 amino acid of chain C formed Pi-Alkyl as well as Pi-Sigma bonds, and LEU 37 of chain B formed Pi-Donor hydrogen bond and Pi-Sigma bond with the ligand. While THR B-146 formed a single carbon-hydrogen bond. According to the Bode *et al* [44], Cladosporine posses the antifungal, antitumour and antibacterial properties and dealt with its production procedure. In this research study, we explored the antiviral potential of Cladosporinebiepoxide. With the highest binding energy, Cladosporinebiepoxide can also be introduced to its antiviral characteristic, making it a suitable candidate for antiviral studies (Fig. 5).

5-Dehydrouzarigenin had a BE value of -8.5 kcal/mol. The ligand only formed Alkyl and Pi-Alkyl bonds with chain A of HBc using PRO 25, TRP 102, PHE 110, and ILE 139 amino acids (Fig. 6).

Gummadiol had a BE value of -8.4 kcal/mol and formed Alkyl, Pi-Alkyl, Pi-Sigma, and Conventional Hydrogen bonds with HBc protein. ARG 39 of B chain, and LEU 15 and VAL 120 of C chain HBc protein were involved in forming Alkyl and Pi-Alkyl bonds with the ligand, while LEU 37 of B chain and THR 12 of C chain formed Pi-Sigma bond and Conventional Hydrogen bond respectively. A study done by Bahadur Gurung *et al* [45], Gummadiol showed inhibitory properties against SARS-CoV-2 3CLpro, SARS-CoV 3CLpro and MERS-CoV 3CLpro exhibited by Coronavirus. Gummadiol exhibited its potential antiviral activity through hydrogen bond-interactions with either His41 or Cys145, and also showed the catalytic dyad of SARS-CoV-2 3CL proenzyme. By attaining a similar BE, -8.4 Kcal/mol, Gummadiol proves to be a potential antiviral activity showing candidate (Fig. 7).

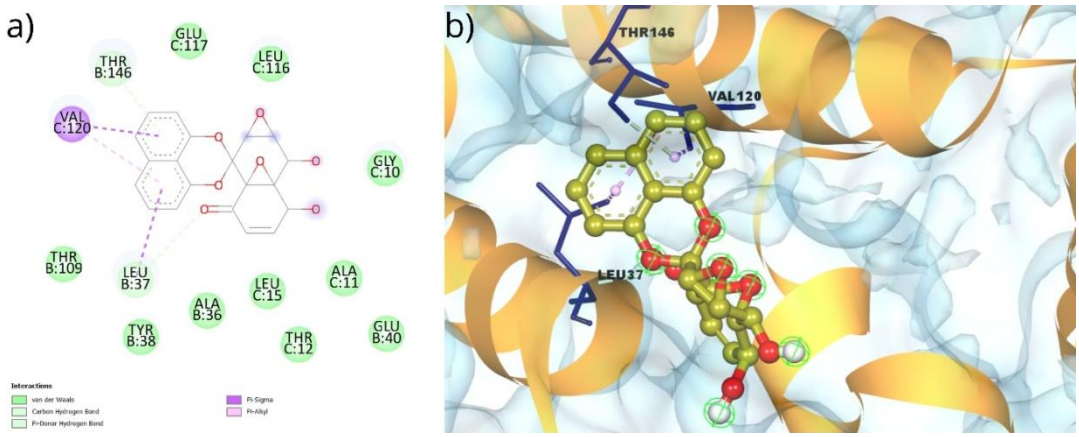

Fig. 5. 2D interaction plot and 3D bonding pattern showing the position of Cladospironebisepoxide within the cavity of Hepatitis B core protein

