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Direct and Indirect Antioxidant Activity of Polyphenol- and Isothiocyanate-Enriched Fractions from *Moringa oleifera*

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Abstract

Moringa oleifera Lam. is a fast-growing, tropical tree with various edible parts used as nutritious food and traditional medicine. This study describes an efficient preparatory strategy to extract and fractionate moringa leaves by fast centrifugal partition chromatography (FCPC) to produce polyphenol and isothiocyanate (ITC) rich fractions. Characterization and further purification of these fractions showed that moringa polyphenols were potent direct antioxidants assayed by oxygen radical absorbance capacity (ORAC), whereas moringa ITCs were effective indirect antioxidants assayed by induction of NAD(P)H quinone oxidoreductase 1 (NQO1) activity in Hepa1c1c7 cells. In addition, purified 4-[(α-L-rhamnosyloxy)benzyl]-isothiocyanate and 4-[(4′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate were further evaluated for their ORAC and NQO1 inducer potency in comparison with sulforaphane (SF). Both ITCs were as potent as SF in inducing NQO1 activity. These findings suggest that moringa leaves contain a potent mixture of direct and indirect antioxidants that can explain its various health-promoting effects.

Keywords

Moringa oleifera; isothiocyanates; 4-[(α-L-rhamnosyloxy)benzyl]isothiocyanate; 4-[(4′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate; polyphenols; NQO1; FCPC; antioxidants

INTRODUCTION

Isothiocyanates (ITCs) are food-derived compounds with strong chemopreventive and anti-inflammatory activities, which can be attributed to modulation of oxidative stress by induction of phase II detoxifying enzymes.[1,2] The plant order Brassicales provides a rich source of these molecules for the human diet.[3] ITCs are formed from glucosinolates (GLSs), having a backbone of glucose attached to sulfonated aldoxime with a variable side chain (Figure 1), by the action of myrosinase (β-thioglucosidase), which is stored in a different compartment in the same plant cells.[4] Although the concentration of ITCs in intact plant

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IR has equity in Nutrasorb LLC, which licensed *Moringa*-related intellectual property from Rutgers University.
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tissue is very low or undetecable,[5] upon tissue disruption such as cutting, chewing, or other mechanical insult, myrosinase and GLSs come in contact and glucose is removed from the molecule, producing an aglycone that is subsequently converted into a corresponding ITC. The bioactivity of ITCs is a function of the –N═C═S group (ITC pharmacophore, Figure 1), which has been reported to cleave disulfide bonds in proteins and react with amino acids in vitro.[6] The rest of the molecule determines its polarity, stability, and volatility.

Most ITCs, including sulforaphane (SF, Figure 1A) isolated from broccoli, are volatile oils, unstable at room temperature, and are spontaneously converted to several inactive intermediates with relatively high degradation rates.[7] In contrast to unstable ITCs from crucifers, the four ITCs formed in the leaves of *Moringa oleifera* Lam. (Moringaceae family, Brassicales order), commonly known as moringa, have emerged as highly stable analogues due to an additional sugar moiety in the aglycone portion of the molecule (Figure 1B).[8] The presence of a rhamnose sugar moiety is unique to moringa ITCs in nature and likely explains their observed stability and solid appearance at room temperature. It has been shown that compound **1** (4-[(α-L-rhamnosyloxy)benzyl]isothiocyanate) and compound **4** (4-[(4′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate) (Figure 1B) from *M. oleifera* Lam. demonstrated 70 and 100% stability, respectively, over 30 days at room temperature.[8] In addition to distinguished stability, studies of moringa ITCs (**1**–**4**) revealed possible pharmacological advantages as anti-inflammatory agents over well-studied crucifer ITCs including SF. For example, compound **1** was found to be a stronger inhibitor of nuclear factor kappa B (NF-κB) expression and myeloma growth in nude mice than SF.[9] ITCs (**1**–**4**) were also shown to reduce nitric oxide (NO) formation at low micromolar concentrations in macrophages.[10] Further studies reported that compound **2** (4-[(2′-*O*-acetyl-α-L-rhamnosyloxy)-benzyl]isothiocyanate) specifically attenuated NO formation more effectively than SF and benzylisothiocyanate.[11] Recently, we showed that both compound **1** and **4** attenuated expression of iNOS and IL-1β and formation of NO and tumor necrosis factor alpha (TNFα) in RAW macrophages at 1 and 5 μM concentrations.[8]

Although there are considerable data on the bioactivity, absorption, metabolism (including hydrolysis in human gut microbiota), and bioavailability of various ITCs from crucifers, for the rhamnose-substituted GLSs and corresponding ITCs of moringa tissues, the available bioactivity data are limited to the above-mentioned examples, and there are no comparable data on the metabolism, absorption, etc. The analysis of GLS content from the leaves of *M. oleifera* has shown that GLS **1** (see Figure 1B for the structure) is present in significant concentrations (ranging between 33.9 and 59.4 mg/g dry weight of the tissue). However, the concentration of its monoacetyl isomers, especially GLSs **2** and **3**, are relatively less abundant (ranging between 1.2 and 5.0 mg/g dry weight of the tissue).[12] The occurrence of these compounds at relatively low levels makes isolation of them and their corresponding ITCs a purification challenge. Herein, we accomplished a large-scale fractionation of moringa leaf extract to yield ITC-rich fractions by fast partition counter current chromatography (FCPC) using a three-phase solvent system, which has been previously used to separate a mixture of plant compounds having a broad range of polarity in a one-step operation.[13] We applied this system followed by solid phase extraction (SPE) to further enrich the ITC content of the fractions, thus enabling the isolation of other, less abundant

monoacetyl isomers. The methanolic extract of moringa was prepared from finely chopped and crushed fresh leaves to activate plant myrosinase for the efficient conversion of GLSs to corresponding ITCs.

The use of moringa leaves as traditional medicine dates back to ancient times. Growing scientific evidence has suggested the effectiveness of moringa in treating inflammation, hyperlipidemia, hyperglycemia, hypertension, bacterial and viral infections, ulcers, and cancer.[14–16] It is now well-known that many of these conditions are brought on and/or exacerbated by oxidative and electrophilic stress.[17] Thus, moringa's ability to prevent and treat such conditions may be due to the high level of antioxidant/bioactive compounds present in the leaves including vitamins, micronutrients, and polyphenols.[14,18] To date, moringa ITCs have not been well studied for their antioxidant activity. Therefore, this study was designed to evaluate and distinguish the role of moringa polyphenols and ITCs in both direct and indirect antioxidant capacities.

Generally, direct antioxidants are redox active, short-lived small molecules that directly scavenge reactive oxygen and/or nitrogen species.[17] On the other hand, indirect antioxidants induce a battery of phase II xenobiotic metabolizing enzymes (XMEs) through a shared Keap1/Nrf2/ARE pathway resulting in increased antioxidant capacity and long-lived protective effect compared to direct antioxidants.[17] Among phase II XMEs, NAD(P)H quinone oxidoreductase 1 [NQO1, EC 1.6.99.2] serves as a cytoprotective marker enzyme to evaluate indirect antioxidant potential of various phytochemicals including ITCs.[19,20] NQO1 catalyzes a two-electron reduction of highly reactive quinone molecules that are found in automobile exhaust, cigarette smoke, and many foods.[21] Numerous studies have shown associations between elevated NQO1 activity and protection against cancer and inflammatory diseases.[22,23]

Assaying NQO1 inducer potency has been used to isolate several chemoprotective agents from plant materials, leading to the synthesis of more effective ITCs and curcumin derivatives.[24] SF was first isolated from broccoli by monitoring NQO1 inducer potency.[25] In the current work, polyphenol-rich fractions, ITC-enriched fractions, compounds **1** and **4**, and SF were tested and compared for indirect antioxidant activity by measuring induction of NQO1 activity in murine hepatoma cells and direct antioxidant activity by measuring the oxygen radical absorbance capacity (ORAC). This study also demonstrates the effectiveness of FCPC and solid phase extraction (SPE) to concentrate polyphenols and ITCs from moringa leaves.

