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Noni (*Morinda citrifolia* L.) fruit juice reverses age-related decline in neural-immune interactions in the spleens of old F344 rats

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ABSTRACT

Ethnopharmacological relevance: Various parts of the tropical plant, *Morinda citrifolia* L. (Noni), have been widely used in traditional medicine in South and Southeast Asia for several centuries. The therapeutic effects of the noni are believed to be mediated through several phytochemicals such as anthraquinones, iridoid, fatty acid glycosides, alcohols, etc.

Aim of the study: The aim of the study is to investigate the effects of *Morinda citrifolia* fruit juice (noni fruit juice; NFJ) on neural-immune interactions through the involvement of intracellular signaling pathways both *in vitro* and *in vivo* in the splenic lymphocytes of young and old male F344 rats.

Material and methods: In the *in vitro* study, splenocytes from young and old F344 rats were isolated and treated with 0.0001–1% concentrations of NFJ for a period of 24 h, while in the *in vivo* study, old F344 rats were orally administered (5 ml/kg body weight) with NFJ (5%, 10% and 20%) twice daily for 60 days. After the treatment period, concanavalin A (Con A)-induced lymphocyte proliferation, cytokines (IL-2, IFN-γ, IL-6, and TNF-α) production, expression of tyrosine hydroxylase (*p*-TH), nerve growth factor (NGF), m-TOR, IκB-α, p-NF-κB (p50 and p65), p-ERK, p-Akt, p-CREB and lipid peroxidation, protein carbonyl formation, nitric oxide (NO) production were examined in the splenocytes.

Results: *In vitro* NFJ incubation of splenic lymphocytes increased Con A-induced lymphocyte proliferation, IL-2 and IFN-γ production, and expression of p-ERK, p-Akt, and p-CREB in young and old rats. *In vivo* treatment of old rats with NFJ increased lymphoproliferation, IL-2 and IFN-γ production, the expression of p-TH, NGF, and NO production, and suppressed IL-6 production, lipid peroxidation, protein carbonyl formation, and the expression of IκB-α and p-NF-κB (p50) in the splenocytes.

Conclusion: Taken together, these results suggest that *Morinda citrifolia* fruit juice enhanced neural-immune interactions and cell survival pathways while inhibiting inflammatory processes that may be useful in the treatment of age-associated diseases.

1. Introduction

An increase in the incidence of cancer, infectious and autoimmune diseases, and neurodegenerative diseases is commonly observed with advancing age due to deficient functioning of neuroendocrine system and immune system (Fulop et al., 2014b; Meites, 1990). Numerous studies have provided a functional basis for the link between the three homeostatic systems, namely nervous, endocrine, and immune systems in health and disease (Bellinger et al., 2008; Nance and Sanders, 2007; ThyagaRajan and Priyanka, 2012). The interactions between these three systems are facilitated via neurotransmitters, hormones, and cytokines and in the periphery, it is through the release of norepinephrine (NE) from noradrenergic (NA) nerve fibers present in the primary and secondary lymphoid organs which interacts with adrenergic receptors on the cells of the immune system (Bellinger et al., 2008; Elenkov et al., 2000; Nance and Sanders, 2007; ThyagaRajan and Priyanka, 2012). However with advancing age, there is loss of NA innervation in the secondary lymphoid organs, spleen and lymph nodes, which is accompanied by reduced populations of naïve T cells that may impair the neural-immune interactions facilitating development of disease (Aspinall and Andrew, 2000; Bellinger et al., 1992; Felten et al., 1987; ThyagaRajan et al., 2011). The pattern and density of tyrosine hydroxylase-positive nerve fibers in the periarteriolar lymphatic sheath in the splenic white pulp rich in T lymphocytes is altered and diminished in older rats which is accompanied by an age-related decline in T cell functions such as lymphocyte proliferation and

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Th (T helper)–1 cytokine production mediated through defective T cell signal transduction pathways (Bellinger et al., 1992; Fulop et al., 2014a; ThyagaRajan et al., 2011). Immunosenescence is accompanied by a low level inflammatory process referred to as inflamm-aging characterized by enhanced proinflammatory status and altered epigenetic mechanisms (Fulop et al., 2016).

One of the plausible reasons for the loss of sympathetic NA nerve fibers and development of immunosenescence may be the generation of free radicals, reactive oxygen/nitrogen species (ROS and RNS), that is regulated by the inflammatory process (Fulop et al., 2014b). Moreover, these processes may also be facilitated by a deficiency in the production of target-derived growth factors, such as nerve growth factor (NGF) and brain derived growth factor (BDNF) that are crucial to the survival and maintenance of neurons. An age-related reduction in NGF content observed in specific brain regions, in human peripheral blood mononuclear cells, and spleen may explain the degeneration of neurons with advancing age (Kale et al., 2014; Nishizuka et al., 1991; Nitta et al., 1993; Priyanka et al., 2013b). NGF receptors that are present on the immune cells such as mast cells, eosinophils, and T and B lymphocytes may be involved in the plasticity of sympathetic neurons and thus, facilitating neural-immune interactions (Otten et al., 1989; Santambrogio et al., 1994; Thorpe et al., 1987). At the molecular level, impaired signal transduction pathways involving ERK, CREB, Akt, and several other transcription factors may contribute to immunosuppression along with pro-inflammatory-like environment in aging (Fallah et al., 2011; Li et al., 2002; Pratap et al., 2016; Priyanka et al., 2013c). Inflammatory process in aging is facilitated by high levels of pro-inflammatory cytokines such as TNF-α that activate several pathways including PI3K, Akt, mechanistic target of rapamycin (mTOR) to regulate the expression of NF-κB through IkB-α phosphorylation and its degradation resulting in the translocation of NF-κB complex into the nucleus (Hoffman and Baltimore, 2006; Sun et al., 1993). Inhibition of mTOR is known to ameliorate age-associated diseases possibly through improved T cell signaling (Johnson et al., 2013; Mannick et al., 2014).

