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ABOUT COVER

Associate editor of World Journal of Hepatology, Dr. Yong-Ping Yang is a Distinguished Professor at Peking University Health Science Center in Beijing, China. Having received his Bachelor’s degree from Yanbian University in 1985, Dr. Yang undertook his postgraduate training at PLA Medical College, receiving his Master’s degree in 1992. He rose to Chief Physician in the Hepatology Division of the Fifth Medical Center of the Chinese PLA General Hospital in 2003 and has held the position since. His ongoing research interests involve liver fibrosis, cirrhosis and hepatocellular carcinoma, with a particular focus on cryoablation and cryo-immunotherapy for hepatocellular carcinoma. Currently, he serves as Chairman of the Department of Liver Disease of the Chinese PLA General Hospital and as President of the Chinese Research Hospital Association for the Study of the Liver Disease. (L-Editor: Filipodia)

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WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

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Basic Study

Screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry

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Abstract

BACKGROUND

Hepatitis C virus genotype 3a (HCV G3a) is highly prevalent in Pakistan. Due to the elevated cost of available Food and Drug Administration-approved drugs against HCV, medicinal natural products of potent antiviral activity should be screened for the cost-effective treatment of the disease. Furthermore, from natural products, active compounds against vital HCV proteins like non-structural protein 3 (NS3) protease could be identified to prevent viral proliferation in the host.

AIM

To develop cost-effective HCV genotype 3a NS3 protease inhibitors from citrus fruit extracts.

METHODS

Full-length NS3 without co-factor non-structural protein 4A (NS4A) and codon optimized NS3 protease in fusion with NS4A were expressed in Escherichia coli. The expressed protein was purified by metal ion affinity chromatography and gel filtration. Citrus fruit extracts were screened using fluorescence resonance energy transfer (FRET) assay against the protease and polyphenols were identified as
INTRODUCTION

Hepatitis C virus (HCV) is responsible for chronic hepatitis C disease in humans\(^1\). HCV infection remains asymptomatic and the virus persists in approximately 80% of untreated cases, which may ultimately lead to liver cirrhosis and finally hepatocellular carcinoma\(^2\). HCV is a major global cause of morbidity and mortality affecting more than 170 million people\(^3\). Annually, 400,000 patients die worldwide due to HCV infection\(^4\). In Pakistan, the prevalence of HCV infection is estimated to range from 4%-6%\(^5\). Seven pathogenic HCV genotypes with subtypes have been identified\(^6\) and genotype 3a is predominant in Pakistan\(^7\).

RESULTS

NS4A fused with NS3 protease domain gene was overexpressed and the purified protein yield was high in comparison to the lower yield of the full-length NS3 protein. Furthermore, in enzyme kinetic studies, NS4A fused with NS3 protease proved to be functionally active compared to full-length NS3. So it was concluded that co-factor NS4A fusion is essential for the purification of functionally active protease. FRET assay was developed and validated by the half maximal inhibitory concentration \(\text{IC}_{50}\) values of commercially available inhibitors. Screening of citrus fruit extracts against the native purified fused NS4A-NS3 protease domain showed that the grapefruit mesocarp extract exhibits the highest percentage inhibition 91% of protease activity. Among the compounds identified by LCMS analysis, hesperidin showed strong binding affinity with the protease catalytic triad having S-score value of -10.98.

CONCLUSION

Fused NS4A-NS3 protease is functionally more active, which is effectively inhibited by hesperidin from the grapefruit mesocarp extract with an \(\text{IC}_{50}\) value of 23.32 µmol/L.

Key Words: Hepatitis C virus genotype 3a; Non-structural protein 3 protease; Fluorescence resonance energy transfer assay; Citrus extract; Mass spectrometry; Hesperidin

Core Tip: The manuscript describes the screening of active metabolites in citrus fruit extracts against hepatitis C virus genotype3a non-structural protein 3 (HCV-G3a NS3) protease. In this study, conditions have been optimized to get highly purified and functionally active protein HCV NS3. Further, fluorescence resonance energy transfer assay was used to screen the citrus extracts against NS3 protease. By using liquid chromatography coupled with tandem mass spectrometry/mass spectrometry analysis and bioinformatics modeling approaches, the observed activity of citrus extracts against HCV genotype3a NS3 protease was ascribed to hesperidin. Fluorescence resonance energy transfer assay confirmed the inhibitory potential of hesperidin against NS4A-NS3 protease domain with an half maximal inhibitory concentration value of 23.32 µmol/L.

