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EVALUATION OF HEPATITIS B SURFACE ANTIGEN SECRETION INHIBITION AND HEPATOPROTECTIVITY BY *HEP-02*, AN HERBAL FORMULATION

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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 – 02 is a herbal formulation used for the treatment of hepatitis B virus. It is also indicated for alleviation of hepatotoxicity. The molecular mechanism involved in the anti-HBV activity of this formulation 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. Suppression of HBsAg production and inhibition were best observed at 150µg/mL and this concentration was used to determine the hepatoprotectivity 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. The results indicate that HEP-02 could thus be beneficial in the treatment of liver inflammation in Hepatitis B.

KEYWORDS: HEP-02, HBsAg secretion, Kupffer cells, LPS, inflammation, hepatoprotectivity.

INTRODUCTION

Hepatitis B Virus (HBV) infection is an emerging threat to the Indian sub-population. With over 40 million Hepatitis B surface antigen (HBsAg) carriers in the country, India is at an intermediate endemic level of HBV when assessed globally. [1] This virus infects around 300-

400 million people worldwide, establishing itself as a major etiological agent for chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. [2] Diverse serological markers present in the course of HBV infection, foremost being the HBsAg that appears two to three weeks prior to manifestation of clinically apparent symptoms. In India, HBsAg positivity is seen in an average of 4.7 percent of the population. [3]

Liver, an organ critical to the management of metabolism in the human body, is made up of basic units termed as hepatocytes. These are primary cells involved in many functions of the liver, including pathogen management, toxicity and inflammation of the organ. [4] This inflammation also includes insult due to alcohols or infections from HBV. Many models are being utilized to understand the stimuli-response mechanism of the hepatocytes in a laboratory controlled *in-vitro* environment. PLC-PRF/5, a continuous human hepatocellular carcinoma cell line is one such model routinely used to study inhibitory activities against HBsAg secretion and expression. [5] The cell line contains an integrated HBV genome and secretes the surface antigen and envelope proteins. Another such model includes the use of Kupffer cells (KCs), which are involved in the defence against infections of the liver. They are the first cells to be exposed to absorbed metabolites in the gastro-intestinal tract. It is also postulated that due to their pivotal location, KCs may operate as antigen presenting cells, taking part in tumour surveillance and processes involved in liver regeneration. [6, 7, 8] Many animal models indicate the implication of Kupffer cells in viral hepatitis, steatohepatitis, alcoholic liver disease, intrahepatic cholestasis, activation or rejection of the liver during liver transplantation and liver fibrosis. [9]

Effective suppression of HBV infection with undeterminable viral load for a prolonged duration is the current goal towards management of chronic viral hepatitis. [10] Ancient Indian medicine globally received as Ayurveda, finds a role in such management. Plants like *Glycyrrhiza glabra* (Licorice),[11,12] *Boerhavia diffusa* (Punarnava)[13,14] and *Emblica officinalis* (Amla). [15] have been reported to exhibit excellent immunomodulatory, anti-oxidant, hepatoprotective and anti-viral activities, at both crude as well as constituent level. In the present study, we have evaluated HEP-02, a novel multi-targeted and multi-faceted herbal formulation used for the treatment of various liver disorders including HBV infection, for its *in-vitro* anti-HBV and hepatoprotective activity.

MATERIALS AND METHODS

Preparation of the test extracts: HEP-02, a novel herbal formulation prepared to fight against Hepatitis B was manufactured by M/s. Charak Pharma Pvt. Ltd., Mumbai. A stock of HEP-02 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 50µg/mL, 100µg/mL, 150µg/mL, 200µg/mL and 250µg/mL.

Hepatitis B Surface Antigen (HBsAg) Binding Inhibition Assay

Serial dilutions of HEP-02 extract 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 (J. Mitra & Co. Pvt. Ltd., India). [5]

Hepatitis B Surface Antigen (HBsAg) Secretion Inhibition 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 $1x10^5$cells/mL and grown to confluency. The medium consisted of Dulbecco's Modified Eagle Medium, (Life Technologies, India) supplemented 10% Fetal Bovine Serum (Life Technologies, India) and 100µM Streptomycin and 100IU/mL Penicillin (Sigma Chemicals, India). The cells were incubated in a humidified atmosphere with 5% CO2 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. [5]

