Physiological significance of Noni phytochemicals on the Neuroendocrine-Immune system

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Foreword

The technical bulletin is the summary of the project entitled 'Physiological Significance of Noni Phytochemicals on Neuroendocrine-Immune System' funded by World Noni Research Foundation. The project is a systematic approach which investigated the molecular mechanisms by which Noni fruit juice modulates the age-related alterations in the neuroendocrine-immune network.

The project, throughout two years [2010-2012], was systematically planned, methodically analyzed and completed successfully. The investigators have demonstrated that Noni fruit juice can be beneficial in preventing aging process.

I appreciate the researchers for their dedicated work in exploring the anti-aging properties of Noni juice and for the preparation of 14th series of the technical bulletin published by World Noni Research Foundation, Chennai.

I place on record our appreciation to the editorial team, Dr. P. Rethinam, Dr. T. Marimuthu and Dr. K. V. Peter for bringing out the 14th Technical Bulletin. The assistance rendered by Mr. T. Thanigai Kumar and Mr. A. Arunachalam in computer setting and printing is appreciated.

Chennai
September, 2014
Chairperson
About the Project

World Noni research foundation is focused on promoting research to unveil the wellness of Noni \textit{(Morinda citrifolia} L\textit{.)}. WNRF funds research organizations to carry out research on crop production, crop protection, pharmacology and clinical sciences besides aspects on food science. The present project is promising since it has proved valuable benefits of Noni as an anti-aging nutritional supplement through in vitro and in vivo experimentation. They have also analyzed the molecular signaling pathways involved in aging, thereby encouraging the use of Noni fruit juice to increase antioxidant activity and maintain a robust immune system which promotes healthy aging.

I congratulate the investigators for their immense efforts and devotion to publish this technical bulletin, 14\textsuperscript{th} in the series.

Chennai
September, 2014

Chairman, Noni BioTech
Preface

The project was envisioned to analyze the role of Noni fruit juice in modulating the neuroendocrine-immune network. I would like to express our sincere gratitude for the constant inspiration extended to us by Dr. Kirti Singh, ChairPerson, Dr. K.V. Peter, Director, Dr. T. Marimuthu, Additional Director and members of Research Advisory Board, World Noni Research Foundation.

During the period of study between 2010 and 2012, in vitro study and the subsequent in vivo studies gave us exciting results that not only extended the knowledge about the intracellular molecules involved in age-related alterations, it also demonstrated how Noni fruit juice can reverse the decline in neuroendocrine-immune system in old age. We have shown that Noni fruit juice strengthen immune system thereby retarding the immunosenescence associated with aging. In addition, it helps the body protect itself from harmful free radicals by enhancing antioxidant enzyme activities. However, we look forward to pursue further clinical studies including human subjects so as to prove Noni as an effective intervention strategy to prevent age-related diseases and cancers.

S. ThyagaRajan
1. Introduction

Aging is a process characterized by deterioration of the physiological functions of an organism in the absence of any disease conditions. Studies from our lab and others have shown that age-related decline in central and peripheral catecholaminergic activity results in age-associated immunosuppression. *Morinda citrifolia* (Noni) has been widely used for the treatment of a variety of diseases in Ayurveda and folk medicine owing to its nutritional and immune-enhancing properties. With the aging population on the rise in the coming future in India, age-associated diseases such as neurodegenerative diseases, cancer, infectious diseases and autoimmune diseases are important concerns. Noni is a potent immune enhancer and can reverse age-related decline in immune and neuronal functions by augmenting antioxidant enzyme functions and reducing lipid peroxidation.

2. State of Knowledge

Bidirectional communication between the neuroendocrine system and immune system through hormones, neurotransmitters and cytokines maintain homeostasis and health status of an individual (Ader *et al.*, 2001; Bellinger *et al.*, 2008). In the periphery, immune responses are regulated through the sympathetic noradrenergic (NA) nerve fibers in the lymphoid organs (bone marrow, thymus, spleen, and lymph nodes) which release norepinephrine (NE) and modulate immune responses by binding to adrenergic receptors on the immune cells (Bellinger *et al.*, 2008). With advancing age, there is an age-associated decline in sympathetic NA innervations in the spleen and lymph nodes along with significant loss of T cell responses including proliferation and cytokine production (Maue *et al.*, 2009; Thyagarajan *et al.*, 2011; Thyagarajan *et al.*, 2012). Effects on cytokine response are especially pronounced, with many studies suggesting that sympathetic activation can inhibit the expression of proinflammatory cytokines, suppress Th1 cytokines in favor of a Th2 profile and inhibit the expression of Type I interferons.

The effects of physiological variation in SNS activity are not properly understood, but direct sympathetic innervations of lymphoid tissues is believed
to represent a primary pathway by which behavioral processes can affect immune function in vivo. Accumulation of free radicals due to loss of antioxidant enzyme activities is one of the major reasons for this sympathetic neurodegeneration in the spleen and lymph nodes (Bolza’n et al., 1995; Salmon et al., 2010). With advancing age, there is a decrease in the level of nerve growth factor. NGF is the most potent growth factor for neurons and it influences the proliferation, differentiation, survival and death of neuronal cells. It is essential for the health and well-being of the nervous system and is a promising candidate for treating Alzheimer's disease. Alterations in NGF levels have been implicated in neurodegenerative disorders, such as Alzheimer's disease and Huntington's disease as well as psychiatric disorders, including depression and substance abuse. Signals emanating from receptors for NGF control practically all aspects of immune defense and, as such, constitute potential targets for therapeutic intervention through rational drug design for neurodegenerative conditions.

