CHAPTER 4

SELECTIVE MODULATION OF AGE-ASSOCIATED IMMUNE FUNCTION AND ANTIOXIDANT ENZYME ACTIVITIES BY PHYTOCHEMICALS IN MORINDA CITRIFOLIA FRUIT THROUGH ERK PATHWAY IN SPLENOCYTES OF MALE F344 RATS

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 by Noni using Bioinformatics tool.

4.1 Rationale

Aging is a process characterized by deterioration of the physiological function with advancing age of an individual. With advancing age, there is increase in the level of reactive oxygen species (ROS) and decrease in noradrenergic (NA) innervation in secondary lymphoid organs that may be responsible for suppressed immunocompetence during aging. Morinda citrifolia (Noni) has been widely used for the treatment of a variety of diseases in Ayurveda and folk medicine owing to nutritional and immune-enhancing properties.

The working hypothesis for this objective is that Morinda citrifolia fruit juice may reverse the decline in immune functions and compensatory mechanisms observed during aging process. The dose-dependent effects of the fruit juice on immune functions, antioxidant enzyme activities, and cell signaling and inflammatory markers will also be investigated. 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, Bioinformatics tools will be used to examine the role phytochemicals in Noni fruit juice in the regulation of intracellular signaling pathways.
4.2 Treatment

4.2.1 Experiment 1

Young (3-4 months), middle-aged (8-9 month) and old (21 months) F344 rats were obtained from the National Institute of Nutrition at Hyderabad. The animals were acclimatized to the animal house at SRM University for a period of one week. Morinda citrifolia fruits were obtained fresh from the National Research Centre for Noni, Salavakkam, Tamil Nadu. The fresh fruits were ground with and without seeds using a blender and the pulp obtained were serially diluted in substituted RPMI medium from 0.0001% to 1% and a dose response curve was generated. Splenic lymphocytes (2 x 10^5 cells/ml) were treated with different dilutions of Noni seedless fruit juice (NSL) and Noni fruit juice with seeds (NWS) in 24 and 96 well-plates in the presence of 0 to 1.25 μg/ml of Con A and incubated in a humidified chamber with 5% CO₂ at 37°C to measure its effects on T lymphocyte proliferation, cytokine production, and antioxidant enzyme activities.

4.2.2 Experiment 2

Docking

Molecular docking was performed by keeping the ERK molecule rigid and ligand-flexible (Rigid Docking). This resulted in different conformations in each run and the best conformer which fits with lowest binding energy (kcal/mol) was chosen and written as a PDB file using UCSF Chimera. Auto Dock4.2.6 was used to automatically dock the ligands to the enzyme. In this version the windows platform, Cygwin was used for our analyses. The enzyme molecule was loaded and stored as a .pdbfile after assigning hydrogen bonds and kollman charges.

The investigation ligand was loaded and their torsions along with rotatable bonds were assigned and the file saved as ligand.pdbqt. Grid menu is toggled, after loading enzyme.pdbqt the map files were selected directly with setting up grid points with 80 X 106X 90 dimensions for the searching of the ligand within the active site of the enzyme molecule. This way the grid parameter files were created. The docking parameter files were then set up with search parameter as genetic algorithm and docking parameter utilizing Lamarckian genetic algorithm. Following up of grid parameter files (gpf) and docking log files (dlg), the command prompt was toggled and commands were typed in stepwise for autogrid and autodock execution. The
docked structures of the inhibitors were generated after a maximum number of evaluations using UCSF Chimera.

4.3 Results
4.3.1 Effects of Noni seedless fruit juice (NSL) and Noni fruit juice with seed (NWS) on age-related proliferation of splenocytes

Lymphocytes isolated from spleen were incubated with 0.1% NWS and significantly increased (p<0.05) Con A-induced lymphocytes proliferative capacity of T cells in young rats and old rats compare to control (Figs. 4.1A and 4.1C). In contrast, there treatment with NSL did not have any effect on lymphoproliferation except that lymphocytes co-incubation with 0.1% dose of NSL significantly decreased (p<0.05) the lymphocyte proliferation in early middle-aged rats (Fig. 4.1B).
Figure 4.1  Con A-induced T lymphocyte proliferation of splenic lymphocytes. Splenic lymphocytes (2 x 10^5 cells/well) were co-incubated with 0.1% dose of NSL and NWS for 72 h. *In vitro* treatment with NWS increased lymphocytes proliferation in young (A) and old (C) rats and treatment with NSL (B) decreased lymphocytes proliferation in early middle-aged rats. *p<0.05 compared to age-matched control.
4.3.2 Effects of Noni seedless fruit juice (NSL) and Noni fruit juice with seed (NWS) on age-related cytokine production