A variety of plants and their extracts have been used to alter the aging process and therefore, the development and incidence of age-related diseases. *Morinda citrifolia* (Noni) is one such plant whose roots, barks, and fruits have been used for the treatment of diseases owing to their immune-enhancing properties (Palu et al., 2008; Potterat and Hamburger, 2007). Recently, we have reported that immunomodulatory effects of *Morinda citrifolia* fruit juice *in vitro* involves specific signaling pathways mediated through distinct phytochemicals in the splenocytes from young and old F344 rats (Pratap et al., 2016). Although the beneficial effects of Noni have been reported through few studies (Potterat and Hamburger, 2007; Wang et al., 2002), the mechanism (s) of action (s) from the perspective of the neural-immune network have not been investigated. Therefore, the present study was conducted to examine the effects of Noni (*Morinda citrifolia*) fruit juice (NFJ) both *in vitro* and *in vivo* on the molecular mechanisms involved in age-related modulation of neural-immune functions in splenocytes from young and old male F344 rats. Following the *in vitro* incubation and *in vivo* treatment of NFJ, splenic lymphocytes were isolated for immunological assays [Con A-induced lymphocyte proliferation and cytokines (IL-2, IFN-γ, IL-6 and TNF-α) production], ELISA (p-ERK, p-CREB, and p-Akt expression), Western blot [(p-TH, NGF, p-mTOR, IκB-α, and p-NF-κB (p50 and p65) expression)], and colorimetric assay for nitric oxide (NO) production.

2. Materials and methods

2.1. Animals

Young and old male F344 rats were obtained from the National Institute of Nutrition, Hyderabad, India and kept in the University animal house for a period of one week. After the period of acclimatization, animals were subjected to either *in vitro* or *in vivo* experiments with noni fruit juice (NFJ). *Morinda citrifolia* fruit juice (NFJ; Divine Noni Gold®, Batch No. 44141) was obtained from World Noni Research Foundation, Chennai. *Morinda citrifolia* fruit juice was made from the Noni fruit grown in National Research Centre for Noni, Salavakkam, Chennai, Tamil Nadu, India and was processed and manufactured by Noni BioTech Pvt, Ltd., Kancheepuram, Tamil Nadu, India. *Morinda citrifolia* L. is an authenticated plant as per the International Plant Names Index (www.theplantlist.com). Phytochemical analysis of Divine Noni® demonstrated the presence of high levels of alkaloids, phenols, terpenoids, and reducing sugars, moderate amounts of saponin, and trace amounts of flavanoids, tannins, and proteins. In addition, iridoid was present at a higher level in the noni fruit juice (Dr. N. Mathivanan, personal communication, unpublished data). Single dose and repeated dose toxicity (14 days) in rats and mice (19 ml/kg body weight) did not cause any mortality nor were there any significant alterations in physical, physiological, hematological, biochemical, and histopathological parameters (Dr. P. Selvam, personal communication, unpublished data). Animals were sacrificed by decapitation at 08:00 h and spleens were aseptically isolated for further processing and use in various assays. All animal experiments were conducted in accordance with the principles and procedures outlined and approved by the University's Institutional Animal Ethics Committee.

2.1.1. Experiment 1: in vitro incubation of NFJ with splenic lymphocytes

After a week of acclimatization in the animal house, young (3–4 months; n=5) and old (18–21 months; n=7) male F344 rats were sacrificed, spleens were isolated, and kept in sterile tubes containing HBSS for *in vitro* experiment with NFJ. *Morinda citrifolia* fruit juice (NFJ; Divine Noni Gold®) was obtained from World Noni Research Foundation, Chennai and serially diluted in substituted RPMI medium from 1% to 0.1%, 0.01%, 0.001%, and finally, to 0.0001% and a dose response curve was generated.

2.1.2. Experiment 2: in vivo administration of NFJ to old rats

After a week of acclimatization, the old (16–17 months) male F344 rats were randomly distributed into a control group that received saline (Old+Saline; n=13), and three Noni-treated groups (Old+NFJ 5%, n=11; Old+NFJ 10%, n=11; and Old+NFJ 20%, n=10). A separate group of 3-month-old male rats (Young; n=8) served as control animals. *Morinda citrifolia* fruit juice (NFJ; Divine Noni Gold®) was obtained from World Noni Research Foundation, Chennai and administered (5 ml/kg body weight) to old F344 male rats twice a day by oral gavage for a period of 60 days. At the end of the treatment period, animals were sacrificed and spleens were isolated aseptically for further processing and use in various assays.

2.2. Isolation of lymphocytes

Lymphocytes in the spleen were isolated as described previously (ThyagaRajan et al., 1998; Priyanka et al., 2013c). A block of spleen was placed in HBSS containing sodium bicarbonate and 4-(2-hydroxyethyl)–1-perazineethanesulfonic acid (HEPES). Spleen tissues were homogenized using a stomacher and the cell suspension was passed through a nylon mesh to remove large aggregates followed by repeated washes with HBSS. Cells were then layered on Histopaque1077 (Sigma-Aldrich, St. Louis, MO) and lymphocytes were removed from the Histopaque/HBSS interface and washed thrice with HBSS. After the final wash, the cells (2×10^5 cells/ml) were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, 5×10^5 M 2-mercaptoethanol, 100 U/ml penicillin streptomycin solution, 24 mM sodium bicarbonate and 10 mM HEPES buffer for *in vitro* culture at 37 °C in a humidified incubator with 5% CO_2 in 24 and 96 well-plates for cytokine and proliferation assays, respectively.

2.3. Lymphocyte proliferation assay

Lymphocytes (2×10^5 cells/well) isolated from spleen were incubated in the presence of 5% CO_2 at 37 °C for 72 h and proliferation was assessed by MTT assay. For the *in vitro* study, lymphocytes were co-incubated with or without 0.1% of NFJ along with 0, 0.5, 1.25, and 5 μg/ml of Concanavalin A (Con A). Only the 0.1% concentration of NFJ was used based on the results from our preliminary studies conducted in the laboratory. In the *in vivo* study, splenocytes of young and old rats were co-cultured with the above-mentioned doses of Con A. A 96 well plate containing 100 μl sample having 2×10^5 cells/ml was treated with MTT reagent, incubated for 3 h and read at 620 nm after completely solubilising it in isopropanol containing 37% HCl. Triplicate wells were used for each experimental condition for establishing the dose response curve for the Noni fruit juice.

