Research Article

Evidence of E ectiveness of Azadirachta Indica Leaves in Chronic Hepatitis C in Addition of Conventional Therapy Failure

Rukhsana Sher[1], Tehseen Abaid[2], Muhammad Zahid[3], Arshad Kamal Butt[4]

[1]Assistant Professor, Department of Pharmacology & Therapeutics, Federal Post Graduate Institute, Sheikh Zayed Hospital, Lahore; [2.] Associate Professor of Pharmacology, D.G Khan Medical College, Dera Ghazi Khan; [3]F o r m e r Professor of Pharmacology, Allama Iqbal Medical College, Lahore; [4]Professor of Gastroenterology & Hepatology, Shaikh Zayed Hospital Lahore

Abstract

Background: HCV is taken as worldwide load in both developed and developing countries. Its treatment depends on HCV genome, dietary factors, pathogenesis and environmental factors.

Aims and Objective: A randomized controlled study was designed to prove the e ectiveness of Azadirachta indica leaves in chronic hepatitis C in addition of conventional therapy failure.

Material and Methods: Twenty patients with history of hepatitis C and failure of conventional therapy were included in the study. E ect of Azadirachta indica leaves and interferon was observed on sample having hepatitis C was observed and seropositivity of HCV was tested.

Results: In vitro study, it was observed that in serum of patients with hepatitis C, there is a protein peptide of 14 Kda is present, which is not observed in serum of normal subjects. Results showed that upon incubation of Azadirachta indica with serum of patients, there was an inhibition of protein peptide of 14 Kda. However when same sample was incubated with interferon alpha there is mild inhibition or decreased activity of of protein peptide of 14 Kda.

In vivo results showed that the extract of neem leaves decrease the titer of 15 patients. While 05 patients showed no response.

Conclusion: Study concluded that the leaves of Azadirachta indica may be e ective for the treatment of hepatitis C. Presence of 14 Kda protein in serum of HCV patients may be a marker of HCV.

Received | 10-11-2017: **Accepted** | 15-12-2018

Corresponding Author | Dr. Rukhsana Sher, Assistant Professor, Department of Pharmacology & Therapeutics, Federal Post Graduate Institute, Sheikh Zayed Hospital, Lahore . **Email:** rsherpharma@gmail.com

Keywords | Azadirachta indica (Neem), Interferon alpha 2 and HCV

Introduction

The hepatitis C virus (HCV) a ects approximately 130–170 million persons (2% –3% of the world's population) are living with hepatitis C virus (HCV) infection world wide.[1]The rate of HCV caused infection in Pakistan is about 5-8% in general population.[3] The prevalence and pattern of various types of viral hepatitis in Pakistan is quite di erent from that of developed countries in the west.[4]

Factors that have been reported to influence the rate of HCV disease progression include age (increasing age associated with more rapid progression), gender (males have more rapid disease progression than females), alcohol consumption (associated with an increased rate of disease progression), HIV coinfection (associated with a markedly increased rate of

disease progression), and fatty liver (the presence of fat in liver cells has been associated with an increased rate of disease progression)[5] to cause infection, HCV must pass from the blood of an infected person into the blood of susceptible person. Risk factors for HCV seropositivity were lower in education level, frequent parenteral injections, blood transfusion, menial occupations, smoking, and age > 50 years.[6]

During the last two decades, several new antivirals have been developed that are active against HCV, allowing sustained cure rates in a significant proportion of patients. All these drugs have side e ects, which may represent a major barrier to achieve cure in many patients in need.[7] However the extremely complex and incompletely understood nature of the HCV lifecycle has complicated the discovery of new therapies.[8] The FDA approved treatment of hepatitis C includes a combination of interferon alpha in combination with ribavirin.[9]

Azadirachta indica, commonly known as neem has attracted worldwide prominence in recent years owing to its wide range of medicinal properties. More than 140 compounds have been isolated from di erent parts of neem. The medicinal utilities have been described especially for neem leaf. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, antifungal, antibacterial, antiviral and, antioxidant properties.[10]

The FDA approved treatment of hepatitis C includes a combination of interferon alpha in combination with ribivarin. We are on the verge of a new era with the introduction of direct acting oral agent that will transform the treatment landscape. Herbal treatment is nowadays a promising treatment to cure many infective diseases.[10]

Present study tried to evaluate the e cacy and safety of the interferon alpha versus Azadirachta indica (herbal treatment) on serum of patients with chronic hepatitis C (in vitro study). Study also used Azadirachta indica leaf extract as a therapeutic treatment of hepatitis C in group of patients (in vivo study).

