Nrf2 Activation by Morinda citrifolia L. (Noni) Fruit Juices

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**Original Article**

Abstract

Objective: The objective of the study was to identify Nrf2 activators from differently treated *Morinda citrifolia* L. fruit juices and their cytotoxicity. Materials and Methods: Noni fruit juices were prepared by different treatments: unripe (T1), ripe (T2), purchased (T3), and Noni juices fermented with *Lactobacillus plantarum* (T4). These extracts were tested for Nrf2 activation and nuclear factor kappa B (NF-kB) inhibition activities. These active extracts were further studied for their nuclear Nrf2 translocation and induction of HO-1 protein expression. Finally, the active extracts were purified using open column chromatography and RP-high pressure liquid chromatography (HPLC) techniques through bioassay-guided separation. Moreover, all Noni juice samples were tested for cytotoxicity using mammalian cell-based methylthiazoltetrazolium (MTT) assay. Results: Only purchased (T3) and ripe Noni fruit juices fermented with LP at 22°C (T4–22) showed strongest Nrf2 activation and NF-kB inhibitory activity. Further, these two extracts enhanced the nuclear accumulation of Nrf2 after 2 h and also promoted Nrf2 and HO-1 nuclear translocation. Induction of HO-1 gene expression of Hep G2/ARE cells treated with T3 confirmed that it is a potent inducer of the Nrf2 target gene HO-1. Bioassay-guided separation resulted in subfractions with high Nrf2 activity. The strongest Nrf2 active subfraction led to the identification of scopoletin as an Nrf2 activator. Moreover, none of the tested samples showed any cytotoxicity for the MTT assay. Conclusions: The presence of potential Nrf2 activators in the Noni fruit juices that were nontoxic in our MTT assay could mitigate the production of harmful reactive species in the biological systems, and thereby, could helpful in alleviating and prevention of chronic diseases.

Keywords: HPLC, noni fruit, Nrf2, nuclear factor kappa B, scopoletin

Introduction

*Morinda citrifolia* L., belonging to Rubiaceae family commonly known as “Noni,” is a small evergreen tree that grows widely in tropical and subtropical climates and native to Southeast Asia and distributed to Australia and South Pacific including Hawaii.[1,2] The whole Noni plant (fruits, leaves, bark, roots, flowers, seeds, etc.) is used for many ailments in the traditional systems of medicine or in folklore including the diseases such as diabetes, cardiovascular diseases: atherosclerosis and blood vessels problems, hypertension, rheumatoid arthritis, infections (microbial or helminthic), gastrointestinal ulcers, depression, menstrual difficulties, headaches, sprains, body aches, muscles aches, colds, cancer, and drug addiction.[3-6] Different preparations of Noni juice have increased the worldwide sales, and it was estimated that approximately more than 400 million US Dollars in the year 2000.[7] Noni fruit juice has been widely used by the people of the South Pacific Islands in the past 2000 years to aid a wide range of health illness in high demand all over the world as an alternative medicine. The review article published by Nerurkar et al. and the group in 2015 has summarized the modern and traditional uses of Noni, among which fruits ranked the most.[6] Since 1990, the products derived from Noni have been sold in the USA as capsules, teas, powders, tinctures, syrups, and fruit juice, which is the most popular and demanded product. The studies of Noni’s phytochemicals such as fatty acid glycosides, flavanol glycosides, iridoids, terpenes, sulfur compounds, and their anti-inflammatory, anti-influenza, and anti-diabetic bioactive properties have boosted the world attention on Noni as dietary supplement.[8] In 2001, the National Institute of Health sponsored a Phase I clinical trial on Noni fruit extracts as a...
treatment for cancer. This was initiated as a result of survival of cancer patients (with invasive adenocarcinoma) taking homemade Noni fruit juice and was able to take regular diet and regain their body weight after 1 month.\footnote{10} Unfortunately, though it was claimed that Noni fruits have the healing power, there are no enough scientific evidences to support the prominence. As a result, in the modern day, researches follow the different preparations of Noni juice prepared by traditional healers used to treat difference diseases and try to prove the scientific rationale [Figure 1] including Hawaii.

