# Betalain pigments: isolation, characterization and *in vitro* antioxidant and anti-inflammatory properties

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. Details of the jointly-authored publications and the contributions of each author are outlined on the next page.

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## Details of authorship contributions

Ganwarige Sumali Nivanthi Fernando: designed the question for literature search, conducted the experiments, collected and analyzed data, drafted and edited the manuscripts, and replied to the comments from reviewers.

Kelly Wood: contributed by supporting to conduct extraction and stability study (manuscript 1).

Emmanouil Papaioannou: contributed by conducting enzyme-assisted extraction of betalains, proofreading and editing manuscript 2.

Maria J. Frutos Fernandez: contributed by providing raw materials, proofreading, and editing manuscript 2.

Nan Jiang: contributed by supporting to conduct mRNA analysis of spray dried beetroot samples in manuscript 4

Christine Boesch, Natalia Sergeeva, and Lisa Marshall: provided supervision and feedback on the experiment methodologies and contributed to the proofreading and editing of the manuscripts.

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## Abstract

Betalains are water-soluble natural pigments which can be classified as red-violet colour betacyanins and yellow-orange colour betaxanthins. Apart from their tinctorial applications, betalains have recently drawn attention due to their biological activities such as antioxidant, anti-inflammatory, and antimicrobial properties which have been associated with health benefits and functional applications. However, the antioxidant and anti-inflammatory properties of individual betalains have not been fully elucidated with one of the reasons being the lack of purified compounds. Therefore, the aim of this study was to optimize betalain extraction while introducing a novel purification method of betalains as well as conducting a comparative assessment of the antioxidant and anti-inflammatory activities of individual betalains.

Low ethanol-water concentrations were found to be most preferable solvent mixtures to extract and support stability of betalains over the 4 weeks of studied period. A flash chromatography-based separation, which is a convenient and fast method to isolate unstable materials, was exploited for the first time to purify betalains from beetroot and prickly pear. Proposed method was successful to obtain high yields of individual betalains betanin, neobetanin, vulgaxanthin I and indicaxanthin in high purity, which were used for biochemical characterization and cellular assays. As the results demonstrate, all purified betalains exhibited antioxidant and free radical scavenging activities, with betanin showing much stronger capacity compared to the other betalains. In addition, all betalains suppressed LPS-induced macrophage inflammatory response determined via expression of interleukin-6 (IL-6), IL-1β, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2) while showing moderate cell-protective properties via induction of nuclear factor erythroid 2-related factor 2 (Nrf2) targets. When compared, betacyanins displayed better antioxidant and anti-inflammatory properties than betaxanthins.

In summary, this research demonstrated that betalains are potent anti-inflammatory and antioxidant compounds, emphasizing their potential for applications beyond colouring purposes. Further, based on the multiple regression analysis, betalains could be assumed as the main contributors to antioxidant activity of betalain extracts while different betalains contribute

differently towards the antioxidant and anti-inflammatory activities which can be related to structural differences.

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# List of Abbreviations

| | |
|---|---|
| ABTS | 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) |
| BCY | Betacyanin |
| BET | Betanin |
| BJ | Beetroot juice |
| BP | Beeetroot powder |
| BW | Beetroot waste |
| BXA | Betaxanthin |
| cDNA | Complimentary deoxyribonucleic acid |
| COX-2 | Cyclooxygenase-2 |
| DAD | Diode array detection |
| DF-DCFH | 2’, 7’-dichlorodihydro fluorescein diacetate |
| DOPA | dihydroxyphenylalanine |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| EAE | Enzyme-assisted extraction |
| ELSD | Evaporative light scattering detector |
| EU | European Union |
| FRAP | Ferric reducing ability of plasma |
| HAT | Hydrogen atom transfer |
| HORSA | Hydroxyl radical scavenging activity |
| HPLC | High performance liquid chromatography |
| IDX | Indicaxanthin |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| ISO | International organization for standardization |
| LPS | Lipopolysaccharide |
| MAE | Microwave-assisted extraction |
| MS | Mass spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NEO | Neobetanin |
| NF-κB | Nuclear factor Kappa B |
| NOX-2 | NADPH oxidase 2 |

| | |
|---|---|
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| PEF | Pulse electric field |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| RT-qPCR | Real time quantitative polymerase chain reaction |
| SD | Standard deviation |
| SET | Single electronic transfer |
| SRB | Sulforhodamine B |
| TBC | Total betalain content |
| TCA | Tricholoacetic acid |
| TEAC | Trolox equivalent antioxidant capacity |
| TLC | Thin layer chromatography |
| TNF-α | Tumour necrosis factor α |
| TPC | Total polyphenol content |
| TPTZ | 2,4,6-Tris(2-pyridyl)-s-triazine |
| UAE | Ultrasound-assisted extraction |
| UV | Ultraviolet |
| VGX | Vulgaxanthin I |

# Chapter 1
# Introduction and method overview

## 1.1 General introduction

Betalains are plant-derived natural pigments that have attracted increasing attention due to their strong tinctorial properties as well as potential bioactive functionality. They are water-soluble nitrogen-containing pigments that are formed by the conjugation of glycosyl derivatives or amino acid derivatives with betalamic acid. This combination leads to the formation of two different types of betalains, violet-coloured betacyanins, and yellow-coloured betaxanthins. Like many other pigments, betalains are distributed in the cytosol and vacuoles of plant cells and can be found in seeds, roots, fruits, and aerial parts of the plant (Martins et al., 2017b). Beetroot (*Beta vulgaris* L.), prickly pear (*Opuntia* spp.), and dragon fruit (*Hylocereus polyrhizus* L.) are the most common edible sources of betalains. Apart from these, betalains naturally occur in swiss chard, amaranth, and a few other plant families of Caryophyllales and some genera of higher fungi within the Basidiomycetes (Strack et al., 2003).

Food and cosmetic industries use colourants to enhance and restore the original colour of their products lost during processing. For this purpose, synthetic dyes are used extensively as they have higher stability and vibrant colour which is not destroyed during processing (Ngamwonglumlert et al., 2017). However, many adverse health-related problems have been reported, such as hyperactivity, sleeping disorders, irritability, and aggressiveness in children, that associate with continuous intake of food products which include synthetic dyes (Amchova et al., 2015; Ngamwonglumlert et al., 2017). Therefore, natural colour pigments are receiving higher attention in the food processing industry as well as the cosmetic industry as alternative ingredients. There is an increasing demand in the food industry for betalains due to their strong tinctorial properties and stability at pH 3 to 7 which is a preferable attribute for their use as a natural colourant in low acidic foods. Although betalains alter their charge over pH changes, they are not subjected to hydrolytic cleavage as compared to anthocyanins and keep their appearance over a broad pH range (Stintzing and Carle, 2004). Apart from the chromatic properties, the food industry uses betalains to improve shelf-life and

the functional properties of processed food products (Martins et al., 2017b). In addition to the food value, it has been shown that betalains possess high antiradical and antioxidant activities even in lower concentrations (Cai et al., 2003; Esatbeyoglu et al., 2015; Garcia-Cruz et al., 2017). Moreover, betalains have been associated with alleviation of oxidative stress, inflammation, and related disorders such as cardiovascular disease and different types of cancers (Matias et al., 2014). For example, it has been reported that oral administration of betalain-rich plant extracts may contribute to reducing total and low-density lipoproteins (LDL) cholesterol, as well as triglycerides, of diabetic rats thus potentially preventing cardiovascular diseases (Clemente and Desai, 2011). However, unlike other natural pigments, there is a lack of studies on the biological properties and mechanism of action of betalains present in edible sources, which makes a strong rationale for further investigation of these compounds.

## 1.2 Rationale and overall research aim

Many biologically active compounds present in food have protective and preventive properties that can be used to promote human health. Betalains are one of the bioactive compound groups naturally found in plants which can be used as a colour pigment and as compound in dietary supplements. Many studies have characterized the betalain composition in different plant sources. However, there are only a few studies looking at the biological activities of purified betalains and there is a lack of information available on the actual contribution of individual betalains. Moreover, there are other reasons limiting research on betalains such as the fact that betalains occur only in a limited number of plant varieties, albeit in high abundance (Azeredo, 2009). Therefore, the aim of this research project was to characterize and purify the main betalain compounds from different common betalain sources such as beetroot, dragon fruit, and prickly pear and investigate the individual compound effects on antioxidant and anti-inflammatory activities using several chemical and biological models.

## 1.3 Thesis objectives

- Optimization of betalain extraction conditions, characterization of betalains, and study of the stability of betalains and polyphenols in selected plant sources.

- Establishing and optimization of a flash chromatography method to purify betalains.

- Characterization of antioxidant and radical scavenging activities of betalain-enriched extracts and purified betalains.

- Assessment of anti-inflammatory and cell-protective properties of purified betalains in RAW macrophage cells.

## 1.4 Thesis hypothesis

This thesis postulated that betalains are highly effective antioxidant and anti-inflammatory compounds that are suitable for use as a natural food additive as well as a therapeutic food. Further, it was assumed that betalains are the main contributors of antioxidant and anti-inflammatory properties of betalain extracts, while different betalains contribute differently to the overall antioxidant and anti-inflammatory activity of the extracts due to structural differences.

## 1.5 General insight of betalains

### 1.5.1 Sources of betalains

Betalains are water-soluble N-heterocyclic pigments found in vacuoles of plant cells in certain plant families of the order Caryophyllales especially in the plants belonging to the family *Amaranthaceae* and *Cactaceae* (Martins et al., 2017b). These pigments provide strong colour to the plant parts such as leaves, stems, flowers, fruits, roots, and seeds by replacing the pigment anthocyanin (Strack et al., 2003). Interestingly, there are no plants found to naturally produce both betalains and anthocyanins together (Polturak and Aharoni, 2018). Moreover, the theory behind the mutual exclusion of two pigments is not entirely understood, although there are several hypotheses to explain this phenomenon (Brockington

et al., 2011). Red beetroot (*Beta vulgaris L*.) is the richest source of betalains and a major commercially exploited betalain crop. Further, there are a few known edible sources of betalains such as coloured swiss chard (*Beta vulgaris* L. *ssp cicla*), cactus fruits (*Opuntia spp*. and *Hylocereus* genera), and grain or leafy amaranth (*Amaranthus spp.)* (Azeredo, 2009).

Total betalain content and composition of betalain present in the plant may vary according to the several abiotic and biotic factors such as cultivar, plant part, ripening stage, insects, oxidative stress, climatic factors, salinity, and farming practice (Celli and Brooks, 2017). The total betalain content of different varieties of beetroot, dragon fruit, prickly pear, and other available betalain sources is summarized in Table 1.1.

**Table 1.1** Total betalain content in different natural sources (according to Celli and Brooks (2017)).

| **Source** | **Total betalain content (mg/ 100 g)** | **Reference** |
|---|---|---|
| Red beetroot (*Beta vulgaris*) | 40 – 77 * | Sanchez-Gonzalez et al. (2013) |
| Yellow beetroot (*Beta vulgaris*) | 568.0 ** | Slatnar et al. (2015) |
| Prickly pear (*O. joconostle*) | 92.7 * | Sanchez-Gonzalez et al. (2013) |
| Red prickly pear (*Opuntia spp*.) | 3.0 * | de Souza et al. (2015) |
| Moroccan Prickly pear fruits (*O. ficus-indica)* | 3.78 * | Khatabi et al. (2016) |
| Purple prickly pear **(***Opuntia ficus indica**)*** | 30.0 * | Robert et al. (2015) |
| Dragon fruit peel (*Hylocereus spp.*) | 73.0 * | Mello et al. (2015) |
| Amaranth | 0.07 – 20.93** | Li et al. (2015) |

* fresh weight basis, **dry weight basis

Beetroot red (E 162) and beetroot powder are the only available natural colourants for use in food products in Europe and the USA and the maximum level that can be used is yet undecided. Beetroot red can be used as a colourant for jams, jellies, sauces, soups, ice creams, tomato products, and yogurts. However, there is limited use of beetroot colour due to their negative impact on

the sensory properties caused by earthy flavour and aroma which is produced due to the formation of geosmin, a bicyclic alcohol produced by soil microbes (Ninfali and Angelino, 2013). Therefore, research focuses to find other suitable alternative natural betalain sources which would not interfere with the sensory quality of the food matrix. For this reason, dragon fruit and prickly pear get more attention as alternative edible betalain sources. Moreover, there is a wider possibility to use these sources as a natural colourant not only for the red colour but also for yellow, purple, and orange colour.

### 1.5.2 Chemical properties of betalains

Betalains are synthesized from an aromatic amino acid named tyrosine in plants through the metabolic pathway called the shikimate pathway which links the metabolism of carbohydrates to the synthesis of aromatic compounds (Tzin and Galili, 2010). Hydroxylated tyrosine (L-DOPA) is subsequently converted into betalamic acid [4-(2-oxoethylidene)-1, 2, 3, 4-tetrahydropyridine-2, 6-dicarboxylic acid] (Figure 1.1a) which is the core structure and chromophore of all betalains (Strack et al., 2003; Slimen et al., 2017). Betalains can be divided into two basic structural groups depending on their colour either betacyanin (red-violet colour pigments) or betaxanthins (yellow colour pigments).

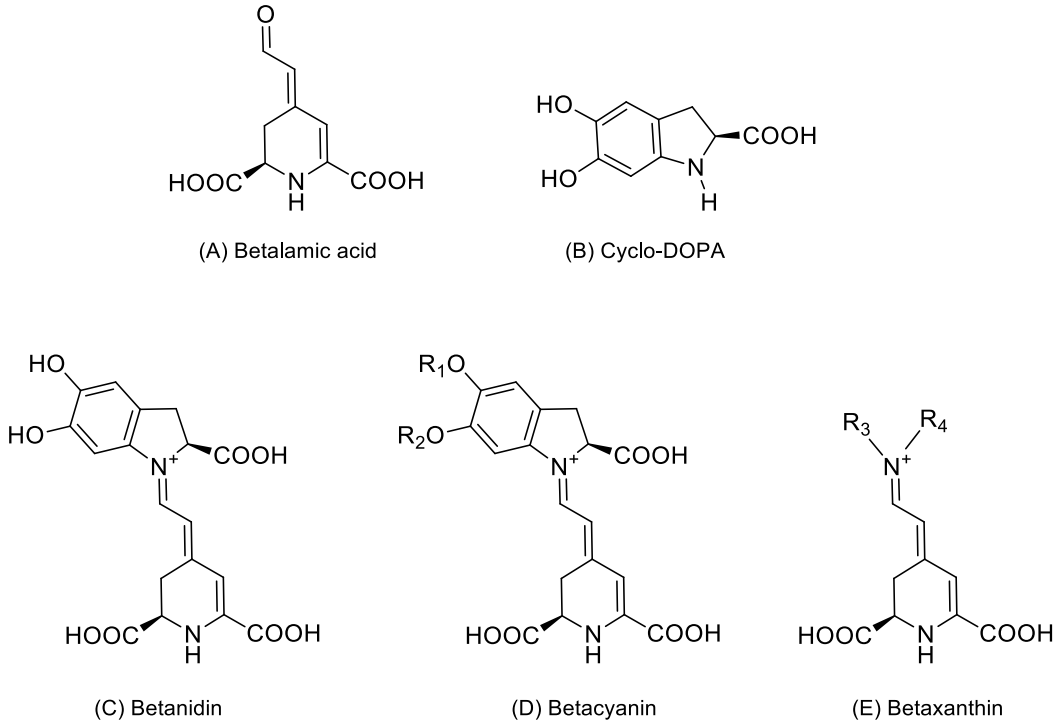
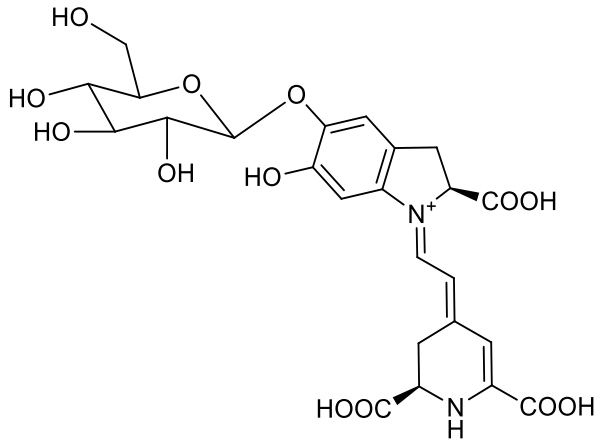



**Figure 1.1** Representative structures of (A) betalamic acid, (B) Cyclo-DOPA, (C) betanidin, (D) betacyanins and (E) betaxanthins where R1 and R2 are sugar moieties and R3 and R4 are amino acid or their derivatives.

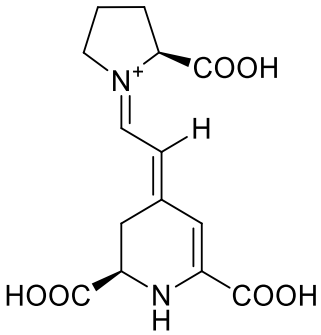
Betacyanins (Figure 1.1D) are composed by condensation of betalamic acid (Figure 1.1A) with cyclo-DOPA [cyclo-L-93, 4-dihydroxyphenylalanine)] (Figure 1.1B) or its glycosyl derivatives. Betanidin (Figure 1.1C) is the core structure of all betacyanins and different glycosylation and acylation substitution of one or both hydroxyl groups which are located at position 5 or 6 results in different types of betacyanins (Azeredo, 2009). The glycosylation of the 5-O-glucoside is very common in betacyanins whereas substitution of both positions (5 and 6) with sugar residues have not been known (Delgado-Vargas et al., 2000). Most of the betacyanins have multiple glycosylations with linkage to glucose, apiose, rhamnose, and xylose sugars (Polturak and Aharoni, 2018). There are four main structural groups in betacyanin i.e. betanin, gomopherenin, amaranthin, and bougainvillein (Slimen et al., 2017). The differences in structural groups is due to the connection of glucosyl groups to the oxygen atom present in the ortho position of cyclo-DOPA moiety (Slimen et al., 2017). Betanin (betanidin-5-O-β-glycoside) (Figure 1.2A) is the most common and most abundant betacyanin found in red beetroot.

Betaxanthins (Figure 1.1E) are formed from conjugation of betalamic acid with amino acids or their derivatives (Khan and Giridhar, 2015; Slimen et al., 2017). The most common betaxanthins found in plant sources are indicaxanthin, (Figure 1.2B) which is the main pigment found in yellow prickly pear, and vulgaxanthin I which is found in beetroots. Indicaxanthin has a proline moiety and vulgaxanthin I has glutamine moiety. The other amino acids that have been reported in contributing to making different betaxanthins are tryptophan, serine, valine, phenylalanine, isoleucine, alanine, histidine, methionine, threonine, arginine, and lysine (Khan and Giridhar, 2015).

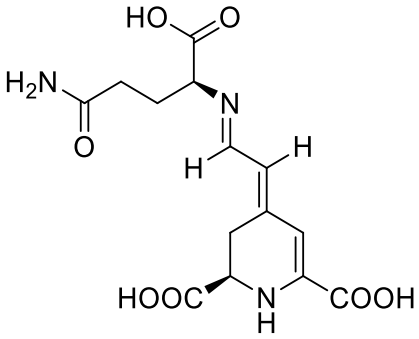
Betacyanins are red in appearance with the chromophore exhibiting its absorption maximum in the 530 to 540 nm range, whereas betaxanthins are yellow in appearance and show their absorption maximum in the range of 470 to 480 nm.




(A) Betanin




(B) Indicaxanthin




(C) Vulgaxanthin I

**Figure 1.2** Structures of (A) betanin, (B) indicaxanthin, and (C) vulgaxanthin I.

Betalain compositions of different plant sources widely vary and major types of betacyanins and betaxanthins present in beetroot, prickly pear, and dragon fruit are summarized in Table 1.2. To date, there are 75 well-known and characterized betalains available (Khan, 2016).

**Table 1.2** Summary of different betalain compounds and other phytochemicals found in beetroot, dragon fruit, and prickly pear.

| Food source | Betalain composition | | Other compounds | Reference |
|---|---|---|---|---|
| | Betacyanin | Betaxanthin | | |
| Beetroot | Betanin<br>Isobetanin<br>Betanidin<br>Isobetanidin<br>Neobetanin | Vulgaxanthin I<br>Vulgaxanthin II<br>Vulgaxanthin IV<br>Miraxanthin<br>Indicaxanthin | Phenolic acids (Quercetin, Myricetin, Kampferol, Ellagic acid)<br>Nitrates ($NO^{3-}$)<br>Ascorbic acid<br>Sugars | Nemzer et al. (2011)<br>Clifford et al. (2015) |
| Prickly pear | Betanin<br>Betanidin<br>Iso betanin<br>Gomphrenin<br>Neobetanin | Indicaxanthin<br>Vulgaxanthin I<br>Vulgaxanthin II<br>Vulgaxanthin III<br>Vulgaxanthin IV | Phenolic acids (Quercetin, Rutin, Myricetin)<br>Ascorbic acid<br>Sugars | Castellanos-Santiago and Yahia (2008)<br>Shetty et al. (2012) |
| Dragon fruit | Betanin<br>Isobetanin<br>Phyllocatin<br>Isophyllocatin<br>Betanidin<br>Isobetanidin<br>Bougainvillein-R-I | Not detectable | Minerals (K, Na, Mg, P)<br>Vitamins C and B3<br>Sugars (glucose and fructose) | Rebecca et al. (2010)<br>Stintzing et al. (2002)<br>Wybraniec and Mizrahi (2002)<br>(Stintzing et al., 2003) |

### 1.5.3 Stability of betalains

The stability of betalains is important to ensure the highest pigment extractability and retention of pigments during storage. Factors that affect betalain stability can be categorized into two main parts; external factors such as temperature, light, oxygen, and internal factor such as pH, and water activity ($a_w$) values (Table 1.3).

**Table 1.3** Favourable conditions for higher pigment stability (Herbach et al., 2006a).

| External factors | Internal factors |
|---|---|
| Low temperature | Low water activity |
| Darkness | pH 3 – 7 |
| Absence of oxygen | Composition of the matrix |
| Absence of metal ions such as iron, copper, tin, and aluminium | The high degree of glycosylation |
| Presence of chelating agents such as ascorbic acid, citric acid | The high degree of acylation |
| Presence of antioxidants ex: ascorbic acid | Absence of endogenous enzymes such as β-glucosidases, polyphenol oxidases, and peroxidases |

Concerning the intrinsic factors that are responsible for pigment stability, several studies reported that betacyanins are more stable compared to betaxanthins and higher pigment concentration increased the overall pigment stability during thermal processing conditions (Moßhammer et al., 2005; Herbach et al., 2006a). Most of the betalains are stable within the pH range 3 to 7 (Stintzing and Carle, 2004), whereas elevated temperatures shift the optimum pH towards 7 in several types of betalains (Herbach et al., 2006a). Further, under aerobic conditions, betalains show higher stability between pH 5 to 6 while anaerobic conditions shift the stable pH towards 4 (Huang and Elbe, 1987). Another key factor that influences betalain stability is the water activity of the matrix. Lower water activity is preferable to increase the stability of the pigments as it reduces the susceptibility of aldimine bond cleavage by reducing the mobility of reactants and oxygen through the food matrix (Delgado-Vargas et al., 2000). For example, hydrolysis of aldimine bond of betanin produced degraded products such as betalamic acid, isobetalamic acid and cyclo-DOPA-5-O-β-glucoside. Moreover, natural matrices are considered more preferable than purified solutions in terms of betalain stability as the sugars, pectin and acids present in natural matrices reduce the $a_w$ value and thereby stabilize the pigments. In addition, the temperature is a crucial factor influencing betalain stability during extraction, storage, and food processing. Generally, betalains are being considered as heat liable pigments and a continuous decline of stability can be seen at temperatures over 50 °C (Havlíková et al., 1983).

It is quite evident that the stability of betalains cannot be attributed to a single factor. Therefore, it is important to consider and manipulate a combination of several factors to ensure the stability of high amounts of pigment in an extract and/or sample over time and under different conditions of extraction and storage.

### 1.5.4 Betalain degradation pathways

Betalains can be degraded during thermal treatments and storage periods by different reversible and irreversible reaction pathways. The degradation of betanin has been studied widely while there was less research focused on the degradation of betaxanthins (Herbach et al., 2006a). The initial degradation pathways of betanin are shown in Figure 1.3. During degradation, betalains can be subjected to processes of isomerization, deglycosylation, hydrolysis, decarboxylation, and dehydrogenation. Some studies have shown that the isomerized products of betalains and decarboxylated products at $C_2$ or $C_{15}$ of betacyanins exhibit similar chromatic properties compared to the original compound (Schwartz and von Elbe, 1983) whereas, hydrolysis decreases the tinctorial properties of the original compound (Herbach et al., 2006a). Further, dehydrogenation of red beetroot juice and dragon fruit juice showed noticeable colour changes during the thermal treatment (Herbach et al., 2006a).

It has to be considered that betacyanins are more stable than betaxanthin, resulting in better colour retention. Moreover, degraded products of betalains (neobetanin, betanidin and decarboxybetanin) appear to be more stable in the food matrix and therefore, it is equally important to study their biological properties (Ravichandran et al., 2013).

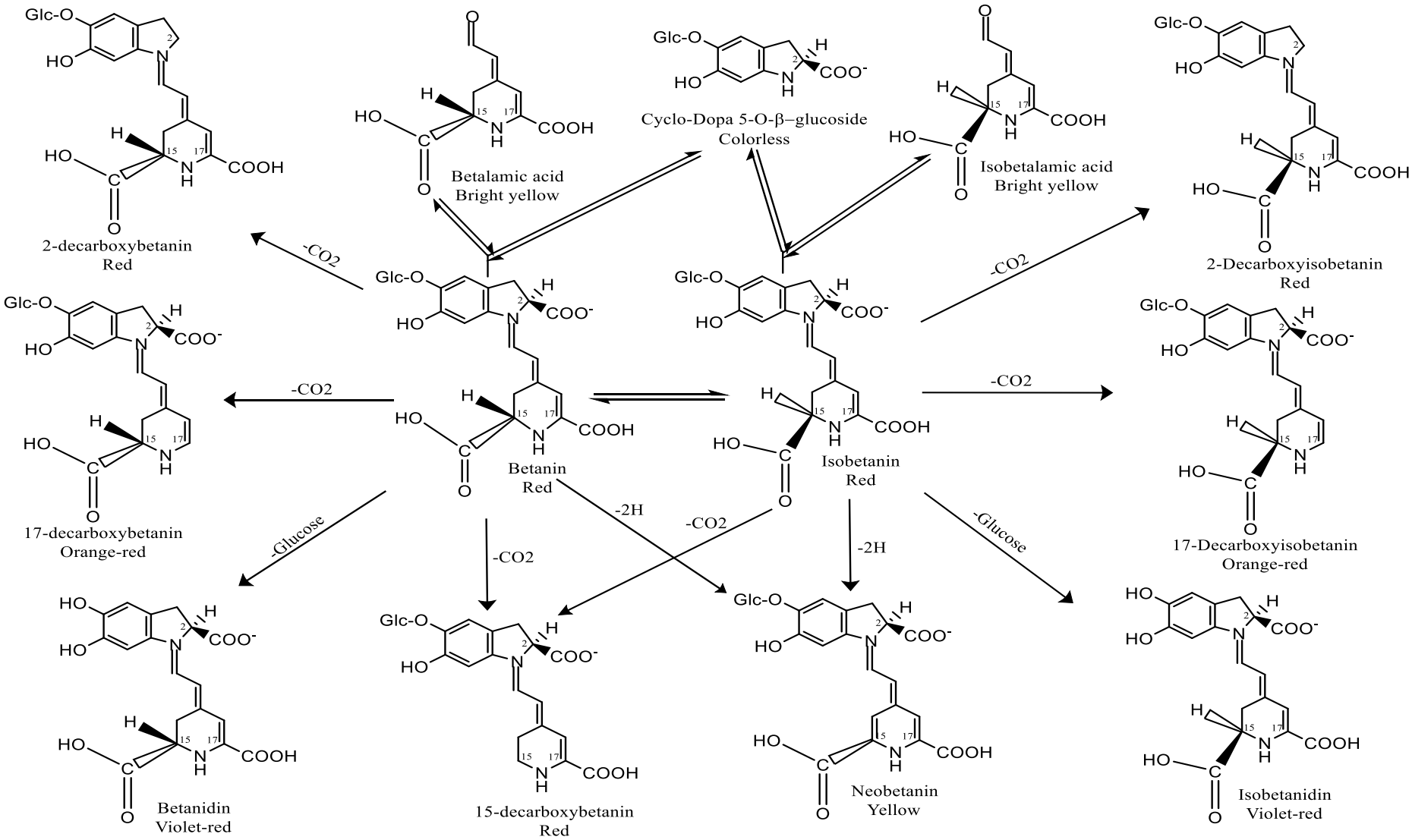



**Figure 1.3** Degradation pathways of betanin and resultant products with their colour. Source: Herbach et al. 2006 (modified from Herbach et al. 2004).

### 1.5.5 Betalain metabolism

The bioavailability of a molecule plays an important role in considerations on its health impact, i.e., whether the molecule is beneficial for human health. Ingested food components pass through the gastrointestinal tract where they may be absorbed and therefore present in the circulation where they can be utilized by different cells/organs. There is very little research available on the absorption, metabolism, excreation and cellular uptake of betalains, and the mechanisms are not fully discovered (Akbar Hussain et al., 2018).

The oral bioaccessibility of betalains from red beetroot juice is considered low in comparison to anthocyanins (Esatbeyoglu et al., 2015). It has been reported that betalains are absorbed in the intestinal tract in their intact form, and that they are excreted in urine, suggesting that hydrolysis of the glycosyl moiety is not a prerequisite for absorption of betacyanins (Tesoriere et al., 2004a). Several human studies have been demonstrated that consumption of betalain-rich cactus pear and red beetroot juice results in micromolar plasma concentrations of betanin and indicaxanthin which may be higher higher than other plant bioactives (Tesoriere et al., 2013). For instance, maximum plasma betanin and indicaxanthin were determined after 3 h at concentrations of 0.2 µmol/L and 6.9 µmol/L, respectively (Tesoriere et al., 2004b), indicating that indicaxanthin may be more available than betanin. As well, Tesoriere et al. (2005) found that betalains can cross the red blood cells with concentrations of betanin and indicaxanthin being 30 µM and 1 µM, respectively, inside the cells. Further, Tesoriere et al. (2008) studied the digestive stability of various types of foods rich in betalain pigments (cactus pear and beetroot) and purified betalains (betanin, isobetanin, indicaxanthin, and vulgaxanthin I) under stimulated oral, gastric and intestinal digestion. It was observed that indicaxanthin was lost only in the gastric environment, whereas vulgaxanthin I was lost in all digestion steps; with no effects seen from the food matrix. In contrast, food matrix prevented degradation of betanin and isobetanin in a simulated gastric environment. During the small intestinal phase of the digestion simulation, betacyanin was detected from both purified and food samples. After digestion of betacyanins,

accumulation of betalamic acid can be seen in the digestion of purified compounds whereas betacyanins from food souces did not show such an accumulation. Betalain stability in the digestive tract is directly related to their bioaccessibility, although other factors, such as the food matrix and processing style, may also affect their bioaccessibility.

## 1.6 Rationale behind the selection of different analytical techniques

### 1.6.1 Information of the samples used in the present study

The following table is providing the details of the samples used in the different chapters in this thesis.

**Table 1.4** Details of origin and processing conditions of the betalain-rich plant sources used in the present study

| No | Sample | Origin/ company | Chapter | Processing conditions |
|---|---|---|---|---|
| 1 | Red beetroot powder | Whole Foods Ltd, Ramsgate, UK Country of origin – Poland | 2 and 3 | Dehydrated whole beetroot with anticaking agent E551 (Silica) |
| 2 | Red Beetroot juice powder | Whole Foods Ltd, Ramsgate, UK Country of origin – Uzbekistan | 2 | Powered from beetroot juice, No information about the processing of juice into powder |
| 3 | Red beetroot powder | Biopower technologies ltd, Milton Keynes, UK | 3 and 4 | No details on processing conditions |
| 4 | Red beetroot waste powder – freeze dried | Biopower technologies ltd (Milton Keynes, UK). | 2 | No compositional details about waste. The waste were freeze-dried and powderized |
| 5 | Beetroot waste powder – air-dried | Biopower technologies ltd, Milton Keynes, UK. | 2 | No compositional details about waste. The waste was air-dried and powderized |
| 6 | Yellow beetroot powder | Biopower technologies ltd, Milton Keynes, UK Country of origin – USA | 3 and 4 | No details on processing conditions |
| 7 | Red beetroot wet pulp | James White Ltd, Ipswich, UK | 2 | No details on processing conditions. Frozen to preserve until experiments done at Lancaster University |
| 8 | Yellow prickly pear samples | Miguel Hernandez University of Elche, Alicante, Spain | 3 | Freeze-dried and powderized |

### 1.6.2 Extraction of betalains from plant sources

Extraction is the most important step in the analysis of plant bioactives as it is necessary to extract desired compounds from the raw material for further separation and characterization. Co-extraction of other compounds such as polyphenols, organic acids, pectin, and sugars is the major challenge of extracting betalains from the crude mixture (Celli and Brooks, 2017). Therefore, it is important to select the appropriate extraction technique/s to obtain maximum extraction yield as well as stability during the processing and storage. The following section discusses the different extraction strategies currently used to extract betalains.