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In recent years, direct acting antiviral drugs have revolutionized HCV treatment. Sofosbuvir (a non-structural protein [NS] 5B inhibitor) and daclatasvir (a non-structural protein 5A [NS5A] inhibitor) have demonstrated sustained virologic response of more than 90% and have far fewer adverse events compared to previous treatment options. Sofosbuvir was the first nucleotide analogue that was found to be effective alone against HCV without interferon combination, which opened new gateways for development of additional direct acting antiviral drugs with excellent therapeutic outcomes\(^1\). The earlier Food and Drug Administration (FDA)-approved NS3 protease inhibitors, boceprevir and telaprevir were only effective against HCV genotype 1 (mainly prevalent in western countries) and less effective against genotype 3a, most prevalent genotype in Pakistan. However, the recently FDA-approved NS3 protease inhibitor glecaprevir has shown broad or pan-genotypic activity against HCV with much reduced side effects, especially in combination with pibrentasvir (ABT-530, NS5A inhibitor) as a single tablet. However, the high cost of protease inhibitors limits their use in resource-limited countries rendering the global eradication of HCV infection a difficult goal.

According to recent World Health Organization report, globally, the landscape for traditional and complimentary medicines has been steadily expanding\(^2\) and a large number of world populations rely on traditional medicines that use natural products for treatment of viral and other infections\(^3\).

Efforts have been made to identify extracts and natural products isolated from citrus family to inhibit HCV genotype 3a NS3 protease, because citrus fruits are considered a treasure trove of several active natural metabolites, including coumarins, alkaloids, flavonoids, limonoids, essential oils, phenolic acids and carotenoids. The anti-oxidative\(^4,5\), anti-inflammatory\(^6,7\), and anti-cancer properties\(^8,9\), as well as cardiovascular\(^10\), neuroprotective\(^11,12\) and hepatoprotective effects\(^13,14\) of citrus and their extracts have been extensively reported\(^15-18\). Moreover, citrus metabolites have been used in many Asian countries as traditional medicinal herbs for treatment of digestive disorders, common cold and influenza, constipation and diarrhea, fluid retention, irritable bowel syndrome, persistent headaches, skin disorders, anxiety, depression, allergies, osteoarthritis, rheumatoid, prostate disorders as well as stomach and breast cancer\(^19-22\).

In the current study, we have successfully produced a highly purified, stable and functionally active HCV NS3 protease of genotype 3a in fusion with its co-factor NS4A. After validating the fluorescence resonance energy transfer (FRET) assay using commercially available protease inhibitors, the extracts from citrus × paradisi (grapefruit), citrus sinensis (orange), citrus aurantum (bitter orange), citrus reticulata (mandarin) and citrus limon (lemon) were collected, enriched and evaluated. The most active extract was analyzed and characterized using high-performance liquid chromatography coupled with tandem mass spectrometry (LCMS/MS). Candidate compounds from most active extracts were docked against the HCV NS3 protease structure to identify the most promising natural product, which was acquired and further evaluated to inhibit HCV NS3 protease using a FRET assay. As a result, this study has pinpointed the most active natural product from citrus family against NS3 protease of HCV genotype 3a.

**MATERIALS AND METHODS**

**Plasmid constructs**

The pET11a-His\(_6\)-NS3 construct was provided by Dr. Ikram Anwar (former PhD student, Drug Discovery and Structural Biology Lab, NIBGE, Pakistan). In pET11a-His\(_6\)-NS3, the nucleotide sequence of full-length NS3 (encoding both protease and helicase domains comprising amino acid 1 to amino acid 631) of hepatitis C virus genotype 3a was cloned into Bam HI/Hind III restriction sites of pET11a vector (Novagen, Madison, WI, United States) using respective restriction enzymes\(^23,24\). The NS3 sequence in the pET11a-His\(_6\)-NS3 construct was placed in the reading frame with the N-terminal His\(_6\)-tag (Supplementary Figure 1A).

Another construct inserted with the NS3 protease domain in fusion with the core of NS4A activator peptide at the C-terminal was named pET11a-His\(_6\)-NS4A-NS3 (Supplementary Figure 1B) and synthesized by GenScript (Piscataway, NJ, United States); further description is given in the Supplementary information.

**Expression of full-length NS3 and NS3 protease in Escherichia coli**

For expression of full-length His\(_6\)-NS3 (contain a serine protease and an RNA helicase)
competent cells of BL21-CodonPlus (DE3)-RIL and Escherichia coli BL21 (DE3) were transformed with pET11a-His6-NS3. For expression of the NS3 protease, pET11a-His6-NS4A-NS3 was transformed into E. coli BL21 (DE3) cells considering the codon-optimized nucleotide sequence of NS3 protease. Further details of the expression experiments are given in the Supplementary information.

