IMMUNOMODULATORY PROPERTIES OF NONI (Morinda citrifolia)

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IMMUNOMODULATORY PROPERTIES OF NONI (Morinda citrifolia)

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Science

by
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May, 2010

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ABSTRACT

Noni (*Morinda citrifolia*) is a popular medicinal plant of family Rubiaceae. Its fruit is rich in various phytochemicals and polysaccharides. Anecdotal evidence indicates that it was used to stimulate the immune system and, thus, to fight various infections. Limited *in vitro* studies also support the immunomodulatory role of Noni, however only one study utilized an animal model. The purpose of this study was to examine the immunomodulatory properties of Noni fruit in the broiler chicken.

Different concentrations of dietary Noni were added to diets and fed to day-old broiler chickens for 3 to 6 weeks. Gut tissue and blood were sampled to determine the expression of selected genes, concentration of immunoglobulins and α1-acid glycoprotein. The results showed that Noni at 6% concentration increased the expression of Toll like receptor -4 (TLR-4) and TLR-5, chemokine (IL-8), interleukin-12 (IL-12), and decreased the expression of IL-6 and TLR-7. At 4% concentration, Noni stimulated the expression of TLR-3. Increased expression of TLR-3, TLR-4 and TLR-5 indicate antiviral and antibacterial properties of Noni fruits in chickens that are further strengthened by increased expression of IL-12 and IL-8. Decreased expression of IL-6 indicates anti-inflammatory properties of Noni. However, Noni did not have effects on serum and gut immunoglobulins or α1-acid glycoprotein concentration. Further investigations will be necessary to explore the active immunomodulatory ingredients of Noni and their effects on immunity during viral and bacterial infections.
DEDICATION

I dedicated this work to my parents and the almighty God who provides me the eternal source of energy to pursue my goals in life.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Denzil Maurice, for his excellent mentoring and his assistance during the course of my graduate program. His encouragement and inspiration provided the source of enthusiasm for completing the task. I highly appreciate his guidance during research that enhanced my thinking. I also appreciate his wife Ms. Diana for providing encouragement during my initial failures in research.

I am highly grateful to Dr. Brandon Moore for providing molecular facilities. Inspite of his busy schedule he taught me the concepts of molecular research. I am thankful to his graduate student Dr. Rucha Karve who provided valuable assistance during the early phase of laboratory work. I appreciate Dr. Joe Toler for discussing statistical concepts of research design and helping me to calculate the statistics not only by software but also manually. I would like to thank Dr. Thomas Scott for serving on my advisory committee, guidance in the early phase of the study, and critical assessment and assistance in the final stages.

I am thankful to my colleagues and/or friends Ms. Rucha, Mr. Tassema, Ms. Erin Curry, Ms. Ashley, Ms. Crystal, Ms. Rachel, Ms. Katie, Ms. Emily, Mr. Jason, Mr. Dale, Ms. Marcia, Ms. Linda, Ms. Heidi, Mr. Bill, Mr. Randheer, Mr. Biswa, Mr. Manoj, Mr. Sahu, Ms. Wooten and Ms. Fiona for encouragement.

Finally, I wish to express my sincere regards to my parents and relatives for providing continuous support, love and encouragement during my study.
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CHAPTER I
INTRODUCTION

Herbal and natural products of folk medicine have been used for centuries in every culture throughout the world. Due to the prohibition of sub-therapeutic use of antibiotics in food animal industry by some countries, the possibility of a ban in other countries, and increasing demand for natural products with health benefits, the importance of medicinal plants have increased in recent years.

Noni \((Morinda citrifolia)\), also known as Indian Mulberry, Ba Ji Tian, Cheese Fruit and various other names in different countries, is a popular medicinal plant. Its fruit is rich in a variety of phytochemicals and polysaccharides. Results from limited studies demonstrated immunomodulatory effects of these constituents in \textit{in vitro} experiments (Hirazumi \textit{et al.}, 1996, 1999, Furusawa \textit{et al.}, 2003, Pawlus \textit{et al.}, 2005 Akihisa \textit{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\textsuperscript{®} Juice, 2002).

Health is directly related to the immune status of an animal. Gut mucosal immunity is important in this regards as the gut represents the largest surface area of the body that contacts with external milieu and thus interacts with diverse chemicals and infectious or non-infectious agents. Gut mucosa have some unique features to cope with these antigens. For example, the presence of the microfold cell (M cell) on mucosa facilitates the uptake of antigen from gut lumen to immune cells, which contain secondary lymphoid organs (e.g., Peyer’s patches) and thus stimulates the mucosal
immunity. Also, the total number of lymphocytes present in mucosal surface is greater than in primary lymphoid organs like spleen and lymph node. Macrophages and dendritic cells, present in these secondary lymphoid organs, are the first cells that interact with antigens, primarily via Toll-like receptors, and produce proinflammatory cytokines that further potentiate the immune response. Other cells, like B-1, of the innate immune system reside in secondary lymphoid tissue and are responsible for the secretion of natural antibodies. These antibodies neutralize a broad range of antigens including polysaccharides. Interestingly, in contrast to adaptive immunoglobulins, the production of natural antibody does not require T cells or prior exposure to the pathogen.

The purpose of this study was to examine the immunomodulatory properties of Noni. The study was conducted in two phases. The first experiment determined whether dietary Noni supplementation enhanced antibody titers, which includes natural antibodies, during induced inflammation. The second experiment examined the effects of dietary Noni supplementation on gene expression of Toll-like receptors, proinflammatory cytokines, and chemokines in gut associated lymphoid tissue as well as systemic and gut associated antibody production. Supplementation of different concentrations of dietary Noni fruit puree to broilers, enhanced gene expression of Toll-like receptors, interleukins and chemokine, however did not effect immunoglobulin concentration. These results indicate immunomodulatory properties of Noni.
CHAPTER II
LITERATURE REVIEW

Introduction

The Noni plant is one of approximately 11,000 species of the family Rubiaceae, some of which are used to treat various diseases. Also known as Indian Mulberry, Ba Ji Tian, Cheese fruit and various other names in different countries, it is the second most popular plant used for curative and preventive purposes in Polynesia (Krauss, 1993) as almost all of its parts in various combinations including root, stem, bark, fruits and leaves have been used to treat various known acute and chronic diseases in this region for 2000 years (Whistler, 1985; Bruggnecate, 1992). However, there are no written scientific documents discussing the ancient use of this fruit as an immunomodulator, even though anecdotal evidence indicates that it was used to fight various infections and therefore likely stimulated the immune system (Earle, 2001). The current research focuses on the effect of the polysaccharides and phytochemicals of Noni on the immune system.

Immunomodulatory Properties of Noni Fruit

The results of recent studies support this role of Noni fruit (Sunder et al., 2007; Li et al., 2008; Zhang et al., 2009). The nutrient composition and chemical analyses of Noni fruit suggest that its polysaccharides and phytochemicals have immunomodulatory effects as evidenced by the results of in vitro experiments (Hirazumi et al., 1996, 1999; Furusawa et al., 2003; Pawlus et al., 2005; Desai et al., 2009).
Immunoglobulins and cytotoxic T-cells are the important components of humoral and cell-mediated immune system, which effectively eliminate the pathogen during infection. Sunder et al. (2007) first reported the effects of Noni fruit juice on humoral and cellular immunity. They found that supplementation to broiler chickens of 5% Noni crude fruit juice in drinking water to 6 weeks of age significantly increased the total antibody titers against goat red blood cells at 3 weeks of age. However, increment of total antibody titers does not necessarily reflect the enhancement of immune status as population of certain types of antibodies are required to neutralize or stimulate complement activation. Cell-mediated immunity was measured by response to T-cell mitogen in terms of skin index to phytohaemagglutinin-P (PHA-P) injection measured 24-h post injection. The response to PHA-P was to be not found significant. To examine whether Noni-stimulated humoral immunity provided sufficient protection against infectious diseases, 3-week old broilers were challenged with infectious bursal disease virus (IBDV), and Noni treatment reduced approximately 75% mortality. However, it is thought that immunity against IBDV correlates with antibody titer, but enhancement of cell-mediated immunity is necessary for protection against the virus which was not affected by Noni in this experiment (Rautenschlein et al., 2002). Sensitivity of the technique to measure cell-mediated immunity and provision of Noni juice in drinking water, as only water soluble compounds were taken by broilers, might be factors that contributed to the lack of response to Noni treatment. The results of this study provide limited information about the immunomodulatory effects of Noni on immunity.
Schafer et al. (2008) utilized dairy calves as an animal model and reported that Noni fruits have antibacterial properties. Feeding 30 ml of Noni fruit puree twice a day per calf to newborn calves for up to 2 weeks enhanced bactericidal activities of blood against *E. coli* in an *in vitro* assay. Their findings need to be further investigated as the conclusion was based on the median differences not the true mean of the data. Based on the results, these authors hypothesized that the bactericidal activity of Noni may be due to the modulation of the neonatal immune system. To examine this hypothesis, Brook et al. (2009) measured the expression of alpha chain (CD25⁺) of IL-2 receptor on CD8⁺ and CD4⁺ T cells and found increased gene expression of CD25⁺ on these cells at 3 days of age. Activated IL-2 receptor transduces cell proliferation signal thus enhance immune cells population. To check whether the higher expression of this receptor correlated with the protection against bacterial infection, they supplemented 30 ml of Noni puree every 12 h in Salmonella Dublin and Salmonella Newport infected calves and found a 54% reduction in total medical treatments per calf as compared to control. This study indicated the positive correlation of CD25⁺ with protection against infection, even though enhanced expression of β and γ subunits are necessary for signal transduction, and these were not examined. Based on these findings, Darien et al. (2009) determined whether Noni puree stimulates cell-mediated immunity or humoral immunity. They found that incubation of 0.1 to 10 mg/ml concentration of Noni puree with isolated peripheral blood mononuclear cells for 4 or 24 h increased the secretion of IL-2, INF-γ, IL-4 and IL-5 in a dose dependent manner. Interestingly, incubation with Noni puree for 4 h induced a greater concentration of these cytokines as compared to 24 h incubation. This *in vitro* study
indicates that Noni puree potentiated cell-mediated, as well as humoral immunity. However, for providing the protection against infections it is necessary to examine the overall outcome of these cytokines in terms of immunoglobulins and different subsets of effector immune cell populations. Also, IL-4 and INF-γ have antagonizing effects on the immune response and thus affects the net outcome of immunity.

