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Therapeutic potential of *Solanum surattense* against hepatitis C virus in liver hepatoma cells

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Abstract: Hepatitis C virus (HCV) causes chronic liver disease (CLD) and severe morbidity and mortality worldwide. For chronically infected HCV patients, direct-acting antivirals (DAAs) are the standard therapy. HCV treatment is still hindered by drug resistance due to resistance-associated substitutions (RASs). New, innovative, and cost-effective anti-HCV drugs must be developed immediately. Therefore, this work employed different extracts of *Solanum surattense* (*S. surattense*) against full-length HCV-3a genome and HCV-NS3 plasmids in liver hepatoma cells at nontoxic concentrations to evaluate the therapeutic potential of the leaves and flowers of *S. surattense*. Data shows that the leaf extract effectively inhibited HCV-3a replication by 62% and 84% at 50µg/mL and 100µg/mL concentrations, respectively, compared to the control daclatasvir, which reduced HCV replication by 70% at 100 nM ($p<0.000$). At a 50 µg/mL concentration, the flower and leaf extracts significantly inhibited HCV-NS3 gene expression ($p<0.000$). Molecular docking investigations confirmed the in vitro results and showed that quercetin 3-glycoside, delphinidin 3,5-diglycoside, and catechin bound to HCV-NS3 protein (helicasee and protease domains) far superior to the positive control. In addition, both herbal extracts showed HepG2 cytotoxicity. The findings suggest that *S. surattense* may be a better HCV and HCC inhibitor and requires further study.

Keywords: Hepatitis C virus (HCV); chronic liver disease (CLD); *S. surattense;* hepatocellular carcinoma (HCC); WST-8 assay

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INTRODUCTION

HCV infection is a worldwide serious health problem with extreme involvement in mortality and morbidity (Petruzziello *et al.,* 2016). Usually, HCV is asymptomatic, but with the advancement of liver disease, liver fibrosis, liver cirrhosis, hepatocellular carcinoma (HCC), and even death occurs ((Javed *et al.,* 2011; Jiang *et al.,* 2019). Recently, the World Health Organization (WHO) revealed in its 2024 report that approximately fifty million HCV infections along with 244000 deaths globally were recorded in 2022, due to HCV-related complications. Pakistan led the globe with 8790812 infections and 24847 fatalities in this regard (Organization 2024). Therefore, abolishing of HCV infection the foremost goal of WHO by 2030 (Davis *et al.,* 2019). HCV is categorized into 8 major genotypes (GTs) and <90 subtypes (Dunford *et al.,* 2012). Amongst these, the treatment of HCV GT3 is particularly challenging due to the extensive genetic diversity in comparison to other GTs (Wang *et al.,* 2024) and is significantly prevalent in the Sindh province of Pakistan (Farooq *et al.,* 2024).

HCV is a single-stranded virus from the hepacivirus genus and *Flaviviridae* family that contains a genome of 9.6 kb (Reed and Rice 2000). It contains two untranslated regions alongside a single open reading frame (ORF) which encodes seven non-structural and three structural proteins (Spitz *et al.,* 2019). The high replication rate of HCV combined with fallible results has stemmed into the great sequence diversity of HCV (Piselli *et al.,* 2021). In addition, multiple barriers to the treatment scale-up embrace the availability of proper healthcare infrastructure, accurate diagnostics and monitoring tests, further making it more challenging to tackle (Amer 2018). Therefore, the available therapeutic intervention against HCV remains compromised because of the various side effects of DAAs (Gonzales 2018) and drug resistance due to the evolution of resistance-associated substitutions (RASs) (Sorbo *et al.,* 2018).

Phytochemicals have proven their substantial roles in both traditional and modern healthcare systems (Ezzat *et al.,* 2019). For example, quercetin from *Embelia ribes* inhibits HCV NS3/4A protease (Bachmetov *et al.,* 2012), epigallocatechin gallate (EGCG) from *Camellia sinensis* inhibits HCV-NS5A (Calland *et al.,* 2012), while flavonolignan phytochemicals silibinin-A and silibinin-B from *Silybum marianum* showed significant inhibition of HCV-NS5B in a cell culture system (Ahmed–Belkacem *et al.,* 2010).Several plant species, including *Solanum surattense* (*S. surattense*), contain more potential

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phytochemicals with good to excellent anti-HCV properties (Ravikumar *et al.,* 2011). *S. surattense* belongs to the Solanaceae family and is commonly known as yellow fruited nightshade or spiny nightshade, while its most common synonym is *Solanum xanthocarpum* (Sahar *et al.,* 2018, Tekuri *et al.,* 2019, Yousuf *et al.,* 2009). It has been practiced in conventional medicine for jaundice, wound healing, cough and liver disorders (Tekuri *et al.,* 2019).

Recently, NS3/4A protease/helicase inhibition studies have attracted significant interest as a potential therapeutic target for developing DAAs to treat HCV infections (Khan *et al.,* 2022), as HCV has produced significant resistance against NS5A and NS5B inhibitors due to the evolution of multiple RASs in its genome (Douglas *et al.,* 2020; Ramirez *et al.,* 2016; Douglas *et al.,* 2020). So the treatment of HCV GT3 is much more difficult as compared to other GTs due to Worldwide genomic variations (Wang *et al.,* 2024).

The NS3 protein of HCV has both helicase and serine protease domains (Wahaab *et al.,* 2021). During the replication of viral RNA, the helicase domain of NS3 utilizes adenosine triphosphate (ATP) as an energy source to unwind double-stranded RNA in a 3′ to 5′ direction (Flechsig 2014; Krishnasarma 2020). Helicase residues Lys 210 and Asp 290, positioned in the conserved motifs Walker A and Walker B, are the key for water-mediated ATP hydrolysis (Appleby *et al.,* 2011; Kim *et al.,* 1998; Lin and Kim 1999; Tai *et al.,* 2001). Hence, the inactivation of Trp 501 completely inhibited the unwinding activity of helicase (Lin and Kim 1999; Tai *et al.,* 2001). The HCV NS3 serine protease domain is responsible for cleaving the viral polyprotein at four specific sites, which are the interfaces between NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 (Wahaab *et al.,* 2021). The incredible activity of the serine protease makes it a promising target for inhibiting HCV replication (Khan *et al.,* 2022; McGivern *et al.,* 2015). The HCV NS3 serine protease domain contains many physiologically active residues (Xue *et al.,* 2014), including a catalytic triad consisting of three essential residues: His 57, Asp 81 and Ser 139 (Patil *et al.,* 2022; Raney *et al.,* 2010). In this context, the present study was aimed to screen the therapeutic potential of the extracts of *S. surattense* and to lay the foundations for the development of an innovative and cost-effective anti-HCV therapy with the least concomitant adverse effects.

MATERIALS AND METHODS

Selection, Collection and identification of S. surattense

The medicinal herb was selected based on the undocumented anti-HCV reports and its extensive traditional uses against liver ailments. The plant specimen was obtained in September 2018 from the Desert of Cholistan, Bahawalpur, Pakistan, and authenticated by the Pakistan Museum of Natural History Islamabad, Pakistan. Its Accession No. on the herbarium sheet was 043720.

Extraction of different parts of the plant

The maceration method was used to extract the leaves and flowers of *S. surattense* (Organization *et al.,* 2008). Plant material was air and shade-dried at room temperature for 15 days and ground with an electric grinder to obtain the coarse powder. After that, 50 grams of each part of the herb was soaked into 500ml 70% ethanol at room temperature, agitated vigorously twice a day for one week and filtered by Whatman filter paper. The filtrates were concentrated, dried in a hot air oven at 40ºC to obtain solid herbal extracts and stored at -4ºC in glass vials until further experimentation (Khalid *et al.,* 2022). Both extracts from different parts of *S. surattense* were labeled as flowers and leaves.

Preparation of stock solutions

A stock solution of each extract was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) with concentrations of 50 mg/ml (Rehman *et al.,* 2011). All stock solutions were filtered using 0.22 µm syringe filters and stored at -20^0C until further experimentation (Ashfaq *et al.,* 2011).

Serum collection

The high-titer serum samples of local HCV-3a patients, used in this study, were taken from GC University Faisalabad, Pakistan. Serum samples were stored at -80ºC until viral inoculation experimentation (Rehman *et al.,* 2011). Quantification of HCV was done by Diascient Diagnostic Laboratory, Faisalabad, Pakistan. Written consent from the patients and approval by the Institutional Ethics Committee were obtained before this study.

Cell culture medium

Hepatic Carcinoma Cell lines, HepG2 & Huh7, were provided by CEMB, Lahore, Pakistan. HepG2 and Huh7 cells were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco-USA) containing 10% fetal bovine serum (FBS, Gibco-USA) and 1% penicillin/streptomycin (Penstrep, Gibco-USA) at 37^0C and 5% CO_2 in the incubator (Bokhari *et al.,* 2007). To subculture the cells, old media was removed from the flask. Cells were washed with 1x phosphate buffer saline (PBS, Gibco-USA) and incubated with trypsin ethylenediaminetetraacetic acid (Trypsin EDTA, Gibco-USA) (1 ml/75cm^2) for 2-5 minutes (Rehman *et al.,* 2016). Following the detachment of cells, trypsin was inactivated by adding FBS containing medium (Ammerman *et al.,* 2008). Subsequently, the solution was centrifuged at 1000 rpm for 5 minutes (Miyamoto *et al.,* 2018) using a temperature-controlled centrifuge machine (HERMLE, Z326K, Germany) to obtain the cell pellet. After that, the supernatant was discarded, the cell pellet was re-suspended in fresh cell culture medium (Myint *et al.,* 2019) and cells were counted via a hemocytometer.

