RESEARCH ARTICLE

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Differential Effects of Betacyanin and Betaxanthin Pigments on Oxidative Stress and Inflammatory Response in Murine Macrophages

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Scope: Betalain pigments are increasingly highlighted for their bioactive and anti-inflammatory properties, although research is lacking to demonstrate contributions of individual betalains. The work herein aimed to compare effects of four main betalains on inflammatory and cell-protective markers and to highlight potential structure-related relationships of the two main subgroups: betacyanins vs betaxanthins.

Methods and results: Murine RAW 264.7 macrophages were stimulated with bacterial lipopolysaccharide following incubation with betacyanins (betanin, neobetanin) and betaxanthins (indicaxanthin, vulgaxanthin I) in concentrations from 1 to 100 µM. All betalains suppressed expression of pro-inflammatory markers IL-6, IL-1β, iNOS, and COX-2 with tendency for stronger effects of betacyanins compared to betaxanthins. In contrast, HO-1 and gGCS showed mixed and only moderate induction, while more emphasized effects were observed for betacyanins. While all betalains suppressed mRNA levels of NADPH oxidase 2 (NOX-2), a superoxide generating enzyme, only betacyanins were able to counteract hydrogen peroxide induced reactive oxygen species (ROS) generation, in alignment with their radical scavenging potential. Furthermore, betaxanthins exerted pro-oxidant properties, elevating ROS production beyond hydrogen peroxide stimulation.

Conclusion: In summary, all betalains display anti-inflammatory properties, although only betacyanins demonstrate radical scavenging capacities, indicating potential differing responses under oxidative stress conditions, which requires further research.

1. Introduction

Betalains are a group of water-soluble natural pigments found in plants of the order *Caryophyllales*[1] with the most common dietary sources of betalains being red beetroot, dragon fruit, prickly pear, and amaranth. There are two main chemical groups of betalains, the red-violet betacyanins and yellow-orange betaxanthins. Both share betalamic acid which is in the case of betacyanins conjugated with cyclo-DOPA and O-glycosylation or acetylation whereas betaxanthins are condensation products of betalamic acid with differing amino acids, e.g., proline in indicaxanthin and glutamine in vulgaxanthin I.[2] Betanin, a glucoside, is the most abundant betacyanin with intense red coloring properties, and red beetroot as a main source while neobetanin is considered as the main thermal degradation product of betanin. Indicaxanthin and vulgaxanthin I are typical representatives of the betaxanthin subgroup predominantly found in yellow and red varieties of prickly pear and beetroot, respectively. Due to their strong tinctorial properties, betalains are generally used as food colorants but have recently gained

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attention as they have shown in vitro and in vivo bioactive properties such as antioxidant,[3] anti-inflammatory, and antimicrobial activities.[4] Indeed, anti-inflammatory properties were addressed in some studies. For example, Reddy, et al.[5] demonstrated a 97% inhibition of cyclooxygenase-2 (COX-2) enzyme activity with 100 μg mL^{-1} betanin in a direct in vitro enzyme inhibition assay, showing greater inhibitory potential compared to cyanidin-3-O-glycoside which inhibited to 59% at the same concentration. Furthermore, Vidal et al.[6] demonstrated dose-dependent inhibition of betanidin, the deglycosylated form of betanin, towards lipoxygenase (LOX) activity with IC_{50} value of 41.4 μM. Previous in vitro data from intestinal Caco-2 cells showed a marked dose-dependent reduction of pro-inflammatory markers such as interleukin 6 (IL-6), interleukin 8 (IL-8), and nitric oxide (NO) following treatment with purified indicaxanthin (5–25 μM).[7] For example, the secretion of IL-6 and IL-8 was reduced by 75% and 65%, respectively, following 24 h incubation with 25 μM indicaxanthin under interleukin-1β-stimulated conditions. However, a recent study in lipopolysaccharide (LPS)-stimulated macrophages demonstrated pro-oxidant effects of indicaxanthin through fourfold higher reactive oxygen species (ROS) production at 100 μM following LPS stimulation, compared to LPS only and non-stimulated control cells.[8] As well, indicaxanthin-induced overproduction of ROS induced both COX-2 and H-PGDS (microsomal PGE2 synthase-1) in LPS-stimulated macrophages.

In addition to in vitro studies, oral administration of beetroot extract (50–500 mg kg^{-1} body weight) has been shown to reduce in vivo oxidative stress, inflammation, and apoptosis in rodent models of acute inflammation.[9,10] Further, Han et al.[11] observed that oral administration of betanin (25 and 100 mg kg^{-1} day^{-1}) attenuated paraquat-induced acute lung injury in rodents in a dose-dependent manner. Similarly, oral administration of indicaxanthin reduced the response to λ-carrageenin-induced acute inflammation in the pleural cavity of rats[12] which was aligned with dose-dependent inhibition of NF-κB transcription factor signaling (0.5–2 μmol kg^{-1}). In addition, a recent human study demonstrated a reduction of pro-inflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, IL-8, interferon (IFN)-γ, and increase of anti-inflammatory cytokine IL-10 in the plasma of healthy human adults after ingestion of yellow cactus pear fruit pulp (2 × 200 g day^{-1} for 2 weeks).[13]

Oxidative stress, which is the excess production of reactive oxygen species (ROS) due to the imbalance between the production of ROS and the availability of antioxidants to scavenge the ROS,[14] is closely related to inflammation. Both processes can easily be induced by each other and are simultaneously found in many pathological conditions.[15] Antioxidant activity against ROS can be a result of the direct scavenging of free radicals or the activation of endogenous antioxidant defence mechanisms by the test compound.[16] The transcription factor Nrf2 (nuclear factor-erythroid factor 2-related factor 2) is a key sensor of the cellular redox environment modulating antioxidant status and detoxification.[17] Indeed, many plant bioactives have been shown to induce Nrf2 signaling[18] and thereby increase expression of Nrf2 target genes such as glutathione peroxidase (GPX) which neutralizes peroxide radicals and gamma-glutamylcysteine synthetase (γGCS) which catalyzes the key step in the synthesis of the intracellular antioxidant glutathione (GSH) that maintains the cellular redox equilibrium. Recently, betalains have been suggested as potential Nrf2 activators. Esatbeyoglu et al.[16] have demonstrated a moderate Nrf2 induction (1.8-fold increase) at 15 μM betanin in a Huh-7 based transient luciferase reporter cell model. In support of this finding, Lu et al.[19] observed that oral administration of red beetroot extract dose-dependently restored the suppressed expression of antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) in different tissues (liver, spleen, and kidney) of gamma-irradiated mice. The betalain-fed irradiated mice also showed decreases in lipid peroxidation compared to the non-betalain irradiation group. These findings are in line with Kanner et al.,[20] who found that the presence of low concentrations of betanin (IC_{50} 0.4 μM) inhibited lipid peroxidation in cell membranes isolated from turkey muscles, even more effectively than catechin (IC_{50} 1.2 μM) and α-tocopherol (IC_{50} 5 μM).

