CHAPTER 1

INTRODUCTION

Globally, the number of old people is expected to increase in all the developed and developing countries, including India in the coming years [1]. The aging population of India is not that high compared to Western countries, but it is predicted to rise in the future [2]. Already, the aging population of India was reported to have increased an incidence of neurodegenerative diseases, infectious diseases, and cancer [3]. This makes it necessary to elucidate the physiological, cellular, molecular mechanisms involved in aging process in order to understand age-associated diseases and create the awareness in the population about the healthy aging. Aging is a phenomenon characterized by an increased incidence of cancer, infectious, and autoimmune diseases, accompanied by a decline in immunological and neuroendocrine functions and diminished responsiveness of central and peripheral catecholaminergic systems [4-6].

A number of studies from our and other laboratories have provided a functional basis for the link between the three systems, namely nervous, endocrine, and immune systems that is essential for maintaining homeostasis and health status of an individual [7-9]. Our studies, as well as studies by others, have demonstrated that in the periphery neural-immune interactions are mediated by the release of norepinephrine (NE) from sympathetic noradrenergic (NA) nerve fibers innervating the primary (bone marrow and thymus) and secondary (spleen and lymph node) lymphoid organs [10-14]. The expression of adrenoceptors on lymphocytes and macrophages provide targets for norepinephrine (NE) to modulate immune responses [15-18]. Nevertheless, loss of NA innervation in spleen with advancing age is accompanied by impaired neural-immune interactions [19-22].

The loss of splenic NA nerve fibers can also be due to a decrease in the number of cell bodies in superior celiac-mesenteric ganglia and the corresponding decline in their ability to regulate the synthesis of NE [23]. Longitudinal study on F344 rats has shown an age-
dependent deterioration of NA fibers in spleen and a significant decrease in cell-mediated immune responses [10, 19, 24]. Furthermore, the number of memory T cells increases and naïve T cells decreases with advancing age leading to susceptibility to various diseases [25].

Age-associated changes in immune system may result in increased mortality rate due to decrease in resistance to infection and diseases such as cancer [26]. Recent evidence shows that immunosenescence is not just accompanied by a gradual decline in immune function, but rather it is a series of alterations in physiological functions, leading to immunosuppression [27, 28]. Physiological changes associated with aging include oxidative stress, mitochondrial dysfunction, synaptic loss, neuronal dysfunction and protein aggregation [29]. Age-associated decline in antioxidant enzyme activities result in accumulation of reactive oxygen species (ROS) that may lead to diminution of NA nerve fibers in the spleen and lymph nodes [30, 31]. Antioxidant enzymes play a crucial role in preventing the harmful effects of free radicals that damage the cellular structure and affect its functions.

Accumulation of toxic free radicals due to repeated release of NE may cause destruction of NA nerve fibers, and consequently interfere with the presence and availability of NE in the spleen [25]. Several studies have established the interaction between sympathetic NA nerve fibers and cells of the immune system through the scattering of tyrosine hydroxylase (TH⁺) nerve fibers on lymphocytes in splenic white pulp region, and the immunomodulatory effects of NE in secondary lymphoid organs [18, 32, 33]. Concurrently, alterations in sympathetic NA neuronal activity are accompanied by an age-related decline in T cell-mediated immune functions, including a decrease in T cell proliferation and IL-2 production in mitogen-stimulated lymphocytes [34].

Furthermore, the attenuation of these NA nerve fibers can also be due to a decline in the production of target-derived growth factors, such as brain-derived growth factor (BDNF) and nerve growth factor (NGF). NGF is a neurotrophic factor that is essential for the survival of the sympathetic neurons distributed in the secondary lymphoid organs and a decline in its level could be responsible for the age-related degeneration of NA nerve fibers in the spleen. NGF receptors are present on lymphoid organs (spleen, lymph nodes, and thymus) and on immune cells such as mast cells, eosinophils, and T and B lymphocytes, which maintain the plasticity of sympathetic neurons [35-37]. Age-related reduction in NGF content observed in specific brain areas and spleen may explain the degeneration of neurons with advancing age [38, 39]. Age associated decline in the expression of p75NTR in the rat splenic dendritic cells
suggests that decrease in T lymphocytes may have been associated with the decline in NGF receptor expression [40].

Figure 1. Sympathetic noradrenergic innervation of spleen in young and old rats.

A number of studies by us and others have shown that expression of molecular markers including ERK, Akt, and CREB are altered with advancing age [41-43]. At the cellular level, age-associated alterations in signal transduction pathways can cause a shift in the balance between pro-inflammatory and anti-inflammatory markers. Age-associated increase in inflammation can lead to multiple pathological conditions such as neurodegenerative disorders. Hence, regulation of inflammatory mechanism can be targeted therapeutically to slow down the age-associated disease [44, 45].