**Purification of full-length NS3 and NS4A-NS3 protease domain**

For purification, E. coli cell paste of pET11a-His6-NS3/BL21-CodonPlus (DE3)-RIL or pET11a-His6-NS4A-NS3/BL21 (DE3) was resuspended in ice-cold 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.6 added with 20% glycerol, 0.5 mol/L NaCl, 2.5 mmol/L β-mercaptoethanol and 0.4% Triton X-100 or 25 mmol/L HEPES (pH 7.5), 1 mol/L NaCl, 25 mmol/L Imidazole and 10% glycerol (buffer A). Both proteins were purified by Nickel affinity chromatography and gel filtration; the detailed procedure is presented in the Supplementary information.

**Activity measurement of full-length NS3 and NS4A-NS3 protease domain**

The FRET assay was performed to determine protease activity, using the depsipeptide substrate Ac-Asp-Glu-Asp-(EDANS)-Glu-Glu-Abu-ψ-[COO]-Ala-Ser-Lys(DABCYL)-NH2 (AnaSpec, San Jose, CA, United States). The NS3 Protease domain cleaves the depsipeptide substrate, which results in the generation of fluorescence that can be read continuously on a fluorescence plate reader (THE SPARK®, TECAN, Morrisville, NC, United States) at their desired excitation (355 nm) and emission (510 nm) wavelengths. The synthetic peptide KKGCVVIVGHIELGK obtained from LifeTein LLC (Somerset, NJ, United States) at their desired excitation (355 nm) and emission (510 nm) wavelengths. The kinetics of enzyme (Km, kcat and kcat/Km) were analyzed using a non-linear regression using GraphPad Prism® software (GraphPad Software Inc., San Diego, CA, United States).

**Validation of NS4A-NS3 protease domain and FRET inhibition assay**

Inhibition experiments using NS4A-NS3 protease domain of genotype-3a were performed according to a previous protocol. After correcting inner filter corrections, the assay was validated using commercial-based inhibitors: Ciluprevir, telaprevir, asunaprevir, and danoprevir (AdooQ Bioscience, Irvine, CA, United States). To determine the kinetics of enzyme (Km, kcat and kcat/Km), the Michaelis-Menton equation was fitted to the data by non-linear regression using GraphPad Prism® software. For inhibition experiments using NS4A-NS3 protease domain, the enzyme was pre-incubated with inhibitor before adding depsipeptide substrate (1 µmol/L). Half maximal inhibitory concentration (IC50) values were obtained using GraphPad Prism® software. The reaction was monitored at wavelengths 355 nm (excitation) and 510 nm (emission) for 20, 30, or 60 min. All activity measurements were done in triplicate. The final percentage of enzyme inhibition was calculated as average from three independent experiments. Errors were calculated as the standard deviation (SD), and IC50 values were calculated as previously described.

**Preparation of extracts**

Five varieties of citrus fruits (citrus × paradisi [grapefruit], citrus sinesis [orange], citrus aurantium [bitter orange], citrus reticulata [mandarin], and citrus limon [lemon]) were used in this study. The different parts of dried and crushed citrus plants materials were provided by Jiaherb Inc. (Pine Brook, NJ, United States) and Sanjiang Bio (Walnut, CA, United States). These powdered extracts were packed in opaque storage bags and stored at -20°C for future use. The extraction procedure is further described in the Supplementary information.

**Inhibition of HCV NS4A-NS3 protease activity by plant extracts**

An opaque 96-well plate was used to perform enzyme inhibition assays as described in the Supplementary information. All extracts were tested at a final 3-fold dilution...
ranging from 1.566 µg/mL to 3.33 mg/mL. The inhibition was calculated as the average from three independent experiments\(^6\). Errors were calculated as the SD, and IC\(_{50}\) values were calculated as mentioned above.

**LCMS analysis of extracts**

For electrospray ionization (ESI)-MS/MS analysis, the dried extracts, which indicated the best inhibition activity by FRET assay, were dissolved in LCMS-grade methanol and subjected to ESI-MS/MS analysis using the LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, United States), equipped with an ESI probe as explained in the Supplementary information.

