Anti-viral effect of Indigoferra gerardiana on core protein of HCV and clinical significance of serum markers in chronic HCV genotype 3a patients in district Peshawar

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Keywords: HCV, Genotypes, viral load, IG, Core gene, anti-viral therapy

Posted Date: February 12th, 2020

DOI: https://doi.org/10.21203/rs.2.23383/v1

Abstract

Background/aim: Hepatitis C virus (HCV) disease is a health challenge due resistance cases globally. Many plant-derived natural compounds have demonstrated antiviral effects. These plants can provide an alternative way to new antiviral. Core protein of HCV has ability to interact with a wide range of viral and cellular proteins, including proto-oncogenes.

Materials and Methods: In this cross-sectional study, total of 421 Hepatitis C Virus (HCV) positive patients were subjected by Polymerase Chain Reaction (PCR). The correlation of genotype with continuous and categorical variables was analyzed. Furthermore, antiviral activity of indigoferra gerardiana (IG) was tested against core protein of HCV. The amplified complete core protein of HCV genotype 3a was sequenced and cloned. IG was extracted and screened against core protein of HCV 3a genotype in cell line.

Results: Genotype 3a was the common genotype type of HCV (n=363, 86%). Significant differences were observed in viral load among 3a genotype of HCV (< P 0.05). Frequently detected age group was 21-40 (59.4%) Sex and age were statistically associated with HCV infection (49% males and 37% in females). The results of RT-PCR demonstrated that methanolic extract of IG showed 57% reduction of infecting cells.

Conclusions: Methanolic extract of IG showed good antiviral activity i.e. 57% reduction of infecting cells.

Introduction

Hepatitis C disease is a worldwide burden that globally affects about 170 million people [1],[2]. Viral clearance was seen in majority of patients. Approximately 60–80% of acute HCV infection persists and may progress into chronic disease. Liver cirrhosis develops by chronic hepatitis c within 10 to 20 years in 10 to 20% of patients. Each year approximately 1 to 5% of these patients are at risk of developing HCC [3]. Certainly, many studies reported the high incidence of HCV as worldwide threat [4]. Clinical findings, viral load and genotypes are basis for HCV infective patients during antiviral therapy [5]. Previous studies tried to relate viral factors (viral load, genotype) and host biochemical factors (AST, ALT and bilirubin) and their relation with liver damage, but no clear outcome were found. HCV is a single-stranded RNA. It encodes polyprotein of 3010 residues. The essential components are the structural proteins (core, E1, E2) and seven nonstructural proteins (p7, NS2, NS3,NS4A, NS4B, NS5A, NS5B) [6]. On the basis of nucleotide heterogeneity, HCV is classified into different genotypes. Genotypes 1 and 3 are circulating world-wide. In Pakistan, genotype 3a is the predominant genotype [7]. Highly conserved, core protein of HCV, encapsidates the genomic RNA. The core protein comprises of basic N-terminal and hydrophobic C-terminus anchors the endoplasmic reticulum. The core protein is translated as an immature protein (22 kDa) and mature protein of 21 kDa due to additional cleavage [8]. The in vitro and invivo studies suggested that core proteins have effect on immune suppression, oxidative stress, carcinogenesis,

steatosis, cell metabolism and lipid metabolism [9]. In patients with chronic HCV, hepatic steatosis developed at a high rate and a close correlation has been noted with intra-hepatic core protein expression level. The growth signaling is disregulated by core protein [10, 11]. Core protein inhibited interferon-α induced nuclear import of STATs [12].

Combination therapy with peg IFN-α and ribavirin is recommended therapy for HCV infection [13]. In some patients, IFN-based therapies are limited by side effects and incomplete response rates, leading to early termination of treatment. The drugs like telaprevir / boceprevir are also given as protease inhibitors for HCV infection. This regimen clears infections more than 70% in HCV genotype-1 infected patients. However drug resistant variants were found to be developed with DAAs [14–16]. Further studies introduced sofosbuvir and simprevir (next generation DAAs) [17, 18]. Some studies reported that various HCV proteins (Core, NS3/4A, E2 and NS5A/5B) provokes antiviral effect of IFN-α [19]. HCV core protein also inhibits nuclear translocation of STAT-1 and IFN induced phosphorylation. [20, 21]. The main target of therapy is to remove the virus and stop complications. The desired outcome of therapy is monitored by SVR [22]. The drug resistant viral species may arises by the use of DAAs against HCV [23]. Many studies are looking for new ways of drug therapy that are used against HCV infection. Different medicinal plants and their phytochemicals have potential uses in HCV therapy. These plants are less toxic, cost effective, easily accessed and have multiple target activities. The phenolic extract obtained from Marrubium peregrinum (Lamiaceae) showed inhibitory effect of flavonoid derivatives, ladanein (BJ486K) on post-attachment entry of HCV with an IC50 value (2.5 μmol) into cultured hepatocytes of human. The inhibitory effect of BJ486K was independent of HCV genotype. Combination of cyclosporine and BJ486K had a synergistic effect in inhibition of HCV infection [24]. Another study reported that the cytotoxic, antitumor and antioxidant effects of Methanolic Extract of I cassioides (MEIC) was evaluated [25]. The IG is generally recognized as Ghoreja, this species of flowering plant belongs to Family Fabaceae (Leguminosae) and its sub-family Faboideae. It is used for relieving abdominal colic and the infectious diseases especially bacterial infections involving skin [26]. In Pakistan, the IG is found in hilly region of Khyber Pakhtunkhwa, Azad Jammu Kashmir, Northern areas of Upper Dir. I.blongifolia possesses antimicrobial, hepatoprotective and lipoxygenase activity [27]. I.aspalathoides effectively suppresses liver tumor induced by DEN and also used against HCV, The flavonoid of I barberi Gamble showed the hepatoprotective effect with histopathological evidence in liver injury caused by paracetamol in rats. Similarly, another specie of Indigoferra i.e I linnaei. demonstrates the potent antitumor and cytotoxic properties [28]. In another research, the flavonoid fraction of methanolic extract of I.tinctoria has been reported to have chemo protective effect against lung cancer [29]. So based on supported links regarding targeted anti-viral activity the current study is carried out to explore another specie Indigoferra gerardiana (IG) for possible antiviral activity [30],[31], [32], [33], [34], [35].