To examine the possibility of Noni influencing neonatal immunity, the diet of dams was supplemented with puree, and gene expression was measured in umbilical cord blood. Noni supplementation during gestation increased gene expressions of Toll-like receptors (TLRs-1,-2,-4,-5, and 10), interleukins (IL-4, IL-17) and their receptors (IL-4R, IL-17RA, IL17RB), chemokine (CXCL11), co-stimulator CD27, TNF-α, and Foxp3. It is interesting that both IL-17 and Foxp3 expression are increased simultaneously, as IL-17 stimulates the innate immune response while Treg cells, which are regulated by Foxp3, have a suppressive effect on immunity.

Preventive as well as prophylactic treatments of mice with fermented Noni exudate or its supernatant showed antitumor activity against S180 tumor cells. Fermented Noni Exudate (fNE) or its supernatant at a dose of 500 µl/day/mouse for 3 days via i.p. injections increased the percentage of granulocytes and Natural Killer (NK) cells, and decreased the CD19+ B cells in spleen, peritoneum and peripheral blood of C57BL/7J mice (Li et al., 2008). These changes in immune responses were positively correlated with the survival of C57BL/7J mice challenged with S180 tumor cells. However they did not determine the expression of CD21 on B cells or dendritic cells to examine the complement modulatory activity of Noni as CD21 binds with the C3d component of
complement system. Interestingly, similar doses of Noni did not reduce the mortality in functional NK deficient beige mice. The results indicated that Noni shows anti-tumor activity via stimulating NK cells. Li et al. (2008) also observed that Noni treatment increased the duration of survival of thymus (T-cell producing organ) deficient nude mice but did not influence survivalibility. The suggestion that Noni may also modulate T cells which may help to eliminate the tumor with NK cells in C57BL/7J mice is supported by the fact that CD8$^+$ cells are increased in C57BL/7J mice peritoneum. However, they also showed that Noni enhanced the memory of tumor immunity but they did not compare the treated data with survived tumor challenged mice from control group. According to the authors, Noni treatment provided long-term protection; i.e., anti-tumor memory development. It is not clear whether this was the result of either tumor challenge or Noni treatment due to insufficient information in control group.

Incubation of pretreated LPS-matured dendritic cells with 1-10% fermented Noni Exudate (fNE) increased proliferation of syngeneic splenocytes that contain a variety of immune cells (Zhang et al., 2009). They further examined a subpopulation of splenocytes and found that B cells and CD4$^+$ T cells increased significantly but that the population of NK cells and CD8$^+$ cells was unaffected. These in vitro findings are contradicted by the in vivo findings of Li et al. (2008) that NK cells are increased in number due to fNE treatment. Proliferation of B cell was not directly stimulated by fNE, but the addition of immature or LPS matured dendritic cells enhanced B-cell proliferation. The highest proliferation was found at the fourth day of incubation. However it is conceivable that the responses to fNE are due to endotoxins such as lipopolysaccharides. The fNE used in the
study was analyzed and found to contain endotoxins and fNE positive for endotoxins stimulated proliferation. The proliferative activity was independent of CD-40 of B cells and required the direct contact between B cells and dendritic cells, and further allowed these cells to grow up to 3 weeks, resulting in increased IgG and IgM immunoglobulin concentrations. These findings suggest that fermented Noni Exudate stimulates the humoral immune response via stimulating LPS matured dendritic cells or endotoxins in fNE induce the response.

**Immunomodulatory Polysaccharides in the Noni Fruit**

Analysis of alcohol precipitated fraction of Noni fruit showed that it contained 80% pectins and 5% protein-bound arabinogalactans (Bui et al., 2006). Similar purified polysaccharide fractions isolated from other plant sources exhibited immunological activities (Luettig et al. 1989; Chintalwar et al. 1999; Lim et al. 2003).

Hirazumi et al. (1996, 1999) studied the effects of polysaccharide rich alcoholic precipitates of Noni on nitrite production by cultured mice peritoneal macrophage cells which contribute to bactericidal activity of macrophage. Fractions precipitated by repeated treatment with ethanol and solubilized in medium enhanced nitrite production. The same dose of solubilized Noni precipitates when combined with 25 U/ml of INF-γ exhibited a synergistic effect on nitrite production, which increased fourfold compared to solubilized precipitates, only. Incubation of different concentrations of precipitates with isolated human peripheral mononuclear cells for 24 hours increased the IL-1β and TNF-α production up to 30-250 pg/ml in a dose dependent manner and the relationship was
linear within the range of 100 to 1000 μg/ml of precipitates. Noni precipitates at 1.25 μg/ml concentration also increased the IL-1β and IL-12 production from mice macrophage when incubated for 16 and 24 h, respectively. Incubation of similar concentration of Noni precipitates with splenocytes in the presence of mitogen inhibited IL-4, enhanced IL-10 production, but had no effect on IL-2 secretion. Similar incubation experiment with thymocytes increased INF-γ secretion. Polysaccharides of Noni also exhibited antitumor activity via modulating immune system (Hirazumi et al., 1999). Prophylactic and therapeutic treatments of Noni precipitates against LLC1 or S180 tumor cells challenged mice increased survivability and life span. Antitumor activity of Noni precipitates was abolished with simultaneous treatment of macrophage, NK cells or T cell inhibitors. Combined with interferon, the antitumor response of precipitates increased synergistically in both prophylactic and therapeutic treatments (Hirazumi et al., 1999; Furusawa et al., 2003). These results suggest that Noni polysaccharides stimulate cell-mediated immunity, but the researchers did not examine the endotoxin contamination of Noni precipitates. The procedure did not eliminate the possibility of bacterial growth during exposure of ripe fruit to sunlight for 1-3 days prior to juice collection and preparation of precipitate.

Peritoneal exudate cells, from mice provided Noni juice at a dose of 100 ml for 15 days, cultured with 3-12 μg/ml concentrations of concanavalin A in the presence of LPS decreased secretion of IL-4 (Palu et al., 2008). In the same study, incubation of polysaccharide-rich Noni precipitates with similar cells suppressed INF-γ production. This observation was contradictory to the finding of Hirazumi et al. (1996, 1999). Palu et
al. (2008) correlated the immune suppression with increased binding of cannabinoid receptor-2 (CB-2) to its ligand by Noni fruit juice concentrate but they did not examine the effect of polysaccharide-rich precipitates on the receptor.

Polysaccharides, hydroalcoholic and aqueous extracts, and alkaloids, and anthraquinone compounds extracted from the dried Noni fruit powder enhanced the phagocytic activity of neutrophils in in vitro assay. Oral dosing of these fractions also increased serum IL-6 in vaccine challenged mice (Nayak et al. 2009).

**Immunomodulatory Properties of the Phytochemicals**

Phytochemicals refer to the chemicals present in plants, which may or may not be nutrients, that affect health (Linus Pauling Institute of Micronutrient Research for Optimum Health). However, limited studies have been examined the effect of Noni fruit phytochemicals, but similar phytochemicals from the different sources have been reported to exhibit immunological properties (Table 2.1). The phytochemicals isolated from the Noni fruit belong to five major classes - iridoids, flavonoids, lignans, anthraquinones and fatty acid esters. (Wang et al., 1999, 2000; Su et al., 2005; Kamiya et al., 2004, 2005; Pawlus et al., 2005; Pawlus et al., 2005; Schripsema et al., 2006; Dalsgaard et al., 2006; Deng et al., 2007; Lin et al., 2007; Zin et al., 2007; Akihisa et al., 2007 and Nayak et al. 2009).
Iridoids

Iridoids are terpenes synthesized from two isoprene (2-methyl-1, 3-butadiene) units and are intermediates in alkaloid biosynthesis. Chemically, iridoids consist of a cyclopentane ring fused with an oxygen heterocycle (Figure 2.1).

Figure 2.1 Chemical structure of iridoids

Although the iridoids in the Noni fruit have not received much research attention, studying similar iridoids extracted from different plant sources have shown regulatory effects on immune cells in an in vitro model. For example, Cimanga et al. (2003) reported that gaertneroside, which structurally resembles the citrifolinoside of Noni fruit, inhibited the classical pathway of the complement system. Complement system is a biochemical pathway that either helps antibodies to eliminate antigen from the body or directly kill the pathogen. However, this study did not explore the affected component of complement pathway by the gaertneroside and thus provides limited information of anti-complementary activity of iridoid. It seems that iridoids also check the adverse reactions of immune cells in the body, since deacetyl asperulosidic acid from Oldenlandia diffusa inhibited by 64% the oxidation of low density lipoproteins. This is one mechanism by which these phytochemicals can reduce atherosclerosis due to the oxidation of lipids by macrophages (Kim et al., 2005). Similarly, Li et al. (2006) reported that asperuloside and deacetylasperulosidic acid extracted from Lasianthus acuminatissimus, inhibit the
secretion of TNF-α by cultured mouse peritoneal macrophage cells, with IC₅₀ values of 0.52 and 1 µg/ml, respectively.

