Triterpene Saponins as Immunomodulators
CHAPTER-1

Effectiveness of triterpene saponins of *Centella asiatica* as immunomodulators

RESULTS

Short-term administration of triterpene saponins extract (EXT) of *Centella asiatica* at doses 100 and 500 mg/kg bodyweight for 10 days did not produce any mortality, change in behavior, body weight, and relative organ weight, hepatic and renal functions when compared with normal untreated rats. *C. asiatica* at 1000 mg/kg body weight dose showed a mild toxicity whereas doses 1500 and 2000 mg/kg body weight exhibited an observable toxicity showing behavior changes, mortality, weight loss, decrease in organ weight, increase in the levels of serum alkaline phosphatase and serum creatinine (Table 3). The lethal dose (LD$_{50}$) was determined as 1000 mg/kg bodyweight. Hence, the experimental dose was selected as one fourth (250 mg/kg bodyweight) of the LD$_{50}$.

The total RBC and WBC counts were determined using complete blood count (CBC) auto analyzer (company) in all groups during the experimental period. The RBC and WBC counts were unchanged in the triterpene saponins extract of *Centella asiatica* (EXT) alone treated rats, where as CYP treatment significantly decreased (P<0.05 compared with controls and EXT alone treated rats) both the counts. With the levamisole treatment to CYP induced immunosuppressed rats, the RBC and WBC counts increased at 60.16 and 31.09% respectively. Interestingly, the triterpene saponins extract of *Centella asiatica* (EXT) also significantly increased the RBC and WBC counts by 59.62% and 21.64% respectively (P<0.05 compared with CYP alone), suggesting the antianemic and antileukopenic activity of triterpene saponins in immunosuppression rats (Table 4). Of the differential WBC, the monocyte and eosinophil counts did not show any significant changes in all the treatment groups compared to controls. CYP induced immunosuppressed rats did not show any significant alterations in the counts of monocytes and eosinophils compared to control rats. On contrary, CYP alone treated rats showed significantly decreased counts of neutrophils and lymphocytes compared to control group. Therapeutic and prophylactic treatment with both EXT and LEV showed significant
increase in neutrophil (15.63% and 27.50%) and lymphocyte (27.41% and 34.67%) counts compared with the CYP group respectively (Table 4). Administration of EXT alone did not produce any significant changes in platelet counts compared to control group. EXT treatment significantly increased platelet counts when compared to the CYP group which exhibited significant reduction by 39.57% compared to the control group. The anti-thrombocytopenic effect of triterpene saponins extract of *Centella asiatica* on platelet counts was on par with that of levamisole (Table 4). Similarly, CYP induced immunosuppressed rats registered a significant decrease (33.79%) in hemoglobin content compared to control rats. However, EXT and LEV treated rats showed an increase of 40.31% & 46.56% respectively for hemoglobin content. Control rats treated with EXT alone did not register any alterations in the concentration of hemoglobin compared to control rats (Table 4).

The Figure 3 and Figure 4 show the relative organ weights of spleen and thymus. CYP treatment drastically reduced the relative organ weights of spleen and thymus. Administration of triterpene saponins at 250 mg/kg body weight, showed a significant (P<0.05) enhancement in the weights of spleen and thymus were observed. The ALP level in liver shows statistically significant reduction in treated groups (CYP+EXT and CYP+LEV) compared to CYP induced immunosuppressed rats. In CYP control, the level of liver ALP was 13.69±0.37 KA, which was significantly (P<0.05) decreased to 12.81±0.35 KA and 12.44±0.32 KA in triterpene saponins extract (EXT) treated at 250 mg/kg body weight and LEV (50 mg/kg body weight) respectively (Figure 5). Rats treated with EXT alone (10.71±0.28) did not show any significant (P<0.05) effect on liver ALP. The elevated level of liver GPT was found to be markedly reduced in triterpene saponins extract (EXT) treated group. The liver GPT level was 46.09±3.32 U/ml in CYP control which were significantly (P<0.05) reduced to 42.53±2.93 U/ml and 41.11±2.72 U/ml in treated (EXT: 250 mg/kg body weight and LEV: 50 mg/kg body weight) animals (Figure 6). The effect of EXT reduction of hike in liver ALP and GPT was on par with that of standard immunomodulator, levamisole.

Lipid peroxidation was determined by evaluating MDA content (Figure 7). A significant (P<0.05) rise in the MDA level was observed in the CYP (86.82%) group
compared with the normal control. The liver MDA level was significantly (P<0.05) decreased in triterpene saponins extract (EXT) 250 mg/kg body weight (40.87%), and levamisole (LEV) 50.0 mg/kg (43.77%) treated groups compared with the CYP. The GSH level of control and experimental animals are shown in Figure 8. The liver GSH level was significantly (P<0.05) reduced on treatment with the CYP compared with the normal control. However, the GSH level was significantly (P<0.05) increased on treatment with triterpene saponins extract (EXT) of *C.asiatica* at 250 mg/kg body weight compared with the immunosuppressed rats (Figure 8). Administration of EXT alone to normal rats demonstrated almost normal levels of GSH.

The quantitative analysis of immunemodulating cytokines in liver was determined in all experimental groups and is shown in the Figure 9 and Figure 10. To understand the mechanism underlying protective effects of triterpene saponins of *C.asiatica* on immunosuppression, we used CYP induced male Wistar rats, as an experimental model. As shown in Figure 9, CYP induced immunosuppressed rats had a significantly (P<0.05) lower (64.45%) expression level of IFN-γ gene in the liver compared to normal control group. When triterpene saponins extract (EXT) of *C.asiatica* was administered CYP+EXT group, the dose of 250 mg/kg body weight significantly increased the IFN-γ mRNA expression (187.42%) in the liver without altering the expression level of β-actin mRNA. LEV treatment at 50 mg/kg body weight also induced the expression of IFN-γ mRNA.