Methods

Twenty male patients with age range 30-50 years with history of hepatitis C and failure of conventional therapy were included in the study. Patients were taken from Out Door Department of Sheikh Zayed Hospital Lahore. Duration of study was from August 2014 to August 2015.

E ect of Azadirachta indica leaves and interferon was observed on serum sample having hepatitis C was observed (in vitro study). 20 age matched health volunteers with no history of any disease considered as controls. Extraction of Azadirachta indica leaves was prepared for in vivo and in vitro study. Seropositivity of HCV was tested before and after the extract of Azadirachta indica leaves using the technique of HCV RNA quantitative analysis by PCR. Detailed protein analysis was carried out to find out the protein peptides in serum of both controls and patients by using 12% SDS-PAGE.

Level of Base line parameters including ALT, AST, total protein, albumin, sodium ion and potassium ion were estimated by standard kit methods.

Study was approved from Institutional Review Board Shaikh Zayed Hospital Lahore Pakistan. Informed written consent was taken from all the patients and subjects who joined the study voluntarily. Patients co-infected with hepatitis A virus, hepatitis B virus, or hepatitis E viruses were excluded. None of the patients had any other cause of liver disease.

Fresh matured leaves of Azadirachta indica were collected from our Institute's (Shaikh Zayed Hospital Lahore, Pakistan) garden and were identified by a pharmacognosy expert.

10 leaves of average size of Azadirachta indica were taken from the Azadirachta indica tree and leaves were soaked in a cup of water overnight and extract was made after boiling the water till a tablespoon left. This extract was given in the fasting state for two weeks to all patients of 30-50 years of age (mean age of 40 years (same dose to all patients) and control group.

Base line parameters including ALT, AST, total protein and albumin were estimated by auto analyzer (Hitachi Tokyo, Japan). Level of sodium and potassium was estimated by flame photometer.

Detailed protein analysis was carried out to find out the prominent protein peptides in serum of both controls and patients by using 12% SDS-PAGE12.

Extract of Azadirachta indica /interferon alpha was incubated with serum samples of patients for 30 min at 37°c. E ect of neem and interferon alpha on serum of patients was studied by 12% SDS gel electrophoresis.

To find out the e ect of Azadirachta indica on serum of HCV patients extract Azadirachta indica leaves were given for two weeks. Seropositivity of HCV was tested before and after the extract of neem leaves using the technique of RT PCR. Statistical analysis was done using SPSS version 17. All quantitative results are expressed as means ± SD. Qualitative variables like PCR positive before and after treatment were described by frequencies. The di erence between drug treated and controls was evaluated by Student's t-test10. Liver function tests between normal and patients were expressed as means ± SD and comparison was made by using T – test. P ≤ 0.05 was considered significant.

Table 1: *Variation in Base Line Biochemical Parameters in Patients with HCV Positive and Normal Subjects Values are Expressed as Mean±SD*

Sr. No.	Parameter	Controls (20)	Patients (20)
1	Total protein (g/dl)	6.42±0.9	6.40±1.01
2	Serum albumin (g/L)	4.60±0.89	3.80±0.9
3	Serum bilirubin (mg/dl)	0.8±0.1	0.9±0.2
4	Serum alanine transferase (U/L)	5.5±2.0	15.84±17.50**
5	Serum alkaline phosphatase (KAU)	7.0±3.1	10.71±5.60*
6	Serum calcium (mg/dl)	9.0±1.1	9.1-12.0
7	Serum potassium (mmol/L)	3.6±1.2	5.0±2.5
8	Serum sodium (mg/dl)	313±102	345±250*

