



PREVENTION OF HEPATITIS B VIRUS (HBV) REPLICATION BY EXTRACTS OF MIMOSA PUDICA, AN UNIQUE INDIAN MEDICINAL PLANT

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Abstract- Hepatitis B virus (HBV) causes acute and chronic viral hepatitis (CVH) and the latter condition could leads to liver cirrhosis and hepatocellular carcinoma which could be fatal. Vaccine against HBV is in practice however the efficacy of the vaccines varies with the individuals. Extensive investigations were done on anti HBV drugs and the common drugs in usage are lamivudine, telbivudine, adefovir, entecavir, and tenofovir and interferon α (IFN- α), an antiviral cytokine. Clinical benefits of medicinal plants against HBV infections has been extensively documented however significant amount of fine tuning is required to increase the responder ratio, optimal drug preparation methodologies and its benefit in terms of the stage of hepatitis. In addition, an optimum drug preparation method without their intrinsic toxicity has yet to be developed. These issues demand to open other alternative avenues to seek for novel anti HBV drugs. With this backdrop an attempt was made to google for the medicinal properties of *Mimosa pudica* (*M. pudica*), a delicate touch sensitive plant with seismonastic response movements and circadian rhythms. In this study, methanolic extracts of *Mimosa pudica* was tested for its anti HBV activity. Anti HBV activity and dose response was measured by HBsAg binding inhibition. *M. pudica* extracts showed a complete inhibition of HBsAg indicating its ability to act as a novel entry inhibitor during HBV infection. Based on the findings it could be concluded that extracts of *M. pudica* could be used as a novel anti HBV drug.

Keywords- *Mimosa pudica*, HBsAg, ELISA, HBV, Hepatitis

Introduction

The Hepatitis B virus (HBV) is a small circular DNA virus containing a nucleocapsid and an envelope and the virus belong to the hepadna virus family. HBV nucleocapsid contains a relatively small and incomplete double stranded (ds) DNA genome 3.2kb in size, a viral polymerase enzyme and core protein. The viral envelope contains surface proteins enclosed by the lipid membrane derived from host cells [1]. Initial or early phase of the viral infection in which the virus enters the cell may be divided into three stages namely: attachment, fusion, and entry. The HBV life cycle begins when the virus attaches itself to the host cell membrane via its envelope (env) proteins. This is followed by the fusion of viral membrane with the cell membrane and the viral genome is released into the cells [2]. Once the viral genome reaches the nucleus, the partial double stranded DNA (dsDNA) genome is converted into covalently closed circle DNA (cccDNA) by the viral polymerase. The cccDNA (covalently closed circular DNA) is considered to be the template for further propagation of pre-genomic RNA, which is in turn responsible for the synthesis of viral DNA and mRNA that encode all other viral proteins [3]. Following the encapsidation of pre-genomic RNA, the HBV core particles are assembled in the cytosol. During the reverse transcription of pre-genomic RNA into complementary DNA, the pre-genomic RNA is degraded. Initially, the HBV surface proteins are synthesized and polymerized in the rough endoplasmic reticulum. Followed by this, these proteins are transported to the post ER and pre-Golgi compartments and budding of the nucleocapsid begins. Finally, the assembled virion and sub-viral particles

are transported to the Golgi for further modification of its glycans in the surface proteins. The complete HBV virions are then are secreted out of the host cell to finish the life cycle [4].