Age-associated changes in inflammatory processes are associated with activation of gene for the expression of proinflammatory markers and increase in the levels of proinflammatory cytokines [46, 47]. Aberrant activation of mTOR with advancing age can lead to immune dysfunction and inhibiting its activation can ameliorate the immune function [48, 49]. Recently, increasing number of evidence have explained about aberrant activation of
mTOR signaling in several age-associated disorders, such as, Alzheimer’s disease, Parkinson’s disease, metabolic diseases, and cancer [50-52].

Previous in vitro and in vivo studies from our laboratories have shown the evidence for the reversal of declined neuroendocrine–immune network in young and old rats treated with deprenyl, brahmi and donepezil [53-57]. In traditional culture and Ayurveda, a number of plants have been identified and their properties have been harnessed for its medicinal properties. Several plant extracts have been under experimentation over the past few decades and several of them were found to have beneficial effects; Morinda citrifolia is one of such plants. Morinda citrifolia, also commonly referred to as Noni, has been widely used since ancient times in traditional medicine in South and Southeast Asia with various parts of the plant; roots, stems, barks, leaves, and fruits finding a variety of use in the treatment of human ailments.

In recent years, it has been increasingly used as a dietary supplement because its more than 150 phytochemical constituents possess multiple biological activities and thus, exert curative effects in a number of diseases such as inflammatory diseases, cardiovascular diseases, infectious diseases, diabetes, cancer, cognitive disorders, and female-specific problems such as menstrual difficulties. Several in vitro and in vivo studies involving cell cultures and rodents, and a few human clinical studies have demonstrated that Noni’s potential health benefits are a result of its antioxidant, immunostimulatory, anti-inflammatory, anti-tumorigenic and pro-apoptotic properties, and the direct modulation of intracellular signaling pathways by its phytochemicals.

Although the beneficial effects of Noni have been reported by a number of studies [58-60], the mechanism(s) of action(s) from the perspective of the neuroendocrine-immune network have not been investigated. Hence, assessing how plant extracts like Morinda citrifolia can modulate the neural-immune network would play a vital role in suggesting natural remedies for autoimmune diseases, neurodegenerative disorders, and cancers that are commonly observed in aging.

Several theories to explain aging have been put forth and neuroendocrinology theory of aging is one of them; According to this theory, altered neurotransmitters levels and signaling molecules associated with impaired receptor sensitivity in the hypothalamus and peripheral organs along with imbalance in hormone concentrations lead to progressive loss of homeostatic functions [61]. Ayurveda considers the aging process as the Swabhava or nature
of the living being, consider it to be a time-bound entity and biologically it comes to an end through senescence and death [62].

In parallel with the progressive age-associated decline in immune function and compensatory mechanism such as antioxidant enzyme activities, there is also a decline in the brain function, concomitant with loss of neuronal plasticity and cognitive functions, which may result in neurodegenerative disorders [62]. Impairment in intellectual, learning, memory, and decision-making abilities are attributed to the alterations in neuronal plasticity in frontal cortex and hippocampus, [63] while age associated alterations in endocrine functions are attributed to dysregulation of hypothalamic functions [64, 65].

An age-related increase in the oxidative stress due to mitochondrial dysfunction and immunosenescence can exacerbate the deterioration of neuronal functions in the neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s diseases [66, 67]. Although in the last few decades, a number of longitudinal studies have been done, the research on aging is at its infancy. Gradually impairment in brain functions due to aging lead to a decline in the release of neurotrophic hormones [68]. Studies have shown the association between cognitive function and androgen level with advancement of age [69, 70]. Moreover, neuroprotective role of androgens including cell survival, axonal regeneration, and dendritic maintenance has been studied extensively [71]. There are shreds of evidence that age-related decrease in testosterone level has direct consequences on cognitive function as well as the quality of life [72]. Age-associated decline in serum testosterone level and decline in cognitive function have been studied extensively in both cross-sectional and longitudinal studies [73, 74].

Among the glucocorticoids, the role of cortisol as a stress hormone and its release as protective mechanism during the stress response has been studied extensively in animal and human studies [75]. On the other hand, increase in stress results in maladaptive alterations in brain functioning, such as elevation in cortisol levels, decrease in BDNF level, hippocampal atrophy, and loss of synapse, leading to neuropathological and cognitive changes in brain [76, 77, 78].

Traditionally, several herbs and phytochemicals have been identified as sources of agents beneficial for slowing down the decline in cognitive functions and for the treatment of age-associated neurodegenerative disorders. It is well known that phytochemicals and their
derivatives can be used as therapeutic agents in neurodegenerative disorders because of their ability to regulate the levels and release of neurotrophins [79].

