



Methanolic Extract of Neem Plant Inhibits NS3 and NS5A Nonstructural Proteins of HCV 3A Genotype

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ABSTRACT

Hepatitis C virus (HCV) has infected an approximate of 170-200 million people worldwide. This unique RNA virus escapes the immunity in most infected people and forms a chronic infection which can progress to liver cirrhosis, fibrosis, hepatic cancer, and death. The goal of this work was to assess the cytotoxicity and *in vitro* inhibitory activities of local medicinal plant *Azadirachta indica* (*A. indica*) extracts on expression of NS3 and NS5A nonstructural proteins of HCV 3a genotype. Owing to the fact that HCV patients can develop HCC, plant extract has also been tested for cytotoxic activity. Colorimetric analysis was done to determine the vitality of HepG 2 cells for 24 h after treatment with methanolic seed extract and minimum inhibitory concentration was calculated. HepG2 cell were transfected stably to express nonstructural proteins NS3 and NS5A of HCV 3a genotype. Western blot and RT PCR confirmed stable protein expression. Immunofluorescence assay was done to check subcellular localization of these nonstructural proteins. The CC₅₀ value for *A. indica* seed extract for HepG2 cells was 650 µg/mL. The extract concentration 500 µg/mL was found to reduce gene and protein expression of NS3 and NS5A by 50 percent. Results of our study shows that phytochemicals of neem seeds have strong antiviral potential against HCV without having any significant cytotoxic effects.

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All authors have accepted responsibility for the entire content of this manuscript and approved its submission. AJS and SR planned the proposed study. AJS supervised the whole project. UH did the lab work. AJS and SR helped in data curation. UH, wrote the manuscript. AJS, SR, MIK and AAS helped in editing and proof reading of the manuscript.

Key words

Azadirachta indica, Cytotoxicity, Nonstructural proteins, Immunofluorescence, HCV 3a genotype

INTRODUCTION

Hepatitis C virus (HCV), also known as a silent killer, after its discovery in late 80s has become a major health concern world-wide affecting approximately 72 million people with chronic infection each year (Dore and Bajis, 2021). Pakistan bears the second largest burden of hepatitis C infection worldwide, where HCV has infected almost 12 million people countrywide. Being a developing country, diagnosis is often delayed, treatment is insufficient,

leading to increased HCV-related morbidity and mortality. Pakistan's HCV epidemic is generalized where adult seroprevalence rate is 4.8% percent (Lim *et al.*, 2020; Qureshi *et al.*, 2010).

HCV possess high genetic variability which has prevented effective vaccine development against this deadly pathogen. To date, seven genotypes and sixty seven subtypes of HCV have been discovered that are distributed geographically (Fernández-Caso *et al.*, 2019). The highly prevalent genotypes of Pakistan are 3a, second by 3b which is followed by 1a as well as mixed genotype infection (Shafique *et al.*, 2020). Genome of HCV is positive strand Ribonucleic acid 9600 nucleotide in length that encodes 3,010 amino acids in the form of a single large poly-protein

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Abbreviations

HCV, hepatitis C virus; PCR, polymerase chain reaction; SVR, sustained virological response; FITC, Fluorescein isothiocyanate; BCIP, 5-Bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; DAAs, direct acting antivirals; HCC, hepatocellular carcinoma; PMSF, phenylmethylsulfonyl fluoride.

producing 10 different individual proteins processed by viral and cellular proteases (Gosert *et al.*, 2003). The coded proteins are envelope and core glycoproteins (E1, E2 and Core) which makes structural proteins of HCV along with nonstructural proteins NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The virus particle is formed by core proteins and envelope glycoproteins (E1, E2) (Lohmann *et al.*, 1999).

All HCV proteins are important and have a key role in viral particle attachment, entry, replication, infectious viral particle formation and interfering with the host cells signaling pathways. Three nonstructural proteins NS3, NS5A and NS5B have been proved to be major targets for new antiviral agents (Suda and Sakamoto, 2021). The NS3 serin protease is a very crucial protein. It has RNA helicase/NTPase activity which is used in replication of HCV. It is responsible for the production of individual viral proteins from translated single polyprotein. Without this protease, viral proteins cannot be processed, hence results in silencing of viral genes (Meewan *et al.*, 2019). The NS5A is a multifunctional phosphoprotein having ability to interact with 3'-UTR of HCV as well helping in new virion assembly (Xie *et al.*, 2017).

A very high mutation rate (almost 10 folds higher than HIV) of this virus has greatly hampered the experimental studies and development of vaccines (Zajac *et al.*, 2019). Studies on viral replication and host response are difficult owing to unavailability of suitable host system for infection, or models which can have a continuous expression of viral proteins (Dennis *et al.*, 2021).