The present study was conducted to evaluate the correlation between LFT's, viral loads and the severity of liver damage in chronic HCV patients and antiviral activity of Indigoferra gerardiana.

Materials And Methods

Study Design

This current study was designed at the Institute of Basic Medical Science (IBMS) Peshawar, Khyber Teaching Hospital, Peshawar, Institute of Biotechnology and Genetic Engineering, Agriculture University Peshawar, Center of Applied and Molecular Biology Lahore. The study duration was 12 months (Jan 2018 to Feb 2019). The institutional ethical committee at the Institute of Basic Medical Science, Peshawar and Institutional Research and Ethical Review Board of Khyber Teaching Hospital approved the study.

Samples Collection and Processing

A total of 421 RNA positive samples (male: 207, female: 156) of chronic HCV infected individuals at Khyber Teaching hospital, Khyber medical college and Khyber medical university Peshawar, KP-Pakistan were selected. Questionnaire was taken from patients who came for HCV initial screening. Informed consent was taken from these patients. The patient responsive to treatment, patient's not giving informed consent and cannot come for follow-up visits were excluded from study. After blood sampling, serum was stored in a refrigerator at -80 °C. A baseline investigation along with genotyping and viral load quantification was done. HCV RNA was detected using quantitative PCR (Q-PCR) according to the operating manual of the test kit (Qiagen, Germany) followed by HCV genotyping [36]. The routine LFTs were assessed for each patient in the hospital laboratory. Furthermore, biochemical tests (ALT, AST, bilirubin) were correlated with viral factors (genotype, viral load). The results were expressed as mean ± S.D (standard deviation) or as percentage. The correlation between categorical variables were assessed using the Chi Square, it was applied to evaluate differences in proportions using SPSS 20.0 software (SPSS Inc., USA). The P value (< 0.05) was labeled significant. HCV genotype determination was carried out as described earlier [36]. For HCV genotype determination, RNA extraction was carried according kit protocol. Isolated RNA was reverse transcribed into complementary DNA (cDNA). The cDNA was amplified in two rounds of type specific nested PCR for isolation of genotype specific PCR product.

Extraction of RNA

Viral RNA was extracted from HCV positive serum of genotype 3a. For extraction, RNA extraction was used as per kit protocol. RNA was immediately stored at -80^{o}C for further analysis.

cDNA synthesis

Complementary DNA (cDNA) was synthesized (Invitrogen cDNA protocol kit) by RT PCR using reverse primers. Briefly, 04 µl RNA and 1 µl of Outer Antisense (OAS) primer was mixed and incubated at 65^{o}C for 5 min and kept immediately on ice. Then 4 µl 5x buffer, 2 µl 10 mM dNTP's, 1 µl RNAase inhibitor and 1 µl reverse transcriptase enzyme was added to a final volume of 15 µl. Mixture was kept in thermal cycler at 42^{o}C for 60 minutes and then termination of reaction at 70^{o}C for 10 minutes.

Conventional PCR for Core Gene Detection

For amplification of HCV Core gene, forward (5'ATGAGCACACTTCCTAAACC3') and reverse primers (5'GACGTATTCCGCCACTCTAG3') were used for amplification. PCR was done in a total reaction volume of 15 µl including 2 µl of cDNA, reverse primer 1 µl, forward primer 1 µl, Master mix (Invitrogen) 7.5 µl and distilled water 3.5 µl. Mixture was kept in the thermal cycler and the temperature was set for initial denaturation at 95^{o}C for 5 min, and then 36 cycles each consisting of denaturation at 95^{o}C for 0.25 sec, annealing done at 55^{o}C for 30 sec and elongation at 72^{o}C for 1 min. Final extension was carried out at 72^{o}C for 10 min.

Gel electrophoresis

The amplified gene specific PCR product (573 bp) was resolved on 1.5% agarose gel (Thermo Fisher Scientific, USA). By using a 100 bp DNA ladder (Fermentas, USA) It was visualized under UV illumination. (Uvitec Limited, Cambridge, UK). The confirmed PCR product was eluted by protocol of kit (Novel Gel/PCR DNA Purification Mini Kit)

Sequencing and Cloning of Core gene

Amplification

For molecular genomic analysis, one sample with high viral titer was selected for isolation and amplification of structural gene. The amplified, sequenced purified PCR product was cloned in pDNA2.1 TA cloning Kit (TOPO TA Cloning Kit Lot no 1506821-invitrogen)

Primer Designing

For amplification of core gene from plasmid both forward (GCGATATCATGAGCACACTTCCTAAA) and reverse (AATCTAGATCATGGCTGCTGGATGAAT) primers were designed. For cloning, primers had restriction sites for EcoRV and Xba1. In all the primers, the start codon was present in the Flag TAG sequence and a stop codon for translation termination was incorporated. The PCR positive clones were used for further confirmation analysis by restriction digestion of the plasmid containing core gene.

Cloning in mammalian expression vector pcDNA3.1

The amplified PCR product was cloned into mammalian expression vector pcDNA3.1. By continuous heat shock, ligation product was transformed into DH5α E Coli (competent cells) Transformed cells were allowed to proliferate for 120 min and incubated at 37^{0}C with shaking. A total of 0.2 ml of the growth medium was spread on fresh LB/AMP and Kanamycin plates. Transformed colonies appeared blue and white after overnight incubation at 37^{0}C. Confirmed clone was sequenced with Big Dye Deoxy Terminator method using vector specific universal outer sense and inner sense primers (UOS, UIS) followed by bidirectional sequencing in DNA sequence (Applied Biosystems).

