CHAPTER – 6:

DISCUSSION

6. DISCUSSION

6.1. IMMUNOMODULATORY ACTIVITIES OF *D. ESCULENTUM*

Only few of the pharmacological activities of *D. esculentum* have been reported so far, and among them little is known about the effect of *D. esculentum* on the immune system. Under various regulatory guidelines, body weight gain is an integral part of the conventional safety evaluation of a test material (Schilter et al., 2003). Significant loss of the body weight is one of the most crucial and a sensitive indicator of an animal's deteriorating health status. Similarly, organ weights are widely accepted in the evaluation of test article-associated toxicities (Wooley, 2003). The choice of the appropriate organ to be weighed in toxicological studies involves the understanding the test article's mechanism of action, metabolism, toxicokinetics and the physiology (Khan et al., 2011). In the present study, significant loss of both the body weight as well as the relative spleen weight indicates the immunotoxic properties of *D. esculentum*. The PFC assay is considered to be one of the most highly predictive single assays for the detection of immunomodulatory/immunotoxic potential of several substances and drugs. It is used to assess the potential modulation of the humoral immune response, which quantifies the number of B cell producing sRBC-specific Immunoglobulin M (Wilson et al., 1999). The dose- and time-dependent decrease in the number of the plaque forming cell as well as the progressive decrease in the degree of the hemagglutination titre in all the treated groups indicate the immunosuppressive potential of *D. esculentum*.

Another important parameter that help in assessing the immunodulatory activity of *D. esculentum* was the counting of the peritoneal macrophages. Macrophages are the important regulatory cells that play an important role in cell-mediated and humoral immunity as antigen-presenting, tumoricidal and microbicidal cells (Cavaillon, 1994). Inactivation of macrophages can, therefore, induce immunosuppression. Significant decreases in the number of peritoneal macrophages in case of BDE treated mice, therefore, represent its immunosuppressive activity.

Counting of the primary cultured splenocytes and MTT assay were used to measure the different parameters of the BDE induced cell proliferation. Splenocyte counting was done to estimate the cell number in the culture after different time intervals, while MTT assay was

performed to determine the metabolic activity of cells. Both of these assays showed that the boiled *D. esculentum* induced the inhibition of cell proliferation, and thereby indicating its immunosuppressive activity.

Hemolysis is due to red blood cell destruction which resulted from the lysis of membrane lipid bilayer. According to Fick's law, diffusion flux from a membrane is proportional to the concentration difference of both sides (Kleszczynska et al., 2005). In the present study, progressive increase in the concentration of BDE in extra cellular membrane causes its diffusion in to the intra cellular membrane up to a specific concentration, which leads to the membrane destruction and thus showing its hemolytic potential.

In continuation of the investigations on immunomodulatory properties of *D. esculentum*, the effects of boiled aqueous preparations of *D. esculentum*, both *in vivo* and *in vitro*, on Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine concentration in mouse have also been studied. IL-2 is a representative cytokine produced by the activated T-cells which leads to the T-cell proliferation and participates in the regulation of other immune cells, including B cells, macrophages and NK cells (Park et al., 2007). IFN-γ is a proinflammatory mediator expressed by the various cells, including Th1, natural killer (NK) and NKT cells. IFN-γ is an important immune-activating cytokine that can prime the macrophages for activation and induce inflammatory responses, such as those observed in delayed-type hypersensitivity and granulomatous lesions (Pacifico et al., 2006). IFN-γ orchestrates leukocyte attraction and directs the growth, maturation, and differentiation of various types of cells in addition to enhancing NK cell activity. IL-2, IL-12, and several other cytokines are known to be the primary cytokines along with the production of IFN-γ by NK cells (Kang et al., 2014). IL-4 is produced by the activated T lymphocytes and mast cells, and can exert both pro- and anti-inflammatory effects (Kleemann et al., 2008). One of the most potent homeostatic regulators of inflammation is the anti-inflammatory cytokine IL-10, which potently inhibits TNF-α production from the macrophages together with the other pro-inflammatory cytokines including IL-1, IL-6, GM-CSF and many chemokines (Brennan et al., 2008). Sub-acute, sub-chronic and chronic oral administration of BDE reduced the body weight and relative spleen weight as well as suppresses the humoral immune response in Swiss albino mouse. Moreover, we observed that BDE

decreased the number of the peritoneal macrophages in mouse. Significant dose-dependent reduction in the level of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine production by T cells in BDE treated mouse indicates the severe immunosuppressiveness in these mice.