MATERIALS AND METHODS

Chemicals

Acetonitrile, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein sodium salt, Folin–Ciocalteu phenol reagent, gallic acid, *n*-hexane, methanol, methyl acetate, β-naphthoflavone (BNF), phosphate-buffered saline (PBS) 10× concentrate, L-sulforaphane (SF), and trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum and α-minimum

essential medium (MEM) were purchased from Invitrogen (Carlsbad, CA, USA). All solvents were of analytical grade. Water used in the experiments was purified using a Millipore water purification system with a minimum resistivity of 18.2 MΩ·cm (Bedford, MA, USA).

Compounds **1** and **4** were previously purified in our laboratory from a methanolic extract of fresh moringa leaves by HPLC as described by Waterman et al.[8] The chemical purity of isolated ITCs was confirmed by LC-MS and ^{1}H NMR analyses.[8]

Plant Material and Preparation of Crude Extract

Fresh leaves from moringa (Indian PKM variety) were shipped overnight from Moringa Farms, Sherman Oaks, CA, USA. A voucher specimen (CW1) was prepared and deposited at the Chrysler Herbarium of Rutgers University (CHRB). The leaves were extracted on the day of arrival. Briefly, 200 g of fresh leaves was finely chopped and crushed by using a pestle and mortar to activate myrosinase enzymes and conversion of GLSs into corresponding ITCs by tissue damaging. The resulting material was extracted in methanol (3 × 300 mL, overnight) at room temperature by continuous shaking. Solvent was removed from extract under reduced pressure by rotary evaporation. The moringa methanol extract (ME) was frozen at −80 °C, lyophilized, and stored at −20 °C.

Selection and Preparation of the Solvent System

The following three-phase solvent system was selected according to the partition coefficient (*K*) of compounds **1** and **4**: *n*-hexane/methyl acetate/acetonitrile/water (4:4:3:4). *K* values were determined as follows: 1 mg of ME was dissolved in 500 μL of a pre-equilibrated two- or three-phase solvent system in a vial with vigorous shaking. After equilibration, equal amounts (100 μL) of upper and lower/middle (in the case of the three-phase solvent system) phases were analyzed by LC-MS using the conditions described under LC-MS Analysis and Quantification of ITCs. The *K* value is defined as the ratio between the peak area of each ITC in the upper phase (UP) and lower phase (LP), ($K_{UP/LP}$) for a two-phase solvent system or as the ratio between the peak area of each ITC in the middle phase (MP) and lower phase ($K_{MP/LP}$) for a three-phase solvent system.

The selected three-phase solvent system of FCPC was equilibrated in a separatory funnel and left for equilibration at room temperature overnight. The three phases were separated and degassed by sonication for 15 min shortly before use.

Fractionation of ME by FCPC

Counter current chromatography was performed by a bench-scale fast centrifugal partition chromatography sytem FCPC1000, v 1.0, from Kromaton (Annonay, France) with a rotor volume of 1000 mL and maximum pressure of 860 psi. For fractionation, the column was first filled with MP and LP in a ratio of (7:3) at a flow rate of 80 mL/min while rotating at 300 rpm. The system was then equilibrated with UP at a flow rate of 10 mL/min and 750 rpm. A 3 g sample of ME was suspended in 10 mL of each phase of the solvent system used for separation. ME was sonicated briefly and filtered through a 0.45 μm pore. As the UP was pumped at 10 mL/min at 750 rpm, the sample was injected through the Rheodyne valve. UV

detection was performed at 227 nm, and 20 mL fractions were collected every 2 min with a CHF122 SC Advantec fraction collector (Dublin, CA, USA).

Enrichment of Fractions by SPE

FCPC fractions, particularly C, D, and E (50–100 mg), were dissolved in 1 mL of water containing 1% acetic acid and sonicated for 10–15 min. A 3 mL SPE column with C18 absorbents (J. T. Baker, Bakerbond) was equilibrated by 3 mL of methanol followed by 3 mL of water containing 1% acetic acid. The sample solution (1 mL) was loaded into the SPE column. The aliquots (1 mL × 3) of water (1% acetic acid) and methanol (1 mL × 3) were successively applied into the SPE tube, and two elutes were collected separately and evaporated to dryness. The methanol elute was used for further characterization of fractions in terms of ITC content, total polyphenol content (TPC), ORAC, and NQO1 activity assays.

LC-MS Analysis and Quantification of ITCs

LC-MS analysis was performed using the Dionex UltiMate 3000 RSLC ultrahigh-pressure liquid chromatography system, consisting of a workstation with Dionex Chromeleon v. 6.8 software package, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector, the eluent flow was guided to a Varian 1200L (Varian Inc., Palo Alto, CA, USA) triple-quadrupole mass detector with electrospray ionization interface, operating in the negative ionization mode. The voltage was adjusted to −4.5 kV, heated capillary temperature was 280 °C, and sheath gas (zero grade compressed air) was used for the negative ionization mode. The mass detector was used in scanning mode from 65 to 1500 atomic mass units. Data from the the Varian 1200L mass detector were collected, compiled, and analyzed using Varian's MS Workstation, v. 6.9, SP2. Compounds were separated on a Phenomenex Luna C8 reversed phase column, size 150 × 2 mm, particle size = 3 μm, pore size = 100 Å. The mobile phase consisted of two components: solvent A (0.5% ACS grade acetic acid in double-distilled deionized water, pH 3–3.5), and solvent B (acetonitrile). The initial conditions of the gradient were 95% A and 5% B. The gradient progressed linearly to 5% A and 95% B over 30 min and then remained isocratic for the next 8 min. During the following 4 min, the ratio of solvents A and B was brought to initial conditions linearly. An 8 min equilibration interval was included between subsequent injections.

ITCs were characterized on the basis of their retention times, UV maxima, and MS fragmentation patterns. The MS data obtained for the ITCs (**1**–**4**) gave the expected *m*/*z* values and fragment ions that were characteristic for these compounds.[8] In the current study, the quantification of ITCs in the fractions were done by using standard curves generated for pure **1** and **4**, which were purified (>98%) previously as described by Waterman et al.[8] Briefly, 1 μL LC-MS injections of compound **1** at 20, 100, and 200 ng/μL generated a standard curve ($y = 123x - 0.098$, $R^2 = 1$), and injections of compound **4** at 10, 50, and 100 ng/μL generated a standard curve ($y = 104.32x - 0.098$, $R^2 > 0.99$). The concentrations of compounds **2** and **3** in the fractions and crude extract were estimated by using the standard curve of compound **4**. All injections were repeated at least three times for either standard

curve generation or determination of ITC content in the fractions. Therefore, results are represented as means ± SD of three independent measurements.

Analyses of TPC and ORAC

TPC was quantified according to a modified Folin–Ciocalteu method[26] as previously described[27] and expressed as mean gallic acid equivalents (GAE) of at least three independent experiments. ORAC was determined by using fluorescein as the fluorescent probe and AAPH as a peroxyl radical generator in a procedure adapted from a previously published method.[28] ORAC results for fractions and ME were expressed as μM Trolox equiv (TE)/g and directly expressed as TE for **1**, **4**, and SF. All samples were assayed in triplicate. Results are presented as means ± SD.