Globally the research on discovery of new drugs from natural source is gaining popularity with almost 30% traded compounds being derived from naturally occurring substances in plants. Research on plant based bioactive compounds has opened a new horizon for the discovery of alternative drugs in the pharma industry. Medicinal plants have been a source of active phytochemicals since beginning of human race (Ahmad *et al.*, 2014). *Azadirachta indica* locally known as Neem, member of family Meliaceae is an indigenous plant of the Indo-Pak subcontinent and has been used in traditional homeopathic and Ayurveda medicines from immemorial times. All parts of neem plants including fruits, leaves, bark, and seeds have been extensively studied and reported to contain a lot of bioactive phytochemicals (Parida *et al.*, 2002). The medicinal potential of this plant has been attributed to its extensive range of phytoconstituents, which include a variety of chemical components, including triterpenoids, Alkaloids, saponin, steroids, tannins, flavonoids and glycosides. Their antibacterial, anti-inflammatory, antiviral, anti-tumor, anti-fungal, pesticidal, and anti HIV activities have been reported (Anyachie, 2009; Benoit *et al.*,

1996; Faccin-Galhardi *et al.*, 2012, 2019; Hao *et al.*, 2014; Ingle *et al.*, 2017; Ong *et al.*, 2014; Parida *et al.*, 2002). Almost 300 bioactive molecules have been extracted and reported for their medicinal activities establishing neem as a holy grail of active medicinal ingredients (Siddiqui *et al.*, 2009).

Natural substances may impart antiviral effect by inhibiting viral replication at one or more phases. They can inhibit infection by interacting directly with the virus, blocking its entry, stimulating the intracellular signals, interacting with viral replication, or triggering / boosting the host immune response. Natural bioactive chemicals have a wide range of pharmacological properties, are inexpensive to produce, and are intrinsically safer than synthesized medications (Rehman *et al.*, 2016). These compounds may possibly give an unconventional and potentially therapeutic opportunity to cure HCV infection in this scenario. As the existing regimens for treatment of HCV are highly costly and produce adverse side effects. Studying the anti- HCV potential of this local medicinal plant might give an insight in the new anti HCV compounds.

The aim of current research was to evaluate cytotoxicity and *in-vitro* inhibition of HCV nonstructural protein expression by methanolic extract of *A. Indica* seeds. For this purpose stable HepG2 cell lines were generated expressing NS3 and NS5A nonstructural proteins and effect of methanolic neem extracts on expression level of nonstructural proteins was studied.

MATERIALS AND METHODS

Preparation of Azadirachta indica seeds extract

Fully ripened fruits were collected from *Azadirachta indica* tree from University of the Punjab, New Campus, Lahore, Pakistan during the month of June. Depulping of fruits was done and seeds were washed thoroughly with water to remove any residual pulp. Seeds were dried at 40°C for one week to reduce water content. Fine powder of seeds was obtained by grinding them in an electrical grinder. Seed powder 30g was used for extraction of phytochemicals by using soxhlet apparatus. Methanol was used as a solvent for extraction in a mass ratio of 1:10 (powder to solvent), because methanol can extract most of the metabolites either polar or semi polar. Extraction was performed thrice. Obtained crude extract were pooled and filtered using whatman filter paper No. 1 and evaporated at 40°C in an oven to get dry crude extract (16.5 g). Dilutions ranging from 100 µg/mL to 1000µg/mL concentration of the extract were made in analytical grade Dimethyl sulfoxide (DMSO).

Cytotoxicity analysis

HepG2 cells were generously provided by Applied and Functional Genomics Lab, Center for Excellence in Molecular Biology. To find out minimum lethal concentration of plant extract, HepG2 cells were seeded in a 96-well plate at density of 2×10^3 cell per well and were incubated overnight so they can achieve log phase of growth. After 24 h, cells were exposed to different concentrations of the plant extracts (from 100 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$). After 24 h of extract addition to the 96-well plate, the cells were observed for their morphology changes under a microscope. The extract and Dulbecco's modified eagle medium (DMEM) mixture both were replaced with fresh media containing 1:100 of Neutral Red dye. After one h incubation at 37°C and 5% CO_2 washing was done with 1x phosphate buffer saline (PBS: $[\text{Na}_2\text{HPO}_4]$: 10 mM, $[\text{KH}_2\text{PO}_4]$: 1.8 mM, $[\text{NaCl}]$: 137 mM, KCl : 2.7 mM) and de-stained for 15 min on a plate shaker with destaining solution (50 % absolute ethanol, 1% glacial acetic acid, 49% double distilled water). Optical density of 96 well plate was measured at 550 nm through an ELISA plate reader using SPECTRA-MAX-384 device. The changing pattern in cell viability and morphology were observed through microscope (Olympus CKX31).