Finally, cells were sub-cultured at a concentration of 2.8 x 10^4 - 3.2 x 10^4 cells/cm^2 in a sterilized cell-culture flask and kept in a CO_2 incubator (Memmert-Germany, Model No. ICO50 Med).

Cell proliferation assay/toxicological analysis of plant extracts by WST-8 assay

WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4disulfophenyl)-2H-tetrazolium, monosodium salt] is reduced into water soluble orange coloured formazan outside the cell by the action of cellular NADPH that transfers electrons to an electron mediator 1-methoxy phenazine methosulfate (1–methoxy PMS), in turn, 1-methoxy PMS transfer these electrons to WST-8 and the formazan readily solubilized in the cell culture medium (Chamchoy *et al.,* 2019).

To determine cell proliferation, 2×10^4 HepG2 cells/well were cultured into a 96-well plate and kept at 37°C and an atmosphere of 5% CO_2 in an incubator (Ganta *et al.,* 2017; Javed *et al.,* 2011). After 24 hours different concentrations of herbal extracts were added (50µg/ml to 400 µg/ml, except for control) and kept in a CO_2 incubator for another 24 hours. After that, media and extracts were replaced with fresh media, and 10 µl WST-8 solution/cell counting kit-8 (CCK-8, Medchem express-USA) was added to each well (Cai *et al.,* 2019; Matsuoka *et al.,* 2000). The plate was wrapped in an aluminum foil and incubated at 37°C for 3 hours. The orange-coloured WST-8 formazan product was analyzed by reading absorbance using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek, USA) at 450 nm (Chamchoy *et al.,* 2019; Chen *et al.,* 2020) and a reference wavelength of 630 nm (Khalid *et al.,* 2022). The viability (%) was measured by the following equation (Rehman *et al.,* 2016).

$$\text{Cell Proliferation/viability (\%)} = \frac{\text{Abs. of Test}\ \ \text{Abs. of Ref. (Test)}}{\text{Abs. of Control}\ \ \text{Abs. of Ref. (Control)}} \times 100$$

Untreated cells were considered as a control and all experiments were performed in triplicate.

Anti-HCV effects of S. surattense against the full-length virus

To evaluate the anti-HCV effects of *S. surattense,* Huh7 cells were cultured at a concentration of 10^5cells/well in 12-well plates (Ratnoglik *et al.,* 2014; Rehman *et al.,* 2011) and incubated in a 5% CO_2 incubator at 37°C for 21 hours. After that, media was replaced and cells were treated with daclatasvir 100 nM (positive control) as well as different nontoxic concentrations of herbal extracts (Wang *et al.,* 2014) in triplicate for 3 hours before inoculation (Bachmetov *et al.,* 2012). In these studies, high titer (3.5×10^6 IU/ml) serum from HCV genotype-3a patients was used as the principal inoculum. After 3-hour treatment, cells were infected with 200µl (7×10^5 IU/well) of serum and incubated for a further 24 hours (Ravikumar *et al.,* 2011).

Extraction of total RNA

After 24 hours, cells were washed with 1x PBS twice and 500µl of triazole reagent (Invitrogen-USA) was added to each well. Each sample (trizol with lysed cells) was transferred into sterilized eppendorf tubes separately and supplemented with 10µl virus-like particles (internal control). Total RNA was extracted according to the protocol described by (Rio *et al.,* 2010). The total RNA pallet was completely dissolved in diethylpyrocarbonate (DEPC) treated water and stored at -20°C till further analysis.

Titration of HCV by RT-qPCR

The amount of extracted RNA was quantified by nanodrop spectrophotometer and equal RNA amounts from each sample were used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis using an RT-qPCR machine (Corbett-Australia, Model No. RG-6000). HCV RT-qPCR kit (Acon, USA) was used to quantify HCV copies (IU/ml) for each sample. RT-qPCR was carried out according to the manufacturer's instructions. Each sample (20µl) was used separately with 20µl of master mix. The following formula was used to calculate the concentration of HCV-RNA for each sample (Javed *et al.,* 2011; Rehman *et al.,* 2016).

$$\frac{\text{Cy3STD/Res}}{\text{Fam.STD/Res}} \times \text{coefficient IC} = \text{IU HCV/ml}$$

IC = internal control which is specific for each lot.

Effects of S surattense extracts on HCV-NS3 protease expression

The effects of extracts on HCV-NS3 were analyzed by the same protocol as previously reported (Javed *et al.,* 2011). Briefly, 5 × 10^4 HepG2 cells were seeded in 24-well plates for 24 hours. The next day, the cells were transfected with pcDNA 3.1 expression vector encompassing HCV-NS3 protease gene using Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA). Alongside, the cells were treated with different concentrations of extracts. After 24-hour incubation, the cells were subjected to tRNA extraction using Trizol reagent (Invitrogen-USA). Subsequently, cDNA was synthesized using Revert Aid™ First Strand cDNA Synthesis Kit (Invitrogen Life Technologies, USA), followed by gene expression analysis via Real-time qPCR (7500 Thermal cycler, Applied Biosystems Inc., USA) using SYBER green master mix (Fermentas). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an indigenous control. These genes were amplified using the following primers.

NS3 forward primer GGACGACGATGACAAGGACT
NS3 reverse primer CCTCGTGACCAGGTAAAGGT
GAPDH forward primer
ACCACAGTCCATGCCATCAC
GAPDH reverse primer TCCACCACCCTGTTGCTGTA
All tests were performed in duplicate.

ETHICAL APPROVAL

Manuscripts containing data has been approved by Institutional Review Board of Government College University Faisalabad Pakistan (Ref. No.GCUF/ERC/ 8237).

STATISTICAL ANALYSIS

In these studies, experiments were performed in duplicates/triplicates, and one-way ANOVA was applied to analyze the results using Statistical Package for Social Sciences (SPSS) version 23.0, Chicago, IL, USA. In this statistical analysis, probabilities of $p<0.05$ were considered significant. Figs. were made using Microsoft Excel 2016.

In silico screening of phytochemicals using the PyRx software package

The objective of this study was to perform the docking of the compounds (ligands) with HCV-NS3 protein (PDB ID: 4a92) (Wadood *et al.,* 2014b) using the PyRx software package. The Biovia Discovery Studio 2021 software was used to visualize the interactions between the HCV-NS3 helicase/protease and the ligands (Ali and Naser 2022).

Preparation of phytochemical library

To achieve the objective, 59 compound structures of particular plants were obtained from the Dictionary of Natural Products 2014 (DNP, 2014) and 30 compound structures from a literature review (Wadood *et al.,* 2014a). Subsequently, the 2D structures of the compounds were retrieved from PubChem and the compounds that were not available in PubChem were drawn using ChemDraw Ultra 14.0 software (Khan *et al.,* 2013; Khan *et al.,* 2022) and saved in sdf format (Patil *et al.,* 2022). Following that, the Open Babel (2020) was employed to model the 3D structure and minimize the energy of the ligands using the Force Field MMFF94X (gradient: 0.05) and the resulting 3D molecules were saved in the pdbqt format to be used as input for molecular docking in the next step (Azeem *et al.,* 2024; Patil *et al.,* 2022). The boceprevir, a well-established drug against HCV, was used as a standard ligand to compare the docking results of phytochemicals. The structures of standard drug (boceprevir) and top three compounds are shown in fig. 4.

Selection and preparation of receptor protein

The solved protein molecule, HCV-NS3 helicase/protease, was obtained from the Protein Data Bank (PDB ID: 4a92) (Wadood *et al.,* 2014b). Polar hydrogen atoms were added and additional ligands, H_2O molecules, and solvent residues were deleted. Subsequently, the protonation, energy minimization, and conversion of the receptor protein, into pdbqt format, was carried out through the Macromolecule tool of PyRx (Azeem *et al.,* 2024).

Molecular docking

The ligands were subjected to molecular docking for the screening of antiviral phytochemicals against HCV-NS3 through the PyRX virtual screening program (Azeem *et al.,* 2024). To determine the accurate conformations of the ligands and obtain structures with minimal energy the ligands were allowed to display flexibility (Wadood *et al.,* 2013). After the docking process, the Biovia Discovery Studio 2021 software was used to visualize the interactions between the receptor protein and significantly active ligands and the most optimal configurations of the ligands were analyzed to determine their best binding interactions. The poses were ranked based on the minimum S-score and compared to the standard drug, boceprevir (Ali and Naser 2022; Azeem *et al.,* 2024).

RESULTS

S. surratense was collected based on its hepatoprotective effects and undocumented reports of antiviral activity against HCV. Herbal materials including flowers and leaves of *S. surattense* were extracted in 70% ethanol. Before extraction material was shade and air dried. Dried material was lost its weight (moisture content) upto 78.40% and 75.96% for leaves and flowers, respectively. Following that dried material was underwent extraction. Yield of extract was found to be 21.92% for leaves and 30.76% for flowers.

Cytotoxic effects of S. surattense against HepG2 cell line by WST-8 assay

Before the antiviral screening against HCV, the nontoxic concentrations of extracts, including 70% ethanolic extracts of flowers and leaves, were determined using the WST-8 assay against HepG2 cells. For this purpose, HepG2 cells were cultured at a concentration of 2×10^4 cells/well in a 96-well plate and placed in a CO_2 incubator for 24 hours. After that, cells were treated with varying concentrations including 50-400µg/ml of plant extracts for a further 24 hours and cell viability was determined through WST-8 assay. The cytotoxicity (%) was found to be highly significant ($p<0.000$) in different concentrations of different extracts. Both flower and leaf extracts showed significant cytotoxicity at concentrations of 150-400 µg/ml ($p<0.000$), when compared to the control, in a concentration dependent manner. In contrast, showed insignificant cytotoxicity at concentrations of 50µg/ml and 100µg/ml ($p>0.05$), when compared to the control. The cell viability (%) of flower and leaf extracts were 67.21±7.34 and 71.31±4.18%, respectively, at a concentration of 150µg/ml. Lower concentrations of plant extracts are related to a higher cell viability (%). Therefore, for further investigation, nontoxic concentrations of the extracts 3.125-100 µg/ml were used to examine our proof of concept (fig. 1).