There is an intercellular communication between neuroendocrine and immune system via signal molecules. Immune system contains receptors for neuroendocrine hormones. These hormones function as endogenous regulators of immune system that convey information from immune to neuroendocrine system. A number of studies have provided evidence for the existence of a bi-directional communication between the central nervous system (CNS) and the immune system. A number of areas in the brain, especially hypothalamus, respond to immune molecules through alterations in the metabolism of neurochemicals. An age-related increase in the level of oxidative stress due to mitochondrial dysfunction and alterations in immune mediators such as cytokines and complement exacerbate the deterioration of neuronal functions in the neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Amor et al., 2010; Axelsen et al., 2010; Wallace et al., 2005).

Noni has a number of micronutrients and has been used for 2000 years as a nutritional supplement to treat various diseases and promote wellness. Till now, more than 158 phytochemicals have been isolated and their property has been identified in Noni, the major components are xeronine, terpene compounds, anthraquinones, morindone, morindin, asperuloside, acubin,
caproic acid, caprylic acid, damnacanthal, scopoletin, polysaccharide and alkaloids (Heinicke, 1985). Heinicke et al., attribute the beneficial effects of *Morinda citrifolia* to another phytochemical proxeronine (Heinicke, 2001). Proxeronine is the molecule that is used to synthesize xeronine which may be an alkaloid essential to life. Results from limited studies demonstrated immunomodulatory effects of these constituents in *in vitro* experiments (Furusawa et al., 2003; Hirazumi et al., 1996; Kuninaga et al., 2007).

Considering the health benefits of Noni juice, the European Commission of Health and Consumer Protection accepted it as a novel food (Opinion of the Scientific Committee on Food on Tahitian Noni® Juice, 2002). Various parts of the plant *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 (Potterat and Hamburger, 2007; Wang et al., 2002). These effects are believed to be exerted through a number of phytochemicals such as anthraquinones in roots and iridoids, fatty acid glycosides and alcohols in fruits.

Several studies from others and our laboratories have provided a functional basis for the link between the three homeostatic systems namely, nervous system, endocrine system and immune system (Thyagarajan and Felten, 2002). A number of studies have provided evidence for the existence of a bi-directional communication between the central nervous system (CNS) and the immune system (Thyagarajan and Felten, 2002). Many of the psychosomatic diseases have autoimmune phenomena in their etiology, a number of stressors precipitate the pathogenesis of neoplasms and psychosocial factors such as depression, marital separation, divorce are associated with immunosuppression. However, the interaction and cross-regulation between these three systems is poorly understood. Although the beneficial effects of Noni have been reported through few studies (Palu et al., 2008), the mechanism(s) of action(s) from the perspective of the neuroendocrine-immune network have not been investigated.
3. Objectives

The present longitudinal study was conducted to assess the effects of *Morinda citrifolia* (Noni) on the age-related modulation of antioxidant status and immune functions *in vitro* and *in vivo*. Three specific objectives have been identified:

i. *In vitro* effects of *Morinda citrifolia* fruit juices Noni juice without seeds, Noni juice with seeds and (Divine Noni Gold) Noni shelf fruit juice on splenic lymphocytes

   a. Immune Functions
   b. Antioxidant status

ii. *In vitro* effects of *Morinda citrifolia* fruit juice (Divine Noni Gold) Noni shelf fruit juice on lymphocytes isolated from draining lymph nodes:

   a. Immune Functions
   b. Antioxidant status

iii. *In vivo* effects of *Morinda citrifolia* fruit juice (Divine Noni Gold) on the lymphoid organs and brain

   a. Splenic and Draining Lymph Node Lymphocyte functions and antioxidant status
   b. Antioxidant status of various brain areas.

4. Experimental details

Animals

Young (3-4 months), middle-aged (8-9 months) and old (16-17 months) male F344 rats were obtained from the National Institute of Nutrition at Hyderabad for the study. The animals were acclimatized to the animal house at SRM University for a period of one week.
**Experiment 1A:**

*In vitro* effects of *Morinda citrifolia* fruit juices Noni juice without seeds, Noni juice with seeds and (Divine Noni Gold) Noni shelf fruit juice on splenic lymphocytes

*Morinda citrifolia* (Noni) fruits were obtained fresh from the National Research Centre for Noni, Salavakkam, Tamil Nadu. The fruits were stored intact for up to three days for use during the study. The fresh fruits were ground with and without seeds using a blender and the pulp obtained were serially diluted in substituted RPMI medium from 10% to 0.01% and a dose response curve was generated. In addition, different dilutions of the shelf juice provided by WNRF ranging from 10% to 0.01% in RPMI medium was also used. The RPMI medium had 5% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 0.01mM nonessential amino acids, 5x10⁻⁵ M 2-mercaptoethanol, 100U/ml penicillin streptomycin solution, 24mM sodium bicarbonate and 10mM Hepes buffer. The proliferation of lymphocytes treated with (0.0001%, 0.01%, and 1%) Noni fruit juice was assessed using the MTT assay in the presence and absence of different doses of Con A ranging from 0.5 µg/ml to 5 µg/ml by maintaining appropriate controls. Replacement of medium containing Noni fruit dilutions was performed every day. The concentration of Noni was determined on the basis of preliminary studies conducted in the laboratory that enabled to establish the dose response curve for the different juices. Lymphocytes treated with different dilutions of Noni fruit juice for 24 hours were pelleted for antioxidant enzyme assays and the supernatants were frozen immediately for cytokine assays.