RNA extraction and complementary DNA synthesis

Blood samples of the patients chronically infected with HCV 3a genotype were collected from Jinnah hospital Lahore. The patients were informed properly about the study and samples were collected after getting consent. RNA isolation kit (Gentra, USA) was used according to manufacturer's protocol for RNA extraction. Purity and integrity of extracted RNA was checked by Nano drop. MMLV reverse transcriptase (Moloney murine leukemia virus RT; Invitrogen USA) was used to reverse transcribe individual genes by using oligo dT primers. Forward and reverse primers for individual genes were synthesized by using primer 3 software. Sequence of Pakistani HCV isolate with Accession number GU294484.1 was used as a reference to synthesize primers. NS3 Forward primer, 5'-GGATCCGCCACCATGGGCGTGAGGG 3' NS3 Reverse primer 5' GCGGCCGCGGTGGTTACTTC-CAGATCAG 3' were used for amplification of NS3 gene. NS5A Forward Primer: 5' GGATCCGCCACCATGAG-CGACGATTG 3' NS5A Reverse Primer: 5' GCGGCCG-CGACGAGACCACGCTCGATG 3' were used for amplification of NS5A gene.

This cDNA was used to individually and completely amplify both genes. Recognition sites for restriction enzymes NotI and Bam HI were introduced at both ends of each gene through polymerase chain reaction. The amplified products were electrophoresed on 1.2 percent

agarose gel having 0.5mg/mL ethidium bromide and were visualized under UV light.

Construction of mammalian expression plasmid

Mammalian expression vector pcDNA 3.1 was used for cloning of amplified HCV NS3 and NS5A genes. Genes were cloned in the multiple cloning site of plasmid spanning BamHI and NotI restriction sites. Constructed plasmids of both genes were confirmed through PCR by using gene specific as well as plasmid specific (T7 and BGH) primers. Restriction digestion also confirmed successful cloning. Sequencing of both the constructed clones was done and by using BLAST sequence was compared with existing HCV3a genotype NS3 and NS5A sequences

Stable transfection in mammalian cells

HepG2 cells were propagated and grown in Dulbecco's modified eagle medium (DMEM) containing fetal bovine serum 10% (Sigma Aldrich, USA) and penicillin; streptomycin 100 $\mu\text{g/mL}$ at 37°C with 5% CO_2 . A day before cells were seeded in a 6-well (5×10^4 /well) plate and incubated overnight to achieve 75-80 percent confluency. Mammalian expression plasmid was linearized upstream of the CMV promoter region using BglII restriction enzyme. Linearized plasmid was purified by using Gene Get Quick Gel Extraction kit (Invitrogen) as per manufacturer's protocol. About 4-5 μg linearized and purified pcDNA/NS3 and pcDNA/NS5A constructs were used for transfection into HepG2 cells by using turbofect transfection Reagent (Thermo Scientific). Briefly 4 μg of the linearized plasmid per each well of the six well plate was mixed in 400 μL of DMEM (serum free). Turbofect (transfection reagent) was briefly vortexed before use and 8-10 μL was added to the diluted plasmid, mixed thoroughly by gentle pipetting. The transfection mixture was incubated at room temperature (25-30°C) for 30 min. The mixture was poured in to each well drop wise and plates were gently rocked to mix the transfection mixture with media for even distribution of the transfection complexes. Cells were grown in transfection media (without antibiotics and FBS) for 7-8 h. Afterwards media was changed to 10% DMEM growth medium.

Total RNA extraction of transfected cells

After transfection cells were allowed to grow for 72 h. Using TRIzol reagent total RNA from transfected cells was extracted. Cells were trypsinized and harvested by centrifugation for 5 min at 1000 rpm. Cell pellet was washed with 1x PBS and homogenized in 800 μL of TRIzol by vortexing, incubated at room temperature for 8-10 min. 500 μL chloroform was added to facilitate phase separation.

Centrifugation was done at 4°C and 12000 rpm. Upper aqueous phase was collected and RNA was precipitated with isopropyl alcohol. This RNA was used for cDNA synthesis by using reverse primers for each gene. This cDNA was used in RT PCR for confirmation of transcription of viral genes at mRNA level. Negative control consisted of untransfected HepG2 cells and was proceeded in the same manner for RNA extraction and PCR.