#### 1.6.2.1 Conventional extraction methods

Generally, betalains are extracted by solid-liquid conventional extraction techniques such as maceration and soxhlet extraction methods as they facilitate the dispersion of betalain pigments into the extraction medium. Water, methanol, and ethanol are the main solvents used to extract the betalains from macerated or powdered plant materials. Some researchers have highlighted that pure/high concentrations of methanol or ethanol reduce the ability to extract betalains from the crude extract (Righi Pessoa et al., 2018). However, it has been found that 20% - 50% (v/v) methanol and ethanol solutions can improve the extraction yield of the betalains (Solovchenko et al., 2019). The combination of ethanol and water can increase the extraction efficiency as ethanol reduces the polarity of the water and thereby ideally match for the hydrophilicity of betalains (Sivakumar et al., 2009; Righi Pessoa et al., 2018). Moreover, the addition of ascorbic or citric acid to the extraction solvent can make a slightly acidic medium to minimize the betacyanin degradation potentially by polyphenol oxidase (De Azeredo et al., 2009; Prakash et al., 2013; Slimen et al., 2017). Conventional extraction methods are usually associated with a few shortcomings such as relatively low yield, lengthy extraction time, contamination of extracts and high consumption of solvents (Wang and Weller, 2006).

Several studies have attempted to optimize the extraction process of betalains by varying the extraction conditions such as solvents, temperature, and pH (Celli and Brooks, 2017). However, there is no standard method such as optimized solvent or

conditions to extract a higher amount of betalains and betalains without other phytochemicals such as polyphenols, sugars, and organic acids (Celli and Brooks, 2017), which shows that the extraction technique is greatly dependent on the different matrices of the plant sources.

#### 1.6.2.2 Novel extraction methods

Some studies use novel techniques, to improve and overcome the problems involved with conventional extraction methods. Ultrasound-assisted extraction (Righi Pessoa et al., 2018), microwave-assisted extraction (Cardoso-Ugarte et al., 2014), and pulsed electric field extraction (Loginova et al., 2011) were some alternatives that were proposed to increase the extraction efficiency of betalains.

Microwave-assisted extraction (MAE) was utilized for red beetroot by Cardoso-Ugarte et al. (2014) and compared against a conventional method. The results demonstrated that the extraction percentage of pigment obtained from MAE was higher than 50% whereas conventional extraction showed 10 – 20% extraction. However, the authors observed the degradation of betalains due to prolonged exposure to the high temperature (80 °C) and suggested incorporating antioxidants such as ascorbic acid (0.040 mol/L) into the extraction solvent to minimize the pigment degradation. Further, the authors showed that the amount of betalains extracted under the conventional method using 20 min can be obtained after a 2-minute extraction using MAE.

Pulsed electric field (PEF) is another novel technique that can be applied to extract betalains. Fincan et al. (2004) used PEF treatment to enhance the extraction efficiency of betalains from red beetroot. They observed that PEF treatment allows extraction of both pigment and ions present in the sample, by damaging the tissue. Therefore, the sample matrix and the electrical conductivity of the sample may affect the extraction efficiency. Another promising technology to extract the betalains is the use of polymers to selectively extract the target compound from a mixture which is known as molecular imprinting polymer technique. This technique involves creating artificial recognition sites in polymeric matrices that are compatible with the template

in terms of size, shape, and arrangement of functional groups. For example, the study conducted by Nestora et al. (2016) develop a one-step method for selective cleanup of betalains from crude beetroot.

Ultrasound-assisted extraction (UAE) was successfully used to extract plant bioactives such as polyphenols, aromatic compounds and carotenoids from plant sources and reported a higher yield than conventional extraction methods (Kumar et al., 2021). Comparatively, UAE has advantages such as less energy and time consumption, being cost-effective, uses mild temperatures, and can be used with a wider range of solvents (Medina-Torres et al., 2017). Maran and Priya (2016) optimized UAE conditions for red beetroot stalks using response surface design and the conditions were 53 °C, 89 W, and 1:19 (w/v) solid to solvent (distilled water) ratio for 35 min. In another study, Sivakumar et al. (2009) showed that a higher amount of betalain yield (1.4 fold) can be obtained using UAE compared to the magnetic stirring process. Righi Pessoa et al. (2018) observed significantly higher (~14%) betacyanin and betaxanthin contents from UAE compared to conventional methods (orbital shaker and metabolic shaker). They used 165 W and a frequency of 25 kHz at 52 and 37 °C with 25% ethanol in water for 90 min. Ramli et al. (2014) compared maceration with UAE to extract betalains from red dragon fruit. They used water as the solvent for both maceration and UAE while using 120 min at 50 °C conditions for maceration and 30 min at 25 °C for UAE. The results showed that the highest extraction yield from the peel (95.25%) was obtained using the conventional method whereas UAE gave the highest yield for flesh (90.08%). According to the researchers, the reason of having low betalain yield from peel using UAE was the higher amount of pectin present in the peel that disturbed the release of the pigment during the 30 min period.

In the present study, the ultrasound-assisted extraction (UAE) method (**Chapters 2 and 3**) was used to extract betalains and polyphenols from different plant matrices. The principle of UAE is connected to the acoustic cavitation formed by the system (Figure 1.4) (Strieder et al., 2019). The acoustic energy waves create compression and rarefaction of the molecules in the medium. This causes pressure changes in

the medium and the formation and collapse of microbubbles result in a microjetting effect. Microjetting can cleavage the cell surfaces, break down the particles, and surface erosion which promotes extraction efficiency.

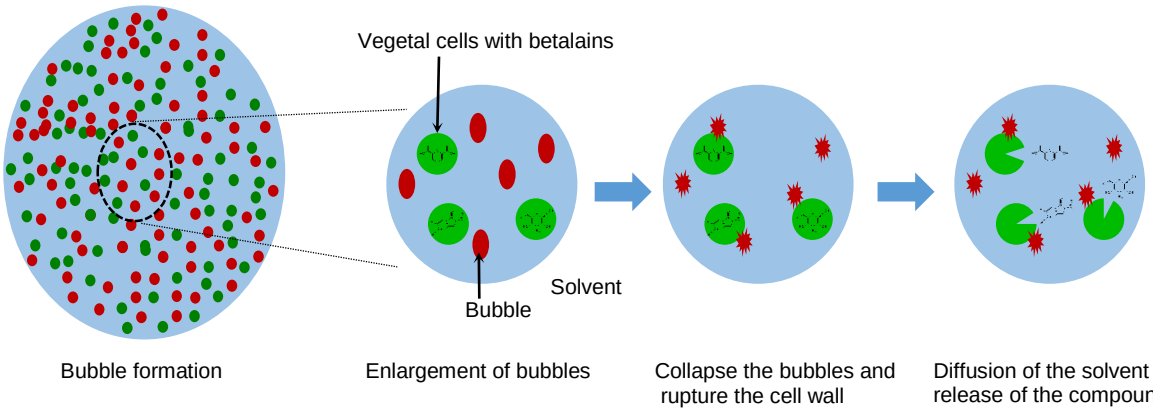



**Figure 1.4** Graphical representation of acoustic cavitation; bubble formation, collapse, and release of betalains.

Generally, UAE is carried out using an ultrasonic probe or ultrasonic bath and the present study used an ultrasonic bath to extract the betalains. Other main factors that contribute to increase the effectiveness of UAE are ultrasonic power, liquid to solid ratio, temperature, pH of the solvent, and time (Kumar et al., 2021).

In addition to the UAE method, an enzyme assisted extraction (EAE) method was also tested in the present study to extract betalains from plant sources. EAE is getting attention due to the current trend of finding sustainable and eco-friendly extraction technologies. EAE is based on the ability of enzymes to hydrolyze components of cell walls and disrupt the structural integrity under mild processing conditions, thereby allowing the efficient extraction and release of the compounds (Nadar et al., 2018). This method reduces the use of hazardous extraction solvents as well as requiring a short extraction time. Further, extraction is conducted under controlled temperature conditions allowing the extraction of thermo-sensitive compounds such as pigments and flavours. The common enzymes used to extract

polyphenols from plant sources are cellulase (for anthocyanins and lignans), pectinase (for starch, quercetin) and hemicellulase (plant pigments) enzymes. These enzymes can act as a catalysts to extract complex bioactives by degrading the plant cell walls and membranes, therefore EAE approach is more efficient to extract bioactives that are associated with the cell membrane e.g. cellulose. The extraction efficiency depends on the solvent system, temperature, mode of action of the enzyme, extraction duration, enzyme loading, substrate availability and pH conditions (Nadar et al., 2018). However, the conditions used in the present study were unsuccessful to extract betalains from beetroot pulp (**Chapter 2**). The reasons may be the degradation of betalains due to the change of pH of the medium and activity of the enzymes. Even though there are many techniques to extract betalains, still, the full recovery of betalains from the plant matrix is challenging.

### 1.6.3 Characterization of betalains

#### 1.6.3.1 Total betalain content

Total betalain content of the extracts was quantified using the spectrophotometric method initially described by Nilsson (1970) (**Chapters 2 and 3**). Chemical compounds are able to absorb, transmit or reflect the electromagnetic radiation over a certain range of wavelength. The spectrophotometer is an instrument that can be used to measure the intensity of light absorbed or transmitted by the chemical compounds once it passes through the sample. Generally, a spectrophotometer consists of four major components such as light source, monochromator, sample component, and detector.

The combination of deuterium and tungsten/halogen lamp is used as a light source in the spectrophotometer. Deuterium lamp emits light in the ultraviolet range (150 nm – 400 nm) and tungsten lamp emits light in the visible and near-infrared region (340 nm – 3500 nm) (Marczenko and Balcerzak, 2000). A monochromator serves to the dispersion of radiation emitted by the light source and convert polychromatic radiation (broad range of wavelengths) into monochromatic radiation (single or narrow range of wavelengths) and pass to the sample that is placed in the cuvettes. The cuvettes are optical transparent cells made out of glass, plastics, or quartz.

Plastic and glass cannot be used for measurements in the UV region as they absorb the UV light at 310 nm. Quartz cuvettes are ideal for taking measurements in both UV and visible light regions (UV-Vis) as they absorb light below 190 nm. The present study used quartz cuvettes to monitor the absorbance as absorbance maxima of polyphenols (280 nm) and betalains (480 – 540 nm) which are ranged in the UV-Vis region.

Betacyanins and betaxanthins can absorb light at ~536 nm and ~486 nm respectively and quantification of total betalain content was done using the Beer-Lambert law. Briefly, Beer-Lambert's law defines a linear relationship between the absorbance and the concentration of the sample (Mayerhöfer et al., 2019). The following formula is used to calculate the pigment concentration:

$$A = \varepsilon c l \quad (1)$$

where A is absorbance, ε is molar absorption coefficient which is a sample-dependent factor, c is the molar concentration and l is the optical path length which is typically 1 cm. This method is widely used, simple, cost-effective, and convenient method to calculate the pigment concentration of the samples. However, the absorbance characteristics of pigments depend on the origin of the samples and the different forms of certain pigments (Picazo Mozo et al., 2013). Research studies have shown that the percentage error of the spectrophotometric method was less than 10 percent when compared with a High-Performance Liquid Chromatography (HPLC) quantification method (Gonçalves et al., 2012; Picazo Mozo et al., 2013).

#### 1.6.3.2 HPLC-evaporative light scattering detector (ELSD) analysis of sugar

The ELSD-based HPLC analysis was used to identify and quantify the sugars present in the samples **(Chapter 3)**. The ELSD system is able to form a fine spray by converting the target component via a nebulizer. This spray is then heated to evaporate the mobile phase from the droplets when the target compounds are less volatile than the mobile phase. Light is then directed on the remaining compounds and the amount of scattered light is detected (Figure 1.5). The advantage of using

ELSD is that it can detect the compounds which cannot be detected using UV spectroscopy such as carbohydrates, sugars, and surfactants. For instance, UV absorption of sugars can only be detected around 190 nm to 195 nm and therefore, direct detection of UV absorption is limited to this low wavelength range (Shimadzu, 2021).

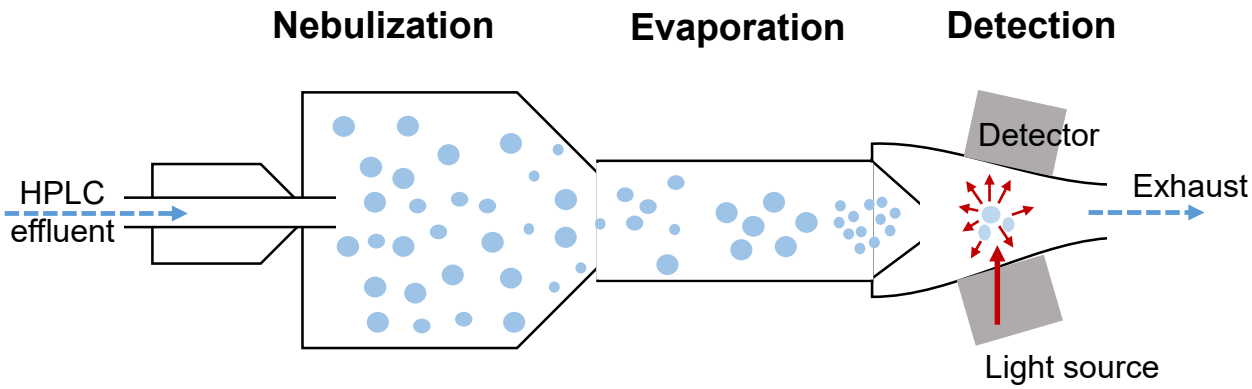



**Figure 1.5** Schematic representation of evaporative light scattering detector principle.

In the present study, the presence of sugar in the purified fractions was checked to confirm the purity of the compounds obtained from the flash chromatography method.

### 1.6.4 Purification of betalains

Purification of betalains is important before their quantitative and qualitative assessment to remove the possible interferences from other compounds such as sugars, polyphenols, soluble dietary fibre, and other soluble phytochemicals. There are different analytical methods available to purify chemical mixtures such as adsorption column chromatography, partition chromatography, membrane filtration, gel filtration chromatography, ion-exchange chromatography, molecular distillation, preparative gas chromatography, supercritical fluid chromatography, and molecular imprinted technology (Zhang et al., 2018a). Chromatographic techniques, especially column chromatography are widely used methods to purify phytochemicals from crude mixtures. However, a higher purity of the target compound can be achieved

by selecting the purification technique based on the chemical and physical properties of a particular compound (Zhang et al., 2018a).

#### 1.6.4.1 Flash chromatography

In the present study, a flash chromatography method was used to purify betalains from different betalain sources (**Chapter 3**). In conventional column chromatography, the column is packed with a solid support (most often silica gel) and introduces the sample to be purified on the top of the column. Then the column is filled with the solvent (mobile phase) and various components present in the sample travel under the forces of gravity and separate at different rates. However, the separation rate is very slow under these conditions. In flash chromatography, air pressure is used to accelerate the flow of solvent and reduce the time taken for the separation (Stevens and Hill, 2009).

In modern flash chromatography, the traditional glass columns are replaced with pre-packed cartridges and the flow rates can be increased by pushing the solvents through the column using pumps. Further, the chromatography system is now connected to the detectors and fraction collectors introducing gradient pumps which allows for faster separation.

#### 1.6.4.2 Reverse-phase cartridge

Reverse-phase flash chromatography was used in this study as the betalains are polar compounds. The pre-packed reusable reverse-phase cartridge was filled with HP–sphere C18 25-micron spherical silica particles. The principle behind this separation is based on the conversion of polar active sites of silica into the neutral, lipophilic sites which allows the compounds to either bind to the silica or less bind to the silica and can be retained or elute using the water-based solvent system (Biotage, 2015).

In an automated flash chromatography system, a mixture of compounds is loaded into the column with a suitable mixture of solvents (mobile phase). When the mixture

reaches the cartridge, the non-polar compounds present in the mixture are strongly attracted to the stationary phase while the polar compounds elute at different rates.

### 1.6.5 Identification of compounds

#### 1.6.5.1 High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis of betalains and polyphenols

Chromatography is a widely used separation technique that can be used to separate target compounds from a mixture. High-Performance Liquid Chromatography (HPLC) is a column chromatography method which uses a stationary phase that is porous solid (glass, silica, or alumina) and packed in a column, and a mobile phase for dissolving the sample and carrying the sample through the stationary phase. There are mainly two primary HPLC separation modes based on the polarity of the tested compounds such as normal phase and reverse phase HPLC (Shabir, 2010). Normal-phase HPLC uses a polar stationary phase with a non-polar mobile phase where the reverse-phase HPLC uses the vice-versa.

The principle behind the separation based on the polarity is that the similar polarity compounds tend to be attracted to each other while the different polarity compounds repel or less attractive to each other. Based on this basis, the mobile phase should be well attracted by the compounds of interest, but not strongly as they cannot elute during the separation process (Waters, 2014). Betalains and most of the polyphenols are polar molecules and reverse-phase HPLC is the method of choice for separation and quantification of betalains and polyphenols **(Chapter 2 and 3)** present in the plant extracts. When the mass spectrometer is coupled with HPLC, it helps to increase the determination capacity of individual compounds and structures of the compounds present in the sample.

#### 1.6.5.2 High-Resolution Mass Spectrometry (HRMS)

Mass spectrometry (MS) is an analytical tool that assists in identifying the compounds present in the sample based on their mass to charge ratio (m/z) and relative abundance. The process of MS primarily involves converting the sample into gas-phase ions with a beam of energetic electrons (electron ionization). These

gaseous ions are broken up into many charged fragments, which are positive or negative charge ions. Those charged ions then pass through a magnetic and electric field which are then separated based on their mass to charge ratio (Figure 1.6). The deflection of the ions depends on their mass and charge. For example, lighter ions are deflected more than heavier ions while ions with two positive charges are deflected more than an ion with one positive charge. The separated ions are then identified using the detectors such as an electron multiplier and display a plot of a spectrum of relative abundance versus mass to charge ratio. In this thesis spectrometry was used to identify the samples purified using the flash chromatography technique **(Chapter 3**).

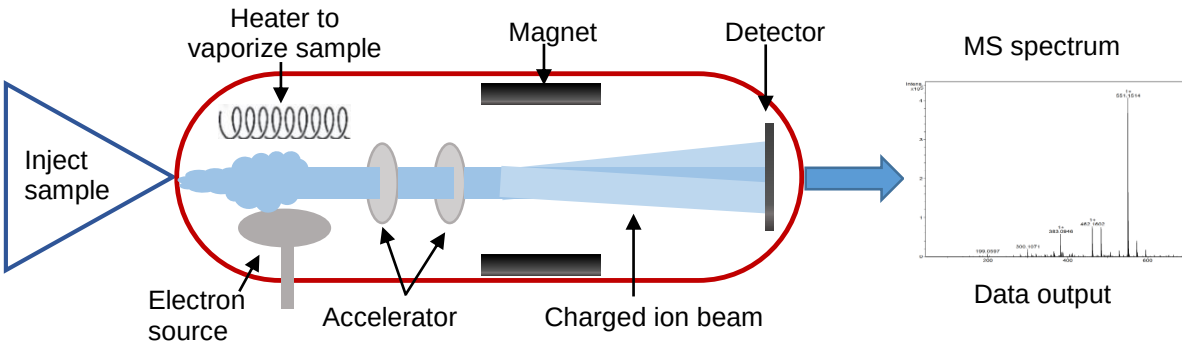



**Figure 1.6** Schematic representation of the principle of mass spectrometry.

### 1.6.6 Assessment of antioxidant activities

An antioxidant is a molecule that can delay or prevent the oxidation of a substrate when present in a low concentration (Prior et al., 2005). Antioxidant compounds can scavenge free radicals, and reduce the oxidation deterioration of food and pharmaceutical products during processing and storage. Also, they can suppress the effects of free radicals and reactive oxygen species (ROS) present in the human body and thereby, retard the development of chronic disease (Lobo et al., 2010).

Antioxidant activity assays can be categorized into 2 major groups depending on their chemical reaction mechanism of deactivating radicals as (i) single electron transfer (SET) and (ii) hydrogen-atom transfer (HAT) reaction-based assays (Bibi Sadeer et al., 2020). SET-based assays detect the capability of antioxidants to

donate an electron to reduce an oxidant, such as radicals, metals, or carbonyls (Figure 1.7) (Prior et al., 2005).

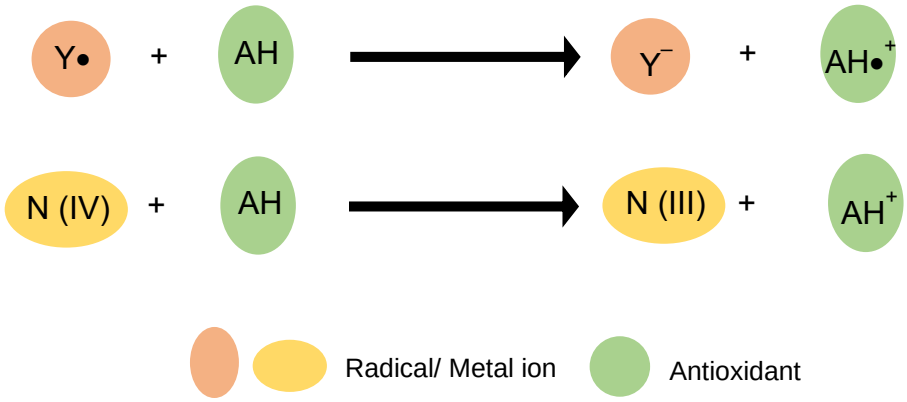



**Figure 1.7** Representation of SET mechanism-based assays.

HAT-based assays involve a hydrogen donation ability of an antioxidant to scavenge free radicals present in the substrate (Figure 1.8).

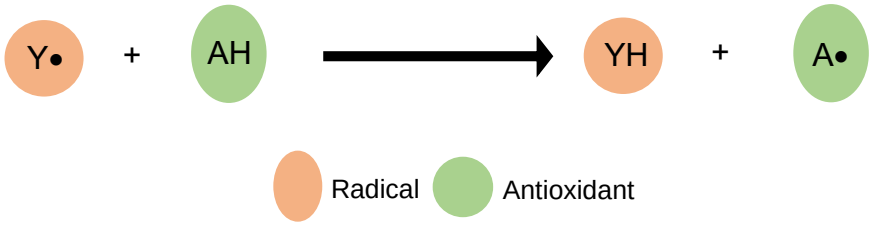



**Figure 1.8** Representation of HAT mechanism-based assays.

Examples for SET and HAT-based assays are FRAP and ORAC (Oxygen radical absorbance capacity), respectively, while TEAC and DPPH assays are considered as mixed-mode assays (both HAT and SET). Antioxidants present in the sample may act by single or multiple chemical reaction mechanisms depending on the reaction system and different radical or oxidant sources. For example, phenolics are very good scavengers of peroxyl radicals relative to the carotenoids, while carotenoids are very effective quenchers of singlet oxygen compared with most of the polyphenols (Prior et al., 2005). Therefore, it is necessary to perform different antioxidant assays as each method will provide different types of information about the antioxidant capacity of the sample. To date, there are many *in vitro* chemical-

based assays developed to measure general and specific antioxidant activity, but none have universal acceptance. In the present study, different antioxidant activity assays were performed to evaluate the potential radical scavenging activity of betalain enrich extracts and purified betalains (**Chapters 2 and 3**). All the assays used in this study were based on colorimetric methods and the principles of each assay are described in sections 1.6.5.1-1.6.5.5.

### 1.6.6.1 Total polyphenol content by Folin-Ciocalteu assay (TPC)

Quantification of total polyphenol content of the samples **(Chapter 2)** was done using the Folin assay. The principle behind the assay is the oxidation of phenolic compounds under basic conditions which reduces the yellow colour molybdotungstophosphate heteropolyanion reagent (Mo (VI)) (Folin reagent) to a blue colour complex (Mo (V)) with an absorbance maximum at 765 nm (Figure 1.9) (Berker et al., 2013). Phenolic compounds can dissociate and produce phenolate anion only under alkaline conditions (pH ~ 10). Folin assay is considered a simple, fast, reproducible, and robust method, however, the bottleneck of this method is that other reducing agents such as some amino acids, sugars, and ascorbic acids can obstruct the analysis by reducing the Folin reagent and may result in overestimation of total phenolic content.

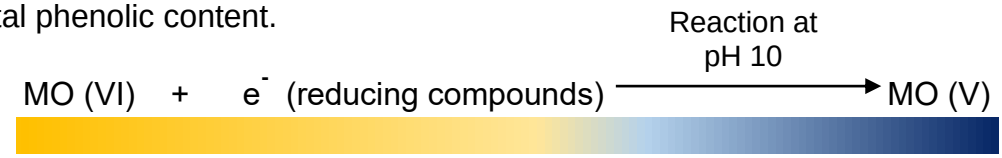



**Figure 1.9** Reaction mechanism of Folin assay.

### 1.6.6.2 Trolox equivalent antioxidant activity (TEAC assay)

TEAC assay is a simple and convenient method of determining the total antioxidant capacity of the sample. ABTS+ radicals which have strong absorption at 734 nm generated from ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) incubate with potassium peroxodisulfate. ABTS+ radicals are blue-green colour and the colour is decreased when mixing with antioxidants (Figure 1.10). The degree of suppression of colour production is proportionate to the concentration of antioxidants. The radical scavenging activity is measured after mixing ABTS+

radicals with the sample for a defined time period (6 min) and compared with the Trolox standard curve (Amorati and Valgimigli, 2015).

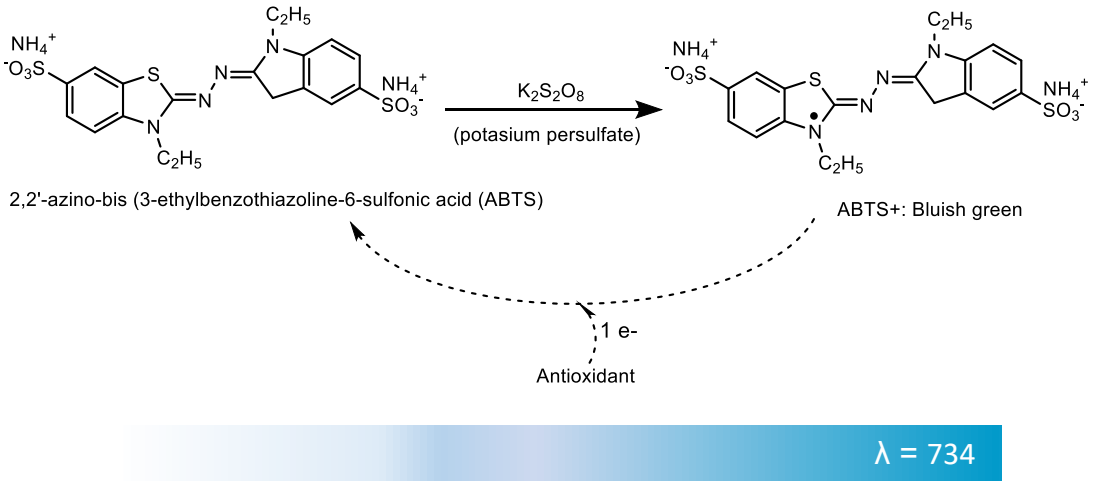



**Figure 1.10** Reaction mechanism of TEAC assay.

Compared with other antioxidant activity assays TEAC assay is simple and can be used to assess the radical scavenging activity of both hydrophilic and hydrophobic antioxidants.

### 1.6.6.3 Ferric reducing ability of plasma (FRAP) assay

The principle of the FRAP assay is associated with the reduction of ferric tripyridyltriazine ($Fe^{3+}$-TPTZ) complex into ferrous tripyridyltriazine ($Fe^{2+}$-TPTZ) by antioxidants at low pH (3.6) (Amorati and Valgimigli, 2015). The $Fe^{2+}$-TPTZ has blue colour with absorption maxima at 593 nm and the absorbance change is proportionate to the concentration of antioxidants present in the sample (Figure 1.11) (Rubio et al., 2016). FRAP assay is a quick, simple and highly reproducible assay compared with other methods.

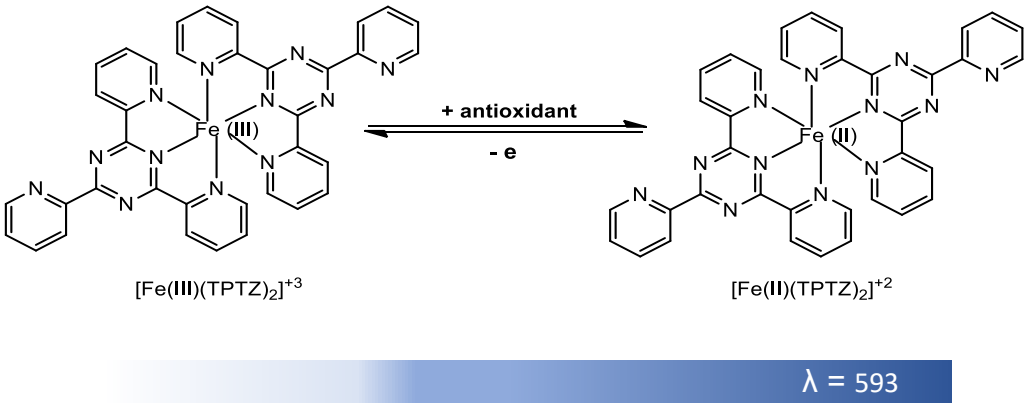



**Figure 1.11** Mechanism of the FRAP assay.

### 1.6.6.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

DPPH is a popular *in vitro* antioxidant activity assay used by many researchers. The DPPH radical is purple colour and shows an absorption maximum at 517 nm. When these radicals react with antioxidants, DPPH accepts hydrogen atoms and loses its purple colour to a white-yellow colour (Figure 1.12). The DPPH scavenging activity is mainly dependent on the solvent properties and pH of the medium (Romulo, 2020).

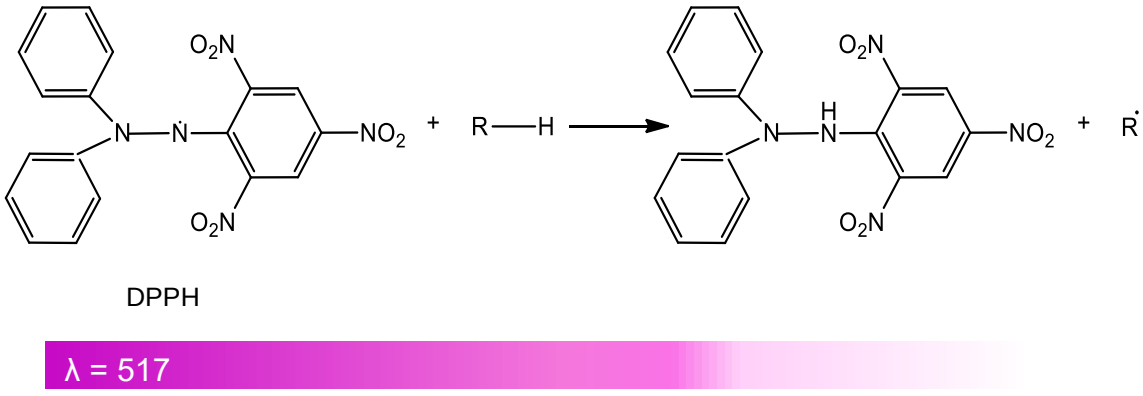



**Figure 1.12** Mechanism of DPPH assay.

DPPH assay is also considered as a simple, accurate, and economical method to determine the antioxidant activity of compounds. Further, results are reproducible and comparable with the other methods. There are few major limitations of this method such as DPPH is only soluble in organic solvents and the absorbance

interference from the coloured samples may be a problem to quantitative analysis (Arnao, 2000).

#### 1.6.6.5 Hydroxyl radical scavenging activity assay (HORSA)

This assay is based on the reaction of ferrous ion ($Fe^{+2}$) with 1,10-phenanthroline. Phenanthroline (phen) is a chelator and can generate a red-orange colour complex with Fe (II) (Figure 1.13). This complex has a maximum absorbance at 536 nm.