2.4. Con A-induced cytokine production

Lymphocytes (2×10^5 cells/well) were co-cultured with 1.25 μg/ml of Con A in 24-well plates and kept at 37 °C in an incubator with 5% CO_2 for 24 h. After 24 h, the supernatants and pellets were collected and stored at −80 °C for cytokine assays (IL-2, IFN-γ, IL-6, and TNF-α) using ELISA kits (R & D Systems, Minneapolis, Minn., USA), and molecular markers, respectively.

2.5. Intracellular signaling pathway markers

Lysis of the cell pellets co-incubated with Con A (1.25 μg/ml) was done using 5 mM Tris buffer with phenylmethylsulfonyl fluoride (PMSF) and orthovanadate (Pratap et al., 2015; Priyanka et al., 2013b). Homogenization of the spleen tissues was done using 5 mM Tris buffer with phenylmethylsulfonyl fluoride (PMSF) and orthovanadate. After homogenization, samples were centrifuged and supernatants were used for analyzed of molecular markers. The samples were analyzed for p-ERK/Total ERK, p-CREB/Total CREB, p-Akt/Total Akt using ELISA (R & D Systems, Minneapolis, Minn., USA) as described below. The primary antibodies (0.2 μg/ml) was coated on Corning COSTAR ELISA plates and incubated at 37 °C overnight. On the following day, the plates were washed (PBS with 0.05% Tween), blocked (PBS with 5% BSA Fraction V) for 2 h, washed again, and incubated with 100 μl of the cell lysates or appropriate standards for 2 h at 37 °C. After 2 h, the plates were washed, treated with appropriate detection antibody (0.2 μg/ml) for 1 h, followed by treatment with HRP-tagged secondary antibody and TMB (3,3′,5,5′-tetramethylbenzidine; Sigma, St. Louis, MO, USA) substrate for quantification of the TMB-HRP colorimetric reaction. The reaction was stopped 20 min after substrate addition using 2 N sulphuric acid and the plates were read at 450 nm using microplate reader. The amount Akt and p-Akt in samples was determined by extrapolation to the standard curve (2000 pg/ml to 20 pg/ml).

2.6. Western blot analysis

Western blot analysis has been described previously (Priyanka et al., 2013a). Briefly, spleen tissues were homogenized in lysis buffer, centrifuged at 1500 rpm for 10 min and the supernatants obtained were used for Western blotting. Protein concentration was estimated using Folin and Ciocalteu's phenol reagent (Sigma, St. Louis, MO). Thirty (30) μg of total protein from fraction was electrophoresed on 10% SDS-polyacrylamide gels and blotted on 0.2 μm nitrocellulose membranes (Sigma, St. Louis, MO). Primary antibody [p-TH (Ser 40; 1:750), NGF (M-20; 1:750); p-mTOR(Ser; 2448), p-IκB-α (B-9) (Ser;32, 1:750), p-NF-κB (p50; Sc-114; 1:750), p-NF-κB (p65; Ser 536; 1:750), and β-Actin (C4; 1:3000)] (Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-anti rabbit IgG and anti-mouse IgG (1:10000) (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibody were used and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System (Sigma, St. Louis, MO). Experiments were performed for at least 7 samples per group in duplicates and signal intensity was quantified using densitometry with ImageJ 1.45 software (NIH) in terms of relative intensity of the blots with reference to control.

2.7. Lipid peroxidation

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

2.8. Protein carbonyl formation

Oxidative modification of proteins was measured using 2, 4-Dinitrophenylhydrazine (DNPH) based assay Reznick and Packer (1994). Homogenized spleen tissue was treated with 10 mM DNPH solution and incubated at room temperature for 1 h. Following incubation an equal volume of 20% trichloroacetic acid solution was added, incubated for 5 min and centrifuged at 10,000g for 10 min. The pellet thus obtained was re-suspended in 1 ml of ethanol/ethyl acetate solution (1:1, v/v) and centrifuged at 10,000*g* for 10 min at 4 °C. The ethanol/ethyl acetate wash was repeated twice and the pellet thus obtained was resuspended in 500 μl of 6 M guanidine hydrochloride solution (pH 2.3) and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatants obtained were loaded in microplates and the absorbance was measured at 370 nm. Protein carbonyl content was expressed as nanomoles/ml.

2.9. Nitric oxide production

The total NO production was measured using the Greiss reagent system (Fiddler, 1977) in the Con A-induced cell culture supernatants. The sample was incubated with equal volume of 0.1% N-1-napthylethylenediamine dihydrochloride in water and 1% sulphanilamide in 5% phosphoric acid for 10 min. The purple color obtained was read at 520 nm in a spectrophotometer. Standard curve was obtained using serial dilutions of 0.1 M sodium nitrite in water. The results were expressed in terms of μg equivalents of sodium nitrite/ml.

2.10. Statistical analysis

Differences between groups were measured using ANOVA by SPSS software package. Parameters that attained significance ($p < 0.05$) with ANOVA were further analyzed by Fisher's least significant difference test. All values are expressed as mean ± S.E.M.

3. Results

3.1. In vivo administration of NFJ on the body weight of old F344 rats

An age-associated decline ($p < 0.05$) in body weight was observed in old rats irrespective of whether treated with saline or NFJ (Fig. 1). The absence of alterations in the body weights of NFJ-treated old rats demonstrated that its treatment with 3 doses was safe to the animals.

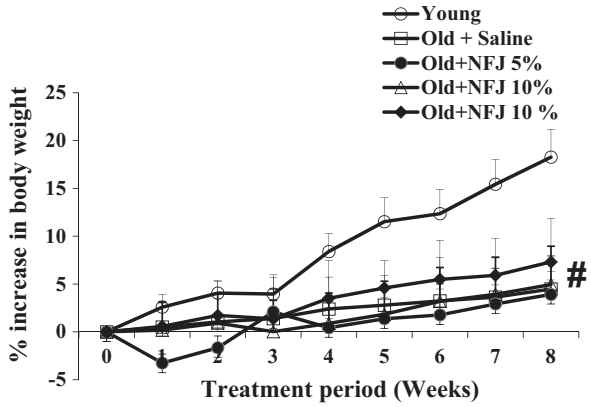

Fig. 1. Body weight of old F344 rats after 60 days of oral administration of *Morinda citrifolia* fruit juice (NFJ). Age-associated decrease in the body weight of old rats were not reversed by treatment with NFJ. #p < 0.05 compared to Young.