Protein expression studies

After 72 h of transfection cells were harvested and were solubilized in protein lysis buffer (1M Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF and mammalian protease inhibitor). Total protein was extracted after incubation on ice for 30 min. Almost 30-40 µg of protein sample was run on 12 percent SDS gel and was blotted on nitrocellulose membrane Hybond-C using Biorad semi dry apparatus. Blocking was done by 5% BSA (bovine serum albumin) in 1x PBS-T (Tween-20; 0.05-%) for 1 h. Primary monoclonal antibodies for NS3 (C44D Invitrogen) and NS5A (H110B Invitrogen) at a concentration of 1:50 in 1xPBS-T were added and membranes were incubated at room temperature for 2 h. Washing of membranes was done thrice with 15 ml PBS-T for 10 min each. A secondary antibody Alkaline Phosphatase conjugated rabbit anti mouse IgG (Sigma) was used at a concentration of 1:10000 in 1xPBS-T and membranes were incubated for 1.5 h at room temperature on orbital shaker. Washing with PBS-T was done thrice to remove excessive secondary antibody. Alkaline phosphatase substrate tablet (NBT/BCIP) was dissolved in 10 ml of water and added on membrane. Incubation for 20-30 min at room temperature was given to develop signal.

Generation of stable cell lines

For selection of cells stably expressing HCV nonstructural proteins G418 di-sulphate salt (Geneticin sigma) was used as selection marker. Initial selection was given at a concentration of 1 mg/ml. G418 resistant clones were selected and further grown for one month at 1mg/ml to check consistent expression. G418 resistant colonies of both genes were picked using sterile micropipette tip and transferred to 24 well plates separately after 2 weeks. Selection media (DMEM without having streptomycin and penicillin) was supplemented with 20 % FBS to allow isolated cells to divide and propagated rapidly. After 24 well plate cells were transferred to 12 well plates and afterwards to 6 well plates and 25 cm² flasks as cell propagated over a period of four weeks. Media was changed after every 72 h.

Immunofluorescence assay

After one month of selection HepG2 cell lines stably

expressing NS3 and NS5A proteins were grown in 12 well plate for 24 h. After 24 h cells were washed with 1x PBS and were fixed using ice chilled methanol at -20 degrees for 10 min. 5 % BSA in PBS was used as blocking agent and plate was incubated in blocking solution at room temperature for 30 min. Primary anti bodies for NS3 (C44D Invitrogen) and NS5A (H110B Invitrogen) were used at the concentration of 1:10 and incubated for 2 h at 30-35°C. Washing of wells with PBS was done thrice for 10 min each. Secondary anti body Goat anti-mouse IgG-FITC (sc-2010) conjugated was used for NS3. Goat Anti-Mouse IgG Antibody, Rhodamine conjugate (Chemicon-API24R) anti body was used for NS5A. After 2 h incubation with secondary antibody counter staining by using DAPI was performed. Cells were mounted and visualized within 1 h.

Effects of A. indica seed extracts on HCV protein expression

After calculation of the minimum lethal concentration of the methanolic extract from seeds of *A. indica*, its effect on HCV NS3 and NS5A protein expression was checked. Stable Hepg2 clones of both genes were seeded in 6-well plates at a cell density of 3×10^3 and were incubated overnight at 37°C and 5 % CO₂. After 24 h Cells were treated with nontoxic doses of the extract for 48 h. Effects of neem seed methanolic extracts on expression of HCV NS3 and NS5A genes was checked at both mRNA and protein levels. Total RNA from cells was extracted by using trizole reagent and RT-PCR was done by using reverse primers for each gene respectively. Expression of GAPDH was also checked as internal control. Total protein was extracted by using protein lysis buffer. Protein samples were loaded on 12 % SDS page and western blot was done to analyze effects of neem seed methanolic extracts on the expression of HCV proteins as described in the previous section. Untreated cells were used as control.

RESULTS

Cytotoxic effects of A. indica seed extracts

Cytotoxic effects of the methanolic neem seed extract were checked on HepG2 cells. Neutral red assay indicated that 90% cells were viable at high concentration of 500 µg/mL, 80-85 % cells were viable even at the concentration as high as 600 µg/mL.

The extract concentration higher than 800 µg/mL was significantly toxic for HepG2 cells. 80 % of the cells could not survive at this concentration of the extract. Thus the methanolic extracts of *A. indica* seeds were considered nontoxic at 500 µg/mL concentration. Figure 1 is showing the cytotoxicity profile of different concentrations of extract against viable cells (%age Viability of the HepG2 cells after 24 h of exposure to increasing concentration

of *A. indica* extract). CC_{50} (concentrations at which 50 percent of the cells are viable) was found to be 650 $\mu\text{g/mL}$.