There was a significant (p<0.05) age-associated decline in the cytokine (IL-2 and IFN-γ) production by splenocytes isolated from F344 rats (Figs. 4.2A-4.2D). Treatment of splenocytes isolated from young (all the 3 concentrations) and old (1%) rats with NSL significantly (p<0.05) decreased IL-2 production, while no alteration in IL-2 production was observed early middle-aged rats (Fig. 4.2A). However, treatment with NWS significantly (p<0.05) increased IL-2 production by lymphocytes from early middle-aged rats (0.0001 and 0.01%) alone while a significant decrease in IL-2 production was observed in young (0.01 and 1%) and old (1%) F344 rats (Fig. 4.2B). Treatment with NSL significantly (p<0.05) decreased IFN-γ production in old rats (0.01 and 1%) alone (Fig. 4.2C). In contrast, NWS treatment significantly (p<0.05) increased IFN-γ production in young (0.0001%), early middle-aged (all the 3 concentrations), and old (all the 3 concentrations) rats (Fig. 4.2D).
Figure 4.2  Con A-induced IL-2 and IFN-γ production of splenic lymphocytes. Splenic lymphocytes (2 x 10^5 cells/well) were incubated with 1.25 µg/ml of Con A and NSL and NWS for 24 h. Age-related decline in IL-2 and IFN-γ production by lymphocytes from the spleen was observed. *In vitro* treatment with NSL decreased IL-2 production in young and old rats (A) and decreased IFN-γ production in old rats (C). NWS (B) treatment increased IL-2 production in middle-aged rats but suppressed IL-2 production in young and old rats while it enhanced IFN-γ production in young, early middle-aged rats and in old rats (D). #p<0.05 compared to young, *p<0.05 compared to age-matched control.

4.3.3 Effects of Noni seedless fruit juice (NSL) and Noni fruit juice with seed (NWS) on age-related cellular antioxidant status

Superoxide dismutase (SOD) activity (units/min/mg protein) significantly (p<0.05) declined in the splenocytes of old rats in comparison to young rats (Figs. 4.3A and 4.3B). Treatment of splenocytes with NSL (1%) increased SOD activity in old rats alone (Fig. 4.3A). NWS treatment however, significantly (p<0.05) enhanced SOD activity in splenic lymphocytes from young (0.01% to 1%), early middle-aged (0.0001%, 0.01% and 1%) and old (0.0001%, 0.01% and 1%) rats compared to age-matched controls (Fig. 4.3B).
Catalase (CAT) activity (units/min/mg protein) significantly (p<0.05) decreased with NSL treatment in splenocytes isolated from young (0.0001%) and early middle-aged (0.0001% and 0.01%) rats (Fig. 4.3C), while there was a significant (p<0.05) increase in old rats (1%) compared to age-matched controls. Co-incubation of splenocytes with NWS increased CAT activity in young (0.01% and 1%), early middle-aged, (1%) and old (0.01% and 1%) F344 rats compared to age-matched controls (Fig. 4.3D).

Glutathione peroxidase (isoform GPx1) also showed an age-dependent decrease in its activity (units/min/mg protein) in the lymphocytes from old rats compared to young controls (Figs. 4.3E and 4.3F). There was a significant (p<0.05) decrease in the activity of GPx in NSL-treated splenic lymphocytes isolated from young (0.01% and 1%), and early middle-aged (0.0001%, 0.01% and 1%) while there was an increase in GPx activity in lymphocytes from old (0.0001%) rats (Fig. 4.3E). Similarly, GPx activity decreased significantly (p<0.05) in the NWS-treated splenic lymphocytes from young (0.0001%, 0.01%) and early middle-aged (0.0001%, 0.01%) rats while there was an increase in NWS-treated old rats (1%) alone (Fig. 4.3F).

