Hesperidin identified from Citrus extracts potently inhibits HCV genotype 3a NS3 protease

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Abstract

Background: Hepatitis C virus infection is the main cause of liver ailments across the globe. Several HCV genotypes have been identified in different parts of the world. Effective drugs for combating HCV infections are available but not affordable, particularly to infected individuals from resource-limited countries. Hence, cost-effective drugs need to be developed against important HCV drug targets. As Citrus fruits naturally contain bioactive compounds with antiviral activities, the current study was designed to identify antiviral inhibitors from Citrus fruit extracts against an important drug target, NS3 protease, of HCV genotype 3a which is found predominantly in South Asian countries.

Methods: The full-length NS3 protease alone and the NS3 protease domain in fusion with the cognate NS4A cofactor were expressed in Escherichia coli, and purified by chromatographic techniques. Using the purified protein as a drug target, Citrus extracts were evaluated in a FRET assay, and active ingredients, identified using ESI–MS/MS, were docked to observe the interaction with active site residues of NS3. The best interacting compound was further confirmed through the FRET assay as the inhibitor of NS3 protease.

Results: Fusion of the NS3 protease domain to the NS4A cofactor significantly improved the purification yield, and NS3-NS4A was functionally more active than the full-length NS3 alone. The purified protein (NS3-NS4A) was successfully employed in a validated FRET assay to evaluate 14 Citrus fruit extracts, revealing that the mesocarp extract of Citrus paradisi, and whole fruit extracts of C. sinesis, C. aurantinum, and C. reticulata significantly inhibited the protease activity of HCV NS3 protease (IC50 values of 5.79 ± 1.44 µg/mL, 37.19 ± 5.92 µg/mL, 42.62 ± 6.89 µg/mL, and 57.65 ± 3.81 µg/mL, respectively). Subsequent ESI-MSn analysis identified a flavonoid, hesperidin, abundantly present in all the afore-mentioned Citrus extracts. Importantly, docking studies suggested that hesperidin interacts with active site residues, and acts as a potent inhibitor of NS3 protease, exhibiting an IC50 value of 11.34 ± 3.83 µg/mL.

Conclusions: A FRET assay was developed using NS3-NS4A protease, which was successfully utilized for the evaluation of Citrus fruit extracts. Hesperidin, a compound present in the Citrus extracts, was identified as the main flavonoid, which can serve as a cost-effective potent inhibitor of NS3 protease, and could be developed as a drug for antiviral therapy against HCV genotype 3a.

Keywords: NS3 protease, HCV genotype 3a, FRET assay, Citrus plant extract, Hesperidin, Mass spectrometry
liver cirrhosis, hepatocellular carcinoma, and liver failure [1, 2], ultimately leading to the death of infected individuals [3]. It has been estimated that each year ~ 1.5 million people develop chronic HCV infections, resulting in the death of ~ 290,000 infected individuals worldwide [3].

HCV harbors a 10 kb-long RNA genome which displays a high level of genetic variation due to the lack of a proof-reading activity of its RNA-dependent RNA polymerase enzyme, resulting in the emergence of different HCV genotypes and subtypes across the globe [4]. So far, at least six (6) genotypes of HCV have been identified, with multiple subtypes [5]. Different HCV genotypes are prevalent in different parts of the world. Genotypes 1 and 3 are the two most common genotypes of HCV [6, 7]. Genotypes 2, 3, 4, 5, and 6 are found predominantly in West Africa and Americas, the Indian sub-continent and Southeast Asia, Central and East Africa, South Africa, and Southeast Asia, respectively [5].

The HCV genome encodes 3 structural (core and envelope (E1 and E2) proteins) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [8]. HCV proteins which have so far been targeted for the discovery of direct-acting anti-HCV drugs include NS3-NS4A protease, NS5A, and NS5B polymerase [9, 10]. Recently, NS3 protease has gained enormous attention as an important drug target for the development of direct-acting anti-HCV drugs. NS3 protease is a multifunctional enzyme that can perform a serine protease activity (through its N-terminal domain) and an RNA helicase activity (through its C-terminal domain), and is implicated in viral processing and maturation [11]. For performing the protease activity, an activator (cofactor) peptide, NS4A, is also required. Inhibiting HCV maturation by blocking the activity of NS3 protease is, therefore, a promising therapeutic strategy. It has been shown that direct-acting drugs developed against NS3 protease, or other drug targets, are more effective and less harmful in combating HCV infections than the previous treatment option, which was based on pegylated interferon and ribavirin [9, 10, 12–14]. Recently, a drug (glecaprevir) with a pan-genotype activity against HCV has been identified. Together with an NS5A inhibitor (pibrentasvir), the United States Food and Drug Administration (FDA) has approved glecaprevir for the treatment of HCV. However, a major drawback which limits the widespread use of glecaprevir for the treatment of HCV is its high cost, limiting the affordable access to therapy for a large proportion of infected individuals in resource-limited and developing countries. Furthermore, the number of HCV infections is still increasing despite the availability of effective drugs, making it highly challenging to achieve the World Health Organization’s (WHO) target of hepatitis C elimination by 2030 [3]. Therefore, there is an urgent need to develop highly efficacious and cost-effective drugs for the treatment of HCV, especially for use in resource-limited and developing countries of the world.