**Flavonoids**

Flavonoids are a group of pigments present in plants. Chemically, flavonoids consist of two benzene rings linked with either a heterocycle pyran or pyrone ring (Middleton *et al.*, 2000; Figure 2.2).

![Figure 2.2 Chemical structure of flavonoids](image)

Quercetin, kaempferol and catechin isolated from different plants or in a chemically pure form have been investigated concerning their possible immunological properties in an *in vitro* model. The results indicate that quercetin inhibits antigen-stimulated histamine release by human basophils and mast cells, over a range of 5 to 50 µM in a dose dependent manner (Middleton *et al.*, 1981; Grossman *et al.*, 1988). Quercetin also inhibits leukocyte migration, and leukotriene-B4 (LTB-4) and prostaglandin-E2 (PGE2) concentrations in carrageenan-induced pleural exudates (Mascolo *et al.*, 1988). In further investigation, these authors found that similar compounds inhibited the synthesis of ionophore-stimulated leukotriene B4 (LTB-4) in leukocytes. The phytochemicals, quercetin and kaempferol triglycosides extracted from
the leaves of *Morinda morindoides* (of the Noni genus) have been found to exhibit their anti-complementary activity on the classical and alternative pathways in an *in vitro* assay (Cimanga *et al.*, 1995). However, this study did not examine the mechanism by which quercetin and kaempferol inhibited complement pathways and thus provides limited information of anti-complementary effects of these phytochemicals.

Flavonoids have also been found to affect cytokine production. Data obtained by Matsunaga *et al.* (2001) showed that epigallocatechin gallate reduced production of IL-10, while at the same time stimulating production of IL-12 and TNF-α, and stimulating gene expression of INF-γ by bacterial induced cultured macrophages. Preincubation or coincubation of the polymeric or dimeric form of catechin or epicatechin reduced nitrous oxide secretion by stimulated macrophages in a concentration dependent manner, with an IC₅₀ value of 50 μg/ml (Terra *et al.*, 2007). Similarly, Shim *et al.* (2008) reported that epigallocatechin decreased T-cell proliferation and IL-2 secretion by inhibiting the MAPK and phospholipase-C pathways, via high affinity binding to ZAP-70 kinase (Kd of 0.6 μM).

**Lignans**

Lignans are important class of phytoestrogen found in plants. These are polyphenolic chemicals derived from phenylalanine and monolignols (Figure 2.3, Checker *et al.*, 2007).
Figure 2.3 Chemical structure of lignans

Epidemiological studies have suggested that lignans might be helpful in reducing the risk of breast, ovarian and prostate cancers in humans (Linus Pauling Institute of Micronutrient Research for Optimum Health). This potential anticancerous property may be due to their anti-proliferative effect on cancer cells. It appears that lignans may also suppress proliferation of immune cells. No lignans of Noni fruit have been studied for their immunomodulatory activities. However, a study conducted by Gredel et al. (2008) suggested that lignans can suppress the immune system. These researchers found that the coincubation of 10 µM of matairesinol inhibited the proliferation of peripheral mononuclear cells. They also reported that matairesinol and secoisolariciresinol had an inhibitory effect on IL-4, INF-γ and TNF-α secretion by the same cells at 50 µM (Gredel et al., 2008).

**Anthraquinones**

Anthraquinones contain two fused benzene rings with an oxygen heterocycle (Figure 2.4) and are abundantly found in the roots of a variety of plants. Based on mice and rat studies, conducted by the National Toxicological Program, anthraquinones are considered to be carcinogens (http://ntp.niehs.nih.gov/files). However, these are
considered non toxic in Noni fruits, since they are present in a trace amount (Pawlus et al., 2005).

For example, under in vitro conditions 0.9 pM concentration of 2-methoxy-1,3,6 trihydroxyanthraquinone extracted from Noni fruit was required to double the activity of the phase II antioxidative enzyme, quinone reductase, as compared to 0.34 µM of the positive control L-sulforaphane. Based on these results, the concentration of Noni anthraquinone required to inhibit 50% of cell growth (ID₅₀) for this compound was 7 times higher than L-sulforaphane. These results indicate that the anthraquinones found in Noni fruit have extremely low toxic potential and may even have a beneficial effect on health (Pawlus et al., 2005).

**Fatty Acid Esters**

The fatty acid esters found in Noni fruit are primarily short chain fatty acids with a glucopyranose ring (Figure 2.5).
Akihisa et al. (2007) determined the anti-inflammatory effect of fatty acid esters isolated from Noni fruits by examining of the anti-inflammatory activity of the esters utilized mouse an animal model. They found that topical application of these esters [2-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose, 2-O-(β-D-glucopyranosyl)-1-O-hexanoyl-β-D-glucopyranose, 6-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose and 2,6-di-O(β-D-glucopyronosyl)-1-O-octanoyl-β-D-glucopyronose] inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear inflammation in mice with ID$_{50}$ values, of 0.46-0.79 mg per ear.

**Miscellaneous Phytochemicals**

In addition to these 5 classes of phytochemicals, Noni fruits contain various other phytochemicals which also have immunomodulatory activities. For example, Su et al. (2005) found that the glycosidic form of β-sitosterol palmitate showed strong anti-complementary activity against the classical pathway in an *in vitro* assay with IC$_{50}$ of 1µM. This concentration was approximately 77 times lower than the control (Yoon et al.,
In further research, Desai et al. (2009) studied the effect of this phytochemical on immune cells, reporting that a 4 µM concentration of β-sitosterol inhibited the release of IL-10 secretions from peripheral blood mononuclear cells isolated from multiple sclerosis patients compared to healthy individuals. However, it enhanced IL-10 secretion and reduced the proliferation of peripheral blood mononuclear cells isolated from healthy individuals at the same concentration compared to control.

In addition, vanillin (4-hydroxy-3-methoxybenzaldehyde) detected in the alcoholic extracts of Noni fruit inhibited carrageenan-induced paw edema of rats in a dose-dependent manner, ranging from 12.5 mg/kg to 50 mg/kg per os. This inhibitory effect was correlated with the antioxidative activity of the compound in an in vitro assay (Lee et al., 2006). Similarly, Murakami et al. (2007) demonstrated that 100-500 µM concentrations of vanillin were potent inhibitors of LPS-stimulated cyclooxygenase-2 (COX-2) expression in macrophages.
## Immunomodulatory Properties of Noni Fruit

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Immune response</th>
<th>Anti-complementary</th>
<th>Anti-inflammatory</th>
<th>Anti-oxidation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Iridoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asperuloside</td>
<td>-</td>
<td></td>
<td></td>
<td>+</td>
<td>Li et al. (2006)</td>
</tr>
<tr>
<td>Deacetylasperulosidic acid</td>
<td>-</td>
<td></td>
<td></td>
<td>+</td>
<td>Kim et al. (2005) Li et al. (2006)</td>
</tr>
<tr>
<td><strong>Flavonoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaempferol triglycosides</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Cimanga et al. (1995)</td>
</tr>
<tr>
<td>Catechin</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>Terra et al. (2007)</td>
</tr>
<tr>
<td><strong>Lignans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3’ bisdemethylpinoresinol</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Kamiya et al. (2004)</td>
</tr>
<tr>
<td>Americanol A</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>Kamiya et al. (2004)</td>
</tr>
<tr>
<td>Americanin A</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Kamiya et al. (2004)</td>
</tr>
<tr>
<td>Americanoic acid A</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Kamiya et al. (2004)</td>
</tr>
<tr>
<td><strong>Anthraquinones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methoxy-trihydroxyanthraquin</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Pawlus et. al. (2005)</td>
</tr>
</tbody>
</table>

---

Table 2.1 Noni fruit phytochemicals and their biological activities
<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty Acid Ester</strong></td>
<td></td>
<td></td>
<td>Akihisa <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B sitosterol</td>
<td>-/+</td>
<td>+</td>
<td>Yoon <em>et al.</em> (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Desai <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Vanillin</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lee <em>et al.</em> (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Murakami <em>et al.</em> (2007)</td>
</tr>
</tbody>
</table>

Note: + and – sign and indicates the stimulatory and inhibitory effects on the response or activity. +/- sign indicates both effects.
CHAPTER III

EFFECTS OF DIETARY NONI SUPPLEMENTATION ON IMMUNE RESPONSES AFTER LPS CHALLENGE IN BROILERS

Materials and Methods

Animals and Husbandry

Day-old broiler chicks were obtained from a commercial hatchery. Chicks were provided with free access to water and practical corn-soy diet during the experiment. All procedures with chickens were approved by Clemson University Institutional Animal Care and Use committee.