NQO1 Induction Assay: Cell Culture and Treatment

Hepa1c1c7 mouse hepatoma cells (CRL-2026, American Type Culture Collection, Manassas, VA, USA) were plated in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) and grown at 37 °C in a humidified incubator with 5% CO_2 to near confluence in α-MEM without ribonucleosides or deoxyribonucleosides (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin (Cellgro, Manassas, VA, USA), and 10% (v/v) fetal bovine serum. Confluent cells were washed two times with PBS (1×) and incubated in serum-free α-MEM overnight (18 h). Cells were treated in triplicate with BNF (0.05–5 μM), SF (5 μM), extract/fractions (ME, A–C, F, 1, 5, and 10 μg/mL; D, E, 1, 2.5, and 5 μg/mL), and compounds **1** (1, 5, and 10 μM) and **4** (1, 2.5, and 5 μM) in serum-free media for 48 h. The NQO1 activity in the cultured cells was assayed in duplicate at the end of the incubation by using an Abcam NQO1 activity assay kit (Abcam, Cambridge, MA, USA). Briefly, cells were washed with PBS (1×) twice and solubilized in 0.2 mL of extraction buffer supplied by the manufacturer. Cells were scraped and incubated on ice for 15 min. Then, lysates were centrifuged at 18000*g* for 20 min at 4 °C. Supernatant was assayed immediately or stored at −80 °C. The protein concentration in the supernatant was quantified by the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The NQO1 activity was measured at 440 nm for 5 min. The linear rate of increase in absorbance was examined over time. The NQO1 activity was normalized to total protein content of each sample to eliminate sample-to-sample variability. NQO1 fold induction was expressed as specific activity of treatment over those of control. Results are means ± SD of three independent experiments.

Cell Viability

Hepa1c1c7 cells were plated into 24-well plates at 1×10^5 cells/well and grown for 48 h (90% confluent), and then confluent cells were washed two times with PBS (1×) and incubated in serum-free α-MEM overnight (18 h). ME (1–25 μg/mL), fractions (A, B, 10 and 25 μg/mL; C–F, 1–25 μg/mL), and compounds **1** and compound **4** (both 5 and 10 μM), dissolved in ethanol (0.25% v/v final concentrations), were added to cells together with vehicle control (ethanol alone) and incubated for an additional 48 h. The effect of treatments on cell viability was examined by using MTT (TCI, Portland, OR, USA).[29] MTT working solution (5 mg/mL in 1× PBS) was filtered through a 0.22 μm cellulose membrane (Corning,

Corning, NY, USA) and added to treated cells during the last 3 h of treatment (48 h). Media were carefully aspirated; cells were dissolved in DMSO, and the absorbance was read at 570 nm. Results are means ± SD of three independent experiments.

Statistical Analysis

Data were analyzed by one-way ANOVA followed by a Dunnett or Wilcoxon test as indicated. Pearson's correlation coefficients for ORAC results with TPCs were calculated using means of triplicate measurements. For statistical analysis GraphPad Prism version 6.02 for Windows (GraphPad Software, Inc.) was used.

RESULTS AND DISCUSSION

Optimization of FCPC Solvent System

Various two-phase solvent systems for FCPC separations were tested and *K* values measured for compounds **1** and **4** (Table 1). Three two-phase solvent systems *n*-butanol/ethyl acetate/water, methanol/ ethyl acetate/water, and *n*-hexane/ethyl acetate/methanol/water at different volume ratios could not provide acceptable partition coefficients, ideally 0.5–2.0 for efficient separation.[30] The three-phase solvent system composed of *n*-hexane/methyl acetate/acetonitrile/water (4:4:3:4) was selected for the current study as the best solvent system on the basis of the optimal partition coefficient of ITCs between the MP and LP. Accordingly, the ideal $K_{MP/LP}$ value obtained for **1** (1.61) together with a larger value for **4** (5.14) suggest that relatively less hydrophilic **4** should be eluted first by MP, followed by more hydrophilic **1** by LP. With this strategy, **1** and **4** were separated completely within a single FCPC run even though their structure differed only by the presence of one acetyl group.

Separation by FCPC

The UV absorption of compounds separated by FCPC in ME is shown in Figure 2. The starting mobile UP was eluted for 100 min to obtain hydrophobic compounds and then switched to MP to elute moderately hydrophilic compounds for 100 min. Finally, the highly polar compounds were separated by eluting the column with LP for 150 min. LC-MS analyses revealed that fractions A, collected between 0 and 92 min (393 mg; 13.1%), and B, collected between 94 and 172 min (231 mg; 7.7%), were composed of nonpolar compounds such as fatty acids and chlorophylls. Fractions C, collected between 176 and 196 min (111 mg; 3.7%), D, collected between 198 and 204 min (123 mg; 4.1%), and E, collected between 206 and 224 min (108 mg; 3.6%), contained high levels of compounds **1**–**4** (Figure 3). The UV profile of fraction C showed compound **4** representing 94% of the total peak area (Figure 3A) and fraction E with compound **1** at 87% of total peak area (Figure 3C), demonstrating efficient separation of these two structurally similar compounds by FCPC. Fraction D contained all ITCs (**1**–**4**) together with niazicin A (see enlarged representation in Figure 3B). Fraction F (2.014 g, 67%), eluted between 226 and 360 min (Figure 2), contained the majority of polyphenolic compounds.

Enrichment by SPE

ITCs **1**–**4** were quantified in fractions C, D, and E before and after SPE by LC-MS analysis as previously described[6] and expressed as percent of fraction dry weight (DW). There was significant enrichment in the total concentration of ITCs (**1**–**4**), after SPE in all three fractions (Figure 4). The concentration of ITCs (**1**–**4**) was increased by 3.7-fold in fraction C-SPE, yielding concentrations of 1.62 ± 0.07% (**1**), 1.50 ± 0.01% (**2**), 1.66 ± 0.11% (**3**), and 5.4 ± 0.08% (**4**) of fraction DW. Fraction D-SPE was enriched by 2.1-fold yielding concentrations of 6.41 ± 0.01% (**1**), 4.00 ± 0.05% (**2**), 1.85 ± 0.05% (**3**), and 5.18 ± 0.06% (**4**) of fraction DW. The highest enrichment (15-fold) was observed in fraction E, initially containing 2.0% (±0.14) of **4** and increasing to 30% (±0.43) of fraction DW after SPE. Overall, fractions obtained by FCPC/SPE contained higher percentage values of **1**–**4** when compared to the crude extract, which contained 1.46% (±0.05) of **1**, 1.36% (±0.07) of **4**, and undetectable amounts of **2** and **3**. These results demonstrated an efficient method to concentrate ITCs for bioassay applications or for further purification by HPLC.

TPC and ORAC

Generally, phenolic compounds are direct antioxidants, which can effectively scavenge and/or chelate readily oxidizable reactive compounds. Moringa extracts and fractions containing phenolic acids (chlorogenic acid, ellagic acids) and flavonoids (mostly quercetin, kaempferol, and rutin) possess direct antioxidant activity in several in vitro assays including β-carotene bleaching, ferric reducing, DPPH/super-oxide/hydroxyl radical scavenging, and lipid peroxidation.[18,31] However, there have not been any studies evaluating the role of moringa ITCs in direct antioxidant activity. Therefore, we evaluated the direct antioxidant capacity of moringa FCPC fractions containing different classes of compounds ranging from essential oils and chlorophylls to ITCs and polyphenols by using the in vitro ORAC assay. ORAC provides an improved analysis technique for direct antioxidant capacity by measuring the inhibition of the peroxyl radical (predominant free radical in biological system) damage over a time course and combining these factors into a single “area under curve” (AUC) quantity.[32] We also measured TPC of each fraction to establish the contribution of polyphenol components of moringa to direct antioxidant capacity (Figure 5A).

