



## In-Vitro Evaluation of the Anti-Hepatitis (HBV) and Hepato-Protective Activity of Herbal Extracts

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### ABSTRACT

Hepatitis B virus (HBV) infects around 350 million of the global population. The current antivirals are laden with side effects as well as expense burden. An alternative medication based on Ayurveda is being proposed to counter this scenario. Hep-1 to Hep-6 are herbal formulations used for the treatment of hepatitis B virus. They are also indicated for alleviation of hepatotoxicity. The molecular mechanism involved in the anti-HBV activity of these formulations is being studied using *in-vitro* models for the first time. Inhibition of Hepatitis B surface antigen (HBsAg) secretion from the transfected human hepatocarcinoma PLC/PRF/5 cells, as well as inhibition of the surface antigen binding was taken up in the present study. These anti-HBV activities were detected by HBsAg specific antibody-mediated enzyme assay (ELISA) at concentrations ranging from 50 to 250µg/mL. The results indicate that the suppression of HBsAg production and inhibition were best observed at 150µg/mL across the extracts. This concentration was used to determine the hepatoprotectivity of these extracts in a Kupffer cell-hepatocyte *in-vitro* co-culture; determined by the expression of cytokines TNF-α, IL-6 and IL-10, in presence of bacterial lipopolysaccharide. Hep-1 was found to be the most suitable formulation showing both anti-HBV activity and optimum hepatoprotectivity during the study. This extract could thus be beneficial in the treatment of liver inflammation.

**Keywords:** Hepatoprotective, Herbal extracts, IL-6, IL-10, Kupffer Cells, TNF-α.

### INTRODUCTION

Liver is a discrete organ and many of its various functions interrelate to one another. The basic units (hepatocytes) are the primary cells involved in pathogenicity and inflammation of liver.<sup>1</sup> This inflammation can be due to chemicals like carbon tetrachloride, alcohols or infections from microorganisms, mainly Hepatitis B virus.

Hepatitis B virus (HBV) infection is a serious health problem worldwide. More than 350 million are chronic carriers of HBV.<sup>2</sup> India is at the intermediate endemic level of hepatitis B with more than 40 million HBsAg carriers. This virus is a major cause of chronic hepatitis, liver cirrhosis and hepato-cellular carcinoma.<sup>3</sup> The virus infects around 300-400 million people globally, detected by the presence of Hepatitis B Surface Antigen (HBsAg) in peripheral blood. In India, HBsAg positivity is seen in an average of 4.7 percent of the population.<sup>4</sup>

PLC/PRF/5 is a continuous human hepatocellular carcinoma cell line in which genome contains integrated HBV DNA and secretes two of the hepatitis B virus envelope proteins. These cells are used to study the effects of drugs on HBsAg expression and secretion.<sup>4</sup> Kupffer cells are involved in the defense against infections of the liver. Their major role in the host defense and the prognosis of liver infection is indicated by studies in experimental models of sepsis. Kupffer Cells (KCs) are the first cells to be exposed to materials absorbed from the gastrointestinal tract. Due to their key location, KCs might function as antigen-presenting cells and participate in

tumor surveillance and the regeneration processes of the liver.<sup>5-7</sup> The evidence derived mostly from animal models, indicates that Kupffer cells may be implicated in the pathogenesis of various liver diseases including viral hepatitis, steatohepatitis, alcoholic liver disease, intra-hepatic cholestasis, activation or rejection of the liver during liver transplantation and liver fibrosis.<sup>8</sup> Thus blocking of Kupffer cell function reduces hepatotoxicity.<sup>9</sup> Rat Kupffer cells co-cultured with rat hepatocytes are used to create an inflammation model to evaluate hepatotoxicity.

Several antivirals are currently available for the treatment of HBV which includes IFN-α, Lamivudine, Entecavir, Teltrivudine and Tenofovir. These drugs frequently cause adverse effects which are of big concern. Moreover, these antiviral drugs are expensive. In India, traditional systems of medicines inclusive of Ayurveda have been used in the treatment of liver disorders. Herbal compounds from plant origin are leading hits for new drug discovery against many infectious, especially viral diseases.<sup>10</sup> Hep-1 to Hep-6 are novel multi-targeted and multi-faceted herbal formulations used for the treatment of various liver disorders including HBV infection. Some of the ingredients of these six formulations are *Phyllanthus niruri*, *Andrographis paniculata*, *Cyperus rotundus*, *Glycyrrhiza glabra* etc. in various concentrations.