Plants are a rich source of natural compounds with diverse pharmaceutical properties [15, 16]. In particular, different parts of plants naturally contain abundant amounts of polyphenols (such as flavonoids), which have demonstrated antimicrobial activities against a wide range of pathogens, including viruses [17–28]. Plant-derived flavonoids have also been shown to possess anti-HCV activities, and could be used as cost-effective alternatives for the treatment of HCV [29–31]. As Citrus fruits naturally contain bioactive metabolites, especially flavonoids, which exhibit diverse bioactivities such as anti-oxidative, anti-cancer, anti-inflammatory, and antiviral properties [32], we focused our efforts on the identification of compounds from Citrus fruit extracts that can potently inhibit NS3 protease of HCV genotype 3a. For this purpose, extracts from 14 Citrus fruits were prepared and evaluated against the purified NS3 protease enzyme, complexed with the NS4A cofactor, of HCV genotype 3a in a FRET assay. Citrus extracts exhibiting anti-HCV properties were subsequently analyzed through the ESI–MS/MS technique for the identification of potent compounds, and insights into the molecular bases for the anti-HCV NS3 protease properties of identified compounds were gained through molecular docking studies.

**Methods**

**Plasmid constructs**

The plasmid (pET11a-His$_C$-NS3) (Drug Discovery and Structural Biology Lab, NIBGE, Pakistan) encoding the full-length NS3 protease of HCV genotype 3a fused to an oligohistidine (His$_6$) tag at the N-terminus was used for protein expression, as described previously [33]. A synthetic construct (pET11a-His$_C$-NS3-NS4A) encoding NS3 protease of HCV genotype 3a covalently linked to the NS4A cofactor (at the C-terminus) and an oligohistidine (His$_6$) tag (at the N-terminus) was commercially purchased (GenScript, Piscataway, NJ, United States).

**Heterologous expression of target proteins in Escherichia coli**

Target proteins (the full-length NS3 and NS3-NS4A) fused to oligohistidine tags were expressed in *Escherichia coli* BL21 (DE3) (Novagen, USA) or *E. coli* BL21-CodonPlus (DE3)-RIL cells (Agilent, USA). For protein expression, an overnight culture of transformed cells was prepared by inoculating 1.5 mL of the LB medium (Sigma Aldrich), supplemented with ampicillin (125 µg/mL) (Sigma Aldrich), with a single transformed colony and incubating the medium overnight at 37 °C with
shaking at 225 rpm. Then, 150 mL of the LB medium, supplemented with ampicillin (125 µg/mL), taken in a 1-L flask, was inoculated with the overnight culture, and the flask culture was continued until OD_{600} reached 0.5–0.6. The culture was subsequently incubated at 4 °C for 30 min, and supplemented with 100 µM zinc chloride (Sigma Aldrich). Expression of recombinant proteins was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Thermo Fisher Scientific), and the cells were cultivated for 16 h (at 18 °C) and 4 h (at 30 °C) for the expression of the full-length His_{6}-NS3 and His_{7}-NS3-NS4A, respectively. The cells were harvested by centrifugation (12,000 × g) at 4 °C for 30 min. After discarding the supernatant, the cell pellet was stored at -80 °C until further use. For the analysis of the protein expression at a small scale, cells were lysed using a bacterial protein extraction reagent (B-PER, Thermo Fisher Scientific), and proteins were analyzed on a 4–12% Bis–Tris NuPAGE gel using an X-Cell Surelock Mini-cell electrophoresis system (Invitrogen).

**Protein purification through affinity chromatography and gel filtration**

The cell pellet was resuspended in buffer A (25 mM HEPES (Thermo Fisher Scientific) pH 7.5, 1 M NaCl (Riedel), 10% glycerol (Invitrogen), and 25 mM imidazole (Sigma Aldrich)) to prevent non-specific binding of proteins to the column during affinity chromatography, and lysed through a high-pressure homogenizer (pressure: 10 kpsi; 2 passes; APV-1000 homogenizer, Invensys APV Products, Denmark). The cell lysate was centrifuged at 30,000 × g (30 min), and the supernatant was clarified by passing it through a 0.45 µm membrane filter. The clarified cell extract was loaded on a His-Trap column (GE, Healthcare), connected to an NGC system, pre-equilibrated with buffer A. After washing the column with buffer A to the baseline, proteins were eluted by applying a linear imidazole gradient (25 mM to 250 mM for His_{6}-NS3, and 25 mM to 500 mM for His_{7}-NS3-NS4A). The proteins were dialysed against the dialysis buffer (25 mM HEPES, pH 7.6, 0.2% Triton X-100, 20% glycerol, 0.4% Triton X-100 (Thermo Fisher Scientific), and 10 mM β-mercaptoethanol (Roth, Germany)) at 4 °C overnight, and concentrated using an Amicon Ultracentrifugal filter (10 kDa molecular-weight cut-off; EMD Millipore Corporation, Billerica, MA). The His_{6}-NS3 protein was subjected to size-exclusion chromatography using a 120 mL HiPrep 16/60 Sephacryl S-200 column (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ), pre-equilibrated with the dialysis buffer. The His_{7}-NS3-NS4A protein was diluted using buffer (25 mM HEPES, pH 7.5, 1 M NaCl, 10% glycerol) to decrease the imidazole concentration to 25 mM, and then incubated with His_{6}-tagged TEV protease at 4 °C overnight, followed by incubation at the room temperature for 4 h, in order to cleave the oligohistidine tag. The target protein (NS3-NS4A) was separated from the cleaved tag and His_{6}-tagged TEV protease through reverse affinity chromatography by applying the digestion mixture on a fresh His-Trap column (GE, Healthcare), pre-equilibrated with buffer A. The flow-through from the column was concentrated using an Amicon Ultracentrifugal filter (10 kDa molecular-weight cut-off; EMD Millipore Corporation, Billerica, MA), and subjected to size-exclusion chromatography using a 120 mL HiPrep 16/60 Sephacryl S-200 column (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ), pre-equilibrated with buffer A. The purified proteins (His_{6}-NS3 and NS3-NS4A) were concentrated using Amicon Ultracentrifugal filters (10 kDa molecular-weight cut-off; EMD Millipore Corporation, Billerica, MA), and were either immediately used for the enzymatic assay or flash-frozen in liquid nitrogen and stored at -80 °C until further use. The protein concentration was estimated using extinction coefficients (calculated through the ExPasy ProtParam tool) of 71,500 M⁻¹ cm⁻¹ and 18,700 M⁻¹ cm⁻¹ for His_{6}-NS3 and NS3-NS4A, respectively.

