Anti Hepatitis B Viral Activity of *Phyllanthus reticulatus*

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Abstract

Phyllanthus reticulatus is a reputed medicinal plant used in Bangladesh and India for the treatment of gastric complaints including colic, constipation etc. The aim of this study was to evaluate the antiviral activity of this plant against hepatitis B virus (HBV) using HBsAg positive serum sample from hepatitis B virus infected patients. Two semi-purified organic fractions designated as PR1 and PR2 of the fat free ethanolic extract were tested at both lower and higher concentrations (20 mg/ml and 40 mg/ml respectively) for their anti hepatitis B virus surface antigen (anti-HBsAg) activity using an *in vitro* system by Reverse Passive Haemagglutination (R-PHA) method. SERRODIA-Anti-HBsAg-Diagnostic kit was used for detection of Anti-HBsAg antibody. Both fractions showed anti-HBsAg activity. But it was found the fractions have little inhibitory action on HBsAg at lower concentration whereas at the higher concentration they have prominent inhibitory action on the antigen. To the best of our knowledge this is the first report of the antiviral activity of *Phyllanthus reticulatus* against HBV. The Anti-HBsAg activity observed by the fractions may be due to the binding of the agents with the antibody binding sites present on HBsAg. Thus the fractions might be the potential sources of the active principles responsible for antiviral activity.

Keywords: *Phyllanthus reticulatus,* organic fractions, ethanol extract, HBsAg, antiviral activity.

Introduction

Hepatitis B virus (HBV) infection, in both its acute manifestations and its chronic consequences, is a major public health problem throughout the world (Sherlock, 1981). 10-15% of the population in developing countries are the common carriers of HBV (Sherlock, 1981). In spite of tremendous advancement of medical science and technology there is no effective therapy available for the treatment of various liver ailments (Sherlock, 1981; London *et al.,* 1982). But there are claims in the literatures of traditional medicines (Ayurveda and Unani) that a number of medicinal plants are effective in the treatment of liver diseases (Satyavati *et al.,* 1987). *Phyllanthus* is a medicinally important genus in the Euphorbiaceae family. A number of plants of this family are observed to be beneficial for the treatment of liver ailments. In the Indian subcontinent, plants like *P. niruri, P. emblica, P. urinaria* etc. have been reported to be effective (Satyavati *et al.,* 1987). Like, aqueous extract of the plant *P. niruri,* was found to inhibit DNA polymerase of hepatitis B virus and also to bind to the surface antigen of hepatitis B virus *in vitro* (Venkateswaran *et al.,* 1954). In another study, crude extract of *P. niruri* showed significant *in vitro* inactivation of HBsAg as tested by both counter immuno-electrophoresis (CEP) and reverse passive haemagglutination (RPHA) methods (Nadkarni, 1954). *P. reticulatus* (Bengali name - Panjuli; Family-Euphorbiaceae) is a climbing shrub which grows all over Bangladesh (Ghani, 2003). This plant has not been studied for the anti-viral activity, but the biological studies on this plant showed hypotensive effects and its folkloric use in gastric complaints including constipation, colic, etc (Rav *et al.,* 1964) and chemical studies demonstrated the presence of octacosanol, teraxerol acetate, friedelin teraxerone, betulin, sitosterol, etc (Joshi, 1991). Besides plants of this genus are reported to contain lignans, flavonoids, triterpenoids, alkaloids, polyphenolic compounds which possess significant activity against hepatitis B virus responsible for hepatotoxicity causing fatal liver diseases (Anjenenlu *et al.,* 1973: Joshi *et al,.* 1991; Yoshida *et al.,* 1982). As a part of our continuing interest in the study of biological activities of plant

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extracts or fractions, we herein, report the results of *in vitro* evaluation of *P. reticulatus* for its activity against hepatitis B virus (HBV).

Materials and Methods

Plant material: P. reticulatus is a subscandent shrub, which is widely distributed elsewhere in Bangladesh and can reach 2-3 m. The plant is straight with slender branches and leaves are distichous. The fruit is berry and the seeds are superimposed. The aerial parts (leaves with branches) of *P. reticulatus* were collected from Gazipur, Dhaka, Bangladesh and was taxonomically identified with the help of Bangladesh National Herbarium.

Preparation of plant extracts and semi-purified fractions: The air-dried and ground powder (500 mg) was defatted with petroleum-ether (60°-80°) and then it was extracted with ethyl acetate and subsequently with ethanol (95%) by means of Soxlet apparatus. The extracts, in all cases were filtered off and evaporated to dryness by using the rotary evaporator to get a concentrated gummy mass. The ethanol extract was subjected to vacuum liquid chromatography (VLC) for fractionation over silica gel 60H (VLC grade) using solvents of increasing polarity (first with 100% toluene, then ethyl acetate- toluene, followed by methanol-ethyl acetate system and finally with 100% methanol). Twenty fractions designated by 1-20 were collected in separate beakers and screened by TLC. The fractions showing components with similar R_f values and similar color reaction with vanillin/sulphuric acid spray reagent were bulked together. In this way two partially purified fractions designated as PR1 and PR2 were obtained. The PR1 contained five components (R_f values: 0.83, 0.74, 0.69, 0.55, 0.53) and PR2 contained four components (R_f values: 0.74, 0.69, 0.58, 0.35).

