Why you and your family should use Divine Noni?
Reason 1

Noni has powerful immunostimulant activity, which helps to improve immunity against deadly viral infections.

Scientific Reference :

Immunostimulant activity of the extracts and bioactives of the fruits of *Morinda citrifolia*

Smita Nayak, and Sushma Mengi
Department of Pharmacology, C.U. Shah College of Pharmacy, S.N.D.T. Women's University, Santacruz (West), Mumbai. Maharashtra, India

Abstract

The present investigation evaluates the immunostimulant effects of the extracts and the bioactive fractions, namely, polysaccharides, anthraquinones, and alkaloids, of the fruits of *Morinda citrifolia* Linn. (Rubiaceae). The extracts and the fractions were evaluated for their effect on *in vitro* phagocytosis of *Candida albicans* spores by neutrophils obtained from pooled human blood. The maximum activity was demonstrated by the hydroalcoholic extract (79.25% at 1.0 mg mL−1, $p < 0.05$) and the polysaccharide fraction (60.0% at 0.2 mg mL−1, $p < 0.05$) obtained from the fruits of the plant. The hydroalcoholic extract and the polysaccharide fraction were evaluated for their effect on serum interleukin-6 (IL-6) levels in rats sensitized by intraperitoneal injection of Bacillus Calmette Guerin (BCG) vaccine I.P. The hydroalcoholic extract (910.82 pg mL−1 at 200 mg kg−1, $p < 0.05$) and the polysaccharide fraction (556.82 pg mL−1 at 40 mg kg−1, $p < 0.05$) significantly increased serum IL-6 levels in the antigenically challenged rats as compared to the vehicle control group (75.04 pg mL−1) and standard reference herbal drug, *Withania somnifera* (L. Dunal) (Solanaceae) (396.38 pg mL−1). The studies indicated the potential of *Morinda citrifolia* as an immunostimulant herbal drug.

Keywords: *Immunostimulant;* Morinda citrifolia*; phagocytic activity; neutrophils; cell-mediated immune response; serum IL-6 levels*

Introduction

The fruits of *Morinda citrifolia* Linn. (Rubiaceae) have been traditionally used for over 2000 years by ancient Polynesians as well as Indians in the treatment of a wide array of diseases and are reported to have antibacterial, antiviral, antifungal, antitumor, anthelmintic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Bhandari, 1985; DerMarderasian, 1999; Iwu, 1993; Kirtikar &

Basu, 1975; Wang & Su, 2001; Wang et al., 2002). *Morinda citrifolia*, commonly known as "noni", is one of the herbal medicines most commonly used by ancient Hawaiians (Abbott & Shimazu, 1985). A review of the traditional uses of the plant in Hawaii revealed that fruits of *Morinda citrifolia* were being used as poultices on wounds, boils, pimples; as a purgative; as a blood purifier; in the treatment of tuberculosis, diabetes, heart ailments, and high blood pressure (Elkins, 1998). In addition, a survey by McClatchey (2002) revealed a host of claims made by producers of commercial noni products in Hawaii including treatment of gastric ulcer, indigestion, diabetes and high blood pressure, prevention of AIDS, prevention of Epstein-Barr virus activation, and as an anti-cancer agent. Phytochemically, the plant is found to be rich in anthraquinones, glycosides, flavones glycosides, carboxylic acids, iridoids, coumarins, essential oils, and sterols (Farine et al., 1996; Kamiya et al., 2005; Krishnamoorthy & Reddy, 1970; Nandhasri et al., 2005; Singh & Tiwari, 1976; Tiwari & Singh, 1977; Wang et al., 2000; Takashima et al., 2007). Hirazumi et al. (1994) evaluated the anti-cancer activity of the plant in mice injected with Lewis lung carcinoma (LLC) cells. The ethanol-precipitable fraction of noni fruit juice (noni-ppt) was employed and the mice were observed to survive for a longer time as compared to untreated mice. However, in the *in vitro* studies conducted by Hirazumi et al. (1996), noni-ppt failed to exert any cytotoxic effects in LLC cultures. Investigation of the underlying mechanism ultimately revealed that suppression of tumor growth was due to activation of the immune system. This was confirmed in another study by Wang et al. (2002) wherein rats administered noni juice orally demonstrated an increase in thymus weight. This led to the conclusion that the anti-cancer activity of the plant was due to enhanced immunostimulation. Thus, although immunostimulation is the underlying mechanism for the anti-cancer activity of the plant, scientific studies to elucidate the immunostimulant mechanism are few (Palu et al., 2008). Hence, an attempt has been made to investigate the immune-enhancing claims made in Polynesian and Indian literature as well as Hawaiian folklore. The present study explores the immunostimulant potential of *Morinda citrifolia* extracts and fractions by evaluating their effects on enhanced phagocytosis of *Candida* spores by neutrophils. Additionally, serum IL-6 levels in rats antigenically challenged with BCG vaccine have been estimated.