**Activity measurement of purified proteins**

For measuring the protease activity, a FRET assay was performed using a depsipeptide substrate, Ac-Asp-Glu-Asp-(EDANS)-Glu-Glu-Abu-ψ-[COO]-Ala-Ser-Lys(DABCYL)-NH2 (AnaSpec,US). The fluorescence signal generated upon cleavage of the depsipeptide substrate by the NS3 protease domain was continuously recorded using excitation and emission wavelengths of 355 nm and 510 nm, respectively, and a fluorescence microplate reader (TECAN, US). In the case of the full-length NS3 protease, the reaction buffer (50 mM HEPES, pH 7.5, 0.4% Triton X-100, 10 mM DTT (Thermo Fisher Scientific), 3% DMSO (Thermo Fisher Scientific), and 40% glycerol) was pre-incubated (at 30 °C for 10 min) with 25 µM of a synthetic peptide, KKGCVIVGVHIELGK (purchased from LifeTein LLC, US), representing the core of the NS4A cofactor required for the catalytic activity [34, 35]. For the FRET assay, 1 nM of the full-length NS3 protease was used. In the case of NS3-NS4A, the protein (0.5 nM) was incubated in the reaction buffer at 30 °C for 10 min (without the synthetic peptide). The reaction was initiated by the addition of the substrate (10 µL) in a two-fold serial dilution up to a concentration of 2 µM. Reaction wells without the substrate were used as negative controls. For correction of the inner filter effect, a previously described procedure was followed [36]. Kinetic parameters (such as kcat, Km, and kcat/Km) were calculated
using the Michaelis–Menten equation. Data were fit
to the equation by non-linear regression using the
GraphPad Prism® software (version 7.04, GraphPad
Prism®, Inc., USA).

Validation of FRET assay
For validation of the FRET assay, commercially available
inhibitors (asunaprevir, ciluprevir, danoprevir, and tel-
aprevir (purchased from AdooQ® Bioscience, US) were
used to inhibit the activity of the target enzyme (NS3-
NS4A protease of HCV genotype-3a), as described previ-
ously [37]. Briefly, 1 µM of the depsipeptide substrate was
added to the protein, pre-incubated with a given inhibitor,
and fluorescence measurements were recorded for
20, 30, or 60 min using 355 nm and 510 nm as excitation
and emission wavelengths, respectively. The IC₅₀ value,
representing the half-maximal inhibitory concentra-
tion, of each commercial inhibitor was calculated using
the GraphPad Prism® software (version 7.04, GraphPad
Prism®, Inc., USA) [38]. The experiment was performed
in triplicates, and the average value obtained was consid-
ered as the IC₅₀ value [39].

For calculation of assay parameters (such as the linear-
ity equation, the R² value, the limit of detection (LOD),
and the limit of quantification (LOQ)), a standard curve
was drawn between the EDANS concentration (the dye
attached with the FRET substrate as a fluorophore) and
the fluorescence signal. Moreover, the accuracy and pre-
cision of the assay were calculated using telaprevir and
danoprevir (commercial inhibitors).

Preparation of extracts
Extrakts from different fruit/seed parts of the following
varieties of Citrus plants were commercially obtained in
a powdered form either from Jiaherb Inc., US or Sanji-
ang Bio, US: Citrus aurantium [bitter orange], C. limon
[lemon], C. paradisi [grapefruit], C. reticulata [manda-
rin], and C. sinesis [orange]. The pomegranate pericarp
extract, previously reported to have potent inhibitory
activity against HCV NS3 protease [40, 41], was used as
a positive control. Four solvents: 70% ethanol in water,
etanol, ethyl acetate, and n-hexane were used to prepare
the extracts, as described elsewhere [42]. Briefly, the pow-
dered sample (1 g each) was extracted with 100 mL each
of the above-mentioned solvents by shaking (150 rpm)
in darkness at ~25 °C for 24 h. After filtration of the super-
natant, solvents from extracts were evaporated in vacuo
using a rotatory evaporator (Buchi R 210) set at 200 rpm
and 30 °C. The samples were stored under the cover of
Argon or oxygen-free nitrogen amber glass vials in an air-
tight environment at -20 °C until further use.

Evaluation of the inhibitory effect of plant extracts
against HCV NS3-NS4A protease
Reaction mixtures (300 µL) were prepared in the assay
buffer (50 mM HEPES, pH 7.5, 0.4% Triton X-100,
10 mM DTT, and 40% glycerol). For initial screening,
3.33 mg of the plant extract was dissolved in 1 mL
DMSO, and was transferred to the reaction buffer such
that the final concentration of DMSO was 3%. The reac-
tion was initiated by adding 0.5 nM NS3-NS4A protease,
and the released RET S1 FRET substrate was monitored
using a fluorescence microplate reader (TECAN). Reaction
mixtures (also containing 3% DMSO) without any
plant extract were used for recording blank measure-
ments. The background fluorescence contributed by the
plant extract was also recorded. For calculation of the
calibration curve, spiking with known amounts (0.25 µM,
0.5 µM, 1 µM, and 2 µM) of the free FRET substrate (RET
S1) was made while keeping the reaction mixture at the
same volume, containing all the constituents (the plant
extract, the assay buffer as well as the enzyme). Slope of
the curve was correlated with that obtained from free
FRET substrate concentrations without any plant extract
as well as the substrate. Initial reaction rates were calcu-
lated, and the percentage (%) enzyme inhibition was calcu-
lated using the equation: 100 x [a-b/a] where “a” refers
to the fluorescence value generated by the reaction mix-
ture only (without the plant extract) and “b” is the solu-
dion fluorescence in the presence of the plant extract.