**Modeling predictions of compounds using bioinformatics software**

Compounds identified by ESI-MS/MS analysis were tested in docking studies using molecular operating environment (MOE) software by means of the following communications, i.e. Intel [R] xenon [R] CPU E5620@2.40 GHz system, which has 3.8GB RAM having 11.4 [X 86_64] operating system\(^6\). Structures of identified compounds were constructed using chembiodraw ultra 14.0 software. HCV NS4A-NS3 protease domain (receptor protein) was modeled by SWISS-MODEL. Three dimensional (3D) protonation and energy minimization were performed using standard MOE parameters. Then docking analysis with default MOE parameters was used to check the interaction of selected ligands to the receptor protein and find the correct ligand conformation. After docking, best conformations were analyzed depending on least S-score values for hydrogen bonding/π-π interactions\(^6\).

**Evaluation of pure natural product (hesperidin)**

After the identification of specific natural product (hesperidin) by ESI-MS/MS analysis and its further interaction with NS3 protease by molecular docking, hesperidin (90% pure; Jiaherb) was subjected to inhibitory activity analysis. Inhibition experiments using the NS4A-NS3 protease domain were performed as previously described\(^7\) and IC\(_{50}\) values were calculated.

**RESULTS**

**Expression and purification of full-length NS3 and NS4A-NS3 protease domain**

To test the efficacy of selected inhibitors against the HCV-NS3 protease domain, full-length NS3 (containing the serine protease and an RNA helicase domains) and a NS4A-NS3 protease domain were produced by recombinant means. To this end, expression of full-length His\(_{6}\)-NS3 from pET11a-His\(_{6}\)-NS3 expression vector (Supplementary Figure 1) was performed in E. coli BL21 (DE3) and BL21-CodonPlus (DE3)-RIL as described in the Methods section. High expression levels of His\(_{6}\)-NS3 were obtained in BL21-CodonPlus (DE3)-RIL compared to BL21 (DE3) cells, as detected by the appearance of a 68.3 kDa band on a Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Figure 1A and 3). Therefore, further expression of His\(_{6}\)-NS3 was performed in BL21-CodonPlus (DE3)-RIL. For the NS4A-NS3 protease domain (22.5 kDa), high expression levels were achieved in E. coli BL21 (DE3) using the pET11A-His\(_{6}\)-NS4A-NS3 expression vector (Figure 1C, Supplementary Figures 1 and 2).

Full-length His\(_{6}\)-NS3 and His\(_{6}\)-NS4A-NS3 protease domain were purified to homogeneity by Ni-NTA affinity chromatography. A high level of purity (> 95%) was achieved for the His\(_{6}\)-NS4A-NS3 protease domain compared to His\(_{6}\)-NS3, as determined by Coomassie blue-stained SDS-PAGE gels (Supplementary Figure 3). To remove impurities from the affinity-purified His\(_{6}\)-NS3 samples, gel filtration was performed that yielded a protein of high purity (Figure 1D, Peaks 1 and 2). For the His\(_{6}\)-NS4A-NS3, native NS4A-NS3 protease domain of high purity was obtained by cleaving the His\(_{6}\)-tag from the protein with TEV protease and purification by nickel-affinity chromatography and gel filtration (Supplementary Figures 1D and 4). Purification yield of full-length His\(_{6}\)-NS3 and NS4A-NS3 protease domain 0.55 and 6 mg per liter culture volume was obtained, respectively.

**Activity analyses of full-length His\(_{6}\)-NS3 and NS4A-NS3 protease domain**

The activity of the purified full-length His\(_{6}\)-NS3 and NS4A-NS3 protease domain were measured using a FRET-based assay as described in the Methods section. An increase in activity of the NS4A-NS3 protease domain was observed upon an increase in
Figure 1 Expression and purification of full-length His6-NS3 and non-structural protein 4a-non-structural protein 3 protease domain. A: Analysis of the full-length His6-NS3 protein (68.3 kDa) expressed in Escherichia coli BL21 (DE3) using pET11a-His6-NS3. Lane M: SeeBlue™ Pre-Stained Protein Marker (LC5625); Lane UI: Uninduced BL21 (DE3) cells and Lane I: Induced BL21 (DE3) cells; B: Analysis of soluble and insoluble fractions of the His6-NS3 protein expressed in E. coli BL21-CodonPlus (DE3)-RIL cells using pET11a-His6-NS3. Lane M: Invitrogen Cat 1891868 See Blue® Plus2 Pre-Stained ladder; Lane UI: Uninduced cells; Lane I: Induced cells; Lane T: Total cell lysate of induced cells; Lane S: Soluble fraction of lysed cells; C: Analysis of soluble and insoluble fractions of the His7-NS4A-NS3 protease domain expressed in E. coli BL21 (DE3) cells using pET11a-His7-NS4A-NS3. Lane M: SeeBlue™ Pre-Stained Protein Marker (LC5625); Lane UI: Uninduced cells; Lane I: Induced cells; Lane T: Total cell lysate of induced cells; Lane S: Soluble fraction of lysed cells; D: Analysis of the Ni-NTA His6-NS3 protein by gel filtration, presence of proteins in peak 1, 2 and 3 is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; and E: Analysis of the native NS4-NS3 protease domain by gel filtration, presence of proteins in peak 1 and 2 is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