*P ≤ 0.05= Significant di erence
**P ≤ 0.001= highly significant di erence

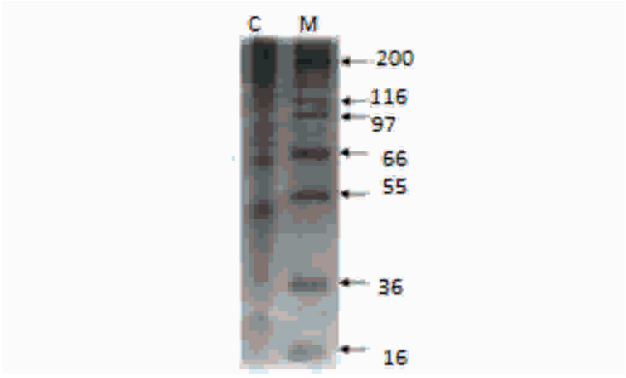


Fig 1: *SDS-PAGE Analysis of Serum Sample from Normal Subjects C and Standard Markers with*

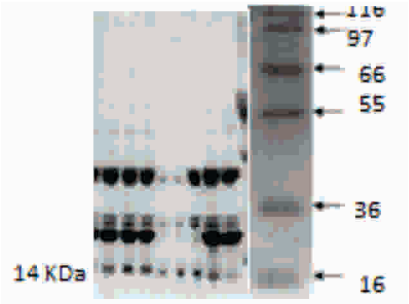
Table 2: *HCV Sero Positivity in HCV Patients before and after the Extract of Azadirachta Indica Leaves*

HCV seropositivity Before Azadirachta indica leaves	HCV seropositivity After Azadirachta indica leaves
80±35 IU/ml (20)	30±15.5 IU/ml** (15)

**P ≤ 0.001= highly significant di erence

Table 3: *Identification of Specific Marker Proteins in Patients of HCV.*

Patients	Controls (10)	Patients (20)
Marker proteins	60-70 kda (++)	60-70 kda (++)
	30 kda (+)	30 kda (+)
	-	14 KDa (+++)



Molecular Weight 200-16 Kda

Figure 2: *SDS-PAGE Analysis of Serum Samples from HCV Subjects.*

(Wells1-8 contains patient' serum, well 9 contains standard marker with proteins of 116-16 Kda).

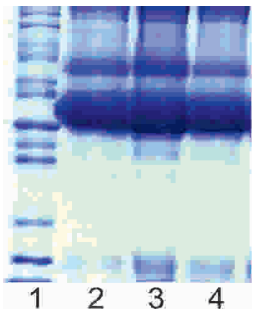


Figure 3: *Clear Image SDS-PAGE Analysis of Patient's Serum Samples against Neem Extract and IFN Alpha.*

From left to right. Lane 1: (standard marker with proteins of 116-16 Kda). Lane 2: (patient sample + neem leaves), Lane 3 (patient sample, before treatment.), Lane 4: (Patient sample + interferon alpha)

Results

Variation in base line biochemical parameters in

patients with HCV positive and normal subjects is tabulated as table 1. It was observed that the level of serum protein and albumin was non significantly decreased in patients as compared to controls. Non-significant change was observed in the level of serum bilirubin and serum calcium. Liver enzyme ALT and the enzyme of alkaline phoshptase were significantly decreased in patients as compared to control with P-value < 0.001 and 0.05 respectively. Level of serum sodium and potassium were increased in patients, but significant di erence was only observed in the level of serum sodium compared with the level of serum sodium of controls.

HCV sero positivity in HCV patients before and after the extract of Azadirachta indica leaves is tabulated as table 2. It was observed that out of 20 patients, in 15 patients the seropositivity of HCV was significantly decreased and attained to cut o values of HCV (50 IU/ml). While in 05 patients no response of neem leaves were observed.

Identification of specific marker proteins in patients of HCV was presented as table 3 and Fig 1& 2. It was observed that in serum of HCV patients a 14 KDa protein was observed which was not seen in serum of control subjects (Table 3), Figure 2 showed that serum of HCV infected patients 14 kda protein is most prominent while 60 kda and 30 kda proteins were at same level.

Figure 3 showed SDS-PAGE analyses of patient's serum samples against Azadirachta indica extract and IFN alpha, Well No. 2 showed the results of incubating mixture i.e. serum of HCV patients + Azadirachta indica extract. It was observed that specific 14 Kda proteins of HCV patients were completely inhibited by neem extract. While well no 4 which contain the serum of HCV patients incubated with interferon alpha, it was observed that there is a decreased density of 14 Kda proteins only.