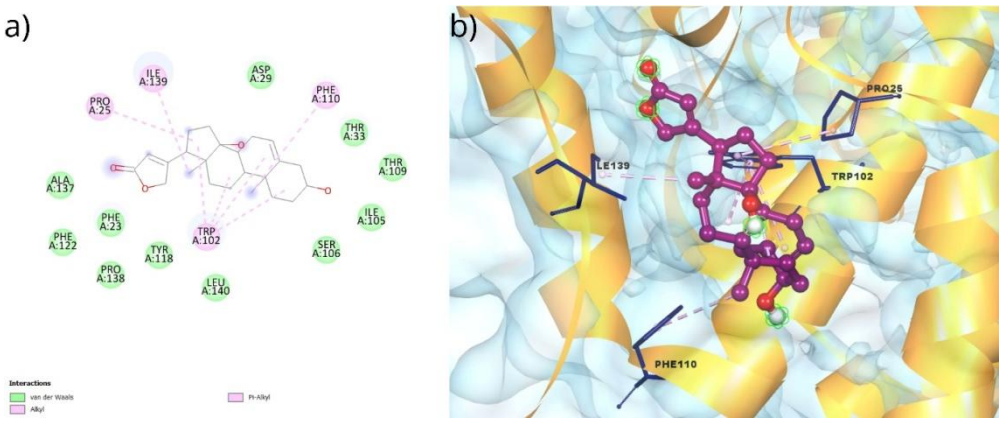

Fig. 6. 2D interaction plot and 3D bonding pattern showing the position of 5-Dehydrouzarigenin within the cavity of Hepatitis B core protein

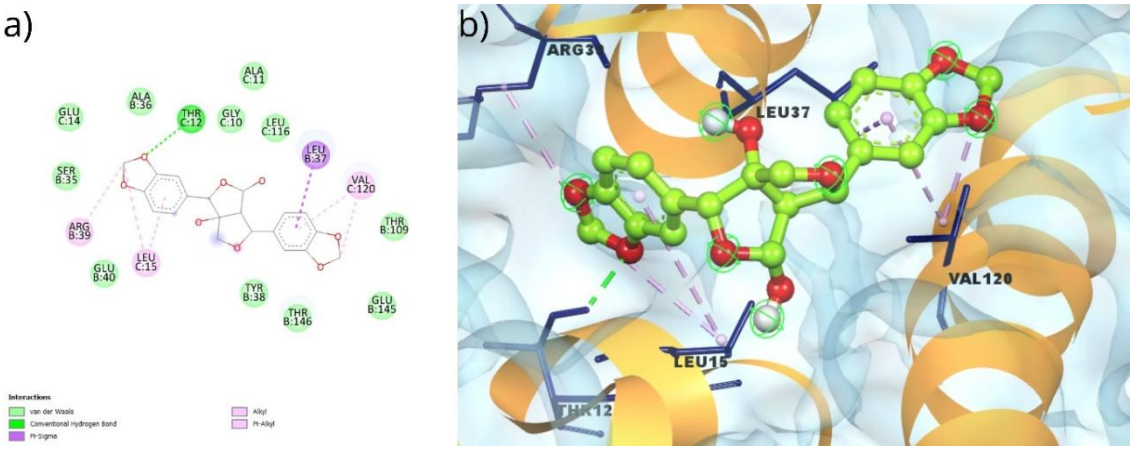

Fig. 7. 2D interaction plot and 3D bonding pattern showing the position of Gummadiol within the cavity of Hepatitis B core protein

4-Hydroxysesamin had BE of -8.3 kcal/mol. THR B-146 and LYS C-7 formed the two Conventional hydrogen bonds in the interaction. VAL C-120 formed Alkyl and Pi-Alkyl bonds, and LEU B-37 formed the Pi-Sigma bond with the ligand. ALA 36 of B chain formed an Unfavourable Acceptor-

Acceptor bond in the interaction as well. The ligand based phytochemical compound showing direct corelation to sesamin, hasn't been explored for its antiviral properties in any previous molecular docking research studies (Fig. 8).

Sesamolin had -8.2 kcal/mol as its BE value and formed only a single hydrogen bond with amino acid GLU 40 of the B chain. It also formed alkyl and pi-alkyl bonds with amino acids VAL C-120 and ARG B-39, and a single pi-sigma bond with LEU 37 belonging to the B chain of HBc protein In a study performed by Anuj Kumar, et al.[46] inhibitory activity of Sesamolin against Main protease protein of SARS-CoV-2 was evaluated. Sesamolin exhibited a binding energy of -6.4 kcal/mol and molecular interactions included Hydrogen bond: ARG105 (6.03 Å), GLN110 (4.52 Å), SER158 (4.08 Å) and Pi–sigma: VAL104 (4.89 Å). Since, current study using sesamolin against Hbc protein have resulted in a comparatively higher binding energy, it can be considered as a good candidate for antiviral studies. (Fig. 9).