In healthy individual, there is a balance between the systemic generation of oxidants and biological antioxidants’ capacity to remove the oxidants or to restore the oxidative balance. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that controls the expression of anti-oxidant and detoxifying enzymes that maintain a healthy cellular redox state by neutralizing or eliminating the toxic foreign chemical substances.\footnote{10} Uncontrolled production of oxidants (reactive oxygen or nitrogen species) in the cellular systems results oxidative stress that impairs cellular functions and contributes to the development of cancer, chronic disease such as Alzheimer, diabetes, arthritis, fibrosis, and toxicity. Nrf2 is targeted for the prevention of cancer and other chronic diseases and autoimmune disorders.\footnote{10,11} Hence, activation of Nrf2 to restore oxidative balance could be used for the prevention of cancer, chronic, and autoimmune diseases. On one hand, the abnormal activation or accumulation of Nrf2, which is common in many tumors, gives cancer cells a selective advantage and is associated to malignant progression, therapy resistance, and poor prognosis. Hence, it is worth mentioning that Nrf2 activators could be used for the prevention of chemical carcinogenesis, whereas Nrf2 inhibitors could be used for cancer treatment.\footnote{12}

In a previous study, we identified 4-methyl catechol (4-MC), 4-ethyl catechol (4-EC), and 4-vinyl catechol (4-VC) as Nrf2 cofactors in Noni juice.\footnote{10} These compounds were just partially accounted for the Nrf2 activation since the yields of these three alkyl catechols are very low in Noni.\footnote{10} One possibility is that 4-VC, 4-EC, and 4-MC degrades as soon as they are purified. In crude extracts, these three compounds (and other catechol analogs) might be stabilized by other antioxidants, but they are not stable when it is pure since they are sensitive to heat and light. Further, the most potent compound 4-VC can be oxidized to 4-vinyl-1,2-benzoquinone. Both 4-VC and 4-vinyl-1,2-benzoquinone can polymerize to form polymers (e.g., poly-4-vinyl catechols/1,2-benzoquinones with different lengths).\footnote{10} The present study undertakes to process Noni fruit juices in different preparations, evaluate their Nrf2 activity and cytotoxicity in the Nrf2 luciferase and methylthiazoltetrazolium (MTT) assays, respectively, fractionate highly active Nrf2 Noni juice preparation through high-performance liquid chromatography (HPLC) and assess these active subfractions, and identify more Nrf2 activators though bioassay-guided separation.

**Materials and Methods**

**Collection of Noni fruits and preparation of Noni juices**

Fresh unripe Noni fruits was collected from Isacc Hale Beach Park, Big Island, Hawaii in 2017. A voucher specimen (DKICP2017001) was deposited at Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo. Fresh fruits were then transported to the laboratory and washed with clean water and then hot water to remove surface dust and debris. Then, the following treatments were carried out to prepare fruit juices. Meanwhile, commercially available Noni fruit juice sample was taken as a treatment.

**Treatment 1 – unripe noni juice**

Air-dried unripe Noni fruits were ground without incorporating additional water and filtered using a cheese cloth. The filtrate was centrifuged at 10,000 rpm for 30 min. The supernatant was filtered using a 0.22 μm sterilized membrane filter to remove the bacteria and stored at 4°C refrigerator.

**Treatment 2 – ripe Noni juice**

Cleaned fruits were kept for about 7 days to fully ripe naturally and followed the similar procedure as in Treatment 1.