An immune response requires the coordinated actions of both innate immunity and the more powerful and flexible acquired immunity. Innate immunity mediated by neutrophils is the first line of defense against invasion by pathogens and, therefore, the role of *Morinda citrifolia* in increasing the phagocytic activity of neutrophils has been evaluated. In addition, T-cells principally involved in cell-mediated immunity have a pivotal role to play in the generation of an immune response. Reports of the cytokine IL-6 being released by activated T-lymphocytes prompted us to estimate IL-6 levels of rats antigenically challenged with BCG vaccine (Cruse & Lewis, 1999; Leung et al., 2004; Zhang & Huang, 2005).

Materials and methods

Plant material and extraction procedure

Dried fruits of *Morinda citrifolia* were obtained from M/s Anju Phytochemicals (Bangalore, India) and authenticated by Vinayak Naik, at Nicholas Piramal Research Center (Mumbai, India). A voucher specimen (no. 4944) was deposited in the herbarium of the institute.

The dried fruits were pulverized in a hammer mill and the fruit powder passing through a 40-mesh sieve was used for the extraction procedure.

Aqueous extract

The fruit powder (50 g) was refluxed with 200 mL of distilled water for 12 h. The extract was filtered, cooled and evaporated to dryness under reduced pressure in a rotary evaporator. The yield of the aqueous extract was 23.7% w/w of the dried powder.

Hydroalcoholic extract

Dried *Morinda citrifolia* fruit powder (50 g) was continuously extracted in a Soxhlet 70-75°C) with 300 mL of 50% v/v ethanol till extraction was complete. The extract was filtered, cooled, and evaporated to dryness under reduced pressure in a rotary evaporator. The yield of the 50% v/v ethanol extract was 25.4% w/w of the dried powder.

Polysaccharides

Morinda citrifolia fruit powder (50 g) was defatted using methanol (300 mL) and refluxed with distilled water (300 mL) for 12 h. The aqueous extract was filtered and concentrated to 100 mL. The polysaccharides were precipitated by pouring the concentrate into 500 mL of acetone. The crude polysaccharide (1 g) was then dissolved in 50 mL of water. To this solution 25 mL of 12% w/v aqueous trichloroacetic acid was added and the protein impurities were filtered off. The residue was poured into 500 mL acetone to precipitate pure polysaccharides.

The precipitate was filtered off under vacuum and air-dried (Chintalwar et al., 1999). From the dried powder, 6.24% w/w of the purified polysaccharide was obtained.

Anthraquinones

Morinda citrifolia fruit powder (50 g) was refluxed with a mixture of 100 mL methanol and 150 mL water for 3 h. This extract was then acidified by addition of 2 mL of concentrated hydrochloric acid and 5 mL of 5% methanolic solution of ferric chloride and refluxed for 6 h. The anthraquinones were extracted in chloroform by shaking the extract with an equal volume of chloroform in a separating funnel. The chloroform layer was separated and evaporated to dryness (Brain & Turner, 1975). The yield of anthraquinones was 0.47% w/w of the fruit powder.