For calculation of the IC₅₀ values, extracts were tested at
a final three-fold dilution ranging from 1.56 µg/mL to
3.33 mg/mL.

Mass spectrometric analysis of extracts
Based on the kinetic studies, four Citrus extracts (C. par-
adisi (mesocarp), C. sinesis (fruit), C. aurantium (fruit),
and C. reticulata (fruit)) which exhibited a strong inhibi-
tory effect on the activity of NS3-NS4A protease were
selected for a detailed investigation of their content using
a mass spectrometer (LTQ XL Linear Ion Trap, Thermo
Fisher Scientific, Waltham, MA, United States), fur-
nished with an Electrospray Ionization (ESI) probe. Each
extract (~5 mg) was dissolved in LC–MS grade metha-
nol (5 mL), after passing through a PTFE membrane filter
(0.45 µm), were injected to ESI–MS using Direct Syringe
Pump while keeping the flow rate at 10 µL min⁻¹. The
sample scanning was done at negative as well as positive
ionization modes by selecting the range of m/z 50–2,000.
At the positive ion mode, capillary and source voltages
were set at 35 kV and 4.2 kV, respectively. Various parameters, i.e.,
capillary temperature (280 °C), sheath gas flow rate (N2)
protein was found to be more than 95% pure as analyzed through SDS-PAGE (Fig. 1B). The obtained purification yield of NS3-NS4A was 6 mg per liter of the bacterial culture.

Activity analysis of NS3-NS4A through FRET assay
A concomitant increase in the activity of the fusion complex (NS3-NS4A protease) was observed upon increasing the concentration of the depsipeptide substrate in the FRET assay (Fig. 2). However, a corresponding increase in the activity was not observed when the full-length His7-NS3 was used in the assay (see Supplementary Materials, Figure S1 & S2), suggesting that NS3-NS4A represents a catalytically active complex that could be employed for enzyme inhibition studies. Furthermore, kinetic parameters calculated for NS3-NS4A (Fig. 2B) indicated a very high substrate binding affinity (Km 8.79 µM) as well as a high catalytic efficiency (kcat/Km 0.019 µM⁻¹.s⁻¹), suggesting that NS3-NS4A is suitable for conducting inhibitor screening assays.

The FRET assay was validated using commercially available inhibitors (asunaprevir, ciluprevir, danoprevir, and telaprevir), and IC₅₀ values in the nanomolar range were obtained (Table 1), confirming that reaction conditions for the FRET assay are properly optimized and are suitable for conducting inhibition studies using natural extracts/compounds from *Citrus* plants.

Various parameters (such as accuracy, precision, the limit of detection (LOD), and the limit of quantitation (LOQ)) calculated for the developed assay are given in Tables S1-S4 & Figure S3.

FRET-based screening of *Citrus* extracts
FRET-based screening of 14 extracts from *Citrus* plants revealed that the maximum inhibition (IC₅₀ value of 5.79 ± 1.44 µg/mL) of NS3-NS4A protease was exhibited by the *C. paradisi* mesocarp extract, followed by fruit extracts of *C. sinensis* (IC₅₀ value of 37.19 ± 5.92 µg/mL), *C. aurantiunum* (IC₅₀ value of 42.62 ± 6.89 µg/mL), *C. reticulata* (IC₅₀ value of 56.65 ± 3.81 µg/mL), and *C. limon* (IC₅₀ value of 66.83 ± 4.92 µg/mL), while the IC₅₀ values obtained for the remaining extracts ranged from 71.94 ± 2.39 µg/mL to 196.40 ± 2.92 µg/mL (Table 2).

All the afore-mentioned extracts displayed IC₅₀ values in the micro-molar range, suggesting that these natural extracts contain potent inhibitors of HCV NS3-NS4A protease (Fig. 3, Table 2). The pomegranate pericarp extract (used as a positive control in the current study) significantly inhibited the activity of NS3-NS4A, exhibiting an IC₅₀ value of 5.52 ± 0.74 µg/mL (Figure S4).
Mass spectrometric analysis of *C. paradisi* (mesocarp), *C. sinesis* (fruit), *C. aurantinum* (fruit) and *C. reticulata* (fruit) extracts

