ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

Harnessing Nature's Gifts: *Salix nigra* and Its Potential for Combating Hepatitis C Virus (HCV)

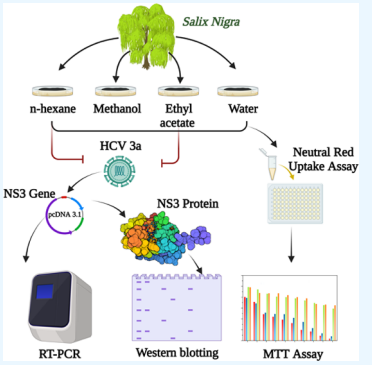
Sadia Bibi, Mohammad Nisar,* Shazia Rafique,* Muhammad Waqas, Muhammad Zahoor, Muhammad Idrees, Nausheen Nazir, Mohammad Ihsan, Saleh H. Salmen, Sulaiman Ali Alharbi, Ajmal Khan, and Ahmed Al-Harrasi*

Cite This: *ACS Omega* 2023, 8, 42987−42999

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Hepatitis C virus (HCV) causes various liver complications, including fibrosis, cirrhosis, and steatosis, and finally progresses toward hepatocellular carcinoma (HCC). The current study aimed to explore the antiviral activity of the traditional Pakistani medicinal plant *Salix nigra* (*S. nigra*) known as black willow against the hepatitis C virus (HCV). The anti-HCV activity of *S. nigra* was established against stable Hep G2 cell lines expressing the HCV NS3 gene. Various plant-derived compounds with anti-HCV activity were identified, making phytotherapy a promising alternative to conventional treatments due to their cost-effectiveness and milder side effects. The two extraction methods (Maceration and Soxhlet) and four solvents (*n*-hexane, methanol, ethyl acetate, and water) were used to obtain crude extracts from *S. nigra*. Cytotoxicity testing showed that methanol (CC_{50} 25 μg/mL) and water (CC_{50} 30 μg/mL) extracts were highly toxic, while ethyl acetate and *n*-hexane (CC_{50} > 200 μg/mL) extracts were nontoxic at low concentrations (10−50 μg/mL), making them suitable for further anti-HCV investigations. Stable transfection of the NS3 gene was successfully performed in Hep G2 cells, creating a cellular expression system for studying virus−host interaction. The ethyl acetate extract of *S. nigra* exhibited significant inhibition of NS3 gene expression (mRNA and protein levels). The phytochemical analysis of *S. nigra* was also performed using the high-performance liquid chromatography (HPLC) technique. The phytochemical analysis identified several polyphenolic substances in the extracts of *S. nigra*. Our results concluded that the extracts of *S. nigra* have significantly reduced the expression of the NS3 gene at mRNA and protein levels. These findings contribute to the global efforts to combat hepatitis C by offering plant-based treatment options for HCV management.



1. INTRODUCTION

Hepatitis C virus (HCV) infection is the most important *Flaviviridae* infection with major global health problems.[1] HCV is the principal cause of liver cirrhosis and fibrosis that finally progresses toward hepatocellular carcinoma.[2] Globally 170−200 million people are infected with HCV, including 10−17 million in Pakistan.[3,4] About six major HCV genotypes and multiple subtypes have been identified globally.[5] In Pakistan, the seroepidemiology of HCV among the general public is 6.8%, while 6% of the population was seen with active HCV infection.[6] The HCV genome (~9.6 kb in length) encodes a single large open reading frame, producing ~3000 amino acid polypeptides that are co- and post-translationally cleaved by viral and host proteases into structural (Core, E1 & E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B).[7] The small protein p7, which is generated by cleavage and serves as an ion channel and viroporin, is thought to be present in the virion.[8] E1 and E2 both contribute to attaching the viral particle to the host. NS3-NS5 are necessary for viral replication in addition to their functions in particle formation and secretion. NS3 is a nonstructural dual-function protein having a helicase domain at its C-terminus and a protease domain at its N-terminus.[8] According to studies, NS3's helicase activity is necessary for viral replication. The unfolding of secondary structures within the HCV genome, the unwinding of the duplex generated by the positive and negative strands during replication, and the displacement of proteins that might prevent viral replication are some of the proposed functions of NS3 helicase.[9] NS3 protein is included in the DExH helicase family, which falls under superfamily 2. It moves in the 3′ to 5′ direction to exert unwinding action on RNA or DNA substrates.[9]

Medicinal plants are an important source of phytochemicals that are inclusively used in drug industries as raw materials.[10] Natural products are preferably used as synthetic drugs, which

Received: August 21, 2023
Accepted: September 26, 2023
Published: October 30, 2023

are associated with many side effects, whereas the former has low or no side effects. A number of natural compounds have been reported to have antiviral activities as they interfere with the virus life cycle, its entrance into the host, and replication.[11] Substantial studies have been conducted on the extracts and compounds isolated from medicinal plants that have activity against different microorganisms causing human infections.[12,13] A wide variety of natural compounds have shown anti-HCV activities[12,14] and have shown hepato-protective effects as described for silymarin, epigallocatechin-3-gallate, caffeine, and naringenin.[15] Therefore, phytotherapies against antiviral infections are considered as an alternative approach to explore further.[16] Natural compounds of medicinal plants have a characteristic low cost of production, high structural diversity, and milder or no side effects than conventional therapies.[17] Furthermore, more than half of the drugs administered presently in treating various diseases have been derived from microorganisms and plants.[18] Nearly 70−80% of individuals globally depend on medicinal plants to cure multiple human diseases including viral infections.[19] Multiple traditional medicine systems like Chinese medicine and Ayurveda have gained popularity and are utilized in different parts of the globe.[20] Direct-acting antiviral (DAAs) drugs have been used for HCV treatment.[21] However, the high costs and potential for developing resistance presented by current therapy demonstrate the need for developing highly efficient novel antivirals or combined therapies that target different stages of the viral pathways.[17] Recently high relapse rates of current therapy have been reported against HCV patients in Pakistan[22] inferring the need for local novel strategic therapies to find alternative potent and targeted therapies for the eradication of this deadly infection. The search for new and effective treatments for HCV infection has therefore become a major research priority. One promising approach is the use of medicinal plants with potential anti-HCV activity. Several medicinal plants and their active compounds have been reported to exhibit anti-HCV activity through various mechanisms including inhibition of viral entry, replication, and assembly. For example, the extract of Phyllanthus niruri, a plant used in traditional medicine for liver diseases, has been shown to inhibit HCV replication by targeting the viral RNA polymerase.[23] Curcumin, a polyphenol extracted from turmeric, has been shown to inhibit HCV entry by blocking viral attachment to host cells.[24] The Black Willow, *Salix nigra*, is a deciduous tree belonging to the family Salicaceae. In North America, black willows may be found all across wetlands, marshes, and floodplains. It has a long history of usage in many forms of folk medicine, and its potential medicinal effects are generally accepted.[25] The range of black willow (*S. nigra*), a species of willow that is threatened in North America, extends from the southern United States to the southeast of Canada. It prefers damp locations and rapidly spreads into new ones along riverbanks, stream margins, and wetland habitats. Its hydrophilia originates from its usefulness in preventing soil erosion and preserving riverine habitats. Due to their perceived medicinal value, different parts of the Black Willow tree have been included in therapies by indigenous communities and traditional medical systems. Black willow bark has traditional medicinal uses for aches and pains.[26] Salicin, a component of the plant, is an analgesic and anti-inflammatory agent with characteristics similar to those of aspirin. Infusions and decoctions made from black willow bark have been used for centuries to cure fevers and reduce body temperature, much like salicin. Inflammatory illnesses, including arthritis and joint pain, have been treated with Black Willow bark due to the salicin content's potential to suppress inflammatory responses. Salicin has analgesic properties. Hence, Black Willow bark decoctions have been used historically to treat headaches and migraines.[27] Wounds, burns, and skin irritations have been treated by using poultices or infusions made from Black Willow bark, which may hasten the healing process and decrease local inflammation. Historically, people have used remedies made from black willow to ease their stomachaches. The antipyretic and febrifuge properties of Black Willow bark have been the subject of several ethnomedical practices, suggesting that it may be useful in treating malaria and other febrile disorders. Traditional wisdom may have uncovered some promising medicinal uses for Black Willow, but further study is required to validate these uses, understand how it works, and find appropriate dosages.[28] The current study focuses on the antiviral activity of the *S. nigra* medicinal plant by targeting the HCV NS3 gene, which is crucial for viral replication.

2. METHODOLOGY

2.1. HCV Genotype 3a Sample Collection. Those Patients who tested positive for HCV-3a were selected for blood sample collection. The method described by Idrees et al. (2008)[29] was used for the determination of HCV genotyping. Those samples which were confirmed for genotype 3a were selected for isolation of RNA. The blood was used to isolate the important nonstructural gene, namely, NS3, which is an important antiviral target in HCV therapeutics. The purpose of the study was fully explained to the participating patients, and written consent was signed from each patient. The following factors were taken into consideration during the sample collection.

2.2. Criteria for Inclusion. Patients having HCV must be at least 18 years old. Patients tested positive for HCV through RNA and ELISA tests. Patients whose DNA and ELISA tests for HBV were negative. Patients showed no signs of liver failure. Patients did not receive any treatment therapy for HCV during or before the time of enrollment.

2.3. Blood Samples Collection. BD Vacutainer tubes were used to collect about 5 mL of blood from patients who tested positive for HCV. Serum was obtained by centrifugation for 10 min at 2000*g*. Serum was isolated through serum separation tubes. The isolated serum was stored at −70 °C.

2.4. Complementary DNA Synthesis and Amplification of NS3 Gene. The patients with genotype 3a who were chronically infected with HCV were selected. The RNA from the HCV-positive patients was extracted using a commercially available RNA isolation kit (Gentra, Life Technologies) according to the manufacturer's instructions. The RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen Life Technologies, CA). The gene was amplified from cDNA using additional gene-specific primers. The primers were designed using Primer3 software with reference sequence GU294484.1 as a template. After web and neb cutter analyses were performed, restriction sites and Kozak sequences were introduced. The primer sequences are shown in Table 1.