CYP treatment of rats induced inhibition of IL-2 mRNA expression (68.27±2.71 % of control) when elucidated by semiquantitative RT-PCR analysis (Figure 9). It was found that IL-2 mRNA is constitutively expressed in the liver of experimental rats of all groups. When immunosuppressed rats were treated with 250 mg/kg body weight of EXT, a significant elevation of IL-2 gene expression (P<0.05, 108.55±2.95 % of control) was observed. Similar, enhancement of IL-2 mRNA expression (116.52±2.96 % of control) by LEV administration was also observed in CYP+LEV group. EXT alone had no effect the expression (95.99±2.54 % of control) of gene encoding the IL-2 in the liver of rat. The experimental animals of CYP group expressed lower levels (40.13%) of GM-CSF as compared with normal
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animals. As shown in Figure 10, the treatment of CYP induced immunosuppressed rats with 250 mg/kg body weight of triterpene saponins extract of *C.asiatica* significantly (P<0.05) elevated the expression (80.92%) of GM-CSF mRNA. Levamisole, a synthetic immunomodulator also abolished the down-regulatory influence of CYP on the expression of GM-CSF mRNA (77.46%) in liver of experimental rats (CYP+LEV group).

Increased hepatic mRNA levels (P<0.05, 195.38±5.08 % of control) of TNF-α was seen in CYP induced immunosuppressed rats as compared with control. The results of densitometry data analysis are given in Figure 10. There was no significant difference in the mRNA expression of TNF-α between control (100±4.01 % of control) and control rats treated with EXT (104.13±4.40 % of control). A noticeable and significant decline (P<0.05, 116.68±4.83 % of control) in the expression levels of TNF-α was observed when the triterpene saponins extract of *C.asiatica* (EXT) at dose of 250 mg/kg body weight was co-administered to immunosuppressed rats. Similar to the above findings, it is statistically obvious that, the TNF-α mRNA expression was decreased significantly (P<0.05, 109.78±4.90 % of control) by 43.81% in immunosuppressed rats treated with LEV compared to CYP control rats. The semiquantitative analysis of immunomodulatory cytokines (IFN-γ, IL-2, GM-CSF and TNF-α) gene expression in the animals of all experimental groups was carried out in the context of co-amplification of β-actin (Figure 9 & 10) and no significant alterations was observed in the expression level of hepatic β-actin mRNA.

Histopathological analysis of intestine of CYP induced immunosuppressed rats showed severe damage to the intestinal villi when compared to the normal controls (Figure 11.B). The lengths of the villi were markedly reduced and the crypt architecture was largely destroyed. This was inhibited by the triterpene saponins extract of *C.asiatica* (Figure 11.D). The intestinal wall structure of control (Figure 11.A) and EXT alone treated rats (Figure 11.C) was normal and intact. Intestinal section of levamisole treated rats also showed normal structure (Figure 11.E). The tunica serosa was found to be distorted considerably in the CYP alone treated
control rats and the treatment with triterpene saponins extract of *C.asiatica* at a dose of 250 mg/kg body weight modulated the disintegration effects of CYP.

Light microscopic observation of the liver sections of normal control rats showed characteristic features of radiating hepatic cells around a normal central vein with narrow sinusoid, with no sinusoidal congestion and mild cellular swelling (Figure 12.A). On the contrary, in CYP induced immunosuppressed rats destructive changes were more evident; the CYP rats exhibited nonradiating sinusoids, severe scattered necrotic cells with pyknotic nuclei, infiltration of some inflammatory cells, severe degenerations in the hepatocytes, kupffer cells hyperplasia and cellular swelling (Figure 12.B). Treatment with the triterpene saponins extract (EXT) (Figure 12.D) and levamisole (LEV) (Figure 12.E), showed improvement in histological structure of the liver sections of the immunosuppressed rats with normalized appearance in the CYP+EXT group and mild degree of injuries in the LEV treated animals.

The effects of triterpene saponins extract of *C.asiatica* on the CYP induced immunosuppressed rat spleen are shown in Figures 13.B. The normal control rats demonstrated normal architecture, vascular organization, cellular composition of white pulp and red pulp of spleen (Figure 13.A). In contrast, the spleen of untreated immunosuppressed rat revealed congested red pulp, severe depletion of white pulp lymphocytes, extensive hemorrhages in the red pulp and hyaline degeneration of the wall of splenic arterioles with edema (Figure 13.B). Interestingly, the cellular components and architecture of the spleen in CYP+EXT (triterpene saponins extract of *C.asiatica* treated; Figure 13.D) and CYP+LEV (levamisole treated; Figure 13.E) groups showed significant restoration compared to the immunosuppressed control group. Histopathological examination of EXT alone treated group showed non-significant changes in the spleen as seen Figure 13.C when compared to the normal architecture of the control group.

**DISCUSSION**

*Centella asiatica* has been used traditionally as tonic for improving memory accelerating nervous activity, wound healing, tuberculosis, leprosy, anticancer and
furthermore to modulate various physiological functions that may play beneficial roles in some diseases (Babu et al., 1995; Shukla et al., 1999; Physicians’ Desk Reference, 2000; Kapoor, 2005; Jamil et al., 2007). It is evident from the information presented above that quite a few therapeutic properties of C. asiatica have been studied in some detail, while there are very limited studies on the immunemodulating potential although there are indications either from Ayurvedic & Unani systems of medicine or from preliminary studies published (Jayathirtha and Mishra, 2004).

