5.
DISCUSSION

5. Discussion

The present study was concentrated on the medicinal, immunological and phytochemical investigations of the ethnomedicinal plant *Nerium indicum* Mill. *N. indicum* is a well-known name in the ethnopharmacological domain all over the world especially in India (Dutt, 1922; Dastur, 1985; Muthu, *et al*., 2006; Khare, 2007; Ghosh, 2008) and Chinese (Ji, 1999; Ding, *et al*., 2003; Sreenivasan, *et al*., 2003; Fu, 2005; Gayathri, *et al*., 2013) traditional medicinal systems. Though numerous studies exists on the medicinal properties of the plant, but the present study for the first time have evaluated and compared the medicinal properties of the three major parts of the plant i.e. leaf, stem and root in a more comprehensive way and correlated the results with the phytochemical composition to highlight its physiological implications.

The central idea of the present study was to investigate the ethnopharmacological claim of the medicinal properties of *N. indicum* through pharmacological perspectives and also to elucidate the mechanisms and underlying rationale behind these bioactivities. Hydromethanolic extracts of *N. indicum* were chosen as the test material because in ethnophatmacology and traditional system, medicines are prepared mostly in polar solvents. Besides, many ethnopharmacological medicines are actually tinctures prepared by hydroalcoholic solvent extraction processes. Moreover, it is a well-known fact that phenolic and flavonoid phytochemical species are the primary mediators of bioactivities of herbal medicines. Initial screening of *N. indicum* revealed presence of high quantity of phenolic and flavonoids in the 70% methanolic extracts and that is why the extracts were chosen for the present study. Besides, according to Harborne (1998), one of the best method of total phenolic species isolation from the plant source is performed with aqueous-alcoholic solvent system such as hydromethanolic extraction. Therefore, considering the aforementioned facts, 70% hydromethanolic extracts of *N. indicum* were chosen for the bioactivity study.

Immunomodulatory evaluation of NILE, NISE and NIRE were performed using both *in vivo* and *in vitro* methods. Though the immunomodulatory potentialities of *N. indicum* have been claimed in the traditional medicinal systems, but very few studies were performed to evaluate such claim. Initial evaluation of the stimulation of murine humoral immune system was performed by PFC assay and corresponding IgM level estimation. The PFC assay is based on complement mediated lysis of foreign antigen (sRBC) through immunoglobulin activity (Bondada & Robertson, 2003). sRBC is a particulate T-cell dependent natural antigen which activates T-cell to induce activation of enough B-cells in whole splenocyte population. Thus,

further challenge with the same antigen (sRBC) evokes IgM mediated immune response at higher multitude followed by the complement mediated lysis of sRBC. IgM binds guenia pig complement more efficiently, thus the PFC value is influenced by the generation of IgM level. The plaque forming cells are detected under binocular microscope through IgM-complement complex mediated lysis of sRBC, which remained surrounded to the antibody secreting cells in the Cuningham chamber (Bondada & Robertson, 2003). sRBC mediated antibody response are also routinely used in immunotoxicological studies (Roy, *et al*., 2013; Ladics, 2007). In the present study, only NILE and NIRE demonstrated gradual dose-dependent stimulation of the murine humoral immune system as evaluated by PFC and IgM assay. The significant increase ($P<0.001$) of the PFC value of NILE and NIRE at 200 mg/kg dose were 1.57 fold and 1.50 fold higher than that of the control. The PFC value without any antigenic stimulation usually remains at basal level (5-30 PFC/10^6 cells) (Bondada & Robertson, 2003). Furthermore, the secreted IgM levels from the same assay, estimated using ELISA corroborated the results of the PFC assay. The increase in IgM level for NILE and NISE at 200 mg/kg were 1.76 fold and 1.42 fold respectively, compared to control. NISE did not demonstrated any significant ($P>0.05$) stimulation of the humoral immune system. Increased PFC value and IgM level at 200 mg/kg NISE were mere 1.01 fold and 1.09 fold, respectively compared to control.

Following the PFC assay, hemagglutination (HA) titre assay was also performed to measure the stimulation of murine humoral immune system by different extracts of *N. indicum*. HA titre assay measures the relative concentration of antibody which is expressed as titre value. Therefore, if PFC assay is the qualitative test, then HA titre is considered as the quantitative test for assessment of stimulation of the humoral immunity. On encounter with antigen, naive B-cells proliferate to generate plasma cells which are programmed to secret antibodies against the antigen. Stabilization of antigen takes place through a complex cross-linking latex formation with the antibody. The insoluble agglutination complex is later internalized and digested by the phagocytic cells. In the present study, on initial immunization, murine naive B-cells generate antibodies against the foreign particle i.e. sRBC. The antibodies present in the serum specific to sRBC later recognize and bind to the sRBC and from agglutination complex in the Khan tubes. Detection of antigen-antibody agglutination complex at higher dilution reflects not only the stimulation of the humoral immune response but also highlights immunogenicity of the plant extracts. Compared to control, only NILE and NIRE showed visible hemagglutination at higher dilutions of the antiserum. Antibody titre at 50 mg/kg, 100

mg/kg and 200 mg/kg dose of NILE were 1/80, 1/160 and 1/320, respectively. In case of NIRE, antibody titre at 100 mg/kg and 200 mg/kg dose were 1/80 and 1/160, respectively. Therefore, NILE and NIRE demonstrated stimulation of murine humoral immunity as evidenced from the significant increase in the humoral antibody titre. Hemagglutination is a routinely used method to evaluate the effect of phytomedicins on the B-lymphocyte function, because B-lymphocytes are most crucial for promoting antibody based immunity against invading pathogens such as bacteria. This method on the other hand, provides evidence that certain phytocompounds could be used as immunogenic adjuvants along with conventional vaccines to provide better protection by stimulating the immune response. Using similar approach, Makare, *et al*., (2001) demonstrated that oral administration of a polyphenol rich fraction from *Mangifera indica* in mice elevated the anti-sRBC antibody titre multiple folds. Moreover, different monoterpene compositions from fruits were demonstrated to elevate the anti-sRBC hemagglutination titre value and the data was validated by increase in anti-sRBC PFC response (Raphael & Kuttan, 2003).

In the present study, various experiments were performed using murine peritoneal exudate macrophages to demonstrate the immunomodulatory activities of NILE, NISE and NIRE. The peritoneum cavity is populated with two sub-sets of macrophages. Around 90% of them are large peritoneal macrophages, expressing CD11b and F4/80 surface markers which are typical to macrophages. These cells disappear rapidly from the peritoneal cavity following lipopolysaccharide (LPS) or thioglycolate stimulation (Ghosn, *et al*., 2010). The remaining 10% are small peritoneal macrophages which express CD11b and F4/80 in lower levels but express MHC-II at higher extent. The *in situ* non-adherent murine peritoneal macrophages have higher expression of inducible NO synthase and IL-12 compared to the macrophages of the splenic origin (Liu, *et al*., 2006). Based on the morphology and surface molecular characteristics, the peritoneal macrophages are highly mature, possess greater phagocytic capacity than splenic macrophages. Wang, *et al*., (2013) demonstrated that macrophages of peritoneal origin are of larger size compared to splenic and bone marrow derived macrophages. Besides, peritoneal macrophages express significantly low levels of CD-80 which regulates T-lymphocyte activation and survival, CD-86 which regulates T-lymphocyte activation and survival, CD115 which is a colony stimulating factor-1 and Gr-1 which is myeloid differentiation antigen. Whereas expression of B7-H1 which regulates T- and B-lymphocyte activation or inhibition was found to be higher in peritoneal macrophages compared to other macrophage sub-sets (Wang, *et al*., 2013).

The present study demonstrated the effect of orally administered NILE, NISE and NIRE on the total peritoneal macrophage count in mice. The results demonstrate that only NILE stimulated significant ($P<0.001$) proliferation of macrophages. The increase in macrophage count at 200 mg/kg NILE, NISE and NIRE were 1.70 fold, 1.05 fold and 1.20 fold respectively compared to control. Similar immunostimulatory activity of other plant material like the edible tuber of *Dioscorea alata* L. demonstrated the elevation in murine peritoneal macrophage count (Dey, *et al*., 2014) and immunosuppressive activity by of *Diplazium esculentum* (Koenig ex Retz.) Sw demonstrated the dose dependent decrease in murine peritoneal macrophage count (Roy, *et al*., 2013). In a similar approach, Garcia, *et al*., (2002) demonstrated the modulation of peritoneal macrophage count by intraperitoneal injection of *Mangifera indica* extracts in male Wistar rats.

Phagocytosis is one of the first line of defence of the immune system which is chiefly mediated by phagocytes such as macrophages. In the present study, the effect of *N. indicum* extracts were evaluated for possible effect on the phagocytic activity of the murine peritoneal macrophages. The effect of NILE, NISE and NIRE on the reticulo-endothelial system comprising of mononuclear mobile and fixed-tissue macrophage was evaluated by the carbon-clearance test. These phagocytes play a profound role in the clearance of non-specific foreign particulates from the systemic circulation (Gokhale, *et al*., 2003). On injection of the colloidal Indian ink containing carbon particle through the tail vein, the rate of macrophage medicated clearance of the particles from the blood stream occurs at an exponential rate which could be measured in a time dependent manner using spectrophotometry. Gradual decrease in absorbance at 650 nm with time indicates the rate of carbon-clearance from the systemic circulation. Furthermore, peritoneal macrophages isolated from *N. indicum* extract treated mice were subjected to yeast phagocytic assay. Macrophages were co-cultured with heat killed yeast cells, which resulted in phagocytosis of the yeast cells by the macrophages. Among the three extracts, only NILE at 200 mg/kg demonstrated significant ($P<0.01$) increase in phagocytic capacity which was 1.43 fold higher than the control. In case of NISE, the phagocytic capacity was slightly (0.92 fold) decreased at the highest dose. Similar trend was observed in case of the phagocytic index with dose-dependent significant ($P<0.05$) increase of PI of NILE and NIRE at the highest dose. In a preliminary study, Bor and his group (1988) demonstrated that oleander extract induces phagocytosis in dog neutrophil leucocytes and predicted that the extract may promote healing process through efficient phagocytic process. Previously Muller, *et al*., (1991) showed stimulation in phagocytic

activity by a polysaccharide rich fraction from the aqueous extract of the oleander leaves. Moreover, Al-Farwachi (2007) showed that subcutaneous injection of aqueous leaf extract of Nerium stimulated phagocytic activity in experimental rabbit model. The present study therefore, remained in concurrence with the previous findings that Nerium leaf extract possess stimulatory activity on the phagocytes.