### MATERIALS AND METHODS

#### Reagents

The PLC/PRF-5 cells were procured from National Centre for Cell Sciences, Pune. The growth media comprising of



Dulbecco's minimal essential medium (DMEM) and Fetal Bovine Serum (FBS) were procured from M/s. Life Technologies. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Lipopolysaccharide (*E.coli* origin) and Dimethyl sulfoxide (DMSO) were procured from M/s. Sigma Chemicals. Sprague Dawley rat hepatocytes (primary cells) and compatible Kupffer cells (rat liver macrophages) were commercially obtained from M/s. Life Technologies, to set up the inflammation model. The growth media for these primary cells, viz. Williams E medium with Hepatocyte Plating and Maintenance Supplement Pack were also procured from M/s. Life Technologies. Quantitation of TNF- $\alpha$ , IL-6 and IL-10 was performed using appropriate rat ELISA kits manufactured by M/s. Diaclone, France.

## Methodology

### Preparation of the test extracts

A stock of each of the extracts was prepared (using the percentage of the active component as given by the manufacturer) in sterile Distilled Water, so as to give the final concentration of the active components as 10mg/mL. This stock was then serially diluted to give 50 $\mu$ g/mL, 100 $\mu$ g/mL, 150 $\mu$ g/mL, 200 $\mu$ g/mL and 250 $\mu$ g/mL.

### Hepatitis B Surface Antigen (HBsAg) Binding Inhibition Assay

Serial dilutions of each of the Hep (1-4) extracts were mixed with an equal volume of cell supernatant containing HBsAg (from PLC/PRF/5). The mixture was incubated for 2 hours at 37°C. This mixture was assayed directly for HBsAg using the HepaLISA Ultra kit.<sup>4</sup>

### Hepatitis B Surface Antigen (HBsAg) secretion Assay

For assaying the effect of the extract on HBsAg expression, PLC/PRF/5 (hepatocellular carcinoma) cells were seeded in a 96-well tissue culture plate at a cell density of around 1x10<sup>5</sup> cells/mL and grown to confluency. The medium consisted of DMEM supplemented 10% FBS and 100 $\mu$ M Streptomycin and 100 IU/mL Penicillin. The cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cells were then washed twice with serum-free medium and incubated with various dilutions of the extracts in serum free DMEM for 24 hours. The culture supernatants were then collected and the HBsAg in the culture medium was quantified by ELISA kit as mentioned earlier.<sup>4</sup>

### Cytotoxicity Assay

After removal of the culture supernatants, the viability of the cells was determined by the MTT Formazan Assay (Mossmann, 1983). In brief, 100 $\mu$ L of MTT (0.5mg/mL) in DMEM without phenol red was added to each well and the plate was incubated at 37°C for 4 hours. After 4 hours, 100 $\mu$ L of dimethylsulfoxide (DMSO) was added to all the wells to dissolve the formazan crystal, and the optical density (OD) was measured at 550 nm.

## Hepatoprotectivity Assay

### Co-culturing of rat hepatocytes and rat Kupffer cells

Sprague Dawley rat hepatocytes (primary cell culture) and compatible Kupffer cells (rat liver macrophages) were commercially obtained. The Hepatocyte/Kupffer cell co-culture was plated and maintained in Williams E medium with Hepatocyte Plating and Maintenance Supplement Pack respectively, as per manufacturer's instructions.<sup>11</sup>

### Induction of Lipopolysaccharide based inflammation

On day 2 of the co-culture, the Kupffer cells were activated using Lipopolysaccharide (LPS, *E.coli* origin), 24 hours prior to start of the assay of the herbal extracts. The six extracts were used at a concentration of 150 $\mu$ g/mL for assaying on the co-culture. Aliquots of the cell culture supernatant were drawn at a time frame of 24 hours, 48 hours and 72 hours after induction with LPS. Parallel to this assay, the cell control (culture without LPS induction), as well as LPS control (culture with LPS induction, but without the extracts) were also monitored and supernatant was aliquoted at the above mentioned time points.<sup>11</sup>

### Estimation of cytokines

Three cytokines, viz. TNF  $\alpha$ , Interleukin-6 and Interleukin-10 were estimated from the cell culture supernatants, aliquot at each time interval. These estimations were carried out in duplicate using respective rat cytokine quantitation kits.

## RESULTS

Four of the six extracts (Hep-1 to Hep-4) were able to bind to the HBsAg in an aqueous *in-vitro* system, thus preventing its further activity (Figure 1). However, though all extracts were able to inhibit the secretion of the HBsAg from PLC/PRF-5 cells, the inhibition was dynamic till the extract concentration of 150 $\mu$ g/mL (Figure 2).