Our knowledge on the usefulness of medicinal plants will increase if detailed studies are carried with plants, which have not been subjected to detailed investigation. The aim of the present study was to screen C. asiatica about which there is no detailed scientific confirmation of the claim for its immunemodulating activity. To the best of our knowledge, this study is the first to show the immunostimulatory potential of C. asiatica. Traditionally a large number of plants used in medicine have been shown to possess immunemodulating activities (Choi et al., 2004) and are being extensively explored for their potential in the treatment and prevention of chronic disease.

Immunomodulatory agents of plant origin increase the immune responsiveness of the body against pathogen by activating immune competent cells (Sharma et al., 2012). There is strong requirement of the drugs which can boost immune system to combat the immunosuppressive consequences caused by stress, chronic diseases like obesity, ischemia, diabetes, tuberculosis, conditions of impaired immune responsiveness (e.g. AIDS) etc. (Wagner, 1999; Sunil et al., 2014). One of the most well-studied plant species in terms of medicinal plant is C. asiatica, found widely in tropical and subtropical countries from which the triterpene saponins as major components (asiatic acid, madecassic acid, asiaticoside and madecassoside) has been identified. The medicinal values of this plant are mainly attributed to the presence of triterpene saponins (Ling et al., 2000; Ling, 2004). Triterpene saponins have also been regarded as its biomarker components (Zheng and Qin, 2007). Therefore, triterpene saponins have been chosen for their immunemodulating potential.
Animal models of immune function assessment are very useful in the study of prevention, diagnosis and therapy design for immunosuppression (House, 2000). Immunosuppression induced by cyclophosphamide in male Wistar rats is the animal model most frequently used (Hou et al., 2007; Roman and Anna, 2010). This model has been used by several groups to study the immunemodulating effects of bioactive molecule (Hou et al., 2007; Pratheeshkumar and Girija, 2010a). Thus, the present study evaluates immunemodulating potential of triterpene saponins from *Centella asiatica* against the immunosuppressive effects of cyclophosphamide in male Wistar rats.

Cyclophosphamide is a cytotoxic chemotherapeutic drug that acts as an alkylating agent producing reactive carbonium ions, which reacts with DNA (Colvin, 2001; Pratheeshkumar and Girija, 2010b). Most of the chemotherapeutic drugs available today are immunosuppressant’s and exerts a variety of side effects. Administration of CYP leads to immunosuppression, which at times leads to life threatening situations. Initial activation reaction of CYP is carried out by microsomal oxidation system in liver producing 4-hydroxy CYP, a cytotoxic metabolite, which diffuses from hepatocytes into plasma and distributed throughout the body. Then, 4-hydroxy CYP is further converted to some other cytotoxic metabolites, phosphoramid mustard and acrolein are among them (Elkhalifa and Weiner, 2010; Pratheeshkumar and Girija, 2010a). Phospharamide mustard is known to cause myelosuppression and immunosuppression (Berger, 1993; Wang et al., 2011).

To mention, the modulation of immune response through stimulation may help in maintaining a disease free state. Thus, the bioactive metabolites of medicinal plants are being reviewed for their immunomodulating activities (Praveen et al., 1996). In the present study, the effect of triterpene saponins extract of *C. asiatica* on immunosuppression seen during CYP treatment in rats was analyzed. Administration of EXT alone did not produce any significant change in the hematological parameters studied compared to control group. When compared with the CYP induced immunosuppressed animals, the total RBC and WBC, neutrophil, lymphocyte and platelet counts were increased by the administration of triterpene
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Saponins extract of *C. asiatica* thereby, providing supportive evidence for their antianemic, antileukopenic and antithrombocytopenic activities. The possible mechanism for the observed effects of CYP treatment may be due to loss of stem cells and inability of bone marrow to regenerate new blood cells (Sanjeev et al., 2012). Bone marrow is a site of continued proliferation and turnover of blood cells and is a source of cells involved in immune activity. A high degree of cell proliferation renders bone marrow a sensitive agent, particularly to cytotoxic drugs. In fact bone marrow is the organ most affected during immunosuppression (Pelczar et al., 1990).

In accordance to the above findings, the methanolic extract of *Cardiospermum halicacabum* found rich in saponins opposed the myelosuppressive effects induced by CYP. The increased in the levels of bone marrow cells and α-esterase positive cells further supported its immunestimulative potential during CYP toxicity (Pratheeshkumar and Girija, 2010b; Danilo, 2012). Dammarane sapogenins (DS) has a protective function against CYP-induced myelosuppression. Yang et al., (2011) described the protective role of dammarane sapogenins, an active fraction from ginseng, on myelosuppression induced by CYP in mice. Its mechanism might be related to stimulating hematopoiesis recovery, as well as enhancing the immunological function. The pretreatment with methanolic extract of *Pongamia glabra* Vent counteracted the cyclophosphamide induced myelosuppression in experimental animals through modulating the bone marrow activity. The potentiated activity of the extract is due to the presence of excess number of bioactive compounds viz., saponins in the *P. glabra* (Sanjeev et al., 2012). However, LEV treatment also reversed the myelosuppression induced by the cyclophosphamide. Of the differential WBC, the monocyte and eosinophil counts did not show any significant changes in both treated and untreated animals.