3.2. NFJ enhanced Con A-induced proliferation of splenocytes

Con A-induced proliferation of lymphocytes from the spleen demonstrated a significant ($p < 0.05$) decline in old rats compared to young rats (Fig. 2A and B). *In vitro* incubation of splenocytes from young rats with 0.1% NFJ significantly ($p < 0.05$) increased Con A-induced proliferative capacity of T lymphocytes while there was no significant difference in the proliferative capacity of lymphocytes from old rats co-cultured with NFJ (Fig. 2A). However, splenocytes isolated from old F344 rats after 60 days of oral administration of NFJ (10% and 20%) significantly ($p < 0.05$) enhanced con A-induced lymphocyte proliferation compared to saline-treated old rats (Fig. 2B).

3.3. NFJ augmented IL-2 and IFN-γ production in vitro and in vivo but inhibited IL-6 only in vivo

Age-related decline in IL-2 and IFN-γ production by the splenocytes was observed both *in vitro* and *in vivo* (Fig. 3A–D). *In vitro* incubation with NFJ significantly ($p < 0.05$) increased IL-2 production by splenic lymphocytes from young (0.1% and 1%) and old (0.1%) rats (Fig. 3A). Similarly, long-term oral administration of NFJ to old rats reversed the age-related decline in IL-2 production by splenocytes (Fig. 3B). *In vitro* co-culturing of splenocytes with NFJ did not alter IFN-γ production in young rats while all the concentrations of NFJ enhanced its production in old rats (Fig. 3C). *In vivo* treatment of old rats with NFJ also resulted in preventing age-associated decline in IFN-γ production and increased its production by splenocytes (Fig. 3D).

In vitro incubation of splenocytes with NFJ (0.0001%, 0.001%, 0.01%, and 1%) significantly ($p < 0.05$) increased the TNF-α production in splenocytes isolated from young and old F344 rats while *in vivo* treatment with NFJ did not have any significant effect in TNF-α production (Fig. 3E and F). Similar to TNF-α production, co-incubation with NFJ increased IL-6 production by splenocytes from young (0.1% and 1%) and old (1%) rats (Fig. 3G). In the *in vivo* study, there was an age-related increase in IL-6 production by the splenocytes in saline-treated old rats (Fig. 3H). In contrast, treatment of old rats with all 3 doses of NFJ for 60 days decreased IL-6 production compared to old saline-treated rats.

3.4. In vivo administration of NFJ on the expression of p-TH, NGF, p-mTOR, p-IκB-α, and p-NF-κB (p50 and p65)

Treatment with NFJ (10%) significantly ($p < 0.05$) increased the expression of p-TH in the spleen of old rats compared to saline-treated old rats (Fig. 4A and B). The age-related decline ($p < 0.05$) in the splenic NGF expression was reversed by treatment with NFJ (10% and 20%). Treatment with NFJ did not have any significant effect in the

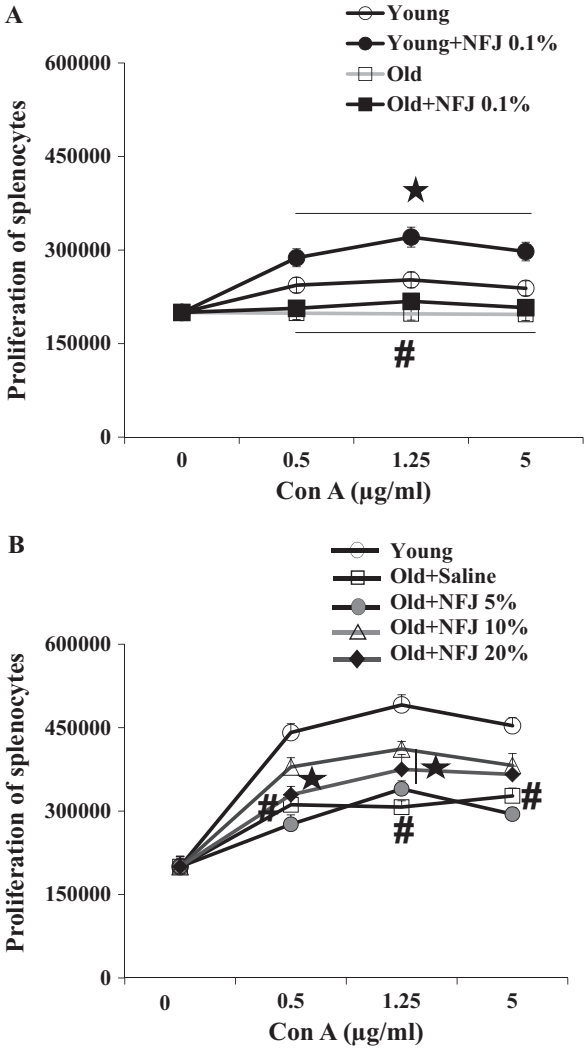

Fig. 2. Concanavalin (Con A)-induced proliferation of splenocytes after *in vitro* (A) and *in vivo* (B) treatment with *Morinda citrifolia* (Noni) fruit juice (NFJ) in young and old F344 rats. Splenocytes isolated from young and old F344 rats were incubated with 0, 0.5, 1.25, and 5 µg/ml of Con A for 72 h and proliferation was measured by MTT assay. NFJ reversed age-associated decline in splenocyte proliferation both *in vivo* and *in vitro*. # p < 0.05 compared to young, * p < 0.05 compared to age-matched control.

expression of p-mTOR in old rats.

