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Isolation and synergism of *in vitro* anti-inflammatory and quinone reductase (QR) inducing agents from the fruits of *Morinda citrifolia* (noni)

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ABSTRACT

The tropical fruits and fruit products of *Morinda citrifolia*, commonly known as noni, are consumed as a food or dietary supplement with purported health benefits. The objective of this study was to investigate the potential anti-inflammatory and cancer preventive effects of noni fruit puree extracts. Bioassay-guided fractionation of an ethyl acetate (EtOAc) extract of noni, comprising ~2% noni puree solids, led to the isolation of scopoletin (**1**), rutin (**2**), and quercetin (**3**). Quantitative HPLC analysis of the EtOAc extract revealed levels (dry weight basis) of scopoletin at 0.62 μmol/g, quercetin at 0.26 μmol/g and rutin at 0.045 μmol/g. Scopoletin and quercetin inhibited the production of nitric oxide (NO) in a concentration-dependent manner in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells and exhibited quinone reductase (QR) induction in cultured Hepa 1c1c7 cells. Increases in QR activity in induced cells were associated with increases in QR protein as confirmed by Western blots. Combinations of scopoletin and quercetin at a low (<10 μM) concentration resulted in synergistic suppression in nitric oxide (NO) production and down-regulated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expressions in LPS-induced RAW 264.7 macrophage cells. These results suggest that the combinations of noni compounds with different groups of chemical structures might be useful to efficiently suppress inflammatory and carcinogenic processes related to iNOS and COX-2 gene overexpression. These findings may provide some basis for the purported *in vitro* anti-inflammatory and anti-cancer effects of noni fruits as functional foods and dietary supplements.

1. Introduction

Morinda citrifolia, commonly called noni, is a plant typically found in the Pacific Islands, Southeast Asia, and other tropical areas. Noni fruits have been used as folk medicine for thousands of years for the alleviation of many diseases including cancer, colds, diabetes, flu, hypertension, and pain (Wang et al., 2002). Furthermore, noni juice, which is commonly prepared as a drip/exudate from senescing fruits held in fermentation vessels, is widely consumed today as a dietary supplement or food for the purported prevention of several diseases such as diabetes, high blood pressure and arteriosclerosis. So far, over 100 compounds have been identified in noni fruits. The structures of many of these are classified as anthraquinones, coumarins, glycosyl-fatty acid esters, flavonoids, polysaccharides, sterols and sulfur-containing compounds. Coumarins and flavonoids are specific chemotypes of the broader chemical group of polyphenols, which are abundant antioxidant components of fruits and vegetables (Faller & Fialho, 2009). Biological testing of both crude extracts and several pure constituents of noni fruit has been performed, revealing anti-inflammatory (Akihisa et al., 2007; Deng et al., 2007), antioxidant (Su et al., 2005), antibacterial, and Phase II enzyme inducing (Pawlus, Su, Keller, & Kinghorn, 2005) activities. However, many of the isolated compounds remain unexamined in this regard. Published reports suggest that noni fruits have chemopreventive activity against some types of cancers (Taskin et al., 2009; Wang et al., 2009) but which compound(s) from noni are responsible has not been determined.

Two biological effects that influence the disease status of animals includes the up-regulation of "vitagenes", predominantly coded for by the antioxidant response element (ARE) (Calabrese et al., 2008; Song et al., 1999; Talalay, Fahey, Holtzclaw, Prestera, & Zhang, 1995), and down-regulation of signaling pathways related to chronic inflammation, as inflammation is considered a pathological factor for many diseases (Surh & Na, 2008). Phase II enzyme induction provides for protection against oxidative stress in general and carcinogenesis in particular. Phase II enzymes are involved in metabolism of potentially harmful xenobiotics by reduction of electrophilic quinones through quinone reductase (QR; NAD(P)H oxidoreductase), or conjugation with polar groups through glutathione-, glucuronidyl- and sulfotransferase enzymes (Song et al., 1999; Talalay et al., 1995). Other

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ARE-encoded enzymes and proteins that defend cells against oxidative stress include hemeoxygenase-1 (HO-1), thioredoxin reductase, sirtuins, glutamate cysteine ligase, and several heat shock proteins (Calabrese et al., 2008; Russo, 2007). The regulatory regions of inducible ARE genes are activated upon binding of the nuclear factor E2-related protein 2 (Nrf2) transcription factor. Nuclear translocation of Nrf2 has been shown to be essential in the up-regulation of these protective genes in response to oxidative stress, electrophiles, and some phytochemicals (ARE inducers) (Eggler, Gay, & Mesecar, 2008).

