IMMUNOPHARMACOLOGICAL INVESTIGATION OF AN EDIBLE FERN, *DIPLAZIUM ESCULENTUM* (KOENIG EX RETZ.) SW., AVAILABLE IN NORTH BENGAL REGION

A THESIS SUBMITTED TO THE

UNIVERSITY OF NORTH BENGAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

SUBMITTED BY:

SUBHRAJYOTI ROY

GUIDED BY:

PROF. TAPAS KUMAR CHAUDHURI

DEPARTMENT OF ZOOLOGY

UNIVERSITY OF NORTH BENGAL

AUGUST 2016

Dedicated

to

My parents

DECLARATION

I declare that the thesis entitled, "IMMUNOPHARMACOLOGICAL INVESTIGATION OF AN EDIBLE FERN, *DIPLAZIUM ESCULENTUM* (KOENIG EX RETZ.) SW., AVAILABLE IN NORTH BENGAL REGION" has been prepared by me under the guidance of Prof. T. K. Chaudhuri, Department of Zoology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Subhrajyoti Roy

(Subhrajyoti Roy)
Department of Zoology
University of North Bengal
Raja Rammohunpur, Dist. – Darjeeling

Dated: 03.08.2016

UNIVERSITY OF NORTH BENGAL

DEPARTMENT OF ZOOLOGY

DST-FIST & UGC-SAP Sponsored

ENLIGHTENMENT TO PERFECTION

P.O. North Bengal University,
Raja Rammohunpur, Dt. Darjeeling,
West Bengal, India, PIN - 734 013

CERTIFICATE

This is to certify that the thesis entitled, "IMMUNOPHARMACOLOGICAL INVESTIGATION OF AN EDIBLE FERN, *DIPLAZIUM ESCULENTUM* (KOENIG EX RETZ.) SW., AVAILABLE IN NORTH BENGAL REGION" is an original investigative study carried out by Mr. Subhrajyoti Roy for the award of Doctor of Philosophy (Ph.D.) degree in Science (Zoology) of the University of North Bengal, under my supervision. He has carried out the wok in the Department of Zoology, University of North Bengal.

He is conversant with the techniques and the literature cited in the dissertation and carried out the work thoroughly. In character and disposition, Mr. Subhrajyoti Roy is fit to submit the thesis.

T. K. Chaudhuri 03/08/2016

Supervisor
Prof. T. K. Chaudhuri
Cellular Immunology Laboratory
Department of Zoology
University of North Bengal

Phone: (0353) 2776353, Fax: (0353) 2699 001, E-mail: zoology_nbu@rediffmail.com, Visit us at: www.nbu.ac.in

PREFACE

I started my research work in 2010 which has been documented in this dissertation entitled, "IMMUNOPHARMACOLOGICAL INVESTIGATION OF AN EDIBLE FERN, *DIPLAZIUM ESCULENTUM* (KOENIG EX RETZ.) SW., AVAILABLE IN NORTH BENGAL REGION" under the supervision of Prof. T. K. Chaudhuri, Department of Zoology, University of North Bengal, West Bengal, India.

Diplazium esculentum, the vegetable fern, is extensively used as a palatable food throughout Asia, Oceania and especially in the Northern part of West Bengal, India where the present study was performed. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon season which continues for almost five months. Pickles made from this plant are used as an appetizer. This plant is used to counteract constipation, indigestion and spermatorrhea. But, interestingly, this fern is rejected as food by animals including cattle and insets. So, it happened in our mind that this fern may have certain toxic substances for which cattle and insects avoid it and therefore, it may also have some detrimental effects to the human body.

Therefore, in the present study, an attempt was made to elucidate the immunopathological, haematological, biochemical, antifertility and neuromodulatory activities of crude (unboiled) and cooked (boiled) *Diplazium esculentum* by investigating several *in vivo* and *in vitro* parameters. We have conducted this pilot study to investigate the health deteriorating effects, if any, of the boiled aqueous preparation of *D. esculentum*, keeping in mind the fact that the local people consume this plant as food after cooking, not as raw material. The findings of the present study are published in various research journals and are presented and discussed in details in the Results and Discussion part of this dissertation.

ABSTRACT

Context

Diplazium esculentum (Koenig ex Retz.) Sw. (Family – Athyriaceae) is one of the most commonly consumed edible ferns throughout the world. It is pantropical in distribution and occurs widely and commonly throughout India, China, Cambodia, Laos, Thailand, Vietnam and Malaysia. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon season which continues for almost five months.

Objective:

Very few researchers have focused on the health impacts of this edible fern. Therefore, the aim of the present study was to find out the possible health deteriorating as well as health promoting properties in this fern. If the fern contains any toxic properties, then the people can be made aware of the harmful effect of consumption of this fern. Apart from its possible harmful effects, *D. esculentum* may have some health promoting effects too as this fern has been used as ethnomedicine among some tribes in India. Therefore, identification of potential therapeutic prospect of this fern is also a major concern of this study.

Materials and methods:

In the present study, an attempt was made to elucidate the immunological, haematological, biochemical, antifertility and neuromodulatory activities of crude (unboiled) and cooked (boiled) *Diplazium esculentum* by investigating several *in vivo* and *in vitro* parameters. To investigate the immunomodulatory as well as hemolytic activities of this plant, the body weight, relative spleen weight, plaque forming cell assay, hemaggutination antibody (HA) titer assay and macrophage

counting were performed in *D. esculentum* treated mice and respective control groups within a span of 180 days, and *in vitro* assays such as counting of cultured splenocytes, splenocytes proliferation assay and hemolytic assay were performed to justify the immunomodulatory as well as hemolytic activities of *D. esculentum*. Both serum and culture supernatant were used for cytokine determination by enzyme-linked immunosorbent assay (ELISA) for different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines. MTT reduction assay was performed for the assessment of the viability of spermatozoa of adult Swiss albino mice. The absolute- and relative testis weight, relative weight of other organs, their biochemical parameters, hypo-osmotic swelling test (HOST) of spermatozoa, testis histology, and fertility and fecundity tests were performed to justify the toxic effects of *D. esculentum* on male reproductive functions. Moreover, acute, sub-acute, sub-chronic and chronic toxicity studies of *D. esculentum* have been performed and its effect on some major organs of mouse, viz. liver and kidney, has been investigated. Biochemical studies of serum were carried out for liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP), γ-glutamyl transferase (GGT), total bilirubin), and kidney function (urea, creatinine). Finally, the *D. esculentum* extract was investigated for its acetylcholinesterase and NADH oxidase inhibitory and total antioxidant activities, as well as its effect on scavenging of different free radicals. The qualitative analysis of different phytochemicals was also carried out for *D. esculentum*, to identify the presence of different phyto-constituents.

Results:

Body weight and relative spleen weight were significantly decreased in BDE fed mice. Significant decreases were observed in the number of plaques formed, HA titer value and in the

number of peritoneal macrophages within a span of 180 d. Significant dose-dependent decrease was observed in the number of cultured splenocytes. Significant dose-dependent increases in the percentage inhibition of splenocyte proliferation as well as percentage of hemolysis were evident by *in vitro* assays. Results indicated significant decreases ($p < 0.05$, $p < 0.01$, and $p < 0.001$) in both Th1 and Th2 cytokine concentrations when compared with their respective controls. Dose dependent inhibition of the viability of sperm was observed in case of all *D. esculentum* fed mice when compared with the controls. The inhibition was statistically significant ($p < 0.001$) and directly proportional to the dose of the BDE. After 135 days and 180 days of treatment, at 320mg/kg body weight, the percentage inhibition of sperm viability was 40.51% and 53.12%, respectively. Significant dose- and time-dependent decreases were observed in absolute- and relative testis weight, relative weights of other organs and their biochemical parameters, percentage of live spermatozoa, and percentage of fertility and fecundity in BDE fed mice. Significant decreases were observed in diameter, perimeter and area of the seminiferous tubules of mice treated for 180 days. The percentage of empty seminiferous tubules was increased significantly in BDE treated mice when compared to the controls. The *D. esculentum* extract also inhibited acetylcholinesterase and NADH oxidase in a dose-dependent manner, with IC_{50} values of 272.97±19.38 and 265.81±21.20 µg/ml, respectively. The extract also showed a potent DPPH radical scavenging activity with an IC_{50} value of 402.88±12.70 µg/ml. Moreover, the extract showed 27.41% and 33.22% of total antioxidant activities determined by FTC and TBA methods, respectively. The single-dose study in mice revealed no untoward physiological events that may arise out of an acute exposure of *D. esculentum* in target species. In the subacute, subchronic and chronic toxicity studies, the data showed significant loss of feed intake and body weight of treated group mice. Gross examination of vital organs such as liver, kidney and testis

of mice from treated groups, and microscopic examination of tissue sections prepared from these organs reveal significant alterations in their histological architecture that could be attributed to *D. esculentum* intake at different doses. Moreover, the activities of serum enzymes such as AST, ALT, LDH, ALP, ACP and GGT (which indicate liver function) were significantly increased after repeated oral dosing of *D. esculentum*. The concentrations of urea and creatinine (which indicate kidney function) have been found to increase significantly in subchronic and chronic doses when compared to the respective control groups.

Discussion and Conclusion:

These results suggest that the intake of *D. esculentum*, even after cooking, may evoke immune dysfunction as well as may cause destruction of erythrocytes even after cooking. *D. esculentum* appears to alter the Th1 and Th2 cytokine balance, and therefore, may induce severe immunosuppression. It shows spermicidal properties which may cause infertility by altering the male reproductive function. It may also alter different liver and kidney parameters. Moreover, results indicated that 70% methanolic extract of *D. esculentum* effectively inhibited the enzymes acetylcholinesterase and NADH oxidase and acted as a potent antioxidant and free radical scavenger, which may be helpful in preventing the progression of various neurodegenerative disorders associated with oxidative stress. Therefore, it can be concluded that crude as well as boiled *D. esculentum* possess several phytochemicals, some of which is beneficial to health. But, the phytochemicals like steroids, tannins or saponins may interfere with the cell metabolism and therefore may be considered as toxic upon consumption. This may be a good reason of avoidance of this fern among insects and cattle. Therefore, people who consume this fern in a regular fashion should be aware about the hazards of its consumption.

ACKNOWLEDGEMENTS

Completion of this doctoral dissertation was possible due to the support of several people. I am grateful to those people who have contributed towards shaping this thesis. I take this opportunity to appreciate and acknowledge the help of each one of these wonderful people in this section of my thesis.

First and foremost I express my sincere gratitude to my respected supervisor, Prof. Tapas Kumar Chaudhuri, Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, for giving me the opportunity to work under his supervision. He has taught me, both consciously and unconsciously, how good experimental immunology is done. I am thankful for all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. The enthusiasm he has for his research was contagious and motivational for me, even during tough times in the Ph.D. pursuit.

I am thankful to the Head of the Department of Zoology, University of North Bengal, for allowing me to use the central instrumentation facility and the departmental animal house during the course of my study. I also extend my deep sense of gratitude to all my teachers of the Department, Prof. Joydeb Pal, Prof. Ananda Mukhopadhyay, Prof. Sudip Barat, Prof. Min Bahadur, Dr. Soumen Bhattacharjee, Dr. Dhiraj Saha and Mr. Tilak Saha for their valuable suggestions and kind help.

I express my sincere gratitude to Prof. Nripendranath Mandal, Division of Molecular Medicine, Bose Institute, Kolkata for giving me the opportunity to work in his laboratory, and also for his whole-hearted support and guidance to carry out a major part of my dissertation.

My fellow lab members with whom I have worked with have contributed immensely to my personal and professional time at the Cellular Immunology Laboratory, Department of Zoology, University of North Bengal. This group has been a source of friendships as well as good advice and collaboration. I am grateful to Dr. Manoj Lama, Department of Zoology, University of Gour Banga, Malda and Mr. Pokhraj Guha for their kind help especially during the

initial stages of my research work. I have had the utmost pleasure to work with or alongside of Dr. Priyankar Dey, Mr. Somit Dutta, Mr. Avishek Das and Mr. Bijoy Mohanto.

I extend my sincere gratitude to my senior colleagues, Prof. Shyamapada Mandal and Dr. Kaushik Chakraborty, Department of Zoology, University of Gour Banga, Malda for their constant encouragement and support to complete my work.

I express my sincere thanks to all the non-teaching, laboratory/office staff of the Department of Zoology, University of North Bengal for their services and kind help.

I like to sincerely acknowledge the University Grants Commission (UGC), New Delhi, India, for providing me the financial assistance to carry out my research work.

It is an opportunity to express my gratefulness to my parents for all their love and inspiration. They have supported me in all my pursuits. Today, I stand here because of their never ending support and encouragement.

Last but certainly not the least, my wife, Bratati Das Roy, deserves a special word of appreciation for her moral support, patience, understanding and love. She has supported me in every possible way to see the completion of this work. Thank you.

Subhrajyoti Roy

Dated: 03.08.2016 (Subhrajyoti Roy)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER – 1:

INTRODUCTION

1. INTRODUCTION

Wild edible plants are used to be one of the commonest food sources for mankind from the time immemorial. Utilization of the wild edible plants as a source of food and medicine is an integral part of the culture of the indigenous people throughout the world. Till date, the agricultural societies did not eliminate the use of non-cultivated resources. Though most of the plant foods for human consumption is based on rather limited number of crops (12 crops contribute more than 85–90% of worlds caloric intake), the use of wild plants are not negligible rather increasing in many parts of the world (Misra et al., 2008). These plants are used as the substitutes and fill the gap of food deficiency.

It was estimated that though a total of 82 species commodities, or 103 species taxonomically, contribute 90% of supplies of food plants in the world (Prescott-Allen & Prescott-Allen, 1990), the usage of wild plant is still a tradition that has survived in many local communities (Turner, 2011). Researchers have emphasized on the diversity and value of traditional vegetables (Misra et al., 2008). Some studies indicated that the nutritional values of traditional leafy vegetables are higher than several known common vegetables (Sundriyal & Sundriyal, 2001; Nordeide et al., 1996; Shackleton et al., 1998; Orech et al., 2007). Most of these traditional leafy vegetables have a potential for income generation but fail to compete with exotic vegetables at present due to the lack of awareness (Misra et al., 2008). Consumption of traditional vegetables has been reported to have many beneficial effects such as prevention of some age related degenerative diseases – arteriosclerosis, stroke, etc. (Lindeberg et al., 2003). According to the several reports, wild green leafy vegetables increase the amount of blood in the body which is likely to be due to the high iron content in them (Misra et al., 2008).

Apart from the beneficial effects, several natural toxins may also be present in these wild edible plants as a result of natural selection and new breeding methods that enhance these protective mechanisms (Risk Assessment Section, 2007). It is noted that information on the possible toxic effects of most of the wild edible plants is little. Therefore, the information

documented on nutritional values and possible side effects of these plants are highly required to make the people aware about the hazardous effects of the consumption of these plants.

Ferns are one of the most widely used wild edible groups of plants throughout the world. Ferns provide food, medicine, fiber, crafts and building material, abrasives and decoration. Ferns constitute the primitive vascular plant group which is found scattered all over the world. Since medicinal uses of some ferns of India were well described, much research works have been done in the field of ethnobotany (Sen & Ghosh, 2011). But very few studies have been done so far on the pharmacological activities of this group of plant.

Since ferns and fern allies have survived from Paleozoic times, they have adapted with many more various changes of environment than the other primitive vascular plants (Wallace et al. 1991). Therefore, ferns are expected to have many useful phytochemicals than other plants. Ferns were reported to have many useful phytochemicals such as flavonoids, steroids, alkaloids, phenols, triterpenoid compounds, varieties of amino acids and fatty acids (Zeng-fu et al., 2008). It is interesting to note that not all the ferns are edible and only a few of them are used as food throughout the world. Edible ferns are some of the most common wild food plants collected by people around the world. The fern stems, rhizomes, leaves, young fronds and shoots, and sometimes the whole plants are used for food (Liu et al., 2012). In recent years, more and more researches have reported the food and ethnomedicinal uses of ferns in different parts of the world but very few studies have been conducted so far to assess the pharmacological/ toxicological impact of this group of plant on human health. If we take the example of bracken fern (*Pteridium aquilinum* var. *latiusculum*), it is observed that bracken fiddleheads are considered as a nutritionally rich food in Korea. They contain significant amounts of protein, fiber, vitamins, and minerals. However in many countries, brackens are known as poisoning plants because of their carcinogenic and antithiamin properties. The carcinogenic substance of bracken is ptaquiloside (Hirono et al. 1984). Ptaquiloside is very carcinogenic in mammals, especially ruminants, which repetitively ingest huge amounts of bracken, and may be very harmful to humans if large and repetitive doses of bracken are taken (Ham, 2004).

Apart from the bracken fern, *Diplazium esculentum* (Koenig ex Retz.) Sw. (Family – Athyriaceae) is one of the most commonly consumed non-bracken edible ferns throughout the

world. Some of the studies have been performed so far to assess the antioxidant, antimicrobial, antitumor or other beneficial activities of this fern (Nanasombat & Teckchuen, 2009; Tongco et al., 2014; Kaushik et al., 2012; Seal, 2013) but very few studies have been performed so far to determine the possible pharmacological and toxicological impacts of this fern on human and animal health. Study conducted on rabbits and guinea pigs demonstrated systemic toxicity and several pathological effects of this fern. Young fronds of *D. esculentum* collected from the high-altitude area of Harsil–Gangotri of Northern India were found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria in hill cattle in India and elsewhere (Somvanshi et al., 2006). A moderate amount of Pta was also found in *D. esculentum* sample that was prepared by freeze drying and shade drying methods (Somvanshi et al., 2006). Moreover, Pta rich frozen- and shade-dried crude *D. esculentum* have already been shown to cause poor growth, decreased body weight, increased spontaneous (vertical and horizontal) and decreased forced motor activity, alterations in values of blood glucose, erythrocyte sedimentation rate, mean corpuscular volume, mean corpuscular hemoglobin, total leukocyte count, neutrophil, lymphocyte and monocyte count, and increased blood SGOT level in both rats and guinea pigs (Gangwar, 2004). Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats (Smith et al., 1994).

It is observed that this fern grows abundantly in the marshy land and also in the wet shabby places where lot of insects are available. But interestingly, insects consuming the leafy portion of this fern have never been found. Leaves are all intact, not consumed by any insect and even cattle. We also observed that the Rajbanshi population, the original inhabitants of this region, consumes this fern regularly after cooking. It seemed that they are aging at a much faster rate as they looked much older than that of their actual age. As we did not get convincing data regarding the toxicity of this fern (if any) to human, we performed the experiments using inbred strains of mouse as an animal model to establish the immunopharmacological as well as toxicological basis of this edible fern.

Since early days, local people were consuming the unmodified plant parts (coiled frond and young leaf) or boiling the plant materials of *D. esculentum* in water. But, sometimes, even

boiling may not completely destroy the toxic materials that are harmful to the body. Therefore, the present study on the pharmacological properties of this wild edible fern may serve as a baseline data for the future studies on the nutritional values and possible side effect of this plant in the study area and elsewhere.

CHAPTER – 2:

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The Review of Literature of this thesis balances the foundational material of immunology and pharmacology with the latest and updated information of the study concerned. However, the content has been winnowed to allow thoughtful updating. In selecting material to be included in this chapter, we have tried to be comprehensive, not encyclopedic.

This chapter starts with an overview of the immune system and association of the immune system with other systems (viz. nervous, reproductive). Then a brief note on the concept of immunomodulation and the role of plants and their secondary metabolites on human and animal health are described. Thereafter, a brief description of the usage of ferns by humans, different bioactive potentials of ferns, and various phytochemicals present in them are described in details. Finally, a brief description of established reports of the edible fern, *Diplazium esculentum* has been provided.

2.1. AN OVERVIEW OF THE IMMUNE SYSTEM

Immune system is designed to protect against foreign organisms or substances (antigens). Immunity involves both humoral and cellular components. Humoral immunity combats pathogens via antibodies, which are produced by B cells and can be found in bodily fluids, and can be transferred passively between individuals to provide immune protection. Cell-mediated immunity involves primarily antigen-specific T lymphocytes, which act to eliminate pathogens or otherwise aid other cells in inducing immunity. The vertebrate immune response can be divided into two interconnected arms of immunity: innate and adaptive. Innate responses are the first line of defense, utilizing germline-encoded recognition molecules and phagocytic cells. Innate immunity is fast, non-specific but rather constitutes a first line of defense, which includes anatomic, physiologic, endocytic, phagocytic, and inflammatory barriers. Adaptive immune responses exhibit four immunologic attributes: specificity, diversity, memory, and self/non-self recognition. It relies upon the B- and T-cell receptors that are randomly generated by DNA rearrangements in developing B and T cells. Innate and adaptive immunity operate in cooperative and interdependent ways. The activation of innate immune responses produce signals that stimulate and direct subsequent adaptive immune responses. Therefore, these two

branches of the immune system often overlap. The high degree of specificity in adaptive immunity arises from the activities of molecules (antibodies and T-cell receptors) that recognize and bind specific antigens. Antibodies recognize and interact directly with antigen. T cell receptors recognize only antigen that is combined with either class I or class II major histocompatibility complex (MHC) molecules. The two major subpopulations of T lymphocytes are the CD4+ T helper (T_H) cells and CD8+ T cytotoxic (T_C) cells. T_H cells secrete cytokines that regulate immune response upon recognizing antigen combined with class II MHC molecule. T_C cells recognize antigen combined with class I MHC and give rise to cytotoxic T lymphocytes (CTLs), which display cytotoxic ability. Exogenous (extracellular) antigens are internalized and degraded by antigen-presenting cells (macrophages, B cells, and dendritic cells); the resulting antigenic peptides complexed with class II MHC molecules are then displayed on the cell surface. Endogenous (intracellular) antigens (e.g., viral and tumor proteins produced in altered self-cells) are degraded in the cytoplasm and then displayed with class I MHC molecules on the cell surface. (Goldsby et al., 2003)

Innate and adaptive immune systems are regulated by a complex network of chemical signals, including enzymes, immunoglobulins and cytokines (Calder, 2006; Li et al., 2007). A bi-directional communication pathway between the immune and endocrine systems supports health and optimal growth (Carroll, 2008). Dysfunctions of the immune system include common maladies such as allergies, asthma, and autoimmune disease (overly active or misdirected immune responses) as well as immune deficiency (insufficient immune responses). Transplanted tissues and cancer present unique challenges to clinicians, because the healthy immune system typically rejects or destroys non-self proteins, such as those encountered in most transplant situations, and tolerates self cells (Owen et al., 2013).

2.2. ASSOCIATION OF IMMUNE SYSTEM WITH OTHER SYSTEMS OF THE BODY

2.2.1. Involvement of the cholinergic nervous system in immunity (The Cholinergic Anti-Inflammatory Pathway)

Acetylcholine (ACh) is a ubiquitous neurotransmitter and found even in the roundworm *Caenorhabditis elegans*, one of the simplest organisms with a nervous system (Rakowski et al.,

2013; Kosinski & Zaremba, 2007). Humans have a large percentage of cholinergic nervous systems including the CNS. Cholinergic nerves also form a major part of the sympathetic and parasympathetic nervous systems (Pohanka, 2012; Bellier & Kimura, 2011). The wider significance of ACh is in understanding the biological effects of tested toxins and/or medical drugs: as any immunological effects of Acetylcholinesterase (AChE) inhibitors can involve both CNS and PNS, this has to be taken into consideration in interpreting any findings (Pohanka, 2014).

The cholinergic system is tightly associated with the cholinergic anti-inflammatory pathway dominantly located in blood and mucosa. This pathway is a regulatory link between nerve terminations in blood and macrophages expressing the α7 nicotinic acetylcholine receptor (α7 nAChR) on their surface (Pohanka, 2012; Wessler & Kirkpatrick, 2008; Rosas-Ballina & Tracey, 2009). Discovery of the cholinergic anti-inflammatory pathway allows us to understand how the CNS is involved in the regulation of innate immunity (Pohanka, 2014). AChE bound on erythrocytes plays an important role in termination of cholinergic anti-inflammatory pathway activation (Pohanka, 2012; Silva-Herdade & Saldanha, 2013). AChE activity is typically low in Alzheimer's disease (AD) patients treated with AChE inhibitors (Coin et al., 2012). Compared to AChE, BChE is constituted in the liver and secreted into the plasma where the enzyme is dissolved (Iwasaki et al., 2007). Apart from the fact that the conversion rate of ACh by BChE is lower than the conversion by AChE, BChE can substitute for AChE and split the neurotransmitter once they make contact (Karlsson et al., 2012; Pohanka, 2011). The effect of BChE became relevant once the cholinergic anti-inflammatory pathway was studied as BChE plays a greater role in the blood than in the nervous system (Pohanka, 2014).

The cholinergic anti-inflammatory pathway is one-way: the CNS can attenuate inflammation mediated by macrophages or any other immune cells having α7 nAChR. ACh released from the vagus nerve agonizes α7 nAChR, which responds by opening a central channel allowing an influx of Ca^{2+} into macrophages (Pohanka, 2012; Noelker et al., 2013; Lee et al., 2014). Increased levels of Ca^{2+} activate the nuclear factor κB (NFκB) resulting in suppression of inflammatory cytokine production including tumor necrosis factor α (TNFα), high mobility group box of proteins and interleukin 6 (IL-6) (Sun et al., 2013; De Haan et al., 2013). The blood

AChE and plasma BChE are able to terminate the stimulation of the cholinergic anti-inflammatory pathway due to splitting of ACh. The principle of the pathway is depicted in following illustration (Pohanka, 2014).

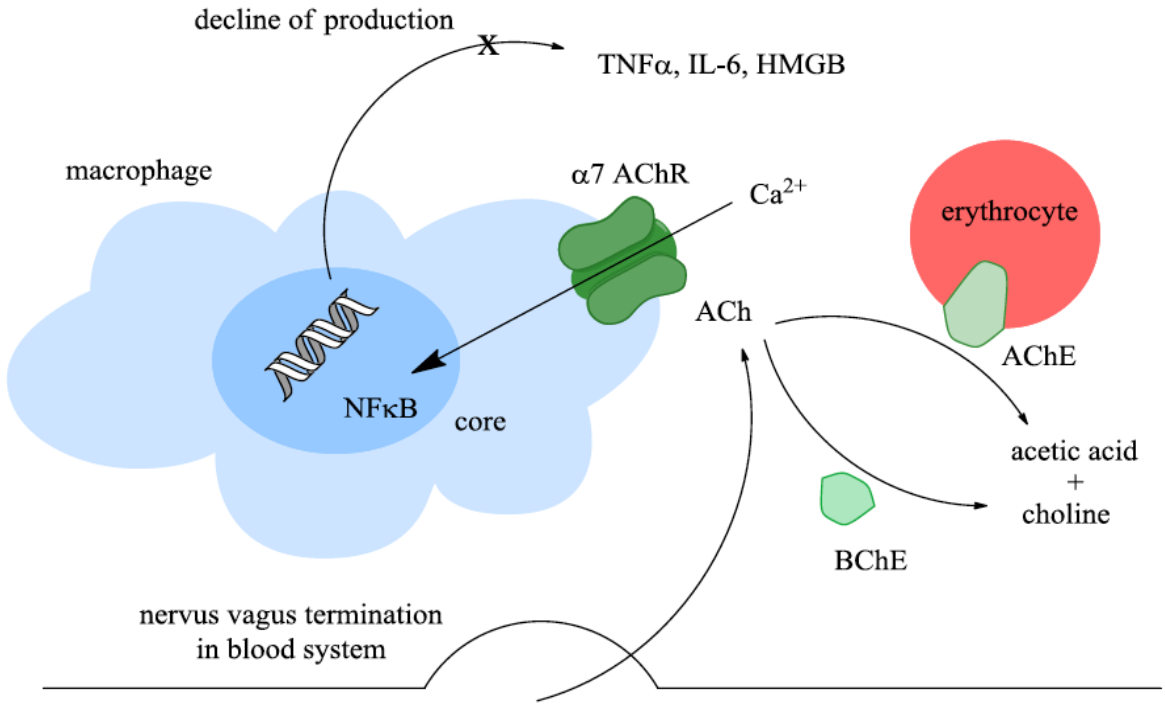


Figure 1. Principle of the cholinergic anti-inflammatory pathway; abbreviations: ACh-acetylcholine; AChE-acetylcholinesterase; BChE-butyrylcholinesterase; HMGB-high mobility group box; IL-6-interleukin 6; NFκB-nuclear factor kappa B; TNFα-tumor necrosis factor alpha (Pohanka, 2014).

Several recent studies indicate that these inhibitors can cause a significant modulation of immunity as a side effect (Pohanka, 2011; Starec et al., 1997; Pohanka, 2013). Figure 1 indicates the modulation of the cholinergic anti-inflammatory pathway via protection of ACh from splitting by cholinesterases and thus enhancing the pathway. The mechanism may be relevant when inhibitor of cholinesterases is used in large amounts and/or for a long time such as patients suffering from AD (Pohanka, 2014).

Apart from the regulation processes, some inhibitors can influence the immune system via forming antigens by reacting with e.g., plasma proteins. The immune system is thus activated

and the stimulation counteracts the anti-inflammatory action. This effect is, however, very weak but it can play a role in forming antibody modified by nerve agents (Smirnov et al., 2013).

2.2.2. Interaction of the reproductive system with the immune system

It is now accepted that the neuroendocrine system (including its reproductive component) and the immune system have a reciprocal regulatory influence on the development and functioning during pre- and postnatal ontogeny. The key role in the interaction of the reproductive and immune systems is played by the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) and sex hormones. During the perinatal period, they regulate the growth and differentiation of various fetal tissues, including the lymphoid tissue. GnRH regulates the secretion of pituitary gonadotropins, which regulate secretion of sex hormones. GnRH is also involved in regulation of sexual behavior, transmission of olfactory signals, and control of humoral and cell-mediated immunity. Sex hormones, in turn, regulate GnRH production in the hypothalamus and therefore, secretion of pituitary gonadotropins and also its production in the thymus and spleen. On the other hand, mediators of the immune system such as thymic peptides and proinflammatory cytokines have a role in controlling the development and functioning of the reproductive system (Zakharova & Izvolskaia, 2012).

Interactions of the reproductive and immune systems during early ontogeny are prerequisite to their normal functioning in adult life. Changes in the normal levels of GnRH and sex steroids in the developing fetus or newborn and their exposure to adverse environmental factors cause disturbances in long-term programming of the regulatory mechanisms of both reproductive and immune systems (Zakharova & Izvolskaia, 2012).

The Sertoli cells of the seminiferous epithelium, and the steroidogenic Leydig cells, together with the testicular macrophage population, have been directly implicated in suppressing or regulating immune responses to antigens located within the testicular environment. It is increasingly evident that these immunological control mechanisms are also impinged upon, and may even participate in the regulation of normal testicular function, spermatogenesis, and steroidogenesis. Conversely, failure of immune privilege is a significant cause of disease in the male tract, leading to chronic inflammation, infertility, and pain (Hedger, 2012).

Many cytokines are crucially important for reproductive processes and their role has been investigated in reproduction. Cytokines are immunoregulatory molecules responsible for determining the nature of immune response. It has been suggested that lower index of Th1/Th2 immune response is supportive for physiological pregnancy. IL-1 and IL-18 are the important factors in embryonic endometrial dialogue, subsequent invasion, neoangiogenesis and embryo implantation, which is an essential step in mammalian pregnancy. Other cytokines like IL-1-beta, IL-6 and TNF-alpha are essential in ovarian cycle regulation and play an important function during growth and development of ovarian follicle. In addition to that, increased production of Th1 cytokines such as TNF-alpha plus IFN-gamma compared to the Th2 cytokine IL-10 is linked to infertility and recurrent spontaneous abortion (RSA) (Mahdi, 2011).

Cytokine tumor necrosis factor alpha (TNF-alpha), produced by Th1 cells, is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages that has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function and evidence was supported by previous studies in which higher serum levels of TNF-alpha were detected in RSA groups and reproductive failure. It is suggested that Th1 cytokines trigger thrombotic/inflammatory processes at the maternal utero-placental blood vessels by activation of vascular endothelial cell procoagulant. The administration of TNF-alpha to normal pregnant mice significantly increased fetal resorption (Mahdi, 2011).

2.3. THE CONCEPT OF IMMUNOMODULATION, IMMUNOMODULATORS AND THEIR CLASSIFICATION

The term immunomodulation means the alteration of the immune response which may increase or decrease the immune responsiveness. Enhancement in the immune responsiveness is called immunostimulation and reduction in the immune responsiveness is called immunosuppression. An immunomodulator may be defined as a substance, biological or synthetic, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response (Kumar et al., 2012). Clinically, immunomodulators can be classified into the following three categories:

2.3.1. Immunostimulants

They are inherently non-specific as they are envisaged as enhancements to a body's resistance to infection. They can act through innate as well as adaptive immune responses. In healthy individuals, the immunostimulants are expected to serve as prophylactic and promoter agents, i.e., as immunopotentiators, by enhancing the basic level of immune response. In the individual with impairment of immune response, they are expected to act as immunotherapeutic agents (Kumar et al., 2012).

2.3.2. Immunosuppressants

They are structurally and functionally heterogeneous group of drugs, which are often concomitantly administered in combination regimens to treat various types of organ transplant rejection and autoimmune diseases (El-Sheikh, 2008).

2.3.3. Immunoadjuvants

They are used to enhance the efficacy of vaccines and therefore could be considered as specific immune stimulants. Immunoadjuvants hold the promise of being the true modulators of the immune response. They can be exploited as selectors between the cellular and humoral helper T1 (Th1) and helper T2 cells (Th2), immunoprotective, immunodestructive, and reagenic [immunoglobulin E (IgE)] versus IgG type immune responses – posing a real challenge to vaccine designers (Alfons & Patrick, 2001).

2.4. IMMUNOMODULATION BY NATURAL PLANT PRODUCTS

Generation of an effective immune response typically involves the critical steps of antigen presentation, activation of T- and/or B-lymphocytes and the resultant secretion of immune effector molecules such as antibodies and cytokines. The development of such effective immune responses following infection or vaccination can be influenced by plant-derived immunomodulators. The ability to modulate immune function offers many advantages from maintaining health through immunity to stimulating or suppressing beneficial or deleterious immune responses. The following illustration describes how the plant immunomodulators could mediate such effects through targeted modulation of key cellular and molecular interactions of

antigen-presenting cells (e.g. dendritic cells), T-lymphocytes and B-lymphocytes (Licciardi & Underwood, 2011).

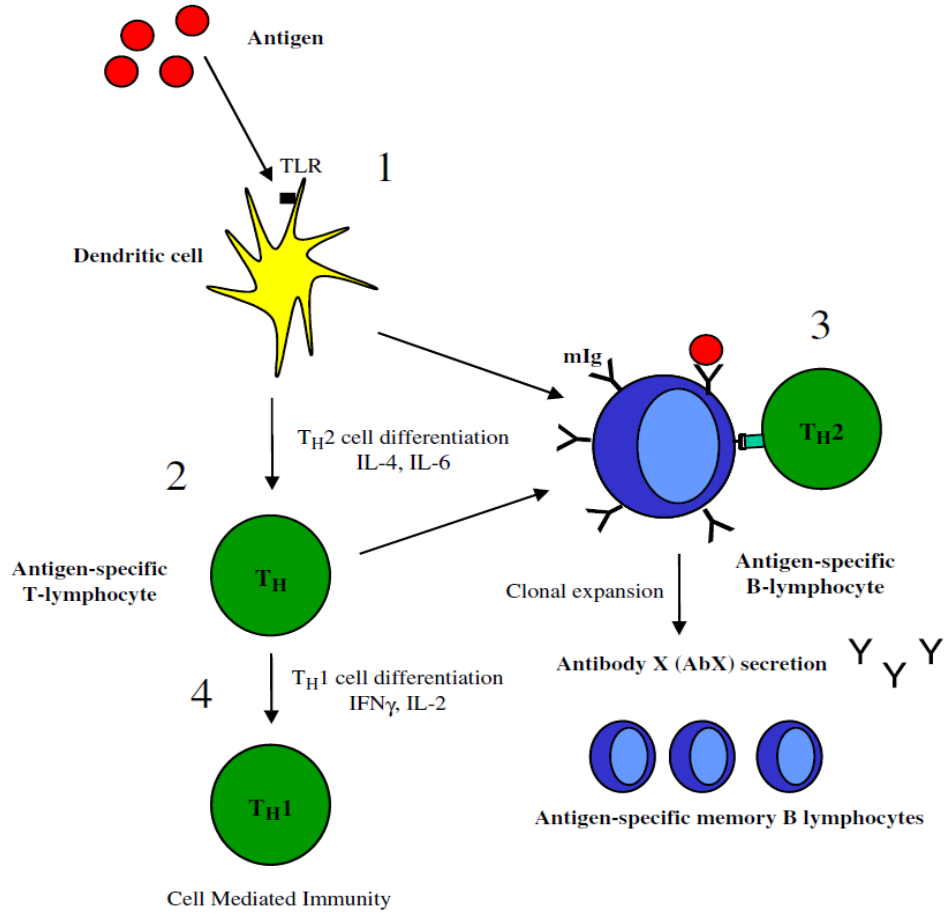


Figure 2. Paradigm for the immunomodulatory activity of plant-derived medicines. Major parameters of the immune response that may be influenced by plant-derived medicines include 1) uptake and processing of antigen by specialised APC such as dendritic cells (DCs) via TLRs; 2) presentation of antigen peptide: MHC by DCs to TH2-lymphocytes (T-dependent pathway) in association with TCR and co-stimulation; or 3) presentation of antigen directly to B-lymphocytes (T-independent pathway). Alternatively, differentiation into TH1 CD8+ T-lymphocytes may occur depending on the cytokine microenvironment and/or the type of infection (4). Such activities of plant-derived medicines may be exploited in the discovery of novel human vaccine adjuvants (Figure adapted from Licciardi & Underwood, 2011).

Very few studies have been done so far with the plant-derived medicines that influence the important biological effects on the immune system. Identification of such immunomodulatory properties will be crucial in the discovery of novel, clinically-relevant

compounds for augmenting existing immunotherapy. Perhaps more importantly, immunomodulatory plant-derived medicines may be critical in achieving a better health status through enhanced protective immunity (Licciardi & Underwood, 2011).

Plant-derived medicines in healthcare have been used since the dawn of every ancient human civilization. Many plant-derived medicines are said to provide a 'tonic' effect that assists the body in the maintenance of health. From this, it may be reasonable to presume some immune basis for this effect, since improved immunity would enhance human health (Licciardi & Underwood, 2011). Researchers, from the beginning of the early-1970s, became increasingly aware of the importance of structurally diverse compounds not directly involved with the essential or "primary" roles of photosynthesis, respiration, growth and development of plants (Rosenthal and Janzen, 1979; Cozier et al., 2006). Phytochemicals, many of which accumulate in surprisingly high concentrations in plants, not belonging to this "primary" category were considered "secondary" and waste products of plant metabolism (Rosenthal and Berenbaum, 1992). We now know that these compounds protect plants from consumers and pests, and they serve as attractants for pollinators and seed dispersing animals, as allelopathic agents, and as filters for UV radiation. They also help plants recover from injury, persist and adapt (Rosenthal and Janzen, 1979; Palo and Robbins, 1991; Cozier et al., 2006).

At high doses, secondary compounds can adversely affect the cellular and metabolic processes, reduce forage intake, and cause weight loss and even death (Cheeke and Shull, 1985; Cheeke, 1988). However, secondary compounds can also adversely affect the harmful bacteria, parasites and fungi that inhabit herbivores' bodies and cause decreases in health status (Lozano, 1998). Thus, at certain concentrations and in appropriate mixtures, secondary compounds can have beneficial effects on herbivores (Provenza, 2008). The nature of this dual action (i.e., toxin/medicine) is merely a matter of dosage and a consequence of the animal's tolerance and current physiological state (Plotkin, 2000).

2.5. EFFECTS OF SOME COMMON SECONDARY COMPOUNDS OF PLANT ON IMMUNE FUNCTION AND OVERALL HEALTH STATUS

2.5.1. Flavonoids

Flavonoids are phenolic substances, widely found in fruits and vegetables. The main effects of flavonoids on immune responses may be due to their actions such as protein binding, active site interference, or antioxidant effects. Several flavonoids specifically affect the function of the enzymes involved in generating inflammatory responses. Dietary flavonoids have been reported to modulate the inflammatory response and have inhibitory effects primarily on T lymphocytes (Middleton et al., 2000). Some flavonoids also alter immune responses which could be involved in immunosurveillance of tumors. Quercetin suppresses antigenic stimulation of cytotoxic T-lymphocytes and inhibits natural killer cell mediated cytolysis (Nichenametla et al., 2006).

2.5.2. Anthocyanins

Condensed tannins or proanthocyanidins are widely distributed in plants from grasses and legumes to browse leaves and fruits (Mueller-Harvey, 2006). Eating plants, high in tannins, is a way for herbivores to reduce internal parasites (Min and Hart, 2003), and tannins alleviate bloat by binding to proteins in the rumen (Waghorn, 1990). This high-quality protein bypass effect has the potential to enhance the immune response and increase the resistance to gastrointestinal nematodes (Min et al., 2004). Bypassing amino acids like arginine, glutamine and cysteine can enhance immune responses as these amino acids regulate activation of T and B lymphocytes, natural killer cells and macrophages, gene expression and lymphocyte proliferation, and the production of antibodies, cytokines and other cytotoxic substances (Li et al., 2007).

Changes in populations of commensal bacteria in the gastrointestinal tract may stimulate gut-associated myeloid tissues and consequently modulate T- or B-cell-mediated immune responses. Thus, through their effects on intestinal bacteria, tannins may have probiotic effects that indirectly impact the immune system. Moreover, the selective effects of tannins on bacteria, both in the rumen and in the intestines, may be an important avenue for research regarding the impact of tannins on intestinal immune responses (Provenza & Villalba, 2010).

2.5.3. Alkaloids

Alkaloids are a diverse group of nitrogen-containing compounds, present in about 20% of plant species, mostly derived from amino acids. Several alkaloids have been tested for their impacts on immune function. Some alkaloids have been reported to enhance the activity of natural killer cell, antibody-dependent cell mediated cytotoxicity, as well as reduction of pro-inflammatory cytokines (Manu and Kuttan, 2007). Some alkaloids have been used to treat many acute and chronic diseases. They directly or indirectly activate macrophages through T cell-associated effects (Furusawa and Wu, 2007). Some alkaloids may have immunosuppressant effects and this may be due to the altered antioxidant status, including vitamin E, in both the plant and the animal (Dawe et al., 1997).

2.5.4. Terpenes

Terpenes are a large and varied class of hydrocarbons derived biosynthetically from units of isoprene. They represent one of the most diverse classes of secondary metabolites of plant (Cozier et al., 2006). Over 30,000 compounds have been identified from fragrances and antibiotics to insect attractants and antifeedants. Information on the immunomodulatory effects of terpenes is scarce. Terpenes have been reported to enhance the cytotoxic activity of NK cell *in vitro* and *in vivo* (da Silva et al., 2007), reduce the production of some proinflammatory cytokines (Li, 2000), function as immunosuppressive agents (Okada et al., 1996), and have bacteriostatic and bactericidal properties (Oh et al., 1970; Nagy & Regelin, 1977). They also have selective effects on ruminal bacteria (Villalba et al., 2006). The selective effects of terpenes, as with tannins, on the gastrointestinal tract could have indirect effects on immunomodulation (Provenza & Villalba, 2010).

2.6. BRIEF DESCRIPTION OF THE USAGE OF FERNS BY HUMANS

2.6.1. Foods

A fern like ostrich fern, *Matteuccia struthiopteris*, is used as food in Europe and America, whereas in Asia and Oceania, several fern species are used as foods and traded in market place. In Korea, bracken fiddleheads are considered as a nutritionally rich food. The dried and steamed

fiddlehead of brackens are important as ancestral service food for different occasions every year in Korea. In USA, boiled fiddleheads are commonly prepared with butter, cider or wine vinegar and a bit of pepper, or pickles with fiddleheads. The cooked fiddleheads have a soft and rich taste; sometimes they taste a bit like asparagus but are much tougher. They contain significant amounts of protein, fiber, vitamins, and minerals (Lee, 2011).

Among other ferns, *Diplazium esculentum* is one of the top preferred edible ferns in the Himalayas, the whole Southeast Asia, China and Japan. In many parts of Eastern Southeast Asia, people use this mineral- and energy-rich edible species by cooking the upper shoots/fronds as vegetables. Available literature indicates that the edible fronds are rich in iron, phosphorus, potassium and protein, richer than that of many conventional vegetables and many wild edibles. The mineral content has also been reported to be several times greater than that of many commercial fruits. The most common recipe using *D. esculentum* involves cooking the dried fronds in oil or butter, using them in a vegetable curry is less preferred. In the north-eastern India, especially in Sikkim, and in the Central and north-western Himalayan states, Himachal Pradesh and Uttarakhand, the local folk relish both vegetables and pickles from *D. esculentum*. Natives consider these recipes effective both to counteract constipation and as an appetizer, especially as a pickle (FAO, 2010).

2.6.2. Cosmetic ingredient

Dryopteris spp. exhibit strong antimicrobial activities against *Propionibacterium acnes*, known as a main factor of acne (pimple) (Kim et al. 2006). Many ferns and fern allies contain considerable amount of phenolic compounds which are known to have beneficial effects on skin such as the prevention of UV-induced skin damage, anti-wrinkle, skin-whitening, etc. Phenolic compounds are currently used as ingredients of natural body and facial cosmetics such as cleanser, toner, moisturizer, shampoo, conditioner, and so on (Lee, 2011).

Ecdysone, a phytoecdysteroids present in several ferns, show the effects on cell regeneration, skin texture refinement and skin barrier strengthening (Lin and Lin 1989; Meybeck et al. 1997). A facial scrub product including spores of bracken is patented in Korea (Jin et al. 2005). The scrub including spores does not cause the skin abrasion due to its small particle size. Therefore

scrubbing the face or body with spores could promote blood circulation and remove dead skin cells smoothly. The ground or skimmed spores of *Lygodium japonicum* are used to produce face mask, powder foundation and compact powder which could increase the effects of skin refinement, increase color expression and control discoloration due to the absorption of sweat and sebum (Son et al. 1999). So, various parts of ferns could be effectively used as natural cosmetic ingredients for skin healing, skin smoothening, anti-acne and protection from aging or UV damage. However, as there are fewer natural cosmetics with ferns as main ingredients than with flowering plants, more research of ferns for application to cosmetic material is required (Lee and Shin, 2011).

2.6.3. Air Purifier

Many fern species show strong air purification activities by removal of volatile formaldehyde, according to the research by NASA and KRDA (Korea Rural Development Administration). According to NASA's report, *Nephrolepis exaltata* (Boston fern) and *Nephrolepis obliterata* (Kimberly queen fern) have benefits for reducing the indoor air pollution such as formaldehyde, xylene and toluene. Furthermore, *N. exaltata* is reported as the most efficient species for removing formaldehyde (Lee and Shin, 2011).

Formaldehyde is the most common indoor volatile organic compound (VOC) with substantially high concentrations. Formaldehyde has several side effects to human beings such as nausea, sore throat, watery eyes, eye burning sensations, headaches, fatigue, and so on (Olsen and Dossing, 1982; US CPSC, 1997). Therefore, the formaldehyde-absorbing ability is one of the most effective functions of ornamental plants. Also according to KRDA, several ferns and fern allies are highly efficient in formaldehyde removal. The formaldehyde removal efficiency has been tested in 84 species of plants. *Osmunda japonica,* similar to royal fern, showed the best formaldehyde removal in chamber. In addition, *Selaginella tamariscina*, *Davallia mariesii*, *Polypodium formosanum* and *Pteris multifida* have been ranked as highly efficient formaldehyde-removing plants. Other ferns, such as *Pteris dispar*, *Cyrtomium caryotideum* var. *koreanum*, and *Sceptrium ternatum*, showed better formaldehyde removal than areca palm tree, the best air purifying plant ranked by NASA. So, ferns could purify the air just by keeping them indoors or outdoors (Lee and Shin, 2011).

2.6.4. Ethnomedicine and other uses

The apatani and Nyishi tribe of Arunachal Pradesh, India uses the frond of *Diplazium esculentum* plant as medicine for constipation and indigestion, and sometimes to cure skin ailments (Kala, 2005; Das et al., 2009). Study conducted in the villages of the Parvati valley, Himachal, India revealed that, of 50 consumed wild edibles, *D. esculentum* is used as a vegetable/pickle by an average of 66 percent of the inhabitants (Kala, 2005). In many Himalayan areas, including the present study area, the dried leaves are used as cattle bedding.

2.7. BIOACTIVE POTENTIAL OF FERNS

2.7.1. Natural antioxidant

Ferns and fern allies are thought to be effective antioxidant agents for protection against aging and chronic diseases. Antioxidant activities, DPPH radical and ABTS radical cation scavenging activities of frond and rhizome extracts of several genus such as *Davallia*, *Diplazium*, *Hypolepis*, *Pteridium*, *Cytominum*, *Dryopteris*, *Polystichum*, *Dicranopteris*, *Lycopodium*, *Osmunda*, *Adiatum*, *Coniogramme*, *Polypodium*, *Pyrrosia*, *Pteris*, *Lygodium*, *Selaginella*, *Thelypteris*, *Athyrium*, *Matteuccia*, *Onoclea* and *Woodsia* were analyzed (Shin, 2010). Ferns belonging to the family Dryopteridaceae, Osmundaceae, Woodsiaceae exhibit powerful antioxidant activities. Crude extracts obtained from these ferns showed antioxidant activities more than vitamin C or BHT (synthetic antioxidant). So, most of the ferns have been thought to possess huge potential abilities as antioxidants.

2.7.2. Natural antimicrobial agents

The extracts obtained from ferns and fern allies have effective antimicrobial activities against fungi, Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*) (Banerjee and Sen 1980; Vincent and Kanna 2007; Lee et al. 2009). Especially, the genus *Dryopteris* showed vigorous antibiotic activities. *D. crassirhizoma* and *D. filix-mas* can be used against MRSA (Methicillin resistant *S. aureus*) (Lee et al. 2009), and *D. cochleata* against Gram positive and negative bacteria and fungi (Banerjee and Sen 1980). *D. crassirhizoma* is patented as an anti-tooth decay substance because of its high activity against *Streptococcus* in Korea (Park et al. 1995).

Therefore, ferns and fern allies can be used as efficient natural antimicrobial ingredients. They could be developed into antibiotic sprays, packing material, toothpaste, hand wash, etc. for protecting the human body and our living environment from undesirable microbials. Most Pteridophytes are known to contain antimicrobial substances such as polyphenols and flavonoids (Francisco and Cooper-Driver, 1984).

2.7.3. Antiulcer activity of ferns

In the region of Sao Paulo, Brazil, the local population makes use of a tea prepared from rhizomes of *Micogramma squamulosa* (Kaulf.) Sota., for the treatment of ulcers (Suffredinia et al., 1999). The action of a crude extract of this species has been evaluated against acute ulcers caused by ethanol and hydrochloric acid using misoprostol and cimetidine as reference substances in both the tests. The extracts showed significant activity against sub-chronic ulcers, but not against acute ulcers. The mechanism of action must be related to the presence of tannins in the extracts, which would induce a stringent action or to the presence of flavonoids, through a systemic action perhaps similarly to the cimetidine action.

2.7.4. Analgesic activity of ferns

Adiantum cuneatum Langsd. and Fisch, (=*Adiantum raddianum* C. Presl), which is used as an ornamental plant is famous for its medicinal properties, mainly in the treatment of pain. Phytochemical examination of the crude extract of this species showed high amount of triterpenes in this species. Fractionation of this extract led to the isolation of the triterpenes filicene, filicenal, adiantol and isoadiantone (Bresciani et al*.,* 2003). Stems of the species *Equisetum arvense* L., furnished a hydro-alcoholic extract that has shown analgesic effect against chemical models of pain perception (acetic acid induced writhing syndrome), but not in thermal models (hot plate) with central and peripheral action (Do Monte et al., 2004).

Oral administration of *Drynaria quercifolia* produced significant inhibition of carrageenan-induced paw oedema and granuloma formation in rats, almost comparable to that caused by indomethacin. *Drynaria quercifolia* significantly attenuated acute and delayed phases of formalin-induced pain and acetic acid-induced writhing episodes in mice. The analgesic activity was comparable to that of sodium salicylate and aspirin respectively (Anuja et al., 2010).

2.7.5. Anti-inflammatory and antinociceptive activity of ferns

Nonato *et al*. (2009) evaluated the antinociceptive and anti-inflammatory actions of the methanolic crude extract of blades of *Blechnum occidentale* L. The study also demonstrated that systematic administration of the methanolic extract of *B. occidentale* did not produce any motor performance alteration. The hydro-alcoholic extract of stems of the species *Equisetum arvense* L. showed anti-inflammatory activity (do Monte et al. 2004). *Cyathea phalerata* Mart. is used to treat various diseases associated with inflammatory processes. Phytochemical investigations of this plant showed the presence of an active flavonoid kaempferol-3-neohesperidoside, which possess hypoglycaemic activity (Pizzolatti *et al.* 2007).

2.7.6. Cytotoxicity of ferns

Different pteridophytes viz. *Adiantum australasicum*, *Adiantum plantagineum*, *Lycopodium reticulatum*, *B. lonchophora*, *Microsorum commutatum*, and *Lycopodium ernua* exhibited cytotoxic activity in a brine shrimp lethality assay conducted at the Gump Research Station on Moorea. Differences between cytotoxicity of root, leaf extracts, ethanol and water extracts were found in the cytotoxicity experiments, suggesting a diversity of compounds within the pteridophytes (Baltrushes, 2005). *Selaginella* has many cytotoxic species and each species with such activity contains bioflavonoids. *Pityrogramma calomelanos*, a moorean fern, is cytotoxic and contains flavonoids (Star et al., 1971**,** Sukumaran et al., 1991). *Peris semipinnata* and *Pteris multifada*, both have cytotoxic properties and contain diterpenes (Li et al., 1998, Li et al., 1999). The ethyl acetate, butanol and water fractions of *Blechnum orientale* Linn. (*Blechnaceae*) possessed cytotoxic activity towards the human colon cancer cell HT-29. Phytochemical analysis of this fern revealed the presence of flavonoids, terpenoids and tannins. Ethyl acetate and butanol fractions showed highest total phenolic content (Lai et al., 2010).

2.7.7. Hepatoprotective activity of ferns

The hepatoprotective potential of *Lygodium flexuosum* (L.) Sw. was evaluated in male Wistar rats against carbon tetrachloride-induced liver damage in preventive and curative models. Rats pre-treated with *Lygodium flexuosum* remarkably prevented the elevation of serum AST, ALT, LDH and liver lipid peroxides in CCl_4-treated rats. Rats treated with the extract after the

establishment of CCl_4 induced liver injury showed significant protection of liver as evident from normal AST, ALT, LDH and MDA levels. Hepatic glutathione levels were significantly increased by the treatment with the extracts in both the experimental groups. Histopathological changes induced by CCl_4 were also significantly reduced by the extract treatment in preventive and curative groups. Phytochemical studies revealed the presence of saponins, triterpenes and sterols, and therefore, *Lygodium flexuosum* extract could be responsible for the hepatoprotective action (Wills and Asha, 2006).

The rhizomes of *Helminthostachys zeylanica* (L.) are used by the Kattunaikan tribe of Kerala for the treatment of various hepatic disorders. The effect of the methanolic extract of *Helminthostachys zeylanica* rhizomes on carbon tetrachloride (CCl_4)-induced liver damage in Wistar rats was studied. The results showed significant hepatoprotective effect against CCl_4-induced liver damage by oral administration of *Helminthostachys zeylanica* methanolic extract. The extract was effective in increasing the choleretic activity of anaesthetised normal rats. It also shortened hexobarbitone induced sleeping time in mice, which was increased by CCl_4 treatment, besides showing significant antilipid peroxidant effect *in vitro*. This provides a scientific rationale for the traditional use of this plant in the management of liver diseases (Suja et al., 2004).

2.8. POSSIBLE HARMFUL EFFECTS OF FERN CONSUMPTION

Bracken fiddleheads are considered as a nutritionally rich food in Korea. They contain significant amounts of protein, fiber, vitamins, and minerals. However, in many countries brackens are known as poisoning plants because of their carcinogenic and antithiamin properties. The carcinogenic substance of bracken is ptaquiloside (Hirono et al. 1984). Ptaquiloside is very carcinogenic in mammals, especially ruminants, which repetitively ingest huge amounts of bracken. However, bracken consumption does not lead to carcinogenesis in humans, because people eat bracken in smaller quantities than animals and do not eat the bracken repetitively. When people eat 350 g fiddlehead of bracken every day, it can cause cancer (Ham 2004). However, people could not get cancer by consuming bracken because nobody can eat more than 350 g of fiddlehead every day.

The antithiamin activity of bracken is extinguished during washing in running water after boiling with or without ashes or sodium hydrogen carbonate. Furthermore, the antithiamin substances in brackens, such as astragalin, isoquercitrin, rutin, caffeic acid, tannic acid, etc., are known as useful natural substances for anticancer or antioxidant in the present time (Kweon 1986; Cai et al. 2004; Katsube et al. 2006). So, the fiddlehead of bracken can be used as a tasty side dish helpful to human health.

While toxicities caused by carcinogenesis and antithiamin activities of bracken fern have been highlighted, the pharmacological effects of the fiddleheads or whole plants of ferns and fern allies are underestimated. However, several healthy effects of ferns and fern allies are currently well known. For example, the glycoprotein isolated from bracken fiddlehead has immune function (Park et al. 1998), and the acidic polysaccharides isolated from the hot water extract of dried bracken fiddlehead have anti-complementary activity (Oh et al. 1994).

2.9. PHYTOCHEMICALS PRESENT IN FERNS

Phytochemicals are chemical compounds or chemical constituents formed in the plant's normal metabolic processes. The chemicals are often referred to as "secondary metabolites" of which there are several classes including alkaloids, steroids, terpenoids, catecholamines, tannins, saponins, anthraquinones, coumarins, fats, flavonoids, glycosides, gums, iridoids, mucilages etc. The naturally occurring phytochemicals offer promise to be used as safe alternatives. The pteridophytes constitute the primitive vascular plant groups which are found scattered all over the world. Although, not much consideration has been given towards the utility of pteridophytes yet these possess equal economic importance including medicinal ones. Caius (1935) is supposed to be the first man who has described medicinal uses of some ferns of India. Filicin, isolated from the rhizome of *Dryopteris filix-mas,* is a potential insecticide. Filicin has got anti-helminthic properties also. The young fronds of *Pteris vittata* L., a common fern found all over the world, are used traditionally as an astringent. Its decoction is reported to be used in dysentery and the rhizome is eaten as a tonic after boiling in water (Anonymous, 1969). The species has not been studied thoroughly for its pharmacological properties.

2.9.1. Alkaloids in ferns

Alkaloids, a group of naturally occurring chemical compounds which mostly contain basic nitrogen atoms, produced by bacteria, fungi, plants and animals and are part of groups of natural products called secondary metabolites which display a variety of marked effects on animals. Alkaloids often act on the nervous system as stimulators, and sometimes as poisons. Cocaine, which exhibits an anaesthetic effect; atropine, which affects motor nerves; and curare, which has been used by South American natives to cause paralysis of prey. Certain *Lycopodium* alkaloids, which occur naturally in *Lycopodium* and other pteridophytes, have been investigated for their medicinal properties. Alpha-onocerin and lycoperine A, for example, exhibit acetylcholinesterase inhibition activity (Zhang et al., 2002, Hirasawa et al., 2003). Huperzine A, an alkaloid, isolated from *Huperzia* species and other members of Lycopodiaceae, has been shown to enhance memory in animals and is also being investigated for treatment of Alzheimer's disease (Ma et al., 2004).

2.9.2. Terpenoids in ferns

The terpenoids have been utilized for their essential oils isolated from plants and their use as fragrances for over two thousand years. These are mainly classified as sesquiterpenoids, monoterpenoid, diterpenoids, and triterpenoids, depending on whether they contain two, three, four or six isoprene units. Terpenoids are of many skeletal types and 40 pteridophytes contain triterpenoids like hopane triterpenoids, epoxytriterpenoid, serratene triterpenoid, diterpenoids, hemiterpene glycosides, and clerodane diterpene glycosides. Many terpenoids are medicinally significant for a wide range of treatments. For example, triterpenoids isolated from *Erica andevalensis* are cytotoxic against human cancer cell lines (Cordero et al., 2001). Two new hopane triterpenoids, *viz.* 4alpha-hydroxyfilican-3-one and fern-9(11)-en-12beta-ol, and olean-18-en-3-one and olean-12-en-3-one, the first example of oleanane compounds from *Adiantum* ferns, were isolated along with many other known triterpenoids from *Adiantum capillusveneris* of China and Egypt (Nakane et al., 2002). A new triterpenoid, 22,29ξ-epoxy-30-norhopane-13β-ol was isolated together with six known compounds viz., fern-9(11)-en-6α-ol, fern-9(11)-ene, fern-9(11)-en-25-oic acid, fern-9(11)-en-28-ol, filicenol-B, adiantone and oxidation product of fern-9(11)-en-6α-ol obtained as 6-oxofern-9(11)-ene from the whole plant of *Adiantum*

lunulatum, and their structures were elucidated by means of spectroscopic analysis and antibacterial evaluation of these compounds were conducted (Reddy et al ., 2001).

2.9.3. Flavonoids in ferns

The flavonoids constitute another very important group of secondary metabolite**,** contain two benzene rings separated by a propane unit and are derived from flavone. They are generally water-soluble compounds and are found in majority of plants. Only a fraction of flavonoid subdivision is represented in pteridophytes. These are biflavonoids, homoflavonoids, flavone glycosides, and flavonol glycosides. Many flavonoids have medicinal properties. Amentoflavone and ginkgetin, flavonoids found in *Selaginella*, exhibit neuroprotective activity against cytotoxic stress. This property suggests their possible use in treatment of neurodegenerative diseases such as stroke and Alzheimer's (Kang et al., 2005). Several studies have reported the presence of leucocyanidin, leucodelphinidin, the flavone ester apigenin 7-O-p-hydroxybenzoate in *P. vittata* L. (Salantino et al., 1998; Imperato, 2006).

The fronds of the fern, *Asplenium trichomanes* contain kaempferol 3, 7-dirhamnoside and the new compounds kaempferol 3-O-*a*-rhamnoside-7-O-*a*-arabinoside and kaempferol 3-O-*a*-arabinoside-7-O-*a*-rhamnoside. The presence of all of the above mentioned flavonoids have been shown by spectroscopic methods and chemical degradations (Imperato, 2005).

2.9.4. Glycosides in ferns

Glycosides, a group of natural product in which a sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. Many such plant glycosides are used as medications. There are only three species that fit into this category. Benzophenones, ent-pimarene type glycosides, and lactone glycosides are the compounds identified so far from these plants. Perhaps the most interesting is the benzophenone that has been isolated in *Davallia solida* (Rancon et al., 2001). Benzophenones are involved in the P-glycoprotein removal of harmful substances and may act in the detoxification function of the body (Thews et al., 2006). A number of glycosides are apigenin, leutolin, isocutellarein-8-O-methyl-ether, kaempherol and quercetin

(Salantino et al., 1998; Imperato, 2006).

2.9.5. Ptaquiloside in ferns

Bracken fern (*Pteridium* spp.), one of the most abundant plants on the planet, is well known to cause cancer naturally in cattle. At certain places, it contains extremely high concentrations of ptaquiloside (Pta), which almost certainly is its major environmental carcinogen. There is epidemiological evidence that the bracken carcinogen, in special situations, may cause cancer in man. Pta in animal models of carcinogenesis also offers a good tool for the study of cancer. Bracken contains many obnoxious metabolites which contribute to its status as one of the five worst weeds in the world. It has been well proved that regular consumption of bracken fern causes haematuria and cancer in cattle in endemic areas. Pta, a water-soluble, norsesquiterpenoid glycoside is reported to be clastogenic, mutagenic and teratogenic. It is activated in the alkaline urinary pH of the bovine urinary bladder, thus causing tumours of the urinary bladder in cattle. It can alkylate DNA *in vitro* causing modifications both of bases and phosphate leading to cleavage of DNA notably at N3 alkyl adenine in a sequence-specific manner (Pathania et al., 2012).

2.9.6. Quercetin in ferns

Another potential mutagen present in bracken is quercetin (3,3,4,5,7-pentahydroxyflavone), a well-known flavonoid which has been found to be genotoxic and mutagenic, but its role in carcinogenesis has not been studied extensively. Quercetin is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. It is classified as IARC group-3 substance (no evidence of carcinogenicity in humans). In cattle, there is a synergistic interaction between bovine papillomavirus-2 (BPV-2) infection and exposure to quercetin, promoting bladder neoplasia, clinically presenting as enzootic haematuria (Pathania et al., 2012).

The mechanism of action of quercetin includes antioxidative direct radical scavenging, inducible nitric oxide synthesis inhibitory action, xanthine oxidase inhibitory action, modulation of gene expression and interaction with other enzyme systems. Quercetin raises a paradox in living cells in that the antioxidant directs oxidative damage selectively to thiol arylation. The quercetin paradox is that in the process of offering protection it is converted into a potential toxic

product. Study in Himachal Pradesh and Uttarakhand, India revealed the presence of considerably high amount of Pta and flavonoid in several nonbracken fern species and they are suspected in the causation of enzootic bovine hematuria along with BPV-2 (Pathania et al., 2012).

2.10. *DIPLAZIUM ESCULENTUM*: DISTRIBUTION, NATURAL OCCURRENCE AND BRIEF MORPHOLOGICAL DESCRIPTION

D. esculentum is an edible fern, pantropical in distribution and occurs widely and commonly throughout India, China, Cambodia, Laos, Thailand, Vietnam and Malaysia. It grows in gregarious colonies in open marshy areas, stream banks and canals from sea level to 2,300 m. The rhizome is erect, often forming a slender leaning black trunk to 1 m tall, scaly at the apex. Scales are 1 cm long, dark brown, margins finely toothed, apex long-acuminate. Fronds 1–2 m long, 0.5–1 m wide, erect to arcuate. Stipe black and scaly at the base, paler above. Lamina 2–3-pinnate, 0.5–1.5 m long, 0.5–1 m wide, dark green. Secondary pinnae variable in size, commonly 5–8 cm long, 1.5–2.5 cm wide, margins very shallowly lobed, lobes are toothed, basal lobes longer than the rest, glabrous beneath, veins are simple or forked, lowest 3–5 pairs of adjacent vein groups anastomosing. Sori spreading along most veins; indusium thin, dark brown, margins becoming uneven with age.

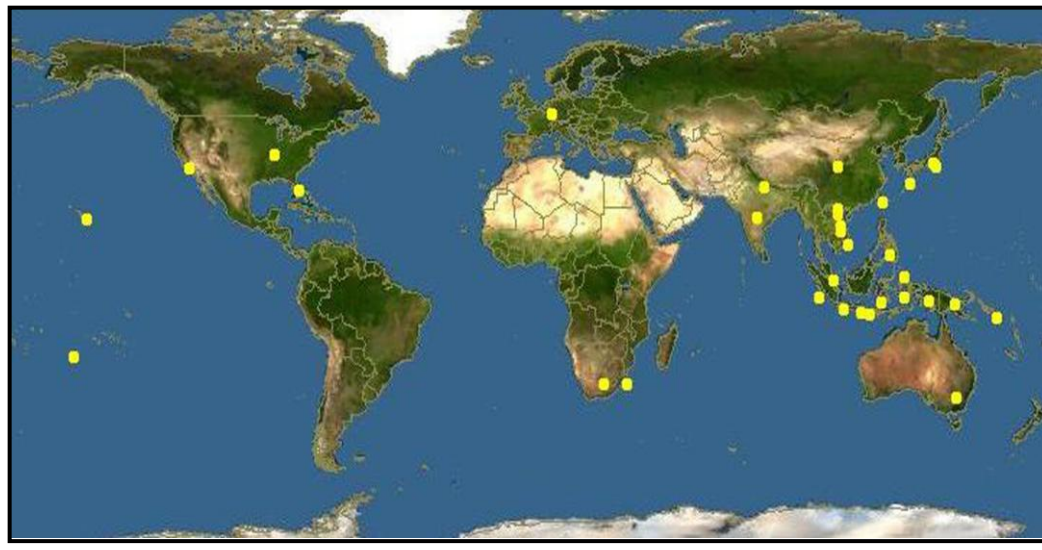


Figure 3. The distribution of *D. esculentum* throughout the world (Image adapted from Discover Life, Designed by The Polistes Corporation)

Figure 4. *Diplazium esculentum* in its natural habitat and collected frond (the main edible part)

2.11. TAXONOMIC CLASSIFICATION

Kingdom: Plantae

Division: Pteridophyta

Class: Polypodiopsida

Order: Polypodiales

Family: Athyriaceae

Genus: *Diplazium*

Species: *D. esculentum*

Scientific name: *Diplazium esculentum* (Koenig ex Retz.) Sw.

2.12. COMMON USES OF *D. ESCULENTUM*

2.12.1. Food

Diplazium esculentum is one of the most common varieties and the most commonly consumed fern throughout Asia and Oceania. In India, young fronds of *D. esculentum* are popularly known as lingra in Northern India, rukja and lochanch in North Eastern India and dheki sak in West Bengal, India. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon season which continues for almost five months. The frond of this fern is generally cooked in oil or butter; using them in a vegetable curry is less preferred. In the north-eastern India, especially in Sikkim, and in the central and north-western Himalayan states of India (Himachal Pradesh and Uttarakhand), the local folk relish both vegetables and pickles from *D. esculentum*. Natives consider these recipes effective both to counteract constipation and as an appetizer, especially as a pickle. (FAO, 2010)

2.12.2. Ethnomedicine

The apatani and Nyishi tribe of Arunachal Pradesh, India uses the frond of this plant as medicine for constipation and indigestion (Kala, 2005). It has been shown that the local

inhabitants of Similipal Biosphere Reserve, Orissa, India used to take honey with decoction of boiled water extract of *D. esculentum* in empty stomach twice a day for 15 days to cure spermatorrhea (Kala, 2005).

2.13. BRIEF DESCRIPTION ON THE PHARMACOLOGICAL REPORTS OF *DIPLAZIUM ESCULENTUM*

Though there are some literatures on the ethnobotanical and ethnomedicinal studies of this edible fern, only few studies have been done on its pharmacological properties. The epidemiological studies of this fern have not yet been attempted. The studies done so far on this fern were mainly concerned with its beneficial effects either *in vitro* or *in vivo* in small laboratory animals. Very few researchers have focused on its health impacts.