2.5. Cloning of the NS3 Gene in the Mammalian Expression Vector and Sequencing. Following amplification, the gene was cloned into pcDNA3.1 at designated restriction sites (Invitrogen Life Technologies, CA). Plasmid pcDNA3.1 contains the genetic code for the whole NS3

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

Table 1. Primers Used for the HCV NS3 Genotype Including a Gene with Housekeeping Gene GAPDH and Estimated Product Size

serial no.	primer name	sequences (5′- 3′)	product size bp
1	NS3-F	GGCCGTGAGGTGTTGTTGG	1953
2	NS3-R	TGGTTACTTCCAGATCGGCTG	
3	GAPDH-F	TTTGGCTACAGCAACAGGGT	224
4	GAPDH-R	ACATGACAAGGTATGCCCCT	

protein. The PCR method was used to insert *Bam*HI and NotI recognition sites at the gene's 3′ and 5′ ends. Following enzyme digestion, the gene was subcloned into pcDNA3.1 (Invitrogen Life Technologies, CA). Each plasmid containing a distinct HCV-3a gene was verified by using PCR, restriction digestion, and sequencing. The sequence of the NS3 gene (accession number **OQ915126**) has been submitted to GenBank.

2.6. Cell Culture and Transfection. Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mg/mL streptomycin, penicillin, and 10% fetal bovine serum was used to cultivate and sustain Hep G2 cell lines for the investigation (FBS; Sigma-Aldrich). After seeding 6-well plates with 5×10^5 cells per well, we cultured the cells until they reached 70−80% confluence. When the cell confluence reached 75%, they were transfected with 10 mg of linearized pcDNA3.1/NS3 using lipofectamine reagent (Invitrogen Life Technologies, CA). After 6−8 h of incubation, the transfection medium was changed out for media with 100 mg/mL of penicillin, streptomycin, and 10% fetal bovine serum.

2.7. Extraction of RNA from Cell Lines and Expression Analysis. Using an RNA extraction kit (Gentra, Life Technologies), total RNA was extracted from Hep G2 cells to verify efficient transfection and expression of NS3 in Hep G2 cell lines. Next, cDNA was synthesized from total RNA using antisense primers specific to each gene of interest. To verify mRNA, we amplified the reverse-transcribed RNA for each gene using Taq polymerase and gene-specific primers (Table 1). All PCR results were run on an agarose gel of 1.2% concentration, stained with ethidium bromide (0.5 mg/mL), and photographed under UV illumination.

2.8. Western Blotting and Protein Isolation from Hep G2 and Stably Transfected Cell Lines. After 72 h, Hep G2 cells were lysed, and total cell lysate protein was extracted for protein expression analysis. After washing in PBS, pellets of cells were centrifuged at 8000 rpm for 3 min to remove debris. Protein lysis buffer (60 mL) was added, and the mixture was vortexed for 5 s before being placed on ice for 15 min. After being spun for 10 min, it was spun at 13,000 rpm for 15 min at 48.0 °C. 60 μg of the extracted protein was transferred to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel at 16 V for 90 min using a semidry blotting system (Bio-Rad). A Hybond-C additional nitrocellulose membrane was substituted for the previous membrane. After two washes in PBS-Tween and 1 h in a 5% skim milk-blocking solution, the membrane proteins spent the night incubated with monoclonal antibodies that recognize the NS3 gene (NS3-sc-69938). The primary antibodies were detected by incubating the blot at room temperature for 1 h with a 1:1000 dilution of a specific antimouse secondary antibody conjugated to alkaline phosphatase (Sigma). After being rinsed three times with PBST, the membrane was incubated with Nitro-blue tetrazolium and 5-bromo-4-chloro-30-indolyphosphate (NBT/BCIP) substrate for 15−30 min at 37 °C.

2.9. Evaluation via Immunofluorescence. Overnight, cloned NS3 gene containing Hep G2 cell lines was cultured on glass coverslips at 20 °C before being fixed with 4% formaldehyde. Inhibition of fixed cells was achieved with 1% donkey serum in 1PBS. The NS3 protein was detected by treating cells with the particular gene−antibody (1:100). After incubating the cells for 2 h in PBS, the secondary donkey antimouse IgG antibody conjugated with fluorescein isothiocyanate was added (FITC, 1:100; Chemicon). After the coverslips were cleaned and mounted on slides, a fluorescence microscope was used to examine the samples.

2.10. Hep G2 NS3 Genotype 3a Stable Cell Line Generation and Establishment. Hep G2 cells were transfected with 10 mg of the linearized plasmid DNA construct (pcDNA3.1/NS3). At 72 h post-transfection, the cells were split onto 60 mm culture plates and subjected to selection with G418 sulfate (Gibco), initially at a concentration of 1 mg/mL for around 5 weeks. The medium was switched after 72 h. Stable cell lines were generated by selecting, cultivating, and multiplying G418-resistant colony clones. The expression of HCV genotype 3a NS3 protein in these cells was confirmed using RT-PCR, Western blotting, and immunofluorescence assays.

2.11. Selection of Plant. Several native plants are found in Asian and Pakistani pharmacopeias that have long been utilized for the treatment of liver issues. However, the effectiveness of these conventional medicinal plants is yet to be found.[30] This research work was designed for the evaluation of cytotoxicity and anti-HCV activity of a local Pakistani plant namely, *S. nigra*, studied for their activity against the nonstructural protein NS3 of HCV-3a.

2.12. Plant Extract Formation. Plants that were fully grown, healthy, and free of infection were chosen for the study. Various plant parts, such as samples of leaves and roots, were cleaned using distilled water to get rid of any dirt and allowed to air-dry for 3 weeks at room temperature. An electric grinder was used to ground the fully dried plant components into a fine powder. The plant materials underwent two processes, maceration and Soxhlet extraction for crude extraction. With increasing polarities, *n*-hexane, methanol, ethyl acetate, and water were utilized as the four extraction solvents.

2.13. Solvent Extraction by Maceration. For crude extraction, 30 g of the plant sample was diluted in 300 mL of all four solvents (*n*-hexane, methanol, ethyl acetate, and water) using a sterilized beaker. The beaker was wrapped in parafilm and set in a magnetic stirrer. The solution was agitated on the magnetic stirrer every 16 h to achieve full extraction for 7 days. Whatman filter paper grade-1 was used to filter the extraction solutions, and the solvent was evaporated through a rotary evaporator. After the entire evaporation of the solvent, the dried extract was collected with a spatula. Extract yield was determined after the extract. The crude extraction process was performed in triplicate, and the resulting extracts were collected in a sample bottle.

2.14. Soxhlet Extraction. 30 g of the plant sample powder was loaded into the thimble. Whatman filter paper grade-1 was used for making a thimble. The thimble was then adjusted in the main chamber. Now, the apparatus has been set up. 300 mL of the chosen solvents was added to a round-bottom flask and placed onto the heating mantle. Wax was used to seal the joints. A reflux condenser was attached to the extractor. The

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

condenser was connected to an inlet and an outlet pipe. The extracting solvent was heated until boiling and left to extract for the required amount of time. This process was carried out for 4−10 cycles. The extract was then transferred from the round-bottom flask to the Petri plate and evaporated at 40 °C in the rotary evaporator until a drop of solvent did not leave a residue. The extract was collected and weighed, and the extract yield was determined. The extraction was performed in triplicate, and the resulting crude extracts were combined in a sample bottle. The Soxhlet and maceration extracts were combined and used for further processing.

2.15. Cell Viability Assay. The cytotoxicity of each plant extract was monitored on Hep-G2 cell lines. The 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or neutral red uptake assay[31] with some modifications was performed to calculate the minimum lethal dose for each plant extract. Neutral red uptake assay is an effective method for assessing a compound's potential cytotoxicity or the viability of treated cells. It employs a colorimetric approach that aids in determining the number of living cells, based on the idea that living cells will take up this dye as their lysosomes can attach to the supravital dye, while nonviable cells are unlikely to keep the dye. Living cells will be able to keep the pH stable through oxidative phosphorylation. After the cells were incubated with the dye, a spectrophotometric study was used to quantify the dye that binds to the cells by measuring the absorbance of light at a wavelength between 540 and 560 nm. The absorbance indicates the amount of dye that has accumulated. A higher absorbance means more dye has accumulated, whereas a lower absorbance suggests less dye has accumulated and perhaps more serious cell damage and death. Solutions for staining and destaining were first made to perform a neutral red uptake experiment. After about 24 h of incubation at 37 °C, the Neutral Red dye solution was employed following syringe filtering. Both solutions were stored in brown bottles at room temperature. In brief, the cells were added at a density of 2 × 103 cells per well in a 96-well plate and cultured in DMEM at 37 °C and 5%CO2. The next day, the cells were exposed to plant extracts at increasing concentrations ranging from (10−100 μg/mL) and sterilization was done through a 0.2 μm syringe filter. Extract dilutions were prepared in such a manner that the total amount of DMSO in every single well was kept to a maximum of 0.1%. The cells of the control group also received treatment of 0.1% DMSO. Following incubation for 24 h, the morphology of the cells was determined by microscopical examination. After extracting the extract and DMEM solution, 150 μL of a 1−100 DMEM-diluted Neutral Red dye mixture was poured into each well. The plate was then incubated for 60 min at 37 °C. After carefully removing the NR media, the cells were next washed with 1X PBS. After gentle aspiration of the PBS, 200 μL of the destaining solution was added to each well of the plates. The Bio-Tec microplate reader was used to read the plates after they had been gently spun on a plate shaker for 15 min. Calculating the 50% cytotoxic concentration (CC50) required an absorbance measurement at 540 nm. This is the lowest concentration at which cell viability is reduced to 50%. Using DMSO control cells, each experiment's percentage of cell viability was calculated using the following formula:

$$\%Cellviability = \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100 \quad (1)$$

2.16. Inhibition in NS3 Region of HCV by *S. nigra* in Hep-G2 Cells. Hep-G2 cells were cultured in 24-well plates for 24 h prior to transfection studies. After the medium was discarded, 1 mL of PBS was used to wash the cells. Lipofectamine TM 2000 (Invitrogen Life Technologies, Carlsbad, CA) was used to transiently transfect cells with expression plasmids expressing HCV NS3 protease in the presence and absence of *S. nigra* 100 g extract and interferon. Total RNA was extracted using Trizol reagent following the protocol provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). cDNA was generated from 1 g of RNA using a Revert Aid TM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot/Germany) in order to examine the impact of *S. nigra* on the HCV NS3 gene. PCR (Applied Biosystems, Inc.) was used to examine gene expression using a 2 PCR Mix (Fermentas). The amplified DNA samples were analyzed on an agarose gel at a concentration of 2%. The DNA bands could be seen in direct UV light, allowing the gels to be photographed by using the gel documentation system. After 48 h of transfection, the cells were lysed and proteins were extracted from the total cell lysate. Transfected Hep-G2 cells were employed as a negative control. We separated 60 μg of protein on an SDS-PAGE gel at 10% and then transferred the protein to a nitrocellulose membrane.