There was a significant ($p < 0.05$) age-related increase in expression of p-IκB-α in the spleens of old rats compared to young rats (Fig. 4A and B). However, treatment with NFJ (5% and 10%) significantly ($p < 0.05$) decreased the expression of p-IκB-α in old rats. Similarly, treatment with all 3 doses of NFJ significantly ($p < 0.05$) decreased the expression of p-NF-κB (p50) in old rats compared to saline treated old rats while no such alterations were observed in the expression of p-NF-κB (p65).

3.5. NFJ enhanced p-ERK/Total ERK, p-CREB/Total CREB, and p-Akt/Total Akt expression in vitro and in vivo

Age-associated decline in the expression of p-CREB/Total CREB and p-Akt/Total Akt in the splenic lymphocytes was observed both *in vitro* and *in vivo* (Fig. 5C–F) while the expression of p-ERK/Total ERK decreased only in old saline-treated rats (Fig. 5B).

In vitro incubation of splenic lymphocytes with of NFJ significantly ($p < 0.05$) enhanced the expression of p-ERK1/2/Total ERK and p-CREB/Total CREB in young (0.01% to 1%) and old (0.0001% to1%) F344 rats (Fig. 5A and C). Similarly treatment with NFJ significantly

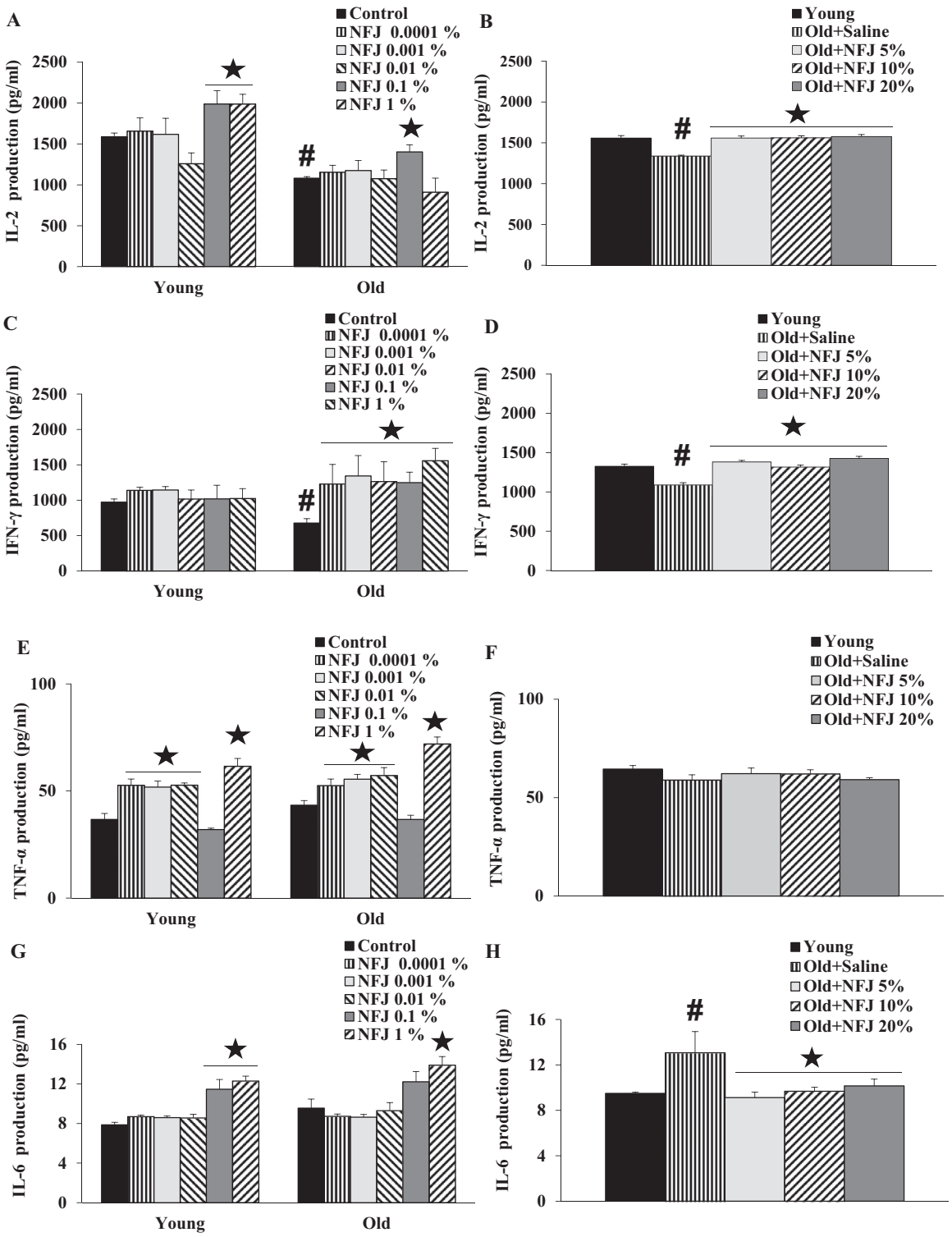

Fig. 3. Con A-induced cytokines (IL-2, IFN-γ, IL-6 and TNF-α) production by splenocytes after *in vitro* (A, C, E, and G) and *in vivo* (B, D, F, and H) treatment with *Morinda citrifolia* (Noni) fruit juice (NFJ) in young and old F344 rats.. # p < 0.05 compared to young, * p < 0.05 compared to age-matched control.

($p < 0.05$) increased the p-Akt/Total Akt expression in the splenocytes isolated from young (0.0001% and 1%) and old (0.0001%, 0.01% and 1%) rats (Fig. 5E).

In vivo treatment with NFJ (10%) significantly ($p < 0.05$) increased p-ERK1/2/Total ERK expression in old rats (Fig. 5B). Similarly, treatment with 5% dose of NFJ significantly ($p < 0.05$) increased p-CREB/Total CREB expression (Fig. 5D) while 10% and 20% dose of NFJ significantly ($p < 0.05$) increased the expression of p-Akt/Total Akt in old rats compared to saline treated old rats (Fig. 5F).