Up-regulation of the ARE pathway is also believed to attenuate activation of the inflammatory pathway (Chen & Kong, 2005; Li et al., 2008). This "cross-talk" relationship was further revealed in a study that correlated potency among members of a series of established Phase II enzyme inducers with the relative ability to suppress inflammatory signaling (Liu, Dinkova-Kostova, & Talalay, 2008). Nitric oxide (NO) and prostaglandin E_2 (PGE_2) are two mediators of the inflammatory process, and are respectively synthesized by the inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). Induction of these enzymes occurs largely through the nuclear factor *kappa*B (NF-κB) pathway, mediated by various pro-inflammatory cytokines, including interferon-γ, tumor necrosis factor-α, and interleukins-1 and -6 (Clancy & Abramson, 1995). The expression of the COX-2 and iNOS is up-regulated in acute/chronic inflammatory diseases, and this link is particularly strong for COX-2 induction and colorectal cancer (Crew, Elder, & Paraskeva, 2000). Moreover, NO has been found to activate COX-2, further increasing prostaglandin production that may enhance inflammation or promote tumorigenesis (Cianchi & Masini, 2005). Therefore, suppression of iNOS/COX-2 expression and their activities is a widely recognized target for preventing inflammatory diseases and cancer (Surh & Na, 2008). Many natural constituents from fruits and vegetables have been identified as *in vitro* inhibitors of COX-2 and iNOS activities, and this provides a basis for continuing to assess the potential for evaluating their anti-inflammatory effects (Lantz et al., 2007).

In the present study, bioassays for potential anti-inflammatory and Phase II inducing activities were used to direct the isolation of bioactive constituents from noni fruits. These isolated noni compounds were then tested alone and in combination in cultured cells to determine mechanistic features of their action related to potential anti-inflammatory and cancer chemopreventive effects.

2. Material and methods

2.1. Chemicals

Scopoletin, quercetin, rutin, alizarin, lipopolysaccharide (LPS) and anti-β-actin antibody were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade ethyl acetate (EtOAc), ethanol (EtOH), methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Chicago, IL). Anti-iNOS, anti-COX-2, anti-NQO1 antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Mouse macrophage cells (RAW 264.7), a commonly used model for inflammatory processes (Kim et al., 2004; Mu et al., 2001; Murakami, Takahashi, Hagihara, Koshimizu, & Ohigashi, 2003; Park et al., 2006; Surh & Na, 2008), and human hepatocellular liver carcinoma (HepG2) cells obtained from ATCC (Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA)). The cells were grown in a humidified incubator at 37 °C and 5% CO_2. Cultured murine hepatoma cells (Hepa 1c1c7, ATCC) were grown in α-minimal essential medium (MEM, Invitrogen) supplemented with heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C and 5% CO_2.

2.3. Noni extraction and isolation

Noni puree was obtained from Tahitian Noni International (American Fork, UT), stored at ~4 °C for up to 10 months, freeze-dried and ground into a fine powder using a mortar and pestle prior to use. Freeze-dried noni powder (~100 g) was first extracted with ~500 ml EtOAc at ~77 °C under reflux conditions for 4 h. The residue was subjected to further sequential extracts by refluxing with EtOH, and finally aqueous extraction at 40 °C for 1 h. The purpose of a sequential extraction protocol was to enrich the bioactive agent(s) in at least one of the crude solvent extracts on the basis of polarity to facilitate isolation efforts. For each extract, the solvent was removed by vacuum rotary evaporation or lyophilization to obtain extracted dry matter. A ~2 g portion of the most active crude extract, the EtOAc soluble fraction (details in results), was flash-chromatographed on a silica gel column (5.5 cm × 60 cm) using a step gradient of 30 to 100% acetone in hexane to afford 4 fractions, F1–F4 (details in results) (Fig. 1) that were resolved on the basis of segregation of material absorbing at 254 nm determined with an optical microtiter plate spectrophotometer (Spectramax Plus, Molecular Devices, CA) Preparative thin layer chromatography (TLC) on silica gel using a developing solvent of 2% MeOH in CH_2Cl_2 was used to resolve fraction F3 obtained from the silica gel step. Fraction F4 was subjected to flash C-18 column chromatography using 40–70% MeOH in water to afford 4 fractions (F4-1, F4-2, F4-3, F4-4). The first two fractions, eluting at 30% MeOH (F4-1) and 50% MeOH (F4-2), were further subjected to Sephadex LH-20 column (2.5 cm × 75 cm, particle size 25–100 μM) chromatography by elution with 50% aqueous MeOH. Structures of the isolated compounds were determined using ^{1}H NMR and MS by comparison to previously reported profiles (Li, Lin, Wu, Lee, & Wu, 2004; Siddiqui, Sattar, Ahmad, & Begum, 2007; Wang et al., 1999). NMR spectra were collected on a Varian Unity-Inova 400 MHz and 500 MHz NMR spectrophotometer (Analytical Instrumentation Center, School of Pharmacy, UW-Madison). High resolution ESI-MS analyses were conducted on Agilent ESI-TOF Mass Spectrometer (Mass spectrometry/Proteomics Facility, Biotechnology Center, UW-Madison).