depsipeptide substrate concentration (0.0625 to 6 µM) whereas with full-length His6-NS3, no obvious activity was detected although the experiment was repeated several times (Supplementary Figure 5A and B). Moreover, the kinetic data revealed that the NS4A-NS3 protease domain exhibited a Km of 6.39 µM and catalytic efficiency ($k_c / K_m$) of 0.015 µM$^{-1}$s$^{-1}$ (15 nM$^{-1}$s$^{-1}$) (Figure S5C) and qualified for conducting assays for an intensive search for inhibitors.

Furthermore, before embarking upon inhibition of the NS4A-NS3 protease domain by natural extracts/compounds, validation of the inhibition assay was done using commercial inhibitors. BILN 2061, VX-950, asuanaprevir and danoprevir inhibited the activity of the NS4A-NS3 protease domain with IC$_{50}$s of 52.28 ± 13.08, 69.59 ± 13.42, 69.42 ± 13.48 and 24.42 ± 8.04 nM, respectively (Figure 2). The obtained IC$_{50}$ values validated the assay and suggested that the activity of the purified NS4A-NS3 protease
Screening of citrus extracts against NS4A-NS3 protease domain of genotype 3a using the FRET assay

After validating the FRET assay, 14 extracts of citrus fruit varieties were collected. Extracts from different parts of citrus (described in methods) were evaluated against the NS4A-NS3 protease domain of genotype 3a. Among them, top five extracts exhibited highest percentage inhibition of protease activity were selected. Selected extracts strongly inhibited the activity of NS4A-NS3 protease domain, i.e. grapefruit mesocarp extract, showed highest 91% inhibition of protease with an IC_{50} value of 7.51 ± 0.87 µg/mL; orange extract (exhibited 86% inhibition of protease with an IC_{50} value of 33.39 ± 1.52 µg/mL; bitter orange extract showed 85% inhibition of protease with an IC_{50} value of 40.24 ± 1.60 µg/mL; mandarin extract showed 82% inhibition with an IC_{50} value of 65.40 ± 1.81; and lemon extract inhibited 80% of protease activity with an IC_{50} value of 74.60 ± 1.86 µg/mL. Among these extracts, grapefruit mesocarp extract showed highest percentage inhibition (91%) with lowest IC_{50} value of 7.51 ± 0.87 µg/mL against NS4A-NS3 protease domain of genotype 3a using the FRET assay (Figure 3).

ESI-MS/MS analysis of grapefruit mesocarp extract

The grapefruit mesocarp extract exhibiting the best activity against NS4A-NS3 protease domain was subjected to ESI-MS/MS analysis to reveal the identification of active natural product(s). Among the tested extraction solvents, n-hexane proved to be the best in minimizing the noise and showing maximum polyphenol natural products at negative ion mode (Figure 4). The full scan mass spectrum followed by the fragmentation through collision induced dissociation (CID) of the ion peaks of extract in negative ion mode [M-H]^- revealed the presence of aliphatic and aromatic organic acids (with and without glycans), lactones (bergapten at m/z 215 and (R)-marmin at m/z 331), flavonoids, i.e. alpinetin at m/z 269, hesperitin at m/z 301, polymethoxyflavone at m/z 435, naringenin arabino furanose at m/z 535, naringin at m/z 579, hesperidin at m/z 609, neohesperidin at m/z 610 and anthocyanin (cyanidin-3-O-sophoroside chloride at m/z 645). At positive ionization mode [M+H]^+ the synephrine, limonene and tangeretin were identified. These compounds were recognized by tandem mass spectrometry and hesperidin analysis data (as a representative) as described below.
Among all of the identified compounds, hesperidin flavonoid was the most abundant natural product in grapefruit mesocarp extract, giving a base peak at m/z 609.3 in negative ion mode. To confirm the hesperidin structure, the molecular ion at m/z 609.3 was fragmented (@CID 15.0) that yielded the daughter ions at m/z 463 (as a minor peak) and at m/z 301 as a base peak by losing one and two glycans, respectively, as described in Figure 5A. The peak at m/z 301 (hesperitin) was further subjected to MS³ fragmentation (@CID20.0), which produced 19 daughter ions, i.e. m/z 286 (-CH₃), further generating the fragments at m/z 268 (-H₂O), m/z 258 (-CO) and m/z 257 after double bond rearrangement (Figure 5B). The ion peak at m/z 257 generated two signals at m/z 151 and m/z 125 by the loss of B and C rings, respectively. The hesperitin (m/z 301) generated a peak at m/z 283 by loss of H₂O producing the alkyne adduct and/or a fragment with extended conjugation on C ring, which further produced an entity at m/z 241 after losing CO. The alkyne adduct at m/z 283 can yield m/z 268 by losing a methyl radical. The ion peaks at m/z 257, m/z 242 and m/z 227 were produced by the loss of CO₂ followed by that of CH₃ and O₂, respectively, from hesperitin (m/z 301). The ion peak at m/z 151 can be produced directly from m/z 301 and/or any of the other adducts having C ring intact through retro Diels-Alder reactions. Notably, m/z 151 accompanied by m/z 125 are the signature ion peaks generated from most of the flavonoid aglycones during their tandem mass spectrometry[47]. The fragment at m/z 301 also generated fused ring adducts at m/z 259, m/z 215, m/z 199 and m/z 185. All of the ion peaks confirmed...
the hesperidin structure\[^{[47,48]}\]. Additionally, the data were fully correlated with fragmentation of authentic hesperidin sample.