Although some evidence on the anti-inflammatory and other properties of betalains/betalain-containing extracts is available, there is a lack of studies addressing and comparing the effects of individual betalains on a cellular and molecular level. Importantly, many betalain (-containing) samples used by other researchers are lacking characterization and purity details, these results could therefore be biased by the presence of other compounds such as organic acids, sugars, and phenolics. Given the lack of detailed knowledge on individual compound effects, the current study focused on the comparison of anti-inflammatory and cellular antioxidant properties of four main betalalains representing the two classes: 1) betacyanins, through betanin and the main degradation product neobetanin, and 2) betaxanthins, represented by indicaxanthin and vulgaxanthin I. These four compounds, shown in **Figure 1**, have been purified recently by our group using a newly proposed flash column chromatography method.[21]

Macrophages were selected as a model of cellular inflammation and oxidative stress, as they play important roles to initiate and regulate inflammatory response.[22] Stimulation of macrophages with inflammatory triggers, such as LPS, releases a range of pro- and anti-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-10, which were investigated as part of this study. We further determined markers of antioxidant and oxidative stress, such as heme oxygenase (HO-1) and γGCS, as well as impact of betalains on exogenous and endogenous generation of reactive oxygen species (ROS).

2. Experimental Section

2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g L^{-1}, without/with phenol red), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, and 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Thermo Fisher Scientific (Loughborough, UK). DuoSet ELISA (TNF-α) was purchased from R&D Systems (Abingdon, UK). Trisure reagent and SensiFast Hi-ROX SYBR Green reagent were obtained from Bioline (Nottingham, UK) and iScript cDNA synthesis kit from Bio-Rad (Hertfordshire, UK). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, Sulforhodamine B Sodium salt (SRB), Trizma base, Trichloroacetic acid (TCA), acetic acid, hydrogen peroxide

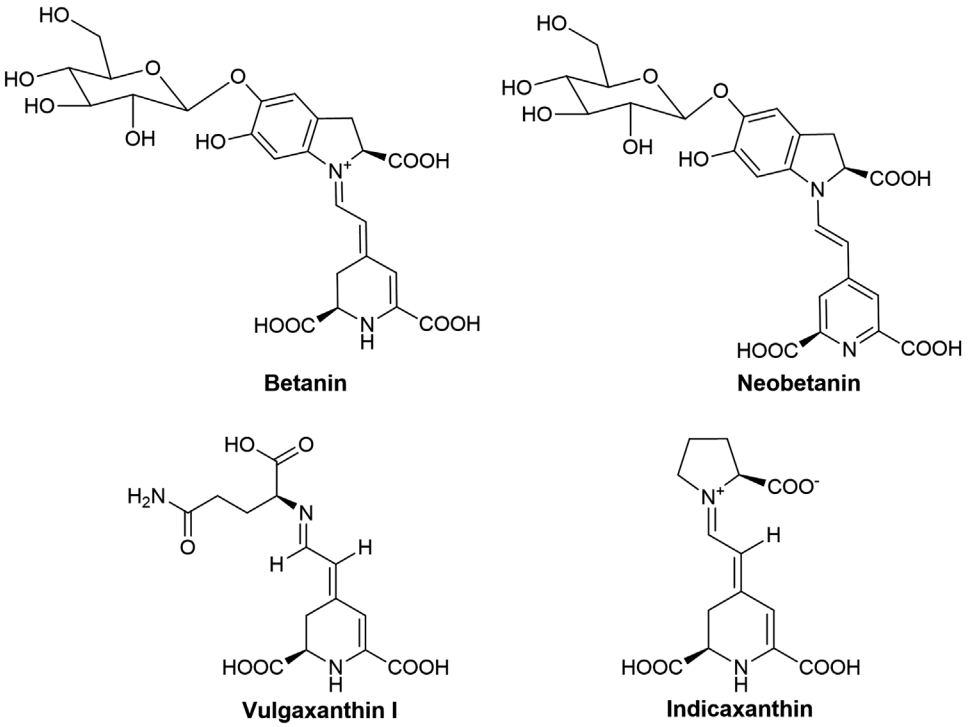

Figure 1. Chemical structures of betalains: betanin, neobetanin, vulgaxanthin I, and indicaxanthin.

(H_2O_2, 30 wt%), DMSO, resazurin sodium salt, and all other reagents at analytical grade were purchased from Sigma-Aldrich (Dorset, UK) unless specified otherwise.

2.2. Betalain Samples (Purified Betalains and Crude Extracts)

Betanin (BET, CAS 7659-95-2), neobetanin (NEO, CAS 71199-29-6), vulgaxanthin I (VGX, CAS 904-62-1), and indicaxanthin (IDX, CAS 2181-75-1), were previously isolated from red beetroot and yellow prickly pear[21] with purities of 97%, 52%, 79%, and 95% respectively (as per HPLC). All compounds were dissolved in 30% v/v ethanol to prepare 10 mM stock solutions and stored at −20 °C for further experiments. Further, extracts from red and yellow beetroot powder (kindly provided by Biopower Ltd., Milton Keynes, UK) were prepared using ultrasound-assisted extraction as previously described Fernando et al.[23] followed by spray drying of the extract using Mini Spray dryer (Buchi, Switzerland) with the following drying conditions: inlet air temperature 121 °C, outlet air temperature 76 °C, and pump pressure 30%. The resulting powdered samples were stored at 4 °C after replacing the air gap with N_2 gas.

2.3. Cell Cultivation

Murine RAW 264.7 macrophages, purchased from the European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK, were cultured in high glucose DMEM medium supplemented with 10% heat-inactivated FBS, and 1% penicillin/streptomycin and maintained in a humidified incubator under standard conditions (37 °C, 5% CO_2). The medium was changed every 2 days and cells were passaged when reaching around 70–80% confluence. For experiments, macrophages were seeded the day before into either 12, 24, or 96 well plates at a density of 1.1×10^5 cells cm^{-2}.

2.4. Cell Viability Assay

Cell viability was determined by a resazurin-based assay.[24] To this end, cells were seeded into 24-well plates and upon reaching 70–80% confluence, they were incubated for 24 h with the test compounds. Then, the resazurin stock solution (44 mM, dissolved in DPBS; sterile filtered, 0.2 µm) was diluted with DMEM medium to prepare 44 µM resazurin working solution. The cell culture medium was discarded and replaced by resazurin-containing medium. After 3 h incubation, aliquots of the supernatants (2 × 200 µL per sample) were transferred into wells of a black 96-well plate and fluorescence intensity measured at 560 and 590 nm, excitation and emission wavelengths, respectively, using Tecan SparkTM 10 M multimode microplate reader (TECAN, Männedorf, Switzerland). Cell viability was expressed in % of untreated cells.