2.4. HPLC analysis

EtOAc crude extracts were profiled by analytical HPLC (model 1100 system, Agilent, Wilmington, DE) using 250 × 4.6 mm, 5 μM, Discovery C18 column (Supelco, Bellefonte, PA) generally using a previously reported protocol (Potterat, Felten, Dalsgaard, &

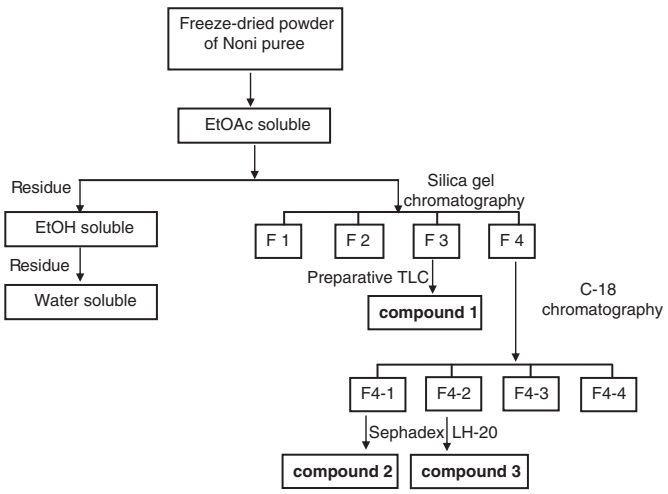

Fig. 1. Flow diagram of noni extraction and purification process.

Hamburger, 2007). The mobile phase consisted of 0.1% formic acid in water (eluant A) and ACN (eluant B). The gradient of solvent B was used as follows: from 5% to 25% B over 7.5 min, from 25% to 80% B over the next 5.5 min, from 80% to 100% B over the next 2 min, and from 100% to 5% B in 1 min, with an isocratic hold at 5% B for 4 min. Peak detection was achieved by monitoring absorbance at 254 nm. Peak identification (by co-elution) and quantification was obtained based on the instrument response to external standard curves prepared with authentic standards.

2.5. Bioassays

2.5.1. Nitric oxide (NO) assay

Mouse macrophage (RAW 264.7) cells were cultured in DMEM phenol red-free medium in 96-well plates at 5×10^3 cells per well for 24 h (Imm, Zhang, Chan, Nitteranon, & Parkin, 2010). The medium was then replaced with fresh DMEM containing either 1 μg/ml lipopolysaccharide (LPS) alone or LPS with various concentrations of test compounds (50–200 μg/ml) and the cells were incubated for another 24 h. NO was measured as nitrite in the culture supernatant using the Griess reagent with absorbance at 542 nm (Park et al., 2006) determined using an optical microtiter plate spectrophotometer. Results were expressed as % inhibition relative to control (100× the ratio of [(nitrite in LPS-treated control cells) − (nitrite in isolate + LPS-treated cells] ÷ [nitrite in LPS control treated cells]). The level of isolated required to inhibit NO evolution by 50% was defined as an IC_{50} value and was interpolated from the dose–response results.

Cell viability was evaluated by the 3-(4,5-dimethyl-thiazol-2,5-diphenyltetrazolium bromide (MTT) assay (Denizot & Lang, 1986). Cells were seeded in 96-well plates in the absence and or presence of noni compounds for 24 h as described in the preceding section. At the end of the incubation period, 100 μl of an MTT solution (0.1 mg/ml in PBS buffer) was added to each well and the plate was incubated for 4 h further. Then 200 μl of DMSO was added to each well to dissolve/extract tetrazolium dye and after 15 min of gentle agitation at 37 °C, absorbance was determined at 550 nm.

2.5.2. Quinone reductase (QR) induction assay

Increases in quinone reductase (QR) specific activity were used as a biomarker for Phase II enzyme induction as described (Imm et al., 2010; Prochaska & Santamaria, 1988). Hepa 1c1c7 cells (5×10^3)/well in 96-well plates were cultured in MEM supplemented with 10% FBS and 100 U/ml penicillin + 100 μg/ml of streptomycin in duplicate 96-well microtiter plates for 24 h at 5% CO_2 and 37 °C. The medium was then replaced with fresh MEM medium containing test compound, and the cells were incubated for an additional 48 h at 5% CO_2 and 37 °C.

After induction, one plate of cells was used for QR activity assay. Reduction of tetrazolium dye was continuously measured over 10 min as changes in absorbance at 490 nm using an optical microtiter plate spectrophotometer. A duplicate plate was used for cell protein measurement using crystal violet stain and measuring absorbance at 610 nm, which allowed calculation of relative QR specific activity in control and induced cells ([ΔAbs_{490nm} for QR assay ÷ Abs_{610nm} for protein assay], with the ratio for control cells set to 1.0). The potency of the compounds was compared by the CD value (the Concentration required to Double specific QR activity).