The secreted cytokines of type 1 CD4+ T helper cells (Th1), such as IL-2 and IFN-γ are considered as proinflammatory, whereas Th2 cytokines such as IL-4 and IL-10 can counteract Th1 cytokine production and activity (Kleemann et al., 2008). IFN-γ enhances Th1 generation but inhibits Th2 generation, whereas Th2 cells and their cytokine, IL-4, promotes Th2 generation but inhibits Th1 generation. In physiological condition, Th0 cells differentiate in to Th1 and Th2 cells proportionally and keep their amount in a relative dynamic balance. Diseases will occur whenever this balance is disturbed (Guo et al., 2014). It has been demonstrated that Th1/Th2 balance plays important roles as anti-tumor immunity in which Th1 cells produce IL-2 and IFN-γ that are essential for inducing cellular and tumor immunity, whereas Th2 cells, producing IL-4 and IL-6, are associated with the suppression of cytolytic activity (Nakamori et al., 2003; Nishimura et al., 1999). Under aberrant conditions, a Th1/Th2 imbalance occurs and various cytokines are thought to cause the autoimmune diseases, such as autoimmune diabetes, rheumatoid arthritis and Crohn's disease (Abbas et al., 1996). Findings from the present study indicate that *D. esculentum* when given in chronic doses, can induce Th1/Th2 imbalance, resulting in severe immunosuppression. This may directly or indirectly induce several metabolic diseases and age-related degenerative disorders as well as may also increase the risk of infection to the people who regularly consumes this fern. This may induce a state of immunodeficiency as an unwanted consequence and therefore, may also become responsible to the growth of tumors.

6.2. EFFECT OF *D. ESCULENTUM* ON THE REPRODUCTIVE FUNCTIONS OF MOUSE

Studies on the effects of plant products on the male reproductive system and fertility are comparatively few and far fetched (Kumari et al., 2012). In the present study, the effect of boiled aqueous preparation of *D. esculentum* (BDE) on the metabolic activity of the spermatozoa of adult Swiss albino mice clearly establishes that BDE can affect the male reproductive system and cause infertility through its spermicidal properties. Mosmann (1983) used MTT tetrazolium salt to assess the cellular viability, proliferation, and cytotoxicity of lymphocytes. Additionally, the

MTT assay has been used in many studies to evaluate the viability of different cells (Carmichael et al., 1987; Campling et al., 1988; Freimoser et al., 1999). The present study provides the new information on the MTT assay for sperm viability assessment in *D. esculentum* fed adult Swiss albino mice. Formation of MTT formazan granules or spikes around the midpiece region of spermatozoa showed that mitochondria contain a succinate dehydrogenase system which converts MTT to formazan. The presence of formazan granules in the midpiece region identifies the viability of spermatozoa. Results indicated a strong correlation between the MTT reduction rate and the viability of spermatozoa. A strong correlation between MTT reduction and the viability of spermatozoa has also been found in bovines, stallions, boars, fowl, and humans (Aziz et al., 2005; Aziz, 2006; Byun et al., 2008; Hazary et al., 2001; Naser-Esfahani et al., 2002). The MTT reduction rate was taken successfully after 1 h of incubation time. This is due to the fact that the spermatozoa are very active cells and rich in mitochondria; therefore, the reduction of MTT by spermatozoa is faster than other cells. Other studies have already revealed that sperm viability is positively related to the sperm quality parameters like acrosome integrity and mitochondrial activity. These parameters also correlate positively with the fertility (Garner et al., 1997). The male accessory sex organs, viz. epididymis and vas deferens are androgen dependent target organs that manifest differential sensibility to androgens for the maintenance of their structure and function. Any change in the circulating androgens would affect the internal microenvironment of epididymis and thereby lead to the alteration of sperm motility and metabolism (Khan & Awasthy, 2003). Present study showed that the rate of MTT reduction decreased gradually with the increase of dose in all the groups. After 135 days and 180 days of the treatment at the dose of 320mg/kg b. w., the percentage inhibition of sperm viability was increased remarkably up to 40.51% and 53.12%, respectively. Ethanolic extract of *Sarcostemma secamone* treated adult male rat showed the reduction in number of female impregnation, number of implantation and also the number of viable fetuses when mated with fertile females (Kumari et al., 2012). These could be due to the decrease in sperm density, viability and motility which supports our findings of having reduced sperm viability due to the treatment of boiled aqueous preparation of *D. esculentum*, and therefore, indicated that *D. esculentum*, may possess antifertility activity, probably due to its spermicidal properties.