3.6. NFJ treatment decreased the extent of lipid peroxidation in vitro and in vivo

Age-associated increase in the extent of lipid peroxidation was observed both *in vitro* and *in vivo* in the splenic lymphocytes of old F344 rats (Fig. 6A and B). *In vitro* incubation of splenic lymphocytes with of NFJ significantly ($p < 0.05$) decreased the extent of lipid peroxidation in young (0.001%, 0.01% and0.1%) and old (0.0001% to1%) F344 rats (Fig. 6A). Similarly, *in vivo* treatment with NFJ (10%

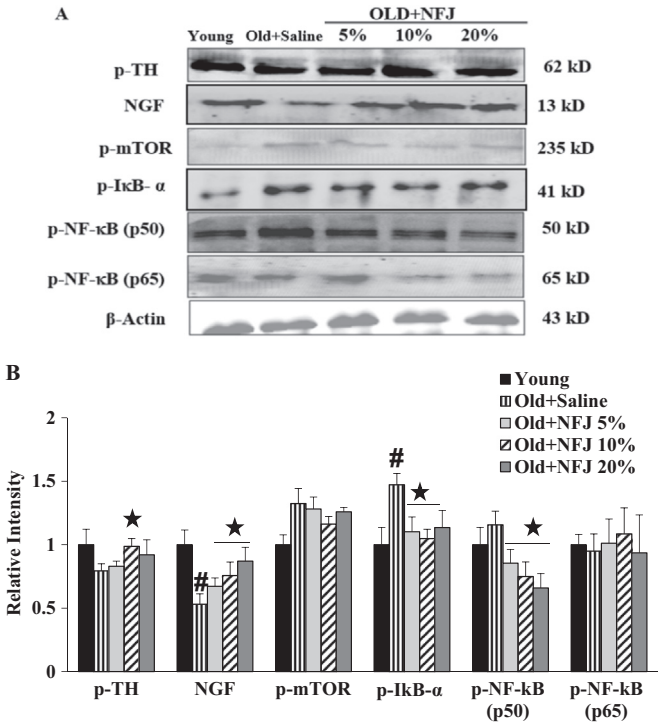

Fig. 4. Western immunoblots probed with antibodies against p-TH, NGF, p-IκB-α, and p-NF-κB (p50 and p65) expression in the splenocytes isolated from old rats after 60 days of oral administration of *Morinda citrifolia* fruit juice (NFJ). Equal amounts of total protein (30 μg) were immunoblotted for the indicated proteins (A). β-actin was used as an internal control. Lower panel are the bar graphs representing the relative density of the indicated proteins that were normalized with β-actin (B). # $p < 0.05$ compared to young, * $p < 0.05$ compared to age-matched control.

and 20%) significantly ($p < 0.05$) decreased extent of lipid peroxidation in old rats (Fig. 6B).

3.7. In vivo administration of NFJ decreased protein carbonyl formation

An age-associated significant ($p < 0.05$) increase in protein carbonyl formation was observed in spleens of old rats (Fig. 7). However, treatment with NFJ (20%) significantly ($p < 0.05$) decreased the protein carbonyl formation in spleen of old rats compared with old saline treated rats.

3.8. In vivo administration of NFJ increased nitric oxide (NO) production

Although there was no age-related decline in NO production, treatment with NFJ (10% and 20%) significantly ($p < 0.05$) increased the NO production in splenocytes from old rats compared with old saline treated rats (Fig. 8).

4. Discussion

In this study, noni (*Morinda citrifolia*) fruit juice (NFJ) enhanced cell-mediated immunity [increase in splenocyte proliferation and Th1 cytokine (IL-2 and IFN-γ) production] and suppressed proinflammatory status [decrease in IL-6 production and the expression of p-IKB-α and p-NF-κB (p50)]. These alterations were accompanied by an increase in p-TH and NGF expression, and an increase in intracellular signaling factors, p-ERK, p-CREB, and p-Akt in the splenocytes suggesting that NFJ is capable of reversing immunosenescence associated with inflammatory process possibly by improving neural-immune interactions involving intracellular signal transduction pathways.

Immunocompetence of both innate and acquired immunity is compromised in aging. Alterations in innate immunity may contribute more to disease pathogenesis because of the inflammatory processes