2.6. Western blot analysis

Macrophage (RAW 264.7) cells were cultured (10^6 cells/ml) in 6-well plates for 24 h. The medium was replaced with a fresh medium containing different concentrations of noni compounds with LPS (0.5 μg/ml) or LPS alone, and cells were incubated for another 24 h. For the whole-cell extract preparation, cells were quickly washed with ice-cold PBS and solubilized in 100 μl of RIPA buffer (50 mM Tris-base, 150 mM NaCl, 0.1% Triton x-100, 0.1% SDS) with a protease inhibitor (10×, Calbiochem, San Diego, CA). Supernatants were collected and the protein concentration in samples was estimated using the Bio-Rad (Hercules, CA) protein assay reagent. For Western blot analysis (Park et al., 2006), 50 μg extracted cellular protein was separated on pre-cast SDS polyacrylamide gel (Bio-Rad). Proteins in the gel were transferred onto a nitrocellulose membrane (Bio-Rad) by electroblotting. The nitrocellulose membrane was blocked with 5% skim milk solution in [Tris-Buffered Saline Tween-20] (TBST) buffer for 1 h at 20–21 °C and then probed with the primary antibodies in 3% skim milk in TBST buffer. Blots were washed three times with TBST and incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at 20–21 °C. Blots were washed again three times with TBST and then visualized by chemiluminescence detection reagent (Thermo Scientific, Rockford, IL), and the membrane was exposed to X-ray film. The membrane was re-probed with β-actin antibody for the loading control.

HepG2 cells were cultured (10^6 cells/ml) in 6-well plates for 24 h. The medium was replaced with a fresh medium containing various concentration of scopoletin (6.25 to 50 μM), and cells were incubated for another 24 h. Total protein and Western blotting were conducted as described in the preceding paragraph. Signal intensities were evaluated by densitometric analysis (Quantity One, Bio-Rad).

2.7. Statistical analysis

All bioassay experiments were done 3 times, each in duplicate or triplicate. The results were expressed as the means ± standard deviation (SD). The statistical significance of different between groups was assessed by a Student's *t*-test ($P<0.05$).

3. Results and discussion

3.1. Noni extraction and bioassay-guided fractionation

The yield of the EtOAc crude extract was ~2% from noni (puree) powder on a dry matter basis. Initial results using Hepa and macrophage bioassays indicated the EtOAc-extracted material was over an order of magnitude more potent than crude EtOH or water extracts. Thus, anti-inflammatory and QR inducing components obtained from dried noni puree became enriched in EtOAc extractable solids. The EtOAc-extracted material inhibited the production of NO in LPS-activated RAW 264.7 macrophage cells with an IC_{50} value of ~200 μg/ml and could double QR induction in cultured Hepa 1c1c7 with a CD value of 25–50 μg/ml. The EtOAc extract was fractionated on silica gel column, yielding 4 major fractions, F1–F4 which eluted from 30–100% acetone in hexane (Fig. 2). Yields of dry matter for each fraction relative to the dry matter loaded were: F1: 29.7%, F2: 9.1%, F3:

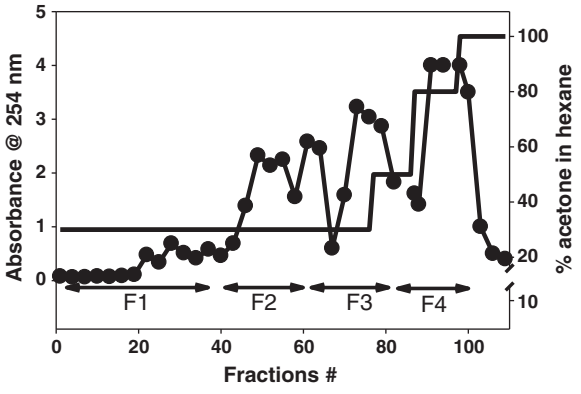

Fig. 2. Silica gel chromatographic resolution of the EtAOc crude extract of noni fruit with 30–100% acetone in hexane. Fractions were collected according to absorbance at 254 nm.

2.1% and F4: 10.2%. NO inhibitory and QR induction bioassays were conducted for each of the four fractions. Fractions F1 and F2 exhibited weak NO inhibiting activity, whereas estimated IC_{50} values for inhibition of NO evolution by fractions F3 and F4 were ~50 ± 25 and ~160 ± 15 μg/ml, respectively (Fig. 3). The NO inhibitory activity of F3 and F4 was not due to a cytotoxic effect as cell viabilities remained>90% (data not shown). For the QR induction assay, fractions F1 and F4 were active with CD values 25–50 μg/ml (Fig. 4). Fractions F2 and F3 were about an order of magnitude more potent than fractions F1 and F4 with CD values 2.5–3.1 μg/ml ($P<0.05$). Compounds with CD values of <10 μg/ml are considered attractive opportunities for further evaluation of potential as chemopreventive agents (Cuendet, Oteham, Moon, & Pezzuto, 2006). Fractions F2 and F3 are likely to contain potent QR inducing components; however since F2 exhibited weak NO inhibitory activity, efforts toward further isolation of active components were limited. Further resolution by TLC of QR inducing compounds from F2 revealed a major component (purity unknown) that exhibited major signals from ^{1}H NMR consistent with the presence of octanoyl residues. These components are likely to be glycosyl esters of octanoic acid ("noniosides") which are abundant in noni tissues (Potterat et al., 2007; Wang et al., 1999). However, follow-up studies will be required to identify the active component(s) in this isolate.