A significant decrease in body weight as well as relative testis weight of BDE treated male mouse was observed. This may be due to the possible adverse effect of BDE on somatic cells or indirectly through the central nervous system, which controls the feed and water intake and regulates the endocrine function (Yousef et al., 1995). Testis acts as endocrine gland as well as a reproductive organ, responsible for the production of hormones and male gametes, and an important target for endocrine disruption. Testis consists of two types of tissues: seminiferous tubules, supported by sertoli cells and the interstitial compartment, comprised of leydig cells (Fisher, 2004; Akingbemi, 2005). Testicular functions, i.e., spermatogenesis, steroidogenesis, etc. are regulated by the hypothalamic-pituitary-testicular (HPT) axis which involves the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Dettin et al., 2003; Jana et al., 2006). Testicular functions are proposed to be regulated by a number of hormones and growth factors, in addition to FSH, LH, and androgens, including insulin-like growth factor, oxytocin, and transforming growth factor- and estrogens (Pryor et al., 2000). BDE caused various structural abnormalities in testes as indicated by the histopathological examinations. Seminiferous tubules were shrunken and appeared to be displaced, diameter of the lumen became increased and vacuolization occurred in the interstitial spaces. This may probably explain the reason behind the decrease in the weight of the testis.

Cholesterol, containing a mono atomic alcohol and one double bond, is considered to be the most important precursor of all the steroid hormones including androgens (Chang et al., 2004). Testes and other tissues actively synthesize cholesterol. Sharpe & Shakkebaek (1993) speculated that most of the tissues of the body are dependent on dietary cholesterol as their source, while testis relies heavily on its endogenous synthesis. Since cholesterol is known to be a precursor of the synthesis of androgen in the testis, changes in the testicular cholesterol levels are considered to be important, as it is implicated in the inhibition/stimulation of spermatogenesis (Meroni et al., 2002). Androgens are very essential for normal functioning of the accessory reproductive organs. In the present study, BDE reduced the cholesterol content significantly. This may lead to decrease in the testosterone level in the testes and blood, increase in the blood levels of the signaling luteinizing hormone (LH), alteration in the mitochondrial membranes in leydig cells, change in the gene expression which controls important proteins and reduced sperm health and numbers (Zhang et al., 2007).

The present study showed significant difference between the treated and untreated groups in epididymis α-glucosidase activity in caudal epididymis. Alpha-glucosidase is a normal constituent of semen, produced mainly in the epididymis. It is significantly correlated to sperm count. Its activity is low in cases of epididymal obstruction. A decrease in fructose level in the testis of treated animals was also observed. Since the function of fructose is to induce the glycolytic metabolism of spermatozoa, it can be suggested that the decrease in fructose content due to BDE treatment hampers the glycolytic metabolism of spermatozoa. This in turn may lead to the abnormal sperm function which ultimately may cause the complete male sterility (Sarkar et al., 2000).

Carbohydrates are stored in the animal tissue in the form of glycogen, which acts as an energy producing source. Glucose plays a major role in energy metabolism and is stored as a readily available energy source in the form of glycogen in cells during various developmental and physiological stages (Thong and Graham, 2004; Sinclair et al., 2003; Gruetter, 2003; Ferrer et al., 2003). Glucose has also been shown to be an essential substrate for maintaining tissue integrity, ATP production and protein synthesis in rat testis (Bajpai et al., 2008). Klip et al., (1994) observed that the testicular interstitial cells are a good source of glycogen. In early pubertal period, spermatogenesis takes place, in which glycogen is degraded to release glucose which is used for the metabolism of actively growing tissue. In the present work, a highly significant decrease ($p < 0.001$) in glycogen content was observed in the BDE treated mice, which could affect energy requirements of cells. It is interesting to note that the protein content in serum, testis and epididymis were decreased significantly in BDE treated mice compared to the control mice. This is in accordance with the view of Zuping et al., (2009), who speculated that protein synthesis in spermatogenic cells is dependent upon glucose. Hence a decrease in the glycogen content could affect protein synthesis and thus subsequently inhibit spermatogenesis.