2.5. Determination of Reactive Oxygen Species

Intracellular ROS production was determined using a recently described method by Ng and Ooi[25] with normalization of DCF values to the respective protein level using SRB assay. Cells were seeded in black clear-bottom 96-well plates. After 24 h, the cells

Table 1. Murine primer sequence information.

Gene	Gene Bank Accession No	Forward primer (5' – 3')	Reverse primer (5' – 3')
β-actin	NM_007393.5	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
HO-1	NM_010442.2	GAGCCTGAATCGAGCAGAAC	AGCCTTCTCTGGACACCTGA
iNOS	NM_0013 13922.1	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
IL-6	NM_0013 14054.1	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
γGCS	NM_010295.2	AGTTCCGACCAATGGAGGTG	TCTCGTCAACCTTGGACAGC
COX-2	NM_011198.5	TTCAACACACTCCTATCACTGGC	AGAAGCGTTTGCGGTACTCAT
IL-10	NM_010548.2	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
NOX-2	NM_007807.5	CACTTTGCTGTGCACCATGA	CGCCTATTGTGGTGTTAGGG
IL-1β	NM_008361.4	CAGGCAGGCAGTATCACTCA	AGCTCATATGGGTCCGACAG

were incubated with 20 μM H_2DCFDA diluted in phenol red-free medium for 30 min at 37 °C. Then, the macrophages were treated with different concentrations of BET (0, 0.4–100 μM) or the other betalains (1, 5, 25 μM) for 1 h, followed by addition of H_2O_2 (250 μM) to initiate oxidative stress. Betalain concentrations were chosen based on the literature Allegra et al.,[8] with an initial maximum of 100 μM for BET, and lowered to establish lowest effective concentration. The fluorescence intensity was measured using Tecan microplate reader at 485 nm and 535 nm excitation and emission wavelengths, respectively, after 30–60 min in scanning mode. Subsequently, the medium was removed and 50 μL of SRB-TCA (0.004% SRB in 10% TCA, both w/v) added to each well. After incubating for 15 min at 4 °C, SRB-TCA was removed and carefully washed with 200 μL of 1% acetic acid. Finally, 100 μL of 10 mM Trizma base solution was added to each well and incubated for 5 min at room temperature (RT). The fluorescence intensity was measured at excitation 540 nm and emission 590 nm. DCF and SRB values were corrected for background values using cell-free controls. The ratio of DCF to SRB was calculated for individual wells to normalize ROS data to cellular protein content.

2.6. RNA Isolation and Quantitative Real-Time PCR

Cells in 12-well plates, 70–80% confluent, were incubated with different concentrations of purified betalains (0, 1–100 μM), spray-dried extracts (10, 50, 400 μg mL^{-1}) or positive control (curcumin, 10 μM) for 1 h, followed by incubation with LPS (100 ng mL^{-1}) for 6 h. Concentrations of betalains and spray-dried extracts were chosen based on the literature and preliminary experiments, respectively. Subsequently, RNA was isolated using Trisure reagent according to the manufacturer's instructions. Total RNA quantity and quality were determined by Tecan plate reader using Nanoquant plate application. RNA was reverse transcribed into cDNA using iScript cDNA synthesis kit according to the manufacturer's instructions. Amplification of target gene mRNA was conducted using SensiFast SYBR Green reagent using StepOne Real-Time PCR machine (Thermo Fisher) with recommended settings. Primer sequences were designed using NCBI tools, reported in previous research[26,27] and shown in **Table 1**. Target gene expression was normalized to β-actin (reference gene) and relative gene expression was calculated using the $2^{-\Delta\Delta C}{}_T$ method described by Livak and Schmittgen.[28]

2.7. Quantification of TNF-α

RAW 264.7 macrophages, grown 70–80% confluent in 24-well plates, were treated with different concentrations of betalains, or curcumin (10 μM) for 1 h and subsequently added LPS (100 ng mL^{-1}) for 24 h. The TNF-α secretion into the medium was determined by immunoassay following the manufacturers' instructions.

2.8. Electron Paramagnetic Resonance Spectroscopy (EPR)

EPR spectra of DPPH- and trapped superoxide-radicals were measured using a JEOL X320 spectrometer (X-band) in glass capillaries (0.8 mm ID) as previously described by Wang et al.[29] Briefly, DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl assay was conducted by mixing purified betalains (final concentration 20 μM) and DPPH (50 μM) in aqueous ethanol (30%). The EPR signal intensity of the mixture was monitored after 1 min at specific time points (0–67 min). The superoxide assay was conducted by combining 550 μM hypoxanthine, 150 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and 1 mM deferoxamine in aqueous ethanol. Purified betalains or 30% ethanol were added into the above mixture followed by addition of 2 U mL^{-1} of xanthine oxidase to initiate the reaction, resulting in 20 and 50 μM as final betalain concentrations in the reaction mixtures.

2.9. Statistical Analysis

Data were presented as mean ± SEM of three independent experiments performed in duplicates or triplicates. Graphs were created using GraphPad Prism Version 9.0 for Windows (GraphPad Software, La Jolla, CA, USA). One-way ANOVA test was conducted and followed by Dunnett's test to compare treatments with the control group; and $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered as significant.

3. Results and Discussion

3.1. Effects of Betalains on Expression of Pro-Inflammatory Cytokines

Betalain rich sources have been associated with beneficial effects on inflammation, blood pressure, endothelial dysfunction, and