Alkaloids

The powdered dried material (25 g) was refluxed with 100 mL of a mixture of ethanol-chloroform (1:3) containing 2% v/v of strong solution of ammonia for 6 h. The resultant mixture was extracted three times with 20 mL portions of 2 N hydrochloric acid. The acid extracts were combined and the pH adjusted to 8.0 by dropwise addition of strong ammonia solution. The resultant alkaloids were extracted into chloroform. The chloroform layer was washed with 20 mL of water and evaporated to dryness (Vishin & Gupta, 1996). The yield of alkaloids was 0.12% w/w of the fruit powder.

Chemicals

Saboraud dextrose broth and minimum essential medium (MEM) was obtained from HiMedia Laboratories (Mumbai, India). Dextran solution (20% w/w) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bacillus Calmette Guerin (BCG) vaccine I.P. manufactured by Serum Institute of India (Pune, India) was used as antigen for immunizing the rats. Enzyme-linked immunosorbent assay (ELISA) rat kits were procured from Bender Med Systems (Vienna, Austria). All other chemicals and reagents were of pure analytical grade obtained from local suppliers.

Animals

Wistar rats of either sex, weighing 180–200 g were used. They were kept in standard environmental conditions and fed with rodent diet and water ad libitum. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of C.U. Shah College of Pharmacy (Mumbai, India).

Effect of Morinda citrifolia *extracts and fractions on in vitro phagocytic activity of neutrophils*

About 10 mL of pooled human blood was collected in a sterile test tube containing two drops of heparin. Neutrophils were isolated from the blood by a density gradient separation technique using 10 mL dextran and transferred to MEM.

Neutrophil count was adjusted to 1 × 106 cells mL−1 using a Neubauer chamber. *Candida* spores were subcultured in Sabouraud dextrose broth and incubated for 18 h. The broth was then centrifuged and the spores transferred to MEM. Spore count was adjusted to 1 × 106 cells mL−1 using a Neubauer chamber (Akbay et al., 2003; Capsoni et al., 1988).

The neutrophils (125 µL, count adjusted to 1 × 106 cells mL−1) were incubated with 125 µL of *Candida albicans* (count adjusted to 1 × 106 cells mL−1) and different concentrations of the extracts/fractions at 37°C for 1 h in a carbon dioxide (CO2) incubator. The system was centrifuged and the supernatant was discarded. The lower neutrophil layer was smeared on a clean, dry glass slide. The smear was air-dried and stained with Giemsa stain.

The slides were mounted under a microscope and 100 cells observed for phagocytic activity.

Effect of Morinda citrifolia *extracts and fractions on serum IL-6 levels in rats sensitized with BCG vaccine I.P.*

BCG vaccine I.P. is a live freeze-dried vaccine derived from attenuated strain of *Mycobacterium bovis*. Each vial containing between 10 × 105 and 330 × 105 Colony Forming Units (CFU) was reconstituted with 4 mL of pyrogen-free sterile saline and 0.1 mL of this reconstituted solution was used as the antigen for eliciting a cellmediated immune response. The rats were dosed for five days. All the rats were immunized on the third day of dosing by injecting 0.1 mL of the antigen intraperitoneally and challenged on the tenth day by injecting the same amount intraperitoneally. Serum IL-6 levels were measured on the eleventh day (24 h after challenge) using ELISA rat kits. A standard curve was generated by using standard IL-6 provided in the kit. IL-6 levels in the rat serum were estimated using the standard curve and expressed as cytokine concentrations (pg mL−1).

Statistical analysis

Values are expressed as mean values ± SEM. The statistical significance of differences between the mean values was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test and Bon Ferroni test. A p value of < 0.05 was considered to be significant.