3.2. Anti-inflammatory and QR inducing activities by noni compounds

One of the most potent fractions, fraction F3 which was enriched in both NO inhibiting and QR inducing activity, was subjected to further purification. Preparative TLC of this fraction yielded one major spot as visualized under UV lamp at 254 nm. Silica gel from this band was scraped, extracted with 2% MeOH in CH_2Cl_2 and subjected to ^{1}H NMR and MS analyses: ^{1}H NMR (400 MHz, in $CDCl_3$): δ 3.95 (3H, s, H-6′), 6.26 (1H, d, J = 9.2 Hz, H-3), 7.58 (1H, d, J = 9.6 Hz, H-4), 6.84 (1H, s, H-5), 6.92 (1H, s, H-8); ESI-MS *m/z*: 191.0358 ($[M-H]^-$) (calcd. for $C_{10}H_7O_4$: 192.0344). These results confirmed the identity to be scopoletin (compound **1**; Fig. 5) by comparison with previously reported spectral analyses (Siddiqui et al., 2007; Wang et al., 1999) . Scopoletin is a derivative of coumaric acid, and both the isolated scopoletin and a commercial standard of this compound inhibited the production of NO in LPS-activated macrophages in a concentration-dependent manner with an IC_{50} of 0.26 mM (data not shown). Previous studies have reported scopoletin to inhibit NO and PGE_2 production in LPS-stimulated RAW 264.7 cells through the inhibition of iNOS and COX-2 expression at the mRNA and protein level (Kang et al., 1999; Kim et al., 2004). Scopoletin also suppresses the production of inflammatory cytokines through inhibition of the nuclear factor (NF)-κB, which is an important transcription factor for iNOS and COX-2, in a human mast cell line (HMC-1) (Moon et al., 2007). For the QR induction assay, the purified scopoletin from

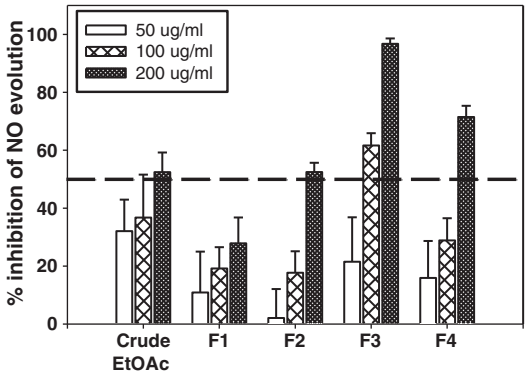

Fig. 3. Effects of crude EtOAc extract and fractions F1–F4 in inhibiting NO production in LPS-activated RAW 264.6 cells.

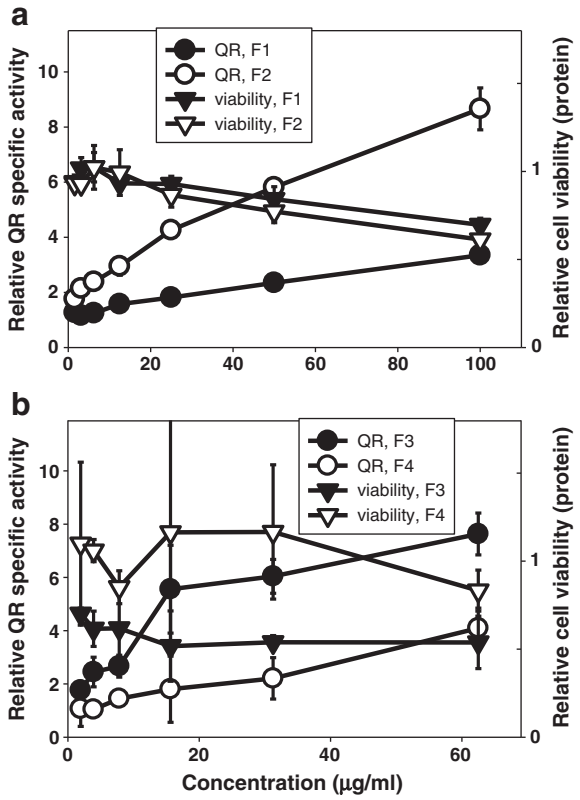

Fig. 4. QR induction assay and cell viability in cultured Hepa 1c1c 7 cell treated with (a) Fractions F1, F2 and (b) Fractions F3, F4.

fraction F3 and the commercial standard could induce QR specific activity in cultured Hepa 1c1c7 cells with a CD value of 6.25 μg/ml (~30 μM), while cell viability was maintained>50% (data not shown). In addition, Western blot analysis showed that scopoletin induced expression of QR protein in a dose-dependent manner (Fig. 6). In fact, there was a close agreement in the levels of scopoletin required to double both QR protein (Western blot) and cellular activity (in the bioassay). In a previous study, isolated scopoletin from *Sida acuta* (Morning Mallow) was found to be a QR inducer with a CD value 3.9 μg/ml (20.3 μM) (Jang et al., 2003) but underlying mechanisms have not been studied. The mechanism of scopoletin induction of Phase II enzymes is likely mediated by the Nrf2 and ARE, but the exact influence or site(s) of action of scopoletin on this pathway remains to be determined. Scopoletin is suggested to contribute significantly to the antioxidant properties to other botanical products, especially Chinese White Olive (*Canarium album Raeusch*) (Liu, Qiu, Ding, & Yao, 2008).