HBV is known for deceiving the immune system and thus escape the immune surveillance and which helps the virus to cause productive infection. During infection, HBV acts like a stealth virus early during infection, remaining undetected while at the same time spreading until the onset of the adaptive immune response several weeks later. The ability of HBV to remain invisible to the innate sensing machinery of the cells probably reflects its replication strategy with the replicating viral genome being sheltered within viral capsid particles in the cytoplasm. However, HBV can be controlled when properly activated HBV-specific CD8⁺ T cells gain entry into the liver, recognize viral antigens and kill infected cells. They also secrete IFN γ which triggers a broad-based cascade that amplifies the inflammatory process and has non cytopathic antiviral activity against HBV [4]. However, establishment of an effective adaptive antiviral immune response is dependent on CD4⁺ T cells and their priming early in infection most likely triggered by the subviral antigens present in the inoculum rather than by the infectious virions. The failure to trigger early CD4⁺ T cell responses, which often occurs in low dose infections, induces functionally impaired CD8⁺ T cell responses resulting in the establishment of chronic persistent infection. As a result of inefficient immune response to HBV during chronic HBV infection, low-level liver cell destruction and regeneration over long periods of time occurs, eventually leading to fibrosis, cirrhosis, steatosis, and finally hepatocellular carcinoma [5].

Hepatitis B virus (HBV) infections are ubiquitous. An estimated 400 million people are chronically infected and about half a million people die each year of cirrhosis and hepatocellular carcinoma caused by chronic HBV infections [6]. Despite the availability of a safe and effective vaccine against HBV, chronic infection with HBV remains a major challenge in drug discovery [7]. Several drugs are currently available for the treatment of chronically infected patients, namely conventional and pegylated interferon-alpha (INF- α) therapy and treatment with nucleoside/nucleotide analogues like lamivudine, adefovir, entecavir, telbivudine and tenofovir. Some of them such as entecavir have been shown to possess activity against both HBV and HIV. Nevertheless, treatment is limited by the adverse effects of INF and the emergence of nucleoside/nucleotide-resistant mutants [6]. There is a great deal of information available on the anti HBV property of traditional medicines especially the ones of Indian origin however their success was found to be hampered by a variety of issues and toxicity. Previous experience with these herbal medications are equivocal and these drugs preparation methodologies warrant further optimization and over hauling and a considerable amount of fine tuning is required to increase the responder ratio, best drug formulation with minimum dilution effect and its benefit towards all stages of viral hepatitis. This study was conducted on the anti HBV activity of *Mimosa pudica*, which is popular for its unique seismonastic response movements and circadian rhythms [8].

Mimosa pudica (*M. pudica*), Linn. [Fig-1] belongs to the family *Mimosaceae* and is a common plant in moist waste ground, lawns, open plantation and weedy thickets. A curious feature of this plant is that the leaves close and the petiole hangs down in response to certain stressors such as a wound, wind, vibration and touch. Hot or cold stimulus, drought or change in illumination may also result in such changes [Fig-1].



Fig. 1- Structure of *Mimosa pudica* with compound leaves which get sensitive on touching, spinous stipules and globose purple or pinkish flower heads. Grows as a weed in countryside.

Seismonastic movements such as response to touch, are regulated by electrical and chemical signal transduction which spreads the stimulus throughout the plant [9]. *M. pudica* is also known as Lajjalu in Hindi, sensitive plant/touch-me-not in English and Thottal sinjuri in Tamil. The plant contains mimosin (an alkaloid), free amino acids, beta-sitosterol, linoleic acid and oleic acid. The drug is also found to be rich in tannins and the total tannin content was reported

to be 10% (W/W) [10]. The known medicinal properties of *M. pudica* are antivenom properties [11], arresting bleeding, skin diseases, wound healing activity [10], alleviating headache, insomnia, diarrhea, dysentery, fever, piles, fistula, anticonvulsant activity [12], anti-hyperglycemic and hepatoprotective activity [8] and anti viral activity [13]. In traditional medicine, the plant is known to cure many neurological disorders [12]. In this paper we report that *M. pudica* possessed anti HBV activity and this is possibly due to interference with the virus binding to its receptor.