Collection and extraction of medicinal plant

The leaves of Indigoferra gerardiana (IG) were collected from northern area of KPK, confirmed by the Botany department of Peshawar university. The leaves were shade dried, segregated and crushed in grinder to form coarse powder. Methanol was added at the ratio of 1:20 (w/v) and was macerated for 72hrs. Whartsman filter paper (no-1/ cloth filter) was used for the filtration of supernatant. The solvent was evaporated using a vacuum rotary evaporator (Heidoloph Rotavapor) under controlled temperature (40 °C) and reduced pressure (204 mbar). Double extractions was done for the collection of residue [37]. Methanolic extract was further fractionized on the basis of polarity, in n-hexane, chloroform and acetone. The results revealed that viral titer was blocked by acetone extract to a greater extent.

Stock solution preparation

The dried plant extract (100 mg) was suspended in 01 ml of Dimethylsulfoxide (DMSO) for stock concentration (100 mg/ml). Then make dilutions in 5% DMSO (5 ml DMSO + 95 ml H_2O) inside the laminar flow hood sieving (by using 0.22 um filter) done of the above solution and stored at (-20 °C).

Cell lines

The Huh-7 cell line (University of Lahore) was cultured in Dulbecco's modified Eagle medium (DMEM). It was supplemented with 100 µg/ml streptomycin, fetal bovine serum (10%) and penicillin (100iu/ml) at 37 °C in an atmosphere of CO_2 (5%).

MTT Assay for Toxicity

Toxicological effect was determined through MTT assay. In living cells, the MTT substance is reduced to purple formazan crystals (insoluble in water) by mitochondrial succinic dehydrogenases. The absorption of dissolved formazan correlates with the number of alive cells. To determine the cellular toxicity, different concentrations of herbal extract was added into 96-well plates (2×10^4 cells/well), the plate was incubated (37 °C) in an atmosphere of CO_2 (5%) and sealed in aluminum foil for 24hrs. At end of extraction, test compounds and media were removed. 20 µl of MTT solution (5 mg/ml in PBS) and fresh media (100 µl) were added to all wells. The plate was wrapped in aluminum foil and then it was incubated at 37 °C for 04 hours. DMSO of 100 µl was added to dissolve the formazan crystals after media was removed. MTT-formazan product was accessed by measuring absorbance-reader at a test wavelength of 570 nm by Enzyme Linked Immunosorbent Assay (ELISA) plate.

Analysis of plant extracts in cell line

To establish, in-vitro replication of HCV, -Huh-7 cell line was used. The same protocol was used for viral inoculation as done by Rehman S in 2011. In these experiments, HCV patients of 3a genotype with high viral titer > 1×10^9iu/ml was used as inoculums. Huh-7 cells were maintained in six well culture plates and washed two times with serum-free medium. Different concentrations of drug containing medium was added to the cell culture replaced after every 24 h. after incubation at 370C for 48 h, DMEM containing MTT at the final concentration of 1 mg/ml was added to each well, after 1 h of it was replaced with

100 ul of DMSO to solubilize the formazan crystals. Using spectrophotometer, the surviving cells of each well were measured at 570 nm by optical density (OD) (30). Transfected cells were maintained in 5% CO_2 for 24 h at 37 °C. On next day, adherent cells were washed 03 times with 1 × PBS buffer, medium was added and again incubated for 48 hrs. Cells were harvested and assessed by RT-PCR. To analyze the effect of medicinal plant extracts on HCV, transfected Huh-7 cells were seeded after 02 days of infection in 24 well plates in both absence and presence of herbal extracts which were then grown to 80 percent confluence. The cells viability was noted after 24hrs. Cells were then lysed by cell lysis solution containing 5 µl internal control (Sacace, Italy). RNA pallet was solubilized in 1% DEPC (Diethyl Pyro Carbonate) treated water. HCV RNA quantifications were determined by RT-PCR using kit protocol.

Effect of IG extract on gene expression in cell line through Real Time PCR

The anti-viral effect of plant extract on HCV core gene were detected by using specific-primers of HCV core genes via quantitative PCR (Fermentas, USA). The GAPDH gene was used ror internal control. Each RT PCR assay was performed in triplicate.

Components and concentrations of PCR reaction

To analyzed the anti-viral effect of plant extract on HCV core gene, from transfected Huh-7 cells. Semi-quantitative RT PCR product was amplified by using forward and reverse primers of the GAPDH and primer of the HCV Core gene under controlled condition. The PCR products were subjected to electrophoresis. Briefly, reagents of PCR were; PCR product-2ul, 10xPCR buffer 2.5 ul, $Mgcl_2$-2ul, dNTPs (10 mM) 2 ul.core, outer reverse primer 1 ul, core inner forward primer 1.0 ul and Taq polymerase-1.0 ul, dist water was added to reaction mixture to make volume up to 20 ul.

Results

Distribution of HCV genotype in chronic HCV patients

The results of the current research work revealed that out of 421 HCV positive patients, genotype HCV 3a was predominant (86%), followed b1a (08%), untypable (03%), 1b (01%), 3b (0.20%), and mixed cases were 0.40%. Out of patients, 241 were male and 180 patients were female while the high number of patients (389) were in age group of 20–40 years (table I).

Liver function test in HCV Genotype

The results of liver function test of HCV revealed that 85.7% (361/421) of patients demonstrated normal values while 14% (59/421) showed abnormal liver function tests. No significant correlation was recorded between LFT's and HCV genotypes ($p = > 0.05$) as shown in table II.

Viral load of HCV in genotype 3a

The present study reported that the HCV RNA viral load was detected in 363 patients having genotype 3a. Out of 363 patients, 273 had high viral load (> 800,000) and 90 patients had viral load less than 800,000. The P value of significant difference ($p < 0.05$) was calculated using chi square test (table III).

PCR amplification of core gene

The core gene of HCV was amplified by optimized conditions of the primers. Amplified product was resolved on TBE agarose gel (1.5%) with DNA marker (100 bp) as presented in figure I.

Blast Analysis

Chromas and Bioedit-software were used on sequencer generated files. In FASTA format samples were copied and analyzed through Blast software.

Sequence Translation

After sequencing, the obtained nucleotide sequences by using Expasy software were translated into amino acid sequences. The obtained open reading frame of amino acids was further considered for expression analysis. Through Blast software, further confirmation was carried out by protein Blast as shown in figure II.