Experimental Design and Dietary Treatments

The study was conducted using 108 day-old commercial broiler chickens distributed at random to 18 wire-floor pens (six birds per pen). A practical corn-soy diet was supplemented with either 0, 1 or 2% (w/w) concentrations of Noni crude fruit preparation to determine the minimum dose requirement of Noni that modulates immunity (Whole Noni fruit puree, 047-058-01, Morinda Agriculture Products Inc., Utah), and the three dietary treatments were assigned at random to the 18 pens to provide replications per treatment (n=6). The diets were fed from day-old to the 21 days of age. At 21 days of age, two pairs of birds were randomly selected from each pen, and each pair treated with either 2% lipopolysaccharide (LPS) (L 7261, Sigma-Aldrich, St. Louis, MO) dissolved in normal saline or normal saline at 2mg/kg via intraperitoneal (i.p.) route. Blood and tissue samples were collected at 6 h and 24 h post LPS challenge.
Sample Collection

To assess the systemic immunoglobulin concentration and serum α1-acid glycoprotein, blood samples were collected via cardiac puncture of birds at 6 and 24 hours after LPS challenge. Serum was separated by centrifugation at 500 X g for 30 minutes and stored at -4 °C until further analysis. For detection of gut tissue immunoglobulins, approximately 1 centimeter length of the gut tissue, along with its content, was cut from right side of Meckel’s diverticulum (towards the ceca) after opening the abdominal cavity of the birds aseptically, and was stored at -80°C until further analysis. Feed consumption, body weight, liver, spleen, and bursa weights were also recorded.

Serological and Gut Tissue Analysis

Serum IgA, IgM and IgG, and intestinal IgA antibodies were estimated by a sandwich enzyme-linked immunosorbent assay (ELISA) using kit (E30-102,-103,-104, Bethyl Laboratories, Montgomery, TX). Briefly, 96-well flat bottomed microtiter plates (01-010-3855, Dynatech, Chantilly, VA) were coated with 100 µl of a 1:100 dilution of goat antichicken IgG in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated for 1 h. Plates were then washed five times with wash solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) and blocked for 30 minutes using 200 µl of blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) to avoid non-specific binding on well surface. After washing, 100 µl of diluted serum (1: 20000, 1: 10000 and 1: 5000 times for IgG, IgM, and IgA, respectively) in sample diluent (50 mM Tris, 0.14
M NaCl, 0.05% Tween-20, 1% BSA, pH 8.0) and gut tissue supernatant in phosphate buffer saline (1:200) were then added and plates were incubated for 1 h. All samples were added in duplicate. Blanks (no test sample) and serial dilutions of chicken IgG, IgM or IgA standards were also included on each plate. Samples, those absorbance values were above than upper limit of standards’ absorbances or that had more than 10% variations in absorbance between duplicates were run again. Standards were run in each plate. Following incubation, plates were washed 5 times with wash solution and subsequently, 100 µl horseradish peroxidase conjugated chicken IgG, IgM or IgA detection (diluted 1:10000 in sample diluents) antibodies were added and incubated for 1 h. After incubation, plates were washed five times with wash solution and 100 µl of substrate, ABTS [2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)], was then added and plates were incubated for 15 minutes. The absorbance was measured at 405 nm using a microplate reader (Thermomax model 1989) and calculated concentration using standard curve. All procedures were done at room temperature. The minimum detection limit of ELISA essay for IgA, IgG and IgM immunoglobulins were 15.625, 3.12, and 3.9 ng/ml, respectively. Intestinal IgA was extracted as described by Haghighi et al. (2005), (Appendix 1), and results were expressed in terms of total soluble protein measured by the bicinchoninic acid (BCA) method (232-23,-24, Thermo Scientific, Rockford, IL). Serum α1- acid glycoprotein (AGP) was measured by chicken α1- acid glycoprotein measurement kit (P0801-1, The Institute for Metabolic Ecosystem, Miyagi, Japan). Briefly, plates with chicken anti-AGP antibody in agarose were used to measure AGP concentration by radial immunodiffusion assay. Five microliter test sample was applied to individual wells in
plate. Chicken AGP standards were also applied to each plate and the plate placed in a humidified chamber at 37°C to incubate for 48 h. After incubation, outer diameter of each precipitin ring was measured and AGP concentration was calculated using the standard curve.

**Statistical Analysis**

Data collected were subjected to analysis of variance (ANOVA) using the general linear model procedure (GLM) of SAS (Version 9.1, SAS, Cary, NC). Response variables were analyzed using the CRD linear model, \( Y_{ij} = \mu + \alpha_i + e_{ij} \), where \( Y_{ij} \) = variable measured; \( \mu \) = overall mean; \( \alpha_i \) = the effect of \( i^{th} \) level of dietary treatment; \( e_{ij} \) = random error for \( j^{th} \) pen receiving \( i^{th} \) level of dietary treatment. Hartley's \( F_{\text{max}} \) test was used to examine homogeneity of variance assumption. The difference between LPS challenge and control for each pen constituted the response variable in the case of organ weights, serum and gut immunoglobulins, and \( \alpha_1 \)-acid glycoprotein.

Orthogonal polynomial contrasts were used to examine relationships between response variables and dietary levels of Noni. Hypothesis tests for linear and quadratic responses were examined using \( \alpha = 0.05 \).
Results showed that performance in terms of body weight, feed conversion ratio and livability, and organ weights were not affected by Noni ($P>0.05$; all contrasts) (Table 3.1). Dietary Noni did not affect serum IgG, IgM and IgA, and gut IgA concentrations after 6 h or 24 h post LPS challenge ($P>0.05$; all contrasts) (Table 3.2 and 3.3). A statistically significant departure from linearity was detected in serum IgM 24 hours post LPS challenge ($P<0.05$) (Table 3.3). Serum IgA 24 h post LPS challenge exhibited a trend in deviation from linearity ($P = 0.07$) (Table 3.3). Linear or non-linear (quadratic) relationship was insignificant between other response variables and levels of Noni at 6 h or 24 h ($P>0.05$) (Table 3.1, 3.2 and 3.3)
Table 3.1 Performance and relative organ weights of broiler chicks fed dietary Noni from day-old to 3 weeks of age

<table>
<thead>
<tr>
<th>Diet(^1) (%Noni)</th>
<th>Body weight (g)</th>
<th>Feed conversion (g/g)</th>
<th>Liver(^2) (g/g) 6 h</th>
<th>24 h</th>
<th>Spleen(^2) (mg/g) 6 h</th>
<th>24 h</th>
<th>Bursa(^2) (mg/g) 6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>805(^3)</td>
<td>1.28</td>
<td>0.11</td>
<td>0.26</td>
<td>0.026</td>
<td>0.020</td>
<td>-0.068</td>
<td>0.010</td>
</tr>
<tr>
<td>1</td>
<td>811</td>
<td>1.26</td>
<td>0.19</td>
<td>0.27</td>
<td>0.002</td>
<td>0.032</td>
<td>-0.049</td>
<td>-0.045</td>
</tr>
<tr>
<td>2</td>
<td>794</td>
<td>1.29</td>
<td>0.01</td>
<td>0.23</td>
<td>0.182</td>
<td>0.002</td>
<td>-0.022</td>
<td>-0.014</td>
</tr>
<tr>
<td>± SEM</td>
<td>9.1</td>
<td>0.010</td>
<td>0.10</td>
<td>0.24</td>
<td>0.009</td>
<td>0.015</td>
<td>0.043</td>
<td>0.033</td>
</tr>
<tr>
<td>Linear</td>
<td>0.52</td>
<td>0.94</td>
<td>0.60</td>
<td>0.89</td>
<td>0.75</td>
<td>0.35</td>
<td>0.33</td>
<td>0.46</td>
</tr>
<tr>
<td>Quadratic</td>
<td>0.36</td>
<td>0.38</td>
<td>0.96</td>
<td>0.35</td>
<td>0.06</td>
<td>0.89</td>
<td>0.90</td>
<td>0.64</td>
</tr>
</tbody>
</table>

\(^1\) Noni animal feed supplement (Morinda\(^\text{®}\)) added to the diet (w/w). Survival was 100% for all diets.

\(^2\) The relative organ weights are expressed as the mean difference between birds challenged with lipopolysaccharide (2 mg/kg body weight, i.p.) and controls that received saline measured at 6 and 24 hours post challenge.
Table 3.2  Immune responses of broiler chickens fed dietary Noni from day-old to 3 weeks of age 6 h post LPS challenge.

<table>
<thead>
<tr>
<th>Diet(^1) (%Noni)</th>
<th>Serum α- acid Glycoprotein(^2) ((\mu g/ml))</th>
<th>Serum Immunoglobulin(^2) (mg/ml)</th>
<th>Intestinal IgA(^2) (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>0</td>
<td>79(^3)</td>
<td>0.14</td>
<td>0.45</td>
</tr>
<tr>
<td>1</td>
<td>66</td>
<td>0.16</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>-0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>± SEM</td>
<td>31.8</td>
<td>0.24</td>
<td>0.26</td>
</tr>
</tbody>
</table>

\(^1\) Noni animal feed supplement (Morinda\(^\text{®}\)) added to the diet (w/w).

\(^2\) The response variables are expressed as the mean difference between birds challenged with lipopolysaccharide (LPS, 2 mg/ kg body weight, i.p.) and controls that received saline measured at 6 hours post challenge.

\(^3\)
Table 3.3 Immune responses of broiler chickens fed dietary Noni from day-old to 3 weeks of age 24 h post LPS challenge.

```
<table>
<thead>
<tr>
<th>Diet¹ (%Noni)</th>
<th>Serum α- acid Glycoprotein² (µg/ml)</th>
<th>Serum Immunoglobulin² (mg/ml)</th>
<th>Intestinal IgA² (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>0</td>
<td>218</td>
<td>0.30</td>
<td>-1.88</td>
</tr>
<tr>
<td>1</td>
<td>311</td>
<td>0.13</td>
<td>-0.19</td>
</tr>
<tr>
<td>2</td>
<td>347</td>
<td>0.40</td>
<td>0.58</td>
</tr>
<tr>
<td>± SEM</td>
<td>126</td>
<td>0.18</td>
<td>1.17</td>
</tr>
</tbody>
</table>

² The response variables are expressed as the mean difference between birds challenged with lipopolysaccharide (LPS, 2 mg/ kg body weight, i.p.) and controls that received saline measured at 24 hours post challenge.

¹ Noni animal feed supplement (Morinda®) added to the diet (w/w).

P value

| Linear   | 0.44 | 0.28 | 0.19 | 0.12 | 0.60 |
| Quadratic| 0.82 | 0.05 | 0.97 | 0.07 | 0.94 |
```
Discussion

In the present study, concentration of dietary Noni at 1% and 2% did not influence performance and immune responses in broiler chickens. The only significant response was a departure from linearity in serum IgM 24 hours post LPS challenge and a trend in deviation from linearity for serum IgA 24 hours post LPS challenge. The former is due to the lack of response to LPS challenge in birds fed 1% Noni and the latter due to non-responsiveness to LPS challenge in birds fed 1 or 2% Noni.