I-asarinin scored -8.1 kcal/mol BE during the docking study with HBc protein, and it formed three conventional hydrogen bonds with ARG B-39, GLU B-40, and THR C-12 amino acids. VAL 120 of the C chain formed a pi-sigma bond and another alkyl and pi-alkyl bond, while LEU B-37 and LEU C-116 both formed alkyl and pi-alkyl bonds each with the ligand. According to a study conducted by Shradha Lakhera et al.,[47] I-asarinin showed a binding affinity of -10.8 kcal/mol with 3 conventional hydrogen bonds towards the target protein (receptor protein 4OVZ) of SARS CoV-2 in an in silico investigation of phytochemicals derived from *Piper Longum.* From this it could be seen that I-asarinin has a greater potential as an antiviral drug against SARS Cov-2 than Hepatitis B infection (Fig. 10).

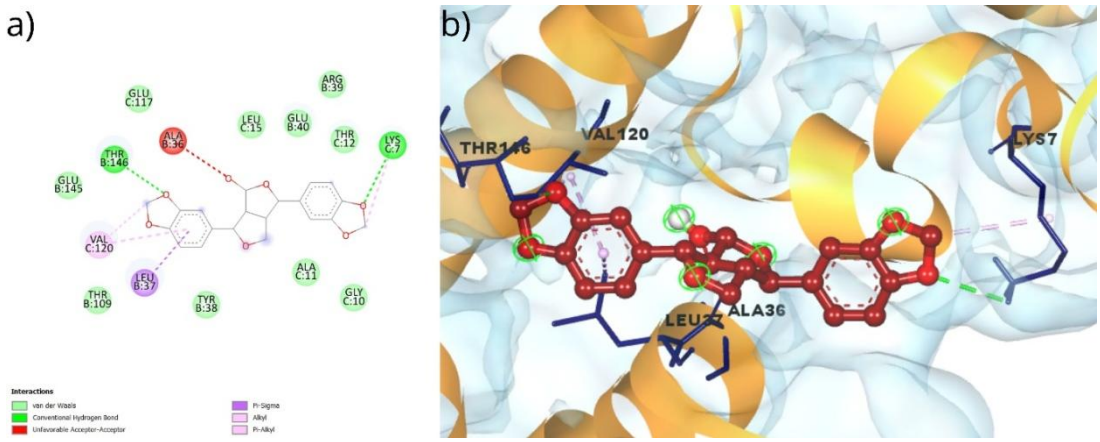

Fig. 8. 2D interaction plot and 3D bonding pattern showing the position of 4-Hydroxysesamin within the cavity of Hepatitis B core protein

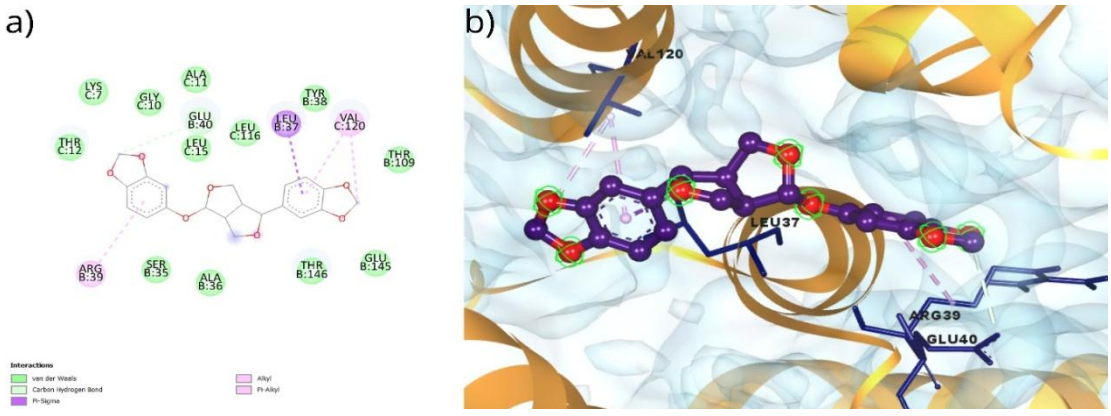

Fig. 9. 2D interaction plot and 3D bonding pattern showing the position of Sesamolin within the cavity of Hepatitis B core protein