Studies have shown that the decline in the level of neurotrophins have been associated with the pathology of numerous neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease [80, 81]. Among the neurotrophins, NGF and BDNF have been studied extensively due to their association with neurodegenerative diseases. BDNF supports the neuronal growth, survival and synaptic plasticity and it is highly available in cortex and hippocampus [82, 83]. The notion that a decrease in the levels of neurotropic factors may result in neurodegenerative disorders has generated an enormous interest in the investigation of BDNF as a potential therapeutic agent.

Insulin-like growth factor-1 (IGF-1) has emerged as a key growth factor for the neuronal plasticity, neurotransmission, and adult neurogenesis [81, 84, 85]. IGF-1 is reported to be involved in signaling pathway responsible for inducing the expression of BDNF and to mediate regulation of genes involved in BDNF-related neurogenesis [84]. A number of age-related impairments occurring in brain, such as atrophy, metabolic dysfunction, cognitive impairments, and accumulated deleterious substances can be attributed to reduced brain IGF-1 input and linked to age-associated neurodegenerative disorders such as Alzheimer's disease [85, 86].

Recent studies involving neurodegenerative diseases have shown that loss of serotonergic and noradrenergic neuronal cell bodies besides the basal forebrain cholinergic neurons also exacerbates the disease [87, 88]. This age-associated degeneration in multiple brain areas leads to progressive loss of memory and cognitive impairment in Alzheimer’s disease [89-91]. During aging, brain undergoes several changes that are influenced by the cellular signaling pathways. A number of studies have investigated the effects of aging on cell signaling pathways along with the pathophysiological implications of these changes in immune function and aging brain [92, 93]. The age-associated modification in cellular signaling pathways can lead to several neurological disorders due to decline in cognitive functions and increased oxidative stress [62].

Mitogen-activated protein kinases (MAPKs), PI3K/Akt, and CREB pathways have been demonstrated to play an important role in diverse cellular function including cell proliferation, neuronal survival, differentiation and cognitive function in the brain [94-98]. Aberrant activation of PI3K/Akt/m-TOR pathway has a major impact in a number of diseases
associated due to aging such as, cancer, type 2 diabetes mellitus (T2DM) and neurodegeneration and now emerging as a potential therapeutic targets for brain aging and neurodegenerative diseases [99, 100]. Likewise, CREB signaling is essential for maintaining long-lasting changes in synaptic plasticity for the conversion of short-term memory to long-term memory, and maintaining cognitive function. Any alteration in CREB signaling can lead to several age-associated deficits in cognitive functions as observed in aging and in several neurodegenerative disorders [101, 102].

With progressing age, there is a decline in the cholinergic neurotransmission, with concomitant loss of neuronal plasticity and cognitive functions of the brain which may result in neurodegenerative disorders. The cholinergic neurotransmission is regulated by the key enzymes—choline acetyltransferase (ChAT), which synthesizes the neurotransmitter, acetylcholine (ACh) and cholinesterase hydrolyses and terminates the activity of ACh. The cholinesterase enzyme subtypes, including acetyl cholinesterase (AChE) and butyryl cholinesterase have been demonstrated to alter with advancing age [103]. Research has shown the role of acetylcholine (ACh) as a neurotransmitter in the behavior, learning and memory and as an anti-inflammatory molecule [104]. Moreover, inhibition of cholinesterase enhances the activity of cholinergic neurons in the central nervous system (CNS) and leads to suppression of inflammation [105].

Nitric oxide (NO) is another neurotransmitter synthesized in the CNS which is involved in memory and learning process. NO is synthesized in the brain in cognitive demand and functions by stimulating soluble guanylyl cyclase to form the second messenger molecule, cyclic guanosine monophosphate (cGMP) in the target cells [106]. *Morinda citrifolia* fruit juice have beneficial effect in age-associated decline in cognitive functions by decreasing cholinesterase activity and increasing NO production in various brain areas [107].

Neuroprotective effects of *Morinda citrifolia* is another important property that may be critical to the treatment of neurological disorders such as memory impairment, cognitive dysfunctions, neurodegenerative disorders in the elderly, especially when the population of elderly is on the rise in several countries of the world [108]. These beneficial effects of Noni are exerted through a number of phytochemicals such as iridoids, fatty acid glycosides, and flavonoids in fruits and anthraquinones in roots [60].