2.17. Preparation of the Extracts for HPLC-UV Analysis. As per the published method, HPLC-UV characterization and quantification were performed. About 1 g of the powdered plant sample was diluted in 20 mL each of methanol and water (1:1) to prepare the extract for HPLC analysis. After being heated at 70 °C in a water bath for an hour, the mixture was centrifuged for 10 min at 4000 rpm. Following that, 2 mL of the plant sample was filtered using Whatman filter paper into HPLC vials and designated with the appropriate codes. The samples were analyzed using an Agilent 1260 infinity high-performance liquid chromatography (HPLC) system, and the bioactive compounds were separated using an Agilent Zorbax Eclipse XDB-C18 column with gradient system consisting of solvents A (methanol, acetic acid, and deionized water, v/v, 900:20:80) and solvent B (methanol, acetic acid, and deionized water, v/v, 100:20:880). For the investigation of phenolic compounds, the ultraviolet array detector (UVAD) was programmed at 250 nm. The retention durations, UV spectra, and relevant standards were used to identify phenolic compounds, while the % peak area of the samples and the reference standard were used for quantification by employing the given formula

$$Cx = \frac{Ax \times Cs(^{\mu g}/_{ml}) \times V(ml)}{As \times Sample(wt.\ ing)} \quad (2)$$

where ***Cx*** is the sample concentration; ***As*** is the standard peak area; ***Ax*** is the sample peak area; and ***Cs*** is the standard concentration (0.09 μg/mL).

2.18. Statistical Analysis. The experiments were carried out in triplicate to test the reliability of the results. The statistical comparisons were made by one-way ANOVA following the Tukey HDS test. Results will be considered significant at $p < 0.05$.

3. RESULTS

3.1. Patient Enrollment Criteria. Only HCV-infected patients were enrolled in the current study after being screened for HCV genotype 3a infection.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

3.2. Amplification of the Nonstructural NS3 Gene of HCV-3a. The FavorPrep Viral Nucleic Acid Extraction Kit was used to isolate RNA from the serum samples. Reverse transcription was performed to generate negative sense cDNA of the extracted RNA using primers that were unique to the HCV gene. The cDNA was synthesized by using the 3′UTR antisense primer. The nonstructural gene NS3 of the HCV genotype 3a of the Pakistani isolate was amplified using the created cDNA as a template. The primer sets that provide complete gene amplification were employed in subsequent investigations after the primers were adjusted for the ideal amplification of the HCV NS3 gene. The NS3 HCV gene's estimated size was 1953 base pairs. NS3A gene amplification of the HCV-3a genotype is given in Figure 1. The predicted

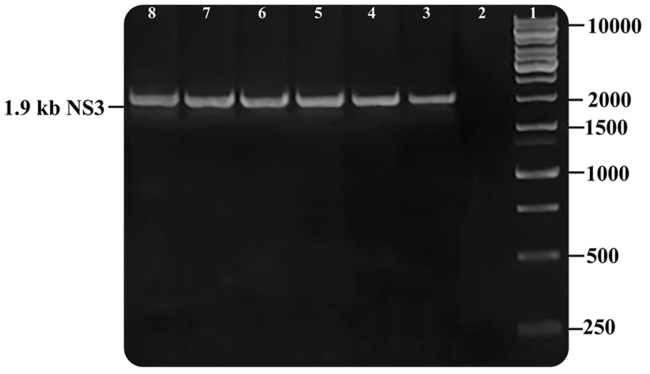


Figure 1. NS3 gene amplification of the HCV-3a genotype. Lane 1, 1kb ladder, Lane 2, negative control, and Lanes 3−8, NS3 gene.

product size of the NS3 gene, 1956 bp, was identified. The NS3 gene of the locally isolated HCV-3a genotype was submitted to the gene bank with accession code OQ915126. This verified sequence NS3 of local HCV-3a was utilized for additional research.

3.3. Construction of Plasmids. Mammalian expression vectors were designed to examine the efficacy of an anti-HCV test based on the production of the nonstructural gene (NS3) of HCV genotype 3a (Figure 2). In this study, we used the mammalian expression vector pcDNA3.1. The amplified gene was cloned into the pcDNA3.1 mammalian expression vector inside the designated restriction sites after digestion by the relevant restriction enzymes. Successful pcDNA3.1/NS3 clones were confirmed by restrictive digestion. The cytomegalovirus (CMV) promoter is included in the pcDNA3.1 vector, which facilitates the transduction of eukaryotic cells for both transient and long-term expression studies.

3.4. Selection of Mammalian Cell Lines. The constructed plasmids of the NS3 gene were initially transiently transfected into three distinct cell lines, HEK-293, Hep-G2, and Hep-G2, to optimize transfection conditions and subsequently assess the expression level of the targeted gene in these hosts. We chose the cell line whose gene expression level was higher for stable transfection at the mRNA and protein levels. The Hep-G2 cell line showed higher expression than the other two cell lines. So, Hep-G2 cell lines were picked for stable transfection.

3.5. Generation/Establishment of Stable Cell Line. Hep-G2 cell lines were transfected with a linearized expression vector using the lipofectamine reagent. The transfected cells were cultured with 1 mg/mL G418 added for selection at 24 h post-transfection. Approximately 4 weeks of culture at this concentration resulted in most of the cells displaying no symptoms of G418 resistance. From colonies of G418-resistant cells, cell lines were selected and grown at a 500 mg/mL dosage. When the cell lines were grown separately, there were some differences in their growth rates.

3.6. Expression Analysis of NS3 Gene in Stable Cell Lines. To determine whether NS3 RNA was present in transfected G418-resistant cells, total RNA was isolated from independently produced cell lines of the targeted HCV gene and untransfected Hep-G2 cells (control cells). Extracted RNA was reverse-transcribed into complementary DNA (cDNA) with the use of reverse transcriptase enzyme (MMLV) and gene-specific primers. Additional processing was performed on the cDNA so that gene-specific primers are used to amplify the NS3 gene. Figure 3 shows the expected size of the HCV-3a NS3 gene.

3.7. NS3 Protein Expression Analysis in Stable Cell Line. Western blotting was used for evaluating the protein expression. The stable clones confirmed by RT-PCR were grown further and subjected to protein extraction and Western blot analysis. Untransfected Hep G2 cells served as a negative control. Utilizing monoclonal antibodies specific to NS3, the 68−70 kDa protein bands for NS3 were confirmed (Figure 4). No substantial decrease in protein expression was noticed, suggesting that stable cell lines for the NS3 gene were generated. Following verification through RT-PCR and Western blotting, these stable cell lines were preserved as cryovials and used in subsequent experiments.

3.8. Stable Cell Lines Immunofluorescence Assay. With fluorescent-labeled antibodies, immunofluorescence was used to visualize expressed proteins in compartments within the cells. NS3-specific monoclonal primary antibodies were used to treat stable cell lines, followed by fluorescent conjugate secondary antibodies. Confirmation of living cells was done after counterstaining with DAPI. Mostly proteins of each gene expressed in the cytoplasm of the cells, with minor leakage expression, were also noticed when the cells expanded to 80−100% confluence. Cell lines expressed HCV nonstructural protein in the cytoplasm at both 20−30 and 80−90% confluence, as demonstrated by immunofluorescence assay. Immunostaining was done to detect NS3 protein expression in 20 and 80% confluent stable cell lines (Figure 5). Untransfected cells as negative control received the same treatment with anti-NS3 primary antibodies and fluorescently labeled secondary antibodies. Stable cell lines of Hep G2 cells showing good expression of the NS3 gene further proceeded for cytotoxicity and antiviral studies.