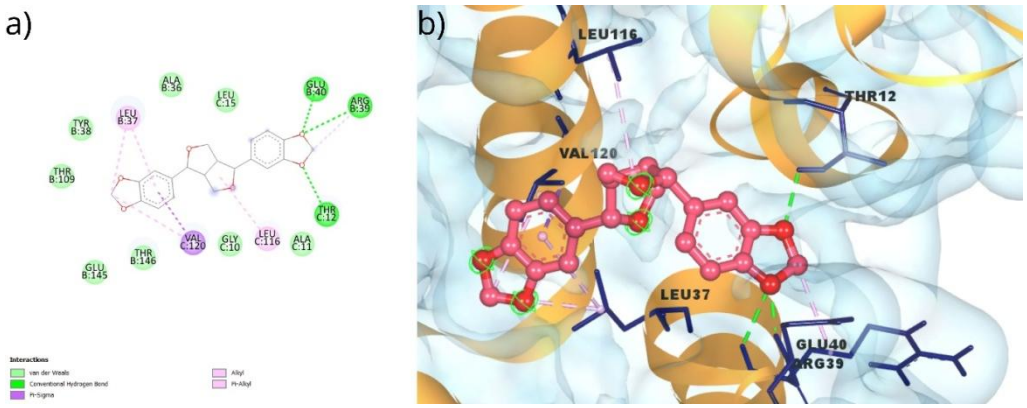

Fig. 10. 2D interaction plot and 3D bonding pattern showing the position of l-asarinin within the cavity of Hepatitis B core protein

Hetisinone had a BE value of -8.1 kcal/mol and formed 2 prominent types of bonds with chain A of HBc protein. It formed a conventional hydrogen bond with THR 33 amino acid, and alkyl and pi-alkyl bonds with LEU 30, TRP 102, ILE 105, and PHE 110 amino acids. In a study done by [48], Hetisinone showed the best BE being 8.46 kcal/ mol among the selected phytochemicals for their activity against ACE2, Importin subunit α-5, and Importin subunit β-1 of SARS-CoV-2. Hetisinone's broad spectrum showcasing its mode of action also presents a promising potential for it to become an antiviral drug against Hepatitis B infection as well (Fig. 11).

Shinjulactone scored -8.1 kcal/mol BE in its docking interaction with HBc protein and formed 2 types of bonds. LYS C-7 and PRO D-45 formed alkyl bonds with the phytochemical, while LEU B-37, GLU B-40, and ALA C-11 amino acids formed conventional hydrogen bonds. Belonging to the family of Quassinoids, Shinjulactone shows a promising anti-HIV activity with >266 µm Half-maximal inhibitory concentration (IC50) [49]. When compared, we can see that Shinjulactone is effectively inhibiting the Hepatitis B infection as well but with better and lower Toxicity levels. This makes the Shinjulactone a much better antiviral agent for Hepatitis B infection (Fig. 12).

Withaferin interacted with only the D chain of the protein and had a BE of -8.0 kcal/mol while forming 3 types of bonds. ASP 22 and SER 106 amino acids formed the conventional hydrogen bonds and PHE 23, TRP 125, ALA 137, ILE 139, and LEU140 formed an alkyl and pi-alkyl bonds with the ligand. A similar insilico study was performed where Withaferin was interacted with the cellular receptor Glucose regulated protein 78 (GRP78) of SARS-CoV 2 which formed a BE of -8.7 Kcal/mol [50]. This indicates that Withaferin is effective, although less affinitive towards Hbc protein than the cellular receptor Glucose regulated protein 78 (GRP78), antiviral agent for Hepatitis B Infection (Fig. 13).

Ingenol scored a BE value of -8.0 kcal/mol and formed 3 types of bonds, one of which is an unfavourable acceptor-acceptor bond with SER 35 amino acid of the B chain of HBc protein. LEU 15 amino acid of chain C formed an alkyl bond with the ligand. The third type of bond formed was a conventional hydrogen bond with the amino acids ALA 36, LEU 37, TYR 38, ARG 39, GLU 40, and ALA 41 of the B chain (Fig. 14).

Both 5-Dehydrouzarigenin and Ingenol phytochemicals have no previous antiviral potential exploration studies done before this research. 5-Dehydrouzarigenin presenting the second best interaction and a BE of -8.5 Kcal/mol with the Hbc core protein, and Ingenol being one of the best affinitive phytochemicals towards Hbc core protein are introduced to their antiviral characteristics, becoming suitable candidates for antiviral studies against Hepatitis B infection.