Use of flavonoids as a dietary supplement has shown protective effects in improving cognitive function by enhancing neuronal function and neuronal regeneration [109]. Studies
have shown that flavonoids are able to cross the blood-brain barrier (BBB) [110-112] and by traversing the BBB and entering into the brain, flavonoid may be a potent therapeutic candidate for the neuroprotective effects and neuromodulatory actions. Experimental evidence has demonstrated that treatment with *Morinda citrifolia* fruit juice protected the mice from stress-induced impairment of cognitive function [113]. Also, another study showed that *Morinda citrifolia* fruit juice has preventive effects against cerebral ischemic stress in ddY mice [114].

Even though there are such experimental evidence exists, it is important that pharmacological and clinical benefits of the phytochemicals at the cellular level in various diseases have to be explored to further understand mechanism(s) of action(s) of already and yet to be determined phytochemicals of *Morinda citrifolia*. Noni, including the roots, barks, and fruits have been used for the treatment of a number of diseases for the past several centuries. The spectrum of its health benefits ranges from antibacterial, antiviral, antifungal, antitumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects [59, 60].

The present set of experiments were designed to examine the role of *Morinda citrifolia* fruit juice on neuroendocrine-immune interactions in aging and the mechanisms involved so that it can be used as a potential therapeutic agent for age-related diseases.

**Our hypothesis** is that the altered homeostatic functioning of the neuroendocrine-immune network, especially the neuroendocrine outflow of the sympathetic noradrenergic innervation is diminished in the secondary lymphoid organs which is accompanied by immunosuppression and therefore, promotes aging. This dysregulation in the neuroendocrine-immune system caused due to the reduced sympathetic nervous system (SNS) activity and responsiveness to catecholamines, decline in hormonal levels and in compensatory mechanisms, such as decrease in antioxidant enzyme activities and growth factors can be modulated by phytochemicals from medicinal plants such as *Morinda citrifolia*. 
The following questions were designed to test our hypothesis:

1. What is the role of *Morinda citrifolia* fruit juice on reactive oxygen species (ROS) generated inside the cells?
2. How treatment with *Morinda citrifolia* fruit juice can modulate the neural-immune interactions?
3. What are the molecular mechanisms by which *Morinda citrifolia* fruit juice mediates its effects on secondary lymphoid organ and in various brain areas?
4. What are the target sites through which *Morinda citrifolia* fruit juice mediates its effects?
5. What is the role of *Morinda citrifolia* fruit juice on age-associated alteration in endocrine functions, and growth factors in various brain areas of old rats?

On the basis of this hypothesis, the following specific objectives have been identified.

**Specific Objective 1:** To investigate dose response of *Morinda citrifolia* fruit juices on lymphoproliferation and antioxidant enzyme activities in splenic lymphocytes of young rats. The aim of this objective is to establish ideal dose of *Morinda citrifolia* fruit juices on splenic lymphocytes of young male Wistar rats and to examine its lymphoproliferation and antioxidant enzyme activities.

**Specific Objective 2:** To examine the effects of *Morinda citrifolia* fruit juice on age-associated modulation of immune function and the possible intracellular molecular targets influenced using Bioinformatics tool. The working hypothesis for this objective is that with the advancement in age, there is a decline in immune function and compensatory mechanisms. Our focus is to examine the immunomodulatory effect of Noni fruit juices on splenic lymphocytes isolated from young, middle-aged, and old F344 rats. Further, to examine the role of phytochemicals present in Noni fruit juice and how it regulates the intracellular signaling pathways by the use of bioinformatics tools.
Specific Objective 3: To examine the effects of *Morinda citrifolia* fruit juice on neural-immune interaction in the spleen of aged F344 rats. Altered homeostatic functioning of neuroendocrine-immune network leads to the degeneration of noradrenergic (NA) nerve fibers in the secondary (spleen and lymph nodes) lymphoid organs and alters neural-immune interactions. With advancing age, alterations in the cellular and molecular pathways also occur that leads to dysregulation of several cellular responses to the given stimuli. Examining the altered neural-immune interactions in the spleen will help us to understand the role of Noni fruit juice in regulating these interactions through selective molecular pathways.

Specific Objective 4: To investigate the role of *Morinda citrifolia* fruit juice on age-associated alterations of endocrine function, growth factors, and signaling mechanisms in various brain areas of old F344 rats. In the aging brain, there is a decline in cognitive function, release of growth factors, and increase in oxidative stress, which can lead to an increase in vulnerability of the brain to several disorders. Investigating the role of Noni fruit juice on the cholinergic system, growth factor, and other compensatory mechanisms can provide us the information about the role of Noni fruit juice in remodeling of neural functions in the aged brain. Noni fruit juice as an intervention therapy for the augmentation of the neuroendocrine system can be a beneficial remedy in the aged-population and thus, ameliorating the cognitive dysfunctions.