Cellular Toxicity through MTT cell proliferation assay

Before assessing the antiviral activity of IG on cell viability, the effects on cell line were evaluated. For this purpose, MTT calorimetric and cell counting assay was employed. Serially diluted different concentrations of the herbal extracts were incubated with Huh-7 cell line. Absorbance readings were taken after incubation using microplate spectrophotometer. The results of cytotoxicity of effective extract were found at nontoxic dose of 100 µg (figure III). For assessing the anti-HCV activity of extract IG, cell lines were transfected and incubated for 24 h. After incubation, expression of gene of interest was detected by the Q-PCR assay (figure IV) and relative to the levels of cells incubated without compound (control).

Anti-viral activity of extract of IG against HCV Infected cell line

The solvent extract from IG was tested against core gene of HCV to determine the antiviral activity. The results of RT-PCR revealed that IG showed good antiviral activity against HCV i.e. IG inhibited 57% of cell line at nontoxic concentration as shown in figure V.

Methanolic extract of IG in different solvents

Methanolic extract of IG was fractionized by different solvents, based upon their polarity content (n-hexane, chloroform, and acetone) while acetone extract inhibited viral titer on large scale as shown in figure V.

Discussion And Conclusions

HCV is a highly prevalent, blood- borne pathogen. Patients infected with HCV have various clinical results, which ranges from acute resolving hepatitis to chronic liver disease (hepatocellular carcinoma or liver cirrhosis). [38]. Almost 80% HCV patients develop chronic infection and in about 20% of these subjects, progression to cirrhosis occurs [39]. Liver disease develops slowly over numerous decades in most of the patients. In Pakistan, chronic HCV is a foremost healthcare challenge resulting liver inflammation, tissue scarring, liver cirrhosis and HCC without treatment [40, 41]. Many studies conducted to determine the relationship between severity of biochemical abnormalities and serum viral load and they have produced contradictory results.[42]. Although raised serum level of ALT and AST levels indicate liver damage, in 20–30% of patients of chronic HCV, the S. AST and ALT levels return back to normal within 12 weeks of infection in spite of progression of disease. Few studies revealed that most of the patients with normal or near normal S.ALT levels, show mild liver lesion on histology and have slow progression of the disease. Some researchers revealed no relation between serum ALT, HCV viral load and the level of hepatic damage while others found major relation between HCV RNA load, serum ALT and degree of hepatic inflammation [43]. As PCR is pricey technique as compare to LFTs, so the current study was conducted to evaluate the relation between LFTs, liver damage severity and serum HCV RNA positivity in chronic HCV patients. Our results of LFT's of HCV showed that 85.7% (361/421) of patients demonstrated normal values while 14% (59/421) showed abnormal liver function tests. No significant correlation between LFT's and HCV genotypes ($p = > 0.05$) was reported. A similar study was conducted in which no significant difference was recorded in LTF's and genotypes of HCV infected patients. Similar observation was also noted in our study ($p = > 0.05$). Another finding in the same study was a useful correlation of viral loads with genotypes of HCV which is in accordance with our study results showing significant correlation of genotype 3a with HCV Viral load ($p < 0.05$) The increase incidence of HCV is found in Intravenous Drug Users (IDUs). Majority of people acquire this infection through reuse of syringes, needles and lack of sterilization practices in IDUs. We observed that there is increase prevalence of hepatitis c disease in males in comparison to female (207, 156 respectively). These observations may also be attributed to the IDU, as males were more affected [44] [45]. Also there is more exposure of males to the environment as compared to females, in our area of study, which may be another contributing factor and hence males were main target. A study conducted in 2014, had got similar results. They had showed that hepatitis c patients with higher levels of ALT have more active immune response to chronic viral infection [46]. To give reason for the poor association between the severity of liver damage and ALT level is not very simple. It was proposed in general terms that hepatocytes apoptosis has an important role. Liver damage and the loss of hepatocytes by apoptosis, could occur in hepatitis c-infected cases without changes in ALT level. ALT is released either by an immune mediated mechanism or by direct cytopathic effect of virus. Few studies suggested that the immune response in hepatitis c infected patients, with persistent normal ALT levels is less activated than in cases with abnormal ALT levels.[1].As hepatitis C infection is also related with several extra hepatic manifestations involving renal system, cutaneous, haemopoietic and neurologic system. Many autoimmune phenomenons are observed in these patients. Autoimmune hemolytic anemia (AIHA) was observed in HCV genotype 4 [47].

If baseline viral load is less than 399,000-850,000 IU/mL, the course of treatment, in genotype-1 and 4 may be reduced to 24 weeks and to 12–16 weeks in genotype 2 or 3 individuals.[48]. Many researchers have also observed that low viral load (599,000-799,000 IU/mL) is a good indicator of SVR. SVR decreases with elevation in viral load [49]. In deciding the response of host to antiviral therapy, viral genotypes have played a very important role. These genotypes and subtypes of HCV have variables that are distributed world widely. In Europe and United States, the genotype 1a and 1b are prevalent, respectively. In Africa countries, genotype 4 is present while HCV genotype 2 is abundantly found in West Africa countries and genotype 3a in Australia and South Asia. Apart from that, Asia and South Africa have genotypes 5 and 6, respectively. All these genotypes of HCV exhibit 30% heterogeneity in their nucleotide sequences. With the passage of time, the presence of HCV genotypes changes in Pakistan. The epidemiological studies may expose the distribution pattern of HCV genotype in order to facilitate the preventive strategies and treatment alternatives [50].

The HCV infection is the health challenge for clinicians globally. The main objective of the WHO is to reduce incidence of hepatitis by 90% and to reduce mortality by 65% by 2030 [51].In Asia and Africa, highest prevalence was observed [2].In Asia-pacific regions, the distribution pattern is inconsistent from 4–12%. [52, 53]. About 10 million people have been infected with HCV in Pakistan [54]. The prevalence rate vary in China, from 1% 31.86%.[55], while in Saudi Arabia the prevalence rate is 1.8% [56].

There is significant prevalence of HCV infection and till now no vaccine available. Lack of less compatibility of antiviral therapy and virus specific treatment opens the gateway to explore and use the medicinal plants for treatment. Currently, available agents target the NS3-4A protease and NS5B polymerase but still there is a chance of high resistance. Various studies are being conducting for new treatment options against HCV infection to overcome this alarming situation and to discover other imminent viral targets.