Cytotoxicity Assay

After removal of the culture supernatants, the viability of the cells was determined by the MTT Formazan Assay with modifications. [16] In brief, 100µL of 0.5mg/mL MTT (Sigma Chemicals, India) in DMEM without phenol red was added to each well and the plate was incubated at 37°C for 4 hours. After 4 hours, 100µL of dimethylsulfoxide (DMSO) was added to all the wells to dissolve the formazan crystals, 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 (Life Technologies, India). 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. [17]

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 extract HEP-02. The extract was used at a concentration of 150µg/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. [17]

Estimation of cytokines

Three cytokines, viz. Tumor Necrosis Factor (TNF-α), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were estimated from the cell culture supernatants, aliquot at each time interval. These estimations were carried out in duplicates using respective rat cytokine quantitation kits as mentioned elsewhere. [18] The negative baseline for the assay was the cell control (without LPS or any extract). The positive control was the cells inflamed using lipopolysaccharide (1µg/mL).

RESULTS AND DISCUSSION

Cytotoxicity

No cytotoxicity was observed on the PLC/PRF/5 cells in the extract concentrations employed in the assays (0µg/mL to 250µg/mL), as observed from the MTT Formazan assay.

HBsAg binding and secretion inhibition assay

Both the inhibitory activities of HEP-02 against HBsAg, viz. inhibition of secretion from transfected cells, as well as inhibition of binding to a suitable antibody, were observed to increase with increasing concentrations of the extract. At concentrations higher than 100µg/mL, more than 90% inhibition was observed in the secretion of HBsAg from

PLC/PRF-5 cells. However, since there was no cytotoxicity observed, it can be inferred that HEP-02 brought about a specific inhibition of translation of the HBV genome responsible for production of new HBsAg molecules. However, at best, only 60% of binding of HBsAg could be inhibited in the concentration range studied (Figure 1).

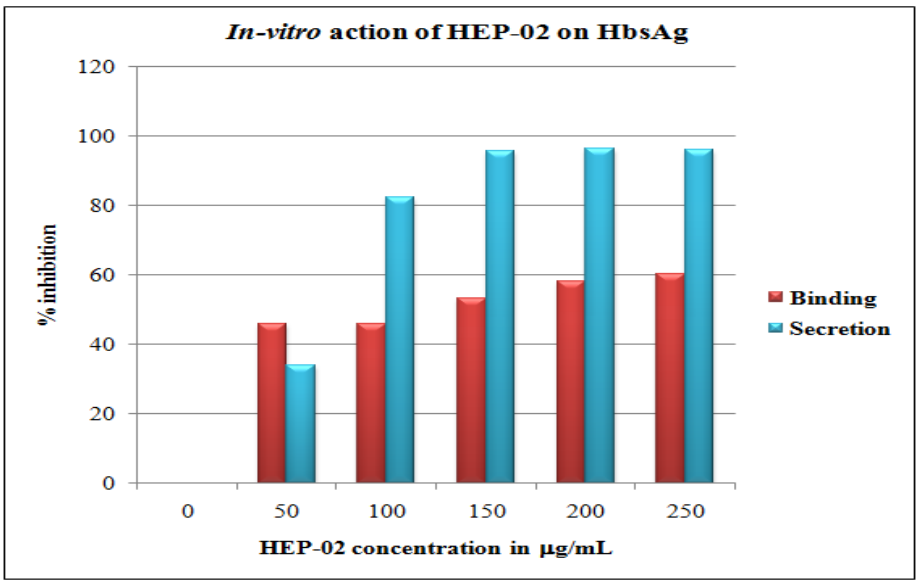

Figure 1: Effect of HEP-02 on the secretion and binding of HBsAg in-vitro.

Hepato-protective potential of HEP-02

Interleukin-6 is a marker for acute phase response. While sudden surge may aid in tissue damage, a controlled expression is also indicated in liver healing and regeneration. [19] LPS induction was validated by an upsurge in the IL-6 levels in the cell free supernatant, 24 hours post induction (Figure 2). HEP-02 was able to minimize this damage via controlled expression of IL-6 during the study period of 72 hours. This activity also indicates the hepatoprotective potential of the extract under investigation. Decrease in the levels of IL-6 that induces the systemic inflammatory response indicates at the immunomodulatory activity of the extracts.[20, 21] Thus it can be hypothesized that HEP-02 has a potential to aid in hepatoprotectivity.