As seen in Figure 5A, the highest ORAC value was obtained for ME (12721 ± 310 μmol TE/g extract) with the highest TPC (18.1 ± 0.3 mg GAE/100 mg). The ORAC of ME was higher than reported values for coffee powder (10007 μmol TE/g), black tea leaves (1566–1629 μmol TE/g), grape seeds (11681–11889 μmol TE/g), and blueberry extracts (2441–2792 μmol TE/g), which have been noted for their high antioxidant values.[32,33] Although TPC of fraction F was 2.6 times less than TPC of ME, the ORAC of fraction F (12452 ± 488 TE/g) was not significantly different from the ORAC of the ME. The LC-MS analysis of fraction F showed much higher peak areas of rutin, chlorogenic acid, and quercetin-malonylglucoside compared to the ME, suggesting these polyphenols concentrated in fraction F play a significant role in the observed high direct antioxidant activity.

In SPE-enriched ITC fractions C, D, and E, the ORAC values were found to be moderate, almost 2–3 times less than that of fraction F. Statistical analyses performed for these

fractions revealed that, although ORAC values were significantly correlated ($r = 0.99$, $p < 0.05$) with TPC, they did not correlate with total ITC content. These findings support the role of moringa polyphenols as direct antioxidants, however, leaving the role of ITCs uncertain. For further investigation, we determined the ORAC of compounds **1** and **4** in comparison to SF, which has no direct antioxidant activity.[34] Figure 5B reports the linear regression analyses of trolox, compounds **1** and **4** (ranging from 1.25 to 12.5 μM), and SF (12.5–62.5 μM) with respect to their net AUC value. SF was found to have the lowest ORAC value (0.09 TE); however, both **1** (0.54 TE) and **4** (0.38 TE) had 6 and 4.2 times higher ($p < 0.0001$) ORAC compared to SF. This relatively high antioxidant capacity of compounds **1** and **4** most likely can be attributed to the presence of an aromatic side chain and increased stability of molecules due to the presence of a rhamnose sugar moiety (Figure 1B). On the basis of previous papers, ORAC values of **1** and **4** are considerably lower than those of other direct acting phenolic compounds such as quercetin (7.28 TE), rutin (6.01 TE), and chlorogenic acid (3.14 TE).[33] On the other hand, the similarity of our data to the ORAC of some physiologically active endogenous antioxidants such as glutathione (0.57 TE) and ascorbic acid (0.75 TE)[35] suggests that moringa ITCs may act as direct antioxidants.

Effects of ME, Fractions, and ITCs on NQO1 Catalytic Activity

Up-regulation of phase II XMEs by indirect antioxidants has been widely accepted as one of the preventive mechanisms of action against several pathological conditions. Among these enzymes, NQO1 has been accepted as a biomarker in the discovery of natural chemopreventive and anti-inflammatory agents due its widespread presence in all mammalians, high magnitude of response toward inducers, and ease of enzymatic detection.[36] The measurement of NQO1 activity in Hepa1c1c7 mouse hepatoma cells provides a reliable, specific, and reproducible method for determining inducer potency of pure[25,37,38] as well as complex bioactive (mixtures or plant extracts) compounds.[39,40] In the current work, cell viability was tested over a range of 1–25 μg/mL for fractions/ME and 5–10 μM for pure ITCs to determine doses that maintain cell viability of >95% (see Figure S1 in the Supporting Information), confirming that the observed induction was due to potency of inducers rather than an artifact of cytotoxicity in the NQO1 assay. Accordingly, ME, SPE fractions, and pure ITCs were all tested at at least three noncytotoxic doses (Figures 6A and 7). The ME (1, 5, and 10 μg/mL) significantly ($p < 0.001$) induced NQO1 activity up to 4.8-fold compared to untreated-control cells in the mouse Hepa1c1c7 cell line (Figure 6A). The three SPE-enriched ITC fractions, C (1–10 μg/mL), D, and E (both at 1–5 μg/mL), also significantly ($p < 0.001$) elevated NQO1 activity. Figure 6B represents induction level of fractions and ME at a fixed dose of 5 μg/mL (common noncytotoxic dose for all) together with total ITC yield. The highest level of induction in NQO1 activity was detected in fraction E containing the highest total ITC yield (30%, ITCs), followed by fractions D (17.4% ITCs) and C (11.5% ITCs). The correlation of ITC yields in the fractions with their NQO1 induction capacity confirms that the compounds with ITC pharmacophore (–

ASSOCIATED CONTENT

Supporting Information

Figure S1 presenting results of MTT assay in Hepa1c1c7 cells. This material is available free of charge via the Internet at http://pubs.acs.org

N═C═S group) are highly effective in inducing indirect antioxidant activity. Fractions A and B, which do not contain ITCs, showed NQO1 activity at the same low level as the control, indicating the fatty acids and chlorophylls do not have any indirect antioxidant activity.

Similarly, fraction F did not induce NQO1 activity even at the relatively high dose (10 μg/mL; Figure 6A), indicating polyphenols present in this fraction (predominantly hydroxylated flavones, quercetin 3-*O*-rutinoside, quercetin 3-*O*-(6″-malonylglucoside), and 5-caffeoylquinic acid) do not possess indirect antioxidant capacity, confirming a recent study which showed that hydroxylated flavones are ineffective at inducing cytoprotective enzymes, despite having substantial direct antioxidant activity.[41] However, other classes of polyphenols, including methoxylated flavones, have been shown to significantly increase NQO1 and elicit cytoprotective enzyme activity.[20,38] Accordingly, we observed a clear dose-dependent induction pattern in cells treated with β-naphthoflavone (5,6-benzoflavone, BNF) (Figure 6B), a classical phenolic inducer of NQO1,[42] confirming the sensitivity of the cells to certain polyphenols under our experimental conditions.

As seen in Figure 7, in addition to the ME and FCPC fractions, compounds **1** (1–10 μM) and **4** (1–5 μM) were tested for their NQO1 inducer capacities in comparison to SF (5 μM). Previously, it was shown that SF is the most potent inducer of NQO1 activity, reaching a maximum induction of 3–5-fold between 2.5 and 8 μM concentrations.[25,37,43] In accordance with these studies, the current work showed 5 μM SF induced NQO1 activity 4.2-fold. At this concentration, compounds **1** and **4** induced NQO1 activity 4.3-and 3.9-fold, respectively, showing that SF and ITCs (**1**, **4**) from moringa are equipotent inducers of NQO1. For compound **1**, concentrations of 10 μM did not cause any greater induction than 5 μM did. Similarly, a dose response could not be observed from 1 to 5 μM for compound **4**, indicating that a clear dose-dependent response occurs under 1 μM dose range. To our knowledge, this is the first study demonstrating that moringa ITCs can significantly induce NQO1 activity at such low concentrations; therefore, it may be a practical dietary alternative to SF from broccoli due to increased stability as previously shown.