In this study, we investigated antiviral compounds of plant extract against HCV core protein to detect potential antiviral agents. The extracts of IG and its fractions were analyzed against HCV core protein for their antiviral activity. The core protein has 3 domains; 1st basic domain (aa 1-117) involves in binding to the viral RNA, 2nd less basic (118–174 aa) links with the lipid droplet and 3rd domain confines between 175-aa 191aa. Two forms of core protein i.e. the longer form p21 and the shorter form p19 which is derived from p21, have been identified [57].

A methanolic extract of leaves when evaluated with RT-PCR revealed the evidence of showing antiviral activity against HCV RNA expression. Specifically targeting steps of HCV life cycle in infected cells is a good approach for the development of new antiviral. The flavonoid (Apigenin) has diverse biological effects that includes improvement of the cancer cell response to chemotherapy [58], anti-platelet activity and tumorigenesis present in many vegetables and fruits [59–61]. Apigenin also the block the maturation of a subset of miRNA. [62]. The inhibitory acitivity of Apigenin on HCV replication was reported in 2018 [63]. Herbs have a key role in managing different disorders of liver. Due to diverse pharmacological

effects such as hepatoprotective and antioxidant activity, plants derived products (flavonoid, steroids and terpenoids) have gained considerable attention in recent years [64, 65]

Pakistan has tested (in vitro) numerous medicinal plants i.e. glycyrrhizin (GL) in recent years, that have inhibitory effects on HCV titer. It also showed synergistic effects with IFN. GL have been recommended as a future drug to help in decreasing the viral titer of HCV because it inhibited the core protein of genotype 3a at the mRNA [66]. The same research group have tested another plant (Silybum marianum (SM)) and confirmed through western blotting technique that two of its fractions exhibited inhibition of HCV core protein of genotype 3a. The researchers proposed that the combination of SM with IFN shows synergistic results in treatment of HCV [67]. It was possibly explained that a component of miRNA-generating complexes through impaired mitogen activated protein kinase activation Micro RNA122 (miR122), are essential for the HCV RNA stability and propagation by decreasing the levels of mature miR122 through the inhibition of the phosphorylation of TRBP. Many studies are still in progress that are looking for new ways of drug therapy against HCV infection. On cell viability effects the antiviral activity of IG were evaluated by incubating serially dilutes extracts along with transfected Huh-7 cells. MTT calorimetric and Cell counting assay was employed. By using the above mentioned assays the results of cytotoxicity assessment of effective extracts were found non-toxic at a concentration of 100 µg dose. The fractionation of methanolic extract of IG leaves into different solvents leads to the further specification. Assay was done similar to Rehman et al in 2011. The results revealed that acetone IG extract direct inactivated HCV. The natural products are small molecules that have capability of being absorbed and drug like activity are biologically active in different assays. The expanse of such medicines are much cost effective than that of producing through combination chemistry. Various herbal plants have been used for different infectious diseases globally and still more effective. The identify the molecular targets and structure-activity correlation, are mandatory in order to depict conclusion.

Our study concludes that HCV 3a genotype is highly prevalent in Peshawar, Pakistan. Due to poverty in Pakistan, doctors generally do not suggest the genotype testing. Importance of genotyping cannot be neglected. This study revealed that there is no relationship between liver function test and the grade of activity or fibrosis stage of liver in chronic HCV patients. The results of RT-PCR revealed that methanolic extract of IG showed more than 50% reduction of infecting cells. By selecting different molecular targets, specific structure relation can be obtained. To establish the importance of IG as antiviral drug against HCV, further research is required for the recognition and isolation of antiviral compound in IG.

Declarations

Ethics approval and consent to participate

The current study was approved by ASRB at the Institute of Basic Medical Science, Peshawar and IRB of Khyber Teaching Hospital approved the study. The patient provided consent for the current study.

Consent for publication

Written informed consent was obtained from the patient for the current study.

Availability of data and materials

All data generated or analyzed during this study are included in the current study.

Competing interests

The authors declare that they have no competing interests.

Funding source

This research was not funded by any funding agency, public, commercial or not-for-profit organization.

Author's contributions

Study concept and design, Shahina Mumtaz, Jawad Ahmed, Shafiq Ahmad Tariq; analysis and interpretation of data; Shahina Mumtaz, Waheed Iqbal, Sami Siraj, Tahir Sarwar, drafting of the manuscript; Shahina Mumtaz, Shafiq Ahmad Tariq, Waheed Iqbal, Sami Siraj, Tahir Sarwar; critical revision of the manuscript; Shahina Mumtaz, Jawad Ahmed, Shafiq Ahmad Tariq, Noor Rehman

Acknowledgements

We also appreciate Mr. Ali Said and Mr. Muhammad Imran for providing technical help in our laboratory work, storage and take care of study samples.

Abbreviations

ALT: Alanine transaminase; AST: Aspartate amino transaminase; HCV: Hepatitis C virus; IRB: Institutional Review Board; ASRB: Advanced study review board; HCV: Hepatitis c virus; PCR: Polymerase chain reaction; Q-PCR: Quantitative polymerase chain reaction.

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Tables

Table 1: Gender wise distribution of HCV genotypes among age groups (n=421).

HCV Genotype	Gender		Different age groups	
	Male	Female	20-40 yrs	≥ 40 yrs
3a	207(49%)	156 (37%)	338 (93.11%)	25 (6.8%)
1A	22(5%)	13(3%)	33 (94.2%)	2(5.7%)
3b	1(0.20%)	1(0.20%)	2 (100%)	0
1b	2(0.40%)	3(0.70%)	5 (100%)	0
Untypable	8(2%)	7(2%)	10 (66.6%)	5 (33%)
Mixed	1(0.20%)	0(0.0%)	1 (100%)	0
Total	241(57.24%)	180(42.76%)	389(92.4%)	32(7.6%)

Table 2: Mean and standard deviation of AST, ALT and total bilirubin in HCV genotypes.