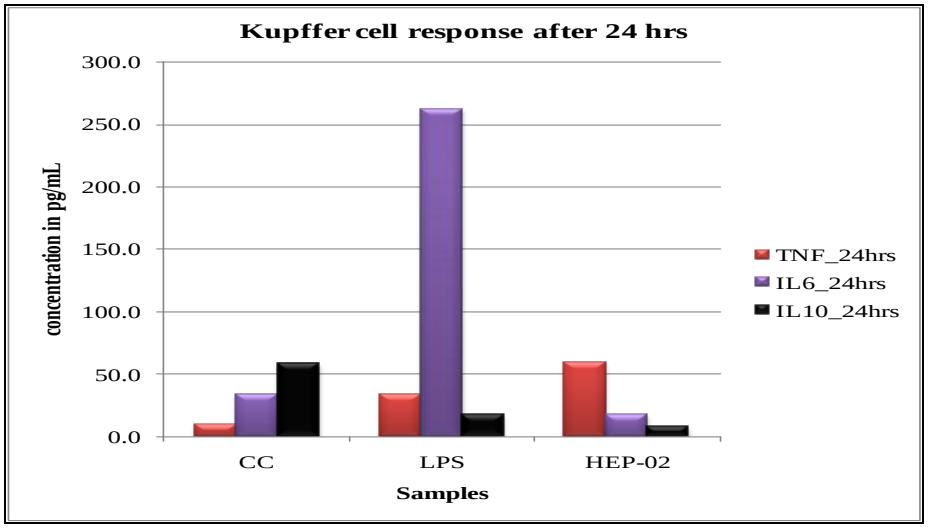

Figure 2: Cytokine expression of Kupffer cells + hepatocyte co-culture after exposure to LPS and LPS+ HEP-02 for 4 hours. The cytokines have been estimated 24 hours post the induction period. CC= cell control, TNF=TNF-α.

It was observed that a release of TNF-α peaked by the 72nd hour of assay, as compared to a 48 hour peak in both cell control as well as LPS induced cells (Figures 3 and 4). This implies that HEP-02 was able to delay the rise in TNF-α level, thus displaying its ability to counter inflammation. However, the levels of TNF-α were always higher than those of LPS and cell controls, which may be indicative of the stress impounded on the cells due to extract. TNF-α also plays a critical role in liver regeneration via promoting the proliferation of hepatocytes [22] We may hypothesize that there is a sustained release of this cytokine rather than any inhibition, which is more suitable in liver injury.

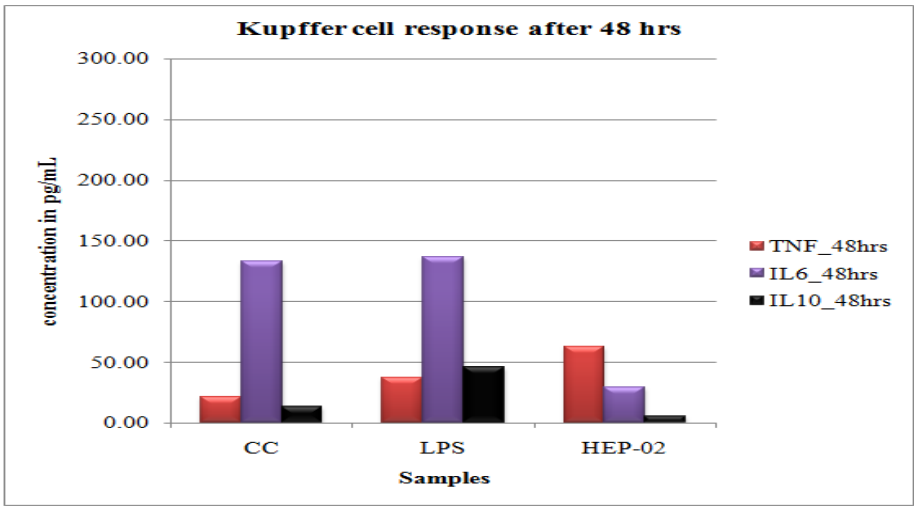

Figure 3: Cytokine expression of Kupffer cells + hepatocyte co-culture after exposure to LPS and LPS+ HEP-02 for 4 hours. The cytokines have been estimated 48 hours post the induction period. CC= cell control, TNF=TNF-α.