The present study investigated the *in vitro* myeloperoxidase, nitric oxide inhibitory and respiratory burst activities of the isolated murine peritoneal macrophage. Recognition and internalization of invading bacteria is the primary function of the macrophages. Inside the phagosome, activation of NADPH oxidase results in generation of superoxide anion, which is deprotonated to from O_2 and H_2O_2, from which the highly reactive hypochlorous acid (HOCl) is generated through the myeloperoxidase reaction (Hampton, *et al*., 1998). Generation of a plethora of oxygenated radical such as superoxide radical, H_2O_2, hydroxyl radical, singlet oxygen, HOCl, chloramines, nitric oxide, peroxynitrite, in order to kill the internalized pathogen, is termed as respiratory burst activity. Although the respiratory burst process is quintessential in acute inflammation, but constitutive release of these free radicals cause local tissue damage. In practice, LPS is utilized to activate macrophages to secrete pro-inflammatory mediator such as NO, TNF-a and IL-1b (Coligan, 2005). NO is released from the activated macrophages and functions as marker for inflammatory progression and cytotoxic activity (MacMicking, *et al*., 1997). LPS cause the activation inducible NO synthase (iNOS) to catalyse the conversion of L-arginine to L-citrulline by oxidizing the guanidino nitrogen of L-arginine to release NO. NO itself possess bactericidal activity and coupling with superoxide radical further generates highly reactive peroxynitrite radical. Therefore, suppression of NO release during inflammatory process has been a central idea behind the functioning of anti-inflammatory drugs (Liu, *et al*., 2012; Lee, *et al*., 2013; Saad, *et al*., 2011; Hung, *et al*., 2011). In the present study, the elevated level of NO due to LPS (20 µg/ml) was significantly ($P<0.001$) lowered by NILE, NISE and NIRE in a dose-dependent manner. The level of NO inhibition at 80 µg/ml was 0.47 fold, 0.51 fold and 0.61 fold for NILE, NISE and NIRE respectively. Dong and his group (2010) previously showed that a polysaccharide fraction from *N. indicum* flower stimulates NO production in macrophage RAW264.7 cells. However, the present study demonstrated the potent activity of *N. indicum* extracts to inhibit the expression of NO in LPS stimulated macrophages. The same has also been demonstrated on murine splenic lymphocytes stimulated with concanavalin A, which is discussed later.

NILE, NISE and NIRE were further studied for their effect on the respiratory burst activity and myeloperoxidase (MPO) level on murine peritoneal exudate macrophages. It was observed that although the respiratory bust activity was significantly increased ($P<0.001$) only in case of NILE, but the MPO level was reduced significantly ($P<0.001$) for all the extracts. Respiratory burst activity was measured at 630 nm, where increase in absorbance signifies increase in respiratory burst activity. The increase of respiratory burst in case of NILE, NISE and NIRE were 1.69 fold, 1.32 fold and 1.31 fold, respectively. On the other hand, the percentage of inhibition of MPO level at 100 μg/ml for NILE, NISE and NIRE were 16.00±1.64%, 7.17±1.68% and 10.64±0.83%, respectively.

It is interesting to note, that the increase of phagocytic activity and respiratory burst activity were not accompanied by the MPO release, which might seem to be paradoxical. However, it has been previously demonstrated that phagocytic activity elevates in MPO deficient granulocytes (Hasui, *et al*., 1991; Stendahl, *et al*., 1984; Gerber, *et al*., 1996). Respiratory burst is actually a process in which various free radicals are generated such as superoxide radical, NO, H_2O_2, peroxynitrite, hypochlorous acid (HOCl), hydroxyl radical etc (Hampton, *et al*., 1998). In this regard, it is essential to note that NILE, NISE and NIRE have also demonstrated potent free radical scavenging activity which has been discussed later. Gerber and her group (1996) have hypothesised that the phagocytic activity in the MPO deficient cells may be enhanced due to the increased receptor expression such as of complement 3b- or Fc-receptor which could be translocated from intracellular pool to the cell surface which is comparatively easier in the MPO deficient cells (O'Shea, *et al*., 1985). Stendahl, *et al*., (1984) further demonstrated that the extent of complement 3b- and Fc-receptor mediated phagocytosis is decreased in zymozan-activated MPO deficient cells when induced with extracellular MPO.

Murine peritoneal macrophages, under stimulation with NILE, NISE and NIRE had demonstrated significant ($P<0.001$) inhibition of cell-adhesion properties. The percentage inhibition of cell-adhesion were 21.52±2.06%, 10.32±1.32% and 9.92±2.41% respectively. During inflammatory situation due to microbial invasion in the body, the circulatory macrophages are recruited to the site of inflammation and enters the target tissue by adhering and passing between the endothelial cell lining of the blood vessels in an innate immune response termed as extravasation. P- and M-selectins and their carbohydrate counter ligands initially mediate rolling and tethering of the macrophages (Middleton, *et al*., 2002). Thereafter, the integrins and their ligands mediated firm cell adhesion. In this process, various

mediators such as IL-8 and macrophage inflammatory protein (MIP-1b), TNF-α, IL-1β and different chemokines play a vital role in activating the integrins on the surface of the macrophage (Carveth, *et al*., 1989; Detmers, *et al*., 1990). The present study demonstrated the inhibition of cell adhesion properties due to *N. indicum* extracts, which remains in accordance with other studies. Previous reports suggests that plant extracts with immunomodulatory or anti-inflammatory properties possess the potentiality to down-regulate the cell adhesion properties in phagocytes either by inhibiting expression of vascular cell adhesion protein-1 (VCAM-1) or P-selectin (Thounaojam, *et al*., 2012; Jadeja, *et al*., 2012; Kim, *et al*., 2014). Therefore, from the present study, it is quite evident that *N. indicum* holds the potentiality to modulate cellularity, phagocytic activity, respiratory burst, MPO release and cell adhesion properties of murine peritoneal macrophages.

The anti-inflammatory activity was evaluated on murine splenic lymphocytes, which were stimulated with Concanavalin A (Con A). Spleen functions primarily as a blood filtration apparatus in the body. However, it generates and maintains immunologically active cells which participate in recognition and elimination of foreign invading pathogens. Splenic lymphocytes predominantly regulate the pro-inflammatory conditions through cytokine production and modulation of the immunocompetent cells (Semaeva, *et al*., 2010). The white pulp of the spleen remains chiefly populated with the B- and T-lymphocytes. Con A is a lectin from the plant *Canavalia brasiliensis* and functions as a potent T-lymphocyte polyclonal activator (Andrade, *et al*., 1999) and also as a potent mediator of chronic inflammation through JAK/STAT3 pathway (Akla, *et al*., 2012). Con A at 5 µg/ml is considered the optimal dose for blastoid differentiation and activation of the lymphocytes (Chaudhuri & Chakravarty 1981; Forni, *et al*., 1987). Liu (2004) demonstrated that 5 µg/ml Con A results in optimal augmentation of proliferation of the T-lymphocytes. Therefore, Con A is routinely used in similar studies to activate lymphocytes and to study the possible immunomodulatory and anti-inflammatory activities of plant extracts and bioactive compounds (Checker, *et al*., 2012; Amro, *et al*., 2013; Jin, *et al*., 2013; Kenny, *et al*., 2013; Moriyama, *et al*., 2003; Shi, *et al*., 2012). Besides, con A is also routinely used to induce hepatitis in experimental mice model (Tiegs, *et al*., 1992; Tegris, 1997).

NO is a potent pro-inflammatory mediator. Plant lectins such as Con A induces murine mononuclear cells to express NO especially when both adherent and non-adherent cells are co-cultures (Andrade, *et al*., 1999). Various chronic diseases such as multiple sclerosis, arthritis, juvenile diabetes, asthma, psoriasis, systemic sclerosis and ulcerative colitis are

associated with chronic release of nitric oxide (Kroncke, *et al*., 1998). The present study demonstrated the inhibitory effect of *N. indicum* extracts on the release of NO from Con A stimulated murine lymphocytes. The inducible form of NO is primarily stimulated by pro-inflammatory signals such as TNF-α, IL-1β, IFN-γ (Andrade, *et al*., 1999). However, in this case, under 5 μg/ml Con A stimulation and in presence of *N. indicum* extract (0-80 μg/ml), the level of TNF-α was down-regulated, whereas the level of IFN-γ was up-regulated. Under same condition, the inversed expression of NO with IFN-γ (R^2= -0.988, -0.845, -0.958) signifies that the inhibition of NO was not IFN-γ mediated. In the present study, NILE, NISE and NIRE had already demonstrated the ability to inhibit NO expression *in vitro*, in addition to *in vitro* capacity to inhibit LPS induced NO expression in murine peritoneal macrophages. The dose-dependent correlation of NO expression with IL-10 was negative (R^2= -0.965, -0.717, -0.973), while TNF-α was positive (R^2= 0.978, 0.697, 0.926). Similar observations were incurred by two independent studies who demonstrated that the inhibition of iNOS is medicated by suppression of TNF-α through IL-10 activity (Gazzinelli, *et al*., 1992; Oswald, *et al*., 1992). IL-10 is a potent anti-inflammatory meditator. Besides, IL-10 is well-known inhibitor of NO biosynthesis (Cuhna, *et al*., 1992; Hamid, *et al*., 1993; Kallio, *et al*., 1997; Huang, *et al*., 2002). Ameredes, *et al*., (2001) showed the elevation of NO production in IL-10 knockout mice. Therefore, from the present observation, it could be stated that down-regulation of NO was probably mediated by NILE, NISE and NIRE influenced inhibition of TNF-α as well as up-regulation of IL-10. The dual effect of *N. indicum* extracts to down-regulate both NO and IL-4 may prove beneficial in asthma patients since both the factors are critically associated with asthmatic conditions (Batra, *et al*., 2007).