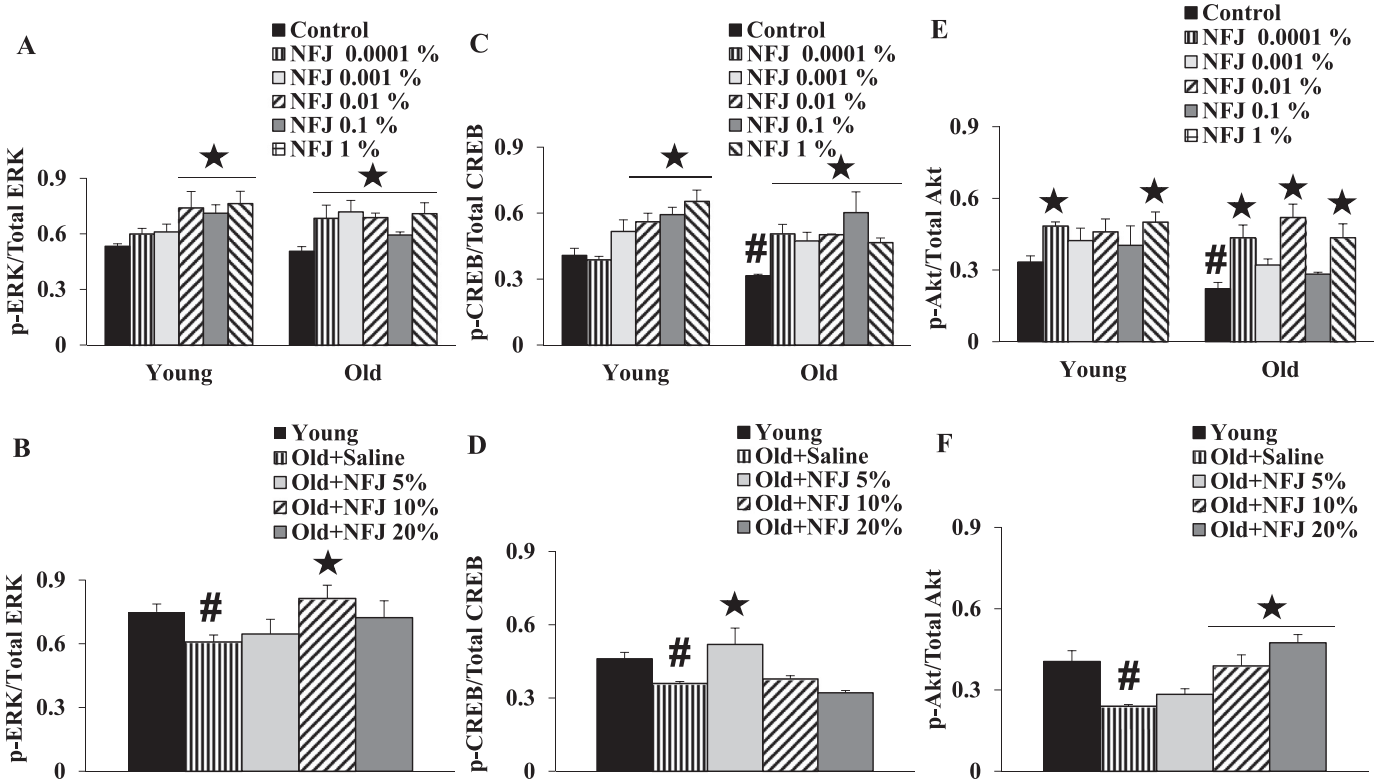

Fig. 5. Expression of p-ERK/Total ERK, p-CREB/Total CREB and p-Akt/Total Akt in splenocytes after *in vitro* (A, C and E) and *in vivo* (B, D and F) treatment with *Morinda citrifolia* (Noni) fruit juice (NFJ) in young and old F344 rats. # $p < 0.05$ compared to young,.* $p < 0.05$ compared to age-matched control.

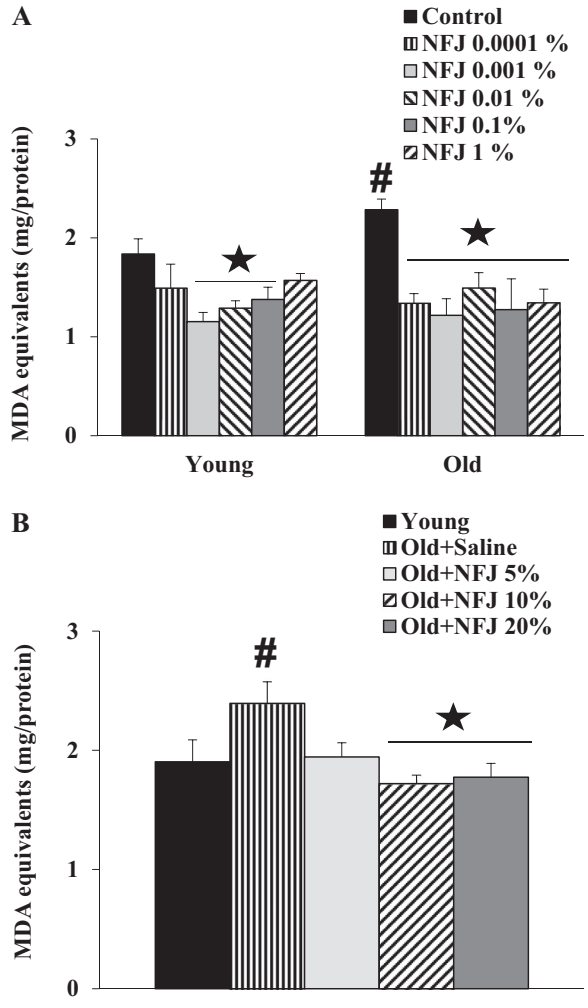

Fig. 6. Extent of lipid peroxidation in splenocytes after *in vitro* (A) and *in vivo* (B) treatment with *Morinda citrifolia* (Noni) fruit juice (NFJ) in young and old F344 rats. #p < 0.05 Compared to young, * p < 0.05 compared to age-matched control.

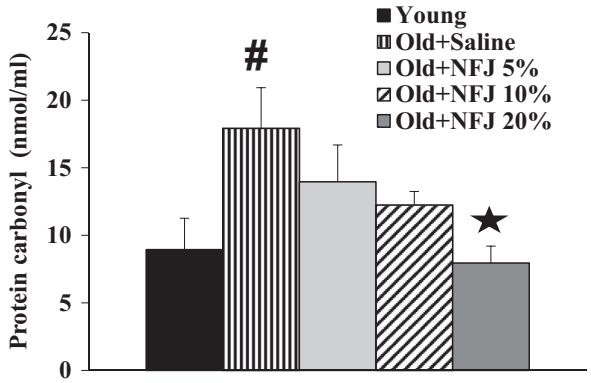

Fig. 7. Protein carbonyl formation in spleen after 60 days of oral administration of *Morinda citrifolia* (Noni) fruit juice (NFJ) in old F344 rats. #p < 0.05 Compared to young, *p < 0.05 Compared to age-matched control.

characterized by altered effector functions of neutrophils and monocytes/macrophages including phagocytosis, chemotaxis and cytotoxicity, and impaired signaling involving nucleotide-binding oligomerization domain (NOD) receptors and Toll-like receptors (Shaw et al., 2011). Ultimately, these alterations activate NF-κB resulting in increased secretion of proinflammatory immune molecules such as TNF-α and IL-6 and thus, promoting inflammation during aging (Salminen and Kaarniranta, 2010). Administration of NFJ to old F344 rats for a