3.8.1. Plant Selection. *S. nigra* belongs to family Salicaceae, a medicinal plant utilized in Pakistani pharmacopeia, was chosen in the current study for having the potential to inhibit the nonstructural NS3 protein of HCV. The plant samples were gathered while conducting an ethnobotanical survey of Dir lower, Malakand Division Khyber Pakhtunkhwa, Pakistan, in May−August 2020, Because of their significance and widespread usage as herbal medicine by local Ayurvedic and homeopathy practitioners, the plant species was identified with taxon number 242417201 (http://www.efloras.org/). The current study focuses on the selected plants' fully grown and matured leaves. The selection of plant components was made considering their proven therapeutic uses in homeopathic and Ayurvedic medicine, particularly for their potential role in hepato-protective or antiviral therapies.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

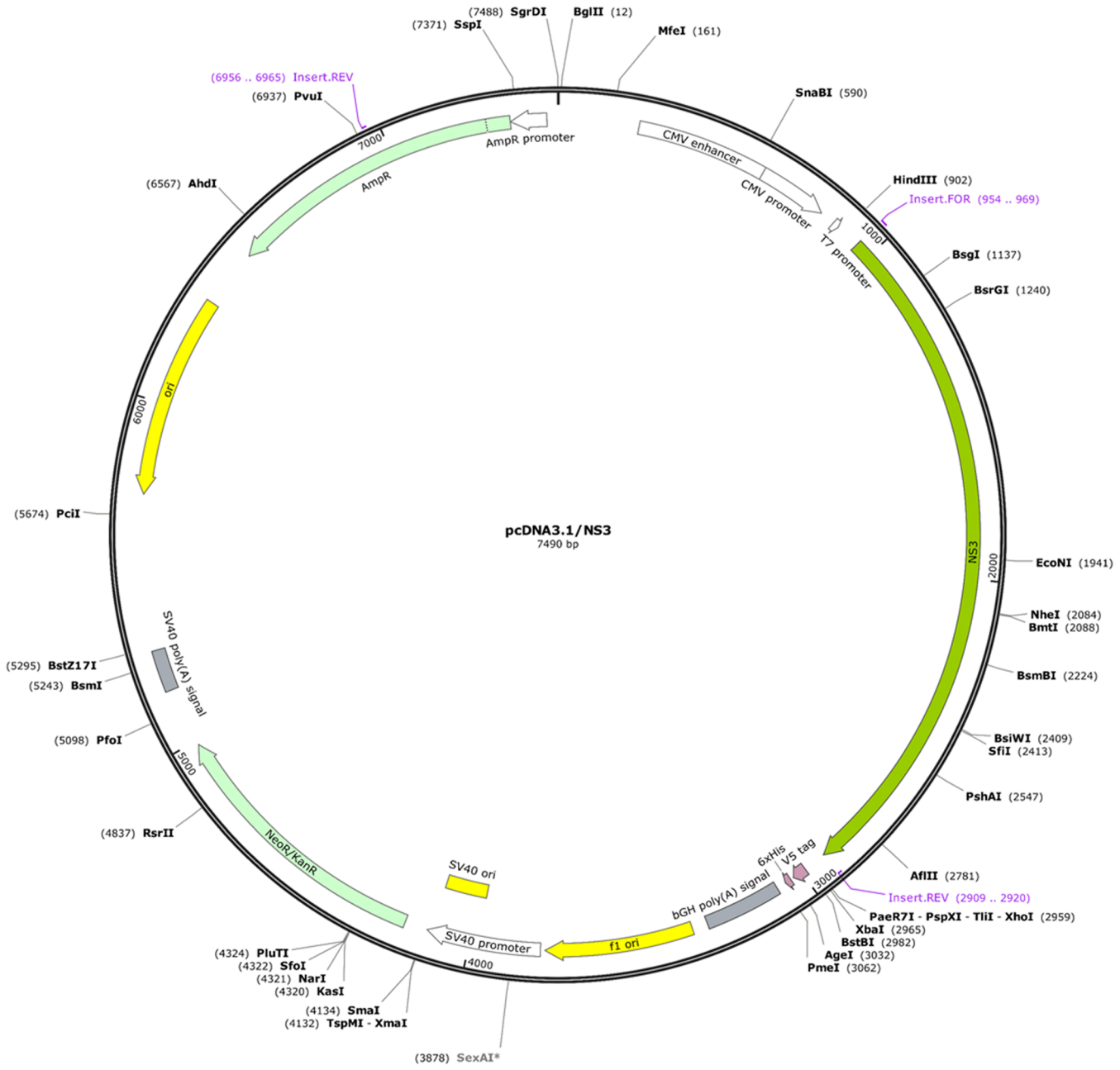


Figure 2. Map of cloning vector pcDNA3.1 with the cloned HCV NS3 gene (Green) at the *Bam*HI and NotI restriction sites.

3.9. Plant Sample Collection and Extract Formation.

During an ethnobotanical trip to Dir lower, Pakistan, *S. nigra* leaves were collected. The selection of this plant was based on the knowledge that its freshly harvested leaves are utilized to make herbal infusion for the treatment of liver-related problems in the northern regions, including Dir lower of Pakistan. It is also used in homeopathic medications and has been reported to possess bioactivity. The leaves were washed and air-dried for a week. The leaves were then ground into a fine powder using an electric grinder. For crude extraction, 30 g of powdered leaves was added to each solvent (*n*-hexane, methanol, ethyl acetate, and water). Two procedures (maceration and Soxhlet extraction) were used for crude extraction, and the procedures were repeated three times to prevent phytochemicals from losing their bioactivity due to high temperature in the Soxhlet method. Whatman filter paper grade-1 was used to filter the extraction solution, and the solvent was evaporated in an oven at 35−40 °C. The crude extracts were combined, and the %yield of crude extract was determined by employing eq 2. Overall, methanol produced the highest yield of crude extract from *S. nigra*, which may extract most of the polar and semipolar components by penetrating the cell wall and membrane. Then, water extracts polar components and ethyl acetate extracts semipolar components. When compared with the maceration method, the quantity of extracts achieved through Soxhlet's hot extraction was greater for all solvents. This may be because higher temperatures loosen cell walls more than cold maceration, resulting in a higher concentration of the extracted phytochemicals. Table 2 demonstrates the % yield of *S. nigra* extracted using separate solvents and 2 independent procedures of extraction.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

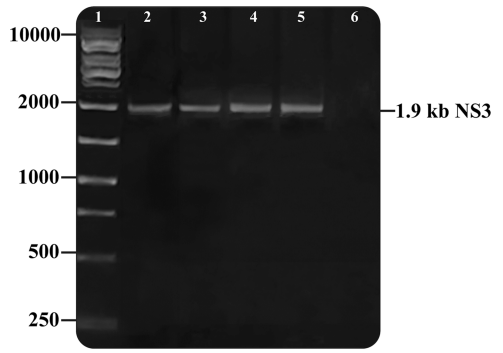


Figure 3. RT-PCR test result of NS3 stable transfected Hep G2 cells. Lane 1, 1kb DNA ladder, Lanes 2−5, chosen NS3 cell lines following propagation and selection on 1 mg/mL G418 for a month, Lane 6, negative control.

3.9.1. Cytotoxicity Analysis of S. nigra Plant Extracts on Hep G2 Cells. Hep G2 cells were exposed to extracts of *S. nigra* at different concentrations of 10−100 and 100−1000 μg/mL for cytotoxicity analysis. The initial screening was conducted with methanol extract, followed by water extract, ethyl acetate extract, and *n*-hexane extract. The procedures were replicated, and the mean results were used to plot the dose−response profile of the extracts. Neutral Red assay was used to detect the amount of cell viability after 24 h of treatment with *S. nigra* extracts. Based on the results of the spectrophotometric analysis of the Neutral Red assay, a cytotoxicity plot was generated for the extract as shown in Figure 6. Water and methanol extracts have an average CC_{50} value of 25−30 μg/mL and cause significant cell death; therefore, they were deemed toxic and not used in antiviral activity studies. Within 16−24 h of exposure, cells shrank, detached from the plate, and exploded, demonstrating that methanol and water extracts are toxic. The CC_{50} value of ethyl acetate and *n*-hexane extracts is greater than 100 μg/mL; they were determined as nontoxic and their concentrations ranging from 10 to 50 μg/mL were used to study the antiviral activities of these extracts on the stable cell lines (Hep G2) expressing HCV NS3 gene.

3.10. Anti-HCV Potential of *S. nigra* Extracts. *3.10.1. NS3 Gene Expression Analysis of Extract-Treated Stable Cell Lines.* The anti-HCV activity of *S. nigra* extracts was checked on the expression of the NS3 gene of stable cell lines. Six-well plates loaded with 400 μg/mL G418 media were used to propagate and maintain the stable Hep G2 cell lines expressing the targeted gene. The cells, after achieving 70% confluence, were exposed to various nontoxic doses of *S. nigra* extracts, and the expression of targeted gene was observed after 24 h of treatment. Stable untreated Hep G2 cell lines served as a positive control. The experiments were replicated for verification. Cytotoxicity testing revealed that no extract of *S. nigra* was toxic to Hep G2 cells at doses up to 50 μg/mL. The IC50 values for all methanol and water extracts were 37 and 42 μg/mL, respectively, while the IC50 values for *n*-hexane and ethyl acetate were equal to 100 μg/mL. Stable Hep G2 cells expressing the NS3 gene were treated with sterilized *S. nigra* extracts of all 4 solvents at concentrations ranging from 10 to 50 μg/mL to find the anti-HCV activity of these extracts. The dried crude extracts were diluted with analytical-grade DMSO.