Discussion

The global scenario is now supporting the development of modern drugs from less toxic plant products with proven medicinal properties. Azadirachta indica or neem leaves are now known to contain nimbin, nimbinene acetylnimbinase, nimbandial, nimbolide and quercentin. Neem leaves are also reported to remove toxins, purify blood and prevent damage caused by free radical in the body by neutralizing them. 10% aqueous extract of tender leaves has been found to show anti-viral properties.[11]

Base line parameters of patients with HCV positive were compared with the parameters of normal controls. It was observed that the mean level of alanine transferase (ALT), alkaline phosphatase, ions like serum sodium and potassium were markedly increased in patients with HCV positive as compared to the controls but significant di erence was observed in case of ALT, alkaline phosphatase and sodium. Level of serum albumin was decreased insignificantly. However no e ect was seen on the level of serum bilirubin, protein and serum calcium.

Number of studies is inline with our study. One of the study found that in liver disease, especially in advanced liver disease, the level of the serum albumin was decreased[12]. A study is stated that serum alkaline phosphatase predict relapse hepatitis C patients[13]. According to a study the increased level of serum ALT, alkaline phosphatase and decreased level of albumin shows fatty liver that may lead to dysfunction of liver[14]. It is proposed that ALT levels are released by direct virus-related cytopathic activity and/or by an immune-mediated process.[15]

Specific marker proteins in patients with hepatitis C and of controls were observed by using the technique of electrophoresis. Our study was observed that both normal subjects and patients with hepatitis C have the same density of 60-70 Kda protein. Two proteins of 30 Kda and of 14 Kda were observed in patients. However serum of control subjects protein of 14 KDa is were not present.

No study was carried out to find the markers protein in HCV patients. However, it is said that in individuals with week immune system, HCV infection causes significant damage to liver[16] and some proteins may convert into short peptides by HCV virus as infection lasting more than 6 months[17]. HCV sero positivity in HCV patients before and after the extract of Azadirachta indica leaves was also estimated. It was observed that out of 20 patients, in 15 patients the seropositivity of HCV was significantly decreased and attained to cut o values of HCV (50 IU/ml). While in 05 patients no response of neem leaves were observed. Our study is in accord in with a study who reported that in acute phase of infection, a spontaneous clearance is possible and 20–50% of patients gain immediate resolution.[18] Additionally a study reported that aqueous Azadirachta indica leaf extracts for viral infection has been used as a drug in Nigeria.[19]

According to our study, SDS-PAGE analysis of normal individuals and of patient serum samples showed that in serum of HCV infected patients 14 kDa protein is most prominent while 60 kDa and 30 kDa proteins were present in both patient's and control serum. Our study is inline with in vitro study showed that Azadirachta indica bark and leaf extracts reduced the production of viral proteins in cells, indicating that Azadirachta indica may inhibit replication of the virus.[20] On the other hand, interferon

alpha decreased the density of 14 kDa protein, a marker protein of HCV disease. A study reported IFNα plays a central role in the innate immune system by inducing an antiviral state in target cells, most notably for HCV by inhibiting viral replication.[19]

Conclusion

Study concluded that the leaves of Azadirachta indica may be e ective for the treatment of hepatitis C and may represent an alternative of interferon alpha for chronic hepatitis C patients who are unable to tolerate conventional treatment. Presence of 14 Kda protein in serum of HCV patients may be a marker of HCV. This article provides an entirely new frontier in research, namely, to look forward to the therapeutic benefit of neem therapy in HCV patients. However further research is needed on large number of patients to see and compare the e ect of neem with interferon to reach a better conclusion.