Glutathione-S-transferase (GST) activity was also reduced (p<0.05) in an age-associated manner in the splenic lymphocytes similar to SOD and GPx (Figs. 4.3D and 4.3D). GST activity increased significantly (p<0.05) in the splenocytes following NSL treatment in young (0.01 and 1%), early middle-aged (0.01 and 1%) and old (0.0001%, 0.01 and 1%) rats compared to their age-matched controls (Fig. 4.3G). NWS treatment (all the 3 concentrations) significantly increased (p<0.05) GST activity in the splenocytes from all the 3 age groups (Fig. 4.3H).

There was an age-dependent increase in the extent of lipid peroxidation in old rats (Figs. 4.3I and 4.3J). NSL treatment (all the 3 concentrations) of splenic lymphocytes from early middle-aged rats showed significant (p<0.05) decrease in lipid peroxidation while lymphocytes from old rats had a significant increase in lipid peroxidation compared to their age-matched controls (Fig. 4.3I). In contrast, NWS (all the 3 concentrations) decreased lipid peroxidation significantly (p<0.05) in splenic lymphocytes of early middle-aged and old rats (Fig. 4.3J).
**Figure 4.3**  Antioxidant status of splenic lymphocytes from young, early middle-aged and old rats treated with NSL and NWS.

**SOD activity** significantly increased in splenic lymphocytes of young, middle-aged, and old rats treated with NSL and NWS (A and B).

**CAT activity** decreased in splenic lymphocytes treated with NSL in young and middle-aged but it increased in old rats (C). In contrast, treatment of splenocytes with NWS showed a significant increase in CAT activity in all the age groups (D).

**GPx activity** decreased in splenocytes treated with NSL in young and middle-aged rats while it increased the activity in old rats (E). Treatment of lymphocytes with NWS increased the GPx activity at higher doses in all the age groups (F).

**GST activity** increased in splenocytes treated with NSL and NWS in all age groups (G and H).

Splenic lymphocytes treated with NSL showed increased in extent of lipid peroxidation in young and old rats while it was decreased in middle-aged rats (I). Treatment with NWS suppressed the extent of lipid peroxidation in middle-aged and old rats (J).

*p<0.05 compared to young, *p<0.05 compared to age-matched control.

### 4.3.4 Effects of NSL and NWS on age-related expression of p-ERK/Total ERK, p-CREB/Total CREB, and p-Akt/Total Akt in splenocytes

An age-related decline (p<0.05) in p-ERK/Total ERK expression was observed in old rats (Figs. 4.4A and 4.4B). Although the treatment with NSL (0.0001%, 0.01 and 1%) significantly (p<0.05) enhanced the p-ERK/Total ERK expression in the splenic lymphocytes isolated from old rats (Fig. 4.4A), NWS (0.0001%, 0.01 and 1%) treatment significantly (p<0.05) decreased its expression in lymphocytes of young, early middle-aged and old rats compared to age-matched controls (Fig. 4.4B).

The expression of p-CREB/Total CREB significantly declined in young and early middle-aged rats treated with NSL (0.0001%, 0.01 and 1%) (Fig. 4.4C). Treatment of splenocytes with NWS decreased p-CREB/Total CREB expression in young (0.0001%, 0.01%) rats but its expression was increased in old rats (0.0001%, 0.01% and 1%) (Fig. 4.4D).

A significant (p<0.05) increase in p-Akt/Total Akt expression was observed in NSL-treated splenocytes from young (0.0001%), early middle-aged and old (all the 3 concentrations) rats (Fig. 4.4E). Similarly, NWS-treated splenic lymphocytes from young (0.0001%), early middle-aged (0.0001%, 0.01% and 1%) and old (0.0001%, 0.01% and 1%) rats showed an increased expression of p-Akt/Total Akt (Fig. 4.4F).
Figure 4.4  Expression of p-ERK/Total ERK, p-CREB/Total CREB and p-Akt/Total Akt in splenic lymphocytes from young, early middle-aged and old rats treated with NSL and NWS.

**p-ERK/Total ERK:** Treatment with NSL reversed the age-related decline in the expression of p–ERK/Total ERK in old rats (A). However, treatment with NWS decreased the expression of p-ERK/Total ERK in all the age groups (B).

**p-CREB/Total CREB:** An age-related decline in p-CREB/Total CREB expression was observed in splenic lymphocytes (C and D). It decreased after treatment with NSL and NWS in young but increased following NWS treatment in old rats.