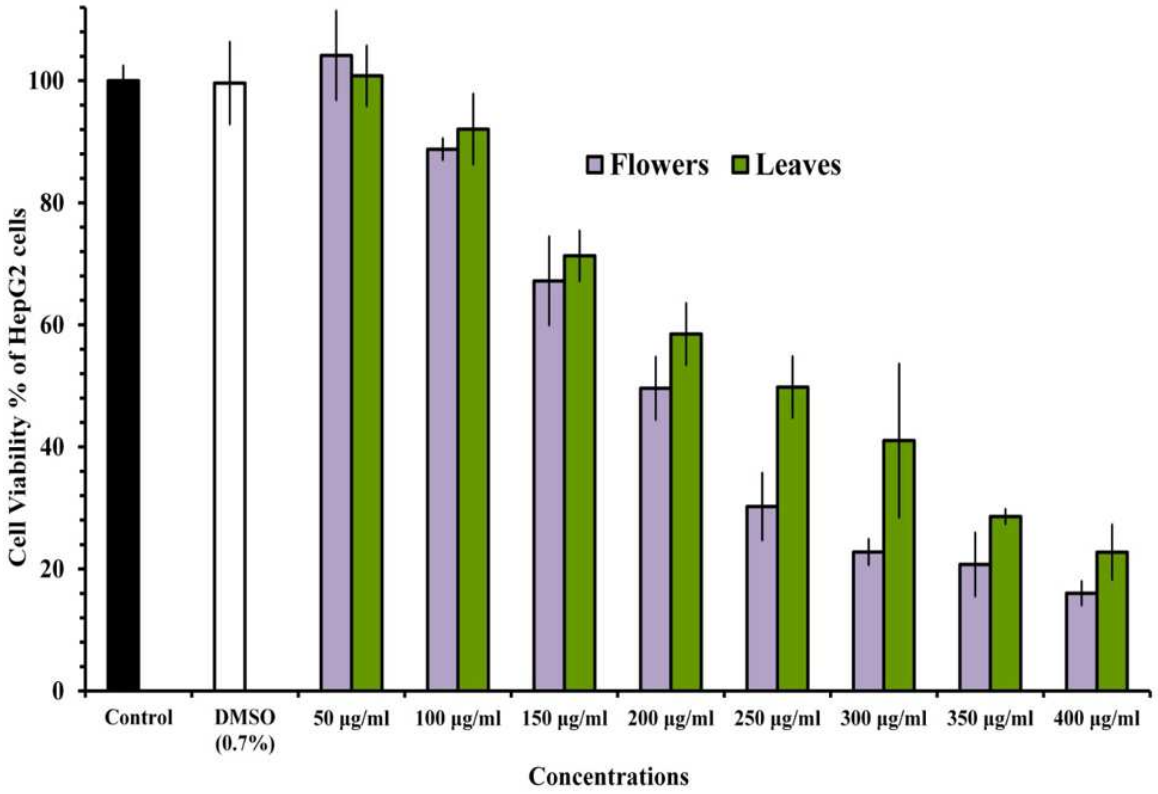

Fig. 1: Toxicological analysis of the extracts of herb using WST-8 cell proliferation assay: Details are mentioned in the Methods (2.6. and 2.9.) and results (3.1.) sections and tables 3S & 4S.

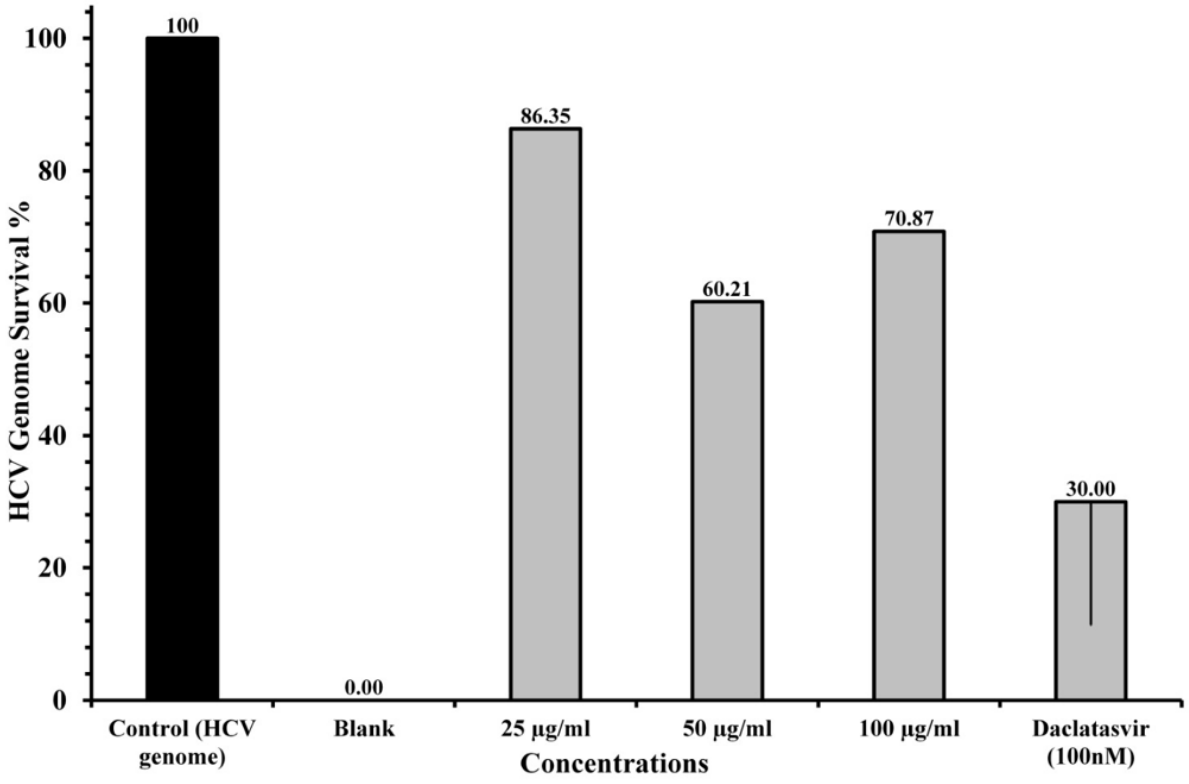

Fig. 2A: Antiviral activity of flower extract against HCV-3a genome: Details are mentioned in the Methods (2.7. and 2.9.) and results (3.2.1.) sections.

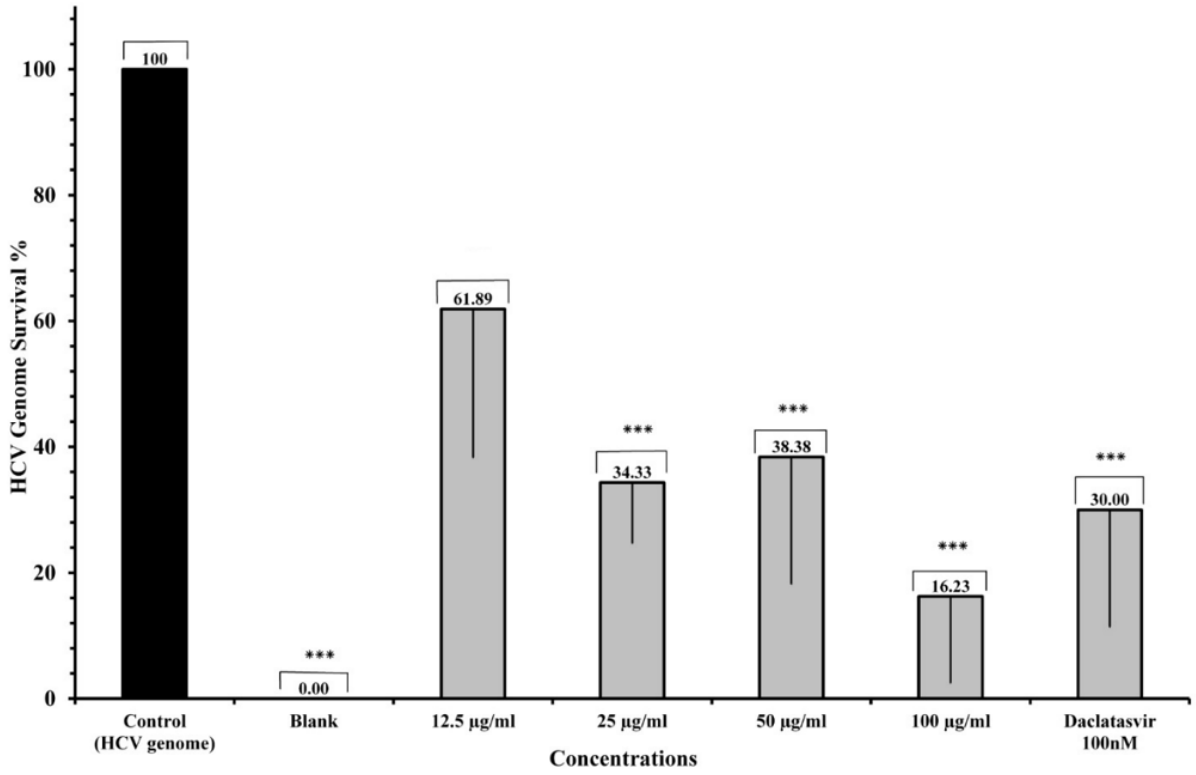

Fig. 2B: Antiviral activity of leaves extract against HCV-3a genome: Details are mentioned in the Methods (2.7. and 2.9.) and results (3.2.1. and 3.2.2) sections and table 5S.