3.10.2. Analysis of NS3 Gene Expression in Cells Treated with S. nigra Extracts. Total RNA was extracted from Hep G2 cells treated with *S. nigra* extracts at various doses to track the expression of the NS3 gene at the mRNA level. The same procedure was carried out for the positive control samples. TRIzol reagent was used to extract total RNA and the extracted RNA was quantified through Nanodrop. MMLV reverse transcriptase enzyme was used to synthesize cDNA from the extracted RNA samples. This procedure was also carried out for untransfected negative control samples. The expression was normalized with GAPDH (internal control). For semiquantitative analysis of changes in gene expression at the mRNA level, the cDNA samples were used in PCR studies employing gene-specific primers. Stable cells that had not been treated were employed as the positive control, whereas

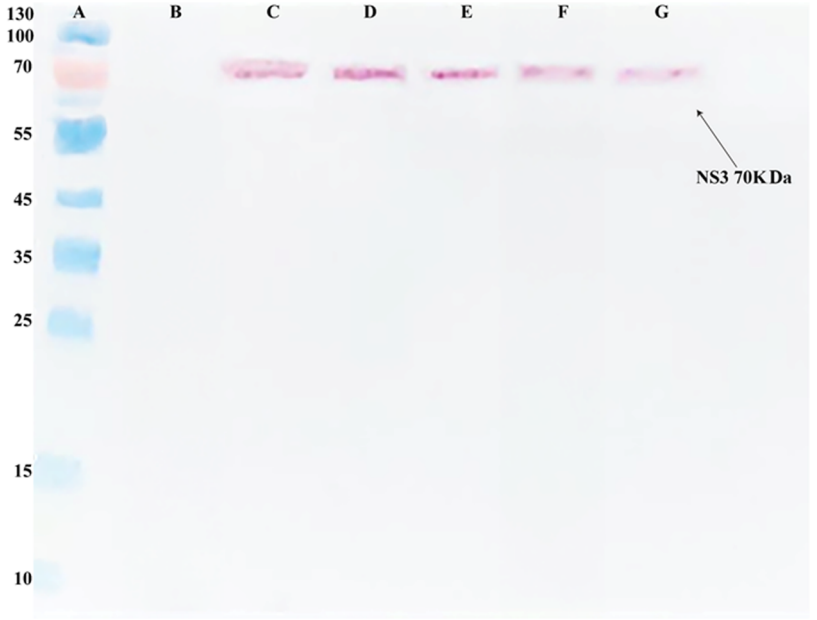


Figure 4. NS3 stable cell line Western blotting result: Lane A, protein Ladder; Lane B, negative control (untransfected cells), and Lane C-G, positive NS3 clones.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

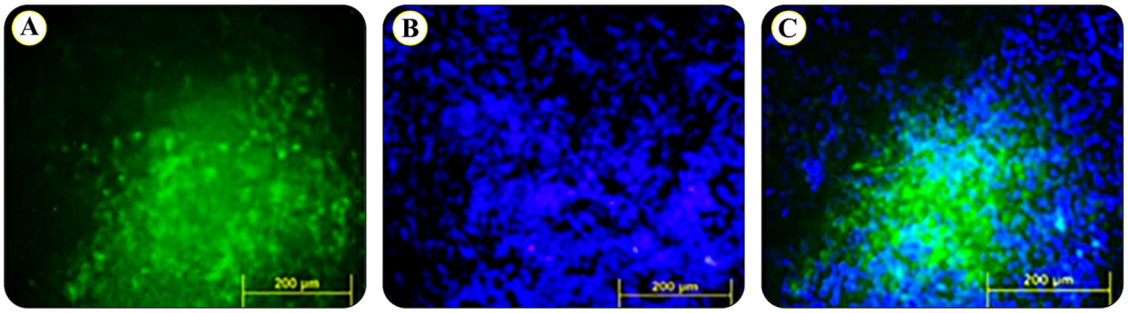


Figure 5. Stable NS3 cell lines immunofluorescence. (A) Immunostaining with anti-NS3 antibodies and a secondary antibody labeled with fluorescence, (B) counterstaining with DAPI, and (C) cells with 80% confluency.

Table 2. % Yield of *S. nigra* Extracts in Various Solvents

sr.,no.	*S. nigra*	distilled water	methanol	ethyl acetate	*n*-hexane
1	Soxhlet extraction	20%	20%	18%	10%
2	maceration	15%	15%	7%	5%

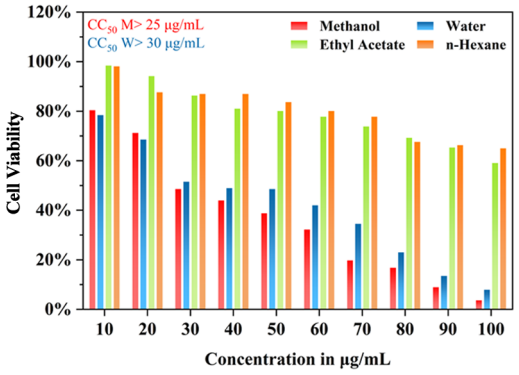


Figure 6. Analysis of the cytotoxicity of herbal extract of *S. nigra* on Hep G2 cells: Evaluation of various *S. nigra* extracts at varying concentrations from 10 to 100 μg/mL. Neutral Red assay suggested that methanol and water extracts exhibited cytotoxic properties at concentrations greater than 10 μg/mL, with CC_{50} values of 25 μg/mL for methanol, 30 μg/mL for water, 100 μg/mL for ethyl acetate, and *n*-hexane extracts. As the CC_{50} value of ethyl acetate and *n*-hexane extracts is greater than 100 μg/mL, they were determined as nontoxic and their concentrations ranging from 10 to 60 μg/mL were used to study the antiviral activities of these extracts on the stable cell lines expressing HCV NS3 gene.

untransfected cells were the negative control. 1.2% Agarose gel was used to analyze PCR products.

3.10.3. Influence of S. nigra Extracts on the Expression of NS3 Gene at the mRNA Level. Total RNA was extracted from Hep G2 cells treated with various *S. nigra* extracts after 24 h of treatment, and RT-PCR was used to analyze the expression of the NS3 gene at the mRNA level. RT-PCR results showed decreased expression of the NS3 gene in cells treated with ethyl acetate extracts (Figure 7), with no effect on the internal control GAPDH expression (Figure 8). Untreated cells were used as positive control. Cells treated with ethyl acetate and *n*-hexane extracts showed no significant effect on the expression of NS3 gene. The RT-PCR results confirmed the anti-HCV activity of *S. nigra* extracts by suppressing the expression of the NS3 gene in Hep G2 cells treated with ethyl acetate extracts.

3.10.4. Treatment of Stable Cell Lines with S. nigra Extracts for Analysis of Protein Expression. The stable Hep

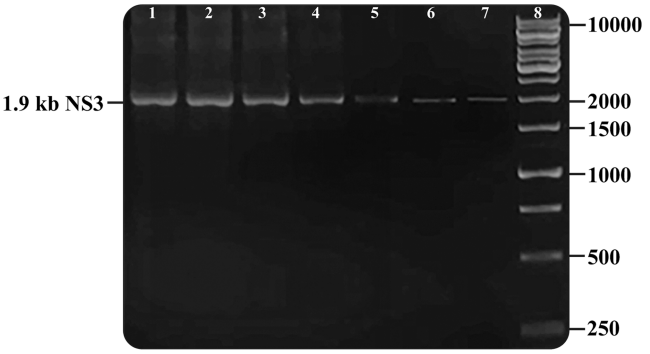


Figure 7. *S. nigra* extract effects on NS3 gene expression at varying concentrations: results of RT-PCR: Lanes 1−2: untreated cells as positive controls, Lanes 2−7: extract at increasing doses (10, 20, 30, 40 and 50 μg/mL), Lane 8: DNA 1kb ladder. At 30 and 50 μg/mL concentrations of ethyl acetate extracts of *S. nigra*, NS3 gene expression decreased by 50 and 80%−90%, respectively. Band intensity decreased, confirming the inhibitory impact of the *S. nigra* extract on NS3 gene expression. Band intensity decreased, confirming the inhibitory impact of *S. nigra* extract on NS3 gene expression.

G2 cell lines expressing NS3 protein were seeded in 60 mm Petri plates with a density of 4×10^3 cells per plate and grown on 400 μg/mL of G418 selection medium. The cells after reaching 70% confluency were treated with nontoxic concentrations of *S. nigra* extracts. The protein expression of stable NS3 cell lines was analyzed after 48 h of treatment with nonlethal concentrations ranging from 10 to 50 μg/mL of various *S. nigra* extracts (*n*-hexane, methanol, ethyl acetate, and water extracts) to check their anti-HCV capability. The extracts were sterilized by passing them through a 22 mm syringe filter. Different dilutions (10−50 μg/mL concentrations) of dried crude extracts were made using analytical-grade DMSO. After 48 h of treatment, Total proteins were isolated from extract-treated cells and used for further protein expression analysis. Proteins from untreated cells (positive control) were also isolated. The procedures were replicated three times.

3.10.5. Protein Expression Analysis of Extract-Treated Stable Cell Lines. Total proteins were extracted after treating the cells with *S. nigra* extracts for 48 h. Proteins were also isolated from the positive and negative control samples. The extracted proteins were quantified, run on a 12% SDS gel, transferred to nitrocellulose membranes, and subjected to Western blotting. The procedures were replicated. Monoclonal antibodies of NS3 were used in Western blotting analysis for checking the inhibitory effect of *S. nigra* extracts on the expression of the NS3 protein. Ethyl acetate extracts

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

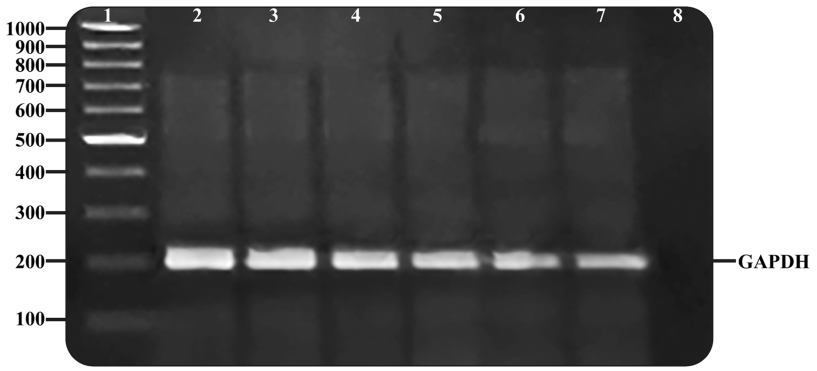


Figure 8. RT-PCR results of stable NS3 cell lines treated with *S. nigra* extracts using GAPDH as an internal control. Lane 1; DNA ladder 100bp, Lanes 2−7: cells exposed to *S. nigra* extracts at rising concentrations from 10 to 50 μg/mL, Lane 8: untreated cells as positive control.