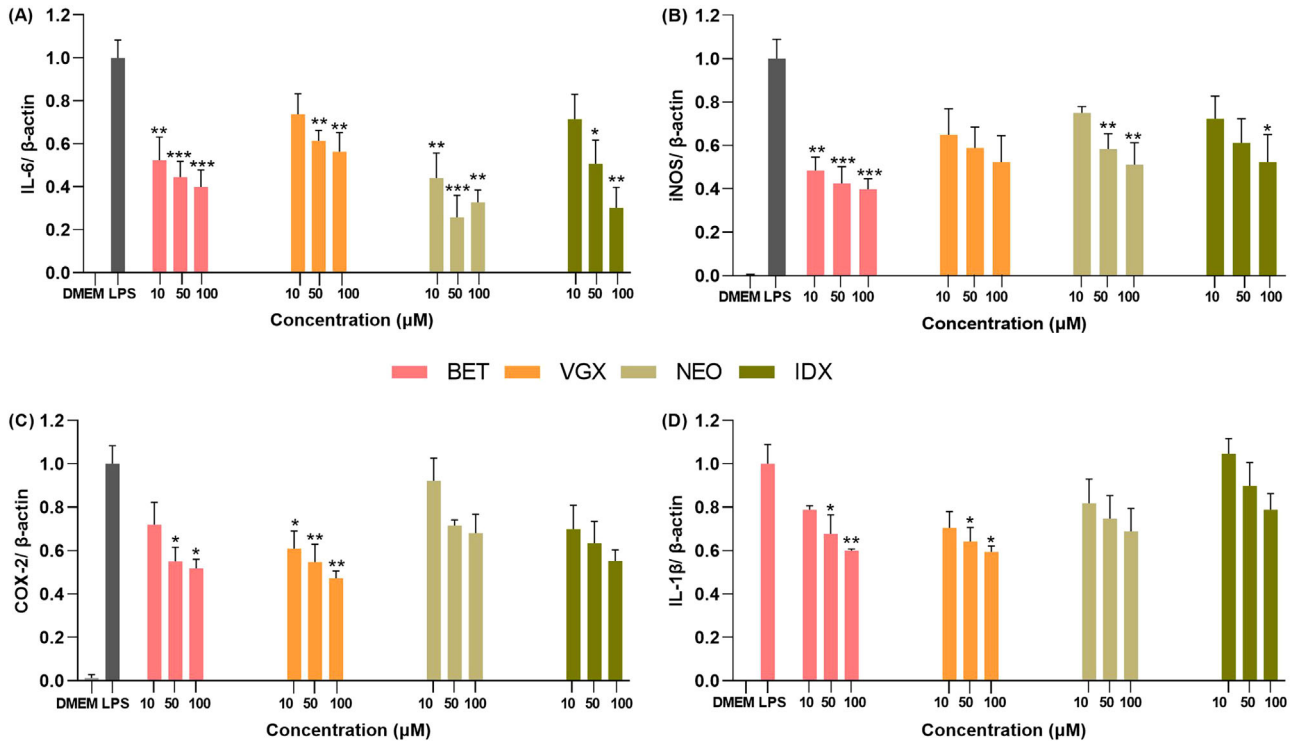

Figure 2. Effects of purified betalains (10, 50, 100 μM) on pro-inflammatory cytokine expression targeting (A) IL-6, (B) iNOS, (C) COX-2, and (D) IL-1β following 6 h LPS stimulation in RAW 264.7 macrophages. Data are mean with SEM of three independent experiments. * Significant difference compared to LPS-stimulated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

apoptosis of tumor cells.[30] However, most studies have applied extracts rather than purified betalains, therefore, the actual contribution of individual betalains is not clear. The present study aimed to establish dose-dependent responsiveness of betalains on inflammation while assessing the differential contribution of selected betacyanins and betaxanthins to inflammatory response and oxidative stress generation. Inflammatory mediators are important to activate immune response and tissue repair, whereas overproduction may lead to induction and progression of chronic inflammatory disease.[31] The transcription factor NF-κB modulates one of the major gene regulatory pathways of inflammatory response thereby stimulating the generation of pro-inflammatory cytokines IL-6, iNOS, IL-1β, COX-2, and others.[32] Therefore, assessing the regulation of inflammatory mediators which relate to NF-κB signaling pathway is an important strategy to recognize the anti-inflammatory potential of different test compounds.

As shown in **Figure 2**, incubations with individual betalains resulted in a dose-dependent down-regulation of pro-inflammatory cytokine mRNA levels at 10, 50, and 100 μM. BET, VGX, NEO and IDX inhibited IL-6 expression by 47.1%, 26.3%, 56.0%, and 28.4%, respectively at 10 μM concentration ($p < 0.05$ for each). While all betalains reduced inflammatory markers, BET was most consistent to inhibit all the tested inflammatory markers. Even at lower concentrations of BET and VGX (1 and 5 μM), cytokine levels were reduced (25–40%), albeit the effects were not significant (Figure S1A–D, Supporting Information).

The results of the present study are in line with Ahmadi et al.[33] who demonstrated that purified BET (500 μM) significantly inhibited ($p < 0.05$) the secretion of IL-6 (50%) and IL-1β (36%) in LPS-induced microglial cells, thereby contribution to reducing neuroinflammation. However, 500 μM is a supraphysiological/pharmacological concentration which is beyond amounts that are bioavailable and circulating in the body. Tesoriere et al.[34] reported a plasma concentration ≈0.2 μmol L^{-1} BET 2 h after consuming 500 g cactus pear fruit (contain 16 mg of BET). In the present study, a concentration range of 1–100 μM of purified betalains was used for inflammatory experiments, and the results demonstrate concentrations as low as 10 μM (Figure 2) and 1 μM (Figure S2, Supporting Information) are efficient to lower inflammatory response. In addition, TNF-α, a primary inflammatory cytokine secreted by inflamed macrophages, was determined in cell culture medium, with a moderate lowering of TNF-α by 18%, 21%, 20%, and 16% following incubation with BET, NEO, VGX, and IDX, respectively, at 5 μM (Figure S2, Supporting Information), compared to the LPS-stimulated control, although there was no dose–response effect with regards to TNF-α secretion of individual betalains.

The influence of purified betalains on cell viability was determined (Figure S3, Supporting Information), as any toxic effects of test compounds may affect immune response and potentially induce oxidative stress. Within the concentration range of betalains that was included in the present study, covering up to 100 μM, no negative effects on cell viability were observed, a finding which is in alignment with the literature.[8,30,33] Although, based on the literature, a concentration of 500 μM purified betanin did not show any toxic effects towards microglial cells, Esatbeyoglu et al.[16] demonstrated toxicity of betanin >15 μM in HT-29 and PON1-Huh7 and >25 μM in Huh7 cells. The source