Fraction F4 from the initial silica column, particularly inhibitory on NO evolution in activated macrophages, was resolved into 4 subfractions using C18 reversed-phase flash chromatography. The first two subfractions respectively eluting in 30 and 50% MeOH in water were resolved further by Sephadex LH-20 to yield two purified compounds. ^{1}H NMR and MS analyses for compound **2**: ^{1}H NMR (500 MHz, in acetone-d_6): δ 6.22 (1H, d, J = 1.7 Hz, H-6), 6.44 (1H, d, J = 1.7 Hz, H-8), 6.91 (1H, d, J = 8.3 Hz, H-5′), 7.54 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 7.69 (1H, d, J = 2.0 Hz, H-2′), 5.35 (1H, d, J = 7.4 Hz, H-Glu-1), 5.12 (1H, d, J = 1.9 Hz, H-Rham-1), 1.00 (1H, d, J = 6.1 Hz, H-Rham-6); ESI-MS *m/z*: 609 ($[M-H]^-$) (calcd. for $C_{27}H_{30}O_{16}$: 610.15), and compound **3**: ^{1}H NMR (500 MHz, in acetone-d_6): δ 6.290 (1H, d, J = 1.5 Hz, H-6), 6.549 (1H, d, J = 1.5 Hz, H-8), 7.013 (1H, d, J = 8.5 Hz, H-5′), 7.715 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 7.854 (1H, d, J = 2.0 Hz, H-2′); ESI-MS *m/z*: 301.1 ($[M-H]^-$) and ($[M-H]^+$) 303.2 (calcd. for $C_{15}H_{10}O_7$: 302.04) revealed the pure compounds to be rutin (**3**) and quercetin (**2**), respectively (Fig. 5). These spectra are consistent with previously reported analyses

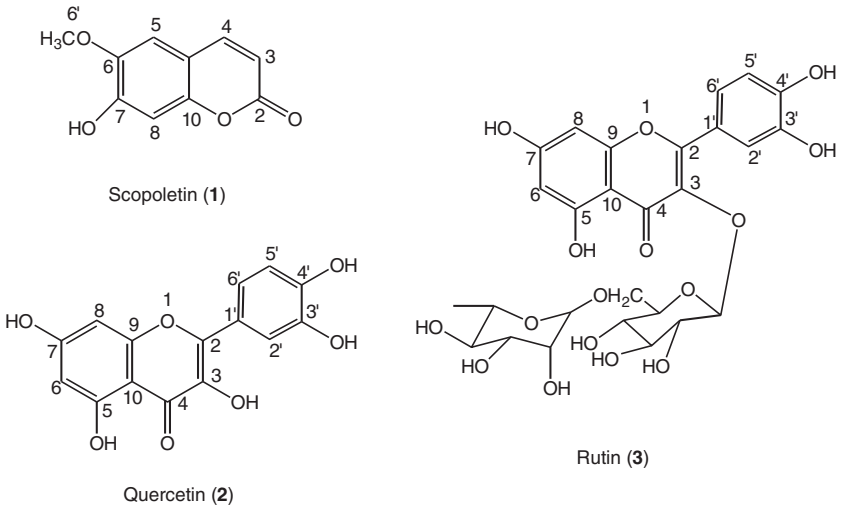

Fig. 5. Chemical structure of noni compounds.

(Fathiazad, Delazar, Amin, & Sarker, 2006; Li et al., 2004; Wang et al., 1999). The isolated quercetin inhibited NO evolution in activated macrophages with an IC_{50} of 66 μM (data not shown). This is consistent with previous reports of quercetin effects on activated macrophages (Hamalainen, Nieminen, Vuorela, Heinonen, & Moilanen, 2007) and LPS-induced NO production in mouse microglial cells (Chen et al., 2005). By comparison, rutin exhibited weak NO inhibition. In the macrophage model, the mechanism of quercetin action involves the reduction in both mRNA and iNOS protein levels (Mu et al., 2001). Quercetin also inhibits the activation of NF-κB transcription factor, and the signal transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS (Hamalainen et al., 2007). In the present study isolated quercetin doubled QR specific activity with a CD value of 0.6 μg/ml (~2 μM) in Hepa 1c1c7 cells which was similar to a CD value of standard (commercial) quercetin (2.5 μM) ($P>0.05$). Others have found quercetin to enhance ARE binding activity and Nrf2 transcription activity and suppress the level of Keap1 protein in the posttranslational level (Tanigawa, Fujii, & Hou, 2007). Quercetin and rutin exhibit other types of cancer chemopreventive activities such as the inhibition of oxidative damage to DNA (Wei, Wei-Jun, Zhi-Rong, & Ya-Ping, 2008).