**Modeling predictions of compounds using bioinformatics software**

The homology model of HCV NS4A-NS3 protease domain was developed using the HCV NS3/NS4A protease crystal structure (PDB ID: 3P8N) as a template that has 76.80% sequence identity with the template confirming a high-quality model. The homology model (Figure 6A) was in a closed flap conformation with GMQE and QMean scores of 0.81 and -1.77, respectively. The model was further validated by the values of the ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/) (98% in the most favored region). Analysis with verify 3D software showed that 84.62% of the residues averaged a 3D-1D score of ≥ 0.2, and the overall quality factor measured ERRAT software was 93.4%.

Among the identified citrus phytochemicals from the grapefruit mesocarp extract, hesperidin exhibited a good docking score [S-score = -10.98] and most importantly, it bound tightly with the catalytic residues of HCV NS4A-NS3 protease domain (HIS 57, ASP 81, and SER 139) (Figure 6B). Hence, it may be worth further exploring against NS4A-NS3 protease domain of HCV genotype 3a.
Evaluation of pure flavonoids

The ESI-MS/MS analysis of the most active extract (grapefruit mesocarp) followed by simulation data, reveals that hesperidin is the most abundant and highly active inhibitor of the NS4A-NS3 protease domain of genotype-3a. The investigations inspired us to acquire hesperidin from commercial sources (Jiaherb) and evaluate its inhibition potential. Inhibition experiments with NS4A-NS3 protease domain of genotype-3a were performed according to the protocol described in methods. Hesperidin was tested at a concentration range of 1.23 µg/mL-1.67 mg/mL in the FRET assay, which gave IC\textsubscript{50} value of 23.32 µmol/L (Figure 6C).
**Figure 6** Inhibition studies of hepatitis C virus non-structural protein 4a-non-structural protein 3 protease domain by hesperidin. A: Hepatitis C virus (HCV) non-structural protein 4a-non-structural protein 3 (NS4A-NS3) protease domain model built by Swiss Model in the blue color cartoon. The active site residues are represented in yellow color for clarification; B: Docking of Hesperidin inside the receptor-binding site of HCV NS4A-NS3 protease domain (HIS 57, ASP 81 and SER 139). Hesperidin is represented in orange color. Blue ribbon represents protease domain of HCV NS3; and C: Inhibition constant (IC\textsubscript{50}) calculation of HCV NS4A-NS3 protease domain by hesperidin.