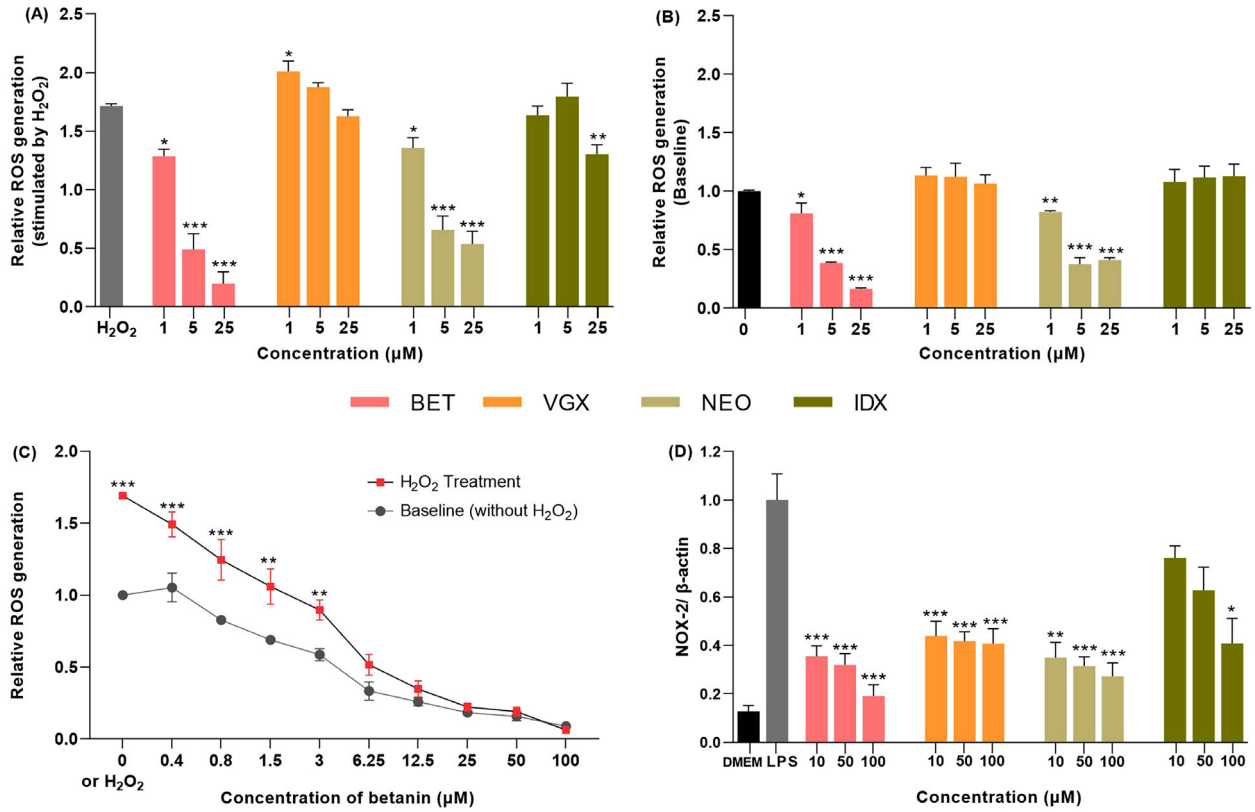

Figure 3. Effects of purified betalains on reactive oxygen species (ROS) formation (A–C) and on mRNA levels of NOX-2, a superoxide generating enzyme (D). Reduction of ROS formation of individual betalains was compared at 1, 5, and 25 μM under (A) H_2O_2-stimulated, (B) baseline conditions and in case of betanin only, over a large range of concentrations (0.4–100 μM) in both conditions. NOX-2 mRNA levels (D) were established following 6 h LPS stimulation. Data are mean with SEM of three independent experiments. * indicates significant difference to H_2O_2/LPS-treated cells (*$p < 0.05$, **$p <$ 0.01, and ***$p < 0.001$).

of betanin in this case was commercially available betanin, diluted with dextrin. While batches may differ, according to our own spectrophotometer-based data, the betanin content of dextrin-diluted betanin was below 2 mg g^{-1} powder indicating presence of considerable amounts of dextrin in the sample, which might have affected cell viability and other outcomes.

3.2. Hydrogen Peroxide-Induced ROS Production and LPS-Stimulated NOX-2 mRNA Expression

Reactive oxygen species (ROS) such as hydrogen peroxide and superoxide are generated during the partial reduction of oxygen during normal metabolism as well as immune response.[35] Accumulation of ROS can result in oxidative stress and contribute to chronic disease etiology and progression, i.e., chronic inflammation and cancer.[36] Further, ROS act as a key signaling molecules as well as mediators of inflammation.[37,38] Cells can modulate their redox status by down-regulating ROS or up-regulating antioxidant defence systems, with the latter benefitting from induction of targets such as γGCS, HO-1, glutathione peroxidase, and superoxide dismutase enzymes that restore cellular glutathione levels and/or support removal of ROS.[31]

The inhibitory effects of betalains on oxidative stress were evaluated by measuring intracellular ROS production following stimulation with hydrogen peroxide (250 μM). As **Figure 3**C indicates, BET decreased ROS generation significantly in a dose-dependent manner ($p < 0.05$), even at low concentrations (0.4–100 μM) which was also evident under baseline (non-stimulated) conditions. In comparison, while neobetanin exerted similar effects to suppress ROS production compared to BET, the betaxanthins, VGX and IDX, did not show reduced ROS production at the further selected concentrations (1, 5, 25 μM), neither at baseline nor under stimulated conditions (Figure 3A,B). In contrast, treatment with VGX at 1 and 5 μM led to 3.8% and 7.5% increases in ROS production, while 5 μM IDX showed 8.2% enhanced ROS production compared to the H_2O_2 stimulated control, indicating pro-oxidant potential of both betaxanthins. Indeed, pro-oxidant properties of IDX were reported by Allegra et al.,[8] demonstrating fourfold higher ROS levels after 12 h incubation under LPS-stimulated conditions. These findings, at relatively high concentration (100 μM) demonstrate a bimodal effect of initial reduction of ROS formation and reduced NF-κB activation; and subsequent significantly higher ROS levels and increased COX-2 and prostaglandin formation. It is assumed that IDX had to be transformed into an active compound in a LPS-dependent reaction which may have triggered anti-inflammatory mediators via prostaglandin metabolism under these conditions. In contrast, the current study used much lower concentrations and different time and experimental settings. Further research is clearly

needed to gain more detailed insight into the dose- and time-dependent molecular changes in response to betaxanthins, and their implication, e.g., for macrophage polarization and inflammatory response.

Pro-oxidant activity of some well-known antioxidants has been observed by other researchers.[39–41] For instance, vitamin C, a strong antioxidant at lower doses (30–100 mg kg^{-1} body weight), shows pro-oxidant activity at high doses (1000 mg kg^{-1} body weight).[42] Reasons for transforming antioxidants into pro-oxidants may be the presence of metal ions or ROS generators such as LPS as well as the concentration of the compound in the matrix and their redox potential.[43]

In addition, direct radical scavenging ability of purified betalains (20 µM) was assessed using electron paramagnetic resonance spectroscopy (EPR), shown in Figures S4A and S4B, Supporting Information. BET and NEO showed 27.1% and 22.4% reduction of EPR signal intensities for superoxide radicals while VGX and IDX demonstrated only 7.7% and 3.2% reduction compared to the control. Moreover, betacyanins showed significant reduction of EPR signal intensities towards DPPH radical formation in comparison to betaxanthins. These results are aligned with the current ROS data showing that betacyanins were more effective to reduce ROS generation while betaxanthins indicate potential for pro-oxidant activity. Esatbeyoglu et al.[16] demonstrated the dose-dependent DPPH- and superoxide radical scavenging activity of BET (diluted with dextrin) (1–10 µM) using EPR which is in line with the results of the present study.