In general, atrophy of immune organs with the use of chemotherapeutic drugs for diseases like cancer, tuberculosis etc. and furthermore the emergence of infectious diseases results in the decrease of the immune organ coefficients, such as thymus coefficient and spleen coefficient, which are often used as hallmarks at organ level to evaluate the success of the animal model for immunosuppression.
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(Richter-Reichhelm et al., 1995; Dean et al., 1998; International Collaborative Immunotoxicity Study, 1998; Center for Drug Evaluation and Research, 2002). Rats treated with CYP alone showed a significant increase in spleen and thymus weights as compared to controls. Treatment with triterpene saponins extract of C.asiatica (EXT) alone to normal rats did not show significant changes; in contrast treatment with EXT to CYP immunosuppressed rats showed a significant increase in the weight of spleen as well as thymus as compared to CYP and LEV treated CYP rats.

In the same context Mohammad et al., (2013) reported that administration of Coffea arabica extract elevated the depressed weights of spleen and thymus that were remarkably decreased by the treatment of CYP. This has been attributed to stimulation of spleen and thymus cells by triterpene saponins and thus enhanced the immune system function. In the case of thymus, this may be partly due to the stimulatory effect of EXT on the lymphocytes and bone marrow hematopoietic cells, which ultimately home in the thymus (Mohammad and Shahid, 2014). Saponins from Aralia taibaiensis significantly antagonized decreases of spleen and thymus weights induced by D-galactose treatment, suggesting the effective protection of saponins (Ying et al., 2014).

CYP is a widely prescribed non-cell-cycle-specific antineoplastic drug which is known to cause hepatotoxicity (DeLeve, 1996). There is pharmacologic evidence that the breakdown of CYP into biologically active alkylating compounds takes place principally in the liver (Brock and Hohorst, 1967; Sheetla et al., 2013). CYP alters liver functions by modulating all liver enzymes (Davila et al., 1989; Sheetla et al., 2013). The liver is the one of the richest sources of both ALP and GPT enzymes (Craig, 1998). In a healthy liver, fluid containing alkaline phosphate and other substances is continually drained away through the bile duct. When the liver is not functioning properly, this bile duct is often blocked, keeping fluid within the liver. Alkaline phosphatase accumulates and eventually escapes into the bloodstream (Craig, 1998; Pratheeshkumar and Girija, 2010a). Furthermore, the alkaline phosphatase of the liver is produced by the cells lining the small bile ducts (ductoles) in the liver. Its origin differs from that of other enzymes called aminotransferases. If the liver disease is primarily of an obstructive nature
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(cholestatic), i.e. involving the biliary drainage system, the alkaline phosphatase will be the first and foremost enzyme elevation. If, on the other hand, the disease is primarily of the liver cells (hepatocytes), the aminotransferases will rise prominently. Thus, these enzymes are very useful in distinguishing the type of liver disease-cholestatic or hepatocellular (Craig, 1998). The primary importance of measuring ALP and GPT is to check the pathological condition of the animal especially, liver injury (Sheetla et al., 2013). Administration of triterpene saponins extract of C. asiatica (EXT) in immunosuppressed rats (CYP group) reduced the liver ALP level, which was elevated during CYP administration indicate the protective effect from hepatotoxicity and immunosuppression. Furthermore, the EXT was also found to decrease the activity of liver GPT in the CYP treated animals support the ameliorating effect of EXT on CYP induced liver damage. It was reported that saponins (triterpenes) isolated from a variety of sources demonstrated antihepatotoxic activity by inhibiting the levels of ALP and GPT (Miyao et al., 1998; Kim et al., 2009; Lijie et al., 2013).

The chemotherapeutic prodrug-CYP is metabolized by liver cytochrome P450 enzymes namely, CYP3A4 and CYP2B6 to yield therapeutically active, cytotoxic metabolites (Roy and Waxman, 2006). The cytotoxic metabolites (acrolein and phosphoramid mustard) formed in the liver are distributed to different tissues by systemic circulation (Premila and Emila, 2007). CYP causes damage to the liver, although liver is the major site of CYP activation to cytotoxic metabolites (Abraham et al., 2007). The metabolism of CYP in the body produces highly reactive oxygen species and free radicals probably due to the formation of acrolein (McDiarmid et al., 1991). It was reported that these free radicals and reactive oxygen species are involved in the CYP induced oxidative stress (Haque et al., 2003; Manda and Bhatia, 2003). Moreover, evidences also indicated that oxidative stress play a predominant aetiological role in CYP induced immunosuppression (Stankiewicz et al., 2002; Manda and Bhatia, 2003; Selvakumar et al., 2005). CYP has a pro-oxidant character, and generation of oxidative stress after CYP administration leads to increase in lipid peroxidation and decrease in the activities of antioxidant enzymes and in liver of rats (Lear et al., 1992, Mathew and Kuttan, 1997, Kaya et al., 1999;
Lipid peroxidation is one of the main manifestations of oxidative damage initiated by reactive oxygen species and it has been linked with impairment of membrane functioning, decreased fluidity, inactivation of membrane bound receptors and enzymes, and increased non specific permeability to ions (Sikka, 2004). In the present study, the triterpene saponins extract of C.asiatica treated rats showed significant reduction in the LPO levels in liver. This indicates the antioxidant activity of triterpene saponins of C.asiatica and ability for reducing CYP induced oxidative stress.