Anti hepatitis B virus surface antigen (anti-HBsAg) activity testing: The fractions PR1 and PR2 were investigated for their anti-hepatitis B virus surface antigen (anti-HBsAg) activity using an *in vitro* system by Reverse Passive Haemagglutination (R-PHA) method in the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh. The test was based on the principle that sensitized red blood cells consisting of fixed chicken erythrocytes adsorbed with highly purified guinea pig anti HBs immunoglobulin (IgG) are agglutinated specifically in the presence of HBsAg in the serum and the test samples may inhibit this agglutination either by binding with antibody binding sites present on HBsAg or by neutralization of HBsAg.

At first strongly HBsAg positive serum was obtained from hepatitis B virus (HBV) infected patients associated with acute and chronic liver diseases. The serum samples were mixed with equal volume of the test samples (0.2 ml serum sample + 0.2 ml test sample). The mixture was incubated for 24 hours at 37°C. Controls comprising only the phosphate buffer saline (PBS) treated sera were used with each set of experiments.

Table 1. Serial dilution of serum samples for testing in microtitre well

Treatment group	Number of rows of microtitre wells											
	1	2	3	4	5	6	7	8	9	10	11	12
Serum diluent (PBS) (µl)	100	25	25	25	25	25	25	25	25	25	25	25
Test serum samples (µl)	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	25 ►	Discard
Serum dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Control cells (µl)	25											
Antibody sensitized cells (µl)		25	25	25	25	25	25	25	25	25	25	
Final Serum dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	

'►' = Amount transferred to the next well as a part of dilution

After 24 hours of incubation period both the sample treated serum and control were simultaneously titrated for HBsAg by utilizing the R-PHA method. In this case, 100 µl of PBS was taken in row 1 and 25 µl of PBS was taken in each of the remaining rows of microtitre wells using micropipette (Table 1). Then 25 µl of test samples was

added in row 1 with the micropipette. The contents of row 1 were mixed well and serial dilution of the serum samples was performed by transferring 25 µl from row 1 to row 2 and so on up to row 11. By using the micropipette 25 µl of control cells was added to row 1 and 25 ml of antibody sensitized cells were added to each of the remaining rows (2 to 11).

Results

Both the fractions (PR1 and PR2) were found to show the anti hepatitis B virus surface antigen (anti HBsAg) activity (Table 2). The fraction PR1 showed the inhibition of agglutination reaction between HBsAg and the antibody in 1:1280, 1:2560 and 1:5120 dilutions at lower concentration (20 mg/ml), while at higher concentration (40 mg/ml), HBsAg negative reaction was observed in 1:320 and 1:640 dilutions in addition to those mentioned before. On the other hand, fraction PR2 showed the inhibitory action at 1:640, 1:1280, 1:2560 and 1:5120 dilutions in both lower (20 mg/ml) and higher (40 mg/ml) concentrations.

The activity might be due to the binding of the agents with the antibody binding sites present on HBsAg. The attachment between the agent and HBsAg did not appear to be strong and significant inactivation did not occur.

Table 2: Effect of PR1 and PR2 on HBsAg present in serum samples of HBV infected patients

Test Sample	Concentration	Results in different dilutions									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
PR1	20 mg / ml	+	+	+	+	+	+	-	-	-	-
	40 mg / ml	+	+	+	±	-	-	-	-	-	-
STC	-----	+	+	+	+	+	+	+	±	-	-
PR2	20 mg / ml	+	+	+	+	+	-	-	-	-	-
	40 mg /ml	+	+	+	+	±	-	-	-	-	-
STC	-----	+	+	+	+	+	+	+	±	-	-

'+' = HBsAg positive, '-' = HBsAg negative, STC = Saline (PBS) treated control

Discussion

In the present study, *P. reticulatus* was investigated for its antiviral activity against hepatitis B virus. *P. reticulatus* is a medicinal plant commonly used in traditional medicine in Bangladesh as well as in India. Besides we recently reported the hepatoprotective and renoprotective activities of this plant against CCl_4-induced liver and kidney injuries respectively. Therefore, we were inspired to study the ant-HBV activity because hepatitis B virus (HBV) is responsible for the hepatotoxicity causing fatal diseases like liver cirrhosis and hepatocellular carcinoma. Although this plant has not previously been investigated for antiviral activity, the plants of this genus especially *Phyllanthus niruri* has been found to possess anti-HBsAg activity. The results indicated that the fractions possessed the anti-HBsAg activity at higher concentration. From the chemical studies it was found that the plants of this genus contain lignans, flavonoids, alkaloids, polyphenolic compounds that possess significant activity against hepatitis B virus. Therefore the results also indicated that the fractions might have the possibility of containing components of such activity.

Conclusion

We have observed the anti hepatitis B virus surface antigen (anti HBsAg) activities of two partially purified organic fractions (PR1 and PR2) of *P. reticulatus,* where the higher concentration was more effective as compared to the lower concentration. Although this plant has been investigated for other biological activities, this is the first report of antiviral activity. The biological evaluation of other fractions is in progress.

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