2.13.1. Beneficial health effects of *D. esculentum*

The nutritional content and phytochemical composition of *Diplazium esculentum* of Philippines have been studied by Tongco et al., (2014). They also quantified the total phenolic and flavonoid contents to further assess the health benefits of the plant species and its potential bioactivities. They showed that *D. esculentum* is high in inorganic minerals (ash content). Additionally, the results showed that *D. esculentum* is high in fiber and protein contents (Tongco et al., 2014). Akhtar et al. (2014) showed that the chloroform and methanol extract of *D. esculentum* exhibits antioxidant, antimicrobial and cytotoxic activity. Different solvent extracts of *D. esculentum* have also been shown to possess anti-inflammatory activity which was screened by measuring the reduction in carrageenan induced hind paw edema in rats (Kaushik et al., 2011). It has been shown that the aqueous leaf extract of *D. esculentum* significantly increased the locomotor activity in mouse. *D. esculentum* has been studied for its CNS stimulant activity and it was found that the aqueous extract of this fern stimulate the CNS function in mouse (Kaushik et al., 2012).

2.13.2. Adverse effects of *D. esculentum* on human and animal health

Study revealed that rats and guinea pigs fed for 30 days with frozen- and shade dried *D. esculentum* showed poor growth, increased spontaneous (vertical and horizontal) and decreased forced motor activity. Haematological studies in rats and guinea pigs showed significant alterations in values of blood glucose and leukocyte count. Serum biochemistry revealed

increase SGOT level in rats as well as in guinea pigs and decrease in other blood parameters in both species of these animals. Tissue biochemistry of visceral organs revealed increase in lipase and SDH. Mortality was increased as 53% guinea pigs that had been fed with frozen plant material were died. Significant alterations were seen in the relative weight of certain visceral organs like brain, lungs and liver in these dead guinea pigs. Shade- and freeze dried samples of *D. esculentum* showed absence of fern toxin ptaquiloside but presence of 10.94 to 16.36 mg/kg pterosin B only in two of the freeze-dried samples by HPLC method. These observations indicate that *D. esculentum* caused mild pathologic effects in rats while feeding of frozen *D. esculentum* induced mortality and moderate type of clinicopathological effects in guinea pigs (Gangwar, 2004).

The young fronds of *D. esculentum*, collected from Harsil, Gangotri, and Uttarkashi of northern India were freeze-dried and analyzed for the presence of ptaquiloside (Pta) by LC-MS. The study revealed the presence of 19 mg/kg of Pta in these samples (Ptaquiloside). Study performed in Lag valley of Kullu district, Himachal Pradesh, India also revealed the presence of moderate level of quercetin in *D. esculentum* samples (Gangwar, 2004).

CHAPTER – 3:

RESEARCH QUESTIONS AND OBJECTIVES OF THE PRESENT STUDY

3. RESEARCH QUESTIONS AND OBJECTIVES OF THE PRESENT STUDY

The overall aim of the present study was to find out the possible health deteriorating as well as health promoting properties in this fern. If the fern contains any toxic properties, then the people can be made aware of the harmful effect of consumption of this fern and thereby to advance the existing knowledge about *D. esculentum* as food in relation to human health. Apart from its possible harmful effects, *D. esculentum* may have some health promoting effects too as this fern has been used as ethnomedicine among some tribes in India. Therefore, identification of potential therapeutic prospect of this fern is also a major concern of this study.

3.1. RESEARCH QUESTIONS

A number of research questions arose from different literature reviewed in the previous section in relation to the different aspects of *D. esculentum*:

1. It is observed that this fern is rejected as food by animals including cattle and insets. This fern grows abundantly in the marshy land and also in the wet shabby places where lot of insects are available. But it is interesting to note that no insect has been found consuming the leafy portion of this fern. Leaves are all intact, not taken by any insect and even cattle. Therefore, the obvious question that comes to mind is that whether this fern contains any toxic metabolites for which the insects as well as other animals avoid consuming this fern.

2. The most popular use of *D. esculentum* is as food. Different literatures revealed that some of local inhabitants of different parts of India and elsewhere in the world use raw, uncooked *D. esculentum* as food. Therefore, it is a very natural question to ask whether this raw, uncooked *D. esculentum* will be suitable as a food or not.

3. In most of the occasions, *D. esculentum* is taken as food after thorough cooking, either boiled or stir fried, among the people who regularly consume this fern. But is it possible to destroy all the toxic metabolites, if any, from this fern during cooking?

4. Is it possible that this fern may have some beneficial properties as it is evident from some studies that apart from its use as a food, *D. esculentum* has been used as ethnomedicine among some tribes in India?

3.2. OBJECTIVES OF THE PRESENT STUDY

1. To study the immunomodulatory activity of *D. esculentum* in mouse.
2. To study the effect of *D. esculentum* on the reproductive functions of mouse.
3. To investigate the neuromodulatory activity of *D. esculentum*, by studying its effect on the cholinergic nervous system of mouse.
4. To investigate the effect of *D. esculentum* on some major organs of mouse, *viz.* liver and kidney, by studying several enzymes, metabolic products and histological examinations.

CHAPTER – 4:

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. COLLECTION AND IDENTIFICATION OF THE PLANT

Diplazium esculentum, especially the young frond portion of the plant was collected from different areas of North Bengal University campus, and also from the adjoining regions of Darjeeling, West Bengal, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and three voucher specimens (Accession No. 9601, 9602 and 9603) were submitted to his herbarium centre.

4.2. PREPARATION OF THE PLANT MATERIAL

4.2.1. Unboiled and Boiled aqueous preparations of the plant material (Crude *D. esculentum*; CDE and Boiled *D. esculentum*; BDE)

Young frond of *D. esculentum* was washed carefully by tap water, then cut into small pieces, and divided in to two parts. The first part was finely mixed by a mixer and dried in an incubator at 40°C until completely dried. This dried plant material (CDE) was then kept at 4°C for future use. CDE has been used in some of the *in vivo* and few of the *ex vivo* experiments to compare the toxic effects (if any), between the CDE and BDE.

The second part of *D. esculentum* (100 g) was boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed using a mixer and dried in an incubator at 60°C until completely dried. This dried plant material (BDE) was then kept at 4°C for future use. BDE has been used in all of the *in vivo* and few of the *ex vivo* experiments, to investigate the effect of heat resistant toxic compound, if any, that may escape heat during cooking, and thereby create possible harmful effects upon ingestion.

4.2.2. Preparation of the methanolic plant extract

Samples were prepared according to a previously described method (Hazra et al., 2008). Briefly, the young fronds of *D. esculentum* were dried at room temperature for 7 days and finely powdered, and used for extraction. The powder (100 g) was mixed with 500 ml methanol:water in a ratio of 7:3 using a shaker for 15 h; then the mixture was centrifuged at 2850× *g* and the

supernatant was decanted. The pellet was mixed again with 500 ml methanol-water and the entire process was repeated once again, i.e., the extraction procedure was done twice. The supernatants, collected from the two phases, were mixed in a round-bottom flask and concentrated under the reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The residue was kept at –20°C for future use. Double-distilled water (MilliQ grade) was used as the solvent for the lyophilized extract in all the experiments. This preparation has been used in most of the *in vitro*/*ex vivo* experiments.

4.3. ANIMALS AND CARE

Both male and female Swiss albino mice (*Mus musculus*) (25 ± 2 g of body weight (b.wt.)) of 6–8 weeks of age were used for all the studies. Animals were housed in polypropylene cages, with dust free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2°C. Guinea pigs (250 g b.w.) were used for obtaining the complement for plaque-forming cell (PFC) assay. Sheep RBC (sRBC) was collected from sheep, maintained in the departmental animal house, and used for sensitizing the mouse. All the experiments were performed after obtaining the approval from the Institutional Animal Ethical Committee (IAEC) (Registration No. 840/ac/04/CPCSEA).

4.4. STUDY OF THE IMMUNOMODULATORY ACTIVITY OF *D. ESCULENTUM*

4.4.1. *In vivo* experiments

4.4.1.1. Dosage

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was sub-divided in to four groups (G 1–4). Therefore, each group contained six mice. All the animals were fed with CDE and / or BDE orally with the help of a syringe specially designed by us. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Mice of Group 2 (G2), Group 3 (G3) and Group 4 (G4) were fed with 0.4 ml of CDE and BDE at the dose of 80, 160 and 320 mg/kg b.wt., respectively. In this way, all groups of S1 were treated daily for 15 d, S2 daily for 45 d, S3 daily for 90 d, S4 daily for 135 d and S5 daily for 180 d.

It is assumed that the average amount of *D. esculentum* consumed by a 60 kg weighed individual is about 20 g/d. Keeping this ratio in mind, we have formulated the different doses for an average adult mouse of 25 g, like 80 mg/kg body weight, i.e. 2 mg/mouse/d; 160 mg/kg body weight, i.e. 4 mg/mouse/d and 320 mg/kg body weight, i.e. 8 mg/mouse/d.

4.4.1.2. Experimental design for in vivo experiments

The *in vivo* experiments were divided in to two parts. At first, some of the CDE and BDE-treated mice were sensitized with sRBC (0.1 ml, 25% suspension in PBS) through lateral tail vein in the following manner: Set 1 mice were sensitized on the 11th day of the experiment whereas, Set 2, Set 3, Set 4 and Set 5 mice were sensitized on 41st day, 86th day, 131st day and 176th day of experiment, respectively. The day of sensitization in each case was designated as day ' 0 '. These sensitized mice were used for PFC assay and hemaggutination antibody (HA) titer assay.

Second part of the *in vivo* experiments included the measurement of the body weight, relative spleen weight, counting of the splenocytes, and counting of peritoneal macrophages of the CDE and BDE mice. Some of the CDE and BDE treated mice from each group were sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose, and the body weight, relative spleen weight and counting of the splenocytes were determined. The remaining CDE and BDE treated mice were used to perform the peritoneal macrophage counting assay.

4.4.1.3. PFC assay

The PFC assay was performed according to the previously described method (Raisuddin et al., 1991) with slight modifications. On the 4th day of sensitization with sRBC, single cell suspension from the spleen of these sensitized mice was prepared in PBS and cells were adjusted at a concentration of 2 x 10^6 cells/ml. For PFC assay, 0.1 ml of this suspension was mixed with 0.05 ml of guinea pigs complement and 0.05 ml of 25% sRBC (prepared in PBS) to prepare the final mixture. Cuningham chambers were prepared using glass slide and bi-gummed tape (Scotch Brand, St. Paul, MN). The chambers were loaded with a known volume of assay mixture, sealed with paraffin and petroleum jelly (1:1) and incubated at 37°C for 4 h. After incubation, the

plaques were counted under a phase contrast microscope and expressed as PFC per 10^6 spleen cells.

4.4.1.4. Collection of serum HA titer assay

The serum was collected from the blood of the sensitized mouse on the 4th day of sensitization with sRBC. Blood was collected from tail vein as a standard method. The mouse was sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) and blood was collected from the heart also. The collected serum was divided in to two parts. First part was stored at -20°C for future use. These serum samples were further used to determine the concentration of immunoglobulin M (IgM). Second part was kept for 45 min at 56°C in a water bath for the inactivation of complement activity. Eight clear Khan tubes were taken in a rack and marked. In the first tube, 0.1 ml of the serum and 0.9 ml of PBS was added. 0.5 ml of PBS was taken to the subsequent tubes. Then 0.5 ml of the mixed solution from the first tube was added to the second tube, and 0.5 ml of the mixture from the second tube was added to the third tube. In this way, eight such dilutions (double fold) were prepared. From the last tube, 0.5 ml of solution was thrown away thus yielding a serial dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280. This is known as double-fold dilution. Now, 0.1 ml of 10% sRBC (prepared in PBS) was added to each tube and the entire set up was incubated at 37°C for 12 h in a humidified atmosphere. After incubation, visible hemagglutination was observed and noted down.

4.4.1.5. Measurement of the body weight, relative spleen weight and counting of the splenocytes

Mouse was sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose of CDE and BDE. Body weight was measured and blood was collected from the heart and serum was separated and stored at -20°C for future use. These serum samples were further used to determine the concentration of different cytokines. The relative spleen weight (spleen weight/100 g of body weight) was also recorded. Single cell suspension of the spleen, prepared in RPMI-1640 medium, was counted using hemocytometer.

4.4.1.6. Counting of peritoneal macrophages

Freund's incomplete adjuvant (0.5 ml) was injected in the peritoneum of the CDE and BDE treated mouse 24 h prior to the experiment. Two ml of PBS was injected in the peritoneum on the next day and the peritoneal exudate cells were collected from the mouse under proper anesthesia. Cells were washed two times with PBS. The pellet was then resuspended in PBS, taken in a glass petridish and incubated for 45 min at 37°C. After incubation, the supernatant was removed and the petridish was washed with chilled PBS to collect the macrophages and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in PBS and a small amount of this cell suspension was mixed with equal volume of neutral red and charged on the hemocytometer to count the number of the live macrophages under a microscope.

4.4.2. *Ex vivo* experiments

4.4.2.1. Effect of CDE and BDE on primary cultured splenocytes

Effect of CDE and BDE on mouse splenocytes were determined according to the previously described method (Yeap et al., 2010), with slight modifications. Splenocyte suspension was prepared in RPMI-1640 medium (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin). The cell number was adjusted to 2×10^6 cells per ml and 1 ml of the cell suspension with 10% of FBS was added in six-well culture plates. Five microliters of concanavalin A (5 μg/ml) and 100 μl of different concentrations (0–200 μg/ml) of CDE and BDE (suspended in RPMI 1640) were added to this and the whole set up was incubated for 24, 48 and 72 h at 37°C in an incubator having 5% CO_2 and 90% humidity. After the incubation period, the cultures were harvested and washed once at 1000 rpm for 5 min. The cell pellet was then resuspended in 0.5 ml of RPMI-1640 medium. Then, 10 μl of cell suspension was mixed with equal volume of 0.4% trypan blue and was counted by using hemocytometer under the phase contrast microscope. Only the viable cells were counted.

4.4.2.2. MTT splenocyte proliferation assay

MTT proliferation assay was carried out according to the previously described method (Mosmann, 1983) with slight modifications. Mouse splenocyte cell suspension was prepared (conc. 2×10^6 cells/ml) in RPMI 1640 medium (containing 50 U/ml penicillin, 50 U/ml

streptomycin and 50 U/ml nystatin). Then in each well of a 96 well microtiter plate, 100 µl of cell suspension was added with 10% of FBS and 100 µl of different concentrations (0–200 µg/ml) of CDE and BDE (suspended in RPMI 1640). The plate was then incubated for 24 h in 37°C incubator having 5% CO_2 and 90% humidity. After the incubation period, 20 µl of MTT solution (5 mg/ml, dissolved in PBS; pH 7.0) was added to each well. The plate was covered and incubated for 4 h at 37°C in an incubator. After incubation, 150 µl of the suspension from each well was taken out without disturbing the bottom layer, and 150 µl of dimethyl sulphoxide (DMSO) was added to each well and mixed thoroughly. Finally, the optical density (O.D.) was taken in a microplate reader at 540 nm.

4.4.2.3. Hemolytic assay

Hemolytic effects of CDE and BDE on mouse erythrocytes were evaluated by using washed erythrocytes (RBCs). A previously described method was followed for the preparation of mouse erythrocytes (Malagoli, 2007). Blood sample from Swiss albino mouse was collected (each weighing 25 ± 2 g) in citrated tubes. The cells were then washed three times with 20mM Tris–HCl containing 144mM NaCl (pH 7.4) and a 2% erythrocyte suspension was prepared. The hemolytic activities of CDE and BDE were tested according to a previously described method under *in vitro* conditions in 96-well plates (Malagoli, 2007). Each well received 100 µl of 0.85% NaCl solution containing 10mM $CaCl_2$. The first well served as negative control containing only solvent. One hundred microliters of CDE and BDE of various concentrations (0–50 µg/ml, suspended in 0.85% NaCl solution containing 10mM $CaCl_2$) were added from the second well. The last well was served as positive control containing 100 µl of 0.1% Triton X-100 in 0.85% saline. Each well then received 100 µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10mM $CaCl_2$. Cells were centrifuged after 30 min incubation at room temperature and the supernatant was collected to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from six assays.

4.4.2.4. Primary culture of splenocytes for cytokine estimation

Spleen was aseptically removed from mouse and cell suspension was prepared in minimum essential medium (MEM), containing penicillin-streptomycin (50 U/ml) and nystatin (50 U/ml).

The cell number was adjusted at 2×10^6 cells per ml and 1 ml of the cell suspension was added in each well of a six-well culture plate. Each well was then supplemented with 10% goat serum (Chaudhuri & Chakravarty, 1983). Five microlitres of concanavalin A (5 μg/ml) was also added to stimulate cytokine production. Finally, 100 μl of different concentrations (0–200 μg/ml) of CDE and BDE (suspended in MEM) were added to each well. The whole set up was then incubated for 48 h at 37°C in an incubator having 5% CO_2 and 90% humidity. Supernatants of cell cultures were collected after 48 h and used for cytokine estimation.

4.4.3. Estimation of IgM and Th1 and Th2 cytokine concentration

Previously collected serum samples and splenocyte culture supernatants were used to determine the concentrations of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (RayBiotech, Inc., USA). Serum samples from sRBC sensitized mouse were used to determine the concentration of IgM. Briefly, a 96-well flat bottom plates were coated with the captured antibody specific to each cytokine and IgM. One hundred microliters of serially diluted specific standards for each cytokine and IgM and 100 μl of the serum/cell culture supernatants (samples) were pipetted into the wells. The specific cytokine and IgM present in the sample were bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-mouse detection antibody specific for each cytokine and IgM were added. After washing away the unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were washed again and TMB substrate solution was added to the wells and the color was developed in proportion to the amount of specific cytokines and IgM bound. Finally, the stop solution was added which changed the color from blue to yellow and the intensity of the color was measured at 450 nm in a microplate reader.

4.5. EFFECT OF *D. ESCULENTUM* ON THE REPRODUCTIVE FUNCTIONS OF MALE SWISS ALBINO MOUSE

4.5.1. Dosage

One hundred twenty (120) male Swiss albino mice were divided in to five sets (S 1-5) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1

(G1) of all the sets were considered as control where 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of CDE and BDE at the dose of 80 mg/kg b.wt., 160 mg/kg b.wt., and 320 mg/kg b.wt., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) were treated daily for 15 days, S2 (G1S2 to G4S2) daily for 45 days, S3 (G1S3 to G4S3) daily for 90 days and S4 (G1S4 to G4S4) daily for 135 days, and S5 (G1S5 to G4S5) daily for 180 days.

4.5.2. Collection of serum sample and preparation of the sperm suspension

Mouse from each group was sacrificed after proper anesthesia (chloroform and ether in 2:1 ratio) 24 h after the last dose. Blood was collected from the heart and serum was separated. The serum samples were used for the determination of total protein content. Caudal epididymis of mouse from each group was separated and minced using a pair of small scissors to release the sperm into 10 ml warmed (37°C) physiological saline. The sperm suspension was used for hypo-osmotic swelling test (HOST) and MTT reduction assay.

4.5.3. Hypo-osmotic swelling test (HOST)

Hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. This was performed by incubating 30 µl of semen with 300 µl of 100 mOsm hypo-osmotic solution (9 g of fructose + 4.9 g of sodium citrate per liter of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a pre-warmed slide. A total of 200 spermatozoa were counted in different fields using a phase-contrast microscope. Sperms with swollen or coiled tails were recorded (Buckett et al., 1997; Revell and Mrode, 1994).

4.5.4. MTT reduction assay of sperm

The sperm suspension was placed in an incubator at 37° C for 10 minutes prior to perform the viability test. The MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) assay was performed according to the previously described method (Mosmann, 1983). Briefly, the sperm suspension was diluted using phosphate buffered saline (PBS) and the number was adjusted at 30×10^6 spermatozoa/ml. Twenty four wells of a 96-well microplate were used to

assess the sperm viability of S1 mice (G1S1, G2S1, G3S1, G4S1). One hundred microliters of sperm suspension from G1S1 mouse was placed in first six wells of first column of the microplate. Similarly, 100 µl of sperm suspension from G2S1, G3S1, and G4S1 mice was placed in the six wells of the second, third and fourth columns of the microplate, respectively. Therefore, a total of 24 wells of the microplate were occupied with the sperm suspension of all the groups of S1 mice. Ten microlitres of MTT stock solution (5 mg/ml, dissolved in PBS; pH 7.0) was added to each of these 24 wells and mixed properly and the rates of MTT reduction (measured as optical density) were then recorded immediately (first reading) using a microplate reader (Bio-Rad, USA). The plate was then incubated at 37°C for 1 h and the optical density was recorded (second reading) again. The MTT reduction rate (change in optical density) was determined by calculating the difference between the first and second reading of the microplate reader, for each group of mouse. MTT reduction rates of the spermatozoa of S2, S3, S4 and S5 mice were evaluated in the similar way as mentioned above.

4.5.5. Tissue biochemistry

4.5.5.1. Testis

Total protein and cholesterol contents were determined in testicular tissue, and the samples were prepared according to a previously described method (Hammami et al., 2008). Briefly, a part of testis (about 0.5 g) was crushed in 2 ml of 0.9% normal saline. The homogenate was centrifuged at 13000 × g for 10 min. The supernatant was collected and used for the determination of total protein and cholesterol contents using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Glycogen content in testis was determined by a previously described method (Montogomery, 1957).

The sample was prepared according to a previously described method (Hammami et al., 2008), with little modification for the determination of acid phosphatase activity. Briefly, 0.5 g of testicular tissue was homogenized in 2 ml of citric acid buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 18000 × g at 4ºC for 30 min. The supernatant was used to determine the acid phosphatase activity using

commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Acid phosphatase activity was expressed as μM/min/g of tissue.

4.5.5.2. Epididymis

Alpha-glucosidase activity in epididymis was measured according to a previously described method (Hammami et al., 2008) with little modification. Briefly, the caudal epididymis of mouse was cut, homogenized in citric acid buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 18000 × g at 4ºC for 30 min. The alpha-glucosidase activity was measured according to a previously described colorimetric method (Wang et al., 1999). The reaction system contained 1.2 ml buffer (69 mM citric acid, pH 6.8), 0.2 ml paranitrophosphateglycerol (PNPG, 23 mM) and 0.2 ml supernatant. The reaction medium was incubated at 37ºC for 4 h and 0.25 ml Na_2CO_3 (0.1 M) was added to stop the reaction. The absorbance was measured at 400 nm in a spectrophotometer and the PNP content was estimated in reference to the PNP standard curve. The α-glucosidase activity was expressed as μmol/min/g of tissue. The supernatant was also used to determine the total protein contents in epididymis using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). The concentration of sialic acid in the epididymis was estimated according to the standard thiobarbituric acid method (Aminoff, 1961).

4.5.5.3. Prostate and seminal vesicle

Sample was prepared from prostate and seminal vesicles according to a previously described method (Hammami et al., 2008). Briefly, 0.2 g of tissues were homogenized in 2 ml of 0.33% perchloric acid at 4ºC and centrifuged at 2500 × g for 10 min. Then, 1 ml of the supernatant was added to 0.5 ml K_2CO_3 (0.75M). The reaction mixture was then centrifuged at 2500 × g for 10 min and supernatant was used for the determination of citric acid content of prostate and fructose content of seminal vesicles. The fructose content was determined according to a previously described protocol (Anderson et al., 1979), with little modifications. Briefly, 100 μl of supernatant was added to distilled water to give a total volume of 0.5 ml. The reaction tube was then placed in a boiling water bath for 7 min, and centrifuged at 10000 × g for 20 min to remove the precipitated material. 0.3 ml of the supernatant was added to 1.5 mM of NADH and sorbitol

dehydrogenase preparation in sodium phosphate (0.1 M, pH 6.8) to give a final reaction mixture volume of 1 ml. The concentration of fructose was determined by comparing the initial rate of decrease in absorbance at 340 nm with that of fructose standard. The concentration of citric acid in prostate was determined according to the World Health Organization semen analysis manual (WHO, 1999).

4.5.6. Histological analysis

A part of the testis was put into Bouin's fluid for fixation, and subsequently embedded in paraffin for the histological sections followed by the microscopic examination in accordance with the routine laboratory procedure. Paraffin sections of 4–5 μm were prepared and stained with haematoxylin and eosin for histological examination. Histomorphometric analysis was performed by KLONK Image Measurement Light software (Version: 13.2.2.12).

4.5.7. Male fecundity/fertility test

Male mice treated for different durations (15, 45, 90, 135 and 180 days) were used for the fertility test. After the end of each treatment, each male was allowed to mate with two fertile females and they were left together for 15 days. This period is sufficient to cover the mouse estrous cycle which takes 4–5 days. After the mating test, each female was observed for delivery (19–21 days following the mating test) as a criterion of successful insemination. After the delivery, the entire litters, number of live pups and any clinical signs and mortalities were recorded. Fecundity was also calculated. Fecundity represents the ratio of the number of male parent of at least one viable pup to the total number of male mice exposed for mating ×100. Pups were followed up until adulthood.

4.6. EFFECT OF *D. ESCULENTUM* ON THE CHOLINERGIC NERVOUS SYSTEM OF MOUSE

4.6.1. *In vivo* experiments

4.6.1.1. Dosage

Forty eight (48) Swiss albino mice were divided in to two sets (S1 and S2) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1 (G1) of

both the sets were considered as control where 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of both the sets were fed with 0.4 ml of CDE and BDE at the dose of 80 mg/kg b.w., 160 mg/kg b.w., and 320 mg/kg b.w., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) and S2 (G1S2 to G4S2) were treated daily for 30 days.

4.6.1.2. Preparation of enzyme source for in vivo assay

CDE and BDE treated mice from each group were sacrificed 24 h after the last dose using proper anesthesia (chloroform and ether in 2:1 ratio). The enzyme, acetylcholinesterase (AChE) was extracted according to a previously described method (Ashraf et al., 2011), with a little modification. A small portion of liver (0.5 g) of mouse from each group was cut, washed with 50 mM Tris-HCl buffer (pH 7.4) and homogenized manually in 10 ml extraction buffer (50 mM Tris-HCl buffer, pH 7.4; 1mM $MgCl_2$; 1mM $CaCl_2$; 0.32M sucrose) in a tissue homogenizer. The sample was homogenized continuously for 15 sec. at a time for 2 consecutive occasions, with an interval of 10 sec. in between. The tube was placed in ice bucket to avoid heating during the homogenization procedure. After homogenization, the contents were filtered through double layers of Whatman filter paper No. 1 and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was used as a source of cholinesterase enzyme. Enzyme source was made fresh everyday and used within 4 hours. Protein was determined by Bradford method and 40-60 µg protein (10 µl) was used per assay.

4.6.1.3. Determination of acetylcholinesterase activity

The AChE activity was determined according to a standard protocol (Ellman et al., 1961), with little modifications. A 0.4 ml aliquot of the supernatant (enzyme source) was added to a test tube containing 2.6 ml of phosphate buffer (pH 8.0, 0.1 M). One hundred microlitre of 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) reagent (0.01 M) was added to it and the absorbance was measured at 412 nm. After that, 20 µl of acetylthiocholine iodide substrate (0.075 M) was added to the test tube. After 15 min incubation, absorbance was measured at 412 nm. Changes in the absorbance were recorded and the rate of change in absorbance per min. was calculated as follows (Ellman et al., 1961):

$$R = 5.74\ (10^{-4}) \times (\Delta A / C_0)$$

Where, R = rate, in moles substrate hydrolyzed per min per g of tissue;

ΔA = change in absorbance per min;

C_0 = original concentration of tissue (mg/ml).

5.74 (10^{-4}) = factor for dilution, extinction coefficient, and changes in units;

4.6.2. *Ex vivo* experiments

For *ex vivo* experiments, 70% methanolic extract of *D. esculentum* (MDE) has been used.

4.6.2.1. Determination of acetylcholinesterase inhibitory activity

Enzyme activity was measured by a previously described method (Ellman et al., 1961), with brief modifications. The method for preparation of the enzyme source was similar to the enzyme preparation step as mentioned in the *in vivo* experiments. The reaction mixture (200 µl) consisted of 160 µl of 50 mM Tris HCl buffer, pH 7.4, with or without the MDE (30 µl) followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate in 96-well plates. The contents were mixed and pre-incubated for 10 min at 25°C. Plates were preread at 412 nm using a plate reader (BioRad, Hercules, USA). The reaction was initiated by the addition of 10 µl of 1 mM DTNB and 3 mM substrate acetylthiocholine iodide. Absorbance was measured at 412 nm within 4-7 min after 15 min incubation. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding enzyme after the addition of DTNB. Absorbance values were subtracted from the control and data presented as percent inhibition of enzyme activity. Experiments were carried out with their respective controls for six times.

4.6.2.2. Determination of NADH oxidase (NOX) inhibitory activity

NOX inhibitory activity was determined according to a previously described method (Ashraf et al, 2011). The reaction mixture (200 µl) consisted of 160 µl 50 mM Tris HCl buffer, pH 7.4 containing 1 mM EDTA disodium salt, with or without MDE (30 µl) followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate. The contents were mixed and pre-incubated for 10 min at 25°C. The reaction was initiated by the addition of 10 µl of 3

mM nicotinamide adenine dinucleotide (reduced). Absorbance was measured at 340 nm using a 96-well plate reader (BioRad, Hercules, USA) after 45 min incubation at 25°C. All the experiments were carried out with the respective controls. Results are mean of six independent determinations.

4.7. ASSESSMENT OF THE ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF *DIPLAZIUM ESCULENTUM*

The 70% methanolic extract of *D. esculentum* (MDE) has been used to assess the antioxidant and free radical scavenging activities.

4.7.1. Antioxidant activity in linoleic acid system

4.7.1.1. Ferric thiocyanate (FTC) method

The method of ferric thiocyanate was followed from a previously described procedure (Kikuzaki & Nakatani, 1993) with little modifications (Mitsuda et al., 1967; Osawa & Namiki, 1981). FTC method was used to determine the amount of peroxide at the initial state of lipid peroxidation. The peroxide reacts with ferrous chloride ($FeCl_2$) to form a reddish ferric chloride ($FeCl_3$) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Four ml of MDE was placed in 4 ml of absolute ethanol. Then, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water were placed in a screw capped vial and then placed in an oven at 40°C in the dark. A small volume (0.1 ml) of this solution was taken and 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate was added in to it. Exactly 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance was measured at 500 nm every 24 h until the absorbance of the control reached maximum. The control and standard were subjected to the same procedures as the sample, except that for the control where only the solvent was added, and for the standard, 4 mg sample was replaced with 4 mg of α-tocopherol.

4.7.1.2. Thiobarbituric acid (TBA) method

TBA value of *D.esculentum* plant extract was determined according to a previously described method (Ottolenghi, 1959). The formation malondialdehyde is the basis for the well-known TBA

method used for evaluating the extent of lipid peroxidation. Malondialdehyde binds TBA to form a red complex at low pH and high temperature (100°C) that can be measured at 532 nm. The increased amount of the red pigment formed correlates with the oxidative rancidity of the lipid. Two ml of 20% trichloroacetic acid and 2 ml of 1 % (w/v) TBA aqueous solution were added to 1 ml of sample solution prepared as in the FTC procedure, incubated in a similar manner. The mixture was then placed in a boiling water bath for 10 min. It was centrifuged after cooling, at 3000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay. Both methods (FTC and TBA) described antioxidant activity by percent inhibition:

% inhibition = [(Absorbance of control on day maximum − Absorbance of sample on the same day)/Absorbance of control on the same day] × 100

All data, about total antioxidant activity, are the average of six replicate analyses.

4.7.2. Total antioxidant activity by ABTS method

The antioxidant activity of *D. esculentum* extract was assayed depending on the ability to scavenge $ABTS^{\bullet+}$ radical cation compared to trolox standard (Re et al., 1999). The $ABTS^{\bullet+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12-16 h in dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the $ABTS^{\bullet+}$ was equilibrated to 0.70 (±0.02) by diluting with water at room temperature. Then 1 ml of $ABTS^{\bullet+}$ was mixed with 10 µl of MDE (Conc. 0.05-10 mg/ml) and the absorbance was measured at 734 nm after 6 min. All experiments were repeated six times. The percentage inhibition of the absorbance was calculated and plotted as a function of the concentration of standard and MDE to determine the trolox equivalent antioxidant capacity (TEAC) which was calculated from dividing the gradient of the plot for the sample by the gradient of the plot for trolox.

4.7.3. Scavenging activity of DPPH radical

The ability of *D. esculentum* extract to scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was determined using the previously reported procedure (Shimada et al., 1992). Briefly, 0.1 mM solution of DPPH in ethanol was prepared. Then, 1 ml of this solution was added to 3 ml of

MDE at different concentrations (0-200 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated the higher free radical scavenging activity. Alpha-tocopherol (α-tocopherol) was used as positive control.

4.7.4. Free radical scavenging activity of *D. esculentum*

4.7.4.1. Hydroxyl radical scavenging activity

This was assayed according to a standard method with slight modification (Elizabeth & Rao, 1990). This assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 µM), EDTA (100 µM), H_2O_2 (1.0 mM), ascorbic acid (100 µM) and various concentrations (0-200 µg/ml) of MDE or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml of 1% aqueous TBA was added, and incubated at 90°C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical OH• scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

4.7.4.2. Superoxide radical scavenging activity

This activity was determined based on the reduction of NBT according to a previously reported method (Fontana et al, 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals that reduce nitro blue tetrazolium (NBT) into a purple-coloured formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (0-20 µg/ml) of MDE. The absorbance was taken at 562 nm against an appropriate blank solution after incubation for 5 min at ambient temperature. All tests were performed six times. Quercetin was used as positive control.

4.7.4.3. Nitric oxide radical scavenging activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illisvoy reaction (Garratt, 1964). The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various concentrations (0-70 µg/ml) of MDE in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Curcumin was used as a standard.

4.7.4.4. Hydrogen peroxide scavenging activity

This activity was determined according to a previously described method (Long et al., 1999) with minor changes. An aliquot of 50 mM H_2O_2 and various concentrations (0-2 mg/ml) of MDE were mixed (1:1 v/v) and incubated for 30 min at room temperature. Then, 90 µl of the H_2O_2-sample solution was mixed with 10 µl of HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were performed six times. Sodium pyruvate was used as the reference compound (Floriano-Sánchez et al., 2006).

4.7.4.5. Peroxynitrite scavenging activity

Peroxynitrite ($ONOO^-$) was synthesized by a previously described method (Beckman et al., 1994). An acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) was mixed with 5 ml of 0.6 M KNO_2 on an ice bath for 1 second and 5 ml of ice-cold 1.2 M NaOH was added. Excess H_2O_2 was removed by treatment with granular MnO_2 prewashed with 1.2 M NaOH and the reaction mixture was left overnight at -20°C. peroxynitrite solution was collected from the top of the

frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}).

The Evans Blue bleaching assay was used to measure the peroxynitrite scavenging assay (Bailly et al., 2000), with slight modification. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 µM Evans Blue, various doses of MDE (0-200 µg/ml) and 1 mM peroxynitrite in a final volume of 1 ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound.

4.7.4.6. Singlet oxygen scavenging activity

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N, N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method (Pedraza-Chaverrí et al., 2004). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2, and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2 , 50 mM histidine, 10 µM RNO and various concentrations (0-200 µg/ml) of MDE in a final volume of 2 ml. It was incubated at 30°C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times.

4.7.4.7. Hypochlorous acid scavenging activity

Hypochlorous acid was prepared immediately before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M^{-1} cm^{-1}. The assay was carried out according to a previously described method (Aruoma & Halliwell, 1987) with minor changes. The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 µM), HOCl (8.4 mM) and increasing concentrations (0-100 µg/ml) of MDE. The assay mixture was incubated at 25°C for 20 min and

the absorbance was measured against an appropriate blank. All tests were performed six times. Ascorbic acid, a potent HOCl scavenger, was used as a reference compound (Pedraza-Chaverrí et al., 2007).

4.7.4.8. Fe^{2+} chelation

The ferrous ion chelating activity was evaluated by a standard method (Haro-Vicente et al., 2006) with minor changes. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations (0-120 µg/ml) of MDE were added to 12.5 µM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 µM). The mixture was shaken vigorously and incubated for 20 min at room temperature, and then the absorbance was measured at 562 nm. All the tests were performed six times. EDTA was used as a positive control.

4.7.4.9. Reducing power

The Fe^{3+}-reducing power of the extract was determined by a previously described method (Oyaizu, 1986) with slight modification. Different concentrations (0-1 mg/ml) of MDE (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1 %), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left over for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All the tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

4.7.4.10. Lipid peroxidation inhibition assay

This assay was carried out according to a previously described method (Kizil et al., 2008), with slight modification. Brain homogenate was prepared by centrifuging Swiss albino mice brain (20 ± 2 g b.w.) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 µl aliquot of the supernatant homogenate was mixed with MDE of various concentrations

(0-25 µg/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, and incubated for 1 h at 37°C. Then 500 µl of 28% TCA was used to stop the reaction and 380 µl of 2% TBA was added with heating at 95°C for 30 min, to generate the colour. Then the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All the tests were performed six times. Trolox was used as the standard.

4.7.4.11. Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to a previously described method (Singleton & Rossi, 1965) with a slight modification. Briefly, MDE (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.75 ml of 0.06% Na_2CO_3 solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. All the tests were performed six times. The phenolic content was evaluated using gallic acid as a standard. The result was expressed as mg of gallic acid equivalent phenolic content present in 1 g sample plant material.

4.7.4.12. Determination of total flavonoid content

The total flavonoid content was determined with aluninium chloride ($AlCl_3$) according to a known method (Zhishen et al., 1999) using quercetin as a standard. The MDE (0.1 ml) was added to 0.3 ml distilled water followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All the tests were performed six times. The flavonoid content was evaluated using quercetin as a standard. The result was expressed as mg of quercetin equivalent flavonoid present in 1 g sample plant material.

4.8. PHYTOCHEMICAL ANALYSIS OF *D. ESCULENTUM*

Qualitative analysis of phytochemicals was carried out for *D. esculentum*, to identify the presence of different phyto-constituents.

4.8.1. Terpenoids

0.5 gm of CDE was dissolved in 5 ml of methanol and filtered. Filtrate was mixed with 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids (Harborne, 1973).

4.8.2. Glycosides

0.5 gm of CDE was dissolved in 5 ml of methanol and filtered. Filter was mixed with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. On addition of 1 ml of concentrated H_2SO_4 to this, a brown ring in the interface indicated a deoxyribose characteristic of cardenolides, a plant glycoside (Dey et al., 2012).

4.8.3. Alkaloids

0.5 g of CDE was defatted with 5% ethyl ether for 15 min. The defatted sample was extracted for 20 min with 5 ml of aqueous HCl on a boiling water bath. The resulting mixture was centrifuged for 10 min at 3000 rpm and divided in to two parts. One ml (First part) of the filtrate was treated with few drops of Mayer's reagent. One milliliter (second part) of the filtrate was treated with with Dragendroff's reagent. Turbidity was observed in both the cases (Harborne, 1973; Trease & Evans, 1996).

4.8.4. Steroids

0.5 g of CDE was dissolved in 5 ml of methanol. 1 ml of the extract was treated with 0.5 ml of acetic acid anhydride and cooled in ice. This was mixed with 0.5 ml of chloroform. One milliliter of concentrated sulphuric acid was then added carefully by means of a pipette. At the separation level of the two liquids, a reddish-brown ring was formed, as an indication of the presence of steroids (Harborne, 1998).

4.8.5. Tannins

About 0.5 g of the CDE was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added. A blue-black precipitation indicated the presence of tannins (Segelman et al., 1969).

4.8.6. Phlobatannins

The CDE was boiled with 1% aqueous hydrochloric acid (HCl) to observe the deposition of red precipitate (Harborne, 1973).

4.8.7. Saponins

0.5 g of CDE was shaken with water in a test tube and it was warmed in a water bath. The persistent of froth indicates the presence of saponins (Kapoor et al., 1969).

4.8.8. Flavonoids

A portion of the CDE was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of diluted ammonia solution. A yellow coloration was observed, indicating a positive test for flavonoids (Edeoga et al., 2005).

4.8.9. Phenols (Ferric chloride test)

Two ml of the MDE was treated with three drops of ferric chloride solution. Formation of bluish black colored solution indicates the presence of phenols (Dey et al., 2012).

4.9. ACUTE, SUB-ACUTE, SUB-CHRONIC AND CHRONIC TOXICITY STUDY OF *D. ESCULENTUM* AS WELL AS ITS EFFECT ON SOME MAJOR ORGANS OF MOUSE

4.9.1. Acute toxicity study

Single-dose study was performed for 15 days following the safety assessment guidelines (Schilter et al., 2003). Both the adult (6-8 weeks old) male and female Swiss albino mice weighing 25 ± 2 g of b.w. were used for the acute toxicity study. Dosages were based on b.w. of the animal (expressed as mg BDE equivalent per g body weight of the animal). Mice were

randomly divided into four groups (Group – A: untreated control, Group – B: – 1 g/kg b.w., Group C – 2 g/kg b.w., Group D – 4 g/kg b.w.), consisting of six animals (n = 6) in each group. BDE was administered orally (using a round-pointed polypropylene microtip fitted onto a graded disposable syringe) as a single bolus dose (800 µl BDE). Control mice received only distilled water (800 µl). Prior to the dosage, mice were fasted overnight to eliminate feed from gastro-intestinal tract. All the mice were thoroughly observed for the onset of any toxic signs immediately and also on each day during 14 days of observation period to record any delayed toxic effects. Survival, feed intake (from day 7 to 15), and body weight (day 0 and every four days) were monitored. Mice were sacrificed under mild chloroform and ether (2:1) anesthesia on day 15, and selected vital organs including liver, kidney, testis, ovary, adrenals, spleen, heart and brain were excised, blotted and weighed.

4.9.2. Subacute (15 and 45 days), subchronic (90 days) and chronic (135 and 180 days) toxicity study

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was sub-divided in to four groups (G 1–4). Therefore, each group contained six mice. All the animals were administered orally as described earlier. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were administered with 0.4 ml of BDE/CDE at the dose of 80, 160 and 320 mg/kg b.w., respectively. In this way, all the groups of S1 were treated daily for 15 days (sub-acute treatment 1), S2 daily for 45 days (sub-acute treatment 2), S3 daily for 90 days (subchronic), S4 daily for 135 days (chronic treatment 1) and S5 daily for 180 days (chronic treatment 2). The body weight of mice was recorded on day '0' and every following week. On 16^{th}, 46^{th}, 91^{st}, 136^{th} and 181^{st} day, mice from each set were sacrificed under proper anesthesia (chloroform and ether in 2:1 ratio), heart was punctured and blood was collected separately for serum separation as well as collected in EDTA vials for hematological examination. Vital organs were excised, blotted and weighed. Liver, kidney, testis and ovary were processed for histopathological examinations. Biochemical studies of serum were carried out for liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP), γ-glutamyl transferase (GGT),

total bilirubin, and kidney function (urea, creatinine), using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India).

4.10. STATISTICAL ANALYSIS

All data are given as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula Y= 100 x A1 / (X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. Differences between two groups (for example, plant extract and standard in DPPH radical scavenging assay) were determined by paired t test, whereas, differences among more than two groups were determined by one-way ANOVA followed by Dunnett's T test. $p < 0.05$ was considered significant.

CHAPTER – 5:

RESULTS

5. RESULTS

This chapter consists of two parts:

- The effect of boiled aqueous preparation of *D. esculentum* (BDE) on different *in vivo* and *ex vivo* parameters of Swiss albino mouse
- Comparative effect of crude- and boiled aqueous preparation of *D. esculentum* (CDE *vs.* BDE) on different *in vivo* and *ex vivo* parameters of Swiss albino mouse

5.1. THE EFFECT OF BOILED AQUEOUS PREPARATION OF *D. ESCULENTUM* (BDE) ON DIFFERENT *IN VIVO* AND *EX VIVO* PARAMETERS OF SWISS ALBINO MOUSE

5.1.1. Effect of BDE on the immune system of mouse

5.1.1.1. Assessment of the humoral immune responses (PFC and HA titre assay)

Significant decreases ($p < 0.001$) in number were observed in the formation of antibody secreting cells (plaques) in case of doses (80, 160, 320 mg/kg b.w) treated for longer durations (135 days and 180 days) (Table 2) when compared with the respective control groups. Table 1 and 2 showed dose- and time-dependent decrease in the HA titre value when they were compared with that of their respective controls. After 180 days of the treatment with different doses of BDE, 16 fold decreases in the titre value were observed when compared with the controls.

5.1.1.2. Measurement of body weight, relative spleen weight and counting of the splenocytes

Significant decreases in the body weights were observed in case of mice that were fed with BDE for longer durations (135 days and 180 days) ($p < 0.05$) when compared with their respective control groups (Group 1) (Table 2). Significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in the relative spleen weights were also observed in case of the treated groups when compared with the respective controls (Table 1 and 2). Results also showed that the number of splenocytes was also significantly decreased ($p < 0.01$ and $p < 0.001$) in case of BDE treated groups when compared with their respective controls (Table 1 and 2).

Table 1. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 15 (S1) and 45 d (S2) of treatment.

	15 d (S1)				45 d (S2)			
Parameters observed (¶)	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
Body weight (g)	25.38 ± 0.49	25.36 ± 0.36	25.30 ± 0.65	25.25 ± 0.39	25.53 ± 0.48	25.35 ± 0.58	25.36 ± 0.53	25.33 ± 0.43
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.008	0.48 ± 0.006	0.48 ± 0.003[a]	0.47 ± 0.005[b]	0.50 ± 0.005	0.48 ± 0.003[c]	0.47 ± 0.005[c]	0.45 ± 0.003[c]
Number of splenocytes (mean ± S.D.) $\times 10^6$/ml	31.84 ± 0.59	31.41 ± 0.47	30.77 ± 0.51[b]	30.50 ± 0.59[b]	31.89 ± 0.52	30.18 ± 0.48[c]	28.96 ± 0.44[c]	28.16 ± 0.45[c]
PFC/10^6 cells	120 ± 6.32	118.33 ± 4.08	110.83 ± 4.92[a]	107.5 ± 5.24[b]	120.83 ± 7.36	116.67 ± 2.58	110.83 ± 4.92[a]	106.67 ± 5.16[c]
HA titer value	1:160	1:160	1:160	1:80	1:160	1:80	1:40	1:40
Number of Macrophages (mean ± S.D.) $\times 10^6$	15.94 ± 1.05	15.57 ± 1.73	15.36 ± 1.78	15.14 ± 0.87	15.30 ± 1.05	14.56 ± 0.59	14.56 ± 1.00	14.18 ± 1.24

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

Table 2. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 90 (S3), 135 (S4) and 180 d (S5) of treatment.

	90 d (S3)				135 d (S4)				180 d (S5)			
Parameters Observed (¶)	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.48 ± 0.41	25.28 ± 0.56	25.3 ± 0.42	24.86 ± 0.20	25.55 ± 0.35	25.38 ± 0.51	25.01 ± 0.29[a]	24.93 ± 0.22[a]	25.38 ± 0.51	25.03 ± 0.42	24.93 ± 0.32	24.7 ± 0.43[a]
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.005	0.47 ± 0.006[c]	0.45 ± 0.002[c]	0.43 ± 0.005[c]	0.49 ± 0.010	0.46 ± 0.005[c]	0.43 ± 0.005[c]	0.42 ± 0.005[c]	0.49 ± 0.003	0.44 ± 0.005[c]	0.41 ± 0.005[c]	0.38 ± 0.007[c]
Number of splenocytes (mean ± S.D.) $\times 10^6$/ml	31.84 ± 0.59	29.44 ± 0.83[c]	28.10 ± 0.55[c]	26.77 ± 0.62[c]	32 ± 0.70	28.53 ± 0.55[c]	27.41 ± 0.48[c]	25.76 ± 0.59[c]	31.84 ± 0.87	27.84 ± 0.45[c]	25.97 ± 0.84[c]	23.73 ± 0.74[c]
PFC/10^6 cells	119.17 ± 5.85	105.83 ± 5.85[b]	98.33 ± 4.08[c]	89.17 ± 5.85[c]	112.50 ± 7.58	102.50 ± 8.80	92.50 ± 7.58[c]	69.17 ± 5.85[c]	115.47 ± 4.47	100 ± 7.07[c]	80 ± 7.07[c]	55 ± 4.47[c]
HA titer value	1:160	1:80	1:40	1:40	1:160	1:20	1:20	1:20	1:160	1:10	1:10	1:10
Number of Macrophages (mean ± S.D.) $\times 10^6$	16.05 ± 1.27	15.09 ± 1.39	14.72 ± 1.99	14.08 ± 1.23	16.26 ± 1.42	15.04 ± 1.10	13.22 ± 0.82[c]	14.34 ± 1.39[c]	17.06 ± 0.94	14.45 ± 1.23[b]	13.65 ± 1.48[c]	11.36 ± 0.77[c]

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

5.1.1.3. Effect of BDE on the number of peritoneal macrophages

As indicated in the Table 1 and 2, the number of the peritoneal macrophages decreased significantly in both dose- and time-dependent manner. After 15 day dose duration period, no significant decrease was observed in the mice that were treated with BDE at a dose of 320 mg/kg b.w., whereas after 180 days of treatment, significant decreases ($p < 0.01$ and $p < 0.001$) were observed in case of all the doses when compared with the respective control groups.

5.1.1.4. Effect of BDE on splenocyte proliferation

After 24 h, 48 h and 72 h of incubation, at 0 µg/ml (control), the number of splenocytes was $(11.57 \pm 0.93) \times 10^6$ cells/ml, $(16.16 \pm 0.59) \times 10^6$ cells/ml and $(12.96 \pm 0.59) \times 10^6$ cells/ml, respectively, whereas, at 200 µg/ml (highest dose), the number of splenocytes decreased remarkably to $(2.93 \pm 0.37) \times 10^6$ cells/ml, $(4.10 \pm 0.47) \times 10^6$ cells/ml and $(3.2 \pm 0.49) \times 10^6$ cells/ml, respectively. Therefore, the results clearly indicated that the number of splenocytes was significantly ($p < 0.001$) decreased in a dose-dependent manner in each case when they were compared with their respective controls (Figure 5 and 6). As shown in Figure 7, a significant ($p < 0.001$) dose-dependent increase in the percentage inhibition of the splenocyte proliferation has been observed in case of the BDE treated splenocytes when compared with the control (0 µg/ml). At 200 µg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 µg/ml.

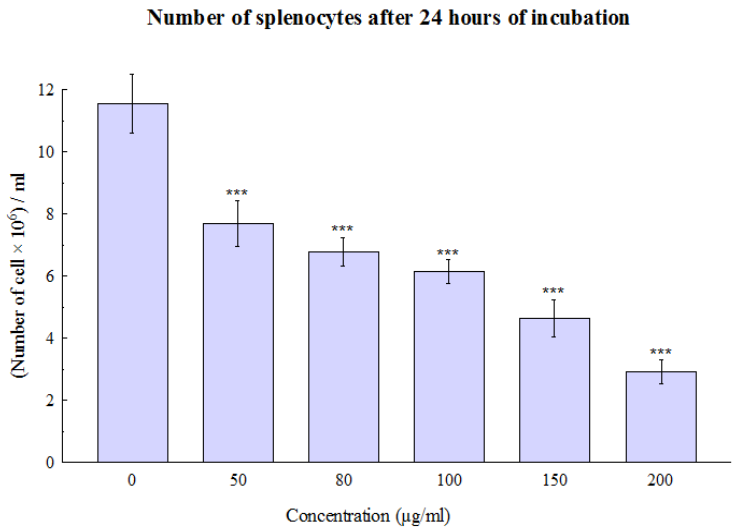


Figure 5. Effect of BDE on the primary cultured splenocytes after 24 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. 0 µg/ml.

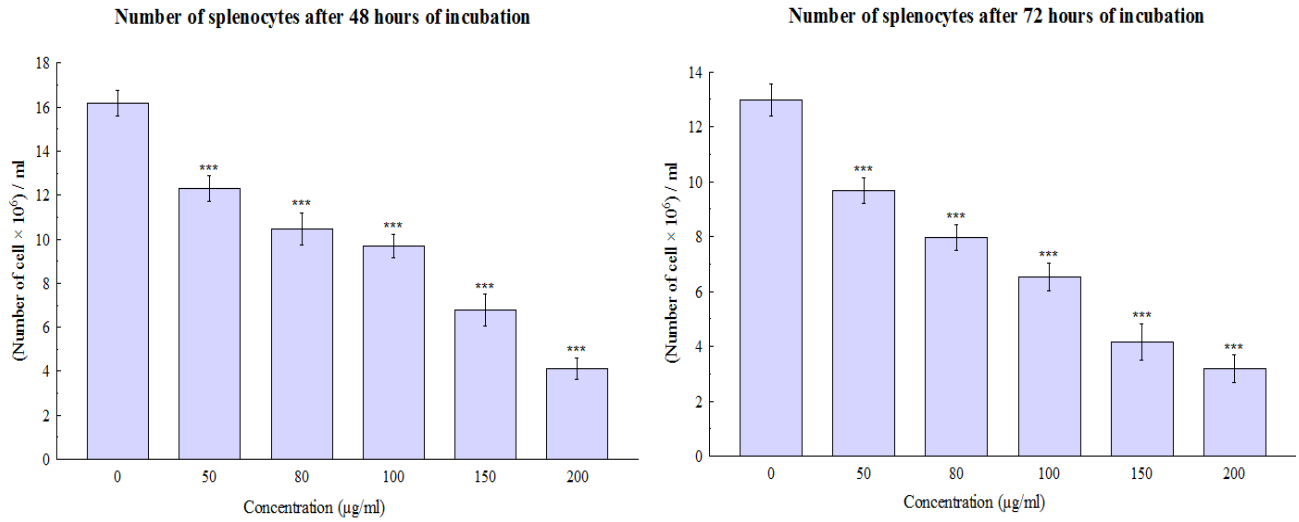


Figure 6. Effect of BDE on the primary cultured splenocytes after 48 h and 72 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

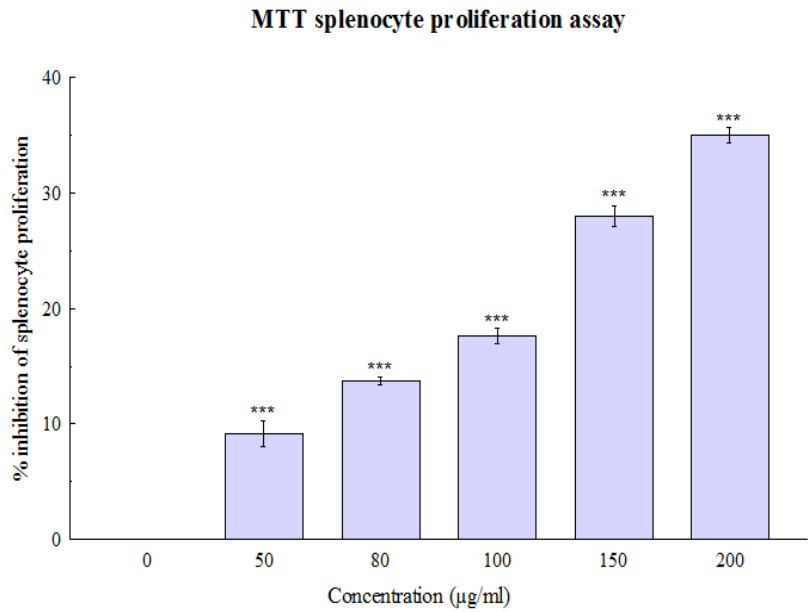


Figure 7. MTT splenocytes proliferation assay demonstrates dose-dependent increase in the percentage inhibition of splenocytes proliferation in the BDE treated splenocytes. At 200 µg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 µg/ml. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

5.1.1.5. Assessment of the effect of BDE on hemolysis

The hemolytic activity of BDE was increased significantly ($p < 0.001$) in a dose-dependent manner in case of mouse erythrocytes (Figure 8). Total hemolysis was obtained using 100 µl of Triton X-100 (0.1%) after 30 min of incubation (not shown in the figure). At 50 µg/ml, the percentages of hemolysis was 40.75%, whereas, the IC_{50} value was 61.78 ± 2.77 µg/ml.

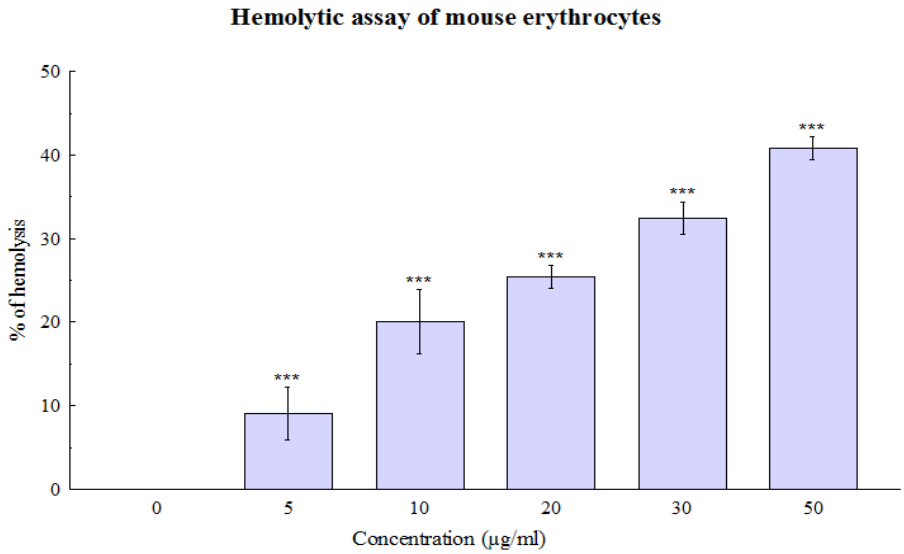


Figure 8. The hemolytic activity of BDE was increased significantly in a dose-dependent manner in case of mouse erythrocytes. At 50 µg/ml, the percentage of hemolysis was 40.75%. The IC_{50} value of BDE was 61.78 ± 2.77 µg/ml. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

5.1.1.6. Effect of BDE on serum concentration levels of Th1 and Th2 cytokines

Significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) were observed in both Th1 and Th2 cytokine concentrations in mice that were treated with different doses of BDE for 90, 135 and 180 days, when compared with their respective control groups (Table 4). After 15 and 45 days of treatment with different doses of BDE, the concentrations of IL-2, IL-4 and IL-10 did not decrease significantly, though after 45 days of treatment with BDE at 160 and 320 mg/kg bw, the concentrations of IFN-γ has been shown to decrease significantly ($p <0.05$) when compared with their respective controls (Table 3). After 180 days of treatment at with 80, 160 and 320 mg/kg bw of BDE, the concentration of all the cytokines as well as serum IgM decreased significantly when compared to their respective controls ($p < 0.001$) (Table 4 & Figure 40).

Table 3. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 15 (S1) and 45 (S2) days.

Concentration of different cytokines[a]	15 days (S1)				45 days (S2)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
IL-2 (pg/ml)	35.59 ± 1.50	35.44 ± 1.45	34.92 ± 0.17	34.74 ± 0.13	35.21 ± 1.18	35.34 ± 1.02	33.92 ± 0.24	35.30 ± 0.21
IFN-γ (pg/ml)	1147.61 ± 8.16	1144.48 ± 8.75	1144.42 ± 8.39	1143.97 ± 11.07	1149.27 ± 1.38	1139.81 ± 7.52	1134.42 ± 8.39*	1130.64 ± 4.68*
IL-4 (pg/ml)	136.81 ± 2.23	136.55 ± 0.76	135.89 ± 1.47	135.81 ± 1.68	136.14 ± 1.81	134.87 ± 2.79	134.55 ± 0.88	133.14 ± 1.14
IL-10 (pg/ml)	3477.09 ± 3.09	3472.06 ± 7.58	3461.05 ± 6.44	3460.42 ± 12.87	3466.42 ± 11.74	3463.73 ± 12.77	3458.05 ± 2.12	3457.42 ± 8.69

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).

Table 4. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 90 (S3), 135 (S4) and 180 (S5) days.

Concentration of different cytokines[a]	90 days (S3)				135 days (S4)				180 days (S5)			
	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
IL-2 (pg/ml)	35.96 ± 1.51	34.56 ± 1.51	32.51 ± 0.61*	32.69 ± 0.22*	35.99 ± 1.55	32.04 ± 0.34**	30.76 ± 1.21***	29.09 ± 0.68***	35.99 ± 0.66	31.70 ± 0.52***	29.09 ± 1.06***	23.61 ± 1.09***
IFN-γ (pg/ml)	1152.27 ± 5.58	1136.48 ± 1.56**	1126.75 ± 5.14***	1119.97 ± 5.09***	1148.61 ± 4.91	1113.15 ± 6.77***	1098.75 ± 5.64***	1078.13 ± 5.34***	1150.94 ± 9.04	1013.15 ± 6.77***	984.73 ± 11.42***	902.99 ± 12.79***
IL-4 (pg/ml)	135.47 ± 1.31	131.22 ± 0.43**	130.22 ± 0.67**	129.81 ± 1.93**	136.81 ± 0.47	129.55 ± 0.76***	128.22 ± 0.46***	124.81 ± 1.93***	136.37 ± 0.87	126.55 ± 1.37***	125.97 ± 3.00***	119.81 ± 1.24***
IL-10 (pg/ml)	3467.09 ± 10.80	3457.06 ± 7.62	3441.38 ± 7.81	3440.76 ± 15.86*	3463.75 ± 4.52	3443.73 ± 3.02**	3434.71 ± 3.83***	3420.76 ± 6.64***	3470.42 ± 6.14	3407.06 ± 18.71***	3328.05 ± 9.52***	3227.42 ± 8.48***

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).
**$p < 0.01$ when compared with Group 1 (Control) (Significantly different).
***$p < 0.001$ when compared with Group 1 (Control) (Significantly different).

5.1.1.7. Effect of BDE on Th1 and Th2 cytokine production from primary cultured splenocytes

Figure 9 indicated significant concentration-dependent IL-2 decrease ($p < 0.001$) in con A induced splenocytes. At 0 µg/ml, the concentration of IL- 2 in splenocyte culture supernatant was 28.33 ± 0.58 pg/ml, whereas, at 200 µg/ml, the concentration of IL-2 decreased to 19.17±0.38 pg/ml. The amount of IFN-γ, IL-4 and IL-10 were also decreased significantly ($p < 0.01$ and $p < 0.001$) in concentration-dependent manner when compared with their respective controls. At 0 µg/ml of BDE, the concentrations of IFN-γ, IL-4 and IL-10 were 913.33 ± 5.77 pg/ml, 127.76 ± 2.21 pg/ml and 2208.33 ± 14.43 pg/ml, respectively, whereas, at 200 µg/ml, the concentrations of IFN-γ, IL-4 and IL-10 has been shown to decrease to 216.67 ± 5.77 pg/ml, 104.79 ± 2.45 pg/ml and 1325 ± 25 pg/ml, respectively (Figure 9).

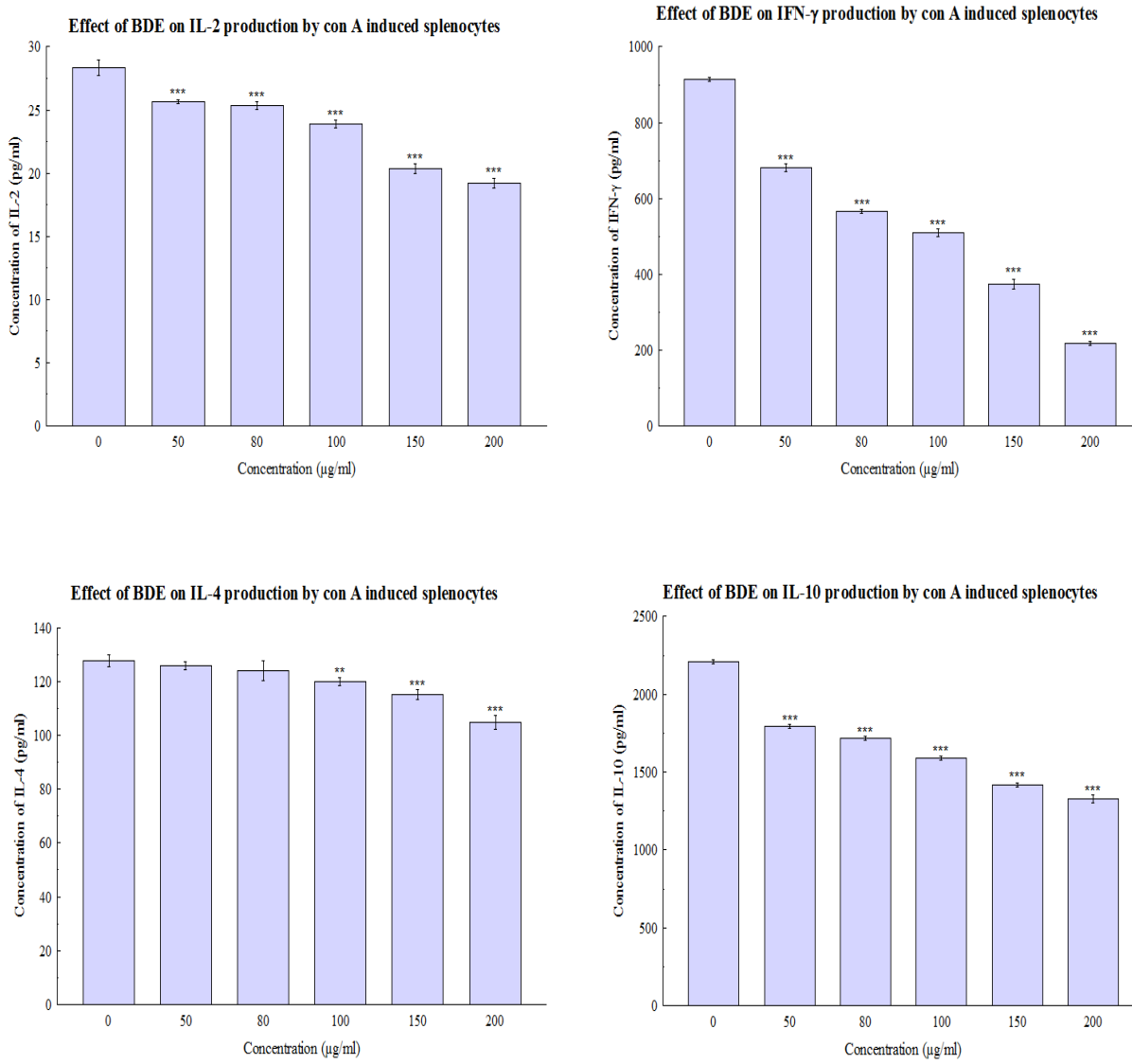
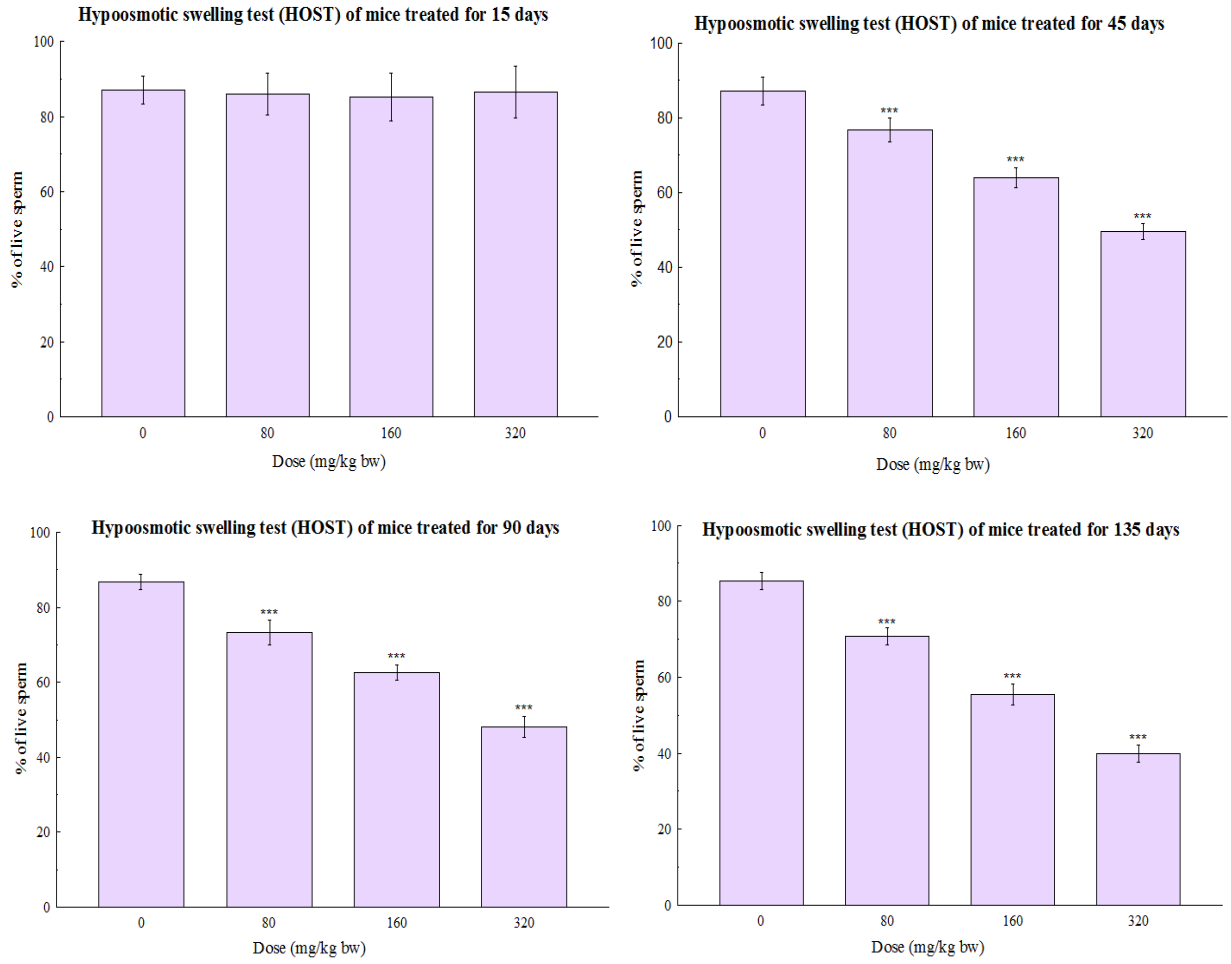


Figure 9: Effect of different concentrations (0-200 µg/ml) of BDE on different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine production by con A induced splenocytes. Data represents significant concentration-dependent decrease in cytokine production. The results are mean ± S.D. of three parallel observations. ** p and ***$p < 0.001$ vs. 0 µg/ml.

5.1.2. Effect of BDE on the reproductive functions of mouse

5.1.2.1. Hypoosmotic swelling test (HOST)

Figure 10 indicated that no significant alterations were observed in sperm viability after 15 days of treatment with BDE. But, at 320 mg/kg bw of treatment with BDE for 45 days, significant decrease ($p < 0.001$) in the number of live sperm was observed when compared with the respective control group. After sub-chronic and chronic treatments (90, 135 and 180 days) with BDE at all the doses (80, 160 and 320 mg/kg bw), sperm viability reduces significantly ($p < 0.001$) when compared with the respective control groups.