3.3 Bioavailability Radar and Toxicity Predictions

To be an effective drug candidate a molecule is required to be bioavailable, reach the target site in sufficient concentration, and not cause any off-site adverse effects. Analyzing the possibility of these off-site adverse effects along with ADME studies are considered as the prerequisites of the drug development process and traditionally

animal models have been used to carry out such toxicity tests. However, in vivo testing has numerous predefined ethical, financial, and time constraints, which makes the in-silico strategies a better option to analyze and predict the toxicity characters of numerous compounds at the same time. Also, many manufacturers prefer to produce oral drug formulations, due to their limited sterility requirements, design flexibility, cost-effectiveness, and administration convenience. When it comes to research and development of novel drug candidates, low aqueous solubility and high lipophilicity are major concern and limit the therapeutic effect of the compound. A molecule with low water solubility will also have low saturation coefficient and will affect its bioavailability. Hence, predicting the bioavailability of a compound is preferred at an early stage, to avoid significant financial losses later in the process of drug development.

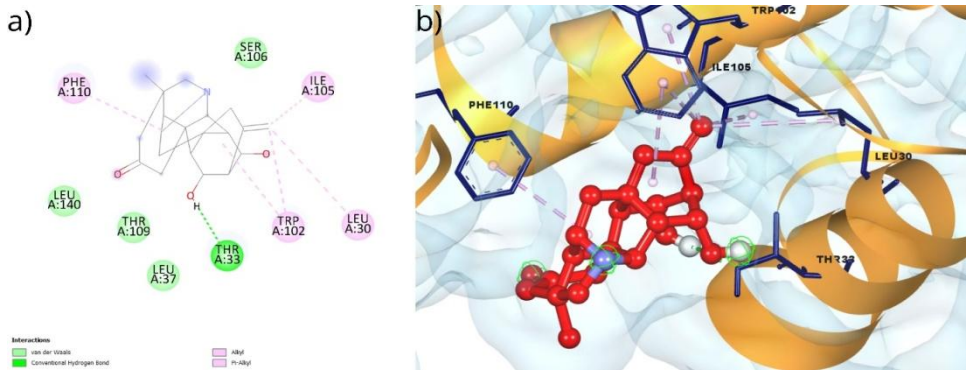

Fig. 11. 2D interaction plot and 3D bonding pattern showing the position of Hetisinone within the cavity of Hepatitis B core protein

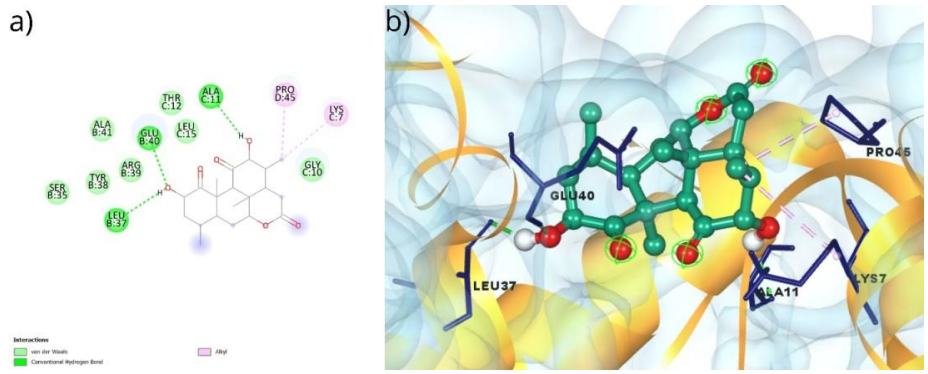

Fig. 12. 2D interaction plot and 3D bonding pattern showing the position of Shinjulactone within the cavity of Hepatitis B core protein

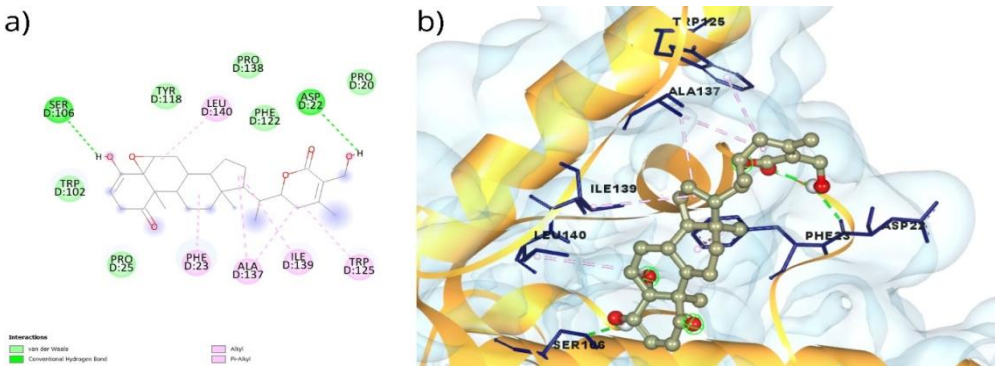

Fig. 13. 2D interaction plot and 3D bonding pattern showing the position of Withaferin within the cavity of Hepatitis B core protein