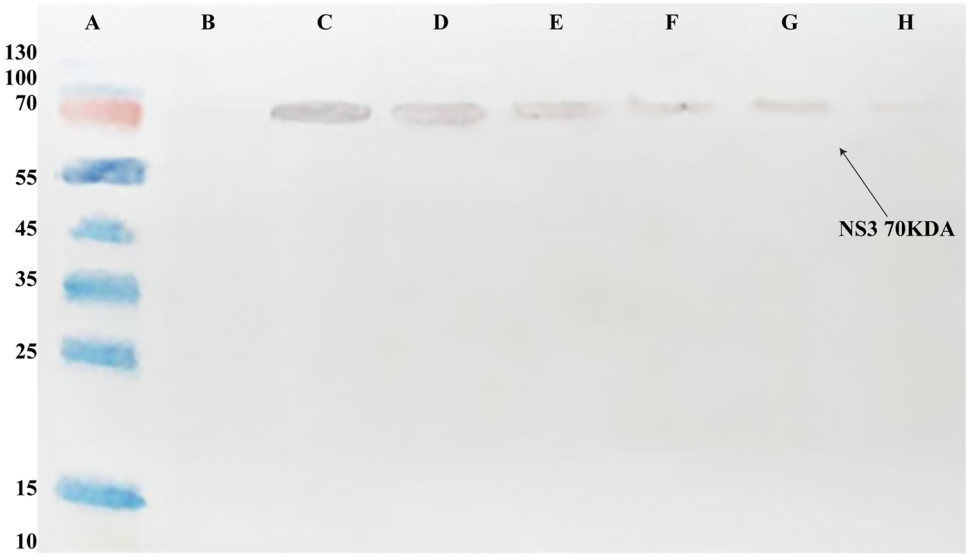


Figure 9. Effect of various *S. nigra* extract doses on nonstructural NS3 protein expression: *S. nigra*-treated NS3 stable cell line Western blot analysis, Lane A: Marker protein, Lane B: negative control, Lane C: untreated cells as positive control, Lanes D-H: extract at increasing doses (10, 20, 30, 40, 50 μg/mL). The expression of the HCV-3a NS3 protein was clearly reduced by approximately 50% at a dose of 30 μg/mL, and by 80 to 90% at a concentration of 50 μg/mL, proving the inhibitory effect of *S. nigra* extracts on the NS3 protein of HCV-3a genotype.

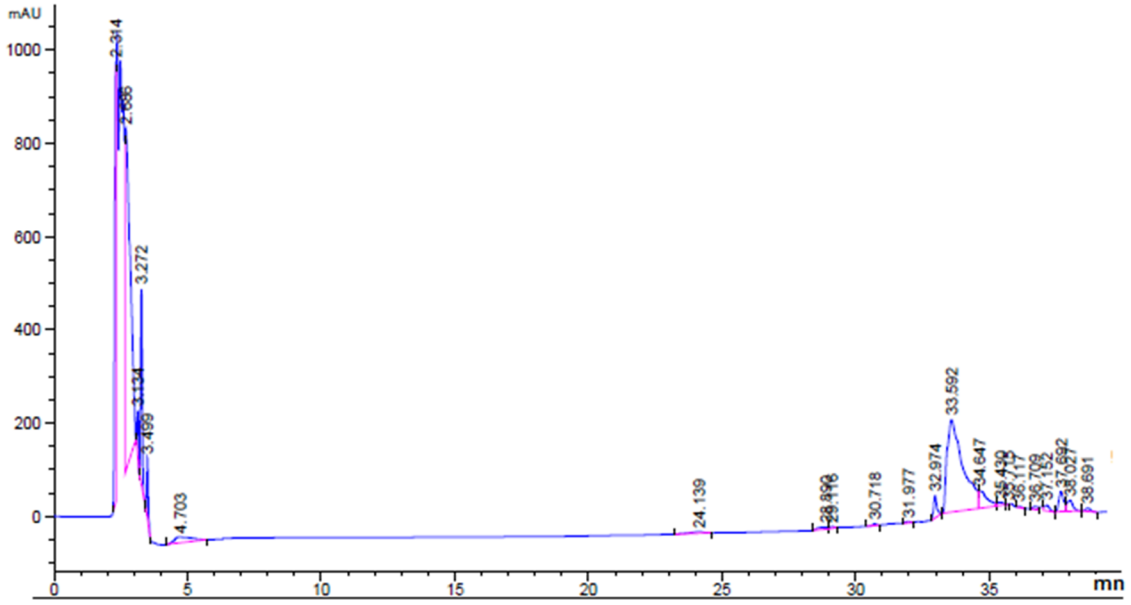


Figure 10. HPLC-UV chromatogram of the *S. nigra* methanol extract.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

Table 3. *S. nigra* Methanolic Extract Compounds Were Identified Using the HPLC-UV

sample extract	no. of peak	retention time (min)	phenolic compounds identity	peak area of sample	peak area of standard	concentration (μg/mL)	identification reference
S. nigra	1	2.6	malic acid	9996.814	40.323	223.126	reference standard
	2	28.8	pyrogallol	37.808	1.014	1076.100	reference standard
	3	30.2	mandelic acid	37.626	72.0	0.470	reference standard

significantly reduced the expression of targeted protein, confirming its inhibitory effect. The NS3 protein was isolated from untreated and treated stable cell lines, and Western blot was used to analyze the results (Figure 9).

3.10.6. Phenolic Compounds Identification in Extracts of S. nigra Using HPLC-UV. Standard HPLC-UV chromatography images of *S. nigra* methanol extracts are displayed in Figure 10. A total of three compounds, malic acid, pyrogallol, and mandelic acid, were identified in the plant *S. nigra*. These three compounds were identified with the reference standard. The identification and quantification of each phenolic component and their peak locations and retention times (Rt) in the chromatogram are listed in Table 3. The typical phenolic constituents in methanol extract were used to identify each phenolic compound in the extract.

4. DISCUSSION

Traditional medicinal plants are common in Pakistan, with more than half of the local population referring traditional medical practitioners, mostly Hakeem (traditional doctors), when confronted with a medical problem because the traditional healthcare system is culturally acceptable, easily accessible, and comparatively inexpensive compared to costly allopathic therapy. Numerous traditional medicinal plants and herbs have been found to have antiviral properties against various viruses.[32] This study aimed to uncover the cytotoxicity and anti-HCV activities of *S. nigra* extracts on the expression of NS3 gene of HCV-3a using stable Hep G2 cell lines. Different compounds derived from plants including Honokiol,[33] ECG (Epicatechin gallate),[34] gallic acid,[35] and EGCG (Epigallocatechin gallate)[34] are identified to have anti-HCV activity. Therefore, phytotherapies against antiviral infections are considered an alternate approach to be explored further.[33] Natural compounds of medicinal plants have a characteristic low cost of production, high structural diversity, and milder or no side effects than conventional therapies.[36] Furthermore, more than half of the bulk of drugs administered presently in treating various diseases have been derived from microorganisms and plants.[37]

The *S. nigra*, or black willow, has been used by Native American communities for centuries as a medicinal remedy.[38] It contains salicin, which has been studied for its potential anti-inflammatory and analgesic effects.[39] Salicin is chemically similar to the active ingredient in aspirin. *S. nigra* bark has been used for centuries as an analgesic. Some Native American communities, such as the Cherokee, relied on it as a folk remedy for things like headaches and muscle tension.[40] Since salicin is contained in *S. nigra*, it is possible that it, like other willow species, has anti-inflammatory properties. It has been used to alleviate the discomfort of inflammation. In addition, it has been used as a fever reducer in traditional medicine. Wounds and other areas experiencing localized pain and inflammation have been treated using *S. nigra* poultices and infusions.[41]

An indigenous medicinal plant utilized in Pakistani pharmacopeia *S. nigra* was chosen to check if it has the potential to inhibit the HCV-3a nonstructural protein NS3 expression in stable generated Hep G2 cell lines. The selection of plant components was made in consideration of their proven therapeutic uses in homeopathic and ayurvedic medicine, particularly for their potential role in hepato-protective or antiviral therapies. Two procedures (maceration and Soxhlet extraction) were used for crude extraction from the selected plants through 4 solvents (*n*-hexane, methanol, ethyl acetate, and water). *S. nigra* extracts were examined for potential cytotoxic properties on Hep-G2 cells before the evaluation of their impact on the level of expression of the HCV nonstructural NS3 gene. The *S. nigra* crude extracts were prepared using DMSO (sigma) at a concentration of 10 mg/mL, and this was used to evaluate the cytotoxicity of the plant extracts. The findings from the cytotoxicity testing revealed that *S. nigra*, methanol, and water extracts were highly cytotoxic having CC_{50} values ranging from 25 to 30 μg/mL. After 24 h of treatment, it was found that up to 95% of extract-treated cells were dead. Only ethyl acetate and *n*-hexane extracts of *S. nigra* up to 200 μg/mL concentration were selected for the inhibition of the NS3 gene.

It is helpful to use stably transformed cell culture models to test new antivirals against viral proteins that have been expressed specifically in the host.[42] With the use of viral vectors, the desired recombinant gene is incorporated into the genome of the host cell during stable transfection. Using a specific marker or antibodies is necessary for stable transfection, which supports gene expression over an extended period of time. While in transient transfection, circular plasmid insertion is used instead of integrating the desired gene into the host genome; as a result, transiently transfected genetic material can be lost by external influences or diluted out during cell division.[43]

Hep G2 cells derived from liver cells were chosen for stable transfection of the NS3 gene because of their cellular structure and machinery resembling the HCV natural host, leading to a better expression of the viral gene than HEK-293 cells, which are derived from kidney cells. Stable transfection was verified by using RT-PCR on the RNA isolated from each cell line of the NS3 gene. The presence of a 1.9 kb fragment of the NS3 gene was confirmed on an agarose gel. Western blotting confirmed the functional expression of NS3, with bands of 70 kDa. Fluorescent-labeled antibodies are useful for the subcellular localization of expressed proteins, and positive immunofluorescence results confirmed the successful generation of stable cellular expression systems of NS3 protein. This cell-culture-based expression system is useful in figuring out how the virus interacts with the host, the pathophysiology of the disease, and how it progresses. It also serves as a model for studies of these targeted HCV genes as potential therapeutic targets for the prevalent genotype in Pakistan.