GSH is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism (Patel, 1987; Sulkowska et al., 1998). Glutathione metabolism is often correlated with cellular sensitivity to cytotoxic agents. Glutathione is protective against drug cytotoxicity. It reacts with toxic endogenous and exogenous substances, including free radicals and cytotoxic agents (Yokomizo et al., 1995). The metabolism of CYP in the body produces highly reactive electrophiles (Haque et al., 2003; Manda and Bhatia, 2003). The glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species (Meister and Anderson, 1983). GSH is an important water-phase antioxidant also acts an important cofactor for antioxidant enzymes taking part in cellular redox reactions (Dickinson and Forman, 2002). Excess lipid peroxidation cause increased GSH consumption (Comporti, 1987; Kailash et al., 2007). GSH, with its sulphydryl group, functions in the maintenance of sulphydryl groups of other molecules (especially proteins), as a catalyst for disulfide exchange reactions, and in the detoxification of foreign compounds, hydrogen peroxide and free radicals. When GSH acts a reducing agent, it’s SH becomes oxidized and forms a disulfide link with other molecules of GSH (Gul et al., 2000).

CYP induced depletion of GSH is primarily mediated by interaction of its reactive metabolite, acrolein with GSH (Kehrer and Biswal, 2000). Acrolein activates intracellular ROS and NO production leading to peroxynitrite production. The formation of peroxynitrite at the site of inflammation is detrimental (Bhatia et al. 2008). As a result of combinative effect of acrolein and peroxynitrite a significant
decrease in antioxidants (GSH) was observed in the liver in CYP induced immunosuppressed animals. Treatment of triterpene saponins extract of *C. asiatica* increased the GSH production. The results of the present study are in agreement with previous studies which reported that triterpene saponins decreases the lipid peroxidation and enhance GSH levels and in turn protect against the damaging effects induced by free radicals (Bian et al., 2012; Kokanova-Nedialkova et al., 2013; Pakdeechote et al., 2014).

Cyclophosphamide weakens both the cell-mediated and humoral immune response; in particular, even a single administration may temporarily impair the immune system (Roman and Anna, 2010). CYP targets fast-dividing cells which replicate rapidly (eg. cancer cells) as they are most sensitive to alkylating agents. However, the action of CYP is not selective, and therefore the drug can also damage the fast-proliferating cells of healthy tissue, including bone marrow cells (in particular the developing blood cells), activated lymphocytes (that proliferate and produce antibodies), immune cells and intestinal epithelial cells (Danysza et al., 1996).

CYP affects cells by alkylating not only DNA but also RNA and enzymes with a protein structure. Therefore, it influences proliferating cells and may also retard their intermitotic functions. The above may inhibit phagocytic activity at various stages of phagocytosis. Phagocyte suppression may result from inhibited synthesis of cytokines and the enzymes which regulate the functions of phagocytes (Roman and Anna, 2010). Furthermore, CYP had an inhibitory effect on protein production, causing a decrease in γ-globulin levels. The above most probably resulted from the adverse side effects of cyclophosphamide on hepatocytes which produce protein. The analyzed drug indirectly affects the levels of ceruloplasmin (and other acute-phase proteins) through the impairment of the synthesis of cytokines which stimulate the production of proteins in the liver, i.e. TNF-α secreted mostly by monocytes and macrophages (Roman and Anna, 2010). Cytokines are low molecular weight extracellular signaling proteins secreted by immune and inflammatory cell populations, chemokines, oncogenes, as well as growth factors and other soluble factors either constitutively or after induction (Desai, 2007). They
are involved in virtually every aspect of immunity and inflammation, including development and functioning of the immune system, cell proliferation and differentiation, cellular recruitment and activation, regulation of cellular interactions with extracellular matrix proteins. Cytokines mediate the communication both locally between cells and tissues and distantly between organs (Nathan and Sporn, 1991; Borish and Steinke, 2003).

CYP reduces the proliferative capacity of T cells and B cells in rats. This suggests that both lymphocyte populations are sensitive to CYP. The decrease in proliferation rates resulted from the action of CYP on cell DNA. Proliferation impairment may be a consequence of CYP’s adverse effect on monocytes/macrophages which participate in regulating the functions of lymphocytes. To mention, CYP affects B cells directly, but also indirectly through the inhibition of T-cells (Roman and Anna, 2010). The lymphokine, IL-2, which was identified as T cell growth factor (Ehrhacat et al., 1997) plays a central role in the maturation and development of lymphocytes and monocytes (Theze et al., 1996), whereas IFN-γ stimulates phagocytic activity of macrophage and differentiation of T cells and cytotoxic effects (Borish and Rosenwasser, 1996).

GM-CSF is a hematopoietic cytokine with well-defined effects on the survival, proliferation, and differentiation of myeloid leukocytes and their precursors (Gasson, 1991). CYP drastically reduced the hepatic mRNA levels of IL-2, IFN-γ, and GM-CSF, whereas treatment of triterpene saponins extract of C.asiatica showed a significantly higher level of IL-2, IFN-γ, and GM-CSF gene expression in the liver than CYP alone treated rats. A similar tendency for LEV treatment to increase the above cytokines mRNA expression was also observed in the liver. Considering that expression of the IL-2, IFN-γ, and GM-CSF genes in the liver was elevated by triterpene saponins, the present results raise the possibility of that triterpene saponins of C.asiatica may produce up-regulatory influence on the production of these cytokine that stimulate the immune system. Moreover, it was found that in contrast to the effect on the above, triterpene saponins failed to affect the expression of the reference gene, β-actin mRNA. Saponins reportedly induced production of cytokines such as interleukins and interferons that might mediate their immunostimulant
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effects (Kensil, 1996; George et al., 2002). Saponins have greater ability to elicit immune responses may be as a result of more efficient uptake by antigen presenting cells via mannose receptors that interact with the carbohydrate chain of saponins. The above may also induce major histocompatibility complex (MHC) class I-restricted cytotoxic T-lymphocyte responses correlates with its capacity to stimulate IL-2 and IFN-γ production (Kensil, 1996; Behboudi et al., 1999). Yesilada et al., (2005) studied the effect of triterpene saponins isolated from Astragalus brachypterus, A.cephalotes, A.microcephalus, A.trojanus and A.oleifolius on in vitro cytokine release. Interestingly, all triterpene saponins tested showed a prominent IL-2 inducing activity.