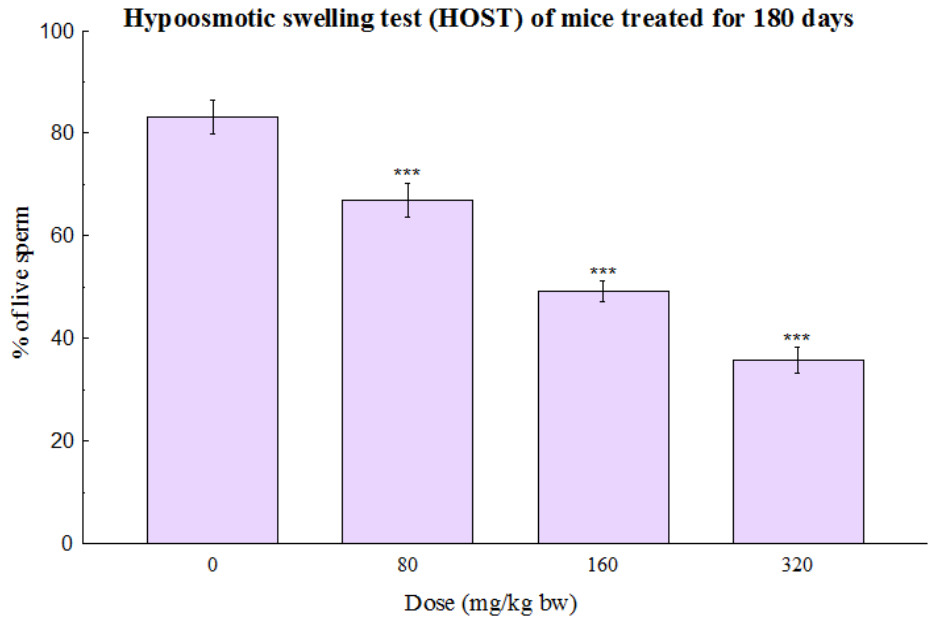
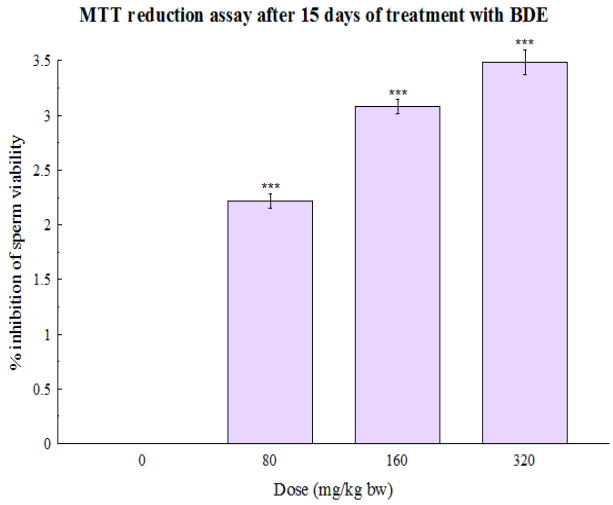


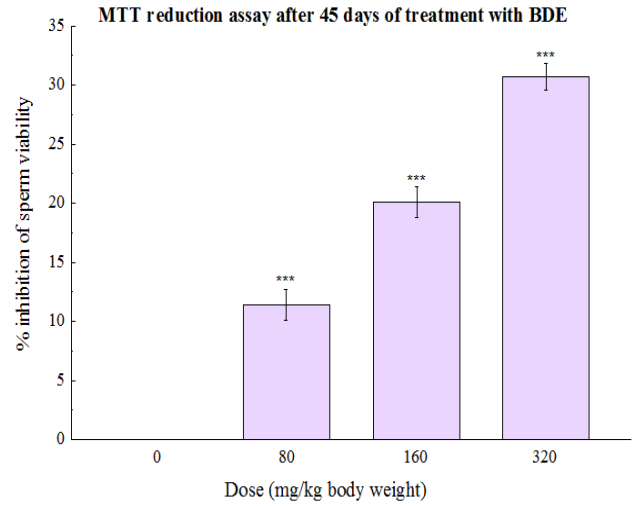
Figure 10: Hypo-osmotic swelling test (HOST) of spermatocytes demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 15, 45, 90, 135 and 180 days. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

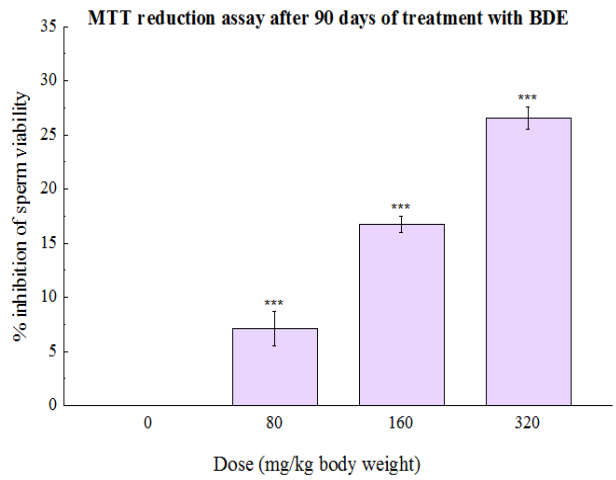
5.1.2.2. MTT reduction assay of live spermatozoa

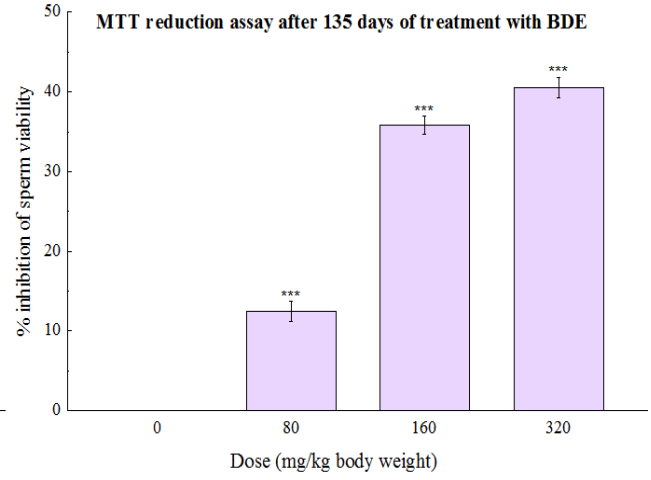
Results of the MTT reduction assay showed significant dose-dependent increased percentage of the inhibition of sperm viability in all of the cases. After 45 days of treatment, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, i.e., at 80 mg/kg bw (11.38%), 160 mg/kg bw (20.07%) and 320 mg/kg bw (30.69%), when compared with the control group (Figure 11). After 90 days of treatment, at 80 mg/kg bw, the percentage inhibition of sperm viability was 7.10%, whereas, at 160 mg/kg bw and 320 mg/kg bw, the percentage inhibitions of sperm viability were 16.69% and 26.56%, respectively. Therefore, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, when compared with the respective control groups (Figure 11). This was also observed significantly after 135 days and 180 days of treatment with BDE. After 135 days of treatment, at 320 mg/kg bw, the percentage inhibition of sperm viability was 40.51% (Figure 11), whereas, after 180 days

of treatment with BDE, at 320 mg/kg bw, the percentage inhibition of sperm viability was increased remarkably up to 53.12% (Figure 11).









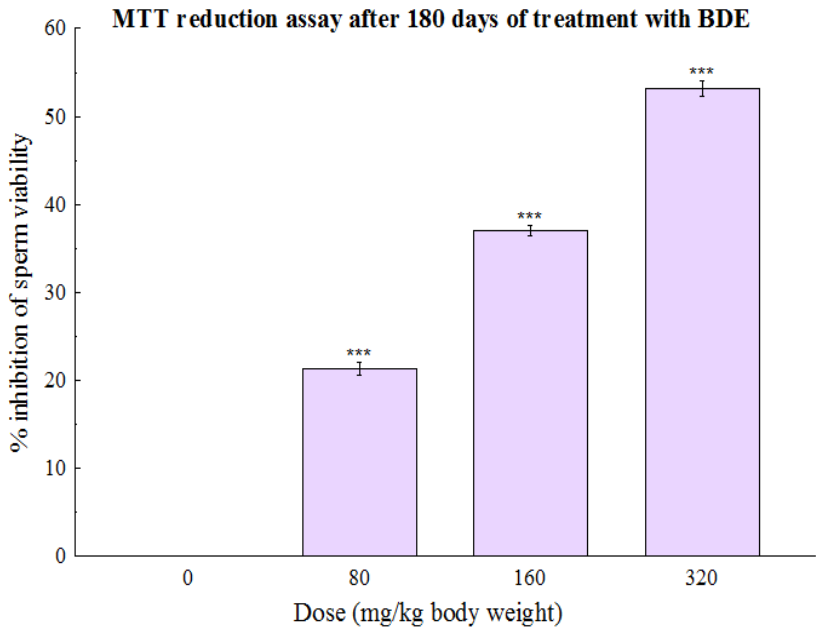


Figure 11: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 15, 45, 90, 135, and 180 days. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. control.

5.1.2.3. Body weight and relative weight of organs

No significant alterations in the body weight and relative organ weight have been observed in mice that were treated with all the doses for subacute and subchronic durations (Table 5). There were a significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in body weight of BDE treated mice (160 and 320 mg/kg bw) after 135 and 180 days of treatment when compared to the control group (Table 6). The relative weights of epididymis, seminal vesicle and prostate of animals treated with the BDE at the dose of 160 and 320 mg/kg bw have been decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) after 135 and 180 days of treatment.

Table 5: Effect of BDE on body weight and relative weight of testis and other sexual organs after subacute and subchronic doses

Parameters	Sub-acute dose I (15 days) (S1)				Sub-acute dose II (45 days) (S2)				Sub-chronic dose (90 days) (S3)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Body weight (g)	25.13 ± 0.62	25.03 ± 0.91	25.05 ± 0.82	25.27 ± 1.03	26.23 ± 0.52	26.03 ± 1.91	26.05 ± 0.82	25.27 ± 1.03	25.83 ± 0.52	25.03 ± 2.93	25.25 ± 0.87	25.27 ± 2.13
Relative testis weight (g/100 g of body weight)	0.98 ± 0.05	0.98 ± 0.07	0.98 ± 0.07	0.97 ± 0.06	0.96 ± 0.08	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.06	0.94 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.94 ± 0.06
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.03	0.34 ± 0.05	0.33 ± 0.02	0.34 ± 0.07	0.35 ± 0.03	0.34 ± 0.05	0.34 ± 0.02	0.33 ± 0.07	0.34 ± 0.03	0.33 ± 0.05	0.33 ± 0.02	0.32 ± 0.07
Relative seminal vesicle weight (g/100 g of body weight)	0.49 ± 0.06	0.48 ± 0.03	0.47 ± 0.05	0.47 ± 0.03	0.48 ± 0.06	0.47 ± 0.03	0.47 ± 0.05	0.46 ± 0.03	0.47 ± 0.06	0.47 ± 0.03	0.46 ± 0.05	0.45 ± 0.03
Relative prostate gland weight (g/100 g of body weight)	0.25 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.24 ± 0.05	0.26 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.25 ± 0.05	0.24 ± 0.01	0.24 ± 0.02	0.24 ± 0.05	0.23 ± 0.05

¶ Data shows no significant differences of the treated groups from the respective controls

Table 6: Effect of BDE on body weight and relative weight of testis and other sexual organs after chronic doses

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.13 ± 0.18	24.86 ± 0.24	24.53 ± 0.15	24.12 ± 0.11	24.83 ± 0.32	23.03 ± 0.93	22.85 ± 0.77	22.27 ± 1.13
Relative testis weight (g/100 g of body weight)	0.95 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.93 ± 0.06*	0.95 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.91 ± 0.01**
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.01	0.31 ± 0.02	0.27 ± 0.01**	0.27 ± 0.01**	0.33 ± 0.02	0.33 ± 0.01	0.27 ± 0.01**	0.24 ± 0.01***
Relative seminal vesicle weight (g/100 g of body weight)	0.48 ± 0.01	0.46 ± 0.01	0.45 ± 0.01*	0.44 ± 0.01**	0.48 ± 0.01	0.43 ± 0.02*	0.42 ± 0.02**	0.37 ± 0.02***
Relative prostate gland weight (g/100 g of body weight)	0.24 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.22 ± 0.01**	0.24 ± 0.01	0.22 ± 0.01*	0.21 ± 0.01**	0.19 ± 0.01***

*$p < 0.05$ when compared with Control (G1).

**$p < 0.01$ when compared with Control (G1).

***$p < 0.001$ when compared with Control (G1).

5.1.2.4. Effect of BDE on different biochemical parameters of sexual organs

Changes of biochemical parameters after different doses of treatment are outlined in Table 7 & 8. Proteins levels in serum, testis and epididymis of BDE treated mice (320 mg/kg bw) decreased significantly ($p < 0.05$) after 90 days of treatment. After 135 and 180 days of treatment at 320 mg/kg bw of BDE, protein contents in serum, testis and epididymis decreased significantly ($p < 0.001$) when compared with the respective controls. Cholesterol level in testis was decreased significantly ($p < 0.001$) in mice receiving BDE at the dose of 160 and 320 mg/kg bw after subchronic and chronic treatments. Fructose and α-glucosidase levels of BDE treated mice (at 160 and 320 mg/kg bw) significantly decreased ($p < 0.001$) after 135 and 180 days of treatment when compared with the control groups. The glycogen level in testis decreased significantly ($p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for subchronic and chronic durations. Sialic acid concentration in epididymis decreased significantly ($p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for 135 and 180 days of treatment when compared with the control groups. The concentration of prostatic citric acid decreased significantly ($p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for subchronic and chronic durations. The testicular acid phosphatase concentration has also been decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in a dose- and time dependent manner within a duration of 180 days of treatment of BDE at different doses, when compared to the respective control groups.

Table 7: Effect of BDE on different biochemical parameters of testis and other sexual organs after sub-acute (15 and 45 days) and sub-chronic doses (90 days)

Parameters	Sub-acute dose I (15 days) (S1)				Sub-acute dose II (45 days) (S2)				Sub-chronic dose (90 days) (S3)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Total Protein (serum) (mg/dl)	7.84 ± 0.75	7.74 ± 0.83	7.73 ± 0.59	7.82 ± 1.11	7.80 ± 0.81	7.56 ± 0.52	7.68 ± 0.27	7.60 ± 0.33	7.52 ± 0.53	7.21 ± 0.28	6.96 ± 0.32	6.91 ± 0.40*
Total Protein(testis) (mg/g)	0.50 ± 0.14	0.42 ± 0.15	0.43 ± 0.17	0.44 ± 0.20	0.52 ± 0.07	0.49 ± 0.07	0.47 ± 0.07	0.43 ± 0.04	0.56 ± 0.03	0.54 ± 0.03	0.52 ± 0.03	0.51 ± 0.01*
Total Protein (epididymis) (mg/g)	0.20 ± 0.07	0.19 ± 0.05	0.18 ± 0.07	0.19 ± 0.08	0.22 ± 0.06	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	0.24 ± 0.05	0.20 ± 0.04	0.19 ± 0.05	0.17 ± 0.04*
Chelesterol (testis) (mg/g)	3.89 ± 0.18	3.79 ± 0.18	3.85 ± 0.16	3.85 ± 0.24	3.82 ± 0.14	3.84 ± 0.11	3.84 ± 0.09	3.75 ± 0.09	3.84 ± 0.11	3.69 ± 0.13	3.71 ± 0.18	3.48 ± 0.06*
α-glucosidase (epididymis) (mU/g)	4.81 ± 0.10	4.79 ± 0.10	4.75 ± 0.06	4.79 ± 0.15	4.82 ± 0.03	4.74 ± 0.10	4.72 ± 0.03	4.74 ± 0.11	4.80 ± 0.01	4.67 ± 0.05*	4.65 ± 0.06*	4.56 ± 0.06**
Fructose (seminal vesicles) (µM/g)	4.54 ± 0.16	4.50 ± 0.10	4.48 ± 0.02	4.49 ± 0.13	4.53 ± 0.08	4.47 ± 0.07	4.52 ± 0.06	4.48 ± 0.08	4.61 ± 0.10	4.54 ± 0.07	4.48 ± 0.08	4.53 ± 0.06
Glycogen (testis) (mg/g)	37.48 ± 2.35	37.66 ± 2.77	37.19 ± 4.30**	37.27 ± 4.58	39.78 ± 1.35	39.66 ± 2.25	39.19 ± 2.30	38.27 ± 3.53	38.52 ± 2.58	37.66 ± 3.25	37.19 ± 3.20	33.27 ± 0.53**
Sialic acid (epididymis) (µM/100 g tissue)	64.25 ± 2.21	64.35 ± 3.37	64.45 ± 2.39	64.25 ± 3.58	65.25 ± 2.21	65.11 ± 0.58	64.87 ± 2.02	64.80 ± 3.01	63.14 ± 0.69	63.02 ± 0.98	62.38 ± 0.36	61.78 ± 0.81*
Prostate citric acid (mg/g)	35.02 ± 2.52	34.75 ± 3.01	34.37 ± 1.36	33.33 ± 1.99	36.42 ± 2.52	36.25 ± 1.71	36.37 ± 1.56	35.33 ± 2.99	36.02 ± 1.52	35.75 ± 1.51	37.37 ± 0.56	31.33 ± 0.99*
Acid phosphatase (testis) (µM/min/g of tissue)	30.09 ± 5.20	28.60 ± 0.66	24.01 ± 1.50	23.83 ± 0.39	31.05 ± 2.79	31.98 ± 2.60	31.65 ± 2.32	22.46 ± 0.96**	30.86 ± 3.87	20.05 ± 2.97	21.65 ± 0.48**	18.50 ± 0.97***

*$p < 0.05$ when compared with Control (G1).

**$p < 0.01$ when compared with Control (G1).

***$p < 0.001$ when compared with Control (G1).

Table 8: Effect of BDE on different biochemical parameters of testis and other sexual organs after chronic doses (135 and 180 days)

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Total Protein (serum) (mg/dl)	7.84 ± 0.49	7.12 ± 0.16**	6.68 ± 0.32***	6.35 ± 0.35***	7.85 ± 0.83	6.78 ± 0.41**	6.06 ± 0.30***	5.64 ± 0.55***
Total Protein (testis) (mg/g)	0.58 ± 0.04	0.53 ± 0.01*	0.51 ± 0.03***	0.48 ± 0.02***	0.56 ± 0.03	0.52 ± 0.03*	0.48 ± 0.02***	0.41 ± 0.01***
Total Protein (epididymis) (mg/g)	0.25 ± 0.04	0.20 ± 0.02*	0.18 ± 0.02***	0.14 ± 0.01***	0.28 ± 0.02	0.19 ± 0.01***	0.13 ± 0.01***	0.08 ± 0.01***
Chelesterol (testis) (mg/g)	3.85 ± 0.13	3.66 ± 0.08	3.53 ± 0.03**	3.42 ± 0.02***	3.82 ± 0.08	3.53 ± 0.12**	3.43 ± 0.07**	3.27 ± 0.06***
α-glucosidase (epididymis) (mU/g)	4.76 ± 0.06	4.63 ± 0.03*	4.61 ± 0.08*	4.52 ± 0.05**	4.77 ± 0.06	4.56 ± 0.06**	4.40 ± 0.05***	4.32 ± 0.06***
Fructose (seminal vesicles) (µM/g)	4.56 ± 0.11	4.46 ± 0.05	4.41 ± 0.07	4.36 ± 0.04*	4.52 ± 0.08	4.42 ± 0.04	4.35 ± 0.06*	4.30 ± 0.04**
Glycogen (testis) (mg/g)	39.58 ± 1.58	38.66 ± 2.25	33.19 ± 1.20**	30.27 ± 0.53***	40.58 ± 1.38	31.46 ± 2.85***	27.19 ± 3.28***	20.27 ± 3.53***
Sialic acid (epididymis) (µM/100 g tissue)	62.12 ± 3.24	56.71 ± 0.93*	50.03 ± 1.14***	41.06 ± 0.81***	63.59 ± 2.24	54.36 ± 3.36***	40.25 ± 3.35***	31.36 ± 2.54***
Prostate citric acid (mg/g)	37.02 ± 1.52	31.75 ± 1.51*	27.37 ± 2.56**	21.33 ± 0.99***	36.58 ± 2.27	25.48 ± 3.35***	21.36 ± 3.69***	18.47 ± 1.36***
Acid phosphatase (testis) (µM/min/g of tissue)	30.07 ± 5.12	23.94 ± 0.88**	20.34 ± 1.44***	16.29 ± 0.05***	33.72 ± 0.38	21.70 ± 0.60***	18.35 ± 1.02***	15.96 ± 1.57***

*p < 0.05 when compared with Control (G1).

**p < 0.01 when compared with Control (G1).

***p < 0.001 when compared with Control (G1).

5.1.2.5. Alterations in the architecture of testes

No significant morphological alterations in the architecture of testes were observed in subacute and subchronic doses and therefore, data was not presented. Morphological alterations of seminiferous tubules were observed only in the groups treated with 320 mg/kg bw of BDE for 135 and 180 days when compared to the control testis. Significant decreases in diameter, perimeter and area of the seminiferous tubules were observed in case of mice treated with 160 and 320 mg/kg bw of BDE for 180 days ($p < 0.01$ and $p < 0.001$) when compared with the control groups. The number of empty seminiferous tubules has been shown to increase with the increasing dose duration. Significant increases ($p < 0.05$ and $p < 0.001$) in the percentage of empty seminiferous tubules were also observed after treatment with 160 and 320 mg/kg bw of BDE for 135 and 180 days when compared with the control groups (Table 9). In the group treated with BDE at 320 mg/kg bw for 180 days, the epithelium was reduced to a single layer in few of the tubules [Figure 14 (A) & (B)]. Seminiferous tubules of mice treated with 320 mg/kg bw of BDE for 135 days [Figure 13 (A) & (B)] and 180 days [Figure 14 (A) & (B)] showed vacuolization in the tubules with reduced epithelial membranes layer, when compared to the control (Figure 12).

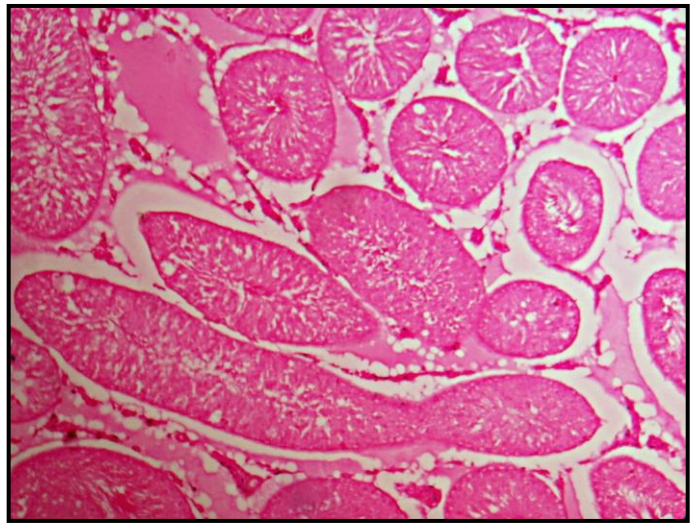


Figure 12: Histology of the testis of control mice (stained with haematoxylin–eosin) showing normal architecture in seminiferous tubules with intact epithelial membranes and no vacuolization.

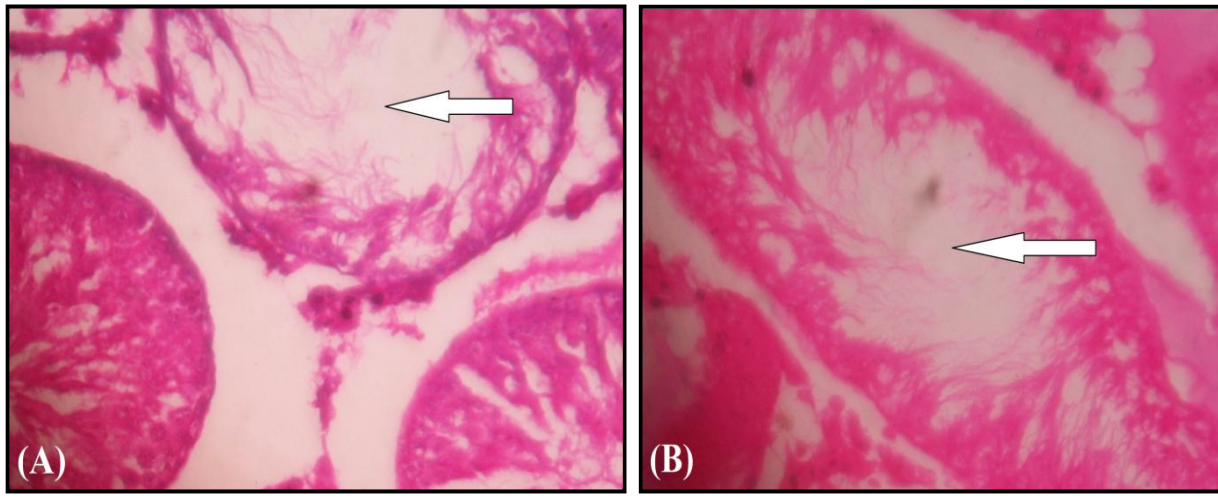


Figure 13 (A & B): Histology of the testis of mice treated with 320 mg/kg bw of BDE for 135 days (observed with a magnitude of ×400 under the microscope, stained with haematoxylin–eosin). Figure shows vacuolization in the seminiferous tubules (indicated by white arrow) with reduced epithelial membranes layer.

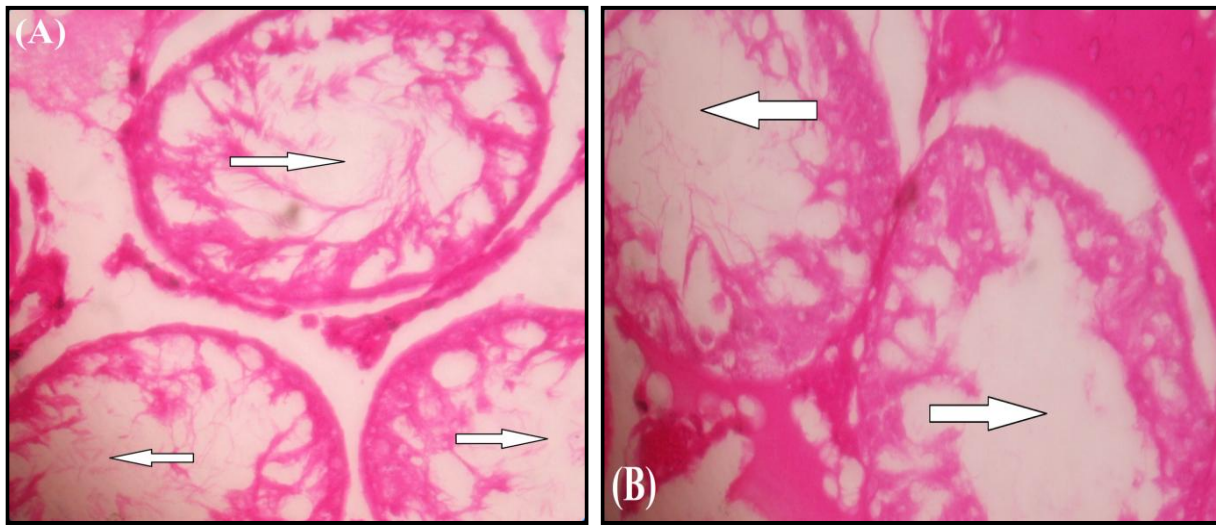


Figure 14 (A & B): Histology of the testis of mice treated with 320 mg/kg bw of BDE for 180 days (observed with a magnitude of ×400 under the microscope, stained with haematoxylin–eosin) showing increased vacuolization in the tubules (indicated by white arrow) with reduced epithelial membrane layers. The number of empty seminiferous tubules as well as inter-seminiferous tubular spaces has been shown to increase with the increased dose duration when compared to the control.

Table 9: Histomorphometric parameters of seminiferous tubules

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Diameter (in µm)	273.44 ± 9.25	256.91 ± 14.50	242.79 ± 14.87	240.76 ± 15.21*	271.92 ± 7.66	268.46 ± 9.73	253.79 ± 8.05	241.45 ± 7.17**
Perimeter (in µm)	964.35 ± 16.50	863.78 ± 12.55***	765.03 ± 15.34***	737.85 ± 12.83***	989.46 ± 30.27	564.31 ± 33.06***	365.11 ± 21.98***	239.64 ± 27.56***
Area (in sq. µm)	61122.62 ± 1927.22***	42978.20 ± 2631.77***	40954.62 ± 1574.45***	37948.97 ± 3270.21***	61583.03 ± 1644.35	50618.27 ± 1559.85***	40007.95 ± 1379.22***	32728.41 ± 3615.11***
Percentage of empty seminiferous tubules	11.60 ± 3.15	12.42 ± 3.13	15.21 ± 3.15	19.96 ± 1.99*	11.62 ± 2.78	16.52 ± 1.92	20.11 ± 2.55*	28.80 ± 3.36***

*p < 0.05 when compared with Control (G1).

**p < 0.01 when compared with Control (G1).

***p < 0.001 when compared with Control (G1).

5.1.2.6. Effect of D. esculentum on fertility and fecundity

As outlined in Table 10 and 11, the fertility as well as fecundity has been decreased in a dose- and time-dependent manner in BDE treated mice when compared to the respective control groups. The percentage of fertility (no. of viable pups) has decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective control groups. After sub-chronic and chronic treatments at 320 mg/kg bw, no viable pups have been recorded. Similarly, percentage of fecundity has been decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective controls. Hundred percent fertility losses observed at 320 mg/kg bw dose of BDE that were treated for subchronic and chronic durations. Similarly, 100% losses of fecundity observed in mice that were treated with 160 and 320 mg/kg bw of BDE for 180 days.

Table 10: Effects of the BDE on fertility of Swiss albino mouse after different days of treatment

Treatment	Ratio of males and females for fertility test	% of fertility (no. of viable pups) after different periods				
		15 days	45 days	90 days	135 days	180 days
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	80
160 mg/kg bw	½	100	80	80	50	50
320 mg/kg bw	½	50	50	0	0	0

Table 11: Effects of the BDE on fecundity of Swiss albino mouse after different days of treatment

Treatment	Ratio of males and females for fecundity test	% of fecundity after different periods				
		15 days	45 days	90 days	135 days	180 days
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	75
160 mg/kg bw	½	100	100	100	75	0
320 mg/kg bw	½	100	100	100	75	0

5.1.3. Effect of BDE on the cholinergic nervous system

5.1.3.1. In vivo experiment

5.1.3.1.1. Determination of acetylcholinesterase activity

The acetylcholinesterase activity of BDE treated Swiss albino mouse was determined by assessing the rate (R) of change in absorbance per min, by a previously described formula (Ellman et al., 1961). Results indicated that R, which was expressed in moles acetylthiocholine iodide (substrate) hydrolyzed per min per g of tissue, decreased significantly ($p < 0.01$ and $p < 0.001$) in a dose-dependent manner, indicating increased dose-dependent inhibition in the acetylcholinesterase activity in the BDE treated mice over a 30 d time period (Figure 15).

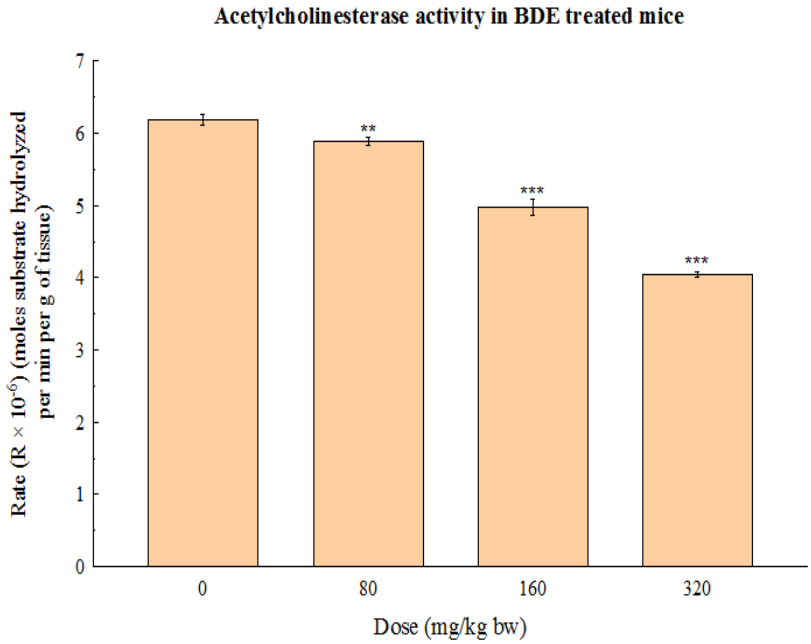


Figure 15: Acetylcholinesterase activity in BDE treated mice. Figure shows dose-dependent decrease in rate (R), which was expressed in moles acetylthiocholine iodide (substrate) hydrolyzed per min per g of tissue. The results are mean ± S.D. of three parallel observations. **p < 0.01 and ***p < 0.001 vs. control.

5.1.3.2. Ex vivo experiment

5.1.3.2.1. Assessment of acetylcholinesterase inhibitory activity

As indicated in Figure 16, a significant ($p < 0.001$) dose-dependent increase in the AChE inhibitory activity of *D. esculentum* has been observed. At 50 and 200 µg/ml, the percentage

inhibition of AChE was 8.63% and 54.39%, respectively. The IC_{50} value of MDE on AChE inhibitory activity has been found to be 272.97 ± 19.38 µg/ml.

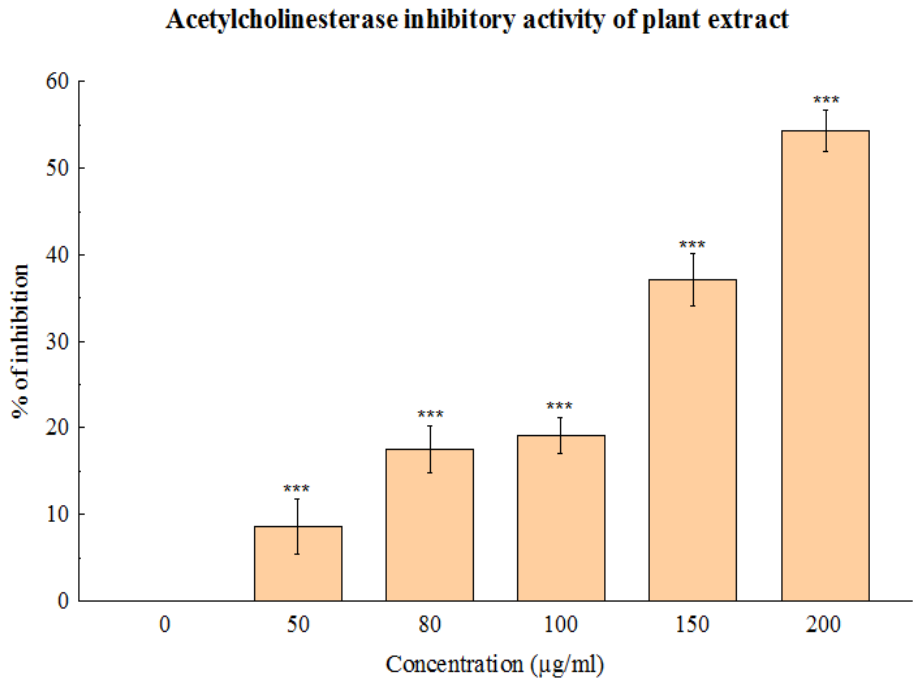


Figure 16: Acetylcholinesterase inhibitory activity of *D. esculentum* extract. The data represent the percentage inhibition of the enzyme acetylcholinesterase. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract was 272.97 ± 19.38 µg/ml.

5.1.3.2.2. Determination of NADH oxidase inhibitory activity

MDE inhibited NADH oxidase significantly ($p < 0.001$) in a dose-dependent manner (Figure 17). At 50 µg/ml, the percentage inhibition of NADH oxidase was 10.43%, whereas at 200 µg/ml, the percentage inhibition of NADH oxidase was increased to 43.99%. The IC_{50} value of MDE on NADH oxidase inhibition was 265.81 ± 21.20 µg/ml.

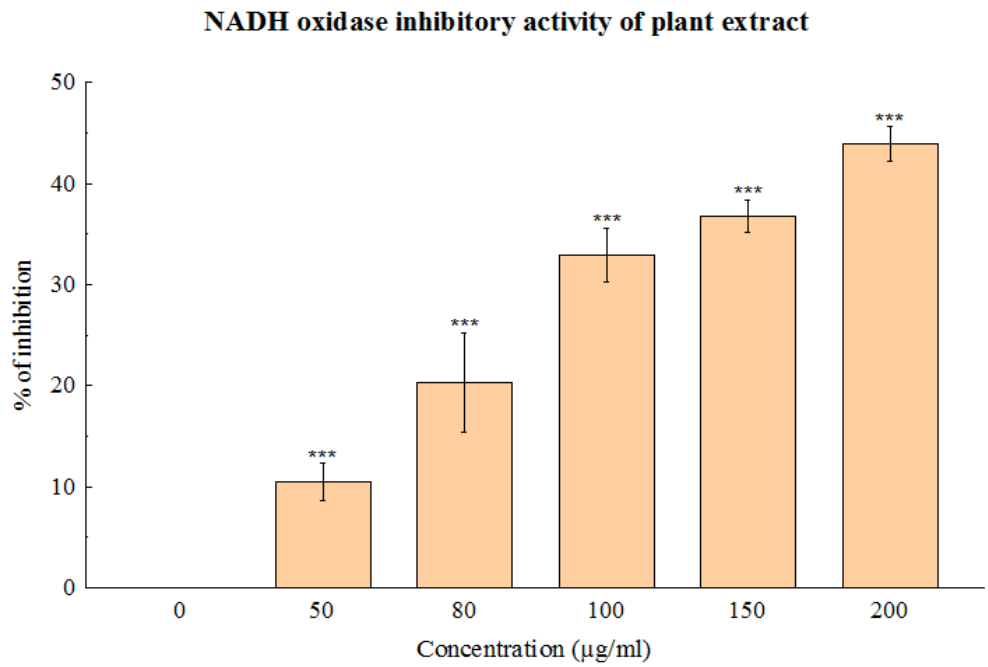


Figure 17: NADH oxidase inhibitory activity of *D. esculentum* extract. The data represent the percentage inhibition of the enzyme NADH oxidase. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract was 265.81 ± 21.20 µg/ml.

5.1.4. Antioxidant and free radical scavenging activities of *Diplazium esculentum*

5.1.4.1. Determination of total antioxidant activity by FTC and TBA method

As shown in Figure 18, the absorbance of the control at 500 nm increased to a maximal value of 0.84 on day 14, whereas vitamin E (α-tocopherol) and MDE increased to 0.57 and 0.61, respectively, on the same day. These differences were found statistically significant than the control ($p < 0.001$). The total antioxidant activity of vitamin E by FTC and TBA methods were 31.85% and 38.97%, respectively, whereas the total antioxidant activity of MDE by FTC and TBA methods were 27.41% and 33.22%, respectively.

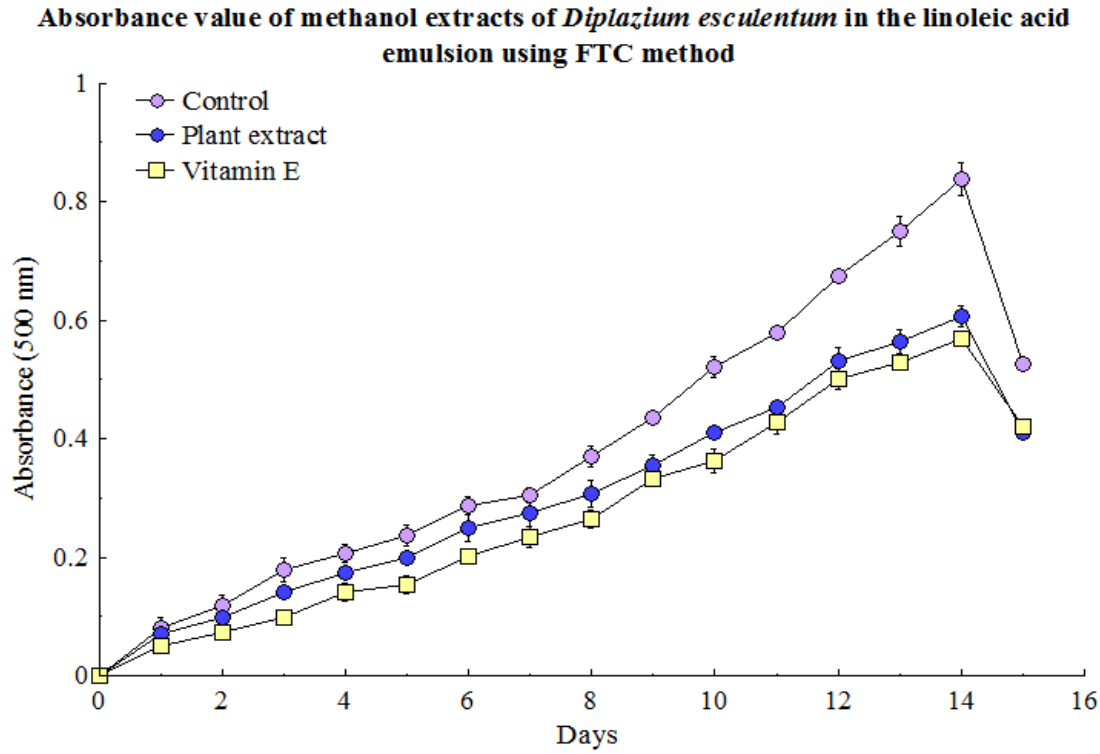


Figure 18: Absorbance value of the MDE in the linoleic acid emulsion using FTC method. The results are mean ± S.D. of six parallel measurements.

5.1.4.2. Total antioxidant activity by ABTS method

The total antioxidant activity of MDE was calculated from the decolorization of $ABTS^{\bullet+}$, which was measured spectrophotometrically at 734 nm. Interaction with the plant extract or standard trolox suppressed the absorbance of the $ABTS^{\bullet+}$ radical cation and the results, expressed as percentage inhibition of absorbance, are shown in Figure 19. The TEAC value of the extract was 0.21 ± 0.02.

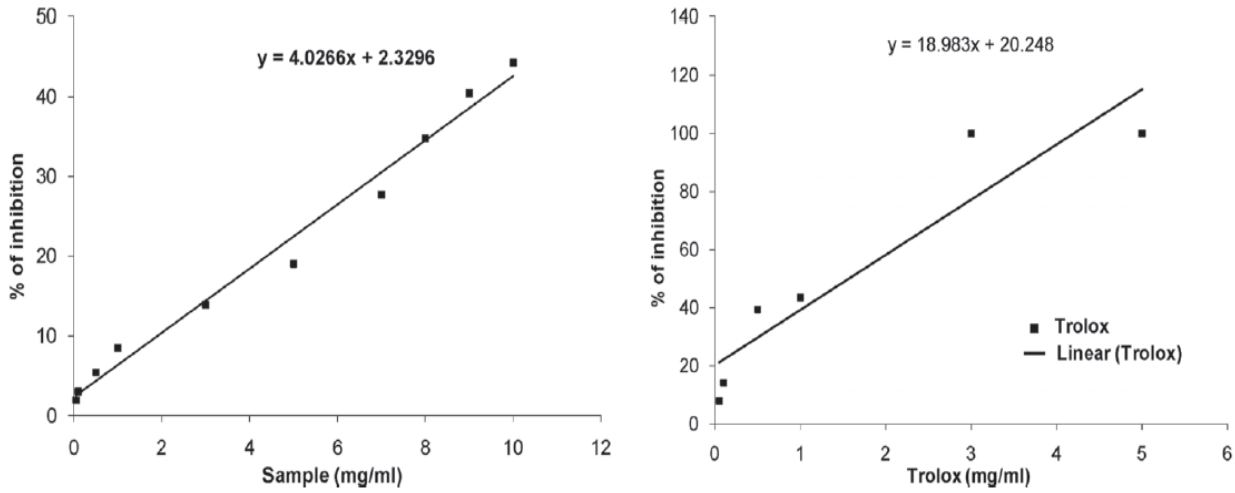


Figure 19: Total antioxidant activity of MDE and reference compound trolox on decolourization of ABTS radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n = 6).

5.1.4.3. DPPH radical scavenging activity

Figure 20 showed a significant ($p < 0.01$ and $p < 0.001$) dose-dependent increase in the percentage of DPPH radical scavenging by MDE, when compared with the standard α-tocopherol. At 50 µg/ml, the percentages of DPPH radical scavenging of the plant extract and standard were 8.75% and 11.07%, respectively, whereas, at 200 µg/ml, the radical scavenging was increased up to 38.12% and 42.69%, for MDE and the standard, respectively. The IC_{50} values of the plant extract and standard on DPPH radical scavenging were 402.88 ± 12.70 µg/ml and 324.86 ± 6.35 µg/ml, respectively.

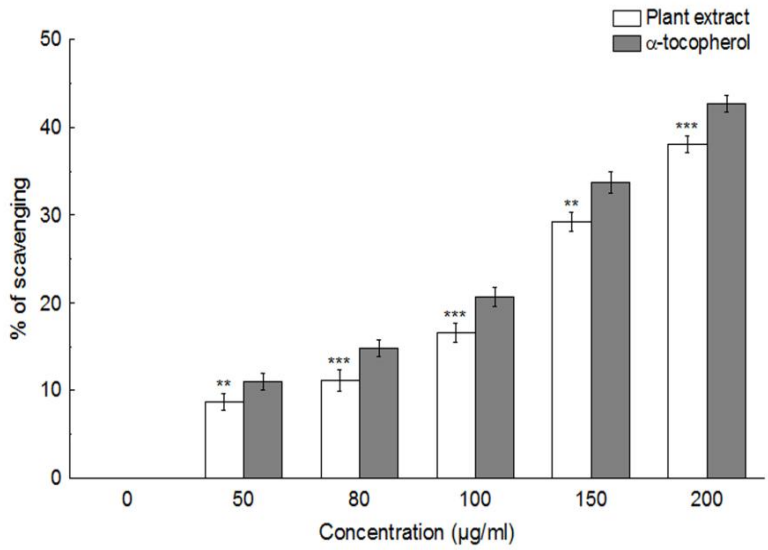


Figure 20: DPPH radical scavenging activity of *D. esculentum* extract. The data represent the effect of *D. esculentum* plant extract and α-tocopherol on the scavenging of DPPH radical. The results are mean ± S.D. of six parallel measurements. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract and the standard (α-tocopherol) were 402.88±12.70 µg/ml and 324.86±6.35 µg/ml, respectively.

5.1.4.4. Hydroxyl radical scavenging activity

This assay shows the abilities of MDE and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Figure 21. The IC_{50} values (Table 12) of MDE and the standard in this assay were 811.00 ± 23.73 µg/ml and 571.45 ± 20.12 µg/ml, respectively. Though the IC_{50} value of MDE was greater than that of the standard, at 200 µg/ml, the percentages of inhibition were 23.4% and 21.9% for *D. esculentum* and mannitol, respectively.

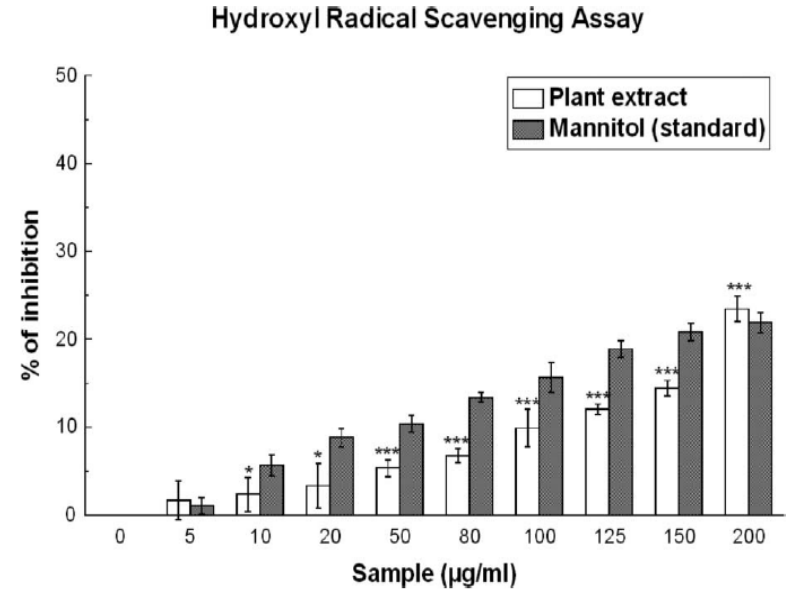


Figure 21: Hydroxyl radical scavenging activities of MDE and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ± S. D. of six parallel measurements. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 811 ± 23.73 µg/ml and 571.45 ± 20.12 µg/ml, respectively.

5.1.4.5. Superoxide radical scavenging activity

Superoxide radicals, generated from the PMS-NADH coupling, can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with MDE and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture (Figure 22), The IC_{50} values (Table 12) of MDE and quercetin on superoxide scavenging activity were 90.39 ± 2.22 µg/ml and 42.06 ± 1.35 µg/ml, respectively. At 20 µg/ml, the percentage of inhibition of the plant extract was 17.8%, whereas that of quercetin was 29.1%.

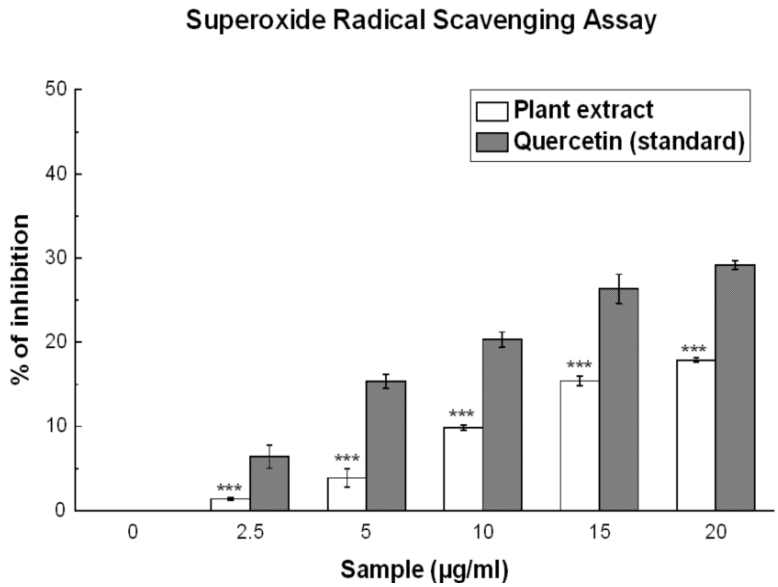


Figure 22: Scavenging effect of MDE and the standard quercetin on superoxide radical. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 90.39 ± 2.22 µg/ml and 42.06 ± 1.35 µg/ml, respectively.

5.1.4.6. Nitric oxide radical scavenging activity

As shown in Figure 23, MDE also caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} value (Table 12) of 204.28 ± 18.31 µg/ml. Curcumin was used as a reference compound and 90.82 ± 4.75 µg/ml curcumin was needed for 50% inhibition. At 70 µg/ml, the percentage of inhibition of MDE was 23.2% whereas that of curcumin was 43.9%.

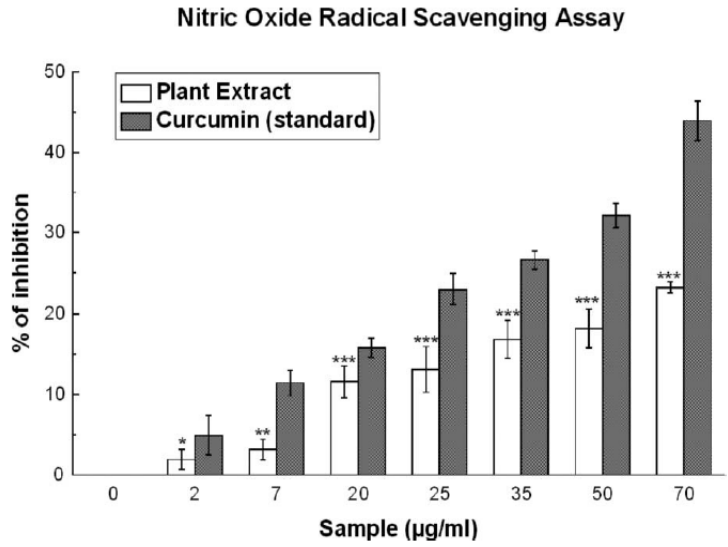


Figure 23: The data represent the percentage nitric oxide inhibition. Each value represents mean ± S.D. (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 204.28 ± 18.31 µg/ml and 90.82 ± 4.75 µg/ml, respectively.

5.1.4.7. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed by the FOX reagent method. Figure 24 shows that MDE has good H_2O_2 scavenging activity (IC_{50} = 4.17 ± 0.86 mg/ml) when compared with the standard sodium pyruvate (IC_{50} = 3.24 ± 0.30 mg/ml) (Table 12). At 2 mg/ml, the percentage of scavenging was 38.4% and 57.5% for *D. esculentum* and sodium pyruvate, respectively. At lower doses viz. 0.5 mg/ml, 0.8 mg/ml, and 1 mg/ml, the percentage of scavenging of the plant extract were 10.5%, 15.2% and 17.0%, respectively which were much higher than that of the sodium pyruvate (1.5%, 5.2% and 11.7% for 0.5 mg/ml, 0.8 mg/ml and 1 mg/ml, respectively).

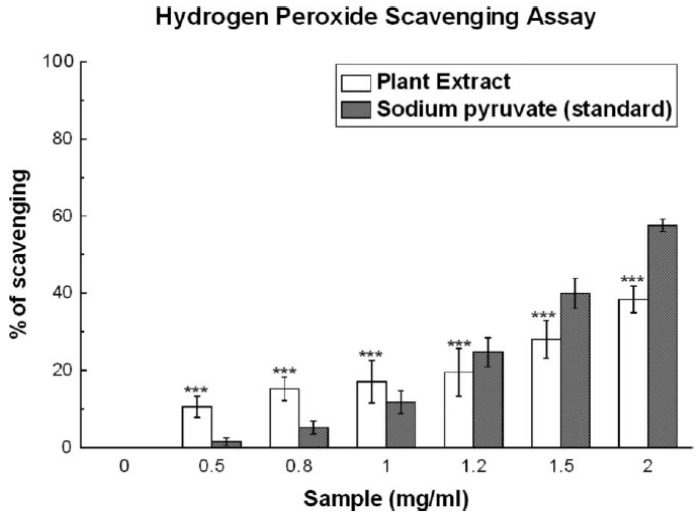


Figure 24: Effect of MDE and sodium pyruvate on the scavenging of H_2O_2. The data represent the percentage H_2O_2 scavenging. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 mg/ml. IC_{50} values of the plant extract and standard are 4.17 ± 0.86 mg/ml and 3.24 ± 0.30 mg/ml, respectively.

5.1.4.8. Peroxynitrite scavenging activity

Figure 25 shows that the peroxynitrite scavenging activity of MDE is concentration dependent. The calculated IC_{50} value was 3.35 ± 0.33 mg/ml which was much greater than that of the reference compound gallic acid (IC_{50} = 0.87 ± 0.05 mg/ml) (Table 12), indicating that MDE is not a good peroxynitrite scavenger when compared to gallic acid. At 200 µg/ml, the scavenging percentages were 7.5% and 15.4% for *D. esculentum* and gallic acid, respectively.

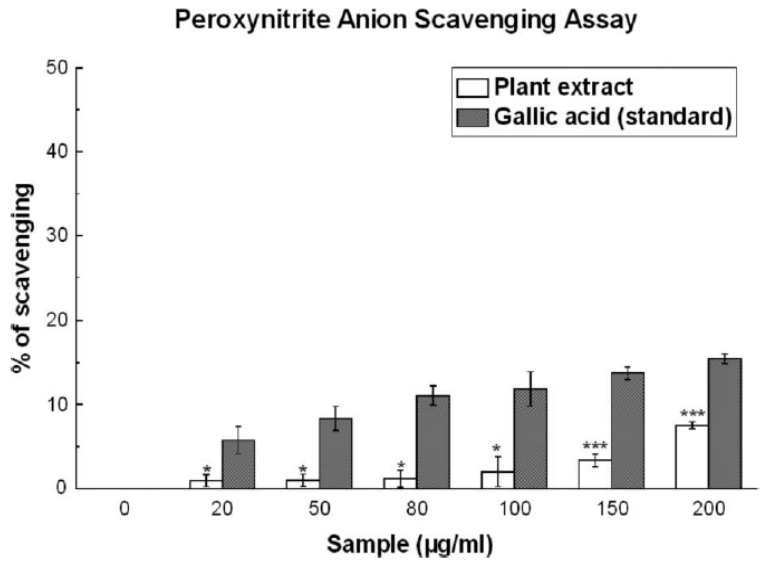


Figure 25: The peroxynitrite anion scavenging activity of MDE and the standard gallic acid. Each value represents mean ± S.D. (n = 6). *$p < 0.05$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 3.35 ± 3.33 mg/ml and 0.87 ± 0.05 mg/ml, respectively.

5.1.4.9. Singlet oxygen scavenging activity

MDE has moderate singlet oxygen scavenging activity when compared to that of lipoic acid (Figure 26). The IC_{50} value (Table 12) of the test sample was 278.88 ± 6.02 µg/ml whereas that of lipoic acid was 46.15 ± 1.16 µg/ml. At 200 µg/ml, the percentage scavenging of MDE was 42.2% whereas that of lipoic acid was 75.3%.

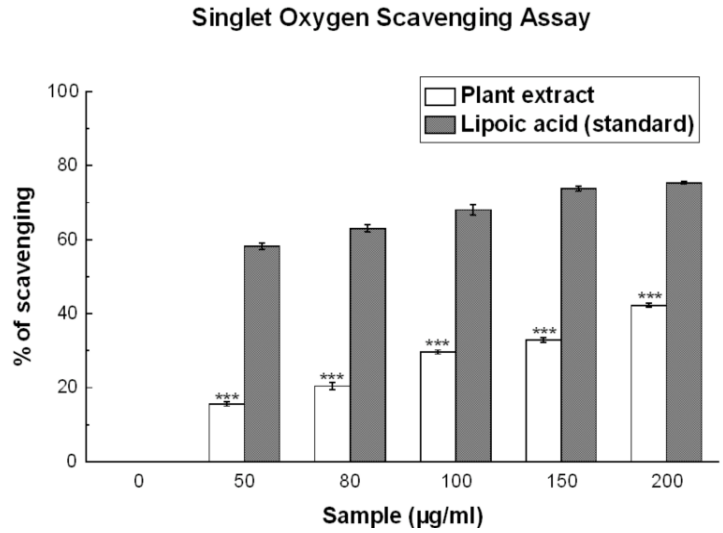


Figure 26: Effects of MDE and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs µg/ml. IC_{50} values of MDE and standard are 278.88 ± 6.02 µg/ml and 46.15 ± 1.16 µg/ml, respectively.

5.1.4.10. Hypochlorous acid scavenging activity

Figure 27 shows that *D. esculentum* possesses an efficient hypochlorous acid scavenging activity (IC_{50} = 338.96 ± 11.60 µg/ml) when compared to that of ascorbic acid (IC_{50} = 235.95 ± 5.75 µg/ml) (Table 12). At 100 µg/ml, the percentage scavenging of MDE was 22.9% whereas that of ascorbic acid was 35.4%. At lower doses viz. 10 µg/ml and 20 µg/ml, the percentage scavenging of MDE were 3.9% and 5.6%, respectively which were higher than that of ascorbic acid (3.4% and 5.0% for 10 µg/ml and 20 µg/ml, respectively).

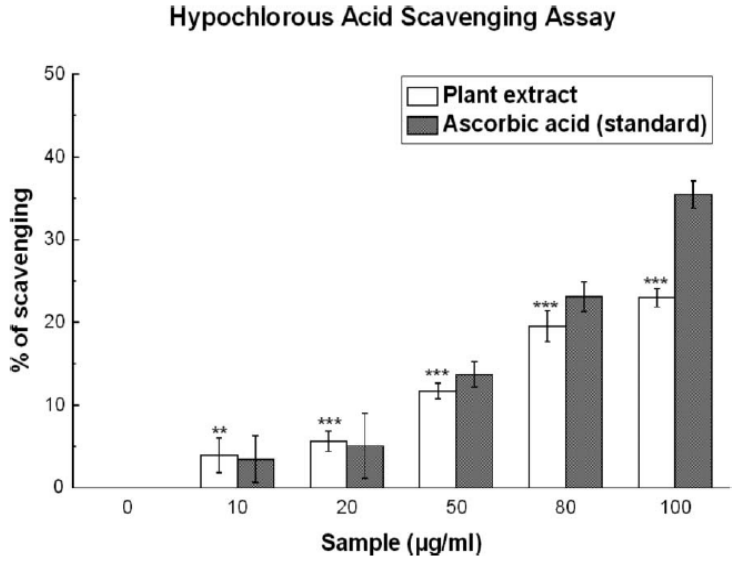


Figure 27: Hypochlorous acid scavenging activities of MDE and the standard ascorbic acid. All data are expressed as mean ± S.D. (n = 6). **$p < 0.01$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 338.96 ± 11.60 µg/ml and 235.95 ± 5.75 µg/ml, respectively.

5.1.4.11. Fe^{2+} chelation

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet colour of the complex is decreased. The results [Figure 28 (a) and Figure 28 (b)] demonstrated that the formation of the ferrozine-Fe^{2+} complex was inhibited in the presence of the test and reference compounds. The IC_{50} values (Table 12) of MDE and EDTA were 1.33 ± 1.13 mg/ml and 0.001 ± 0.000 mg/ml, respectively. At 120 µg/ml, the percentage inhibition of MDE was 14.72% whereas at 20 µg/ml that of EDTA was 99.34%.

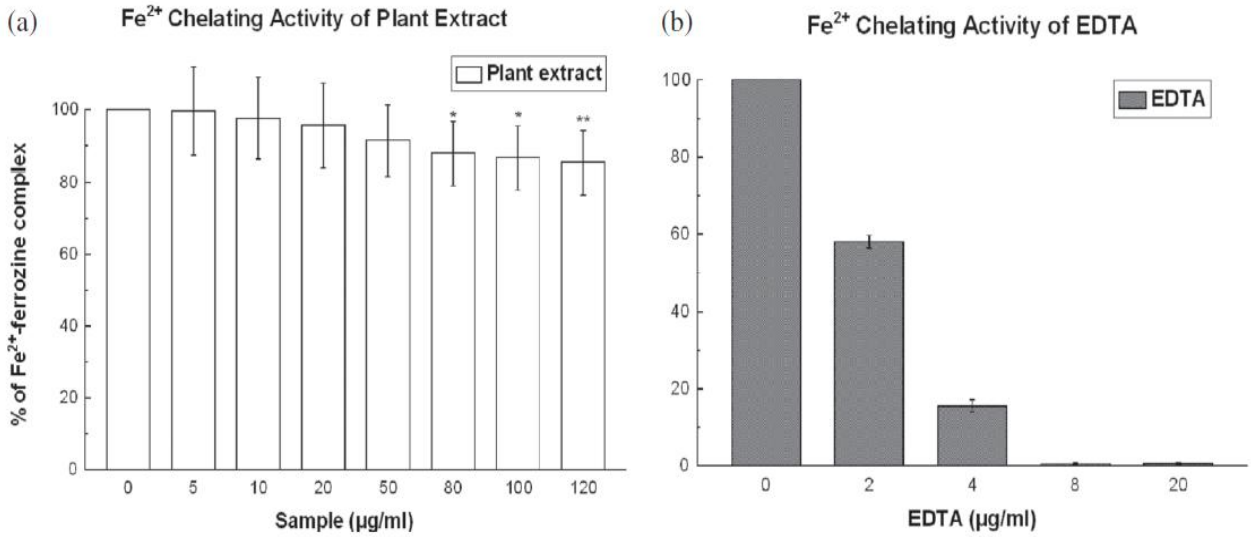


Figure 28: Effects of *D. esculentum* plant extract and EDTA on Fe^{2+}-ferrozine complex formation is shown. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. *$p < 0.05$ and **$p < 0.01$ vs 0 µg/ml. IC_{50} value of the plant extract and the standard were 1.33 ± 1.13 mg/ml and 0.001±0.00005 mg/ml, respectively.

5.1.4.12. Reducing power

As illustrated in Figure 29, Fe^{3+} was transformed to Fe^{2+} in the presence of MDE and the reference compound ascorbic acid to measure the reductive capability. Although the activity of ascorbic acid was better than MDE with absorbance values of 0.05 and 0.47 at 1 mg/ml for MDE and reference compound, respectively, still MDE showed moderate reducing capability.

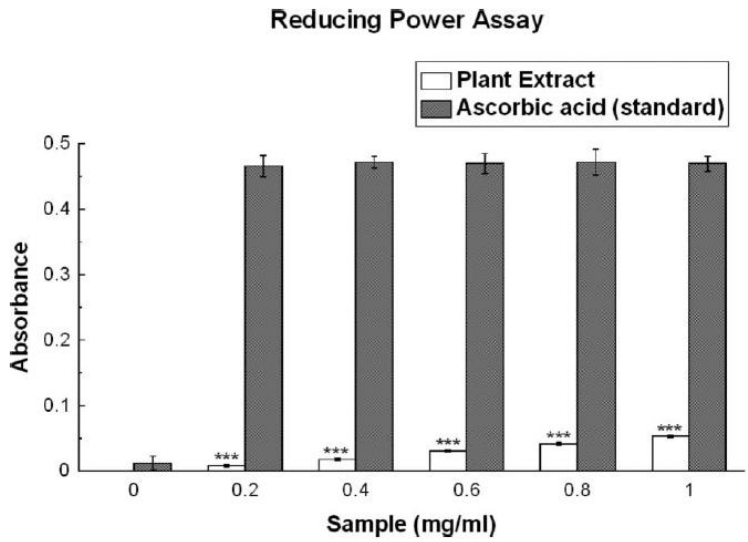


Figure 29: Reducing Power Assay. The reductive abilities of MDE and the standard ascorbic acid. The absorbance (A_{700}) was plotted against concentration of sample. Each value represents mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 mg/ml.

5.1.4.13. Lipid peroxidation inhibition assay

The IC_{50} values (Table 12) of MDE (141.67 ± 4.19 µg/ml) and the standard (6.76 ± 0.17 µg/ml) supported the fact that the inhibitory efficiency of MDE was poor compared to standard trolox. As shown in Figure 30, the increase in lipid peroxidation inhibition with increasing concentration of MDE reflects its antioxidant property.

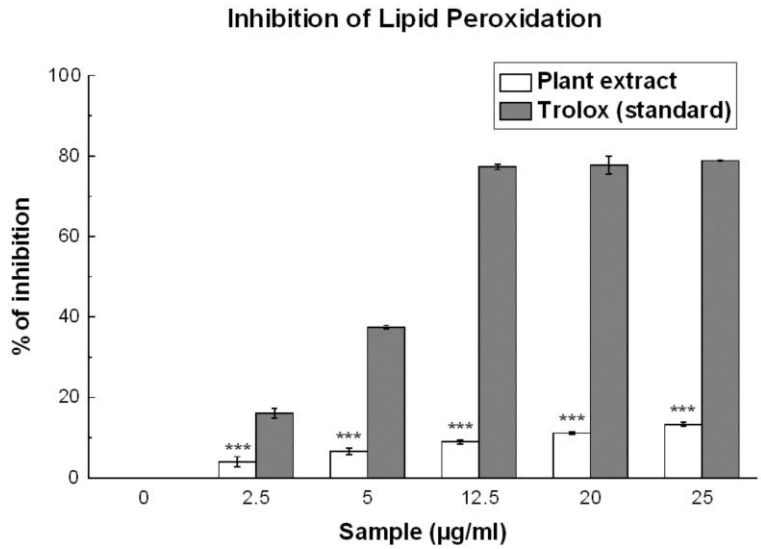


Figure 30: The data is expressed as the percentage of lipid peroxidation inhibition of brain homogenate, induced by Fe^{2+}/ascorbic acid. Each value represents mean ± S.D. (n=6). ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 141.67 µg/ml and 6.76 µg/ml, respectively.

5.1.4.14. Determination of total phenolic content

MDE showed 126.67 ± 8.16 mg gallic acid equivalent phenolic content in 1 g dried plant extract.

5.1.4.15. Determination of total flavonoid content

MDE showed 94.33 ± 6.12 mg quercetin equivalent flavonoid content in 1 g dried plant extract.

Table 12. Scavenging of reactive oxygen species, iron chelating and lipid peroxidation inhibition activity (IC_{50} values) of *Diplazium esculentum* (MDE) and reference compounds.

Activity	Extract/Reference	IC_{50}[#]
Hydroxyl radical (OH•) scavenging	*Diplazium esculentum*	811.00 ± 23.73
	Mannitol	571.45 ± 20.12 (6)***
Superoxide anion ($O_2^{\bullet -}$) scavenging	*Diplazium esculentum*	90.39 ± 2.22
	Quercetin	42.06 ± 1.35 (6)***
Nitric oxide radical (NO) scavenging	*Diplazium esculentum*	204.28 ± 18.31
	Curcumin	90.82 ± 4.75 (6)***
Hydrogen peroxide (H_2O_2) scavenging	*Diplazium esculentum*	4.17 ± 0.86
	Sodium pyruvate	3.24 ± 0.30 (6)[N.S.]
Peroxynitrite ($ONOO^-$) scavenging	*Diplazium esculentum*	3.35 ± 0.33
	Gallic acid	0.87 ± 0.05 (6)***
Singlet oxygen ($^1O_2$) scavenging	*Diplazium esculentum*	278.88 ± 6.02
	Lipoic acid	46.15 ± 1.16 (6)***
Hypochlorous acid (HOCl) scavenging	*Diplazium esculentum*	338.96 ± 11.60
	Ascorbic acid	235.95 ± 5.75 (6)***
Iron chelating	*Diplazium esculentum*	1.33 ± 1.13
	EDTA	0.001 ± 0.000 (6)*
Lipid peroxidation inhibition	*Diplazium esculentum*	141.67 ± 4.19
	Trolox	6.76 ± 0.17 (6)***

[#]Units of IC_{50} for all activities are μg/ml, except H_2O_2 scavenging, peroxynitrite scavenging, and iron chelating where the units are mg/ml. Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.

EDTA: ethylenediamine tetraacetic acid; N.S.: not significant.

*$p < 0.05$; ***$p < 0.001$ vs. *Diplazium esculentum*.