In summary, the separation and concentration of distinct classes of compounds in moringa leaves, namely, phenolics and ITCs, were successfully achieved through FCPC fractionation followed by SPE. These enriched fractions allowed direct and indirect antioxidant analysis, distinguishing which phytochemicals are responsible for differential antioxidant mechanism. We determined that the polyphenol-rich fraction had high ORAC, indicating strong direct antioxidant activity, whereas ITC-enriched fractions, compounds **1** and **4**, effectively induced NQO1 activity, representing their potent indirect antioxidant activity.

The unique combination of direct (polyphenols) and indirect (ITCs) antioxidant compounds in moringa provides justification and support for the therapeutic uses of polyphenol-and ITC-enriched moringa products. One of the major findings of this study is that compounds **1** and **4** are as potent as SF in inducing NQO1 activity. This indirect antioxidant potency of moringa ITCs, combined with their unique chemical stability and anti-inflammatory activity, makes them attractive food-based therapeutic alternatives to unstable ITCs such as SF. Health-related applications will require the purification of ITCs (**1**–**4**) in significant

quantities. The large-scale fractionation followed by SPE enrichment techniques reported here may provide an efficient strategy for ITC isolation.

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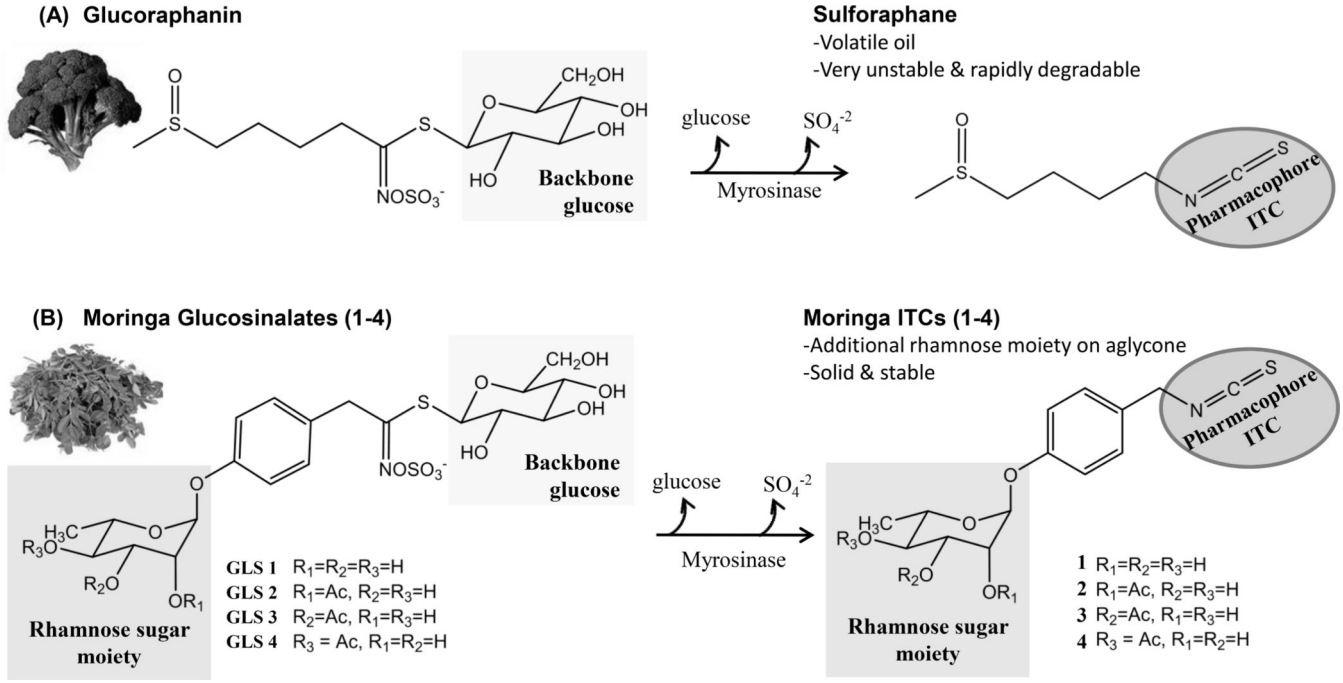

Figure 1.

Enzymatic conversion of (A) glucorophanin from broccoli to sulforaphane (SF) and of (B) moringa glucosinolates (GLSs) to isothiocyanates (ITCs) **1**–**4**.

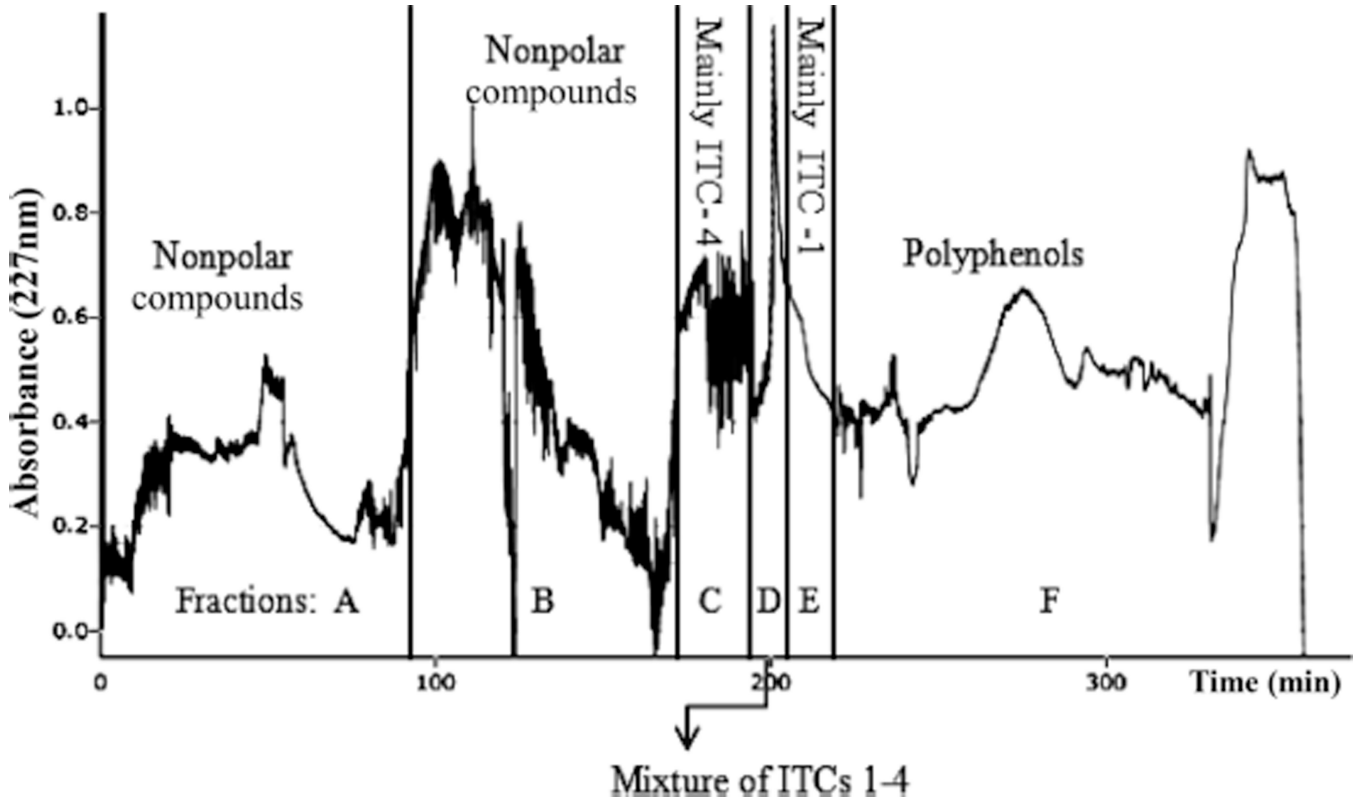

Figure 2.