**p-Akt/Total Akt:** A significant increase in p-Akt/Total Akt expression in splenocytes was observed in young, middle-aged, and old rats treated with NSL and NWS (E and F). #p<0.05 compared to young, *p<0.05 compared to age-matched control.
4.3.5 NSL treatment enhanced p-NF-κB (p50) expression in young rats although decreased p-NF-κB (p50) expression in middle-aged and old rats

The expression of p-NF-κB (p50) significantly ($p<0.05$) increased in young (0.01% and 1%) rats (Figs. 4.5.A and B) while it decreased in early middle-aged (0.01% and 1%) (Figs. 4.5.C and 4.5.D) and old (0.0001% to 1%) rats (Figs. 4.5.E and 4.5.F) after treatment with NSL compared to age matched control groups.

**Figure 4.5**  *In vitro* incubation of splenic lymphocytes with noni seedless (NSL) fruit juices on the expression of NF-κB (p50). The expression of NF-κB (p50) increased in young (A and B) rats while it decreased in middle-aged (C and D) and old (E and F) rats after treatment with NSL. *$p$*<0.05 compared to age-matched control
4.3.6 NWS treatment enhanced p-NF-κB (p50) expression in young rats, middle-aged and old rats

The expression of p-NF-κB (p50) significantly ($p < 0.05$) decreased in young (1%) (Figs. 4.3.6A and B), early middle-aged (0.0001%) (Figs. 4.3.6C and D) and old (0.01% and 1%) (Figs. 4.3.6E and 4.3.6F) F344 rats after treatment with NWS compared to age matched control groups.

**Figure 4.6** *In vitro* incubation of splenic lymphocytes with noni fruit juice with seeds (NWS) on the expression of NF-κB (p50). The expression of NF-κB (p50) decreased in young (A and B), middle-aged (C and D) and old (E and F) rats after treatment with selective doses of NWS. *$p < 0.05$* compared to age-matched control.
4.3.7 Docking of ERK by known Noni phytochemicals-Damnacanthal, myricetin and ursolic acid

On the basis of the data obtained, the enzyme ERK (PDB ID: 2ERK; *Rattus rattus*; (Table 4.1A) which was inhibited by Noni fruit juice was docked with 20 known phytochemical ligands from *Morinda citrifolia*. Amongst these phytochemicals, five compounds showed significant inhibitory activity—damnacanthal, myricetin, quercetin, ursolic acid, and oleanoic acid (Table 4.1B). Analysis of the results obtained demonstrate that damnacanthal binds to the unique Lys112 residue in the P3 site of ERK with a $K_i=95.72$ µM and an energy of -5.48Kcal/mol and to Asp 177 which is close to the catalytic pocket with an energy of -5.21Kcal/mol (Fig.4.7A and 4.7B). Ursolic acid also bound to the unique P3 site at a significant bond distance of 1Å with the Lys112 residue indicating potent inhibitory effect (Figs. 4.8A and 4.8B) with a $K_i=2.42$ µM and an energy of -7.66Kcal/mol. Myricetin also bound to the similar pocket to Met106 with a $K_i=585.58$ µM although its specificity was lesser than the other two phytochemicals (Figs. 4.9A and 4.9B).
Table 4.1 Docking studies of *Rattus rattus* ERK (2-ERK) catalytic and phosphorylation sites (4.1 A) with Noni phytochemicals, listing the interacting amino acids, binding energies and bond distances (4.1 B).

### A

**SEQUENCE HOMOLOGY WITH HUMAN ERK (PDB id: 1WZY)**

<table>
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<th>10</th>
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#### Catalytic Residues
- Asp147, Asp165, Asn152, Lys 149 and Thr 188