5.1.5. Qualitative analysis of phytochemicals

Qualitative phytochemical analysis revealed the presence of secondary metabolites like terpenoids, flavonoids, cardiac glycosides, tannins, etc. (Table 13).

Table 13. Qualitative analysis of the phytochemicals of *D. esculentum*

Phytochemicals analysed	Terpenoid	Gylcoside	Alkaloids	Steroid	Tannin	Phlobatannins	Saponin	Flavonoid	Phenols
Present in *D. esculentum*	+	+	+	+	+	–	+	+	+

5.1.6. Effect of *D. esculentum* on the some major organs of mouse (*viz.* liver and kidney)

5.1.6.1. General condition, symptoms and mortality

All animals appeared to tolerate well the acute BDE dose and no mortality occurred in any of the treatment groups. No abnormal behavior or cases of diarrhea and soft feces were observed except for the mice treated with 320 mg/kg bw BDE in the initial days of chronic toxicity study. In general, dosing of BDE in mice did not induce any clinical signs of toxicity in acute regimen.

5.1.6.2. Acute study

The single-dose study in mice revealed no untoward physiological events that may arise out of an acute exposure of a test material in target species. Both feed intake and body weight gain (data not shown) of treated groups were not significantly different from that of untreated control mice. Feed conversion efficiency of untreated control and BDE administered groups were 15.2%, 14.9%, 14.2% and 13.9%, respectively. Relative weight of organs (Table 14) in mice of control and treatment groups did not show significant difference. No mortality, no alteration in growth and normal state of vital organs of adult mice in this study showed that they can tolerate maximum recommended dose (Schilter et al., 2003) of BDE. Based on this data, we conducted

sub-acute (45 days) and subchronic (90 days) and chronic (135 and 180 days) dietary study in adult mouse.

5.1.6.3. Subacute, subchronic and chronic toxicity studies

The data showed significant loss of feed intake and body weight of treated group mice from that of untreated control mice in subacute and subchronic and chronic toxicity studies (Table 15). The feed conversion efficiency were 14.8%, 13.2% and 12.4% in case of mice that were treated daily for 180 days at 80 mg/kg bw, 160 mg/kg bw and 320 mg/kg bw, respectively, when compared to the control (15.5%), indicating significant ($p < 0.05$) dose-dependent decrease in feed conversion efficiency. The growth of mice was also significantly reduced in treatment groups when compared to that of controls. Gross examination of vital organs such as liver, kidney and testis of mice from treated and control groups, and microscopic examination of tissue sections prepared from these organs reveal alterations in their histological architecture that could be attributed to BDE intake at different doses.

Table 14: Acute toxicity study of BDE on body weight and relative organ weight of Swiss albino mouse

Dosage group (g BDE/kg bw)	Final body weight (g)	Relative organ weight (g/100 g bw)							
		Brain	Heart	Spleen	Liver	Kidney	Adrenal	Testis	Ovary
Group A: untreated control	22.13 ± 0.62	1.83 ± 0.04	0.71 ± 0.05	0.33 ± 0.05	5.31 ± 0.50	1.99 ± 0.07	0.024 ± 0.005	0.98 ± 0.05	0.18 ± 0.02
Group B: 1 g/kg bw	25.03 ± 0.91	1.85 ± 0.05	0.70 ± 0.06	0.31 ± 0.03	5.27 ± 0.43	2.02 ± 0.05	0.025 ± 0.006	0.98 ± 0.07	0.18 ± 0.02
Group C: 2 g/kg bw	25.05 ± 0.82	1.83 ± 0.06	0.69 ± 0.05	0.32 ± 0.04	5.37 ± 0.57	1.98 ± 0.08	0.020 ± 0.004	0.98 ± 0.07	0.17 ± 0.03
Group D: 4 g/kg bw	25.27 ± 1.03	1.81 ± 0.04	0.69 ± 0.03	0.31 ± 0.04	5.42 ± 0.40	1.99 ± 0.10	0.023 ± 0.005	0.97 ± 0.06	0.17 ± 0.03

Table 15: Chronic toxicity study (180 days) of BDE on body weight and relative organ weight of Swiss albino mouse

Dosage group (g BDE/kg bw)	Final body weight (g)	Relative organ weight (g/100 g bw)							
		Brain	Heart	Spleen	Liver	Kidney	Adrenal	Testis	Ovary
Group A: untreated control	22.13 ± 0.62	1.83 ± 0.04	0.71 ± 0.05	0.33 ± 0.05	5.31 ± 0.50	1.99 ± 0.07	0.024 ± 0.005	0.98 ± 0.05	0.18 ± 0.02
Group B: 80 mg/kg bw	22.03 ± 0.91	1.82 ± 0.05*	0.70 ± 0.06	0.31 ± 0.03	5.27 ± 0.43*	1.98 ± 0.05	0.022 ± 0.006	0.96 ± 0.07	0.17 ± 0.02
Group C: 160 mg/kg bw	21.05 ± 0.82**	1.81 ± 0.06**	0.69 ± 0.05	0.30 ± 0.04	5.22 ± 0.57*	1.95 ± 0.08*	0.020 ± 0.004	0.93 ± 0.07*	0.16 ± 0.03
Group D: 320 mg/kg bw	20.27 ± 1.03***	1.80 ± 0.04**	0.68± 0.03**	0.28 ± 0.04**	5.15 ± 0.40**	1.90 ± 0.10**	0.015 ± 0.005*	0.88 ± 0.06**	0.15 ± 0.03*

*p < 0.05 when compared with control (Significantly different).

**p < 0.01 when compared with control (Significantly different).

***p < 0.001 when compared with control (Significantly different).

Table 16 and Table 17 represent serum biochemistry of mice fed with BDE at different doses in the subacute, subchronic and chronic toxicity study. Activities of serum enzymes such as AST, ALT, LDH, ALP, ACP and GGT (which indicate liver function) were significantly increased after repeated oral dosing of BDE. Total bilirubin, which is involved in lipid metabolism, in mice across the treated groups was increased significantly when compared to the control. In untreated control and treated mice, clinical chemical endpoints of kidney function such as creatinine and urea levels were also compared. The concentrations of urea and creatinine have been found to increase significantly in subchronic and chronic doses when compared to the respective control groups.

Table 16: Biochemical measurements in serum of mice fed with BDE for 15, 45 and 90 days

Parameters	Sub-acute dose I (15 days)				Sub-acute dose II (45 days)				Sub-chronic dose (90 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.68 ± 0.05	20.75 ± 0.08	20.73 ± 0.11	20.76 ± 0.09	20.47 ± 0.03	20.47 ± 0.06	20.60 ± 0.05***	20.63 ± 0.04***	20.50 ± 0.08	20.63 ± 0.04**	20.80 ± 0.05***	20.94 ± 0.03***
ALT (Units/ml)	19.73 ± 0.12	19.75 ± 0.10	19.80 ± 0.10	18.79 ± 0.08	19.65 ± 0.10	19.85 ± 0.12**	20.11 ± 0.05***	20.60 ± 0.08***	20.60 ± 0.08	20.89 ± 0.10***	21.98 ± 0.09***	22.77 ± 0.09***
LDH (Units/L)	126.36 ± 0.49	126.42 ± 0.68	126.58 ± 0.54	127.21 ± 0.68	123.90 ± 0.62	124.78 ± 0.47*	126.26 ± 0.52***	127.87 ± 0.46***	124.63 ± 0.87	126.97 ± 0.09***	128.76 ± 0.80***	131.66 ± 0.75***
ALP (K.A. Units)	10.24 ± 0.16	10.23 ± 0.19	10.31 ± 0.14	10.28 ± 0.09	10.24 ± 0.10	10.26 ± 0.13	10.64 ± 0.11***	10.83 ± 0.13***	10.19 ± 0.11	10.38 ± 0.15*	10.78 ± 0.09***	11.29 ± 0.09***
Total ACP (K.A. Units)	1.29 ± 0.06	1.28 ± 0.09	1.32 ± 0.12	1.25 ± 0.12	1.32 ± 0.11	1.31 ± 0.06	1.44 ± 0.10	1.70 ± 0.09***	1.31 ± 0.07	1.52 ± 0.08**	1.81 ± 0.10***	2.24 ± 0.11***
Prostatic ACP (K.A. Units)	0.24 ± 0.01	0.24 ± 0.02	0.23 ± 0.03	0.25 ± 0.05	0.23 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.23 ± 0.01	0.24 ± 0.02	0.24 ± 0.01	0.25 ± 0.01*
γ-glutamyl transferase (Units/L)	13.96 ± 0.58	13.99 ± 0.28	14.09 ± 1.02	14.25 ± 0.58	14.53 ± 1.28	15.65 ± 0.67	15.68 ± 0.86	16.27 ± 1.07*	13.42 ± 1.05	14.17 ± 1.28	16.25 ± 0.97***	17.10 ± 0.73***
Total bilirubin (mg/dl)	0.52 ± 0.03	0.53 ± 0.04	0.51 ± 0.06	0.52 ± 0.02	0.50 ± 0.02	0.52 ± 0.02	0.51 ± 0.02	0.54 ± 0.02**	0.51 ± 0.02	0.52 ± 0.01	0.52 ± 0.01	0.54 ± 0.02**
Urea (mg/dl)	17.62 ± 0.68	17.69 ± 0.64	17.85 ± 0.69	18.12 ± 0.37	17.56 ± 0.80	17.56 ± 0.48	18.45 ± 1.02	19.04 ± 0.81*	17.62 ± 0.65	17.92 ± 0.56	18.62 ± 0.91*	19.41 ± 0.57***
Creatinine (mg/dl)	1.22 ± 0.11	1.23 ± 0.09	1.24 ± 0.11	1.24 ± 0.13	1.20 ± 0.08	1.21 ± 0.12	1.23 ± 0.08	1.26 ± 0.14	1.23 ± 0.06	1.31 ± 0.08	1.38 ± 0.11*	1.41 ± 0.09**

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

Table 17: Biochemical measurements in serum of mice fed with BDE for 135 and 180 days

Parameters	Chronic dose – I (135 days)				Chronic dose – II (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.67 ± 0.04	20.80 ± 0.39	21.88 ± 0.06***	22.84 ± 0.09***	20.90 ± 0.04	21.92 ± 0.09***	23.49 ± 0.09***	25.70 ± 0.05***
ALT (Units/ml)	20.72 ± 0.10	21.87 ± 0.11***	22.93 ± 0.10***	23.62 ± 0.10***	20.71 ± 0.12	22.02 ± 0.09***	23.19 ± 0.08***	24.48 ± 0.09***
LDH (Units/L)	126.26 ± 0.59	128.60 ± 0.87***	132.73 ± 0.89***	137.81 ± 0.87***	124.87 ± 1.20	128.86 ± 1.30***	137.78 ± 1.04***	142.02 ± 1.06***
ALP (K.A. Units)	10.21 ± 0.11	11.18 ± 0.10***	11.31 ± 0.12***	11.84 ± 0.10***	10.21 ± 0.09	11.45 ± 0.14***	12.44 ± 0.09***	13.27 ± 0.05***
Total ACP (K.A. Units)	1.27 ± 0.10	1.99 ± 0.12***	2.68 ± 0.14***	3.49 ± 0.11***	1.27 ± 0.12	2.27 ± 0.12***	3.27 ± 0.10***	4.34 ± 0.14***
Prostatic ACP (K.A. Units)	0.22 ± 0.01	0.24 ± 0.01*	0.28 ± 0.01***	0.29 ± 0.01***	0.22 ± 0.01	0.26 ± 0.01***	0.30 ± 0.01***	0.33 ± 0.01***
γ-glutamyl transferase (Units/L)	13.33 ± 0.57	14.57 ± 1.20	15.68 ± 0.74***	16.87 ± 1.10***	13.80 ± 0.49	15.25 ± 1.09*	16.02 ± 0.69***	17.67 ± 0.85***
Total bilirubin (mg/dl)	0.51 ± 0.01	0.61 ± 0.02***	0.67 ± 0.02***	0.72 ± 0.02***	0.51 ± 0.01	0.81 ± 0.02***	0.92 ± 0.02***	1.62 ± 0.02***
Urea (mg/dl)	17.64 ± 0.51	21.16 ± 0.83***	21.38 ± 0.94***	23.41 ± 1.13***	18.38 ± 0.73	24.69 ± 1.58***	30.62 ± 1.46***	37.33 ± 1.64***
Creatinine (mg/dl)	1.24 ± 0.06	1.36 ± 0.07*	1.45 ± 0.06***	1.48 ± 0.08***	1.24 ± 0.04	1.57 ± 0.13***	1.77 ± 0.11***	2.07 ± 0.13***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

The histological study did not reveal any changes in the in liver and kidney tissue architecture except for the chronic dose, where mice were treated with BDE at 320 mg/kg bw for 180 days. Figure 31 (b) revealed disorganized portal area in the liver of BDE treated mice when compared to the controls [Figure 31 (a)]. Similarly, deformed distal tubules in the kidney have been observed in the BDE treated mice [Figure 32 (b)] when compared to the control group [Figure 32 (a)].

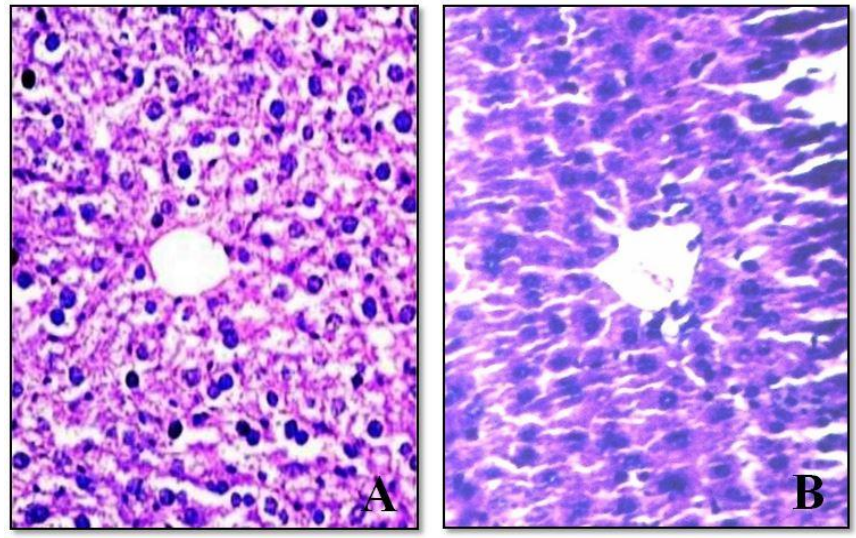


Figure 31: Histological architecture of liver in Control (A) and BDE treated mouse (B). Figure (B) shows liver of mice that received 320 mg/kg of bw of BDE (for 180 days). It displays liver with pleiomorphoinuclei, dilated blood vessels and lymphocytes surrounding ducts.

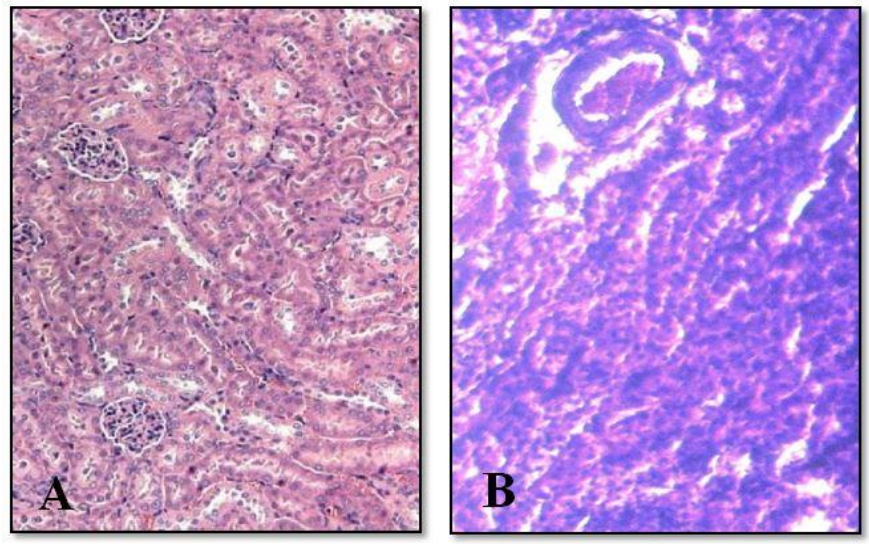


Figure 32: Histological architecture of kidney in Control (A) and BDE treated mouse (B). Figure (B) shows kidney of mice that received 320 mg/kg of bw of BDE (for 180 days). It displays kidney with tubular degeneration, dilation and inflammatory infiltrate.

5.2. COMPARATIVE ANALYSIS OF THE EFFECTS OF CDE AND BDE ON DIFFERENT *IN VIVO* AND *EX VIVO* PARAMETERS OF SWISS ALBINO MOUSE

Several parameters have been investigated to compare the effects of CDE and BDE *in vivo* and *in vitro*. The experimental procedures used to investigate these parameters have already been described in previous sections. The results observed are presented in this section.

5.2.1. Comparison of the effect of CDE and BDE on immunomodulation

5.2.1.1. Splenocyte count

Dose-dependent decreases in splenocyte number have been observed in both in CDE and BDE treated mice after 180 days of treatment, though significant differences ($p < 0.05$, $p < 0.01$, and $p < 0.001$) were there between the cell number of CDE and BDE treated mice at all the doses (Figure 33). At control group, the number of splenocytes for CDE and BDE treated mice were $(31.25 \pm 1.05) \times 10^6$/ml and $(31.84 \pm 0.87) \times 10^6$/ml, respectively, whereas, after 320 mg/kg bw of CDE and BDE, the cell numbers decreased to $(17.07 \pm 1.48) \times 10^6$/ml and $(23.73 \pm 0.74) \times 10^6$/ml, respectively.

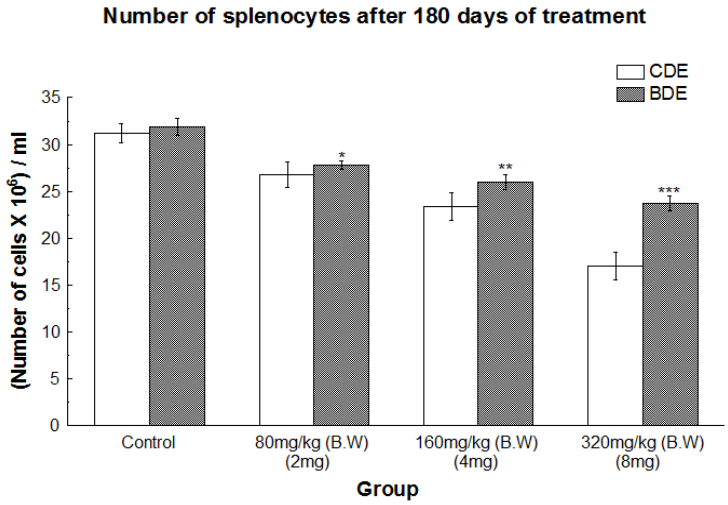


Figure 33: Comparison between the numbers of splenocytes of CDE vs BDE treated mice after 180 days of treatment. *p < 0.05 at 80 mg/kg bw, *p < 0.01 at 160 mg/kg bw, and *p < 0.001 at 320 mg/kg bw (CDE vs BDE).

5.2.1.2. PFC and HA titre assay

Figure 34 indicates the dose-dependent decrease in the number of plaque forming cells in spleen of mice treated with both CDE and BDE, though the decrease was 5% higher in case of CDE treated mice than that of BDE after 180 days of treatment. At 0 mg/kg bw, the numbers of PFC were 496.67 ± 8.16 and 495 ± 10.49, for CDE and BDE, respectively. At 320 mg/kg bw, the PFC numbers have decreased to 386.67 ± 8.16 and 406.67 ± 8.16, for CDE and BDE, respectively. Table 18 showed dose-dependent decrease in the HA titre value in both CDE and BDE treated mice when they were compared with that of their respective controls. After 180 days of the treatment with 360 mg/kg bw of CDE and BDE, the observed titre value of CDE and BDE treated mice were 1/10 and 1/20, respectively, 32- and 16 folds higher than that of their respective controls.

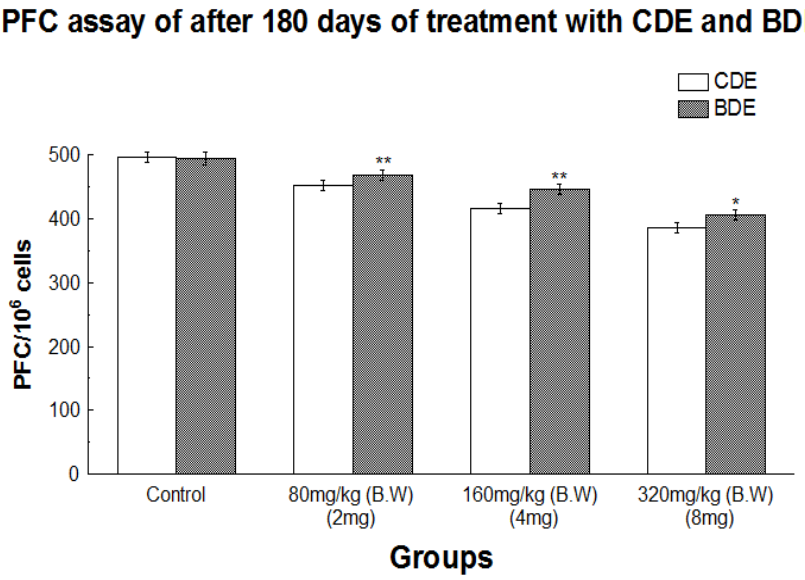


Figure 34: Comparison between the numbers of plaque forming cells in the spleen of CDE and BDE treated mice. **p < 0.01 at 80mg/kg bw and at 160mg/kg body weight (CDE vs BDE) and *p < 0.05 at 320mg/kg body weight (CDE vs BDE).

Table 18: Effect of CDE and BDE on the HA titre value in mice after 180 days of treatment

Group	Titre value	
	CDE	BDE
(I) Control	1 : 320	1 : 160
(II) 80mg/kg (B.W) (2mg)	1 : 160	1 : 10
(III) 160mg/kg (B.W) (4mg)	1 : 40	1 : 10
(IV) 320mg/kg (B.W) (8mg)	1 : 10	1 : 10

5.2.1.3. Comparison of the effect of CDE and BDE on the in vitro splenocyte viability and proliferation (MTT assay) as well as RBC hemolysis

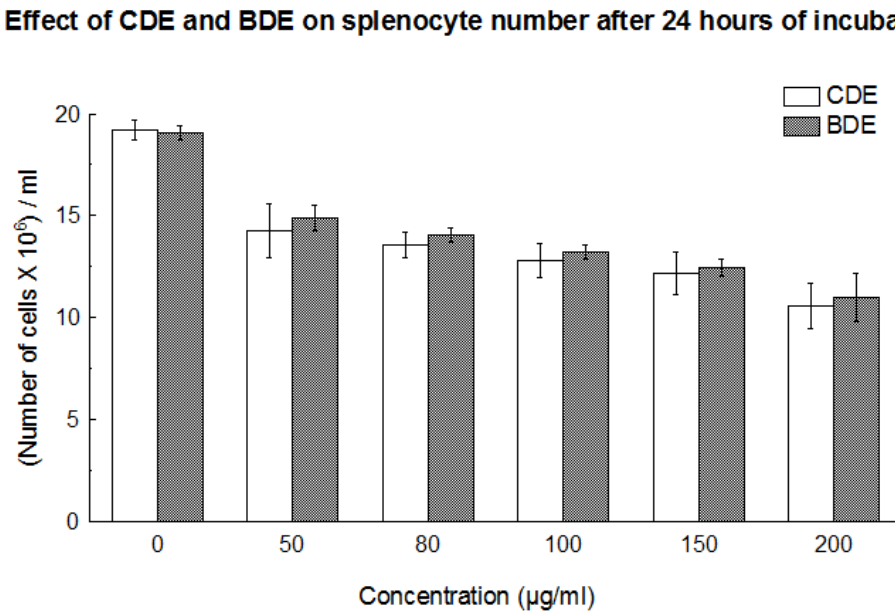


Figure 35: Result shows dose-dependent decrease in the number of splenocyte after 24 hours of incubation. There are no statistical differences between the CDE and BDE treated splenocyte at any of the dose (0-200 µg/ml). At 200 µg/ml of CDE and BDE, the no. of cell were $(10.56 \pm 1.09) \times 10^6$/ml and $(10.97 \pm 1.19) \times 10^6$/ml, respectively.

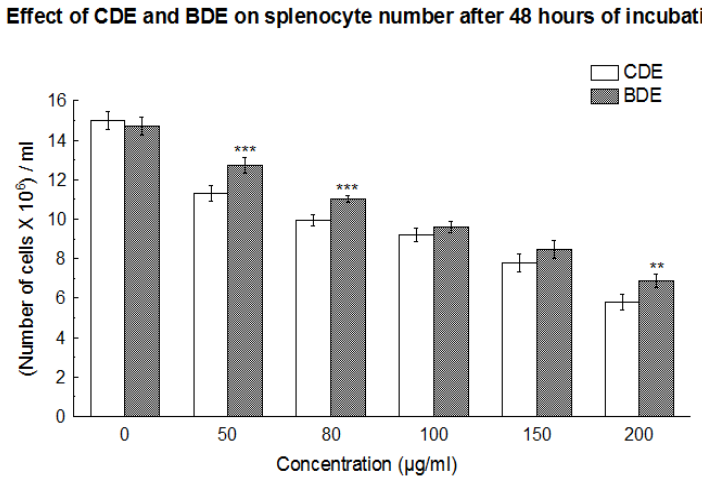


Figure 36: Result shows dose-dependent decrease in the number of splenocyte after 48 hours of incubation. ***$p < 0.001$ at 50µg/ml (CDE vs BDE) and at 80µg/ml (unboiled vs boiled), and **$p < 0.01$ at 200µg/ml (CDE vs BDE). At 200 µg/ml of CDE and BDE, the no. of cell were $(5.81 \pm 0.37) \times 10^6$/ml and $(6.88 \pm 0.34) \times 10^6$/ml, respectively.

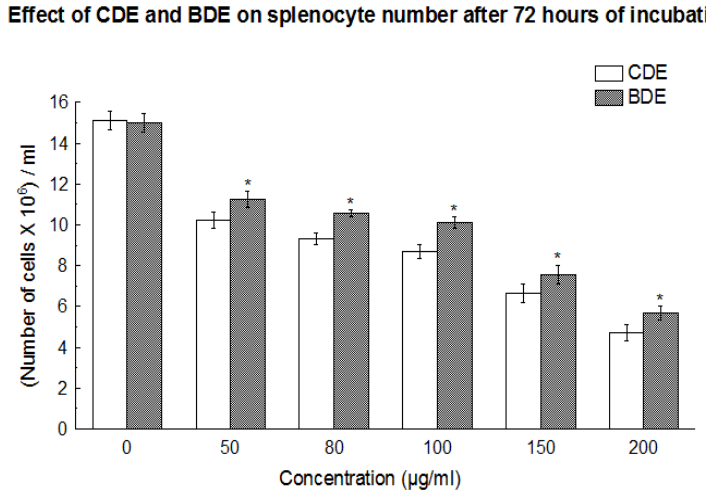


Figure 37: Result shows dose-dependent decrease in the number of splenocyte after 72 hours of incubation. *$p < 0.05$ at all the doses (CDE vs BDE). At 200 µg/ml of CDE and BDE, the no. of cell were $(4.69 \pm 0.37) \times 10^6$/ml and $(5.69 \pm 0.34) \times 10^6$/ml, respectively.

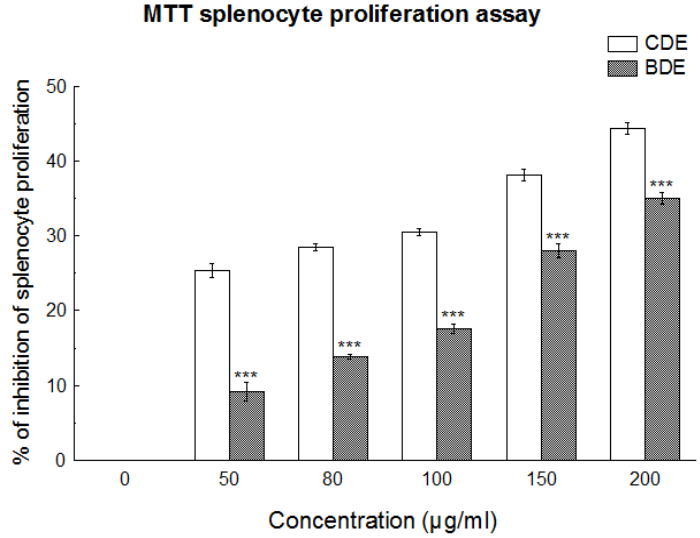


Figure 38: MTT assay showing the effect of CDE and BDE on splenocytes. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ for CDE vs BDE at each concentration (0-200 µg/ml). IC_{50} values of the CDE and BDE are 221.82 ± 3.97 µg/ml and 412.96 ± 12.14 µg/ml, respectively.

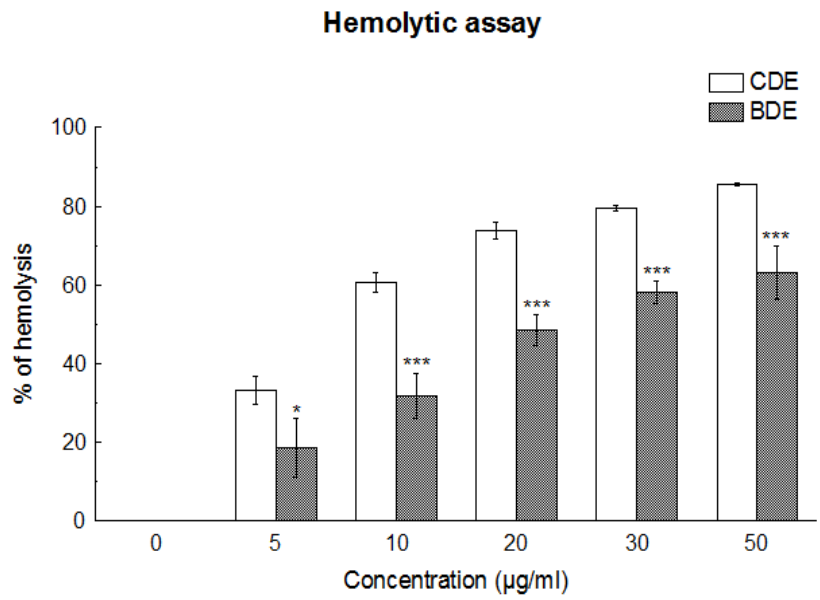


Figure 39: Comparison between the hemolytic activity of CDE and BDE on mouse erythrocytes. *p < 0.05 at 5µg/ml, ***p < 0.001 at 10, 20, 30 and 50 µg/ml. IC_{50} valus of CDE and BDE were 7.81 ± 0.36 µg/ml and 23.06 ± 3.14 µg/ml, respectively.

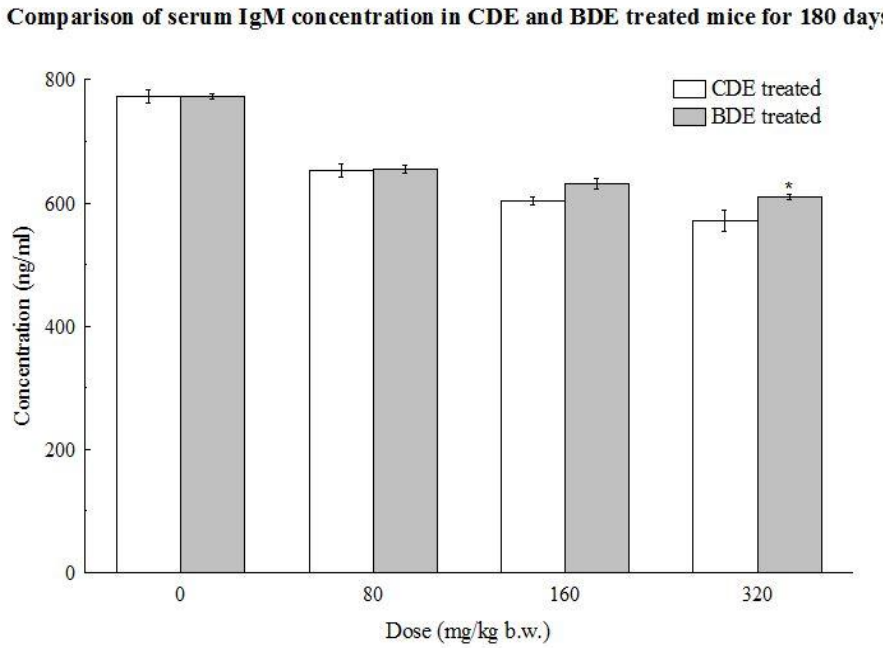


Figure 40: Comparison between serum IgM concentration of CDE and BDE. At 0 mg/kg bw of CDE and BDE treatment, the concentration of serum IgM were 772.66 ± 11.55 ng/ml and 772.74 ± 4.85 ng/ml, respectively, whereas, at 320 mg/kg bw of CDE and BDE treatment, the concentration of serum IgM decreased to 572.01 ± 16.66 ng/ml and 609.63 ± 4.99 ng/ml, respectively.

5.2.2. Comparative analysis of the effect of CDE and BDE on some reproductive functions

5.2.2.1. Hypo-osmotic swelling test of mouse sperm

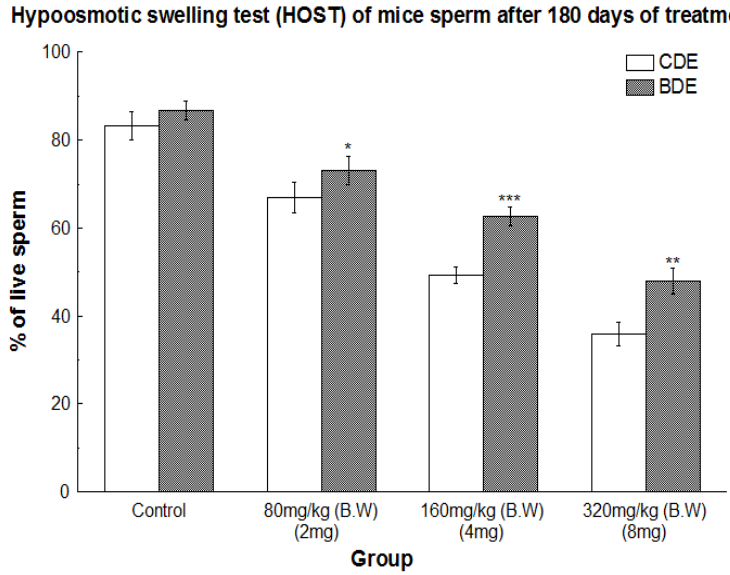


Figure 41: Comparison of the percentage of live sperm between CDE and BDE. Data represents dose-dependent decrease in both CDE and BDE treated mice. All data are expressed as mean ± S.D. (n = 6). *p < 0.05, ***p < 0.001 and **p < 0.01 vs 80mg/kg body weight, 160mg/kg body weight and 320mg/kg body weight, respectively. At 320 mg/kg bw of CDE and BDE, the percentages of live sperm were 35.83% and 48%, respectively.

5.2.2.2. Comparison of the sperm MTT assay between CDE and BDE treated mice

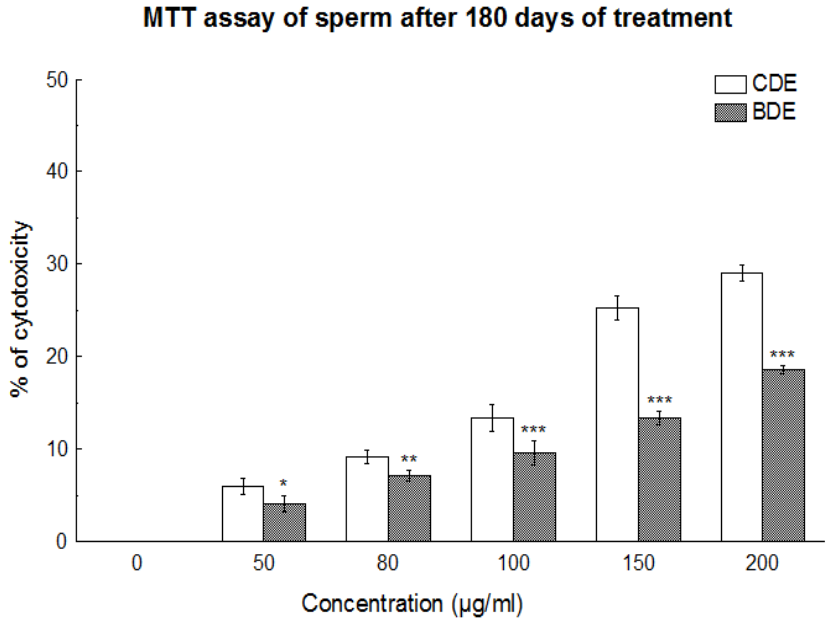


Figure 42: MTT assay showing the effect of CDE and BDE on sperm. All data are expressed as mean ± S.D. (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.001 for CDE vs BDE of each concentration (0-200 µg/ml). IC_{50} values of the CDE and BDE were 537.75 ± 19.13 µg/ml and 941.86 ± 37.63, respectively.

5.2.2.3. Comparative effect of CDE and BDE on body weight, relative organ weight and different biochemical parameters of male reproductive organs

Table 19: Effect of CDE and BDE on body weight and relative weight of male reproductive organs after chronic dose (180 days)

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
Body weight (g)	25.13 ± 0.18	24.86 ± 0.24	23.53 ± 0.15	21.12 ± 0.11*	24.83 ± 0.32	23.03 ± 0.93	22.85 ± 0.77	22.27 ± 1.13
Testis weight (g/100 g body weight)	0.95 ± 0.08	0.94 ± 0.07	0.91 ± 0.07*	0.86 ± 0.06***	0.95 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.91 ± 0.01**
Epididymis (g/100 g body weight)	0.33 ± 0.01	0.27 ± 0.02**	0.22 ± 0.01**	0.18 ± 0.01***	0.33 ± 0.02	0.33 ± 0.01	0.27 ± 0.01**	0.24 ± 0.01***
Seminal vesicles (g/100 g body weight)	0.48 ± 0.01	0.42 ± 0.01*	0.40 ± 0.01**	0.35 ± 0.01***	0.48 ± 0.01	0.43 ± 0.02*	0.42 ± 0.02**	0.37 ± 0.02***
Prostate gland (g/100 g body weight)	0.24 ± 0.01	0.23 ± 0.01	0.22 ± 0.01**	0.17 ± 0.01***	0.24 ± 0.01	0.22 ± 0.01*	0.21 ± 0.01**	0.19 ± 0.01***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

Table 20: Effect of CDE and BDE on different biochemical parameters of male reproductive organs after 180 days of treatment

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
Total Protein (serum) (mg/dl)	7.84 ± 0.49	6.12 ± 0.16***	5.68 ± 0.32***	5.35 ± 0.35***	7.85 ± 0.83	6.78 ± 0.41**	6.06 ± 0.30***	5.64 ± 0.55***
Total Protein (testis) (mg/g)	0.58 ± 0.04	0.50 ± 0.01***	0.48 ± 0.03***	0.39 ± 0.02***	0.56 ± 0.03	0.52 ± 0.03*	0.48 ± 0.02***	0.41 ± 0.01***
Total Protein (epididymis) (mg/g)	0.25 ± 0.04	0.18 ± 0.02***	0.12 ± 0.02***	0.06 ± 0.01***	0.28 ± 0.02	0.19 ± 0.01***	0.13 ± 0.01***	0.08 ± 0.01***
Chelesterol (testis) (mg/g)	3.85 ± 0.13	3.52 ± 0.08**	3.43 ± 0.03***	3.22 ± 0.02***	3.82 ± 0.08	3.53 ± 0.12**	3.43 ± 0.07**	3.27 ± 0.06***
α-glucosidase (Epididymis) (mU/g)	4.76 ± 0.06	4.53 ± 0.03**	4.41 ± 0.08***	4.32 ± 0.05***	4.77 ± 0.06	4.56 ± 0.06**	4.40 ± 0.05***	4.32 ± 0.06***
Fructose (µM/g) (Seminal vesicle)	4.56 ± 0.11	4.46 ± 0.05	4.31 ± 0.07**	4.26 ± 0.04***	4.52 ± 0.08	4.42 ± 0.04	4.35 ± 0.06*	4.30 ± 0.04**
Glycogen (mg/g) (testis)	39.58 ± 1.58	28.66 ± 2.25***	23.19 ± 1.20***	20.27 ± 0.53***	40.58 ± 1.38	31.46 ± 2.85***	27.19 ± 3.28***	20.27 ± 3.53***
Sialic acid (µM/100 g tissue) (epididymis)	62.12 ± 3.24	51.71 ± 0.93***	40.03 ± 1.14***	28.06 ± 0.81***	63.59 ± 2.24	54.36 ± 3.36***	40.25 ± 3.35***	31.36 ± 2.54***
Prostate citric acid (mg/g)	37.02 ± 1.52	21.75 ± 1.51***	17.37 ± 2.56***	11.33 ± 0.99***	36.58 ± 2.27	25.48 ± 3.35***	21.36 ± 3.69***	18.47 ± 1.36***
Acid phosphatase (µM/min/g of testicular tissue)	30.07 ± 5.12	17.94 ± 0.88***	15.34 ± 1.44***	14.29 ± 0.05***	33.72 ± 0.38	21.70 ± 0.60***	18.35 ± 1.02***	15.96 ± 1.57***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

5.2.3. Comparison of the effect of CDE and BDE on the cholinergic nervous system

5.2.3.1. Effect of CDE and BDE on acetylcholinesterase activity

In vivo acetylcholinesterase activity of CDE and BDE had been determined and the results were illustrated in Figure 43. The data represents that both CDE and BDE inhibit the acetylcholinesterase activity *in vivo* in a dose-dependent manner.

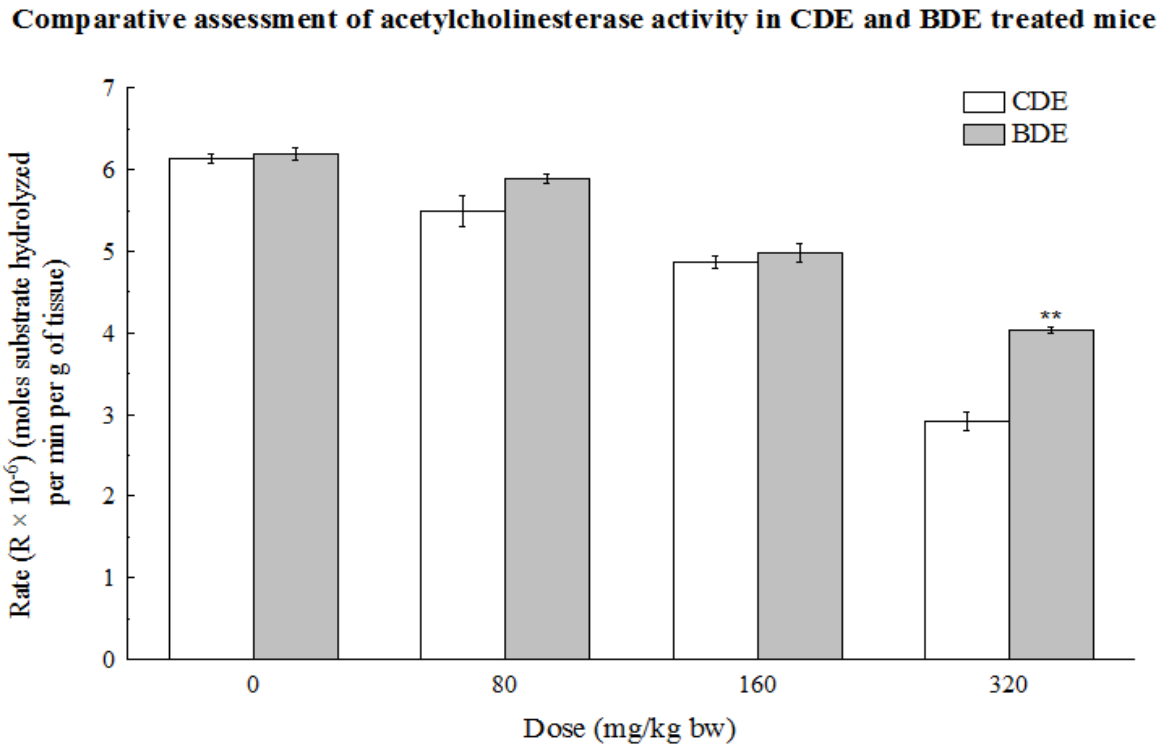


Figure 43: Results indicated dose-dependent decrease in the rate of hydrolysis of acetylthiocholine iodide substrate by acetylcholinesterase. All data are expressed as mean ± S.D. (n = 6). **p < 0.01 for CDE vs BDE at 320 mg/kg bw.

5.2.4. Comparative effect of CDE and BDE on different biochemical parameters of liver and kidney function

Table 21: Comparison of the biochemical parameters in serum of mice fed with CDE and BDE for 180 days

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.67 ± 0.04	22.80 ± 0.39***	23.88 ± 0.06***	25.84 ± 0.09***	20.90 ± 0.04	21.92 ± 0.09***	23.49 ± 0.09***	25.70 ± 0.05***
ALT (Units/ml)	20.72 ± 0.10	22.87 ± 0.11***	23.93 ± 0.10***	25.62 ± 0.10***	20.71 ± 0.12	22.02 ± 0.09***	23.19 ± 0.08***	24.48 ± 0.09***
LDH (Units/L)	126.26 ± 0.59	129.60 ± 0.87***	138.73 ± 0.89***	143.81 ± 0.87***	124.87 ± 1.20	128.86 ± 1.30***	137.78 ± 1.04***	142.02 ± 1.06***
ALP (K.A. Units)	10.21 ± 0.11	12.18 ± 0.10***	13.31 ± 0.12***	14.84 ± 0.10***	10.21 ± 0.09	11.45 ± 0.14***	12.44 ± 0.09***	13.27 ± 0.05***
Total ACP (K.A. Units)	1.27 ± 0.10	2.99 ± 0.12***	3.68 ± 0.14***	4.49 ± 0.11***	1.27 ± 0.12	2.27 ± 0.12***	3.27 ± 0.10***	4.34 ± 0.14***
Prostatic ACP (K.A. Units)	0.22 ± 0.01	0.27 ± 0.01***	0.32 ± 0.01***	0.39 ± 0.01***	0.22 ± 0.01	0.26 ± 0.01***	0.30 ± 0.01***	0.33 ± 0.01***
γ-glutamyl transferase (Units/L)	13.33 ± 0.57	15.57 ± 1.20**	16.68 ± 0.74***	18.87 ± 1.10***	13.80 ± 0.49	15.25 ± 1.09*	16.02 ± 0.69***	17.67 ± 0.85***
Total bilirubin (mg/dl)	0.51 ± 0.01	0.81 ± 0.02***	0.97 ± 0.02***	1.72 ± 0.02***	0.51 ± 0.01	0.81 ± 0.02***	0.92 ± 0.02***	1.62 ± 0.02***
Urea (mg/dl)	17.64 ± 0.51	25.16 ± 0.83***	31.38 ± 0.94***	39.41 ± 1.13***	18.38 ± 0.73	24.69 ± 1.58***	30.62 ± 1.46***	37.33 ± 1.64***
Creatinine (mg/dl)	1.24 ± 0.06	1.66 ± 0.07***	1.85 ± 0.06***	2.48 ± 0.08***	1.24 ± 0.04	1.57 ± 0.13***	1.77 ± 0.11***	2.07 ± 0.13***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

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.

CHAPTER – 7:

CONCLUSIONS

7. CONCLUSIONS

In the present study, an attempt was made to elucidate the immunopathological, haematological, biochemical, antifertility and neuromodulatory activities of crude (unboiled) and cooked (boiled) *Diplazium esculentum* (DE) by investigating several *in vivo* and *in vitro* parameters.

Both unboiled and boiled DE fed mice exhibit poor growth, decreased body weight and relative organ weight. Hematological as well as immunological studies showed significant alternations in blood parameters, such as total and differential leukocyte count, number of plaque forming cells, HA titre values, macrophage number, etc. in both unboiled and boiled DE fed mice. Decreased serum levels of Th1 and Th2 cytokines have been observed in both crude and boiled DE fed mice, whereas mouse lymphocytes incubated *in vitro* with methanolic extract of DE showed significant decrease in cell numbers as well as Th1 and Th2 cytokine production.

Subchronic and chronic doses of crude and boiled DE have been found to decrease the relative testis & other accessory organs weight, decrease the sperm count, sperm viability & motility, fertility index and fecundity, total protein in serum, testis & ovary, decrease the fructose and α-glucosidase level, decrease the cholesterol in testis, etc. Interestingly, the Th1/Th2 cytokine index has been increased significantly in some of the pregnant mice that were treated with both crude and boiled DE, i.e., Th1 cytokine expression is higher than Th2 cytokine in these mice that ultimately causes infertility and recurrent spontaneous abortion (RSA).

Methanolic extract of DE has been shown to possess acetylcholinesterase inhibitory activity. Acetylcholinesterase inhibitors on the other hand can cause a significant modulation of immunity as a side effect. Therefore, DE seems to modulate the cholinergic anti-inflammatory pathways via the protection of ACh from splitting by cholinesterases and thus enhancing the pathway and decline the production of different proinflammatory cytokines like IL-2 and IFN-γ. Methanolic extract of DE has been shown to possess a moderate quantity of flavonoids and phenolic compounds, which may confer the antioxidant and free radical scavenging activities in this plant. But boiled preparation of DE has been found to have trace amount of antioxidant and free radical scavenging activities.

D. esculentum has been found to possess several phytochemicals such as flavonoids, tannins, phenolic compounds, saponins, glycosides, terpenoids, etc. Some of these phytochemicals are beneficial, whereas, some are detrimental in nature. We have observed that both crude and boiled DE 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.

Serum biochemistry revealed increased level of several enzymes and metabolic products of liver and kidney in to the blood of both unboiled and boiled DE fed mice, indicating malfunctioning of these systems that may induces several metabolic diseases and age-related degenerative disorders which are closely associated with the oxidative processes in the body. Histopathologically, disorganized vasculature and other associated changes were seen in liver and kidney, in both crude and boiled DE fed mice. However, only crude DE fed mice showed progressive specific lesions, whereas, in case of boiled DE fed mice, distinct disorganization has been observed only in those mice that are treated with chronic doses (180 days) of boiled DE.

Therefore, it can be concluded that crude as well as boiled DE possess several phytochemicals, some of which is detrimental to health. The phytochemicals like steroids, tannins or saponins may interfere with the cell metabolism and therefore may be considered as toxic upon consumption. This may be a good reason of avoidance of this fern among insects and cattle. It is also important to note that, even after boiling, phytochemicals like tannins and saponins were present in DE, indicating its heat tolerance property, and therefore, people who consume it in a regular fashion, should be aware about the hazards of its consumption.

BIBLIOGRAPHY

Abbas AK, Murphy KM, Sher A. (1996). Functional diversity of helper T lymphocytes. Nature, 383, 787–93.

Ademosun AO, Oboh G. (2014). Anticholinesterase and antioxidative properties of water-extractable phytochemicals from some citrus peels. J Basic Clin Physiol Pharmacol, 25, 199–204.

Adler ID. (1993). Synopsis of the *in vivo* results obtained with the 10 known or suspected aneugens tested in the CEC collaborative study. Mutat Res, 287, 131–37.

Akingbemi, BT. (2005). Estrogen regulation of testicular function. Reprod Biol Endocrinol, 3, 51.

Akter S, Hossain MM, Ara I, Akhtar P. (2014). Investigation of in vitro antioxidant, antimicrobial and cytotoxic activity of Diplazium esculentum (Retz). Sw. Int J Adv Pharm, Biol Chem, 3, 723– 33.

Alfons B, Patrick M. (2001). Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. J Leukoc Biol, 70, 849–60.

Aminoff D. (1961). Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem J, 81, 384–92.

Anderson AR, Reddy JM, Oswald C, Zaneveld LJD. (1979). Enzymic determination of fructose in seminal plasma by initial rate analysis. Clin Chem, 25, 1780–82.

Anonymous. (1969). *Pteris* L. (Pteridiaceae) pp. 300, In The Wealth of India. eds. *Ambastha* sp Publication and Information Directorate, CSIR, New Delhi, India.

Anuja GI, Latha PG, Suja SR, Shyamal S, Shine VJ, Sini S, Pradeep S, Shikha P, Rajasekharan S. (2010). Anti-inflammatory and analgesic properties of *Drynaria quercifolia* (L.) J. Smith. J Ethnopharmacol,132, 456–60.

Aruoma OI, Halliwell B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. Biochem J, 248, 973–76.

Ashraf M, Ahmad K, Ahmad I, Ahmad S, Arshad S, Shah SMA, Nazim FH. (2011). Acetylcholinesterase and NADH oxidase inhibitory activity of some medicinal plants. J Med Plant Res, 5, 2086–89.

Aziz DM, Ahlswede L, Enbergs E. (2005). Application of MTT reduction assay to evaluate equine sperm viability. Theriogenol, 64, 1350–56.

Aziz DM. (2006). Assessment of bovine sperm viability by MTT reduction assay. Anim Reprod Sci, 92, 1–8.

Bailly F, Zoete V, Vamecq J, Catteu JP, Bernier JL. (2000). Antioxidant actions of ovothiol-derived 4-mercaptoimidazoles: Glutathione peroxidase activity and protection against peroxynitrite- induced damage. FEBS Letters, 486, 19–22.

Bajpai M, Gupta G, Setty BS. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol, 138, 322–27.

Baltrushes M. (2005). Medical Ethnobotany, Phytochemistry, and Bioactivity of the Ferns of Moorea, French Polynesia. Ph.D. Thesis. University of California, Berkeley.

Banerjee RD, Sen SP. (1980). Antibiotic activity of pteridophytes. Econ Bot, 34, 284–98.

Barış D, Kızıl M, Aytekin C, Kızıl G, Yavuz M, Çeken B, Ertekin AS. (2011). In vitro antimicrobial and antioxidant activity of ethanol extract of three *Hypericum* and three *Achillea* species from Turkey. Int J Food Prop, 14, 339–55.

Bassey ME, Etuk EUI, Ibe MM, Ndon BA. (2001). *Diplazium sammatii*: athyraceae („Nyama Idim'): Age-related nutritional and antinutritional analysis. Plants Food Hum Nutr, 56, 7–12.

Beckman JS, Chen H, Ischiropulos H, Crow JP. (1994). Oxidative Chemistry of Peroxynitrite. Methods Enzymol, 233, 229–40.

Bellier JP, Kimura H. (2011). Peripheral type of choline acetyltransferase: Biological and evolutionary implications for novel mechanisms in cholinergic system. J Chem Neuroanat, 42, 225–35.

Brennan FM, Green P, Amjadi P, Robertshaw HJ, Alvarez-Iglesias M, Takata M. (2008). Interleukin-10 regulates TNF-alpha-converting enzyme (TACE/ADAM-17) involving a TIMP-3 dependent and independent mechanism. European J Immunol, 38, 1106–17.

Bresciania L, Priebea J, Yunesa R, Magrob J. et al. (2003). Pharmacological and phytochemical evaluation of *Adiantum cuneatum* growing in Brazil, Tubingen, 58, 191–94.

Buckett WM, Luckas MJM, Aird IA, Farquharson RG, Kingsland CR, Lewis-Jones DI. (1997). The hypo-osmotic swelling test in recurrent miscarriage. Fertil Steril, 68, 506–09.

Byun JW, Choo SH, Kim HH, Kim YJ, Hwang YJ, Kim DY. (2008). Evaluation of boar sperm viability by MTT reduction assay in Beltsville thawing solution extender. Asian-australas J Anim Sci, 2, 494–98.

Caius JF. (1935). Medicinal and Poisonus ferns of India. Bombay Nat Hist Soc, 83, 341–61.

Cai Y, Luo Q, Sun M, Corke H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci, 74, 2157–84.

Calder PC. (2006). Branched-chain amino acids and immunity. J Nutr, 136, 288S–293S.

Campling BG, Pym J, Galbraith PR, Cole SP. (1988). Use of the MTT assay for rapid determination of chemo sensitivity of human leukemic blast cells. Leuk Res, 12, 823–31.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. (1987). Evaluation of a tetrazolium-based semi automated colorimetric assay: Assessment of radio sensitivity. Cancer Res, 47, 943–46.

Carroll JA. (2008). Bidirectional communication: growth and immunity in domestic livestock. J Anim Sci, 86, E126–E137.

Cavaillon JM. (1994). Cytokines and macrophages. Biomed Pharmacother, 48, 445–53.

Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H, Yeh S. (2004). Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc Natl Acad Sci USA, 101, 6876–81.

Chaudhuri TK, Chakravarty AK. (1983). Goat serum as a substitute for fetal calf serum in *in vitro* culture of murine lymphocytes. Indian J Exp Biol, 21, 494–96.

Cheeke P, Shull LR. (1985). Natural toxicants in feeds and poisonous plants. Westport, USA: Avi Publishing.

Cheeke, PR. (1988). Natural toxicants in feeds, forages, and poisonous plants, 2nd ed., Danville, USA: Interstate Publ. Inc.

Chemes H. (1986). The phagocytic function of Sertoli cells: A morphological, biochemical, and endocrinological study of lysosomes and acid phosphatase localization in the rat testis. Endocrinol, 119, 1673–81.

Coin A, Perissinotto E, Catanzaro S, Mosele M, de Rui M, Girardi A, Inelmen EM, Toffanello ED, Manzato E, Sergi G. (2012). Effects of 21 months of cholinesterase inhibitors on cognitive and functional decline in demented patients. Aging Clin Exp Res, 24, 14–16.

Costello LC, Franklin RB. (2002). Testosterone and prolactin regulation of metabolic genes and citrate metabolism of prostate epithelial cells. Horm Metab Res, 34, 417–24.

Cozier A, Clifford MN, Ashihara H. (2006). Plant secondary metabolites: occurrence, structure and role in the human diet. Oxford, UK: Blackwell Publishing.

da Silva SL, Figueiredo PMS, Yano T. (2007). Chemotherapeutic potential of the volatile oils from *Zanthoxylum rhoifolium* Lam leaves. Eur J Pharmacol, 576, 180–88.

Dawe DL, Stuedemann JA, Hill NS, Thompson FN. (1997). Immunosuppression in cattle with fescue toxicosis. In: Bacon CW, Hill NS, ed. Neotyphodium/Grass Interactions. NewYork: Plenum Press, 411–12.

De Haan JJ, Hadfoune M, Lubbers T, Hodin C, Lenaerts K, Ito A, Verbaeys I, Skynner MJ, Cailotto C, van der Vliet J, de Jonge WJ, Greve JW, Buurman WA. (2013). Lipid-rich enteral nutrition regulates mucosal mast cell activation via the vagal anti-inflammatory reflex. Am J Physiol Gastrointest Liver Physiol, 305, G383–91.

Dettin L, Ravindranath N, Hofmann MC. (2003). Morphological characterization of the spermatogonial subtypes in the neonatal mouse testis. Biol Reprod, 69, 1565–71.

Dey P, Roy S, Chaudhuri TK. (2012). A quantitative assessment of bioactive phytochemicals of *Nerium indicum*: An ethnopharmacological herb. Int J Res Pharm Sci, 3, 579–87.

Do Monte FH, dos Santos JG Jr, Russi M, Lanziotti VM, Leal LK, Cunha GM. (2004). Antinociceptive and anti-inflammatory properties of the hydroalcoholic extract of stems from *Equisetum arvense* L. in mice. Pharmacol Res, 49, 239–43.

Edeoga HO, Okwu DE, Mbaebie BO. (2005). Phytochemical constituents of some Nigerian medicinal plants. African J Biotechnol, 4, 685–88.

Elizabeth K, Rao MNA. (1990). Oxygen radical scavenging activity of curcumin. Int J Pharmaceut, 58, 237–40.

Ellman GL, Courtney KD, Andres V, Featherstone RM. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, 7, 88-95.

El-Sheikh ALK. (2008). Renal transport and drug interactions of immunosuppressants [thesis]. Nijmegen, Netherlands: Radbound University, pp. 62.

Ferrer JC, Favre C, Gomis RR, Fernandez-Novell JM, GarciaRocha M, de la Iglesia N, Cid E, Guinovart JJ. (2003). Control of glycogen deposition. FEBS Lett, 546, 127–32.

Fisher JS. (2004). Environmental antiandrogens and male reproductive health: Focus on phthalates and testicular dysgenesis syndrome. Reproduction, 127, 305–15.

Floriano-Sánchez E, Villanueva C, Medina-Campos ON, Rocha D, Sánchez-González DJ, Cárdenas-Rodríguez N, Pedraza-Chaverr J. (2006). Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion, and hypochlorous acid and prevents *in vivo* tyrosine nitration in lung. Free Rad Res, 40, 523–33.

Fontana M, Mosca L, Rosei MA. (2001). Interaction of enkephalines with oxyradicals. Biochem Pharmacol, 61, 1253–57.

Food and Agriculture Organization. (2010). A vegetable fern, *Diplazium esculentum* – potential for food security and socio-economic development in the Himalayas. Non-Wood News, 20, 10.

Francisco MS, Cooper-Driver G. (1984). Anti-microbial activity of phenolic acids in *Pteridium aquilinum*. Am Fern J, 74, 87–96.

Freimoser FM, Jakob CA, Aebi M, Tuor U. (1999). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. Appl Environ Microbiol, 65, 3727–29.

Furusawa S, Wu J. (2007). The effects of biscoclaurine alkaloid cepharanthine on mammalian cells: Implications for cancer, shock, and inflammatory diseases. Life Sci, 80, 1073–79.

Gangwar NK. (2004). Studies on the pathological effects of linguda (*Diplazium esculentum*, Retz.) in laboratory rats and guinea pigs. Indian Journal of Veterinary Pathology, 28, 149–50.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. (1997). Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod, 57, 1401–06.

Garratt DC. (1964). The quantitative analysis of drugs, Volume 3. Japan: Chapman and Hall Ltd.

Giacobini E. (2002). Long term stabilizing effect of cholinesterase inhibitors in the therapy of Alzheimer's disease. J Neural Transm Suppl, 62, 181–87.

Gheri G, Vichi D, Thyrion GD, Bonaccini L, Vannelli GB, Marini M, Sgambati E. (2009). Sialic acid in human testis and changes with aging. Reprod Fertil Dev, 21, 25–33.

Goldsby RA, Kindt TK, Osborne BA, Kuby J. (2003). Immunology, 5th ed. New York, USA: W. H. Freeman and Company.

Gruetter R. (2003). Glycogen: The forgotten cerebral energy store. J Neurosci Res. 74, 179–83.

Guo H, Yun C, Hou G, Du J, Huang X, Lu Y, Keller ET, Zhang J, Deng JG. (2014). Mangiferin attenuates Th1/Th2 cytokine imbalance in an ovalbumin-induced asthmatic mouse model. PLoS One, 9, e100394.

Gupta RS, Sharma R, Sharma A, Bhatnager AK, Dobhal MP, Joshi YC, Sharma MC. (2002). Effect of *Alstonia scholaris* bark extract on testicular function of Wistar rats. Asian J Androl, 4, 175–78.

Ham SS. (2004). Wild vegetables: anticancer & healthy life. Seoul, Korea: Human & Books.

Hammami I, Nahdi A, Mauduit C, Benahmed M, Amri M, Amar AB, Zekri S, May AE, May MVE. (2008). The inhibitory effects on adult male reproductive functions of crude garlic (*Allium sativum*) feeding. Asian J Androl 2008, 10, 593–601.

Harborne JB (1973). Phytochemical methods. London, UK: Champman and Hall Ltd.

Harborne JB. (1998). Phytochemical methods: A Guide to Modern Techniques of Plant Analysis. 3rd ed. New York, USA: Chapman and Hall.

Haro-Vicente JF, Martinez-Gracia C, Ros G. (2006). Optimization of *in vitro* measurement of available iron from different fortificants in citric fruit juices. Food Chem, 98, 639–48.

Hazary RC, Chaudhuri DGJ, Wishart GJ. (2001). Application of an MTT reduction assay for assessing sperm quality and predicting fertilising ability of domestic fowl semen. Br Poult Sci, 42, 115–17.

Hazra B, Biswas S, Mandal N. (2008). Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Comp Alt Med, 8, 63.

Hedger MP. (2012). Immune privilege of the testis: meaning, mechanisms, and manifestations, In: Stein-Streilein J, ed. Infection, immune homeostasis and immune privilege. Basel: Springer Basel, 31–52.

Hirasawa Y, Morita H, Shiro M, Kobayashi J. (2003). Sieboldine A, a novel tetracyclic alkaloid from Lycopodium sieboldii, inhibiting acetylcholinesterase. Organic Lett, 5, 3991–93.

Hirono I, Aiso S, Yamaji T, Mori H, Yamada K, Niwa H, Ojika M, Wakamatsu K, Kigoshi H, Niiyama K, Uosaki Y. (1984). Carcinogenicity in rats of ptaquiloside isolated from bracken. Gann 75, 833–36.

Imperato F. (2005). A new flavonoid from the fern *Dryopteris villarii*. P. 12. In Integrative plant biochemistry as we approach 2010. PSNA Annual Meeting, La Jolla CA USA. [Abstract]

Imperato F. (2006). The new flavone ester Apigenin-7-O-oxy-p-hydroxyhenzoate and 3-Di-C-glycosyl flavones from *Pteris vittata*. Amer Fern J, 96, 62–65.

Iwasaki T, Yoneda M, Nakajima A, Terauchi Y. (2007). Serum butyrylcholinesterase is strongly associated with adiposity, the serum lipid profile and insulin resistance. Intern Med, 46, 1633–39.

Jagetia GC, Krishnamurthy H, Jyothi P. (1999). Evaluation of cytotoxic effects of different doses of vinblastine on mouse spermatogenesis by flow cytometry. Toxicology, 112, 227–36.

Jana K, Jana S, Samanta PK. (2006). Effects of chronic exposure to sodium arsenite on hypothalamo-pituitarytesticular activities in adult rats: Possible an estrogenic mode of action. Reprod Biol Endocrinol, 4, 9.

Jin HM, Kang SJ, Lee SH. (2005). Cosmetic composition for scrubbing. PCT Int Appl, WO/2005/082328, KO Appl. 1020050016923.

Kala CP. (2005). Ethnomedicinal botany of the Apatani in the Eastern Himalayan region of India. J Ethnobiol Ethnomed, 1, 11.

Kang H, Ahn K, Oh S, Kim JW. (2014). Genkwadaphnin induces IFN-γ via PKD1/NF-kB/STAT1 dependent pathway in NK-92 cells. PLoS One, 9, e115146.

Kang SS, Lee JY, Choi YK, Song SS, Kim JS, Jeon S, Han YN, Son KH, Han BH. (2005). Neuroprotective effects of naturally occurring biflavonoids. Bioorg Med Chem Lett, 15, 3588–91.

Kapoor LD, Singh A, Kapoor SL, Shrivastava SN (1969). Survey of Indian plants for saponins, alkaloids and flavonoids. Lloydia, 32, 297–304.

Karlsson D, Fallarero A, Brunhofer G, Mayer C, Prakash O, Mohan CG, Vuorela P, Erker T. (2012). The exploration of thienothiazines as selective butyrylcholinesterase inhibitors. Eur J Pharm Sci, 47, 190–205.

Katsube T, Imawaka N, Kawano Y, Yamazaki Y, Shiwaku K, Yamane Y. (2006). Antioxidant flavonol glycosides in mulberry (Morus alba L.) leaves isolated based on LDL antioxidant activity. Food Chem, 97, 25–31.

Kaushik A, Kaushik JJ, Das A, Gemal S, Gaim D. (2011). Preliminary studies on anti-inflammatory activities of *Diplazium esculentum* in experimental animal models. Int J Pharma Sci Res, 2, 1251–53.

Kaushik A, Jijta C, Kaushik JJ, Zeray R, Ambesajir A, Beyene L. (2012). FRAP (Ferric reducing ability of plasma) assay and effect of *Diplazium esculentum* (Retz) Sw. (a green vegetable of North India) on central nervous system. Indian J of Nat Prod Resources, 3, 228–31.

Khan MI, Denny-Joseph KM, Muralidhara M, Ramesh HP, Giridhar P. (2011). Acute, subacute and subchronic safety assessment of betalains rich *Rivina humilis* L. berry juice in rats. Food Chem Toxicol, 49, 3154–57.

Khan PK, Awasthy KS. (2003). Cytogenetic toxicity of neem. Food Chem Toxicol, 41, 1325–28.

Kikuzaki H, Nakatani N. (1993). Antioxidants effects of some ginger constituents. J Food Sci, 58, 1407–10.

Kim HJ, Lim HW, Choi SW, Yoon CS. (2006). Antimicrobial effect of ethanol extract of *Dryopteris crassirhizoma* Nakai on *Propionibacterium acnes*. J Soc Cosmet Sci Korea, 32, 201–08.

Kizil G, Kizil M, Yavuz M, Emen S, Hakimoglu F. (2008). Antioxidant activities of ethanol extracts of *Hypericum Triquetrifolium* and *Hypericum Scabroides*. Pharmaceut Biol, 46, 231–42.

Kleemann R, Zadelaar S, Kooistra T. (2008). Cytokines and atherosclerosis: A comprehensive review of studies in mice. Cardiovasc Res, 79, 360–76.

Kleszczynska H, Bonarska D, Luczynski J, Witek S, Sarapuk J. (2005). Hemolysis of erythrocytes and erythrocyte membrane fluidity change by new lysosmtropic compounds. J Fluoresc, 15, 137–41.

Klip A, Tsakiridis T, Marette A, Ortiz PA. (1994). Regulation of expression of glucose transporters by glucose: A review of studies *in vivo* and in cell cultures. FASEB J, 8, 43–53.

Kosinski RA, Zaremba M. (2007). Dynamics of the model of the *Caenorhabditis elegans* neural network. Acta Phys Pol B, 38, 2201–10.

Kumar D, Arya V, Kaur R, Bhat ZA, Gupta VK, Kumar V. (2012). A review of immunomodulators in the Indian traditional health care system. J Microbiol, Immunol Infec, 45, 165–84.

Kweon MR. (1986). Thermostable antithiamin factor of bracken fern. MS dissertation, Seoul National University, Seoul, Korea.

Kumari TK, Sakthidevi G, Muthukumaraswami S, Mohan VR. (2012). Antifertility activity of whole plant extract of *Sarcostemma secamone* (L) Bennet on male albino rats. Int Res J Pharm, 3, 139–44.

Lai HY, Lim YY, Kim KH. (2010). *Blechnum Orientale* Linn - a fern with potential as antioxidant, anticancer and antibacterial agent. BMC Complementary Alt Med, 10, 15.