**Treatment 3 – purchased Noni juice**

Commercially available Noni juice was purchased from Virgin Noni Juice, [http://www.virginnonijuice.com](http://www.virginnonijuice.com) with an Order Number 809979. According to the vendor’s web site, the virgin Noni juice was made using traditional Hawaiian method known as “dripping.” The finest ripe Noni fruits were carefully hand-picked, washed thoroughly with running water, and placed in large closed containers. The Noni fruit was then fermented for several weeks until the juice was leached from the fruits. The stacked Noni fruits develop a natural positive pressure that further allows the juice to flow under the weight of all the ripe fruits. The pure Noni juice was then strained several times and pasteurized to preserve the most of the nutrients, vitamins, and especially for important active enzymes.\footnote{13}

**Treatment 4 – Lactobacillus plantarum fermented Noni juice**

The Noni juices were fermented with *Lactobacillus plantarum* (LP) (ATCC8014) at 22°C (room temperature) and 37°C at different incubation time periods (12 and 24 h) separately in a shaking incubator (225 rpm). After each

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Figure 1: Noni tree and its fruits
incubation period, juice was centrifuged at 10,000 rpm, and the supernatant was harvested. Further, supernatant was filter-sterilized using a 0.22 µm membrane filter to remove the bacteria and stored at −80°C freezer. LP is one of the mostly studied Lactobacilli in food industry as a potent probiotic and microbial starter. LP incorporated foods and beverages showed improved nutritional and functional food properties.[14]

Preparation of Lactobacillus plantarum inoculum
LP was grown in Lactobacilli MRS broth media (Difco) prepared according to the manufacturer instructions. The overnight grown cultures were centrifuged at 10,000 rpm, washed two times with phosphate buffer saline (pH 7.4) incorporated with glucose (10 mg/mL). Finally, aliquot of bacterial cell suspension (cell density 8 × 10^6 cells/mL) was introduced to the prepared Noni juices under aseptic conditions.

HP20 open column, preparative, and semi-preparative high-performance liquid chromatography
The extracts obtained from the four treatments were fractionated to test their Nrf2 and cytototoxic activities. Twenty milliliters of treated Noni juices were freeze dried. The samples were dissolved in 10 mL of sterilized distilled water and loaded into an open column (HP20 6.6 g, 1.5 × 6.0 cm). HP20 resin is made of rigid polystyrene/divinylbenzene matrix, which gives a controlled pore size distribution and large surface area for separation of molecules. Here, the separation is more similar to the C18 reverse phase chromatography. Where, most polar molecules eluted first followed by moderate polar and finally nonpolar molecules with 100% organic solvent acetonitrile (ACN). Hence, a gradient of solvent systems from 99% water to 100% acetonitrile (1, 10, 30, 50, 70, and 100% ACN) was used. The fractions were dried using SpeedVac to yield six fractions. The active fractions were initially separated and purified with a Thermo Scientific Ultimate 3000 preparative HPLC system connected to diode array detector (HPLC conditions: Phenomenex Luna C18 column (100 Å, 100 × 21.2 mm L × i.d, 5 µm); a gradient of ACN in Milli-Q water over a period of 45 min from 10% to 100% and end up with 100% ACN for another 5 min at a flow rate of 10 mL/min). Thermo Scientific Ultimate 3000 semi-preparative HPLC system equipped with a diode array detector (column: Phenomenex Luna C18 or C8 (100 Å, 100 × 10 mm L × i.d, 5 µm) at a flow rate of 3 mL/min was employed to get the pure compound. In both HPLC systems, 254 nm wavelength was monitored for the isolation process. 1H NMR data were recorded in deuterated methanol (MeOD-d4) on a Bruker 400 MHz NMR. For the determination of molecular weights of the isolates, Agilent 1260 HPLC coupled to 6120 quadrupole liquid chromatography–mass spectrometry spectrometer was used in both positive and negative modes (Column: Phenomenex analytical column C18, 100 Å, 100 × 4.6 mm, 5 µm; Flow-rate: 0.2 mL/min; Solvent A: water with 0.1% formic acid, Solvent B: acetonitrile with 0.1% formic acid, loading at 10% B, increasing the solvent gradient to 100% B in 20 min, and then re-equilibrate the column over 7 min in 10% B). Both NMR and MS data play a major role in structure elucidation of natural products.