Out of 14 Citrus extracts, the top four active extracts against NS3-NS4A protease domain, i.e., *C. paradisi* mesocarp, fruit extracts of *C. sinesis*, *C. aurantinum* and *C. reticulata* were analyzed by ESI-MS for the identification of active natural compound(s). Various solvents (80% ethanol, 100% ethanol, ethyl acetate and *n*-hexane) were used to prepare extracts and among these, *n*-hexane and ethyl acetate gave better results in curtailing the noise and higher intensities of polyphenol peaks. The results related to identification of compounds in these extracts by ESI-MS have been summarized in Table 3. The full scan mass spectrum at the negative ion mode of the *C. paradisi* mesocarp extract gave several ion peaks in the range of *m/z* 100—700 (Fig. 4A, Table 3). To precisely identify natural products in extracts, each peak in negative [M-H]⁻ as well as positive [M+H]⁺ ion modes was subjected to collision induced dissociation (CID) lead fragmentation. The ESI-MS analysis [M-H]⁻ discovered the presence of bergapten (*m/z* 215), aliphatic/aromatic organic acids (*m/z* 255, 279, 281, 283, 367, 383 and 459); aglycone flavonoids, i.e., alpinetin (*m/z* 331), hesperitin (*m/z* 331) and methoxyflavone (*m/z* 435); coumarin, i.e., (R)-marmin (*m/z* 331) &; several glycone...
flavonoids such as naringenin arabinofuranose (m/z 535, 2% abundance), naringin (m/z 579, 6% abundance), hesperidin (m/z 609, 100% abundance), neohesperidin (m/z 610, 29% abundance), di-hydrated adduct of hesperidin [M-H+2H2O]−1 m/z 645, ~19% abundance) and traces of di-hydrated adduct of cyanidin-3-O-sophoroside chloride [M+2H+2H2O]−1. The extracts were also analyzed at the positive ionization mode [M+H]+, which revealed the identification of predominantly methoxylated flavonoids, i.e., limonene, synephrine, and tangeretin. Structures of the identified natural products were confirmed by the ESI-MSn analysis.

Similarly, compounds were identified in fruit extracts of C. sinesis, C. aurantinum and C. reticulata by the ESI-MSn analysis, which gave more or less similar profile patterns of secondary metabolites as spotted in the C. paradisi extract (Fig. 4 & Table 3), except that alpinetin (m/z 269), (R)-marmin (m/z 331), feruric acid di-saccharide (m/z 459) and naringin arabinofuranose (m/z 535)

| Sr. No | Inhibitor   | IC50 value (µg/mL) |
|--------|-------------|--------------------|
| 1      | Telaprevir  | 0.0475 ± 0.0075    |
| 2      | Danoprevir  | 0.0184 ± 0.0036    |
| 3      | Asunaprevir | 0.0511 ± 0.0093    |
| 4      | Ciluprevir  | 0.0412 ± 0.0101    |

Table 1 Half-maximal inhibitory concentration (IC50 values) calculated for commercial inhibitors of HCV NS3 protease using the FRET assay.

Fig. 2 Activity analysis of purified NS3-NS4A. A The activity of NS3-NS4A increased upon the addition of the depsipeptide substrate, indicated by a corresponding increase in the fluorescence intensity. B Kinetic parameters (Km, kcat, and kcat/Km) calculated for NS3-NS4A using the Michaelis Menten equation are presented.
were only identified in *C. paradisi* extract. Whereas, hesperitin (m/z 331) was present in significantly higher abundance in *C. reticulata*, quercetin (m/z 301) and isorhamnetin glucoside (m/z 477) were only spotted in *C. aurantinum*, esculin (m/z 339) and diosmetin-7-O-glucoside (m/z 461) were located in *C. sinesis*.

All four extracts exhibited a high abundance of hesperitin (m/z 609) and hesperidin di-hydrated adduct (m/z 645) (Table 3, Fig. 4). Structures of both the compounds were determined through tandem mass spectrometry. The ion peak m/z 645 was fragmented @CID 3.5 at the negative ion mode, which after losing 2H2O (two molecules of water involved in adduct formation) gave peak m/z 609 as a base peak (Fig. 5A), further MS3 fragmentation of m/z 609 yielded a minor daughter ion (m/z 463) after losing one hexose and a major daughter ion (m/z 301) by dissociating both hexoses. Notably, the native ion peak m/z 609 produced during the full scan of all four extracts as well the daughter ion (m/z 609) of m/z 645 gave a similar fragmentation pattern as described in Fig. 5B. Naringin (m/z 579) was also universally present in all *Citrus* extracts and its fragmentation produced the signature daughter ions, i.e., m/z 473, 459, 339 and 271 ion peaks (Fig. 5C).

Among the aglycone flavonoids, hesperitin and quercetin exhibit the same ion peak at m/z 301. The tandem mass spectrometric analysis enabled us to differentiate and precisely elucidate the structures of hesperitin and quercetin metabolites. Ion peaks produced by each of the four *Citrus* extracts at m/z 301 were subjected to fragmentation, which yielded two types daughter ion peaks patterns as described in Fig. 6. The *C. paradisi* mesocarp extract and fruit extracts of *C. sinesis* and *C. reticulata* yielded the daughter ions matching the hesperitin fragmentation pattern (Fig. 6A). Whereas, *C. aurantinum* ion peak (m/z 301) produced the daughter ion, which correlated with quercitin predominantly (~90%) (Fig. 6B), and only traces of hesperitin daughter ions were spotted in this extract.

Considering the detailed MS2 fragmentation analysis of hesperitin (m/z 301) isolated from full scans as well as the daughter ion of m/z 609 (MS3) (@CID3.5), yielding several daughter/grand-daughter peaks with m/z 286 being a base peak produced by the loss of methyl radical (-CH3•), which subsequently generated ion peaks by losing a -H2O (m/z 268), -CO (m/z 258) and rearrangement of a double bond (m/z 257) (Fig. 6A). The subsequent fragmentation of m/z 258 yielded m/z 151 and m/z 125 by losing the B and the C rings, respectively. The ion peak m/z 301 fragmentation further produced m/z 283 after losing H2O, subsequently yielding the alkyne adduct and C ring extended conjugation, which after losing CO2 formed an ion peak at m/z 241. The ion peak m/z 268 was produced from alkyne adduct (m/z 283) after losing a -CH3•. Whereas, hesperitin (m/z 301) also produced m/z 227, m/z 242 and m/z 257 after losing O•, CH3• and CO2, respectively. The m/z 151 may likely to be produced directly after fragmentation of m/z 301 and/or its subsequent adducts bearing C ring possibly through Retro Diels–Alder reactions. Markedly, the ion peaks m/z 151