TNF-α upregulates key adhesion molecules and chemokines leading to migration and infiltration of the leukocytes, and triggers hepatocytes apoptosis. It is therefore implicated in liver failure. Concomitantly, it arbitrates hepatocyte proliferation and survival, thus making its presence and signalling control essential in the liver. TNF-α thus plays a dichotomous role in the liver, where TNF-α not only acts as a mediator of cell death but also induces hepatocyte proliferation and liver regeneration. [23, 24] It is therefore important to study and achieve controlled release of TNF-α, rather than aiming for its increase/decrease.

Interleukin-10 is a hallmark of anti-inflammatory response mounted by a biological system. One of its main functions is to counter the activity of TNF-α, which it undertakes by inhibiting the synthesis of T-helper type 1 (Th1) cytokines. It also leads to the decreased efficiency in the antigen presenting capacity of macrophages.[25] This may be extremely detrimental while dealing with infectious stimuli to the liver, for e.g. viral hepatitis.

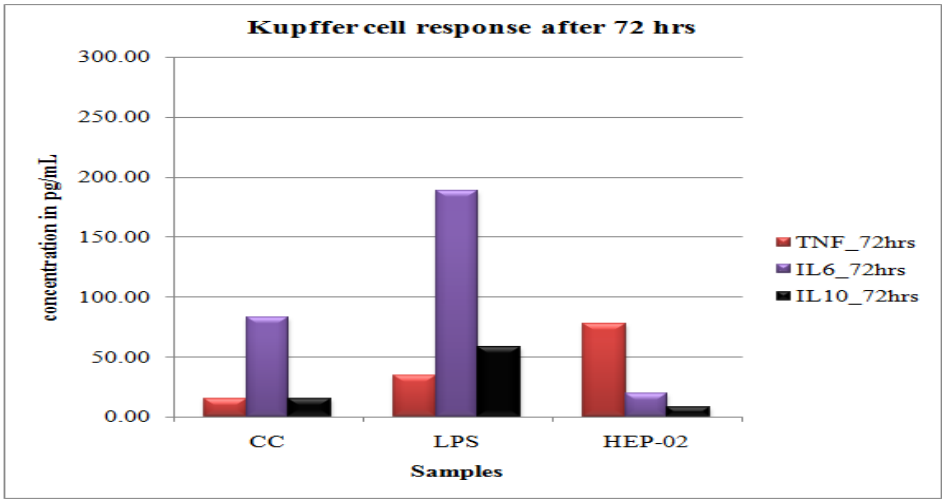

Figure 4: Cytokine expression of Kupffer cells + hepatocyte co-culture after exposure to LPS and LPS+ HEP-02 for 4 hours. The cytokines have been estimated 72 hours post the induction period. CC= cell control, TNF=TNF-α.

IL-10 has been studied in modulating liver injury induced by galactosamine and Lipopolysaccharide. [9, 26] Though there is a surge in the IL-10 levels 48 hours after LPS induction, HEP-02 has been able to bring the IL-10 levels at par with the control (baseline) levels all through the study period.

DISCUSSION

Assessment of immunomodulation is a promising avenue in phytotherapeutic research. Alterations in the host immune mechanism due to aggravation or alleviation of the immune

responses usually determine the outcome of an insult to an organ. Immunomodulators regulate the expression and secretion of cytokines and chemokines, which in turn regulate the T cell and B cell machinery. In liver, Kupffer cells (liver macrophages) have an assigned task for warding off any infection and also protecting its physiology.

HEP-02 is a multicomponent herbal formulation, comprising of extracts of *Glycyrrhiza glabra*, *Boerhavia diffusa*, *Emblica officinalis*, *Zingiber officinale and Cyperus rotundus* as some of the key active principles. This formulation aims to alleviate the problems associated with liver, especially viral hepatitis and its sequelae. HEP-02 has demonstrated its potential for the anti-HBV activity, by inhibiting the secretion of HBsAg from a hepatocellular carcinoma cell line contained integrated HBV genome, as well as inhibiting the binding of HBsAg to its ligand/antibody. *Cyperus rotundus*, an ingredient in the formulation, has been reported to inhibit the expression of HBsAg from human hepatocellular carcinoma cell line models. [27]