Cell culture and condition
Hep G2-Nrf2 ARE reporter cell line (ATCC HB-8065) was purchased from BPS Bioscience (San Diego, CA, USA). Cells were proliferated at 37°C in a humidified incubator with 5% CO₂ in Eagle’s Minimum Essential Medium (MEM, Corning) with nonessential amino acids and supplemented with 10% fetal bovine serum (FBS, Invitrogen), and antibiotics: penicillin and streptomycin (Thermo Fisher, USA). Cells were trypsinsized and split every 6–7 days.

Chemicals exposure and Luciferase assay to measure the Nrf2 activation
Stock solutions of crudes (Treatments 1–4) and HP20 fractions were prepared (5 mg/mL) in sterile dimethyl sulfoxide (DMSO) solution (the final well concentration of DMSO was 5%). Samples were sonicated for better dissolution and then centrifuged. The supernatant was taken for the bioassay. The Hep G2-Nrf2 stable cell line was seeded into 96-well plates at 4 × 10^4 per well in a final volume of 100 µL of MEM media. After 24 h seeding, MEM media were replaced with fresh media and the cells were treated with the test samples (crude extract or fractions). Plates were incubated for another 24 h, and then 100 µL ONE-Step Luciferase reagent (BPS Bioscience) was added. Luminescence was detected using a luminometer (LUMIstar Galaxy BMG), and data are expressed as Relative Luminescence Units emitted from total assays. DL-sulforaphane (Sigma No S4441) was used as the positive control at a concentration of 5 µM. All experiments were performed in triplicate.

Nuclear factor kappa B assay
Construct HEK 293 cells (Panomics) were seeded into 96-well plates at 20 ×10^3 cells/well. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Co.), supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 µg/mL each), and L-glutamine (2 mM). After 48 h incubation, the medium was replaced, and the cells were treated with various concentrations of test samples. Tumor necrosis factor alpha (TNF-α) (human, recombinant, Escherichia coli, and Calbiochem) was used as an activator at a concentration of 2 ng/mL. The plate was incubated for 6 h. Spent DMEM medium was discarded, and the cells were washed once with PBS buffer. Cells were lysed using 50 µL of reporter lysis buffer (Promega) by incubating for 5 min on an orbital shaker and stored at −80°C. The luciferase assay was performed using the Luc assay system purchased from Promega. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light, which was detected using a luminometer (LUMIstar Galaxy BMG). Nuclear factor kappa B (NF-κB) inhibition is expressed as IC₅₀ values (i.e., concentration required to inhibit TNF-α-induced NF-κB activity by 50%). The positive controls of the assay were known NF-κB inhibitors, TPCK (Na-tosyl-l-phenylalanine chloromethyl ketone), and BAY-11-7082 with IC₅₀ values.
of 5.3 ± 0.9 and 11 ± 1.8 μM, respectively. These inhibitors selectively and irreversibly inhibited NF-κB activation by blocking TNF-α-induced phosphorylation without affecting constitutive IκB-α phosphorylation. All experiments were performed in triplicate.

**Immunofluorescence analysis**
The Hep G2/ARE cells were seeded on glass coverslip in a six-well plate at a density of 2 × 10^4 cells/well and then incubated for overnight. The cells were treated with the test samples (40 μg/mL) in different time points (0, 0.5, 1, 2, 6, 8, 12, and 24 h), the cells were fixed with 2 mL of 4% p-formaldehyde solution for 30 min at room temperature, and subsequently cells were permeabilized with 2.0 mL of 0.1% Triton X-100 in PBS for 30 min on ice. Then, the cells were incubated with 5% bovine serum albumin (BSA) for 30 min (for blocking). The cells were then incubated with Nrf2 primary antibody for overnight, next incubated with the secondary antibody (Alexa Fluor 488-conjugated secondary antibody) for 1 h. The cells were washed with PBS buffer before every treatment. The primary and secondary antibodies were diluted with blocking buffer (BSA). The fluorescence images were captured using a Leica TCS SP8 Confocal Laser Scanning Microscope System (Leica, Wetzlar, Germany).