Cytokines are known as in-house immunoregulatory molecules which are not only responsible for the fine tuning of the immune response but also mediates proper communication in immune system for activation, growth, development and functionality of the immunocompetent cells. In reference to their activities during immune response in inflammation, cytokines can be divided into two broad categories i.e. pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines mediate immediate immune response during the acute inflammation but proved to be deleterious in chronic inflammation. Even though the anti-inflammatory cytokines perform critical functions in activating and regulating different sub-sets of immunocompetent cells, but they also play a crucial role in controlling the pro-inflammatory immune response which has proven to be beneficial in protection against chronic inflammations and auto-immune disorders. The present study deals

with the effect of NILE, NISE and NIRE on mitogen activated expression of pro-inflammatory (IL-2, IFN-γ, TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokines in murine splenic lymphocytes. Activation of mast cells, eosinophils and basophils and also promotion of IgM to IgE class switching in B-lymphocytes are mediated by IL-4. It is a potent mediator of allergic inflammation and promotes conditions such as asthma and atopic syndrome. Besides, IL-4 has also been found to be associated with the development of allergic airway inflammation and airway hypersensitivity (Karp, *et al*., 1996) as well as may play a key role in inflammatory arthritis (Ohmura, *et al*., 2005). IL-10 is an anti-inflammatory cytokine which repress pro-inflammatory signals and restricts unnecessary tissue damage during inflammatory response (Ouyang, *et al*., 2011). It is also considered as immunosuppressive cytokine due to its property of indirectly inhibiting antigen-specific T-cell activation (de Vries, 1995). IFN-γ mediates antiviral and anti-tumor environment (Schroder, *et al*., 2004) apart from activation of macrophage, stimulating MHC expression and amplifying leukocyte migration. IFN-γ has clinically been used in the treatment of prophylaxis of chronic granulomatous disease, visceral leishmaniasis and also possess utility in leprosy, cutaneous leishmaniasis, and disseminated atypical mycobacterial infection (Murray, 1996). IL-2 mediates the innate response against microbial infections, proliferation of CD4+ and CD8+ T-lymphocytes and self/foreign antigen recognition is mediated by IL-2. It is also a favourable immunotherapeutic agent for the next generation treatment of metastatic melanoma, acute myelogenous leukemia, and metastatic renal cell carcinoma (Atkins, 2002). TNF-α is a potent mediator of inflammation and exerts tissue damage in sepsis, tumor cachexia and autoimmune diseases (Pfeffer, 2003). Elevated level of TNF-α has been found to be associated with autoimmune conditions such as rheumatoid arthritis, psoriasis and Crohn's disease (Scheinfeld, 2004). In the present study, *N. indicum* demonstrated its potentiality to down-regulate TNF-α and IL-4 expression and up-regulate IL-2, IFN-γ and IL-10 expression in activated lymphocytes *in vitro*. *N. indicum* also down-regulates the TNF-α level in murine hepatocytes which was demonstrated under hepatoprotective evaluation. This has been discussed later.

IL-10 possess inhibitory effects on TH1 cytokine expressions. But in this case, increase in IL-10 expression had no suppressive effect on IL-2 or IFN-γ expression (both TH1 and pro-inflammatory), even though TNF-α was down-regulated significantly. In addition, two major pro-inflammatory mediators, NO and TNF-α were significantly down-regulated by NILE, NISE and NIRE. TNF-α and NO shared positive correlation (R^2= 0.978, 0.697, 0.926).

Moreover, the present study demonstrated the potentiality of *N. indicum* extracts to ameliorate delayed type hypersensitivity reactions in murine paw edema model. Besides, Erdemoglu, *et al*. (2003) also have demonstrated i*n vivo* anti-inflammatory activity of *N. indicum* leaf and flower extracts on paw edema model. Therefore, in the present study, down-regulation of TNF-α and IL-2 and up-regulation of IL-10 may contribute to the anti-inflammatory activities of *N. indicum*.

High negative correlation resided between IL-10 and TNF-α in case of NILE and NIRE (R^2= -0.9827, -0.934) which may prove to be beneficial target in the conditions such as psoriasis, ulcerative colitis, psoriatic arthritis and Crohn's disease (Cutler & Brombacher, 2005). Besides, increase in NILE mediated IL-2 level may prove beneficial in autoimmune colitis, rheumatoid arthritis, allograft rejection, as IL-2 is primarily associated with immune tolerance (Lee & Margolin, 2011).

The complex immunological associations during inflammatory conditions are mediated by fine regulation of pro- and anti-inflammatory cytokines and formation of arachidonic metabolites through the enzymatic cascade of cyclooxygenase (COX). Among the two isoforms of COX, COX-1 is constitutively expressed whereas COX-2, the inducible isoform, is predominantly activated during inflammatory conditions. Prostaglandins (PG) are the metabolites of arachidonic acid which are generated through an enzymatic cascade of COX and PG synthase, that leads to the cardinal signs of inflammation i.e. redness due to increase of blood flow, swelling due to vasodilation and pain due to induction of peripheral sensory neurons (Phipps, *et al*., 1991). PGs are the terminal mediators of hyperalgesia, generation of fever, increase in vascular permeability as well as primarily responsible for vascular diseases and angiogenesis. COX is considered as the central molecule of inflammation and therefore, COX inhibitors are routinely used as therapeutics to inhibit PG synthesis to eliminate inflammation in a wide range of diseases (Chizzolini & Brembilla, 2009). Previously, Singhal (2012) demonstrated COX-1 and COX-2 inhibitory activities of methanolic extract of *N. indicum* flower extract. In the present study, compared to control, the increase of COX-1 and COX-2 expression at 0 µg/ml was attributed to Con A induction. The dose dependent inhibition of both the COX isoforms were evident with increased concentration of *N. indicum* extract. At 80 µg/ml, the extent of inhibition of total COX, COX-1 and COX-2 for NILE were 58.75%, 51.63% and 59.94% respectively; for NISE were 22.70%, 19.50% and 44.89%; for NIRE were 14.10%, 15.00% and 31.47%, respectively. Cardiac glycosides at present are considered as one of the novel candidate for anti-inflammatory drug as well as various such

compounds isolated from natural source such as quabain, digitoxin, digioxin, etc. are effective against different cancer cells (Newman, *et al*., 2008). Afaq, *et al*., (2004) demonstrated that the tropical application of oleandrin, a cardiac glycoside isolated from oleander leaves, provides significant decrease in TPA (skin tumor promoter) induced edema, hyperplasia, epidermal ornithine decarboxylase activity and COX-2 expression in mice. The COX inhibitory effect of NILE, NISE and NIRE were also reflected by the gradual decrease of PGE_2, the level of which is dependent on metabolism of arachidonic acid by COX. At 80 µg/ml, the inhibition of PGE_2 level by NILE, NISE and NIRE were 55.94%, 28.71% and 36.49% respectively. In case of NILE and NISE, high positive correlation (R^2= 0.981 and 0.898) resided between total COX and COX-1 demonstrating that total COX activity was influenced more by COX-1 activity than COX-2. In case of NIRE, COX-2 activity had higher influence on total COX activity compared to COX-1, as shown by high correlation value (R^2= 0.982). NO is a well-known inducer of COX activity (Salvemini, *et al*., 1993). This was even evident in case of NILE and NIRE, where the inhibition of NO has profound effects on down-regulation of COX activities as demonstrated by the high positive correlations for both COX-1 and COX-2 activities. Moreover, the PGE_2 level, which is a direct measure of COX activity, have demonstrated decent correlation with COX activities only in case of NIRE. This signifies that there might have other factors in action for the regulation of PGE_2 level and COX activities.

The anti-inflammatory activity of *N. indicum* was further visually confirmed through *in vivo* DTH study. DTH response is primarily initiated by the activated T_H cells which result in localized inflammatory response upon encountering antigens. An array of cytokine secreted by T_H1 cells promote extravasation of blood mononuclear cells to accumulate at the site of response. Accumulated macrophages result in release of NO, TNF-α and oxygenated free radicals which mediate the effector part of the localized DTH response (Kindt, *et al*., 2008). In the present study, the lowest increase of paw edema were found in case of NILE treated groups and highest in case of untreated control, which signifies potent anti-inflammatory activity of NILE. The results substantiate the findings of Erdemoglu, *et al*., (2003) who previously demonstrated that Oleander flower and leaf extract hold the potentiality to reduce edema formation by sub-cutaneous carrageenan challenge. DTH reaction is basically governed by certain immunological events like NO and pro-inflammatory cytokine release, cell adhesion and formation of chemical species, all of which are inhibited by *N. indicum* extracts at varying degrees. Moreover, *N. indicum* extract mediated suppression of COX

activities and its associated PGE_2 synthesis possibly produced lower edema in extract treated groups compared to control, giving rise to a visible evidence of the anti-inflammatory activity of *N. indicum*.

In last two decades, the basic idea of the causative effects of disease whether microbial infections, autoimmune disorders or inflammatory diseases, their progression, clinical and pathological manifestation had went through tremendous changes. In most of the cases, reactive oxygen and nitrogen species, harmful free radicals and reactive metabolites were found to play a cardinal role in the pathogenesis of diseases. Mechanism based screening of herbal medicines have not only revealed the beneficial effects of herbal medicines through their antioxidative properties, different health-enhancing and disease-preventing foods were also identified which provide antioxidative defences through scavenging of free radicals. Previously, two different groups of researchers back to back studied the free radical scavenging activities of the flower fractions. Gayathri, *et al*., (2011a) studied the ABTS and DPPH radical scavenging assays using glycosidic and nonglycosidic fractions of oleander flower. Thereafter, Singhal and Gupta (2012) studied *in vitro* reducing power, lipid peroxidation, DPPH, ABTS, superoxide anion, hydroxyl radicals and metal chelation activities of methanolic extract of Oleander flower. However, the complete antioxidant and free radical scavenging profiles of the leaf, stem and root remained unexplored.