Lee CH, Shin SL. (2011). Functional activities of ferns for human health. In: H. Fernández et al., ed. Working with Ferns: Issues and Applications, Berlin: Springer, 347–59.

Lee HB, Kim JC, Lee SM. (2009). Antibacterial activity of two phloroglucinols, flavaspidic acids AB and PB, from *Dryopteris crassirhizoma*. Arch Pharm Res, 32, 655–59.

Lee L, Kosuri P, Arancio O. (2014). Picomolar amyloid-beta peptides enhance spontaneous astrocyte calcium transients. J Alzheimers Dis, 38, 49–62.

Li J, Liang N, Mo L, Zhang X, He C. (1998). Comparison of the cytotoxicity of five constituents from *Pteris semipinnata* L. *in vitro* and the analysis of their structure-activity relationships. Yao Xue Xue Bao, 33, 641–44.

Li JH, He CW, Liang NC, Mo LE, Zhang X. (1999). Effects of antitumor compounds isolated from Pteris semipinnata L on DNA topoisomerases and cell cycle of HL-60 cells. Zhongguo Yao Li Xue Bao, 20, 541–45.

Li XY. (2000). Immunomodulating components from Chinese medicines. Pharmaceut Biol, 38, 33–40.

Li P, Yu-long Y, Li D, Kim SW, Wu G. (2007). Amino acids and immune function. Br J Nutr, 98, 237–52.

Licciardi PV, Underwood JR. (2011). Plant-derived medicines: A novel class of immunological adjuvants. Int Immunopharmacol 11, 390–98.

Lin N, Lin W. (1989). β-Ecdysone containing skin-protecting cosmetics. Faming Zhuanli Shenqing Gonkkai Shuomingshu. CN 86106791 (Cl. A61K7/48) (Chemical Abstracts 111: 239323e).

Lindeberg S, Cordain L, Eaton SB. (2003). Biological and clinical potential of a paleolitic diet. J Nutr Environ Med, 13, 149-60.

Liu Y, Wujisguleng W, Long C. (2012). Food uses of ferns in China: A review. Acta Soc Bot Pol 81, 263–70.

Long LH, Evans PJ, Halliwell B. (1999). Hydrogen peroxide in human urine: Implications for antioxidant defense and redox regulation. Biochem Biophys Res Comm, 262, 605–09.

Lozano GA. (1998). Parasitic stress and self-medication in wild animals. In: Moler AP, Milinski M, Slater PJB, ed. Advances in the Study of Behavior. London, UK: Elsevier Science, 291–317.

Ma LQ, Komar KM, Tu C, Zhang W, Kennelley ED. (2001). A fern that hyperaccumulates arsenic. Nature, 409, 579.

Ma X, Gang DR. (2004). The Lycopodium alkaloids. Nat Prod Reports, 21, 752–72.

Mahdi BM. (2011). Role of some cytokines on reproduction. Middle East Fert Soc J, 16, 220–23.

Malagoli D. (2007). A full-length protocol to test hemolytic activity of palytoxin on human erythrocytes. ISJ, 4, 92–94.

Manu KA, Kuttan G. (2007). Effect of punarnavine, an alkaloid from *Boerhaavia diffusa*, on cell-mediated immune responses and TIMP-1 in B16F-10 metastatic melanoma-bearing mice. Immunopharmacol Immunotoxicol, 29, 569–86.

Martín-Cordero C, Reyes M, Ayuso MJ, Toro MV. (2001). Cytotoxic Triterpenoids from *Erica andevalensis*. Z Naturforsch, 56c, 45–48.

Mathur PP, Chattopadhyay S. (1982). Involvement of lysosomal enzymes in flutamide-induced stimulation of rat testis. Andrologia, 14, 171–76.

Meistrich ML, Finch M, da Cunha MF, Hacker U, Au WW. (1982). Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer Res, 42, 122–31.

Meistrich ML. (1986). Relationship between spermatogonial stem cell survival and testis function after cytotoxic therapy. Br J Cancer Suppl, 7, 89–101.

Meroni SB, Riera MF, Pellizzari EH, Cigorraga SB. (2002). Regulation of rat Sertoli cell function by FSH: Possible role of phosphatidylinositol 3-kinase/protein kinase B pathway. J Endocrinol, 174, 195–204.

Meybeck A, Bonte F, Redziniak G. (1997). Use of an ecdysteroid for the preparation of cosmetic or dermatological compositions intended in particular, for strengthening the water barrier function of the skin or for the preparation of a skin culture medium, as well as to the compositions. US Patent 5, 609, 873.

Middleton Jr. E, Kandaswami C, Theoharides T. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol Rev, 52, 673–751.

Min BR, Hart SP. (2003). Tannins for suppression of internal parasites. J Anim Sci, 81, E102–09.

Min BR, Pomroy WE, Hart SP, Sahlu T. (2004). The effect of short-term consumption of a forage containing condensed tannins on gastrointestinal nematode parasite infections in grazing wether goats. Small Rumin Res, 51, 279–83.

Mishra RK, Singh SK. Effect of aqueous leaf extract of *Azadirachta indica* on the reproductive organs in male mice. (2005). Indian J Exp Biol, 43, 1093–1103.

Mishra SB, Mukerjee A, Vijayakumar M. (2010). Pharmacognostical and phytochemical evaluation of leaves extract of *Jatropha curcas* Linn. Pharmacog J, 2, 9–14.

Misra S, Maikhuri RK, Kala CP, Rao KS, Saxena KG. (2008). Wild leafy vegetables: A study of their subsistence dietetic support to the inhabitants of Nanda Devi Biosphere Reserve, India. J Ethnobiol Ethnomed, 4, 15.

Mitsuda H, Yasumoto, Iwani K. (1967). Antioxidant actions of indole compounds during autooxidation of linoleic acid. Eiyo to Shoduryou, 19, 210.

Montgomery R. (1957). Determination of glycogen. Arch Biochem Biophys, 67, 378-89.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 65, 55–63.

Mueller-Harvey I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. J Sci Food Agric, 86, 2010–37.

Nagy JG, Regelin WL. (1977). Influence of plant volatile oils on food selection by animals. Proc Congr Game Biol, 13, 225–30.

Nakamori M, Iwahashi M, Nakamura M, Ueda K, Zhang X, Yamaue H. (2003). Intensification of antitumor effect by T helper 1-dominant adoptive immunogene therapy for advanced orthotopic colon cancer. Clin Cancer Res, 9, 2357–65.

Nakane T, Maeda Y, Ebihara H, Arai Y, Masuda K, Takano A, Ageta H, Shiojima K, Cai S, Abdel-Halim OB. (2002). Fern constituents: triterpenoids from *Adiantum capillus-veneris*. Chem Pharm Bull, 50, 1273–75.

Nanasombat S, Teckchuen N. (2009). Antimicrobial, antioxidant and anticancer activities of Thai local vegetables. J Med Plants Res, 3, 443–49.

Naser-Esfahani MH, Aboutorabi R, Esfandiari E, Mardani M. (2002). Sperm MTT viability assay: A new method for evaluation of human sperm viability. J Assist Reprod Genet, 19, 477–82.

Nichenametla SN, Taruscio TG. Barney DL, Exon JH. (2006). A review of the effects and mechanisms of polyphenolics in cancer. Crit Rev Food Sci Nutr, 46, 161–83.

Nishimura T, Iwakabe K, Sekimoto M, Ohmi Y, Yahata T, Nakui M, Sato T, Habu S, Tashiro H, Sato M, Ohta A. (1999). Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. J Exp Med, 190, 617–27.

Noelker C, Stuckenholz V, Reese JP, Alvarez-Fischer D, Sankowski R, Rausch T, Oertel WH, Hartmann A, van Patten S, Al-Abed Y, Bacher M. (2013). CNI-1493 attenuates neuroinflammation and dopaminergic neurodegeneration in the acute MPTP mouse model of Parkinson's disease. Neurodegener Dis, 12, 103–10.

Nonato FR, Barros TAA, Lucchese AM, Oliveira CEC, dos Santos RR, Soares MBP, Villarreal CF. (2009). Antiinflammatory and antinociceptive activities of *Blechnum occidentale* L. extract. J Ethnopharmacol, 125, 102–07.

Nordeide MB, Hatloy A, Folling M, Lied E, Oshoug A. (1996). Nutrient composition and nutritional importance of green leaves and wild foods in an agricultural district, Koutiala, in Southern Mali. Int J Food Sci Nutr, 47, 455–68.

Oh JH, Jones MB, Longhurst WM, Connolly GE. (1970). Deer browsing and rumen microbial fermentation of Douglas fir as affected by fertilization and growth stage. Forest Sci, 16, 21–27.

Oh BM, Kweon MH, Ra KS. (1994). Isolation and characterization of acidic polysaccharides activation complement system from the hot water extracts of *Pteridium aquilinum* var. *latiusculum*. Korean J Food Nutr, 7, 159–68.

Olsen JH, Dossing M. (1982). Formaldehyde-induced symptoms in day-care centers. Am Ind Hyg Assn J, 43, 366–70.

Okada Y, Matono N, Shiono M, Takai T, Hikida M, Ohmori H. (1996). Suppression of *in vitro* cellular immune response by nitrogen-containing terpene alcohol derivatives. Biol Pharm Bull, 19, 1443–46.

Orech FO, Aagaard-Hansen J, Friis H. (2007). Ethnoecology of traditional leafy vegetables of the Luo people of Bondo district, western Kenya. Int J Food Sci Nutr, 58, 522–30.

Osawa T, Namiki M. (1981). A novel type of antioxidant isolated from leaf wax of *Eucalptus* leaves. Agric Biol Chem, 45, 735–39.

Ottolenghi A. (1959). Interaction of ascorbic acid and mitochondria lipids. Arch Biochem Biophys, 79, 355–63.

Owen J, Punt J, Stranford S, Jones P. (2013). Immunology, 7th ed. New York , USA: W. H. Freeman and Company.

Oyaizu M. (1986). Studies on products of browning reactions: Antioxidant activities of products of browning reaction prepared from glucose amine. Japanese J Nutr, 44, 307–15.

Pacifico L, Di Renzo L, Anania C, Osborn JF, Ippoliti F, Schiavo E, Chiesa C. (2006). Increased T-helper interferon-γ-secreting cells in obese children. European J Endocrinol, 154, 691–97.

Palo RT, Robbins CT. (1991). Plant defenses against mammalian herbivory. Boca Raton, USA: CRC Press.

Park JH, Kim JT, Park YS, Park KY, Lee KY. (1995). Anticariogenic and manufacturing process for anticariogenic foods. KO Appl. 1019950016816.

Park HA, Kweon MH, Han HM, Sung HC, Yang HC. (1998). Effects of the glycoprotein isolated from *Pteridium aquilinum* on the immune function of Mice. Korean J Food Sci Technol, 30, 976–82.

Park KR, Lee JH, Choi CY, Liu KH, Seog DH, Kim YH, Kim DE, Ynu CH, Yea SS. (2007). Suppression of interleukin-2 gene expression by isoeugenol is mediated through down-regulation of NF-AT and NF-κB. Int Immunopharmacol, 7, 1251–58.

Pathania S, Kumar P, Singh S, Khatoon S, Rawat AKS, Punetha N, Jensen DJ, Lauren DR, Somvanshi R. (2012). Detection of ptaquiloside and quercetin in certain Indian ferns. Curr Sci, 102, 1683–91.

Pedraza-Chaverrí J, Barrera D, Maldonado PD, Chirino YI, Macías- Ruvalcaba NA, Medina-Campos ON, Castro L, Salcedo MI, Hernández-Pando R. (2004). S-allylmercaptocysteine scavenges hydroxyl radical and singlet oxygen *in vitro* and attenuates gentamicin induced oxidative and nitrosative stress and renal damage *in vivo*. BMC Clin Pharmacol, 4, 5.

Pedraza-Chaverrí J, Arriaga-Noblecía G, Medina-Campos ON. (2007). Hypochlorous acid scavenging capacity of garlic. Phytother Res, 21, 884–88.

Pizzolatti MG, Brighente, IMC, Bortoluzzi AJ, Schripsema J,Verdi LG. (2007) .Cyathenosin A, a spiropyranosil derivate of proto-catechuic acid from *Cyathea phalerata*. Phytochemistry, 68, 1327–30.

Plotkin MJ. (2000). Medicine quest: In search of nature's healing secrets. New York, USA: Penguin Putnam Inc.

Pohanka M. (2011). Cholinesterases, a target of pharmacology and toxicology. Biomed Pap, 155, 219–29.

Pohanka M. (2012). Alpha7 nicotinic acetylcholine receptor is a target in pharmacology and toxicology. Int J Mol Sci, 13, 2219–38.

Pohanka M. (2013). Role of oxidative stress in infectious diseases. A review. Folia Microbiol, 58, 503–13.

Pohanka M. (2014). Inhibitors of acetylcholinesterase and butyrylcholinesterase meet immunity. Int J Mol Sci, 15, 9809-25.

Prasad AM, Ramnarayan K, Bairy KL. Effect of imatinib on histological parameters in male Swiss albino mice. (2010). Int J Pharm Sci Rev Res, 4, 116–22.

Prescott-Allen R, Prescott-Allen C. (1990). How many plants feed the world? Conserv Biol 4, 365–74.

Provenza FD. (2008). What does it mean to be locally adapted and who cares anyway? J Anim Sci, 86, E271–84.

Provenza FD, Villalba JJ. (2010). The role of natural plant products in modulating the immune system: An adaptable approach for combating disease in grazing animals. Small Rumin Res, 89, 131–39.

Pryor JL, Hughes C, Foster W, Hales BF, Robaire B. (2000). Critical windows of exposure for children's health: The reproductive system in animals and humans. Environ Health Persp, 108, 491–503.

Raisuddin S, Zaidi SIA, Singh KP, Ray PK. (1991). Effect of subchronic aflatoxin exposure on growth and progression of Ehrlich's ascites tumor in mice. Drug Chem Toxicol, 14, 185–206.

Rakowski F, Srinivasan J, Sternberg PW, Karbowski J. (2013). Synaptic polarity of the interneuron circuit controlling *C. elegans* locomotion. Front Comput Neurosci, 7, 128.

Rancon S, Chaboud A, Darbour N, Comte G, Bayet C, Simon PN, Raynaud J, Di Pietro A, Cabalion P, Barron D. (2001). Natural and synthetic benzophenones: interaction with the cytosolic binding domain of Pglycoprotein. Phytochemistry. 57, 553–57.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med, 1999, 26, 1231–37.

Reddy VLN, Ravikanth V, Rao TP, Venkateswarlu Y. (2001). A new triterpenoid from the fern *Adiantum lunulatum* and evaluation of antibacterial activity. Phytochem, 56, 173–75.

Revell SG, Mrode RA. (1994). An osmotic resistance test for bovine semen. Anim Reprod Sci, 36, 77–86.

Risk Assessment Section. (2007). Natural Toxins in Food Plants. Report No. 27, Centre for Food Safety of the Food and Environmental Hygiene Department, Government of the Hong Kong.

Rosas-Ballina M, Tracey KJ. (2009). Cholinergic control of inflammation. J Intern Med, 265, 663–79.

Rosenthal GA, Janzen DH. (1979). Herbivores: Their interaction with secondary plant metabolites. New York, USA: Academic Press.

Rosenthal GA, Berenbaum MR. (1992). Herbivores: Their interactions with secondary plant metabolites, 2nd ed. New York, USA: Academic Press.

Rout SD, Panda T, Mishra N. (2009). Ethnomedicinal studies on some pteridophytes of Similipal Biosphere Reserve, Orissa, India. Int J Med Med Sci, 1, 192–97.

Salatino MLF, Prado J. (1998). Flavonoid glycosides of Pteridaceae from Brazil. Biochem Sys Ecol, 26, 761–69.

Sarkar M, Gangopadhyay P, Basak B, Chakrabarty K, Banerji J, Adhikary P, Chatterjee A. (2000). The reversible antifertility effect of *Piper betle* Linn. on Swiss albino male mice. Contraception, 62, 271–74.

Schilter B, Andersson C, Anton R, Constable A, Kleiner J, O'Brien J, Renwick AG, Korver O, Smit F, Walker R. (2003). Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. Food Chem Toxicol, 41, 1625–49.

Seal T. (2012). Antioxidant activity of some wild edible plants of Meghalaya state of India: A comparison using two solvent extraction systems. Int J Nutr Metabolism, 4, 51–56.

Segelman AB, Farnsworth NR, Quimby MW. (1969). False negative saponins test results induced by the presence of tannins. Lloydia, 32, 52–58.

Sellers RS, Morton D, Michael B, Roome N, Johnson JK, Yano BL, Perry R, Schafer K. (2007). Society of toxicologic pathology position paper: Organ weight recommendations for toxicology studies. Toxicol Pathol, 35, 751–55.

Sen A, Ghosh PD. (2011). A note on the ethnobotanical studies of some pteridophytes in Assam. Indian J Trad Knowl, 10, 292–95.

Shackleton SE, Dzerefos CM, Shackleton CM, Mathabala FR. (1998). Use and trading of wild edible herbs in central lowveld savannah region, South Africa. Econ Botany, 52, 251–59.

Sharpe RM, Shakkebaek NE. (1993). Are estrogen involved in falling sperm counts and disorders of the male reproductive tract? Lancet, 341, 1392–1405.

Shimada K, Fujikava K, Yahara K, Nakamura T. (1992). Antioxidative properties of xanthan on the autooxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem, 40, 945–48.

Shin SL. (2010). Functional components and biological activities of Pteridophytes as healthy biomaterials. Ph.D. dissertation, Chungbuk National University, Cheongju, Korea.

Silva-Herdade AS, Saldanha C. (2013). Effects of acetylcholine on an animal mode of inflammation. Clin Hemorheol Microcirc, 53, 209–16.

Sinclair KD, Rooke JA, McEvoy TG. (2003). Regulation of nutrient uptake and metabolism in pre-elongation ruminant embryos. Reprod Supp. 61, 371–85.

Singh A, Singh SK. (2009). Evaluation of antifertility potential of Brahmi in male mouse. Contraception, 79, 71–79.

Singleton VL, Rossi JA. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Americal J Enol Viticult, 16, 144–58.

Smirnov I, Belogurov A, Friboulet A, Masson P, Gabibov A, Renard PY. (2013). Strategies for the selection of catalytic antibodies against organophosphorus nerve agents. Chem Biol Interact, 203, 196–201.

Smith BL, Seawright AA, Ng JC, Hertle AT, Thomson JA, Bostock PD. (1994). Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium* spp.) from eastern Australia and from cultivated worldwide collection held in Sydney, Australia. Nat Toxins, 2, 347–53.

Somvanshi R, Lauren DR, Smith BL, Dawra RK, Sharma OP, Sharma, VK, Singh AK, Gangwar NK. (2006). Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. Curr Sci, 91, 1547–52.

Son HH, Seo DS, You YC. (1999). Cosmetics compostin comprising spora Lygodii. KO Appl 1019990034870.

Star AE, Mabry TJ. (1971). Flavonoid Frond Exudates from 2 Jamaican Ferns *Pityrogramma tartarea* and *Pityrogramma calmoelanos*. Phytochemistry, 10, 2817–18.

Starec M, Sinet M, Kodym P, Rosina J, Fiserova A, Desforges B, Rouveix B. (1997). The effect of drugs on the mortality of mice inoculated with friend leukaemia virus or *Toxoplasma gondii*. Physiol Res, 46, 107–11.

Suffredini IB, Bacchi EM, Sertie JAAA. (1999). Antiulcer action of Microgramma squamulosa (Kaulf.) Sota. J Ethnopharmacol, 65, 217–23.

Sujaa SR, Lathaa PG, Pushpangadan P, Rajasekharan S. (2004). Evaluation of hepatoprotective effects of *Helminthostachys zeylanica* (L.) Hook against carbon tetrachloride-induced liver damage in Wistar rats.

Sukumaran K, Kuttan R. (1991). Screening of 11 ferns for cytotoxic and antitumor potential with special reference to *Pityrogramma calomelanos*. J Ethnopharmacol, 34, 93–96.

Sundriyal M, Sundriyal RC. (2001). Wild edible plants of the Sikkim Himalaya: Nutritive values of selected species. Econ Botany, 55, 377–90.

Sun P, Zhou KW, Wang S, Li P, Chen SJ, Lin GP, Zhao Y, Wang TH. (2013). Involvement of MAPK/NF-κB signaling in the activation of the cholinergic anti-inflammatory pathway in experimental colitis by chronic vagus nerve stimulation. PLoS One, 8, e69424.

Thews O, Gassner B, Kelleher DK, Schwerdt G, Gekle M. (2006). impact of extracellular acidity on the activity of P-glycoprotein and the cytotoxicity of chemotherapeutic drugs. Neoplasia. 8, 143–52.

Thomas GT, Rebecca S, Andre BA. (2008). The natural history of symptomatic androgen deficiency in men: Onset, progression, and spontaneous remission. J Am Geriatr Soc, 56, 831–93.

Thong FS, Graham TE. (2002). The putative roles of adenosine in insulin and exercise-mediated regulation of glucose transport and glycogen metabolism in skeletal muscle. Can J Appl Physiol, 27, 152–78.

Tongco JVV, Villaber RAP, Aguda RM, Razal RA. (2014). Nutritional and phytochemical screening, and total phenolic and flavonoid content of *Diplazium esculentum* (Retz.) Sw. from Philippines. J Chem Pharm Res, 6, 238–42.

Trease GE, Evans WC. (1996). A textbook of Pharmacognosy, 14th ed. London, UK: Bailliere Tindall Ltd.

Turner NJ, Łuczaj ŁJ, Migliorini P, Pieroni A, Dreon AL, Sacchetti LE, et al. (2011). Edible and tended wild plants, traditional ecological knowledge and agroecology. Curr Rev Plant Sci, 30, 198–225.

US Consumer product safety commission (US CPSC). (1997). An update on formaldehyde. (http:// www.cpsc.gov/cpscpub/pubs/725.pdf)

Villalba JJ, Provenza FD, Olson KC. (2006). Terpenes and carbohydrate source influence rumen fermentation, digestibility, intake, and preference in sheep. J Anim Sci, 84, 2463–73.

Vincent P, Kanna R. (2007). Antibacterial activity of ferns – *Christilla parasitica* and *Cyclosorus interuptus* against *Salmonella typhi*. SiddhaPapers. http://openmed.nic.in/2009/

Viveka S, Udyavar A, Shetty B, Kuriakose S, Sudha MJ. (2015). Histomorphometric effects of gemcitabine on Swiss albino mice spermatogenesis. Adv Biomed Res, 4, 29–34.

Waghorn GC. (1990). Beneficial effects of low concentrations of condensed tannins in forages fed to ruminants. In: Akin DE, Ljungdahl LG, Wilson JR, Harris PJ, ed. Microbial and plant opportunities to improve lignocellulose utilization by ruminants. New York: Elsevier Science Publishing Co.,137–247.

Wallace RA, Sander GP, Ferl RJ. (1991). Biology: the science of life. New York, USA: HarperCollins.

Wang ZP, Gu ZP, Cao L, Xu Y, You GD, Mao BY, Qian SZ. (1999). Effects of tripchlorolide on the epididymides and testes of rats. Asian J Androl, 1, 121–25.

Wessler I, Kirkpatrick CJ. (2008). Acetylcholine beyond neurons: The non-neuronal cholinergic system in humans. Br J Pharmacol, 154, 1558–71.

Wills PJ, Asha VV. (2006). Protective effect of *Lygodium flexuosum* (L.) Sw. extract against carbon tetrachloride-induced acute liver injury in rats. J Ethnopharmacol, 108, 320–26.

Wilson, SD, Munson AE, Meade BJ. (1999). Assessment of the functional integrity of the humoral immune response: the plaque-forming cell assay and the enzyme-linked immunosorbent assay. Methods, 19, 3–7.

Wooley A. (2003). Determination – general and reproductive toxicology. In: A guide to practical toxicology evaluation, prediction and risk. New York: Taylor and Francis, 80–106.

World Health Organization. (1999). WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 4th ed. Cambridge, UK: Cambridge Univ. Press.

Yeap SK, Alitheen NBM, Ho WY, Omar AR, Ali AM, Beh BK, Yousr AHN. (2010). Immunomodulatory role of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell proliferation, cytokine secretion and cytolytic activity. J Med Plant Res 5, 958–65.

Yousef MI, Salem MH, Ibrahim HZ, Helmi S, Seehy MA, Bertheussen, K. (1995). Toxic effect of carbofuran and glyphosate on semen characteristics in rabbits. J Environ Sci Health B, 30, 513–34.

Zakharova LA, Izvolskaia MS. (2012). Interactions between reproductive and immune systems during ontogeny: Roles of GnRH, sex steroids, and immunomediators. In: Kahn SM, ed. Sex steroids. Rijeka, Croatia - European Union: InTech, 221–46.

Zeng-fu LI, Huil H, Hang-yi Z, Jun-chen Z. (2008). Review on the extraction of flavonoids from fern. J Sanm Univ 25, 22.

Zhang SY, Ito Y, Yamanoshita O, Yanagiba Y, Kobayashi M, Taya K, Li C, Okamura A, Miyata M, Ueyama J, Lee CH, Kamijima M, Nakajima T. (2007). Permethrin may disrupt testosterone biosynthesis via mitochondrial membrane damage of Leydig cells in adult male mouse. Endocrinol, 148, 3941–49.

Zhang Z, ElSohly HN, Jacob MR, Pasco DS, Walker LA, Clark AM. (2002). Natural products inhibiting Candida albicans secreted aspartic proteases from *Lycopodium cernuum*. J Nat Prod, 65, 979–85.

Zhishen J, Mengcheng T, Jianming W. (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. Food Chem, 64, 555–59.

Zuping H, Kokkinaki M, Dym M. (2009). Nodal signaling via an autocrine pathway promotes proliferation of mouse spermatogonial stem/progenitor cells through Smad2/3 and Oct-4 activation. Stem cells, 27, 2580–89.

INDEX

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W

KITS USED IN EXPERIMENTS

I. ELISA kits

RayBio®
Mouse IL-2 ELISA Kit

User Manual
(Revised Mar 1, 2012)

RayBio® Mouse IL-2 ELISA Kit Protocol

(Cat#: ELM-IL2-001)

RayBiotech, Inc.

RayBiotech, Inc.

RayBio® Mouse IL-2 ELISA Kit Protocol

ABLE OF CONTENTS

RayBio®
Mouse IFN-γ ELISA Kit

User Manual
(Revised Mar 1, 2012)

RayBio® Mouse IFN-γ ELISA Kit Protocol

(Cat#: ELM-IFNgamma-001)

RayBiotech, Inc.

RayBiotech, Inc.

RayBio® Mouse IFN-γ ELISA Kit Protocol

TABLE OF CONTENTS

RayBio®
Mouse IL-4 ELISA Kit

User Manual
(Revised Mar 1, 2012)

RayBio® Mouse IL-4 ELISA Kit Protocol

(Cat#: ELM-IL4-001)

RayBiotech, Inc.

RayBiotech, Inc.

RayBio® Mouse IL-4 ELISA Kit Protocol

TABLE OF CONTENTS

RayBio®Mouse IL-10 ELISA Kit

User Manual
(Revised Mar 1, 2012)

RayBio® Mouse IL-10 ELISA Kit Protocol

(Cat#: ELM-IL10-001)

RayBiotech, Inc.

RayBiotech, Inc.

RayBio® Mouse IL-10 ELISA Kit Protocol

TABLE OF CONTENTS

II. Biochemical kits for studying liver and kidney functions

Coral Clinical Systems — Quick Reference Guide — **SGOT (ASAT) KIT**

SGOT (ASAT) Kit
Reitman & Frankel's Method

Intended Use:

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood stream. Elevated levels are found in myocardial infarction, cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in pregnancy, beri beri and diabetic ketoacidosis. SGOT (ASAT) kit uses the Reitman & Frankel's method to determine SGOT (ASAT) in serum.

SGOT (ASAT) Kit components:

L1	Substrate Reagent
L2	DNPH Reagent
L3	NaOH Reagent (4N)
S	Pyruvate Standard (2mM)
Other Accessories	Package Insert

Coral Clinical Systems — Quick Reference Guide — **SGPT (ALAT) KIT**

SGPT (ALAT) Kit
Reitman & Frankel's Method

Intended Use:

SGPT is found in a variety of tissues but it is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction. SGPT (ALAT) kit uses the Reitman & Frankel's method to determine SGPT (ALAT) in serum.

SGPT (ALAT) Kit components:

L1	Substrate Reagent
L2	DNPH Reagent
L3	NaOH Reagent (4N)
S	Pyruvate Standard (2mM)
Other Accessories	Package Insert

Coral Clinical Systems

Quick Reference Guide

LACTATE DEHYDROGENASE (P-L) KIT

Lactate Dehydrogenase (P-L) Kit
Mod. IFCC Method

Intended Use:

Lactate dehydrogenase (LDH) is found in many body tissues particularly heart, liver, skelatal muscle, kidney and RBCs. LDH is found in the form of isoenzymes based on their electrophoretic mobility with each isoenzyme being primarily from different organs. Increased levels are found in myocardial infarction, pulmonary diseases, hepatic diseases, hemolytic anemias, renal diseases and muscular dystrophy. LDH (P-L) kit uses the Mod. IFCC Method to determine lactate dehydrogenase in serum.

Lactate Dehydrogenase (P-L) Kit components:

L1	Buffer Reagent
L2	Starter Reagent
Other Accessories	Package Insert

Coral Clinical Systems

Quick Reference Guide

GAMMA GLUTAMYL TRANSFERASE KIT

Gamma Glutamyl Transferase Kit
Carboxy Substrate Method

Intended Use:

Gamma glutamyl transferase (GGT) is an enzyme found mainly in serum from hepatic origin, though the highest levels are in the kidneys. Elevated levels are found in hepatobiliary and pancreatic diseases. Chronic alcoholism, myocardial infarction with secondary liver damage and diabetics. Gamma glutamyl transferase kit uses the carboxy substrate method to determine gamma glutamyl transferase in serum.

Gamma Glutamyl Transferase Kit components:

L1	Buffer Reagent
T1	Substrate Tablets
Other Accessories	Package Insert

Coral Clinical Systems

Quick Reference Guide **ALKALINE PHOSPHATASE KIT**

Alkaline Phosphatase kit
Mod. Kind & King's Method

Intended Use:

Alkaline Phosphatase (ALP) is an enzyme of the hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in the liver, biliary tract epithelium and in bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone diseases. Moderate increases are seen in Hodgkins diseases and congestive heart failure. Alkaline Phosphatase kit uses the Mod. Kind & King's method to determine alkaline phosphatase activity in serum.

Alkaline Phosphatase Kit components:

L1	Buffer Reagent
L2	Substrate Reagent
L3	Color Reagent
S	Phenol Standard (10 mg/dl)
Other Accessories	Package Insert

Coral Clinical Systems

Quick Reference Guide **ACID PHOSPHATASE KIT**

Acid Phosphatase Kit
Mod. King's Method

Intended Use:

Acid Phosphatase (ACP) is an enzyme of the hydrolase class of enzymes and acts in an acidic medium. It is widely distributed and found in high concentrations in the liver, RBC's and the prostrate. Increased levels of the prostatic fraction are associated with prostatic carcinomas. Increased levels of the non prostatic fraction are associated with liver disease, hyperparathyroidism and Paget's disease.

Acid Phosphatase kit is used for the determination of acid phosphatase activity in serum using the Mod. King's Method.

Acid Phosphatase Kit components:

L1	Buffer Reagent
L2	Substrate Reagent
L3	Colour Reagent
L4	Tartrate Reagent
S	Phenol Standard (10 mg/dl)
Other Accessories	Package Insert

Bilirubin Kit
Mod. Jendrassik & Grof's Method

Intended Use:

Bilirubin is mainly formed from the heme portion of aged or damaged RBCs. It then combines with albumin to form a complex which is not water soluble. This is referred to as indirect or unconjugated bilirubin. In the liver this bilirubin complex is combined with glucuronic acid into a water soluble conjugate. This is referred to as conjugated or direct bilirubin. Elevated levels of bilirubin are found in liver diseases (hepatitis and cirrhosis), excessive hemolysis / destruction of RBC (hemolytic jaundice) obstruction of the biliary tract (obstructive jaundice) in the drug induced reactions. The differentiation between the direct and indirect bilirubin is important in diagnosing the cause of hyperbilirubinemia. Bilirubin kit uses mod. Jendrassik & Grof's method to determine direct & total bilirubin in serum.

Bilirubin Kit components:

L1	Direct Bilirubin Reagent
L2	Direct Nitrite Reagent
L1	Total Bilirubin Reagent
L2	Total Nitrite Reagent
S	Artificial Standard (10 mg/dl)
Other Accessories	Package Insert

Urea Kit
Diacetyl Monoxime (DAM) Method

Intended Use:

Urea is the end product of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy. Urea kit uses DAM method to determine urea in serum, plasma & urine.

Urea Kit components:

L1	Urea Reagent
L2	Acid Reagent
L3	DAM Reagent
S	Urea Standard (40 mg/dl)
Other Accessories	Package Insert

Creatinine Kit
Alkaline Picrate Method

Intended Use:

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy. Creatinine kit uses the alkaline picrate method to determine creatinine in serum and urine.

Creatinine Kit components:

L1	Picric acid Reagent
L2	Buffer Reagent
Standard	Creatinine Standard (2 mg/dl)
Other Accessories	Package Insert

System Parameters			
Reaction	: End Point	**Interval**	: ---
Wavelength	: 520 nm	**Sample Vol.**	: 0.20 ml
Zero Setting	: Reagent Blank	**Reagent Vol.**	: 1.10 ml
Incub. Temp	: R.T.	**Standard**	: 2 mg/dl
Incub. Time	: 20 min.	**Factor**	: ---
Delay Time	: ---	**React. Slope**	: Increasing
Read Time	: ---	**Linearity**	: 8 mg/dl
No. of read	: ---	**Units**	: mg/dl

Storage / Stability	Temperature	Duration
Unopened kit	15-30°C	24 Months
Opened kit (Unmixed)	15-30°C	24 Months
In use stability	2-8°C	1 Day

Available Pack Sizes	
15 Tests	35 Tests

Photographs of some of the experiments conducted

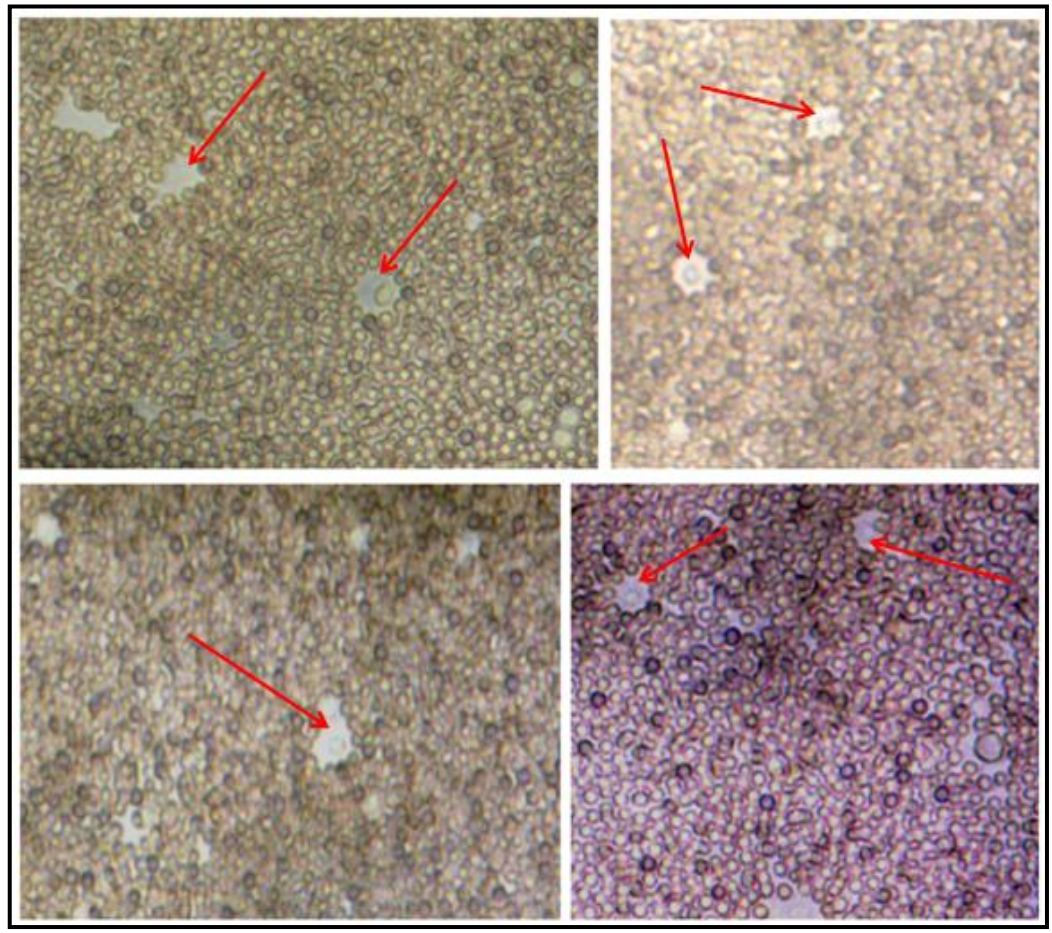


Figure: Images of PFC assay (Red arrow indicate "plaques")

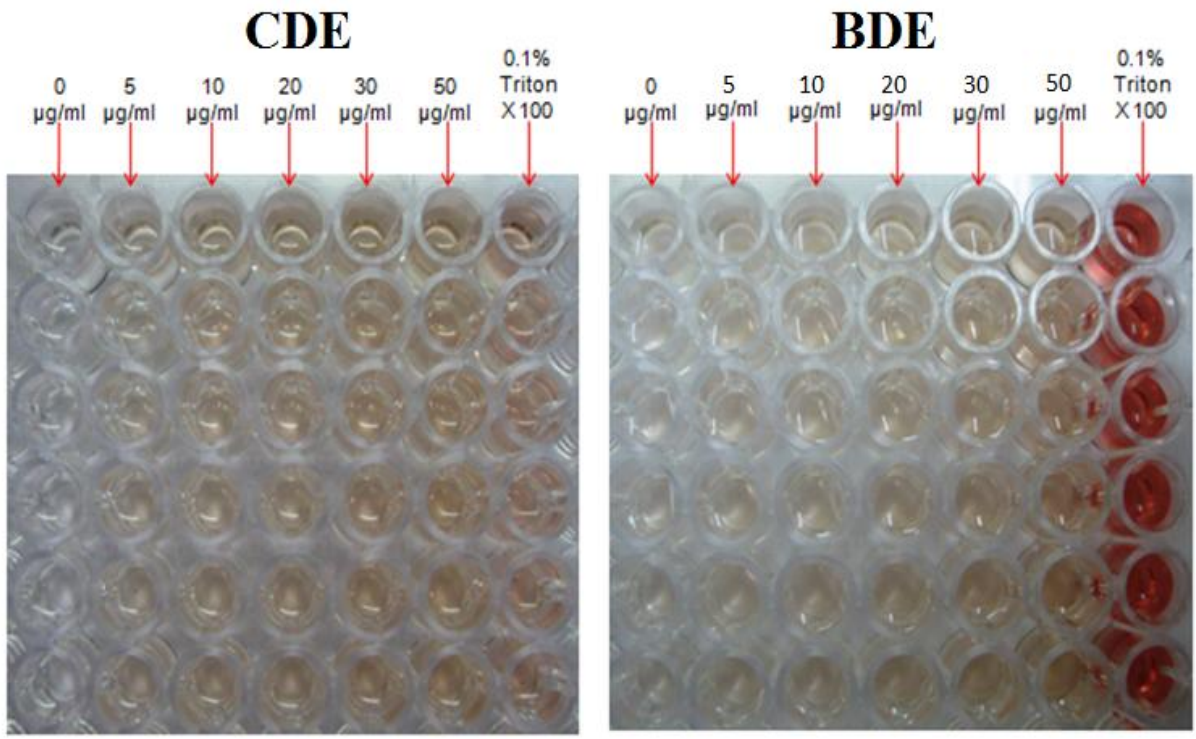


Figure: Dose-dependent increase in the optical density (O.D) of the sample in the microtitre plate. Increase in the O.D indicates liberation of more hemoglobin from the erythrocytes in the suspending medium, hence more the color. Therefore, the above experiment indicates that both CDE and BDE cause hemolysis.

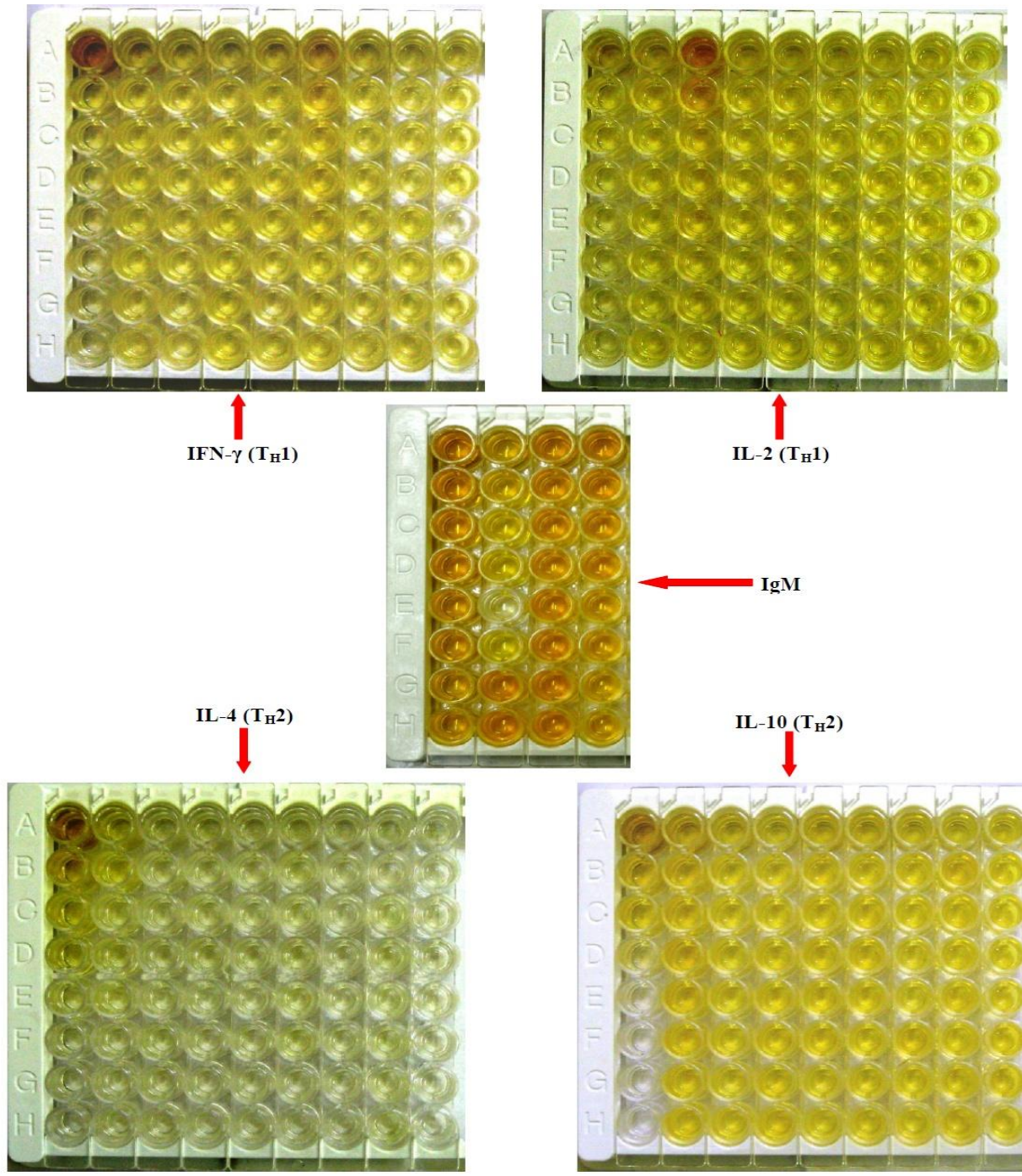


Figure: Photographs of ELISA plates of different TH1 and TH2 cytokines and Immunoglobulin M (IgM)

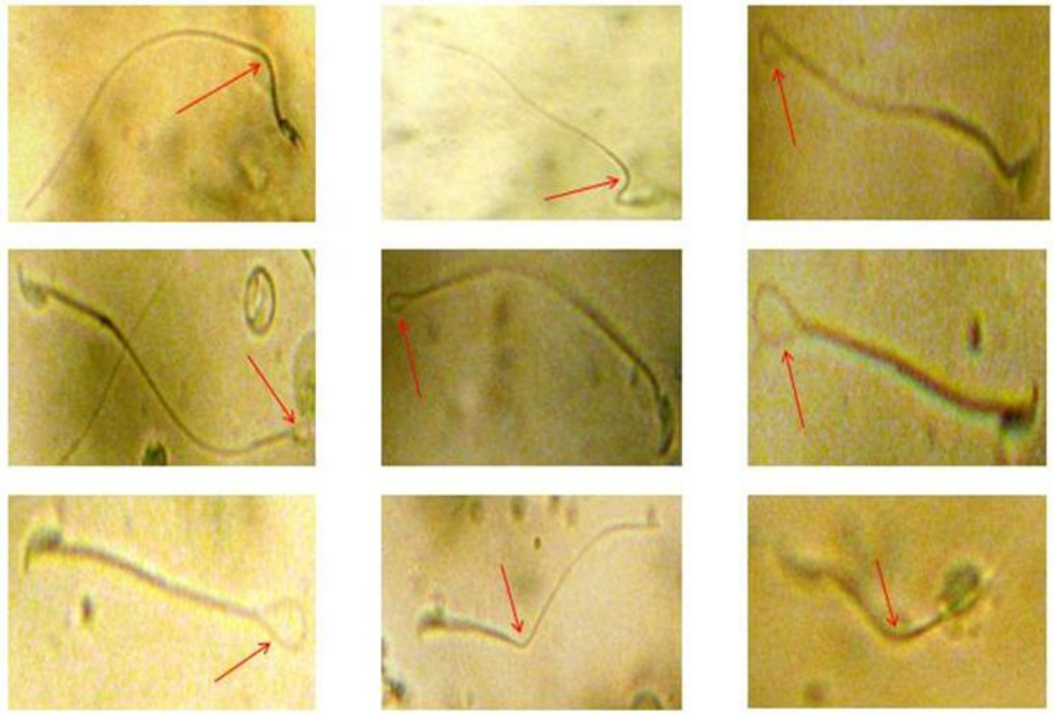


Figure: Some active spermatozoa (indicated by red arrow) showing coiling during HOST

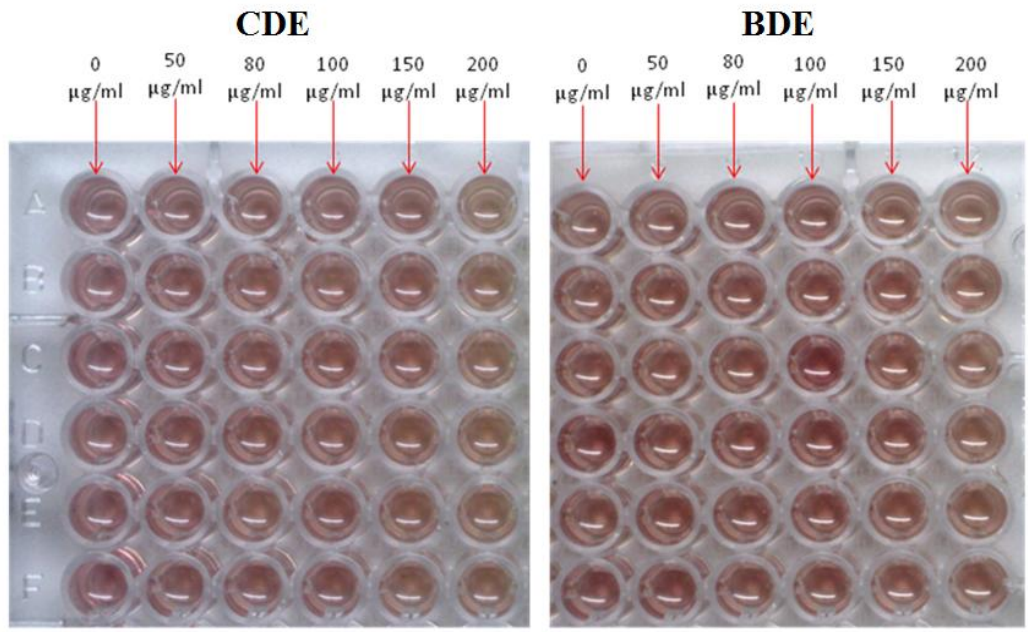


Figure: Photograph of sperm MTT assay after solubilization of formazan

APPENDIX – 3

PUBLICATIONS

1. **Roy S**, Chaudhuri TK. (2016). Toxicological assessment of *Diplazium esculentum* on the reproductive functions of male Swiss albino mouse. Drug and Chemical Toxicology, Published online 9 June 2016.

2. **Roy S**, Chaudhuri TK. (2015). Assessment of Th1 and Th2 cytokine modulatory activity of an edible fern, *Diplazium esculentum*. Food and Agricultural Immunology, 26: 690–702.

3. **Roy S**, Dutta S, Chaudhuri TK. (2015). In vitro assessment of anticholinesterase and NADH oxidase inhibitory activities of an edible fern, *Diplazium esculentum*. Journal of Basic and Clinical Physiology and Pharmacology, 26: 395–401.

4. **Roy S**, Tamang S, Dey P, Chaudhuri TK. (2013). Assessment of the immunosuppressive and hemolytic activities of an edible fern, *Diplazium esculentum*. Immunopharmacology and Immunotoxicology, 35: 365–372.

5. **Roy S**, Hazra B, Mandal N, Chaudhuri TK. (2013). Assessment of the antioxidant and free radical scavenging activities of methanolic extract of *Diplazium esculentum*. International Journal of Food Properties, 16: 1351–1370.

6. **Roy S**, Tamang S, Chaudhuri TK. (2013). Sperm viability assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of Swiss albino mice treated with *Diplazium esculentum*. Asian Journal of Pharmaceutical and Health Sciences, 3: 684–689.

Drug and Chemical Toxicology

www.tandfonline.com/idct
ISSN: 0148-0545 (print), 1525-6014 (electronic)

Drug Chem Toxicol, Early Online: 1–12
 DOI: 10.1080/01480545.2016.1190739

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Toxicological assessment of *Diplazium esculentum* on the reproductive functions of male Swiss albino mouse

Subhrajyoti Roy[1,2] and Tapas Kumar Chaudhuri[1]

[1]*Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India and* [2]*Immunopharmacology and Molecular Cell Biology Laboratory, Department of Zoology, University of Gour Banga, Malda, West Bengal, India*

Abstract

Context: *Diplazium esculentum*, a commonly consumed seasonal vegetable, has been reported to have some pathological effects in some animals. But, its effect on the male reproductive function has not yet been studied. *Objective*: To investigate the effects of boiled *D. esculentum* (BDE), the form which human consumes, on male reproductive functions of Swiss albino mice. *Materials and methods*: Male (120 in no.) and female (80 in no.) Swiss albino mice (6–8 weeks of age) were fed orally with 80, 160 and 320 mg/kg bw of BDE within a span of 180 d. After the treatment, body weight, absolute- and relative-testis weight, relative-weight of other organs, their biochemical parameters, hypo-osmotic swelling test (HOST) of spermatozoa, testis histology and fertility and fecundity tests were performed to justify the toxic effects of *D. esculentum* on male reproductive functions. *Results*: Significant dose- and time-dependent decreases were observed in body weight, absolute- and relative-testis weight, relative-weights of other organs and their biochemical parameters, percentage of live spermatozoa and percentage of fertility and fecundity in BDE fed mice. Significant decreases were observed in diameter, perimeter and area of the seminiferous tubules of mice treated for 180 d. The percentage of empty seminiferous tubules was increased significantly in BDE treated mice when compared to the controls. *Discussion and conclusion*: These results suggest that the intake of *D. esculentum*, even after cooking, may induce infertility by altering the male reproductive function, and therefore, should be evaluated further as a potential antifertility agent.

Keywords

Testicular integrity, fertility and fecundity, reproductive toxicity, male infertility

History

Received 30 August 2015
Revised 24 February 2016
Accepted 20 March 2016
Published online 9 June 2016

Introduction

Wild edible plants are utilized as a food source among the indigenous people throughout the world. The progressive decrease in the stock of cultivated crops leads to the increase in the consumption of these plants. These plants are used as substitutes and fill the gap of food deficiency (Teklehaymanot & Giday, 2010). But information on the possible toxic effects of most of the wild edible plants is too little to make the people aware about the hazardous effects of the consumption of these plants. Till date, very few studies have been conducted to assess the toxicological impact of wild edible plants on male fertility. Male infertility is a common problem nowadays occurring worldwide. According to a study of WHO, the male reproductive capacity was found to be deficient in about 50% of infertile couples (WHO, 2000).

Address for correspondence: Tapas Kumar Chaudhuri, Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India. Tel: +91 9434377127 (M); +91 3532776353 (O). Fax: +91 3532699001. E-mail: dr_tkc_nbu@rediffmail.com

Diplazium esculentum (Koenig ex Retz.) Sw. (Athyriaceae) is one such wild edible plant found commonly throughout Asia and Oceania. It is one of the most commonly consumed wild edible fern, that has been reported to have medicinal/therapeutic as well as toxic properties (Roy et al., 2013a,b,c). The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during the monsoon season, which continues for almost five months in the tropical regions where this plant grows abundantly (Roy et al., 2013b). Our previous study of this fern showed that it possesses trace amounts of flavonoids and phenolic compounds which may confer the antioxidant and free radical scavenging activities of this plant (Roy et al., 2013a). Methanolic extract of this plant has been shown to possess anticholinesterase and NADH oxidase inhibitory activities, thus indicating its neuromodulatory activity (Roy et al., 2015). Another study indicated that the methanol and chloroform extracts of *D. esculentum* possess significant antimicrobial and cytotoxic activity (Akter et al., 2014). This plant has also been reported to possess different phytochemicals such as alkaloids, anthraquinones, anthranol glycosides, cardiac glycosides, cyanidins, saponins, leucoanthocyanins, phytosterols, diterpenes and triterpenes (Tongco et al., 2014). Some of these phytochemicals may not only provide the antioxidant activity to this plant but also aid

in other important pharmacological effects in relation to human health.

To date, very few studies have been conducted so far to assess the pharmacological and toxicological impacts of this fern on animal health. Interestingly, this fern is rejected as food by cattle and insets. We have observed that this fern grows abundantly in the marshy land and also in the wet shabby places where lot of insects are available. But interestingly, we have never found any leaf consumed by the insects and all the leaves are intact throughout the season (Roy et al., 2013b). We previously demonstrated that *D. esculentum*, even boiled, possess potent hemolytic activity and it affects some of the innate and cell-mediated immune responses, by altering Th1/Th2 cytokine balance (Roy et al., 2013b; Roy & Chaudhuri, 2015). Young fronds of *D. esculentum* collected from the high-altitude area of Northern India have been found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria in hill cattle in India and elsewhere (Somvanshi et al., 2006). Moreover, Pta rich frozen- and shade-dried crude *D. esculentum* have already been shown to cause poor growth, decreased body weight, increased spontaneous (vertical and horizontal) and decreased forced motor activity, alternations in values of blood glucose, erythrocyte sedimentation rate, mean corpuscular volume, mean corpuscular hemoglobin, total leukocyte count, neutrophil, lymphocyte and monocyte count, and increased blood SGOT level in both rats and guinea pigs (Gangwar, 2004). Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats (Smith et al., 1994).

Previously we have also demonstrated that the boiled preparation of this fern possess spermicidal property in Swiss albino mouse (Roy et al., 2013c). In the purview of our previous study, this study is an attempt to evaluate the effect of the boiled plant material (boiled *D. esculentum*; BDE) on different variables of reproductive functions of male Swiss albino mouse, viz. testicular, epididymal, prostate and seminal vesicle markers, sperm density and testicular integrity on histological sections. This study was conducted only with cooked (boiled) material, because, the local people consumes it regularly after cooking, not as a raw vegetable.

Materials and methods

Preparation of the plant material

Young *D. esculentum* plants were collected during June–August 2014 from different areas of North Bengal University campus and the adjoining markets of Darjeeling, West Bengal, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal, India, where a voucher specimen (Accession No. 9602) was also deposited.

Young frond of *D. esculentum* (100 g) was washed carefully by tap water, then cut into small pieces, and boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed by a mixer and dried in an incubator at 60 °C until completely dried. This dried plant material (BDE) was then kept at 4 °C for future use.

Animals and care

Both male (120 in no.) and female (80 in no.) Swiss albino mice (25 ± 2 g of body weight [bw]) of 6–8 weeks of age were used for the study. They were housed in polypropylene cages, with dust free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with pellet food (Hindustan Unilever Ltd., Mumbai, India) and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2 °C. All the experiments were performed after obtaining the approval from the Institutional Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

Dosage

Sub-acute (15 and 45 d durations), sub-chronic (90 d duration) and chronic (135 and 180 d durations) toxicological studies were performed in 120 male Swiss albino mice that were fed with different doses of BDE according to a previously described method (Roy et al., 2013b). Briefly, One hundred twenty (120) male Swiss albino mice were divided in to five sets (S 1–5) and each set was subdivided into four groups (G1–4). Therefore, each group contained six mice. All the animals were fed orally with the help of a syringe specially designed by us. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of BDE at the dose of 80, 160 and 320 mg/kg bw, respectively. In this fashion, all groups of S1 (S1G1, S1G2, S1G3 and S1G4) were treated daily for 15 d (Sub-acute dose – I), S2 (S2G1, S2G2, S2G3 and S2G4) daily for 45 d (Sub-acute dose – II), S3 (S3G1, S3G2, S3G3 and S3G4) daily for 90 d (Sub-chronic dose), S4 (S4G1, S4G2, S4G3 and S4G4) daily for 135 d (Chronic dose – I) and S5 (S5G1, S5G2, S5G3 and S5G4) daily for 180 d (Chronic dose – II).

We assume that the average maximum amount of cooked *D. esculentum* consumed by a 60 kg weighed individual is about 20 g/d. Keeping this ratio in mind, we have formulated different doses for an average weighed adult mouse (25 g), like 80 mg/kg bw, i.e. 2 mg/mouse/d; 160 mg/kg bw, i.e. 4 mg/mouse/d; and 320 mg/kg bw, i.e. 8 mg/mouse/d (Roy et al., 2013b).

Body weight, relative-weight of organs and collection of serum

Mouse from each group was sacrificed after proper anesthesia (chloroform and ether in 2:1 ratio) 24 h after the last dose, and body weight, absolute- and relative-testis weight, and relative-weights of epididymis, seminal vesicle and prostate were determined. Blood was collected from the heart and serum was separated. The serum samples were used for the determination of total protein content.

Preparation of the sperm suspension for hypo-osmotic swelling test (HOST)

Caudal epididymis was separated and minced using a pair of small scissors to release the sperm into 10 ml pre-warmed (37 °C) physiological saline. HOST was used to evaluate the

functional integrity of the sperm membrane, based on coiled and swollen tails. This was performed by incubating 30 µl of semen with 300 µl of 100 mOsm hypo-osmotic solution (9 g of fructose + 4.9 g of sodium citrate per liter of distilled water) at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a pre-warmed slide. A total of 200 spermatozoa were counted in different fields using a phase-contrast microscope. Sperms with swollen or coiled tails were recorded (Buckett et al., 1997; Revell & Mrode, 1994).

Tissue biochemistry

Testis

Total protein and cholesterol contents were determined in testicular tissue, and the sample was prepared according to a previously described method (Hammami et al., 2008). Briefly, a part of testis (about 0.5 g) was crushed in 2 ml of 0.9% normal saline. The homogenate was centrifuged at 13000 × g for 10 min. The supernatant was collected and used for the determination of total protein and cholesterol contents using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Glycogen content in testis was determined by a previously described method (Montgomery, 1957).

The sample was prepared according to a previously described method (Hammami et al., 2008), with little modification, for the determination of acid phosphatase activity. Briefly, 0.5 g of testicular tissue was homogenized in 2 ml of citric acid buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 18000 × *g* at 4 °C for 30 min. The supernatant was used to determine the acid phosphatase activity using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). Acid phosphatase activity was expressed as µM/min/g of tissue.

Epididymis

Total protein content in epididymis was determined using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India), according to the manufacturer's instruction. α-glucosidase activity in epididymis was measured according to a previously described method with little modification (Hammami et al., 2008). Briefly, the caudal epididymis of mouse was cut, homogenized in citric acid buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 6.2, supplemented with 0.4% Triton X-100 solution) and centrifuged at 180000×*g* at 4 °C for 30 min. The α-glucosidase activity was measured according to a previously described colorimetric method (Wang et al., 1999). The reaction system contained 1.2 ml buffer (69 mM citric acid, pH 6.8), 0.2 ml paranitrophosphateglycerol (PNPG, 23 mM) and 0.2 ml supernatant. The reaction medium was incubated at 37 °C for 4 h and 0.25 ml Na_2CO_3 (0.1 M) was added to stop the reaction. The absorbance was measured at 400 nm in a spectrophotometer and the PNP content was estimated in reference to the PNP standard curve. The α-glucosidase activity was expressed as µmol/min/g of tissue. The supernatant was also used to determine the total protein contents using commercially available standard biochemical assay kits (Crest Biosystems, Goa, India). The concentration of sialic acid in the epididymis was estimated according to the standard thiobarbituric acid method (Aminoff, 1961).

Prostate and seminal vesicle

Sample preparation from prostate and seminal vesicle was done according to a previously described method (Hammami et al., 2008). Briefly, 0.2 g of tissues were homogenized in 2 ml of 0.33% perchloric acid at 4 °C and centrifuged at 2500×*g* for 10 min. Then, 1 ml of the supernatant was added to 0.5 ml K_2CO_3 (0.75 M). The reaction mixture was centrifuged at 2500×*g* for 10 min and supernatant was used for the determination of citric acid content of prostate and fructose content of seminal vesicle. The fructose content was determined according to a previously described protocol with little modification (Anderson et al., 1979). Briefly, 100 µl of supernatant was added to distilled water to give a total volume of 0.5 ml. The reaction tube was then placed in a boiling water bath for 7 min, and centrifuged at 10000×*g* for 20 min to remove the precipitated material. 0.3 ml of the supernatant was added to 1.5 mM of NADH and sorbitol dehydrogenase preparation in sodium phosphate (0.1 M, pH 6.8) to give a final reaction mixture volume of 1 ml. The concentration of fructose was determined by comparing the initial rate of decrease in absorbance at 340 nm with that of fructose standard. The concentration of citric acid in prostate was determined according to the WHO semen analysis manual (WHO, 1999).

Histological analysis

A part of the testis was put into Bouin's fluid for fixation, and subsequently was embedded in paraffin for the histological sections followed by microscopic examination in accordance with routine laboratory procedure. Paraffin sections of 4–5 µm were prepared and stained with hematoxylin and eosin for histological examination. Histomorphometric analysis was performed by KLONK Image Measurement Light software (Version: 13.2.2.12).

Protocol of male fertility and fecundity

Male mice treated for different durations (15, 45, 90, 135 and 180 d) were used for the fertility and fecundity tests. Eighty (80) female Swiss albino mice (25 ± 2 g of body weight [bw]) of 6–8 weeks of age were used for fertility and fecundity tests. After the end of each treatment, randomly selected male mice were allowed to mate with fertile females (male and female in 1:2 ratios) and they were left together for 15 d. This period is sufficient to cover the mouse estrous cycle which takes 4–5 d. After the mating test, each female was observed for delivery (19–21 d following the mating test) as a criterion of successful insemination. At the delivery, the entire litters, number of live pups and any clinical signs and mortalities were recorded. Fecundity was also calculated. Fecundity represents the ratio of the number of male mice siring at least one viable pup to the total number of male mice exposed for mating × 100 (Mbongue et al., 2011). Pups were followed up until adulthood.

Drug Chem Toxicol, Early Online: 1–12

Table 1. Effect of BDE on body weight, absolute- and relative-weight of testis, and relative-weight of other sexual organs after sub-acute and sub-chronic doses.

	Sub-acute dose – I (15 d) (S1)				Sub-acute dose – II (45 d) (S2)				Sub-chronic dose (90 d) (S3)			
Parameters[¶]	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Body weight (g)	25.13 ± 0.62	25.03 ± 0.91	25.05 ± 0.82	25.27 ± 1.03	26.23 ± 0.52	26.03 ± 1.91	26.05 ± 0.82	25.27 ± 1.03	25.83 ± 0.52	25.03 ± 2.93	25.25 ± 0.87	25.27 ± 2.13
Absolute testis weight (mg)	235.20 ± 5.54	235.03 ± 6.60	233.04 ± 7.04	233.07 ± 4.79	234.47 ± 6.64	235.98 ± 6.92	234.77 ± 5.75	232.83 ± 4.97	233.97 ± 5.02	231.43 ± 6.14	234.47 ± 9.04	232.57 ± 9.89
Relative testis weight (g/100 g of body weight)	0.98 ± 0.05	0.98 ± 0.07	0.98 ± 0.07	0.97 ± 0.06	0.96 ± 0.08	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.06	0.94 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.94 ± 0.06
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.03	0.34 ± 0.05	0.33 ± 0.02	0.34 ± 0.07	0.35 ± 0.03	0.34 ± 0.05	0.34 ± 0.02	0.33 ± 0.07	0.34 ± 0.03	0.33 ± 0.05	0.33 ± 0.02	0.32 ± 0.07
Relative seminal vesicle weight (g/100 g of body weight)	0.49 ± 0.06	0.48 ± 0.03	0.47 ± 0.05	0.47 ± 0.03	0.48 ± 0.06	0.47 ± 0.03	0.47 ± 0.05	0.46 ± 0.03	0.47 ± 0.06	0.47 ± 0.03	0.46 ± 0.05	0.45 ± 0.03
Relative prostate gland weight (g/100 g of body weight)	0.25 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.24 ± 0.05	0.26 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.25 ± 0.05	0.24 ± 0.01	0.24 ± 0.02	0.24 ± 0.05	0.23 ± 0.05

[¶]All values are mean ± SD of three observations. Data shows no significant differences of the treated groups from the respective controls.

Statistical analysis

Data have been presented as mean ± SD of three observations. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Differences in mean ± SD among different groups were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test. $p < 0.05$ was considered significant.

Results

Body weight, absolute testis weight and relative-weight of other organs

No significant alterations in the body weight have been observed in mice that were treated with all the doses for sub-acute, sub-chronic and chronic durations (Tables 1 and 2). The absolute- and relative-weight of testis, and relative-weights of epididymis, seminal vesicle and prostate of animals treated with the BDE at the dose of 160 and 320 mg/kg bw have been decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) after 135 and 180 d of treatment (Table 2).

Hypo-osmotic swelling test (HOST)

Figure 1(a) indicated that no significant alterations were observed in the viability of sperm after 15 d of treatment with BDE. But, after sub-acute (45 d), sub-chronic (90 d) and chronic (135 and 180 d) treatment with BDE at all the doses (80, 160 and 320 mg/kg bw), sperm viability reduced significantly ($p < 0.001$) when compared with the respective control groups (Figures 1b–e). After 180 d of treatment with BDE, at 0 mg/kg bw (Control), the percentage of live sperm was 83.17 ± 3.19, whereas, at 320 mg/kg bw, the percentage of viable sperm decreased to 35.83 ± 2.64.

Effect of BDE on different biochemical parameters of sexual organs

Changes of biochemical parameters after different doses of treatment are outlined in Tables 3 and 4. Protein levels in serum, testis and epididymis of BDE treated mice (320 mg/kg bw) were decreased significantly ($p < 0.05$) after 90 d of treatment. After 135 and 180 d of treatment at 320 mg/kg bw of BDE, protein contents in serum, testis and epididymis were also decreased significantly ($p < 0.001$) when compared with the respective controls. Cholesterol level in testis was decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in mice receiving BDE at the dose of 160 and 320 mg/kg bw after sub-chronic and chronic treatments. Fructose and α-glucosidase levels of BDE treated mice (at 160 and 320 mg/kg bw) significantly decreased ($p < 0.05$, $p < 0.01$ and $p < 0.001$) after 135 and 180 d of treatment when compared with the control groups. The glycogen level in testis decreased significantly ($p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for sub-chronic and chronic durations. Sialic acid concentration in epididymis decreased significantly ($p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for 135 and 180 d of treatment when compared with the control groups. The concentration of citric acid content in prostate decreased significantly

Table 2. Effect of BDE on body weight, absolute- and relative-weight of testis and, and relative-weight of other sexual organs after chronic doses.

Parameters[¶]	Chronic dose – I (135 d) (S4)				Chronic dose – II (180 d) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.13 ± 0.18	24.86 ± 0.24	24.53 ± 0.15	24.12 ± 0.11	24.83 ± 0.32	23.03 ± 0.93	22.85 ± 0.77	22.27 ± 1.13
Absolute testis weight (mg)	234.37 ± 8.01	233.07 ± 8.91	232.66 ± 1.96	219.33 ± 2.05[a] (p=0.042)	233.57 ± 6.54	232.43 ± 5.94	235.30 ± 4.55	221.30 ± 3.65[a] (p=0.048)
Relative testis weight (g/100 g of body weight)	0.95 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.93 ± 0.06[a] ($p = 0.042$)	0.95 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.91 ± 0.01[b] ($p = 0.002$)
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.01	0.31 ± 0.02	0.27 ± 0.01[b] ($p = 0.002$)	0.27 ± 0.01[b] ($p = 0.002$)	0.33 ± 0.02	0.33 ± 0.01	0.27 ± 0.01[b] ($p = 0.002$)	0.24 ± 0.01[c] ($p = 0.000002$)
Relative seminal vesicle weight (g/100 g of body weight)	0.48 ± 0.01	0.46 ± 0.01	0.45 ± 0.01[a] ($p = 0.033$)	0.44 ± 0.01[b] ($p = 0.005$)	0.48 ± 0.01	0.43 ± 0.02[a] ($p = 0.035$)	0.42 ± 0.02[b] ($p = 0.002$)	0.37 ± 0.02[c] ($p = 0.000002$)
Relative prostate gland weight (g/100 g of body weight)	0.24 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.22 ± 0.01[b] ($p = 0.002$)	0.24 ± 0.01	0.22 ± 0.01[b] ($p = 0.002$)	0.21 ± 0.01[c] ($p = 0.00004$)	0.19 ± 0.01[c] ($p = 0.000003$)

[¶]All values are mean ± SD of three observations.
[a]$p < 0.05$ when compared with control (G1).
[b]$p < 0.01$ when compared with control (G1).
[c]$p < 0.001$ when compared with control (G1).