It is also not certain that successful stable transfection will occur if cell lines are grown under optimal selection pressure.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

Because of its own defense system, the host machinery can suppress foreign genes. For choosing the host cell line, 3 different cell lines were transiently transfected, and conditions for stable transfection were optimized.[43] In our study, the *S. nigra* plant and its 4 crude extracts were investigated to evaluate their potential antiviral activity, The *S. nigra* indicated a significant decrease in expression of the NS3 gene. Methanol extracts of *S. nigra* and the ethyl acetate extract of *S. nigra* exhibited antiviral properties, whereas the ethyl acetate extract of *S. nigra* at concentrations ranging from 40 to 50 μg/mL inhibited the expression of the NS3 gene up to 80−90%.

It is always important to know the phytoconstituents of extracts under investigation. Therefore, plants were subjected to a phytochemical analysis. The plants under study were also previously analyzed for phytoconstituents. HPLC-UV technique was employed for the identification of compounds in the extracts of selected medicinal plant. The HPLC technique is an analytical method that is increasingly used since it is both a highly sensitive procedure and one that offers significant information on the analytes.[44] The identification of several polyphenolic substances was made possible by developing the HPLC method to investigate polyphenols in medicinal plants.[44]

The crude extracts of this medicinal plant were selected for further evaluation of their phytochemical analysis by using HPLC. *S. nigra* is a Salicaceae tree with flavonoids, lignin, and phenolic compounds in its leaves. It has been used for its antiperiodic, antipyretic, sterile, antirheumatic, and pain-relieving effects (52). Scientists have studied the antibacterial activity of *S. nigra* leaf extract against various bacterial pathogens, including *Escherichia coli*, *Salmonella Typh*, and *Salmonella paratyphi*. *S. nigra*, is known to possess antimicrobial activity and have biological compounds that are used to treat ailments from the beginning of human civilization.[45,46] Several phytochemicals including alkaloids, resins, and phenols have been found in *S. nigra* ethanolic plant extract. It has shown tremendous antimicrobial activity against pathogenic microorganisms, especially against *Staphylococcus aureus*.[46] Based on our HPLC analysis, three compounds, malic acid, pyrogallol, and mandelic acid, were identified in the *S. nigra* plant extract. The ethyl acetate of *S. nigra* significantly reduced the expression level of NS3 at the mRNA and protein levels. The results of the present investigation will contribute to the achievement of the WHO goal of eliminating the hepatitis C virus and its effects by 2030 by providing potential plant-based treatment options for the management of HCV infection.

CONCLUSION

From these results, it is concluded that *S. nigra* has a significantly reduced expression of NS3 gene at both mRNA and protein levels. The extracts of the selected plant might be utilized as potential candidates for the management of HCV infection. The phytochemical analysis identified several polyphenolic substances in the extracts of *S. nigra*. Plants contain millions of compounds and are considered as factories of natural products; therefore, in the future, more research work is required to study the phytochemical composition of *S. nigra* completely. Moreover, bioassay-guided fractionation procedures are required to isolate bioactive anti-HCV compound(s) from the selected plant. Further, these findings contribute to the global efforts in combating HCV and provide valuable insights into plant-based treatment options for viral infections, supporting the goal of eliminating the hepatitis C virus by 2030.

AUTHOR INFORMATION

Corresponding Authors

Mohammad Nisar − *Department of Botany, University of Malakand, Chakdara 18800 Khyber Pakhtunkhwa,, Pakistan*; Email: mnshaalpk@yahoo.com

Shazia Rafique − *Division of Molecular Virology, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore 54590, Pakistan*; orcid.org/0000-0002-0575-8246; Email: shazia.rafique@cemb.edu.pk

Ahmed Al-Harrasi − *Natural and Medical Sciences Research Center, University of Nizwa, 616 Nizwa, Sultanate of Oman*; orcid.org/0000-0002-0815-5942; Email: aharrasi@unizwa.edu.om

Authors

Sadia Bibi − *Department of Botany, University of Malakand, Chakdara 18800 Khyber Pakhtunkhwa,, Pakistan*

Muhammad Waqas − *Department of Biotechnology and Genetic Engineering, Hazara University Mansehra, Mansehra 2100, Pakistan; Natural and Medical Sciences Research Center, University of Nizwa, 616 Nizwa, Sultanate of Oman*

Muhammad Zahoor − *Department of Biochemistry, University of Malakand, Chakdara 18800 Khyber Pakhtunkhwa,, Pakistan*; orcid.org/0000-0002-4528-8517

Muhammad Idrees − *Division of Molecular Virology, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore 54590, Pakistan*

Nausheen Nazir − *Department of Biochemistry, University of Malakand, Chakdara 18800 Khyber Pakhtunkhwa,, Pakistan*

Mohammad Ihsan − *Department of Botany, University of Malakand, Chakdara 18800 Khyber Pakhtunkhwa,, Pakistan*

Saleh H. Salmen − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Sulaiman Ali Alharbi − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Ajmal Khan − *Natural and Medical Sciences Research Center, University of Nizwa, 616 Nizwa, Sultanate of Oman*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06193

Author Contributions

Conceptualization: M.N., S.R., and A.A.-H.; methodology: S.B. and M.W.; software: S.B. and N.N.; formal analysis: S.B. M.Z. and M.I.; investigation: S.B. and S.H.S; resources: A.A., A.K., and A.A.-H.; data curation: J.U.; writing—original draft preparation: F.A. and A.A.; writing—review and editing: S.B., M.W., and S.A.A; visualization: S.B.; supervision: M.N., S.R., and A.A.-H.; funding acquisition: S.H.S. and A.A.-H.

Funding

The project was supported by grant from The Oman Research Council (TRC) through the funded project (BFP/RGP/EBR/22/021).

Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

■ ACKNOWLEDGMENTS

The project was supported by grant from The Oman Research Council (TRC) through the funded project (BFP/RGP/EBR/22/021).