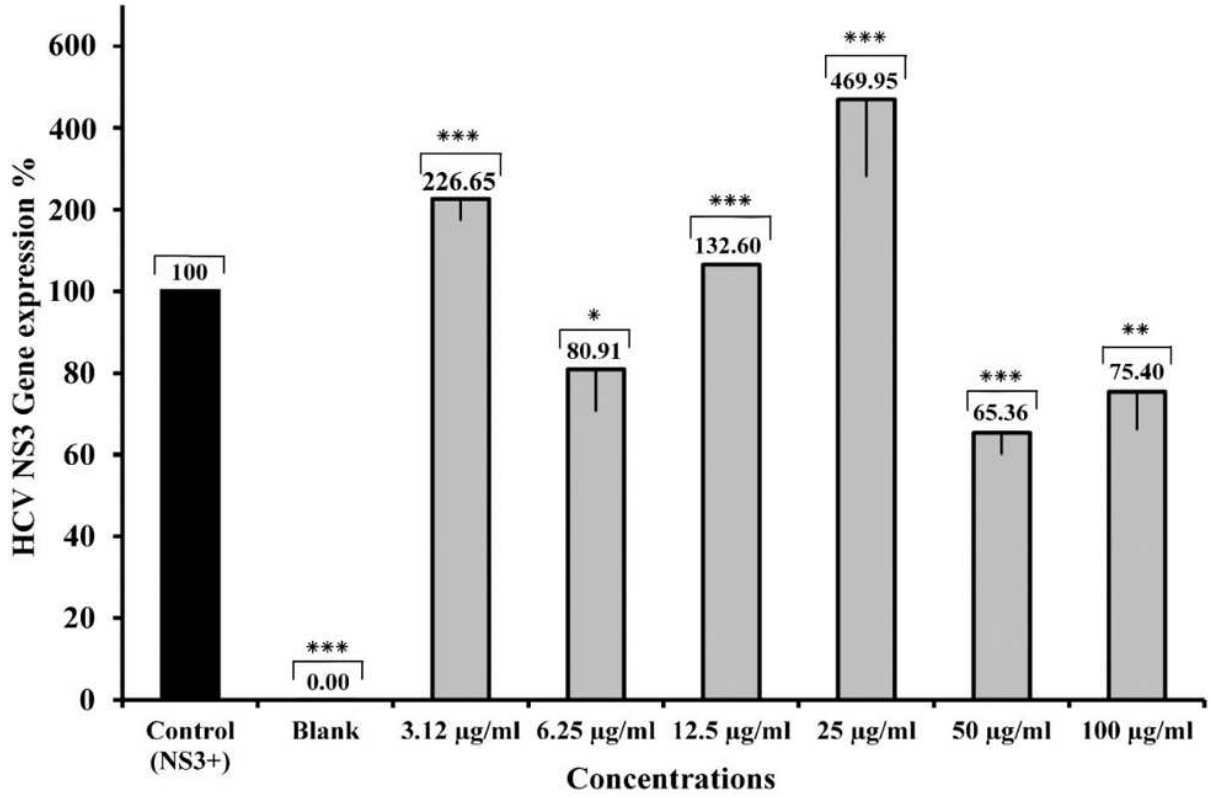

Fig. 3A: Analysis of HCV-NS3 gene expression by flower extract: Details are mentioned in the Methods (2.8. and 2.9.) and results (3.3.1. and 3.3.2) sections and table 6S.

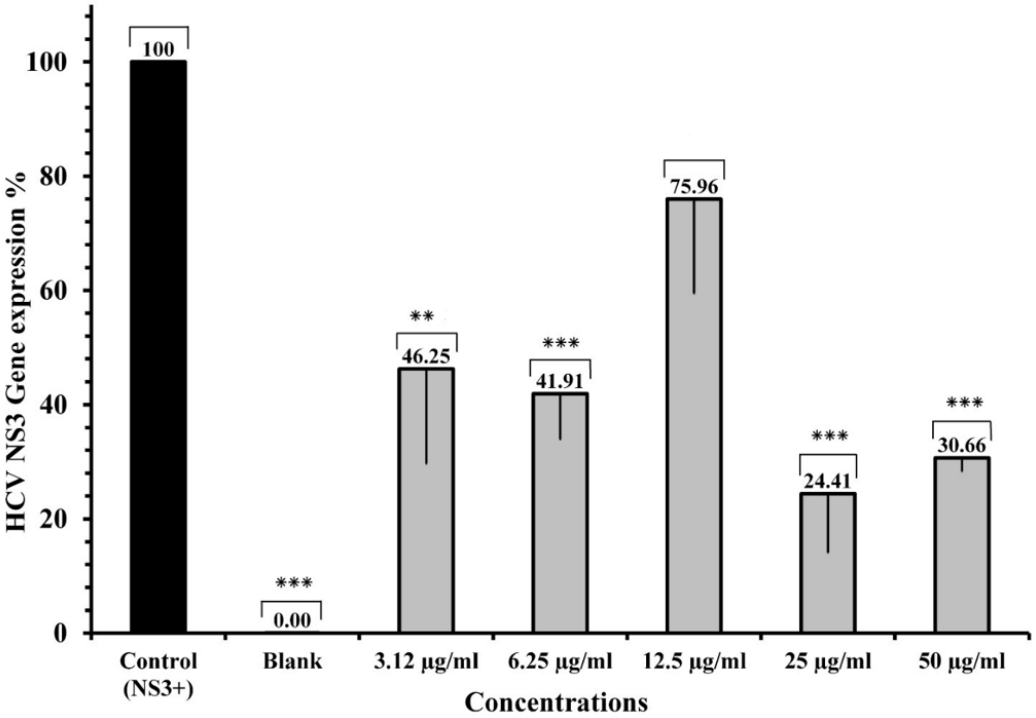

Fig. 3B: Analysis of HCV-NS3 gene expression by leaves extract: Details are mentioned in the Methods (2.8. and 2.9.) and results (3.3.1. and 3.3.2) sections and table 7S.

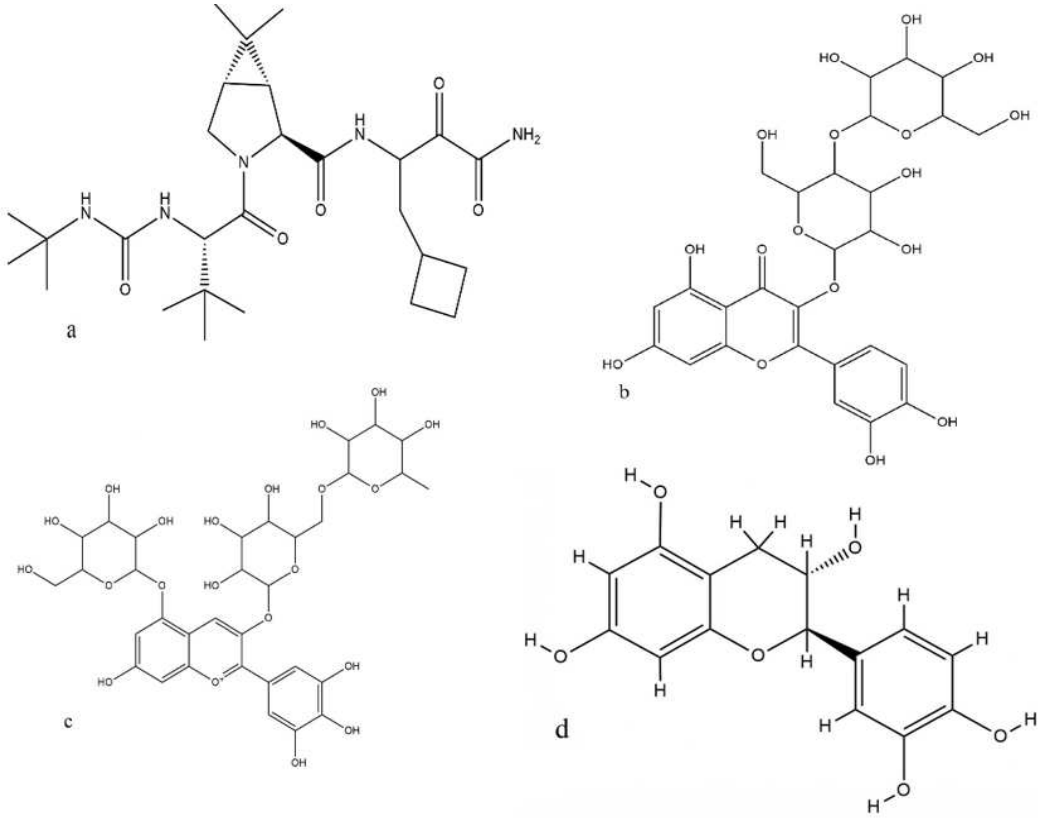

Fig. 4: (a) Standard drug boceprevir (PubChem ID: 10324367), (b) 3-*O*-[β-D-Glucopyranosyl-(1→4)-β-D-mannopyranoside a derivative of quercetin 3-glycoside (CRC No. KWD99-A), (c) 3-*O*-[α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside], 5-*O*-β-D-glucopyranoside a derivative of delphinidin 3,5-diglycoside (CRC No. KWN36-D), (d) catechin (PubChem ID: 9064).