($p < 0.05$, $p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for sub-chronic and chronic durations. The testicular acid phosphatase concentration has also been decreased significantly ($p < 0.01$ and $p < 0.001$) in a dose- and time-dependent manner within a duration of 180 d of treatment of BDE at different doses, when compared to the respective control groups.

Alterations in the architecture of testes

No significant morphological alterations in the architecture of testes were observed in sub-acute and sub-chronic doses, and therefore, data was not presented. Morphological alterations of seminiferous tubules were observed only in the groups treated with 320 mg/kg bw of BDE for 135 and 180 d when compared to the control testis. Significant decreases in diameter, perimeter and area of the seminiferous tubules were observed in case of mice treated with 160 and 320 mg/kg bw of BDE for 180 d ($p < 0.01$ and $p < 0.001$) when compared with the control groups. The number of empty seminiferous tubules has been shown to increase with the increasing dose duration. Significant increases ($p < 0.05$ and $p < 0.001$) in the percentage of empty seminiferous tubules were also observed after treatment with 160 and 320 mg/kg bw of BDE for 135 and 180 d when compared with the control groups (Table 5). In the group treated with BDE at 320 mg/kg bw for 180 d, the epithelium was reduced to a single layer in few of the tubules (Figure 4).Seminiferous tubules of mice treated with 320 mg/kg bw of BDE for 135 d (Figure 3) and 180 d (Figure 4) showed vacuolization in the tubules with reduced epithelial membranes layer, when compared to the control (Figure 2).

Effect of *D. esculentum* on fertility and fecundity

As outlined in Tables 6 and 7, the fertility as well as fecundity has been decreased in a dose- and time-dependent manner in BDE treated mice when compared to the respective control groups. The percentage of fertility (no. of viable pups) has decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective control groups. After sub-chronic and chronic treatments at 320 mg/kg bw, no viable pups have been recorded. Similarly, percentage of fecundity has been decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective controls. Hundred percent fertility losses observed at 320 mg/kg bw dose of BDE that were treated for sub-chronic and chronic durations. Similarly, 100% losses of fecundity observed in mice that were treated with 160 and 320 mg/kg bw of BDE for 180 d.

Discussion

Very few of the pharmacological activities of *D. esculentum* have been reported till date, and especially very little is known about the effect of *D. esculentum* on the male reproductive system. We have previously reported that BDE can affect male reproductive system and cause infertility through its spermicidal properties (Roy et al., 2013c). In continuation of our previous investigations, this work was aimed to investigate the effects of boiled aqueous preparations of *D. esculentum* on testis histology as well as on some

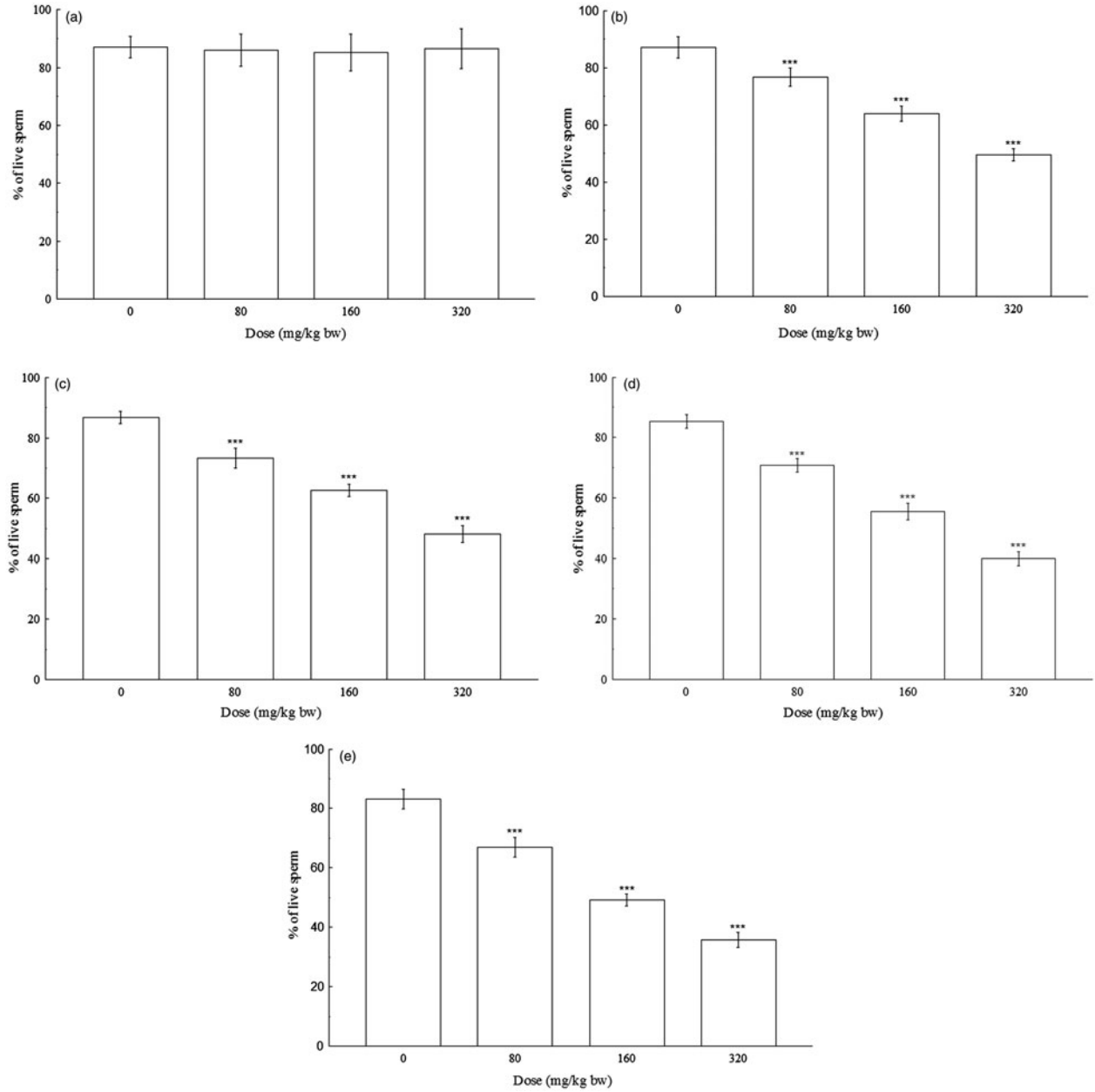


Figure 1. (a) Hypo-osmotic swelling test (HOST) of spermatozoa. Figure shows no significant decrease after 15 d of treatment. The results are mean ± SD of three parallel observations; (b) Hypo-osmotic swelling test (HOST) of spermatozoa demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 45 d. The results are mean ± SD of three parallel observations. ***$p < 0.001$ versus control; (c) Hypo-osmotic swelling test (HOST) of spermatozoa demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 90 d. The results are mean ± SD of three parallel observations. ***$p < 0.001$ versus control; (d) Hypo-osmotic swelling test (HOST) of spermatozoa demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 135 d. The results are mean ± SD of three parallel observations. ***$p < 0.001$ versus control; (e) Hypo-osmotic swelling test (HOST) of spermatozoa demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 180 d. The results are mean ± SD of three parallel observations. ***$p < 0.001$ versus control.

biochemical parameters of testis and other accessory organs of Swiss albino mice.

Body weight increase is considered to be 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 appropriate organ to be weighed in toxicological studies involves understanding the test article's mechanism of action, metabolism, toxicokinetics and the physiology of the test species (Khan et al., 2011). In this study, significant losses of the body weight, absolute- and relative-testis weight and relative-weight of different reproductive organs weight indicate the toxic properties of *D. esculentum*. Significant decreases in body weight, absolute- and relative-testis weight and relative epididymis, seminal vesicles and prostate weight of BDE treated male mouse were observed. This may be due to the possible adverse effects of BDE on somatic cells or indirectly through the central nervous system which controls the feed and water intake and regulates the endocrine function.

DOI: 10.1080/01480545.2016.1190739

Table 3. Effect of BDE on different biochemical parameters of testis and other sexual organs after sub-acute (15 and 45 d) and sub-chronic doses (90 d).

	Sub-acute dose – I (15 d) (S1)				Sub-acute dose – II (45 d) (S2)				Sub-chronic dose (90 d) (S3)			
Parameters[¶]	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Total protein (serum) (mg/dl)	7.84 ± 0.75	7.74 ± 0.83	7.73 ± 0.59	7.82 ± 1.11	7.80 ± 0.81	7.56 ± 0.52	7.68 ± 0.27	7.60 ± 0.33	7.52 ± 0.53	7.21 ± 0.28	6.96 ± 0.32	6.91 ± 0.40[a] ($p = 0.041$)
Total protein (testis) (mg/g)	0.50 ± 0.14	0.42 ± 0.15	0.43 ± 0.17	0.44 ± 0.20	0.52 ± 0.07	0.49 ± 0.07	0.47 ± 0.07	0.43 ± 0.04	0.56 ± 0.03	0.54 ± 0.03	0.52 ± 0.03	0.51 ± 0.01[a] ($p = 0.032$)
Total protein (epididymis) (mg/g)	0.20 ± 0.07	0.19 ± 0.05	0.18 ± 0.07	0.19 ± 0.08	0.22 ± 0.06	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	0.24 ± 0.05	0.20 ± 0.04	0.19 ± 0.05	0.17 ± 0.04[a] ($p = 0.037$)
Cholesterol (testis) (mg/g)	3.89 ± 0.18	3.79 ± 0.18	3.85 ± 0.16	3.85 ± 0.24	3.82 ± 0.14	3.84 ± 0.11	3.84 ± 0.09	3.75 ± 0.09	3.84 ± 0.11	3.69 ± 0.13	3.71 ± 0.18	3.48 ± 0.06[a] ($p = 0.022$)
α-glucosidase (epididymis) (mU/g)	4.81 ± 0.10	4.79 ± 0.10	4.75 ± 0.06	4.79 ± 0.15	4.82 ± 0.03	4.74 ± 0.10	4.72 ± 0.03	4.74 ± 0.11	4.80 ± 0.01	4.67 ± 0.05[a] ($p = 0.042$)	4.65 ± 0.06[a] ($p = 0.021$)	4.56 ± 0.06[b] ($p = 0.0012$)
Fructose (seminal vesicles) (μM/g)	4.54 ± 0.16	4.50 ± 0.10	4.48 ± 0.02	4.49 ± 0.13	4.53 ± 0.08	4.47 ± 0.07	4.52 ± 0.06	4.48 ± 0.08	4.61 ± 0.10	4.54 ± 0.07	4.48 ± 0.08	4.53 ± 0.06
Glycogen (testis) (mg/g)	37.48 ± 2.35	37.66 ± 2.77	37.19 ± 4.30[a] ($p = 0.04$)	37.27 ± 4.58[a] ($p = 0.02$)	39.78 ± 1.35	39.66 ± 2.25	39.19 ± 2.30	38.27 ± 3.53	38.52 ± 2.58	37.66 ± 3.25	37.19 ± 3.20	33.27 ± 0.53[b] ($p = 0.005$)
Sialic acid (epididymis) (μM/100 g tissue)	64.25 ± 2.21	64.35 ± 3.37	64.45 ± 2.39	64.25 ± 3.58	65.25 ± 2.21	65.11 ± 0.58	64.87 ± 2.02	64.80 ± 3.01	63.14 ± 0.69	63.02 ± 0.98	62.38 ± 0.36	61.78 ± 0.81[a] ($p = 0.032$)
Prostate citric acid (mg/g)	35.02 ± 2.52	34.75 ± 3.01	34.37 ± 1.36	33.33 ± 1.99	36.42 ± 2.52	36.25 ± 1.71	36.37 ± 1.56	35.33 ± 2.99	36.02 ± 1.52	35.75 ± 1.51	37.37 ± 0.56	31.33 ± 0.99[a] ($p = 0.023$)
Acid phosphatase (testis) (μM/min/g of tissue)	30.09 ± 5.20	28.60 ± 0.66	24.01 ± 1.50	23.83 ± 0.39	31.05 ± 2.79	31.98 ± 2.60	31.65 ± 2.32	22.46 ± 0.96[b] ($p = 0.003$)	30.86 ± 3.87	26.05 ± 2.97	21.65 ± 0.48[b] ($p = 0.005$)	18.50 ± 0.97[c] ($p = 0.0008$)

[¶]All values are mean ± SD of three observations.
[a]$p < 0.05$ when compared with control (G1).
[b]$p < 0.01$ when compared with control (G1).
[c]$p < 0.001$ when compared with control (G1).

Table 4. Effect of BDE on different biochemical parameters of testis and other sexual organs after chronic doses (135 and 180 d).

	Chronic dose – I (135 d) (S4)				Chronic dose – II (180 d) (S5)			
Parameters[¶]	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Total protein (serum) (mg/dl)	7.84 ± 0.49	7.12 ± 0.16[b] ($p = 0.006$)	6.68 ± 0.32[c] ($p = 0.00003$)	6.35 ± 0.35[c] ($p = 0.00002$)	7.85 ± 0.83	6.78 ± 0.41[b] ($p = 0.002$)	6.06 ± 0.30[c] ($p = 0.00003$)	5.64 ± 0.55[c] ($p = 0.00001$)
Total protein (testis) (mg/g)	0.58 ± 0.04	0.53 ± 0.01[a] ($p = 0.03$)	0.51 ± 0.03[c] ($p = 0.0005$)	0.48 ± 0.02[c] ($p = 0.0002$)	0.56 ± 0.03	0.52 ± 0.03[a] ($p = 0.02$)	0.48 ± 0.02[c] ($p = 0.00002$)	0.41 ± 0.01[c] ($p = 0.00001$)
Total protein (epididymis) (mg/g)	0.25 ± 0.04	0.20 ± 0.02[a] ($p = 0.02$)	0.18 ± 0.02[c] ($p = 0.0002$)	0.14 ± 0.01[c] ($p = 0.00001$)	0.28 ± 0.02	0.19 ± 0.01[c] ($p = 0.00002$)	0.13 ± 0.01[c] ($p = 0.000002$)	0.08 ± 0.01[c] ($p = 0.000001$)
Cholesterol (testis) (mg/g)	3.85 ± 0.13	3.66 ± 0.08	3.53 ± 0.03[b] ($p = 0.003$)	3.42 ± 0.02[c] ($p = 0.0005$)	3.82 ± 0.08	3.53 ± 0.12[b] ($p = 0.008$)	3.43 ± 0.07[b] ($p = 0.0013$)	3.27 ± 0.06[c] ($p = 0.0001$)
α-glucosidase (epididymis) (mU/g)	4.76 ± 0.06	4.63 ± 0.03[a] ($p = 0.044$)	4.61 ± 0.08[a] ($p = 0.024$)	4.52 ± 0.05[b] ($p = 0.002$)	4.77 ± 0.06	4.56 ± 0.06[b] ($p = 0.006$)	4.40 ± 0.05[c] ($p = 0.00015$)	4.32 ± 0.06[c] ($p = 0.00003$)
Fructose (seminal vesicles) (μM/g)	4.56 ± 0.11	4.46 ± 0.05	4.41 ± 0.07	4.36 ± 0.04[a] ($p = 0.027$)	4.52 ± 0.08	4.42 ± 0.04	4.35 ± 0.06[a] ($p = 0.016$)	4.30 ± 0.04[b] ($p = 0.004$)
Glycogen (testis) (mg/g)	39.58 ± 1.58	38.66 ± 2.25	33.19 ± 1.20[b] ($p = 0.006$)	30.27 ± 0.53[c] ($p = 0.0006$)	40.58 ± 1.38	31.46 ± 2.85[c] ($p = 0.0001$)	27.19 ± 3.28[c] ($p = 0.00007$)	20.27 ± 3.53[c] ($p = 0.00002$)
Sialic acid (epididymis) (μM/100 g tissue)	62.12 ± 3.24	56.71 ± 0.93[a] ($p = 0.039$)	50.03 ± 1.14[c] ($p = 0.0004$)	41.06 ± 0.81[c] ($p = 0.000008$)	63.59 ± 2.24	54.36 ± 3.36[c] ($p = 0.00002$)	40.25 ± 3.35[c] ($p = 0.00001$)	31.36 ± 2.54[c] ($p = 0.000002$)
Prostate citric acid (mg/g)	37.02 ± 1.52	31.75 ± 1.51[a] ($p = 0.018$)	27.37 ± 2.56[b] ($p = 0.004$)	21.33 ± 0.99[c] ($p = 0.00002$)	36.58 ± 2.27	25.48 ± 3.35[c] ($p = 0.00004$)	21.36 ± 3.69[c] ($p = 0.00001$)	18.47 ± 1.36[c] ($p = 0.000005$)
Acid phosphatase (testis) (μM/min/g of tissue)	30.07 ± 5.12	23.94 ± 0.88[b] ($p = 0.008$	20.34 ± 1.44[c] ($p = 0.0007$)	16.29 ± 0.05[c] ($p = 0.0001$)	33.72 ± 0.38	21.70 ± 0.60[c] ($p = 0.000003$)	18.35 ± 1.02[c] ($p = 0.000002$)	15.96 ± 1.57[c] ($p = 0.000002$)

[¶] All values are mean ± SD of three observations.
[a] $p < 0.05$ when compared with control (G1).
[b] $p < 0.01$ when compared with control (G1).
[c] $p < 0.001$ when compared with control (G1).

DOI: 10.1080/01480545.2016.1190739

Table 5. Histomorphometric parameters of seminiferous tubules.

	Chronic dose – I (135 d) (S4)				Chronic dose – II (180 d) (S5)			
Parameters[¶]	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Diameter (in µm)	273.44 ± 9.25	256.91 ± 14.50	242.79 ± 14.87	240.76 ± 15.21[a] ($p = 0.047$)	271.92 ± 7.66	268.46 ± 9.73	253.79 ± 8.05	241.45 ± 7.17[b] ($p = 0.005$)
Perimeter (in µm)	964.35 ± 16.50	863.78 ± 12.55[c] ($p = 0.00007$)	765.03 ± 15.34[c] ($p = 0.000003$)	737.85 ± 12.83[c] ($p = 0.000002$)	989.46 ± 30.27	564.31 ± 33.06[c] ($p = 0.00005$)	365.11 ± 21.98[c] ($p = 0.000003$)	239.64 ± 27.56[c] ($p = 0.000002$)
Area (in sq. µm)	61122.62 ± 1927.22	42978.20 ± 2631.77[c] ($p = 0.000003$)	40954.62 ± 1574.45[c] ($p = 0.000002$)	37948.97 ± 3270.21[c] ($p = 0.000001$)	61583.03 ± 1644.35	50618.27 ± 1559.85[c] ($p = 0.000002$)	40007.95 ± 1379.22[c] ($p = 0.000001$)	32728.41 ± 3615.11[c] ($p = 0.000001$)
Percentage of empty seminiferous tubules	11.60 ± 3.15	12.42 ± 3.13	15.21 ± 3.15	19.96 ± 1.99[a] ($p = 0.019$)	11.62 ± 2.78	16.52 ± 1.92	20.11 ± 2.55[a] ($p = 0.012$)	28.80 ± 3.36[c] ($p = 0.0001$)

[¶]All values are mean ± SD of three observations.
[a]$p < 0.05$ when compared with control (G1).
[b]$p < 0.01$ when compared with control (G1).
[c]$p < 0.001$ when compared with control (G1).

Figure 2. Histology of the testis of control mice (observed with a magnitude of ×400 under the microscope, stained with hematoxylin–eosin) showing normal architecture in seminiferous tubules with intact epithelial membranes and no vacuolization.

Figure 3. (a & b) Histology of the testis of mice treated with 320 mg/kg bw of BDE for 135 d (observed with a magnitude of ×400 under the microscope, stained with hematoxylin–eosin). Figure shows vacuolization in the seminiferous tubules (indicated by white arrow) with reduced epithelial membranes layer.

As indicated by the histological examinations, BDE caused various structural abnormalities in testes. Seminiferous tubules were shrunken and appeared to be displaced, lumen diameter was decreased and vacuolization occurred in the interstitial spaces. This may probably explain the decrease in testes weight.

The hypo-osmotic solution results in the loss in permeability of plasma membrane under the stress of swelling. The HOST evaluation determines the resistance of the sperm

plasma membrane to damage, that is induced by the hypo-osmotic treatment (Bucak et al., 2009). Therefore, this test may be useful when testing the membrane stabilizing action against a potential toxic agent. Results of this study clearly showed the dose- and time-dependent decrease in percentages of viable spermatozoa in BDE treated mice when compared to the control groups, indicating the presence of toxic properties in *D. esculentum*.

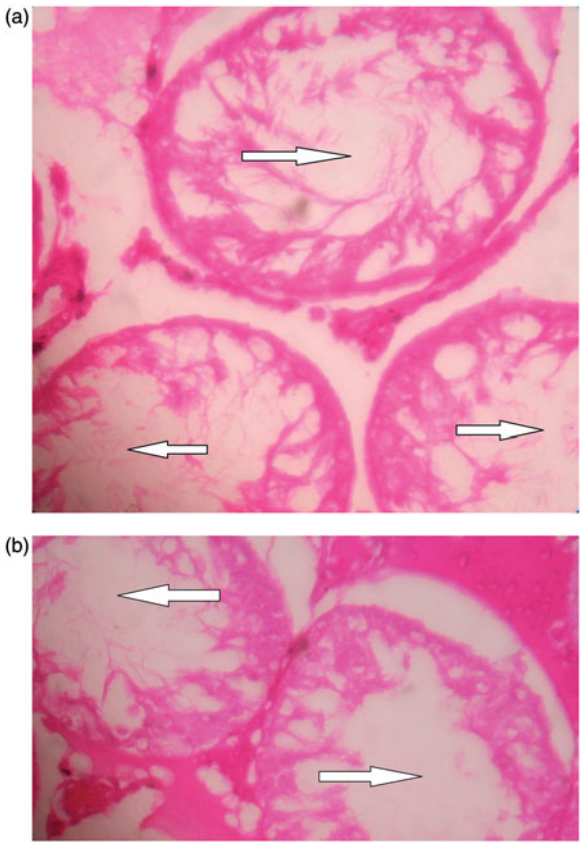


Figure 4. (a & b) Histology of the testis of mice treated with 320 mg/kg bw of BDE for 180 d (observed with a magnitude of ×400 under the microscope, stained with hematoxylin–eosin) showing increased vacuolization in the tubules (indicated by white arrow) with reduced epithelial membrane layers. The number of empty seminiferous tubules as well as inter-seminiferous tubular spaces has been shown to increase with the increased dose duration when compared to the control.

In this study, BDE reduced the cholesterol content significantly. Decreased cholesterol level in testis may possibly lead to the decreased testosterone level in the testes and blood, increased blood levels of the signaling luteinizing hormone (LH), altered mitochondrial membranes in Leydig cells, changed gene expression which controls important proteins and reduced sperm health and numbers (Zhang et al., 2007).

This study showed significant difference between treated and untreated groups in epididymal α-glucosidase activity in caudal epididymis. α-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 (Krause & Bohring, 1999). A decrease in fructose level in the testis of BDE treated animals was also observed. Since the function of fructose is to induce the glycolytic metabolism of spermatozoa, it can be said 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 male infertility.

Glucose is stored in animal tissue in the form of glycogen, which acts as an energy producing source. Glucose has been shown to be an essential substrate for maintaining tissue integrity, ATP production and protein synthesis in the rat testis (Bajpai et al., 1998). It has been observed that the testicular interstitial cells are a good source of glycogen (Klip et al., 1994). In this work, highly significant decreased level of glycogen content was observed in the BDE treated mice when compared to the control, 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

Table 6. Effects of the BDE on fertility of Swiss albino mouse after different days of treatment.

Treatment	Ratio of males and females for fertility test	% of fertility (no. of viable pups) after different periods				
		15 d	45 d	90 d	135 d	180 d
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	80
160 mg/kg bw	½	100	80	80	50	50
320 mg/kg bw	½	50	50	0	0	0

Table 7. Effects of the BDE on fecundity of Swiss albino mouse after different days of treatment.

Treatment	Ratio of males and females for fecundity test	% of fecundity after different periods				
		15 d	45 d	90 d	135 d	180 d
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	75
160 mg/kg bw	½	100	100	100	75	0
320 mg/kg bw	½	100	100	100	75	0

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 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). Alterations in sialic acid level in reproductive tissues indicate changes in the level of glycoprotein/FSH and LH which is needed for normal functioning of the gonads and accessory reproductive organs (Gupta et al., 2002). In this study, sialic acid content of testis significantly decreased in all the dose groups. Depletion in the testicular sialic acid content in the mice possibly reflects the androgen and gonadotrophic deficiency resulting in the inhibition of spermatogenesis, loss of spermatozoa motility and fertilizing ability (Gheri et al., 2009).

This study showed that the reduction in prostate weight was associated with a significant decrease in prostate citric acid content when mice were fed with 320 mg/kg bw of BDE. These results suggest the 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 this study, the decrease in acid phosphatase activity might reflect decreased testicular function in the treated mice, and therefore, may interfere with the secretion of testosterone.

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 this 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 reduced epithelial cell height and lack of sperms in the lumen. As the meiosis in most of the germ cells have halted in early stage, therefore, in this study, it was not possible to differentiate the primary and secondary spermatocytes in most of the tubules. Appearance of intraepithelial vacuolations may be due to the intraepithelial edema and altered intercellular connections, due to chronic cytological toxicity of a particular drug. Similar intraepithelial vacuolations are reported in mice treated with Neem extract (Mishra & Singh, 2005) and Brahmi leaves (Singh & Singh, 2009). Occasional such intraepithelial vacuolations have been observed in this study, indicating its toxicity.

Previous findings revealed that 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, alterations in the values of blood glucose and total leukocyte count, increase in serum glutamic oxaloacetic transaminase and serum dehydrogenases. Feeding of frozen dried sample of *D. esculentum* induced mortality rate in guinea pigs (Gangwar, 2004). Most of the studies done so far were on the freeze- or shade-dried samples of *D. esculentum*, and its effect on rabbits and guinea pigs. But, there is little information available regarding the effect of boiled preparation of *D. esculentum* on different laboratory animals, such as mouse, rabbits or guinea pigs. This study was performed using mouse as this is the standard convention to use inbreed strains of mouse for performing the pharmacological experiments. We performed the study using boiled preparations of *D. esculentum*, to find out the possible effect of temperature on some toxic compounds present, if any, in *D. esculentum*, which may inhibit the male reproductive functions in adult Swiss albino mouse.

As ptaquiloside is a heat labile compound, boiling may probably reduce its toxicity. Results of this study indicated that BDE caused significant reduction in different biochemical parameters of testis and other accessory organs in Swiss albino mouse, as well as it (BDE) altered the histological architecture of testis. Therefore, it is clearly evident that it may possess some compounds besides ptaquiloside that can withstand heat and provide toxicity. Study revealed that *Diplazium sammatii*, a related edible fern, contains moderate amount of tannins which 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 boiled preparation of *D. esculentum*, and may be one of the factors of inhibition of reproductive function.

Conclusion

It can be concluded that *D. esculentum*, even boiled, possesses toxic properties that can hamper the male reproductive functions considering the findings of this study. The chronic feeding of *D. esculentum* altered the testicular and other accessory gland (prostate, epididymis and seminal vesicle) functions. This study was an attempt to report the reproductive dysfunction due to the intake of the edible *D. esculentum*, and thereby to make people aware about the hazards of its consumption and it will advance the existing knowledge in relation to human health. Further studies are, however, needed for a better understanding of the effect of *D. esculentum* on the functional physiology of the male reproductive system.

Declaration of interest

The authors report no declarations of interest. We gratefully acknowledge the financial support (Vide Letter F.No.37–464/2009 (SR) dt.11.01.2010) received from the University Grants Commission (UGC), New Delhi, India to carry out this study.

References

Akter S, Hossain MM, Ara I, Akhtar P. (2014). Investigation of in vitro antioxidant, antimicrobial and cytotoxic activity of *Diplazium esculentum* (RETZ). SW. Available from: http://www.ijapbc.com/files/32-3369.pdf. Accessed on 15 November 2014.

Aminoff D. (1961). Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem J 81:384–392.

Anderson AR, Reddy JM, Oswald C, Zaneveld LJD. (1979). Enzymic determination of fructose in seminal plasma by initial rate analysis. Clin Chem 25:1780–1782.

Bajpai M, Gupta G, Setty BS. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol 138:322–327.

Bassey ME, Etuk EUI, Ibe MM, Ndon BA. (2001). *Diplazium sammatii*: Athyraceae ('Nyama idim'): age-related nutritional and antinutritional analysis. Plant Foods Hum Nutr 56:7–12.

Bucak MN, Tuncer PB, Sarıözkan ve S, Ulutas PA. (2009). Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. Small Rumin Res 81:13–17.

Buckett WM, Luckas MJM, Aird IA, et al. (1997). The hypo-osmotic swelling test in recurrent miscarriage. Fertil Steril 68: 506–509.

Chemes H. (1986). The phagocytic function of sertoli cells: a morphological, biochemical, and endocrinological study of lysosomes and acid phosphatase localization in the rat testis. Endocrinology 119: 1673–1681.

Costello LC, Franklin RB. (2002). Testosterone and prolactin regulation of metabolic genes and citrate metabolism of prostate epithelial cells. Horm Metab Res 34:417–424.

Gangwar NK. (2004). Studies on the pathological effects of linguda (*Diplazium esculentum*, Retz.) in laboratory rats and guinea pigs. Indian J Vet Pathol 28:149–150.

Gheri G, Vichi D, Thyrion GD, et al. (2009). Sialic acid in human testis and changes with aging. Reprod Fertil Dev 21:25–33.

Gupta RS, Sharma R, Sharma A, et al. (2002). Effect of *Alstonia scholaris* bark extract on testicular function of Wistar rats. Asian J Androl 4:175–178.

Hammami I, Nahdi A, Mauduit C, et al. (2008). The inhibitory effects on adult male reproductive functions of crude garlic (*Allium sativum*) feeding. Asian J Androl 10:593–601..

Khan MI, Denny-Joseph KM, Muralidhara, et al. (2011). Acute, subacute and subchronic safety assessment of betalains rich *Rivina humilis* L. berry juice in rats. Food Chem Toxicol 49:3154–3157.

Klip A, Tsakiridis T, Marette A, Ortiz PA. (1994). Regulation of expression of glucose transporters by glucose: a review of studies in vivo and in cell cultures. FASEB J 8:43–53.

Krause W, Bohring C. (1999). Why do we determine alpha-glucosidase activity in human semen during infertility workup? Andrologia 31: 289–294

Mathur PP, Chattopadhyay S. (1982). Involvement of lysosomal enzymes in flutamide-induced stimulation of rat testis. Andrologia 14:171–176.

Mbongue GYF, Kamtchouing P, Dimo T. (2011). Effects of the aqueous extract of dry seeds of *Aframomum melegueta* on some parameters of the reproductive function of mature male rats. Andrologia 44:53–58.

Mishra RK, Singh SK. (2005). Effect of aqueous leaf extract of *Azadirachta indica* on the reproductive organs in male mice. Indian J Exp Biol 43:1093–1103.

Montgomery R. (1957). Determination glycogen. Arch Biochem Biophys 67:378–389.

Revell SG, Mrode RA. (1994). An osmotic resistance test for bovine semen. Anim Reprod Sci 36:77–86.

Roy S, Hazra B, Mandal N, Chaudhuri TK. (2013a). Assessment of the antioxidant and free radical scavenging activities of methanolic extract of *Diplazium esculentum.* Int J Food Prop 16:1351–1370.

Roy S, Tamang S, Dey P, Chaudhuri TK. (2013b). Assessment of the immunosuppressive and hemolytic activities of an edible fern, *Diplazium esculentum.* Immunopharmacol Immunotoxicol 35: 365–372.

Roy S, Tamang S, Chaudhuri TK. (2013c). Sperm viability assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of Swiss albino mice treated with *Diplazium esculentum.* Asian J Pharm Health Sci 3:684–689.

Roy S, Chaudhuri TK. (2015). Assessment of Th1 and Th2 cytokine modulatory activity of an edible fern, *Diplazium esculentum.* Food Agri Immunol 26:690–702.

Roy S, Dutta S, Chaudhuri TK. (2015). In vitro assessment of anticholinesterase and NADH oxidase inhibitory activities of an edible fern, *Diplazium esculentum.* J Basic Clin Physiol Pharmacol 26: 395–401.

Schilter B, Andersson C, Anton R, et al. (2003). Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. Food Chem Toxicol 41:1625–1649.

Singh A, Singh SK. (2009). Evaluation of antifertility potential of Brahmi in male mouse. Contraception 79:71–79.

Smith BL, Seawright AA, Ng JC, et al. (1994). Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium* spp.) from eastern Australia and from cultivated worldwide collection held in Sydney, Australia. Nat Toxins 2:347–353.

Somvanshi R, Lauren DR, Smith BL, et al. (2006). Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. Curr Sci 91:1547–1552.

Teklehaymanot T, Giday M. (2010). Ethnobotanical study of wild edible plants of Kara and Kwego semi-pastoralist people in Lower Omo River Valley, Debub Omo Zone, SNNPR, Ethiopia. J Ethnobiol Ethnomed [Online] Available from: http://www.ethnobiomed.com/content/pdf/1746-4269-6-23.pdf. Accessed on 14 November 2011.

Thomas GT, Rebecca S, Andre BA. (2008). The natural history of symptomatic androgen deficiency in men: onset, progression, and spontaneous remission. J Am Geriatr Soc 56:831–893.

Tongco JVV, Villaber RAP, Aguda RM, Razal RA. (2014). Nutritional and phytochemical screening, and total phenolic and flavonoid content of *Diplazium esculentum* (Retz.) Sw. from Philippines. J Chem Pharm Res [Online] Available from: http://jocpr.com/vol6-iss8-2014/JCPR-2014-6-8-238-242.pdf. Accessed on 28 December 2014.

Viveka S, Udyavar A, Shetty B, et al. (2015). Histomorphometric effects of gemcitabine on Swiss albino mice spermatogenesis. Adv Biomed Res [Online] Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4333484. Accessed on 23 May 2015.

Wang ZP, Gu ZP, Cao L, et al. (1999). Effects of tripchlorolide on the epididymides and testes of rats. Asian J Androl 1:121–125.

Wooley A. (2003). Determination – general and reproductive toxicology. In: A guide to practical toxicology evaluation, prediction and risk. New York: Taylor and Francis, 80–106.

WHO. (1999). WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th Ed. Cambridge (UK): Cambridge Univ. Press.

WHO. (2000). WHO manual for the standardized investigation, diagnosis and management of the infertile male. Cambridge (UK): Cambridge Univ. Press.

Zhang SY, Ito Y, Yamanoshita O, et al. (2007). Permethrin may disrupt testosterone biosynthesis via mitochondrial membrane damage of Leydig cells in adult male mouse. Endocrinol 148:3941–3949.

Zuping H, Kokkinaki M, Dym M. (2009). Nodal signaling via an autocrine pathway promotes proliferation of mouse spermatogonial stem/progenitor cells through Smad2/3 and Oct-4 activation. Stem Cells 27:2580–2589.

Food and Agricultural Immunology, 2015
Vol. 26, No. 5, 690–702, http://dx.doi.org/10.1080/09540105.2015.1007449

Assessment of Th1 and Th2 cytokine modulatory activity of an edible fern, *Diplazium esculentum*

Subhrajyoti Roy[a,b] and Tapas Kumar Chaudhuri[a]*

[a]*Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India;* [b]*Immunopharmacology and Molecular Cell Biology Laboratory, Department of Zoology, University of Gour Banga, Malda 732103, West Bengal, India*

(*Received 1 November 2014; accepted 11 January 2015*)

The present study was conducted to determine the effect of *Diplazium esculentum* on Th1 and Th2 cytokine modulation in Swiss albino mice that were administered orally with different doses of boiled *D. esculentum* (BDE), daily within a span of 180 days. After the treatment, serum was collected. Splenocytes were also cultured *in vitro* with different concentrations of BDE, and culture supernatant was collected. Both serum and culture supernatant were used for cytokine determination by enzyme-linked immunosorbent assay (ELISA) for different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines. Results indicated significant decreases ($p < 0.05$, $p < 0.01$, and $p < 0.001$) in both Th1 and Th2 cytokine concentrations when compared with their respective controls. These results suggest that the intake of *D. esculentum*, even after cooking, may evoke immune dysfunction by altering Th1 and Th2 cytokine balance, may induce severe immunosuppression, and may be considered as alarming.

Keywords: *Diplazium esculentum*; ELISA; cytokine regulation; immunosuppression; wild edible plant

1. Introduction

Wild edible plants are very commonly used as food among the indigenous people throughout the world. There are possibilities of having several toxic substances in these plants, which upon ingestion may produce several harmful effects on human health. But, to date, little information is available that describes the toxicological impacts of these plants on human health. One such popular wild edible plant is *Diplazium esculentum* (Koenig ex Retz.) Sw. (Athyriaceae), the most commonly consumed fern throughout Asia and Oceania. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during the monsoon season, which continues for almost five months in the tropical regions where this plant grows abundantly. Our previous study of this fern showed that it possesses trace amounts of flavonoids and phenolic compounds which may confer the antioxidant and free radical scavenging activities of this plant (Roy, Hazra, Mandal, & Chaudhuri, 2013). Another study indicated that the methanol and chloroform extracts of *D. esculentum* possess significant antimicrobial and cytotoxic activity (Akter, Hossain, Ara & Akhtar, 2014). Moreover, phytochemical analysis indicated the presence of different phytochemicals in *D. esculentum* such as alkaloids, anthraquinones, anthranol glycosides, cardiac glycosides, cyanidins, saponins, leucoanthocyanins, phytosterols,

*Corresponding author. Email: dr_tkc_nbu@rediffmail.com

diterpenes, and triterpenes. Some of these phytochemicals not only provide the antioxidant activity to this plant but also aid in other important pharmacological effects in relation to human health (Tongco, Villaber, Aguda, & Razal, 2014).

To date, very few studies have been conducted so far to assess the pharmacological and toxicological impacts of this fern on animal health. Interestingly, this fern is rejected as food by cattle and insets. We observed that this fern grows abundantly in the marshy land and also in the wet shabby places where lot of insects are available. But interestingly, we have never found any insect consuming the leafy portion and all the leaves are intact throughout the season (Roy, Tamang, Dey, & Chaudhuri, 2013). On the basis of our observation, we have planned to perform the experiments using inbreed mouse as this is the standard convention to use inbreed strains of mouse for performing the immunological experiments.

We previously demonstrated that *D. esculentum*, even boiled, possesses potent hemolytic activity and it affects some of the innate and cell-mediated immune responses (Roy, Tamang, Dey et al., 2013). Boiled preparation of this fern has been shown to possess spermicidal and antifertility activities in Swiss albino mouse (Roy, Tamang, & Chaudhuri, 2013). The study conducted on rabbits and guinea pigs demonstrated systemic toxicity and several pathological effects of this fern. Young fronds of *D. esculentum* collected from the high-altitude area of Harsil–Gangotri of North India has been found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria in hill cattle in India and elsewhere (Somvanshi et al., 2006). Pta was found in *D. esculentum* sample that was prepared by freeze- and shade-drying method. Moreover, the frozen- and shade-dried crude *D. esculentum* have already been shown to cause mild pathological effects in rats, and induce mortality and moderate pathological effects in guinea pigs (Gangwar, 2004). Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats (Smith et al., 1994). However, the effect of this fern on Th1/Th2 cytokine modulation has not yet been studied.

T-cells play a critical role in the pathogenesis of various diseases through the production of a variety of cytokines. Modulation of cytokine secretion by herbal immunomodulators may offer novel approaches in the treatment of a variety of inflammatory diseases (Spelman et al., 2006). Among the cells of the immune system, T-cells play a major role in the inflammatory response. Key regulators of this response are a subset of T-cells called CD4-positive T-helper (Th) cells, which can further be differentiated into two subtypes, Th1 and Th2 cells, by the different cytokines produced by these cells (Pacifico et al., 2006).

In the present study, we have investigated the effect of the boiled plant material (boiled *D. esculentum*; BDE) on the serum Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine levels of Swiss albino mice that were treated with different doses of BDE within a span of 180 days. We have also investigated the effect of BDE on Th1 and Th2 cytokine production by Concanavalin A (con A)-induced splenocytes of Swiss albino mouse. Con A is a stimulator of lymphocyte blast formation and possesses mitogenic activity (Kitao & Hattori, 1977). The present study was conducted with the cooked plant material only, because the local population consumes this fern regularly after cooking, not as raw material. Therefore, the focus of the present study was to find out whether the cooked *D. esculentum* possess any immunomodulatory activity by altering the normal

Th1/Th2 cytokine homeostasis, and thereby to find out the presence of any heat resistant toxic compound in this edible fern.

2. Materials and methods

2.1. Preparation of the plant material

Young *D. esculentum* plants were collected during June–August from different areas of North Bengal University campus and the adjoining markets of Darjeeling, West Bengal, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal, where a voucher specimen (Accession No. 9602) was also deposited.

Young frond of *D. esculentum* (100 g) was washed carefully by tap water, then cut into small pieces, and boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed by a mixer and dried in an incubator at 60°C until completely dried. This dried plant material (BDE) was then kept at 4°C for future use.

2.2. Chemicals

Minimum essential medium (MEM), penicillin-streptomycin solution, nystatin, Con A, and trypan blue were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IFN-γ, IL-4 and IL-10 were procured from RayBiotech, Inc., Norcross, USA.

2.3. Animals and surgical procedures

Both male and female Swiss albino mice (25 ± 2 g of body weight [bw]) of 6–8 weeks of age were used for the studies. They were housed in polypropylene cages, with dust free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2°C. Goat (*Capra bengalensis*) blood was collected aseptically from the jugular vein and serum was collected in aliquots and stored at –20°C until further use. All the experiments were performed after obtaining the approval from the Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

2.4. In vivo *experiments*

2.4.1. Dosage

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was subdivided into four groups (G1–4). Therefore, each group contained six mice. All the animals were fed orally with the help of a syringe specially designed by us. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Group 2 (G2), Group 3 (G3), and Group 4 (G4) of all the sets were fed with 0.4 ml of BDE at the dose of 80, 160, and 320 mg/kg bw, respectively. In this way, all groups of S1 were treated daily for 15 d, S2 daily for 45 d, S3 daily for 90 d, S4 daily for 135 d, and S5 daily for 180 d.

We assume that the average maximum amount of cooked *D. esculentum* consumed by a 60 kg weighed individual is about 20 g/d. Keeping this ratio in mind, we formulate

different doses for an average weighed adult mouse (25 g), namely 80 mg/kg bw, i.e. 2 mg/mouse/d; 160 mg/kg bw, i.e. 4 mg/mouse/d; and 320 mg/kg bw, i.e. 8 mg/mouse/d.

2.4.2. Collection of serum

Mice from each group were sacrificed after proper anesthesia (chloroform and ether in 2:1 ratio) 24 h after the last dose, blood was collected from the heart and serum was separated and stored at –20°C for future use. These serum samples were further used to determine the concentration of different cytokines.

2.5. Ex vivo *experiment*

2.5.1. Primary culture of splenocytes

Spleen was aseptically removed from mouse and cell suspension was prepared in MEM [containing penicillin-streptomycin (50 U/ml) and nystatin (50 U/ml)]. The cell number was adjusted to 2×10^6 cells per ml and 1 ml of the cell suspension was added in six-well culture plates. Each well was then supplemented with 10% goat serum (Chaudhuri & Chakravarty, 1983). Five microliters of Con A (5 μg/ml) was also added to stimulate cytokine production. Finally, 100 μl of different concentrations (0–200 μg/ml) of BDE (suspended in MEM) were added to each well. The whole set-up was incubated for 48 h at 37°C in an incubator having 5% CO_2 and 90% humidity. Supernatants of cell cultures were collected after 48 h and used for cytokine estimation.

2.6. Estimation of cytokine production

Previously collected serum samples and splenocyte culture supernatants were used to determine the concentrations of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines by ELISA according to the manufacturer's instructions (RayBiotech, Inc., USA). Briefly, a 96-well flat bottom plate was coated with the captured antibody specific to each cytokine. One hundred microliters of serially diluted specific standards for each cytokine and 100 μl of the serum/cell culture supernatants (samples) were pipetted into the wells. The specific cytokine present in the sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-mouse detection antibody specific for each cytokine was added. After washing away the unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells, and the color was developed in proportion to the amount of specific cytokine bound. Finally, the stop solution was added which changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.7. Statistical analysis

Data have been presented as mean ± SD of three observations. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Differences in mean ± SD among different groups were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test. $p < 0.05$ was considered significant.

Table 1. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 15 (S1) and 45 (S2) days.

Concentration of different cytokines[a]	15 days (S1)				45 days (S2)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
IL-2 (pg/ml)	35.59 ± 1.50	35.44 ± 1.45	34.92 ± 0.17	34.74 ± 0.13	35.21 ± 1.18	35.34 ± 1.02	33.92 ± 0.24	35.30 ± 0.21
IFN-γ (pg/ml)	1147.61 ± 8.16	1144.48 ± 8.75	1144.42 ± 8.39	1143.97 ± 11.07	1149.27 ± 1.38	1139.81 ± 7.52	1134.42 ± 8.39*	1130.64 ± 4.68*
IL-4 (pg/ml)	136.81 ± 2.23	136.55 ± 0.76	135.89 ± 1.47	135.81 ± 1.68	136.14 ± 1.81	134.87 ± 2.79	134.55 ± 0.88	133.14 ± 1.14
IL-10 (pg/ml)	3477.09 ± 3.09	3472.06 ± 7.58	3461.05 ± 6.44	3460.42 ± 12.87	3466.42 ± 11.74	3463.73 ± 12.77	3458.05 ± 2.12	3457.42 ± 8.69

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).

Table 2. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 90 (S3), 135 (S4) and 180 (S5) days.

Concentration of different cytokines[a]	90 days (S3)				135 days (S4)				180 days (S5)			
	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
IL-2 (pg/ml)	35.96 ± 1.51	34.56 ± 1.51	32.51 ± 0.61*	32.69 ± 0.22*	35.99 ± 1.55	32.04 ± 0.34**	30.76 ± 1.21***	29.09 ± 0.68***	35.99 ± 0.66	31.70 ± 0.52***	29.09 ± 1.06***	23.61 ± 1.09***
IFN-γ (pg/ml)	1152.27 ± 5.58	1136.48 ± 1.56**	1126.75 ± 5.14***	1119.97 ± 5.09***	1148.61 ± 4.91	1113.15 ± 6.77***	1098.75 ± 5.64***	1078.13 ± 5.34***	1150.94 ± 9.04	1013.15 ± 6.77***	984.73 ± 11.42***	902.99 ± 12.79***
IL-4 (pg/ml)	135.47 ± 1.31	131.22 ± 0.43**	130.22 ± 0.67**	129.81 ± 1.93**	136.81 ± 0.47	129.55 ± 0.76***	128.22 ± 0.46***	124.81 ± 1.93***	136.37 ± 0.87	126.55 ± 1.37***	125.97 ± 3.00***	119.81 ± 1.24***
IL-10 (pg/ml)	3467.09 ± 10.80	3457.06 ± 7.62	3441.38 ± 7.81	3440.76 ± 15.86*	3463.75 ± 4.52	3443.73 ± 3.02**	3434.71 ± 3.83***	3420.76 ± 6.64***	3470.42 ± 6.14	3407.06 ± 18.71***	3328.05 ± 9.52***	3227.42 ± 8.48***

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).
**$p < 0.01$ when compared with Group 1 (Control) (Significantly different).
***$p < 0.001$ when compared with Group 1 (Control) (Significantly different).

3. Results

3.1. Effect of BDE on serum concentration levels of Th1 and Th2 cytokines

Significant decreases ($p < 0.05$, $p < 0.01$, and $p < 0.001$) were observed in both Th1 and Th2 cytokine concentrations in mice that were treated with different doses of BDE for 90, 135, and 180 d, when compared with their respective control groups (Table 2). After 15 and 45 d of treatment with different doses of BDE, the concentrations of IL-2, IL-4, and IL-10 did not decrease significantly, though after 45 d of treatment with BDE at 160 and 320 mg/kg bw, the concentrations of IFN-γ have been shown to decrease significantly ($p < 0.05$) when compared with their respective controls (Table 1). After 180 d of treatment at with 80, 160 and 320 mg/kg bw of BDE, the concentration of all the cytokines decreased significantly when compared to their respective controls ($p < 0.001$; Table 2).

3.2. Effect of BDE on Th1 and Th2 cytokine production from primary cultured splenocytes

Figure 1 indicated significant concentration-dependent IL-2 decreases ($p < 0.001$) in Con A-induced splenocytes. At 0 µg/ml, the concentration of IL-2 in splenocyte culture supernatant was 28.33 ± 0.58 pg/ml, whereas, at 200 µg/ml, the concentration of IL-2 decreased to 19.17 ± 0.38 pg/ml. The amount of IFN-γ, IL-4, and IL-10 were also decreased significantly ($p < 0.01$ and $p < 0.001$) in a concentration-dependent manner when compared with their respective controls. At 0 µg/ml of BDE, the concentrations of IFN-γ, IL-4, and IL-10 were 913.33 ± 5.77 pg/ml, 127.76 ± 2.21 pg/ml, and 2208.33 ± 14.43 pg/ml, respectively, whereas, at 200 µg/ml, the concentrations of IFN-γ, IL-4, and

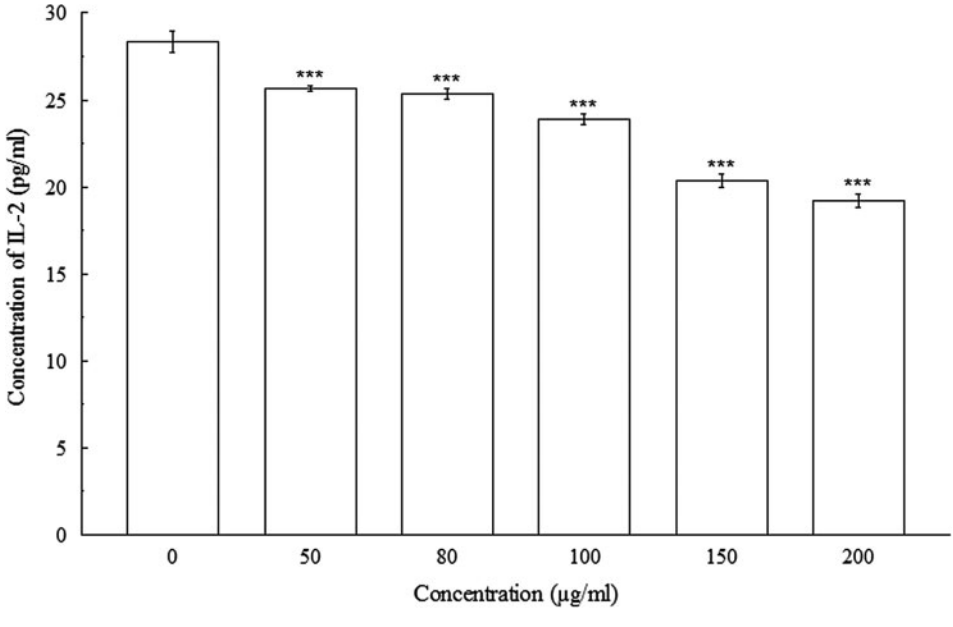


Figure 1. Effect of different concentrations (0–200 µg/ml) of BDE on IL-2 production by con A induced splenocytes. Data represents significant concentration-dependent decrease in IL-2 production. The results are mean ± SD of three parallel observations. ***$p < 0.001$ vs. 0 µg/ml.

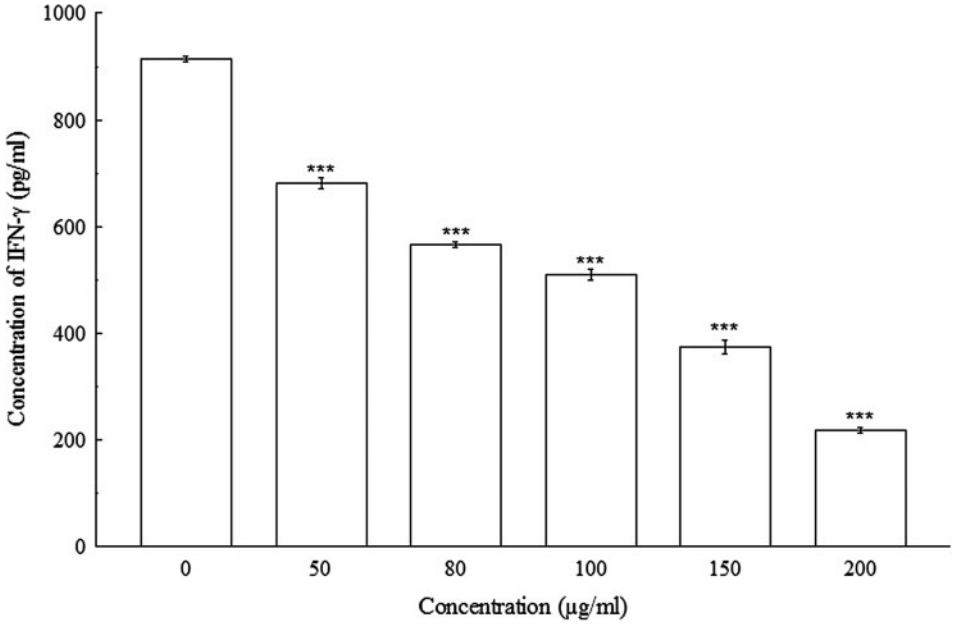


Figure 2. Effect of different concentrations (0–200 μg/ml) of BDE on IFN-γ production by con A induced splenocytes. Data represents significant concentration-dependent decrease in IFN-γ production. The results are mean ± SD of three parallel observations. ***$p < 0.001$ vs. 0 μg/ml.

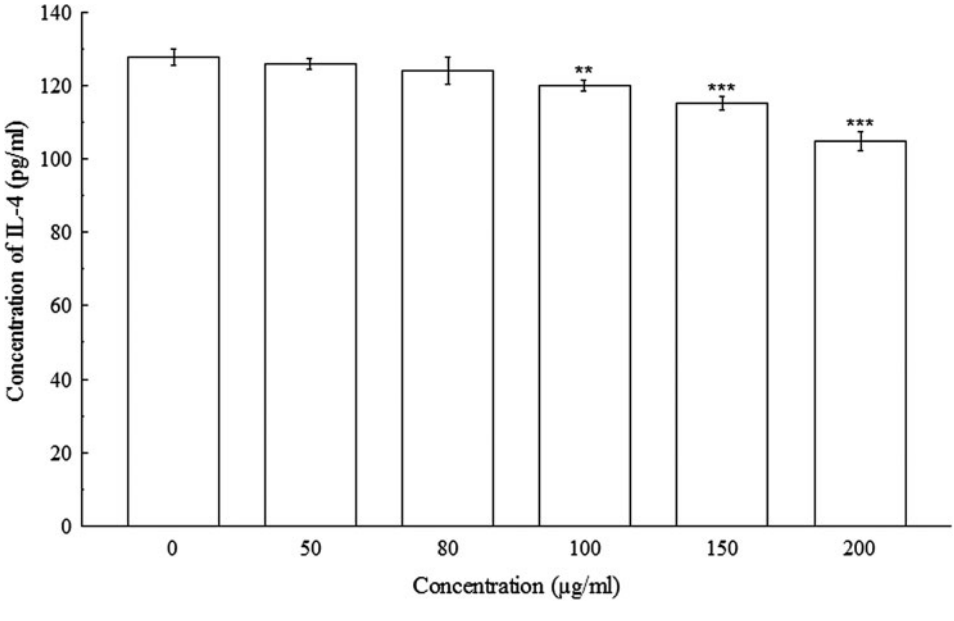


Figure 3. Effect of different concentrations (0–200 μg/ml) of BDE on IL-4 production by con A induced splenocytes. Data represents significant concentration-dependent decrease in IL-4 production. The results are mean ± SD of three parallel observations. **$p < 0.01$ and ***$p < 0.001$ vs. 0 μg/ml.

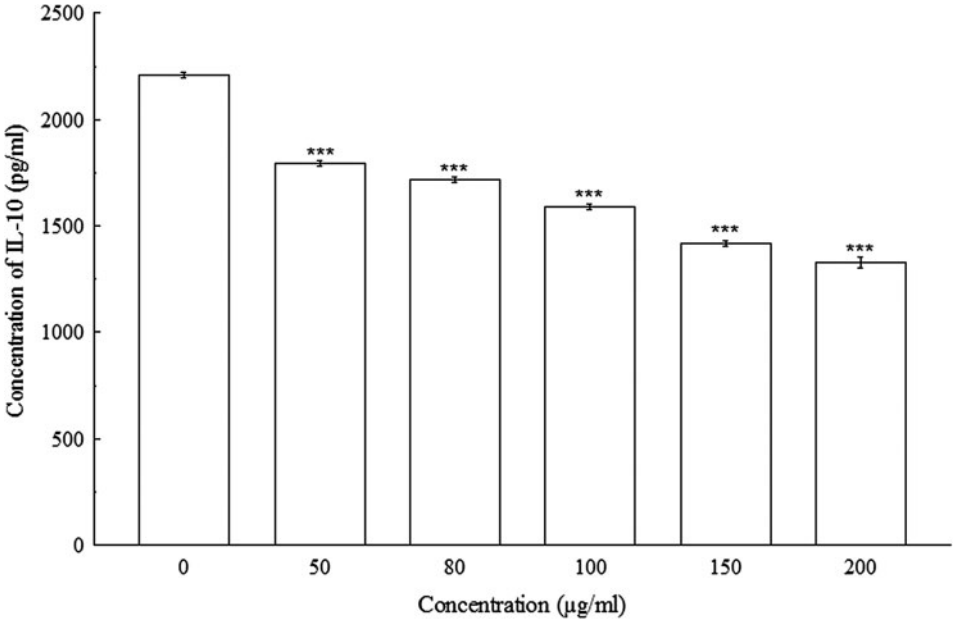


Figure 4. Effect of different concentrations (0–200 µg/ml) of BDE on IL-10 production by con A induced splenocytes. Data represents significant concentration-dependent decrease in IL-10 production. The results are mean ± SD of three parallel observations. ***$p < 0.001$ vs. 0 µg/ml.

IL-10 has been shown to decrease to 216.67 ± 5.77 pg/ml, 104.79 ± 2.45 pg/ml, and 1325 ± 25 pg/ml, respectively (Figure 2–4).

4. Discussion

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. We have previously reported that the sub-acute, sub-chronic, and chronic oral administration of boiled aqueous preparation of *D. esculentum* causes immunosuppression and hemolysis in Swiss albino mouse (Roy, Tamang, Dey et al., 2013). In continuation of our previous investigations, this work was aimed to investigate 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.

IL-2 is a representative cytokine produced by activated T-cells that leads to T-cell proliferation and participates in the regulation of other immune cells, including B cells, macrophages and natural killer (NK) cells (Park et al., 2007). IFN-γ is a proinflammatory mediator expressed by various cells, including Th1, NK, and NKT cells. IFN-γ is an important immune-activating cytokine that can prime 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 much type of cells in addition to enhancing NK cell activity. As intrinsic factors, 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, Ahn, Oh, & Kim, 2014). IL-4 is produced by activated T lymphocytes and mast cells, and can exert both pro- and anti-inflammatory effects (Kleemann, Zadelaar, & Kooistra, 2008). One of the most potent homeostatic regulators of inflammation is the anti-inflammatory cytokine IL-10, which potently inhibits TNF-α production from 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 showed to reduce the body weight and relative spleen weight, as well as suppress the humoral immune response in Swiss albino mouse. Moreover, BDE is also reported to decrease the number of the peritoneal macrophages in mouse (Roy, Tamang, Dey et al., 2013). 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 severe immunosuppression in these mice.

Study revealed that *D. esculentum* collected from high altitude area of Harsil–Gangotri had high quantity of Pta (Somvanshi et al., 2006). Shade- and freeze-dried samples of *D. esculentum* showed absence of fern toxin Pta but the presence of moderate amount of pterosin B in the freeze-dried samples identified by HPLC method (Gangwar, 2004). During metabolism, Pta undergoes a series of reactions and produces a reactive aglycone dienone intermediate, the inactive pterosin B and DNA adducts. Pta 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 and serum dehydrogenases. Feeding of frozen dried sample of *D. esculentum* induced mortality rate in guinea pigs (Gangwar, 2004). All these studies done so far were on the freeze- or shade-dried samples of *D. esculentum*, and its effect on rabbits and guinea pigs. But, there is little information available regarding the effect of boiled preparation of *D. esculentum* on different laboratory animals, such as mouse, rabbits or guinea pigs. We performed the present experiment using mouse as this is the standard convention to use inbreed strains of mouse for performing the immunological experiments. We performed the study using boiled preparations of *D. esculentum*, to find out the possible effect of temperature on some toxic compounds present, if any, in *D. esculentum*, which may alter the Th1 and Th2 cytokine balance. It was found that BDE caused significant dose-dependent reduction in cytokine concentration in both *in vivo* and *in vitro* models.

As Pta is a heat labile compound, boiling may probably reduce its toxicity. As BDE caused significant reduction in Th1 and Th2 cytokine concentration in Swiss albino mouse, it is clearly evident that there may be some compound besides Pta that can withstand heat and provide toxicity. Study revealed that *Diplazium sammatii*, a related edible fern, contains moderate amount of tannins which inhibit protein availability through denaturation (Bassey, Etuk, Ibe, & Ndon, 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 boiled preparation of *D. esculentum*, and may be one of the causes of immunosuppression. Thus, the cytokine inhibitory 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 speculation in future studies.

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 to Th1 and Th2 cells proportionally and keep their amount in a relative dynamic balance. Whenever this balance is disturbed, diseases will occur (Guo et al., 2014). It has been demonstrated that Th1/Th2 balance plays important roles in 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 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 autoimmune diseases, such as autoimmune diabetes, rheumatoid arthritis and Crohn's disease (Abbas, Murphy, & Sher, 1996). Findings from the present study indicate that *D. esculentum*, when given in chronic dose, can induce Th1/Th2 imbalance, resulting in severe immunosuppression, which may directly or indirectly induce several metabolic diseases and age-related degenerative disorders, as well as may increase the risk of infection in the people who regularly consumes this fern; induce a state of immunodeficiency as an unwanted consequence, and therefore, may also be related to the growth of tumors.

5. Conclusion

D. esculentum is extensively used as a palatable food throughout Asia, Oceania, and many other places throughout the globe, including the northern part of West Bengal, India, where we reside. The possible consequences of the immunosuppressive effects of *D. esculentum* on human health may be alarming. Considering the findings of the present study, it may be concluded that *D. esculentum*, even boiled, possesses potent immunosuppressive activities as evident by Th1/Th2 cytokine imbalance. This is the first report on the assessment of the Th1/Th2 cytokine imbalance in the body due to the intake of the edible *D. esculentum*, and thereby to make people aware about the hazards of its regular consumption which might be helpful in advancing the existing knowledge of this fern in relation to human health.

Acknowledgment

We gratefully acknowledge the financial support (Vide Letter F.No.37-464/2009 (SR) dt.11.01.2010) received from the University Grants Commission (UGC), New Delhi, India, to carry out this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Abbas, A. K., Murphy, K. M., & Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature*, *383*, 787–793. doi:10.1038/383787a0

Akter, S., Hossain M. M., Ara, I., & Akhtar, P. (2014). Investigation of *in vitro* antioxidant, antimicrobial and cytotoxic activity of *Diplazium esculentum* (Retz). Sw. *International Journal*

of Advances in Pharmacy, Biology and Chemistry, *3*, 723–733. Retrieved from http://www.ijapbc.com/files/32-3369.pdf

Bassey, M. E., Etuk, E. U. I., Ibe, M. M., & Ndon, B. A. (2001). *Diplazium sammatii*: *Athyraceae* ('Nyama Idim'): Age-related nutritional and antinutritional analysis. *Plant Foods for Human Nutrition*, *56*(1), 7–12. doi:10.1023/A:1008185513685

Brennan, F. M., Green, P., Amjadi, P., Robertshaw, H. J., Alvarez-Iglesias, M., & Takata, M. (2008). Interleukin-10 regulates TNF-α-converting enzyme (TACE/ADAM-17) involving a TIMP-3 dependent and independent mechanism. *European Journal of Immunology*, *38*, 1106–1117. doi:10.1002/eji.200737821

Chaudhuri, T. K., & Chakravarty, A. K. (1983). Goat serum as a substitute for fetal calf serum in *in vitro* culture of murine lymphocytes. *Indian Journal of Experimental Biology*, *21*, 494–496. Retrieved from http://www.niscair.res.in/sciencecommunication/researchjournals/rejour/ijeb/ijeb0.asp

Gangwar, N. K. (2004). Studies on the pathological effects of linguda (*Diplazium esculentum*, Retz.) in laboratory rats and guinea pigs. *Indian Journal of Veterinary Pathology*, *28*, 149–150. Retrieved from http://www.indianjournals.com/ijor.aspx?target=ijor:ijvp&volume=28&issue=2&article=thesis-abs-005

Guo, H.-W., Yun, C.-X., Hou, G.-H., Du, J., Huang, X., Lu, Y., … Deng, J. (2014). Mangiferin attenuates Th1/Th2 cytokine imbalance in an ovalbumin-induced asthmatic mouse model. *PLoS ONE*, *9*(6), e100394. doi:10.1371/journal.pone.0100394.t002

Kang, H.-B., Ahn, K.-S., Oh, S.-R., & Kim, J. W. (2014). Genkwadaphnin induces IFN-γ via PKD1/NF-kB/STAT1 dependent pathway in NK-92 cells. *PLoS ONE*, *9*(12), e115146. doi:10.1371/journal.pone.0115146

Kitao, T., & Hattori, K. (1977). Concanavalin A as a carrier of daunomycin. *Nature*, *265*, 81–82. doi:10.1038/265081a0

Kleemann, R., Zadelaar, S., & Kooistra, T. (2008). Cytokines and atherosclerosis: A comprehensive review of studies in mice. *Cardiovascular Research*, *79*, 360–376. doi:10.1093/cvr/cvn120

Nakamori, M., Iwahashi, M., Nakamura, M., Ueda, K., Zhang, X., & Yamaue, H. (2003). Intensification of antitumor effect by T helper 1-dominant adoptive immunogene therapy for advanced orthotopic colon cancer. *Clinical Cancer Research*, *9*, 2357–2365. Retrieved from http://clincancerres.aacrjournals.org/content/9/6/2357.long

Nishimura, T., Iwakabe, K., Sekimoto, M., Ohmi, Y., Yahata, T., Nakui, M., … Ohta, A. (1999). Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *Journal of Experimental Medicine*, *190*, 617–628. doi:10.1084/jem.190.5.617

Pacifico, L., Di Renzo, L., Anania, C., Osborn, J. F., Ippoliti, F., Schiavo, E., & Chiesa, C. (2006). Increased T-helper interferon-γ-secreting cells in obese children. *European Journal of Endocrinology*, *154*, 691–697. doi:10.1530/eje.1.02138

Park, K.-R., Lee, J.-H., Choi, C. Y., Liu, K.-H., Seog, D.-H., Kim, Y. H., … Yea, S. S. (2007). Suppression of interleukin-2 gene expression by isoeugenol is mediated through down-regulation of NF-AT and NF-κB. *International Immunopharmacology*, *7*, 1251–1258. doi:10.1016/j.intimp.2007.05.015

Roy, S., Hazra, B., Mandal, N., & Chaudhuri, T. K. (2013). Assessment of the antioxidant and free radical scavenging activities of methanolic extract of *Diplazium esculentum*. *International Journal of Food Properties*, *16*, 1351–1370. doi:10.1080/10942912.2011.587382

Roy, S., Tamang, S., & Chaudhuri, T. K. (2013). Sperm viability assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of Swiss albino mice treated with *Diplazium esculentum*. *Asian Journal of Pharmaceutical and Health Sciences*, *3*, 684–689. Retrieved from http://ajphs.com/wp-content/uploads/2013/05/04_AJPHS_V3_I2_Apr-Jun_2013_246.pdf

Roy, S., Tamang, S., Dey, P., & Chaudhuri, T. K. (2013). Assessment of the immunosuppressive and hemolytic activities of an edible fern, *Diplazium esculentum*. *Immunopharmacology and Immunotoxicology*, *35*, 365–372. doi:10.3109/08923973.2013.775588

Smith, B. L., Seawright, A. A., Ng, J. C., Hertle, A. T., Thomson, J. A., & Bostock, P. D. (1994). Concentration of ptaquiloside, a major carcinogen in bracken fern (Pteridium spp.) from eastern Australia and from cultivated worldwide collection held in Sydney, Australia. *Natural Toxins*, *2*, 347–353. doi:10.1002/nt.2620020602

Somvanshi, R., Lauren, D. R., Smith, B. L., Dawra, R. K., Sharma, O. P., Sharma, V. K., … Gangwar, N. K. (2006). Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. *Current Science*, *91*, 1547–1552. Retrieved from http://www.iisc.ernet.in/currsci/dec102006/1547.pdf

Spelman, K., Burns, J. J., Nichols, D., Winters, N., Ottersberg, S., & Tenborg, M. (2006). Modulation of cytokine expression by traditional medicines: A review of herbal immunomodulators. *Alternative Medicine Review*, *11*, 128–150. Retrieved from http://www.nutraxin.com.tr/pdf/AstragalusSpecies/Astragalus_02.pdf

Tongco, J. V. V., Villaber, R. A. P., Aguda, R. M., & Razal, R. A. (2014). Nutritional and phytochemical screening, and total phenolic and flavonoid content of *Diplazium esculentum* (Retz.) Sw. from Philippines. *Journal of Chemical and Pharmaceutical Research*, *6*, 238–242. Retrieved from http://jocpr.com/vol6-iss8-2014/JCPR-2014-6-8-238-242.pdf

Subhrajyoti Roy, Somit Dutta and Tapas Kumar Chaudhuri*

In vitro assessment of anticholinesterase and NADH oxidase inhibitory activities of an edible fern, *Diplazium esculentum*

Abstract

Background: *Diplazium esculentum* is the most commonly consumed edible fern throughout Asia and Oceania. Several studies have been performed so far to determine different functional properties of this plant, but there have been no reports on the anticholinesterase and nicotinamide adenine dinucleotide (NADH) oxidase inhibitory activities of this plant. Therefore, the present study was conducted to determine the anticholinesterase and NADH oxidase inhibitory activities of 70% methanolic extract of *D. esculentum*.

Methods: The *D. esculentum* extract was investigated for its acetylcholinesterase and NADH oxidase inhibitory activities as well as its free radical scavenging and total antioxidant activities in the linoleic acid system. The free radical scavenging activity of the extract was determined by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method. The total antioxidant activity of the extract was evaluated by ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods.