NADPH oxidases (NOX 1–5 and dual oxidases 1–2) are a family of membrane-associated enzymes that generate reactive oxygen species in response to different triggers including infection.[44] NOX-2 is the main enzyme responsible to generate ROS in immune cells such as macrophages, T cells, and neutrophils[45] with NOX-2 derived ROS contribution to generation of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β) through activating NF-κB signaling.[46] In the present study (Figure 3D), all betalains demonstrated a dose-dependent down-regulation of NOX-2 mRNA levels in LPS-activated macrophages (10, 50, 100 µM); in particular, BET, VGX, and NEO showed 65%, 57%, and 66% reduction of NOX-2 at 10 µM, respectively. When investigated further, both BET and VGX showed significant down-regulation of NOX-2 expression, even at 1 and 5 µM (Figure S1E, Supporting Information). In contrast, IDX showed the weakest effect on NOX-2 mRNA with 34%, 37%, and 59% reduction at 10, 50, and 100 µM (Figure 3D). Similarly, Tesoriere et al.[7] found that 25 µM IDX prevented activation of NOX-1 complex resulting in reduced levels of cytosolic NOX-1 in IL-1β-stimulated intestinal Caco-2 cells. Members of the NOX family, i.e., NOX-2, are modulated by NF-κB transcription factor,[47] which explains the alignment of outcomes with inflammatory markers. Interference with NF-κB signaling has also been shown by Manea et al.[48] in TNF-α stimulated smooth muscle cells which is likely the case for all betalains, and in particular for betacyanins. Further, it could be assumed that pro-oxidant tendencies of IDX and VGX might counteract anti-inflammatory mechanisms.

To summarize, the current results indicate that only betacyanins, BET and NEO, can prevent generation of ROS formation directly whereas all betalains demonstrate down-regulation of NOX-2 and indicate capability to suppress inflammation-induced superoxide generation. Higher potency of betacyanins for ROS-inhibition is in alignment with our recent in vitro data and current results from EPR measurements on radical scavenging properties of these four compounds, indicating higher potential of betacyanins in comparison to betaxanthins.[21]

3.3. Effects of Betalains on Expression of Cell-Protective and Anti-Inflammatory Markers

Many bioactive compounds have been shown to modulate the expression of antioxidant enzymes such as HO-1, γGCS, superoxide dismutase, glutathione S-transferase thereby enhancing cell-protective capacities.[49] All of these enzymes are target genes of transcription factor Nrf2 signaling, one of the major signaling pathways that leads to enhanced cell protection against oxidative stress. Nrf2 is inactive in the cytoplasm bound to Keap 1 protein (Kelch-like ECH-associated protein 1) under normal condition.[50] In case of oxidative stress, Nrf2 dissociates from Keap 1 and then translocates into the nucleus to trigger the expression of enzymes such as HO-1 and γGCS involved with antioxidant status and cytoprotective function.[51,52] Indeed, many polyphenols are strong activators of Nrf2 signaling, interrupting Nrf2-Keap 1 binding (as well as being potent anti-inflammatory compounds). The present study sought to establish whether all individual betalains were capable to induce expression of prominent Nrf2 target genes HO-1 and γGCS.

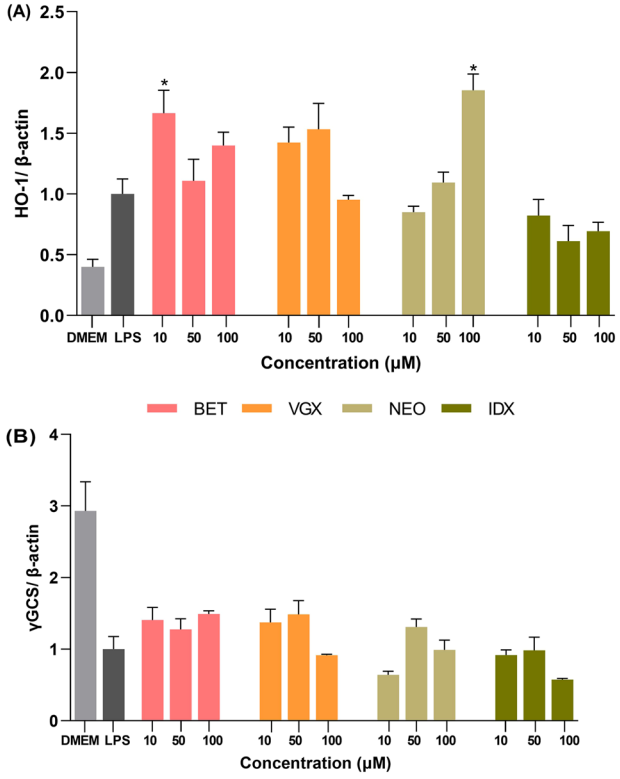

Figure 4. Effects of purified betalains (10, 50, 100 µM) on transcript levels of (A) HO-1 and (B) γGCS following 6 h LPS stimulation in RAW 264.7 macrophages. Data are mean with SEM of three independent experiments. * indicates significant differences to H_2O_2/LPS-treated cells (*$p < 0.05$).

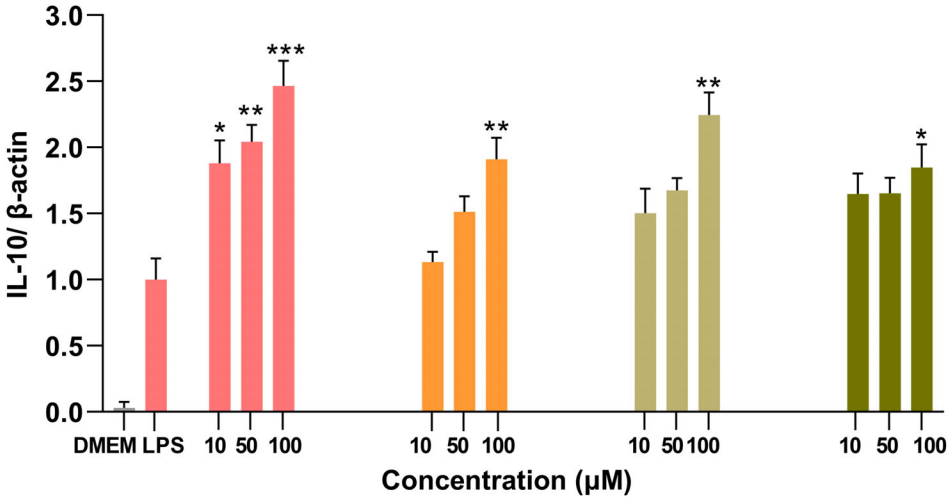

Figure 5. Effect of purified betalains on IL-10 mRNA levels following 6 h LPS stimulation in RAW 264.7 macrophages at 0, 10, 50, and 100 μM. Data are mean with SEM of three independent experiments. * indicates significant differences to H_2O_2/LPS-treated cells (*$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$).