Many factors can influence the determination of humoral immune response against LPS in chickens fed dietary Noni. Genetic differences in breeds (Van der Zijpp et al., 1983; Kundu et al., 1999) and lines (Kundu et al., 1999; Hangalapura et al., 2003; Parmentier et al., 2004) of meat and layer chickens are associated with differences in natural or specific antibody (NAb) response. This finding is strengthened by the fact that at the molecular level, the distribution of Toll-like receptor-4 (TLR-4) is dependent on the genetic make-up of the birds (Dil et al., 2002). However, it is assumed that broilers are genetically similar due to selective line breeding, though, the production of NAb against different or same antigens is affected by linkage between the genes or by genetic-environmental interaction, for example, different quantitative trait loci are associated with natural antibody responses against LPS in chicken (Siwek et al., 2006). Along with genetics, significant individual variation within treatment groups for gut IgA immune response may be ascribed to variability in the location of IgA producing B-cell rich Peyer’s patches within the chicken intestine. The sampling procedure may result in samples with little or no Peyer’s patches.
The present experiment was designed to examine the effect of dietary Noni at 3 weeks of age in chickens as GALT (Gut Associated Lymphoid Tissue) is fully functional two weeks post-hatching in broilers (Shira-Bar et al., 2003). Further, Lactobacillus species in broiler chickens are present predominantly in the small intestines at two weeks of age while some obligatory anaerobes become prominent in the ceca at 25 days of age (Romach et al., 2004). However, the number of CD3+ T cells continue to increase to 4-6 weeks of age (Lillehoj et al., 1992) and the level of NAb in serum increases from 5 to 18 weeks of age in layers (Siwek et al., 2006). These factors may contribute to both individual variation and response of variables measured.

Levels of maternal antibodies transferred to the chicks are directly related to the dam’s circulating concentration of antibodies (Hamal et al., 1996), which may differ among dams due to differences in genetic make-up; also, carry-over antibodies may persist up to 25-27 days in chicks and contribute to individual variation in antibody titer (Mahmud et al., 2007). Hence, serum IgG response in the study may have been affected by maternal antibodies. In addition to these factors, the limits of detection of the ELISA assay may also be a factor that contributes to failure to detect the effect of Noni on NAb as the detection limit of ELISA essay for IgA, IgG and IgM immunoglobulins were 15.625, 3.12, and 3.9 ng/ml, respectively.

To date, the exact function of non-specific NAb in not clear, and it seems that it is less important in domesticated species of birds. Natural immunity was found to be lower in commercial layers as compared to their wild ancestor, Red Jungle Fowl (Mekchey et al., 1999). Hence, in addition to a follow-up study to examine gene expression, the effect
of dietary Noni on vaccine-induced specific immunity against a commercially important specific viral or bacterial disease is warranted.
CHAPTER IV

EFFECTS OF DIETARY NONI SUPPLEMENTATION ON GENE EXPRESSION AND IMMUNOGLOBULINS IN BROILERS

Materials and Methods

Experimental Design and Dietary Treatments

Day-old commercial broiler chicks were distributed at random in wire-floor pens. A pen of six birds was the experimental unit. A practical diet was supplemented with 0, 4 and 6% (w/w) concentrations of Noni crude fruit preparation (whole Noni fruit puree, Lot # 051-011-16, Morinda Agriculture Products Inc., Utah) and fed for 42 days. Treatments were assigned at random to 3 pens to provide 6 replications per treatment (n=6). At day 42, birds from each pen were sampled for blood and tissue collection. To avoid the loss of phytochemicals, the Noni puree was stored at -4°C and mixed in the diet on alternate days during the experiment.

Sample Collection

To assess gene expression of cytokines, chemokine and Toll-like receptors, cecal tonsils were collected from both sides of the ileocecal junction (Appendix B). Blood samples were collected from cutanea ulnaris of the birds. Serum was separated from sampled blood by centrifugation at 500 X g for 30 minutes and stored at -4°C till further analysis. For determination of gut IgA, a 6-cm portion of intact distal ileum (with contents) (approximately 5 cm cranial from the iliocecal junction) was sampled, placed in liquid nitrogen during collection and then stored at -80°C until further analysis. The
mucosal scraping along with gut content of the intestine was used to determine the concentration of immunoglobulins.

**Serum and Gut Immunoglobulins Analysis**

Serum IgM and IgG, and gut (mucosal and luminal) IgA antibodies were estimated by using ELISA assay kit (L30-102,-103,-104, Bethyl Laboratory, Montgomery, TX). Intestinal IgA was extracted from gut content and mucosal scrapping as described by Haghighi et al. (2005) (Appendix A). The absorbance results were expressed in terms of total soluble protein measured by bicinchoninic acid (BCA) method (232-23,-24, Thermo Scientific, Rockford, IL).

**RNA Isolation, Quantification and Real-Time PCR**

Total RNA was isolated from the right cecal tonsil of each sample using RNeasy protect mini kit (74124, Qiagen Inc., Valencia, CA) as described in Appendix C. RNA was quantified at 260 nm wavelength (Eppendorf, Hamburg, Germany) and samples with mRNA purity of about 1.8 (ratio E260/E280) or above 1.8 (ratio E260/E280) were used. Contaminating DNA was digested using the RNase free DNase set (79245, Qiagen Inc., Valencia, CA) during RNA extraction and resulting RNA eluted in 50 µl RNase-free water per 30 mg tissue and stored at -80 °C. Real-Time PCR (QPCR) was done using the QuantiTech™ SYBR Green one step RT-PCR Kit (204243, Qiagen Inc., Valencia, CA) and a BIO-RAD thermocycler (Model IQ5), as described in Appendix D. QuantiTect SYBR Green RT-PCR master mix containing reverse transcriptase together with the
QuantiTect RT Mix containing DNA polymerase allow both reverse transcription and amplification of transcript in a single tube. No-RNA template control and melting curve of gene specific PCR product were analyzed to cross check the primer dimer formation. No-RT control reaction was used to ensure the amplification of gene specific PCR product. The gene specific primers used for PCR are listed in appendix F.

**Semi-Quantitative RT-PCR and Gel Electrophoresis**

RT-PCR was done using ProtoScript M-MuLV Taq RT-PCR kit (E6400S, New England Biolab, Ipswich, MA) and BIO-RAD thermocycler (Model MJ mini, according to Appendix E). Two and one half microgram of total RNA was used for reverse transcription reaction, using 10 units of Moloney murine leukemia virus reverse transcriptase (RT), 2 µl of RT buffer, 2 µl of 50 µM oligo dT primer, 20 units of murine RNase inhibitor, 500 µM of dNTP in a reaction mix of 20 µl RNase free water. Reverse transcription was performed to convert mRNA to cDNA at 42 °C for 60 minutes and 80 °C for 5 minutes.

PCR reaction were performed in a volume of 50 µl containing 2.5-5 µl of cDNA from the reverse transcriptase reaction, 200 nanomoles of each primer, 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTP, 0.05 % Tween-20, 5% glycerol and 1.25 units of Taq DNA polymerase. Before the start of the first cycle, all PCR were preceded by a denaturation step of 1 minute at 94 °C and the last cycle step was followed by a final elongation step of 10 minutes at 68 °C. Thirty five cycles were used for IL-8 and TLR-7 assays and 40 cycles were applied for IL-12 assay. The number of cycles was 33.
optimized to prevent the amount of PCR product from exceeding the upward slope of a typical cycle number-PCR product. The gene specific primers used for PCR reactions were similar to Real-Time PCR primers. Each PCR cycle contained denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and elongation at 68 °C for 60 seconds. Gene specific PCR products was separated in 1.5% agarose gels (32803, USB, Cleveland, OH) using 0.02% ethidium bromide for visualization. Band intensity was examined in ultraviolet light using an illuminometer and quantified using Image J software (www.rsb.info.nih.gov).