**Figure 1.13** Principle of HORSA assay.

Hydrogen peroxide ($H_2O_2$) can oxidize the Fe (II) into Fe (III) and prevent the formation of the coloured complex. If the scavenger is capable of scavenging $H_2O_2$, there is no/ less conversion of ferrous (Fe (II)) to ferric (Fe (III)). This can be detected by adding phen which yields a red-orange complex with ferrous ions. The generation of the red-orange complex will be directly proportional to the ability and concentration of the scavenger.

### 1.6.7 Cell-based experiments

In the present study purified betalains (**Chapter 3**) were applied to RAW 264.7 cells stimulated with lipopolysaccharide (LPS) to evaluate their anti-inflammatory and cell-protective properties. RAW 264.7 cells (Figure 1.16) are macrophage cells derived from BALB/c mice (Taciak et al., 2018). This cell line is often used for initial screening of natural compounds for their bioactivity and to predict their potential anti-inflammatory effects.

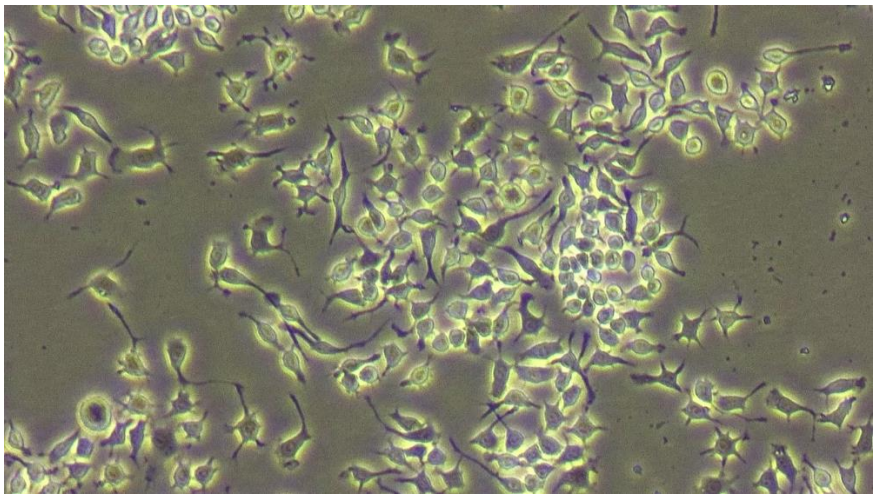


**Figure 1.14** Microscope view of RAW 264.7 cells. Cells maintained in DMEM supplemented with 10% FBS. The image was captured using a light microscope (Oxion Inverso) attached to the camera (Moticam x3) at 10× magnification 24 h after seeding.

RAW 264.7 cells induced with LPS are often use as an inflammatory cell model as they can release a large number of immune-regulatory cytokines such as tumour necrosis factor α (TNF-α), interleukin 1 (IL-1), IL-6, and IL-1β (Hambleton et al., 1996). The different assays used in the present study (**Chapter 4**) are described in sections 1.6.6.1-1.6.6.3.

### 1.6.7.1 Determination of cell viability

Cell viability assays are routinely used to establish whether the compounds of interest exert toxicity towards the cells. Thereby, viability assays can be used as an initial screening method of selecting suitable non-toxic concentrations for further experiments such as ROS measurements and cytokine expression. Generally, cell viability should be between ~80 – 95% in most of the cells lines (Standardization, 2009) after being treated with the compound. Further, ISO 10993-5 described the toxicity of the compounds depending on the cell viability as >80% - non cytotoxicity, 80% - 60% - weak cytotoxicity, 60% - 40% - moderate cytotoxicity and <40% - strong cytotoxicity (López-García et al., 2014). Other than measuring cell viability, these assays are often used to measure the cell proliferation in cancer research. There are different types of viability assays available based on the various functions of cells

such as enzyme activity, cell membrane permeability, adenosine triphosphate (ATP) production, cell adherence, nucleotide uptake activity, and co-enzyme production (Thangaraj, 2016). Cell viability assays can be broadly categorized according to the mechanism and read-out such as dye exclusion, colorimetric, fluorometric, luminometric, and flow cytometric assays (Kamiloglu et al., 2020). Dye exclusion assays are straightforward assays which use dyes such as trypan blues, congo red, and eosin to stain the live cells and counting the live cells using microscope. Colorimetric assays measure some general aspects of metabolism or enzyme activity of viable cells using the spectrophotometric method. These assays are simple, economical, and can be applied to both adherent and suspension cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and neutral red assays are the most commonly used colorimetric assays with many cell lines. MTT assay measures the cell viability based on the ability of the mitochondrial enzyme to reduce the MTT dye whereas neutral red assay measures the ability of accumulation of red dye by lysosomes of the viable cells. Fluorometric assays such as resazurin assay can be performed using a fluorescence microplate reader and these assays are more sensitive compared to the other methods. Flow cytometry is also used to measure the cell viability by characterizing or phenotyping the cells within a liquid flow through lasers. When selecting a cell viability assay, factors such as safety, reliability, speed, effectiveness, sensitivity, and cost should be considered while not interfering with the test compound (Aslantürk, 2017). Despite that, accuracy and reproducibility of the measurements, the concentration of reagents, and incubation time are the other important considerations regardless of the assay chosen (Riss et al., 2004).

Considering the colour absorbance properties of betalains, it was decided to exclude the neutral red and MTT assays which read out at 540 nm and 570 nm respectively, to measure the cell viability. The reasuzurin reduction assay also known as Alamar blue assay was used to measure cell viability (**Chapter 4**). Fluorescence characters of betalains were first observed in natural flowers and reported excitation and emission wavelengths for betacyanin were 521 – 529 nm and 570 – 575 nm respectively whereas betaxanthins showed excitation and emission maxima

between 471 – 474 nm and 548 – 551 nm respectively (Gandía-Herrero et al., 2005; Guerrero-Rubio et al., 2020). Based on the fluorescence characteristics of betalains, it was not expected to interference of betalains for resazurin assay which was therefore used to measure the cell viability experiments.

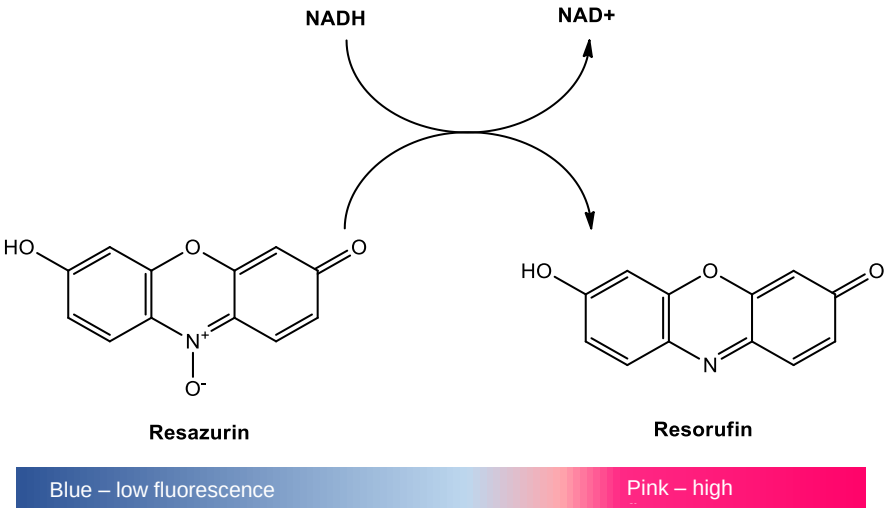



**Figure 1.15** Principle of resazurin assay.

The principle of the assay involves the reduction of non-fluorescent resazurin into highly fluorescent resorufin by the mitochondrial reductase (NADPH dehydrogenase or NADH dehydrogenase) present in the metabolically active viable cells (Kuete et al., 2017). The fluorescence output of resorufin is proportional to the number of viable cells and can be quantified with 560 nm excitation and 590 nm emission filters. The quantity of resorufin produced also can be measured by a change in absorbance; however, measurements are less sensitive than the fluorescence measurements (Riss et al., 2013).

Optimization of the incubation time is very critical to get consistent and reproducible results in this assay. There should be a sufficient incubation time to generate an adequate amount of fluorescent signal above the background. Usually, the incubation time is ranging from 1 to 4 hrs, depending on the cell type, cell density per well, and type of cell culture medium (Riss et al., 2013). Therefore, preliminary studies were conducted to optimize the incubation time and working concentration

of resazurin (Appendix A, Figure A.1). Based on the results, 3 hrs incubation time with 0.44 µM working concentration of resazurin was selected for the further experiments. The advantages of resazurin assay are, it is inexpensive, more sensitive than other available viability assays such as tetrazolium assays, and uses a homogeneous format (do not require removal of buffer or medium from the assay wells to take reading) (Kuete et al., 2017). The limitations are the possibility of interferences from coloured compounds to the fluorescent measurements and failure to notice the direct toxic effects on the cells (Riss et al., 2013).

#### 1.6.7.2 Measuring reactive oxygen species (ROS) generation - DF-DCFH assay

Reactive oxygen species are the reactive radicals generated from molecular oxygen during the mitochondrial electron transport of aerobic respiration or by metal-catalyzed oxidation and oxidoreductase enzyme activities (Hancock et al., 2001). They have a vital role in disease initiation, progression, and severity in the biological system (Griendling et al., 2016). Measurement of ROS production is a challenging task, due to the instability and the short life of most radicals (Zhang et al., 2018b). However, there are several types of methods such as fluorescent, chemiluminescent, spectrophotometric, and chromatographic methods available to measure intracellular ROS production. Among these, fluorescent-dependent methods are widely used due to the easiness of use, availability, sensitivity, and accuracy. In the present study, 2', 7'-dichlorodihydro fluorescein diacetate (DCFH-DA) fluorescent probe was used to measure the intracellular ROS production.

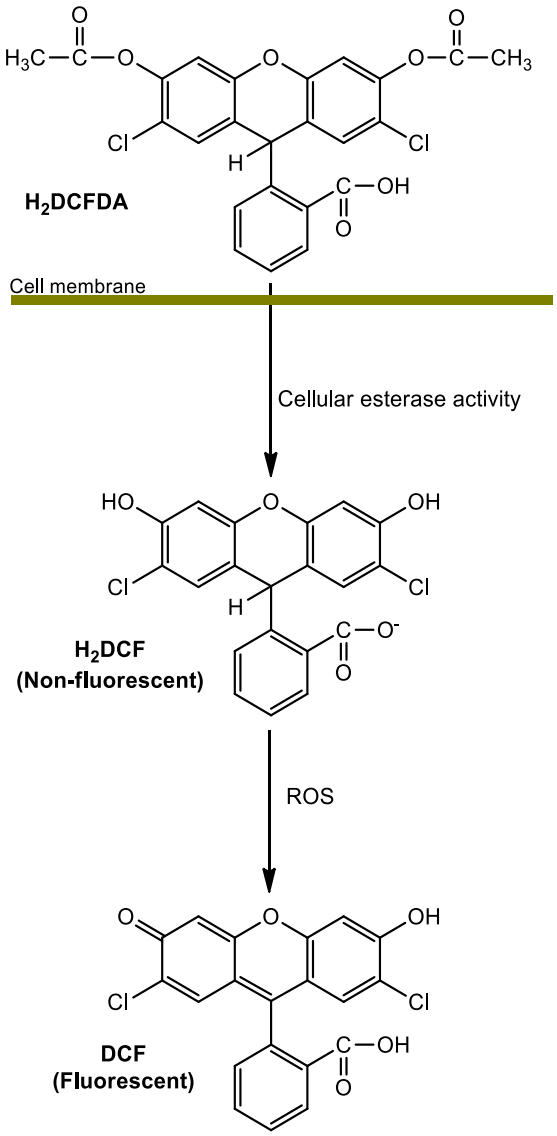



**Figure 1.16** Mechanism of $H_2$DCFDA assay.

After entering the cells, $H_2$DCFDA is deacetylated into non-fluorescent $H_2$DCF by intracellular esterases and then it is oxidized by ROS such as hydroxyl, carbonate, nitrogen, or thiyl radicals into the highly fluorescent DCF (Figure 1.16) (Ng and Ooi, 2021). DCF can be detected by fluorescence spectroscopy with 485 nm and 535 nm excitation and emission filters respectively, and flow cytometry technique. However, due to the short-shelf-life of DCF, its use for detecting the ROS using microscopy and flow cytometry is limited. In the present study, a microplate assay utilizing $H_2$DCFDA probe was used to detect intracellular ROS production. This method was able to overcome the common signal distortions linked to uneven seeding, thermal

gradient effects during incubation, and evaporation. Further, fluorescent background signals from molecules and other reagents can be deducted using cell-free controls (Ng and Ooi, 2021). This ensures the fluorescent characteristics of betalains do not interfere with the assay readouts. Typically, fluorescent signals gained from cell-based microplate assays require to be normalized to cell population per well. There are several assays such as bicinchronic acid (BCA) assay and nuclear DNA stains available to normalize the signal to the cell population. However, those methods have several drawbacks such as lack of endpoint, overestimate populations and radiation stress. Therefore, sulforhodamine B (SRB) assay which is used to determine the cell density based on the cellular protein content was used to normalize the DCF signal to cell population per well. SRB assay provides a stable endpoint measurement of cell density without affecting morphology (Ng and Ooi, 2021). The SRB assay is based on the ability of SRB dye (anionic aminoxanthene protein stain) to bind with basic amino acid residues under acidic conditions and the results are linear with the number of cells and cellular protein (Voigt, 2005). In the present study, the SRB-TCA assay that combines fixation and staining steps was used to normalize the DCF signals to the cell population. Several preliminary experiments were conducted to optimize the cell density and the DCF and SRB fluorescent signals to the RAW 264.7 cells (data not shown).

#### 1.6.7.3 Real-time quantitative polymerase chain reaction (RT qPCR)

Assessment of cytokine expression is important to understand the host responses to infection. Moreover, it helps to elucidate the signaling pathways involved in many inflammatory diseases such as autoimmune reactions and infectious disease (Peinnequin et al., 2004). Cytokines are small protein molecules, which regulate the immune response by managing interactions and communications between cells (Zhang and An, 2007). There are several approaches such as western blot, RNase protection assay, and enzyme-linked immunosorbent assay (ELISA) that have been used to measure the cellular cytokine expression (Ramos-Payán et al., 2003). However, those methods have some limitations such as being time-consuming, need a relatively large quantity of samples, lack of sensitivity, and measure the limited number of cytokines. Real-time PCR is one of the most innovative and widely used

techniques to investigate the mRNA expression of a broader range of cytokines mainly due to its sensitivity and reproducibility (Overbergh et al., 2003). The RT-qPCR method was used in the present study to assess the mRNA expression of inflammatory cytokines with the treatments (**Chapter 4**).

The general steps of performing real-time PCR experiments for gene quantification involve; RNA isolation and characterization, cDNA synthesis, real-time PCR data acquisition, and data analysis. In the present study, a two-step reaction was performed and cDNA was synthesized before using it as a PCR template. Many technical factors need to be considered in all these steps which are essential to have an accurate and reproducible outcome. The purity and integrity of the RNA, primers, and probes used in the experiments are some of the critical considerations. The purity and the concentration of RNA were determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer. The ratio between the absorbance values of 260 nm and 280 nm ($A_{260}/A_{280}$) should be in the range of 1.9 – 2.1 for the pure RNA. Lower or higher ratios indicate the presence of contaminants such as protein or phenol in the isolated RNA. Once the purity of RNA is determined, it should be stored at -80 °C until further use to ensure stability during storage.

TaqMan and SYBR Green are the most popular techniques used in quantitative analysis of gene expression (Tajadini et al., 2014). The TaqMan method uses a single-stranded oligonucleotide fluorogenic probe which binds only with the DNA sequence between two primers (Cao and Shockey, 2012). Therefore, this technique allows fluorescent signals only to be produced from the specific PCR product. SYBR Green is a fluorescent dye that intercalates into double-stranded DNA to detect the amplification of the target gene generated from gene-specific primers (Cao and Shockey, 2012). SYBR Green is widely used to detect and quantify the mRNA due to its simple design, easy setup, and low running cost (Soltany-Rezaee-Rad et al., 2015). Despite that, there are few drawbacks of SYBR Green, such as the dye is non-specific which can give false positive signal to non-specific products present in the assay and the length of the amplicon affects the intensity of amplification (Cao and Shockey, 2012). However, those drawbacks can be minimized using different

technical considerations. One approach to monitoring the SYBR green specificity is the analysis of the melt curve. Melt curve is an indicator of dissociation characteristics of the PCR amplicon during heating and there is a unique melting curve for different primers. Therefore, melt curve analysis is helpful to ensure the production of a single specific product by amplifying the same target gene. The other critical consideration is the design of the primers. In mRNA, some non-coding regions are known as introns, and the protein-coding regions are known as exons. It is preferable to design a primer sequence that crosses an exon-exon junction because those primers are specific for the amplification of cDNA as they only can bind to the exon sequence. Further, primers should be designed to get PCR product length < 200 bp for efficient amplification. Moreover, final PCR amplicons can then be run through electrophoresis with a 2% agarose gel to check the specificity of SYBR Green. A single DNA band of expected size should appear if the primers are only bound to the cDNA. Additionally, the G (guanine) C (cytosine) content of the PCR primer should be maintained between 40 – 60% in order to improve the specificity of primer binding to the complementary sequence.

## 1.7 Outline of the thesis

With this overview, this thesis demonstrates the isolation, characterization, and purification of betalains to elucidate the antioxidant and anti-inflammatory properties of betalains. The outline of this thesis is highlighted in Figure 1.17.

**Chapter 1** includes the general introduction and the rationale for the selection of different analytical techniques described in the experimental chapters.

**Chapter 2** presents the valorization of beetroot waste using an ultrasound-assisted extraction method and studies the efficiency of betalain extraction and stability using different aqueous solvents. An optimized method was applied to extract the betalains from different beetroot sources including pomace and the composition of different beetroot sources was compared.

**Chapter 3** explores a flash chromatography-based methodology for purification of betanin, vulgaxanthin I, and neobetanin from beetroot and indicaxanthin from prickly pear. The antioxidant and radical scavenging properties of purified betalains were compared against each other.

**Chapter 4** evaluates and compares the anti-inflammatory and cell-protective activities of purified betalains in RAW 264.7 macrophage cells. The mechanisms of betalains in suppressing LPS-induced inflammation and $H_2O_2$-induced reactive oxygen species production were investigated.

**Chapter 5** presents a general summary and discussion of the main results of this PhD study including conclusion and emphasis on areas for future studies.

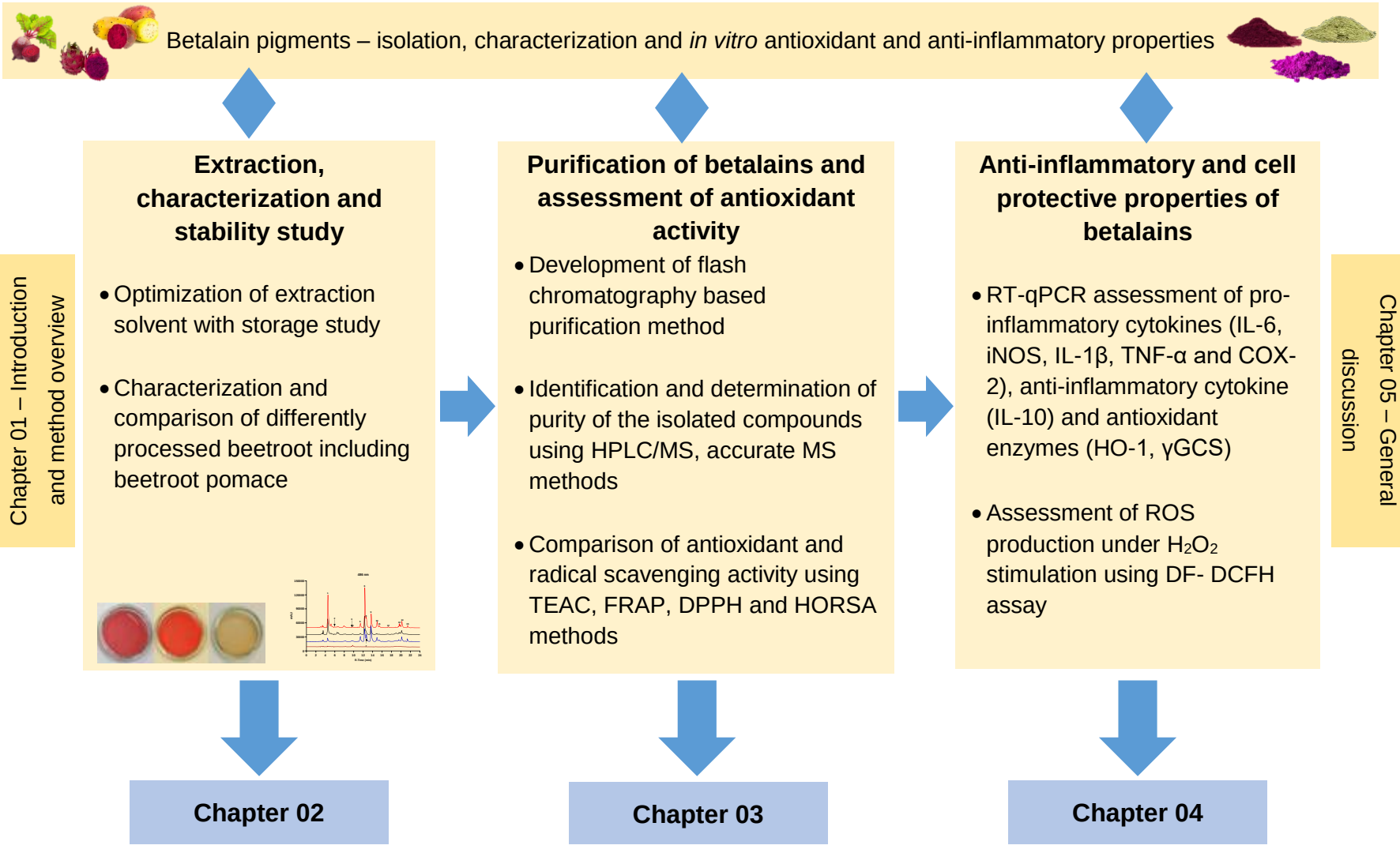



**Figure 1.17** Schematic overview of the main experiments employed in this thesis.

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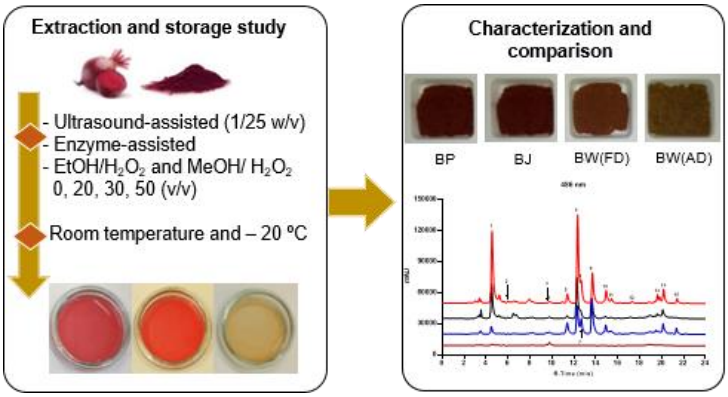
# Chapter 2

# Application of ultrasound-assisted extraction method to recover betalains and polyphenols from red beetroot waste




## Abstract

Agriculture and food industries generate substantial quantities of waste material with huge potential for bioactive ingredients to be recovered and converted into high value chemicals. Red beetroot, known for its high content in betalains, natural red pigments, as well as polyphenols, fibre and nitrate, is experiencing increasing demand, in particular as juice, which is leaving behind large amounts of waste. The present study focused on the recovery of betalains and polyphenols from dried whole beetroot, wet and dried beet pulp waste from the juicing industry. As part of an ultrasound-assisted extraction, ethanol/water-based solvent mixtures were used as they were found to be more effective than single solvents. Enzyme-assisted extraction was initially examined in case of wet pulp, but was not able to retain betalains. Betalains appear to be more stable in dried pulp. Ultrasound-assisted extraction was found more suitable to effectively extract both betalains and polyphenols with high bioactive yield from dried pulp. The total betalain and polyphenol profiles as well as storage stability and antioxidant capacities were evaluated over a period of 4 weeks after extraction from the dried waste. During the 4 week storage, betalains quickly degraded at room temperature in contrast to -20 °C, whereas polyphenols and antioxidative activity were much less influenced by temperature. When compared, dried samples from the beetroot juicing industry

demonstrate good betalain and polyphenol extractability, thus this data indicate that dried beet waste can serve as a good source of betalains for the colour industry and other technological sectors.

**Key words:** Betalains, polyphenols, antioxidant capacity, storage, beetroot waste, ultrasound-assisted extraction, enzyme-assisted extraction

## 2.1 Introduction

The food industry is responsible for the generation of up to 60% of total food waste during their production, distribution and retail process (Mirabella et al., 2014). In Europe, around 90 million tonnes of food waste are generated on a yearly basis, which corresponds to ca. 170 million tonnes of $CO_2$ equivalent emitted per year (Bio Intelligence service, 2010). Of these, juice, canned and frozen fruits and vegetable industries approximately generate 11.5 million tonnes of waste annually excluding the waste from grape and wine industries (Sagar et al., 2018). This waste material has generally a high moisture content (~80% w/w) and is rich in sugars (~75% w/w dry matter) (Arvanitoyannis and Varzakas, 2008), which makes it prone to microbial spoilage. Their incineration has been proven to be unsustainable as it uses high temperatures, has a low energy yield, contributes to waste disposal in landfills, and downgrades organic material which could be used for other purposes. Other treatments such as composting and anaerobic digestion provide more stable final material from a microbiological perspective, but again downgrade the initial organic matter. The management of food waste becomes an increasingly relevant challenge to reduce pollution, increase the industry revenues and improve recycling. So far, most food waste is utilized for the production of biofuels, preparation of fiber and as animal feed (Vulić et al., 2013). However, there is good evidence that food waste could be more effectively used as a source of bioactive compounds with increased value and significance to human nutrition, target compounds being phenolics, pigments, vitamins, peptides, and aromatic compounds (Sagar et al., 2018; Coman et al., 2020). For instance, it was reported that peels and seeds of citrus fruits, grapes, mangoes, avocados, and jackfruit contain over 15% more polyphenols than

the edible parts (Soong and Barlow, 2004). As well, Choi et al. (2016) reported that potato peels contain three times higher chlorogenic acids as compared to the cortex.

Beet (*Beta vulgaris* L.) is a popular crop grown around the world with some cultivars used for food as well as for sugar production. Sugar beet pulp, the main by-product of the sugar beet industry is being extensively utilized (Brachi et al., 2017), and is an excellent source for polyphenols (Mohdaly et al., 2010). In contrast to sugar beet, the waste from red beetroot processing has not been sufficiently considered for its alternative uses. The EU is the largest beetroot global producer (~70%), with the beetroot juice production in the UK alone generating waste corresponding to ~35-40% w/w of the initial biomass (Neelwarne, 2012). Red beetroot is a rich source of betalains, red pigments with strong tinctorial properties, which are receiving increasing popularity for different applications in the food and non-food industries (Choo, 2017). The global beetroot market is expected to significantly increase in the next decade, with the global production of 690,000 tonnes of beet powder (in 2016) being projected to reach 11 million tonnes by 2027 (Insights, 2017). Apart from betalains, red beetroot also contains other bioactive compounds such as polyphenols, betaine, fiber, nitrate, ascorbic acid and carotenoids (Clifford et al., 2015) and is considered as one of the top ten vegetables associated with superior health benefits (Azeredo, 2009). In particular, the industrial production of beet juice, which is increasingly popular due to its blood pressure lowering properties, generates large amounts of pulp waste that are mostly ending up in landfill. In addition to peel and pomace, aerial parts of beet (leaves and stalks) are generally discarded after processing of beets (Ben-Othman et al., 2020). Therefore, valorization of beet processing waste can contribute to reduction of waste generation and thereby support the concept of zero waste.

Extraction and maximum recovery of bioactive compounds are usually complex and require multistep techniques. The choice of solvent is extremely important for extraction of organic molecules from plant tissues such as betalains, polyphenols and other bioactives, with factors such as solubility of the target compounds, solvent polarity, solvent/target compound/waste matrix interaction, toxicity, cost and

availability of solvents needing to be taken into account (Medina-Torres et al., 2017). Commonly, organic solvents are used to extract bioactives and are combined with novel extraction approaches such as ultrasound-, microwave- and enzyme-assisted extraction methods (de Faria et al., 2019; Fu et al., 2020). In the present study, ultrasound- and enzyme-assisted extraction methods were selected as candidates to probe the feasibility of extracting betalains and polyphenols as they are considered more sustainable compared to conventional extraction, due to a reduced extraction time, solvent volume and energy consumption (Medina-Torres et al., 2017). Betalains and polyphenols are located in vacuoles in the plant cells (Martins et al., 2017a) and the acoustic cavitation caused by ultrasound facilitates the breakdown of cell walls and allows betalains as well as phenolic compounds to disseminate into the extraction solvent which can result in higher extraction yield compared to maceration. Further, ultrasound-assisted extraction uses a moderate temperature, which is favorable for extraction of heat-sensitive compounds and can easily be carried out in hybrid with other novel extraction techniques such as supercritical carbon dioxide extraction and microwave treatment (Esclapez et al., 2011). In addition, ultrasound-assisted extraction has been applied to betalain extraction from different plant sources with better performance in comparison to conventional extraction methods such as maceration, magnetic agitation, orbital and metabolic shaking (Sivakumar et al., 2009; Silva et al., 2020b; Ramli et al., 2014). For example, Sivakumar et al. (2009) demonstrated a 1.4 fold higher betalain yield when using ultrasound-assisted extraction (ultrasonication applied with probe) compared to maceration with magnetic stirring, while Righi Pessoa et al. (2018) and Ramli et al. (2014) who used ultrasonic bath found 1.08 and 1.21 increase of extraction yield respectively.

Similarly, enzyme-assisted extraction is receiving an increasing interest and for highly effective extraction under comparatively mild extraction conditions (low temperature and short periods of time) with high recovery of bioactives as it facilitates to retrieve bound compounds (Nadar et al., 2018). Indeed, the wet waste pulp of red beet is a complex matrix and consists mainly of the plant cell wall polysaccharides (pectin, cellulose, and hemicellulose), lignin, other small organic

molecules (such as carbohydrates, betalains and polyphenols) and inorganic ions (such as $Ca^{2+}$, $K^{+}$ and $Na^{+}$). Enzymatic pre-treatment of agri-food waste with appropriate hydrolyzing enzymes is an already established approach (Fockink et al., 2016). For instance, Papaioannou and Karabelas (2012) studied lycopene recovery from tomato peel under mild conditions assisted by enzymatic pre-treatment and non-ionic surfactants, thereby allowing disruption of the cell wall structure for enhanced recovery of compounds from plant cell walls.

The aim of the present study was to establish an efficient and sustainable extraction method for betalain containing plant material. To this end, extraction was established in whole beet powder and applied to other betalain rich samples. In addition to betalain yield, pattern and stability, polyphenol extraction and overall antioxidant activity was determined.

## 2.2 Experimental section

### 2.2.1 Materials

All chemicals and solvents were purchased from Sigma-Aldrich (Dorset, UK) and Fisher Scientific (Loughborough, UK). Betanin standard was obtained from Insight Biotechnology (Wembley, UK). Red beetroot powder (BP) and red beetroot juice powder (BJ) were purchased online from Whole Foods Ltd. **(**Ramsgate, UK). Food-grade beetroot waste powder (micronized, Beet waste (FD) and air-dried, Beet waste (AD)) were provided by Biopower (Milton Keynes, UK). The wet pulp was provided from James White Ltd. The enzymes Celluclast® 1.5 L (cellulase enzyme) and Pectinex® Ultra Mash (pectinase enzyme) were provided by Novozymes A/S, Denmark.

### 2.2.2 Ultrasound- and enzyme-assisted extraction procedures

The ultrasound-assisted extraction of betalains was carried out using the method described by Righi Pessoa et al. (2018) with some modifications. A 1 g sample was mixed with 25 mL of extraction solvent (water and 20, 30, 50% v/v ethanol or methanol) for 2 min using a vortex. The mixtures were then placed in an ultrasonic bath (XUBA3, Grant Instruments, UK) and sonicated at 44 kHz for 30 min at 30 °C.