■ REFERENCES

(1) Fernandez, A. F.; Esteller, M. Viral epigenomes in human tumorigenesis. *Oncogene* **2010**, *29* (10), 1405−1420.
(2) Aziz, H.; Raza, A.; Murtaza, S.; Waheed, Y.; Khalid, A.; Irfan, J.; Samra, Z.; Athar, M. A. Molecular epidemiology of hepatitis C virus genotypes in different geographical regions of Punjab Province in Pakistan and a phylogenetic analysis. *Int. J. Infect. Dis.* **2013**, *17* (4), e247−e253.
(3) Simmonds, P. Genetic diversity and evolution of hepatitis C virus−15 years on. *J. Gen. Virol.* **2004**, *85* (11), 3173−3188.
(4) Idrees, M.; Riazuddin, S. Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. *BMC Infect. Dis.* **2008**, *8* (1), 69.
(5) Zein, N. N.; Persing, D. H. Hepatitis C genotypes: current trends and future implications. In *In Mayo Clinic Proceedings*; Elsevier, 1996; Vol. *71*, pp 458−462.
(6) Inchauspe, G.; Zebedee, S.; Lee, D.-H.; Sugitani, M.; Nasoff, M.; Prince, A. M. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (22), 10292−10296.
(7) Deniz, M. T.; Akhan, S. Hepatitis C Virus Structure and Diagnostic Methods. In *Hepatitis C-Recent Advances*; IntechOpen, 2023.
(8) Li, Q.; An, N.; Yin, X.; Zhang, R.; Shao, H.; Yi, D.; Cen, S. MxB Disrupts Hepatitis C Virus NS5A−CypA Complex: Insights From a Combined Theoretical and Experimental Approach. *Front. Microbiol.* **2022**, *13*, No. 849084.
(9) Isken, O.; Pham, M. T.; Schwanke, H.; Schlotthauer, F.; Bartenschlager, R.; Tautz, N. Characterization of a multipurpose NS3 surface patch coordinating HCV replicase assembly and virion morphogenesis. *PLoS Pathog.* **2022**, *18* (10), No. e1010895.
(10) Yousaf, T.; Rafique, S.; Wahid, F.; Rehman, S.; Nazir, A.; Rafique, J.; Aslam, K.; Shabir, G.; Shah, S. M. Phytochemical profiling and antiviral activity of Ajuga bracteosa, Ajuga parviflora, Berberis lycium and Citrus lemon against Hepatitis C Virus. *Microb. Pathog.* **2018**, *118*, 154−158.
(11) Lin, L.-T.; Hsu, W.-C.; Lin, C.-C. Antiviral natural products and herbal medicines. *J. Tradit. Complementary Med.* **2014**, *4* (1), 24−35.
(12) Mycol, V.; Kotwal, G.; Longum, P. Natural Antivirals against Human Viruses Bitter melon. *Virol. Mycol.* **2014**, *3*, 3−5.
(13) Clercq, E. D. Highlights in antiviral drug research: antivirals at the horizon. *Med. Res. Rev.* **2013**, *33* (6), 1215−1248.
(14) Calland, N.; Dubuisson, J.; Rouillé, Y.; Séron, K. Hepatitis C virus and natural compounds: a new antiviral approach? *Viruses* **2012**, *4* (10), 2197−2217.
(15) (a) Assini, J. M.; Mulvihill, E. E.; Burke, A. C.; Sutherland, B. G.; Telford, D. E.; Chhoker, S. S.; Sawyez, C. G.; Drangova, M.; Adams, A. C.; Kharitonenkov, A.; et al. Naringenin prevents obesity, hepatic steatosis, and glucose intolerance in male mice independent of fibroblast growth factor 21. *Endocrinology* **2015**, *156* (6), 2087−2102. (b) Tipoe, G. L.; Leung, T. M.; Liong, E. C.; Lau, T. Y. H.; Fung, M. L.; Nanji, A. A. Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mice. *Toxicology* **2010**, *273* (1−3), 45−52. (c) Zhang, W.; Hong, R.; Tian, T. Silymarin's protective effects and possible mechanisms on alcoholic fatty liver for rats. *Biomol. Ther.* **2013**, *21* (4), 264.
(16) Khalaf, N.; White, D.; Kanwal, F.; Ramsey, D.; Mittal, S.; Tavakoli-Tabasi, S.; Kuzniarek, J.; El-Serag, H. B. Coffee and caffeine are associated with decreased risk of advanced hepatic fibrosis among patients with hepatitis C. *Clin. Gastroenterol. Hepatolo.* **2015**, *13* (8), 1521−1531. e1523.
(17) Jardim, A. C. G.; Shimizu, J. F.; Rahal, P.; Harris, M. Plant-derived antivirals against hepatitis c virus infection. *Virol. J.* **2018**, *15* (1), 34.
(18) (a) Kitazato, K.; Wang, Y.; Kobayashi, N. Viral infectious disease and natural products with antiviral activity. *Drug Discovovery Ther.* **2007**, *1* (1), 14−22. (b) Mann, J. Natural products in cancer chemotherapy: past, present and future. *Nat. Rev. Cancer* **2002**, *2* (2), 143−148.
(19) (a) Balunas, M. J.; Kinghorn, A. D. Drug discovery from medicinal plants. *Life Sci.* **2005**, *78* (5), 431−441. (b) Brahmachari, G. Natural products in drug discovery: impacts and opportunities−an assessment. In *Bioactive natural products: opportunities and challenges in medicinal chemistry*; World Scientific, 2012; pp 1−199.
(20) (a) Wang, X.; Liu, Z. Prevention and treatment of viral respiratory infections by traditional Chinese herbs. *Chin. Med. J.* **2014**, *127* (07), 1344−1350. (b) Edziri, H.; Mastouri, M.; Mahjoub, M.; Ammar, S.; Mighri, Z.; Gutmann, L.; Aouni, M. Antiviral activity of leaves extracts of Marrubium alysson L. *J. Med. Plants Res.* **2011**, *5* (3), 360−363.
(21) Zeuzem, S.; Dusheiko, G. M.; Salupere, R.; Mangia, A.; Flisiak, R.; Hyland, R. H.; Illeperuma, A.; Svarovskaia, E.; Brainard, D. M.; Symonds, W. T.; et al. Sofosbuvir and ribavirin in HCV genotypes 2 and 3. *N. Engl. J. Med.* **2014**, *370* (21), 1993−2001.
(22) Wahid, B.; Saleem, K.; Ali, A.; Rafique, S.; Idrees, M. Rising relapse rate in hepatitis C virus type 3a-infected patients against sofosbuvir and ribavirin combination therapy: a Pakistani experience. *Eur. J. Gastroenterol. Hepatol.* **2017**, *29* (8), 979−980.
(23) Wahyuni, T. S.; Azmi, D.; Permanasari, A. A.; Adianti, M.; Tumewu, L.; Widiandani, T.; Utsubo, C. A.; Widyawaruyanti, A.; Hafid, A. F.; Hotta, H. Anti-viral activity of Phyllanthus niruri against hepatitis C virus. *Malays. Appl. Biol.* **2019**, *48* (3), 105−111.
(24) Tung, B. T.; Nham, D. T.; Hai, N. T.; Thu, D. K. Curcuma longa, the polyphenolic curcumin compound and pharmacological effects on liver. *Diet. Interventions Liver Dis.* **2019**, 125−134.
(25) Sharma, S.; Sahu, D.; Das, H. R.; Sharma, D. Amelioration of collagen-induced arthritis by *Salix nigra* bark extract via suppression of pro-inflammatory cytokines and oxidative stress. *Food Chem. Toxicol.* **2011**, *49* (12), 3395−3406. From NLM Medline
(26) Ahmed, W.; Ahmad, M.; Rauf, A.; Shah, F.; Khan, S.; Kamal, S.; Shah, S.; Khan, A. Evaluations of some trace metal levels from the leaves of *Salix nigra* in Hayatabad industrial estate Peshawar, Khyber Pakhtunkhwa Pakistan. *Am. J. Biomed. Life Sci.* **2015**, *3*, 21−24.
(27) Wasim, A.; Mushtaq, A.; Nadia, M. Phytochemistry and Anti-Acetyl Cholinesterase Activity of *Salix nigra*. *Int. J. Pharmacogn.* **2014**, *6*, 942−944.
(28) Ahmed, W.; Ahmad, M.; Khan, R. A; Mustaq, N. Promising potential of salix nigra extract against krait snake venom acetylcholinesterase and its role as anticancer and antioxidant agent. *Indian J. Anim. Res.* **2016**, *50*, 317−323.
(29) Idrees, M.; Riazuddin, S. Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. *BMC Infect. Dis.*. **2008**, *8*, 69.
(30) Astutik, S.; Pretzsch, J.; Ndzifon Kimengsi, J. Asian medicinal plants' production and utilization potentials: A review. *Sustainability* **2019**, *11* (19), 5483.
(31) Repetto, G.; Del Peso, A.; Zurita, J. L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* **2008**, *3* (7), 1125−1131.
(32) Ben-Shabat, S.; Yarmolinsky, L.; Porat, D.; Dahan, A. Antiviral effect of phytochemicals from medicinal plants: Applications and drug delivery strategies. *Drug Delivery Transl. Res.* **2020**, *10*, 354−367.
(33) Mandal, A.; Hazra, B. Medicinal plant molecules against hepatitis C virus: Current status and future prospect. *Phytother. Res.* **2023**. DOI: 10.1002/ptr.7936.
(34) Mekky, R. Y.; El-Ekiaby, N.; El Sobky, S. A.; Elemam, N. M.; Youness, R. A.; El-Sayed, M.; Hamza, M. T.; Esmat, G.; Abdelaziz, A. I. Epigallocatechin gallate (EGCG) and miR-548m reduce HCV entry through repression of CD81 receptor in HCV cell models. *Arch. Virol.* **2019**, *164*, 1587−1595.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999

(35) Shakya, A. K. Natural phytochemicals: Potential anti-HCV targets in silico approach. *J. Appl. Pharm. Sci.* **2019**, *9* (8), 094−100.
(36) Süntar, I. Importance of ethnopharmacological studies in drug discovery: role of medicinal plants. *Phytochem. Rev.* **2020**, *19* (5), 1199−1209.
(37) (a) Courdavault, V.; O'Connor, S. E.; Oudin, A.; Besseau, S.; Papon, N. Towards the microbial production of plant-derived anticancer drugs. *Trends Cancer* **2020**, *6* (6), 444−448. (b) Anand, U.; Jacobo-Herrera, N.; Altemimi, A.; Lakhssassi, N. A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites* **2019**, *9* (11), 258. (c) Singh, A.; Singh, D. K.; Kharwar, R. N.; White, J. F.; Gond, S. K. Fungal endophytes as efficient sources of plant-derived bioactive compounds and their prospective applications in natural product drug discovery: Insights, avenues, and challenges. *Microorganisms* **2021**, *9* (1), 197.
(38) Moerman, D. E. *Native American Ethnobotany*; Timber Press. Portland OR, 1998.
(39) Blumenthal, M.; Goldberg, A.; Brinckmann, J. *Herbal medicine. Expanded commission E monographs*; Integrative Medicine Communications, 2000.
(40) Brickell, C. *RHS encyclopedia of plants and flowers*; Dorling Kindersley Ltd, 2019.
(41) Foster, S.; Johnson, R. L. *National geographic desk reference to nature's medicine*; National Geographic Books, 2008.
(42) Wöhnke, E.; Fuchs, W.; Hartmann, L.; Blohm, U.; Blome, S.; Mettenleiter, T. C.; Karger, A. Comparison of the proteomes of porcine macrophages and a stable porcine cell line after infection with African swine fever virus. *Viruses* **2021**, *13* (11), 2198.
(43) Fus-Kujawa, A.; Prus, P.; Bajdak-Rusinek, K.; Teper, P.; Gawron, K.; Kowalczuk, A.; Sieron, A. L. An overview of methods and tools for transfection of eukaryotic cells in vitro. *Front. Bioeng. Biotechnol.* **2021**, *9*, No. 701031.
(44) Wang, L.; Zou, Y.; Kaw, H. Y.; Wang, G.; Sun, H.; Cai, L.; Li, C.; Meng, L.-Y.; Li, D. Recent developments and emerging trends of mass spectrometric methods in plant hormone analysis: a review. *Plant Methods* **2020**, *16* (1), 54.
(45) Mworia, J. K. *Phytochemical Profile, Antipyretic, Analgesic and Anti-Inflammatory Potential of Dichloromethane Leaf Extracts of Eucalyptus Globulus (Labill) and Senna Didymobotrya (Fresenius)*. Kenyatta University, 2021.
(46) Tahir, U.; Fakhar, M.; Zameer, M.; Yasmeen, U.; Mazhar, M.; Hassan, A.; Rafique, A.; Zahra, N.; Gilani, K.; Alyas, S. Antibacterial Activity, Phytochemical Analysis and FT-IR Analysis of *Salix Nigra* Plant Extract Against Gram Positive and Gram Negative Bacteria. *Fresenius Environ. Bull.* **2022**, *31* (8), 7535−7542.

https://doi.org/10.1021/acsomega.3c06193
ACS Omega 2023, 8, 42987−42999