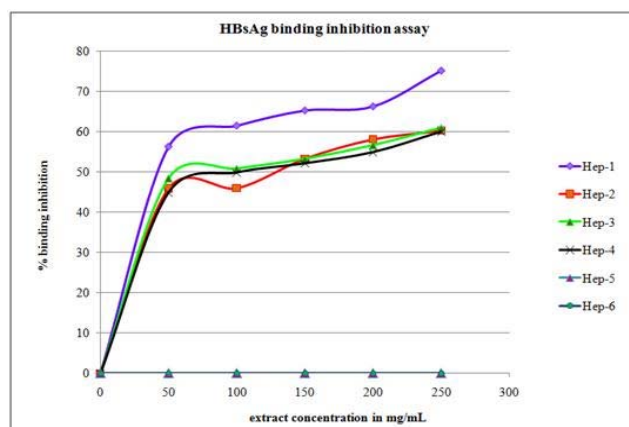
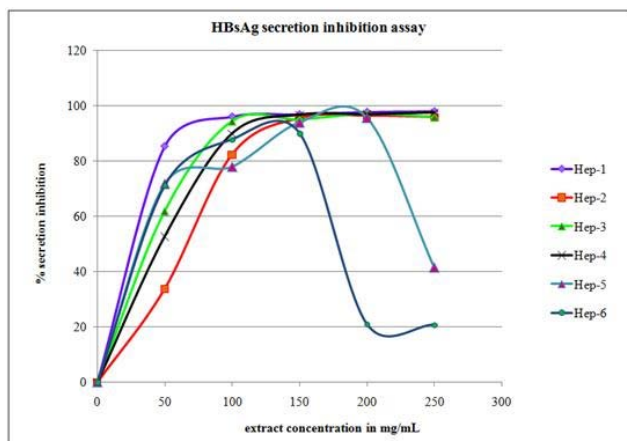


Figure 1: *In-vitro* HBsAg binding inhibition by herbal extracts.

### Cytotoxicity

No cytotoxicity was observed on the PLC/PRF/5 cells in the extract concentrations employed in the assays

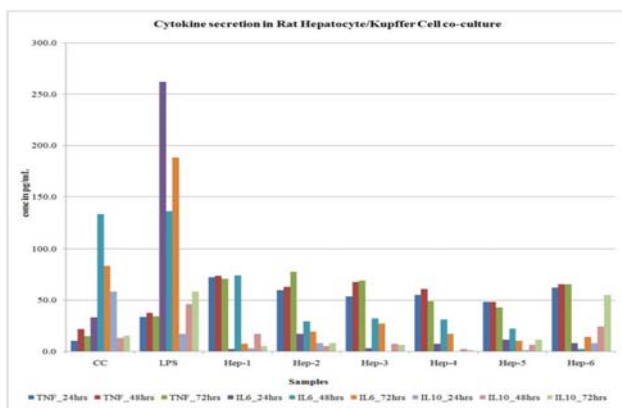
(0 $\mu$ g/mL to 250 $\mu$ g/mL), as observed from the MTT-Formazan assay.



**Figure 2:** *In-vitro* HBsAg secretion inhibition by herbal extracts.

### Hepatoprotective activity of the extracts

The negative baseline for the assay was the cell control (without LPS or any extract). The positive control was the cells inflamed using lipopolysaccharide (1 $\mu$ g/mL). Base line (average over 72 hours) TNF  $\alpha$  value in cell control was 15pg/mL, with a peak of 21pg/mL at 48 hours. Concomitantly, this value was 35pg/mL, with a peak of 37pg/mL again at 48 hours after activation by LPS. It was observed that an additional release of TNF  $\alpha$  occurred by the virtue of the extracts, usually by the 48th hour of assay. This implies that the extracts up regulated the production and release of TNF  $\alpha$  in the test system.



**Figure 3:** Cytokine expression by Hepatocyte/Kupffer cell co-culture.

Base line IL-6 value in cell control was 83pg/mL, with a peak of 133pg/mL at 48 hours. Concomitantly, this value was 195.3pg/mL, with a peak of 262pg/mL, 24 hours after LPS activation. It was thus observed that the release of IL-6 peaked by 48 hours of assay after addition of the extracts. However, it reached neither the LPS induction level nor the cell control baseline level, thus indicating that the extracts down-regulated the production of IL-6 in the test system.

Base line IL-10 value in cell control was 28.7pg/mL, with a peak of 58pg/mL at 24 hours. Concomitantly, this value

was 40.3pg/mL, with a peak of 58pg/mL, 72 hours following activation by LPS. It was thus observed that the release of IL-10 peaked after 48 hours of assay after addition of the extracts. However, it reached neither the LPS induction level nor the cell control baseline level, except in Hep-6, where it reached to 55pg/mL. This indicates that all extracts, barring Hep-6 down-regulated the production of IL-10 in the test system.

Figure 3 depicts the trend of cytokine expression in presence of the various herbal extracts. It is evident that both IL-6 and IL-10 values have been brought to base line levels (cell control values) or below across the extracts. There are two major deviations in the observations. Irrespective of the extract being added, there is a distinct rise in the TNF- $\alpha$  levels; even higher than the LPS. This may be due to two possible reasons. One, while induction of inflammation is occurring at the LPS concentration of 1 $\mu$ g/mL, the extracts are being studied at a concentration of 150 $\mu$ g/mL, as this concentration has shown complete HBsAg binding and secretion inhibition in earlier experiments. Thus it may be the case that the study concentration may be higher than the upper limit of assay, resulting in acute inflammation.

