

# A Study on *in vitro* antiviral activities of lyophilized extracts of *Glycyrrhiza glabra* on Hepatitis B Virus

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## Abstract

The present study is to determine the effect of lyophilized extracts of different solvents of *Glycyrrhiza glabra* on Hepatitis B. The lyophilized plant extracts were collected and studied for its cytotoxicity in HepG2 cell line and *in vitro* antiviral activity of these extracts was investigated by HBs Ag binding Inhibition Assay, Hepatitis B Virus DNA Polymerase Inhibition Assay using fluorescent probes. The results from *Glycyrrhiza glabra* were promising in acting as a potent antiviral agent.

**Keywords:** Hepatitis B, *G. glabra*, HBs Ag Binding Assay, HBV DNA polymerase

## 1. Introduction

Hepatitis B virus is a major global health problem affecting 1/3 of the populations in the world [1]. About 5% of the people with acute infection develop chronic infection which leads to hepato cellular carcinoma and liver cirrhosis [2,3]. Nucleoside analogs like lamivudine, fanciclovir and penciclovir are currently available drugs [4, 5]. Long term usage of these drugs led to the emergence of drug resistant virus [6,7]. This urges for an alternative approach to treat HBV infection.

Medicinal plants and its compounds are widely used as a source to treat various viral infections [8, 9]. *Glycyrrhiza glabra* Linn is an age old plant used in traditional medicine across the globe to cure varieties of ailments from single cough to hepatitis and so on. *Glycyrrhiza glabra* is considered to be the modern medicine for the development of drugs in future [10]. Glycyrrhizin from *Glycyrrhiza glabra* along with interferon inhibit the full length viral particles and HCV core gene expression in a dose dependent manner [11]. The antiviral effects of crude extracts of *G. glabra* on Hepatitis B were proved to be effective by *in silico* analysis. The lead docking score of compounds of *G. glabra* exhibits a better alternative to treat infection [12]. Hence the present study has a special interest on *Glycyrrhiza glabra* to screen the antiviral activity against Hepatitis B.

## 2. Materials & Methods

### 2.1 Preparation of Plant Extracts

The roots of *Glycyrrhiza glabra* were collected, surface sterilized with double distilled water, dried under shade and made into a powder. 20gms of the powder was soaked in 100 ml of different solvents (Aqueous, Ethanol, and Aqueous ethanol) and stored overnight at 4°C. It was filtered through wire gauze. The filtrate was centrifuged with the help of a cooling centrifuge at 3500rpm for 10min. The supernatant was filtered through Seitz filter (0.20m). Seitz filtered extracts were further subjected to lyophilization under high vacuum. The concentrated plant components were scraped and stored in sterile vials until use.

### 2.2 Serum Sample Collection

Positive Serum Samples of Hepatitis B were collected from Medlabs, (VHS) Voluntary Health Service Hospital, Adyar, and Chennai.

### 2.3 Stock preparation of the lyophilized extracts

1mg of the lyophilized extracts was dissolved in water, ethanol or DMSO according to their solubility and made up to 1ml with the Double minimal essential medium (DMEM) then, it was filtered with the help of syringe filter (0.22µm) and subjected to cytotoxic analysis using HepG2 cell line.

#### 2.3.1 Cytotoxic evaluation by MTT Assay

The principle involved in the assay are the reduction of tetrazolium dye MTT 3-(4, 5 – dimethyl thiazol – 2 – yl)

– 2, 5- diphenyl tetrazolium bromide to an insoluble formazan which results in the formation of purple colour. 20µl of MTT (5µg/µl) was added on to the wells and kept for incubation at 37°C for 4 hours after incubation, the medium was gently aspirated and 150µl of DMSO was added onto it to dissolve the formazan crystals and the readings were recorded using ELISA reader at 450nm and 650nm wavelength.