Evaluation of antioxidant capacity through electron transfer methods are the most popular antioxidant assays (Huang, *et al*., 2005). The assays are based on the following reaction: *oxidant + electron from antioxidant → reduced oxidant + oxidized antioxidant.* Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) is one of the widely used electron-transfer assay to screen antioxidant capacity of herbal compounds and functional foods. In this assay, oxidation of ABTS by potassium persulfate generates purple oxidizing chromophore $ABTS^{\bullet -}$, neutralization of which is achieved by the electron donating capacity of the test material. The concentration of test material giving same percentage of inhibition of $ABTS^{\bullet -}$ compared to the same of trolox is the TEAC of the test material. Previously, Gayathri, *et al*., (2011a) showed that the extent of $ABTS^{\bullet +}$ inhibition for hydro-ethanolic extract, glycosidic and non-glycosidic fractions were 92.40±0.69%, 80.28±0.35% and 83.99±1.27%, respectively at 100 µg/µl concentration. In the present study, NILE, NISE and NIRE demonstrated convincing TEAC value of 0.31±0.00, 0.325±0.00 and 0.396±0.00, respectively with the extent of inhibition of 74.83%, 83.98% and 68.65%, respectively at 10 mg/ml. The TEAC values of numerous compounds and dietary

supplements have already been reported (Cao, *et al*., 1998; Pietta, *et al*., 2000; Gil, 2000; Proteggente, *et al*., 2002; Nielsen, *et al*., 2003; Pellegrini, *et al*., 2003). Similarly, different bioactive compounds such as ascorbic acid (1.05), α-tocopherol (0.97), ferulic acid (1.90) and p-coumaric acid (2.00), caffeic acid (1.00), quercetin (3.00) and kaempferol (1.00) also possess high TEAC value (Huang, *et al*., 2005). DPPH (diphenyl-1-picrylhydrazyl) is another simple spectrophotometric assay which evaluates overall antioxidant capacity of test material. DPPH is a stable organic nitrogen radical which has an absorbance maxima at 515 nm. The purple colour of soluble DPPH fades with gradual increased concentration of the antioxidant compounds. In a previous study, Gayathri, *et al*., (2011a) showed that DPPH percentage of inhibition for hydro-ethanolic extract, glycosidic and non-glycosidic fractions of the flower at 100 µg/µl were 72.20±0.69%, 43.23±1.55% and 60.24±0.86% respectively. Singhal and Gupta (2012) also demonstrated that the methanolic fraction of the flower scavenge 95.64% DPPH at 500 µg/ml concentration with an IC_{50} value of 193.37 µg/ml. However, in the present study, NILE, NISE and NIRE demonstrated excellent DPPH scavenging activity with very low IC_{50} value. NISE demonstrated better DPPH scavenging activity than NIRE and NILE.

The ABTS radical is a non-physiological free radical which is not found in the living system. Thermodynamically, any compound possessing lower redox potential than that of ABTS (0.68 V) could scavenge ABTS (Prior, *et al*., 2005). Moreover, the long lived DPPH radical does not possess any similarity with the highly reactive and transient peroxyl radicals in the body, responsible for lipid peroxidation (Huang, *et al*., 2005). Thus, many antioxidants which reacts with peroxyl radicals may react slowly or may even remain insensitive to DPPH. ABTS and DPPH assays evaluate the overall antioxidant capacity of test material however, scavenging assays, precisely aiming towards inhibition of a certain free radical is of current interest, because in the free radical forming cascade in the living system, different free radicals perform different functions and their activity may in turn affect other free radicals.

Hydroxyl radical (OH•) holds tremendous potential to damage cellular components, DNA, initiate lipid peroxidation and cause carcinogenesis, mutation and cytotoxicity (Packer & Ong, 1998). In biological system, OH• is generated through Fenton reaction when hydrogen peroxide (H_2O_2) reacts with iron (Fe^{2+}). OH• is extremely short lived which makes it almost impossible for the antioxidant materials to directly react and scavenge OH•. However, it is possible to restrict the formation of OH• by inhibiting different factors of the Fenton reaction. The strategy includes, deactivation of free metal ions (Fe^{2+}) or breakdown of H_2O_2 to H_2O and

O_2. In this assay, $OH^{\bullet}$ was generated at pH 7.4 by the reaction of ascorbic acid, H_2O_2 and iron-EDTA complex, resulting in degradation of 2-deoxy-2-ribose. Increased concentration of Nerium directly scavenged the OH•, which was evident form inhibition of 2-deoxy-2-ribose degradation. The results of the assay revealed excellent OH• scavenging activity of the plant extracts which were even better than the activity of the classical OH• radical scavenger mannitol. In a previous study (Singhal & Gupta, 2012), methanolic extract of the flower demonstrated IC_{50} value of 211.29 µg/ml. However, the activity of NILE, NISE and NIRE were better than the previous report as evident from the lower IC_{50} values.

Superoxide radical ($O_2•^-$) is generated by addition of one electron to molecular oxygen. In biological system it is generated through enzymatic reaction catalysed by NADPH oxidase, xanthine oxidase and non-enzymatically by redox active compounds such as semi-ubiquinones, present in the mitochondrial electron transport chain (Augusto & Miyamoto, 2011). Mitochondria mediated formation of $O_2•^-$ has been implicated in different derivative process like cardiotoxicity, apoptosis, alcoholism related tissue damage and aging (Turrens, 1997). Oxidation of lipid however, does not get directly initiated by $O_2^{\bullet-}$, rather formation of singlet oxygen from $O_2^{\bullet-}$ results in oxidation to lipid moiety. In most living cells dismutation of $O_2^{\bullet-}$ is catalysed by superoxide dismutase (SOD) enzyme which generates O_2 and H_2O_2. NILE, NISE and NIRE, in the present study, demonstrated comparatively similar $O_2^{\bullet-}$ radical scavenging activities. The activity of standard quercetin was higher than that of the extracts.

Nitric oxide (NO) is a free radical generated by the conversion of L-arginine to citrulline by enzymatic action of NO synthase. As a free radical, NO is relatively non-reactive under physiological and pathophysiological conditions (Beckman & Koppenol, 1996). NO is also a signalling molecule in cardiovascular, immune and nervous systems and play an active role in microbial killing of the host cells. However, NO could couple with $O_2^{\bullet-}$ to generate peroxynitrite radical, which possess immense potential to cause cellular damage. Direct tissue toxicity and septic shock associated vascular collapse are linked to chronic release of nitric oxide radical. Moreover, conditions such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis possess association with NO up-regulations (Miller, *et al*., 1993). In the present study, NILE, NISE and NIRE have demonstrated NO inhibitory activity on LPS stimulated macrophages, Con A stimulated lymphocytes and from hepatic system under *in vitro* evaluation. Similarly in the present assay, the plant extracts have demonstrated potent NO inhibitory activity, which was even effective than the standard curcumin. The NO

inhibitory activity of the extracts would not only cut down the extent of reactive peroxynitrite formation, but also secure low expectancy of chronic inflammatory symptoms.

The simplest peroxide, H_2O_2 is a highly oxidizing agent, capable of inactivating enzymes by directly oxidizing the thiol groups (-SH). Formation of H_2O_2 in biological system could possibly occur through enzymatic reaction catalysed by different oxidases. However, SOD mediated dismutation of $O_2^{\bullet-}$ radical continuously generates H_2O_2. Even though H_2O_2 is a potent two electron-oxidant, most damaging effect of H_2O_2 occurs through the formation of H_2O_2 derived secondary free radicals such as OH• and $NO_2^{\bullet}$, hypochlorous acid (HOCl) and related species (Augusto & Miyamoto, 2011). In phagocytic cells, H_2O_2 mediated uncontrolled generation of HOCl, NO_2^- to $NO_2^{\bullet}$ through the action of MPO, results in tissue injury and contributes to the pathogenesis of several diseases (Klebanoff, 2005; Davies, *et al*., 2008). Under immunological investigations, NILE, NISE and NIRE have shown their potentiality to reduce MPO level significantly in murine macrophage cells. However, the *in vitro* H_2O_2 scavenging activity of the extracts were considerably low compared to the standard sodium pyruvate.

Peroxynitrite anion ($ONOO^-$) though short-lived, but is a highly reactive free radical, generated by the coupling of $O_2\bullet^-$ and $NO^{\bullet}$. Both $ONOO^-$ and its protonated from peroxynitrous acid (ONOOH) can initiate one or two electron oxidation of cellular biomolecules (Szabo, *et al*., 2007). $ONOO^-$ could inhibit antioxidative enzymes like SOD and glutathione reductase, causing inability of cells to combat free radicals. $ONOO^-$ mediated protein aggregation by oxidation of α-synuclein and microtubule associated tau protein results in neurodegenerative complications (Paxinou, *et al*., 2001; Reynolds, *et al*., 2006). $ONOO^-$ activates matrix metalloproteinases, cytochrome c and protein kinase C-ε through nitration, which cause induction of pro-inflammatory cascade and apoptotic pathways. Furthermore, $ONOO^-$ cause impairment in cellular signalling, ionic imbalance, enhancement of cellular inflammation, release of mitochondrial death factors and genotoxic damage through a various activities such as oxidation, nitration, protein activation, dimerization, nitrosation etc (Szabo, 2003; Szabo, *et al*., 2007). One of the most deleterious effect of $ONOO^-$ comes from its ability to cause lipid peroxidation, which generates malondialdehyde, conjugated diene, formation of nitrito-, nitro-, nitrosoperoxo- and nitrated lipid oxidation adducts (Rubbo, *et al*., 1994). In the Evans blue bleaching assay, decolouration of Evans blue resulted due to the oxidation by $ONOO^-$. NILE, NISE and NIRE demonstrated convincing dose-dependent $ONOO^-$ scavenging

activity compared to standard gallic acid, as demonstrated by prevention of Evans blue bleaching with increase in the extract concentrations.

Singlet oxygen ($^1O_2$) is the major cytotoxic species present in eukaryotes (Devasagayam & Kamat, 2002). $^1O_2$ is primarily generated through UV mediated photosensitization reaction resulting in energy transfer from triplet state of photosensitizer to ground state of molecular oxygen (Cadenas, 1989). $^1O_2$ can travel long distance in cellular microenvironment due to its longer half-life and cause damage to biomolecules. Lipid peroxidation caused by $^1O_2$ results in haemolysis of erythrocytes, damage to cardiomyocytes and degradation of lipid membrane. $^1O_2$ mediated oxidation of histidine, tryptophan, methionine and tyrosine generates sulphoxide and short-lived endoperoxides, which are toxic to other cells (Devasagayam & Kamat, 2002). Moreover, the effect of $^1O_2$ on DNA strands are generally strand breakage and/or base substitution. Different plant based compounds are known to scavenge $^1O_2$ primarily through any one of the two mechanisms i.e. through simple energy transfer (carotenoids) or electron transfer (phenolic). In the present study, NILE, NISE and NIRE at 200 μg/ml concentration demonstrated moderate $^1O_2$ scavenging activity. The activity of the reference compound lipoic acid, was higher than that of the extracts.