**Experiment 1B:**

*In vitro* effects of *Morinda citrifolia* fruit juice (Divine Noni Gold) Noni shelf fruit juice on lymphocytes isolated from draining lymph nodes

Lymphocytes isolated from the draining lymph nodes of young, middle-aged and old F344 rats were treated with different dilutions of the shelf juice provided by WNRF ranging from 10% to 0.01% in RPMI medium in the presence and absence of different doses of Con A ranging from 0.5 µg/ml
to 5 µg/ml by maintaining appropriate controls. Replacement of medium containing Noni fruit dilutions was performed every day. Proliferation, cytokine production and antioxidant enzyme activities of Noni shelf juice-treated lymphocytes were assessed.

**Experiment 2:**

**In vivo effects of *Morinda citrifolia* fruit juice (Divine Noni Gold)**

Oral administration of Noni fruit juice (5%, 10% and 20%) was done twice a day for a period of 60 days (5ml/kg body weight) in old (16-17 months) male F344 rats. They were sacrificed by decapitation at 08:00 hr and spleens, draining lymph nodes and brain areas (hippocampus, medial basal hypothalamus, striatum and frontal cortex) were dissected and kept in sterile tubes containing ice cold HBSS. Lymphocytes (2 x 10^5 cells/well) isolated from the spleens and draining lymph nodes were co-incubated with Con A and kept for 72 hours in a humidified atmosphere containing 5% CO₂ at 37°C and Con A-induced proliferation and cytokine production were assessed. Antioxidant enzymes were assessed in unstimulated lymphocytes from the spleen and draining lymph nodes and the brain areas.

**Isolation of Lymphocytes**

Briefly, a block of spleen was placed in HBSS (Hanks Balanced Salt Solution) containing sodium bicarbonate and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES). Spleen tissue was homogenized using a stomacher and the cell suspension was passed through a nylon mesh to remove large aggregates followed by repeated washes with HBSS. After removing the lymphocytes from the Histopaque/HBSS interface and washed thrice with HBSS, the cells were then re-suspended in RPMI 1640 medium supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.01 mM nonessential amino acids, 5 x 10⁻⁵ M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium bicarbonate, and 10 mM HEPES for *in vivo* culture.

**Lymphocyte proliferation assay**

Proliferation of lymphocytes was assessed using the MTT assay as follows. A 96 well plate containing 100 µl samples having 90% confluence was treated
with MTT reagent, incubated for 3 hrs and read at 620 nm after completely solubilising it in isopropanol containing 37% HCl. Triplicate wells were used for each experimental condition.

Con A-induced cytokine production

Lymphocytes (2 x 105 cells/well) isolated from the spleens and draining lymph nodes were co-cultured with 1.25 µg/ml of Con A and kept at 37°C in an incubator with 5% CO₂ for 24 hours. After 24 hours, the supernatants were collected for cytokine assays (IL-2 and IFN-α) using ELISA kits (R&D Systems, Minneapolis, Minn., USA).

Antioxidant enzyme activities

a) Estimation of SOD activity: The superoxide dismutase activity was measured in terms of percentage inhibition of epinephrine auto oxidation (Sun et al., 1978). The tissue was homogenized in a 5:3 ice-cold mixture of ethanol and chloroform, centrifuged and the supernatant thus obtained was used for the assay. The sample was diluted using 0.1M carbonate buffer, pH 10, added equal volumes of 0.6mM EDTA and 1.3mM Epinephrine in carbonate buffer, vortexed. The optical density was read immediately at 412 nm in a spectrophotometer at time intervals of 0', 30' and 60' respectively. The results were expressed in terms of units/ min/ mg of protein.

b) Estimation of CAT activity: The total catalase activity was estimated by the method of Goth L (1991). The total CAT activity was measured by a hydrogen peroxide based assay where it forms a complex with ammonium molybdate. The sample was suspended in 60mM sodium phosphate buffer, pH 7.4, with 65mM hydrogen peroxide solution and incubated for 4 minutes after which the reaction was stopped using 32.4mM ammonium molybdate and the optical density was measured 405 nm. The total catalase activity was expressed in units/ min/ mg of protein.

c) Estimation of GPx activity: The GPx activity was measured using Ellman's Reagent (Ellman, 1959). Briefly, the sample was diluted in 0.4M phosphate buffer pH 7, with 10mM sodium azide, 4mM reduced glutathione, 2.5mM hydrogen peroxide and incubated for 0’, 30’ and 60’ after which the reaction
was stopped with 10% trichloroacetic acid and centrifuged. To the supernatant, added 0.3M disodium hydrogen phosphate solution and 1mM Di-thio-nitro-benzene in 1% sodium citrate. The optical density was read immediately at 412 nm in a spectrophotometer. Standard curve was obtained using serial dilutions of 0.1 Tri-nitrobenzene in water. The results were expressed in terms of units/min/ mg of protein.

d) Estimation of GST activity: The GST activity was measured using 1-chloro 2, 4-dinitrobenzene (CDNB) (Habig et al., 1974). Briefly, the sample was diluted in 0.5 M phosphate buffer pH 6.5, added 25 mM 1-chloro 2, 4-dinitrobenzene in 95% ethanol and 20 mM reduced glutathione. The optical density was read immediately at 340 nm in a spectrophotometer at 30 seconds intervals for 1.5 minutes. One unit of GST activity is defined as the amount of enzyme producing 1 ìM of GS-DNB conjugate/min under the conditions of the assay.