TNF-α is a 17-kDa protein secreted predominantly by macrophages after a variety of stimuli (Bentler and Cerami, 1986; Pratheeshkumar and Girija, 2010a). To date, the mechanisms involved in CYP induced immunosuppression are not completely clarified. It has been proposed that administration of CYP might cause impairment of cellular respiration due to damage of mitochondrial energy converting mechanisms (Souid et al., 2003), which may interfere with hepatic intracellular oxidant/antioxidant balance and lead to accumulation of reactive oxygen species (Zarei and Shivanandappa, 2013). The resultant oxidative stress may then trigger nuclear factor-κB (NF-κB) inflammatory pathway, which increases hepatic proinflammatory cytokines as tumor necrosis factor-α (Hamsa and Kuttan, 2010; Azza and Rehab, 2014).

Of the several transcription factors activated by inflammatory stimuli, NF-κB is known to play critical roles in the expression of pro-inflammatory cytokines, TNF-α (Wong and Tergaonkar, 2009). TNF-α is a key indicator in pathological situations (Dinarello et al. 1986; Dinarello, 2000); and its expression level was drastically elevated after CYP treatment and was reduced by the administration of triterpene saponins of C.asiatica. Yeonju et al., (2012) also proved that Blue cohosh triterpene saponins also exerted anti-inflammatory effect through the inhibition of expression of pro-inflammatory cytokines such as TNF-α. It was found that the triterpene saponins suppress the production of TNF-α (Shah et al., 2009; Li et al., 2013) suggesting that inhibition of NF-κB activation in macrophages may be
attributable to the suppression of TNF-α mRNA expression (Valsala, et al., 2001; An et al., 2011).

Histopathology in our study was carried out on the principle tissue, intestine and revealed marked changes; lengths of the villi were markedly reduced and the crypt architecture and tunica serosa was largely destroyed in the intestinal wall of rats treated with CYP and mild pathological changes, superficial ulceration and minor inflammation in the animals receiving EXT alone and Levamisole. However, it was demonstrated that triterpene saponins extract (EXT) treatment reduced the CYP induced tissue damage. Since the epithelial cells of villi play an important role in mucosal immunity (Kumar et al., 2004) its protection during immunomodulation is very important. These data were similar to those published earlier (Pratheeshkumar and Girija, 2010a). The former studies also found that plants containing high amounts of saponins have been shown to possess intestinal protective activity in several experimental bioassays (Yesilada and Takaish, 1999; Morikawa et al., 2006) probably acting as an activator of mucus membrane protective factors of immunity (Adeola et al., 2011; Inas et al., 2011) CYP is known to cause multiorgan damage that result in severe morbidity and might end fatally (Haubitz, 2007). Most reports focused on studying CYP induced hepatotoxicity (Goldberg and Lidsky, 1985; Hamsa and Kuttan, 2010). To date, the mechanisms involved in CYP induced hepatotoxicity are not completely clarified. It is known that CYP disrupts the redox balance of tissues resulting in induction of oxidative stress (Stankiewicz et al., 2002). Previous studies have indicated that tissue damage results from a disturbance of the oxidation–antioxidation balance; production of oxygen free radicals and its oxidation product is one of the primary causes leading to hepatotoxicity (Akay et al., 2006; Martínez et al., 2011). The resultant oxidative stress has great impact on immune system. Free radical components have a relevant pathophysiological role in several types of immune mediated diseases (Ames et al., 1993; Sudha et al., 2003; Hamsa and Kuttan, 2010).

Histological examination of rat liver treated with CYP showed typical hepatotoxicity characterization, such as nonradiating sinusoids, severe scattered necrotic cells with pyknotic nuclei, infiltration of some inflammatory cells, severe
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degenerations in the hepatocytes, kupffer cells hyperplasia and cellular swelling. Treatment with the levamisole (LEV) showed improvement in histological structure of the liver sections of the immunosuppressed rats with normalized appearance. However, the typical hepatotoxicity characterization was also not shown in EXT alone treated groups, suggesting that triterpene saponins have potential protective effect against liver injury in CYP immunosuppressed rats. The protective effects exhibited by the Centella asiatica in this study might be due to various interactions between the triterpene saponins and the mechanisms involved in CYP induced hepatotoxicity and immunosuppression. Reported antioxidant and hepatoprotective immunomodulating activities of the triterpene saponins may be responsible for their protective activity (Tran et al., 2001; Xi et al., 2010; Elena et al., 2012).

The spleen plays an important role in the immune system and is the major site for mounting the immune response. The spleen is composed of the red and white pulp (Tiron and Vasilescu, 2008). White pulp is lymphatic tissue that mainly consists of lymphocytes called B-lymphocytes and T-lymphocytes that surround arteries. Red pulp consists of venous sinuses and splenic cords. Venous sinuses are essentially cavities filled with blood, while splenic cords are connective tissues containing red blood cells and certain white blood cells (including lymphocytes and macrophages) (Tiron and Vasilescu, 2008). The spleen of the control untreated immunosuppressed (CYP) rats showed congested red pulp, severe depletion of white pulp lymphocytes, extensive hemorrhages in the red pulp and hyaline degeneration of the wall of splanic arterioles with edema similar to other experimentally induced immunosuppressed animal models (Siegal et al., 1986; Kishimoto et al., 1990).