Fractionation chromatogram of moringa methanolic leaf extract (ME) by fast centrifugal partition chromatography (FCPC) using *n*-hexane/methyl acetate/acetonitrile/water (4:4:3:4) three-phase solvent system.

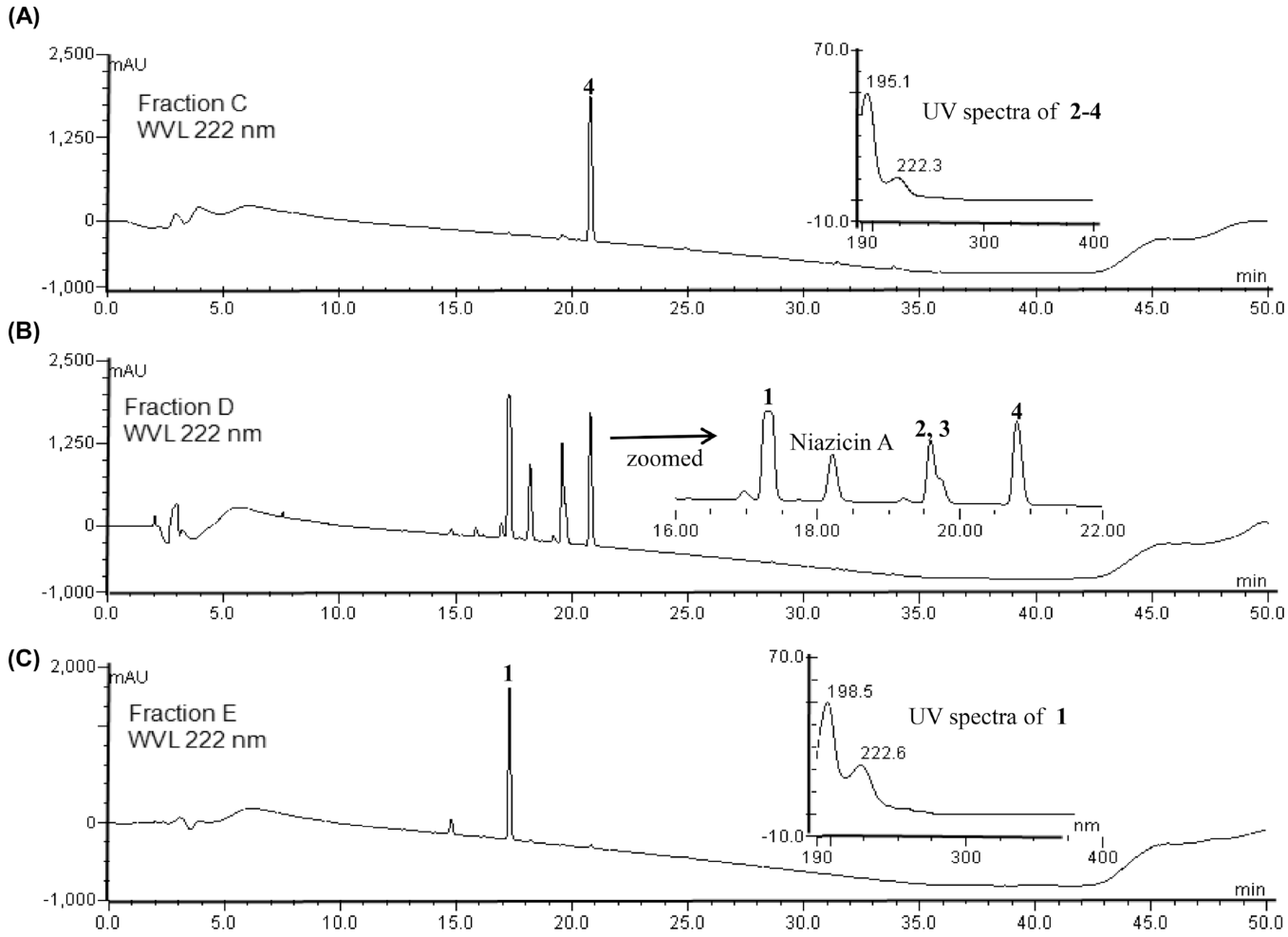

Figure 3.

LC-MS analysis of fractions C, D, and E. (A) Fraction C. Elution peak corresponds to compound **4**; UV spectra of ITCs **2**–**4** (isomers) are also presented on the right. (B) Fraction D. Elution peaks seen in the inset correspond to **1**, niazicin A, and **2**–**4**, respectively. (C) Fraction E. Elution peak corresponds to compound **1**; UV spectra are also presented on the right.