Estimation of Lipid peroxidation

The extent of lipid peroxidation was measured in terms of rate of formation of adducts with thiobarbituric acid according to the method of Yagi, 1976. The sample was treated with ice-cold 10% trichloroacetic acid for precipitation of proteins, incubated for 15 min and centrifuged at 2200g for 15 min. The supernatants obtained were treated with equal volumes 0.67% TBA and incubated in a boiling water bath for 10 min. After cooling, the optical density was measured at 532 nm using a spectrophotometer. Standard curve was obtained using serial dilutions of 1, 1, 3, 3- tetraethoxy-propane or malondialdehyde (MDA) in distilled water. The results were expressed in terms of MDA equivalents/min/mg of protein.

Statistical Analysis

Data was analyzed using ANOVA by SPSS software package. Parameters that attained significance with ANOVA (P<0.05)were further analyzed by post-hoc tests.
5. Experimental findings

Experiment 1A
Effects of Noni Fruit Juice on proliferation and cytokine production of splenic lymphocytes

In young and middle-aged animals, there was a dose-dependent increase in proliferation of lymphocytes isolated from spleen (Fig. 1A). In both the young and the middle aged animals, there was an increase in proliferation with 0.5 µg/ml of Con A (p<0.05), 1.25 µg/ml of Con A (p<0.05) and 5 µg/ml of Con A (p<0.05). However, there was no increase in the proliferation of lymphocytes isolated from the spleens of old animals.

Co-incubation of lymphocytes with Noni juice with seeds and without seeds did not proliferation of lymphocytes in all the three age groups (Figs.1B and 1C). On treatment with Noni shelf juice, there was an increase in proliferation of the lymphocytes from the spleens of young and middle aged rats (p<0.05) while there was no such pattern in the old rats (Fig.1D).
Fig. 1: Effects of age on Con A-induced proliferation of lymphocytes in male F344 rats (A) and the effects of Noni fruit juice without seeds (1B), Noni fruit juice with seeds (1C) and Noni Shelf fruit juice (1D) on proliferation of lymphocytes in male F344 rats. *p<0.05 compared to age-matched control.
Effects of Noni juice on Con A-induced IFN-γ production

There was a significant age-related decline in IFN-γ production in middle-aged and old rats compared to young rats (p<0.05). Treatment with Noni seedless juice (Fig. 2A; 0.1%) significantly enhanced IFN-γ production in middle-aged rats alone. Noni juice with seeds (Fig. 2B) on the other hand significantly enhanced IFN-γ production (p<0.05) in young, middle-aged and old rats in all doses administered. Noni shelf fruit juice treatment (Fig. 2C) also significantly enhanced IFN-γ production in middle-aged (0.01 and 1%) and old (0.0001 and 1%) F344 rats.

Fig. 2: Effects of Noni fruit juices (Noni Juice with seeds, Noni Juice without seeds and Noni shelf fruit juice) on IFN-γ production by lymphocytes in young (2A), middle-aged (2B) and old (2C) male F344 rats. *p<0.05 compared to age-matched control
Effect of Noni juice on Con A-induced IL-2 production

There was a significant age-related decline in IL-2 production in middle-aged and old rats compared to young rats (p<0.05). Treatment with Noni seedless juice (Fig. 3A) significantly enhanced IFN-γ production in young (0.1%) and middle aged (0.00001, 0.001 and 1%) F344 rats alone. Noni juice with seeds (Fig. 3B) on the other hand significantly enhanced IFN-γ production (p<0.05) in middle-aged (0.001, 0.01 and 1%) and old (0.000001 and 0.001%) F344 rats. Noni shelf fruit juice treatment (Fig. 3C) also significantly enhanced IFN-γ production in young (0.1%) middle-aged (all doses) and old (0.000001 and 0.1%) F344 rats.

Fig. 3: Effects of Noni fruit juices (Noni Juice with seeds, Noni Juice without seeds and Noni shelf fruit juice) on IL-2 production by lymphocytes in young (2A), middle-aged (2B) and old (2C) male F344 rats. *p<0.05 compared to age-matched control
Effect of Noni juice on age-related alterations in the antioxidant enzyme activities of lymphocytes from F344 rats

Superoxide Dismutase

The activity of superoxide dismutase (SOD) was found to decline with age (p<0.05; Fig. 4A). However, treatment with 1% to 10⁻³% of the Noni fruit juice with seeds resulted in an increase in the SOD activity of the lymphocytes isolated from all three age groups (p<0.05; Fig. 4B). The shelf juice was also found to increase SOD activities in both middle-aged and old rats at a dilution range of 10⁻¹ to 10⁻⁴% (p<0.05; Fig. 4C). However, treatment with the seedless extract and shelf juice did not affect the SOD activity of young rats significantly. Similar to the activity of the shelf juice, the seedless juice was found to increase the SOD activity in the middle aged and old rats significantly (p<0.05).

Fig. 4: Effects of Noni fruit juice with seeds (3A), Noni fruit juice without seeds (3B) and Noni Shelf fruit juice (3C) on SOD activity in lymphocytes of male F344 rats. *p<0.05 compared to age-matched control
Catalase: The activity of catalase was found to decline with age (p<0.05). However, treatment with 1% to 10^{-3}% of the Noni fruit juice with seeds resulted in an increase in the catalase activity of the lymphocytes isolated from all three age groups (p<0.05; Fig. 5A). The treatment of lymphocytes with 10^{-1} to 10^{-4}% Noni seedless juice showed a significant increase in catalase activities in the case of middle-aged rats (p<0.05; Fig. 5B). However, in the case of young and old rats, there was no significant difference in the catalase activity. The treatment of lymphocytes with Noni shelf juice showed no significant change in the catalase activities of all the three age groups (Fig. 5C).