The ultrasonic bath has an inbuilt temperature control. The temperature was monitored before and during the treatment, which stayed within a 0.5 degrees difference to the target temperature. The nominal power used for the study was 35W and the energy input per unit volume (energy density (J/mL)) was calculated according to the following equation used by Arruda et al. (2019);

$$Energy\ density\ \left(\frac{J}{mL}\right) = \frac{Nominal\ ultrasonic\ power\ (W) \times Extraction\ time\ (s)}{Sample\ volume\ (mL)} \quad (1)$$

For the enzyme-assisted extraction, 17 mL of a 1:1 mixture of pectinase:cellulase enzymes with activity 200 Unit/mL each at pH 5.5 (acetate buffer), was added to 1 g of wet pulp sample and then placed on a controlled heating plate at temperatures 35, 45 and 55 °C with magnetic agitation and left to hydrolyze for 2 h. The same procedure was followed with the pulp macerated in only 17 mL water and this was used as reference. Subsequently, ethanol was added to this mixture to achieve a final concentration of 30% (v/v) and, after an additional incubation for 2.5 h at 30 °C, the resulting extracts were collected and analysed.

The samples of the above mentioned procedures were centrifuged (Centrifuge 5810 R, Eppendorf, Germany) for 10 min at 3500 × g at 4 °C and at each stage supernatants were collected separately and stored at -20 °C until analyzed. The residues were re-extracted as before with the same solvent that was used for the initial extraction stage (water and 20, 30, 50% v/v ethanol or methanol) for maximum pigment recovery. The supernatants were collected and filtered through a 45 µm pore membrane. Aliquoted supernatants used for the stability study were stored at -20 °C and room temperature as indicated in the section below.

### 2.2.3 Quantification of total betalain and polyphenol content

The amount of betalains was determined using spectrophotometry (Specord 210 plus, Analytik Jena, Germany) (Ben Haj Koubaier et al., 2014) after appropriate dilution with distilled water into absorbance range (300 - 800 nm) and calculated using extinction coefficient values for 60,000 $cm^{-1}M^{-1}$ at $\lambda_{max}$ 540 nm and 48,000 $cm^{-}$

$^{1}M^{-1}$ at $\lambda_{max}$ 480 nm for betacyanin and betaxanthin, respectively. The total amount of betalain (in mg per g sample) was calculated by adding the values for betacyanin and betaxanthin.

The total polyphenol content (TPC) in extracts from different solvents was analyzed using 96 well microplate format as recently described (Perez-Hernandez et al., 2020). Gallic acid was used as the reference standard in the concentration range 0 – 250 µg/mL. For the assay, 10 µL of the sample or gallic acid standard was mixed with 40 µL of 10% Folin reagent (v/v) and 150 µL 4% sodium carbonate (w/v) incubated for 30 min at room temperature in the dark. Subsequently, absorbance was measured at 765 nm using a Tecan Spark™ 10M multimode microplate reader (TECAN, Männedorf, Switzerland). All samples and standards were analyzed in triplicate and the results were expressed as mg gallic acid equivalent (GAE)/g sample.

### 2.2.4 Colour measurement in beetroot extracts

The colour of the different extracts was assessed using a portable Datacolor check 3 spectrophotometer (Datacolor, Lawrenceville, New Jersey, USA). The instrument was calibrated using a black trap and white tile before measuring the extracts. Extracts were placed in glass Petri dishes with lid and measurements were taken from three different random places of the petri dish. The readings of L*C*h* were recorded and converted into the L*a*b* values using ColourMine conversion software. The colour parameters were expressed as a mean of triplicate measurements.

### 2.2.5 Antioxidant capacity assays

The extracts were assayed for their potential to inhibit $ABTS^{+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical according to Re et al. (1999) with some modifications. Briefly, $ABTS^{+}$ stock solution (14 mM) was mixed with potassium peroxodisulfate (4.9 mM) at a ratio of 1:1 (v/v), and the mixture was allowed to stand in the dark to formation of radicals at room temperature for 12 – 24 hrs. $ABTS^{+}$ working solution was prepared by diluting the $ABTS^{+}$ stock solution with water to an

absorbance of 0.700 ± 0.020 at 734 nm. A standard solution of Trolox was prepared to cover a range of 0 to 750 µM in ethanol: water (75:25 v/v). Then, 10 µL of sample or Trolox standard were mixed with 300 µL of $ABTS^{+}$ working solution and incubated for 60 min at room temperature in the dark. Subsequently, absorbance was measured at 734 nm using microplate reader.

The ferric reducing antioxidant power assay (FRAP) was performed according to Lotito and Frei (2004) with some modifications. Acetate buffer (300 mM, pH 3.6) was mixed with 10 mM TPTZ and 20 mM $FeCl_3$ at 10:1:1 (v/v) to prepare the FRAP reagent. Trolox was used as the standard and prepared to cover a concentration range of 0 to 1000 µM in ethanol: water (75:25 v/v). Briefly, 10 µL of the sample or Trolox standard were mixed with 300 µL of FRAP reagent and incubated for 15 min at 37 °C. Subsequently, absorbance was measured at 593 nm using the microplate reader. For both assays, samples and standards were run in triplicate and the results were expressed as mean ± standard deviation in µM Trolox equivalent (TE)/g of sample.

### 2.2.6 Identification of betalains and polyphenols

Identification of betalains in the extracts was performed using the Shimadzu application note (Shimadzu, 2015) for betalain analysis with some modifications and polyphenols were analyzed using the method described by Ifie et al. (2016). HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) fitted with an electrospray ionization source (ESI-MS) with a reverse phase Phenomenex Gemini $C_{18}$ column (4.6 mm × 250 mm, 5 µm) was used for both analyses. Both Single Ion Monitoring (SIM) and scan were used in positive mode for betalains and negative mode for polyphenols. The chromatographic conditions for betalain analysis were defined as follows; mobile phase A 2% (v/v) formic acid in water and mobile phase B pure methanol, flow rate 0.95 mL/ min. Betalains were separated using gradient elution mode started with 5-25% B for 15 min, 25-70% B for 4 min, and 70-5% B for last 7.10 min. The temperature of the column oven was set for 40 °C and the injection volume was 10 µL. Betacyanins and betaxanthins were monitored at 536 nm and 486 nm, respectively. The chromatographic

conditions for polyphenol analysis were as follows: mobile phase A 0.5% (v/v) formic acid in water and mobile phase B mixture of acetonitrile, water, and formic acid (50:49.5:0.5, v/v), flow rate 0.5 mL/ min. The gradient conditions were as follows; the initial condition started with 8% B and was increased to 18% B at 5.32 min, 32% B at 27.36 min, 60% B at 42.56 min reaching 100% B at 49.04 min, held at 100% B for 6.08 min and returning to initial conditions for 4.52 min. Identification of different polyphenols present in the extracts was performed using the *m/z* values taken from the literature (Kujala et al., 2001; Ben Haj Koubaier et al., 2014).

### 2.2.7 Data analysis

The data are reported as mean ± standard deviation of three extractions measured in duplicate or triplicate and graphs were drawn using GraphPad Prism version 9.0 for Windows. One-way ANOVA was applied to determine the statistical significance among the extractions at $p < 0.05$ among the different groups. Pearson correlation coefficients were calculated using the GraphPad Prism version 9.0 for Windows.

## 2.3 Results and discussion

### 2.3.1 Effect of different solvents on extraction of betalains using ultrasound

In the present study, different solvents and solvent-water mixtures were initially tested to optimize extraction conditions for betalains from red beetroot samples using ultrasound-assisted extraction and to assess the stability of betalains and polyphenols at different storage temperatures (RT, -20 °C, for 4 weeks). The principle of ultrasound-assisted extraction involves the acoustic cavitation which is resulted in microjetting (Strieder et al., 2019). The microjetting generates the effects such as surface peeling and particle breakdown which can promote higher extraction yield (Ashokkumar, 2015). Use of high nominal power (power provided by the device) creates greater extent of shear force and results in high extraction yield (Strieder et al., 2019). However, there is energy loss in the device during the conversion of mechanical energy into the cavitation (Wang et al., 2018) The nominal power and the energy density during the extraction process of present study were 35 W and 252 J/mL respectively.

Previous studies have reported that aqueous mixtures of organic solvents are most effective for efficient extraction of water-soluble phytochemicals (Do et al., 2014; Altemimi et al., 2017; Fathordoobady et al., 2016). Indeed, different mixtures of solvents miscible with water (20, 30, 50%, v/v) showed superior performance in this study to extract betalains in comparison to pure methanol and ethanol (Figure 2.1). This is mainly due to the polarity of the target compounds. Betalains are hydrophilic pigments; therefore mixing of organic solvents with water increases the extraction yield when compared to pure organic solvents such as alcohols. Although, pure water can improve the betalain yield, it has caused severe difficulties during the solute separation by filtration due to co-extraction of mucilaginous compounds such as pectin (Fathordoobady et al., 2016). The results are in agreement with the findings of Righi Pessoa et al. (2018) who demonstrated total betalain contents in red beetroot ranging from 0.13 mg/g to 6.97 mg/g using different combinations of water with organic solvents. Interestingly, whilst there was no change in betalain yield when extracted with water in comparison to solvent mixtures, some studies suggested that the use of aqueous ethanol or methanol is required to achieve efficient extraction of betalains (Azeredo, 2009; Celli and Brooks, 2017; Fathordoobady et al., 2016). Compared to methanol, ethanol proved to be a better choice as an extraction solvent due to being considered non-toxic; it can also be bio-produced from renewable resources and is thus “greener” in environmental assessments, with the added benefit that it can be readily used in the food industry (Capello et al., 2007). According to the literature ethanol can reduce the co-extraction of pectin, some soluble fiber and proteins (Perussello et al., 2017) while increasing the extraction of compounds of lower molecular weight (Sun et al., 2015), thereby enhancing the overall extraction of bioactives such as polyphenols and betalains. Indeed, the preliminary experiments showed 18.3% lower total values of polyphenols when extraction was performed using water in comparison to 30% ethanol (data not shown).

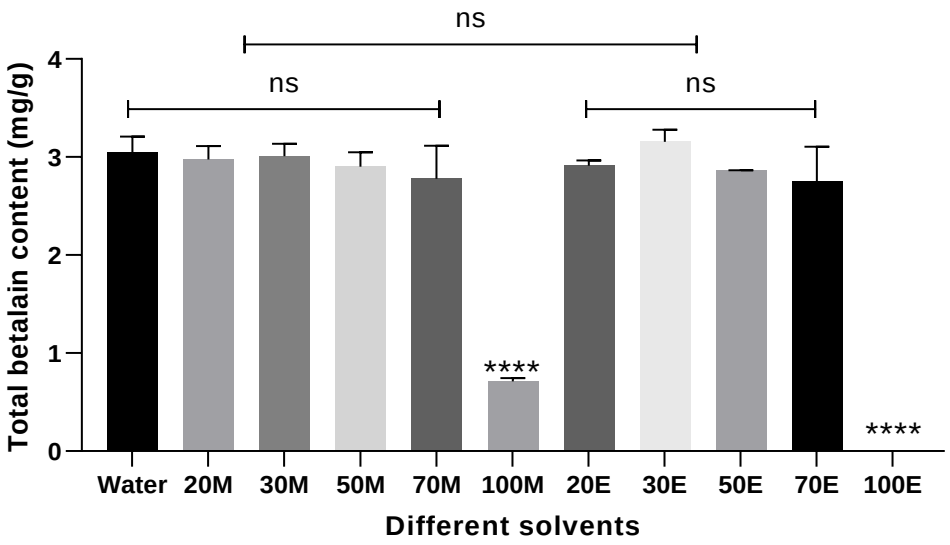



**Figure 2.1** Effect of solvents on total betalain content in dried red beetroot powder extracts. Data are mean with SD of three independent extractions. (M = Methanol v/v%, E = Ethanol v/v%). **** indicates significant difference ($p < 0.05$), Tukey's multiple comparison test.

The further analysis into individual betalain composition of dried red beetroot powder extracts was conducted using HPLC demonstrating the presence of a range of betalains and metabolites in all samples (Appendix B, Figure B.1). As expected, they were the main red pigments betanin and isobetanin as well as the predominant yellow pigment vulgaxanthin I, which is in accordance with the literature (Kujala et al., 2002; Herbach et al., 2006b). A comparison of the peak areas of three main betalain pigments is presented in Figure 2.2, showing a similar pattern for all the samples. Based on peak area analysis, ethanol performed better with regards to extraction of betalains: the total betalain extractability with aqueous ethanol was 7.7% and 19.9% higher in comparison to methanol (both at 30% v/v) and water, respectively (although not significant, $p>0.05$). The variation of the yield can be attributed to a different polarity of the extraction solvents e.g. relative polarity: water (1.000), methanol (0.762) and ethanol (0.654) (Reichardt, 2003). Efficiency of the extraction process depends on the ability to solvate target molecules. The dominant contributors to solvation of polar molecules e.g. betalains are charge-dipole, dipole-dipole, H-bonding, which favor polar solvents. On the other hand, weaker

electrostatic interactions e.g. ion-π, π-π interactions involving neutral or less polar fragments present in betalains will favor extraction solvents of lower polarity. This indicates ethanol to be a better choice to embrace both types of molecular interactions when combined with polar solvent such water. The results demonstrate a preference for 30% v/v ethanol for extraction, which was selected for further experiments.

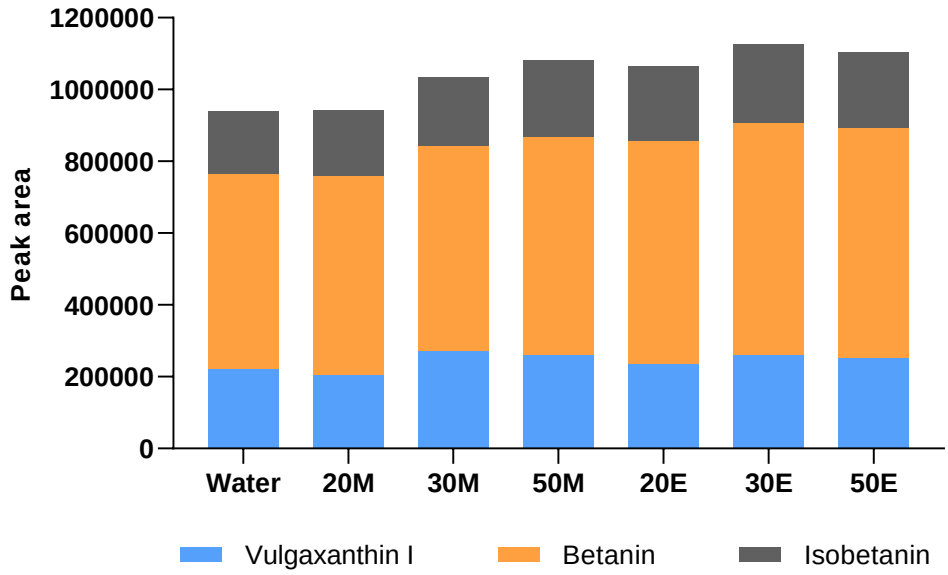



**Figure 2.2** Peak areas of main betalains under different solvent extraction conditions. M = Methanol v/v%, E = Ethanol v/v%. Data are from HPLC with vulgaxanthin I and betanin/isobetanin monitored at 486 nm and 536 nm, respectively.

The same solvent mixtures were used to evaluate the extraction of betalains and polyphenols from the wet pulp (81.5 ± 0.67 % w/w moisture content) under the temperatures of 35, 45 and 55 °C. Similar results were obtained in this case with the 30% v/v aqueous ethanol lead to the recovery of 6.86 ± 0.23 mg/g dry weight polyphenols after three repeating extraction steps, whereas the increase of ethanol in the mixture didn't improve further the polyphenols extraction (data not shown). The amount of polyphenols extracted by 30% v/v ethanol was 19.3% and 71% higher than these of the pure water and pure ethanol, respectively. This is indicative of the extracted polyphenols mixture higher affinity to lower ethanol concentrations (~30%

v/v). Apart from solvent-based extractions, enzyme-assisted extraction, which is considered as a highly effective and sustainable extraction option to achieve high product yields, reduced by-product formation under avoidance of harsh conditions (Nadar et al., 2018) was employed in this study. The enzymes used, cellulase and pectinase, are able to hydrolyze cell wall components and release bioactives that are associated with these, therefore, allowing an overall more efficient extraction of bioactives. In the current study, however, pre-treatment with cellulase and pectinase enzymes was unsuccessful to increase total bioactive recovery, especially betalains from wet pulp. Enzyme treatments were performed at three temperatures, 35, 45 and 55 °C, prior to extraction. There was an enhancement in recovered polyphenols (10.06 ± 0.21 mg/g dry weight) at 45 °C with a net recovery of 3.2 mg/g dry weight as determined by Folin assay compared to extracted polyphenols by maceration. Betalains were not detectable in the macerated wet and enzyme treated samples. Given the absence of the targeted betalains, from these waste material, enzyme-assissted extraction was not further pursued. Betalain absence in the case of wet pulp can be explained by the fact that there are enzymes present which could lead to betalain degradation, whereas in the dry samples these enzymes are not active. Further, high water activity induces the aldimine bond cleavage and promotes the betalain degradation (Fu et al., 2020).

### 2.3.2 Effect of extraction solvent and temperature on betalains, total polyphenols and antioxidant activity during four weeks of storage

There are many internal and external factors such as pH, light, temperature, oxygen and water activity that may influence the stability of betalain pigments during storage (Azeredo, 2009). Storage temperature in particular can be considered as one of the crucial factors that determine betalain stability (Herbach et al., 2006c). Following extraction with different solvents, the present study sought to establish betalain content and pattern, total polyphenol content and antioxidant activity as well as colour measurement as potential indicators of sample deterioration during storage at -20 °C and RT. Betalain content displayed a fast and marked decrease when stored at RT whilst extracts stored at -20 °C remained at the same level (Appendix B, Figure B.2 A and B). This results, covering a period of 4 weeks, are in line with

others indicating that temperatures below 10 °C are required to preserve betalains from degradation (Herbach et al., 2006b; Cejudo-Bastante et al., 2016; Martins et al., 2017a). Only Castellar et al. (2003) observed that betalains in extracts from *Opuntia* varieties were preserved for 19 days at 25 °C. Sapers and Hornstein (2006) reported that the degradation of betalains during storage was mainly depending on the pH and light exposure, and directly proportional to the initial concentration of betalains in the samples. In this study, the storage temperature also had an impact on individual betalains; as shown in Figure 2.3, betalain pattern during storage indicate that vulgaxanthin I was around 20% less prone to degradation in 30% ethanol as compared to methanol at the same percentage of solvent or compared to water. Other betalains displayed much less of a compositional change among the different extracts.

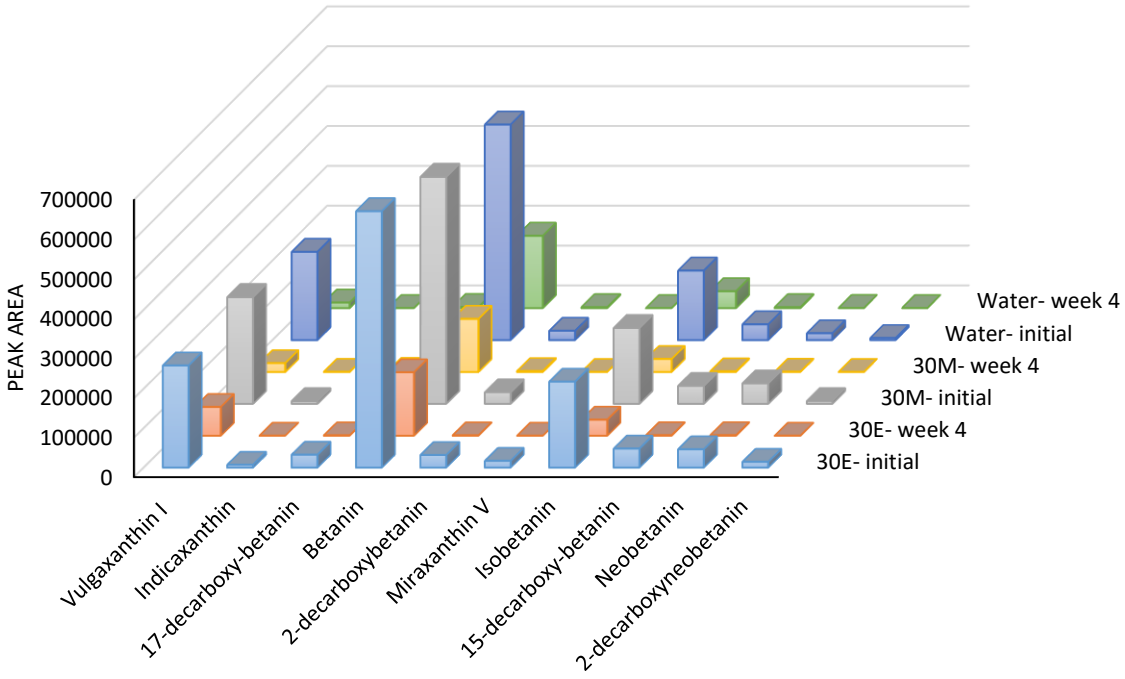



**Figure 2.3** Changes in betalain pattern during 4-week storage of extracts at room temperature. Presented are the peak areas of samples extracted with water, 30% v/v methanol (M) and 30% v/v ethanol (E), initially and at the end of the storage period.

In contrast to betalains, the TPC of the beetroot extracts showed a different pattern. Whilst the initial values of total polyphenols did not differ between the samples (Appendix B, Figure B.3), around 20% of increase was observed up to the second week and then a gradual decline until the end of the storage period, irrespective of the storage conditions; however, this was much more pronounced in samples stored at -20 °C (Appendix B, Figure B.2 C and D). The increased TPC is a phenomenon observed also by other relevant studies associating increases with the release of phenolic compounds bound to proteins or polysaccharides during storage (Klimczak et al., 2007), deglycosylation, new compound formation (Madiwale et al., 2011), and reactions occurring between (oxidized) polyphenols (Castro-López et al., 2016). Indeed, Madiwale et al. (2011) demonstrated activation of phenylalanine ammonia-lyase (PAL), an enzyme which regulates the biosynthesis of polyphenols, during storage which induced the de novo synthesis of secondary metabolites and may therefore contribute to increased phenolic content. Klimczak et al. (2007) observed an increase of free p-coumaric and ferulic acids in orange juice during storage at different temperatures due to the release of free acids from their bound form (at 18, 28 and 38 ºC) which could be a further reason for changes of TPC content. Folin reagent itself is lacking specificity, and some other reducing compounds such as phenolic amino acids and ascorbic acid are known to react with Folin, thereby increasing the TPC values independently of polyphenols (Georgé et al., 2005). In addition, the high polyphenol content during the storage period could be linked to the preferential oxidation of betalains that prevents the degradation of polyphenols present in the samples. In studies involving ABTS+, betanin was 1.5 to 2 times more efficient as a free radical scavenger than anthocyanins at neutral or basic pH (Gliszczyńska-Świgło et al., 2006). It was also observed that among betacyanins such as betanidin, betanin, and phyllocactin, betanidin was the most potent antioxidant against peroxyl radical and nitric oxide indicating that glycosylation decreases the radical scavenging activity of betacyanins (Taira et al., 2015; Slimen et al., 2017).

Antioxidant activities were determined using TEAC assay, commonly used method to assess ABTS+ radical scavenging properties. This assay, as well as others such

as FRAP, ORAC, DPPH, superoxide radical scavenging, have been shown to correlate with betalain content as demonstrated in several studies (Czapski et al., 2009; Georgiev et al., 2010; Ben Haj Koubaier et al., 2014). As shown in Appendix B, Figure B.2 E and F, antioxidant capacity was similar among samples after extraction and remained largely unaffected during storage at -20 °C. In the case of stored samples at RT, there was a successive decline in antioxidant capacity over the four-week period to around 22%. This loss of antioxidant activity was highly correlated with the betalain decline during RT storage ($r=0.7716$, $p < 0.0001$), but no correlation at -20 °C was evident ($r=0.1877$, $p = 0.1198$) (Figure 2.4 A and B). Similarly, the TPC and antioxidant capacity showed a significant correlation at RT ($r=0.3209$, $p = 0.0068$), but not at -20 °C storage ($r=0.1662$, $p = 0.1690$) (Figure 2.4 C and D).

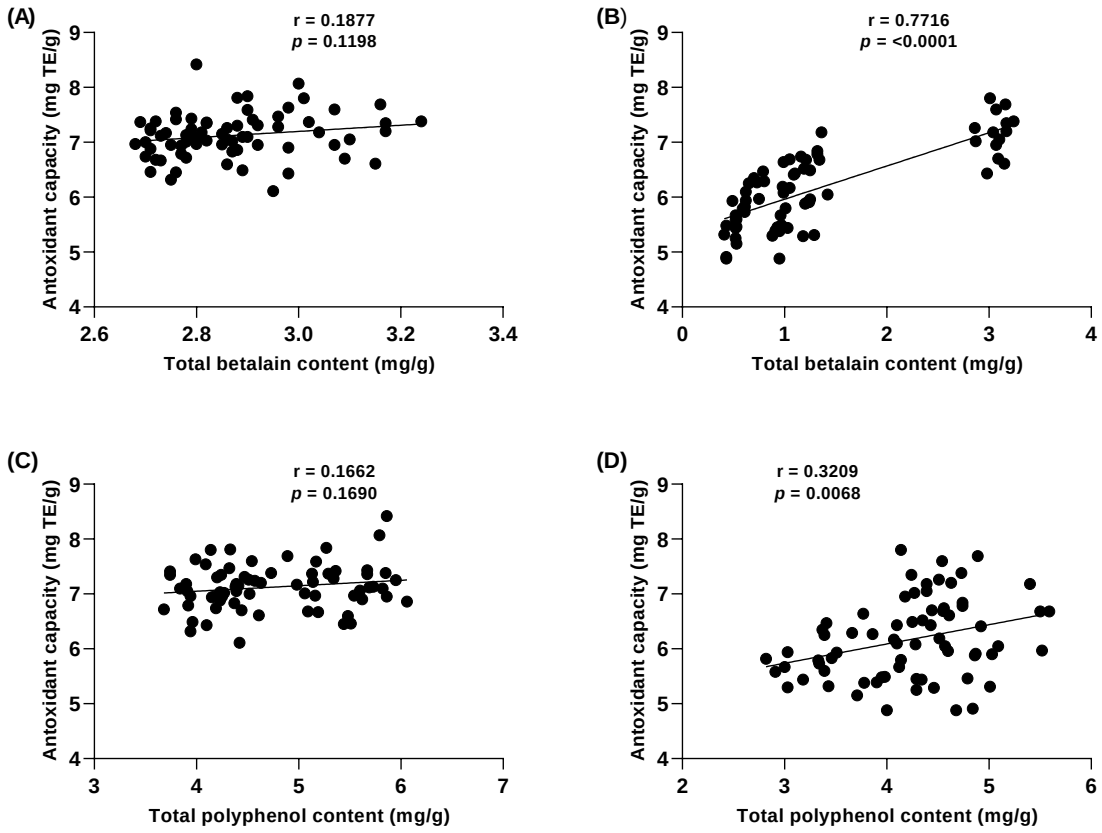



**Figure 2.4** Correlation of betalain content and polyphenol content with antioxidant activity (TEAC -Trolox equivalent antioxidant capacity) of the red beetroot extracts stored in -20 ºC (A and C) and room temperature (B and D).

Results derived from antioxidant capacity measurements are a reflection of overall radical scavenging or reducing capabilities of a sample, which is, similarly to TPC, depending on the composition as well as individual structural features of bioactives in the mixture. Apart from polyphenols, betalains have demonstrated strong radical scavenging activities as compared to known antioxidants such as ascorbic acid, tocopherols and rutin (Swarna et al., 2013; Gliszczyńska-Świgło et al., 2006). Moreover, there is evidence indicating that the degradation products of betalains, such as neobetanin, have even higher antioxidant activity than the betalains themselves (Wootton-Beard et al., 2014; Mikołajczyk-Bator and Czapski, 2018). This appears irrelevant for this study as the neobetanin concentrations were lower after 4 weeks compared to the initial data (Figure 2.3). In addition, other compounds are potentially present in extracts such as betains, carotenoids and dietary nitrate and nitrite and contributing to overall antioxidant activity (Lee et al., 2014; Clifford et al., 2015). The data are demonstrating a substantial decline (80%) of betalains at RT storage, but lower loss of antioxidant activity (22%) emphasizing possible synergetic effects of polyphenols, betalains and their metabolites as well as other compounds present in the extracts in radical scavenging and iron reducing capabilities (Georgiev et al., 2010).

### 2.3.3 Colour measurements as indicators for pigment degradation during storage

Betalains are sensitive to oxidation during storage in solutions, which affects their colour stability as a result of structure changes. Colour stability is a highly important factor when using betalains as natural colorants. Therefore, it is important to measure the colour parameters of extracts as it gives indirect indication on the pigment concentration over time. Considering the colour measurements, the L* value represent the lightness and darkness of the sample whereas the a* and b* values represent the colour direction from red to green and yellow to blue of the samples, respectively (Costa et al., 2017). The initial chromatic properties of the extracts did not show any significant difference ($p > 0.05$). There was a marked reduction of a* values (reduction of red colour) of the RT stored samples during storage compared to the initial values which indicates the degradation of betalains

in the extract, likely due to the decarboxylation of betacyanin and formation of degradation products leading to changes of the red colour to yellow/orange (Wybraniec, 2005). This was confirmed by the increasing values of b* of the room temperature stored samples compared to the initial values which indicates the development of yellow colour in the samples. However, both a* and b* values remained unchanged with the samples stored at -20 °C when compared to the initial colour values. The initial colour results of this study were compatible with data from Prieto-Santiago et al. (2020) on the relationship between the colour and the thermal degradation of beetroot betalain pigments. Pearson correlation coefficients (r) between colour measurements (L*, c*, h*, a* and b*) with TBC are shown in Appendix B, Table B.1. The L* h* and b* values were negatively correlated ($p < 0.0001$) with TBC ($r = -0.9074$, $r = -0.9256$ and $r = -0.8807$ respectively) while c* and a*values showed positive correlation ($p < 0.0001$) with TBC ($r = 0.5903$ and $r = 0.8967$ respectively). Other studies (Prieto-Santiago et al., 2020; Arias et al., 2000; Chandran et al., 2014) have reported that pigment content can be correlated better with the combined colour parameters than the single colour measurements. Therefore, the different combinations of colour numeric values were calculated and shown in Appendix B, Table B.1. The L*a*b* data is a good indicator of visual colour assessment of the samples (Prieto-Santiago et al., 2020) and there was a strong positive correlation between L*a*b* data and total betalain content ($r = 0.9820$, $p < 0.0001$). Additionally, the a/b ratio can be used as a convenient parameter for assess the colour degradation accurately as well as quantitatively (Chandran et al., 2014). Therefore, these correlations indicate that the colour measurement could be used as indirect assessment to determine the betalain pigments as easy and inexpensive method.

### 2.3.4 Application of selected extraction conditions to characterize different red beetroot samples

A further aim of this study was to apply the selected extraction conditions to different beet derived samples, which were, apart from whole beet, beet juice, beet pulp waste from juicing industries as air dried and freeze dried products.

Following extraction using 30% v/v ethanol, betalain values in the four samples ranged from 0-3.06 mg/g as shown in Figure 2.5 A. As indicated in the earlier section, the data of the present study are in the range of others, some authors have shown a higher total betalain content in red beetroot cultivars ranging from 4.43 – 9.60 mg/g dry matter (Kujala et al., 2002), and 7.42 – 8.56 mg/g dry matter (Slatnar et al., 2015). In contrast, Lee et al. (2014) observed relatively low concentrations of betalains (0.65 – 0.80 mg/g fresh weight) in red beetroot cultivars from USA. Variations of results could, apart from extraction and extraction conditions (temperature, pH), be due to differences in beet varieties and growth conditions (Sawicki et al., 2016). The ratio of betacyanin to betaxanthin was 1.12, 1.35 and 1 for the BP, BJ and beet waste (FD) samples respectively, demonstrating that betalain composition of the samples was varied. A similar ratio of betacyanin to betaxanthin has been reported previously for different beetroot sources (Kujala et al., 2002; Wruss et al., 2015).