HCV	Total No. of patients	ALT(IU/L)	AST(IU/L)	Total Bilirubin(mg/dl)
3a	363	44.19 ±10.36	43.64 ± 9.73	1.02 ±.12
1a	35	44.34 ±11.52	45.14 ±10.11	1.04 ±.13
1b	5	39.80 ±14.73	41.40 ±15.32	1.06±.19
3b	2	62.00 ± 4.24	66.00 ±0.00	1.20 ±0.00
Mix	1	44.00 ± 0.00	44.00 ± 0.00	1.00 ± 0.00
Untypable	15	47.73 ±9.33	48.13 ±8.85	1.04 ±0.12

*AST = Aspartate transaminase, *ALT Alanine transaminase

Table 3: HCV Viral loads in genotype3a

Genotype 3a	Low	High	*P Value*
Viral Load	<800,000	>800,000	<0.05
Total Patients	90	273	

Figures

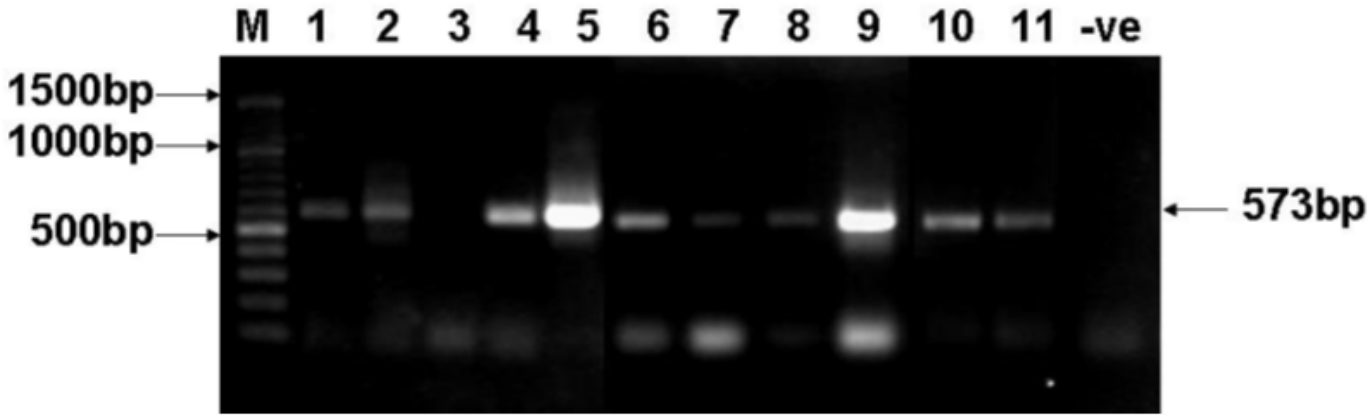

Figure 1

HCV core gene amplification through PCR. Core gene of HCV 3a genotype from patients' serum samples. Sample 1, 2, 4, 5, 6, 7, 8, 9, 10 and 11 shows amplified PCR product of core gene (573bp) 100bp DNA size Marker (M) and PCR negative.