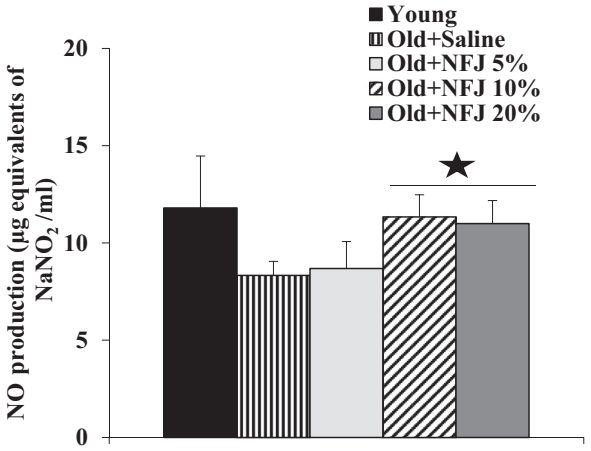

Fig. 8. Nitric oxide (NO) production in the splenocytes isolated from old rats after 60 days of oral administration of *Morinda citrifolia* (Noni) fruit juice (NFJ). * p < 0.05 compared to age-matched control.

period of 2 months suppressed both splenic IKB-α and NF-κB (p50) expression which was associated with a decline in IL-6 production by splenocytes suggesting that it is capable of slowing down the age-associated inflammatory processes. In support of this finding, we had earlier reported that *in vitro* incubation of splenocytes with noni fruit juice with seeds and without seeds suppressed NF-κB expression that may be due to its bioactive compound, damnacanthal, exerting its effects through the inhibition of p56lck tyrosine kinase (Kim and Jeong, 2014; Pratap et al., 2016; Nualsanit et al., 2011).

Age-dependent alteration in T cell activation, Th1/Th2/Tregs/Th17 population and cytokine production, and loss of CD4+ naive cells and CD8+ late-differentiated memory T cells results in inadequate immune response to new antigens and impaired memory response promoting development of diseases and cancer (Fulop et al., 2014b). NFJ treatment reversed age-related decrease in Con A-induced lymphoproliferative capacity and IL-2 and IFN-γ production that was similar to the *in vitro* incubation of splenocytes with NFJ with seeds (Pratap et al., 2016). BALB/c mice treated with *Morinda citrifolia* fruit extract modulated the cell-mediated immune functions by inhibiting the decrease in IL-2 production that may be possibly mediated through iridoid glycoside, deacetylasperulosidic acid (Murata et al., 2014). Other similar phytochemicals such as damnacanthal in noni may also be responsible for the enhanced lymphoproliferation and cytokine production. (Alitheen et al., 2010). Although it is yet to be determined, lectin may be another bioactive compound that may be responsible for the increase in Th1 cytokine production because garlic lectin is known to stimulate the production of IFN-γ and IL-12 in splenocytes via activation of p38-MAPK and ERK pathways (Dong et al., 2011). These effects may also involve improved antigen presentation by dendritic cells and thus, proliferation of splenocytes comprising B cells and CD4+T cells (Zhang et al., 2009).

Treatment of old rats with NFJ partially restored the age-related decline in expression of TH and NGF in the spleen suggesting that it possesses neuroprotective and neurotrophic functions. Age-associated decline in TH expression implies the loss of NA innervations in the spleen of old animals that may have been due to the accumulation of toxic oxidative metabolites of NE and free radicals in the local microenvironment (Bellinger et al., 2008; ThyagaRajan et al., 2011). The age-associated loss of sympathetic NA innervation in the secondary lymphoid organs (spleen and lymph nodes) may also result from a decrease in growth factors and antioxidant enzymes. *Morinda citrifolia* augmented the level of brain derived neurotrophic factor (BDNF) that improved streptozotocin-induced memory impairment (Pachauri et al., 2013). NFJ-induced increase in the expression of nerve growth factor (NGF), an essential neurotrophic factor for the survival of sympathetic

neuron, may also be involved in the proliferation of T and B cells, antibody production, and facilitate lymphocyte migration (Bonini et al., 2003). NFJ decreased the extent of lipid peroxidation and protein carbonyl formation in the spleens isolated from old F344 rats (. The role of NFJ in upregulating the NGF expression and immune responses to restore splenic NA fibers and thus, neural-immune interactions may not only depend on suppression of free radical production but also may be mediated through various intracellular signaling pathways.

In vitro and *in vivo* treatment with NFJ increased the expression of p-ERK, p-CREB, and p-Akt in young and old rats. One of the possible explanations for age associated decline immune responses may be due to impairment of activation of MAPK in stimulated T lymphocytes with advancing age (Gorgas et al., 1997; Whisler et al., 1996; Pratap et al., 2016; Priyanka et al., 2013c). The decrease in ERK expression leads to suppression of lymphocytes proliferation and IL-2 production (Li et al., 2002; Liu et al., 1997). The observed NFJ-induced increase in splenic lymphocyte proliferation and IL-2 and IFN-γ production in this study may have been through upregulated p-ERK/p-CREB pathways leading to improved cell-mediated immunity. Studies from our laboratory have shown that plant extracts of Brahmi can reverse the age-associated decline in immune function by upregulation the cell cytokines production and intracellular signaling pathways such as ERK and CREB in splenocytes isolated from rats (Priyanka et al., 2013a, 2013c). Similar to ERK and CREB, NFJ enhanced Akt expression in the splenocytes of young and old F344 rats with no significant difference in the expression of mTOR. There was a tendency for NO production to decline with age in splenocytes similar to alveolar macrophages that may alter vasodilation resulting in impaired trafficking of immune cells (Koike et al, 1999). In contrast, treatment of old rats with NFJ enhanced NO production that may exert immunostimulatory properties through cGMP-mediated Akt activation (Brown, 2010). The expression of mTOR in the spleen showed a trend to decrease in old rats following *in vivo* treatment with NFJ that may explain improved intracellular signaling in splenocytes and possibly aid in reversing age-related deficits in immunity (Johnson et al., 2013; Mannick et al., 2014).

5. Conclusions

Morinda citrifolia fruit juice has beneficial effects on immunity by increasing the Th1 cytokine production and decreasing the expression of inflammatory markers, and enhancing neural-immune interactions through intracellular signal transduction and preventing free radical generation in the spleen. Findings from our study provide an understanding about its modulatory role in neural-immune interactions that can be used as a therapeutic intervention in the aged population to promote healthy aging. Further studies are needed to explore the mechanisms of *Morinda citrifolia* fruit juice-mediated improvement in immunity by examining the role of phytochemicals in different subsets of immune cells of young and old individuals.

Declaration of interest

The authors declare that they do not have any conflict of interest.

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