Amongst all the mammalian peroxidase, MPO possess the unique capability to catalyse the oxidation of Cl^- to HOCl which possess strong oxidizing and chlorinating capacities (Harrison & Schultz, 1976; Klebanoff, 1999). The process chiefly takes place in activated neutrophils and macrophages at the site of inflammation. The double bonds in the unsaturated fatty acids and cholesterol are primarily attacked by HOCl resulting in peroxidation or formation of chlorohydrin (Spickett, *et al*., 2000). HOCl reacts rapidly with proteins, DNA (Prutz, 1996), lipids (Winterbourn, *et al*., 1992), cholesterol (Carr, *et al*., 1996), free thiols and disulfides (Prutz, 1996). The selective oxidizing nature of HOCl preferentially oxidizes certain amino acids such as methionine, cysteine, tryptophan, lysine etc (Hawkins, *et al*., 2003). The *N. indicum* extracts under immunological evaluation demonstrated the potentiality to down-regulate MPO level in murine peritoneal macrophage. NILE, NISE and NIRE in the present study also demonstrated higher HOCl scavenging activities than that of the standard ascorbic acid. Therefore, the inhibitory effect *N. indicum* extracts confirm reduced HOCl mediated tissue damage during the inflammatory conditions.

In biological microenvironment, transition metals are capable of damaging nuclear proteins, DNA, inhibit enzymes and degrade lipid membrane through oxidative reactions (Stohs &

Bagchi, 1995; Flora, *et al*., 2008) which ultimately results in neurotoxicity, hepatotoxicity and nephrotoxicity (Stohs & Bagchi, 1995; Chen, *et al*., 2001). The dual oxidation state of iron enables it to donate or accept electrons and participate in redox reactions which is crucial for different biological process. However, this property is harmful from the oxidative point of view. In excess, iron could mediate the reaction of H_2O_2 and $O_2^{\bullet -}$ through Haber-Weiss reaction to generate OH• (Valko, *et al*., 2004) which in turn, mediates tremendous damage to cellular biomolecules. Overload of iron is implicated in a wide variety of pathological conditions such as hepatotoxicity, cardiovascular disorders, cancer, neurodegenerative disease, diabetes, hormonal imbalance and immune dysfunction (Valko, *et al*., 2005). Iron-induced oxidative stress primarily has two implications: **(a)** failure in redox regulation resulting in DNA damage, lipid peroxidation and oxidative protein damage; **(b)** free radical-induced activation of signal transduction pathways (Valko, *et al*., 2005). A plethora of evidence suggests the desperate need of metal chelators for the amelioration of metal induced oxidative stress (Reeder, *et al*., 2008). The results of the iron chelation assays suggests decrease in dose-dependent colour formation with ferrozine in presence of NILE, NISE and NIRE, indicating their potent iron chelating properties. The extent of inhibition of Fe^{2+} for NILE, NISE and NIRE were around 38.91, 16.06 and 16.78%, respectively at 120 µg/ml concentration. Even though, the iron chelating capacity and H_2O_2 scavenging activity of none of the extracts were high enough, but from their excellent OH• scavenging activity, it could be postulated that the potent inhibition of OH• resulted from direct inhibition of OH• by donation of proton, but not through inhibition of the Fenton reaction.

A common target for most of the free radicals is the lipid membrane of the cells and organelles, degradation of which causes serious pathophysiological implications in various diseases such as neurodegeneration, inflammation, infection, gastric and nutritional diseases (Repetto, *et al*., 2012). Formation of ferry-perferryl complex or OH• catalysed by iron, accelerates the process of lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. Lipid membranes, due to containing high amount of polyunsaturated fatty acids and transition metals, are vulnerable to oxidative and nitrosative stress (Chance, *et al*., 1979; Halliwell & Gutteridge, 1984). In the present experiment, NILE, NISE and NIRE have demonstrated moderate lipid peroxidation inhibitory activities. The activity of standard Trolox was much higher than the extracts. However, NILE, NISE and NIRE demonstrated convincing *in vivo* lipid peroxidation inhibitory activity when evaluated for anti-diabetic and hepatoprotective capacity.

Among estimated 1200 species of plants used for the treatment of diabetes in traditional and ethnopharmacological practices (Hsu, *et al*., 2009), *N. indicum* is one such plant which is extensively used in ethnomedicine for its anti-diabetic capacities around the world (Bnouham, *et al*., 2002; Hussain, *et al*., 2013; Jouad, *et al*., 2001; Rachid, *et al*., 2012; Tahraoui, *et al*., 2007) as well as mentioned in Ayurveda for the same (Sudha, *et al*., 2011; Middha, *et al*., 2012). It is interesting to note that, very recently in a survey at Malda district of West Bengal, India, by Saha, *et al*., (2014), *N. indicum* was found to be used in one of the anti-diabetic formulations practiced by the local tribal people.

Diabetes is one of the most notorious chronic disorder and a challenge to global healthcare system with an estimated 382 million global cases in 2013 and expected 55% increase (592 million) by 2035 (International Diabetes Federation, 2013). It sounds horrible that every 6 seconds, a person dies from diabetes and it has been estimated that by the end of 2013, globally 5.1 million deaths might have occurred due to diabetes, costing $546 billion USD which accounted for 11% of expense in global healthcare (International Diabetes Federation, 2013). Today, a staggering 80% people suffering from diabetes belong to low- and middle-income countries (International Diabetes Federation, 2013) and due to easy availability and low cost, traditional medicine is the primary therapeutic approach in those countries. Diabetes is a chronic disorder characterized by impairment of carbohydrate, protein and fat metabolism resulting due to deficiency in insulin secretion accompanied by varying degree of insulin resistance (Jarald, 2008). Malfunction in glucose absorption leads to hyperglycaemic condition where uncontrolled elevation in glucose level in blood affects body tissue over time and subsequently turns into a lethal health complication (International Diabetes Federation, 2013).

In the present anti-diabetic evaluation of *N. indicum* extracts, type 1 diabetes was induced in mice by alloxan which is an oxygenated pyrimidine derivative, results in insulin-dependent diabetes mellitus exclusively in rodents. Selective uptake of alloxan as a toxic-glucose analogue in pancreatic β-cells results in different disorders such as inhibition of glucokinase enzyme essential for carbohydrate metabolism, impairment of intracellular calcium homeostasis and free radical mediated tissue toxicity, ultimately leading to necrotic death of β-cells (Jarald, 2008). The present study focuses on the diabetes associated antihyperglycemic, antihyperlipidemic and antioxidative capacities of oleander on alloxanized mice.

Hyperglycaemia in diabetes leads to critical condition and therefore, modulation of carbohydrate metabolism by inhibition of pancreatic α-amylase activity has become an essential therapeutic approach in diabetes. Pancreatic α-amylase, a major enzyme of the digestive system, responsible for the initial step of catalysis of starch to maltose, maltotriose and various α-(1-6) and α-(1-4) oligoglucans. These are further degraded by α-glucosidases yielding glucose, which moves to blood stream upon absorption. Rapidly conversion of the dietary starch to glucose cause elevated post-prandial hyperglycaemia (Sudha, *et al*., 2011). Eichler, *et al*., (1984) previously demonstrated that the post-prandial glucose level directly correlates with the pancreatic α-amylase activity. Therefore, under diabetic condition, regulation of both α-amylase and α-glucosidases remains quintessential from the therapeutic point of view. Ishikawa, *et al*., (2007) reported that anti-hyperglycaemic potentiality of *N. indicum* is through the inhibition of α-glucosidases like maltase and sucrose. They also isolated chlorogenic acid fractions from the hot water leaf extracts of Nerium and demonstrated that the compounds inhibited α-glucosidases through a non-competitive mechanism. The average IC_{50} value was calculated to be 3.05 mM. However, in this respect, the α-amylase remained unchecked. In this study, dose dependent α-amylase inhibitory activity of the extracts concludes that NILE, NIRE and NIRE could be utilized as an anti-nutritional supplement to block the breakdown of carbohydrates to delay the intestinal absorption of glucose. Therefore, the extracts possess inhibitory effect on both the primary enzymes of complex carbohydrate catabolism and thereby cause delay in primary breakdown of carbohydrates to monosaccharides. This leads to slower absorption of glucose and subsequently lower postprandial blood glucose level.

Regulation of blood glucose level is one of the quintessential parameter in anti-diabetic strategy and various plant extracts were reported to exert anti-hyperglycaemic activities through protection of pancreatic β-cells from free radical medicated damage, restoration of β-cells activity, stimulation of insulin release, elevation of peripheral glucose absorption etc (Mohammad, *et al*., 2012). Once hyperglycemia becomes apparent, function of β-cell gradually decreases, glucose-induced insulin secretion becomes low and degranulation of β-cells accompanied with their decrease in number is often detected (Porte, 1990; DeFronzo, *et al*., 1992; Yki-Jarvinen, 1992; Vinik, *et al*., 1996). A previous anti-hyperglycaemic study with *N. indicum* aqueous extract showed no sub-acute glucose reduction at 500 mg/kg dose (Sikarwar, *et al*., 2009). In alloxan induced diabetic model, the researchers demonstrated that after a week of treatment, blood glucose level were around 0.40, 0.27, 0.48 and 0.81 fold

lower in case of *N. indicum* methanol, chloroform, aqueous and petroleum ether extract compared to diabetic control. On the contrary, Ishikawa, *et al*., (2007) showed that *N. indicum* water extract lowers blood glucose level in maltose and sucrose loaded rats but at very high dose of 16 g/kg. They further reported that the blood glucose lowering capacity of *N. indicum* water extract was only specific towards disaccharide and had no effect on glucose loading. Nevertheless, in the present study, blood glucose levels were found to be significantly lower in the treated diabetic animals both after 20 consecutive days of treatment. Among the groups, blood glucose lowering capacity of NILE at 200 mg/kg was found to greater than the reference drug glibenclamide. Similar lowering of blood glucose level was also found under hepatoprotective evaluation.