**Real-Time Data Analysis**

The data obtained from the QPCR, was analyzed using cycle number ($C_t$ values). The cycle number at which the fluorescence crosses an arbitrary line called the threshold (i.e. when the fluorescence signal becomes detectable) is known as $C_t$ value. Following treatment with 4% or 6% dietary Noni supplement, relative fold change of mRNA for the gene of interest was determined as described by Pfaffl (2001), using equation 1. The initial RNA concentration was same for all samples and adjusted by diluting in RNase free water before performing Real-Time PCR.

\[
\text{Fold expression} = \frac{(1 + E_{\text{Target}})^{\Delta C_{t, \text{Target}}}}{(1 + E_{\text{GAPDH, Control}})^{\Delta C_{t, \text{GAPDH}}}}
\]

where,
\[ \Delta C_t, \text{Target} = (C_t, \text{Target, Control} - C_t, \text{Target, Noni treatment}) \]

and

\[ \Delta C_t, \text{GAPDH} = (C_t, \text{GAPDH, Control} - C_t, \text{GAPDH, Noni treatment}) \]

PCR efficiency of the target and reference gene was calculated by plotting the log of the RNA template dilution versus \( C_t \) value using the following equation:

\[ E = 10^{-\left(\frac{1}{s}\right)} - 1 \]  \hspace{1cm} (2)

where \( s \) represents the slope of the line plotted for log of the template dilution versus log of \( C_t \) value. Standard curves were generated using 5 fold serial dilution of total cellular RNA from broiler chickens. Total cellular RNA used ranged from 200 ng to 12.5 ng. The efficiency of standard curves were listed in Table 4.1

Table 4.1: Description of standard curves used in data analysis.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Efficiency</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>0.93</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.10</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.08</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.90</td>
<td>0.98</td>
</tr>
<tr>
<td>IL-12</td>
<td>1.23</td>
<td>0.94</td>
</tr>
<tr>
<td>TLR-3</td>
<td>0.94</td>
<td>0.98</td>
</tr>
<tr>
<td>TLR-4</td>
<td>1.24</td>
<td>0.99</td>
</tr>
<tr>
<td>TLR-5</td>
<td>1.69</td>
<td>0.90</td>
</tr>
<tr>
<td>TLR-7</td>
<td>1.12</td>
<td>0.93</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

ANOVA was performed using the general linear model (GLM) procedure of JMP statistical software (SAS Institute Inc., Cary, NC, USA), and hypothesis testing was
conducted using $\alpha = 0.05$. Response variables were analyzed using the following linear model:

$$Y_{ij} = \mu + \alpha_i + e_{ij},$$

where $Y_{ij} =$ variable measured; $\mu =$ overall mean; $\alpha_i =$ the effect of $i^{th}$ level of dietary treatment; $e_{ij} =$ random error associated with experimental unit. Normalized values of response variables were taken for statistical analysis. Normalization was done by dividing the Ct value of gene of interest to that of Ct value of internal control (GAPDH) of the sample.
Results

The objective of this experiment was to investigate the effects of dietary Noni crude fruit extract on target gene expression in broiler chickens. Genes included cytokines, chemokine, and Toll-like receptors in cecal tonsil, and immunoglobulin concentrations. Gel electrophoresis of PCR product showed the modulatory effects of Noni on IL-8, IL-12 and TLR-7 gene expression (Figures 4.1). Further analysis by Real-time PCR revealed that 4% Noni treatment increased (1.9 fold) TLR-3 gene expression. However did not show any effect on the expression of other genes (Figure 4.2). Noni at 6% inclusion increased the expression of IL-8 (3 fold), IL-12 (4 fold) and TLR-4 (2.6 fold), and decreased gene expression of IL-6 (0.6 fold) and TLR-7 (0.3 fold) (Figure 4.3). TLR-5 gene expression was increased 1.7 and 2.3 fold by 4% and 6% Noni, respectively (Figures 4.2 and 4.3). Neither concentrations of Noni affected IL-1 gene expression (Figures 4.2 and 4.3). The values of coefficient of determination (R²) of standard curve for TLR-7, TLR-5 and IL-12 genes were 0.93, 0.90 and 0.94, respectively (Table 4.1) which were less as than R² values obtained for other measured genes (0.98-0.99) (Table 4.1). Dietary Noni supplementation did not have significant effects on the concentration of serum IgM and IgG or intestinal IgA (Table 4.2)
Table 4.2 Immune responses of broiler chickens fed dietary Noni from day-old to 6 weeks of age

<table>
<thead>
<tr>
<th>Diet&lt;sup&gt;1&lt;/sup&gt; (%Noni)</th>
<th>Serum Immunoglobulin&lt;sup&gt;2&lt;/sup&gt; (mg/ml)</th>
<th>Gut Immunoglobulin&lt;sup&gt;2&lt;/sup&gt; (% Total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>0</td>
<td>0.30</td>
<td>1.32 ± 0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>1.47</td>
</tr>
<tr>
<td>6</td>
<td>0.36</td>
<td>1.42</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.05</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Noni animal feed supplement provided by Morinda<sup>®</sup>, was supplemented to the diet as w/w basis and fed from day-old to 6 weeks of age.

<sup>2</sup> Each pooled mean based on 5 samples except serum IgG in control group where pooled mean is based on 6 samples.
Figure 4.1 Effect of 6% dietary Noni supplement on gene expression in caecal tonsil of broilers fed to 6 weeks of age starting from day-old chicks using semi quantitative RT-PCR. Gel electrophoresis was performed (Panel A). The band densities of gene specific PCR products were quantified using Image J software (http://rsb.info.nih.gov/ij/) and expressed as a percentage of their respective internal control GAPDH (Panel B).
Figure 4.2 Effect of 4% dietary Noni supplement on gene expression in caecal tonsil of broilers fed to 6 weeks of age using Real-Time PCR.

P values are presented above the bar and $P < 0.05$ indicates the significant difference from the control group. Data are presented as mean of 5 samples ($n=5$).

Gene expression of treatment group is expressed relative to control group according to Pfaffl (2001) method.
Figure 4.3 Effect of 6% dietary Noni supplement on gene expression in caecal tonsil of broilers fed to 6 weeks of age using Real-Time PCR.

P values are presented above the bar and $P < 0.05$ indicates the significant difference from the control group. Data are presented as mean of 5 samples (n=5)

Gene expression of treatment group is expressed relative to control group according to Pfaffl (2001) method.
Discussion

The observed increased TLR-3, TLR-4, IL-8 and IL-12 gene expression and decreased IL-6 and TLR-7 gene expression in gut associated caecal tonsil in chickens fed dietary Noni indicated that Noni crude fruit extracts have immunomodulatory properties. The immunomodulatory property of Noni fruit is supported by some in vitro studies (Hirazumi et al., 1996, 1999; Furusawa et al., 2003; Li et al., 2008; Zhang et al., 2009; Brooks et al., 2009). For example, Noni fruit polysaccharides increased IL-1 and TNF-α secretion from human peripheral mononuclear cells in a dose dependent manner and fermented Noni juice pretreated dendritic cells increased immunoglobulin secretion from B cells ((Hirazumi et al., 1996, Zhang et al., 2009)

Noni fruit is rich in phytochemicals and polysaccharides (Cimanga et al., 1995, Sang et al., 2003; Kamiya et al., 2004; Pawlus et al., 2005; Lin et al., 2007; Akisha et al., 2007). Polysaccharides from Noni fruit have been reported to have immunomodulatory properties in vitro (Hirazumi et al., 1996, 1999; Furusawa et al., 2003). Also, some phytochemicals present in Noni fruit were shown to have immunomodulatory activity by modulating NF-kB signal transduction pathway in a dose dependent manner (Desai et al., 2009; Murakami et al., 2007).

In the present study, 4% dietary Noni supplement only increased the expression of TLR-3. This response may be due to the effects of phytochemicals on the TLR-3 signal transduction pathway, which uses interferon regulatory factor-3 (IRF-3) transcription factor to express antiviral products, in contrast to other TLRs that signal via the NF-kB transcription factor (Akira et al., 2004). This is supported by the fact that some
phytochemicals like quercetin which is present in the Noni fruit, stimulate the production of interferon-γ (IFN-γ) an antiviral product (Park et al., 2009). IFN-γ then acts in an autocrine manner and provides positive feedback for expression of TLR-3 via the JAK STAT pathway (Tanabe et al., 2003; Tohyama et al., 2005). However, at higher concentrations of phytochemicals, these might inhibit the expression of TLR-3 due to excessive production of IFN-γ which provides a negative feedback for TLR-3 gene expression (Dai et al., 2006). IFN-γ inhibition is due to production of SOCS (suppressors of cytokine signaling) which inhibits the signaling of IFN-γ via the JAK STAT pathway.

The flavonoid, luteolin, also present in Noni fruit, suppresses the expression of interferon regulatory factor-3 at least at higher concentrations (50 µM). This might be another mechanism by which Noni suppresses gene expression of TLR-3 (Lee et al., 2009). In the present study, the increased TLR-3 gene expression did not show any effect on measured cytokines and chemokine gene expression. This lack of response may be explained by the fact that neither TLR-4 nor TLR-5 were increased, and the increased then TLRs modulate the expression of cytokines (Schaefer et al., 2004).

Noni at 6% increased 2.6 and 2.3 fold gene expression of Toll-like receptor-4 and -5, respectively but decreased the TLR-7 gene expression (Figure 4.4). These TLRs all share the common NF-kB pathways for gene expression. The increased expression of IL-8 and IL-12 suggest that Noni polysaccharides bind to the TLR-4 at cell surface, since plant polysaccharides cannot enter cells. Also, Han et al. (2002, 2003) shown that polysaccharides bind with TLR-2 and -4 on the surface of immune cells and stimulate production of various cytokines and chemokines by the NF-kB pathway. This is further
supported by the findings that several polysaccharides isolated from plants have been shown to have immunostimulatory activities (Kim et al., 1996; Han et al., 1998). The activation of TLR-4 leads to its increased expression by both transcriptional and posttranslational mechanisms (Muzio et al., 2000; Yan et al., 2006). Stimulation of TLR-4 gene expression enhances expression of various proinflammatory cytokines and chemokine, including IL-12 and IL-8 in human mast cells and in broilers heterophils (Ferdous et al., 2008; Kirshenbaum et al., 2008). The results of this study agree with those findings. Our observed increased TLR-5 gene expression might be possibly due to the enhanced levels of cytokines.