#### 2.4 HBs Ag binding Inhibition Assay

The HBs Ag binding Assay was performed according to the instructions of the kit (ERBA LISA HEPATITIS B). The absorbance was measured at 450 and 650 nm as reference wavelength. Triplicate results were recorded and the blank absorbance was deducted from both the test and control wells. Lyophilized *Glycyrrhiza glabra* extracts with different solvents (aqueous, ethanol, aqueous: ethanol) were taken and it is solubilized with appropriate solubilizing agents. Equal volume of HBs Ag positive samples and plant extracts were mixed and incubated at 37°C for 5 days. Positive control (viral samples with lamivudine) & Negative control (without the plant extracts) were set up. The mixture was assayed daily for 5 days. Binding Inhibition of the extracts were analyzed every day and the percentage of inhibition was calculated [13].

#### 2.5 HBV DNA polymerase Inhibition Assay using fluorochromes:

- This assay was performed according to Lofgren *et al* [14] with little modification.
- Fresh positive serum samples were taken and centrifuged at 35,000rpm for 3 h.
- After centrifugation, the supernatant was discarded and the pellet was washed with PBS and centrifuged once again at 35,000rpm for 1 h.
- The supernatant was discarded and the pellet obtained finally was resuspended in PBS and stored at -20°C for future use. Before starting the assay, 2% Mercaptoethanol, and 10% NP 40 (30µl each) was added to viral preparation and kept for 15-30 minutes at room temperature.
- 25 µl of viral preparation was added to 100 µl of reaction mixture containing dATP, dGTP, dCTP and (dTTP)replaced with Fluorescein-12- dUTP (fluorochrome tagged molecule) to achieve the concentration of 0.1µM. Tris HCL (pH -8), MgCl<sub>2</sub>, KCl and plant extracts(400 µg / µl) are incubated at 37°C for 3 hrs. Positive control (Reaction mixture with lamivudine 1 µg / µl) and Negative controls (without the plant extract) were set up.
- The mean of the triplicate counts were used to calculate the percentage of inhibition according to the formula given below.

$$\text{Percentage Inhibition} = 100 - \left[ \frac{\text{Mean CPM}_{\text{test}} \times 100}{\text{Mean CPM}_{\text{negative control}}} \right]$$

The plant extracts showing inhibition greater than or equal to 50% were considered as positive [15].

### 3. Results & Discussion

*In vitro* cytotoxicity of the lyophilized extracts of *Glycyrrhiza glabra* were analyzed after 72 h of incubation.

**Table 1: The *in vitro* antiviral activity of lyophilized extracts of *Glycyrrhiza glabra***

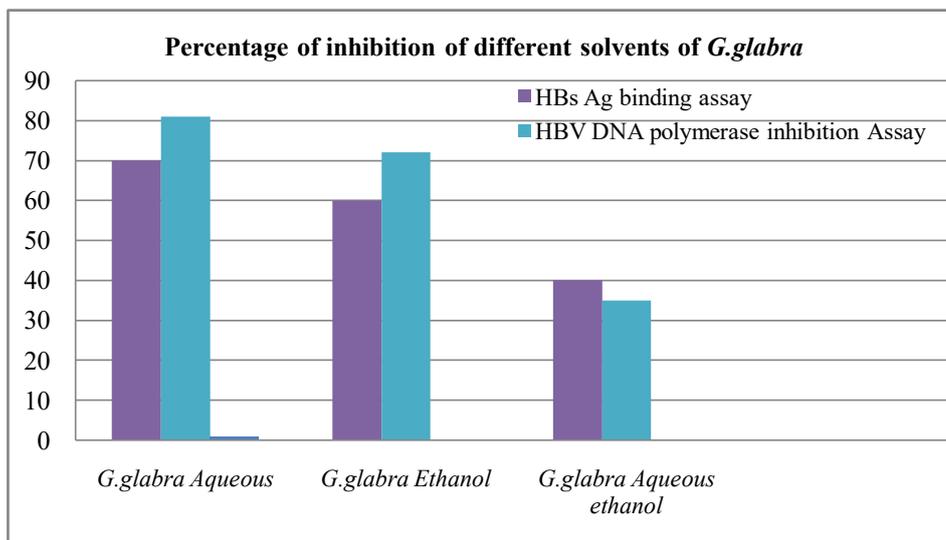
S. No	Plant	MTT Assay (MNTC)	HBs Ag Binding Assay					HBV DNA Polymerase Inhibition Assay
			Day 1	Day 2	Day 3	Day 4	Day 5	
1.	<i>Glycyrrhiza glabra</i> Aqueous	500µg/ml	+++	+++	+++	+++	+++	+++
2.	<i>Glycyrrhiza glabra</i> Ethanol	62.5µg/ml	++	++	++	++	++	+++
3.	<i>Glycyrrhiza glabra</i> Aq. Ethanol	500µg/ml	-	-	-	-	-	-
		++	Positive greater than 60%					
		+++	Positive greater than 70%					

(-) = Non toxic;

(+) = toxic to Hep G2 cell line.