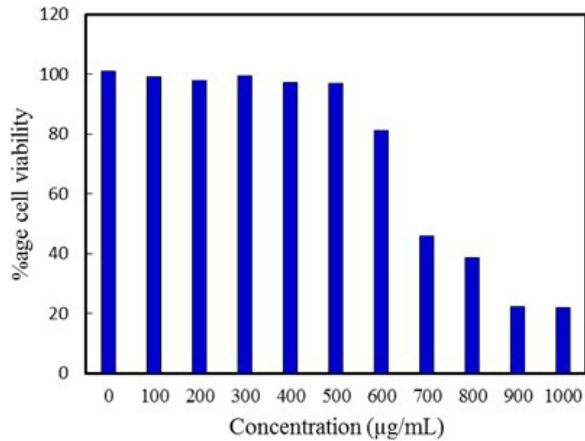


Fig. 1. Cytotoxicity analysis of methanolic extracts of *A. indica* seeds.

Amplification of NS3 and NS5A genes and cloning into expression vector

Figure 2A shows amplified nonstructural genes of exact expected size. For NS3 gene estimated product size was 1954bp and for NS5A estimated gene size was 1355bp. Both nonstructural genes and plasmid pcDNA3.1 were digested with the restriction enzymes BamH I and Not I. Mammalian expression vector was constructed by using T4 DNA ligase. This vector was selected due to presence of CMV promoter which is responsible for high level of protein expression in both transiently and stably transfected eukaryotic cells.

Transfection

The expression vector was linearized using BglII enzyme (Thermo-scientific) for stable integration of pcDNA/NS3 and pcDNA/NS5A construct into the genome of HepG2 cells. Gel purification of the plasmid was performed following linearization. Figure 2B is showing linearized purified cloned plasmid of both genes. After 72 h of transfection selection pressure was given. Initially 1 mg/ml solution of G418 di-sulphate salt was used. Almost 70-80 % cells could not develop resistance against G418. After 7 day of selection it was possible to identify resistant colonies of the transfected cells. These colonies were further propagated and were allowed to grow in the same selection pressure of 1 mg /ml of G418 for about 6 weeks. Following successful selection and maintenance of cell lines at 1 mg/ml selection pressure was decreased to 500 $\mu\text{g/mL}$ for further growth of cell lines. This selection pressure is required for expansion of the cells as G418 is

selectable marker this ensures that cells which retain the gene for selection marker also retain gene of interest. Total RNA was extracted by TRIzol reagent and RT PCR was performed using reverse primers of both genes. No significant decrease in expression level of the proteins was seen even after 6 weeks of selection. Cells were propagated for 6 passages and changes in their expression level was monitored. Figure 3 is showing RT PCR results of both genes from stably expressing HepG2 cell lines.

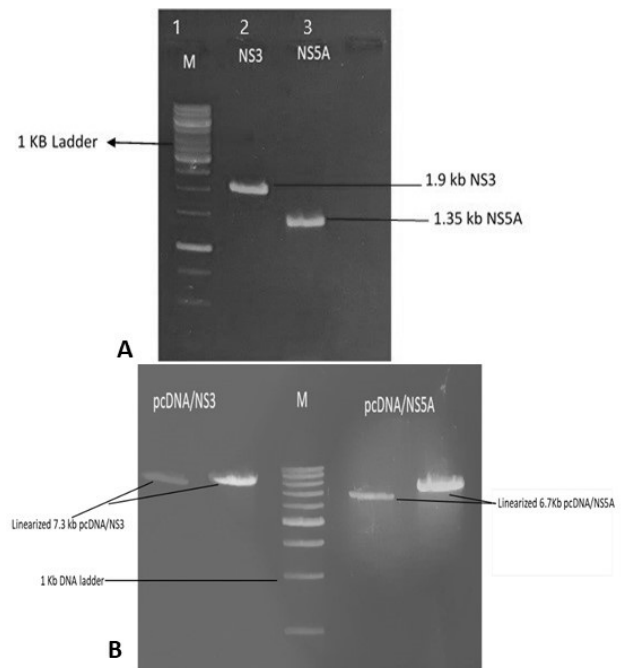


Fig. 2. (A) PCR amplification of NS3 and NS5A; Lane 1 DNA size marker (1 Kb), lane 2 amplified gene product NS3 (1.9 kb) lane 3 amplified gene product NS5A (1.35 kb). (B) Linearized purified plasmid constructs; Lane 1 and 2 linearized pcDNA/NS3 (5.4+1.9=7.3 kb) lane 3 DNA ladder (1 Kb) lane 4 and 5 linearized pcDNA/NS5A (5.4+1.3=6.7kb).