Sialic acid is a carbohydrate component attached with protein to form glycoprotein. It is found at the end of the oligosaccharide chains of many soluble glycoproteins which determine whether the protein will continue to circulate in the blood stream or to be removed by the liver. Sialic acid is also concerned with the stabilization of the plasma membrane, maintenance of sperms in a decapitated state, ionic balance in the epididymal plasma and antigen interaction

between sperm and epididymal epithelium (Thomas et al., 2008). The synthesis and secretion of sialic acid is under androgen control. Gupta et al., (2002) indicated that possible role of androgen dependent sialic acid is to inhibit the stabilization of the acrosome of the maturing spermatozoa by contributing to surface negative charge. Epididymal epithelium is involved in the synthesis and secretion of compounds containing sialic acid. Alteration in sialic acid level in reproductive tissues indicate changes in the level of glycoprotein/FSH and LH which is needed for normal functioning of gonads and accessory reproductive organs (Gupta at al., 2002). In the present study, sialic acid content of testis significantly decreased in all the groups having different doses of CDE or BDE. Depletion in the testicular sialic acid content in the mouse possibly reflects the androgen and gonadotrophic deficiency resulting in the inhibition of spermatogenesis, loss of spermatozoa motility and fertilizing ability (Gheri et al., 2009).

Present study showed that the reduction of the prostate weight was associated with the significant decrease in citric acid content when mice were fed with 320 mg/kg bw of BDE. These results suggest a dysfunction of the prostate gland, which may decrease the testosterone levels, because the secretion of citric acid is regulated by androgens (Costello & Franklin, 2002). In the testis, acid phosphatase is widely distributed in lysosomes of Sertoli cells, spermatogonia and late spermatids (Chemes, 1986). Activities of free lysosomal enzymes have been shown to rise when testicular steroidogenesis is increased (Mathur & Chattopadhyay, 1982). In the present study, the decrease in acid phosphatase activity might reflect the decreased testicular function in the treated mice and therefore may interfere with the secretion of testosterone.

BDE has resulted in statistically significant decrease in weight of the testis of mice and the effect is not reversible, possibly accounted by its chronic toxic effect on the mucosa of digestive tract, the recovery of which was not possible. Reduction is also noted in its visible vascularity. The chronic toxic effect of BDE on the endothelium of the vessels possibly decreases the blood supply to the testis and resulted in gross decrease in its weight. Similar alterations in the weight are also reported in a previous study where researchers observed the effect of 14 different toxics on mice testis and spermatogenesis (Meistrich et al., 1982).

It has been shown in this study that BDE arrests the normal spermatogenesis at early stage (primary spermatocytic cycle) in majority of the seminiferous tubule as evident by the

significant decreases of the seminiferous tubular dimensions and seminiferous epithelial height. The effects are dose and time dependent. The mice exposed to the chronic dose have shown significant disruption of seminiferous tubular morphology than that of the control mice. The changes in the spermatogenic cells have been observed by various authors using array of toxic chemicals, including different plant extracts to physical constraints like prolonged hypoxia on testis. Most of the studies do correlate with humans and therefore, comparable effects can be seen naturally exposed to these chemical and physical agents (Viveka et al., 2015).

In the present study, the spermatogenic cells have been reduced to single layer, showing complete halt of spermatogenesis. In most of the tubules studied in the chronic treatment group mice testis, the spermatogenetic halt was evident by the reduced epithelial cell height and lack of sperms in the lumen. Injection of imatinib mesylate to mice gives similar results in less than 2 weeks (Prasad et al., 2010). In this study it was not possible to differentiate the primary and secondary spermatocytes in most of the tubules as the meiosis in most of the germ cells have halted in early stage.

Present study indicated that some of the seminiferous tubules of the testis of mice treated with 320 mg/kg bw of CDE and BDE for 180 days of treatment show detachment of spermatogenic cells from the basement membrane, which indicates an altered interaction with basement membrane. Appearance of intraepithelial vacuolations may be due to the intraepithelial edema and altered intercellular connections, due to acute cytological toxicity of BDE. Similar intraepithelial vacuolations are reported in mice treated with Neem extract (Mishra & Singh, 2005) and Brahmi leaves (Singh & Singh, 2009).

