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).

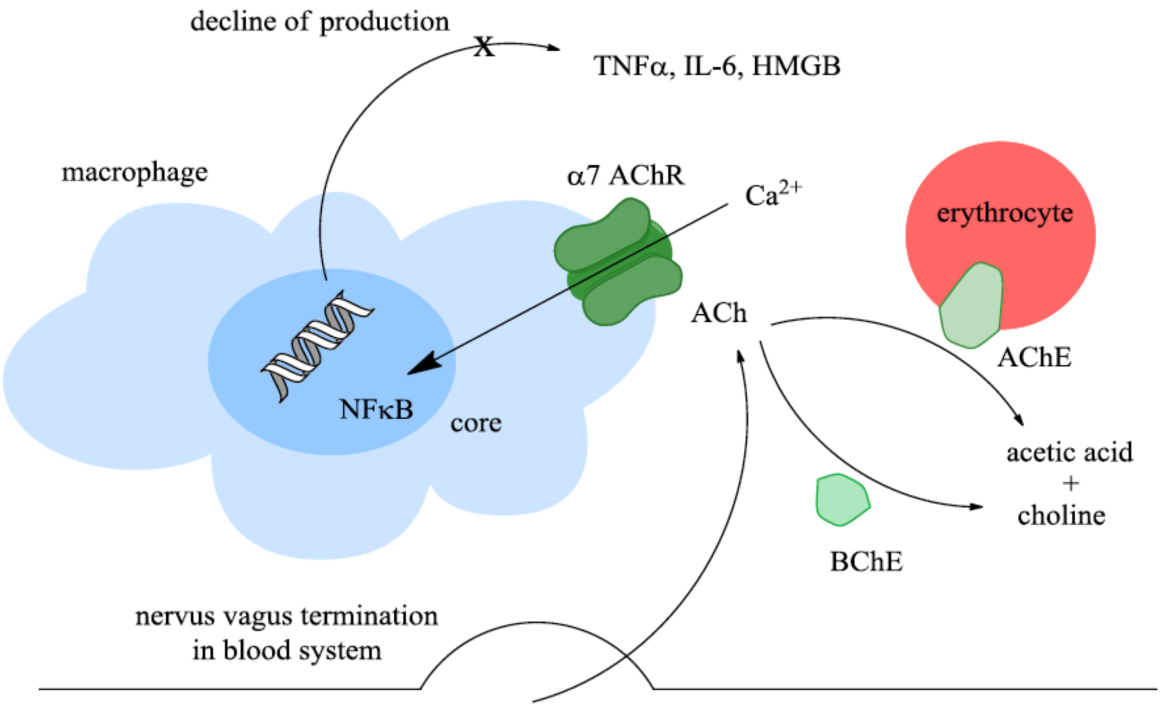

Figure 1. Principle of the cholinergic anti-inflammatory pathway; abbreviations: ACh-acetylcholine; AChE-acetylcholinesterase; BChE-butyrylcholinesterase; HMGB-high mobility group box; IL-6-interleukin 6; NFκB-nuclear factor kappa B; TNFα-tumor necrosis factor alpha (Pohanka, 2014).

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).