3.3. Quantitative analysis by HPLC

To obtain quantitative data on the composition of noni fruits, HPLC analysis was performed to analyze noni constituents, particularly scopoletin, rutin, quercetin and alizarin (an anthraquinone) (Fig. 7). The amount of the identifiable compounds in the EtOAc extract from noni fruit powder (dry weight basis) was scopoletin: 0.62 μmol/g; rutin: 0.045 μmol/g and quercetin: 0.26 μmol/g. Comparison to previous studies (Potterat et al., 2007) indicated that scopoletin was mainly found in juice at various concentrations for different samples, but the ground noni fruit powder (GNFP) contained small amounts of the scopoletin (0.02 mg/g; 0.10 μmol/g). The differences between these samples from our studies may be due to extraction method, variety and maturity of noni fruits and whether the puree or juice was aged or freshly pressed and pasteurized. The rutin content was 0.36 mg/g (0.54 μmol/g) in GNFP (Potterat et al., 2007). This concentration is 12-fold higher than our noni fruit puree EtOAc extract. Aside from the previous factors mentioned, we speculate that residual glycosidase activity in the stored puree, either endogenous in origin or from the resident microflora, could also be partially responsible for the relative lack of rutin. However, alizarin (anthraquinone) was not detected in the EtOAc extract from noni fruit similar to previous studies (Deng, West, Jensen, Basar, & Westendorf, 2009; Potterat et al., 2007). Deng et al. (2009) found only trace amounts of anthraquinone (5,15-dimethylmorindol) in noni fruits and leaves; no other anthraquinones, such as lucidin and alizarin were detected in noni samples. These compounds are considered potentially useful as references for identification and authentication of noni fruits and their commercial products.

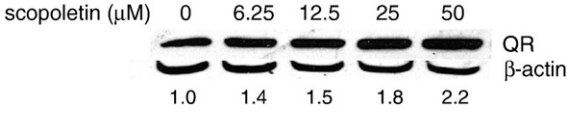

Fig. 6. QR protein expression in scopoletin-treated in HepG2 cells. Scopoletin was treated for 24 h. Data are expressed as a density ratio of QR over the control group (0 μM scopoletin).

3.4. Scopoletin and quercetin synergistically inhibit NO production in activated macrophages

Combinations of drugs or bioactive compounds may exhibit additive or synergistic effects compared to the use of single agents (Murakami, Takahashi, Hagihara, et al., 2003; Murakami, Takahashi, Koshimizu, & Ohigashi, 2003; Ohigashi & Murakami, 2004). In this study, commercial preparations of scopoletin and quercetin from noni fruits were examined for combined effects on inhibition of NO evolution in activated macrophages. The combination at low concentrations of scopoletin (6.3 μM) and quercetin (5 μM) inhibited NO production by ~60%, more than the expected additive effect of 39% ($P<0.05$) (Fig. 8a). A surprising finding was that scopoletin alone at 6.3 μM barely had any inhibitory effects, and such an observation would usually lead one to conclude that it is ineffective. Thus, these results show that even components, or low levels of bioactive components, that may appear void in bioactivity may either modulate the activity of other components or require the presence of other components for their activity to be evoked. At elevated levels (2× the level of the combination showing synergism) of scopoletin:quercetin (12.5:10 μM), the combined NO inhibitory effect was less than additive ($P>0.05$) (Fig. 8b). This is more likely caused by a "saturation" of inhibitory action on relevant targets, rather than any true antagonistic relationship between these compounds. iNOS and COX-2 protein expression were also determined in LPS-activated RAW 264.7 cells treated with scopoletin (6.3, 12.5 μM), quercetin (5 μM), and scopoletin:quercetin mix (6.3:5 and 12.5:5 μM). Western blot analysis showed that the combination of scopoletin and quercetin significantly inhibited iNOS and COX-2 expressions (Fig. 9). The

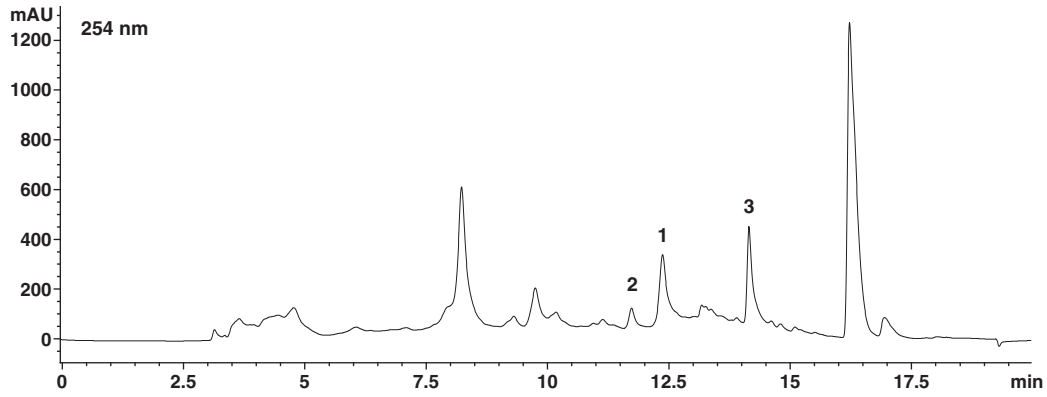

Fig. 7. HPLC chromatographic profile of the EtOAc extract from noni fruits. **1**: scopoletin, **2**: rutin and **3**: quercetin.