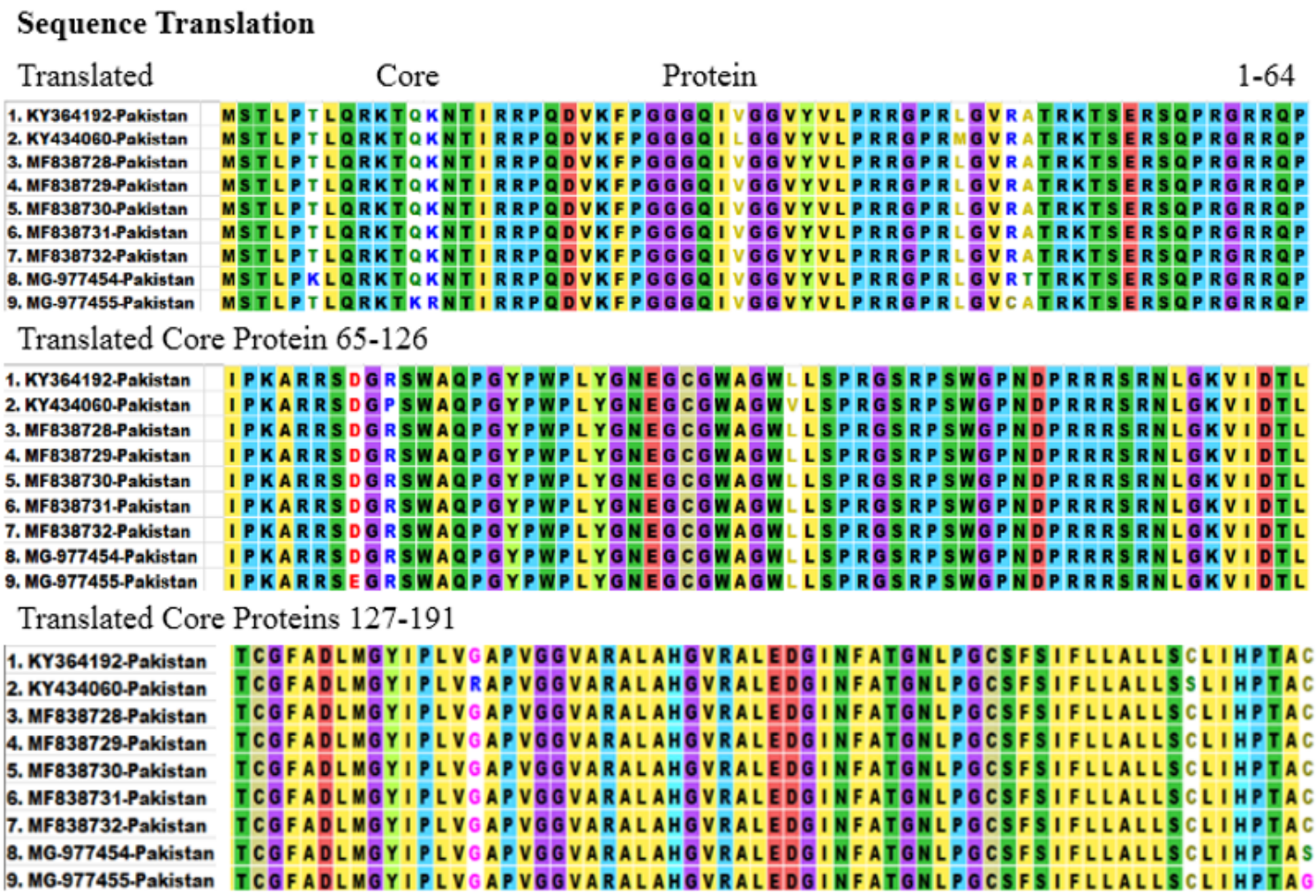

Figure 2

Translated HCV core gene sequences

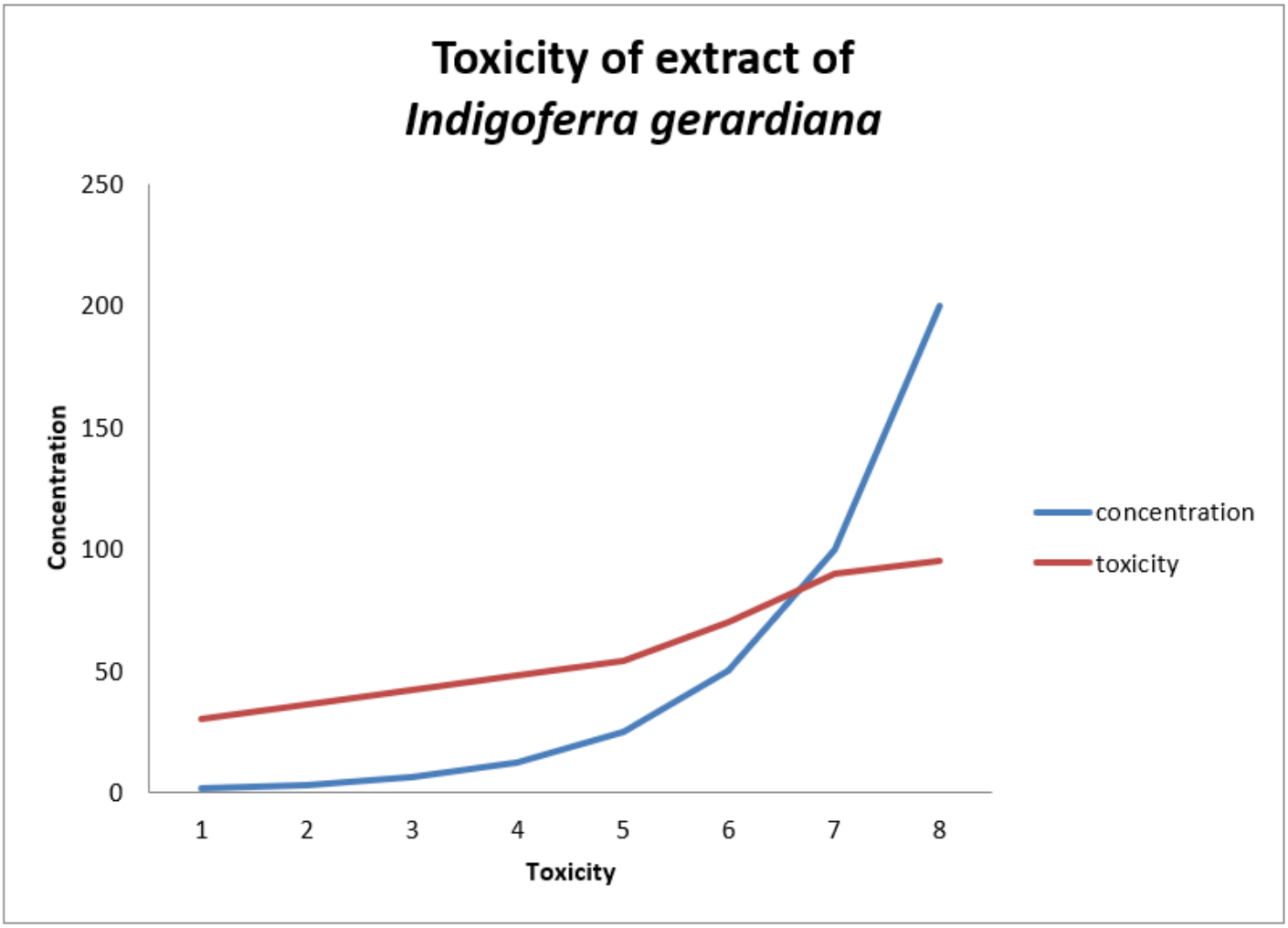

Figure 3

Cellular Toxicity through MTT cell proliferation assay; Toxicity of extract of IG: At different concentrations of the herbal extracts the cell lines were incubated. Absorbance readings were taken through microplate spectrophotometer at the end of incubation period.