#### Phosphate Binding Site
- Lys 52 (forms an ion pair with Glu 69)

#### P1 Pocket
- Arg 192, Ala 187, Arg189, Tyr 231, Gln 234, Leu 235

#### P3 Site
- Lys 112 (distinguishes ERK from cAPK)

#### Phosphorylation Residues
- Thr183, Tyr 185

#### Hydrophobic Pocket
- Val 186, Ile 196, Gly 202, Ile 207

### B

**NONI Phytochemicals**

<table>
<thead>
<tr>
<th>Residues of ERK that interact</th>
<th>Binding energy (Kcal/mol)</th>
<th>Ligand efficiency</th>
<th>Inhibitor constant</th>
<th>Unbound energy (Kcal/mol)</th>
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<tr>
<td><strong>Damnacanthal</strong></td>
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<td>Lys 112</td>
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<td>Asp 177</td>
<td>-5.21</td>
<td>-0.25</td>
<td>154.44 µM</td>
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<td><strong>Myricetin</strong></td>
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<td>-0.17</td>
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Figure 4.7 Docking studies using ERK and damnacanthal. Damnacanthal bound to P3 site of ERK to the Lys112, Met 106 (Ki=95.72 µM) and His 178 residues (A and B) and to Asp177 close to the catalytic pocket indicating potent inhibitory activity.
Figure 4.8  Docking studies using ERK and ursolic acid. Ursolic acid bound to P3 site of ERK to the Lys112 and Met 106 (Ki=2.42 μM) with a bond distance of 1Å and Ile 84 (Ki=3.45 μM) residues (A and B) indicating that ursolic acid could possess potent ERK inhibitory activity.
Docking studies using ERK and myricetin. Myricetin also bound to the P3 site of ERK to Met106 (Ki=585.58 µM) and Asp177 (Ki=1.2 mM) similar to the region of binding showed with damnacanthal and ursolic acid (A and B).
Figure 4.10 An overview of the results depicting immunomodulatory effects of Noni fruit juices with or without seeds on splenic lymphocytes in vitro.
4.4 Discussion

Based on the results from the preliminary study on young male Wistar rats, a longitudinal *in vitro* study was conducted on young, middle-aged, and old male F344 rats to examine the effects of NSL and NWS on age-associated alterations in immune functions, factors involved in compensatory mechanisms such as antioxidant enzyme activities, and role of intracellular signaling pathways in mediating these effects. Results from the study showed that the effects of Noni fruit juice (NSL and NWS) on immune responses varied depending upon the presence or absence of seeds; NSL either had no effect or suppressed Con A-induced lymphocytes proliferation, and cytokines (IL-2 and IFN-γ) production, however NWS reversed the age-associated decline in lymphocytes proliferation and improved the immune responses by up regulating IL-2 and IFN-γ production.

The little effect mediated by Noni fruit juice in old group might be due to the age of rats. Results from the study showed that splenocytes incubated with Noni fruit juice and Con A increased the lymphocyte proliferation in young and middle-aged rats, although no significant difference in lymphocyte proliferation was observed in old rats. No significant differences in the lymphocytes proliferation in old rats might be due the age-associated decline in immune function and co-stimulatory signal molecules. No effect in response to Con A might be due the age-associated decline in immune function in old rats and which was not reversible after being treated with Noni fruit juice or Con A. Parallel to this, an age-associated decline in co-stimulatory signal might have been responsible for the absence of any response to Con A stimulation in splenocytes isolated from old F344 rats.

Noni fruit juice, irrespective of the absence (NSL) or presence (NWS) of seeds enhanced the activities of SOD and GST and Akt expression while suppressing the expression of CREB in F344 rats. Noni fruit juice (NSL and NWS) had diverse effects on the CAT and GPx activities and the expression of ERK 1/2 that were dependent on dosage and age groups. More importantly, NSL increased the extent of lipid peroxidation in the splenocytes of young and old rats, but NWS decreased the lipid peroxide levels in middle-aged and old rats. Similarly, NF-κB expression was decreased in the splenocytes of middle-aged and old rats treated with both NSL and NWS but it was enhanced in the NSL-treated splenocytes of young rats.

Age-associated decline in cell-mediated immune functions and a shift in immunity from Th1 to Th2 may predispose the elderly to a number of neurodegenerative diseases, infectious diseases, and cancer [8, 10, 53-55]. Increased production of Th1 cytokines, IL-2
and IFN-γ, by the NWS may be mediated through CREB and Akt pathways that may reverse the age-associated decline in cell-mediated immunity. Possibly, these effects may have been mediated through the activation of CB2 receptors that is capable of not only suppressing Th2 cytokine, IL-4 production, but enhancing the Th1 cytokine, IFN-γ production [123]. Further supporting these findings, a polysaccharide-rich extract from noni was capable of stimulating several mediator of immune molecules, such as TNF-α, IL-1β, IL-10, IL-12 p70, IFN-γ and nitric oxide, while suppressing the levels of IL-4, that were the direct effects of noni on the murine effector cells to promote antitumor effects in mice [127].