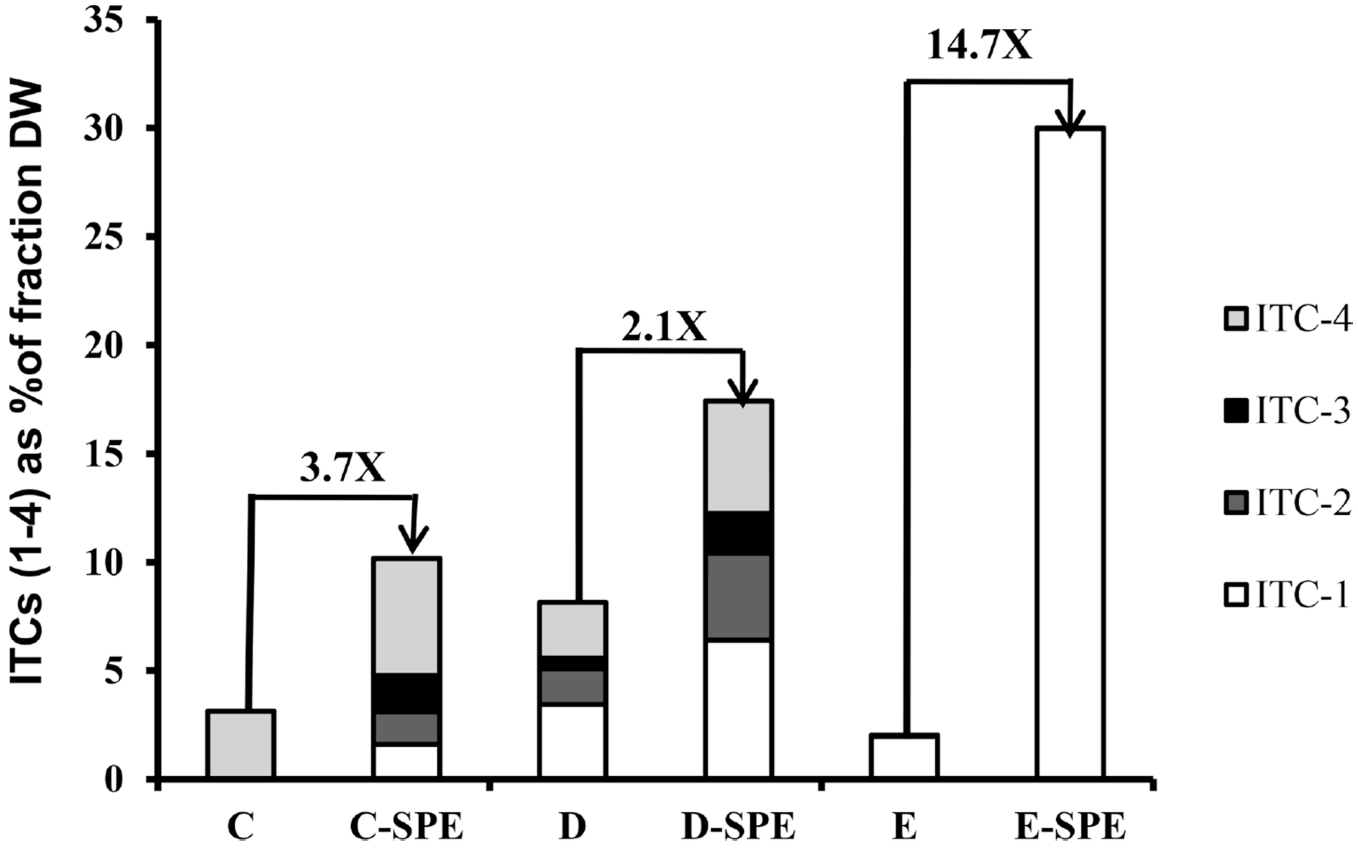

Figure 4.
ITCs (**1**–**4**) as percent of fraction dry weight (DW) before and after solid phase extraction (SPE).

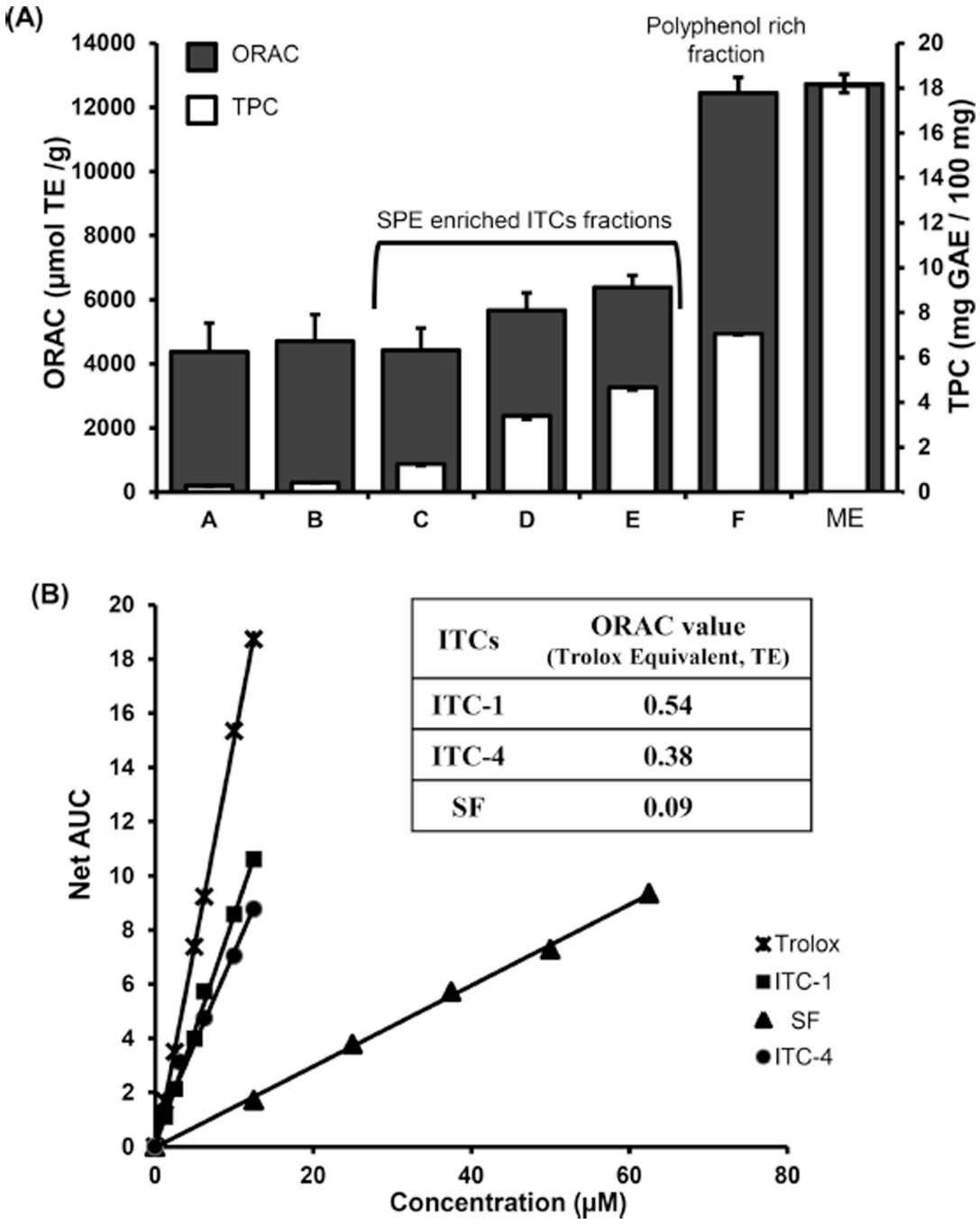

Figure 5.

(A) Oxygen radical absorbance capacity (ORAC) and total polyphenol content (TPC) of fractions (A–F) and moringa methanol extract (ME). ORAC was determined as μmol Trolox equiv (TE)/g. TPC was determined as mg gallic acid equiv/100 mg extract or fractions. (B) Linear regression of ORAC value as a function of the concentration (μM) of Trolox (y = 1.534x ± 0.017; r = 0.999), **1** (y = 0.824x ± 0.042; r = 0.989), **4** (y = 0.576x ± 0.042; r = 0.989), and SF (y = 0.1494x ± 0.0048; r = 0.996). The table on the graph shows TE values of each ITC calculated by slope of compound/slope of trolox. The net AUCs were calculated

by subtracting AUC of blank (75 mM potassium phosphate buffer at pH 7.4) from AUC of sample. Data represent the mean of three independent experiments.