Material and Methods

Mimosa pudica Extracts Preparation

Mimosa pudica was collected from Puducherry union territory, India and authenticated at the Department of Botany, University of Madras. The whole plant was rinsed in sterile distilled water, shade dried, powdered and stored at room temperature until use. Methanol extract of *Mimosa pudica* was prepared by adding 50 gm of whole plant powder in 500 ml of methanol and then filtered using Whatman filter paper (No.1). As a negative control phenolic extract of *Pongamia pinnata* seed was similarly prepared. The filtrate was then allowed to evaporate for about 2-3 days, the dried filtrate collected, weighed and stored at 4°C until use.

Virus Preparation

Hepatitis B virus positive blood was obtained from voluntary health service (VHS) hospital, Adyar, Chennai and virus concentration was determined by measuring the HBsAg content by ELISA (Hapanostika HBsAg ultra, France). The virus was then stored at -86°C until use for the assay.

Determination of HBsAg Inhibition

HBsAg binding inhibition was done by mixing equal volume of pre-titrated HBV and *Mimosa pudica* extract and incubated at 37°C for 5 days. The mixture was assayed on every day up to fifth day for the presence of bound/unbound HBsAg using ELISA kit. In the first set of experiments 5 mg/ml of *M. pudica* extract or control extracts were mixed with varying concentration of HBV (viral dose response analysis). Culture supernatants were collected on every day and assayed for HBsAg concentration. Blockade of HBsAg binding was represented in terms of percentage inhibition. In the next set of experiments, to a fixed concentration of virus different concentration of extract was added and this was to find out the optimum dose of extract required to neutralize HBsAg completely. The third set of experiments were done in checker board format to identify the optimum concentration of extract and the virus required for the complete neutralization of the virus. In all these experiments controls included in the experiment were drug positive control (Elan-PA001) [14] and drug negative control (Nonoxynol-9). Other controls included were the kit positive and negative controls. The ELISA procedure was then performed according to the kit protocol. Briefly, to the anti HBsAg antibody pre-coated plates extract treated HBV virus was added and incubated for 1 hour at 37°C. Then the plates were washed and secondary antibody-HRP conjugate was added and further incubated for 1 hour at 37°C. Then the plates were washed and TMB substrate was added and incubated at room temperature for 30 minutes. To this stop solution was added and the plates were read at 450 nm in ELISA reader (BioTek, USA). Results are represented as ELISA optical density (OD) and percentage (%) inhibition. Percentage inhibition was calculated as per the formula : (OD of the control - OD of Test)/OD of the control x 100 [15].

Result

Inhibition of HBsAg Binding to Its Receptor by *Mimosa pudica* Extract

Varying concentrations of the HBV (150 pg/ml to 0.8 pg/ml) was treated with methanolic extracts of *M. pudica* (5 mg/ml) and incubated at 37°C for 5 days. The supernatants were collected on every day and HBsAg was measured by ELISA. First set of experiments were done to evaluate the efficacy of *M. pudica* in neutralizing the HBsAg of HBV. As illustrated in [Fig-2]. *M. pudica* induced a significant inhibition of HBsAg at 5 days post treatment suggesting *M. pudica* mediated inhibition could be at the virus entry level. For this study 5 mg/ml concentration of drug extract was used in the treatment against varying concentrations of HBV. Virus concentrations used ranged from 150 pg/ml to 0.8 pg/ml. 5 mg/ml concentration was able to neutralize upto 7.5 pg/ml concentration of HBV [Fig-2]. When the virus concentration was increased to 15 pg/ml or above there was no inhibitory activity suggesting that 7.5 pg/ml dose was optimum virus dose for the complete viral neutralization by 5 mg/ml *M. pudica* extract. As shown in the [Fig-2] methanolic extract of *P. pinnata* (an irrelevant drug) failed show any anti HBV activity suggesting that *M. pudica* mediated suppression of HBV is specific. In addition *M. pudica* mediated HBsAg suppression was found as early as 24 hour later treatment and persisted until day 5 (data not shown). In-house drug positive control completely inhibited the HBV as revealed by its 98% inhibition. These data clearly suggest that *M. pudica* extracts completely prevented the HBV replication especially the virus entry level.