Table 2: Inhibitory effect of *Citrus* extracts on the activity of NS3-NS4A. The extracts were screened using the validated FRET assay, percentage inhibition and IC50 values were calculated for each extract. The pomegranate pericarp extract was used as a positive control.

| Sr. # | Scientific name     | Common name          | Plant parts used | % Inhibition (±SD) *100) | IC50 value (µg/mL) |
|-------|--------------------|----------------------|------------------|--------------------------|--------------------|
| 1     | *Citrus paradisi*  | Grapefruit           | Mesocarp         | 91%                      | 5.79±1.44          |
| 2     | *Citrus sinesis*   | Orange               | Fruit            | 86%                      | 37.19±5.92         |
| 3     | *Citrus aurantinum*| Bitter Orange        | Fruit            | 85%                      | 42.62±6.89         |
| 4     | *Citrus reticulata*| Mandarin            | Fruit            | 82%                      | 57.65±3.81         |
| 5     | *Citrus limon*     | Lemon                | Fruit            | 80%                      | 66.83±4.92         |
| 6     | *Citrus aurantinum*| Bitter Orange        | Pericarp         | 79%                      | 71.94±2.39         |
| 7     | *Citrus aurantinum*| Bitter Orange        | Mesocarp         | 78%                      | 77.06±4.26         |
| 8     | *Citrus reticulate*| Mandarin            | Seeds            | 77%                      | 80.02±3.67         |
| 9     | *Citrus sinesis*   | Orange               | Seeds            | 75%                      | 86.93±5.16         |
| 10    | *Citrus reticulate*| Mandarin            | Mesocarp         | 74%                      | 90.59±1.89         |
| 11    | *Citrus paradisi*  | Grapefruit           | Pericarp         | 71%                      | 107.90±5.64        |
| 12    | *Citrus paradisi*  | Grapefruit           | Fruit            | 69%                      | 120.30±2.08        |
| 13    | *Citrus wateride*  | Grapefruit           | Seeds            | 68%                      | 132.23±2.12        |
| 14    | *Citrus aurantinum*| Bitter Orange        | Seeds            | 63%                      | 196.40±2.92        |
| 15    | *Punica granatum*  | Pomegranate          | Pericarp         | 98%                      | 5.52±0.74          |
and m/z 125 are considered as the signature ion fragments produced by most of the flavonoids during the ESI-MSn analysis [47]. Moreover, m/z 301 fragmentation yielded fused ring products (m/z 259, 215, 199 and m/z 185). All of the data analysis of daughter ion peaks confirmed the m/z 609 as a hesperidin structure [47, 48]. To further confirm the structure of ion peak at m/z 609, the fragmentation of authentic hesperidin standard data was found to be the fully correlated with fragmentation pattern of peak identified in the extracts of the C. paradisi mesocarp extract and fruit extracts of C. sinesis and C. reticulata.

Fragmentation of the ion peak m/z 301 spotted in C. aurantinum yielded the signature daughter ions of quercetin, i.e., m/z 273, 257, 179 and 151 ion peaks (Fig. 6B), which fully correlated with peaks produced during fragmentation of the quercetin reference standard.

The detailed mass spectrometric analysis of the pomegranate pericarp extract (used as a positive control for inhibition of NS3-NS4A in the current study) can be found in Figure S5.

Molecular modeling and docking studies of selected compounds
A good quality model of NS3-NS4A (with excellent stereochemical properties such as 98% residues in the most favored region of the Ramachandran plot, a QMean score of -1.77, and an overall quality factor of more than 93%
as measured through the ERRAT software) allowed us to use the generated homology model for docking studies with compounds identified through the ESI–MS/MS analysis.

Results obtained from the docking experiment suggest that the highest affinity for NS3-NS4A protease is exhibited by hesperidin (S-score value of -10.98) among the identified compounds. Hesperidin also interacts with the catalytic triad of the enzyme (Fig. 7), suggesting that hesperidin is a potent inhibitor of NS3-NS4A protease.

Evaluation of pure flavonoids
The ability of hesperidin to inhibit the activity of NS3-NS4A was confirmed using the FRET assay. It was observed that commercially obtained hesperidin significantly inhibited the activity of NS3-NS4A (with an IC_{50} value of 11.34 ± 3.83 µg/mL (Fig. 8), confirming that hesperidin is a potent inhibitor of the enzyme. Ellagic acid (Jiaherb, USA), identified from the pomegranate pericarp extract (positive control) (Figure S6- S8), exhibited an IC_{50} value of 29.62 ± 1.47 µg/mL against HCV NS3-NS4A protease (Figure S9).

Discussion
Despite the availability of effective direct-acting anti-HCV drugs, HCV infections are continuously increasing across the globe [3]. This is in part due to the high cost of the approved drugs, making it difficult for HCV-infected individuals, particularly from resource-limited and developing countries, to access the treatment option. Moreover, HCV, similar to other RNA viruses, is prone to mutations due to the lack of a proof-reading activity of its RNA polymerase, ultimately leading to the emergence of resistant HCV variants to currently available drugs [4].
Fig. 4 Full scan LC–MS analysis of top 4 potentially active Citrus extracts. **A** *C. paradisi* mesocarp extract, **B** *C. sinesis* fruit extract, **C** *C. aurantium* fruit extract, and **D** *C. reticulata* fruit extract.
Hence, there is a need to develop new anti-HCV drugs in order to combat HCV infections.