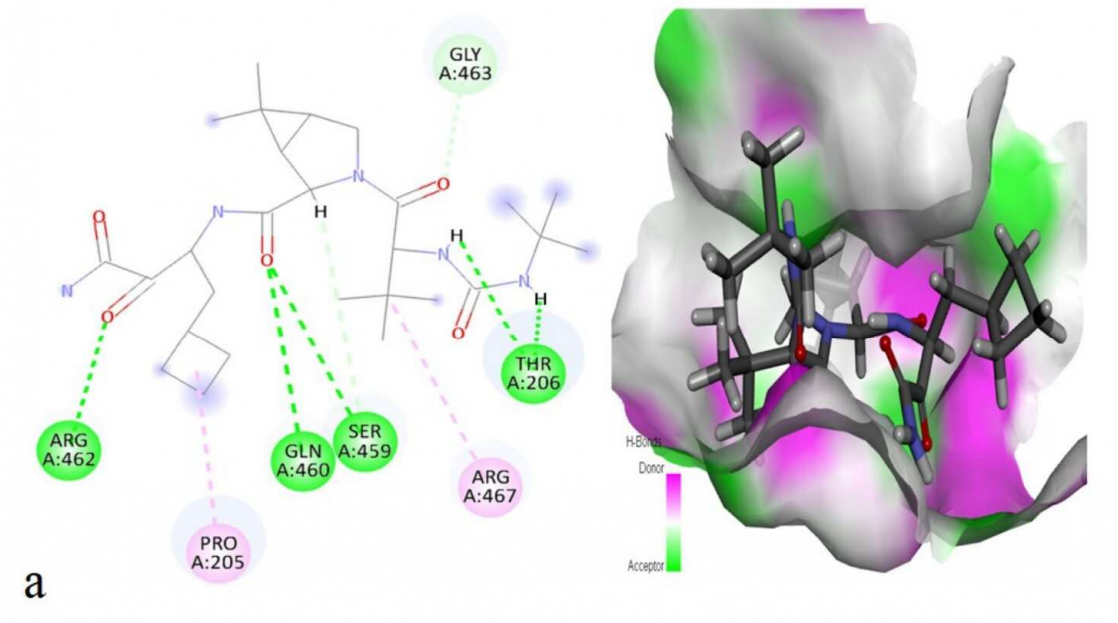

Fig. 5A: The figure shows the 2D and 3D interactions and conformations of reference drud Boceprevir, which scored -7.2 kcal/mol after interacting with Arg 462 and Gln 460, the most conserved residues of the helicase/ATPase domain of HCV-NS3 protein.

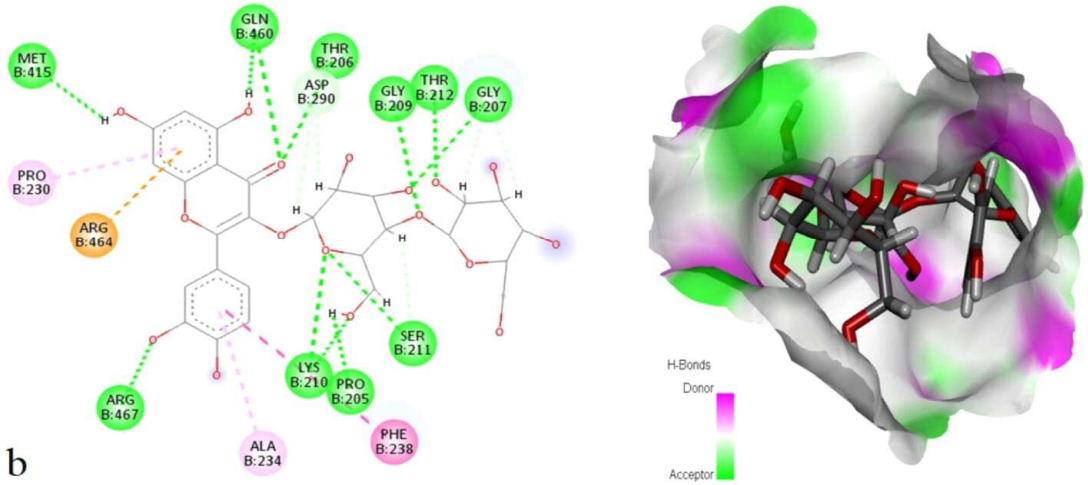

Fig. 5B: The figure shows the 2D and 3D interactions and conformations of Quercetin 3-glycoside, which scored -9.9 kcal/mol while interacting with Lys 210 and Asp 290, the most conserved and functionally active residues of the helicase/ATPase domain of HCV-NS3 protein.

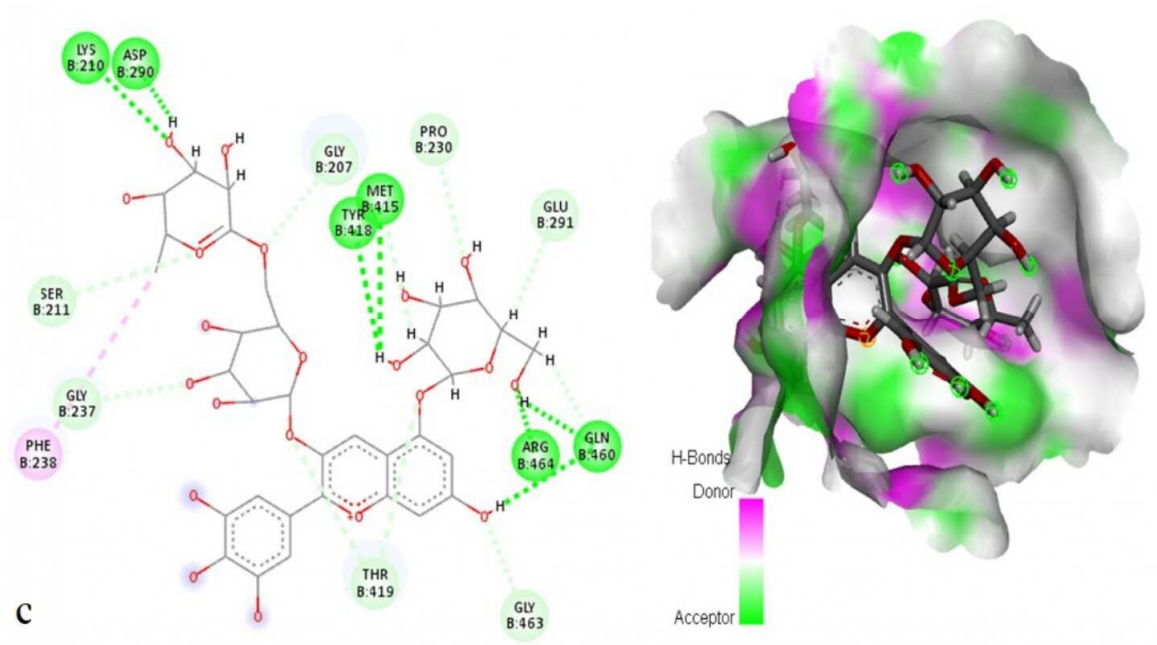

Fig. 5C: The figure shows the 2D and 3D interactions and conformations of Delphinidin 3,5-diglycoside, which scored -9.1 kcal/mol while interacting with Asp 290 and Lys 210, the most conserved and functionally active residues of the helicase/ATPase domain of HCV-NS3 protein.

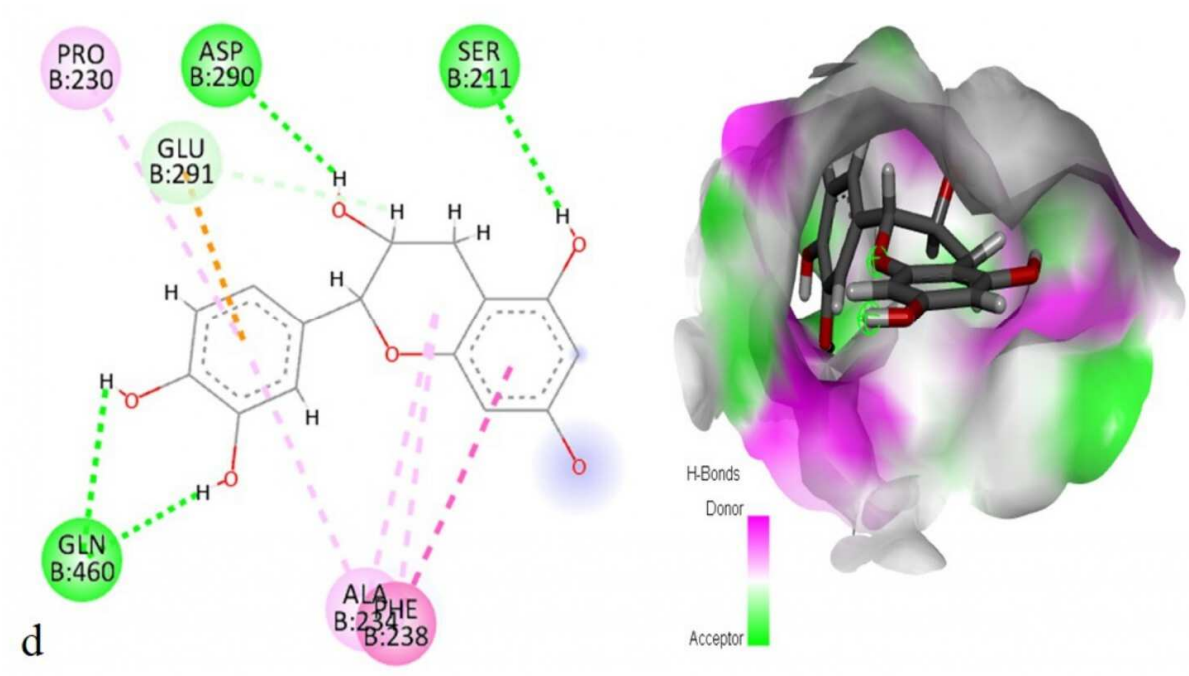

Fig. 5D: The figure shows the 2D and 3D interactions and conformations of Catechin, which scored -8.2 kcal/mol when it bonded to Asp 290 and Gln 460, the most conserved and functionally active residues of the helicase/ATPase domain of HCV-NS3 protein.