Fig. 5: Effects of Noni fruit juice with seeds (3A), Noni fruit juice without seeds (3B) and Noni Shelf fruit juice (3C) on catalase activity in lymphocytes of male F344 rats. *p<0.05 compared to age-matched control
Glutathione Peroxidase

The glutathione peroxidase activity was found to increase significantly with age. Treatment of lymphocytes with Noni juice with seeds (Fig. 7A) and Noni seedless juice (Fig. 7B) was found to increase the GPx activity in all age groups (p<0.06). However, treatment with Noni shelf juice significantly reduces the GPx activity in all the three age groups. (p<0.05; Fig. 7C).

![Figure 7: Effects of Noni fruit juice with seeds (3A), Noni fruit juice without seeds (3B) and Noni Shelf fruit juice (3C) on GST activity in lymphocytes of male F344 rats. *p<0.05 compared to age-matched control](image)

Glutathione-s-transferase

There was no age-related decline in glutathione s transferase activity. Treatment of lymphocytes with Noni juice with seeds (Fig. 6A) significantly enhanced the GST activity in young (0.0001 to 1%), middle-aged (0.1 and 1%) and old (all doses) F344 rats. Noni seedless juice (Fig. 6B) treatment was found to increase the GST activity in young (0.00001% to 1%), middle-
aged (0.1 and 1%) and old (0.001, 0.001, 0.1 and 1%) F344 rats. Finally, treatment with Noni shelf fruit juice also significantly increases GST activity in young (0.01 and 1%), middle-aged (0.1%) and old (0.0001, 0.001 and 0.1%) F344 rats (p<0.05; Fig. 6C).

Fig. 6: Effects of Noni fruit juice with seeds (3A), Noni fruit juice without seeds (3B) and Noni Shelf fruit juice (3C) on GPx activity in lymphocytes of male F344 rats. *p<0.05 compared to age-matched control

**Lipid Peroxidation**

With age there is a significant increase in the extent of lipid peroxidation in the lymphocytes (p<0.05). Treatment with Noni juice with seeds significantly reduces the extent of lipid peroxidation in the middle aged and old rats compared to age matched controls (p<0.05; Fig.8A). Treatment with Noni shelf juice showed a significant decrease in the thiobarbiturate reactive substances in all the three age groups (p<0.05; Fig. 8B). On the other hand the seedless juice showed no significant effect on lipid peroxidation in the young and the middle aged rats. However, in the old rats, treatment with Noni seedless juice significantly increased lipid peroxidation (p<0.05; Fig. 8C).
Experiment 1B

Effects of Noni Fruit Juice on in vitro proliferation and cytokine production by lymphocytes isolated from draining lymph nodes

Proliferation of T-lymphocytes from the draining lymph nodes did not show any age-related alterations (Fig. 9A). In young animals, there was a dose-dependent increase in proliferation of lymphocytes isolated from draining lymph nodes 72 hours after treatment with Noni fruit juice (Fig. 9A). In contrast, there was a significant increase in the proliferation of lymphocytes treated with 0.01% Noni fruit juice alone in middle-aged rats while lymphocyte proliferation increased when treated with 1% Noni fruit juice in old rats. There was a significant increase (p<0.05) in IL-2 production in young, middle-aged and old rats following treatment with all the doses of Noni fruit.
juice (Fig. 9C). Similarly, IFN-α production was increased in old (0.0001%, 0.01%, and 1%) rats thereby reversing the age-associated decline in the cytokine production, although in middle-aged (0.01%) and young rats there was a significant decrease with all the three doses of Noni fruit juice (Fig. 9B).

**Fig. 9: Effects of Noni Shelf Juice on lymphocyte proliferation and cytokine production: Treatment with Noni Shelf Juice significantly (p<0.05) increased the proliferation of lymphocytes isolated from draining lymph nodes in young (10^6 to 1%) , middle-aged (10^5%) and old (1%) F344 rats (A). Treatment with Noni Shelf Juice (10^6 to 1%) significantly increased (p<0.05) IL-2 production in young, middle-aged and old rats (C). Noni Shelf Juice treatment significantly reversed the age-related decline in IFN-γ production (B) in middle-aged (10^6 to 1%) and old (10^6 to 1%) rats, although in young rats there was a significant decrease (10^6 to 1%) (C). *Significantly different from age-matched control at p<0.05; **Significantly different from young control at p<0.05

**Effects of Noni fruit juice on Con A-induced cytokine production**

Treatment with Noni fruit juice (0.1%) significantly increased (p<0.05) Con A-induced IL-2 production (Fig. 10B) and IFN-γ production (Fig. 10A) in young, middle-aged and old rats.
Effects of Noni fruit juice on age-related alterations in the antioxidant enzyme activities of lymphocytes from F344 rats:

Superoxide dismutase

The activity of superoxide dismutase (SOD) did not show any age-related alterations (Fig. 11A). Noni fruit juice (0.0001%, 0.01%, and 1%) treatment
significantly (p<0.05) decreased the SOD activity in young, middle-aged, rats but there was no significant change in old F344 rats.

**Catalase**

The activity of catalase in the draining lymph nodes lymphocytes was found to decline (p<0.05) in old rats compared to young rats. However, treatment with Noni fruit juice significantly increased the catalase activity in young (0.0001%) and old (0.0001%, 0.01%, and 1%) F344 rats but there were no change in catalase activity old F344 rats (Fig. 11B).

**Glutathione peroxidase**

A significant age-related decrease in GPx activity (Fig. 11C) was also found. The GPx activity was also significantly decreased following Noni fruit juice treatment in young (0.01% and 1%), middle-aged (0.0001%, 0.01%, and 1%) and old (0.01%) F344 rats (Fig. 3C).