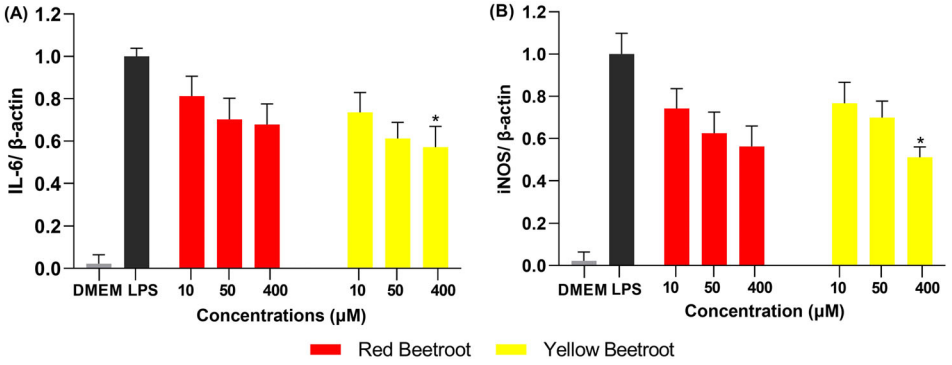

Figure 6. Effect of red and yellow beetroot samples (10, 50, 400 μM) on (A) IL-6 and (B) iNOS mRNA levels following 6 h LPS stimulation in RAW 264.7 macrophages. Data are mean with SEM of three independent experiments. * indicates significant difference to LPS-treated cells ($p < 0.05$).

As shown in **Figure 4**, effects on both targets were somewhat varied and moderate with few marked differences, e.g. increased HO-1 expression by 39% and 85% for BET (10 μM) and NEO (100 μM), respectively. Further, there was no induction of HO-1 with purified betalains under non-stimulated conditions (Figure S5, Supporting Information).

There is overall little knowledge on betalain effects on Nrf2 signaling and target gene expression. Esatbeyoglu et al.[16] has demonstrated a 1.4-fold increase of HO-1 with 25 μM BET compared to the control which is not considered a strong response. Furthermore, Krajka-Kuźniak et al.[53] demonstrated that 2, 10, and 20 μM BET increased cytosolic Nrf2 mRNA levels in THLE-2 cells by 21–30%. Moreover, the same study showed that the protein levels of nuclear Nrf2 were significantly increased by the same doses of BET compared to the untreated cells. In the present study, moderate inductions of HO-1 and γGCS mRNA levels were observed following incubations with purified betalains, however, the results were inconsistent across different concentrations (Figures 4 and S6, Supporting Information).

Further, BET demonstrated a dose-dependent increase of IL-10 mRNA levels by 88%, 104%, and 140% compared to LPS-stimulated cells at 10, 50, and 100 μM, respectively ($p < 0.05$ for each, **Figure 5**). NEO, VGX, and IDX were weaker in their capacity to induce IL-10 which was only significant at 100 μM with 124%, 91%, and 84% ($p < 0.05$), respectively. Lower concentrations of BET and VGX (1 and 5 μM) did not markedly change IL-10 gene expression (Figure S7, Supporting Information). IL-10 is considered as anti-inflammatory cytokine, therefore, increases in IL-10 expression are associated with generalized beneficial effect on immunoregulation and inflammatory response. While not demonstrated for betalains earlier, polyphenols such as apigenin, kaempferol, and resveratrol, have demonstrated promotion of IL-10 production in RAW264.7 macrophages.[54]

Overall, the chemical structure of betalains greatly affects their anti-inflammatory properties. The structural differences can be assumed to impact the compound reactivity which was clearly highlighted in one of our previous studies using in vitro cell free antioxidant activity assays.[55] For instance, betanin showed better attenuation of pro-inflammatory cytokine secretion and ROS production could be linked to its ability to donate hydrogen and electrons in basic solutions when changing from cationic to deprotonated states.[56]

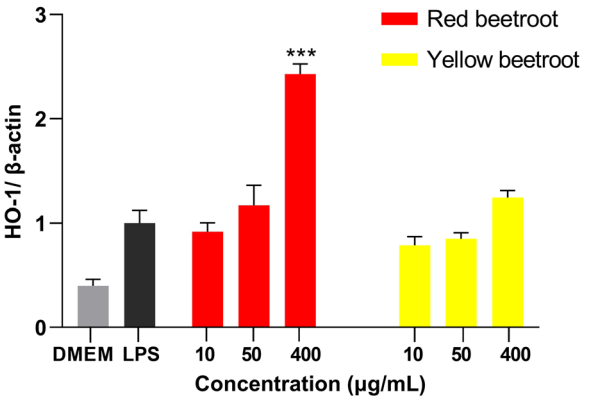

Figure 7. Effects of red and yellow beetroot samples on HO-1 expression following 6 h LPS stimulation in RAW 264.7 cells. Data are mean with SEM of three independent experiments. *** indicates significant difference to LPS-treated cells ($p < 0.001$).

3.4. Effects of Betalain-Containing Extracts on Expression of Selected Pro-Inflammatory and Cell Protective Markers

Further experiments were conducted using extracts from red and yellow beetroot samples (SRB, SYB) which were similarly exposed to macrophages and subsequently stimulated with LPS. These extracts represent more complex samples containing betalains as well as polyphenols and other ethanol/water soluble compounds, and have been used by other authors.[57,58] A dose-dependent down-regulation of IL-6 and iNOS was observed for both, SRB and SYB extracts, demonstrating a 35% and 48% reduction for IL-6 at 400 μg mL^{-1} (**Figure 6**A) whereas iNOS was down-regulated by up to 44% and 49% (Figure 6B), respectively, at this concentration, although only significant for SYB for both markers. With regards to the betalain content, the highest concentration of 400 μg mL^{-1} SRB contained only 1.11 μM equivalent betacyanins (the majority being betanin and isobetanin) and 1.41 μM equivalent betaxanthins (majority vulgaxanthin I) whereas the same concentration of SYB