Similarly, aqueous and hydroalcoholic extracts of *Morinda citrifolia* increased the proliferation of splenocytes on and delayed type hypersensitivity reaction, emphasizing its immunostimulatory properties [128]. Although the effects of noni fruit juices on dendritic cells (DC) was not measured in this study, it is possible that the increased T cell proliferation was facilitated through DC, because treatment of DC with fermented noni exudate increased the proliferation of splenocytes [129]. Some of the differences in the parameters examined in our study with NSL and NWS may be because of the absence and presence of specific phytochemicals.

Damnacanthal, a phytochemical present in Noni, was able to increase the production of IL-2 and IL-12 in human peripheral blood mononuclear cells (PBMCs) and also, its proliferative capacity similar to the NWS-mediated effects on these immune parameters in the present study [32]. Immunosenescence and subsequent increase in the incidence of diseases and cancer may be the result of oxidative stress due to increased cellular insults and damage over the years by the formation of free radicals. The present study provides crucial evidence that some of the immunostimulatory effects of noni may be mediated through the improvement in antioxidant enzyme activities in the lymphocytes.

Results from the study demonstrated that irrespective of the absence (NSL) or presence (NWS) of seeds, Noni fruit juices increased the activities of SOD and GST in splenocytes. Noni fruit juice (NSL and NWS) had diverse effects on the CAT and GPx activities that were dependent on dosage and age groups used in this study. In addition, there was a marked difference in affecting the extent of lipid peroxidation; NSL enhanced while NWS suppressed it suggesting that again this effect may be mediated through specific phytochemicals present in the seeds.

Previous studies from our laboratory showed the age-dependent increase in the extent of lipid peroxidation in rodents and women [57, 116]. Noni fruit juice (NSL and
NWS) mediated increases in antioxidant enzyme activities might be through activation of Antioxidant Response Element (ARE) in the nucleus that might be responsible for the increase in immune function and down-regulation of NF-κB expression. There is not much information about the intracellular mechanism through which Noni modulates the immune function and antioxidant enzyme activities.

Results from *in vitro* study showed the diverse effect of NSL and NWS on expression of p-ERK, p-Akt and p-CREB in young, middle-aged and old F344 rats. Noni fruit juice down regulates manganese-induced hypoxia inducible factor-1α (HIF-1α), and also, down regulates the expression of ERK-1/2, Akt, JNK-1, S6, and eIF-2α in lung cancer cells [130]. The results are in agreement with our present study, especially ERK 1/2 inhibition by NWS but there was an increase in Akt expression by both NSL and NWS. These varied actions of NSL and NWS on the intracellular signaling pathways necessitate further investigation as these effects may be due to different phytochemical components in the NSL and NWS exerting unique effects within the cell.

Further, to know more about the mechanism, by which Noni fruit Juice (NSL and NWS) exerts differential effect of on signaling pathways, docking studies were done to examine the role of various phytochemicals in interacting with ERK protein. Results from the docking study demonstrated the varied effects of NSL and NWS on the intracellular signaling pathways. Our study warrants further investigation as these effects may be due to different phytochemical components in the NSL and NWS exerting unique effects within the cell and thus, influencing the immunity and lipid peroxidation.

Docking studies of damnacanthal, ursolic acid, and myricetin among several Noni phytochemicals with ERK protein demonstrated that these specific phytochemicals can directly inhibit the activity of the ERK protein by binding to its phosphorylating and catalytic sites. Ursolic acid among the ligands tested not only had the lowest energy of binding, but also the highest inhibition constant. The fact that many compounds were successful in binding to the phosphorylation cleft suggests that ERK might be significantly inhibited by these interactions. There is a possibility that different concentrations of these specific phytochemicals in NSL and NWS may have influenced the ERK activity.