**Western blot analysis**
Total protein was extracted from the cells and prepared with RIPA (radioimmunoprecipitation assay) buffer. Protein samples were boiled for 5 min at 95°C. Equal amounts of the protein concentrations were quantified by the bicinchoninic acid assay (Pierce). In brief, cell proteins were separated on an SDS (sodium dodecyl sulphate)–polyacrylamide gel. The proteins were then electrophoretically transferred at 15 V at RT onto a NC membrane (Bio-Rad, USA). The blotted membranes were blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated overnight at 4°C with the indicated primary antibodies. After three times wash with TBST, the membranes were then incubated with corresponding secondary antibodies and bands were visualized and analyzed by the LI-COR Odyssey image system (LI-COR Biosciences, USA). Each Western blot analysis was performed in triplicate.

**Methylthiazol tetrazolium-based cytotoxicity assay**
Cellular cytotoxicity was evaluated by in vitro MTT-based cytotoxicity assay (Sigma-Aldrich, St Louis, USA). Briefly, ARE repporter-Hep G2 cells (4 × 10^4 cells/well) were seeded in a 96-well plate in 100 μL MEM media and incubated for 12 h. Culture medium was replaced and cells were treated with crude (80 μg/mL), subfractions (80 μg/mL), and pure compounds (40 μg/mL) for another 24 h. The medium of each well was replaced by 200 μL of fresh medium with 50 μL of the MTT solution (5 mg/mL in PBS). The plates were incubated at 37°C for 4 h. The absorbance was measured at 570 nm using a Bio-Rad 680 plate reader. DL-Sulforaphane was used as a control at a concentration of 5 μM. All experiments were performed in triplicate.

**Data representation and statistical analysis**
Each data value represents a minimum of three (n = 3) replicate experiments, and all assays were conducted in at least three times (triplicate). All the data are expressed as mean ± standard deviation. P < 0.05 (P < 0.05) was considered statistically significant. All analyses were performed with the Student t-test using GraphPad Prism 5.1 (GraphPad, La Jolla, CA, USA).

**Results**

**Nrf2 activation and nuclear factor kappa B inhibition by Noni juice treatments and subfractons**
Initial concentration of 5.0 mg/mL of crude of treated Noni juices were tested for their Nrf2 and NF-κB activity, together with PBS buffer as the negative and DL-sulforaphane (Nrf2) as the positive controls, respectively. As shown in Figure 2, different treatments of Noni juices have the ability to activate or deactivate Nrf2 and NF-κB. All treatments activated Nrf2 except ripe noni juice (T2) and ripe Noni juice fermented by LP at 37°C for 12 h (T4–37-1). Purchased Noni juice (T3), ripe Noni juice fermented with LP at 22°C (T4–22-1) and 37°C (T4–37-1) at 12 h demonstrated inhibition of NF-κB. Ripe Noni juice fermented with LP at 22°C (T4–22) showed the strongest Nrf2 activation after 12 h and 24 h. Ripe Noni juice fermented with LP at 37°C after 12 h (T4–37-1) exhibited both Nrf2 and NF-κB inhibition. Unripe Noni without fermentation (T1) showed both Nrf2 and NF-κB activation.

In the following studies, we used purchased Noni juice (T3) as the sample for further study due to its stronger induction of Nrf2 translocation to the nuclei [Figures 3 and 4] and its commercial availability. In the case of Nrf2 nuclear translocation, T4–22 together with T3 was also evaluated.