**Glutathione-S-transferase**

With age, there a significant age-related decrease in GST activity in the lymphocytes isolated from draining lymph nodes. Treatment with Noni fruit juice significantly (p<0.05) increased the glutathione-S-transferase activity in middle-aged (0.0001 %) and old (0.0001%, 0.01%, and 1%) F344 rats (Fig.11D).

**Lipid peroxidation**

With age there is a significant increase in the extent of lipid peroxidation in the lymphocytes (p<0.05). Noni fruit juice treatment significantly (p<.05) reduced the age-related increase in lipid peroxidation in young (1%), middle-aged and old (0.0001%, 0.01%, and 1%) F344 rats (Fig. 11E).
Fig. 11: Effects of Noni Shelf Juice on antioxidant enzyme activities and extent of lipid peroxidation: Treatment with Noni Shelf Juice significantly increased the Catalase activity in young (10% to 1%) and old F344 rats (A). Noni Shelf Juice treatment significantly decreased the SOD activity in young (10% to 10%), middle-aged (10% and 1%) and old (10% to 1%) F344 rats (B). The GPx activity was also significantly decreased following Noni Shelf Juice treatment in young (10% to 1%), middle-aged and old (10% to 1%) rats (C). A significant age-related decline in catalase activity (A) and age-related increase in GPx activity (C) was also found. Similarly, a significant (p<0.05) increase in glutathione-S-transferase activity was found in middle-aged (10% to 1%) and old (10% to 1%) rats following noni shelf juice treatment (D). Noni shelf juice treatment significantly (p<0.05) reduced the age-related increase in lipid peroxidation in young (1%), middle-aged and old (10% to 1%) F344 rats (E). *Significantly different from age-matched control at p<0.05; **Significantly different from young control at p<0.05
Experiment 2

Noni treatment on age-related lymphocyte proliferation and cytokine production

There was an age-related decline in Con A-induced proliferative capacity of T lymphocytes in old rats (Figs. 12A and 12B). Co-incubation of lymphocytes isolated from old rats following the Noni treatment from old rats did not alter the proliferative capacity of T lymphocytes (Fig. 12A). In *in vivo* study, there was no significant difference in Con A-induced proliferative capacity of T lymphocytes in old rats (Figs. 12A and 12B).

There was a significant (P<0.05) age-related decline in the cytokine (IL-2 and IFN-γ) production in lymphocytes isolated from both spleen and draining lymph nodes of old male F344 rats (Figs. 13A, 13B, 13C, and 13D). Noni treatment significantly (P<0.05) increased the IL-2 and IFN-γ production in lymphocytes isolated from old rats co-incubated with Con A (1.25 µg/ml) in all doses (5%, 10% and 20%) of NFJ.
Noni on the age-related activities of antioxidant enzymes in spleen and draining lymph nodes

**Superoxide Dismutase**

Superoxide dismutase (SOD) activity (units/min/mg protein) declined significantly (P<0.05) in the lymphocytes and tissue isolated from old rats in comparison to age-matched controls. There was no significant difference in SOD activity in spleen following 60 days treatment of NFJ in old rats compared to aged-matched control (Figs.14A). SOD activity was found to be increased significantly (P<0.05) in lymphocytes isolated from draining lymph nodes following 60 days treatment of NFJ in old rats in all doses (5%, 10% and 20%) (Fig.14B).
An age-associated decline \((P<0.05)\) in the activity (units/min/mg protein) of catalase (CAT) was evident in old male F344 rats (Figs. 15A and 15B). CAT activity in spleen increased significantly \((P<0.05)\) with Noni (5% and 20%) and in draining lymph nodes too a significant increase was seen in Noni (10%) treated old rats as compared to age-matched controls.
Glutathione Peroxidase

Glutathione peroxidase (isoform GPx1) also showed an age-dependent decrease in its activity (units/min/mg protein) in the lymphocyte and tissue isolated from old F344 rats (Figs. 16A and 16B). GPx activity increased significantly (P<0.05) in spleen and draining lymph nodes following Noni (10%) administration in old rats compared to age-matched controls.

![Fig. 16: GPx (Isoform 1) activity in the spleen and lymphocytes from draining lymph nodes isolated from old male F344 rats treated with NFJ (5ml/kg BW) in various dilutions (5%, 10% and 20%). A significant age-related decline in GPx activity was also found. GPx activity (units/min/mg protein) was significantly increased in spleen and draining lymph nodes of old rats treated with Noni (Figs. 16A and 16B). *P<0.05 compared to age-matched control, §P<0.05 compared to young control.]

Glutathione-S-Transferase

Similar to other antioxidant enzymes, glutathione-S-transferase (GST) activity was reduced in an age-associated manner in old rats (Figs. 17A and 17B). GST activity was significantly (P<0.05) increased in spleen and draining lymph nodes following Noni treatment in (5%) dose compared to age-matched controls.
Fig. 17: GST activity in the spleen and lymphocytes from draining lymph nodes isolated from old male F344 rats treated with NFJ (5ml/kg BW) in various dilutions (5%, 10% and 20%). There was an age-related decrease in GST activity in spleen and draining lymph nodes of old rats (Figs. 17A and 17B). *P<0.05 compared to age-matched control, §P<0.05 compared to young control

**Lipid Peroxidation**

Extent of lipid peroxidation was found to increase significantly (P<0.05) with advancing age compared to young rats. (Fig. 18A and 18B). In spleen, 5%, 10% and 20% dose of NFJ was found to reverse the age-dependent increase in the extent of lipid peroxidation (Fig 17A). In draining lymph nodes 5% and 10% dose of NFJ was found to reverse the age dependent increase in the extent of lipid peroxidation (Fig 18B).