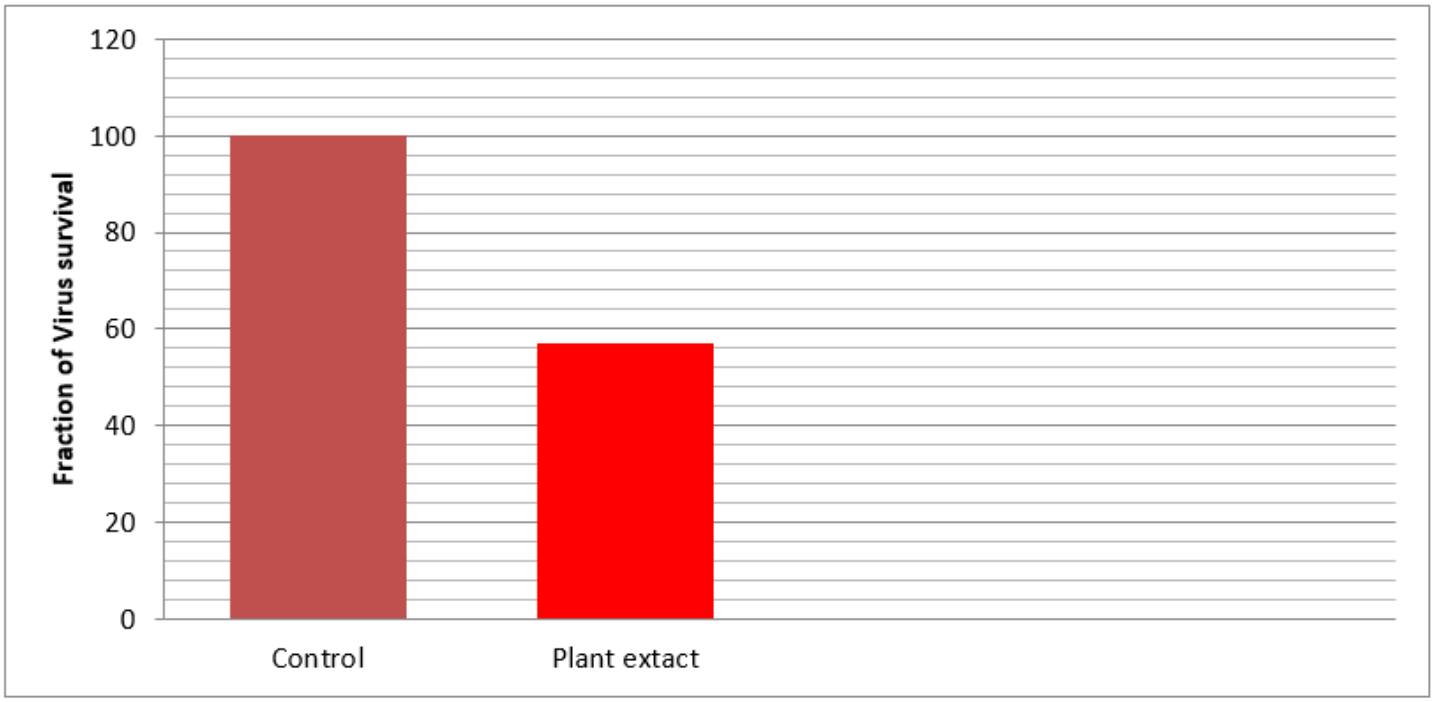

Figure 4

Anti-HCV activity of extract IG. Cell lines were transfected and incubated for 24 h. At the end of incubation period, expression of gene of interest was determined by the Q-PCR assay and are shown in Fig 4 relative to the levels of cells incubated without compound (control) The results demonstrated that IG resulted in 57% inhibition of cell line at nontoxic concentration.

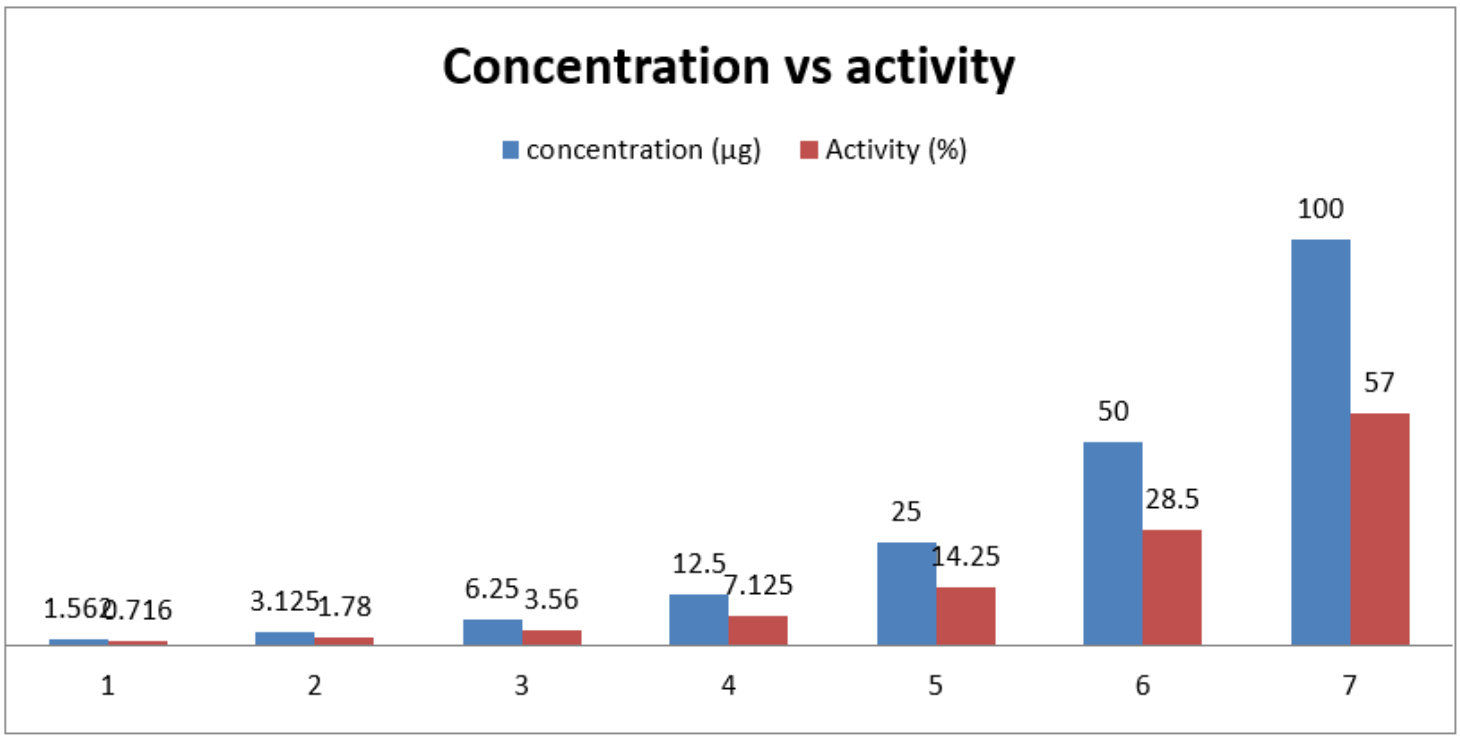

Figure 5

Different concentrations of acetone extract impeded viral titer to 57%.