![Figure 2: Nrf2 activation and nuclear factor kappa B inhibition of treated Noni fruit juices](image-url)

Figure 2: Nrf2 activation and nuclear factor kappa B inhibition activities of the different treatments of Noni fruits juices (T1: Unripe Noni fruit juice; T2: Ripe Noni fruit juice; T3: Purchased Noni fruit juice; T4: Noni juice fermented with Lactobacillus plantarum; T4–22: Noni fruit juice fermented with Lactobacillus plantarum at 22°C (1-at 12 h; 2-at 24 h); T4–37: Noni fruit juice fermented with Lactobacillus plantarum at 37°C (1-at 12 h; 2-at 24 h); Neg Ctrl: PBS buffer; Pos Ctrl: DL-sulforaphane (Nrf2). The bars with different letters (a-c) represent statistical significant difference (P < 0.05) compared with each group.
Manavalan, et al. Nrf2 activators, NF-κB inhibitors, Noni fruit juice, HepG2/ARE, HO-1, MTT

Nrf2 translocation in Hep G2/ARE: Immunofluorescence analysis of Noni fruit juices

Our findings with Hep G2/ARE that purchased Noni juice (T3) and ripe Noni juice fermented with LP at 22°C (T4–22) activated Nrf2, suggested that T3 and T4–22 may promote Nrf2 translocation to the nucleus. Hence, to test for the translocation of Nrf2 into nuclei, we stimulated Hep G2/ARE with T3 and T4–22 (final concentration of 40 μg/mL) and stained for Nrf2 with immunohistochemistry. As shown in Figure 3, T3 clearly promoted nuclear accumulation of Nrf2 after 2 h, consistent with Nrf2 activation [Figure 2]. At 12 h, translocation of Nrf2 to nuclei was obviously observed. We further tested purchased noni juice (T3) and ripe Noni juice fermented with LP at 22°C (T4–22) for the promotion of Nrf2 and heme oxygenase-1 (HO-1) nuclear translocation at 24 h. The results showed that both significantly enhanced the nuclear accumulation of Nrf2 and HO-1 at 24 h [Figure 4].

Western blotting analysis of Noni fruit juice samples

Next, with Western blotting of Hep G2/ARE and HeLa cells, we examined the induction of HO-1 protein expression by purchased noni juice (T3), 4-MC (positive control), 4-EC (positive control), and PSB (negative control). As shown in Figure 5, Western blotting and its protein quantification results (histogram) clearly indicate that T3 is a potent inducer of the Nrf2 target gene HO-1 and it was almost as active as the previously isolated and identified Nrf2 activator 4-EC from the Noni fruits[10] at the tested concentration (indicated in red arrow). This indicated that there was (or were) a compound (or compounds) responsible in T3 for the observed activity, which led us to the further study of T3 to isolate and identify the natural products that accounted for the activity. As a result, bioassay-guided separation of T3 extract was carried out.

Bioassay-guided separation and identification of Nrf2 activators

Due to the unavailability of considerable amount of Nrf2-active crude extracts of T2 and fermented (T4–22) products of Noni fruit juice, bioassay-guided isolation of the Nrf2 activators was carried out only in treatment 3 (T3: purchased Noni fruit juice) extracts that showed strong Nrf2 activation and NF-κB inhibition. Therefore, further fractionation of the crude of T3 with HP20 open column eluted with water and ACN yielded six fractions (ACN% [%w/w]: 1 [36.8], 10 [5.43], 30 [6.25], 50 [5.13], 70 [9.25], 100% [15.67%]). All six fractions were subjected to Nrf2 luciferase activity at the concentration of 40 μg/mL [Figure 6]. According to the assay results, the fraction eluted with 30% ACN was more active than the other fractions and the parent T3 extract. Furthermore, 10% ACN fraction was moderately active in the Nrf2 luciferase assay. Both fractions showed Nrf2 activity at 40 μg/mL.