The triterpene saponins extract of Centella asiatica (EXT) significantly reversed CYP induced histopathological alterations in the spleen. This may possibly be due to the immunomodulating potential of triterpene saponins as previously hypothesized (Behboudi et al., 1996, 1997; Khajuria et al., 2007; Elena et al., 2012). Furthermore, the standard immunomodulator levamisole also significantly attenuated the extent of spleenic damages. In conclusion, the results of the above experiments strongly suggest the triterpene saponins of Centella asiatica are strong immunomodulators. Therefore, triterpene saponins may provide curative and/or
preventive treatment options against diseases wherein a depleted immune status contributes to the pathological processes. Further mechanistic studies are required to suggest the appropriate mechanism for the immunostimulating effect of the triterpene saponins.

However, the main limiting factor for natural product drug discovery is the ability to produce enough material for clinical applications. The biological activity and potency of these bioimmunomodulators are derived largely from their complex structures involving region-specific functionalization and chirality. Total synthesis poses many challenges for chemists, although often unsuccessful due to many separation steps, and has very little practical value for the development of these complex molecules for research and therapeutics. Recently, challenging hypotheses related to the role of fungal endophytes in enhancing in planta production and natural products have emerged. Thus it can be speculated that the fungal endophytes standardizes yield fluctuation issues and offers significant promise for a scalable means to provide sufficient quantities of a triterpene saponins, the natural immune modulators. Noteworthy, the plant species sampled from unique ecological niches species are known to harbor potential fungal endophytes. Thus, the interests continued in this direction made me to study the fungal endophytes associated with the Tirumala Hills of Seshachalam Biosphere Reserve in Eastern Ghats, India in order to exploit them as potential in planta bioenhancers of immunomodulators.
### Table 3. Toxicological evaluation of triterpene saponins extract of *Centella asiatica* in male Wistar rats

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>Control</th>
<th>100 mg/kg body weight</th>
<th>500 mg/kg body weight</th>
<th>1000 mg/kg body weight</th>
<th>1500 mg/kg body weight</th>
<th>2000 mg/kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (D/T)</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>3/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Behavior changes</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Hypo activity</td>
<td>Hypo activity</td>
</tr>
<tr>
<td>Body weight (gm)</td>
<td>176.66±6.81</td>
<td>177.82±5.95</td>
<td>175.71±6.81</td>
<td>165.72±6.92</td>
<td>157.94±5.26</td>
<td>152.83±5.41</td>
</tr>
<tr>
<td>Liver weight (gm)</td>
<td>5.42±0.75</td>
<td>5.49±0.95</td>
<td>5.38±0.69</td>
<td>5.02±0.57</td>
<td>4.59±0.42</td>
<td>4.37±0.39</td>
</tr>
<tr>
<td>Spleen weight (gm)</td>
<td>0.89±0.12</td>
<td>0.93±0.16</td>
<td>0.91±0.14</td>
<td>0.78±0.09</td>
<td>0.66±0.06</td>
<td>0.59±0.07</td>
</tr>
<tr>
<td>Thymus weight (gm)</td>
<td>0.76±0.09</td>
<td>0.78±0.07</td>
<td>0.74±0.09</td>
<td>0.62±0.05</td>
<td>0.51±0.08</td>
<td>0.44±0.06</td>
</tr>
<tr>
<td>Serum ALP (U/ml)</td>
<td>14.27±1.36</td>
<td>14.13±1.44</td>
<td>14.32±1.27</td>
<td>16.86±1.66</td>
<td>22.67±2.18</td>
<td>25.75±2.42</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.81±0.11</td>
<td>0.83±0.09</td>
<td>0.89±0.13</td>
<td>0.95±0.15</td>
<td>1.22±0.11</td>
<td>1.37±0.17</td>
</tr>
</tbody>
</table>

Values are the mean±SD.

All the treated animals were carefully examined for 14 days for any signs of toxicity (behavioral changes and mortality)

D/T: Dead/treated mice, None: No toxic symptoms were seen during the observation period

* P≤0.05 significantly different from control
### Table 4. Effect of triterpene saponins extract of *C. asiatica* on hematological parameters in control and experimental animals

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>GROUP I CON</th>
<th>GROUP II CYP</th>
<th>GROUP III EXT</th>
<th>GROUP IV CYP+EXT</th>
<th>GROUP V CYP+LEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (million/mm³)</td>
<td>6.08±0.77</td>
<td>3.69±0.51</td>
<td>5.93±0.33</td>
<td>5.89±0.86</td>
<td>5.91±1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-39.30)</td>
<td>(-2.46)</td>
<td>(+59.62)</td>
<td>(+60.16)</td>
</tr>
<tr>
<td>WBC (thousand/mm³)</td>
<td>14.47±0.80</td>
<td>9.10±0.36</td>
<td>13.03±0.45</td>
<td>11.07±0.78</td>
<td>11.93±0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-37.11)</td>
<td>(-9.95)</td>
<td>(+21.64)</td>
<td>(+31.09)</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>71.33±1.53</td>
<td>53.33±2.08</td>
<td>70.00±1.73</td>
<td>61.67±3.06</td>
<td>68.00±3.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-25.23)</td>
<td>(-1.86)</td>
<td>(+15.63)</td>
<td>(+27.50)</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>44.50±1.29</td>
<td>31.00±2.16</td>
<td>41.50±1.29</td>
<td>39.50±1.29</td>
<td>41.75±3.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-30.33)</td>
<td>(+6.74)</td>
<td>(+27.41)</td>
<td>(+34.67)</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>2.75±0.50</td>
<td>1.25±0.50</td>
<td>2.50±0.58</td>
<td>2.00±0.82</td>
<td>2.25±0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-54.54)</td>
<td>(-9.09)</td>
<td>(+60.00)</td>
<td>(+80.00)</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>6.33±0.58</td>
<td>4.33±0.58</td>
<td>5.67±1.15</td>
<td>5.33 ±1.15</td>
<td>6.00±1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-31.59)</td>
<td>(-10.42)</td>
<td>(+23.09)</td>
<td>(+38.56)</td>
</tr>
<tr>
<td>Platelet (lakh/mm³)</td>
<td>3.74±0.48</td>
<td>2.26±0.34</td>
<td>3.56±0.67</td>
<td>3.17±0.55</td>
<td>3.43±0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-39.57)</td>
<td>(-4.81)</td>
<td>(+40.26)</td>
<td>(+51.76)</td>
</tr>
<tr>
<td>Haemoglobin (gms%)</td>
<td>14.50±0.79</td>
<td>9.60±1.08</td>
<td>14.13±0.45</td>
<td>13.47±1.16</td>
<td>14.07±1.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-33.79)</td>
<td>(-2.55)</td>
<td>(+40.31)</td>
<td>(+46.56)</td>
</tr>
</tbody>
</table>