Reduced IL-6 gene expression might be one contributing factor for reduced TLR-7 gene expression, since previous research has shown that IL-6 cytokine selectively increased the TLR-7 gene expression in humans (Zarember et al., 2002). Decreased IL-6 expression might be associated with unaltered IL-1 gene expression, as IL-6 gene expression is mainly induced by IL-1 and TNF-α (Benveniste et al., 1990; Miyazawa et al., 1998). Interestingly, both IL-1 and TNF-α were decreased in a graded manner when dietary Noni treatment increased from 4 to 6%. Also, the location of TLR-7 on internal cellular endosomes might limit its stimulation by Noni polysaccharides, as they can not enter through the cell. In contrast, a number of phytochemicals that are present in Noni can easily enter the cell. These might inhibit the basal expression level of TLR-7 and IL-6. At the molecular level, the basal expression of TLR-7 and IL-6 is negatively regulated by SOCS, phosphatases, and some other inhibitory enzymes, any of which might be stimulated by Noni phytochemicals. Interestingly, in contrast to mammals, TLR-7 in
chicken is not able to induce type I interferon in chicken and seems less important in viral infection (Philbin et al., 2005).

In conclusion, the selectively increased TLR-3, TLR-4 and TLR-5 indicate that Noni fruit induces antiviral and antibacterial responses in chickens which is further strengthened by increased expression of IL-12 and IL-8 (Philbin et al., 2005). At the same time, decreased expression of TLR-7 and IL-6 indicated anti-inflammatory properties of Noni. Further investigations will be necessary to explore the active immunomodulatory ingredients of Noni and its effects during viral and bacterial infections.

Noni at 4% and 6% could increase the concentration of serum IgG and IgM immunoglobulins. However, our lack of observing a significant effect might be due to the contribution of natural antibodies (NAb) on the total measured IgG or IgM. The NAb level against different or same antigens is affected by the linkage between the gene-environment interactions, and different genetic origins of breeds, strains, and lines of chickens (Van der Zijpp et. al., 1983; Kundu et al., 1999; Parmentier et al., 2004, Hangalapura et al., 2003, Siwek et al., 2006). However it is assumed that broilers are genetically similar due to selective line breeding but polygenic inheritance may be associated to natural antibody response against antigen in chicken (Siwek et al., 2006) and thus the interaction between more than two genes lead to the differences in natural antibody concentration among broilers.

Further investigation is necessary to examine the effect of Noni using genetically uniform lines, or strains with reduced genetic variation with increased number of sample
size in order to extend the observation of apparent antiviral/antibacterial properties by Noni after vaccination.
APPENDICES
Appendix A

Extraction of IgA Antibody from Chicken Gut Tissue

Materials required:
1. Phosphate buffer saline tablet (Sigma-Aldrich, P4417) 0.01 M phosphate buffer, pH 7.4.
2. Trypsin inhibitor (Sigma-Aldrich, T-9003) @100 µg/ml in PBS.
3. Glass beads of 1 mm diameter (Biospec Product Inc. 11079110).
4. Centrifuge (Beckman- Coulter Microcentifuge 22R).

Procedure:
1. Dissolve one tablet of phosphate buffered saline in 200 ml of distilled water to obtain 0.01 M phosphate buffer saline. Trypsin inhibitor is added to PBS @ 100 µg/ml.
2. Weigh whole frozen intestinal tissue and its content as quick as possible to avoid thawing.
3. Put Intestine tissue along with its content into 1.5 ml plastic vial. Add 1 ml of trypsin inhibited phosphate buffer saline into the vial and bring to final volume with glass beads. Tightly cap the vial and make sure there are no bubbles present.
4. Homogenize the tissues in bead beater at 4800 rpm for 3 minutes. The homogenate gets warm after 3 minutes. To minimize heating during the extraction treatment prechill beads and buffer and stop for 60 seconds after each 60 seconds blending. Immediately place homogenates on ice.
5. Decant homogenate or remove by pipetting after the beads settle to the bottom and transfer into the 1.5 ml high “g” micro centrifuge tubes.
6. Centrifuge homogenate at 14000 X g for 10 minutes at 4 ºC.
7. Remove the supernatant from the pellet and discard the pellet.
8. Store the supernatant at -80 ºC until further analysis.

Appendix B

Collection of Tissue for RNA Extraction

Introduction:
RNases are present in cells and even the surrounding environment. They do not require a cofactor to cleave the RNA in a non-specific or specific manner. However, tissue RNA can be degraded by other non-specific mechanism, if samples are not stored properly. To prevent degradation of RNA, samples must be preserved either by storage at very low temperature (-80°C), in stabilizing reagents (RNAlater, Qiagen Inc.), or using a combination of both.

Materials required
1. Sterilized 5 ml RNA free collection vials
2. Sterilized scissors
3. Sterilized forceps
4. Concentrated EtOH (95%)
5. Test tube stand
6. Permanent markers
7. RNAlater RNA stabilization reagent
8. Insulator box with ice
9. Gloves
10. Sterilized syringe/droppers
11. Sterilized normal saline solution
12. Beaker 500 ml capacity (autoclaved and treated with 0.1% diethylpyrocarbonate water)
13. Analytical balance

Things to be considered before collection of tissue
- RNAlater RNA stabilization reagent may form a precipitate during storage below room temperature (15-25°C). Before using the RNAlater RNA stabilization reagent, redissolve the precipitate by heating to 37°C with agitation.
- Only fresh unfrozen tissue can be stabilized using RNAlater RNA stabilization reagent.
- Amount of reagent needed per sample: Around 180-250 mg samples need approximately 2.5 ml of RNAlater RNA stabilization reagent.
- Storage container should be wide enough so that the reagent covers the entire tissue.
- The procedures for tissue harvesting and RNA stabilization should be carried out as quickly as possible.
- Sample must be cut into slices less than 0.5 cm thick and immediately put into the stabilization reagent (If the slices are thicker than 0.5 cm the reagent will
diffuse too slowly into the interior of the sample and RNA degradation will occur).

**Protocol for collection of tissue:**
1. Sterilize work area and instruments with 70% EtOH.
2. Treat glass vials with 0.1% DEPC to eliminate RNAase. Pipette 10 x (estimated tissue mass) RNA later RNA stabilization reagent into each vial and cap the vials.
3. Collect tissue, take a suitable mass of sample, chop and place in vial with stabilization reagent. Make sure the level of stabilization reagent is well above the tissue.
4. Place vials overnight at 2-8°C, then store at -20°C.

**Precaution:**
1. Preparation of normal saline (0.89% W/V) in sterile water (water must be autoclaved and 0.1% diethylpyrocarbonate treated).
2. Collection vials should be RNase free. Use glass vials and make them RNase free by 0.1% diethylpyrocarbonate treatment.
3. If transporting tissue samples in RNA later reagent, ensure that the tissue always remains submerged in the reagent. Either keep the tubes upright during transport or fill the tubes completely with RNA later reagent.
4. Stored tissue can be thawed at room temperature and frozen again for up to 20 freeze-thaw cycles without affecting RNA quality or yield.

**Source:**
Appendix C

Extraction of Total RNA

Introduction:
Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini spin column. The total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 50 μl water.

Materials required:
Qiagen RNeasy® protect mini kit with required buffers (RLT, RPE, RW1 and RDD)
2 ml RNase free polypropylene tubes
Disposable blades
Pestle and mortar
Micropipettes / RNase free tips
Liquid nitrogen
Ethanol (96-100% and 70%)
Centrifuge
QIAshredder columns
RNase-free DNase set
Glass vials
Thermocol box with ice (for maintain the temperature during the DNase preparation)

Things to do before starting experiment:
- Buffer RLT is prepared by adding 10 microliters of mercaptoethanol per 1 ml of RLT. It can be stored at room temperature for up to 1 month.
- Before using for the first time, add 4 volumes of ethanol (96-100%) in concentrated buffer RPE as indicated to obtain a working solution.
- 70% ethanol is prepared using RNase free water.
- DNase digest buffer is prepared by adding lyophilized DNase in buffer RDD.

Protocol:

1. Remove RNA later stabilized tissue from the reagent using forceps and remove any crystals that may have formed.

2. Weigh the piece to be used (20 mg) directly into microcentrifuge tube. If necessary, cut tissue on a clean sterile surface with a sterile scalpel.

3. Immediately place the weighed tissue in liquid nitrogen and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free,
liquid nitrogen-cooled, 2 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

4. Add 600 µl buffer RLT, vortex it and pipette the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 minutes at full speed at room temperature.

5. Discard the QIA shredder column.

6. Centrifuge the lysate for 3 minutes at full speed. Carefully remove the supernatant by pipetting and transfer it to a new microcentrifuge tube. Use only this supernatant (lysate) in subsequent steps.

7. Add 1 volume of 70% ethanol to the cleared lysate and mix immediately by pipetting. Do not centrifuge. Immediately proceed to step 8 (precipitate may be visible after addition of ethanol. This does not affect the procedure)

8. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied by Qiagen). Centrifuge for 15 s at 10,000 rpm. Discard the flow through. If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

9. Add 350 µl of buffer RW1 to the RNeasy spin column and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow through. Retain the collection tube for reuse in step 11.

10. Add 80 µl DNase I directly to the RNeasy spin column membrane, and place on the bench top (for 15 minutes). Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the wall or the O-ring of the spin column.

11. Add 350 µl Buffer RW1 to the RNeasy spin column and centrifuge for 15 s at more than or equal to 10,000 rpm. Discard the flow-through. Transfer RNeasy column to a new 2 ml collection tube.