Cytotoxicity to pancreatic β-cells due to alloxan induced DNA alkylation and production of free radicals, leads to preliminary islet inflammation followed by gradual infiltration of macrophages and lymphocytes. This results in downfall of insulin level and eventually leads to persistent hyperglycaemic condition. Direct free radical mediated cytotoxicity to β-cells cause lower insulin level, leading to abnormal glucose metabolism. Alloxan inhibits glucokinase enzyme causing inhibition of glucose induced insulin secretion. Besides, it causes reduction in glucose oxidation and ATP formation that further leads to the inhibition of insulin secretion (Lenzen, 2007). In a previous study of anti-hyperglycaemic activity of oleander, Bas, *et al*., (2012) demonstrated that the insulin level in diabetic mice with high fat diet was elevated around 3.30 fold compared to control, which was attenuated around 50% by treatment with oleander distillate at 750 μg/ml dose. In the present study, insulin level in the an untreated diabetic animals was decreased 0.35 fold compared to non-diabetic animals. The insulin level, due to treatment with 200 mg/kg dose of NILE, NISE and NIRE were elevated around 2.07 fold, 1.34 fold and, 1.38 fold, respectively compared to diabetic animals. In fact, NILE high group resulted in 1.04 fold higher insulin level than standard glibenclamide treated group. Improvement of the insulin level accompanied with α-amylase inhibitory activity would aid in slower release of glucose and its faster absorption in blood, thereby ensuring a better regulation of hyperglycaemic condition.

Oral glucose tolerance test (OGTT) is crucial to evaluate body's efficiency to handle sugar and concludes regarding the risk of getting diabetes. Around 30% cases of previously undiagnosed diabetes remains undetected when evaluated alone using fasting glucose level parameter (WHO, 2006). OGTT is the only means for detection of impared glucose tolerance in asymptomatic people. In a previous report, however, with lacking data of the diabetic

control, Sikarwar, *et al*., (2009) demonstrated that treatment with chloroform extract of *N. indicum* leaves exerts higher glucose tolerance in rats compared to methanolic, aqueous and petroleum ether extract of the same. NILE, NISE and NIRE in the present study, resulted in the improvement in glucose tolerance in the diabetic animals, compared to control. The blood glucose level on 3 h post-glucose oral feeding were 79.00±14.93 mg/dl, 325.66±12.34 mg/dl, 76.66±13.42 mg/dl, 135.33±9.71 mg/dl and 151.33±7.02 mg/dl, respectively for normal control, diabetic control, NILE high, NISE high and NIRE high group. This suggests improvement of peripheral glucose utilization after 20 consecutive days of treatment. This resulted due to the amelioration of impaired glucose metabolism and insulin secretion, which further increased the peripheral insulin sensitivity and the rate of glucose clearance from the blood in the extract treated animals.

Glycated haemoglobin (HbA1c) is formed irreversibly by non-enzymatic coupling of blood glucose with the amino group of valine and lysine residue of haemoglobin. HbA1c is a direct measure of blood glucose as the amount of HbA1c is proportional to glucose load in blood. The correlation between HbA1c and retinopathy is similar to that of glucose load (WHO, 2011). In current study, the elevated HbA1c level in diabetic control was eventually lowered due to administration of the extracts. Compared to diabetic control, HbA1c level was 0.72 fold, 0.84 fold and 0.86 fold lowered in 200 mg/kg dose of NILE, NISE and NIRE. Bas, *et al*., (2012) reported similar result with oleander shoot distillate witch demonstrated similar HbA1c lowering capacity in streptozotocin-induced diabetic rats. The results implicated lower glucose carriage by the erythrocytes which may have resulted due to better absorption of glucose by improved insulin level. The extracts may thus, cut the risk of microvascular complications such as retinopathy, nephropathy, and neuropathy which arise from higher HbA1c level.

Usually decrease of body weight is found associated with diabetic conditions. However, the present study demonstrated body weight gain in diabetic animals, which might have occurred as a result of elevated triglyceride level in untreated diabetic animals. Earlier, correlation between body weight and triglyceride level has already been shown (Despres, *et al*., 1989; Bray, 2004). Moreover, higher level of uric acid has also been seen associated with elevated body mass index (Oyamada, *et al*., 2006) as well as pathogenesis of diabetic conditions (Johnson, *et al*., 2013). In the extract treated groups, drastic increase in body weight change has not been observed in any case. A previous report of Gayathri, *et al*., (2011b) might answer the cause, which demonstrated body weight lowering capacity of oleander extract through

triglyceridemic activity. Moreover, leptin, which trends to be higher in obese individuals (Considine, *et al.*, 1996), was found to be much lower in oleander distillate treated animals compared to untreated diabetic animals (Bas, *et al.*, 2012).

Glucose is stored in liver and skeletal muscle in the form of glycogen and insulin regulates glucose utilization. Liver glycogen level increase due to elevated glycogenesis and/or decrease in glycogenolysis. Moreover, the activity and amount of glucokinase and phosphofructokinase is influenced by insulin resulting hepatic glycolysis (Dodamani, *et al*., 2012). Insulin promotes glycogen deposition by regulating glycogen synthase and inhibiting glycogen phosphorylase. Low hepatic glycogen content in poorly controlled diabetic patients or in case of reduced gluconeogenesis or increased peripheral glucose hyperinsulinemia, may result in hypoglycaemia in type 1 diabetic patients (Riddell, 2012). Prolonged hypoinsulinemia or hyperglycaemic conditions might also leads to lower glycogen level followed by dehydration and electrolyte imbalance (Jimenez, *et al*., 2007). The present investigation exhibited significant ($P<0.001$) lowering of hepatic glycogen (0.42 fold) due to alloxan diabetes and its restoration by the *N. indicum* extract treatments. At 200 mg/kg dose, the extent of hepatic glycogen restoration by NILE, NISE and NIRE were 1.48 fold, 1.25 fold and 1.39 fold, respectively compared to diabetic control. The extracts may have restored the alloxan toxified β-cells by protecting through free radical scavenging activity which eventually resulted in gradual increase in insulin level and influence the restoration of hepatic glycogen. Besides, the increased glucose absorption as demonstrated by OGTT, contributed in the conversion of blood glucose to glycogen and its further restoration in hepatic tissue.

Free radical mediated metabolic abnormalities are responsible for microvascular complications such as retinopathy, nephropathy, and neuropathy in hyperglycaemic and diabetic conditions. In different tissues, ROS are chiefly produced through the glycation reaction in diabetes (Sakurai & Tsuchiya, 1988; Hunt, *et al*., 1990). During this process, glucose in enediol form is oxidized to an enediol radical anion in a transition metal dependent process, the anion in turn is thereafter converted into reactive ketoaldehydes and $O_2^{\bullet-}$ (Maritim, 2003). Oxidative stress in diabetic conditions may causes various forms of tissue damage in patients with diabetes (Kaneto, *et al*., 1999). Elevated blood glucose level generates overproduction of superoxide radicals ($O_2^{\bullet-}$) and H_2O_2 (Ceriello, *et al*., 2002) which in turn interferes with nitric oxide metabolism in endothelial cells as well as held responsible for microvascular complications in hyperglycaemia (Scott & King, 2004). Impairment of the major antioxidant enzymes such as catalase, peroxidase and superoxide results in induction of

diabetic complications (Sindhu, *et al*., 2004). Alloxan participates in a cyclic redox reaction with intracellular thiols to produce reactive oxygen species (ROS) leading to β-cell toxicity. Autoxidation of alloxan derived dialuric acid generates $O_2^{\bullet-}$, H_2O_2 and hydroxyl radical ($OH^{\bullet}$) (Lenzen, 2007). Superoxide dismutase converts superoxide radical ($O_2^{\bullet-}$) to H_2O_2, which in turn is converted to O_2 and H_2O by catalase (CAT) and peroxidase (PX) activity. Hyperglycemia is found to promote peroxidation of low density lipoprotein through a superoxide-dependent pathway (Tsai, *et al*., 1994; Kawamura, *et al*., 1994). Furthermore, diabetes mellitus has been recognized to be associated with free radical mediated lipid peroxidation leading to atherosclerotic and cardiovascular mortality. In this autocatalytic process, polyunsaturated fatty acids in the plasma membrane undergo degradation forming lipid hydroperoxides such as MDA (Tangvarasittichai, *et al*., 2009). The reactive carbonyl compound MDA is a natural lipid peroxidation derivative and MDA concentration increases in the circulation in diabetes mellitus conditions. The *N. indicum* extracts under antioxidant evaluation has already demonstrated $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ scavenging capacity in addition to inhibition of lipid peroxidation activity *in vitro*. Moreover, the extracts have also demonstrated inhibition of lipid peroxidation in association with normalization of liver catalase and peroxidase activities under hepatoprotective evaluation. In this case, ROS mediated oxidative stress in alloxan induced diabetes was confirmed through the increase of MDA in the alloxanized mice and the extracts had demonstrated stabilization of CAT and PX activities in liver, kidney and skeletal muscle in addition to protection from ROS mediated lipid peroxidation in terms of decrease in MDA content in systemic circulation. The present outcomes corroborated the previous findings of Gayathri and her group (2011a), who showed that hydromethanolic extract of the oleander prevented lipid peroxidation by means of normalization of antioxidative enzymes and scavenging of free radicals. Besides, Singhal and Gupta (2012) also demonstrated capacity of oleander flower methanolic extract to normalize liver SOD and inhibit lipid peroxidation under *in vivo* hepatoprotective evaluation.