Results

Effect of Morinda citrifolia *extracts and fractions on in vitro phagocytic activity of neutrophils*

Studies carried out in our laboratory indicated that the extracts and fractions of the dried fruits of *Morinda citrifolia* were capable of enhancing the *in vitro* phagocytosis of *Candida* spores by neutrophils (Table 1). The neutrophils demonstrated a significant increase in phagocytic activity when incubated with *Candida* spores in the presence of the aqueous and the hydroalcoholic extracts at concentrations ranging from 0.25 to 1.0 mg mL−1. The aqueous extract (0.25 mg mL−1) demonstrated a significant ($p < 0.05$) increase in phagocytic activity (51.0 ± 3.1%) when compared to vehicle control (32.5 ± 0.56%). Increasing the concentration further to 0.5 and 1.0 mg mL−1 resulted in a dose-dependent increase in phagocytosis. These responses were statistically similar to the responses elicited by the standard test material *Withania somnifera* (L. Dunal) (Solanaceae) (64.75 ± 2.57%).

The hydroalcoholic extract elicited significant phagocytic responses ($p < 0.05$) as compared to the vehicle control at all the concentrations tested, i.e., 0.25, 0.5, and 1 mg mL−1. These responses were found to be dose-dependent. The response elicited by 1 mg mL−1 of the hydroalcoholic extract (79.25 ± 3.75%) was significantly greater ($p < 0.05$) in magnitude than that elicited by the standard herbal drug, *Withania somnifera* (64.75 ± 2.57%).

Table 1. Effect of extracts and fractions of dried fruits of *Morinda citrifolia* on phagocytic activity of neutrophils.

Treatment	**Concentration (mg mL−1)**	**Phagocytosis (%)**
Vehicle control	-	32.5 ± 0.56
Aqueous extract	0.25	51.0 ± 3.1*
	0.50	64.75 ± 3.45*
	1.0	70.00 ± 2.57*
Hydroalcoholic extract	0.25	57.75 ± 1.42*
	0.50	71.0 ± 2.74*
	1.0	79.25 ± 3.75*
Polysaccharide	0.05	31.11 ± 4.19
	0.1	44.56 ± 1.02*
	0.2	60.0 ± 2.16*
Anthraquinone	0.005	39.5 ± 1.79
	0.01	42.5 ± 1.94*
	0.02	52.4 ± 1.86*
Alkaloid	0.001	42.7 ± 2.80*
	0.002	42.7 ± 3.41*
	0.004	55.5 ± 1.56*
Standard herbal drug		

(*Withania somnifera*)	0.25	64.75 ± 2.57*

All values are expressed as mean ± SE, n = 6. *Statistically significant difference at p <0.05 as compared to vehicle control as seen by applying the Dunnett's test followed by Bon Ferroni's test.

The polysaccharide fraction of the dried fruits of *Morinda citrifolia* failed to enhance the activity of the neutrophils at the concentration of 0.05 mg mL−1. However, with an increase in the concentration to 0.1 mg mL−1, a significant increase (p < 0.05) in phagocytic activity (44.56 ± 1.02%), as compared to the vehicle control (32.5 ± 0.56%), was seen. Increasing the concentration to 0.2 mg mL−1 led to a further increase in response (60.0 ± 2.16%). The responses elicited by 0.1 and 0.2 mg mL−1 of the polysaccharides were statistically similar to the responses elicited by *Withania somnifera* (64.75 ± 2.57%).

The anthraquinone fraction at the concentration of 0.005 mg mL−1 did not stimulate phagocytosis of *Candida* spores by neutrophils. A further increase in concentration to 0.01 mg mL−1 and 0.02 mg mL−1 led to statistically significant responses (p < 0.05), as compared to vehicle control. Statistical analysis of the data revealed the response elicited by 0.02 mg mL−1 of the anthraquinone fraction was statistically similar to the response of the standard *Withania somnifera*. The alkaloidal fraction of the dried fruits of *Morinda citrifolia* revealed immunostimulant activity at all the concentrations tested. These responses were statistically similar to the responses elicited by *Withania somnifera*, but were not dose-dependent.