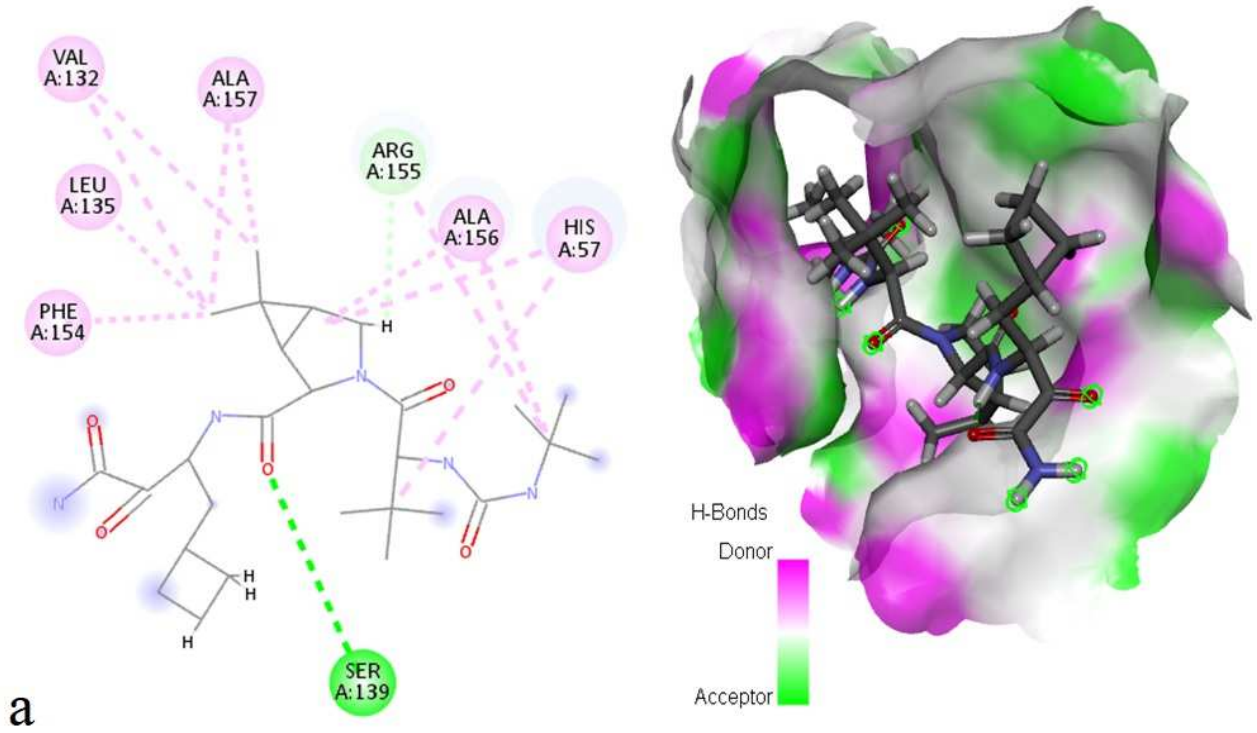

Fig. 6A: The figure represents the interactions between the reference drug Boceprevir and the protease domain of HCV-NS3 protein, emphasizing the catalytic triad, which exhibited a binding score of -6.1 kcal/mol towards Ser 139 and His 57.

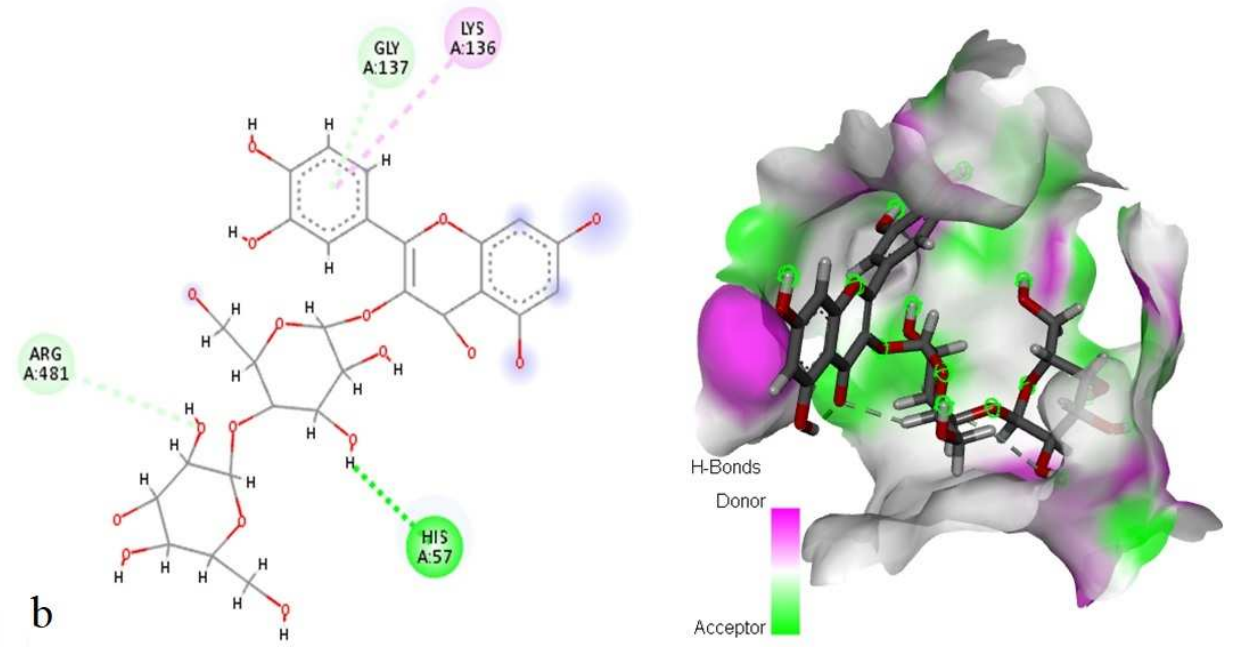

Fig. 6B: The figure shows the interactions between the Quercetin 3-glycosid and the protease domain of HCV-NS3 protein, focusing on the functionally active residues, which indicated a binding score of -6.4 kcal/mol towards His 57 and Gly 137.

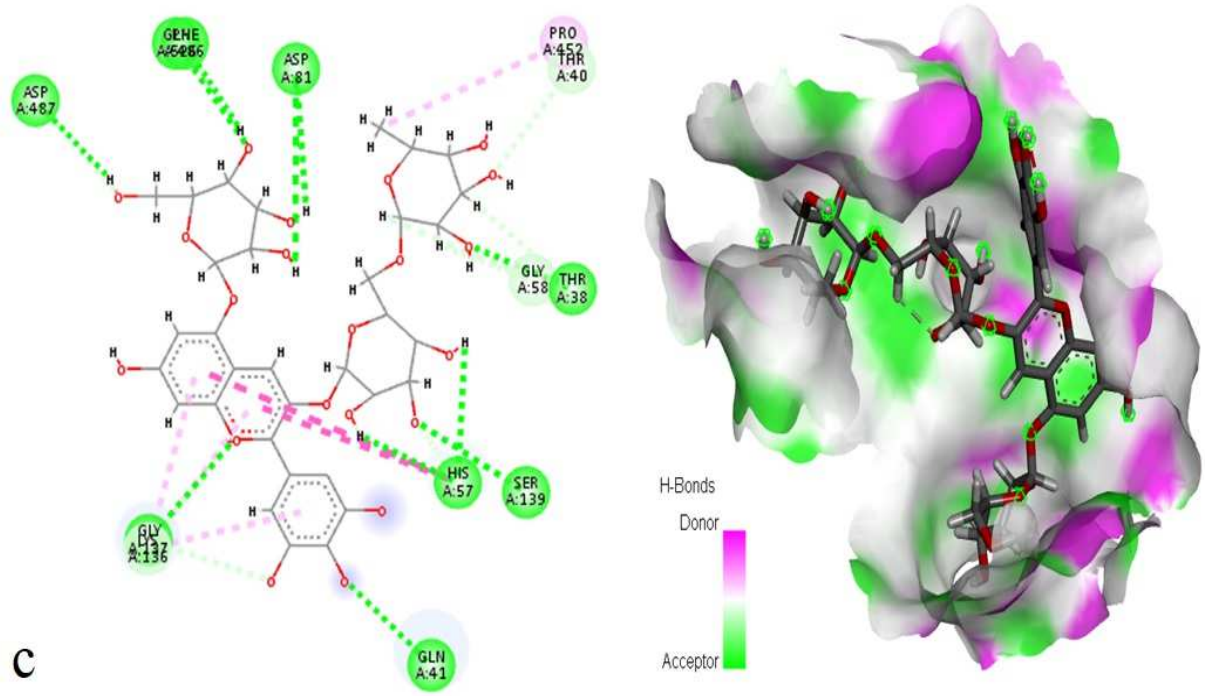

Fig. 6C: The indicates the interactions between the Delphinidin 3,5-diglycoside and the protease domain of HCV-NS3 protein, focusing on the catalytic triad, which showed an excellent binding score of -8.5 kcal/mol towards Asp 81, His 57 and Ser 139.