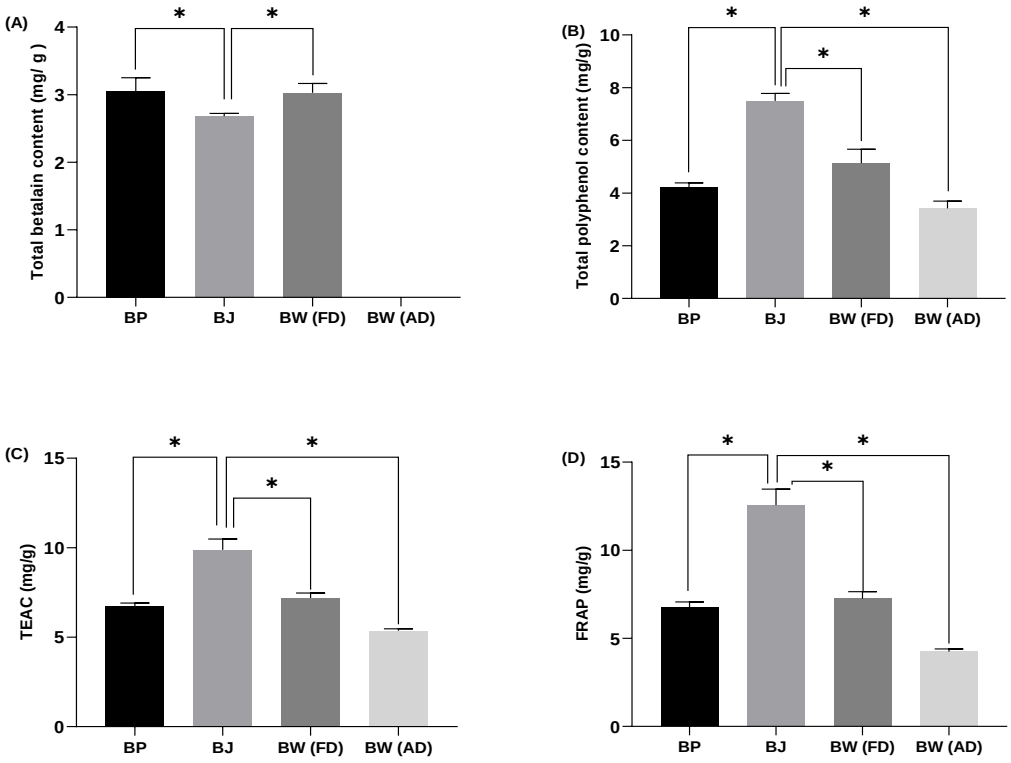



**Figure 2.5** Total betalains (A), total polyphenols (B) and antioxidant activities (C, D) in extracts of different beetroot samples after ultrasound-assisted extraction. BP – red beetroot powder, BJ – beetroot juice powder, BW (FD) – freeze-dried red beetroot waste powder and BW (AD) – air-dried red beetroot waste powder. Data are mean with SD of three independent extractions. * indicates significant difference ($p < 0.05$), Tukey's multiple comparison test.

Betalain peaks were identified using individual retention times, interpretation of MS fragmentation spectrum (m/z values) and $\lambda_{max}$ values compared with previously published data (Nemzer et al., 2011). The red beetroot sources examined in the present study contained sixteen different betalain compounds with eleven of them belonging to the betacyanin group and five to betaxanthins Figure 2.6. However, some previously reported betanin derivatives and betaxanthins could not be detected. Sawicki et al. (2016) reported the presence of eighteen betacyanins with twelve betaxanthins in thirteen Polish varieties of red beetroot. In comparison, only three betalains (betanin, isobetanin and vulgaxanthin I) were identified in red beet cultivars grown in USA and Finland (Kujala et al., 2002; Lee et al., 2014). Differences in betalain content and pattern may be due to varietal diversity, local growth and climate conditions as well as post-harvest conditions (Wiczkowski et al., 2014). The most prominent peaks identified in the current study were betanin, isobetanin, vulgaxanthin I and neobetanin. Further, not all samples contained all betalains that had been identified. The sample that had been originally air dried was devoid of most peaks indicating large-scale degradation of betalains, likely UV and temperature facilitated, whereas the peak areas in beetroot waste FD sample were more similar to the BP sample which is derived from whole beet. In general, the betalain content (betacyanins and betaxanthins) is highest in the peel of red beet in comparison to the inner rings (Sawicki et al., 2016; Kujala et al., 2002; Slatnar et al., 2015), which is also evident in the present study. Peak areas of vulgaxanthin I and betanin are much lower in the beet juice sample as compared to samples comprising the whole beet (BP) and pomace fraction (beet waste, FD) (Figure 2.6). In summary, the results of betalain analysis demonstrate that the dried beetroot waste from juicing industries can be a good source of betalain pigments, with regards to betalain yield equivalent to whole beet and beet juice.

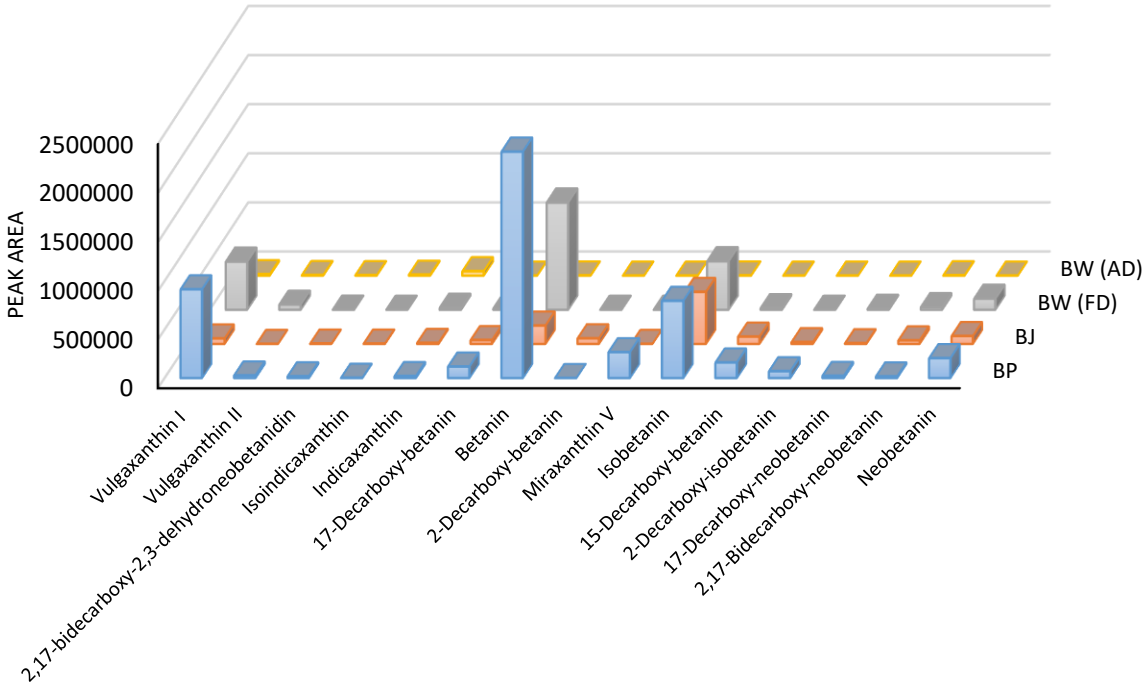



**Figure 2.6** Comparison of betalain peaks present in different beetroot sources (BP – red beetroot powder, BJ – beetroot juice powder, BW (FD) – freeze dried red beetroot waste powder, BW (AD) – air dried red beetroot waste powder)

Generally, betalains are quantified using the spectrophotometric method based on the absorption at a single wavelength and the molar extinction coefficient of the prominent betacyanin and betaxanthin present in the extracts. However, the problems arising in spectrophotometric analysis of such complex mixtures have been highlighted in the literature and attributed mainly to overlapping peaks of betacyanins and betaxanthins, and absorption by the other interfering substances present in the extract (Strack et al., 2003; Gonçalves et al., 2012). In the present study, air dried beet waste (AD) did not show any peaks around 486 nm or 536 nm in UV-vis spectrum (Appendix B, Figure B.4), but some betalains were observed in the HPLC chromatogram (Appendix B, Figure B.5). Therefore, HPLC is the method of choice for most accurate quantification of betalains, by eliminating the aforementioned problems associated with spectrophotometry. However, the standards have to be isolated from the plant materials in case of quantification of

betalains using HPLC due to lack of commercial availability (Stintzing et al., 2003). Thus, despite the relatively high discrepancy (~15%) in calculation between the two methods, HPLC and UV-vis spectroscopy, the latter remains the most convenient and fastest method to quantify betalains (Gonçalves et al., 2012). In the present study, peak areas were used as basis for comparing individual samples, which has been applied by many other groups (Ben Haj Koubaier et al., 2014; Slatnar et al., 2015).

In contrast to betalains, there are detectable polyphenols in all samples, however, the total polyphenol content is much higher in BJ compared to BP and Beet waste samples Figure 2.5. Current TPC values (3.42 ± 0.27 – 7.50 ± 0.28 mg/g) are in the range that others have reported from 0.51 ± 0.07 to 15.5 ± 0.1 mg/g (Kujala et al., 2000; Ben Haj Koubaier et al., 2014; Kavalcová et al., 2015; Vasconcellos et al., 2016; Guldiken et al., 2016) which include as main polyphenols gallic, syringic, caffeic and ferulic acids (Ben Haj Koubaier et al., 2014). In the present study twelve different polyphenols were identified of which seven were hydroxycinnamic acid derivatives, four belonging to the flavonoids group and one trihydroxybenzoic acid (Table 2.1). Similar polyphenol composition was reported by the other studies which analyzed the polyphenol composition of different varieties of beetroot including juice, roots and stem extracts (Kujala et al., 2002; Wruss et al., 2015; Płatosz et al., 2020).

**Table 2.1** HPLC-MS data (negative ionization mode) for identification of polyphenols present in different beetroot sources

| No | Compound | Retention time (min) | $\lambda_{max}$ | $[M-H]^-$ | Beetroot sources | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | BP | BJ | BW (FD) | BW (AD) |
| 1 | Catechin | 4.29 | 282 | 289 | + | + | + | + |
| 2 | Cochliophilin A | 4.98 | 283 | 281 | + | + | + | nd |
| 3 | *p*-coumaric acid | 5.33 | 282 | 163 | + | + | + | + |
| 4 | Caffeic acid | 5.67 | 265 | 179 | + | + | + | + |
| 5 | *N-trans*-feruloylmethoxytyramine | 6.13 | 278 | 342 | + | + | + | + |
| 6 | Ferulic acid | 6.39 | 274 | 193 | + | + | + | + |
| 7 | Chlorogenic acid | 7.77 | 281 | 353 | + | + | + | nd |
| 8 | Gallic acid | 9.25 | 282 | 169 | + | + | + | + |
| 9 | Rosmarinic acid | 16.65 | 265 | 359 | + | + | + | nd |
| 10 | *N-trans*-feruloyltyramine | 21.75 | 274 | 312 | + | + | + | nd |
| 11 | Quercetin | 52.40 | 361 | 301 | + | + | + | + |
| 12 | Betavulgarin | 56.42 | 279 | 311 | + | + | + | nd |

nd – not detected

In line with the polyphenol content, the antioxidant activity of BJ, determined as TEAC and FRAP, was 32% and 46% higher compared with BP and 27% and 42% higher than beet waste (FD) and 45% and 66% higher than beet waste (AD), respectively (Figure 2.5 C and D). A highly significant correlation ($p$<0.05) was observed between the total polyphenol content with TEAC assay (r= 0.9845) and FRAP assay (r= 0.9753). Interestingly, the betalain content did not show any significant correlation with TEAC (r= 2196, $p$= 0.5314) and FRAP (r=0.2078, $p$=0.5442) assays ($p$>0.05). Several studies determined a strong relationship between radical scavenging activity and betalains as well as polyphenols present in a range of fruits and vegetables (Kähkönen et al., 1999; Gil et al., 2000; Swarna et

al., 2013). For instance, Čanadanović-Brunet et al. (2011) observed a significantly high linear correlation between hydroxyl ($r > 0.81$) and superoxide ($r > 0.92$) radical scavenging activities with betacyanins and betaxanthins extracted from beetroot pomace.

## 2.4 Conclusion

To conclude, effective combined extraction of betalains and polyphenols from red beet dried powder has been demonstrated in an ultrasound-assisted approach establishing low ethanol concentrations (30% ethanol) as the most suitable solvent combination compared to the enzyme-assisted extraction method from wet pulp. The stability of betalains, in contrast to polyphenols, was strongly affected by storage temperature leading to a rapid loss of betalains over the observation period of four weeks at room temperature, irrespective of the solvent used, a finding that is in good correlation with colour measurements. The comparatively moderate loss of antioxidant activity *vs* betalain content over time emphasizes the potential contribution of betalains and polyphenols as well as their metabolites and/or degradation products to antioxidant activity. These comparative extraction results indicate that the samples derived from the beetroot industry can provide good pigment yield, after their initial drying, similarly to whole beet samples.

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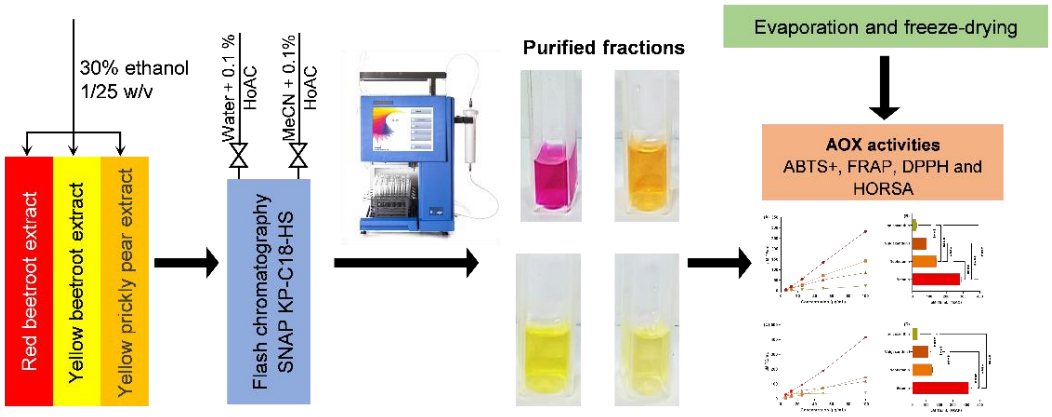
# Chapter 3

## Novel approach for purification of major betalains using flash chromatography and comparison of radical scavenging and antioxidant activities




## Highlights

- Application of flash chromatography for the first time to purify betanin and other major betalains.
- Purification yield of betalains ranged from 120 – 487 mg per 100 g of powdered raw material.
- Betanin yield from flash chromatography was 13 times higher than preparative HPLC.
- Betanin fraction exhibited higher radical scavenging activity compared to other betalains.

## Abstract

The present study focused on the development of a new purification protocol suitable for betanin and other major betalains, vulgaxanthin I, indicaxanthin and neobetanin, using flash chromatography with silica which is a convenient and fast method to isolate unstable materials. Following preliminary tests, a gradient procedure using 0-60% acetonitrile, with 0.1% (v/v) formic acid as mobile phase, was selected for the purification. Different fractions were collected based on UV detection at 254 and 280 nm and purities were confirmed by reverse-phase HPLC analysis to be 97%, 95%, 79% and 52% for betanin, indicaxanthin, vulgaxanthin I, and neobetanin, respectively, with pigment yields ranging from 120 – 487 mg per 100 g of powdered raw material. Comparative assessment of antioxidant and radial scavenging properties of individual betalains indicated highest potential for betanin followed by neobetanin, vulgaxanthin I and indicaxanthin.

**Key words**: Betalains, purification, flash chromatography, HPLC, antioxidant and radical scavenging activity

## 3.1 Introduction

Betalains are a group of natural pigments responsible for red, pink, and yellow-orange colours of plants belonging to the families of *Amaranthaceae* and *Cactus*. According to their chemical structure, two types of betalains can be distinguished: betacyanins such as betanin and isobetanin, and betaxanthins such as vulgaxanthin I and indicaxanthin. Betalains are present in abundance particularly in red beetroot, prickly pear and dragon fruit; however, total betalain content and betalain composition of any given plant species is distinctive and depends on different factors such as cultivar, plant part, ripening stage, salinity and farming practices (Celli and Brooks, 2017). Betalains have gained increasing attention in recent years not only due to their high tinctorial strength but also due to their promising bioactive properties. It has been demonstrated that betalain extracts from different plant sources have stronger antioxidant and radical scavenging potential comparable to the typical antioxidants such as ascorbic acid, rutin, catechin and tocopherol (Slimen et al., 2017).

Recent studies on antioxidant activity suggested that betalains should be considered as a new category of dietary antioxidants, although there is inconsistency on whether betacyanin or betaxanthins are more potent. Gomphrenin-type betacyanins from *Amaranthacea* have been shown to reduce DPPH radical formation by 74%, whereas betaxanthins showed around 50% inhibition (Cai et al., 2003). In contrast, the lipoperoxyl radical scavenging of betaxanthin-rich fractions were higher compared to betacyanin-rich fractions (Zakharova and Petrova, 1998). Most of the bioactivity studies have been conducted with crude plant extracts with little to no pigment purification (Czapski et al., 2009; Georgiev et al., 2010; Čanadanović-Brunet et al., 2011; Vulić et al., 2014). Crude plant extracts contain not only betalains but also other bioactive compounds such as polyphenols, organic acids, and therefore, it is difficult to clearly allocate bioactivities to individual compounds. In addition to major betalain pigments, Mikołajczyk-Bator and Czapski (2017) identified neobetanin, a degradation product of betanin, as a strong antioxidant and established that increasing concentrations of neobetanin significantly increased antioxidant activity of beetroot juice. Despite the emerging evidence on betalain bioactivities, there is a lack of studies comparing the radical scavenging activities of individual betalains (Cai et al., 2003; Butera et al., 2002). Studying purified betalains is highly important in order to understand their individual potential and the detailed mechanisms by which they act on biological processes, and hence allow development of betalain-based novel applications.

Purification of individual betalains has been reported using different techniques, such as column chromatography and preparative HPLC (Kusznierewicz et al., 2021). The common challenges identified in the purification of phytochemicals including betalains, are the co-extraction of compounds bearing the same polarity, low purification yield, time consuming procedures and impacts on the stability of the pure compounds (Aznar and Rai, 2020). Gonçalves et al. (2012) compared seven different techniques previously used for betalain purification by other researchers (Butera et al., 2002; Stintzing et al., 2002) and concluded, based on yield and purity, that reversed-phase HPLC and ion exchange chromatography are the best methods to purify betanin from red beetroot. The purification yields using above methods were

14.7 ± 2.7 and 16.2 ± 2.9 mg per 100 g of raw material (red beetroot powder), respectively, with around 99% purity achieved with both methods. Butera et al. (2002) purified betanin and indicaxanthin from prickly pear using a gel filtration based method and showed purification yields of 5.12 ± 0.51 and 8.42 ± 0.51 mg per 100 g edible pulp, respectively. Similar to the above studies on betalain extraction, low yields of polyphenols were obtained using the column chromatography and preparative HPLC method. Minh et al. (2019) was able to purify 4.58 mg/g of gallic acid and methyl gallate mixture as well as 0.6 mg/g of fraxetin using dried methanol extract from stem bark of *Jatropha podagrica* by normal phase column chromatography method. Liu et al. (2020) purified 12 different polyphenols from 200 g of dried roots of *Heshouwu* (*Polygonum multiflorum* Thunb) using a combination of high-speed countercurrent chromatography and preparative HPLC which resulted in purification yields ranging from 4.1 - 20.2 mg. Although these methods demonstrated high compound purity, they are time-consuming, result in low purification yields, and require large quantities of raw materials and solvents.

Flash chromatography is a simple and robust column chromatography based technique that is heavily used in the pharmaceutical industry for drug discovery, peptide and antibiotic purification (Sandesh et al., 2021). Compared to other chromatographic methods, flash chromatography uses relatively high flow rates with low pressure either by normal or reverse phase separation and high sample loading which contributes to good separation in a short time (Weber et al., 2011). Further, flash chromatography is easily applicable to scale up to meet industrial applications (Bickler, 2018). Whilst this method has been more frequently applied to purification of synthetic compounds, there is an increasing interest in utilizing flash chromatography as part of natural compound purification approaches. For example, flash chromatography was used as part of the fractionation work flow to purify four main bioactive compounds (two proanthocyanidins, *p*-hydroxybenzoic, and hyperoside) from extracts of *A. columbrina* leaves (Rodrigo Cavalcante de Araújo et al., 2019). Further, recent literature demonstrated the purification of quercetin and other individual compounds from *Chenopodium album* (Arora and Itankar, 2018), as well as rosmarinic acid in *Origanum majorana* (Hossain et al., 2014). Therefore, flash

chromatography is considered a comparatively effective technique to achieve high yields using low solvent and raw material input.

The present study aimed to develop a flash chromatography-based protocol for the purification of betanin from red beetroot which is not currently available, and to apply the method to isolate other major pigments, vulgaxanthin I and indicaxanthin, from yellow beetroot and prickly pear sources, respectively. Furthermore, we aimed for the isolation of neobetanin, a major betanin degradation product that frequently evolves during storage, which has not been attempted earlier. Ultimately, the comparative assessment of the four purified betalains with regards to their antioxidant and radical scavenging activities is targeted to provide more detailed information on the potential of individual betalains.

## 3.2 Materials and methods

### 3.2.1 Chemicals and materials

Red and yellow beetroot, originating from UK and US sources, respectively, were kindly provided by Biopowder Ltd., Milton Keynes, UK in dried and powdered form (moisture content < 2% (w/w)). Yellow prickly pear samples (freeze dried) were sourced from Spain (Miguel Hernandez University of Elche, Alicante, Spain). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl hydrate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium peroxodisulfate, sodium acetate-trihydrate, iron (III)-chloride-hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine, 1,10-phenanthroline, ferrous sulfate heptahydrate, 30% hydrogen peroxide, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dehydrate were purchased from Sigma-Aldrich (Dorset, UK). All other chemicals and solvents were purchased from Fisher Scientific (Loughborough, UK). All aqueous solutions were prepared using deionized water purified by Milli-Q water purification system (Millipore, MA, USA).

### 3.2.2 Sample preparation

Extraction of betalains was carried out using ultrasound-assisted extraction as recently described (Fernando et al., 2021). This method uses 30% (v/v) ethanol instead of pure water to extract the betalains because pure water can cause severe difficulties during the solute separation by filtration stage due to the co-extraction of mucilaginous compounds such as pectin (Fernando et al., 2021). Moreover, the removal of water is more challenging than alcohol when concentrating the samples and pure water could lead to degradation of pigments in the extract.

Briefly, beetroot and prickly pear powder samples (10 g) were mixed with 250 mL of 30% (v/v) ethanol. The mixture was vortexed for 2 min, then placed in an ultrasonic bath (XUBA3, Grant Instruments, UK) and sonicated at 44 kHz for 30 min at 30 °C. The mixture was subsequently centrifuged for 10 min at 3500 × g and the supernatant decanted. The residue was re-extracted with the same amount of solvent for maximum recovery of pigments. The extracts were filtered using 0.22 µm pore membrane filter and narrowed down to 20% of initial volume using Genevac centrifugal evaporator (SP industries Inc., Warminster, Pennsylvania). Extracts were stored at -20 °C until further use. Initial betalain content of the crude extracts was determined using spectrophotometry (Specord 210 plus, Analytik Jena, Germany) and calculated using extinction coefficient values for 60,000 $cm^{-1}M^{-1}$ at $\lambda_{max}$ 540 nm and 48,000 $cm^{-1}M^{-1}$ at $\lambda_{max}$ 480 nm for betacyanin and betaxanthin, respectively. The total amount of betalain (in mg per g sample) was calculated by combining the values for betacyanin and betaxanthin.

### 3.2.3 Purification of betalains

#### 3.2.3.1 Preparative High Performance Liquid Chromatography (Reversed-phase)

Initially, the purification of betanin was performed using the Agilent 1260 preparative HPLC system (Santa Clara, United States) with a reverse-phase Kinetex® 5 µm C18 100 Å, AXIA packed LC Column 100 × 21.2 mm (Part number: OOD-4601-PO-AX) following a method described by Gonçalves et al. (2012) with modifications. The program consisted of 40 min gradient elution with 0.1% (v/v) acetic acid in water

(solvent A) and 0.1% acetic acid in 60% acetonitrile (solvent B) with a flow rate of 5 mL/min. A sample volume of 5 mL (concentrated red beetroot extract), which is the maximum volume with this equipment, was used per run. The chromatographic conditions were defined as follows: gradient elution mode with 2-20% B for 30 min, 20-100% B for 5 min, and 100-5% B for the last 5 min. Elution monitoring was performed at 280 nm. Eluted fractions were stored separately at -20 °C until used for identification and lyophilization. The fractions were identified as betanin, betaxanthin and a mixture of degraded products of betanin (neobetanin, 15-decarboxy-betanin, 2-decarboxyisobetanin, and 2-decarboxyneobetanin), based on the HPLC/MS analysis. Further, UV-Vis monitoring of three main fractions showed absorbance maxima ($\lambda_{max}$) at 530 nm, 479 nm, and 478 nm (data not shown) which is characteristic to betacyanins, betaxanthins and degraded products of betanin, respectively.

#### 3.2.3.2 Flash chromatography

Thin layer chromatography was used to pre-determine the separation of betalains in two different solvent systems. Briefly, red beetroot extract was run in acetonitrile (0 – 100% v/v) with 0.1% formic acid or with acetic acid on TLC Silica gel 60 $F_{254}$ aluminium plates (Merck, Darmstadt, Germany). The retention factor of the compounds was calculated by dividing travel distance by the solvent front. The solvent system containing 60% acetonitrile with 0.1% formic acid showed the best separation (Appendix C, Figure C.1) and was therefore used with flash chromatography to purify the betalains.

Concentrated extracts of red beetroot, yellow beetroot, and yellow prickly pear were submitted to a reversed-phase flash chromatography procedure using a KP-C18-HS Biotage SNAP cartridge that was mounted on a fully automated Biotage Isorela system (Isorela One, Biotage, Sweden). This reverse-phased cartridge was suitable for separation of polar compounds and the cartridge was packed with silica, amorphous (irregular). Average particle size was 50 microns with 100A pore diameter and 500 $m^2/g$ surface area with good resolution. For the purification, 15 mL of concentrated extract was loaded onto the cartridge. The elution process was

performed at a flow rate of 50 mL/min. The chromatographic procedure was run using a linear-gradient solvent system with mobile phase A with water and 0.1% formic acid, and mobile phase B with acetonitrile and 0.1% formic acid. The elution started with 1 column volume (CV) of 0% (v/v) of solvent B for equilibrating the cartridge and then eluted with 10 CV of the solvent B starting from 0% to 60% (v/v). UV absorption was monitored at 254 nm and 280 nm wavelength. Eluted fractions (20 mL) were stored separately at -20 °C until further use. Flash purification process was repeated a minimum of three times per compound.

The flash chromatograms of each purified compound using crude extracts as input material are shown in Appendix C, Figure C.2. For example, a total of 19 fractions were collected using red beetroot extract while 22 fractions were collected using yellow beetroot extract (Appendix C, Table C.1 – C.4). All the fractions were analysed by HPLC/MS, described under section 3.2.4, and the fractions bearing the same mass and spectral characteristics were combined together. The combined fractions were then evaporated to remove the solvent and freeze-dried.

### 3.2.4 Identification of purified betalains

Identity and purity of the target compounds in collected fractions were determined with HPLC/MS and accurate mass methods.

HPLC/MS method described in Fernando et al. (2021) was used to identify the purified fractions. The HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a 4.6 mm × 250 mm, 5 µm Phenomenex Gemini C18 column was used for the analysis. Run conditions were as follows: gradient elution mode started with 5-25% B for 15 min, 25-70% B for 4 min and 70-5% for the last 7.10 min. The injection volume was 10 µL and the flow rate of 0.95 ml/min was used. Betacyanins and betaxanthins were monitored at 536 nm and 486 nm, respectively. HPLC elution profiles of the purified fractions were identified using m/z values and retention times from the literature (Hayet et al., 2014).

The Maxis Impact HD Mass Spectrometer (Bruker MaXis impact, Bremen, Germany) was used to confirm the results of analytical HPLC/MS characterization in purified fractions by determining the accurate molecular formula of the compounds. Samples were submitted to electrospray ionization (ESI) mass spectroscopy and a positive ion ESI mass spectrum was recorded.

Based on HPLC/MS identification, fractions of major betalains from different flash chromatography runs were pooled (betanin neobetanin, vulgaxanthin I and indicaxanthin). Acetonitrile was evaporated from the samples using Genevac centrifugal evaporator followed by lyophilization (Labconco FreeZone 2.5, Labconco cooperation, Kansas City, United States). Freeze dried samples were stored at -20 °C until further analysis.

### 3.2.5 Assessment of purity of the purified betalains

#### 3.2.5.1 Sugar analysis of purified fractions

Presence of sugars in purified fractions was determined using UFLC$_{XR}$ system (Shimadzu, Kyoto, Japan) with evaporative light scattering detector (ELSD). The samples were separated on a Grace Davison Prevail Carbohydrate Es column (5 µm, 250 mm × 4.6 mm) using isocratic conditions. The mobile phase was 75% acetonitrile (v/v) and the flow rate was 0.5 mL/min with a sample volume of 10 µL. The operating parameters for ELSD were signal, 0.00, gain 4 and pressure 350 kPa with the 40 °C drift tube temperature. Peak identification and quantification were carried out based on the comparison of retention time with external standards of sucrose, glucose and fructose ranged between 100 to 3000 µg/mL.

#### 3.2.5.2 Polyphenol analysis of purified fractions

The presence of polyphenols in purified fractions was tested using HPLC/MS method described by Ifie et al. (2016). The same HPLC/MS instrument described in 2.4 section was used for the analysis. The mobile phase consisted of 0.5% (v/v) formic acid in water (solvent A) and a mixture of acetonitrile, water, and formic acid (50:49.5:0.5, v/v) (solvent B), with a flow rate of 0.5 mL/min. The gradient elution started with 8% B and was increased to 18% B at 5.32 min, 32% B at 27.36 min,

60% B at 42.56 min reaching 100% B at 49.04 min, held at 100% B for 6.08 min and returning to initial conditions for 4.52 min, with total run time of 60.04 min. Presence of polyphenols was checked using DAD data and m/z values taken from the literature (Hayet et al., 2014).

### 3.2.6 Antioxidant activity assays

Purified betalains were subjected to antioxidant and radical scavenging assays using Trolox equivalent antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP), 2,2,-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and hydroxyl radical scavenging activity assays. Stock solutions (5 mg/mL) of each purified compound were prepared in 30% ethanol and further diluted according to the assay requirement (between 0 – 100 µg/mL concentrations).

The methods described in Han et al. (2021) were used to perform TEAC and FRAP assays. Briefly, $ABTS^{\bullet+}$ was prepared by reacting ABST stock solution with potassium peroxodisulfate. ABTS radical scavenging activity was measured adding 300 µL of diluted ABTS radical solution into 10 µL of sample. Absorbance values were recorded after 6 min incubation at 734 nm. For the FRAP assay, $FeCl_3$ solution in sodium acetate buffer was prepared adding 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ) solution and acetate buffer (FRAP reagent). Reducing capability was measured mixing 10 µL of sample with 300 µL FRAP reagent. Absorbance was monitored at 594 nm after incubation at 37 °C for 15 min. Trolox standard curve was used in both assays and results were expressed as mg Trolox equivalents per g purified pigments.

The DPPH assay was conducted based on the method described by Yu et al. (2019) with some modifications. Briefly, 0.1 mM DPPH solution in methanol (200 µL) was added to 20 µL of sample and incubated for 30 min in the dark at room temperature. Absorption was measured at 517 nm. The hydroxyl radical scavenging activity assay (HORSA) was carried out using the method described by Arise et al. (2016). Samples and 1,10 phenanthroline (3 mM) were dissolved in 0.1 M phosphate buffer, pH 7.4, while $FeSO_4$ and 0.01% hydrogen peroxide were each prepared in distilled

water. Scavenging activity was measured adding 50 µL of sample/standard or buffer into the 96-well plate followed by 50 µL of 1, 10-phenanthroline and 50 µL of $FeSO_4$. Then, 50 µL of $H_2O_2$ was added into each well to start the reaction and measured the absorbance at 536 nm every 10 min for a period of 1 h at 37 °C. The absorbance of blank (without $H_2O_2$) and control (without sample) was also determined. Antioxidant activities were given as percentage DPPH and HORSA scavenging and calculated as: [(blank absorbance – sample absorbance)/ (blank absorbance)] × 100. All plate based absorbance measurements were conducted using a Tecan Spark™ 10M multimode microplate reader (TECAN, Männedorf, Switzerland).