Results: The *D. esculentum* extract inhibited acetylcholinesterase and NADH oxidase in a dose-dependent manner, with IC_{50} values of 272.97±19.38 and 265.81±21.20 μg/mL, respectively. The extract also showed a potent DPPH radical scavenging activity with an IC_{50} value of 402.88±12.70 μg/mL. Moreover, the extract showed 27.41% and 33.22% of total antioxidant activities determined by FTC and TBA methods, respectively.

Conclusions: Results indicated that 70% methanolic extract of *D. esculentum* effectively inhibited the enzymes acetylcholinesterase and NADH oxidase and acted as a potent antioxidant and free radical scavenger. These in vitro assays indicate that this plant extract is a significant source of natural antioxidants, which may be helpful in preventing the progression of various neurodegenerative disorders associated with oxidative stress.

Keywords: acetylcholinesterase; antioxidant; *Diplazium esculentum*; NADH oxidase; neurodegenerative disorders.

DOI 10.1515/jbcpp-2014-0100
Received September 7, 2014; accepted December 19, 2014; previously published online February 18, 2015

***Corresponding author: Tapas Kumar Chaudhuri,**
Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India, Phone: +91-9434377127 (M), +91-353-2776353 (O), Fax: +91-353-2699001, E-mail: dr_tkc_nbu@rediffmail.com
Subhrajyoti Roy: Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India; and Immunopharmacology and Molecular Cell Biology Laboratory, Department of Zoology, University of Gour Banga, Malda 732103, West Bengal, India
Somit Dutta: Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India

Introduction

Acetylcholine is involved in the signal transfer in the synapses. Acetylcholine is hydrolyzed to the acetyl and choline groups in a reaction catalyzed by the enzyme acetylcholinesterase (AChE) after being delivered in the synapses [1]. In recent years, AChE has been targeted for therapeutic interventions in several diseases. The inhibition of AChE has been proved to be a strategy for the treatment of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease [2]. There are a few synthetic medicines, for example, tacrine, donepezil, and the natural product-based rivastigmine for the treatment of cognitive dysfunction and memory loss associated with AD [3]. All of these compounds have been reported to have their adverse effects, including gastrointestinal disturbances and problems associated with bioavailability [4, 5], which necessitate the interest in finding out better AChE inhibitors from natural resources.

The search for the anticholinesterase activity from natural products has got tremendous importance in recent years for the treatment of different neurodegenerative disorders, but the literature survey has revealed

little report on the nicotinamide adenine dinucleotide (NADH) oxidase (NOX) inhibition studies of plant extracts [6]. NOX catalyzes the two-electron reduction of oxygen to peroxide or the four-electron reduction of oxygen to water. They share the ability to reduce oxygen; their physiological role is to reduce oxygen and to catalyze different reactions [7]. NOX is becoming a potential target for therapeutic interventions in vascular diseases [8]. It was investigated that the action of natural products against parasitic infections involves the NOX inhibition, which therefore may be an underlying mechanism for cytotoxicity in parasites [9].

Recent studies have pointed out that AD is associated with inflammatory processes. β-Amyloid peptides contained in the senile plaques found in the brain of AD patients can induce these inflammatory processes in which reactive oxygen species (ROS) are liberated, among other components [10, 11]. ROS are able to damage cellular constituents and act as a secondary messenger in inflammation. Antioxidants can scavenge ROS and can also attenuate inflammation pathways. Antioxidants may be useful in the treatment of AD [12, 13]. Therefore, natural drug candidates with antioxidant properties in addition to cholinesterase and NOX inhibitory activity could be regarded as especially desirable for the treatment of different cognitive disorders [14]. In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders. Ethnopharmacological approach and bioassay-guided isolation have provided a lead in identifying potential anticholinesterases and NOX inhibitors from plant sources, including those for memory disorders [15].

Diplazium esculentum (Koenig ex Retz.) Sw. (family: Athyriaceae) is an edible fern distributed throughout Asia and Oceania. It is known as paco in the Philippines, linguda in northern India (referring to the curled fronds), and dheki shak in the northern region of West Bengal, India. *Diplazium esculentum* is one of the most common varieties and perhaps the most commonly consumed fern. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during the monsoon season, which continues for almost 5 months in the tropical regions where this plant grows abundantly. We previously determined the total antioxidant and different free radical scavenging activities of 70% methanolic extract of *D. esculentum*. The extract acted as an iron chelator and also possessed reducing power. It also inhibited lipid peroxidation. Moreover, the extract yielded high phenolic and flavonoid content, which may confer the antioxidant activity to this plant [16].

Therefore, the current study is an attempt to evaluate the anticholinesterase and NOX inhibitory activities of the methanolic extract of *D. esculentum* and to find out the potential therapeutic importance of this edible plant, if any, against different neurodegenerative and neuropharmacological disorders. The extract was also examined for its DPPH radical scavenging activity and its total antioxidant activity by the peroxidation of linoleic acid using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods.

Materials and methods

Plant material

Young *D. esculentum* plants were collected from different areas of North Bengal University campus, Darjeeling, India. These were identified by Professor A.P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal, and a voucher specimen (accession no. 9601) was submitted to him.

Chemicals

Tris-HCl, magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sucrose, Coomassie brilliant blue G-250, sodium hydroxide, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide, ethylenediaminetetra acetic acid disodium salt (EDTA-Na_2), NADH (reduced), linoleic acid, and ammonium thiocyanate were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. Ethanol, methanol, phosphoric acid, and hydrochloric acid were procured from Merck, New Delhi, India. Trichloroacetic acid, thiobarbituric acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and α-tocopherol were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. Ferrous chloride was procured from Sigma-Aldrich, St. Louis, Missouri, USA.

Preparation of the plant extract

Samples were prepared according to a previously described method [16]. The young fronds of *D. esculentum* were dried at room temperature for 7 days, finely powdered, and used for extraction. The powder (100 g) was mixed with a 500 mL methanol-water solution (7:3) using a shaker for 15 h. Then the mixture was centrifuged at 2850×*g* in a centrifuge (Eppendorf, Hamburg, Germany), and the supernatant was decanted. The pellet was mixed again with a 500 mL methanol-water solution, and the entire process was repeated once again, that is, the extraction procedure was performed twice. The supernatants collected from the two phases were mixed in a round-bottom flask and concentrated under reduced pressure in a rotary evaporator (Buchi, Flawil, St. Gallen, Switzerland). The concentrated extract was then lyophilized in a lyophilizer (LaboGene, Lynge, Allerød, Denmark). The residue was kept at –20 °C for future use. Double-distilled water (MilliQ grade) was used to dissolve the lyophilized extract in all the experiments, except FTC and TBA methods.

Preparation of enzyme source

The enzyme was prepared according to a previously described method [6], with a little modification. Fresh chicken liver (1 g) was purchased from the local market, washed with 50 mM Tris-HCl buffer (pH 7.4), and homogenized in 10 mL extraction buffer (50 mM Tris-HCl buffer, pH 7.4; 1 mM $MgCl_2$; 1 mM $CaCl_2$; and 0.32 M sucrose) in a homogenizer for 15 s each after 10-s intervals. The test tube was placed in an ice bucket to avoid heating. Contents were filtered through double layers of Whatman filter paper no. 1 and centrifuged at 15,000 rpm for 15 min at 4 °C in a cooling centrifuge (Eppendorf, Hamburg, Germany). The supernatant was used as a source of enzyme. The enzyme source was made fresh everyday and used within 4 h. Protein was determined using the Bradford method, and 40–60 μg protein (10 μL) was used per assay.

Determination of AChE inhibitory activity

Enzyme activity was measured by a previously described method [17]. The reaction mixture (200 μL) consisted of 160 μL of 50 mM Tris-HCl buffer, pH 7.4, with or without the plant extract (30 μL), followed by the addition of 10 μL enzyme (40–60 μg protein) from fresh chicken liver homogenate in 96-well plates. The contents were mixed and preincubated for 10 min at 25 °C. Plates were preread at 412 nm using a plate reader (Bio-Rad, Hercules, CA, USA). The reaction was initiated by the addition of 10 μL of 1 mM DTNB and 3 mM substrate acetylthiocholine iodide. After 15 min of incubation, absorbance was measured at 412 nm within 4–7 min. Control experiments were conducted to correct nonenzymatic hydrolysis by adding enzyme after the addition of DTNB. Absorbance values were subtracted from the control, and data were presented as the percentage inhibition of enzyme activity. Experiments were conducted with their respective controls for six times.

Determination of NOX inhibitory activity

NOX inhibitory activity was determined according to a previously described method [6]. The reaction mixture (200 μL) consisted of 160 μL 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA disodium salt with or without the plant extract (30 μL), followed by the addition of 10 μL enzyme (40–60 μg protein) from fresh chicken liver homogenate. The contents were mixed and preincubated for 10 min at 25 °C. The reaction was initiated by the addition of 10 μL of 3 mM NADH (reduced). After 45 min of incubation at 25 °C, absorbance was measured at 340 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). All experiments were conducted with their respective controls. Results are mean of six independent determinations.

Scavenging activity of DPPH radical

The ability of the extracts to scavenge DPPH was determined using a previously reported procedure [18]. Briefly, 0.1 mM solution of DPPH in ethanol was prepared. Then 1 mL of this solution was added to 3 mL of each extract solution at different concentrations (0–200 μg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (Shimadzu Corp., Kyoto, Honshu, Japan). The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. α-tocopherol was used as a positive control.

Antioxidant activity in the linoleic acid system

FTC method: The FTC method was followed from a previously described procedure [19] with little modification [20, 21]. The FTC method was used to determine the amount of peroxide at the initial state of lipid peroxidation. The peroxide reacts with ferrous chloride ($FeCl_2$) to form a reddish ferric chloride ($FeCl_3$) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Four milliliters of methanolic extract was placed in 4 mL of absolute ethanol; 4.1 mL of 2.52% linoleic acid in absolute ethanol, 8 mL of 0.05 M phosphate buffer (pH 7.0), and 3.9 mL of water were placed in a vial with a screw cap and then placed in an oven at 40 °C in the dark. A total of 9.7 mL of 75% ethanol and 0.1 mL 30% ammonium thiocyanate were added to 0.1 mL of this solution. Exactly 3 min after the addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid (HCl) to the reaction mixture, the absorbance was measured at 500 nm in a spectrophotometer (Shimadzu Corp., Kyoto, Honshu, Japan) every 24 h until the absorbance of the control reached maximum. The control and the standard were subjected to the same procedures as the sample, except only the solvent was added for the control and the 4-mg sample was replaced with 4 mg of α-tocopherol for the standard.

TBA method: The TBA values of the plant extract were determined according to a previously described method [22]. The formation of malondialdehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. At low pH and high temperature (100 °C), malondialdehyde binds TBA to form a red complex that can be measured at 532 nm. The increased amount of the red pigment formed correlates with the oxidative rancidity of the lipid. Two milliliters of 20% trichloroacetic acid and 2 mL of 1% (w/v) TBA aqueous solution were added to 1 mL of sample solution. This solution was prepared as in the FTC procedure and incubated in a similar manner. The mixture was then placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min in a centrifuge (Eppendorf, Hamburg, Germany), and the absorbance of the supernatant was measured at 532 nm in a spectrophotometer (Shimadzu Corp., Kyoto, Honshu, Japan). Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay. Both methods (FTC and TBA) described antioxidant activity by the percentage inhibition, as follows:

$$\%\ \text{inhibition} = [(\text{absorbance of control on day maximum} - \text{absorbance of sample on the same day}) / \text{absorbance of control on the same day}] \times 100$$

All data about total antioxidant activity are the average of six replicate analyses.

Statistical analysis

All data are presented as the mean±SD of six measurements. Statistical analysis was performed using the KyPlot version 2.0 beta 15 (32 bit) © 1997–2001 by Koichi Yoshioka (available at http://

www.qualest.co.jp). IC_{50} values were calculated using the following formula: $Y=100\times A1/(X+A1)$, where $A1=IC_{50}$, Y=response (Y=100% when X=0), and X=inhibitory concentration. Differences between two groups (plant extract and standard in DPPH radical scavenging assay) were determined by a paired t test, whereas differences among more than two groups were determined by a one-way analysis of variance followed by Dunnett's t test. $p<0.05$ was considered significant.

Results

Assessment of AChE inhibitory activity

As indicated in Figure 1, a significant ($p<0.001$) dose-dependent increase in the AChE inhibitory activity of *D. esculentum* has been observed. At 50 and 200 µg/mL, the percentage inhibition of AChE was 8.63% and 54.39%, respectively. The IC_{50} value of the *D. esculentum* extract on AChE inhibitory activity has been found to be 272.97±19.38 µg/mL.

Determination of NOX inhibitory activity

Diplazium esculentum extract inhibited NOX significantly ($p<0.001$) in a dose-dependent manner (Figure 2). At 50 µg/mL, the percentage inhibition of NOX was 10.43%, whereas at 200 µg/mL, the percentage inhibition of NOX was increased to 43.99%. The IC_{50} value of the *D. esculentum* extract on NOX inhibition was 265.81±21.20 µg/mL.

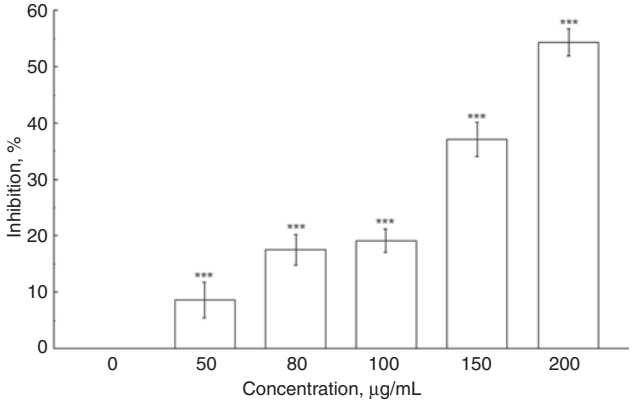


Figure 1: AChE inhibitory activity of the *D. esculentum* extract. Data represent the percentage inhibition of the enzyme AChE. Results are presented as mean±SD of six parallel measurements. ***$p<0.001$ vs. 0 µg/mL. The IC_{50} value of the plant extract was 272.97±19.38 µg/mL.

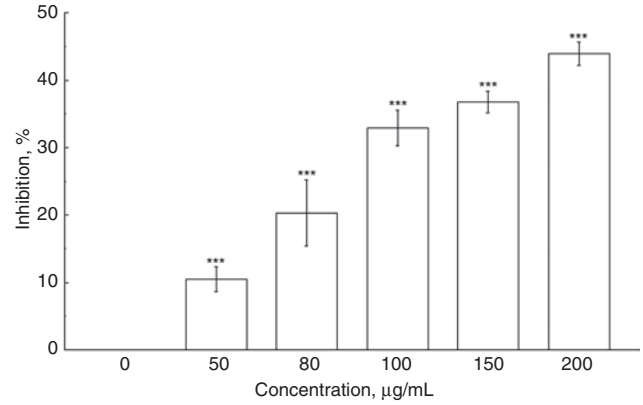


Figure 2: NOX inhibitory activity of the *D. esculentum* extract. Data represent the percentage inhibition of the enzyme NOX. Results are presented as mean±SD of six parallel measurements. ***$p<0.001$ vs. 0 µg/mL. The IC_{50} value of the plant extract was 265.81±21.20 µg/mL.

DPPH radical scavenging activity of the plant extract

Figure 3 showed a significant ($p<0.01$ and $p<0.001$) dose-dependent increase in the percentage of DPPH radical scavenging by the plant extract when compared with the standard α-tocopherol. At 50 µg/mL, the percentage of the DPPH radical scavenging of the plant extract and standard was 8.75% and 11.07%, respectively, whereas at 200 µg/mL, radical scavenging was increased up to 38.12% and 42.69% for the plant extract and the standard,

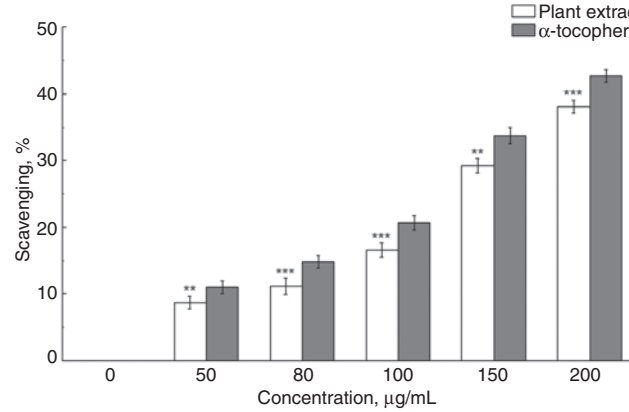


Figure 3: DPPH radical scavenging activity of the *D. esculentum* extract. Data represent the effect of *D. esculentum* plant extract and α-tocopherol on the scavenging of DPPH radical. Results are presented as mean±SD of six parallel measurements. **$p<0.01$ and ***$p<0.001$ vs. 0 µg/mL. The IC_{50} values of the plant extract and the standard (α-tocopherol) were 402.88±12.70 and 324.86±6.35 µg/mL, respectively.

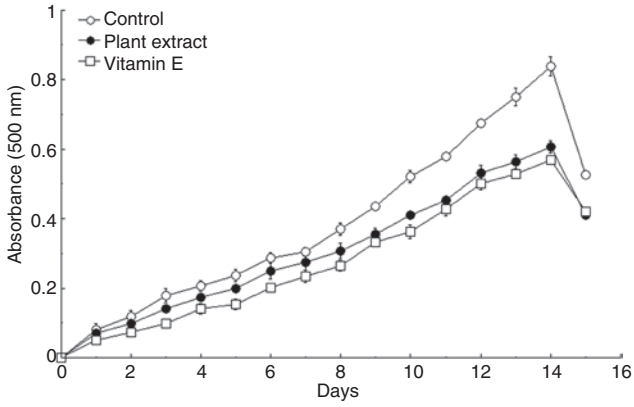


Figure 4: Absorbance value of the methanol extracts of *D. esculentum* in the linoleic acid emulsion using the FTC method.
Results are presented as mean±SD of six parallel measurements.

respectively. The IC_{50} values of the plant extract and standard on DPPH radical scavenging were 402.88±12.70 and 324.86±6.35 μg/mL, respectively.

Determination of total antioxidant activity by FTC and TBA methods

As shown in Figure 4, the absorbance of the control at 500 nm increased to a maximal value of 0.84 on day 14, whereas vitamin E (α-tocopherol) and methanol extracts of *D. esculentum* increased to 0.57 and 0.61, respectively, on the same day. These differences were found statistically significant than the control ($p<0.001$). The total antioxidant activity of vitamin E by FTC and TBA methods were 31.85% and 38.97%, respectively, whereas the total antioxidant activity of the methanolic extract of *D. esculentum* by FTC and TBA methods were 27.41% and 33.22%, respectively.

Discussion

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 [6]. The enzyme inhibition assays have prompted us to carry out the AChE and NOX inhibitory activities of the methanolic extracts of *D. esculentum*. In the present study, significant dose-dependent increases in the AChE and NOX inhibitory activities as well as low IC_{50} values for AChE and NOX inhibition of the plant extract were observed, indicating its effectiveness as a good anticholinesterase and NOX inhibitor. Cholinesterase and NOX inhibitory therapies may be considered by their pharmacological nature as a simple symptomatic short-term intervention. It has previously been suggested that 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 and slowing down one of the major pathological steps of the disease progression [23]. 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 AChE inhibitor [24]. Our study plant, *D. esculentum*, being an edible fern, may also be a good dietary source of AChE and NOX inhibitors and thereby can be used for the management of oxidative stress-related neurodegenerative disorders.

It has long since been known that certain phytochemicals such as flavonoids and phenolic compounds confer antioxidant activity. Antioxidants can scavenge ROS and attenuate inflammatory pathways; therefore, it can also act as AChE and NOX inhibitors. Both of these classes of compounds have good antioxidant potential because of their radical scavenging abilities, and their effects on human nutrition and health are considerable. We have previously demonstrated that *D. esculentum* possesses high amounts of flavonoid and phenolic compounds [16] and therefore may be a good source of AChE and NOX inhibitors. We have investigated the DPPH radical scavenging property as well as the total antioxidant activities in the 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 [25]. It measures the capacity of the extract to scavenge free radicals in the solution. In the present study, the DPPH scavenging potential of the *D. esculentum* extract was evaluated. Although the plant extract was not as potent as the standard tocopherol, as indicated in the Figure 3, the IC_{50} value of the plant extract shows that the plant extract possesses moderate free radical scavenging activity.

Our previous study on *D. esculentum* demonstrated that the methanolic extract of *D. esculentum* possesses

scavenging activities against different 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 lipid peroxidation. Previously, the total antioxidant activity of the extract was evaluated using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method as a trolox equivalent antioxidant capacity value [16]. In the present study, the total antioxidant activity was measured 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 FTC method measures the amount of peroxide at the primary stage of linoleic acid peroxidation, whereas the TBA method measures it at the secondary stage [25]. The total antioxidant activity of the 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 the plant extract and vitamin E in both FTC and TBA methods.

Conclusions

On the basis of the results obtained in the present study, it can be concluded that 70% methanolic extract of *D. esculentum* shows the inhibitory activity of the enzymes AChE and NOX and possesses radical scavenging and antioxidant activities. These in vitro assays indicate that this plant extract is a significant source of natural antioxidants, which may be helpful in preventing the progression of various oxidative stress associated neurodegenerative disorders. Further, it is interesting to note that *D. esculentum*, which has been used for a long time as food throughout the globe, has in fact properties that may suggest new applications. Finally, investigations on the isolation and identification of antioxidant components in the plants may lead to chemical entities with potential for clinical use.

Acknowledgments: The authors gratefully acknowledge the financial support [Vide Letter F.No.37-464/2009 (SR) dt.11.01.2010] received from the University Grants Commission (UGC), New Delhi, India, to carry out this study. They would also like to extend their thanks to Dr. Nripendranath Mandal, Division of Molecular Medicine, Bose Institute, Kolkata, for his assistance and guidance to prepare sample for the study.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Voet D, Voet JG. Biochemistry, 4th ed. New Jersey: John Wiley & Sons, Inc., 2011:527.
2. Rahman AU, Choudhary MI. Bioactive natural products as a potential source of new pharmacophores. A theory of memory. Pure Appl Chem 2001;73:555–60.
3. Oh MH, Houghton PJ, Whang WK, Cho JH. Screening of Korean herbal medicines used to improve cognitive function for anticholinesterase activity. Phytomedicine 2004;11:544–8.
4. Schulz V. Ginkgo extract or cholinesterase inhibitors in patients with dementia: what clinical trial and guidelines fail to consider. Phytomedicine 2003;10:74–9.
5. Melzer D. New drug treatment for Alzheimer's diseases: lessons for healthcare policy. Br Med J 1998;316:762–4.
6. Ashraf M, Ahmad K, Ahmad I, Ahmad S, Arshad S, Shah SM, et al. Acetylcholinesterase and NADH oxidase inhibitory activity of some medicinal plants. J Med Plant Res 2011;5:2086–9.
7. Serve WM, Kengen JO, Willem MV. Molecular characterization of H_2O_2-forming NADH oxidases from Archaeoglobus fulgidus. Eur J Biochem 2003;270:2885–94.
8. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK. Angiotensin II mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone. J Clin Invest 1996;97:1916–23.
9. Scotti L, Ferreira EI, Da Silva MS, Scotti MT. Chemometric studies on natural products as potential inhibitors of the NADH oxidase from Trypanosoma cruzi using the VolSurf approach. Molecules 2010;15:7363–77.
10. Vina J, Lloret A, Orti R, Alonso D. Molecular bases of the treatment of Alzheimer's disease with antioxidants: prevention of oxidative stress. Mol Aspects Med 2004;25:117–23.
11. Stuchbury G, Munch G. Alzheimer's associated inflammation, potential drug targets and future therapies. J Neural Transm 2005;112:429–53.
12. Calabrese V, Scapagnini G, Colombrita C, Ravagna A, Pennisi G, Stella G, et al. Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated

with oxidative stress: a nutritional approach. Amino Acids 2003;25:437–44.
13. Gibson GE, Huang HM. Oxidative stress in Alzheimer's disease. Neurobiol Aging 2005;26:575–8.
14. Mathew M, Subramanian S. In vitro screening for anti-cholinesterase and antioxidant activity of methanolic extracts of ayurvedic medicinal plants used for cognitive disorders. PLoS One 2014;9:e86804.
15. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. Phytomedicine 2007;14:289–300.
16. Roy S, Hazra B, Mandal N, Chaudhuri TK. Assessment of the antioxidant and free radical scavenging activities of methanolic extract of Diplazium esculentum. Int J Food Prop 2013;16:1351–70.
17. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
18. Shimada K, Fujikava K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autooxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992;40:945–8.
19. Kikuzaki H, Nakatani N. Antioxidants effects of some ginger constituents. J Food Sci 1993;58:1407–10.
20. Mitsuda H, Yasumoto K, Iwani K. Antioxidant actions of indole compounds during autooxidation of linoleic acid. Eiyo to Shoduryou 1967;19:210.
21. Osawa T, Namiki M. A novel type of antioxidant isolated from leaf wax of Eucalptus leaves. Agric Biol Chem 1981;45:735–9.
22. Ottolenghi A. Interaction of ascorbic acid and mitochondria lipids. Arch Biochem Biophys 1959;79:355–63.
23. Giacobini E. Long term stabilizing effect of cholinesterase inhibitors in the therapy of Alzheimer's disease. J Neural Transm Suppl 2002;62:181–7.
24. Ademosun AO, Oboh G. Anticholinesterase and antioxidative properties of water-extractable phytochemicals from some citrus peels. J Basic Clin Physiol Pharmacol 2014;25:199–204.
25. Barış D, Kızıl M, Aytekin C, Kızıl G, Yavuz M, Çeken B, et al. In vitro antimicrobial and antioxidant activity of ethanol extract of three Hypericum and three Achillea species from Turkey. Int J Food Prop 2011;14:339–55.

Immunopharmacology and Immunotoxicology

http://informahealthcare.com/ipi
ISSN: 0892-3973 (print), 1532-2513 (electronic)
Immunopharmacol Immunotoxicol, 2013; 35(3): 365–372

DOI: 10.3109/08923973.2013.775588

informa healthcare

RESEARCH ARTICLE

Assessment of the immunosuppressive and hemolytic activities of an edible fern, *Diplazium esculentum*

Subhrajyoti Roy[1,2], Suman Tamang[1], Priyankar Dey[1], and Tapas Kumar Chaudhuri[1]

[1]*Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India and* [2]*Immunopharmacology and Molecular Cell Biology Laboratory, Department of Zoology, University of Gour Banga, Malda, West Bengal, India*

Abstract

Context: *Diplazium esculentum* is the most commonly consumed fern throughout Asia and Oceania. Systemic toxicity and pathological effects on its consumption have already been demonstrated. But, the immunosuppressive and hemolytic activities of the boiled *Diplazium esculentum* (BDE), the form in which human consumes it, have not yet been studied.
Objective: To investigate the immunosuppressive as well as hemolytic activities, if any, of BDE in Swiss albino mice.
Materials and methods: Body weight, relative spleen weight, plaque forming cell assay, hemaggutination antibody (HA) titer assay and macrophage counting were performed in BDE treated mice and respective control groups within a span of 180 days, and *in vitro* assays such as counting of cultured splenocytes, splenocytes proliferation assay and hemolytic assay were performed to justify the immunomodulatory as well as hemolytic activities of *D. esculentum*.
Results: Body weight and relative spleen weight were significantly decreased in BDE fed mice. Significant decreases were observed in the number of plaques formed, HA titer value and in the number of peritoneal macrophages within a span of 180 d. Significant dose-dependent decrease was observed in the number of cultured splenocytes. Significant dose-dependent increases in the percentage inhibition of splenocyte proliferation as well as percentage of hemolysis were evident by *in vitro* assays.
Discussion: These results suggest that the intake of *D. esculentum* may evoke immune dysfunction as well as may cause destruction of erythrocytes even after cooking.
Conclusion: Therefore, the consumption of *D. esculentum* is alarming and may act as immunosuppressive agent.

Keywords

Hemagglutination antibody titer, immune dysfunction, immunotoxicity, macrophage inhibition, splenocyte proliferation

History

Received 2 December 2012
Revised 3 February 2013
Accepted 8 February 2013
Published online 14 May 2013

Introduction

Utilization of wild edible plants as a source of food is an integral part of the culture of indigenous people throughout the world. Increase in the consumption of these plants is due to the progressive decrease in the stock of cultivated crops. These plants are used as substitutes and fill the gap of food deficiency[1]. But, information on the possible toxic effects of most of the wild edible plants is little. Therefore, the information documented on nutritional values and possible side effects of these plants are highly required to make the people aware about the hazardous effects of the consumption of these plants.

Diplazium esculentum (Koenig ex Retz.) Sw. (Family – Athyriaceae) is one of the most common varieties and the most commonly consumed fern throughout Asia and Oceania. In India, young fronds of *D. esculentum* are popularly known as lingra in Northern India[2], rukja and lochanch in North Eastern India[3] and dheki sak in West Bengal, India[4]. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon season which continues for almost 5 months.

Very few studies have been conducted so far to assess the toxicological impact of this fern on human health. Interestingly, this fern is rejected as food by animals including cattle and insets. We observed that this fern grows abundantly in the marshy land and also in the wet shabby places where lot of insects are available. But interestingly, we have never found any insects consuming the leafy portion. Leaves are all intact, not taken by any insect and even cattle. We also observed that the Rajbanshi population, the original inhabitants of this region, consumes this fern regularly after cooking. It seemed that they were aging at a much faster rate as they looked much older than that of their actual age. As we did not get convincing data regarding the toxicity of the fern to human, we performed the experiment using mouse as this is the standard convention to use inbreed strains of mouse for performing the immunological experiments. Study conducted on rabbits and guinea pigs demonstrated systemic toxicity and several pathological effects of this fern[5]. Young fronds of *D. esculentum* collected from the high-altitude area of

Address for correspondence: Tapas Kumar Chaudhuri, Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri – 734013, West Bengal, India. Tel: +91-9434377127 (M); +91-353-2776353 (O). Fax: +91-353-2699001. E-mail: dr_tkc_nbu@rediffmail.com

Harsil–Gangotri of North India had been found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria in hill cattle in India and elsewhere[6]. Ptaquiloside was found in *D. esculentum* sample that was prepared by freeze drying and shade drying method. Moreover, the frozen and shade dried crude *D. esculentum* has already been shown to cause mild pathological effects in rats, and induce mortality and moderate clinic-pathological effects in guinea pigs[7]. Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats[8].

However, the immunomodulatory activities as well as the effect of this plant on erythrocytes have not yet been studied. It happened in our mind that this fern may have certain toxic substances which may be detrimental to the human body. Therefore, we have conducted this pilot study to investigate the immunosuppressive as well as hemolytic activities, if any, of the boiled aqueous preparation of *D. esculentum* (BDE), keeping in mind the fact that the local people consume this plant as food after cooking, not as raw material.

Till date, there is no study that indicates the presence of ptaquiloside or any other related compounds in *D. esculentum* after it was boiled. As ptaquiloside is heat labile, it is most possible that the effect of heat during cooking may interfere with the effect of ptaquiloside. The present study was conducted only with cooked material, because, the local population consumes it regularly after cooking. Therefore, the objective of the present study was to find out whether the cooked *D. esculentum* possess any immunomodulatory as well as hemolytic activities, and thereby to find out the presence of any toxic compound, if any, that is heat resistant. For this reason, our main focus was on the cooked or boiled plant material, rather than the study of the effect of crude *D. esculentum* on immunomodulation.

Materials and methods

Preparation of the plant material

Young *D. esculentum* plants were collected from different areas of North Bengal University campus, from the market those are taken by the local people and also from the adjoining regions of Darjeeling, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and a voucher specimen (Accession No. 9602) was submitted to him.

Young frond of *D. esculentum* (100 g) was washed carefully by tap water, then cut into small pieces, and boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed by a mixer and dried in an incubator at 60 °C until completely dried. This dried plant material (BDE) was then kept at 4 °C for future use.

Chemicals

Alsever's solution was purchased from Sigma-Aldrich, St. Louis, MO. Dimethyl sulphoxide (DMSO), Tris–HCl, sodium chloride (NaCl), boric acid, calcium chloride ($CaCl_2$) and Triton X-100 were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Paraffin wax and incomplete adjuvant were obtained from Merck, Mumbai, India. Phosphate buffered saline (PBS), RPMI-1640, penicillin, streptomycin, nystatin, fetal bovine serum (FBS), concanavalin A (Con A), trypan blue, and (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Animals and care

Both male and female Swiss albino mice (25 ± 2 g of body weight (b.wt.)) of 6–8 weeks of age were used for all the studies. They were housed in polypropylene cages, with dust-free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2 °C. Guinea pigs (250 g) were used for obtaining the complement for plaque-forming cell (PFC) assay. Sheep RBC (sRBC) was collected from sheep, maintained in the departmental animal house and used for immunizing the mouse. All the experiments were performed after obtaining the approval from the Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

Dosage (for *in vivo* experiments)

One hundred twenty (120) Swiss albino mice were divided in to five sets (S 1–5) and each set was sub-divided in to four groups (G 1–4). Therefore, each group contained six mice. All the animals were fed orally with the help of a syringe specially designed by us. Group 1 (G1) of all the sets were considered as control where 0.4 ml of distilled water was given. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of BDE at the dose of 80, 160 and 320 mg/kg b.wt., respectively. In this way, all groups of S1 were treated daily for 15 d, S2 daily for 45 d, S3 daily for 90 d, S4 daily for 135 d and S5 daily for 180 d.

We assume that the average maximum amount of cooked *D. esculentum* consumed by a 60 kg weighed individual is about 20 g/d. Keeping this ratio in mind, we formulate different doses for an average weighed adult mouse (25 g), namely, 80 mg/kg body weight, i.e. 2 mg/mouse/d; 160 mg/kg body weight, i.e. 4 mg/mouse/d and 320 mg/kg body weight, i.e. 8 mg/mouse/d.

In vivo experiments

Experimental design for *in vivo* experiments and immunization

The *in vivo* experiments were divided in to two parts. At first, some of the control and BDE-treated mice were immunized with sRBC (0.1 ml, 25% suspension in PBS) through lateral tail vein in the following manner: Set 1 mice were immunized on the 11th day of the experiment whereas, Set 2, Set 3, Set 4 and Set 5 mice were immunized on 41st day, 86th day, 131st day and 176th day of experiment, respectively. The day of immunization in each case was designated as day ''0''. These immunized mice were used for PFC assay and hemaggutination antibody (HA) titer assay.

Second part of the *in vivo* experiments included the measurement of the body weight, relative spleen weight,

counting of the splenocytes, and counting of peritoneal macrophages of the unimmunized mice. Some of the unimmunized mice from each group were sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose, and the body weight, relative spleen weight and counting of the splenocytes were determined. The remaining unimmunized mice were used to perform the peritoneal macrophage counting assay.

PFC assay

The PFC assay was performed according to the previously described method[9] with slight modifications. On the 4th day of immunization with sRBC, single cell suspension from the spleen was prepared in PBS and cells were adjusted at a concentration of 2×10^6 cells/ml. For PFC assay, 0.1 ml of this suspension was mixed with 0.05 ml of guinea pigs complement and 0.05 ml of 25% sRBC (prepared in PBS) to prepare the final mixture. Cuningham chambers were prepared using glass slide and bi-gummed tape (Scotch Brand, St. Paul, MN)[10,11]. The chambers were loaded with a known volume of assay mixture, sealed with paraffin and petroleum jelly (1:1) and incubated at 37 °C for 4 h. After incubation, the plaques were counted under a phase contrast microscope and expressed as PFC per 10^6 spleen cells.

HA titer assay

The serum was collected from the blood of the immunized mice on the 4th day of immunization with sRBC. Blood was collected from tail vein as a standard method. When the animal was sacrificed, blood was also collected from the heart. The collected serum was kept for 45 min at 56 °C in a water bath for the inactivation of complement activity. Eight clear Khan tubes were taken in a rack and marked. In the first tube, 0.1 ml of the serum and 0.9 ml of PBS was added. 0.5 ml of PBS was taken to the subsequent tubes. Then 0.5 ml of the mixed solution from the first tube was added to the second tube, and 0.5 ml of the mixture from the second tube was added to the third tube. In this way, eight such dilutions (double fold) were prepared. From the last tube, 0.5 ml of solution was thrown away thus yielding a serial dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280. Now, 0.1 ml of 10% sRBC (prepared in PBS) was added to each tube and the entire set up was incubated at 37 °C for 12 h in a humidified atmosphere. After incubation, visible hemagglutination was observed and noted down.

Measurement of the body weight, relative spleen weight and counting of the splenocytes

Mice were sacrificed after proper anesthesia (Chloroform and ether in 2:1 ratio) 24 h after the last dose. Body weight and relative spleen weight (spleen weight/100 g of body weight) were recorded. Single cell suspension of the spleen was prepared in RPMI-1640 medium and the number was counted by hemocytometer.

Counting of peritoneal macrophages

Freund's incomplete adjuvant (0.5 ml) was injected in the peritoneum of the unimmunized mouse 24 h prior to the experiment[12,13]. Next day, 2–3 ml PBS was injected in the peritoneum. The peritoneal exudate cells were collected from the mouse under proper anesthesia, collected in centrifuge tube and washed three times with PBS. The pellet was then resuspended in PBS, taken in a glass petridish and incubated for 45 min at 37 °C. After incubation, the supernatant was removed and the petridish was washed with chilled PBS and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in PBS and the solution was mixed with equal volume of neutral red and charged on the hemocytometer to count the number of the live macrophages under a phase contrast microscope.

Ex vivo experiments

Effect of plant extract on primary cultured splenocytes

Effect of BDE on mouse splenocytes was determined according to previously described method[14], with slight modifications. Splenocyte suspension was prepared in RPMI-1640 medium (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin). The cell number was adjusted to 2×10^6 cells per ml and 1 ml of the cell suspension was added in six-well culture plates. To this, 1 ml of FBS, 5 µl of con A (5 µg/ml) and 100 µl of different concentrations (0–200 µg/ml) of BDE (suspended in RPMI 1640) were added and the whole set up was incubated for 24, 48 and 72 h at 37 °C in an incubator having 5% CO_2 and 90% humidity. After the incubation period, the cultures were harvested and washed once at 1000 rpm for 5 min. The cell pellet was then resuspended in 0.5 ml of RPMI-1640 medium. Then, 10 µl of cell suspension was mixed with equal volume of 0.4% trypan blue and was counted by using hemocytometer under phase contrast microscope. Only viable cells were counted.

MTT splenocyte proliferation assay

MTT proliferation assay was carried out according to the previously described method[15] with slight modifications. Mouse splenocyte cell suspension was prepared (Conc. 2×10^6 cells/ml) in RPMI 1640 medium (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin). Then in each well of a 96 well microtiter plate, 100 µl of cell suspension with 10% of FBS was added with 100 µl of different concentrations (0–200 µg/ml) of BDE (suspended in RPMI 1640). The plate was then incubated for 24 h in 37 °C incubator having 5% CO_2 and 90% humidity. After the incubation period, 20 µl of MTT solution (5 mg/ml, dissolved in PBS; pH 7.0) was added to each well. The plate was covered and incubated for 4 h at 37 °C in an incubator. After incubation, 150 µl of the suspension from each well was taken out without disturbing the bottom layer, and 150 µl of DMSO was added to each well and mixed thoroughly. Finally, the optical density (O.D.) was taken in a microplate reader at 540 nm.

Hemolytic assay

Hemolytic effect of BDE on mouse erythrocytes was evaluated by using washed erythrocytes (RBCs). For the preparation of mouse erythrocytes, a previously described

method was followed[16]. Blood samples from Swiss albino mice were collected (each weighing 25 ± 2 g) in citrated tubes. The cells were then washed three times with 20 mM Tris–HCl containing 144 mM NaCl (pH 7.4) and a 2% erythrocyte suspension was prepared. The hemolytic activity of BDE was tested according to a previously described method under *in vitro* conditions in 96-well plates[16]. Each well received 100 µl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well served as negative control containing only solvent. From the second well, 100 µl of BDE of various concentrations (0–50 µg/ml, suspended in 0.85% NaCl solution containing 10 mM $CaCl_2$) were added. The last well served as positive control containing 100 µl of 0.1% Triton X-100 in 0.85% saline. Each well then received 100 µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After 30 min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from six assays.

Statistical analysis

Data have been presented as mean ± SD of six observations. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit) © 1997–2001 by Koichi Yoshioka (available at http://www.qualest.co.jp). Differences in mean ± SD among different groups were statistically analyzed using one way ANOVA followed by Dunnett's test. The IC_{50} values were calculated according to a previous described formula, $Y = 100 * A1/(X + A1)$, where $A1 = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration[17,18]. $p < 0.05$ was considered significant.

Results

Assessment of the humoral immune responses (PFC and HA titer assay)

Significant decreases ($p < 0.001$) were observed in the formation of antibody secreting cells (plaques) in case of doses treated for the longer duration (135 and 180 d) (Table 2) when compared with the respective control groups. Tables 1 and 2 showed dose- and time-dependent decrease in the HA titer value when they were compared with that of their respective controls. After 180 d of the treatment with different doses of BDE, 16-fold decreases in the titer value were observed when compared with the controls.

Measurement of the body weight, relative spleen weight and counting of the splenocytes

Significant decreases in the body weight were observed in case of mice that were fed with BDE for longer durations (135 and 180 d) ($p < 0.05$) when compared with their respective control groups (Group 1) (Table 2). Significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in the relative spleen weight were also observed in case of the treated groups when compared with the respective controls (Tables 1 and 2). Results also showed that the number of splenocytes was also significantly decreased ($p < 0.01$ and $p < 0.001$) in case of BDE treated groups when compared with their respective controls (Tables 1 and 2).

Table 1. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 15 (S1) and 45 d (S2) of treatment.

	15 d (S1)				45 d (S2)			
Parameters observed (¶)	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
Body weight (g)	25.38 ± 0.49	25.36 ± 0.36	25.30 ± 0.65	25.25 ± 0.39	25.53 ± 0.48	25.35 ± 0.58	25.36 ± 0.53	25.33 ± 0.43
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.008	0.48 ± 0.006	0.48 ± 0.003[a]	0.47 ± 0.005[b]	0.50 ± 0.005	0.48 ± 0.003[c]	0.47 ± 0.005[c]	0.45 ± 0.003[c]
Number of splenocytes (mean ± S.D.) × 10^6/ml	31.84 ± 0.59	31.41 ± 0.47	30.77 ± 0.51[b]	30.50 ± 0.59[b]	31.89 ± 0.52	30.18 ± 0.48[c]	28.96 ± 0.44[c]	28.16 ± 0.45[c]
PFC/10^6 cells	120 ± 6.32	118.33 ± 4.08	110.83 ± 4.92[a]	107.5 ± 5.24[b]	120.83 ± 7.36	116.67 ± 2.58	110.83 ± 4.92[a]	106.67 ± 5.16[c]
HA titer value	1:160	1:160	1:160	1:80	1:160	1:80	1:40	1:40
Number of Macrophages (mean ± S.D.) × 10^6	15.94 ± 1.05	15.57 ± 1.73	15.36 ± 1.78	15.14 ± 0.87	15.30 ± 1.05	14.56 ± 0.59	14.56 ± 1.00	14.18 ± 1.24

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

DOI: 10.3109/08923973.2013.775588

Table 2. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 90 (S3), 135 (S4) and 180 d (S5) of treatment.

	90 d (S3)				135 d (S4)				180 d (S5)			
Parameters Observed (¶)	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.48 ± 0.41	25.28 ± 0.56	25.3 ± 0.42	24.86 ± 0.20	25.55 ± 0.35	25.38 ± 0.51	25.01 ± 0.29[a]	24.93 ± 0.22[a]	25.38 ± 0.51	25.03 ± 0.42	24.93 ± 0.32	24.7 ± 0.43[a]
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.005	0.47 ± 0.006[c]	0.45 ± 0.002[c]	0.43 ± 0.005[c]	0.49 ± 0.010	0.46 ± 0.005[c]	0.43 ± 0.005[c]	0.42 ± 0.005[c]	0.49 ± 0.003	0.44 ± 0.005[c]	0.41 ± 0.005[c]	0.38 ± 0.007[c]
Number of splenocytes (mean ± S.D.) $\times 10^6$/ml	31.84 ± 0.59	29.44 ± 0.83[c]	28.10 ± 0.55[c]	26.77 ± 0.62[c]	32 ± 0.70	28.53 ± 0.55[c]	27.41 ± 0.48[c]	25.76 ± 0.59[c]	31.84 ± 0.87	27.84 ± 0.45[c]	25.97 ± 0.84[c]	23.73 ± 0.74[c]
PFC/10^6 cells	119.17 ± 5.85	105.83 ± 5.85[b]	98.33 ± 4.08[c]	89.17 ± 5.85[c]	112.50 ± 7.58	102.50 ± 8.80	92.50 ± 7.58[c]	69.17 ± 5.85[c]	115.47 ± 4.47	100 ± 7.07[c]	80 ± 7.07[c]	55 ± 4.47[c]
HA titer value	1:160	1:80	1:40	1:40	1:160	1:20	1:20	1:20	1:160	1:10	1:10	1:10
Number of Macrophages (mean ± S.D.) $\times 10^6$	16.05 ± 1.27	15.09 ± 1.39	14.72 ± 1.99	14.08 ± 1.23	16.26 ± 1.42	15.04 ± 1.10	13.22 ± 0.82[c]	14.34 ± 1.39[c]	17.06 ± 0.94	14.45 ± 1.23[b]	13.65 ± 1.48[c]	11.36 ± 0.77[c]

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

Effect of BDE on the number of peritoneal macrophages

As indicated in Tables 1 and 2, the number of the peritoneal macrophages decreased significantly in both dose- and time-dependent manner. After 15 d dose duration period, no significant decrease was observed in the mice that were treated with BDE at a dose of 320 mg/kg b.wt., whereas after 180 d of treatment, significant decreases ($p < 0.01$ and $p < 0.001$) were observed in case of all the doses when compared with the respective control groups.

Effect of BDE on splenocyte proliferation

After 24, 48 and 72 h of incubation, at 0 μg/ml (control), the number of splenocytes was $(11.57 \pm 0.93) \times 10^6$ cells/ml, $(16.16 \pm 0.59) \times 10^6$ cells/ml and $(12.96 \pm 0.59) \times 10^6$ cells/ml, respectively, whereas, at 200 μg/ml (highest dose), the number of splenocytes decreased remarkably to $(2.93 \pm 0.37) \times 10^6$ cells/ml, $(4.10 \pm 0.47) \times 10^6$ cells/ml and $(3.2 \pm 0.49) \times 10^6$ cells/ml, respectively. Therefore, the results clearly indicated that the number of splenocytes was significantly ($p < 0.001$) decreased in a dose-dependent manner in each case when they were compared with their respective controls (Figure 1a–c). As shown in Figure 2, a significant ($p < 0.001$) dose-dependent increase in the percentage inhibition of the splenocyte proliferation has been observed in case of the BDE-treated splenocytes when compared with the control (0 μg/ml). At 200 μg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 μg/ml.

Assessment of the effect of BDE on hemolysis

The hemolytic activity of BDE was increased significantly ($p < 0.001$) in a dose-dependent manner in case of mouse erythrocytes (Figure 3). Total hemolysis was obtained using 100 μl of Triton X-100 (0.1%) after 30 min of incubation (not shown in the figure). At 50 μg/ml, the percentages of hemolysis was 40.75%, whereas, the IC_{50} value was 61.78 ± 2.77 μg/ml.

Discussion

Under various regulatory guidelines, body weight gain is an integral part of the conventional safety evaluation of a test material[19]. 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[20]. The choice of appropriate organ to be weighed in toxicological studies involves understanding the test article's mechanism of action, metabolism, toxicokinetics and the physiology of the test species[21]. 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 detection of the 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[22]. The dose- and time-dependent decreases in the number

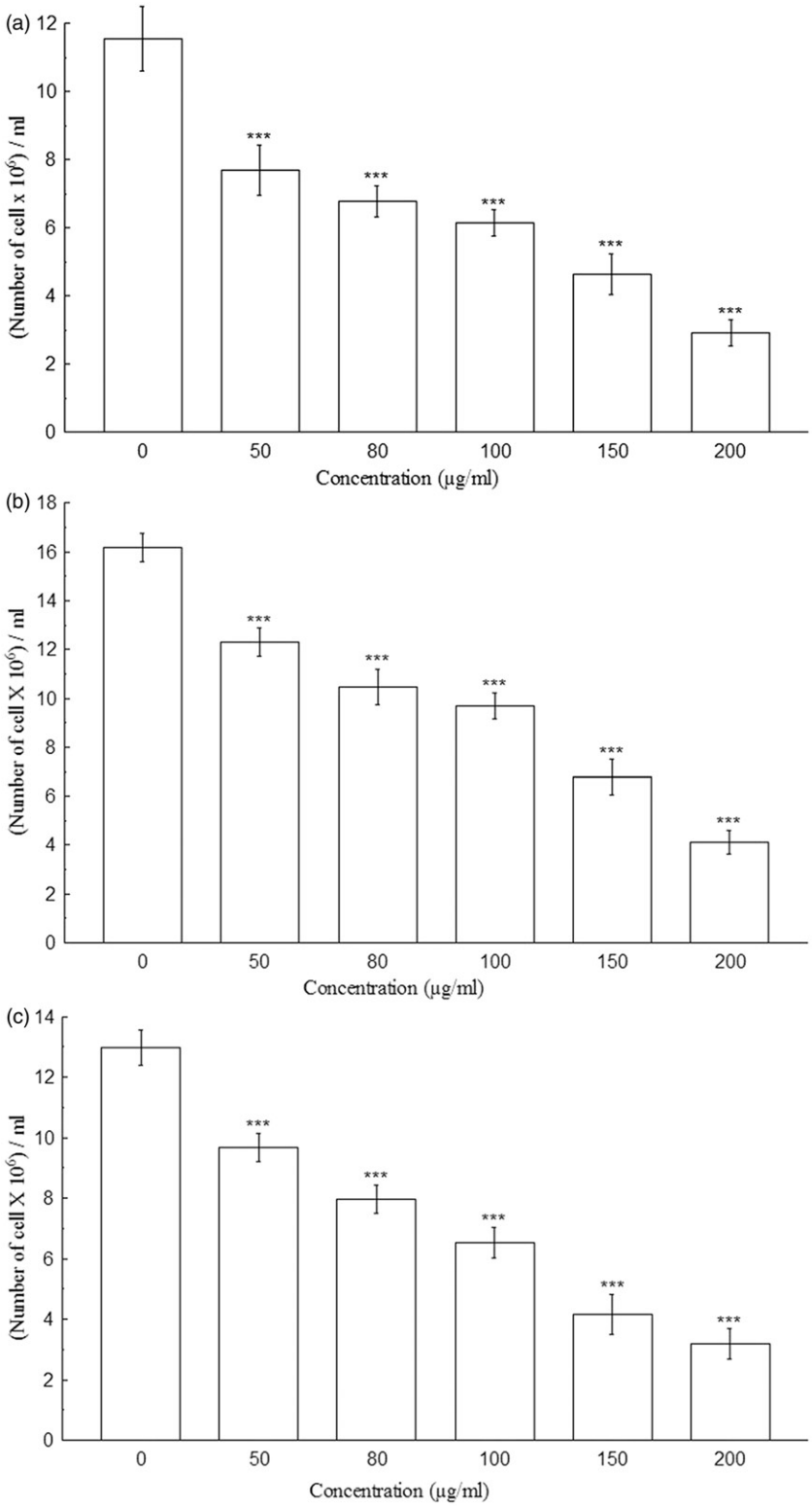


Figure 1. (a) Effect of BDE on the primary cultured splenocytes after 24 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ versus 0 µg/ml. (b) Effect of BDE on the primary cultured splenocytes after 48 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ versus 0 µg/ml. (c) Effect of BDE on the primary cultured splenocytes after 72 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ versus 0 µg/ml.

of the PFCs as well as the progressive decrease in the hemagglutination titer values in all the treated groups indicate immunosuppressive potential of *D. esculentum*.

Another important parameter that could help in assessing the immunodulatory activity of *D. esculentum* was the counting of the peritoneal macrophages. Macrophages are the important regulatory cells that are central to cell-mediated and humoral immunity as antigen-presenting, tumoricidal and microbicidal cells[23]. 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

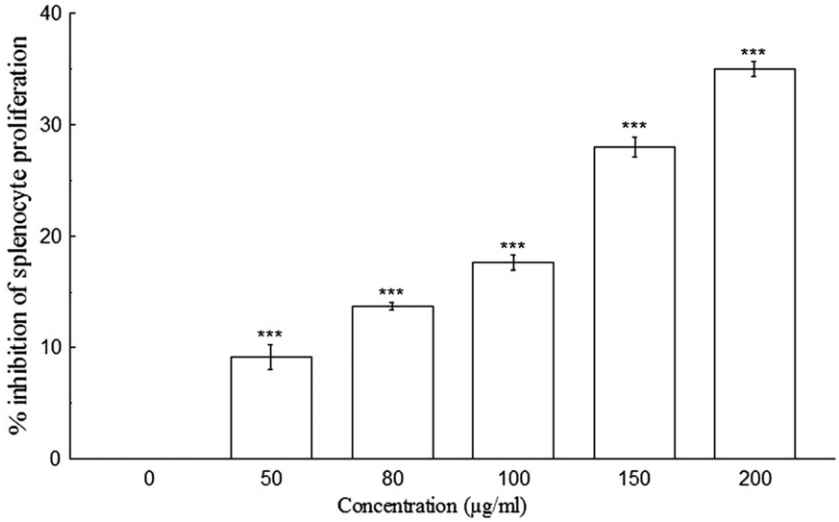


Figure 2. MTT splenocytes proliferation assay demonstrates dose-dependent increase in the percentage inhibition of splenocytes proliferation in the BDE treated splenocytes. At 200 μg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 μg/ml. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ versus 0 μg/ml.

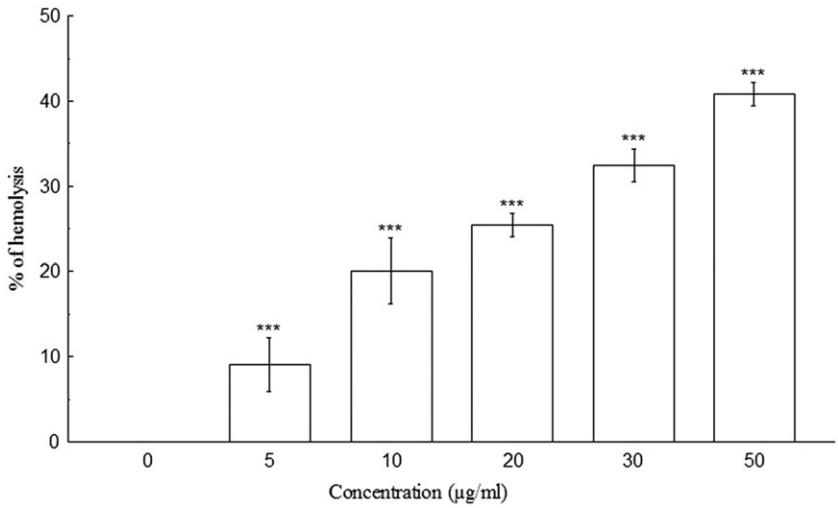


Figure 3. The hemolytic activity of BDE was increased significantly in a dose-dependent manner in case of mouse erythrocytes. At 50 μg/ml, the percentage of hemolysis was 40.75%. The IC_{50} value of BDE was 61.78 ± 2.77 μg/ml. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ versus 0 μg/ml.

the metabolic activity of cells. Both of these assays showed that the BDE induced the inhibition of cell proliferation, thereby indicating its immunosuppressive activity.

Hemolysis is due to red blood cell destruction which resulted from lysis of membrane lipid bilayer. According to Fick's law, diffusion flux from a membrane is proportional to concentration difference of both sides[24]. In the present study, progressive increase in the concentration of the BDE in extra cellular membrane causes its diffusion in to the intra cellular membrane up to a specific concentration, which leads to membrane destruction, thus showing its hemolytic potential.

Study revealed that *D. esculentum* collected from high-altitude area of Harsil-Gangotri had 19 mg/kg Ptaquiloside[6]. Shade- and freeze-dried samples of *D. esculentum* showed absence of fern toxin ptaquiloside but the presence of 10.94–16.36 mg/kg pterosin B only in two of the freeze-dried samples by HPLC method[7]. 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[8]. 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 and serum dehydrogenases. Feeding of frozen dried sample of *D. esculentum* induced 53% mortality in guinea pigs[7].

All the studies done so far were on the freeze- 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 performed the experiment using mouse as this is the standard convention to use inbreed strains of mouse for performing the immunological experiments. However, experiments using rabbits and guinea pigs may be performed in future once it is established that this fern is toxic as food.

As ptaquiloside is a heat labile compound, boiling may probably reduce its toxicity. Present study showed several toxic effects of this fern on the immune system of 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 g. Tannins inhibit protein availability through denaturation[25]. 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 boiled preparation of *D. esculentum*, and may be one of the causes of immunotoxicity. Thus, the immunotoxic effects observed in our study could be related to tannins and other heat stable compounds.

We have used different doses of food for different time periods, so that low and high levels of 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 d and also for a long period like 6 months. As it may so happen that consumption of this fern for a shorter period may not cause any problem, therefore we had to choose 15 d period. But, as food consumption was not evaluated in the present study, it is not possible to assure that BDE itself induces an immunotoxic effects on animals. If food consumption is reduced, nutritional status may interfere with immune response.

The possible consequences of the immunosuppressive effects of *D. esculentum* on human health may be alarming. Immunosuppression may directly or indirectly induce several metabolic diseases and age-related degenerative disorders, as well as may increase the risk of infection in the people who regularly consumes this fern; induce a state of immunodeficiency as an unwanted consequence, and therefore, may also be related to the growth of tumors.

Conclusions

Diplazium esculentum, the vegetable fern, is extensively used as a palatable food throughout Asia, Oceania and especially in the Northern part of West Bengal where we reside. Considering the findings of the present study, it can be concluded that *D. esculentum*, even boiled, possesses potent immunosuppressive and hemolytic activities. This is the first report on the assessment of the immune dysfunction due to the intake of the edible *D. esculentum*, and thereby to make people aware about the hazards of its consumption and it will advance the existing knowledge in relation to human health.

Declaration of interest

The authors report no declarations of interest.

We gratefully acknowledge the financial support (Vide Letter F.No.37-464/2009 (SR) dt.11.01.2010) received from the University Grants Commission (UGC), New Delhi, India to carry out this study.

References

1. Teklehaymanot T, Giday M. Ethnobotanical study of wild edible plants of Kara and Kwego semi-pastoralist people in Lower Omo River Valley, Debub Omo Zone, SNNPR, Ethiopia. J Ethnobiol Ethnomed 2010;6:23–30.
2. Uniyal SK, Awasthi A, Rawat GS. Developmental processes, changing lifestyle and traditional wisdom: analyses from western Himalaya. Environmentalist 2003;23:307–12.
3. Angami A, Gajurel PR, Rethy P, et al. Stats and potential of wild edible plants of Arunachal Pradesh. Indian J Trad Knowl 2006;5: 541–50.
4. Sen A, Ghosh PD. A note on the ethnobotanical studies of some pteridophytes in Assam. Indian J Trad Knowl 2011;10:292–5.
5. Somvanshi R, Devi V, Gounalan S, Kataria M. Preliminary clinicopathological observations on effects of linguda *(Diplazium esculentum*) feeding in laboratory rabbits. Indian J Toxicol 1998; 5:7–11.
6. Somvanshi R, Lauren DR, Smith BL, et al. Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. Current Sci 2006;91:1547–52.
7. Gangwar NK. Studies on the pathological effects of *linguda* (*Diplazium esculentum*, Retz.) in laboratory rats and guinea pigs. Indian J Vet Pathol 2004;28:149–50.
8. Smith BL, Seawright, AA, Ng JC, et al. Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium* spp.) from eastern Australia and from cultivated worldwide collection held in Sydney, Australia. Nat Toxins 1994;2:347–53.
9. Raisuddin S, Zaidi SIA, Singh KP, Ray PK. Effect of subchronic aflatoxin exposure on growth and progression of Ehrlich's ascites tumor in mice. Drug Chem Toxicol 1991;14:185–206.
10. Cunningham AJ, Szenberg A. Further improvements in the plaque technique for detecting single antibody forming cell. Immunol 1968;14:599–601.
11. Williams CA, Chase MW. Methods in immunology and immunochemistry. Vol 5. New York: Academic Press; 1976.
12. Landahl CA. Ontogeny of adherent cells involved in immune response [Ph.D. Thesis]. Madison, WI: University of Wisconsin; 1974.
13. Chakraborty AK, Chakravarty AK. Antibody mediated immune response in the bat, *P.giganteus*. Dev Comp Immunol 1984;8: 415–23.
14. Yeap SK, Alitheen NBM, Ho WY, et al. Immunomodulatory role of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell proliferation, cytokine secretion and cytolytic activity. J Med Plant Res 2010;5:958–65.
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
16. Malagoli D. A full-length protocol to test hemolytic activity of palytoxin on human erythrocytes. ISJ 2007;4:92–4.
17. Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Compl Alt Med 2008;8:63–72.
18. Hazra B, Sarkar S, Biswas S, Mandal N. Comparative study of the antioxidant and reactive oxygen species scavenging properties in the extracts of the fruits of *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*. BMC Compl Alt Med 2010;10:20–34.
19. Schilter B, Andersson C, Anton R, et al. Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. Food Chem Toxicol 2003;41:1625–49.
20. Wooley A, ed. Determination – general and reproductive toxicology. In: A guide to practical toxicology evaluation, prediction and risk. New York: Taylor and Francis, 2003: 80–106.
21. Khan MI, Denny-Joseph KM, Muralidhara, et al. Acute, subacute and subchronic safety assessment of betalains rich *Rivina humilis* L. berry juice in rats. Food Chem Toxicol 2011;49:3154–7.
22. Wilson, SD, Munson AE, Meade BJ. Assessment of the functional integrity of the humoral immune response: the plaque-forming cell assay and the enzyme-linked immunosorbent assay. Methods 1999; 19:3–7.
23. Cavaillon JM. Cytokines and macrophages. Biomed Pharmacother 1994;48:445–53.
24. Kleszczynska H, Bonarska D, Luczynski J, et al. Hemolysis of erythrocytes and erythrocyte membrane fluidity change by new lysosmtropic compounds. J Fluoresc 2005;15:137–41.
25. Bassey ME, Etuk EUI, Ibe MM, Ndon BA. *Diplazium sammatii*: athyraceae ('Nyama Idim'): age-related nutritional and antinutritional analysis. Plants Food Hum Nutr 2001;56:7–12.

International Journal of Food Properties, 16:1351–1370, 2013

ISSN: 1094-2912 print / 1532-2386 online
DOI: 10.1080/10942912.2011.587382

Taylor & Francis
Taylor & Francis Group

ASSESSMENT OF THE ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF METHANOLIC EXTRACT OF *DIPLAZIUM ESCULENTUM*

Subhrajyoti Roy[1], Bibhabasu Hazra[2], Nripendranath Mandal[2], and Tapas Kumar Chaudhuri[1]

[1]*Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India*
[2]*Division of Molecular Medicine, Bose Institute, Kolkata, India*

The present study was carried out to determine the antioxidant and different free radical scavenging activities of 70% methanolic extract of* Diplazium esculentum*. Total antioxidant activity of the extract was evaluated as trolox equivalent antioxidant capacity value. The IC_{50} values for scavenging of different free radicals indicated its efficient free radical scavenging properties. The extract acted as an iron chelator and also possessed reducing power. It also inhibited lipid peroxidation. Moreover, the extract yielded high phenolic and flavonoid content. Therefore, the results indicated that 70% methanolic extract of* D. esculentum *acted as a potential antioxidant and free radical scavenger.

Keywords: *Antioxiodant,* **Diplazium esculentum***, Lipid peroxidation, Phenolic content.*

INTRODUCTION

It is now well known that free radicals, the byproducts of aerobic metabolism, are fundamental in modulating various physiological functions.[1] Free radicals are produced by endogenous sources, such as mitochondrial leak, respiratory burst, enzyme reactions, and auto-oxidant reactions, and environmental sources, such as smoking, pollutants, ultraviolet and ionizing radiations, and xenobiotics.[2] They have unpaired electrons and seek stability through electron pairing with biological molecules, such as protein, lipid, and DNA in healthy cells, and cause protein and DNA damage along with lipid peroxidation. All these factors result in the disruption of the antioxidant defense mechanism of the body, which may lead to 'oxidative stress', bringing about a variety of pathophysiological conditions, such as coronary heart disease, neurodegenerative disorders, diabetes, arthritis, inflammatory diseases, lung damage, aging, and cancer.[3–5]

The body has effective antioxidant defense systems, which constitute enzymes, such as superoxide dismutase (SOD), and catalase and compounds, such as ascorbic acid, tocopherol, and glutathione.[6] But all these endogenous antioxidants are not sufficient in

Received 24 February 2011; accepted 8 May 2011.
Address correspondence to Tapas Kumar Chaudhuri, Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, Siliguri, West Bengal 734013, India. E-mail: dr_tkc_nbu@rediffmail.com

protecting the body against oxidative stress. Therefore, dietary supplementation through natural antioxidants in place of synthetic antioxidants is necessary in strengthening the antioxidant system of the body by inhibiting free radical generation and thus preventing chronic diseases. Recently, much attention has been directed towards exploring natural antioxidants because they are natural products that are considered to be a safe source.

Diplazium esculentum (Koenig *ex* Retz.) Sw. (Family—Athyriaceae) is an edible fern distributed throughout Asia and Oceania. It is known as paco in the Philippines, linguda in northern India referring to the curled fronds, and dheki shak in the northern region of West Bengal, India. The young fronds are stir-fried as a vegetable or used in salads. The genus *Diplazium* includes 400 known species. *Diplazium esculentum* is one of the most common varieties and perhaps the most commonly consumed fern. Several studies have been performed to determine the antioxidant activity of this plant using different methodologies,[7–10] but there have been no reports on various endogenously produced free radicals scavenging activities by this plant.

Therefore, the objective of the present study was not just to evaluate the total antioxidant potential but also the scavenging activities of different endogenously produced free radicals that comprise various reactive oxygen species (ROS) and reactive nitrogen species (RNS), including hydroxyl, superoxide, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen, and hypochlorous acid, using 70% methanolic extract of *D. esculentum*. The extract was also examined for iron chelating capacity, measurement of reduction power, lipid peroxidation inhibition ability, and for total phenolic and flavonoid content.

MATERIALS AND METHODS

Chemicals

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche Diagnostics, Mannheim, Germany. 6-Hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), ethylenediamine tetraacetic acid (EDTA), ascorbic acid, 2-deoxy-2-ribose, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride (NED), L-histidine, lipoic acid, sodium pyruvate, quercetin, and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Hydrogen peroxide, potassium hexacyanoferrate, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), butylated hydroxytoluene (BHT), sodium hypochloride (NaOCl), aluminium chloride ($AlCl_3$), ammonium iron (II), sulfate hexahydrate ($(NH_4)_2Fe\ (SO_4)_26H_2O$), potassium nitrite ($KNO_2$), N,N-dimethyl-4-nitrosoaniline, and xylenol orange were obtained from Merck, Mumbai, India. Gallic acid and curcumin were obtained from MP Biomedicals, Illkirch, France. Ferrous sulfate and catalase were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Evans Blue was purchased from BDH, London, England. Mangese dioxide was obtained from SD Fine Chemicals, Mumbai, India. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd., Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Sodium nitrite was obtained from Qualigens Fine Chemicals, Mumbai, India.

Plant Material

Young *D. esculentum* plants were collected from different areas of North Bengal University campus, Darjeeling, India. These were identified by Professor A.P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and a voucher specimen (Accession No. 9601) was submitted to him.

Animals

Inbred adult Swiss albino mice (20 ± 2 g) were used for lipid peroxidation inhibition assay. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12-h dark/light cycle at an environmental temperature of 25 ± 2°C. The experiment was performed after obtaining approval from the Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

Sample Preparation

Samples were prepared according to a previously described method.[11] The young fronds of *D. esculentum* were dried at room temperature for 7 days, finely powdered, and used for extraction. The powder (100 g) was mixed with 500 ml methanol:water (7:3) using a shaker for 15 h; then the mixture was centrifuged at $2850\times$ *g* and the supernatant was decanted. The pellet was mixed again with 500 ml methanol-water and the entire process was repeated once again, i.e., the extraction procedure was done twice. The supernatants collected from the two phases were mixed in a round-bottom flask and concentrated under reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The residue was kept at -20°C for future use. Double-distilled water (MilliQ grade) was used as the solvent for the lyophilized extract in all the experiments.

Total Antioxidant Activity

The antioxidant activity of the *D. esculentum* extract was assayed depending on the ability to scavenge $ABTS^{\bullet+}$ radical cation compared to the trolox standard.[12] The $ABTS^{\bullet+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12–16 h in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the $ABTS^{\bullet+}$ was equilibrated to 0.70 ($\pm$0.02) by diluting with water at room temperature. Then 1 ml of $ABTS^{\bullet+}$ was mixed with 10 µl of the test sample (0.05–10 mg/ml) and the absorbance was measured at 734 nm after 6 min. All experiments were repeated six times. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant capacity (TEAC), which was calculated from dividing the gradient of the plot for the sample by the gradient of the plot for trolox.

Hydroxyl Radical Scavenging Activity

This was assayed according to a standard method with a slight modification.[13] This assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system

(Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM); and various concentrations (0–200 μg/ml) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical $OH^{\bullet}$ scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

Superoxide Radical Scavenging Activity

This activity was determined based on the reduction of NBT according to a previously reported method.[14] The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals that reduce nitro blue tetrazolium (NBT) into a purple-colored formazan. The 1-ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM), and various concentrations (0–20 μg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance was taken at 562 nm against an appropriate blank solution. All tests were performed six times. Quercetin was used as the positive control.

Nitric Oxide Radical Scavenging Activity

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illisvoy reaction.[15] The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4), and various concentrations (0–70 μg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Curcumin was used as a standard.

Hydrogen Peroxide Scavenging Activity

This activity was determined according to a previously described method[16] with minor changes. An aliquot of 50 mM H_2O_2 and various concentrations (0–2 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 μl of the H_2O_2 sample solution was mixed with 10 μl of HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were performed six times. Sodium pyruvate was used as the reference compound.[17]

Peroxynitrite Scavenging Activity

Peroxynitrite ($ONOO^-$) was synthesized by a previously described method.[18] An acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) was mixed with 5 ml of 0.6 M KNO_2 on an ice bath for 1 s and 5 ml of ice-cold 1.2 M NaOH was added. Excess H_2O_2 was removed by treatment with granular MnO_2 prewashed with 1.2 M NaOH and the reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

The Evans Blue bleaching assay[19] was used to measure the peroxynitrite scavenging assay, with slight modification. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various doses of plant extract (0–200 μg/ml), and 1 mM peroxynitrite in a final volume of 1 ml. After incubation at 25°C for 30 min, the absorbance was measured at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound.