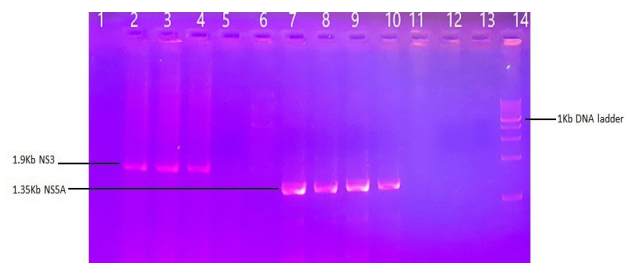


Fig. 3. RT PCR of stable cell lines; Lane 2-4 NS3 1.9 kb positive stable clones, lane 5-6 negative control NS3, lane 7-10 positive stable clones of NS5A (1.35 kb) lane 11-13 negative control NS5A, lane 14 1Kb DNA ladder.

Western blot analysis

Expression of NS3 and NS5A proteins in stably transfected HepG2 cells was checked by performing western blot using gene specific monoclonal antibodies. Bands of specific protein size NS3 70 KDa and NS5A 56-58 KDa of both genes were identified confirming successful expression of both nonstructural genes of HCV 3a genotype. Negative control comprised of protein lysate from un-transfected HepG2 cells. **Figure 4A** and **B** showing western blot results of NS3 and NS5A proteins of HCV, respectively.

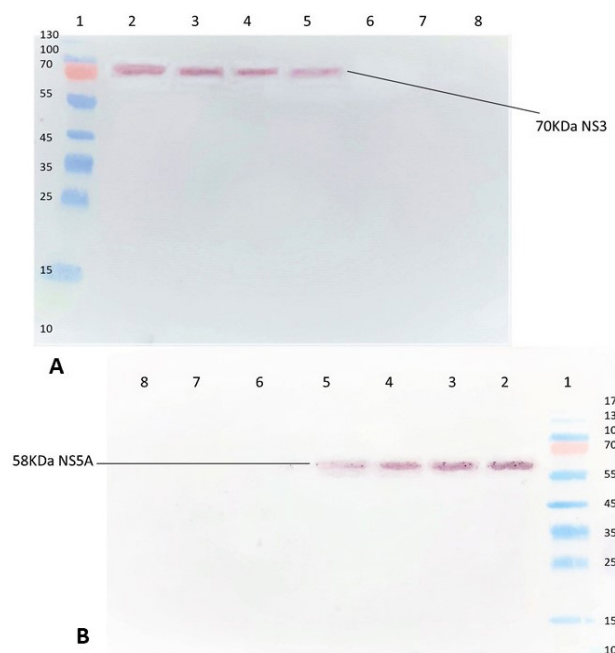


Fig. 4. Western blot analysis of HepG2 NS3 (A) and NS5A (B) stable cell lines; Protein marker lane 1. Lane 2-5 positive NS3 clones, while lane 1 in B is positive NS5A clones lane 2-5 negative control 6-8.

Immunofluorescence assay

For cellular localization and direct visualization of both nonstructural proteins, immunofluorescence assay was performed using FITC and Rodamin labelled fluorescent secondary antibodies for NS3 and NS5A proteins respectively. Counter staining with DAPI confirmed the cellular viability. Proteins were successfully visualized in the stable cell lines also counter staining confirmed that cells were alive and successfully producing viral proteins. **Figure 5A, B** are showing results of immunofluorescence of NS3 and NS5A proteins respectively. **Figures 6A, B** are showing results of negative control with Goat anti mouse IgG FITC and Goat anti mouse IgG Rodamine, respectively.

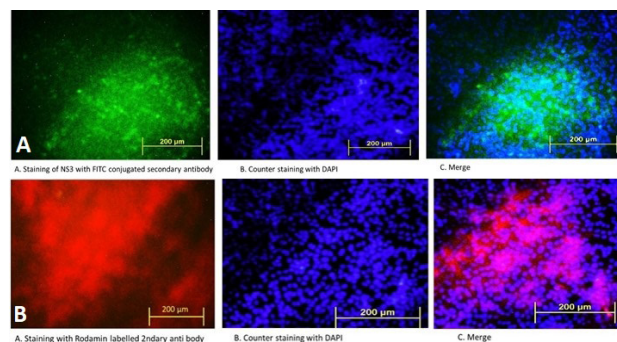


Fig. 5. Immunofluorescence assay for NS3 protein (A) and NS5A protein (B) A NS3 staining with FITC labelled secondary antibody, counter staining with DAPI, merge. B NS5A staining with rodamin labelled secondary antibody, counter staining with DAPI, merge.