Since the main objective of this study is to isolate highly active Nrf2 activators, only the most active fraction was further separated. Therefore, the 30% ACN fraction that...
was highly active against Nrf2 from the HP20 open column was further fractionated using a semi-preparative HPLC. Each peak was collected as a fraction detected by the ultraviolet (UV)-diode array detector at 254 nm and tested for the Nrf2 activity [Figure 7] as described above. Among these, subfractions 18, 19, 20, and 21 showed strong Nrf2 luciferase activity, which was similar to the positive control DL-sulforaphane. The main compound responsible for the Nrf2 activation in subfraction 20 was identified as scopoletin (about 2 mg/L). The structure characterization was carried out using molecular mass-based dereplication of natural products databases: SciFinder and the Dictionary of Natural Products in combination of the $^1$H NMR data with the published data. Scopoletin: $^1$H NMR (400 MHz, MeOH-d$_4$) $\delta$: 3.90 (3H, s, 6-OCH$_3$), 6.18 (1H, d, $J$ = 9.5 Hz, H-3), 6.82 (1H, s, H-8), 7.10 (1H, s, H-5), 7.90 (1H, d, $J$ = 9.5 Hz, H-4); LC-ESIMS: [M + H]$^+$ 193.2 

**Evaluation of cellular cytotoxicity of treated Noni fruit juices**

To evaluate the cytotoxic potential of treated Noni fruit juices, Nrf2 luciferase active fractions, subfractions, and the isolated compound (scopoletin) were subjected to methylthiazol tetrazolium (MTT)-based cytotoxicity assay. None of the crude, fractions, subfractions, or pure compound showed any toxicity to Hep G2/ARE reporter cells at 40 and 80 μg/mL. All the tested samples showed 100% cell viability similar to the DMSO control.

**Discussion**

Nrf2 and NF-κB are two important key transcription factors that regulate cellular responses to oxidative stress and inflammation, respectively. It is known that there is a functional cross-link between these two pathways. NF-κB can control the activity of Nrf2 positively and negatively on gene expression. Activation of Nrf2 is a promising therapeutic strategy for several chronic diseases such as neurodegenerative, cardiovascular, and metabolic diseases, which are related to oxidative stress and inflammation. The transcription factor NF-κB modulates many aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses. Hence, activation of Nrf2 and inhibition of NF-κB are two good approaches for the prevention of some chronic diseases, for examples, cancer, Alzheimer, and diabetes. Both purchased Noni juice (T3) and ripe Noni juice fermented with LP at 22°C (T4–22) after 12 h treatment displayed Nrf2 activation and NF-κB inhibition [Figure 2]. While on the other hand, in cancer, where Nrf2 provides a survival advantage to constituted tumors that was supported by many clinical studies, which is the “dark side of Nrf2” as it was reported, Nrf2 inhibition is desired. Ripe Noni juice fermented with LP at 37°C (T4–37) for 12 h strongly inhibited Nrf2 and NF-κB, indicating that it might be worthy to investigate T4–37 for Nrf2 inhibitors.

Upregulation of Nrf2 (a master regulator of antioxidant defense) nuclear translocation would promote the expression of antioxidant enzymes and phase II detoxifying enzymes, thus protecting body from many chronic diseases. Among the enzymes upregulated by Nrf2, HO-1 has remarkable anti-inflammatory as well as anti-oxidative properties. Upregulating HO-1 prevents the inflammatory response in various inflammatory conditions. Nrf2 activation should induce nuclear localization of Nrf2 and HO-1 expression. Nuclear accumulation of Nrf2 and HO-1 promoted by purchased Noni juice (T3) and ripe Noni juice fermented with LP at 22°C (T4–22) confirmed the activation of Nrf2.
by these two treatments [Figures 3 and 4]. T3 induced more Nrf2 translocation to nuclei than T4–22, while T4–22 increases the HO-1 expression more than T3, but both T3 and T4–22 enhanced the nuclear localization of Nrf2 and HO-1 expression, indicating that the commercially purchased fermented Noni juice (T3) and the self-fermented Noni juice (T4–22) may contain the same Nrf2 activators but with different concentrations.