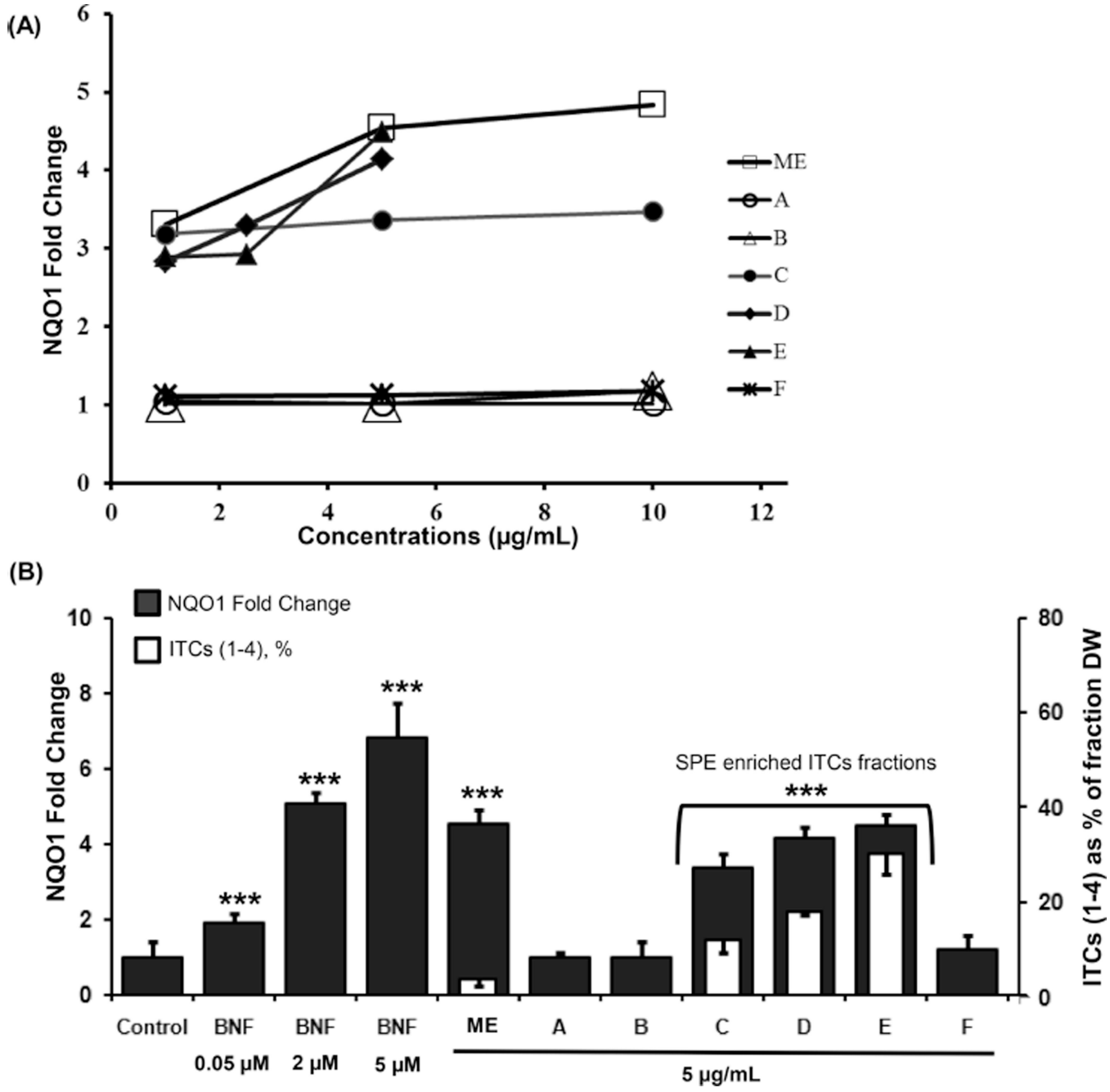

Figure 6.

Effect of moringa methanol extract (ME) and its FCPC fractions (A–F) on the induction of NQO1 catalytic activity (treated/untreated) in Hepa1c1c7 cells (A) at three different noncytotoxic doses and (B) at fixed common noncytotoxic doses (5 μg/mL) in comparison with corresponding ITC concentration (**1**–**4**, % by DW) in each fraction. β-Naphthoflavone was used as a positive control. Data represent the mean of three experiments ± SD; ***, $p < 0.001$.

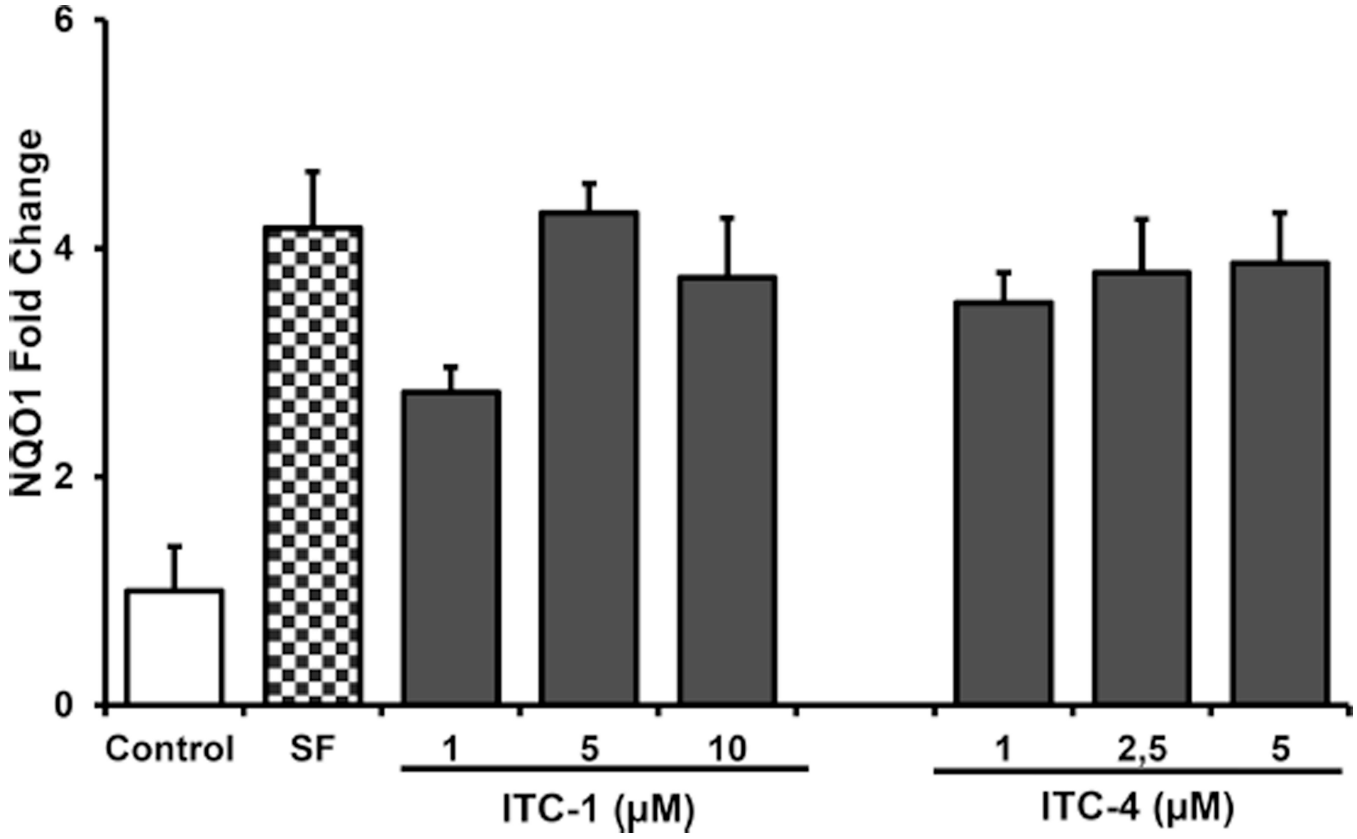

Figure 7.

Effect of compounds **1** and **4** and sulforaphane (SF) on the induction of NQO1 catalytic activity (treated/untreated) in Hepa1c1c7 cells. Data represent the mean of three experiments ± SD, which are significantly different (***, $p < 0.001$) for all compared to control.

Table 1

Partition Coefficients (*K*) of 1 and 4 in Various Solvent Systems

		partition coefficient[a] (*K*)	
solvent system	**ratio**	**1**	**4**
n-butanol/ethyl acetate/water	1:1.5:2.5	28.5	74.5
methanol/ethyl acetate/water	1:3:2	3.97	9.66
	1:2:2	3.97	8.24
n-hexane/ethyl acetate/methanol/water	4:6:4:6	0.01	0.03
	3:7:4:6	0.09	0.37
	3:7:3:7	0.02	0.10
	35:100:35:100	0.02	0.07
n-hexane/methyl acetate/acetonitrile/water[b]	4:4:3:4	1.61[c]	5.14[c]

[a] Expressed as concentration at upper phase/concentration at lower phase.

[b] Solvent system used in the current study.

[c] Three-phase solvent system: concentration at middle phase/concentration at lower phase.