### 3.2.7 Folin-Ciocalteu method

The Folin-Ciocalteu method described by Fernando et al. (2021) was used with different dilutions of purified compounds (0 – 100 µg/mL). Gallic acid was used as the reference standard. Briefly, 10 µL of sample or gallic acid standard was mixed with 40 µL of 10% (v/v) Folin reagent and 150 µL of 4% (w/v) sodium carbonate followed by a 30 min incubation at room temperature in the dark. Absorbance was measured at 765 nm using Tecan microplate reader.

### 3.2.8 Data analysis

The data are reported as mean ± standard deviation of three or more independent experiments and graphs were drawn using GraphPad Prism version 9.0 for Windows. One-way and two-way ANOVA with Tukey's post-hoc multiple comparison test were applied to determine the statistical significance among the different purified compounds and within the different treatments of purified compounds at $p < 0.05$. ChemDraw Ultra version 12.0.2. for Windows were used to draw the chemical structures and calculate the exact mass values.

## 3.3 Results and discussion

### 3.3.1 Purification of betalains

Betanin purification was carried out by preparative HPLC method in the first instance to assess the yield and purity of the compound. The chromatogram of preparative HPLC showed three main peaks with retention times of 2.52, 5.92, and 33.74 min

(Appendix C, Figure C.3) which were corresponds to betanin, betaxanthins and degraded products of betanin respectively.

The total betalain content (betacyanin and betaxanthin) of initial red beetroot extract was 8.67 ± 0.30 mg/g and the yield of the purified betanin using preparative HPLC was 0.37 ± 0.01 mg/g which corresponds to 4.2% of the initial betalain content based on the UV-Vis spectrophotometric measurements. The betanin yield achieved here was similar to da Silva et al. (2019) who reported a betanin yield of 0.384 mg/g. However, this yield is fairly low and preparative HPLC is a lab-intensive and time-consuming process to obtain purified betalains. The aim of the present study was to develop a quantitative purification method for betalains, which can deliver a cost- and resource effective solution for betalains in amounts that are adequate for further experiments i.e. identification and bioactivity confirmation. Therefore, automated flash chromatography was selected as a fast, effective and economical method compared with other purification techniques, suitable for separating large quantities. Flash chromatography has indeed been successfully applied to purification of groups of compounds as well as individual compounds. For example, using this technique, Arora and Itankar (2018) obtained yields up to 7.34 mg/g purified flavonoid from *Chenopodium album* and Hossain et al. (2014) demonstrated the successful purification of rosmarinic acid and arbutin from *Origanum majorana L*. To our knowledge, this method has not been applied for betalain purification.

In the present study, flash chromatography was initially used to purify betanin from red beetroot extracts. The method optimized for betanin was subsequently applied to separate neobetanin, vulgaxanthin I and indicaxanthin from red beetroot, yellow beetroot and yellow prickly pear extracts, respectively. Preliminary experiments using TLC were conducted to test solvent effects on separation effectiveness of the target compounds since it is a simple and rapid method to generate separation fingerprints of crude extracts. The mobile phase that resulted in $R_f$ (retention factor) values between 0.2-0.5 was selected for flash separation (Bedikou et al., 2020). $R_f$ values indicate the solubility of the particular compound in the solvent by measuring the compound movement on the TLC plate. The solvent system composed of 60%

acetonitrile with 0.1% formic acid (v/v) showed better separation compared to other tested solvent systems (Appendix C, Figure C.1). Acetonitrile with 0.1% formic acid showed better separation compared to the equivalent with 0.1% acetic acid addition. It has been well documented that formic acid is ideal for improving the peak shape and separation and is available in higher purity compared to acetic acid which can be easily degraded and contaminated (Do et al., 2020).

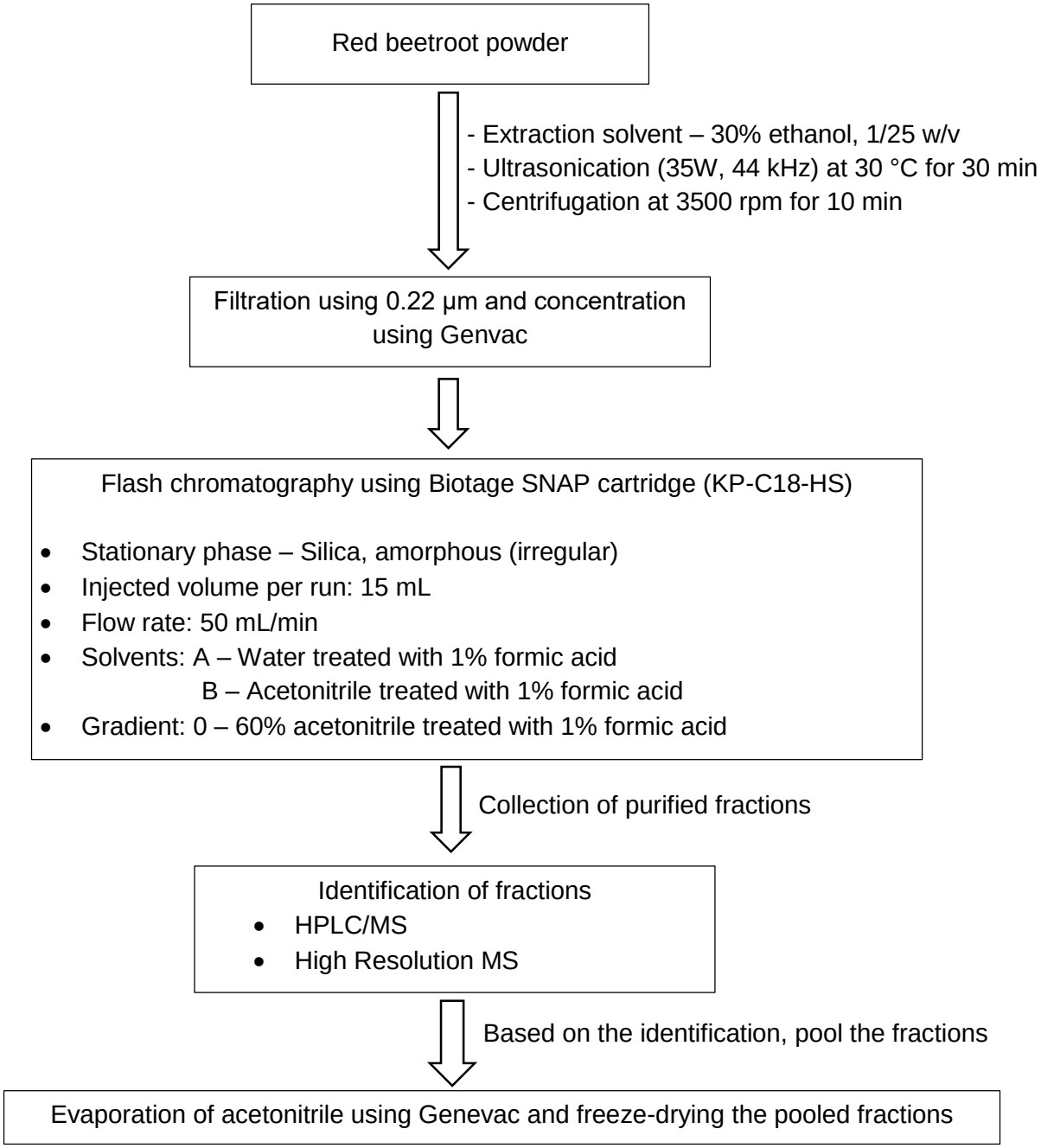



**Figure 3.1** Work flow of extraction, purification, and identification of betanin using flash chromatography method.

The purification workflow is shown in Figure 3.1. In the present study reversed-phase 50 µM C18 SNAP cartridge was used for the purification and this high surface silica cartridge provides the improved loading capacity and effective separation of betalains from crude extracts. In addition, the present study used the UV region to monitor the separation of compounds due to the unavailability of detectors for visible region in the instrument. Betalains are typical organic compounds, which have characteristic bands in visible as well as UV regions. Therefore, both regions can be used for efficient detection and separation of the pigments. The average purification yields using flash chromatography were 4.87, 1.87, 3.02 and 4.09 mg/g for betanin, neobetanin, vulgaxanthin I and indicaxanthin, respectively (Table 3.1). Compared to the preparative HPLC, purification via flash chromatography has resulted in a 13.2 times higher betanin yield. In this case flash chromatography is a faster, cheaper and equivalent separation performance compared to the preparative HPLC due to several features such as the cartridges are relatively shorter and wider with larger particle sizes (25 or 50 microns), so they can withstand a higher sample load with reduced operating pressure at the elevated flow rates, they are reusable with comparatively low replacement and operating costs. Furthermore, it is possible to scale up flash chromatography separation to industrial-scale compared to the other pigment separation techniques such as preparative HPLC. The effective scale up of flash purification can be achieved controlling the factors such as sample concentration, solvent, form of sample (dry or liquid), stationary phase and solvent flow linear velocity.

**Table 3.1** Yield, purity (%), calculated m/z values, observed HRMS molecular formula and m/z values of purified betalains obtained through flash chromatography.

| Compound | Molecular weight (g/mol) | Yield (mg) using 10 g of raw material | Formula from HRMS | Calculated [M + H] (*m/z*) | Observed [M + H] (*m/z*) | % purity based on HPLC |
|---|---|---|---|---|---|---|
| Betanin[a] | 550.5 | 48.7 ± 2.12 | M + H | 551.1508 | 551.1507 | 96.85 |
| Neobetanin[a] | 548.5 | 18.7 ± 1.69 | M - $CH_2OH$ + H + $Na^+$ | 541.1070 | 541.1524 | 51.86 |
| Vulgaxanthin I[a] | 339.3 | 30.2 ± 2.89 | M + $H_2O$ + H + $Na^+$ | 381.0910 | 381.0788 | 79.17 |
| Vulgaxanthin I[b] | | 12.0 ± 1.06 | | | | 61.55 |
| Indicaxanthin[c] | 308.3 | 40.9 ± 2.26 | M + H | 309.1082 | 309.1069 | 95.32 |

Source: a – red beetroot powder, b- yellow beetroot powder, c- yellow prickly pear powder

The betanin as well as indicaxanthin yields from the present study were higher compared to Butera et al. (2002) who used gel filtration on a Sephadex G-25 column and obtained 5.12 mg betanin and 8.42 mg indicaxanthin from 100 g edible pulp of red and yellow prickly pear respectively. Further, current betanin yield was higher than Gonçalves et al. (2012) who purified betanin using seven different purification methods including normal and reverse phase chromatography, as well as aqueous two-phase extraction methods, resulting in purification yields ranging between 4.9 ± 1.4 – 20.5 ± 2.4 mg/ 100 g of red beetroot powder. Vulgaxanthin I yield in the present study was 3.7 times higher compared to Tesoriere et al. (2008), who used liquid chromatography on a Sephadex G-25 column (80 × 2.2 cm) and obtained 81.6 mg/100 g from red beetroot, which was quantified by spectrophotometry without indication of purity. Overall, results of the present study indicate superior purification yield using flash chromatography when compared to other betalain purification methods.

### 3.3.2 Identification of purified betalain fractions

In the present study several analytical techniques such as HPLC-DAD (Diode-Array Detector), quadrupole MS and HRMS were used for the determination and profiling of betalains while confirming the purity of the isolated compounds that were obtained from flash chromatography. These are standard analytical techniques utilized in characterizing of chemical compounds. Initial identification of the purified fractions was performed using HPLC/MS. Further, HPLC-DAD chromatograms were monitored at 536 nm and 486 nm for betacyanin and betaxanthin, respectively (Figure 3.2) in order to calculate the purity of the isolated compounds. The retention times for vulgaxanthin I, indicaxanthin, betanin and neobetanin were 5.2, 9.5, 12.1 and 20.1 min, respectively in HPLC-DAD chromatograms. Additionally, chromatographic profiles of betalains in crude extracts (red beetroot, yellow beetroot and yellow prickly pear) were monitored at 536 nm and 486 nm (Appendix C, Figure C.4) to compare the purification effectiveness. Purity of the isolated compounds was calculated using peak area of DAD chromatograms observed at 536 nm for betanin and 486 nm for neobetanin, vulgaxanthin I and indicaxanthin (Table 3.1). A high purity (> 95%) was noted for betanin and indicaxanthin while vulgaxanthin I showed

only 79% purity under these conditions. The purity of neobetanin was found to be 52% with approximately 10% of betanin and isobetanin identified in the sample.

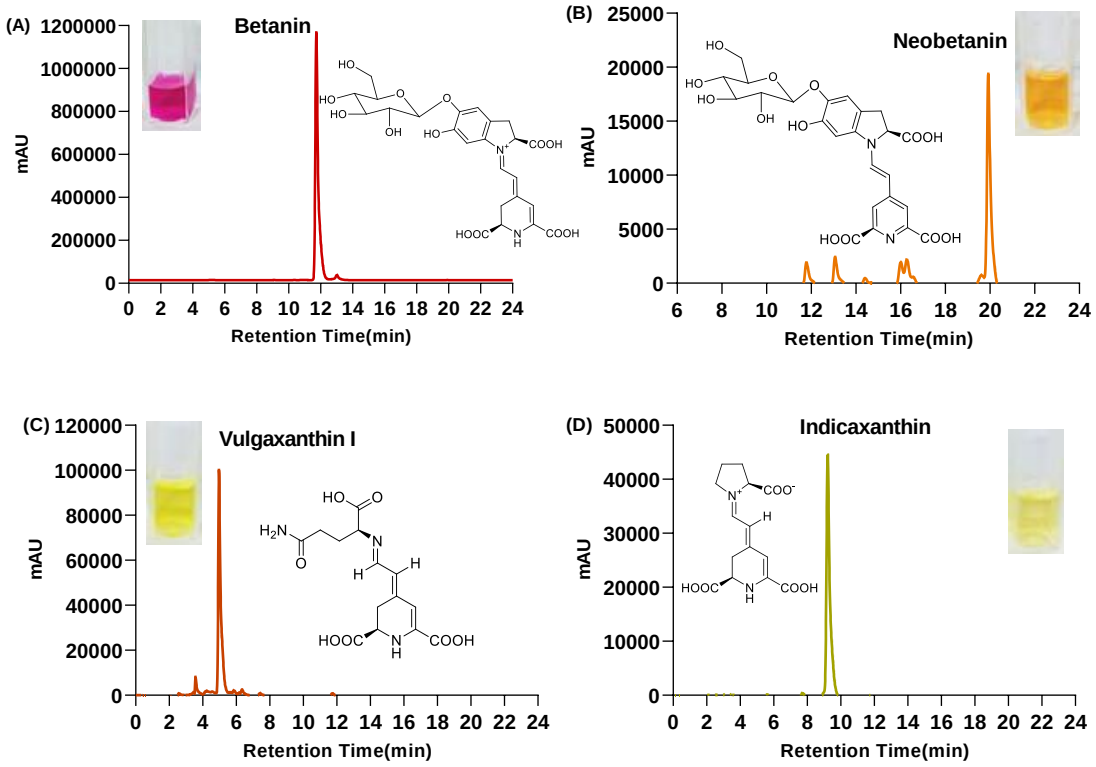



**Figure 3.2** HPLC-DAD chromatograms of purified betalains (A) betanin at 536 nm, (B) neobetanin, (C) vulgaxanthin I, and (D) indicaxanthin at 486 nm. Chromatographic conditions: mobile phase A - water/formic acid (98:2 v/v), B – methanol; flow rate: 0.95 mL/min; column oven: 40 °C.

Furthermore, identification of purified betalain fractions was performed using high resolution mass spectroscopy (HRMS) which is shown for betanin in Appendix C, Figure C.5. The pseudo-molecular ion having an m/z value of 551.1507 which corresponds to betanin and having a fragmentation ion with an m/z of 389.0973 corresponding to betanidin, can be observed in the MS spectrum and provided confirmation of betanin presence. The same fragmentation pattern of betanin was observed in studies conducted by Gonçalves et al. (2012) and da Silva et al. (2019). Table 3.1 shows the calculated m/z values using ChemDraw (Calculated m/z) and

observed m/z values in HRMS of all purified betalains confirming the identity and purity of the collected fractions.

In the present study, reverse-phase cartridge which contains hydrophobic stationary phase (C18) and hydrophilic mobile phases (water/ acetonitrile) was used to perform flash chromatography. Therefore, sugars (hydrophilic compounds) pass through the column and elute first. Further, retention times of sugars are shorter than the retention time of the pigment and sugars elute before the pigments in the flash chromatography system under the chromatographic conditions applied in this study. However, to ensure absence of sugars that might interfere with subsequent analyses, purified betalain samples were analysed via HPLC-ELSD. The results demonstrate that all samples were free from sugars except vulgaxanthin I which was isolated from both red and yellow beetroot extracts, which showed a sucrose peak in the purified sample (318.03 ± 22.5 mg/g). Attempts to remove sugars from the yellow beetroot extract prior to flash chromatography e.g. using SPE cartridge (Oasis MAX 3 cc Vac cartridge, 60 mg sorbent, Waters Corporation, U.K), or adjustment of elution gradient during chromatography run were unsuccessful which is likely due to the similar polarities of vulgaxanthin I and sucrose resulting in co-elution, which is particularly evident with low pigment concentrations in the initial extract. Indeed, the yield and purity of vulgaxanthin I from yellow beetroot were significantly lower ($p<0.05$) compared to red beetroot extract (yield: 12 vs 30.2 mg/g; purity: 61 vs 79%) with starting concentrations of total betaxanthins in yellow beetroot being 6.27 times lower compared to red beetroot.

### 3.3.3 Antioxidant activity assays

The antioxidant and radial scavenging potential of betalains has been highlighted by a number of studies, however, very few have utilized purified betalains (Gandia-Herrero et al., 2016; Cai et al., 2003). The focus of previous research was on betanin and indicaxanthin, and to the best of our knowledge, none of the studies included purified vulgaxanthin I and neobetanin. The current study therefore focused on evaluating the antioxidant properties of purified betanin compared to neobetanin, vulgaxanthin I and indicaxanthin using several commonly used *in vitro* assays that

assess radical scavenging (ABTS, DPPH, hydroxyl radical) and iron reduction capacities (FRAP). Given the different mechanisms and radical probes as well as limitations of each individual assay, it is of advantage to run several assays to determine the overall antioxidant capacity of a particular compound (Mu et al., 2018).

Scavenging effects of purified betalains with different radical systems are shown in Figure 3.3. ABTS+ and DPPH radical scavenging assays determine the reducing capabilities of antioxidants either by electron transfer or radical quenching via H atom transfer while FRAP assay measures the ability of an antioxidant to transfer one electron to reduce the $Fe^{3+}$ into $Fe^{2+}$ (Nimse and Pal, 2015). All purified betalain compounds appeared to exhibit a dose-dependent response in radical scavenging assays (ABTS+, DPPH and FRAP). When compared at 100 µg/mL, betanin showed significantly higher ABTS+ and FRAP reducing antioxidant activity ($p<0.001$) than neobetanin, vulgaxanthin I and indicaxanthin (Figure 3.3B and 3.3D). Current results are in agreement with Butera et al. (2002) who demonstrated that betanin was more potent scavenger of ABTS+ radicals compared to indicaxanthin.

Similar to the above results, betanin showed higher DPPH scavenging activity compared with other betalains, however, a plateau can be observed at 25 µg/mL and above (Figure 3.3E). Olszowy and Dawidowicz (2018) examined the possible usage of ABTS+ and DPPH assay to estimate the antioxidant activity of coloured compounds. They exhibited that reliable results from the above assays can be obtained if a test compound does not coincide with the wavelength used in the assay. The DPPH assay used a 517 nm wavelength to monitor the radical scavenging activity and the absorption maxima ($\lambda_{max}$) of betanin is ~536 nm. Therefore, absorbance scans with and without DPPH radicals were examined (Appendix C, Figure C.6) to assess the possible colour interference of purified betanin. The results showed that higher concentration of betanin interfered with the assay results while lower concentrations (< 25 µg/mL) showed less interference. The possible reason could be that any remaining betanin which does not react with DPPH radicals at the moment of the measurement also absorbs at 517 nm wavelength and therefore,

does not allow the exact monitoring of DPPH radical formation (Olszowy and Dawidowicz, 2018).

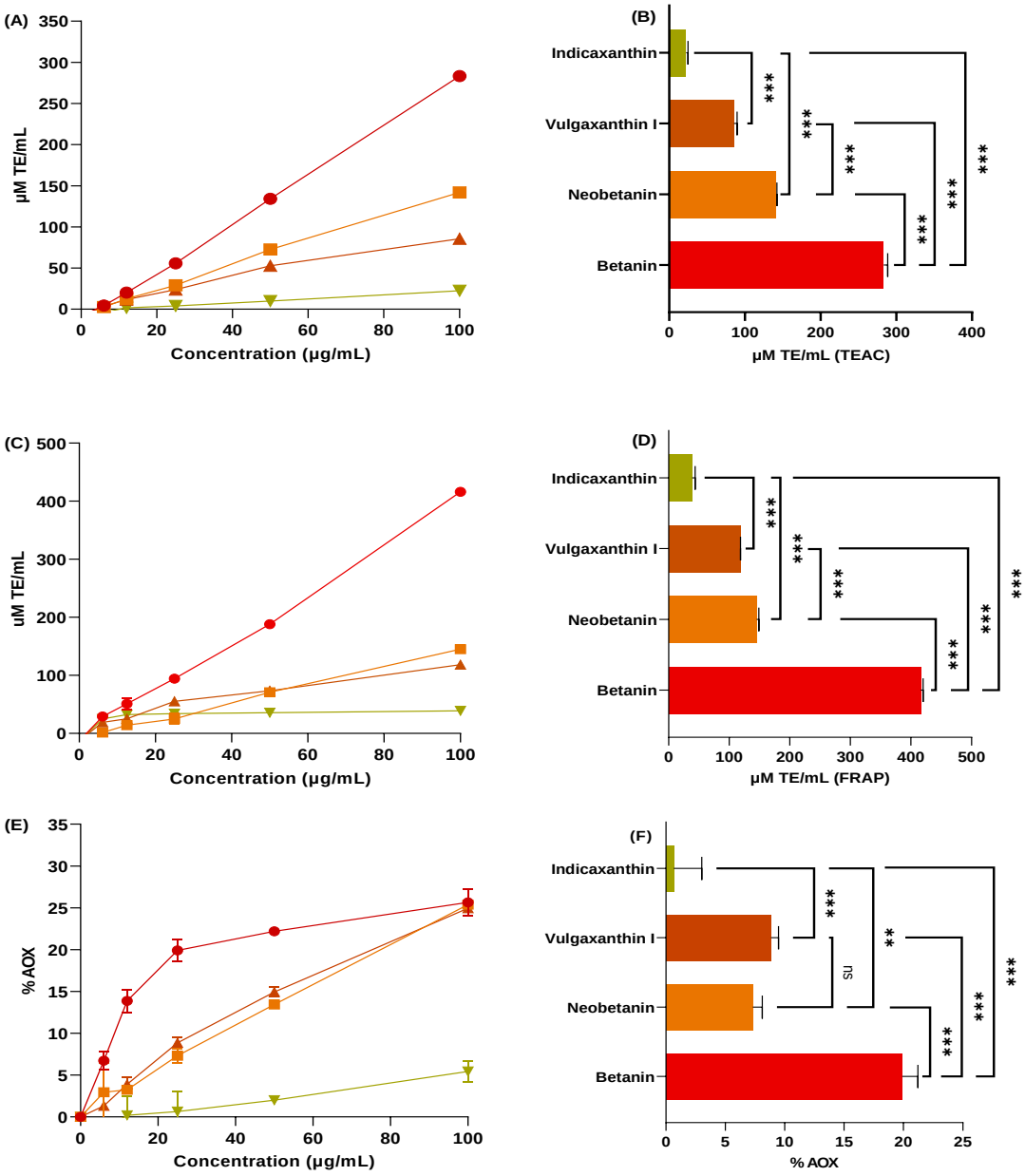



**Figure 3.3** Dose-dependent antioxidant activity of purified betalains demonstrating (A) ABTS+ radical scavenging activity, (C) Ferric reducing antioxidant power (FRAP), and (E) DPPH radical scavenging activity. Comparison of ABTS+ radical scavenging activity (B), FRAP (D) at 100 µg/mL and DPPH (F) at 25 µg/mL of individual betalains. Data are presented as mean (A, C, E) and mean with SD (B, D, F) of at least three independent experiments performed in duplicate. * indicates significant difference, Tukey's test: *$p < 0.05$, ** $p<0.01$, and *** $p<0.001$.

All other purified samples were tested with and without DPPH radicals and they did not show any interference (data not shown). Indeed, when compared at 25 µg/mL, the DPPH response pattern of betanin is similar to to ABTS+ and FRAP assays indicating superior radical scavenging activity to all other betalains ($p<0.001$; Figure 3.3F). In addition, we tested the application of HORSA assay to assess hydroxyl radical scavenging properties of betalains, however, due to wavelength interference at 536 nm it was only possible to monitor vulgaxanthin I and indicaxanthin. In line with other radical scavenging assays, vulgaxanthin I showed 31.4% higher hydroxyl radical scavenging activity compared to indicaxanthin (data not shown). These results highlight limitations of absorbance based assays when testing pigmented samples, and alternative methods such as Electron Spin Resonance (ESR) could be advantageous. In fact, a recent study conducted by Esatbeyoglu et al. (2014) demonstrated a dose-dependent scavenging of betanin against DPPH-, galvinoxyl-, superoxide- and hydroxyl- radicals using ESR. In summary, antioxidant activity of purified betalains in the present study decreased in the following order betanin > neobetanin > vulgaxanthin I > indicaxanthin.

The chemical structure of the betalain molecules greatly influence their antioxidant activity. The structures of the betalains marked with potentially active groups are shown in Figure 3.4. Generally, higher antioxidant activity in betalains is connected with the increasing number of hydroxyl groups and the presence of other hydrogen donation groups such as amino (-NH), and thiol (-SH) groups (Cai et al., 2003) as well as imino and acylation (Esatbeyoglu et al., 2015). Further, the degree and position of glycosylation influence the radical scavenging abilities of betalains. In particular, 6- *O*- glycosylated betacyanins have shown higher scavenging capabilities compared to the 5- *O*- glycosylated betacyanins (Cai et al., 2003; Esatbeyoglu et al., 2015). Gandía-Herrero et al. (2010) emphasized that the radical scavenging activity of betalains not only link with the number of hydroxyl groups present in the molecule but also with the electronic resonance system supported between two nitrogen atoms. In the case of betanin as the most studied pigment, its high antioxidant activity has been linked with the hydrogen and electron donation

ability when changing from cationic state to different deprotonated states in basic solutions (Gandía-Herrero et al., 2010; Slimen et al., 2017) .

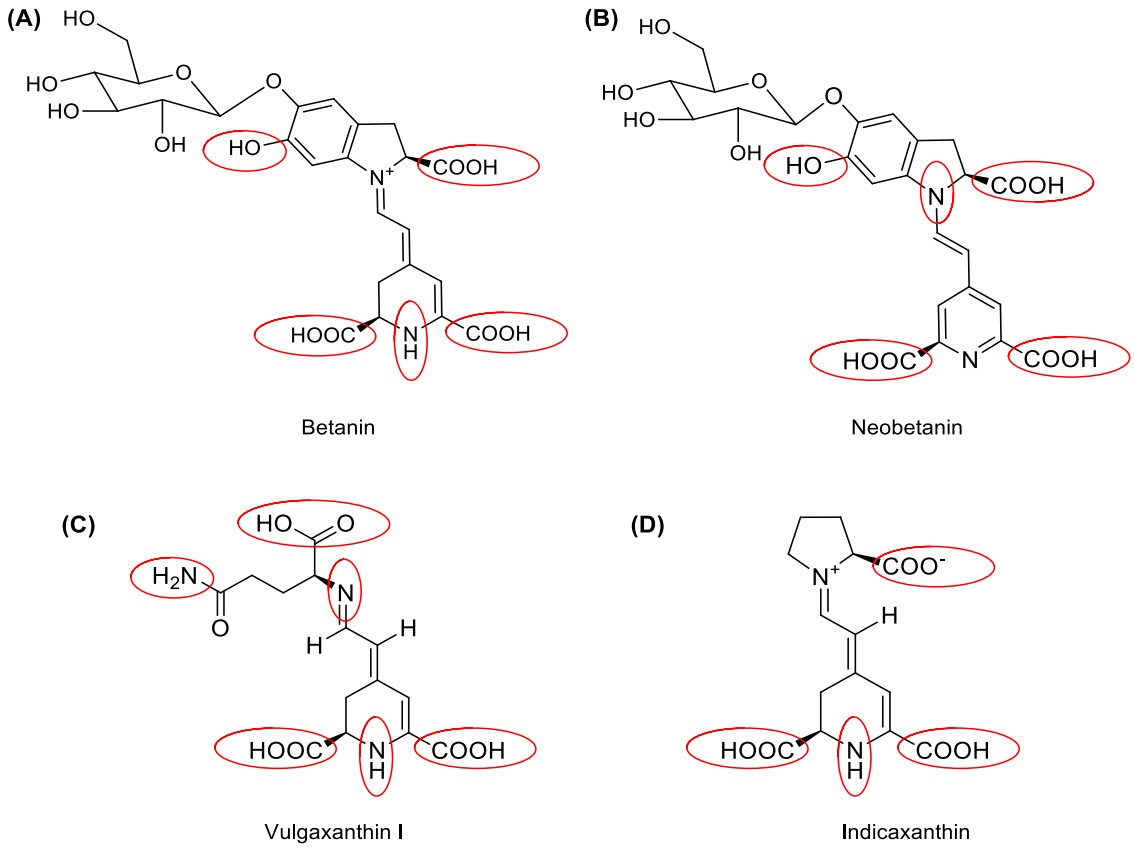



**Figure 3.4** Structures of purified betalains with potential active groups marked in red circles. (A) betanin, (B) neobetanin, (C) vulgaxanthin I and (D) indicaxanthin

To the best of our knowledge, radical scavenging capacities of vulgaxanthin I and neobetanin have not been demonstrated so far. Vulgaxanthin I is formed by conjugation of betalamic acid with glutamine (Figure 3.4C). Neobetanin is a degradation product of betanin formed by removal of two hydrogen atoms from the betanin structure (Figure 3.4B). In principle, both vulgaxanthin I and neobetanin are good radical scavengers while vulgaxanthin I has more active groups than neobetanin. However, in the current study, neobetanin showed higher radical scavenging activity compared to vulgaxanthin I. The reason could be that the presence of indoline-like substructures (Appendix C, Figure C.7B) may significantly enhance the radical scavenging properties of the neobetanin (Gandía-Herrero et al.,

2010). Further, the presence of the phenolic hydroxyl group in neobetanin can significantly enhance the radical scavenging activity in comparison to vulgaxanthin I (Gandía-Herrero et al., 2010). The lower potency of neobetanin compared to betanin could be explained by the ability of betanin to create a stable carbocation through the electron resonance system shared between the imino and the tetrahydropyridine groups (Appendix C, Figure C.8A) as well as stabilization of tetrahydropyridine through hydrogen bonds with adjacent carboxylic groups (Slimen et al., 2017).

To summarize, present data demonstrate that among betaxanthins, vulgaxanthin I was more potent compared to indicaxanthin. The reason could be that the charged imino group present in indicaxanthin can decrease the scavenging activity of the molecule. Overall, betacyanins (betanin, neobetanin) are more potent than betaxanthins (vulgaxanthin I, indicaxanthin) due to the presence of the indole substructure (Appendix C, Figure C.8B) and hydroxyl group.