Comparison of the activity of the extracts and the bioactives of the fruits of *Morinda citrifolia* revealed that the hydroalcoholic extract demonstrated maximum increase in phagocytic activity of the neutrophils to the extent of 79.25%.

Effect of Morinda citrifolia *extracts and fractions on serum IL-6 levels in rats sensitized with BCG vaccine*

The hydroalcoholic extract and the polysaccharide fraction were investigated for their role in stimulating the *in vivo* release of IL-6 by activated T-lymphocytes in rats antigenically challenged with BCG (Table 2). Rats sensitized with BCG vaccine, when treated with the hydroalcoholic extract (50 mg kg−1), demonstrated a significant increase (p < 0.05) in serum IL-6 levels (409.00 ± 10.41 pg mL−1) as compared to the untreated rats (75.04 ± 11.42 pg mL−1). Increasing the dose to 100 mg kg−1 led to a slight increase in serum IL-6 levels to 520.38 ± 46.39 pg mL−1. However, this increase was not statistically significant. When the dose was increased to 400 mg kg−1, a significant increase (p < 0.05) in serum IL-6 levels (910.82 ± 87.48 pg mL−1)was observed This response was significantly greater (p

<0.05) than serum IL-6 levels elicited in rats treated with *Withania somnifera* (396.38 ± 8.10 pg mL−1). Pretreatment of rats with 10 mg kg−1 of the polysaccharide fraction failed to elevate serum IL-6 levels (113.49 ± 42.79 pg mL−1). Increasing the dose to 20 mg kg−1 led to a significant increase ($p < 0.05$) in serum IL-6 levels (342.33 ± 18.56 pg mL−1), as compared to the vehicle control group (75.04 ± 11.42 pg mL−1). This response was statistically similar to that elicited by the standard, *Withania somnifera* (396.38 ± 8.10 pg mL−1). An increase in dose to 40 mg kg−1 elevated IL-6 levels further to 556.82 ± 49.98 pg mL−1. This response was significantly greater than IL-6 levels elicited by rats treated with the standard, *Withania somnifera.*

Evaluation of the data revealed that oral dosing of the antigenically challenged rats with 200 mg kg−1 of the hydroalcoholic extract led to maximum induction of serum IL-6 levels, to the extent of 910.82 ± 87.48 pg mL−1.

Table 2. Effect of hydroalcoholic extract and polysaccharide fraction of dried fruits of *Morinda citrifolia* on rat serum IL-6 levels.

Treatment	**Dose (mg kg−1) (pg mL−1)**	**Serum IL-6 levels**
Vehicle control	-	75.04 ± 11.42
Hydroalcoholic extract	50	409.00 ± 10.41*
	100	520.38 ± 46.39*
	200	910.82 ± 87.49*
Polysaccharide fraction	10	113.49 ± 42.79
	20	342.33 ± 18.56*
	40	556.82 ± 49.98*
Standard herbal drug (*Withania somnifera*)	100	396.38 ± 8.10*

All values are expressed as mean ± SE, n = 6. *Statistically significant difference at $p < 0.05$ as compared to vehicle control as seen by applying the Dunnett's test followed by Bon Ferroni's test.

Discussion

In the present study, we have carried out a preliminary screening of the extracts and the fractions of the dried fruits of *Morinda citrifolia* for their immunostimulant potential employing the *in vitro* phagocytic activity of neutrophils against *Candida albicans* spores. A study has also been designed to evaluate the potential of the plant in modulating cytokine IL-6 levels which play a pivotal role in adaptive immune

defense system. Being an important marker of cell-mediated immunity, IL-6 was estimated in order to confirm the role of the plant as an immunostimulant.