Data represent mean ± standard deviation (SD) of 6 individual rats
Values in the parenthesis are % change (ª compare to CON, º compared to CYP)
Values are not sharing a common superscript (a,b,c) differ significantly at P≤0.05 (DMRT)
Figure 3. Effect of triterpene saponins extract of *C. asiatica* on spleen weight in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are not sharing a common superscript (a,b,c,d) differ significantly at P≤0.05 (DMRT)

Figure 3.1. % change of spleen weight in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are % change (*compare to CON, *compared to CYP)
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Figure 4. Effect of triterpene saponins extract of *C.asiatica* on thymus weight in control and experimental animals

![Bar graph showing effect of triterpene saponins extract on thymus weight](image)

Data represent mean ± standard deviation (SD) of 6 individual rats. Values are not sharing a common superscript (a, b, c) differ significantly at P≤0.05 (DMRT).

Figure 4.1. % change of thymus weight in control and experimental animals

![Bar graph showing % change of thymus weight](image)

Data represent mean ± standard deviation (SD) of 6 individual rats. Values are % change (*compare to CON, *compared to CYP).
Figure 5. Effect of triterpene saponins extract of *C. asiatica* on alkaline phosphatase level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are not sharing a common superscript (a,b,c,d) differ significantly at P≤0.05 (DMRT)

Figure 5.1. % change of alkaline phosphatase level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are % change (compare to CON, compared to CYP)
Figure 6. Effect of triterpene saponins extract of C. asiatica on glutamate pyruvate transaminase level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are not sharing a common superscript (a,b,c,d) differ significantly at P≤0.05 (DMRT)

Figure 6.1. % change of glutamate pyruvate transaminase level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are % change (*compare to CON, †compared to CYP)
Figure 7. Effect of triterpene saponins extract of *C. asiatica* on lipid peroxidation in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats

Values are not sharing a common superscript (a,b,c) differ significantly at P≤0.05 (DMRT)

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Figure 7.1. % change of lipid peroxidation in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats

Values are % change (*compare to CON, *compared to CYP)
Figure 8. Effect of triterpene saponins extract of *C. asiatica* on glutathione level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats. Values are not sharing a common superscript (a, b, c, d) differ significantly at $P \leq 0.05$ (DMRT).

Figure 8.1. % change of glutathione level in control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats. Values are % change (*compares to CON, *compares to CYP).
Figure 9. Effect of triterpene saponins extract of *C.asiatica* on mRNA expression of IFN-γ, IL-2 and β-actin in liver of control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats. Values are not sharing a common superscript (a,b,c,d,e) differ significantly at P≤0.05 (DMRT)
Figure 10. Effect of triterpene saponins extract of *C. asiatica* on mRNA expression of GM-CSF, TNF-α and β-actin in liver of control and experimental animals

Data represent mean ± standard deviation (SD) of 6 individual rats
Values are not sharing a common superscript (a,b,c,d) differ significantly at P≤0.05 (DMRT)
Figure 11. Photomicrographs of intestine tissues of rats from different experimental groups
(a) CON: Control; (b) CYP: Cyclophosphamide; (c) EXT: Extract; (d) CYP+EXT: Cyclophosphamide+Extract; (e) CYP+LEV: Cyclophosphamide+Levamisole. (IV)-Intestinal villi; (IC)- Intestinal crypts; (TS)- Tunica serosa.
Figure 12. Photomicrographs of liver tissues of rats from different experimental groups
(a) CON: Control; (b) CYP: Cyclophosphamide; (c) EXT: Extract; (d) CYP+EXT: Cyclophosphamide+Extract; (e) CYP+LEV: Cyclophosphamide+Levamisole. (CV)-central vein; (He)-Hepatocytes; (Ne)-Necrosis; (Si)-Sinusoids; (Kc)-Kupffer cells; (Pn)-Pyknotic nuclei; (In)-Inflammatory cell infiltration
Figure 13. Photomicrographs of spleen tissues of rats from different experimental groups
(a) CON: Control; (b) CYP: Cyclophosphamide; (c) EXT: Extract; (d) CYP+EXT: Cyclophosphamide+Extract; (e) CYP+LEV: Cyclophosphamide+Levamisole. (RP)-Red pulp; (WP)- White pulp; (DL)- Severe depletion of lymphocyte in WP; (EH)- Extensive hemorrhages in RP; (HD)- Hyaline degeneration; (ED)- Edema