Clumping of the sperms inside the seminiferous tubules was also observed in the present study specially in the testis of mice treated with 320 mg/kg bw of CDE and BDE for 180 days, which is an indication of halt of the normal spermatogenesis, loss of junctional complexes between the adjacent Sertoli cells, mitochondrial membrane damage, plasma membrane damage with profound disturbances in the membrane functions of spermatozoa in the lumen as a result of hypoxic and hyponutritive environments prevailing in the seminiferous tubules under the influence of BDE. Aggregates of mouse sperms are well‑documented in many spermatogenesis studies (Adler, 1993; Meistrich, 1986; Jagetia et al., 1996).

One of the interesting observations in the present study was the association of the reproductive function with Th1/Th2 cytokine homeostasis. Interestingly, the Th1/Th2 cytokine index has been increased significantly in some of the pregnant mice that were treated both with crude and boiled *Diplazium esculentum*, i.e., Th1 cytokine expression is higher than Th2 cytokine in these mice that ultimately causes infertility and recurrent spontaneous abortion (RSA).

All the abnormalities that have been observed in the mice treated with 320 mg/kg bw of CDE and BDE for 180 days did not show any sign of reversal after 60 days interval, as evident by the seminiferous tubular morphology where statistically significant difference exist with the control group even after 60 days of interval. The permanent loss of spermatogonial stem cells may explain lack of recovery.

6.3. Neuromodulatory activity of *D. esculentum*

Enzymes are the primary targets for the development of new drugs because of the simplicity of enzyme based assays. The inhibitor interacts with the enzyme or enzyme-substrate complex with a decrease in the rate of reaction (Ashraf et al., 2011). The results of the *in vivo* acetylcholinesterase activity indicated the dose-dependent decrease in the rate of the conversion of the substrate acetylthiocholine iodide in to acetyl- and choline group by the enzyme acetylcholinesterase. This enzyme inhibition assays have prompted us to carry out the acetylcholinesterase and NADH oxidase inhibitory activities of the methanolic extracts of *D. esculentum*. In the present study, significant dose-dependent increases in the acetylcholinesterase– and NADH oxidase inhibitory activities, as well as low IC_{50} values for acetylcholinesterase– and NADH oxidase inhibition of the plant extract were observed, indicating its effectiveness as a good anticholinesterase and NADH oxidase inhibitor. The Cholinesterase inhibitory therapy and NADH oxidase inhibitory therapy may be considered, by its pharmacological nature, as a simple symptomatic short-term intervention. It has previously been suggested that the anticholinesterase effects may be due to the interaction of the cholinesterase inhibitor with the amyloid cascade, influencing the expression and/or the metabolic processing of the amyloid precursor protein (APP) and slowing down one of the major pathological steps of the disease progression (Giacobini, 2002). In traditional practices,

numerous plants have been used to treat cognitive disorders, including different neurodegenerative diseases. Water-extractable phytochemicals from some citrus peels of Nigeria have been shown to possess potent anticholinesterase and antioxidative properties, and therefore, make the peels a good dietary source of natural acetylcholinesterase inhibitor (Ademosun & Oboh, 2014). Our study plant, *D. esculentum*, being an edible fern, may also be a good dietary source of acetylcholinesterase– and NADH oxidase inhibitor and thereby, can be used for the management of oxidative stress-related neurodegenerative disorders.

It is known since long back that certain phytochemicals, such as flavonoids and phenolic compounds confer antioxidant activity. Antioxidants can scavenge ROS and can also attenuate inflammatory pathways, and therefore can act as acetylcholinesterase– and NADH oxidase inhibitor. Both of these classes of compounds have good antioxidant potential due to their radical scavenging abilities and their effects on human nutrition and health are considerable. We have demonstrated that *D. esculentum* possesses high amount of flavonoid and phenolic compounds, and therefore, may be a good source of acetylcholinesterase– and NADH oxidase inhibitor. We have investigated for DPPH radical scavenging property as well as total antioxidant activities in linoleic acid system of the plant extract to support our findings. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. The mechanism involved in the reduction of DPPH free radicals is based on the capacity of some compounds to donate hydrogen. Some plants are rich in secondary metabolites, such as, flavonoids, phenolic acids and tannins. These phenolic compounds are able to donate hydrogen, presenting antiradical activity (Barış et al., 2011). It measures the capacity of the extract to scavenge free radicals in solution. In the present study, DPPH scavenging potential of the *D. esculentum* extract was evaluated. The IC_{50} value of the plant extract shows that the plant extract possesses moderate free radical scavenging activity, though the plant extract was not as potent as the standard tocopherol.