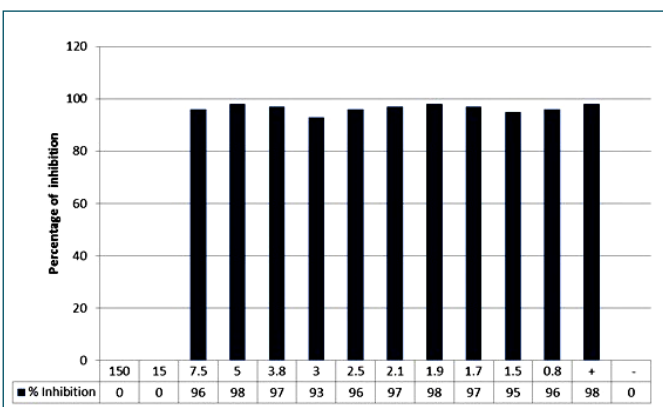


Fig. 2- Varying concentration of HBV was treated with 5 mg/ml concentration of *Mimosa pudica* methanolic extract.

Results are presented as percentage(%) Inhibition. Virus dose range was 150 - 0.8pg/ml. (-) drug negative control, (+) drug positive control

Inhibition of HBV by *M. pudica* was Viral Dose Dependent

The next set of experiments were done to find the optimum drug concentration that is required to bring about the maximum HBV inhibition. For this varying concentration of *M. pudica* was treated with fixed concentration of HBV (7.5 pg/ml). As shown in the [Fig-3] HBV concentration of 7.5 pg/ml was completely neutralized by 5 mg/ml or higher doses. Since the inhibitory activity peaked to its maximum at 5 mg/ml the levels of inhibitory activity remained plateau with higher drug concentrations. It is important to note that HBV inhibitory activity was not noticed at concentrations below 5 mg/ml.

When the virus concentration was increased to 15 pg/ml the anti HBV activity was abrogated whereas at this virus concentration a higher dose of drug (50 pg/ml) was required to bring about the com-

plete neutralization of the virus [Fig-3]. When the virus concentration was further escalated to 30 pg/ml or 150 pg/ml the anti HBV property of *M. pudica* was completely abrogated. There appears to be a threshold level for *M. pudica* and if the virus concentration elevated beyond this point the anti HBV property was lost. This warrants further optimization and better methodologies which would completely suppress HBV replication even at higher viral concentration.

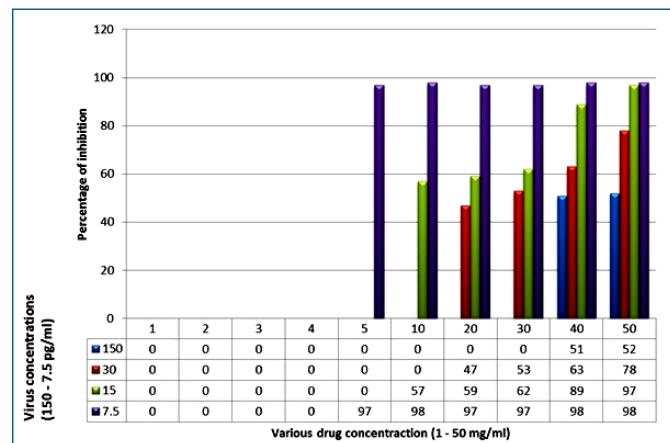


Fig. 3- AntiHBV activity of *Mimosa pudica* was tested by checker board method. varying Concentrations off HBV (150-7.5pg/ml) was tested with varying concentrations of *M. pudica* (50-1mg/ml).