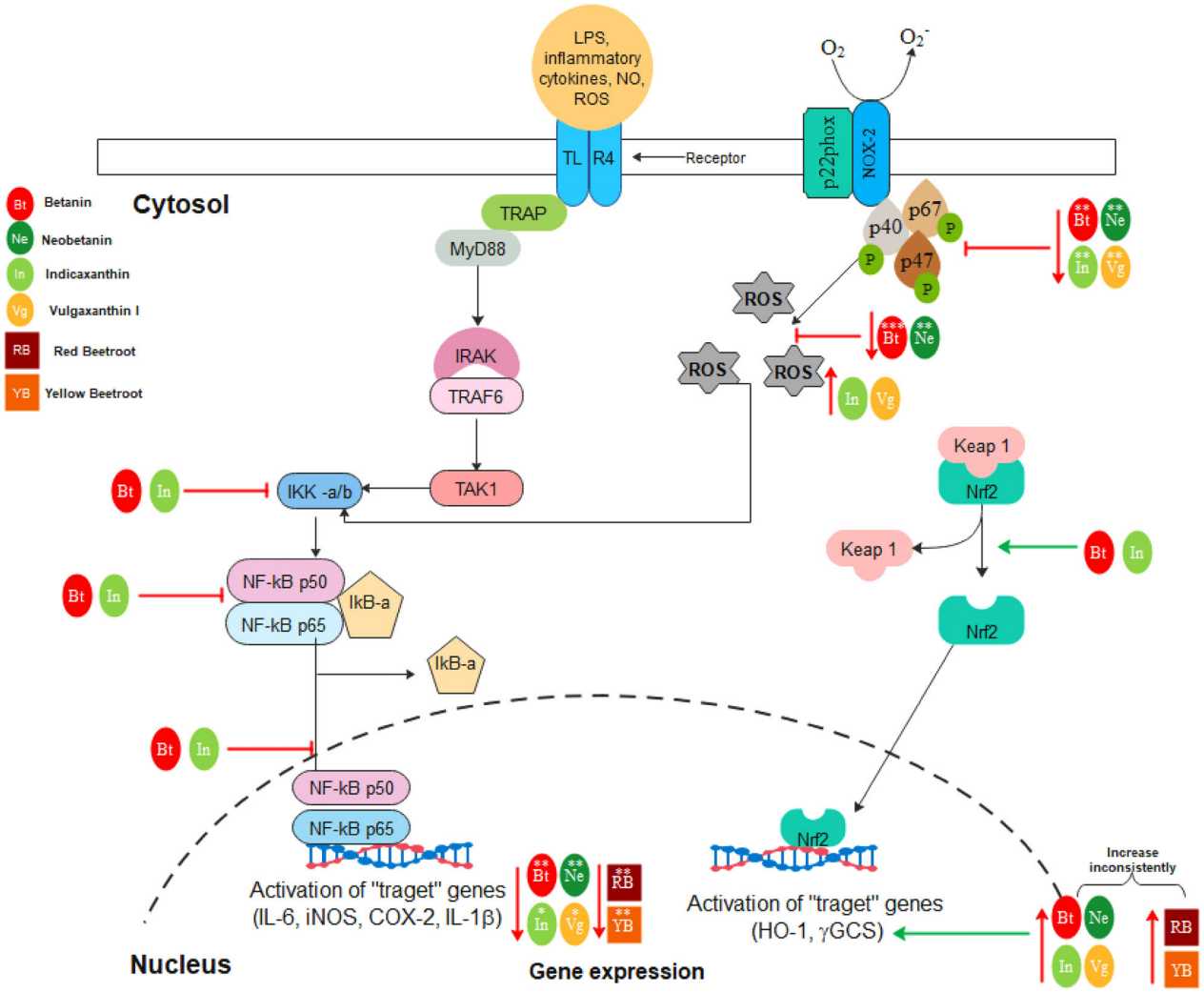

Figure 8. Signaling pathways involved in inflammation and proposed mechanistic scheme illustrating possible targets of betalains. NF-κB pathway which is responsible to increase the gene expression of inflammatory cytokines. Nrf2 signaling pathway which attenuates ROS production and induces the production of antioxidant enzymes.

contained no betacyanins and 1.18 μM equivalent betaxanthins (majority vulgaxanthin I). Overall, SRB contained more betaxanthins (13%) compared to SYB as well as higher amounts of polyphenols, which were more than double in SRB compared to SYB (Table S1, Supporting Information). The main polyphenol compounds found in SRB were chlorogenic acid, gallic acid, and rosmarinic acid (Table S2, Supporting Information). Based on these considerations, it can be assumed that components other than betalains, such as polyphenols, might have considerably contributed to the inflammatory response. Our findings emphasize the importance of conducting experiments using pure compounds in order to be able to distinguish between the actual effects of targeted compounds. We also compared HO-1 induction in these extract samples and were only able to confirm a significant induction (2.2-fold compared to stimulated control) following incubation with red beet extract. Again, given the comparably much higher betalain as well as polyphenol content in SRB versus SYB, this effect is likely a joint response of different bioactive ingredients but not likely due to betalains (**Figure 7**).

Based on the results of the present study and the available literature, **Figure 8** has been created to provide an overview on possible interactions of betalains with anti-inflammatory and antioxidant signaling pathways. In the presence of LPS and other pro-inflammatory triggers, the activation of NF-κB signaling cascade leads through multiple steps to increased translocation of p65 thereby inducing the expression of a range of pro-inflammatory mediators.[50] Betalains have shown to prevent the progression of NF-κB signaling at several key points and are able to promote the dissociation of Nrf2 and Keap 1 bond thereby triggering the expression of cell-protective markers. In addition, it has been demonstrated that the intracellular presence of betalains, i.e., betacyanins, interferes with ROS generation.

Some limitations should be considered when interpreting the results reported in this study. For instance, the lower purity of neobetanin and vulgaxanthin I might have impacted their efficacy on cellular responses, although both demonstrate anti-inflammatory properties and results of both compounds are in line with distinct betanin and indicaxanthin responses on ROS production and radical scavenging properties. Further, betalains are lacking stability, and a possible degradation during incubation cannot be excluded, which may interfere with cell responses. In addition, further studies need to be conducted to translate the in vitro findings on anti-inflammatory and oxidative effects of betalains into in vivo conditions.

4. Concluding Remarks

In summary, the present data emphasize the anti-inflammatory properties of all betalains although betacyanins appear to be somewhat more effective than betaxanthins to suppress expression of pro-inflammatory cytokines in macrophages. Importantly, in contrast to betacyanins, betaxanthins did not prevent formation of ROS. The current findings positively support the potential use of betalains as natural anti-inflammatory compounds which could be utilized as part of preventative and/or therapeutic strategies against chronic diseases. The results of VUL and NEO should be interpreted with caution considering their relatively low purity (79% and 52%, respectively). Moreover, further studies need to be carried out to better understand the detailed molecular mechanisms and cellular targets of individual betalains in different tissue environment and under different conditions of challenge. Establishing the overall and specific effects of betalains is of high relevance to understand their potential beneficial contribution to maintain and increase human health, and to develop targeted applications for betalain pigments.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

G.S.N.F. – conceptualization, methodology, validation, formal analysis, investigation, writing - original draft, writing - review & editing; C.B. – conceptualization, methodology, validation, writing - original draft, writing - review & editing, supervision; N.N.S. – methodology, validation, writing - review & editing, supervision; L.J.M. – validation, writing-review & editing, supervision; N.V. – methodology and editing; V.C. – methodology and editing.

Data Availability Statement

Data available on request from the authors.

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betalains, inflammation, oxidative stress, pro-inflammatory cytokines

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