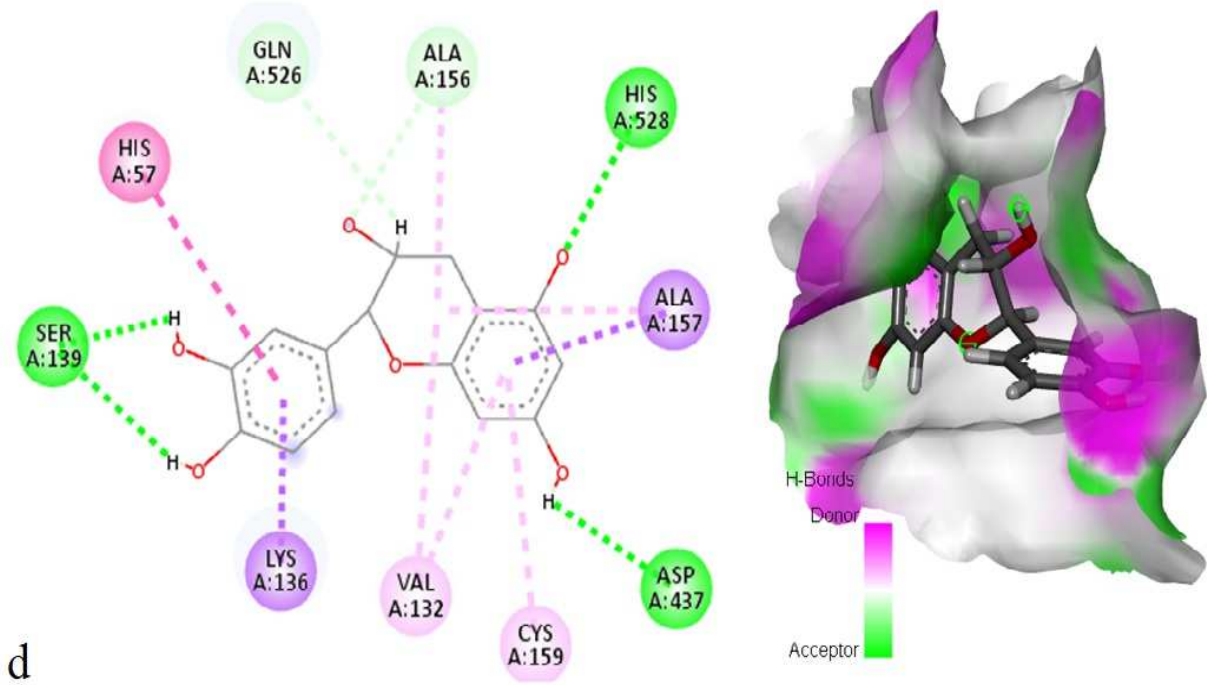

Fig. 6D: The figure illustrates the interactions between the Catechin and the protease domain of HCV-NS3 protein, emphasizing the catalytic triad, which demonstrated a binding score of -6.8 kcal/mol for Ser 139 and His 57. It indicates moderate interaction affinity between the ligands and receptor protein.

In-vitro antiviral activity against HCV-3a (full-length) genome in a cell culture system

Anti-HCV activity in Huh7 cells

To investigate the antiviral effects of the extracts against the HCV full-length genome, HCV-3a from patients' serum was obtained and inoculated into Huh7 cells. The nontoxic concentrations of extracts and positive control (daclatasvir 100nM) were used to treat the cells 3 hours before inoculation. Amongst these, the extract of leaves (25, 50 and 100µg/ml) and daclatasvir (100nM) effectively inhibited HCV replication with a higher significance level ($p<0.000$). The RT-qPCR findings indicated that the flower extract, at concentrations of 25, 50, and 100µg/ml, exhibited inhibitory effects of 13.65%, 39.79% and 29.13% against HCV genome replication, respectively, as shown in fig. 2A. Furthermore, the leaf extract at concentrations of 50 and 100µg/ml caused significant inhibition of HCV genome replication, with percentages of 61.62±20.17% ($p<0.001$) and 83.77± 13.74% ($p<0.000$), respectively. Similarly, the administration of the positive control daclatasvir at a concentration of 100nM resulted in a considerable decrease in HCV replication by 70±18.61% (p 0.000) when compared to the control, as shown in fig. 2B.

Dose response of the leaf extract against HCV-3a genome

Following the antiviral activity of *S. surattense* extracts and positive control daclatasvir (100nM) against HCV, the 50% inhibitory concentration (IC_{50}) of leaves extract against the full-length HCV-3a genome was evaluated. The dose-response assay showed significant inhibition ($p <0.000$) of HCV-3a genome replication in a concentration-dependent manner with IC_{50} of 19.69±2.73 µg/ml (fig. 2 B).

Inhibition of HCV-NS3 proteases gene expression

Inhibitory effects of extracts on HCV-NS3 proteases gene expression

Following the antiviral analysis of *S. surattense* against the full-length genome of HCV-3a, the effects of the extracts on HCV-NS3 protease expression were also independently observed. In our studies, HepG2 cells were transfected with NS3 protease plasmid and treated with different concentrations of plant extracts. Untreated transfected cells were taken as NS3 control. The results showed that the flower extract (50 and 100µg/ml) caused 34.64±5.17 and 24.6±9.24% inhibition of HCV-NS3 gene expression, respectively (fig. 3A). Whereas, leaves extract (25 and 50µg/ml) caused 75.59±10.28 and 69.34±2.34% inhibition of HCV-NS3 gene expression, respectively (fig. 3B).

Dose response of the flower and leaf extracts against HCV-NS3 protease

As shown above, the flower and leaf extracts caused highly significant inhibition of HCV-NS3 gene expression. Therefore, based on their marvellous inhibitory activity against the HCV-NS3 gene, IC_{50} value against the HCV-NS3 gene was determined. We found that they significantly inhibited HCV-NS3 gene expression at concentrations of 25 and 50µg/ml with IC_{50} of 7.24±0.56µg/ml (fig. 3A) and 5.42±2.38 µg/ml (fig. 3B) for flower and leaf extracts, respectively.

Molecular docking

The docking study on the bioactive compounds from *S. surattense* against the HCV-NS3 protein reveals intricate details about their potential inhibitory effects, particularly focusing on interactions within the critical helicase/ ATPase and protease domains. Quercetin 3-glycoside [3-O-[β-D-Glucopyranosyl-(1→4)-β-D-mannopyranoside], a key compound, showed a notable binding affinity in both domains. In the helicase/ATPase domain, it scored -9.9 kcal/mol, interacting primarily with residues like Asp290, Lys210 and Gln460 (fig. 5b). These residues are pivotal for the protein's ATPase activity, where Arg464 and Arg467 stabilize ATP's phosphate groups necessary for helicase function. Its ability to bind effectively suggests that it could significantly inhibit the viral replication process by disrupting ATP binding and energy transduction. In the protease domain, quercetin 3-glycoside demonstrated a docking score of -6.4 kcal/mol, binding to residues like His57, Gly137 and Lys136 (fig. 6b). His57, being a part of the catalytic triad, plays a crucial role in the proteolytic processing of the viral polyprotein, and thus, its interaction with this compound could suggest a strong inhibition of the protease activity, thereby, halting viral replication.

Delphinidin 3,5-diglycoside [3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside], 5-O-β-D-glucopyranoside] also exhibited significant binding across both domains. It scored -9.1 kcal/mol binding affinity in the helicase/ATPase domain by interacting with residues such as Arg464 and Gln460, which are critical for stabilizing the helicase structure, while Lys210, Asp290 and Glu291 play a key in ATPase activity (fig. 5c). Therefore, their involvement indicates that delphinidin 3,5-diglycoside could potentially destabilize the domain and inhibit viral RNA unwinding. In the protease domain, this compound showed a stronger binding affinity with a score of -8.5 kcal/mol by interacting with residues of the catalytic triad including Asp81, His57 and Ser139 (fig. 6c), further emphasizing its role in inhibiting the protease function crucial for viral replication. Catechin, another compound, demonstrated a docking score of -8.2kcal/mol in the helicase/ATPase domain (fig. 5d) and -6.8kcal/mol in the protease domain (fig. 6d). Its interactions with residues such as Asp290, Gln460 and Glu291 in the helicase/ATPase domain and Ser139, Asp437, and His57 in the protease domain indicate its potential as a competitive inhibitor that could obstruct substrate binding to the active site.

Boceprevir, the positive control, showed lower binding affinities across both domains compared to the natural compounds. In the helicase/ATPase domain, it scored -7.2 kcal/mol with interactions involving Arg462, Arg467 and Gln460 (fig. 5a), suggesting a moderate inhibition capacity. In the protease domain, boceprevir had a docking score of -6.1 kcal/mol, interacting with residues such as Ser139, His57 and Arg155 (fig. 6a). Although it is an effective inhibitor, its lower binding affinity compared to Delphinidin 3,5-diglycoside and Quercetin 3-glycoside suggests that these natural compounds might provide enhanced inhibition of both enzymatic activities essential for viral replication. The detailed residue interactions and docking scores across these four compounds underline the potential of S. surattense bioactives as powerful dual inhibitors targeting both the helicase/ATPase and protease domains of the HCV-NS3 protein, making them promising candidates for further exploration in antiviral drug development. This comprehensive interaction profile offers a compelling case for advancing these compounds into more detailed preclinical studies (table 1S).