Hepatotoxicity and nephropathy are accentuated results of diabetes mellitus. Liver disease is one of the leading cause in diabetes. In the population-based Verona diabetes study, liver cirrhosis was found to be the fourth leading cause of mortality which accounted for around 4.4% of diabetes related deaths (de Marco, *et al*., 1999). Liver cirrhosis accounted for 12.5% mortality in diabetic patients in another prospective cohort study (Balkau, *et al*., 1991). Virtually the entire spectrum of liver disease such as abnormal liver enzymes, nonalcoholic fatty liver disease (NAFLD), cirrhosis, hepatocellular carcinoma, and acute liver failure are

found associated with diabetes. Hence, patients with diabetes show high prevalence of liver disease and *vice versa* (Tolman, *et al*., 2007). Hepatic damage leads to leakage to various liver marker enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) into the blood circulation. Several prospective studies have reported positive correlation between hepatic transaminase and diabetes mellitus (Ahn, *et al*., 2014). In the present study, substantial decrease in liver marker enzymes such as ACP, ALP, AST, ALT due to *N. indicum* treatment in alloxanized animals corroborates the findings of Singhal and Gupta (2012) who formerly demonstrated under hepatoprotective evaluation that oleander flower extract normalizes AST, ALT and ALP levels possibly through antioxidative mechanism. Excess free fatty-acids in serum, peroxisomal β-oxidation, ROS mediated production of toxic lipid peroxidation by products and hepatic inflammation results in elevated transaminase level (Harris, 2005). Besides, increase in hepatic phosphatase activity due to excessive hepatic phosphorylation in alloxanized animals is well established (Drabkin & Marsh, 1947). Furthermore, alloxan induced free radical mediated hepatic inflammation was minimized due to the anti-inflammatory and antioxidative effects of *N. indicum* extracts, these properties has already been demonstrated by NILE, NISE and NIRE under anti-inflammatory and antioxidant evaluation. The present observation that SGOT and SGPT levels were significantly elevated in the diabetic control group corroborates with the previous findings that chronic elevation of hepatic transaminases are common in diabetic patients (Ahn, *et al*., 2014). Additionally, renal morbidity and mortality resulting due to diabetic nephropathic symptoms such as renal atherosclerosis, urinary tract infections, papillary necrosis and glomerular lesions causes chronic kidney failure. Elevated serum urea, uric acid and creatinine levels are the hallmark of kidney dysfunction. In fact, low serum insulin level in diabetes condition results in inadequate carbohydrate derived energy leading to increase in protein catabolism, causing glomerulo-dysfunction, which may be the root to elevated urea, uric acid and creatinine levels in serum. Therefore, *N. indicum* mediated increase in insulin level and improvement in carbohydrate metabolism contributed in amelioration of diabetic nephropathy. The antioxidative protection rendered by *N. indicum* may likewise functioned in safeguarding the renal system as free radical facilitated oxidative stress possess serious implications on kidney disease in diabetes (Forbes, *et al*., 2008). *N. indicum* has demonstrated to elevate the urea, uric acid and creatinine levels in diabetic animals which is a positive sign in amelioration of diabetic nephropathy.

Diabetes associated cardiovascular diseases such as angina, myocardial infarction, stroke, peripheral artery disease, and congestive heart failure are the most prevalent cause of mortality among diabetic patients results due to high blood glucose and hyperlipidemia (International Diabetes Federation, 2013). Severe retinopathy leading to complete blindness due to blockage of blood vessels of eye may result in diabetes due to high cholesterol level. Elevation of cholesterol and triglyceride levels during diabetic condition is the demarcation of abnormality in lipid metabolism causing hyperlipidemia. Marked increase in liver triglyceride is seen in non-alcohol fatty liver disease in diabetes (Harris, 2005). In fact, malfunction of β-cells in insulin production or insulin resistant condition may give rise to hyperlipidemia as insulin possess inhibitory effect on 3-hydroxy-3-methyl-methylglutaryl coenzyme-A, a key enzyme of cholesterol biosynthesis (Ghoul, *et al*., 2012). Severe retinopathy leading to complete blindness due to blockage of blood vessels of eye may result in diabetes due to the high cholesterol. In the present study, the increased levels of triglyceride and cholesterol were brought back to normal by administration of the extracts, which represented anti-hyperlipidemic role of *N. indicum*. The present findings correlates with two previous interconnected studies by Gayathri and her group (2011b, 2013) who demonstrated antihyperlipidemic activity of oleander flower by studying cholesterol, triglyceride, very low-density lipoprotein (VLDL) and lipolytic enzymes. Thus, diabetes associated cardiovascular disorders could be attenuated by *N. indicum* treatment, attributed to its anti-hyperglycaemic and antihyperlipidemic activities.

In the present immunopharmacological evaluation of *N. indicum*, NILE, NISE and NIRE were tested for their potentiality to ameliorate haloalkane xenobiotic induced hepatotoxicity in murine model. The hepatoprotective potentiality of *N. indicum* directly correlates with its antioxidant and anti-diabetic activities.

Liver is associated with most of the metabolic and physiological process including growth, immunity, nutrition, energy metabolism and reproduction. Liver performs central role in the transformation and metabolism of xenobiotic compounds, which in turn, damages the liver itself (Subramoniam & Pushpangadan, 1999). Other extrinsic causative agents behind hepatic injury include chronic alcoholism, viral infections, hepatocarcinoma etc (Lu, *et al*., 2012). Halogenated alkanes are organic xenobiotics which are among the most harmful environmental toxicants (Belkin, 1992) capable of exerting tremendous hepatic injury. Susceptibility of liver to xenobiotic induced injury is because of its portal location within the blood circulation, central role in metabolism and the physiological structure of the liver

(Sturgill & Lambert, 1997; Jones, 1996). Though liver possess tremendous regenerative capacity but very often subclinical live injury occurs due to various xenobiotics such as toxic chemicals, drugs and their metabolic intermediates. Around the globe, drug induced hepatotoxicity has emerged as a serious medical concern where 10% of cases of acute liver failures were found to be associated with idiosyncratic xenobiotic-reactions (WHO, 1998). Liver is one of the crucial organs of the body and therefore, requires safeguard from the harmful xenobiotics.

CCl_4 is a potent environmental toxin (Thomas & Aust, 1986). The hepatotoxicity of the haloalkane is primarily governed by trichloromethyl radical ($CCl_3^{\bullet}$) which is a derivative of cytochrome activated CCl_4 (Weber, *et al*., 2003). $CCl_3^{\bullet}$ promotes lipid peroxidation, steatosis and hepatocarcinoma. It further couples with diatomic oxygen to form highly reactive trichloromethylperoxy radical ($CCl_3OO^{\bullet}$) which in turn affects mitochondria, endoplasmic reticulum and plasma membrane permeability resulting in loss of cytosolic Ca^{2+} sequestration and homeostasis (Weber, *et al*., 2003). CCl_4 is a model hepatotoxic inducer while silymarin from milk thistle (*Silybum marianum*), remains an universal positive control to assay hepatoprotective activity of the natural compounds. Use of herbal remedies as hepatoprotective therapy has been practised in various traditional medicinal systems for ages. There are around 77 herbal formulations mentioned in Ayurveda to have hepatoprotective capacity (Selim & Kaplowitz, 2003). *N. indicum* one such Ayurvedic plant with hepatoprotective potentials (Singhal & Gupta, 2012).

Hepatic injury and hepatotoxicity are primarily characterized by leakage of hepatobillary enzymes i.e. transaminases and phosphatases form liver to the blood circulation (Amacher, 2002). In the present study, CCl_4 toxicity have demonstrated considerable increase in serum ACP, ALP, bilirubin, cholesterol, GGT, glucose, LDH, GOT, GPT and urea levels, whereas serum albumin and protein levels were decreased. The results of *in vitro* analysis are comparable with the *in vivo* study, demonstrating similar pattern of changes in the enzymatic and biochemical parameters. Similar results were found by Singhal and Gupta (2012) who demonstrated anti-hepatotoxic activity of *Nerium* flower extract by down-regulation of AST, ALT, APL and bilirubin level in serum. Furthermore, Gayathri and her group (2011a) previously demonstrated that a bioactive fraction of *Nerium* flower prevented elevation of LDH, GGT, AST, ALT, ALP and creatinine levels on isoproterenol-induced oxidative stress in rats. In addition, Gayathri, *et al*., (2011b) in an interconnected study, further showed that *Nerium* flower extract possess hypolipidemic capacity which corroborates the LDH and

cholesterol lowering potentials of *N. indicum* extracts in the present study. The *N. indicum* extracts also demonstrated significant glucose level lowering capacity which correlated with its hypoglycaemic activity demonstrated under anti-diabetic evaluation.

CCl_4 toxicity in the experimental animals resulted in significant weight loss as well as highest relative liver weight among all the groups. The extent of increase in body weight was lower in NILE, NISE and NIRE treated groups compared to control. This may have resulted due to body weight lowering capacity of *Nerium* contributing to its potent anti-hyperlipidemic activity as demonstrated by Gayathri *et al*., (2011b). Lower relative liver weight in NILE treated animals compared to CCl_4 group reflected prevention of fatty liver formation on CCl_4 toxicity (Becker, *et al*., 1987).

In spite of high recovering potential, administration of drugs often cause tremendous hepatocellular necrosis. On activation by cytochrome, CCl_4 muddles cellular homeostasis by primary and secondary bond formation. In the present study, the potentiality of *N. indicum* was evaluated to prevent CCl_4 mediated cell necrosis by MTT method. This is a routinely used system to evaluate *in vitro* hepatoprotective activity of plant extracts on primary hepatocytes and/or HepG2 cells (Pramyothin, *et al*., 2007; Oskoueian, *et al*., 2014; Chen, *et al*., 2011) because cultured hepatocytes can be considered biochemically equivalent to intact hepatic system (Weber, *et al*., 2003). In this assay, the succinate-tetrazolium reductase enzyme of the mitochondrial respiratory chain of metabolically active hepatocytes reduced the tetrazolium salt to form purple formazan, the absorbance (540 nm) of which was proportional to the viable hepatocytes. The results indicate that number of viable cells were significantly higher in the extract treated groups compared to CCl_4 treated group. This demonstrated the capacity of *N. indicum* to prevent haloalkane induced direct hepatocyte necrosis.