Glycyrrhizin, a key component of *Glycyrrhiza glabra* has been demonstrated to aid in reduction of blood levels of TNF-α and IL-6 in inflammation induced mouse models for allergic rhinitis. [28] Its hepatoprotective activity in carbon tetrachloride insulted mice has also been demonstrated in earlier studies. [29] Punarnavine, an alkaloid from *Boerhavia diffusa* has been observed to reduce significantly the LPS induced serum levels of TNF-α, IL-6 and IL-1β in BALB/c mice. [30] It also finds reference in Ayurvedic literature, and possesses extraordinary antioxidant, hepatoprotective, antibiotic, anti-diabetic and anti-carcinogenic properties. [31] *Emblica officinalis* also shows similar properties, decreasing TNF-α and IL-6 levels. [32] Additionally, it also aids in reduction of IL-10 levels. [33] Earlier reports are available in literature demonstrating the decrement in TNF-α and IL-6 levels in alleviation of the clinical symptoms of chronic Hepatitis C, by using a natural/herbal product. [34] *Zingiber officinale*, a component of HEP-02 is also hypothesized as having anti-oxidant activity, leading to reduction in inflammation and apoptosis, hence curbing the manifestations of liver cirrhosis. [35] Our present study is in agreement with these findings, where we have demonstrated a controlled increase in TNF-α over a 72 hour time period at hepatocyte level. However, both IL-6 levels show a negative trend while IL-10 is more or less constant, but lower than cell control.

CONCLUSION

The present study thus elucidates the hepatoprotective potential of HEP-02 along with its ability to counter the activities of Hepatitis B virus, thus rendering it important for treatment in liver inflammation in HBV infection.

REFERENCES

1. Hou J, Liu Z, Gu F. Epidemiology and Prevention of Hepatitis B Virus Infection, Int J Med Sci, 2005; 2(1): 50-57.
2. Rao MB. The prevalence of hepatitis B in India and its prevention with Ayurveda- a revisit, J New Appr Med Health, 2012; 19(4).
3. Varma SR, Sundaram R, Gopumadhavan S, Vidyashankar S, Patki PS. HD-03/ES: A Herbal Medicine Inhibits Hepatitis B Surface Antigen Secretion in Transfected Human Hepatocarcinoma PLC/PRF/5 Cells, Hep Res Treat, 2013. (http://dx.doi.org/10.1155/2013/125398).
4. Guyton AC, Hall J., The liver as an organ. Text book of Medical Physiology 10th edition, W. B. Saunders Company, Philadelphia, 2000; 800-801.
5. Yeh SF, Gupta M, Sarma DNK, Mitra SK. Down regulation of Hepatitis B Surface Antigen expression in human hepatocellular carcinoma cell lines by HD-03, a polyherbal formulation, Phytother. Res, 2003; 17: 89-91.
6. Nolan JP, Endotoxin, reticuloendothelial function and liver injury. Hepatology, 1981; 1: 458-465.
7. Bayon LG, Izquierdo MA, Sirovich I, van Rooijen N, Beelen RH, Meijer S. Role of Kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver, Hepatology, 1996; 23: 1224-1231.
8. Fausto N, Laird AD, Webber EM. Liver regeneration 2. Role of growth factors and cytokines in hepatic regeneration. FASEB J, 1995; 9: 1527-1536.
9. George K, Vassilis V, Elias K. Role of Kupffer cells in pathogenesis of liver disease, World J Gastroenterol, 2006; 12(46): 7413-7420.
10. Lai CL, Yuen MF. Chronic hepatitis B—new goals, new treatment. N Engl J Med, 2008; 359: 2488–91.
11. Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, Bielenberg J. Antiviral effects of Glycyrrhiza species. Phytother Res, 2008; 22(2): 141-8.
12. Adianti M, Aoki C, Komoto M, Deng L, Shoji I, Wahyuni TS, Lusida MI, Soetjipto, Fuchino H, Kawahara N, Hotta H. Anti-hepatitis C virus compounds obtained from

Glycyrrhiza uralensis and other Glycyrrhiza species. Microbiol Immunol, 2014; 58(3): 180-7.