Treatment with NSL and NWS suppressed NF-κB expression in the lymphocytes of middle-aged and old rats, whereas NSL increased and NWS decreased NF-κB expression in the young rats. Noni extract and its bioactive component, damnacanthal, exhibited its anti-inflammatory effects by inhibiting lipopolysaccharide (LPS)-induced NF-κB activity, expression of proinflammatory cytokines, cyclooxygenase-2, and inducible nitric oxide
synthase [131]. Similarly, methanolic extract of the roots of *Morinda officinalis* inhibited NF-κB expression, production of nitric oxide, prostaglandin E2 and TNF-α in LPS-stimulated macrophages, suggesting that Noni has potent anti-inflammatory properties [132]. Hence, the individual component (lectin and similar phytochemicals) that is responsible for the selective activation of immune cells must be further investigated.

In summary, NWS and NSL exerted differential effects on cytokine production and antioxidant enzyme activities of the splenocytes from young, early middle-aged, and old F344 rats by modulating the intracellular signaling pathways. Altered immunomodulatory effects of Noni fruit juice with seeds may be attributed to altering phytochemical and protein profiles of seed components that need to be investigated further. Further bioavailability of these phytochemicals in Noni fruit juice needs to be determined in order to predict the efficacy of the fruit juice *in vivo*.

*Morinda citrifolia* has diverse actions in the body, but there have been limited studies to understand its role in influencing the interactions between neurochemicals, hormones and immune molecules such as cytokines and the intracellular signaling mechanisms in the maintenance of homeostasis and their roles in the aging process in human beings. The results from the present study show that the mechanism(s) of action(s) of these systems in the aging process and secondly, the results from the treatment of Noni fruit juice in various age groups provide information about the role of cellular and intracellular targets in aging. However, further studies are warranted to determine if there are differences in the concentrations of these phytochemicals based on the absence or presence of seeds in Noni fruit juices.

Reason for the variability in these results is due to the presence or absence of a number of phytochemicals in the seeds and pulp of the fruit. Till now more than 200 phytochemicals have been identified from the various parts of *Morinda citrifolia* plant [58]. Various categories of phytochemicals have been identified in the seed, such as anthraquinone, iridoids, lignans, flavonols, triterpenes, linoleic acid, ursolic acid, oleic and palmitic acids, scopoletin, as well as campesterol, stigmasterol, tocopherol and some other phytochemicals [60, 133]. There are some phytochemicals that are present in the seed but absent or available in less amount in fruit pulp such as dammacanthal, myricetin, quercetin, dammacanthal, americanin. For example linoleic acid, ursolic acid, scopoletin and a number of other phytochemicals that are present in the seeds have anti-inflammatory properties. Americanin A and quercetin present in seeds are potent antioxidant and shows superoxide dismutase (SOD)-like activity. Presence of these phytochemicals in seed might be
responsible of the increase in SOD and other antioxidant enzyme activities in splenocytes and in various brain areas of F344 rats [134, 135].

The results from the present study provide preliminary information about the benefits of Noni fruit juice as an effective anti-aging supplement.

4.5 Key findings

Effects of Noni fruit juice (NSL and NWS) on immune responses were more specific, depending on the absence or presence of seeds; NSL had no effect or suppressed Con A-induced splenocyte proliferation, IL-2 and IFN-γ production while NWS reversed the age-related decline in lymphocyte proliferation and enhanced the immune responses by up regulating the IL-2 and IFN-γ production.

Results from the study demonstrated that irrespective of the absence (NSL) or presence (NWS) of seeds, Noni fruit juice increased the antioxidant enzyme activities in old F344 rats. In addition, there was a marked difference in affecting the extent of lipid peroxidation. Treatment with NSL and NWS decreased the p-NF-κB (p50) expression in old rats.

Treatment with NWS had inhibitory effects in ERK expression but there was an increase in Akt expression by both NSL and NWS. These varied actions of NSL and NWS on the intracellular signaling pathways necessitate further investigation as these effects may be due to presence of different phytochemicals components and their concentrations in the NSL and NWS exerting unique effects within the cell.

Results from the docking study demonstrated the diverse effects of NSL and NWS on the intracellular signaling pathways. Docking studies of dammancanthal, ursolic acid, and myricetin among several Noni phytochemicals with ERK protein demonstrated that these specific phytochemicals can directly inhibit the activity of the ERK protein by binding to its phosphorylating and catalytic sites.

The results from the present study provided preliminary information about the beneficial effects of Noni fruit juices as an effective anti-aging supplement. However, further research is needed to isolate the active compounds in Noni and examine their effects on the neural-immune parameters.