Fig. 18: Noni fruit juice treatment significantly (P<0.05) reduced the age-related increase in lipid peroxidation in spleen and lymphocytes from draining lymph nodes of old rats (Figs. 18A and 18B). *P<0.05 compared to age-matched control, §P<0.05 compared to young control
Noni on the age-related activities of antioxidant enzymes in the brain areas

Superoxide Dismutase

Treatment of male F344 rats with NFJ (5ml/kg BW) significantly increased SOD activity in medial basal hypothalamus following 5% and 10% doses of NFJ (Fig.19A). In frontal cortex, there was a significant increase in superoxide dismutase activity in all the dosages (5%, 10% and 20%) (Fig. 19B), but it decreased with increasing dosages of Noni fruit juice. In hippocampus and striatum, 5% dose of Noni fruit juice significantly increased the SOD activity (Figs. 19C and 19D).

Fig. 19: Superoxide dismutase (SOD) activity in the brain areas of male F344 rats treated with Noni. SOD activity (units/min/mg protein) was significantly increased in medial basal hypothalamus, frontal cortex, hippocampus and striatum. (Fig. 19A, 19B, 19C and 19D). *P<0.05 compared to age-matched control, § P<0.05 compared to young control.
Catalase

Catalase activity was found to increase significantly (P<0.05) in medial basal hypothalamus following 20% dosage of NFJ (Fig 20A). In frontal cortex, there was a significant (P<0.05) increase in catalase activity in the all dosages (Fig. 20B). In hippocampus, there was no significant difference in catalase activity (Fig. 20C). However, in striatum, 5% dose of Noni fruit juice significantly (P<0.05) increased the catalase activity (Fig. 20D).

Fig. 20: CAT activity in the brain areas isolated from old male F344 rats treated with NFJ (5ml/kg BW) in various dilutions (5%, 10% and 20%). There was a significant increase in catalase activity in medial basal hypothalamus, frontal cortex and in striatum (Fig 20A, 20B, and 20D); however in hippocampus CAT activity was unaltered (Fig. 20C) *P<0.05 compared to age-matched control, §P<0.05 compared to young control.
Glutathione Peroxidase

The activity of glutathione peroxidase (units/min/mg/protein) was found to decrease significantly (P<0.05) in medial basal hypothalamus following 5% and 20% dosage of NFJ (Fig. 21A). However, in frontal cortex and hippocampus, there was no significant difference in glutathione peroxidase activity in the dosages (5%, 10% and 20%) of NFJ (Fig. 21B and 21C). In striatum, 5% dose of Noni fruit juice significantly (P<0.05) increased in GPx activity (Fig. 21D).

Fig. 21: GPx (Isoform 1) activity in the brain areas isolated from old male F344 rats treated with NFJ (5ml/kg BW) in various dilutions (5%, 10% and 20%). GPx activity was decreased by Noni treatment in medial basal hypothalamus (Fig. 21A). However, in frontal cortex and hippocampus there was no significant difference (Fig. 21B and 21C). In striatum, lower dose of Noni (5%) increased GPx activity (Fig. 21D). *P<0.05 compared to age-matched control, §P<0.05 compared to young control.
Glutathione-S-Transferase

The activity of glutathione-S-transferase was found to increase significantly (P<0.05) in medial basal hypothalamus following all (5%, 10% and 20%) dosage of NFJ (Fig. 22A). However, in frontal cortex, there was no significant difference in GST activity, whereas in hippocampus 5% dose of NFJ significantly increased GST activity (Fig 22B and 22C). In striatum, 10% and 20% dose of Noni fruit juice significantly (P<0.05) increased the GST activity (Fig. 22D).

Fig. 22: GST activity in the brain areas isolated from old male F344 rats treated with NFJ (5ml/kg BW) in various dilutions (5%, 10% and 20%). Noni treatment significantly increased GST activity in medial basal hypothalamus (Fig. 22A) whereas GST activity was unaltered following Noni treatment in frontal cortex and in hippocampus (Fig. 22B and 22C). However, in striatum, GST activity decreases with increasing dose of NFJ (Fig. 22D). *P<0.05 compared to age-matched control, §P<0.05 compared to young control.
Lipid Peroxidation

In medial basal hypothalamus, age-associated extent of lipid peroxidation was found to be reversed in old male F344 rats following the treatment of Noni fruit juice (10%) (Fig. 23A). In frontal cortex, 20% dose of NFJ significantly (P<0.05) decreased the extent of lipid peroxidation (Fig. 23B) however, in hippocampus, there was no significant (P<0.05) difference with respect to age-matched control (Fig 23C). NFJ was found to decrease the extent of lipid peroxidation in striatum following all the dosages (5%, 10% and 20%) in old male F344 rats (Fig. 23D).
6. Summary and conclusions

The current study confirms and provides additional findings on the immunomodulatory role of noni fruit juice on splenic lymphocytes and lymphocytes isolated from the draining lymph nodes of young, middle-aged and old F344 rats. Since the present study was a longitudinal study, the age-associated decline in immune function and antioxidant enzyme activities of the lymphoid organs and the brain areas were clearly shown. The results from the present study demonstrate that Noni fruit juice enhanced lymphocyte proliferation (spleen and draining lymph nodes) in the presence and absence of Concanavalin A stimulation in young, middle-aged and old F344 rats. There was a concurrent increase in the IFN-γ and IL-2 production on treatment with Noni shelf juice in young, middle-aged and old rats in the presence and absence of con A. Unpublished data from our laboratory have shown that Noni juices increase the proliferation of lymphocytes in the case of young Wistar rats. The findings of the present study are also concurrent with these findings and other studies that have shown the immune-enhancing effects of the Noni fruit extracts (Thyagarajan et al., 2012). Since plant extracts are known to have lectins that can stimulate proliferation in the lymphocytes, the increase in proliferation observed with the Noni extract could also be due to a lectin in the juice. The individual component (lectin and similar phytochemicals) that is responsible for the proliferative action must be ascertained and its selective activation of immune cells must be investigated.