In the current study, we selected NS3 protease of HCV genotype 3a as a drug target due to its pivotal role in processing of viral proteins [11]. In order to identify potent inhibitors of NS3 protease, we employed the purified NS3 protease from HCV genotype 3a in fusion with...
its cognate NS4A cofactor in a FRET assay for screening of natural extracts from *Citrus* plants. Initially, a FRET assay was established using the purified NS3 protease of HCV genotype 3a. As expected, our efforts to establish a FRET assay using His<sub>6</sub>-NS3 protease alone (without a fused NS4A cofactor) did not prove fruitful (Figure S2). However, the fusion complex (NS3-NS4A) displayed a significantly high catalytic activity in the presence of a depsipeptide substrate in the FRET assay, suggesting that HCV genotype 3a NS3 is catalytically active only in fusion with the NS4A cofactor (Fig. 2). The FRET assay was validated using commercially available inhibitors (asuanaprevir, ciluprevir, danoprevir, and telaprevir) which significantly decreased the activity of the enzyme.
Furthermore, the IC$_{50}$ values of commercial inhibitors (such as telaprevir (0.0475 ± 0.0075 µg/mL) and danoprevir (0.0184 ± 0.0036 µg/mL)) obtained in the current study are very close to the IC$_{50}$ values (0.0475 µg/mL and 0.0146 µg/mL, respectively) reported elsewhere [49, 50] (Table S3). In agreement with previous studies [40, 41], the positive control (the pomegranate pericarp extract) significantly inhibited the activity of HCV NS3-NS4A, displaying an IC$_{50}$ value of 5.52 ± 0.74 µg/mL (Figure S4). The validated FRET assay was used for screening of natural extracts from Citrus plants, which are rich in antiviral compounds such as flavonoids [29–31]. Inhibitory activities of flavonoids are well-documented against multiple viruses such as herpes simplex virus [51], respiratory syncytial virus [52, 53], poliovirus [54, 55], sindbis virus [56], and dengue virus [57]. Our selection of Citrus plant extracts was based on previous studies, which demonstrated that Citrus plants are a rich source of flavonoids (such as apigenin, quercetin, and naringenin) exhibiting anti-HCV activities [29–31]. Our screening campaign revealed that the C. paradisi mesocarp extract displayed the lowest IC$_{50}$ value (5.79 ± 1.44 µg mL$^{-1}$), against NS3-NS4A protease of HCV genotype 3a out of 14 Citrus fruits extracts tested in the current study (Fig. 3A, Table 2).

Subsequent analysis by LC–MS/MS revealed hesperidin as the most abundant constituent of the Citrus extract (Fig. 4, Table 3). Structures of hesperidin, hesperitin, and quercetin were confirmed through the ESI-MS$^n$ technique (Fig. 5 & 6). Notably, the orange extract,
the bitter orange extract, and the mandarin extract exhibited the 2nd, 3rd and 4th best inhibitory activity against NS3-NS4A protease (Fig. 3B, 3C & 3D), and the afore-mentioned extracts also revealed the presence of hesperidin, as demonstrated by the ESI–MS analysis (Fig. 4&Table 3). In order to confirm that hesperidin is a potent inhibitor of NS3-NS4A, commercially available hesperidin (90% pure; obtained from Jiaherb, USA) was evaluated in the FRET assay. In agreement with results obtained from screening of Citrus extracts, hesperidin inhibited the activity of NS3-NS4A with high potency.

Notably, the broad-spectrum antiviral activity of hesperidin has previously been reported against a range of viruses such as poliovirus, parainfluenza virus, herpes simplex virus, vesicular stomatitis virus, vaccinia virus, rotavirus, human immunodeficiency virus, canine distemper virus, hepatitis B virus, and sindbis virus [56, 58–63]. Recently, it has been shown that hesperidin actively inhibits SARS-CoV-2 replication in the cell culture model [64]. Hesperidin is one of the safest flavonoids for human use that can help i) normalize glucose metabolism and mitigate the diabetes mellitus, ii) alleviate hyperlipidemia, iii) diminish retinal and plasma abnormalities, iv) improve liver inflammation and hepatocellular steatosis, and v) prevent cardiovascular diseases [65–71]. The pharmacological effects of hesperidin are also due to its ability to scavenge free radicals, and it has been demonstrated that hesperidin also plays an important role in cutaneous wound healing, skin whitening by inhibiting melanogenesis, and protection against Alzheimer’s disease and various types of cancers [63, 72–76].

Natural compounds with demonstrated inhibitory potential against HCV NS3 protease include excoecariphenol D, corilagin, 3-hydroxy caruiglan C, quercetin, epigallocatechin-3-gallate, and honokiol [30, 77–82]. However, hesperidin displays the lowest IC₅₀ value 11.34 ± 3.83 µg/mL (Fig. 8) compared to the above-mentioned compounds. The IC₅₀ value of hesperidin against HCV NS3-NS4A is better than that of ellagic acid (29.62 ± 1.47 µg/mL; Fig. 8 & S9), the bioactive compound identified in the pomegranate extract [40, 41]. In order to gain insights into the molecular interaction of hesperidin with HCV NS3 protease, molecular docking studies were performed, which suggested that hesperidin inhibits NS3 protease by blocking active site residues (Fig. 7).