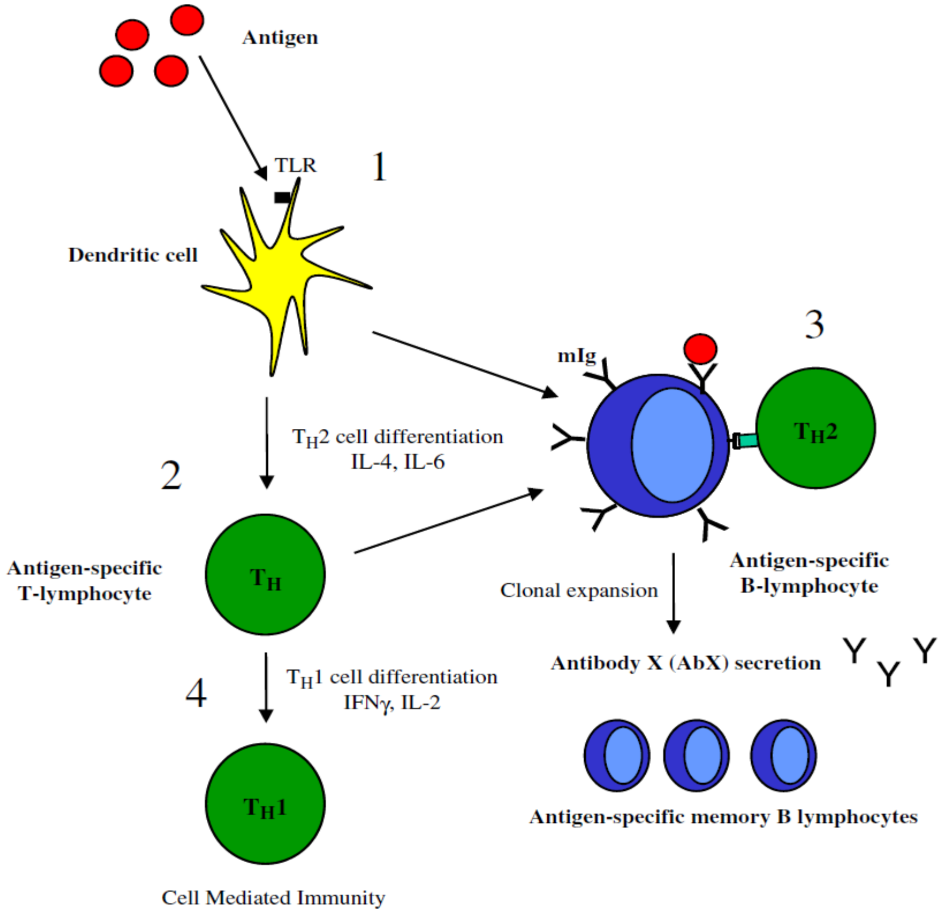

Figure 2. Paradigm for the immunomodulatory activity of plant-derived medicines. Major parameters of the immune response that may be influenced by plant-derived medicines include 1) uptake and processing of antigen by specialised APC such as dendritic cells (DCs) via TLRs; 2) presentation of antigen 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.

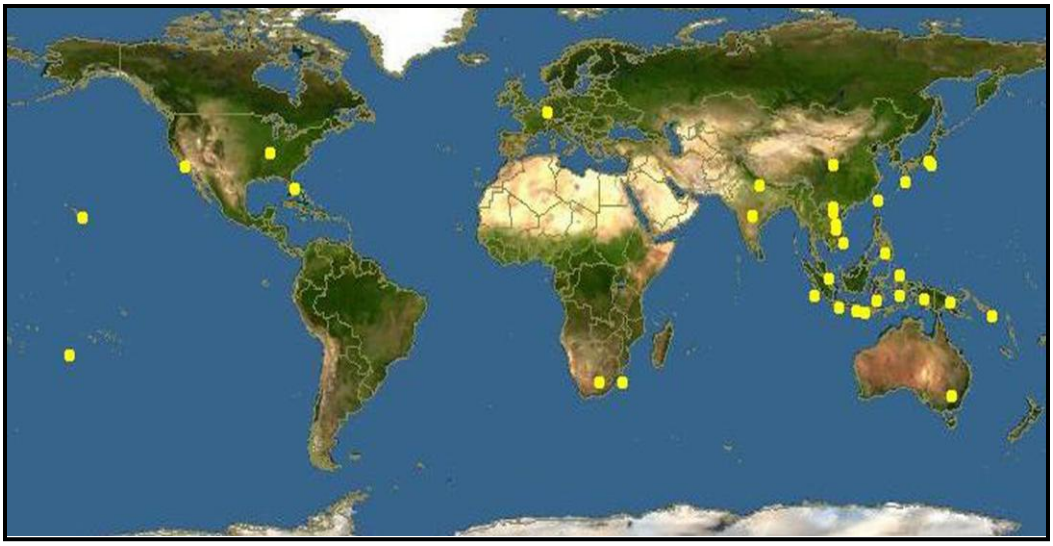

Figure 3. The distribution of *D. esculentum* throughout the world (Image adapted from Discover Life, Designed by The Polistes Corporation)

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).