Apart from its potent immune-enhancing effects, Noni shelf juice was found to reverse the age-associated decline in Catalase, SOD and GST in middle-aged and old rats when compared with age-matched controls. Similarly, the age-associated increase in the extent of lipid peroxidation and glutathione peroxidase activity were significantly reduced in middle-aged and old rats following noni shelf juice treatment. Treatment with Noni juices significantly reduced the activity of glutathione peroxidase in all the three age groups probably because of the reduction in free radicals due to the scavenging effect of Noni. Plant extracts are known to be rich in flavonoids and other free-radical scavengers that contribute to their antioxidant properties (Ader et al., 2001). Thus Noni fruit juices may contribute to enhancing the immune
function by increasing the antioxidant enzyme activities, reducing the age-dependent increase in free-radicals and through possible lectins.

In humans, immunosenescence is associated with loss of native T cells, concomitant increase in memory T cell population, suppressed T lymphocyte proliferation, decrease in the populations of B lymphocytes and subsets of T lymphocytes, CD4+ and CD8+ and an increase in the markers of inflammation (Larbi et al., 2008). In addition to age-related inhibition of the activities of antioxidant enzymes, the extent of lipid peroxidation increased with advancing age that may also contribute to immunosenescence (Fuente et al., 2005; Miquel et al., 2006). The results from the present study demonstrate that Noni fruit juice (Divine Noni Gold) enhanced proliferation and cytokine production in lymphocytes isolated from draining lymph nodes and spleen in the presence and absence of Concanavalin A in old rats. There was a concurrent increase in the IFN-γ production in draining lymph nodes and in spleen after treatment with Noni fruit juice in all doses in old rats in the presence of Con A. Further, IL-2 production was also increased in the lymphocytes isolated from the draining lymph node and spleen of old rats after treatment with Noni fruit juice when compared to age-matched controls treated with con A.

Age-associated decline in immunity may also be caused by impaired activity of sympathetic noradrenergic (NA) nerves that are present in primary and secondary lymphoid organs of rodents which influence immune reactivity through the release of norepinephrine (NE) and direct synaptic associations with lymphocytes (Bellinger et al., 2001; Thyagarajan and Felten, 2002). NA sympathetic nerve fibers in primary and secondary lymphoid organs undergo age-associated alterations in their density, distribution in the parenchyma of lymphoid organs, NE concentrations, NE uptake and release, number of lymphocytes and β-adrenergic receptor-induced signaling in rodents (Thyagarajan and Felten, 2002). Lymphocytes and NA fibres share a bi-directional communication; while the neurotransmitters released from these nerves modulate the proliferation and functions of T lymphocytes, the lymphocytes secrete target-derived growth factors like NGF and aid in sustaining these fibres (unpublished data). Previously, we have demonstrated that sympathetic NA innervation and NE levels increased with aging in the
thymus, declined in spleen and mesenteric lymph nodes and was accompanied by significant reductions in NK cell activity, IL-2 and IFN-$\gamma$ production, and T and B cell proliferation in old female rats with profound changes occurring in middle-aged female rats (Thyagarajan et al., 2011).

The compensatory mechanisms such as antioxidant enzymes and growth factors are critical to the removal of reactive oxygen species, maintenance of neuronal functions, and thus, crucial to the homeostatic functioning of immune system. Prevention of oxidative stress and maintenance of antioxidant status at the cellular level is critical for normal physiological functions and inhibition of age-associated disorders (Salmon et al., 2010). The results from the present study demonstrated that Noni had distinct effects on the antioxidant enzyme (SOD, CAT, GPx and GST) activities in the brain areas (frontal cortex, medial basal hypothalamus, striatum, and hippocampus) and lymphoid organs (spleen, and draining lymph nodes).

Aging is characterized by loss of neuroendocrine functions and immunosuppression resulting in the development of cancer, infectious and autoimmune diseases, age-associated disorders and neurodegenerative diseases. In the periphery, immunosenesence is accompanied by the loss of sympathetic NA innervations in the spleen and lymph nodes (Bolzán et al., 1995; Maue et al., 2009). Previously, we had demonstrated that deprenyl, a monoamine oxidase inhibitor can reverse this age-related decline in sympathetic NA innervations along with cell-mediated immune responses in old and tumor-bearing rats (Thyagarajan and Felten, 2002). The results from the present study on the spleen, draining lymph nodes and brain areas of old age groups suggests that Noni fruit juice may have similar effects on neurogenesis in the spleen through diverse pathways or by modulation of antioxidant enzyme activity. Thus Noni fruit juice may contribute to enhancing the immune function by increasing the antioxidant enzyme activities, reducing the age-dependent increase in free-radicals and possibly through lectins which may also contribute to the similar effect. With advancing age there is decrease in the level of cytokines that are released from TH-1 cells and there is a shift from cell mediated to humoral immunity. Further, a strong role is played by the TH-1 cytokines as shown by increase in IL-2 and IFN-$\gamma$ in contributing to this effect. It is important to examine the extent of
influence exerted by Noni phytochemicals in modulating the bidirectional communication between the neuroendocrine system and immune system to alter the aging process (Palu et al., 2008).

References