Among the tested Citrus extracts, the highest IC₅₀ value against NS3-NS4A is exhibited by the bitter orange seeds extract (Table 2). The LCMS analysis of the bitter orange seeds extract reveals that the weak inhibitory effect of the extract against HCV NS3-NS4A protease could be attributed to a very low relative abundance of hesperidin in the extract (Figure S10). Moreover, the docking analysis of the other identified compounds (palmitic acid, linoleic acid, and cerebronic acid) from the bitter orange seeds extract suggests that these compounds do not interact with the active site residues (the catalytic triad: His57, Ser139, and Asp81), except linoleic acid which interacts only with His57 from the catalytic triad (Figure S11). In contrast, hesperidin and telaprevir inhibit the activity of HCV NS3-NS4A protease by blocking the active site residues (Fig. 7 & Figure S11). As hesperidin is naturally present in abundant quantities in grapefruit and other plants, especially in the Citrus family, hence, hesperidin could be pursued as a cost-effective drug candidate against HCV genotype 3a.

Conclusions
In the current study, we demonstrated that NS3 protease from HCV genotype 3a is functionally active in the NS4A-fused form, which can be employed for the development of a FRET assay for the discovery of anti-HCV inhibitors. Using a validated FRET assay, plant extracts from the Citrus family were evaluated for their inhibitory effect on the activity of NS3-NS4A. Results of the FRET assay together with ESI–MS/MS and molecular docking analyses revealed that a flavonoid, hesperidin which is abundantly present in the grapefruit mesocarp, orange, bitter orange, and mandarin whole fruit extracts, could act as an effective inhibitor of HCV NS3-NS4A protease, suggesting that Citrus fruit extracts are a rich source of cost-effective natural products with antiviral bioactivities.
Additional file: Figure S1. Cloning strategy, expression and purification of the full-length NS3 of HCV genotype 3a. (A) The gene encoding the full-length NS3 was cloned in the pET11a plasmid under the control of the T7 promoter using BamHI and HindIII restriction sites. The construct was used for heterologous expression of the full-length NS3 fused to a polyhistidine (His$_6$) tag in Escherichia coli. (B) Expression analysis of the full-length NS3 in E. coli BL21 (DE3) cells (lanes 1 and 2). The cell lysate was analyzed on a 4-12% Bis-Tris NuPAGE gel. Lanes 1 and 2 represent the soluble fraction of the cell lysate from the uninduced and induced cells and IPTG-induced cells, respectively. Lanes 3 to 9 represent samples not related to the current study. The black arrow indicates the band corresponding to the estimated molecular weight (~ 66 kDa) of the full-length NS3. Lane M represents the mobility of proteins with known molecular weights (SeeBlue Pre-stained Protein Marker). (C) Expression analysis of the full-length NS3 in BL21-CodonPlus(DE3)-RIL cells (lanes 1 and 2). The cell lysate was analyzed on a 4-12% Bis-Tris NuPAGE gel. Lanes 1 and 2 represent the soluble fraction of the cell lysate from the uninduced and induced cells and IPTG-induced cells, respectively. Lane 3 represents the sample collected during stringent washing of the HisTrap column. Lanes 4, 5, and 6 represent the samples collected during the elution step from the HisTrap column, pooled samples after gel filtration, and the last fraction collected during gel filtration, respectively. Samples were analyzed on a 4-12% Bis-Tris NuPAGE gel. **Figure S2.** Activity analysis of purified full-length NS3. Upon increasing the substrate concentration, no detectable increase in the fluorescence intensity was observed in the case of the full-length His$_6$-NS3, suggesting that the full-length His$_6$-NS3 is non-functional in the absence of a fused N54A cofactor. **Figure S3.** A standard calibration curve plotted between various concentrations of EDANS and the generated fluorescence signal. A linear line having an R$^2$ value of 0.996 was obtained. **Figure S4.** Inhibitory effect of the pomegranate pericarp extract (used as a positive control) on the activity of NS3-N54A. The pomegranate extract significantly inhibited the activity of NS3-N54A (IC$_{50}$ value of 5.52 ± 0.74 µg/mL) as measured through the validated FRET assay. **Figure S5.** ESI-MS/MS analysis of P granatum pericarp methanol extract in negative ion mode. **Figure S6.** Proposed fragmentation of ellagic acid hexoside based on quasi-ESI-MS$^+$ spectra in negative ion mode. **Figure S7.** Proposed fragmentation of ellagic acid based on quasi-ESI-MS$^+$ spectra in negative ion mode. **Figure S8.** Proposed fragmentation of ellagic acid pentoside based on quasi-ESI-MS$^+$ spectra in negative ion mode. **Figure S9.** Inhibitory effect of ellagic acid on the activity of NS3-N54A. Ellagic acid significantly inhibited the activity of NS3-N54A as measured through the FRET assay, yielding an IC$_{50}$ value of 29.62 ± 1.47 µg/mL. **Figure S10.** ESI-MS/MS analysis of the bitter orange seeds extract in the negative ion mode. **Figure S11.** Docking of strong (telaprevir) and weak (palmitic acid, linoleic acid, and cerebrosic acid) inhibitors in the active site of HCV genotype 3a N53 protease. The region around the active site selected for docking of compounds is depicted as a blue circle. Active site residues (His57, Asp81, and Ser139) are shown in red, while docked compounds are shown as orange sticks (telaprevir), magenta sticks (palmitic acid), green sticks (linoleic acid), and blue sticks (cerebrosic acid). Hydrogen bonds are depicted as blue dotted lines. The interaction of telaprevir, palmitic acid, linoleic acid, and cerebrosic acid with active site residues is presented in (A), (B), (C), and (D), respectively. **Table S1.** Inhibition trials of telaprevir against NS3-N54A protease. **Table S2.** Calculation of Linearity, LOD, and LOQ using standard curve. **Table S3.** Comparison of IC$_{50}$ values of commercial inhibitors used in study with IC$_{50}$ reported in the literature. **Table S4.** Inhibition trials of danoprevir against NS3-N54A protease
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