The extracts Hep-1 to Hep-5 targeted for hepatoprotective activity contain *Phyllanthus spp* (niruri) as one of the constituents. An earlier study reports that various indices of activation and functions murine bone marrow-derived macrophages were significantly enhanced by pre-treatment with the extract, including phagocytosis, lysosomal enzymes activity, and TNF- $\alpha$  release.<sup>12</sup>

In Hep-6, there is an increase in the IL-10 levels at the end of 72 hours of study. This extract contains *Cyperus spp.* as one of the constituents. Known for their hepatoprotective activity; *Cyperus spp.* also present with an anti-inflammatory activity, leading to wound healing.<sup>13</sup> In another model of lentivirus, it has been shown that over-expression of IL-10 decreases the inflammatory response to injury, creating an environment conducive for regenerative adult wound healing.<sup>14</sup> It may be therefore hypothesized that *Cyperus spp.* may be involved in increasing the levels of IL-10 in the test system.

In all the extracts, there is a distinct composition of the binding agents / fillers, which comprise the inactive component. Though these components might not have a significant role in the anti-HBV activity, their role in eliciting an immune response in the test system cannot be undermined. It is hence essential that the extracts and the fillers may be independently evaluated for the potential to alleviate and enhance inflammation of the hepatocytes.

### DISCUSSION

All extracts under study have presented with partial or total anti-HBV activity, by inhibiting the binding of HBsAg to its ligand/antibody and/or inhibiting the secretion of the HBsAg from a hepatocellular carcinoma cell line

transfected with incomplete HBV genome. TNF- $\alpha$  signaling is particularly important in the liver. While it mediates hepatocyte survival and proliferation, it is also implicated in liver failure, since it triggers hepatocyte apoptosis and leads to upregulation of key adhesion molecules and chemokines involved in leukocyte migration and infiltration.<sup>15</sup> TNF- $\alpha$  plays a dichotomous role in the liver; where TNF- $\alpha$  not only acts as a mediator of cell death but also induce hepatocyte proliferation and liver regeneration.<sup>16,17</sup> Thus increase in the TNF- $\alpha$  levels may have a significant role in hepatoprotectivity, though a lower concentration might be required to prove the same.

IL-6 is one of the major physiological mediators of acute phase reaction, which rises after LPS induction, as is also seen with the present study. Decrease in the levels of IL-6 that induces the systemic inflammatory response

indicates at the immunomodulatory activity of the extracts.<sup>18,19</sup> Both these activities have been observed for extracts Hep-1 to Hep-6.

IL-10, a potent anti-inflammatory cytokine is important for hepatoprotective action, has been studied in modulating liver injury induced by galactosamine and lipopolysaccharide.<sup>8,20</sup> However, there is also an immediate acute rise in the IL-10 levels following LPS induction, along with the increase in TNF- $\alpha$  levels. The extract labeled Hep-6 has been able to bring the IL-10 levels LPS induced levels.

However, there is also an immediate acute rise in the IL-10 levels following LPS induction, along with the increase in TNF  $\alpha$  levels. The extract labeled Hep-6 has been able to bring the IL-10 levels LPS induced levels. These details are summarized in Table 1.

**Table 1:** Hepatoprotective and anti-HBV activity of herbal extracts

Extracts (150 $\mu$ g/mL)	Anti HBV activity (HBsAg inhibition)		Cytotoxicity	Hepato-protective Activity (compared with LPS induced levels after extract addition)		
	Binding inhibition	Secretion inhibition		TNF $\alpha$	IL-6	IL-10
Hep-1	+++	+++	-	↑↑↑	↓↓	↓↓
Hep-2	+++	+++	-	↑↑↑	↓↓	↓↓
Hep-3	+++	+++	-	↑↑↑	↓↓	↓↓
Hep-4	+++	+++	-	↑↑↑	↓↓	↓↓
Hep-5	-	++	-	↑↑↑	↓↓	↓↓
Hep-6	-	++	-	↑↑↑	↓↓	↑↑

(+): observed, (-): not observed, (↑): up regulation, (↓): down regulation

Thus from the present study, it may be hypothesized that the extracts show activity against Hepatitis virus (antigen), as well as putative hepatoprotective activity. In the present study, Hep-1 seems to be the most promising extract for the activities under investigation. However, to confirm the same, testing of all extracts at a lower concentration range for TNF- $\alpha$  levels is recommended. This will help validate our hypothesis of stress to due high concentration. Testing of the extracts in the pure/unblended form, which is without any binders or fillers for the hepatoprotective activity, also needs to be undertaken for further evaluation.

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