Singlet Oxygen Scavenging Activity

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method.[20] Singlet oxygen was generated by a reaction between NaOCl and H_2O_2, and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM histidine, 10 μM RNO, and various concentrations (0–200 μg/ml) of sample in a final volume of 2 ml. It was incubated at 30°C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of the sample was compared with that of lipoic acid, which was used as a reference compound. All tests were performed six times.

Hypochlorous Acid Scavenging Activity

Hypochlorous acid was prepared immediately before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of $100\ M^{-1}\ cm^{-1}$. The assay was carried out according to a previously described method[21] with minor changes. The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM), and increasing concentrations (0–100 μg/ml) of plant extract. The assay mixture was incubated at 25°C for 20 min and the absorbance was measured against an appropriate blank. All tests were performed six times. Ascorbic acid, a potent HOCl scavenger, was used as a reference compound.[22]

Fe^{2+} Chelation

The ferrous ion chelating activity was evaluated by a standard method[23] with minor changes. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various

concentrations (0–120 μg/ml) of plant extract were added to 12.5 μM ferrous sulfate solution and the reaction was initiated by the addition of ferrozine (75 μM). The mixture was shaken vigorously and incubated for 20 min at room temperature, and then the absorbance was measured at 562 nm. All tests were performed six times. EDTA was used as a positive control.

Reducing Power

The Fe^{3+}-reducing power of the extract was determined by a previously described method[24] with slight modification. Different concentrations (0–1 mg/ml) of the extract (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water, and 0.1 ml of $FeCl_3$ solution (0.01%) was added. The reaction mixture was left over for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

Lipid Peroxidation Inhibition Assay

This assay was carried out according to a previously described method,[25] with slight modification. Brain homogenate was prepared by centrifuging Swiss albino mouse brain (20 ± 2 g) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 μl aliquot of the supernatant homogenate was mixed with the plant extract of various concentrations (0–25 μg/ml), followed by the addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, and incubated for 1 h at 37°C. Next, 500 μl 28% TCA was used to stop the reaction and then 380 μl 2% TBA was added with heating at 95°C for 30 min to generate the color. Then the samples were cooled on ice, centrifuged at 8000 rpm for 2 min, and the absorbance of the supernatant was taken at 532 nm. All tests were performed six times. Trolox was used as the standard.

Determination of Total Phenolic Content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to a previously described method[26] with a slight modification. Briefly, the plant extract (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.75 ml of 0.06% Na_2CO_3 solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. All tests were performed six times. The phenolic content was evaluated using gallic acid as a standard. The result was expressed as mg of gallic acid equivalent phenolic content present in 1 g sample plant material.

Determination of Total Flavonoid Content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to a known method[27] using quercetin as a standard. The plant extract (0.1 ml) was

added to 0.3 ml of distilled water followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All tests were performed six times. The flavonoid content was evaluated using quercetin as a standard. The result was expressed as mg of quercetin equivalent flavonoid present in 1 g of sample plant material.

Statistical Analysis

All data are given as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula $Y = 100^*A1/(X + A1)$, where $A1 = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired *t* tests. $p < 0.05$ was considered significant.

RESULTS

Total Antioxidant Activity

The total antioxidant activity of the extract was calculated from the decolorization of $ABTS^{\bullet+}$, which was measured spectrophotometrically at 734 nm. Interaction with the plant extract or standard trolox suppressed the absorbance of the $ABTS^{\bullet+}$ radical cation and the results, expressed as percentage inhibition of absorbance, are shown in Figs. 1a and 1b, respectively. The TEAC value of the extract was 0.21 ± 0.02.

Hydroxyl Radical Scavenging Activity

This assay shows the abilities of the extract and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Fig. 2. The IC_{50} values (Table 1) of the extract

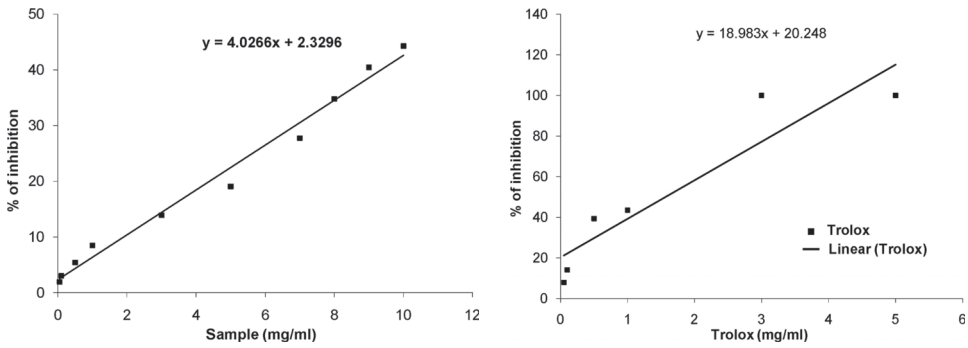


Figure 1 (a) Total antioxidant activity of *D. esculentum* extract on decolorization of ABTS radical cation. The percentage inhibition was plotted against the concentration of the sample. All data are expressed as mean ± S.D. ($n = 6$). (b) Total antioxidant activity of reference compound trolox on decolorization of ABTS radical cation. The percentage inhibition was plotted against the concentration of the sample. All data are expressed as mean ± S.D. ($n = 6$).

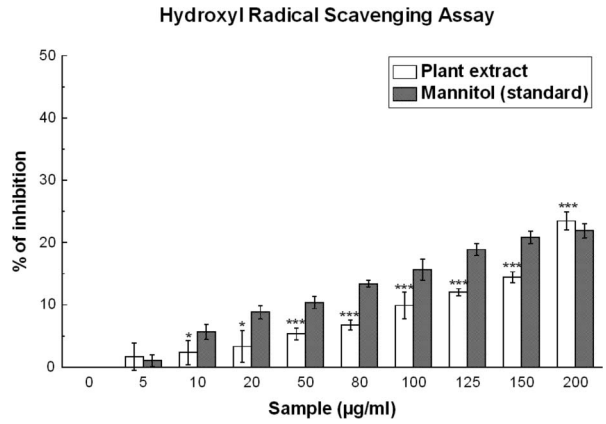


Figure 2 Hydroxyl radical scavenging assay. Hydroxyl radical scavenging activities of the *D. esculentum* extract and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ± S.D. of six parallel measurements. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 811 ± 23.73 and 571.45 ± 20.12 µg/ml, respectively.

Table 1 Scavenging of reactive oxygen species, iron chelating, and lipid peroxidation inhibition activity (IC_{50} values) of *Diplazium esculentum* and reference compounds.

Activity	Extract/Reference	IC_{50}[#]
Hydroxyl radical (OH•) scavenging	*Diplazium esculentum*	811.00 ± 23.73
	Mannitol	571.45 ± 20.12 (6)***
Superoxide anion ($O_2^{\bullet -}$) scavenging	*Diplazium esculentum*	90.39 ± 2.22
	Quercetin	42.06 ± 1.35 (6)***
Nitric oxide radical (NO) scavenging	*Diplazium esculentum*	204.28 ± 18.31
	Curcumin	90.82 ± 4.75 (6)***
Hydrogen peroxide (H_2O_2) scavenging	*Diplazium esculentum*	4.17 ± 0.86
	Sodium pyruvate	3.24 ± 0.30 (6)N.S.
Peroxynitrite ($ONOO^-$) scavenging	*Diplazium esculentum*	3.35 ± 0.33
	Gallic acid	0.87 ± 0.05 (6)***
Singlet oxygen ($^1O_2$) scavenging	*Diplazium esculentum*	278.88 ± 6.02
	Lipoic acid	46.15 ± 1.16 (6)***
Hypochlorous acid (HOCl) scavenging	*Diplazium esculentum*	338.96 ± 11.60
	Ascorbic acid	235.95 ± 5.75 (6)***
Iron chelating	*Diplazium esculentum*	1.33 ± 1.13
	EDTA	0.001 ± 0.000 (6)*
Lipid peroxidation inhibition	*Diplazium esculentum*	141.67 ± 4.19
	Trolox	6.76 ± 0.17 (6)***

[#]Units of IC_{50} for all activities are µg/ml, except H_2O_2 scavenging, peroxynitrite scavenging, and iron chelating where the units are mg/ml. Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.

EDTA: ethylenediamine tetraacetic acid; N.S.: not significant.

$^{*}p < 0.05$; $^{***}p < 0.001$ vs. *Diplazium esculentum*.

and the standard in this assay were 811.00 ± 23.73 and 571.45 ± 20.12 µg/ml, respectively. Though the IC_{50} value of the extract was greater than that of the standard, at 200 µg/ml, the percentages of inhibition were 23.4 and 21.9% for *D. esculentum* and mannitol, respectively.

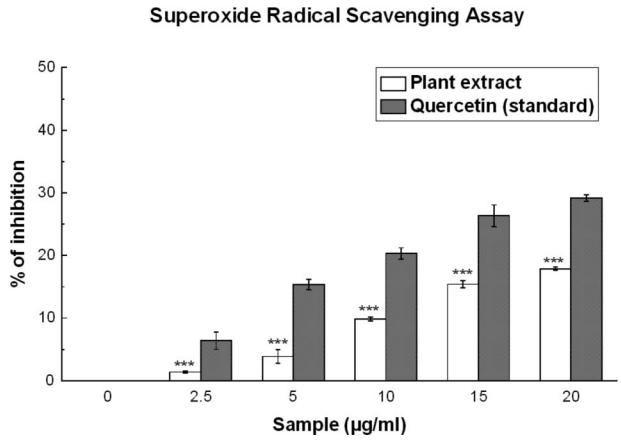


Figure 3 Superoxide radical scavenging assay. Scavenging effect of *D. esculentum* plant extract and the standard quercetin on superoxide radical. All data are expressed as mean ± S.D. ($n = 6$). $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 90.39 ± 2.22 and 42.06 ± 1.35 µg/ml, respectively.

Superoxide Radical Scavenging Activity

Superoxide radicals, generated from the PMS-NADH coupling, can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in Fig. 3, the IC_{50} values (Table 1) of the plant extract and quercetin on superoxide scavenging activity were 90.39 ± 2.22 and 42.06 ± 1.35 µg/ml, respectively. At 20 µg/ml, the percentage of inhibition of the plant extract was 17.8%, whereas that of quercetin was 29.1%.

Nitric Oxide Radical Scavenging

As shown in Fig. 4, *D. esculentum* extract also caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} value (Table 1) of 204.28 ± 18.31 µg/ml. Curcumin was used as a reference compound and 90.82 ± 4.75 µg/ml curcumin was needed for 50% inhibition. At 70 µg/ml, the percentage of inhibition of the plant extract was 23.2% whereas that of curcumin was 43.9%.

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide scavenging activity was assayed by the FOX reagent method. Figure 5 shows that the plant extract has good H_2O_2 scavenging activity ($IC_{50} = 4.17 \pm 0.86$ mg/ml) when compared with the standard sodium pyruvate ($IC_{50} = 3.24 \pm 0.30$ mg/ml) (Table 1). At 2 mg/ml, the percentage of scavenging was 38.4 and 57.5% for *D. esculentum* and sodium pyruvate, respectively. At lower doses, viz. 0.5, 0.8, and 1 mg/ml, the percentages of scavenging of the plant extract were 10.5, 15.2, and 17.0%, respectively, which were much higher than that of the sodium pyruvate (1.5, 5.2, and 11.7% for 0.5, 0.8, and 1 mg/ml, respectively).

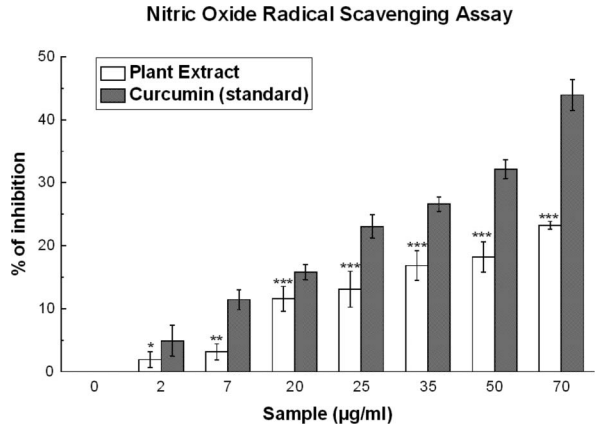


Figure 4 The nitric oxide radical scavenging activity of *D. esculentum* extract and the standard curcumin. The data represent the percentage nitric oxide inhibition. Each value represents mean ± S.D. ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 204.28 ± 18.31 and 90.82 ± 4.75 µg/ml, respectively.

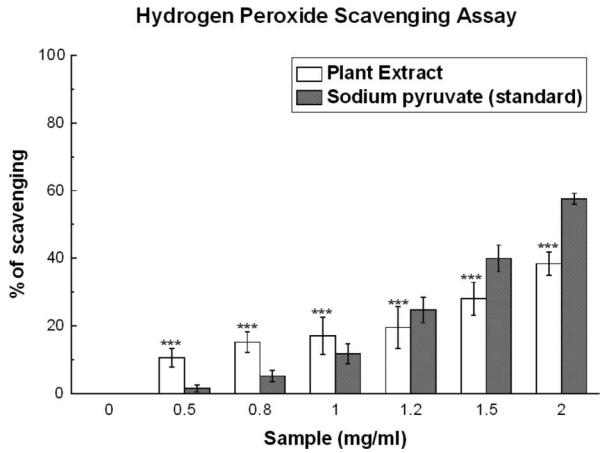


Figure 5 Hydrogen peroxide scavenging assay. Effect of *D. esculentum* plant extract and the sodium pyruvate on the scavenging of H_2O_2. The data represent the percentage H_2O_2 scavenging. All data are expressed as mean ± S.D. ($n = 6$). $^{***}p < 0.001$ vs. 0 mg/ml. IC_{50} values of the plant extract and standard are 4.17 ± 0.86 and 3.24 ± 0.30 mg/ml, respectively.

Peroxynitrite Scavenging Activity

Figure 6 shows that the peroxynitrite scavenging activity of the plant extract is concentration dependent. The calculated IC_{50} value was 3.35 ± 0.33 mg/ml, which was much greater than that of the reference compound gallic acid (IC_{50} = 0.87 ± 0.05 mg/ml) (Table 1), indicating that the plant extract is not a good peroxynitrite scavenger when compared to gallic acid. At 200 µg/ml, the scavenging percentages were 7.5 and 15.4% for *D. esculentum* and gallic acid, respectively.

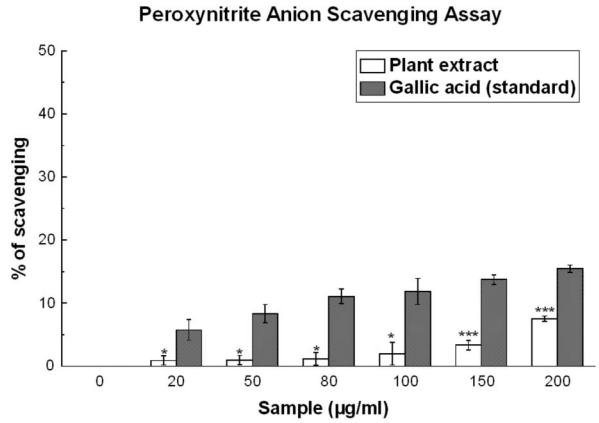


Figure 6 Peroxynitrite anion scavenging assay. The peroxynitrite anion scavenging activity of *D. esculentum* plant extract and the standard gallic acid. Each value represents mean ± S.D. ($n = 6$). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 3.35 ± 3.33 and 0.87 ± 0.05 mg/ml, respectively.

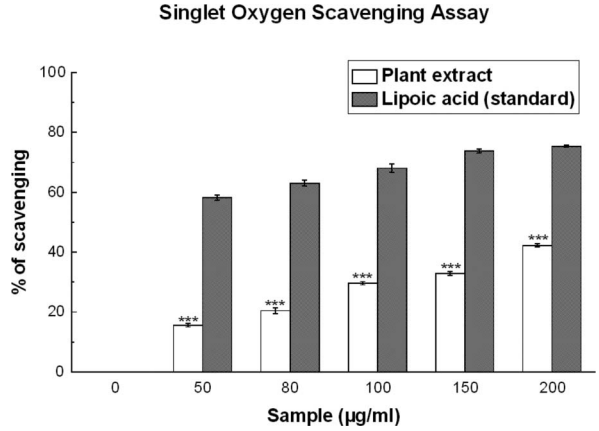


Figure 7 Singlet oxygen scavenging assay. Effects of *D. esculentum* plant extract and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. $^{***}p < 0.001$ vs. µg/ml. IC_{50} values of the plant extract and standard are 278.88 ± 6.02 and 46.15 ± 1.16 µg/ml, respectively.

Singlet Oxygen Scavenging Activity

D. esculentum extract has moderate singlet oxygen scavenging activity when compared to that of lipoic acid (Fig. 7). The IC_{50} value (Table 1) of the test sample was 278.88 ± 6.02 µg/ml, whereas that of lipoic acid was 46.15 ± 1.16 µg/ml. At 200 µg/ml, the percentage scavenging of plant extract was 42.2%, whereas that of lipoic acid was 75.3%.

Hypochlorous Acid Scavenging Activity

Figure 8 shows that *D. esculentum* possesses an efficient hypochlorous acid scavenging activity ($IC_{50} = 338.96 \pm 11.60$ µg/ml) when compared to that of ascorbic acid

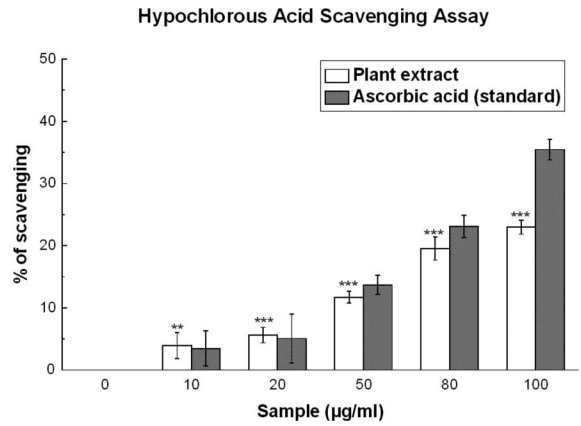


Figure 8 Hypochlorous acid scavenging activities of *D. esculentum* plant extract and the standard ascorbic acid. All data are expressed as mean ± S.D. ($n = 6$). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 338.96 ± 11.60 and 235.95 ± 5.75 µg/ml, respectively.

($IC_{50} = 235.95 \pm 5.75$ µg/ml) (Table 1). At 100 µg/ml, the percentage scavenging of the plant extract was 22.9% whereas that of ascorbic acid was 35.4%. At lower doses, viz. 10 and 20 µg/ml, the percentages scavenging of the plant extract were 3.9 and 5.6%, respectively, which were higher than that of ascorbic acid (3.4 and 5.0% for 10 and 20 µg/ml, respectively).

Fe^{2+} Chelation

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted, and as a result, the violet color of the complex is decreased. The results (Figs. 9a and 9b) demonstrate that the formation of the ferrozine-Fe^{2+} complex is inhibited in the presence of the test and reference compounds. The IC_{50} values (Table 1) of the plant extract and EDTA were 1.33 ± 1.13 and 0.001 ± 0.000 mg/ml, respectively. At 120 µg/ml, the percentage inhibition of the plant extract was 14.72%, whereas at 20 µg/ml that of EDTA was 99.34%.

Reducing Power

As illustrated in Fig. 10, Fe^{3+} was transformed to Fe^{2+} in the presence of *D. esculentum* extract and the reference compound (ascorbic acid) to measure the reductive capability. Although the activity of ascorbic acid was better than the sample with absorbance values of 0.05 and 0.47 at 1 mg/ml for the plant extract and reference compound, respectively, still the plant extract showed somewhat moderate reducing capability.

Lipid Peroxidation Inhibition Assay

The IC_{50} values (Table 1) of the sample (141.67 ± 4.19 µg/ml) and the standard (6.76 ± 0.17 µg/ml) support the fact that the inhibitory efficiency of the plant extract is poor compared to standard trolox. As shown in Fig. 11, the increase in lipid peroxidation inhibition with increasing concentration of the plant extract reflects its antioxidant property.

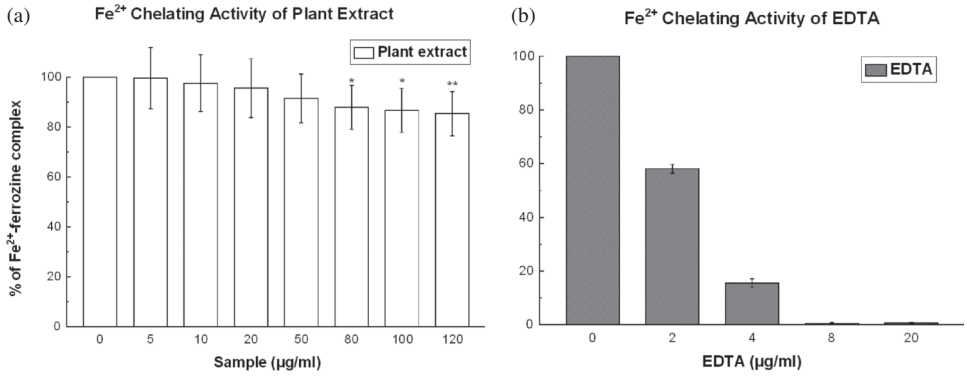


Figure 9 (a) Fe^{2+} chelation assay of *D. esculentum*. Effects of *D. esculentum* plant extract on Fe^{2+}-ferrozine complex formation is shown. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. 0 µg/ml. IC_{50} value of the plant extract was 1.33 ± 1.13 mg/ml. (b) Fe^{2+} chelation assay of standard EDTA. Effects of EDTA on Fe^{2+}-ferrozine complex formation is shown. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. IC_{50} value of the standard was 0.001 ± 0.00005 mg/ml.

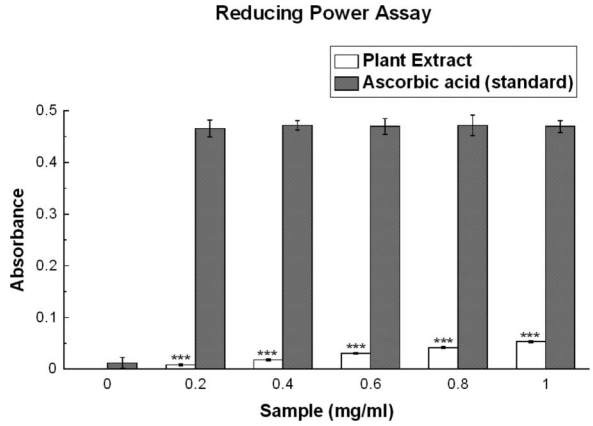


Figure 10 Reducing power assay. The reductive abilities of *D. esculentum* extract and the standard ascorbic acid. The absorbance (A_{700}) was plotted against concentration of the sample. Each value represents mean ± S.D. ($n = 6$). $^{***}p < 0.001$ vs. 0 mg/ml.

Determination of Total Phenolic Content

The 70% methanolic extract of *D. esculentum* showed 126.67 ± 8.16 mg gallic acid equivalent phenolic content in 1 g of dried plant extract.

Determination of Total Flavonoid Content

The 70% methanolic extract of *D. esculentum* showed 94.33 ± 6.12 mg quercetin equivalent flavonoid content in 1 g of dried plant.

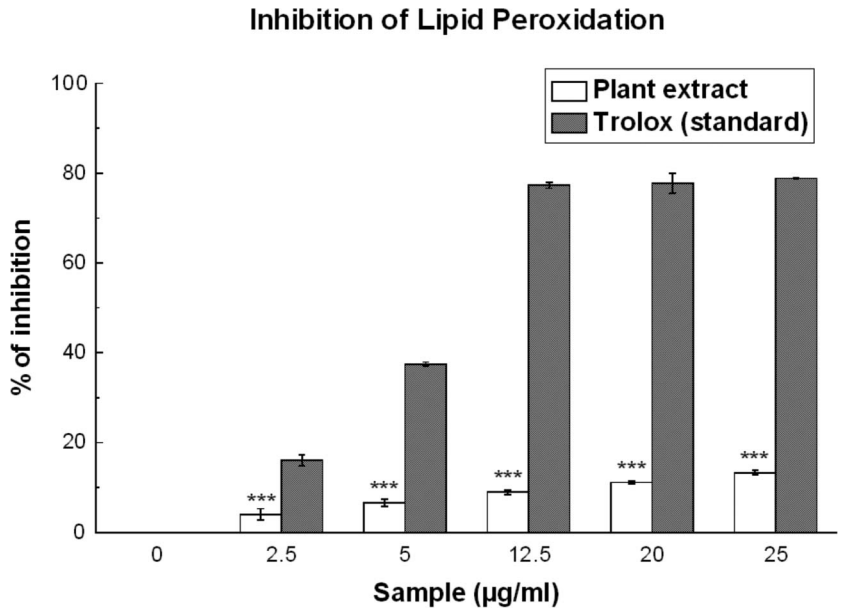


Figure 11 Inhibition of lipid peroxidation by *D. esculentum* extract and the standard trolox. The data is expressed as the percentage of lipid peroxidation inhibition of brain homogenate, induced by Fe^{2+}/ascorbic acid. Each value represents mean ± S.D. ($n = 6$). $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} values of the plant extract and standard are 141.67 and 6.76 µg/ml, respectively.

DISCUSSION

Free radicals play a crucial role in the pathogenesis of various diseases, especially degenerative diseases, and extensive lysis.[28] They are constantly generated in the living system and responsible for the damage of cellular biomolecules, such as proteins, enzymes, nucleic acids, lipids, and carbohydrates, and adversely affect the immune function.[29] Antioxidants inhibit the production of free radicals and also play a key role to inactivate them. All human cells protect themselves against oxidative damage by various antioxidant mechanisms, but sometimes these are not sufficient to prevent the damage caused by free radicals. Therefore, many synthetic drugs are used to protect against oxidative damage caused by free radicals but they have adverse side effects. For this reason, natural antioxidants in the form of common food supplements from plant origin have drawn much attention in recent years, as several investigators have reported that the plant-derived antioxidants have beneficial effects on human health.[30,31]

In the present study, the total antioxidant capacity of the young frond extract of *D. esculentum* was measured by an ABTS radical cation decolorization assay that has been commonly used because of its good reproducibility and easy quality control.[32] The reaction between ABTS and potassium persulfate results in the production of a blue colored chromophore, $ABTS^{\bullet+}$. After addition of the plant extract this preformed radical cation was converted to ABTS in a dose dependent manner. The result is compared with trolox and the TEAC value demonstrates the extract as a potent antioxidant.

Hydroxyl radical is one of the major reactive oxygen species that causes lipid peroxidation and DNA damage.[33,34] It was produced by incubating Fe^{3+}-EDTA with ascorbic acid and H_2O_2 at pH 7.4. Hydroxyl radicals formed in this reaction were then

reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound formed a pink chromogen upon heating with TBA at low pH. Additions of *D. esculentum* extract to the reaction mixture removed hydroxyl radicals from the sugar and prevented further damage. The dose dependent increase in the scavenging activity indicates its potential as a good hydroxyl radical scavenger. Observation also showed that at a higher dose, the plant extract works as a better hydroxyl radical scavenger than the standard mannitol.

Superoxide anion radical ($O_2^{\bullet-}$) originates from the one-electron reduction of free molecular oxygen by nicotinamide adenine dinucleotide phosphate oxidase, which is the membrane-bound enzyme.[35,36] In a cell, it changes to other harmful reactive oxygen species like hydrogen peroxide and hydroxyl radical.[37] It indirectly initiates lipid oxidation by generating singlet oxygen. On excessive production, this radical causes tissue injury and organ dysfunction during sepsis and septic shock.[38–40] Xanthine oxidase-derived superoxide radical has been linked to the postischemic tissue injury and generation of neutrophil chemotoxins. Elimination of superoxide radical anion generated by this enzymatic pathway would be beneficial in the case of ischemia.[41] The present study indicates the dose dependent increasing superoxide radical scavenging activity of the plant extract. Therefore, superoxide radical scavenging activity of *D. esculentum* extract could play a therapeutic role in superoxide radical mediated ischemia.

Nitric oxide (NO), an important bioregulatory molecule, plays an important role in diverse physiological conditions, including neural signal transduction, control of blood pressure, platelet function, and antitumor and antimicrobial activity.[42] But sustained levels of production of NO are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions, including diabetes, multiple sclerosis, arthritis, and ulcerative colitis.[43] The toxicity increases greatly when NO reacts with a superoxide radical and forms highly reactive peroxynitrite anion ($ONOO^-$) implicated in the pathogenesis of diseases, such as heart diseases, Alzheimer's disease, and atherosclerosis.[44] The methanolic extract of *D. esculentum* shows significant nitric oxide scavenging activity in a dose dependent manner. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract directly competes with oxygen to react with nitric oxide, thus inhibiting nitrite formation. Reducing the generation of nitric oxide in the digestive tract was found to be effective in preventing the reactions of nitrate with amines and amides to form carcinogenic nitrosoamines and nitrosoamides.[45] Hence, the NO scavenging activity of the plant extract could play a preventive role against nitrosoamine-mediated carcinogenesis.

Hydrogen peroxide (H_2O_2) is a weak oxidizing agent that belongs to a non-radical form of ROS. The H_2O_2, formed by the two-electron reduction of O_2, is not a free radical but is an oxidizing agent. In the presence of O_2 and transition metal ions, H_2O_2 can generate hydroxyl ion via the Fenton reaction. H_2O_2 at micromolar levels is poorly reactive. However, higher levels of H_2O_2 can attack some energy-producing systems. H_2O_2 inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. In addition, H_2O_2 also forms hydroxyl ion in the presence of metal ions and oxygen facilitates this reaction. Hence, metal chelating and H_2O_2 scavenging processes are important for living organisms.[46] The present study clearly shows the dose dependent increasing H_2O_2 scavenging activity of the *D. esculentum* extract. It is interesting to note that at lower concentrations, the plant extract possesses much higher H_2O_2 scavenging activity than that of the standard sodium pyruvate, thus indicating its efficiency as a potent H_2O_2 scavenger.

Peroxynitrite is relatively stable compared to other free radicals but once protonated it forms the highly reactive peroxynitrous acid (ONOOH).[47] It has strong oxidizing properties toward various cellular constituents, including sulphydryls, lipids, amino acids, and nucleotides, and causes cell death, lipid peroxidation, carcinogenesis, and aging.[48] It has been shown that the systemic inflammatory response and lung injury occur following intestinal ischemia-reperfusion (IIR) with an increase of nitric oxide, inducible isoform of NO synthase (iNOS) expression, and the formation of peroxynitrite in the lung. Inhibition of iNOS prevented the lung injury, suggesting that excessive NO production and peroxynitrite formation are cytotoxic to the cell and tissue and are involved in the secondary lung injury induced by the IIR.[49] Therefore, inhibiting peroxynitrite may be a novel pharmacological approach to prevent cell injury and multiple organ failure. The methanolic extract of *D. esculentum* exhibits significant dose dependent increasing peroxynitrite scavenging activity indicating its effectiveness as a good peroxynitrite scavenger for the prevention of peroxynitrite mediated diseases.

Singlet oxygen, a high energy form of oxygen, is generated in the skin by ultraviolet radiation. Singlet oxygen induces hyperoxidation, oxygen cytotoxicity, and decreases the antioxidative activity.[50] Results indicate that *D. esculentum* extract has good singlet oxygen scavenging activity but is not as efficient as the standard lipoic acid.

Hypochlorous acid (HOCl) is a major oxidant produced by neutrophils and monocytes, via the myeloperoxidase-catalyzed oxidation of chloride by hydrogen peroxide.[51] HOCl is a potent oxidant capable of damaging host tissue during inflammation. Inappropriate and/or excessive activation of neutrophils leads to oxidative stress and collateral damage of surrounding tissues. Cysteine and methionine residues in proteins and reduced glutathione (GSH) appear to be the main targets for HOCl, thereby altering the structure and function of proteins and lowering the antioxidant status in the cell. HOCl has the ability to inactivate the antioxidant enzyme catalase through breakdown of the heme prosthetic group. In the present study, we observed that catalase inactivation is inhibited in the presence of the *D. esculentum* extract, signifying its HOCl scavenging activity. The result also suggests that at lower doses the extract is more efficient in HOCl scavenging than the standard ascorbic acid.

In excess, iron can react with superoxide anion and hydrogen peroxide and convert them in to hydroxyl radical ($OH^{\bullet}$) (Haber-Weiss reaction) that cause severe injury to membranes, proteins, and DNA. It decomposes lipid hydro-peroxides into peroxyl and alkoxyl radicals responsible for the chain reaction of lipid peroxidation. To date, there are no definitive examples in the literature of *D. esculentum* acting to bind heavy metal ions. According to the result of the present study, though the plant extract is not as good as the standard EDTA in iron chelating, but the decrease in concentration dependent color formation in the presence of the extract indicate that it has iron chelating activity to some extent.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom.[52] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.[53] The result obtained from the experiment indicates that the plant extract has some reducing capacity, thus justifying its antioxidant activity.

In the process of lipid peroxidation, the iron catalyzed generation of ferry-perferryl complex or hydroxyl radicals accelerates peroxidation by decomposing lipid

hydroperoxides in to peroxyl and alkoxyl radicals. The highly reactive hydroxyl radicals react with polyunsaturated fatty acid moieties of cell membrane and yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA.[54] With the addition of the sample extract or standard, the generation of MDA is reduced, thus indicating the ability of the sample, although less than the standard, to inhibit lipid peroxidation.

The results indicate that *D. esculentum* plant extract contains significant amounts of flavonoids and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging or chelation.[55] Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.[56]

CONCLUSION

On the basis of the results obtained in the present study, it can be concluded that the 70% methanolic extract of *D. esculentum* contained a high amount of flavonoids and phenolic compounds, exhibited good antioxidant and free radical scavenging activities, and also possessed reducing power, iron chelating capacity, and lipid peroxidation inhibition ability. These *in vitro* assays indicated that this plant extract was a significant source of natural antioxidants, which might be helpful in preventing the progression of various oxidative stress associated diseases. Therefore, further studies should be carried out to isolate the bioactive components of this plant that confer the antioxidant property. Furthermore, the *in vivo* antioxidant activity of this extract needs to be studied thoroughly prior to clinical use.

ACKNOWLEDGMENTS

University Grants Commission (UGC), New Delhi, India has provided the financial support to carry out this study (Vide Letter F. No. 37-464/2009 (SR) dt.11.01.2010). The authors would also like to extend thanks to Mr. Rhitajit Sarkar, Division of Molecular Medicine, Bose Institute, Kolkata, for his assistance in operating the instruments throughout the study.

REFERENCES

1. Halliwell, B. Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. American Journal of Medicine **1991**, *91*, S14–S22.
2. Young, I.; Woodside, J. Antioxidants in health and disease. British Medical Journal **2001**, *54*, 176.
3. Braca, A.; Sortino, C.; Politi, M.; Morelli, I.; Mendez, J. Antioxidant activity of flavonoids from *Licania licaniaeflora*. Journal of Ethnopharmacology **2002**, *79*, 379–381.
4. Maxwell, S.R. Prospects for the use of antioxidant therapies. Drugs **1995**, *49*, 345–361.
5. Stadtman, E.R. Protein oxidation and aging. Science **1992**, *257*, 1220–1224.
6. Niki, E.; Shimaski, H.; Mino, M. *Antioxidantism-Free Radical and Biological Defense*; Gakkai Syuppn Center: Tokyo, Japan, 1994; 3–16.
7. Phomkaivon, N.; Areekul, V. Screening for antioxidant activity in selected Thai wild plants. Asian Journal of Food and Agro-Industry **2009**, *2* (4), 433–440.
8. Wong, S.P.; Leong, L.P.; Koh J.H.W. Antioxidant activities of aqueous extracts of selected plants. Food Chemistry **2006**, *99* (4), 775–783.

9. Nanasombat, S.; Teckchuen, N. Antimicrobial, antioxidant and anticancer activities of Thai local vegetables. Journal of Medicinal Plants Research **2009**, *3* (5), 443–449.
10. Rahmat, A.; Kumar, V.; Fong, L.M.; Endrini, S.; Sani, H.A. Determination of total antioxidant activity in three types of local vegetables shoots and the cytotoxic effect of their ethanslic extracts against different cancer cell lines. Asia Pacific Journal of Clinical Nutrition **2003**, *12* (3), 308–311.
11. Hazra, B.; Biswas, S.; Mandal, N. Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Complementary and Alternative Medicine **2008**, *8*, 63.
12. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine **1999**, *26*, 1231–1237.
13. Elizabeth, K.; Rao, M.N.A. Oxygen radical scavenging activity of curcumin. International Journal of Pharmaceutics **1990**, *58*, 237–240.
14. Fontana, M.; Mosca, L.; Rosei, M.A. Interaction of enkephalines with oxyradicals. Biochemical Pharmacology **2001**, *61*, 1253–1257.
15. Garratt, D.C. *The Quantitative Analysis of Drugs*, Volume 3; Chapman and Hall Ltd: Japan, 1964; 456–458.
16. Long, L.H.; Evans, P.J.; Halliwell, B. Hydrogen peroxide in human urine: Implications for antioxidant defense and redox regulation. Biochemical Biophysical Research Communications **1999**, *262*, 605–609.
17. Floriano-Sánchez, E.; Villanueva, C.; Medina-Campos, O.N.; Rocha, D.; Sánchez-González, D.J.; Cárdenas-Rodríguez, N.; Pedraza-Chaverrí, J. Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion, and hypochlorous acid and prevents *in vivo* tyrosine nitration in lung. Free Radical Research **2006**, *40*,523–533.
18. Beckman, J.S.; Chen, H.; Ischiropulos, H.; Crow, J.P. Oxidative chemistry of peroxynitrite. Methods in Enzymology **1994**, *233*, 229–240.
19. Bailly, F.; Zoete, V.; Vamecq, J.; Catteu, J.P.; Bernier, J.L. Antioxidant actions of ovothiol-derived 4-mercaptoimidazoles: Glutathione peroxidase activity and protection against peroxynitrite- induced damage. FEBS Letters **2000**, *486*, 19–22.
20. Pedraza-Chaverrí, J.; Barrera, D.; Maldonado, P.D.; Chirino, Y.I.; Macías-Ruvalcaba, N.A.; Medina-Campos, O.N.; Castro, L.; Salcedo, M.I.; Hernández-Pando, R. S-Allylmercaptocysteine scavenges hydroxyl radical and singlet oxygen *in vitro* and attenuates gentamicin induced oxidative and nitrosative stress and renal damage *in vivo*. BMC Clinical Pharmacology **2004**, *4*, 5.
21. Aruoma, O.I.; Halliwell, B. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. Biochemical Journal **1987**, *248*, 973–976.
22. Pedraza-Chaverrí, J.; Arriaga-Noblecía, G.; Medina-Campos, O.N. Hypochlorous acid scavenging capacity of garlic. Phytotherapy Research **2007**, *21*, 884–888.
23. Haro-Vicente, J.F.; Martinez-Gracia, C.; Ros, G. Optimization of *in vitro* measurement of available iron from different fortificants in citric fruit juices. Food Chemistry **2006**, *98*, 639–648.
24. Oyaizu, M. Studies on products of browning reactions: Antioxidant activities of products of browning reaction prepared from glucose amine. Japanese Journal of Nutrition **1986**, *44*, 307–315.
25. Kizil, G.; Kizil, M.; Yavuz, M.; Emen, S.; Hakimoglu, F. Antioxidant activities of ethanol extracts of *Hypericum triquetrifolium* and *Hypericum scabroides*. Pharmaceutical Biology **2008**, *46*, 231–242.
26. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture **1965**, *16*, 144–158.
27. Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. Food Chemistry **1999**, *64*, 555–559.

28. Halliwell, B.; Gutteridge, J.M. *Free Radicals in Biology and Medicine*; Oxford University Press: Oxford, 1998.
29. Nilsson, J.; Stegmark, R.; Akesson, B. Total antioxidant capacity in different pea (*Pisum sativum*) varieties after blanching and freezing. Food Chemistry **2004**, *86*, 501–507.
30. Gungor, N.; Sengul, M. Antioxidant activity, total phenolic content and selected physicochemical properties of white mulberry (*Morus alba* L.) fruits. International Journal of Food Properties **2008**, *11*, 44–52.
31. Okmen, B.; Sigva, H.O.; Mutlu, S.; Doganlar, S.; Yemenicioglu, A.; Frary, A. Total antioxidant activity and total phenolic contents in different Turkish eggplant (*Solanum melongena* L.) cultivars. International Journal of Food Properties **2009**, *12*, 616–624.
32. Lee, B.W.; Lee, J.H.; Gal, S.W.; Moon, Y.H.; Park, K.H. Selective ABTS radical-scavenging activity of prenylated flavonoids from *Cudrania tricuspidata*. Bioscience, Biotechnology, and Biochemistry **2006**, *70* (2), 427–432.
33. Aurand, L.W.; Boone, N.H.; Giddings, G.G. Superoxide and singlet oxygen in milk lipid peroxidation. Journal of Dairy Science **1977**, *60*, 363–369.
34. Packer, L.; Ong, A.S.H.; Eds. *Biological Oxidants and Antioxidants: Molecular Mechanisms and Health Effects*; AOCS Press: Champaign, IL, 1997.
35. Badwey, J.A.; Karnovsky, M.L. Active oxygen species and the functions of phagocytic leucocytes. Annals of Internal Medicine **1980**, *93*, 480–489.
36. Babior, B.M. Phagocytes and oxidative stress. American Journal of Medicine **2000**, *109*, 33–44.
37. Al-Mamun, M.; Yamaki, K.; Masumizu, T.; Nakai, Y.; Saito, K.; Sano, H.; Tamura, Y. Superoxide anion radical scavenging activities of herbs and pastures in northern Japan determined using electron spin resonance spectrometry. International Journal of Biological Sciences **2007**, *3* (6), 349–355.
38. Brigham, K.L. Oxygen radicals—An important mediator of sepsis and septic shock. Klinische Wochen-Schrift **1991**, *69*, 1004–1008.
39. Takeda, K.; Shimada, Y.; Okada, T.; Amano, M.; Sakai, T.; Yoshiya, I. Lipid peroxidation in experimental septic rats. Critical Care Medicine **1986**, *14*, 719–723.
40. Walker, P.D.; Shah, S.V. Reactive oxygen metabolites in endotoxin induced acute renal failure in rats. Kidney International **1990**, *38*, 1125–1132.
41. Selloum, L.; Reichl, S.; Müler, M.; Sebihi, L.; Arnhold, J. Effects of flavonols on the generation of superoxide anion radicals by xanthine oxidase and stimulated neutrophils. Archives of Biochemistry and Biophysics **2001**, *395*, 49–56.
42. Kumaran, A.; Karunakaran, J. Nitric oxide radical scavenging active components from *Phyllanthus emblica* L. Plant Foods for Human Nutrition **2006**, *61*, 1–5.
43. Tylor, B.S.; Kion, Y.M.; Wang, Q.I.; Sharpio, R.A.; Billiar, T.R.; Geller, D.A. Nitric oxide downregulates hepatocyte-inducible nitric oxide synthase gene expression. Archives of Surgery **1997**, *132*, 1177–1183.
44. Ischiropoulos, H.; al-Mehdi, A.B.; Fisher, A.B. Reactive species in ischemic rat lung injury: Contribution of peroxynitrite. American Journal of Physiology **1995**, *269*, 158–164.
45. Boone, C.W.; Kelloff, G.J.; Malone, W.E. Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: A review. Cancer Research **1990**, *50*, 2–9.
46. Aruoma, O.I. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of Americal Oil Chemists' Society **1998**, *75*, 199–212.
47. Balavoine, G.G.; Geletti, Y.V. Peroxynitrite scavenging by different antioxidants. Part1: Convenient study. Nitric Oxide **1999**, *3*, 40–54.
48. Choi, H.R.; Choi, J.S.; Han, Y.N.; Bae, S.J.; Chung, H.Y. Peroxynitrite scavenging activity of herb extracts. Phytotherapy Research **2002**, *16* (4), 364–367.
49. Zhou, J.L.; Jin, G.H.; Yi, Y.L.; Zhang, J.L.; Huang, X.L. Role of nitric oxide and peroxynitrite anion in lung injury induced by intestinal ischemia-reperfusion in rats. World Journal of Gastroenterology **2003**, *9* (6), 1318–1322.

50. Kochevar, E.I.; Redmond, W.R. Photosensitized production of singlet oxygen. Methods in Enzymology **2000**, *319*, 20–28.
51. Winterbourn, C.C.; Vissers, M.C.; Kettle, A.J. Myeloperoxidase. Current Opinion in Hematology **2000**, *7*, 53–58.
52. Geckil, H.; Ates, B.; Durmaz, G.; Erdogan, S.; Yilmaz, I. Antioxidant, free radical scavenging and metal chelating characteristics of propolis. American Journal of Biochemistry and Biotechnology **2005**, *1* (1), 27–31.
53. Matsusighe, K.; Basnet, P.; Kadota, S.; Namba, T. Potent free radical scavenging activity of dicaffeoyl quinic acid derivatives from propolis. Journal of Traditional Medicine **1996**, *13*, 217–228.
54. Hazra, B.; Sarkar, S.; Mandal, S.; Biswas, S.; Mandal, N. Studies on antioxidant and antiradical activities of dolichos biflorus seed extract. African Journal of Biotechnology **2009**, *8* (16), 3927–3933.
55. Cook, N.C.; Samman, S. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. Journal of Nutritional Biochemistry **1996**, *7*, 66–76.
56. Diplock, A.T. Will the good fairies please prove to us that vitamin E lessens human degenerative disease? Free Radical Research **1997**, *27*, 511–532.

Asian Journal of Pharmaceutical and Health Sciences
www.ajphs.com

Sperm viability assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of Swiss albino mice treated with *Diplazium esculentum*

Subhrajyoti Roy[1,2], Suman Tamang[1], Tapas Kumar Chaudhuri[1]*

1 Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri - 734013, West Bengal, India.
2 Immunopharmacology and Molecular Cell Biology Laboratory, Department of Zoology, University of Gour Banga, Malda - 732103, West Bengal, India.

ARTICLE HISTORY

Received: 10.01.2013

Accepted: 31.01.2013

Available online: 10.05.2013

Keywords:

Diplazium esculentum; Sperm viability; MTT assay; Male infertility

***Corresponding author:**

Email : dr_tkc_nbu@rediffmail.com
Tel : +91 9434377127

ABSTRACT

Diplazium esculentum is the most commonly consumed fern throughout Asia and Oceania. Systemic toxicity and pathological effects on its consumption have already been demonstrated. But the spermicidal properties of the boiled *Diplazium esculentum* (BDE) have not yet been investigated. Here an attempt was made to investigate the effect of boiled *Diplazium esculentum* (BDE) on the viability of spermatozoa of adult Swiss albino mice, if any.3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed for the assessment of the viability of spermatozoa of adult Swiss albino mice. Dehydrogenase, present in the mitochondria of the midpiece region of spermatozoa converts the yellow colored insoluble tetrazolium salt to purple colored water soluble formazan. This was measured spectrophotometrically using a microplate reader in the present study. Rates of MTT reduction were recorded before and after the incubation at 37°C for 1 h. The MTT reduction rate (change in optical density) for each group was determined by calculating the difference between the first and second reading of the microplate reader. Dose dependent inhibition of the viability of sperm was observed in case of all the treated animals when compared with the controls. The inhibition was statistically significant ($p < 0.001$) and directly proportional to the dose of the BDE. After 135 days and 180 days of treatment, at 320mg/kg body weight, the percentage inhibition of sperm viability was 40.51% and 53.12%, respectively. These results suggest that *D. esculentum*, even after boiling, possess spermicidal properties which may cause male infertility. Therefore, the consumption of *D. esculentum* is alarming and may act as an antifertility agent.

INTRODUCTION

Declination of the male fertility has been a great concern as male infertility accounts for about 30% of infertility cases worldwide. Male infertility is an important issue, a common problem occurring worldwide [1]. There has been a steady accumulation of information regarding the screening of plants having antifertility properties in males. But, very few studies have been conducted so far to assess the effect of wild edible plants on male fertility. Increase in the consumption of these plants is due to the progressive decrease in the stock of cultivated crops [2]. But information on the possible toxic effects of most of the wild edible plants is too little to make the people aware about the hazardous effects of the consumption of these plants.

Diplazium esculentum (Koenig ex Retz.) Sw. (Family Athyriaceae) is one of the most common varieties and the most commonly consumed fern throughout Asia and Oceania. In India, young fronds of *D. esculentum* are popularly known as lingra in Northern India [3], rukja and lochanch in North Eastern India [4] and dheki sak in West Bengal, India [5]. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon which continues for almost five months.

Very few studies have been conducted so far to assess the toxicological impact of this fern on human health. Interestingly, this fern is rejected as food by animals including cattle and insets. Studies conducted on rabbits and guinea pigs demonstrated systemic toxicity and several pathological effects of this fern [6]. Young fronds of *D. esculentum* collected from the high-altitude area of HarsilGangotri of North India had been found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria (EBH) in hill cattle in India and elsewhere [7]. Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats [8]. However, the antifertility activity of this plant has not yet been studied.

Therefore, it happened in our mind that this fern may have certain toxic substances which may be associated with antifertility especially with male sterility. Assessment of the metabolic status of spermatozoa can provide valuable information regarding the viability as well as characteristics of spermatozoa, which is directly correlated with male fertility. The reduction activity of spermatozoa depends on the ability of metabolically active spermatozoa to reduce specific stains. The ability of spermatozoa to reduce the resazurin redox dye [9-10] and the methylene blue dye [11] has been used to evaluate semen quality in boars and bulls, respectively. Yellow 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), a water-soluble tetrazolium salt dye, is converted to water-insoluble purple formazan by the succinate dehydrogenase system of an active mitochondria by the reductive cleavage of its tetrazolium ring [12]. Thus, the amount of formazan formed can be determined spectrophotometrically and can serve as an estimate of the number of active mitochondria, and hence the viable cells, in a sample [13-14]. The MTT assay has been evaluated successfully in different animal species [15-17], whereas literature pertaining to the use of this technique to evaluate the viability of the spermatozoa of *D. esculentum* fed adult Swiss albino mice is still lacking.Therefore, we have conducted this pilot study to investigate whether the boiled aqueous preparation of *D. esculentum* (BDE) affect the viability of the spermatozoa of adult Swiss albino mice, keeping in mind the fact that the local people consume this plant as food after cooking, not as raw material.

MATERIALS AND METHODS

Preparation of the plant material

Young *D. esculentum* plants were collected from different areas of North Bengal University campus, from the market where from the local people procure and also from the adjoining regions of Darjeeling, India. Plants were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and a voucher specimen (Accession No. 9602) was submitted to him.

Young frond of *D. esculentum* (100 g) was washed carefully by tap water, then cut into small pieces, and boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed by a mixer and dried in an incubator at 60^0C until completely dried. This dried plant material (boiled *Diplazium esculentum*, BDE) was then kept at 4^0C for future use.

Animals and care

Male Swiss albino mice (25 ± 2 gm of body weight (b.wt.)) of 6-8 weeks of age were used for all the studies. They were housed in polypropylene cages, with dust-free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2°C. All the experiments were performed after obtaining the approval from the Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

Dosage

Ninety six (96) male Swiss albino mice were divided in to four sets (S 1-4) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1 (G1) of all the sets were considered as control and 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of BDE at the dose of 80 mg/kg b.wt., 160 mg/kg b.wt., and 320 mg/kg b.wt., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) were treated daily for 45 days, S2 (G1S2 to G4S2) daily for 90 days, S3 (G1S3 to G4S3) daily for 135 days and S4 (G1S4 to G4S4) daily for 180 days.

Preparation of the sperm suspension and MTT reduction assay

Mice were sacrificed 24 h after the last dose by using proper anesthesia (chloroform and ether in a ration of 2:1). Sperm suspension was prepared according to the previously described method [18]. Caudal epididymis was separated and minced using a pair of small scissors to release the sperm into 10 ml warmed (37° C) physiological saline. The sperm suspension was placed in an incubator at 37° C for 10 minutes prior to perform the viability test. The MTT assay was performed according to the previously described method [19]. Briefly, the sperm suspension was diluted using phosphate buffered saline (PBS) and adjusted the number as $30x10^6$ spermatozoa/ml. To assess the sperm viability of S1 mice (G1S1, G2S1, G3S1, G4S1), twenty four wells of a 96-well microplate were used. One hundred microliters of sperm suspension from G1S1 mouse was placed in the first six wells of the first column of the microplate. Similarly, 100 µL of sperm suspension from G2S1, G3S1, and G4S1 mice was placed in the six wells of the second, third and fourth columns of the microplate, respectively. Therefore, a total of 24 wells of the microplate were occupied with sperm suspensions of all the groups of S1 mice. Then, 10 µL of MTT stock solution (5 mg/mL, dissolved in PBS; pH 7.0) was added to each of these 24 wells and mixed properly. The rates of MTT reduction (measured as optical density) were recorded immediately and after incubation at 37°C for 1 h using a microplate reader (Bio-Rad, USA). The MTT reduction rate (change in optical density) for each group was determined by calculating the difference between the first and second reading of the microplate reader. MTT reduction rates of the spermatozoa of S2, S3 and S4 mice were evaluated in the similar way as mentioned above.

Statistical analysis

Data have been presented as mean ± SD of six observations. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit) software. Differences in mean ± SD among different groups were statistically analyzed using one way ANOVA followed by Dunnett's test. A probability value of $p < 0.05$ was considered significant.

RESULTS

Results of the MTT reduction assay showed significant dose-

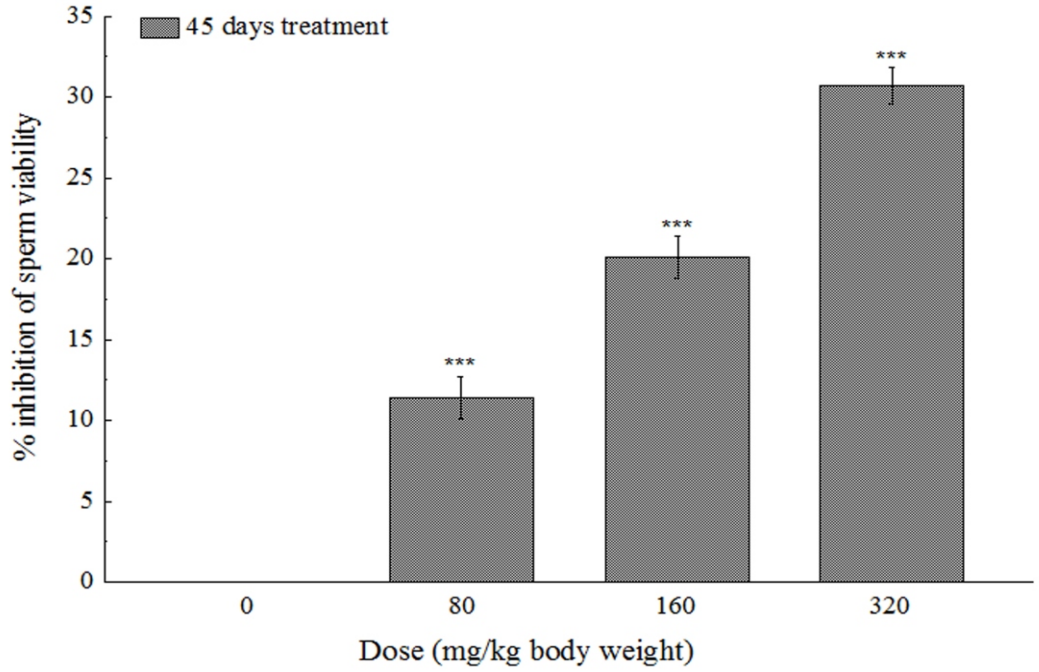


Figure 1: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 45 days. At 320 mg/kg b. wt., the percentage inhibition of sperm viability was 30.69%. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. Control.

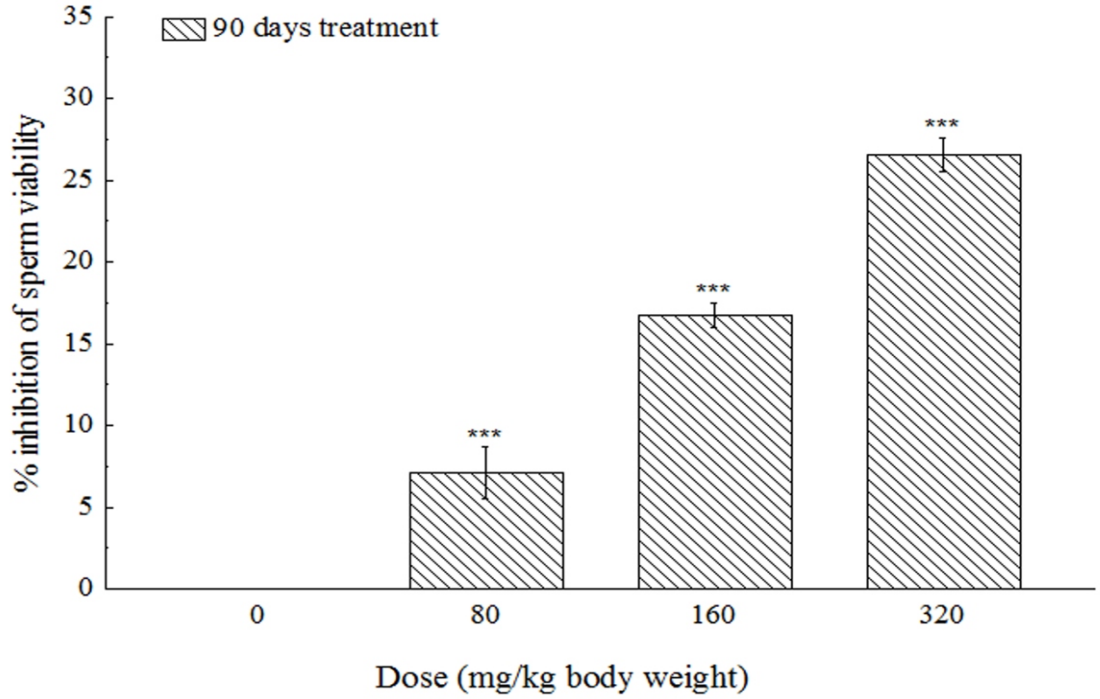


Figure 2: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 90 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 26.56%. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. control.

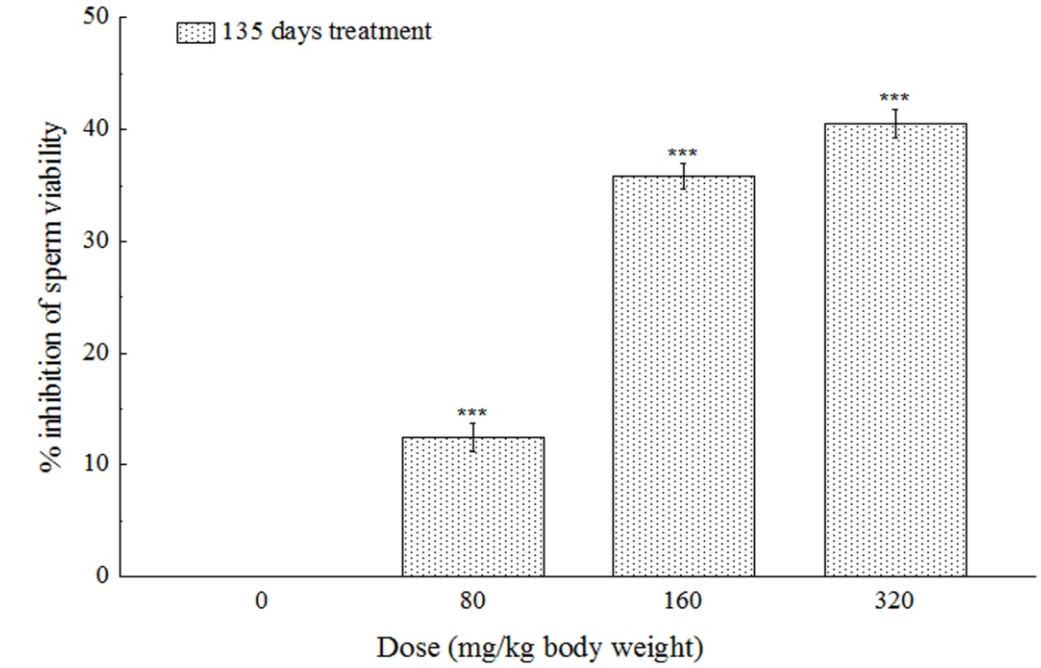


Figure 3: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 135 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 40.51%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

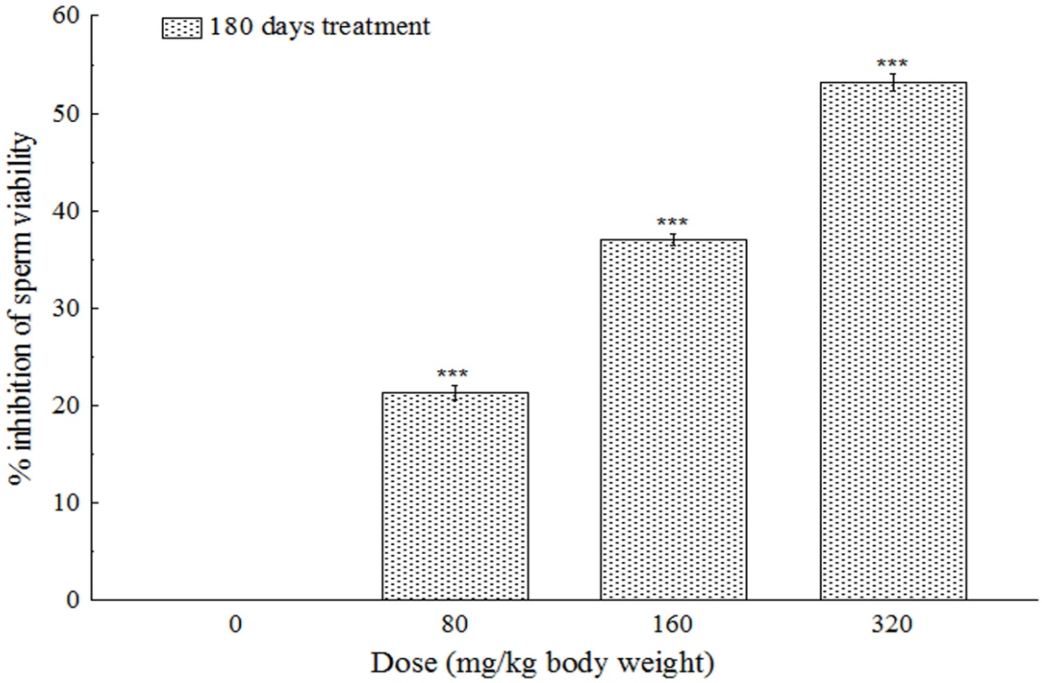


Figure 4: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 180 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 53.12%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

dependent increased percentage of the inhibition of sperm viability in all of the cases. After 45 days of treatment, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, i.e., at 80mg/kg b. wt. (11.38%), 160mg/kg b. wt. (20.07%) and 320mg/kg b. wt. (30.69%), when compared with the control group (Figure 1). After 90 days of treatment, at 80mg/kg b. wt., the percentage inhibition of sperm viability was 7.10%, whereas, at 160mg/kg b. wt. and 320mg/kg b. wt., the percentage inhibitions of sperm viability were 16.69% and 26.56%, respectively. Therefore, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, when compared with the respective control groups (Figure 2). This was also observed significantly after 135 days and 180 days of treatment with BDE. After 135 days of treatment, at 320mg/kg b.wt., the percentage inhibition of sperm viability was 40.51% (Figure 3), whereas, after 180 days of treatment with BDE, at 320mg/kg b.wt., the percentage inhibition of sperm viability was increased remarkably up to 53.12% (Figure 4).

DISCUSSION

Studies on the effects of plant products on the male reproductive system and fertility are comparatively few and far fetched [20]. 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 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 [21-23]. The present study provides 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 that 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 [15-17] [24-25]. The MTT reduction rate was taken successfully after 1 h of incubation time. This is due to the fact that 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 sperm quality parameters like acrosome integrity, mitochondrial activity and these parameters also correlate positively with fertility [26]. 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 alter the sperm motility and metabolism [27]. Present study showed that the rate of MTT reduction decreased gradually with the increase in dose, in all the groups. After 135 days and 180 days of treatment, at the dose of 320mg/kg b. wt., the percentage inhibition of sperm viability was increased remarkably up to 40.51% and 53.12%, respectively. Treatment with the ethanolic extract of *Sarcostemma secamone* to adult male rat has been shown to reduce the number of female impregnation, number of implantation and also the number of viable fetuses, when mated with fertile females [20]. 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. The differences in the mean values among the treatment groups were greater than would be expected by chance; there were statistically significant differences ($p < 0.001$). To isolate the group or groups that differ from the others, we use a multiple comparison procedure. All pair-wise multiple comparison procedures (Dunnett's method) were also performed for the authentication of the results.

CONCLUSION

Diplazium esculentum, the vegetable fern, is extensively used as a palatable food throughout Asia, Oceania and especially in the Northern part of West Bengal where we reside. Considering the findings of the present study, it can be concluded that *D. esculentum*, even boiled, possesses potent spermicidal properties. This is the first report on the assessment of the reproductive dysfunction due to the intake of the edible *D. esculentum*, and thereby to make people aware about the hazards of its consumption and it will advance the existing knowledge of this fern in relation to human health.

ACKNOWLEDGEMENT

We gratefully acknowledge the financial support (Vide Letter F.No.37-464/2009 (SR) dt.11.01.2010) received from the University Grants Commission (UGC), New Delhi, India to carry out this study.

REFERENCES

1. Nwangwa EK. Antifertility effects of ethanolic extract of *Xylopia aethiopica* on male reproductive organ of Wistar rats. Am. J. Med. and Med. Sci. 2012:2(1): 12-15.
2. Teklehaymanot T, Giday M. Ethnobotanical study of wild edible plants of Kara and Kwego semi-pastoralist people in Lower Omo River Valley, Debub Omo Zone, SNNPR, Ethiopia. J. Ethnobiol. Ethnomed. 2010:6:23.
3. Uniyal SK, Awasthi A, Rawat GS. Developmental processes, changing lifestyle and Traditional wisdom: analyses from western Himalaya. The Environmentalist 2003:23:307-312.
4. Angami A, Gajurel PR, Rethy P, Singh B, Kalita SK. Stats and potential of wild edible plants of Arunachal Pradesh. Indian J. Trad. Knowl. 2006:5:541-550.
5. Sen A, Ghosh PD. A note on the ethnobotanical studies of some pteridophytes in Assam. Indian J. Trad. Knowl. 2011:10:292-295.
6. Somvanshi R, Devi V, Gounalan S, Kataria M. Preliminary clinicopathological observations on effects of linguda (*Diplazium esculentum*) feeding in laboratory rabbits. Indian J. Toxicol. 1998:5:7-11.
7. Somvanshi R, Lauren DR, Smith BL, Dawra RK, Sharma OP, Sharma VK, Singh AK, Gangwar NK. Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. Current Sci. 2006:91:1547-1552.
8. Smith BL, Seawright, AA, Ng JC, Hertle AT, Thomson JA,

Bostock PD. Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium spp.*) from eastern Australia and from cultivated worldwide collection held in Sydney, Australia. Nat. Toxins 1994:2:347-353.

9. Foote RH. Resazurin reduction and other tests of semen quality and fertility of bulls. Asian J. Androl. 1999:1:109-114.
10. Zrimsek P, Kunc J, Kosec M, Mrkun J. Spectrophotometric application of resazurin reduction assay to evaluate boar semen quality. Int. J. Androl. 2004:27:5762.
11. Chandler JE, Harrison CM, Canal AM. Spermatozoal methylene blue reduction: An indicator of mitochondrial function and its correlation with motility. Theriogenol. 2000:54:261-271.
12. Slater TF, Swyer B, Straeuli U. Studies on succinatetetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. Biochim. Biophys. Acta 1963:77:383-393.
13. Denizot F, Lang G. Rapid colorimetric assay for cell growth and survival. J. Immunol. Methods 1986:89:271-277.
14. Song XX, Park CK, Piao YJ, and Niwa K. Effect of monosaccharide l-fucose and polysaccharide fucoidan on sperm l-fucosidase activity and relation to sperm-oocyte interaction in pig. Asian-australas. J. Anim. Sci. 2007:20:351-358.
15. Aziz DM, Ahlswede L, Enbergs E. Application of MTT reduction assay to evaluate equine sperm viability. Theriogenol. 2005:64:1350-1356.
16. Aziz DM. Assessment of bovine sperm viability by MTT reduction assay. Anim. Reprod. Sci. 2006:92:18.
17. Byun, JW, Choo SH, Kim HH, Kim YJ, Hwang YJ, Kim DY. Evaluation of boar sperm viability by MTT reduction assay in Beltsville thawing solution extender. Asian-australas. J. Anim. Sci. 2008: 2: 494-498.
18. Linder RE, Strader LF, McElroy WK. Measurement of epididymal sperm motility as a test variable in the rat. Bull. Environ. Contam. Toxicol. 1986:36:317-324.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983:65:55-63.
20. Kumari TK, Sakthidevi G, Muthukumaraswami S, Mohan VR. Antifertility activity of whole plant extract of *Sarcostemma secamone* (L) Bennet on male albino rats. Int. Res. J. Pharm. 2012:3(11):139-144.
21. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semi automated colorimetric assay: Assessment of radio sensitivity. Cancer Res. 1987:47:943-946.
22. Campling BG, Pym J, Galbraith PR, Cole SP. Use of the MTT assay for rapid determination of chemo sensitivity of human leukemic blast cells. Leuk. Res. 1988:12:823-831.
23. Freimoser FM, Jakob CA, Aebi M, Tuor U. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. Appl. Environ. Microbiol. 1999:65:3727-3729.
24. Hazary RC, Chaudhuri D, Wishart GJ. Application of an MTT reduction assay for assessing sperm quality and predicting fertilising ability of domestic fowl semen. Br. Poult. Sci. 2001:42:115-117.
25. Naser-Esfahani MH, Aboutorabi R, Esfandiari E, Mardani M. Sperm MTT viability assay: A new method for evaluation of human sperm viability. J. Assist. Reprod. Genet. 2002:19:477-482.
26. Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 1997:57:1401-1406.
27. Khan PK, Awasthy KS. Cytogenetic toxicity of neem. Food Chem. Toxicol. 2003: 41:1325-1328.