DISCUSSION

HCV infection is a severe global health concern that requires effective treatment (Permanasari *et al.,* 2021) because a protective vaccine against HCV infection is not yet available due to intensive strain variations (Ashfaq and Idrees 2014) and 0.4 million people die each year due to HCV related complications (Permanasari *et al.,* 2021). Eight major genotypes of HCV (Bhattacharjee *et al.,* 2021) and more than 87 sub-genotypes (Hafid *et al.,* 2017) along with millions of quasispecies have yet to be identified (Bhattacharjee *et al.,* 2021).

According to the mechanism of action, four types of DAAs including, first generation Protease inhibitors (Telaprevir and Boceprevir), Second generation Protease inhibitors (Simeprevir, Faldaprevir, Paritaprevir and Ritonavir), Nucleos(t)ide polymerase inhibitors (Sofosbuvir), Non-nucleos(t)ide polymerase inhibitors (Dasabuvir) and NS5A inhibitors (Daclatasvir, Ledipasvir and Ombitasvir) are available (Bertino *et al.,* 2016; Alghamdi *et al.,* 2024). But, despite the extensive prevalence of antiviral drugs containing generic formulations, the available therapeutic intervention against HCV remains compromised and partial advancement has been accomplished in the global adoption of HCV treatment (Sollima *et al.,* 2016; Sorbo *et al.,* 2018), because of the various side effects of DAAs (Gonzales 2018) and drug resistance due to the RASs (Sorbo *et al.,* 2018). Furthermore, many patients cannot afford antiviral drugs because they are highly expensive, especially in developing nations (Khan *et al.,* 2022, Smith *et al.,* 2019). Additionally, the risk of HCC development cannot be mitigated by DAAs in individuals with advanced liver cirrhosis, even after the complete eradication of HCV (Crouchet *et al.,* 2018). Therefore, there is a dire need to find new, novel and cost-effective antiviral therapies/agents.

Fortunately, several plant species contain more potential compounds which have excellent antiviral properties. This indicates that they might be beneficial as broad-spectrum antiviral pharmaceuticals against HCV (Ravikumar *et al.,* 2011, Siew *et al.,* 2024). Furthermore, clinical investigations also showed that many traditional medicinal plants have therapeutic potential against chronic HCV infections (Javed *et al.,* 2011; Yang *et al.,* 2019). Therefore, in the present study, we aimed to screen the therapeutic potential of *S. surattense* against HCV infections.

Our studies show that 70% ethanolic extract of the leaves has robust activity against the full-length HCV-3a genome and HCV-NS3 gene expression as well. The effects of extracts of the flowers and leaves of S. surattense, on HCV replication in an HCV cell culture system showed that 70% ethanolic leaf extract was the most efficient ($p<0.000$) against full-length HCV-3a with 83.77% inhibition at a concentration of 100µg/ml with IC_{50} of 19.69±2.73µg/ml. In comparison, the positive control (daclatasvir 100nM) showed 69.86% inhibition as compared to the control (fig. 2B). These results reflect that leaf extract at 100µg/ml observes inhibition better than the control daclatasvir at 100nM. It clearly demonstrates the presence of antiviral constituents in the crude leaf extract, if purified and use in the assay, it may give parallel results with that of the control daclatasvir. It is further emphasized that there may be more than one antiviral constituents in the extract that may possess multi-target activities. Further research is warranted in this regard.

In addition, advances in analytical approaches, such as synthetic biology, computer-aided applications and databases, are providing valuable resources to recognize phytochemicals as new therapeutics. Amongst these, molecular docking is a prominent method used to investigate the interactions between proteins and ligands, to comprehend the relative arrangement of molecules, when they are docked together. The molecular docking strategies are beneficial to discovering prospective therapeutic compounds that can target a particular domain of concern (El Bakri *et al.,* 2023). Before the molecular docking investigations, it is important to understand the task to determine whether a derivative or analog surpasses the original compound. For instance, studies have shown that Kaempferol-7-glucoside is more effective in impeding the replication of HIV than Kaempferol, while also causing less cytotoxicity. Even then, it does not meet the criteria of almost all drug-likeness filters (Siew *et al.,* 2024).

In the aforementioned context, we performed molecular docking to determine the phytochemicals that can inhibit

the helicase and protease domains of HCV-NS3 separately. The molecular docking study of the HCV-NS3 protein with phytochemicals related to *S. surattense* reveals its potential to inhibit the virus by selectively targeting both the helicase/ATPase and protease domains. The active phytochemicals, including quercetin 3-glycoside, exhibited remarkable binding affinities of -9.9 kcal/mol and -6.4 kcal/mol towards the helicase/ATPase and protease domains, respectively. This suggests that it has the potential to disrupt crucial enzymatic activities and prevent viral replication (Azeem *et al.,* 2024). Delphinidin 3,5-diglycoside experienced a substantial inhibitory impact on the protease enzyme, as seen by its docking affinity of -8.5 kcal/mol. This compound specifically interacted with His57, along with other crucial residues that are essential to the proper functioning of the protease. Catechin follows the competitive inhibition model in both enzymes and increases its antiviral activity. In comparison, boceprevir, a standard drug, also shows stronger binding affinities but lower than our predicted compounds (fig. 5 & 6). These docking studies support our in vitro data findings and dictate further experimentation to inter-relate the therapeutic applications regarding antiviral properties of the targeted compounds.

In previous studies, the antiviral effects of several medicinal plants against HCV infections have already been proven. For example, research has demonstrated that several ethanolic extracts from Indonesian medicinal plants have shown tremendous Potential to inhibit HCV in a cell culture system (Wahyuni *et al.,* 2013). An active compound called tannin procyanidins-A2, derived from *Carapa procera* (Meliaceae) and *Pericopsis laxiflora* (Fabaceae), has been discovered to be effective against HCV when administered at nontoxic concentrations (Bamba *et al.,* 2021). Furthermore, apigenin and caffeic acid have inhibitory effects on HCV replication, whereas rutin, curcumin, EGCG and gallic acid have been shown to prevent HCV entry (Bose *et al.,* 2017; Hsu *et al.,* 2015; Wang *et al.,* 2020). Similarly, quercetin derived from *E. ribes,* EGCG from *C. sinensis,* and silibinin-A and silibinin-B from *S. marianum* have exhibited inhibitory effects against HCV-NS3 (Bachmetov, Gal-Tanamy *et al.,* 2012), HCV-NS5A (Calland *et al.,* 2012) and HCV-NS5B (Ahmed–Belkacem *et al.,* 2010), respectively. It has also been shown that some flavonoids including quercetin and apigenin from *S. surattense* have both anti-HCV and anti-cancer activities in different concentrations as multi-target bioactive phytochemicals. Therefore, it is unsurprising that different parts of *S. surattense* have both anti-HCV and anti-cancer activities (Khalid *et al.,* 2022, Kumar 2021) but to different extents and concentrations. The differential phytochemical distribution in separate parts of the herb should explicate this phenomenon. Two opposite therapeutic strategies to treat diseases exist, including multi-drug and multi-target therapy. The first one is used extensively to date, e.g., the detailed studies of the HCV life cycle have revealed almost five targets to inhibit HCV (Crema *et al.,* 2015; Reed and Rice 2000; Scheel and Rice 2013). Therefore, different drugs have been developed for each target and are combined to treat HCV infections (Ganesan and Barakat 2017; Yang *et al.,* 2019).

On the other hand, multi-target therapy is in its evolutionary process. For example, the flavonoids apigenin and quercetin have both anti-HCV and anti-cancer activities but in different concentrations (Bachmetov *et al.,* 2012; Calland *et al.,* 2012; Korga *et al.,* 2019; Pi *et al.,* 2016; Shibata *et al.,* 2014). In this context, it is quite possible that phytochemicals can inhibit HCV at its early as well as most advanced stage of infection, which is known as HCC (Jiang *et al.,* 2019). Previous studies have also revealed the anti-HCV-NS3 activity of many phytochemicals. But the question is, either these HCV-NS3 inhibitors inhibit or not the other targets of HCV? If the answer is yes, then what should be the 50% inhibitory concentration (IC_{50}) of the compound/drug for each target and the cytotoxic concentration as well? For example, quercetin and many other phytochemicals from S. surattense showed significant cytotoxicity against hepatoma liver cells (anti-HCC activity) (Khalid *et al.,* 2022) which also demonstrated anti HCV-NS3 activity but at low (non-toxic) concentratins, comparatively (Bachmetov *et al.,* 2012). Therefore, it is a dire need of the hour to work on these lines to explore the therapeutic potential of these novel, cost-effective, and multi-target phytochemicals which might be a better choice for the treatment of such diseases (like cancers and viral infections, etc.), whose treatment is very difficult and costly. In conclusion, recent studies have shown that S. surattense has promise as a possible treatment for HCV and HCC. However, additional research is needed to confirm its therapeutic potential and ensure its safety for clinical application.

CONCLUSION

Hydro-alcoholic leaf extract of *S. surattense* showed significant antiviral activity in nontoxic concentrations against the full-length HCV genome in Huh7 cells and HCV-NS3 gene in HepG2 cells as compared to the positive control. Nonetheless, these extracts showed significant cytotoxicity in different concentration levels indicating that this plant has also a potential against HCC. The results showed that the bioactive phytochemicals from *S. surattense* exhibited strong binding affinities with both the helicase/ATPase and protease domains of the HCV-NS3 protein. The phytochemicals, including quercetin 3-glycoside, delphinidin 3,5-diglycoside and catechin, exhibited higher docking scores than the positive control, boceprevir. This suggests that these phytochemicals can act as dual inhibitors, which further

support the plant's therapeutic potential against HCV and HCC.

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