Data is represented as percentage inhibition

Discussion

Lack of safe allopathic medicines to offer complete cure for HBV infection serves as an alluring platform for the discovery of novel anti-HBV drugs from natural resources. In this study the mechanism of action of *M. pudica* was evaluated. The results of the study strongly suggest that *M. pudica* is the optimal drug of choice to control HBV infections. *M. pudica* has clearly inhibited the 7.5 pg/ml of the virus and this was achieved with 5 mg/ml. While the study required further optimization potential usage of *P. pudica* against HBV infections is an eye opener and a positive lead in the light of magnitude of chronic viral hepatitis world-wide. An overview of literature indicate that so similar study done previously and hence results are discussed in the context of using *M. pudica* as a possible anti HBV drug.

Chronic HBV infections can lead to liver cirrhosis and hepatocellular carcinoma and hence the need for proper treatment modalities to fight against human HBV infection. The HBV surface antigen (HBsAg) is the one that helps the virus to adhere to the target tissue [16] and hence is a major component of the virion. In this report we showed that *M. pudica* extract completely inhibited the virus possibly through interfering with HBsAg. Importance of HBsAg is multi-fold and it is highly immunogenic. HBsAg is also known as "australia antigen" and based on this antigen HBV can be divided into 4 phenotypes namely adw, ayw, adr, and ayr and each phenotype is epidemiologically important. Presence of HBsAg in a patient is an indication that it is a recent infection and antibodies to HBs (anti HBs antibody) are efficient in clearing the HBV. Besides that there are two other important antigens namely HBeAg and HBcAg are important for the complete clearance of the virus during chronic infections. In chronic HBV infection both HBsAg and antibodies to HBs (anti HBs) are found in the patients and presence of HBsAg

helps in the new infection of hepatocytes [14] which leads to viral hepatitis.

Receptors for HBV is not fully known and it is speculated that preS domain of surface protein of the virus bind to carboxypeptidase D molecules found on hepatocytes [4]. Besides glycine decarboxylase (DGD) believed to be the secondary receptor. Thus the surface antigen (HBsAg) plays an important role in virus attachment to the hepatocytes and any methodology that would interfere with this initial binding can prevent the virus attachment to the host tissue. Our investigation has shown that *M.pudica* extract inhibits HBsAg binding to its receptor and in this study anti HBs antibody act as the receptor. In this study we clearly demonstrated that 5 mg/ml concentration of the extract inhibited the virus binding and this inhibition was noticed up to 7.5 pg/ml and lower concentration of the virus.

Exploring novel drugs to combat HBV binding especially the chronic HBV hepatitis is important. Traditional medicines serve as potential treasure hunt platform for unique drug development that could be used to treat viral hepatitis. Our study clearly show *M. pudica* inhibited HBsAg binding to its receptor and in this study anti-HBs antibody acted as the receptor. In this study varying concentration of *M. pudica* was tested for the anti-HBV activity and it was found that 5 mg/ml concentration of *M. pudica* completely neutralized the HBV suggesting its medicinal scope to treat viral hepatitis. However its need to be recorded that virus concentration of above 7.5 pg/ml was not neutralized and to achieve that more *P. pudica* extracts was needed. This suggest that further fine tuning is required to achieve a complete cure. Furthermore drug toxicity study need to be conducted to make sure the drug is safe and *P. pudica* offers a fertile ground to further explore on the above discussed issues. Thus *M. pudica* is a novel drug with strong anti HBV activity.

Conclusion

Mimosa pudica (*M. pudica*), a delicate touch sensitive plant is known for its seismonastic response movements and circadian rhythms. Its bioactivities has been well documented and in this manuscript we record its anti HBV activity. As shown in the results, *M. pudica* exhibited anti HBV activity at 5 mg/ml. However, further research is needed to identify the active compound responsible for the anti-HBV activity of *Mimosa pudica*. This could open new avenues to target HBV replication and thus develop powerful tool for the treatment of this dreaded disease. In this age where new viral diseases are emerging in all corners of the globe, there is a great need to better explore nature's products for their medicinal properties.

Conflicts of Interest: None declared.

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