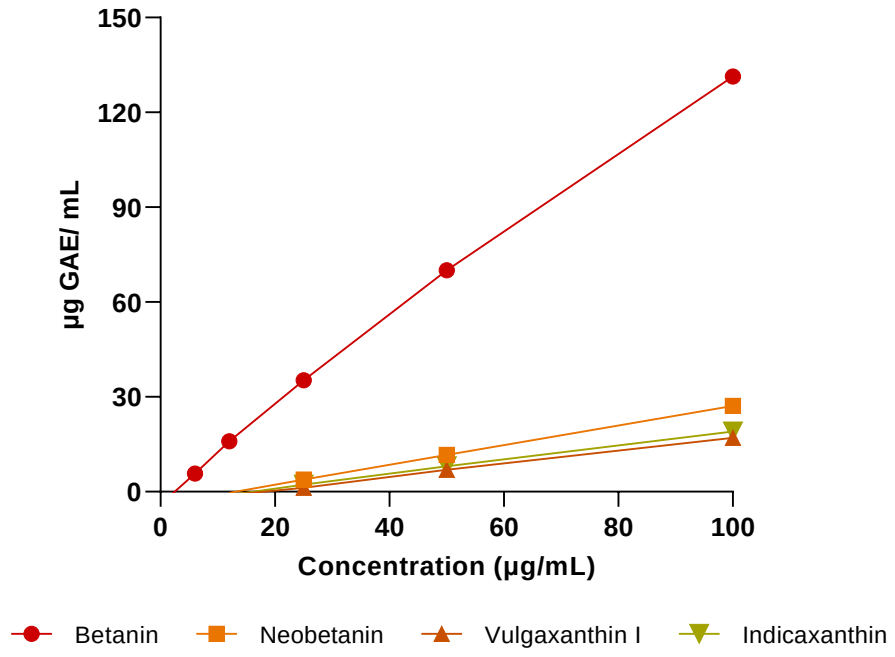



**Figure 3.5** Dose dependent response of purified betalains for the Folin assay.

The Folin assay is the most commonly used method to evaluate the total polyphenol content in plant and food extracts (Sánchez-Rangel et al., 2013). The lack of specificity of the Folin assay is well known, as the Folin reagent can be reduced by other compounds such as reducing sugars (glucose, fructose), dehydroascorbic acid and amino acids present in a sample (Sánchez-Rangel et al., 2013). The current study examined whether betalains could react with the Folin reagent and therefore affect the specificity of the Folin assay to determine the polyphenol content in a mixture. As shown in Figure 3.5, betanin demonstrated a strong dose-dependent increase in signal in contrast to neobetanin, vulgaxanthin I and indicaxanthin. When compared at 50 µg/mL, the corresponding values for PP content were 70.06, 11.66, 6.99, 8.08 µg GAE/ml for betanin, neobetanin, vulgaxanthin I and indicaxanthin, respectively. It can be assumed that the results are a good representation of betalain response as polyphenol analysis with HPLC/MS confirmed the absence of polyphenols in the purified betanin and indicaxanthin samples (data not shown). In the case of purified vulgaxanthin I, trace amounts (<1%) of catechin and epichatechin were found while neobetanin contained trace amounts (<1%) of n-trans-feruloyltyramine; a lower accuracy can therefore not be excluded for these two compounds. Based on these findings, it needs to be taken into account that the application of the Folin assay to determine the TPC in betalain-containing samples may be significantly confounded, in particularly in samples with high betanin content.

## 3.4 Conclusions

The flash chromatography system is capable of purifying betanin, neobetanin, vulgaxanthin I, and indicaxanthin from crude extracts of beetroot and prickly pear sources effectively, using a single chromatographic step. Given the current purification yields of 1.87 – 4.87 mg/g and percentage purity of betanin, neobetanin, vulgaxanthin I, and indicaxanthin of 97%, 52%, 79%, and 95%, flash chromatography demonstrates strong potential with significant advantages compared to other methods. Further work should be done to increase purity of vulgaxanthin I and neobetanin, and apply the method to purification of other betalains. Importantly, all purified betalains demonstrated antioxidant and free radical scavenging activities, with betanin being showing much stronger capacity as

compared to the other betalains. Overall, betacyanins were more potent as antioxidants in comparison to betaxanthins which indicates a strong structure-function relationship of different betalains, and further studies should investigate the relevance of these findings.

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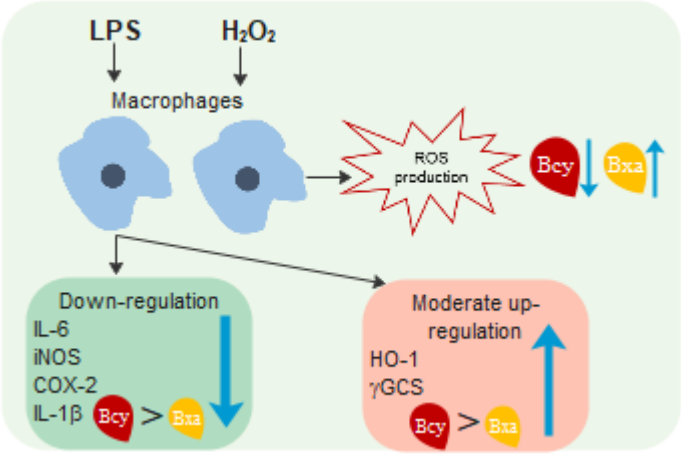
# Chapter 4

# Differential effects of betacyanin and betaxanthin pigments on oxidative stress and inflammatory response in LPS-stimulated murine macrophages




## Abstract

Betalains are a group of natural pigments that have been increasingly highlighted for their bioactive properties and anti-inflammatory potential, although there is a lack of research available on the contributions of individual betalains and their mechanisms of action. The work herein compares the effects of four main betalains on inflammatory and cell-protective markers while aiming to identify the structure-activity relationship of two main subgroups of betalains (betacyanins versus betaxanthins). Individual betacyanins (betanin, neobetanin) and betaxanthins (indicaxanthin, vulgaxanthin I) as well as betalain-rich extracts (from red and yellow beetroot) were applied to murine macrophages, a cellular model of inflammation, to determine inflammation response and redox status. 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, Nrf2 targets HO-1 and γGCS showed mixed and only moderate induction, but again, more emphasized for betacyanins. Whilst all betalains suppressed mRNA levels of NADPH oxidase 2 (NOX-2), a superoxide generating enzyme, only betacyanins

were able to counteract hydrogen peroxide induced intracellular generation of reactive oxygen species (ROS). Betaxanthins even displayed pro-oxidant properties, elevating ROS production beyond hydrogen peroxide stimulation. In summary, our data confirmed anti-inflammatory properties for all individual major type of betalains, which includes neobetanin, a major degradation product of betanin, commonly formed during food storage. Inflammation-inhibitory properties could also be demonstrated for betalain-containing complex samples, indicating the potential for beetroot to contribute to alleviating inflammation, which should be followed up in further studies. Further, the implications of the lacking protective properties of betaxanthins towards acute oxidative stress should be investigated further.

**Keywords**: Inflammation, macrophages, pro-inflammatory cytokines, betalains and oxidative stress

## 4.1 Introduction

Betalains are a group of water-soluble natural pigments found in plants of the order *Caryophyllales* (Choo, 2017) 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 such as red-violet betacyanins and yellow-orange betaxanthins. Due to their intrinsic tinctorial properties, betalains are generally used as food colourants but have recently gained attention as they have shown *in vitro* and *in vivo* bioactive properties such as antioxidant (Gandia-Herrero et al., 2016), anti-inflammatory, and antimicrobial activities (Gengatharan et al., 2015).

A range of studies considered different mechanistic aspects of betalains. Reddy et al. (2005) investigated direct enzyme inhibition *in vitro*, showing a 97% inhibition of cyclooxygenase-2 (COX-2) enzyme activity through betanin at 100 µg/mL, which was greater compared to the cyanidin-3-O-glycoside which was 59% inhibition at the same concentration. Furthermore, Vidal et al. (2014) demonstrated that betanidin, the deglycosylated form of betanin, dose-dependently inhibited 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) (Tesoriere et al., 2014). The secretion of IL-6 and IL-8 was reduced by 75% and 65%, respectively, following 24 h-incubation with 25 µM indicaxanthin under IL-1β-stimulated conditions. However, a recent study in LPS-stimulated macrophages demonstrated pro-oxidant effects of indicaxanthin through four-fold higher ROS production at 100 µM following LPS stimulation, compared to non-stimulated control cells (Allegra et al., 2014a). 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 adminstration of beetroot extract (50 – 500 mg/Kg body weight) has been shown to reduce *in vivo* oxidative stress, inflammation, and apoptosis in rodent models of acute inflammation (El Gamal et al., 2014; Adhikari et al., 2017). Further, Han et al. (2015) observed that oral administration of betanin (25 and 100 mg/kg/day) 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 (Allegra et al., 2014b)aligned with the dose-dependent inhibition of NF-κB transcription factor signalling (0.5 - 2 µmol/kg). In addition, a recent human study demonstrated a reduction of pro-inflammatory cytokines TNF-α, IL-1β, IL-8, INF-γ and increase of anti-inflammatory cytokine, IL-10 in the plasma of healthy human adults after ingestion of cactus pear fruit pulp (2 × 200 g/day for two weeks) (Attanzio et al., 2018).

Oxidative stress, which is the excess production of reactive oxygen species due to the imbalance between the production of ROS and the availability of antioxidants to scavenge the ROS (Chatterjee, 2016), is closely related to inflammation. Both processes can easily be induced by each other and are simultaneously found in many pathological conditions (Biswas, 2016). 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 (Esatbeyoglu et al., 2014). 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 (Ma, 2013). Indeed, many plant bioactives have been shown to induce Nrf2 signalling (Gugliandolo et al., 2020) 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. (2014) 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. (2009) 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. These 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. (2001), who found that low concentrations of betanin ($IC_{50}$ 0.4 µM) inhibited lipid peroxidation more effectively than catechin ($IC_{50}$ 1.2 µM) and α-tocopherol ($IC_{50}$ 5 µM) in cell membranes isolated from turkey muscles.

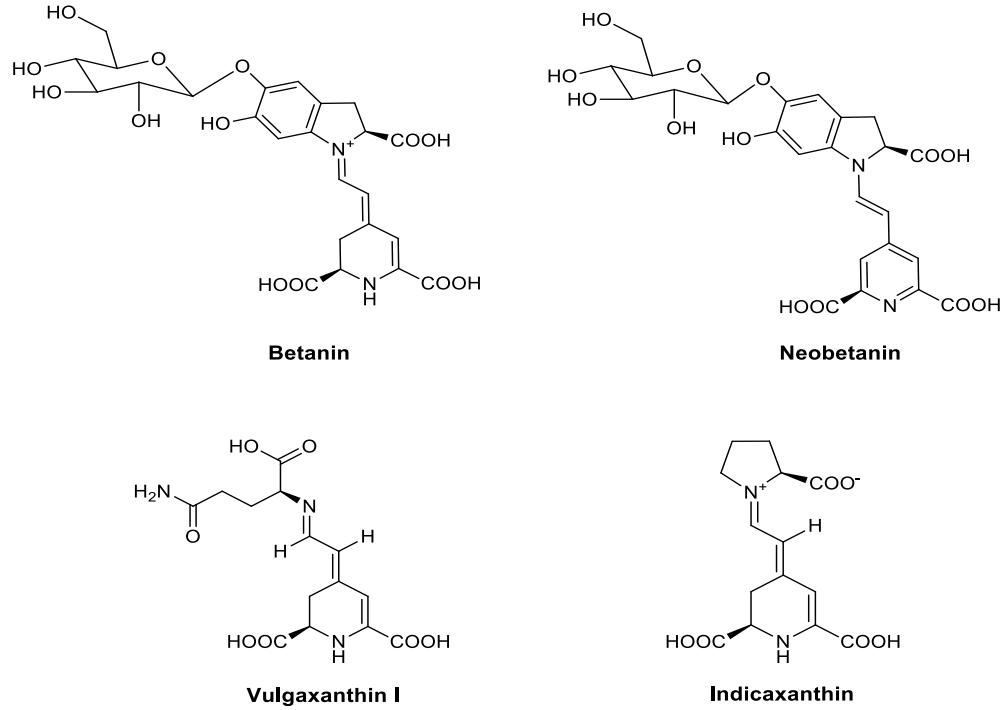



**Figure 4.1** Chemical structures of betalains: betanin, neobetanin, vulgaxanthin I and indicaxanthin.

Although some evidence on the anti-inflammatory and other properties of betalains/betalain-containing extracts is available, there is a lack of studies addressing the effects of individual betalains on a cellular and molecular level. Importantly, many betalain (-containing) samples used by other researchers are lacking characterization and indication of purity, these samples may therefore potentially contain other bioactive compounds such as organic acids, sugars and polyphenols, which could be responsible for the bioactive properties. The lack of detailed knowledge on individual compound effects is limiting the progress in the field and the development of downstream applications.

Therefore, the current study aimed to investigate and compare the anti-inflammatory and cell-protective activities of four main betacyanins (betanin, neobetanin) and betaxanthins (indicaxanthin, vulgaxanthin I), shown in Figure 4.1, which have been purified using a newly established flash-chromatography based procedure described in Chapter 2 (Fernando et al. 2021b). Whilst betanin, vulgaxanthin I and indicaxanthin are main betalains present in red, yellow beet and prickly pear, these have not been directly compared. Further, neobetanin is the main degradation product of betanin, therefore formed during storage and likely present in many betalain containing food products. Macrophages were selected as a model of cellular inflammation and oxidative stress, as they can play important roles in initiating and regulating inflammatory response and maintenance of *in vivo* homeostasis (Nakagawa et al., 2012). Stimulation of macrophages with inflammatory triggers, such as LPS, can release a range of pro-and anti-inflammatory cytokines such as tumor necrosis factor (TNF-α), IL-6, IL-1β, and IL-10, and other inflammatory mediators such as CCL4 and monocyte chemoattractant protein-1 (MCP-1).

## 4.2 Experimental section

### 4.2.1 Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM) high glucose (4.5 g/L, without/with phenol red), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, and $H_2$DCFDA were purchased from Thermo Fisher Scientific (Loughborough, UK). DuoSet ELISA (TNF-α) was purchased from R&D

Systems (Abingdon, UK). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, Sulforhodamine B Sodium salt (SRB), Trizma base, Trichloroacetic acid (TCA), acetic acid, 30% wt. hydrogen peroxide ($H_2O_2$), DMSO, resazurin sodium salt, and all other reagents were purchased from Sigma-Aldrich (Dorset, UK) unless specified otherwise.

### 4.2.2 Cell line

The murine RAW 264.7 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS, and 1% Pen/Strep (penicillin and streptomycin) and maintained in a humidified incubator under standard conditions (37 °C, 5% $CO_2$).

### 4.2.3 Materials (purified betalains and crude extracts)

Betanin (BET), neobetanin (NEO), vulgaxanthin I (VGX), and indicaxanthin (IDX), were previously isolated from red beetroot and yellow prickly pear (Fernando et al., 2022) with a purity 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.

Red and yellow beetroot were extracted using ultrasound-assisted extraction method as previously described in Chapter 2 (Fernando et al. (2021) 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%. Powdered samples were collected and stored at 4 °C after replacing the air gap with $N_2$ gas.

### 4.2.4 Cell viability assays

Cell viability was determined by a resazurin-based assay (Riss et al., 2013). Cells were seeded at $4 \times 10^5$ per well in a 24-well plate and once 70-80% confluent, incubated for 24 h with the test compounds. The next day, 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 1 mL resazurin-containing medium per well and incubated for 3 h. Then, aliquots of the supernatants were transferred into wells of a black 96-well plate and fluorescence intensity measured at 560 nm and 590, excitation and emission wavelengths, respectively, using Tecan SparkTM 10M multimode microplate reader (TECAN, Männedorf, Switzerland). Cell viability was expressed in % of untreated cells.

### 4.2.5 Production of reactive oxygen species

The assessment of intercellular ROS production was performed using a recently described method by Ng and Ooi (2021) with normalization of DCF values to the respective protein level using a rapid SRB assay. Cells were seeded at $6 \times 10^4$ cells/well in black clear-bottom 96-well plates. After 24 h, the cells were incubated with 20 µM DCFH-DA (2'-7'dichlorofluorescin diacetate) (100 µL) diluted in phenol red-free medium for 30 min at 37 °C. Then, the macrophages were treated with different concentrations of betanin (0, 0.4 - 100 µM) or the other betalains (1, 5, 25 µM) for 1 h, followed by addition of 250 µM of $H_2O_2$ to initiate oxidative stress. The fluorescence intensity (FI) was measured using a Tecan microplate reader at 485 nm and 535 nm excitation and emission wavelengths, respectively, after 30 min and 1 h in well-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). Fluorescence intensity was measured at excitation 540 nm and emission 590 nm in scanning mode. DCF and SRB values were corrected using cell-free controls. The ratio of DCF to SRB was calculated for individual wells to normalize ROS data to cellular protein content.

### 4.2.6 RNA isolation and quantitative real-time PCR

Cells were seeded at $4 \times 10^5$ per well in a 12-well plate and incubated for 24 h until reaching 70% confluence. Briefly, cells were incubated with different concentrations of purified betalains (1 -100 µM), spray-dried extracts (10, 50, 400 µg/mL) or positive

control (curcumin, 10 μM) for 1 h and then incubated with LPS (100 ng/mL) for 6 h. Subsequently, RNA was isolated using Trisure reagent (Bioline, Nottingham, UK) according to the manufacturer's instructions. Total RNA quantity and quality were determined by Tecan plate reader using Nanoquant plate. RNA reverse transcribed into cDNA using iScript$^{TM}$ cDNA synthesis kit (Bio-Rad, Hertfordshire, UK) 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) using recommended settings. Primer sequences were designed using NCBI tools and previous research (Wagner et al., 2012) which are shown in Table 4.1. Target gene expression was normalized to β-actin (housekeeping gene) and relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen (2001).

**Table 4.1** Murine primer sequence information

| Gene | Forward primer (5' – 3') | Reverse primer (5' – 3') |
|---|---|---|
| β-actin | CCTCTATGCCAACACAGTGC | CCTGCTTGCTGATCCACATC |
| HO-1 | GAGCCTGAATCGAGCAGAAC | AGCCTTCTCTGGACACCTGA |
| iNOS | GGCAGCCTGTGAGACCTTTG | GCATTGGAAGTGAAGCGTTTC |
| IL-6 | AGTTGCCTTCTTGGGACTGA | CAGAATTGCCATTGCACAAC |
| γGCS | AGTTCCGACCAATGGAGGTG | TCTCGTCAACCTTGGACAGC |
| COX-2 | TTCAACACACTCCTATCACTGGC | AGAAGCGTTTGCGGTACTCAT |
| IL-10 | GCTCTTACTGACTGGCATGAG | CGCAGCTCTAGGAGCATGTG |
| NOX-2 | CACTTTGCTGTGCACCATGA | CGCCTATTGTGGTGTTAGGG |
| IL-1β | CAGGCAGGCAGTATCACTCA | AGCTCATATGGGTCCGACAG |

### 4.2.7 Enzyme-linked immunosorbent assay (ELISA) for TNF-α

RAW 264.7 cells (70% confluent, 24 well plates) were treated with different concentrations of betalains, or positive control (curcumin, 10 μM) for 1 h and then incubated with LPS (100 ng/mL) for 24 h. The TNF-α secretion into the medium was determined by ELISA kit following the manufacturers' instructions.

### 4.2.8 Statistical analysis

Data are presented as mean ± SEM of three independent experiments and graphs were created using GraphPad Prism Version 9.0 for Windows (GraphPad Software, La Jolla, CA). One-way ANOVA test was used to perform the analysis followed by Dunnett's multiple comparison test to compare treatments with the control group and *$p < 0.05$, ** $p<0.01$, and *** $p<0.001$ were considered as significant.

## 4.3 Results and discussion

### 4.3.1 Effects of betalains on expression of pro-inflammatory cytokines

Betalains are natural pigments that have shown beneficial effects on inflammation, blood pressure, endothelial dysfunction, and apoptosis of tumor cells (Rahimi et al., 2019). However, most of the studies have used extracts rather than purified betalains, therefore, the actual contribution of individual betalains remains unclear. The present study demonstrated the beneficial effects of betalains on inflammation while assessing the differential contribution of major subgroups of betalains (betacyanins and betaxanthins) to attenuate inflammation and oxidative stress. Inflammatory mediators are important to activate the immune response and repair the tissues, whereas overproduction leads to the induction and progression of some inflammatory diseases (Hwang et al., 2019). NF-κB is one of the major gene expression regulatory pathways of inflammatory responses and stimulates the generation of pro-inflammatory cytokines such as IL-6, iNOS, IL-1β, and COX-2 (Dai et al., 2018). Inflammatory triggers such as LPS activates the phosphorylation of NF-κB p65 and translocates it from the cytoplasm to the nucleus and thereby activates the generation of pro-inflammatory mediators (Silva et al., 2020a) (Figure 4.8). Therefore, assessing the regulation of inflammatory mediators is an important strategy to recognize the anti-inflammatory potential of different test compounds.

As shown in Figure 4.2, incubations with individual betalains resulted in a dose-dependent down-regulation of pro-inflammatory cytokine mRNA levels. Whilst all betalains demonstrated reduction of inflammatory markers, in particular betanin (BET) was consistent to inhibit IL-6, iNOS, IL-1β and COX-2 by 60.0%, 60.2%, 48.1%, and 39.9%, respectively, at 100 µM ($p < 0.05$ for each). Even at lower

concentrations of BET and VGX (1 µM and 5 µM) 25 - 40% lower cytokine levels were found, albeit the effects were not significant (Appendix D, Figure D.1 A-D).

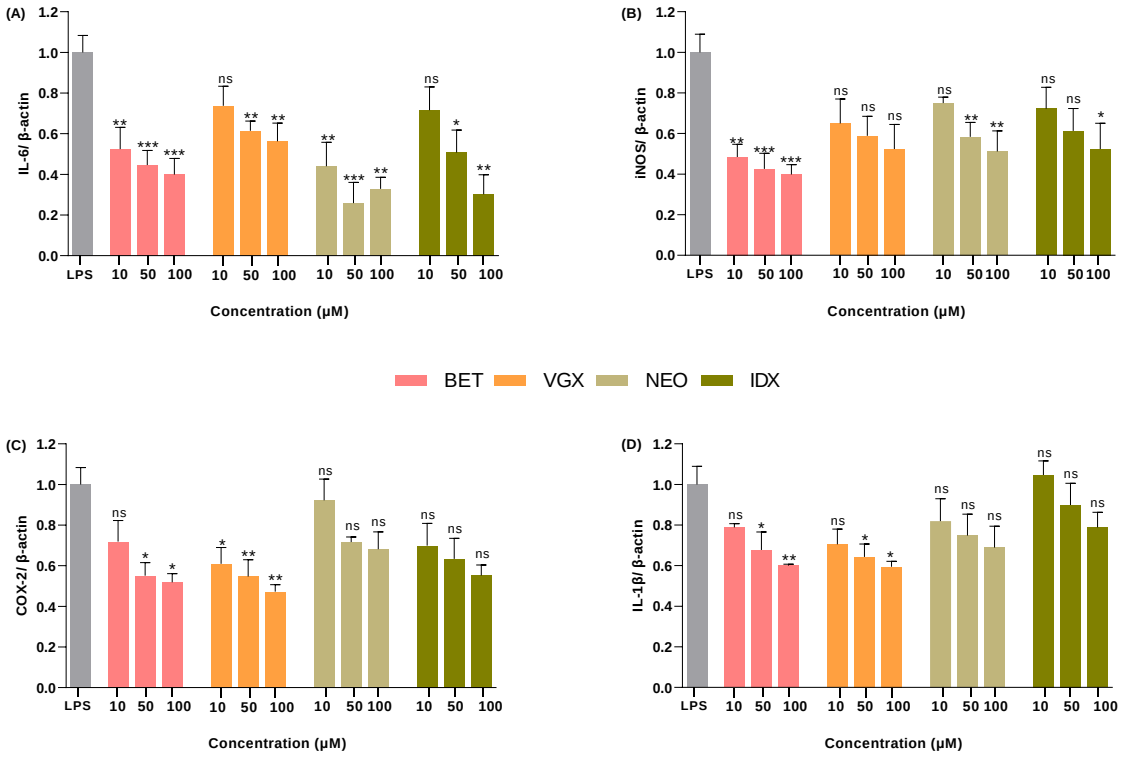



**Figure 4.2** Effects of purified betalains (10 µM, 50 µM and 100 µM) on pro-inflammatory cytokine expression targeting (A) IL-6, (B) iNOS, (C) COX-2 and (D) IL-1β in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *p < 0.05, ** p<0.01, and *** p<0.001.

The results are in line with Ahmadi et al. (2020) who demonstrated that BET (500 µM) significantly inhibited (p<0.05) the secreted levels of IL-6 (50%) and IL-1β (36%) in LPS-induced microglial cells, thereby contribution to reducing neuroinflammation. Further, Allegra et al. (2014b) also showed anti-inflammatory effects of IDX in pleural inflammation in rats. They demonstrated that pre-treatment with 2 µmol/kg IDX inhibited the expression of IL-1β (35%), COX-2 (88%), and iNOS (75%) mRNA levels in carrageenan-induced rat pleurisy.

Given that TNF-α is a primary inflammatory cytokine secreted by macrophages upon LPS stimulation, we sought to determine TNF-α in the medium via ELISA. Indeed, BET, NEO, and VGX showed significant inhibition ($p<0.05$) of TNF-α secretion at 50 µM concentration (Appendix D, Figure D.2), with inhibition of 24.9%, 16.8%, and 17.1%, respectively compared to the cells treated only with LPS. The influence of purified betalains on cell viability was assessed (Appendix D, Figure D.3), as any toxic effects of the test compounds may affect immune response and potentially induce cellular oxidative stress. No negative effect on cell viability in the presence of purified betalain compounds (0 – 100 µM) was observed which is in alignment with the literature (Ahmadi et al., 2020; Rahimi et al., 2019; Allegra et al., 2014a). However, Esatbeyoglu et al. (2014) demonstrated toxicity of betanin above 15 µM for HT-29 and PON1-Huh7 and above 25 µM for Huh7 cells. Betanin in this case was commercially available betanin, diluted with dextrin. According to our own data obtained by spectrophotometer, the betanin content of commercially available betanin standard is below 0.1 mg/mL in a 50 mg/mL solution, indicating that there might be considerably large amounts of dextrin present in the sample, which might have affected cell viability as well as contributed to the outcomes.

### 4.3.2 Effects of betalains on 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 (Sekhar et al., 2015). Accumulation of ROS can result in oxidative stress and be causative of several degenerative diseases such as cancers and chronic inflammation (Ames et al., 1993). Further, ROS can act as a key signaling molecules as well as a mediators of inflammation (Mittal et al., 2014; Herb and Schramm, 2021). Cells can regulate oxidative stress mainly by down-regulation of ROS or up-regulation of antioxidant defence system e.g. through HO-1 and γGCS induction (Hwang et al., 2019) in order to maintain redox equilibirium in the cell.

The inhibitory effects of betalains on oxidative stress were evaluated by measuring intracellular ROS production following stimulation with hydrogen peroxide. As Figure

4.3C indicates, BET significantly decreased ($p<0.05$) ROS generation from low concentrations onwards (0.4-100 µM) in a dose-dependent manner following hydrogen peroxide stimulation (250 µM) which was also evident under baseline conditions. In comparison, Figure 4.3A and B, which shows ROS generation for all betalains at concentrations of 1, 10 and 25 µM, highlighted that, whilst neobetanin exerted a similar effect to suppress ROS production in comparison to BET, whereas betaxanthins VGX and IDX did not show reduced ROS production, neither at baseline nor under stimulated conditions. In contrast, treatment with 1 µM and 5 µM of VGX showed 3.8% and 7.5% increment of ROS production while IDX showed 15.9% and 16.9% increment of ROS production indicating they could have pro-oxidant potential.

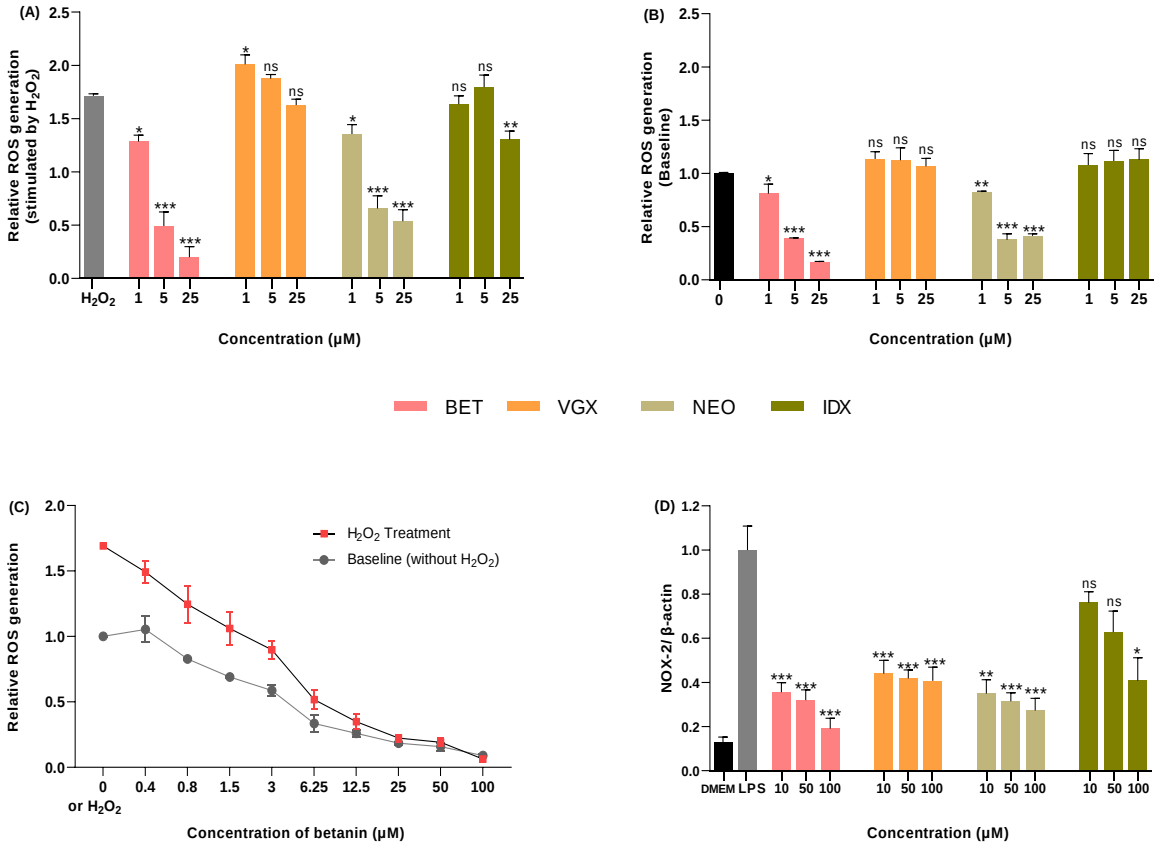



**Figure 4.3** Dose-dependent effects of betalains on reactive oxygen species (ROS) production (A-C) and NOX-2 mRNA levels (D) in murine macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to $H_2O_2$/LPS-treated cells: *$p < 0.05$, ** $p<0.01$, and *** $p<0.001$.

The observation of pro-oxidant effects is in alignment with Allegra et al. (2014a) who demonstrated dose- and time dependent pro-oxidant activity of 100 µM IDX. In their study, IDX showed time and concentration-dependent pro-oxidant activity and 100 µM IDX showing a four-fold higher final ROS level (after 12 h) than the control cells under LPS stimulated conditions. The pro-oxidant activity of some well-known antioxidants was also observed by other researchers (Halliwell, 2008; Blumberg and Block, 1994; Estela Guardado Yordi et al., 2012). For instance, vitamin C can act as an antioxidant at lower doses and show pro-oxidant activity at high doses (Seo and Lee, 2002). The reasons of transforming antioxidants into pro-oxidants may be the presence of metal ions, presence of other ROS generators such as LPS and concentration of the compound in the matrix and their redox potential (Sotler et al., 2019).

NADPH oxidases (NOX) are membrane enzymes that stimulate intracellular reactive oxygen species in response to infection (Yu et al., 2018). NOX group compromise of several members such as NOX 1-5 and dual oxidase (Duox) 1-2 while NOX-2 is the main enzyme responsible to generate ROS in immune cells such as macrophages, T cells, and neutrophils (Panday et al., 2015). NOX-2 derived ROS can induce the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β activating the NF-κB signaling pathway (Kim et al., 2017). All the tested betalains in the present study showed a strong dose-dependent down-regulation of NOX-2 mRNA levels in activated macrophages although IDX showed the weakest effect on down-regulation of NOX-2 compared to the other purified compounds (Figure 4.3D). BET, VGX, NEO, and IDX showed 65%, 57%, 66%, and 24% reduction of NOX-2 at 10 µM concentration, respectively. In addition, both BET and VGX at 1 µM and 5 µM concentrations showed significant ($p<0.05$) down-regulation of NOX-2 expression (Appendix D, Figure D.1-E). In line with our data, Tesoriere et al. (2014) observed that 25 µM IDX was able to prevent activation of the NOX-1 complex in Caco-2 cells stimulated by IL-1β. Manea et al. (2010) observed that inhibition of activation of NF-κB pathway was able to reduce the up-regulation of NOX isoforms expression in smooth muscle cells exposed to TNF-α. The results of the present study provide support to this finding providing evidence that the reduction of NOX-2 mRNA levels

which was aligned with the reduction of pro-inflammatory cytokines which are regulated by NF-κB pathway. Further, some polyphenols are reported to prevent the activation of NOX-2 by inhibition of association between subunits (p40, p47, and p60) and membrane subunit (p22phox) which is essential to create the NOX complex (Maraldi, 2013) (Figure 4.8). Therefore, further study needs to be conducted to assess the mechanism by which betalains prevent the NOX-2 activation.