Further, the induction of HO-1 protein expression by T3 was examined by the Western blotting technique [Figure 5]. Both T3 and 4-MC strongly induced HO-1 in Hep G2 liver cells and HeLa cervical cells. 4-EC also increased the HO-1 expression in HeLa cells, but it did not enhance the HO-1 expression in Hep G2 as much as in HeLa cells. This result indicated that different compounds have different HO-1 activities in different cell lines.

Bioassay-guided separation of T3 resulted in six fractions depending on the ACN percentage of the eluent. Among that the 30% ACN fraction was more active than the others and the parent T3 extract. Further, HPLC purification on Nrf2-active fraction furnished UV active plant metabolite scopoletin as the strongest Nrf2 activator. Scopoletin [6-methoxy-7-hydroxycoumarin, Figure 8] is a Coumarin type of molecule and known as one of the major chemical biomarker in the Noni fruits. There are published research works on scopoletin for Nrf2-ARE nuclear translocation and transactivation genes to protect from oxidative stress-related diseases.[18] Therefore, the compound scopoletin is well established for their therapeutic potential for the prevention of oxidative stress-related diseases such as antidiabetic,[18,19] antiobesity,[19] antipsychotics,[20] antidiabetic,[20] antigastroesophageal inflammatory diseases,[21,22] anticancer,[23] anti-infective,[24] memory-enhancing activity,[25] reduce liver damages,[19] and Alzheimer’s diseases.[26] In 2015, Nam and Kim reported that scopoletin could regulate the genes responsible for the regeneration. Therefore, it is well documented that the transient activation of Nrf2 target genes by scopoletin to fight against oxidative stress-related diseases and influence the activation of transcription factors such as p53, which is a tumor suppressor.[28] Therefore, scopoletin like natural Nrf2 activators abundantly found in Noni fruits could be used for the prevention of chemically and radiation (UV)-induced carcinogenesis.[29] It was reported in in vivo rat model and in vitro neuroblastoma SH-SY5Y cell study that scopoletin protected against oxidative stress and apoptosis induced by rotenone through Nrf2 activation. It was found that scopoletin increased the nuclear translocation of Nrf2.[27] Moreover, another study showed that scopoletin protects methylglyxal-induced hyperglycemia and insulin resistivity in a mice study.[18] Here, methylglyxal is metabolized into lactic acid as a result of the activation of Ser40 phosphorylation through Nrf2. As a result, accumulation of side products of protein glycation in the liver was reduced.[18] Further, Chang et al. show that scopoletin downregulated the expression of protein tyrosine phosphate 1B, thus alleviating the insulin resistivity. Thus, the scopoletin has the therapeutic potential as an antidiabetic and antiglycation agent.

Importantly, none of these extracts, fractions, or the scopoletin showed toxicity against mammalian cells in the MTT assay. Therefore, consumption of Noni fruit juice may offer good health benefits as a safe food supplement to mitigate oxidative stress-related diseases such as cancer, inflammation, diabetes, and Alzheimer’s diseases.

**Conclusions**

Extensive studies of biological activity have been reported on Noni fruit juices, our study was focused on bioassay-guided isolation of Nrf2 activators from differently treated Noni fruit juices. Purchased Noni juice and LP fermented ripe Noni at 22°C showed the strongest Nrf2 activation and NF-kB inhibitory activity. Further these two extracts show the nuclear accumulation of Nrf2 after 2 h and also the promotion of Nrf2 and HO-1 nuclear translocation at 24 h. Induction of HO-1 gene expression of Hep G2/ARE cells treated with purchased Noni juice confirmed that purchased Noni juice is a potent inducer of the Nrf2 target gene HO-1. Further, bioassay-guided separation of purchased Noni juice over multiple chromatographic techniques furnished scopoletin as the strongest Nrf2 activator. Due to no toxicity against mammalian cells in our assay, Noni fruit juice could be a safe food supplement and offer good health benefits to mitigate oxidative stress-related diseases such as cancer, inflammation, diabetes, and Alzheimer’s diseases.
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Conflicts of interest
There are no conflicts of interest.

REFERENCES