13. Olaleye MT, Akinmoladun AC, Ogunboye AA, Akindahunsi AA. Antioxidant activity and hepatoprotective property of leaf extracts of Boerhaavia diffusa Linn against acetaminophen-induced liver damage in rats. Food Chem Toxicol, 2010; 48(8-9): 2200-5.
14. Manu KA, Kuttan G. Immunomodulatory activities of Punarnavine, an alkaloid from Boerhaavia diffusa. Immunopharmacol Immunotoxicol, 2009; 31(3): 377-87.
15. Shivananjappa MM, Joshi MK. Influence of Emblica officinalis aqueous extract on growth and antioxidant defense system of human hepatoma cell line (HepG2). Pharm Biol, 2012; 50(4): 497-505.
16. Mossman, T. Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Meth, 1983, 65: 55-63.
17. Protocol for Plating Rat Cryopreserved Kupffer cells and Rat Kupffer-Hepatocyte Co-cultures, Life Technologies Inc, 2013. (doi:http://www.lifetechnologies.com/in/en/home/references/protocols/drug-discovery/adme-tox-protocols/plating-rat)
18. Kothari S, Gohil D, Sangar V, Patil M, Sasibhushan V, Chowdhary A. In-Vitro Evaluation of the Anti-Hepatitis (HBV) and Hepato-Protective Activity of Herbal Extracts. Int. J. Pharm. Sci. Rev. Res, 2014; 24(1:17): 96-100.
19. Streetz K. Luedde T, Manns M, Trautwein C. Interleukin 6 and liver regeneration. Gut, 2000; 47: 309–312.
20. Fausto N, Laird A, Webber E, Liver regeneration 2. Role of growth factors and cytokines in hepatic regeneration, FASEB J, 1995; 9: 1527-1536.
21. Ling W, Yuan L, Qin M, Decoction decreases IL-6 levels in patients with acute pancreatitis, Univ-Sci B iomed & Biotechnol, 2011; 12(12): 1034-1040.
22. Satoh M, Adachi K, Suda T, Yamazaki M, Mizuno D. TNF-driven inflammation during mouse liver regeneration after partial hepatectomy and its role in growth regulation of liver. Mol Biother, 1991; 3(3): 136-47.
23. Bradley J, TNF-mediated inflammatory disease, Journal of Pathology, 2008; 214: 149-160.
24. Robert FS, David AB, Mechanisms of Liver Injury-I. TNF-α induced liver injury: role of IKK, JNK, and ROS pathways, Am J Physiol Gastrointest Liver Physiol, 2006; 290: G583–G589.

25. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries J. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med, 1991; 174(4): 915-24.
26. Louis H, Le Moine O, Peny MO, Hepatoprotective role of interleukin 10 in galactosamine/lipopolysaccharide mouse liver injury, Gastroenterology, 1997; 112(3): 935-942.
27. Rajkumar JS, Sekar MG, Mitra SK, Safety and efficacy of oral HD-03/ES given for six months in patients with chronic hepatitis B virus infection World J Gastroenterol, 2007; 13(30): 4103-4107.
28. Xiao-Lan Li and Ai-Guo Zhou, Evaluation of the Immunity Activity of Glycyrrhizin in AR Mice, Molecules, 2012; 17: 716-727.
29. Lee C, Park S, Kim Y, Kang S, Kim J, Lee S and Lee SM, Protective Mechanism of Glycyrrhizin on Acute Liver Injury Induced by Carbon Tetrachloride in Mice, Biol. Pharm. Bull, 2007; 30(10): 1898-1904.
30. Manu K and Kuttan G, Immunomodulatory activities of Punarnavine, an alkaloid from Boerhaavia diffusa, Immunopharmacology and Immunotoxicology, 2009; 31(3):377–387.
31. Khan M, Ansari I, Ahmad S, Akhter F, Hashim A, Srivastava A, Chemotherapeutic potential of Boerhaavia diffusa Linn: A review, J Appl Pharm Sci, 2013; 3(01): 133-139.
32. Rao T, Okamoto T, Akita N, Hayashi T, Yasuda N and Suzuki K, Amla (Emblica officinalis Gaertn.) extract inhibits lipopolysaccharide-induced procoagulant and pro-inflammatory factors in cultured vascular endothelial cells, British J Nutr, 2013; 110(12): 2201-2206.
33. Sidhu S , Pandhi P, Malhotra S, Vaiphei K, Khanduja K, Beneficial effects of Emblica officinalis in L-arginine-induced acute pancreatitis in rats. J Med Food. 2011, 14(1-2):147-55.
34. Gomez EV, Perez YM, Sanchez HV, Forment GR, Soler EA, Bertot LC, et.al., Antioxidant and immunomodulatory effects of Viusid in patients with chronic hepatitis C. World J Gastroenterol, 2010; 16(21): 2638-2647.
35. Bardi DA, Halabi MF, Abdullah NA, Rouhollahi E, Hajrezaie M, and Abdulla MA, In Vivo Evaluation of Ethanolic Extract of Zingiber officinale Rhizomes for Its Protective Effect against Liver Cirrhosis. BioMed Research International, 2013; (http://dx.doi.org/10.1155/2013/918460).