Due to the formation of metabolic intermediates and subsequent alteration in cellular biochemical response, different reactive species are generated in hepatotoxicity which eventually leads to cellular damage. The mammalian antioxidant defence enzymes remains inactivated or dominated in such stressful conditions. CCl_4 toxicity generates free radicals and cause intense lipid peroxidation which inactivate these enzymes causing reduction in antioxidative defence activity (Tsai, *et al*., 2009). Peroxidase is an essential enzyme in detoxification of xenobiotic induced hepatic injury, which functions in reduction and conversion of hydroperoxides and lipid peroxides into harmless species. Catalase scavenge H_2O_2 and protects cellular biomolecules from the generation of highly reactive hydroxyl

radical. Inhibition of these enzymes during hepatocellular stress cause accumulation of $O_2^{\bullet -}$ and H_2O_2 which accentuate a cascade of free radical formation (Muller, *et al*., 2007). Furthermore, the CCl_4 derived trichloromethylperoxy radical ($CCl_3OO^{\bullet}$) abstracts proton from polyunsaturated fatty acids and initiates lipid peroxidation (Weber, *et al*., 2003). The reactive carbonyl compound MDA is a natural lipid peroxidation by-product. Increase in MDA level in the serum due to CCl_4 administration is the hallmark of oxidative stress in liver. NILE, NISE and NIRE had demonstrated various free radical scavenging activities including neutralization of OH•, NO, $O_2^{\bullet -}$, $^1O_2$, $ONOO^-$ as well as demonstrated *in vitro* inhibition of lipid peroxidation under evaluation of antioxidant activities. We have also demonstrated normalization of catalase and peroxidase activities in liver, kidney and skeletal muscle in mice as well as decreased MDA content in serum under anti-diabetic evaluation. In addition, Singhal and Gupta (2012) have also previously shown that *Nerium* extract normalizes hepatic superoxide dismutase (SOD) level on CCl_4 induced hepatotoxicity. Gayathri, *et al*., (2011b) further showed that *Nerium* flower prevented lipid peroxidation by maintaining the levels of SOD, glutathione peroxidase, reduced glutathione and nitrite. The present study thus, corroborated the previous findings which demonstrated antioxidative potential of *Nerium*. Suppression of catalase and peroxidase activity due to CCl_4 administration was evident by increased oxidative stress, resulting in lipid peroxidation. Increased MDA and decreased antioxidant enzymatic activity was subsequently attenuated by NILE treatment hence, reflecting on its potent antioxidant mediated hepatoprotective activity.

CCl_4 induces inflammation in hepatocytes causing releases of the pro-inflammatory cytokine TNF-α. Due to CCl_4 exposure, protein synthesis is severely hindered, thereby initiating TNF-α mediated pro-inflammatory response (Weber, *et al*., 2003). Up-regulation of TNF-α has been identified in case of various acute liver diseases and its association has been found with CCl_4 mediated hepatotoxicity. Gabele, *et al*., (2009) have demonstrated through bile duct ligation model that TNF-α actively potentiates hepatotoxicity and fibrogenesis. During CCL_4 mediated hepatotoxicity, liver Kupffer cells are activated which secret a vast array of cytokines (IL-1, IL-6, IL-8, TNF-α) and chemokines (MIP-2, IP-109, MCP-1, KC/GRO), many of which initiates hepatic inflammation and induce toxicity either by direct cellular damage or by chemoacttracting neutrophils and lymphocytes (Afford & Lalor, 2006). Due to CCl_4 treatment, 1754.58% increase in TNF-α expression was observed in cultured hepatocytes, which was down-regulated significantly ($P<0.001$) due to NILE, NISE and NIRE. NILE high, NISE high and NIRE high groups demonstrated 6.83 fold, 3.48 and 2.60

fold decrease in the TNF-α expression respectively compared to CCl_4 group. The efficiency of TNF-α suppression by the extracts were higher compared to silymarin. NO is another inflammatory mediator of CCl_4 induced hepatotoxicity and it's overproduction is associated with endotoxin shock and various modes of inflammatory hepatic injury (Al-Shabanah, *et al*., 2000). TNF-α stimulates inducible nitric oxide synthase (iNOS) in liver parenchymal and non-parenchymal cells to form NO, which causes nitrosative stress. In mitochondria, excess NO may react with superoxide radical ($O_2^{\bullet-}$) to produce reactive peroxynitrite ($ONOO^-$) radical. NILE in this regard proved to be potent inhibitor of CCl_4 induced NO production and thus, hepatoprotective in nature. Moreover, in the present study the *N. indicum* extracts demonstrated the significantly inhibition of free radical NO. In addition, the extracts have also significantly inhibited the NO level in Con A stimulated lymphocytes and LPS stimulated macrophages *in vitro*. Therefore, with the inhibition of TNF-α and NO in hepatocytes, the *N. indicum* extracts exerted anti-inflammatory effects on hepatocytes and thereby protects further tissue damage.

Comparative analysis of the various signs of liver damage demonstrated extensive hepatic tissue damage occurred in CCl_4 group. Usually the earliest histological evidence of liver tissue degeneration emerges around 6 h after CCl_4 administration which is evident from prominent signs of necrosis (Lockard, *et al*., 1983) followed by central zonal necrosis around 12 h post CCl_4 administration. Increased microsomal fatty concentration is generally seen 3 h post CCl_4 administration (Recknagel & Anthony, 1959). Treatment with both silymarin and the extracts demonstrated the renewal of healthy liver histology with much lesser injury score than the CCl_4 group. Prominent nucleus containing hepatocytes and well conserved cytoplasm were visible in control, while deformed nucleus in amoeboid overlapped hepatocytes were observed in CCl_4 group. The loosely packed cells were the evidence of loss of tissue integration in CCl_4 group. The pro-inflammatory cytokine TNF-α, a secondary mediator of CCl_4 induced tissue damage, was down regulated by the extracts. This was histologically evident as the inflammatory signs were less in silymarin and extract treated groups. Presence of fatty infiltration in CCl_4 group reflected the extensiveness of lipid peroxidation. Vascular congestion and deformity in hepatic nodular structures were evident in CCl_4 group and its retrieval was visible in the silymarin and extract treated liver sections. In general, the normal liver architecture was well preserved in case of silymarin and extract treated groups than the CCl_4 group.

In the present study, chemical characterization of NILE, NISE and NIRE were performed using biochemical and spectrophotometric methods, Fourier Transform Infrared Spectroscopy (FTIR), High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The results identified the presence of various classes of phytocompounds which directly correlated with the bioactivities of the *N. indicum* extracts. Initial phytochemical qualitative screening identified the presence of tannin, phlobatannins, terpenoids, glycosides, phenolics, flavonoids, alkaloids, saponins, proteins and carbohydrates. Cholesterol, compound steroids and anthraquinones were only identified respectively in leaf, stem and root. These are the essential constituents of herbal medicine and also commonly detected in most of the angiosperms.

Alkaloids are ubiquitous in plant system and the pharmacological activities of different alkaloids, isolated from plants, are well known. Most alkaloids from plants primarily modulates the neurotransmitters (Robetrs & Wink, 1998). However, different alkaloids isolated from plants such as opium, strychnine, piperine, reserpine, caffeine, quinine, cinchonine, colchicine and vinca alkaloids are well established for their wide range of pharmacological activities. In recent years, several bioactive alkaloids were identified which demonstrated their potent anti-inflammatory activities by inhibiting COX activities (Souto, *et al*., 2011). Plant phenolics are broadly categorized into very common phenolic acids, flavonoids, tannins and less abundant stilbenes and lignans. Phenolics are considered as potent free radical scavengers than vitamin C, vitamin E and carotenoids (Dai & Mumper, 2010). Reduced risk of cardiovascular disorders, cancers and osteoporosis are associated with the consumption of phenolic rich fruits and vegetables. Numerous phenolics are identified which possess potent anti-diabetic (Asgar, 2013) and hepatoprotective (Madrigal-Santillan, *et al*., 2014) activities and their activities are chiefly governed by their antioxidant and free radical scavenging properties. Moreover, phenolics are considered as the alternative to conventional anti-inflammatory therapeutics in case of chronic inflammatory diseases (Sergent, *et al*., 2010). Flavonoids are one of the most ubiquitous phenolic compounds found in plants and are associated with diverse bioactivities such as anti-hyperglycaemic, antioxidant, hepatoprotective, immunomodulatory, cardioprotective, anti-microbial activities and more (Tapas, *et al*., 2008). Glycosides are diverse classes of compounds which are natural derivatives of vitamins, phenols, alkaloids, glycopeptides, cardiac glycosides, steroid, terpenoid etc. with various bioactivities. Different cardiac glycosides have already been isolated from *N. indicum* which have already demonstrated promising anti-cancer activities

(Newman, *et al*., 2008). Besides, three essential micronutrient i.e. thiamine, riboflavin and ascorbic acid were identified in *N. indicum* which not only play profound role in maintaining the normal health status but also are capable of scavenging harmful free radicals through their potent antioxidant activities.

HPLC analysis was employed to identify and quantify various phenolic metabolites present in *N. indicum* extracts. Vanillic acid (VA) was identified in all the extracts, however, syringic acid (SA), ferulic acid (FA) and myricetin (MY) were found both in NISE and NIRE. Apart from that, NILE contained gallic acid (GA), 4-hydroxy benzoic acid (4HBA), p-coumaric acid (PCA), jasmonic acid (JA), rutin (RU), o-cresol (oCRE) and 3,4-xylenol (XYL); NISE contained 4HBA. Previously, Newman and his group (2001) identified different carbohydrates in aqueous extract of oleander leaf using HPLC method. Furthermore, cinnamic acid, chlorogenic acid, RU, catechin the epicatechin, quercitin, quenonic acid and shikimic acid were identified in the leaves using HPLC analysis (Siham, *et al*., 2014). Nigam and Niranjan (2014) had isolated RU, gossypin and different glycosides of quercetin and kaempferol from different varieties of oleander. Very recently ellagic acid, methyl gallate, catechin and reserpine were identified in 70% hydro-methanolic extract of *N. indicum* leaf (Ghate, *et al*., 2015). Moreover, several other bioactive phytochemicals such as phytol, tochoferol, vanillin , methylparaben, stigmasterol, sitosterol etc. were also identified using GC-MS analysis. A detailed account the known bioactivities of the identified compounds are presented in Appendix C. The aforementioned compounds has also been reported in various other bioactive plant extracts. In the present study, the antioxidant, anti-diabetic, hepatoprotective, immunomodulatory and anti-inflammatory activities demonstrated by 70% hydromethanolic extracts of *N. indicum* resulted possibly due to the synergistic activities of these bioactive phytochemicals.