In the present study, we have demonstrated that the methanolic extract of *D. esculentum* possesses scavenging activities against different reactive oxygen species (ROS) and reactive nitrogen species (RNS), including hydroxyl, superoxide, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen, and hypochlorous acid. Moreover, the extract acted as an iron chelator and also possessed reducing power. It also inhibited the lipid peroxidation. In the

present study, the total antioxidant activity of the extract was evaluated by ABTS method as trolox equivalent antioxidant capacity value as well as by FTC and TBA methods. Peroxide is gradually decomposed to lower molecular compounds during the oxidation process and these compounds were measured by FTC and TBA methods. The amount of peroxide at the primary stage of linoleic acid peroxidation was measured by FTC method, whereas TBA method measures at the secondary stages (Barış et al., 2011). The total antioxidant activity of methanolic extract of *D. esculentum* was determined by the peroxidation of linoleic acid using the FTC and TBA methods. During linoleic acid peroxidation, peroxides were formed, and these compounds oxidized Fe^{2+} to Fe^{3+}, which had a maximum absorbance at 500 nm. Thus, in the present study, a high absorbance value was an indication of high peroxide formation during the emulsion incubation, thereby showing high percentages of the total antioxidant activity of both the plant extract and Vitamin E in both FTC and TBA methods.

Phytochemical analysis shows the presence of many pharmacologically important secondary plant metabolites like terpenoids, cardiac glycosides, saponins, flavonoids, phenolic compounds, etc. which indicate that the plant possesses high profile values and can be used to treat various kinds of diseases. The qualitative phytochemical investigation gave the valuable information about the different phytoconstituents present in the extracts, which help the future investigators regarding the selection of the particular extract for further investigation of isolating the active principle (Mishra et al., 2010). We have observed that both the crude and boiled *D. esculentum* possess hemolytic activity. Saponins have the capacity to destroy cell membrane, therefore may be related to the hemolytic potential. On the other hand, tannins inhibit protein availability through denaturation. Tannins are heat resistant compounds that can withstand high temperature during boiling. Thus, the toxic effects observed in our study could be related to tannins and other heat stable compounds.

6.4. Acute, sub-acute, sub-chronic and chronic toxicity study of *D. esculentum*

Results of the present study indicate that BDE alter the growth process. Under various regulatory guidelines, gain of the body weight is an integral part of the conventional safety evaluation of a test material (Schilter et al., 2003). Significant loss of the body weight is considered to be one of the most sensitive indicators of an animal's deteriorating health status

(Schilter et al., 2003). Similarly, organ weights are widely accepted in the evaluation of test article-associated toxicities (Wooley, 2003). The choice of appropriate organ to be weighed in toxicological studies involve understanding the test article's mechanism of action, metabolism, toxicokinetics and the physiology of the test species (Khan et al., 2011). In the present study, significant losses of the body weights as well as the relative organ weights indicate the toxic properties of *D. esculentum*. Organs as targets for this study were selected according to the Society of Toxicologic Pathologists (STP) recommendations (Sellers et al., 2007).

Biochemical determinations in serum serve as an indicator of toxicity of a test material (Schilter et al., 2003). AST is an enzyme found mainly in liver cells, heart muscles, skeletal muscles and kidneys. Injury to these tissues results in the release of this enzyme in the blood stream. Elevated levels are found in myocardial infarction, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases, etc. Decrease levels may be found in pregnancy, Beri Beri, and diabetic ketoacidosis. SGPT is found in variety of tissues but mainly in liver. Increased levels of ALT are found in hepatitis, cirrhosis, obstructive jaundice, and myocardial infarction. LDH is found mainly in liver, heart, kidney, skeletal muscle and RBC. LDH is found in the form of isoenzymes based on their electrophoretic mobility with each isoenzyme being primarily from different organs. Increased levels of LDH are found in myocardial infarction, pulmonary diseases, hepatitis diseases, hemolytic anemia, renal diseases, and muscular dystrophy. GGT is found mainly in serum from hepatic origin, though the highest levels are found in kidneys. Elevated levels of GGT are found in hepatobiliry and pancreatic diseases, chronic alcoholism, myocardial infarction with secondary liver damage, and diabetes. ALP is found in high concentration in liver, biliary tract epithelium, and in bones. Normal levels of ALP are age-dependent and increased with bone development. Increased levels are associated mainly with the liver and bone diseases. ACP is widely distributed and found in high concentrations in liver, RBC and the prostate. Increased levels of prostatic fraction are associated with the prostatic carcinomas. Increased levels of nonprostatic fraction are associated with the liver diseases, hyperparathyroidism, etc. Elevated levels of bilirubin are found in liver diseases (hepatitis, cirrhosis), excessive hemolysis/destruction of RBC (hemolytic jaundice), obstruction of the biliary tract (obstructive jaundice), and in drug induced reactions. It was clear from these data that *D. esculentum* affect the metabolic activity of mice, which is considered to have resulted