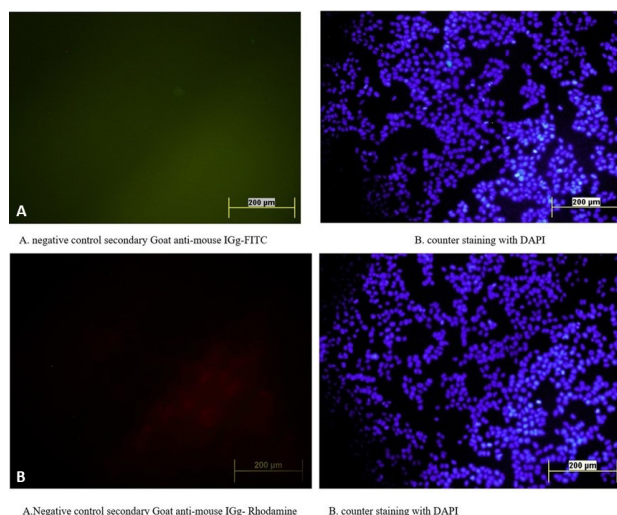


Fig. 6. Stable cell line control immunofluorescence assay. A: staining with goat anti mouse secondary antibody FITC in A and Rodamin in B; B: counter staining with DAPI.

A. indica methanolic seed extract inhibit NS3 and NS 5A gene and protein expresion

The RT-PCR results of NS3 and NS5A genes depicts that *A. indica* seed extract downregulated expression of both genes in a concentration dependent manner. With almost 80% reduction in gene expression of NS3 and almost 60% reduction of NS5A mRNA expression at 500 μg/mL concentration without having any cytotoxic effects on cell viability. **Figure 7A, B** are showing the RT-PCR results of extract treated cells on mRNA expression of NS3 and NS5A, respectively.

Western blot analysis also confirmed the strong inhibitory action of methanolic neem extract on the expression of both genes. **Figure 8A, B** show western

blot results of both protein expressions in treated cells at different concentrations. Untreated cells were used as controls for both RT-PCR and western blot analysis.

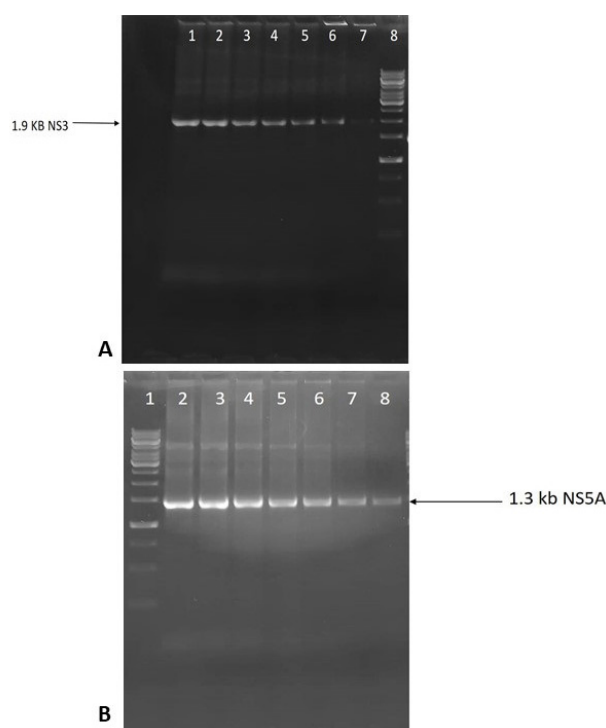


Fig. 7. Effect of concentration of *A. indica* seed extract on expression of nonstructural gene NS3 (A) and NS5A (B). RT-PCR results: lane 1 in A positive control, lane 2-7: increasing extract concentrations (100, 200, 300, 400, 500, 600 µg/mL), lane 8: 1kb DNA ladder. B., lane 1: 1kb DNA ladder, lane 2: positive control, lane 3-8: increasing extract concentrations (100, 200, 300, 400, 500, 600 µg/mL).

DISCUSSION

Pakistan bears second largest burden of HCV infection in the world. A huge burden on the health care system for a developing country. Infected persons are either not diagnosed timely due to poor socioeconomic conditions or if diagnosed might not have access to proper treatment strategies (Butt *et al.*, 2011). Due to different geographical distribution of hepatitis C virus the treatment strategies available in Pakistan are not genotypically specific for local HCV genotype resulting in lower sustained virological response as well as relapse of disease even after complete treatment with DAAs (Khan *et al.*, 2020). Genetic mutability of HCV and emerging drug resistance is another barrier in complete suppression of this virus from Pakistan. According to a recent report published in the Lancet Global Health Pakistan needs to

invest almost 10 percent of the annual health budget in scaling up the screening and management of HCV for its complete eradication by 2030 as part of the WHO plan (Lim *et al.*, 2020). During this time of Covid-19 pandemic this criteria appears to be extremely difficult to meet.