12. Add 500 µl of buffer RPE to the RNeasy spin column and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow through.

13. Add another 500 µl of buffer RPE to the RNeasy spin column and centrifuge for 2 minutes at 10,000 rpm to wash the spin column membrane.

14. Place column in a new 2 ml collection tube and discard the flow through after centrifugation for 1 minute.
15. Transfer column to a new 1.5 ml collection tube (supplied in kit). Add 50 μl of RNase free water directly onto the column and keep it for 1 minute at room temperature. Close tube and centrifuge at 10,000 rpm for 1 minute to elute.

Source:
Appendix D

Conversion of mRNA to cDNA and Amplification of cDNA

Introduction:
Use of the QuantiTect SYBR Green RT-PCR master mix containing reverse transcriptase together with the QuantiTect RT Mix containing DNA polymerase allow both reverse transcription and amplification of transcript in a single tube.

Material required:
QuantiTect SYBR Green RT-PCR Kit
Primers
96 well plates for the reactions
Plate covers
Ice box with ice
RNase free water
Pipettes: 2 µl, 20 µl, 200 µl and 1000 µl
RNase free tips for measurements
Real time cycler
Extracted RNA
RNase free water
Gloves
Sterile 1.5 ml of centrifuge tubes

Things to do before starting:

- Adjust the RNA concentration in RNase free water in such a manner that all RNA concentration must be equal to 10 ng per 10 µl of volume.
- Calculate the amount of master mix needed for the reactions according to Table 1. Multiply the individual volumes with number of samples.
- Make the PCR plate template and set temperature and time program in the computer software attached with thermocycler day before planning to run samples. Check the direction of putting PCR plate in the Real time cycler.
- Thawing should be performed at ice temperature.
- Dilute the final concentration of primer to 0.5 µM in RNase free water.

Protocol:
1. Thaw 2x QuantiTect SYBR Green RT-PCR Master Mix (if stored at -20°C), template RNA, primers, and RNase- free water at ice. Mix the individual solutions, and place them on ice. The QuantiTect RT Mix should be taken from -20°C immediately before use, always kept on ice, and returned to storage at -20°C immediately after use.
Table 1: Different volumes of 25 μl reaction mixture.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x QuantiTect SYBR Green RT-PCR Master Mix</td>
<td>12.5 μl</td>
<td></td>
</tr>
<tr>
<td>Primer forward</td>
<td>0.5 μl</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>Primer backward</td>
<td>0.5 μl</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>QuantiTect RT Mix</td>
<td>0.25 μl</td>
<td>0.25 μl / reaction</td>
</tr>
<tr>
<td>RNase- free water</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>Template RNA ( added at step 5)</td>
<td>10 μl</td>
<td>1ng / μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 μl</td>
<td></td>
</tr>
</tbody>
</table>

2. Vortex the Primers and master mix, and mix the RT solution by gentle tapping. Do not centrifuge any of them.

3. Each mixed component is then dispensed in 96 well PCR plate as following order and final volume adjusted to 25 μl with RNase free water:
   A. RNase free water  B. master mix  C. RT (reverse transcriptase)

4. Add template RNA to the individual PCR wells containing reaction mixture. This may be done prior to step 3.

5. Seal the plate with sealer

6. Program the Real time cycler according to the program outlined:
   One cycle of
   - 50°C for 30 minutes (Reverse transcription)
   - 95°C for 15 minutes (DNA polymerase is activated, reverse transcriptases are deactivated and template cDNA is denatured)

   Followed by 40 cycles of
   - 94°C for 15 seconds (Denaturation)
   - 57°C for 20 seconds (anneling)
   - 72°C for 20 seconds (extension)

7. Data acquisition should be performed during the extension step.

8. Keep samples on ice until the Real time cycler is programmed. Place the PCR tubes or plates in the thermal cycler and start the cycling program.

Source:
Qiagen kit manual for quantitative, real time, one step RT-PCR, November, 2000
Appendix E

Conversion of mRNA to cDNA and Amplification of cDNA for Gel Electrophoresis

Introduction:
Reverse transcriptase is used to convert the mRNA to cDNA using oligo dT primers. The resulting cDNA are amplified using specific primers for the specific gene expression in separate tube.

Material required:
Protoscript ® II RT-PCR Kit
Primers
Polyacrylamide gel 1.5%
Camera
Illuminometer
PCR tubes
100 bp Ladder Aluminium foil
Ice box
RNase free water (DEPC treated water)
Dye (Ethidium bromide)
6X loading dye
Thermocycler

First Strand cDNA Synthesis:
Proper precautions should be used to avoid ribonuclease contamination. This includes the use of autoclaved tubes, baked glassware, ultra-pure solutions, sterile pipette tips and latex gloves during manipulations

1. Thaw system components and place on ice. The 10X RT Buffer can be warmed briefly at 42°C and vortexed to dissolve any precipitate. (Note: It is important to set up a -RT control reaction (no reverse transcriptase) to insure there is no DNA contamination).

2. Make the RNA/primer/dNTP mix by combining the following components in a PCR tube:
   
   \[
   \begin{align*}
   & \text{Total RNA} \quad : \quad 1-10 \, \mu l \ (2 \, \mu g) \\
   & \text{Primer dT}_{23} \, VN \quad : \quad 2 \, \mu l \\
   & \text{dNTP Mix} \quad : \quad 4 \, \mu l \\
   & \text{Nuclease-free water} \quad : \quad \text{variable} \\
   & \text{Total volume} \quad : \quad 16 \, \mu l
   \end{align*}
   \]

3. Heat for 5 minutes at 70°C. Spin briefly and promptly chill on ice.

4. Add the following components to the 16 µl RNA/primer/dNTP solution and mix well
by pipetting up and down:

- RT control

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume 1</th>
<th>Volume 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>2 μl</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.5 μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1 μl</td>
<td>–</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>0.5 μl</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20 μl</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

4. Incubate the 20 μl cDNA synthesis reaction mixture at 42°C for one hour.

5. Inactivate the enzyme at 80°C for 5 minutes.

6. Bring the reaction volume to 50 μl with water. The cDNA product should be stored at –20°C.

**PCR Amplification:**

1. Mix the following in a PCR tube on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume 1</th>
<th>Volume 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Taq</em> 2X Master Mix</td>
<td>25 μl (mix well by inverting before use)</td>
<td></td>
</tr>
<tr>
<td>Forward Primer (10 μM)</td>
<td>1 μl (final concentration 200 nm)</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer (10 μM)</td>
<td>1 μl (final concentration 200 nm)</td>
<td></td>
</tr>
<tr>
<td>Diluted cDNA</td>
<td>2.5 or variables</td>
<td></td>
</tr>
<tr>
<td>H₂O variable</td>
<td>20.5 or variables</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

2. Mix gently and allowed the reaction to proceed under the following condition:

<table>
<thead>
<tr>
<th>Reaction Step</th>
<th>Temp</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>35-40 Cycles</td>
<td>94°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>57°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>1 minute per kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>68°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

3. Optimize cycle numbers to prevent the amount of PCR product from exceeding the upward slope of a typical cycle number-PCR product. Apply 35 cycles for IL-8 and TLR-7 assays and 40 cycles for IL-12 assay.

**Quantification:**

1. Separate gene specific PCR products in 1.5% agarose gels using 0.02% ethidium bromide for visualization.

2. Quantify band intensity in ultraviolet light using illuminometer and Image J software (www.rsb.info.nih.gov). Target gene is expressed relative to the internal control (GAPDH).
Source:
## Appendix F

### Primer Sequences

<table>
<thead>
<tr>
<th>Genes</th>
<th>Direction</th>
<th>*Primer sequences (5‘-3’)</th>
<th>Accession no.</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>GCTCTACATGTCGTGTGTGATGAG</td>
<td>Y15006</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>TGTCGATGTCGCCGCATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>ATGTGCAAGAAGTTCACCCTGTGC</td>
<td>AJ309540</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>TCCAGGATGGCTGAAAGCGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward</td>
<td>TTTCAAGCTGTCTGCGTCCCAGTA</td>
<td>DQ393272</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>GCCCGCAGCCTTCCCCCATCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>Forward</td>
<td>TGTCTCACCTGCTATTTGCCCTAC</td>
<td>NM_213571</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>CAGCCTCTGCTCTCTAAAGTTCCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-3</td>
<td>Forward</td>
<td>ACCCGGATTGCAAGTCAGTCACTCAGTAC</td>
<td>NM_001011691</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>TGGTCTTCAAGGTGATCAGTCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-4</td>
<td>Forward</td>
<td>GTTGCATTGCTGCAGGAGTTTCCT</td>
<td>AY064697</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>GCAGGATCCACAGCTCATCTCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-5</td>
<td>Forward</td>
<td>TGAACCTCGAGACACACTCAGGT</td>
<td>NM_001024586</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>CGCAGGATGCTGATTCTCTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-7</td>
<td>Forward</td>
<td>ATCTGACGTTGTTGATCCTTG</td>
<td>FJ915600</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>AGCGCTGTGATTGCTCTTACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>ATGCCATCACAGCCACACAAGAAGA</td>
<td>NM_204305</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Backward</td>
<td>GCACACGGGAAGCCATCCAGTAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Primers were designed using PrimerQuest software (www.idtdna.com, integrated DNA Technology, Iowa)


http://ec.europa.eu/food/fs/sc/scf/out151_en.pdf


Linus Pauling Institute of Micronutrient Research for Optimum Health (http://lpi.oregonstate.edu/infocenter)


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www.rsb.info.nih.gov/ij/