**Table 2: Comparative Analysis of HBsAg binding and HBV DNA polymerase inhibition Assay**

S. No	Lyophilized extracts	HBs Ag Binding Assay	HBV DNA Polymerase Inhibition Assay
		Percentage of Inhibition	
1.	<i>Glycyrrhiza glabra</i> (Aqueous)	70	81
2.	<i>Glycyrrhiza glabra</i> (Ethanol)	60	72
3.	<i>Glycyrrhiza glabra</i> (Aq. Ethanol)	>50	>50



**Fig.1. Percentage of Inhibition of different solvents of *Glycyrrhiza glabra***

The cytotoxicity of the lyophilized aqueous, ethanolic and aqueous ethanolic extracts of *Glycyrrhiza glabra* was analyzed using HepG2 cell line to study the toxic free concentrations of the extracts. The maximum non toxic concentrations in aqueous extracts and ethanolic extracts of *Glycyrrhiza glabra* found to be 1000 µg/ml whereas in the ethanolic extract, the MNTC of 125 µg/ml were determined.

HBs Ag binding inhibition assay was performed to study the effect of lyophilized aqueous and ethanolic extracts of *Glycyrrhiza glabra* by screening the surface antigen of Hepatitis B. It shows maximum effectiveness in the aqueous extracts at the concentration of 70%, followed by ethanolic extracts showing 60% inhibition. The aqueous ethanolic extracts did not possess any antiviral activity (Table 1). Similar study was carried out using HD-03/ES, an herbal medicine proved to be effective in acting against Hepatitis B [16]. In the present study, of the three extracts, aqueous and ethanolic extracts shows maximum binding inhibition.

DNA polymerase is one of the key enzymes critical for DNA replication [17]. The inhibition of this enzyme by the extract could suggest the antiviral activity of Hepatitis B. These extracts were further ascertained by using HBV DNA polymerase Inhibition assay following the method of Lofgren et al., with little modification in detection replacing radio nucleotides with fluorescent labeled probes was carried out for aqueous and ethanolic extracts of *Glycyrrhiza glabra*. DNA Polymerase Inhibition assay was performed using fluorochromes since radioactive assay faces the problem of disposal. Fluorometric assay is an inexpensive and easy method to measure the activity of the enzymes [18]. The fluorescent labeled probes were used for detection of DNA polymerase enzyme. Fluorescein dUTP nucleotides are efficient in incorporating the enzyme [19]. The anti HIV-1 RT activity was determined by fluorometric method using Pico green has been described [20]. The lyophilized aqueous extracts of *Glycyrrhiza glabra* were proved to be effective showing 81% inhibition followed by ethanolic extracts of *Glycyrrhiza glabra* with 72% inhibition in HBV DNA Polymerase inhibition assay (Table 2 and Fig.1). The inhibition was greater in aqueous extracts followed by ethanolic extracts which correlates with that of the HBs Ag binding inhibition assay. Similar study was reported on the inhibition of Hepatitis B virus using HBs Ag binding assay and HBV DNA polymerase Inhibition assay by radiometric method in medicinal plants [21,22]. This study emphasizes the importance of *Glycyrrhiza glabra* as an antiviral agent against hepatitis B.

#### 4. Conclusion

In the present study, the aqueous extracts of *Glycyrrhiza glabra* found to be more effective against Hepatitis B virus followed by ethanolic extracts. Further phytochemical analysis of this extract may reveal the interesting compounds possessing antiviral activity. DNA Polymerase Inhibition assay using fluorescent nucleotides for detection avoid the risk of disposal of radio nucleotide and also an easy, quick and inexpensive method measured by fluorimeter. This is a novel technique used for the first time to detect HBV DNA using fluorescent nucleotides.

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