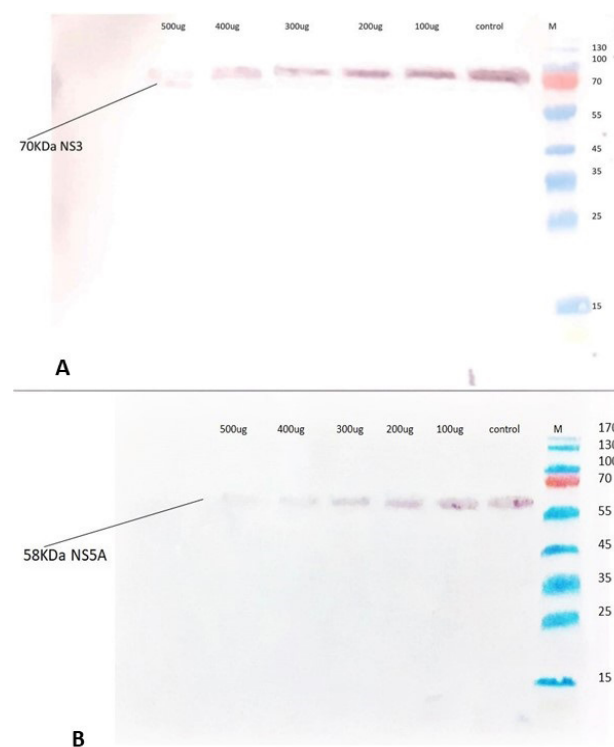


Fig. 8. Effect of concentration of *A. indica* seed extract on expression of nonstructural protein NS3 (A) and NS5A (B). Western blot analysis of *A. indica* treated NS5A stable cell lines, (right to left) lane 1: protein marker, lane 2: positive control, Lane 3-7: increasing extract concentrations (100, 200, 300, 400, 500 µg/mL).

Development of a cellular expression systems of specific HCV proteins which are major targets for antiviral drugs will help in testing of new compounds for their antiviral potential against the local prevailing genotype of HCV (Butt *et al.*, 2011). Several cell cultured base systems have been established for studying viral pathogenesis and replication around the world for genotypes prevailing in that particular region (Sharma *et al.*, 2017). Therefore, it is need of the h to generate a cell culture system for local Pakistani genotype 3a, and discovery of new antiviral compounds which are cheap, safe and effective simultaneously.

Pakistan is enriched in diverse medicinally important flora. *Azadirachta indica* a local evergreen tree has been used since many centuries for treating various ailments.

Every part of this plant contains ingredients which have been proven to be, anti inflammatory, anti viral, anti bacterial, pesticidal, anti allergic and anti tumor. Its role as anti viral agent against HBV, dengue, chicken gonorrhea, coxsackie virus, influenza, HIV, have already been reported (Anyachie, 2009; Benoit *et al.*, 1996; Faccin-Galhardi *et al.*, 2012, 2019; Hao *et al.*, 2014; Ingle *et al.*, 2017; Ong *et al.*, 2014; Parida *et al.*, 2002). To the best of our knowledge no research group has reported *in vitro* studies on inhibition of HCV by this plant. Our study showed the inhibitory potential of methanolic neem seed extract on the expression of NS3 and NS5A nonstructural proteins of HCV. Expression of both genes is reduced in a dose dependent way in the presence of neem seed extracts as compared to untreated cells. This inhibition effect could be a synergistic effect of the saponins limonoids and phenolic compounds present in the extract. Further investigation on the active ingredients may result in identification of a single novel anti HCV compound from *Azadirachta indica* plant.

CONCLUSION

We successfully generated stable hepatoma cell lines producing two non-structural proteins NS3 and NS5A of HCV 3a genotype. This cell culture model will be an efficient tool in understanding of host-viral protein interactions as well as their role in development of liver cirrhosis, fibrosis, and hepatocellular carcinoma. We investigated the effects of *Azadirachta indica* methanolic seed extract on expression of NS3 and NS5A. Our studies show this extract have a positive inhibition effect on the expression of both genes at mRNA and protein levels. Further investigations on bioactive ingredient may lead to discovery of a new anti HCV drug with better results and no side effects; a step toward total eradication of HCV by 2030 as per WHO action plan.

Statement of conflict of interest

The authors have declared no conflict of interest.

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