Table 3. Table representing the Toxicity prediction of best ligands

Ligands	Class	LD 50 (mg/kg)	Hepato-toxicity	Carcino-genicity	Immuno-toxicity	Muta-genicity	Cyto-toxicity	Conventional hydrogen bonds
Cladospironebisepoxide	2	34	Inactive	Active	Inactive	Active	Inactive	-
5-Dehydrouzarigenin	2	34	Inactive	Inactive	Active	Inactive	Inactive	-
Gummadiol	3	1500	Inactive	Inactive	Active	Inactive	Inactive	1
4-Hydroxysesamin	3	1500	Inactive	Active	Active	Inactive	Inactive	2
Sesamolin	4	1500	Inactive	Active	Active	Inactive	Inactive	-
l-asarinin	3	1500	Inactive	Active	Active	Inactive	Inactive	3
Hetisinone	4	500	Inactive	Inactive	Inactive	Inactive	Inactive	1
Shinjulactone	5	3900	Inactive	Inactive	Active	Inactive	Inactive	3
Withaferin	3	300	Inactive	Inactive	Active	Inactive	Active	2
Ingenol	4	665	Inactive	Inactive	Active	Active	Inactive	6

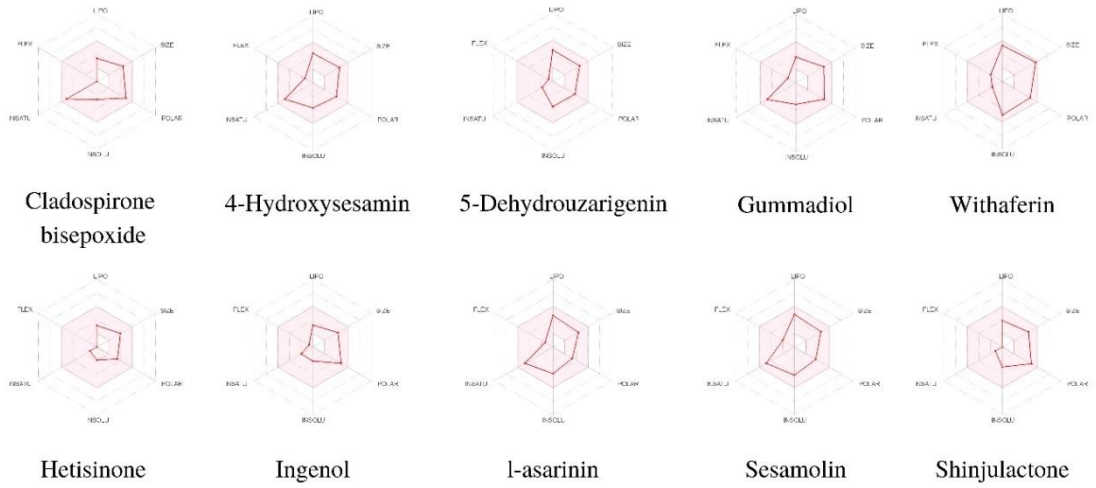

Fig. 15. Bioavailability Radar diagram of the best ligands

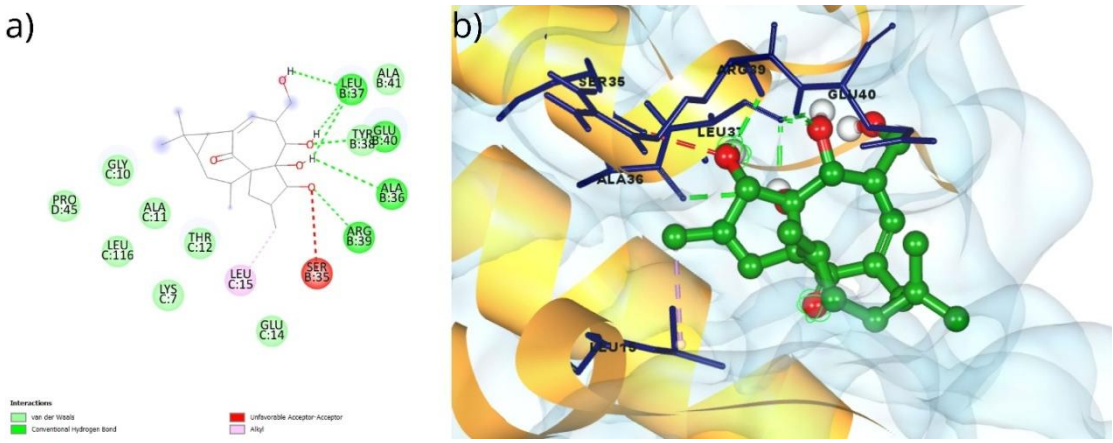

Fig. 14. 2D interaction plot and 3D bonding pattern showing the position of Ingenol within the cavity of Hepatitis B core protein

Following the ADME analysis, molecular docking, and molecular dynamic simulations, the phytochemicals were investigated for their bioavailability and toxicity profiles. Bioavailability radar- an expository tool- was used to predict the drug-likeness of the phytochemicals [44]. The tool analyses six physicochemical properties of a compound to determine its bioavailability, namely size, solubility, lipophilicity, flexibility, polarity, and saturation. All the analyzed phytochemicals were found to be orally bioavailable (Fig. 15) and were further assigned for in-silico toxicity testing using PROTOX-II webtool [33]. The toxicity analysis was based on the Toxicity class (Oral Toxicity), Predicted LD50, Hepatotoxicity, Carcinogenicity, Immunotoxicity, Mutagenicity, and Cytotoxicity which is represented in Table3.

Considering the toxicity and bioavailability profiles of the chosen phytochemicals, Ingenol can be inferred as the best candidate among the ten, for drug development, and to be used as a potential molecule therapeutic intervention against HBV. In comparison to other ligands, it formed the maximum number of hydrogen bonds, held a high LD50 value (665 mg/kg), and belonged to toxicity class IV. However, it expressed immunotoxic and mutagenic activity, which makes it inappropriate for oral consumption. Further analysis needs to be carried out to reduce its toxic characters and for making it a lead molecule for formulation development. Both I-asarinin and Withaferin formed a good number of hydrogen bonds with the target protein, and could also be considered for formulation development, but the dosage of these phytochemicals must be calculated as they possessed certain toxic characters and fall into class III based on the toxicity profile.