Ethical Approval: Given
Conflict of Interest: None
Funding Source: None

References

1. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology. 2013;57(4):1333-42.
2. Al Kanaani Z, Mahmud S, Kouyoumjian SP, Abu-Raddad LJ. The epidemiology of hepatitis C virus in Pakistan: systematic review and meta-analyses. R Soc Open Sci.
3. Review of medical microbiology and immunology. 12th Ed: . Warren Levinson; 2018. p. 324-33.
4. Fotiou A, Kanavou E, Antaraki A, Richardson C, Terzidou M, Kokkevi A. HCV/HIV coinfection among people who inject drugs and enter opioid substitution treatment in Greece: prevalence and correlates. Hepatol Med Policy. 2016 Aug 25;1(1):9.
5. Sharhani Asaad, Mehrabi Yadollah, Noroozi A, Nasirian M, Higgs P, Hajebi A, Hamzeh B, Khademi N, Noroozi M, Shakiba E, Etemad K. Hepatitis C virus seroprevalence and associated risk factors among male drug injectors in Kermanshah, Iran. Hepat Mon. 2017 October;17(10)
6. Negro F, Alberti A. The global health burden of hepatitis C virus infection. Liver Int. 2011;31:1-3.
7. Ajugwo AO, Erhabor TA, Eledo BO et al. Prevalence of transfusion transmissible infections in a Nigerian tertiary hospital. J Transm Dis Immun. 2017;2:1-3.
8. Lee JH, Cho J, Kim YJ, Im SH, Jang ES, Kim J, Kim HB, Jeong S. Occupational blood exposures in health care workers: incidence, characteristics, and transmission of bloodborne pathogens in South Korea. BMC Public Health. 2017;17(1):827
9. Xu J, Song X, Yin ZQ, Cheng AC, Jia RY, Deng YX, Ye KC, Shi CF, Lv C, Zhang W. Antiviral activity and mode of action of extracts from neem seed kernel against duck plague virus in vitro. Poult Sci. 2012;91(11):2802-07.
10. Galán RJ, Cidoncha EC, Martin M. Fet al. Antiviral regimen complexity index as an independent predictor of sustained virologic response in patients with chronic hepatitis C. J Manag Care Pharm. 2013;19(6):448-53.
11. Andersohn F, Claes AK, Kulp W, Mahlich J, Rockstroh JK. Simeprevir with pegylated interferon alfa 2a plus ribavirin for treatment of hepatitis C virus genotype 1 in patients with HIV: a meta-analysis and historical comparison. BMC Infect Dis. 2015;16(1):10.
12. Tiwari Ruchi, Verma Amit Kumar, Chakraborty Sandip. Neem (Azadirachta indica) and its Potential for Safeguarding Health of Animals and Humans: a review. J Biol Sci. 2014;2:110-23.
13. Idrees Sobia, Ashfaq Usman A. HCV infection and NS-3 serine protease inhibitors. Virol Mycol. 2013;2:112.
14. Pfe er Lawrence M, Li Kui, Fleckenstein Jaquelyn F, Marion Tony N, Diament Joel, He Yang CH, Pfe er Susan R, Fan Meiyun, Handorf Elizabeth, Handorf CR"* et al. An interferon response gene signature is associated with the therapeutic response of hepatitis C patients. PLOS ONE. 2014
15. García-Sastre A. Ten strategies of interferon Evasion by viruses. Cell Host Microbe. 2017;22(2):176-84.
16. WILKINS THAD, AKHTAR MARIAM, EUNICE GITITU MD et al. Diagnosis and management of hepatitis C. Am Fam Phys. 2015;91(12):835-42.
17. Amer Khorshed Alam, Hossain Monir, Hossain Ahmed, Hasnat Nur E, Sultana Khatun Chand. Phytochemical analysis, anti-oxidant and analgesic activity investigation of methanolic extract of Azadirachta indica Leaves. J Pharmacogn Phytochem. 2017;6(4):1699-702.
18. Ikechukwu Nwobodo Emmanuel, Nwosu Dennis C, Nwanjo Harrison U et al. Vitamins C and E levels are enhanced by Azadirachta indica leaves aqueous extract in paracetamol induced hepatoxicity in Wistar rats.2106. Vol. 10(24); June 2016. p. 338-43.
19. Mbah AU, Udeinya IJ, Shu EN, Chijioke CP, Nubila T, Udeinya F, Muobuike A, Mmuobieri A, Obioma MS. Fractionated neem leaf extract is safe and increases CD4+ cell levels in HIV/AIDS patients. Am J Ther. 2007;14(4):369-74.
20. Heim Markus H. Innate immunity and HCV. J Hepatol. 2013;58(3):564-74
21. Morozov VA, Lagaye S. Hepatitis C virus: morphogenesis, infection and therapy. WJH. 2018;10(2):186-212.