from different organ and system failure and we have also demonstrated the pathological evidences that can support it. Gross examination of vital organs such as liver and kidney of mice from treated groups, and microscopic examination of tissue sections prepared from these organs revealed the alterations in their histological architecture that could be attributed to *D. esculentum* intake at different doses.

Previous study revealed that *D. esculentum* collected from the high-altitude area of Harsil-Gangotri (Northern India) had 19 mg/kg Ptaquiloside (Somvanshi et al., 2006). Shade- and freeze dried samples of *D. esculentum* showed the absence of fern toxin ptaquiloside but the presence of 10.94 to 16.36 mg/kg pterosin B only in two of the freeze-dried samples by HPLC method (Gangwar, 2004). During metabolism, ptaquiloside undergoes a series of reactions and produces a reactive aglycone dienone intermediate, the inactive pterosin B and DNA adducts. Ptaquiloside is activated at alkaline pH, which is considered as the reason for the location of tumors in the urinary bladder of ruminants and the ileum of rats (Smith et al., 1994). Feeding of frozen- and shade dried samples of *D. esculentum* to rats and guinea pigs showed decreased body weight, increased spontaneous and decreased forced motor activity. Hematological and biochemical studies in rats and guinea pigs fed with frozen- and shade dried *D. esculentum* showed significant alterations in the values of blood glucose and total leukocyte count, increase in serum glutamic oxaloacetic transaminase (SGOT) and serum dehydrogenases (SDH). Feeding of frozen dried sample of *D. esculentum* induced 53% mortality in guinea pigs (Gangwar, 2004).

All the studies done so far were on the freeze dried or shade dried samples of *D. esculentum*, and its effect on rabbits and guinea pigs. But, there was no information available regarding the toxic effect of boiled preparation of *D. esculentum* on rabbits and guinea pigs. We have performed the experiment using the mouse as this is the standard convention to use inbreed strains of mouse for performing the pharmacological experiments. However, experiments using rabbits and guinea pigs may be performed in future once it is established that this fern is toxic as food.

The aim of the present study was to conduct different experiments with cooked (boiled) material, because the local people consume it regularly after cooking, not as a raw vegetable. Ptaquiloside is one of the major compounds present in *D. esculentum*. As ptaquiloside is a heat

labile compound, boiling may probably reduce its toxicity. Present study showed several toxic effects of this fern on Swiss albino mouse, which clearly indicated that there may be some compound that, can withstand heat and provide toxicity. Study on a related edible fern, *Diplazium sammatii* revealed that this fern contains 42.4 mg tannins/100 gm. Tannins inhibit protein availability through denaturation (Bassey et al., 2001). Tannins are heat resistant compounds that can withstand high temperature during boiling. As *D. esculentum* and *D. sammatii* are of same genus, we can assume that tannins may also be present in the boiled preparation of *D. esculentum*, and may be one of the causes of toxicity. Thus, the toxic effects observed in our study could be related to tannins and other heat stable compounds. Standard tannins (tannic acid) may be applied in the splenocyte cultures to reduce the speculation in future studies.

We have used different doses of *D. esculentum* for different time periods, so that low level and high level of the food intake may be covered. The periods were divided in such a manner so that the effect can be visualized, if any, even after treating for the nominal period like 15 days and also for a long period like 6 months. We had to choose 15 days period as it may happen that consumption of this fern for a shorter period may not cause any problem. But, as food consumption was not evaluated in the present study, it is not possible to assure that BDE itself induces any toxic effect on animals. If food consumption is reduced, nutritional status may interfere with observed parameters.