4. CONCLUSION

Despite the availability of vaccines and medications that can be used to manage HBV infections and even limit viral transmission, it continues to be a leading healthcare burden throughout the world. To solve this problem we need to find novel viral inhibitors and develop creative therapeutics in the form of formuations and analogs to work against the viral targets. In this study, we used in-silico strategies to screen phytochemicals as novel inhibitors of HBc protein. Nowadays such tests are used to reduce the time required to identify and pipeline new molecules as potential drug candidates in an effective and reliable manner.

In the first round of screening, the phytochemicals were shortlisted based on their pharmacokinetic properties and ADME characters using Lipinski's rule of five, which aided to identify the compounds, through a long list of molecules to be assigned for further investigation. Molecular docking was carried out on these shortlisted compounds using PyRx tool technologies, results of which revealed the best ligand-target pair binding conformations. Binding energies for the top ten selected ligands were way better than the BE values of the drugs currently used for HBV treatment. This inturn signified the potential of the natural compounds to be used directly or as analogs for therapeutic development. Further binding simulation analysis of the top ligand-target pairs gave their bonding patterns and presence of bond types in the interaction. Ingenol among all had the highest number of hydrogen bonds, which are crucial in predicting the ADME, metabolic, and inhibitory characters of the ligand. Further the toxicity and

bioavailability profiles were developed using in-silico tools to identify the potential compounds to be assigned for drug development pipeline. All the top compounds chosen for analysis proved to be necessarily bioavailable but a few among these also possessed certain toxic characters. Upon complete analysis, Ingenol, followed by I-asarinin and Withaferin were determined as the best candidates to be developed as effective therapeutic interventions. Hetisinone was found to be inactive for all the toxicological parameters, however, the ligand and target protein interaction exhibited lowest number of conventional hydrogen bonds which are essential for target interaction and activity. Hence, Ingenol was found to be the best candidate for therapeutic use but should be administered at concentrations below its LD50 value. In this way, in silico analysis expands the drug development possibilities and further experimental tests must be conducted to confirm the effectivity and determine the dosage limits of the chosen candidates.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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