The role of neutrophils as phagocytes against infectious agents has been amply cited in the literature (Dewitt & Hallet, 2002; Gisbergen et al., 2005; Kobayashi et al., 2005, Xing & Remicki, 2004). Upon stimulation, they release chemoattractants such as cathepsins and defensins that stimulate T-cell accumulation at the sites of inflammation (Taub et al., 1996). They also trigger the activation of T-cells leading to the release of cytokines (Bhattacharjee & Akira, 2005; Pedraza-Sanchez et al., 2006). Thus, they are important cellular components of the immune system and act as the first line of defense against antigens such as whole microorganisms, insoluble particles, injured and dead cells, and cellular debris. Several workers have employed neutrophils to confirm the immunostimulant activity of plants (Capsoni et al., 1988; Daswani & Yegnanarayan, 2002, Thatte et al., 1992). We, in our study, have demonstrated the immunostimulant activity of the extracts and fractions of dried fruits of *Morinda citrifolia* by evaluating their role in enhancing the phagocytosis of *Candida* spores by neutrophils.

Our studies revealed that both the aqueous and the hydroalcoholic extracts elicited an increase in the phagocytic activity of the neutrophils. Scrutiny of the data revealed that the hydroalcoholic extract demonstrated an activity that was significantly greater ($p < 0.05$) than that elicited by the standard herbal drug, *Withania somnifera*. When individually tested, each of the three fractions, namely, polysaccharide, anthraquinone and alkaloid, were found to possess immunostimulant potential. However, the magnitude of the effect was less than that seen with the hydroalcoholic extract. The hydroalcoholic extract and the polysaccharide fraction were selected for evaluation of their potential to activate T-cells *in vivo* by estimating serum IL-6 levels in rats antigenically challenged with BCG vaccine. BCG vaccine was selected as the antigen to induce a cell-mediated immune response since neutrophils enhance lymphocyte recruitment to the sites of mycobacterial infection (Fulton et al., 2002). BCG induced strong Th1 type responses leading to IL-2 production (Ichim, 2005; Lagranderie et al., 1996). IL-2 acts as a critical autocrine growth factor for T-cells and augments the release of IL-6 by activated T-cells (Walsh, 1998; Zhang & Huang, 2005). A correlation between IL-2 levels and IL-6 levels has been reported by a group of workers (Chen et al., 2006). IL-6 acts on B-cells as a differentiation factor and leads to terminal differentiation of B-cells into immunoglobulin secreting cells (Kishimoto, 1992). IL-6 also acts on resting T-cells as an activation factor (Goodrich & McGee, 1999). Thus, IL-6 has pleiotropic effects and is an important marker of cell-mediated immune response.

Several groups of workers have employed the release of IL-6 by isolated T-lymphocytes as a parameter to prove the immunostimulant activity of several polysaccharides of plant and animal origin (Leung et al., 2004; Nair et al.,

2004; Zhang & Huang, 2005). This prompted us to measure serum IL-6 levels in rats antigenically challenged with BCG as a marker of cell-mediated immunity.

In our study, both the hydroalcoholic extract and the polysaccharide fraction of *Morinda citrifolia* produced a significant elevation in serum IL-6 levels in rats. The observed elevation of IL-6 levels indicates that the underlying mechanism could be *in vivo* activation of T-cells by *Morinda citrifolia*. The response elicited by 200 mg kg−1 of the hydroalcoholic extract was significantly greater ($p < 0.05$) than the response elicited by the vehicle control group, the polysaccharide fraction and the standard herbal drug, *Withania somnifera*.

Our studies clearly indicated that *Morinda citrifolia* potentiated the host defense mechanism by stimulating neutrophils, an important component of the first line of defense and mediated the release of IL-6, an important marker of cell-mediated immunity. Of the two extracts (hydroalcoholic and aqueous) and the three bioactive fractions of *Morinda citrifolia* (polysaccharide, anthraquinone, and alkaloid) tested, the hydroalcoholic extract significantly ($p < 0.05$) enhanced the phagocytic activity of neutrophils to the extent of 79.25% and elevated rat serum IL-6 levels to 910.82 pg mL−1. The plausible explanation could be that the multiplicity of compounds found in the unfractionated extract may provide a greater immune stimulatory potential (Burger et al., 1997). In the light of the above findings, it is evident that *Morinda citrifolia* is an immunostimulant of great promise.

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