**DISCUSSION**

A multifunctional NS3 of HCV comprising of serine protease domain at N-terminal and a C-terminal RNA helicase domain. The protease domain requires the NS4A activator peptide to cleave 3 of its 4 polyprotein processing sites\cite{49,50} and therefore provides an attractive target for inhibition of viral replication\cite{51}; the protease is much more efficient at cleaving the fourth in the presence of NS4A. In the present study, full-length NS3 without cofactor NS4A and NS3 protease domain in fusion with co-factor NS4A from a codon-optimized NS4A-NS3 protease domain open reading frame were expressed in *E. coli* and purified (Figure 1). The NS4A-NS3 protease domain showed a higher purification yield than full-length NS3. Full-length NS3 did not display any detectable protease activity. This might be because the NS4A was not fused with the sequence of full-length NS3 and a separate synthetic NS4A peptide was used in activity assay buffer which may not bind to the proper site in NS3 to increase protease cleavage efficiency\cite{52}. Another possible reason is that the fusion provides a higher effective local concentration of the activator. On the other hand, the native NS4A-NS3 protease domain, containing fused cofactor, showed significant activity with $K_{\text{m}}$ and $k_{\text{cat}}/K_{\text{m}}$ of 6.39 µmol/L and 0.015 µM$^{-1}$s$^{-1}$, respectively. Moreover, inhibition of the NS4A-NS3 protease domain using known commercial inhibitors (BILN 2061, VX-950, asunaprevir and danoprevir) validated the activity assay (Figure 2), which suggests that native purified protein can be employed to search for natural inhibitors/compounds against HCV.

Citrus fruits are important for human health because of their highly nutritious and...
Because hesperidin is an important bioflavonoid widely found in natural products and to identify potent phytochemicals, which inhibits HCV patients in resource limited and developing countries. So, there is a need to study available in market, they are very expensive and are not affordable for many HCV patients (IC\textsubscript{50} value = 23.32 µmol/L). Additionally, hesperidin was reported to be active against sindbis virus infection, by plaque assay\textsuperscript{59} and dengue virus\textsuperscript{84}. Because of their abundance and possible medicinal uses, citrus extracts were included in the present study. Different plant parts (mesocarp, seeds, pericarp and pulp) of grapefruit, orange, bitter orange, mandarin and lemon were used to extract the bioactive compounds from them. FRET assay inhibition study of the tested extracts revealed that grapefruit mesocarp extract proves to be the more active against NS4A-NS3 protease domain having lowest IC\textsubscript{50} value (7.51 ± 0.87 µg/mL)\textsuperscript{(Figure 3).} ESI-MS/MS analysis of the grapefruit mesocarp extract demonstrated significantly higher abundance of hesperidin as compared to other metabolites\textsuperscript{(Figure 4).} The structure of hesperidin was confirmed using the tandem mass spectrometric technique\textsuperscript{(Figure 5).} Molecular docking studies of the identified flavonoids against the NS4A-NS3 protease domain (HCV 3a) revealed that hesperidin demonstrated a potential inhibitory activity against the NS4A-NS3 protease domain, exhibiting S-score value of -10.98 and strong binding affinity with the catalytic site residues (his 57, asp 81 and ser 139)\textsuperscript{(Figure 6A and B).} These assumptions were proved when hesperidin (90% purity) was evaluated against NS4A-NS3 protease domain using the FRET assay, which gave sub-nanomolar IC\textsubscript{50} (23.32 µmol/L)\textsuperscript{(Figure 6C).} This indicated that the predominant inhibition of grapefruit mesocarp extract was due to the presence of hesperidin in it. Notably, hesperidin was tested against different viruses, \textit{i.e.} sindbis virus, vaccinia virus, parainfluenza virus, herpes simplex virus types 1 and 2, poliovirus, and vesicular stomatitis virus\textsuperscript{59,60,61}. Hesperetin has exhibited an inhibitory effect with an IC\textsubscript{50} value of 20.5 µg/mL (about 68 µM) against sindbis virus infection, by plaque assay\textsuperscript{89}. Moreover, previously docking studies on hesperidin and narirutin showed their inhibition potential against avian influenza virus H1N1\textsuperscript{91}, which were consistent with our results, and their inhibitory activity was experimentally confirmed in another study\textsuperscript{84}. In this study, we found that hesperidin actively inhibited HCV NS3 protein with an IC\textsubscript{50} value of 23.32 µmol/L. Additionally, hesperidin was reported to be active involving host-factor, \textit{i.e.} suppression of mitogen-activated protein kinase signaling pathways in H1N1 infection and this pathway is also activated by HCV\textsuperscript{59-60}. Various natural products exhibit the potential to be anti-HCV protease inhibitors. Among them, epigallocatechin-3-gallate targets both viral cell entry and RNA replication steps\textsuperscript{62} into both primary human hepatocytes and hepapoma cell lines\textsuperscript{63}. It also exhibits an antiviral activity against all HCV genotypes, tested in the HCV pseudotyped particles (HCVpp system)\textsuperscript{64}. Other natural products including quercetin, honokiol, 3-hydroxy caruilignan C and excoecariphenol D corilagin exhibit anti-HCV behaviors, which have been tested in vivo or in cellular models. Quercetin exhibited inhibition of HCV NS3 protease\textsuperscript{65}. It has an ability to minimize the production of virus by inhibiting both NS3 as well as heat shock proteins which are required for the replication of HCV\textsuperscript{66,67}. Honokiol prevents HCV infection by interfering with their cell entry and replication process\textsuperscript{68,69}, 3-hydroxy caruilignan C also exhibited anti-HCV activity at both RNA and protein levels\textsuperscript{70}. Excoecariphenol D corilagin inhibited HCV NS3-4A protease with an IC\textsubscript{50} values within a range of 3.45-9.03 µmol/L, whereas excoecariphenol D and corilagin significantly showed potential inhibition of HCV RNA in huh 7.5 cells\textsuperscript{71}. Our findings suggested that hesperidin showed lowest IC\textsubscript{50} value = 23.32 µmol/L\textsuperscript{(Figure 6C) as compared to previously reported natural products including epigallocatechin-3 gallate (IC\textsubscript{50} value = 5-21 µmol/L), honokiol (IC\textsubscript{50} value = 4.5 µmol/L), 3-hydroxy caruilignan C (IC\textsubscript{50} value = 37.5 µmol/L) and excoecariphenol D corilagin (IC\textsubscript{50} value = 12.6 and 13.5 µmol/L)\textsuperscript{59}. Although FDA-approved, direct-acting antiviral drugs such as mavyret are available in market, they are very expensive and are not affordable for many HCV patients in resource limited and developing countries. So, there is a need to study natural products and to identify potent phytochemicals, which inhibits HCV replication and can be developed into inexpensive anti-HCV drugs. In conclusion, the present study identified hesperidin as a potential new inhibitor of HCV protease. Because hesperidin is an important bioflavonoid widely found in grapefruit mesocarp\textsuperscript{94}, its cheap availability may make it an interesting candidate as anti-HCV drug prospects which might replace other more expensive drugs.
**CONCLUSION**