synergistic and antagonistic effects of the combinations may depend on the chemical structures of the compounds and the possible formation of stable intermolecular complexes (Peyrat-Millard & Berset, 2003). Furthermore, these two compounds likely act at different targets and/or with different mode of actions in a manner that efficiently suppresses inflammatory-related gene expression. For example, scopoletin could inhibit iNOS expression through NF-κB transcription factor but quercetin also suppresses iNOS expression through NF-κB and STAT-1 transcription factors (Hamalainen et al., 2007). Similarly, Cheung, Khor, and Kong (2009) reported that the combination of curcumin (anti-inflammatory effect) and sulforaphane (Phase II enzyme inducer) could be more effective than either used alone in preventing inflammation. Evidence of synergistic action between multiple bioactive food agents serves to reinforce the paradigm that a diversity of food products from botanical origin may be more effective at promoting health than single highly potent compounds ("silver bullets"). In this regard, it would be interesting to determine if the ratio and levels of many of the bioactive compounds in noni fruit would yield even greater synergism than the binary mixtures used in this study.

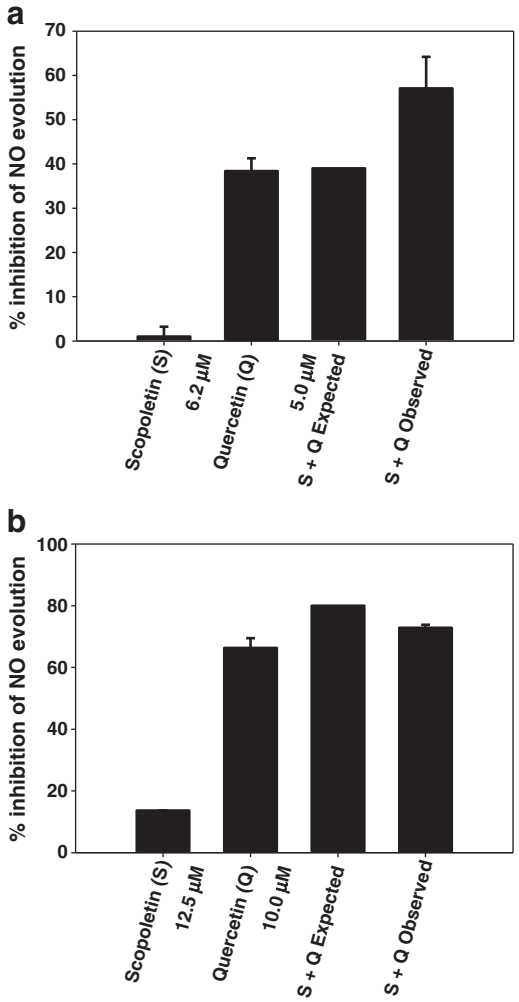

Fig. 8. Effect of commercial sources of scopoletin, quercetin and their combination with various concentrations on LPS-induced RAW 264.7 macrophage in inhibiting NO production. (a) scopoletin 6.25 μM and quercetin 5 μM, (b) scopoletin 12.5 μM and quercetin 10 μM.

4. Conclusion

In this study, scopoletin and quercetin were isolated from noni fruit puree as potentially beneficial components related to anti-inflammatory and anti-cancer activities. In the anti-inflammatory bioassay, a synergistic relationship between these two components (at ~5 μM each) at the same ratio they are present in the active extract of noni puree. The combined actions of these compounds likely involve multiple mechanisms of biological effect. Given the presence of many other bioactive components identified in noni fruit, it is possible that even lower (<1 μM) or even trace levels may contribute in a significant way to the overall biological effect of this fruit *in vitro* or *in vivo*. Further efforts should focus on examination of the biological effects of more complex mixtures of bioactive noni components, in the context of designing combinations where components are known to act by different mechanisms. Such studies may improve our understanding and knowledge of potential beneficial effects of consuming noni fruits as food or and dietary supplement.

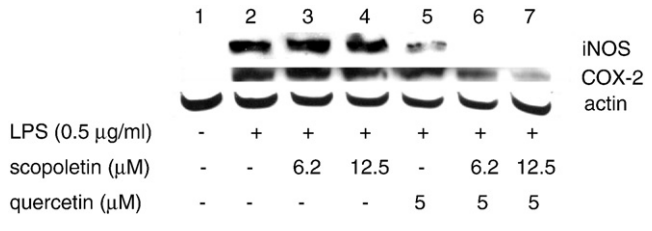

Fig. 9. Effect of commercial sources of scopoletin, quercetin and their combination on LPS-induced iNOS and COX-2 protein in RAW 264.7 cells, lane 1: untreated- RAW 264.7, lane 2: LPS-treated cells, lane 3: 6.3 μM scopoletin, lane 4: 12.5 μM, lane 5: 5 μM quercetin, lane 6: scopoletin:quercetin (6.3:5 μM) and lane 7: scopoletin:quercetin (12.5:5 μM).

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