In this study, HCV NS3 protease fused with co-factor NS4A was found to be functionally more active compared to full-length NS3. Citrus fruit extracts were screened using FRET assay against NS4A fused protease. Among these extracts, grapefruit mesocarp showed the highest percentage inhibition (91% of protease activity). LCMS data revealed the high abundance of hesperidin in the most active extract, which was subsequently subjected to docking studies showing strong binding interaction of hesperidin with the catalytic site residues of NS4A-NS3 protease domain (S-score = -10.98). Hesperidin inhibited NS4A-NS3 protease domain with an IC<sub>50</sub> value of 23.32 µM in FRET assay.

**ARTICLE HIGHLIGHTS**

**Research background**

Hepatitis C virus genotype 3a (HCV G3a) is highly prevalent in many countries including Pakistan. FDA-approved drugs have significantly contributed in effective control of the disease but are expensive and not affordable to a large proportion of the infected population.

**Research motivation**

Medicinal natural products having antiviral potential could be screened for the cost-effective treatment of the disease. Using such products, inhibition assays against vital viral proteins like non-structural protein (NS) 3 protease could be developed to prevent viral proliferation in the host.

**Research objectives**

This study developed cost-effective HCV G3a NS3 protease inhibitors from citrus fruit extracts.

**Research methods**

Codon optimized NS3 protease domain fused with NS4A as well as full-length NS3 constructs were cloned in pET11a expression vector. Both constructs were expressed in *Escherichia coli* BL21 (DE3) cells and purification was performed using Ni-affinity chromatography followed by gel filtration. The fluorescence resonance energy transfer assay was developed and validated using commercial inhibitors. Furthermore, extracts from different citrus species, were screened on the basis of percentage inhibition. The components of the most active extract were identified using electro spray ionization-mass spectrometry/mass spectrometry technique. Docking was performed with Molecular operating environment software to screen out the potent natural product, which was acquired in purified form and evaluated against NS3/4A protease using fluorescence resonance energy transfer assay.

**Research results**

We successfully overexpressed and purified genotype 3a NS3 protease domain fused with NS4A and the yield was also higher than full-length NS3 protein. Inhibition of NS3 protease fused with NS4A protein was tested against different citrus extracts and grapefruit mesocarp extract showed highest percentage inhibition of protease activity (91%). Hesperidin was identified as the inhibiting compound in the extract having docking S-score value of -10.98.

**Research conclusions**

NS3 protease fused with co-factor NS4A was found functionally more active. Hesperidin from the grapefruit mesocarp extract showed the inhibition against NS4A-NS3 protease domain with an IC<sub>50</sub> value of 23.32 µmol/L.

**Research perspectives**

Hesperidin flavonoid may be further explored as potential antiviral agent against HCV as an affordable option for infected population.
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