To summarize, the results of the present study indicate that only BET and NEO (betacyanins) are able to prevent generation of ROS formation directly whereas all betalains are capable of downregulating NOX-2 and therefore supressing of superoxide generation.

### 4.3.3 Effects of betalains on expression of cell-protective and anti-inflammatory cytokines

Many bioactive compounds have been shown to modulate the expression of antioxidant enzymes such as heme-oxygenase (HO-1), gamma-glutamylcysteine synthetase (γGCS), superoxide dismutase, glutathione S-transferase thereby enhancing cell-protective capacities (Ferrari et al., 2016). Nrf2 is one of the major signaling pathways that protect the cells against oxidative stress. Under normal conditions, Nrf2 is inactive in the cytoplasm by bounding with Keap 1 protein (Kelch-like ECH-associated protein 1) (Silva et al., 2020a). When there is oxidative stress Nrf2 dissociation from Keap 1 and then translocates into the nucleus to promote the production of some enzymes such as HO-1 and γGCS involve with antioxidant and cytoprotective effects (Tonelli et al., 2018; Saha et al., 2020) (Figure 4.8). Therefore, to investigate the potential role of cell-protective and anti-inflammatory markers, differential expression of HO-1, γGCS, and IL-10 was evaluated on mRNA level. Effects of purified betalains on expression of HO-1 and γGCS are shown in Figure 4.4. BET and NEO demonstrated 39% and 85% increment of HO-1 expression while VGX and IDX showed 5% and 31% reduction, respectively at 100 µM concentration. Further, BET and VGX showed 40% and 37% induction of γGCS at 10 µM concentration, whereas NEO and IDX showed 36% and 1% reduction of γGCS at the same concentration. Moreover, BET at 1 µM and 5 µM concentrations did not

show induction of either HO-1 or γGCS, whereas VGX at 1 µM and 5 µM showed 153% and 115% increment of HO-1 and 111% and 115% increment of γGCS compared to the cells only treated with LPS (Appendix D, Figure D.4). Further, there was no induction of HO-1 with purified betalains under non-stimulated conditions (Appendix D, Figure D.5).

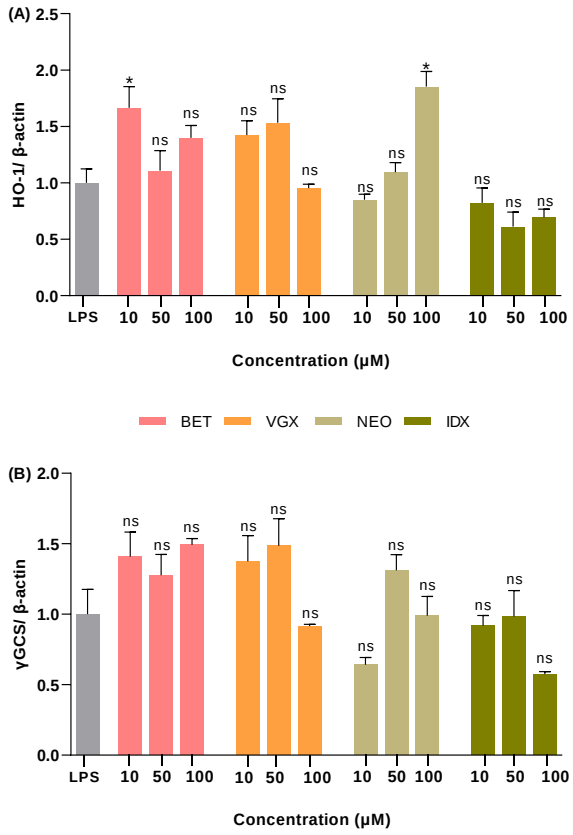



**Figure 4.4** Effects of purified betalains (10, 50 and 100 µM) on transcript levels of (A) HO-1 and (B) γGCS in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to $H_2O_2$/LPS-treated cells: *p < 0.05, ** p<0.01, and *** p<0.001.

Whilst HO-1 induction was reported in a previous study, referring to a 1.4 fold increase of HO-1 levels at 25 µM BET compared to the control (Esatbeyoglu et al., 2014), this not considered a strong response. The present study, also observed moderate inductions of mRNA levels of HO-1 and γGCS by purified betalains even though the results were not consistent across different cocentrations.

The IL-10 expression of BET showed 88%, 104%, and 140% increment ($p<0.05$) compared to LPS-stimulated cells at 10, 50, and 100 µM concentrations, respectively (Figure 4.5). In comparison, NEO, VGX and IDX showed 124%, 91% and 84% increase ($p<0.05$) at 100 µM, respectively. Lower concentrations (1 µM and 5 µM) of BET and VGX did not show up-regulation of IL-10 gene expression (Appendix D, Figure D.6). Whilst effects of betalains on expression of IL-10 may not have been determined earlier, IL-10 is considered as anti-inflammatory cytokine with increased induction being considered beneficial as part of an immune response.

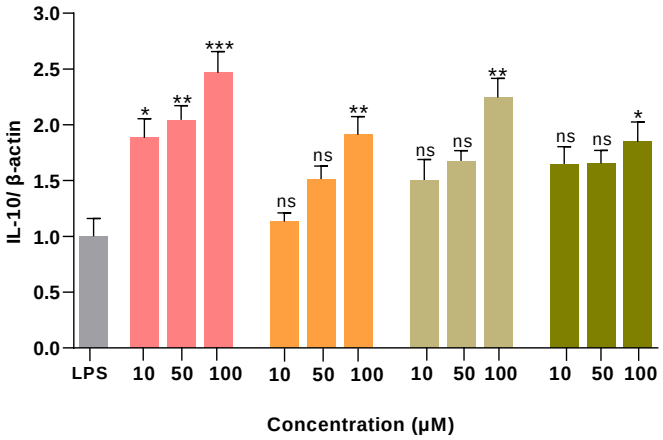



**Figure 4.5** The effect of purified betalains on IL-10 mRNA expression in LPS-stimulated RAW 264.7 macrophages at concentrations of 10, 50 and 100 µM. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to $H_2O_2$/LPS-treated cells: *$p < 0.05$, ** $p<0.01$, and *** $p<0.001$.

### 4.3.4 Effects of crude extracts on expression of selected pro-inflammatory and cell protective markers

In addition to the purified compounds, crude extracts from beetroot samples were used to examine the effects of betalain-rich extracts on the mRNA profiles of selected markers IL-6, iNOS and HO-1. The spray-dried red (SRB) and yellow beetroot (SYB) extracts showed a dose-dependent down-regulation of IL-6 by up to 35% and 48% (Figure 4.6-A) whereas iNOS was down-reglated by up to 44% and 49% (Figure 4.6-B), respectively, at the highest concentration (2520 and 1230 µM, respectively). However, there was no significant difference ($p>0.05$) of cytokine expression between the SRB and SYB.

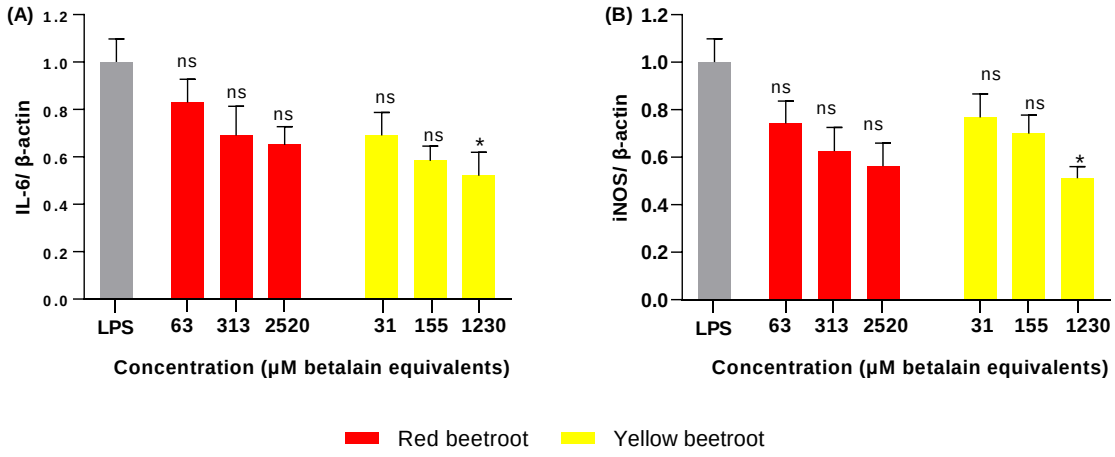



**Figure 4.6** The effect of red (63, 313 and 2520 μM) and yellow beetroot samples (31, 155 and 1230 μM) on gene expression related to pro-inflammatory cytokines (A) IL-6 and (B) iNOS in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. *$p < 0.05$ indicates significant differences to LPS-treated cells.

Considering the strength of beetroot extracts, the highest concentration of SRB contained 1.11 mM equivalent betacyanins (with majority betanin and isobetanin) and 1.41 mM equivalent betaxanthins (majority vulgaxanthin I) whereas the same concentration of SYB contained 1.23 mM equivalent betaxanthins (majority vulgaxanthin I). The betalain content was determined using HPLC. Overall, SRB contained more betaxanthins (13%) compared to SYB. The composition of SRB and SYB is more complex (Appendix D, Table D.1, D.2, and Figure D.7) and the actual amount of betalains present in the samples is quite low. It can therefore be assumed that other components than betalains, such as polyphenols, might have contributed to the anti-inflammatory response. This finding emphasizes the importance of conducting experiments using pure compounds to be able to distinguish between actual effects of target compound group.

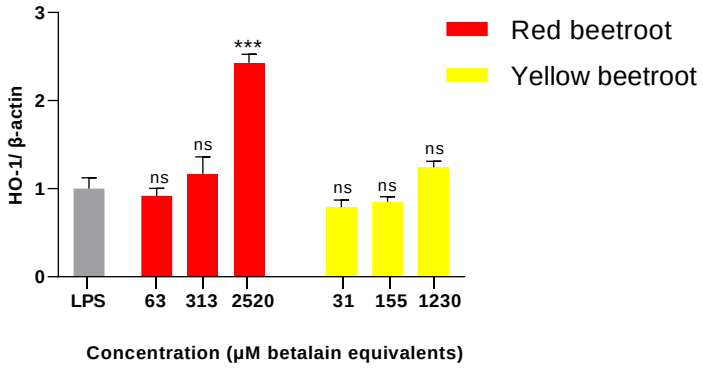



**Figure 4.7** Effects of red and yellow beetroot samples on gene expression of HO-1 in LPS-stimulated RAW 264.7 cells. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *** $p<0.001$.

In addition to inflammatory markers, HO-1 mRNA levels were determined. Whilst SRB demonstrated a significant 2.5 fold increase, SYB incubation did not affect HO-1 mRNA levels (Figure 4.7). As indicated earlier, the modulation of target gene expression in beetroot extracts could be linked with the effects of betalains as well as other bioactive compounds present in the samples. Therefore, it might be the case that the anti-inflammatory properties of betalains are not directly associated with the HO-1 and γGCS activity.

Overall, the present study showed that the betacyanins (BET, NEO) had higher tendency of effective modulation of pro-inflammatory cytokines and other inflammatory markers compared to the betaxanthins (VGX, IDX) (Appendix D, Figure D.9 and D.10). Also, bioavailability of a compound needs to be considered when assessing its cellular properties, with the assumption that the molecule should be present in the circulation in order to demonstrate health beneficial effects in the body. There are limited studies available on the bioavailability of betalains which are not sufficiently clear. Tesoriere et al. (2013) demonstrated that indicaxanthin was more bioavailable than betanin using the *in vitro* Caco2 cell monolayer model suggesting that both pigments are absorbed mostly in their un-metabolized form.

Based on the results of the present study and available literature, the Figure 4.8 can be speculated as an overview of possible intervention pathways of betalains as an anti-inflammatory and antioxidant compound assuming that betalains can mediate anti-inflammatory effects by interfering with pro-inflammatory signaling cascades.

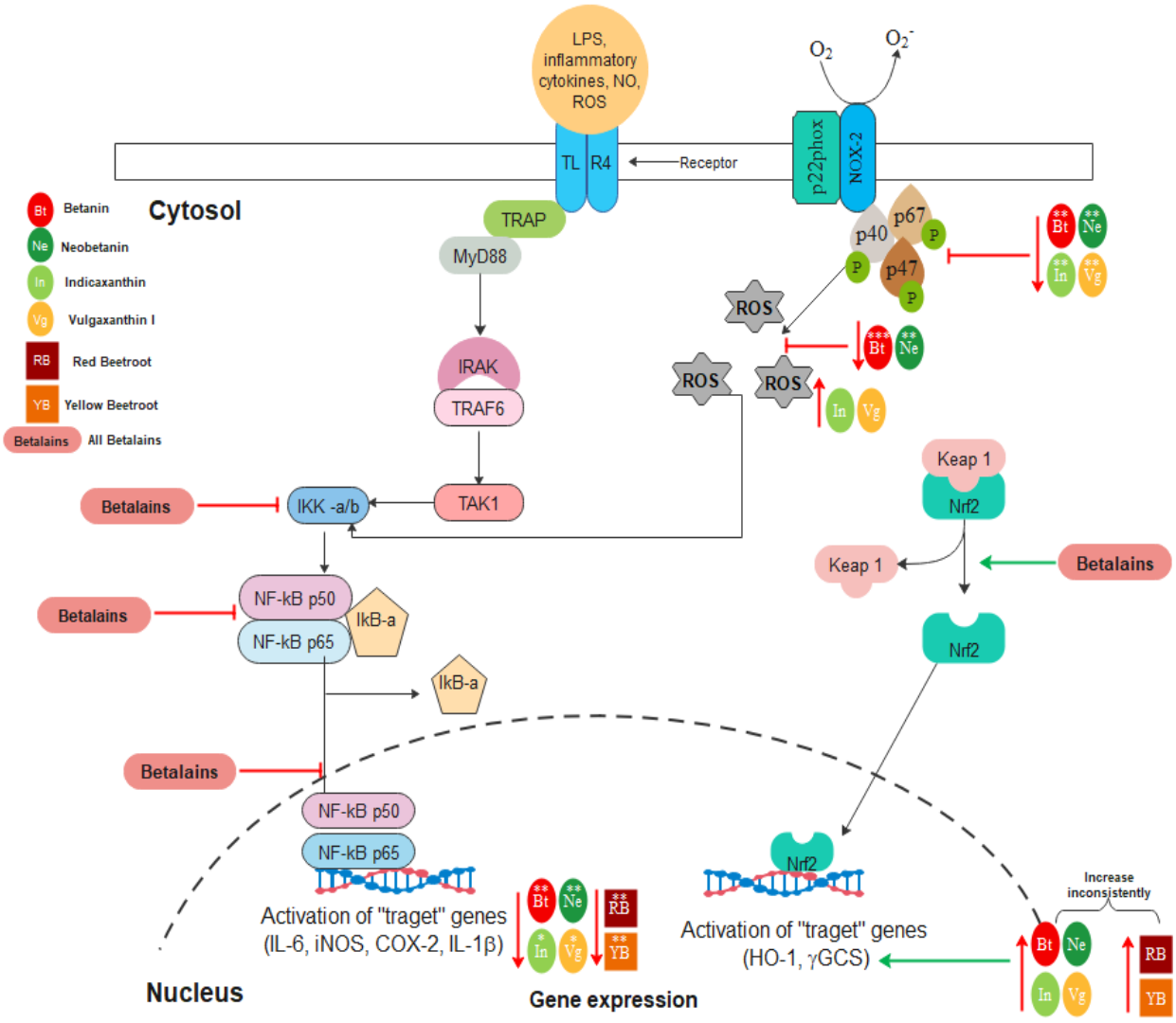



**Figure 4.8** Overview on major signaling pathways involved in inflammation and illustrating possible targets of betalains. NF-κB signalling, activated by inflammatory triggers, is leading to rapid increase in pro-inflammatory markers. Activation of Nrf2 signaling attenuates ROS production and leads to induction of antioxidant target genes. *** - high expression, ** - moderate expression, * - low expression.

## 4.4 Concluding remarks

In summary, the present data emphasized 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 towards chronic disease. Further studies need to be carried out to better understand the detailed molecular mechanisms and cellular targets of individual as well as mixtures of betalains, their effects in different tissues and systemically to elucidate the beneficial potential role of betalains in a cellular but also more complex context. Establishing the overall effects of certain bioactive sources are important in the view of diet, although understanding individual contributions and their interactions will drive the design of more tailored and safe solutions.

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# Chapter 5
# General Discussion

Betalains have gained increasing attention as a new class of dietary antioxidants in recent years. Betalain pigments are mainly using as a natural dye in food, pharmaceutical, and cosmetic products due to their strong tinctorial properties (Sadowska-Bartosz and Bartosz, 2021). In particular, betalains are water-soluble and stable at pH 3-7, which makes them very suitable to use as a food additive (Slimen et al., 2017). It has been reported that the addition of 0.1-1% w/w betalains is sufficient to generate the desired colour of most processed food products such as frozen pizza, tomato paste, jelly, yoghurt, and strawberry ice cream (Sadowska-Bartosz and Bartosz, 2021). In addition, there is an emerging knowledge on the biological activities of betalains such as antioxidant, anti-cancer, anti-lipidemic, anti-diabetic, antimicrobial, and antiviral properties (Gengatharan et al., 2015; Madadi et al., 2020) which have been associated with chronic disease, and betalains may therefore have potential to be considered for preventative and/or therapeutic approaches. For instance, beetroot as a most popular betalain-rich dietary source, contains betalains as well as a variety of other bioactive phytochemicals such as flavonoids, polyphenols, saponins, carotenoids and inorganic nitrates ($NO_3$) which provide potential contributions to the biological activity. Moreover, it is a rich source of minerals such as potassium, sodium, phosphorous, calcium, magnesium, copper, iron, zinc, and manganese (Bhupinder Singh and Hathan, 2014). Human studies have shown that chronic and acute beetroot supplementation lowers systolic and diastolic blood pressure, inhibits platelet aggregation, improves vascular and endothelial function, decreases blood sugar levels and improves insulin sensitivity, as well as providing renoprotective benefits (Mirmiran et al., 2020). Specially, nitrates which are abundantly present in beetroot have been demonstrated to lower blood pressure and vasculature related diseases (Clifford et al., 2015). However, there is a lack of knowledge on the relevance and contribution of other compounds in beetroot on health and disease-related outcomes, research is therefore warranted to understand contributions of individual compounds, such as betalains. Further, current knowledge on functional properties of betalains is insufficient compared to

the other natural pigments such as anthocyanins, carotenoids and chlorophylls. Therefore, it is important to understand the actual contribution of individual betalains on cellular and molecular mechanisms to understand the basis of their potential health benefits.

This thesis has addressed three key areas of betalain research: isolation and purification of betalain sources, characterization of betalains/ betalain sources and evaluation of the biological properties (antioxidant and anti-inflammatory) of individual betalains using cell-free and cell-based methods. Primarily, efficient and sustainable extraction and characterization protocol for betalain sources was suggested followed by providing comparative information of differently processed beetroot sources such as total betalain content, antioxidant activity, betalain and polyphenol profiles. Secondly, a novel workflow to purify different betalains with higher yield and purity using flash chromatography was proposed. Finally, antioxidant and anti-inflammatory properties of purified betalains were assessed and compared.

## 5.1 Ultrasound-assisted extraction combined with aqueous ethanol as a sustainable method to extract betalains avoiding degradation

Betalains are commonly extracted using solid-liquid extraction methods. However, the combination of solid-liquid extraction with novel techniques such as microwave, ultrasound, and pulsed electric field extraction can enhance pigment extraction efficiency which has been detailed in **Chapter 1**. The studies conducted by Sivakumar et al. (2009); Maran and Priya (2016), and Righi Pessoa et al. (2018) showed that ultrasound-assisted extraction can result in higher betalain yield compared to conventional extraction methods such as maceration and shaking. In the present study, ultrasound-assisted extraction was applied and the betalain yields are comparable with the aforementioned studies. In addition, the extraction solvent plays a vital role to achieve higher pigment yields. However, there are no universal extraction conditions to extract betalains from different dietary sources, and each source may have unique profile of compounds with different polarities and a complex

sample matrix. Therefore, the present study tested the extraction efficiency using different solvent types (water, ethanol, and methanol). Water is the ideal solvent to extract betalains as betalains are polar compounds. However, poor pigment stability as well as co-extraction of other polar compounds (Perussello et al., 2017) such as sugars and polyphenols, leads to assessment of alternative solvents to extract betalains. Based on the results of the present study, 30% ethanol (v/v) can be highlighted to efficiently dissolve betalains from red beetroot samples and to sustain stability over the storage period, which is an important consideration when using betalains as a potential functional food ingredient or nutraceutical.

There are a number of studies on the extraction and characterization of different betalain sources as summarized in **Chapter 1**. However, the present study provides new data on the betalain and phenolic composition as well as biological properties of different beetroot sources including pomace obtained from the juice industry (**Chapter 2**). Further, approaches and results of the present study should be beneficial for other researchers who want to analyze complex matrices which contain betalains depending on their objectives and available equipment.

Enzyme assisted extraction (EAE) is an innovative extraction method which is increasingly applied to extract the cell-wall bound polyphenols and bioactives in plant sources (Gligor et al., 2019). This method had been considered as a part of this research through a collaborative project with Lancaster University (N8 Agrifood project). However, EAE was not successful in improving betalain yield in the present study. This may be due to the location of betalains in plant cells which are mainly found in epidermal and subepidermal tissues, and accumulated in the vacuoles of the vegetative and reproductive tissues, as well as the enzymes used, and the conditions that were applied in this study. The present study used a combination of pectinase and cellulase enzymes (1:1) which has been reported as successful to increase the yield of anthocyanins (Benucci et al., 2017), 6-gingerol (Nagendra et al., 2013), and secoisolariciresinol (Renouard et al., 2010) from withered grape skin, ginger, and flax hull, respectively. Another reason for low betalain recovery may be the degradation of betalains by cellulase enzyme which is responsible for

hydrolyzing the bond between glucose and betanidin molecules. Further, degradation of betalains may have occurred during the enzymatic reaction conditions (pH 5.5, temperature 55 °C) thereby decreasing betalain yield. It would therefore be important to further optimize the EAE method to extract betalains using the different combinations of pectinase, cellulase, tannase with modified conditions of pH and temperature.

Overall, there are common limitations when comparing the findings of extraction and characterization studies. The first limitation is reporting the outcomes in an inconsistent manner; typically, different units are provided such as the total betalain content which is frequently reported in extraction volume (mg/mL, mg/L), or sample weight (mg/g), but results are only comparable when calculated to weight and the information of fresh/dry matter added. Inconsistency of starting material is another limitation. There is a large variation of betalain content in different beetroot cultivars due to differing growth conditions, seasons, fertilizer, and geography (Sawicki et al., 2016). There are more than 20 beetroot varieties available with Avenger, Bulls blood, Detriot dark red, Red ace, Crosby and Ruby queen are being some of the common red beetroot varieties grown in Europe and the USA. However, details of variety/cultivar are not always available when working with industrial/commercial samples. For example, in the present study we used beetroot samples provided by industry and they may be mixed batches of different beetroot varieties. Further, when working with commercial samples, there is mostly little or no information available about sample processing, transportation and storage conditions: For instance, the present study used beetroot samples ordered online as well as provided by industry. The processing, transportation, and storage conditions of the samples prior to analysis were not known and those conditions may affect the stability of betalains and other compounds (polyphenols and organic acid) present in the sample. For example, **Chapter 2** of the present study showed that air drying caused to degradation of betalain pigments present in the red beetroot pomace. Lack of information on full or partial extraction of pigments and parts of the plant sources used for the extraction is another common limitation. The distribution of betalains in the plant parts is widely different. For instance, a higher amount of pigments can be

extracted using the roots of the beet compared to the stem and the leaves (Hayet et al., 2014). The extraction yield is therefore different when using only the roots or the whole plant.

Due to these limitations, the results of the present study are difficult to compare with most of the available literature. It is important to emphasize the need for detailed information on starting material, plant parts, extraction procedure (full or partial pigment extraction), and processing conditions when reporting the betalain content.

## 5.2 Flash chromatography as a novel approach to purify betacyanins and betaxanthins

A number of different attempts have been made to purify betanin and indicaxanthin using different analytical techniques such as column chromatography and preparative HPLC method (Gonçalves et al., 2012; Gandía-Herrero et al., 2012; Allegra et al., 2014b). However, those methods consume more time, solvent, give low purification yield, and involve several steps that lead to degradation of the compounds. The advantages and disadvantages of popular purification techniques are summarized in Table 5.1.

**Table 5.1** Advantages and disadvantages of popular purification techniques.

| Technique | Advantages | Disadvantages |
|---|---|---|
| Silica gel column chromatography | Inexpensive<br>Can be used a broader range of mobile phases | Time-consuming<br>Low separation power<br>Higher quantities of solvent usage<br>Automation makes more complex and costly |
| Preparative High-Performance Liquid Chromatography – MS or UV | One injection<br>Sharp peak shape<br>High accuracy<br>High sensitivity and resolution | The sample might be lost<br>Expensive and complex<br>Peak broadening |

| | | |
|---|---|---|
| High-speed concurrent chromatography | Low solvent usage<br>Recycling the solvents<br>Used for unstable natural compounds<br>Can be used both normal and reverse phases together | Lower efficiency<br>Results broader peaks<br>Single run operates relatively narrow polarity window<br>Time-consuming |
| Flash chromatography | A larger amount of samples can be separated at one time<br>Rapid and economical<br>A wide range of mobile phases can be used<br>Both reverse and normal phases can be employed | Due to an open system, it can be affected by some factors such as temperature, humidity<br>Required more amount of solvent depending on column size |
| Supercritical fluid chromatography | Green extraction technique<br>No need any solvents<br>Low operating temperatures | Very expensive and complex equipment<br>High power consumption<br>No polar substances can be extracted |

Flash chromatography-based purification method was successfully applied to purify other bioactives such as polyphenols from plant sources (Arora and Itankar, 2018; Hossain et al., 2014) which is described in **Chapter 1** and **Chapter 3**. To our knowledge, this method has not been applied in the field of betalains. Therefore, in order to overcome the limitations of other purification methods and to utilize the advantages of flash purification, this method has been tested to purify betalains. In the present study, betanin, vulgaxanthin I and indicaxanthin which are predominant compounds present in common betalain-rich dietary sources beetroot and prickly pear as well as neobetanin, one of the major degraded products of betanin, were purified (**Chapter 3**).

The chemical composition of these three betalain sources is distinct from each other (Appendix E, Table E.1 and E.2 – HPLC/MS analysis of betalains and polyphenols present in beetroot and prickly pear). The developed flash chromatography method was able to isolate the different betalain types from two different complex chemical matrices and the purification yield of betanin, neobetanin, vulgaxanthin I, and indicaxanthin were 487, 187, 302 and 409 mg from 100 of powdered raw material

respectively. Kanner et al. (2001) purified betanin using a C18 column and obtained 60 mg of purified betanin from 100 g of red beetroot. Further, Gonçalves et al. (2012) used seven different techniques to purify betanin and the purification yield ranged between 8.9 – 30.6 mg from 100 g of fresh beetroot while the percentage purity (calculated using HPLC chromatograms) ranged between 86 – 99%. Therefore, the betanin yield using the flash chromatography method is about eight times higher than the maximum value that has been reported in the other studies. There are a few studies that have used purified indicaxanthin but they did not provide the purification yield or the purity of indicaxanthin (Butera et al., 2002; Allegra et al., 2014a; Allegra et al., 2014b). The purity of betanin and indicaxanthin in this study was 96% and 95% respectively; purity > 90% is generally considered as high, while the purity of vulgaxanthin I and neobetanin was 79% and 52% respectively. The reason for the low purity percentage in vulgaxanthin I was co-elution of sucrose (318.03 ± 22.5 mg/g) at the same time, which is likely due to the same polarity of both compounds. Further, it is assumed that in the case of neobetanin, the presence of ~10% betanin and isobetanin and some other degraded products of betanin were the reason for the low purity. Changing the flash chromatography conditions such as introducing a step gradient, modifying the pH of the mobile phase, increasing run time, changing the flow rate and sample loading volume as well as pre-treatment of crude extract to remove sugars (solid-phase extraction) may be the next steps to increase the purity of vulgaxanthin I and neobetanin. Removal of other possible bioactive compounds such as polyphenols and nitrates is important as they may show synergistic and/or antagonistic effects with betalains. Further, presence of sugars in the purified fractions could induce hyperglycaemic cell stress and changes in osmotic pressure. The effects of sugars on the expression of inflammatory markers can be tested with the sugar-matched controls parallel to the low purity fractions. Therefore, the purification of betalains with high purity and yield is of high relevance in order to study and understand their individual effects and biological activities.

## 5.3 Betacyanins possess higher antioxidant activity than betaxanthins

Several studies have demonstrated the antioxidant properties of betalain pigments using chemical and biological models (Slimen et al., 2017). In agreement with that, the present study demonstrated the radical scavenging and antioxidant activity of betalain extracts (**Chapter 2**) as well as purified betalains (**Chapter 3**) upon various types of free radicals such as ABTS+, DPPH+, and hydroxyl radicals.

Betalains have high radical scavenging activity as shown by Escribano et al. (1998) and Gandía-Herrero et al. (2010) in red beetroot, in *Opuntia ficus-indica* by Butera et al. (2002), and in Amaranthaceae by Cai et al. (2003). In all studies, betalains showed higher radical scavenging activity as compared to ascorbic acid, rutin, catechin, and Trolox. Further, Gandía-Herrero et al. (2012) demonstrated that betalamic acid, the core structure of all betalains showed higher $ABTS^{+}$ radical scavenging activity than Trolox at pH 7. The scavenging activity was found to be increased 0.4 times and 1.6 times when the betalamic acid was connected with an aromatic ring and indole-like substructure, respectively (Gandía-Herrero et al., 2010).

In betacyanins, acylation improves the scavenging activity while glycosylation decreases the scavenging activity. In betaxanthins, antioxidant activity depends on the number of imino and hydroxyl groups attached to the compound (Slimen et al., 2017). Hence, the attachment of different chemical groups to betalamic acid affects the radical scavenging activity of betalains. Another factor that affects betalains' antioxidant activity is pH. Escribano et al. (1998) and Gliszczyńska-Świgło et al. (2006) showed that the free radical scavenging ability of betanin is much higher at basic pH (pH > 4) compared to acidic pH. This pH-dependent free radical scavenging activity is observed not only in betanin but also in other different natural and synthetic betalains (Gandía-Herrero et al., 2010). According to Gliszczyńska-Świgło et al. (2006), betanin exhibits in a cationic state at low pH, and when the pH increases betanin is converted into mono-, di- and tri-deprotonated forms. Further, there was a clear decrease of bond dissociation energy and ionization potential of betanin

when increasing the pH and thereby possess higher radical scavenging activity at a higher pH. Butera et al. (2002) compared the reduction potential of betalains, which indicates the ability of a molecule to donate its electrons, and observed that betacyanins (betanin -0.4 V) have more reduction potential than betaxanthins (indicaxanthin -0.6 V). The present study is in line with these findings, by providing evidence that betacyanins are more potent than betaxanthins in scavenging different types of radicals.

Due to the presence of a complex mixture of bioactive compounds such as betalains, polyphenols, and organic acids make it difficult to conclude the individual contribution of betalains on the antioxidant activity of betalain-rich crude extracts. Georgiev et al. (2010) suggested that the higher antiradical activity of the betalain-rich extracts may be due to the synergistic effects of polyphenols with betalains. The present study therefore assessed the antioxidant activity of purified compounds and provided comparison data on *in vitro* antioxidant activity of betacyanins and betaxanthins using several radicals.

The results of the present study showed that antioxidant activity of purified betalains increased in the following order indicaxanthin < vulgaxanthin I < neobetanin < betanin. These findings are in line with the results presented by Butera et al. (2002) who demonstrated that betanin was more active to provide radical scavenging properties compared to indicaxanthin, both of which were isolated from prickly pear. The reason for this could be the higher H-atom or electron donation ability of betanin due to the monophenol nature and production of reducing intermediates during the oxidation process (Escribano et al., 1998).

In addition, antioxidant activity of betalain-rich extracts from red beetroots, yellow beetroot and yellow prickly pear also measured. Further, multiple linear regression was calculated to predict the antioxidant activity of betalain extracts based on their total betalain content (TBC) and total polyphenol content (TPP). Significant regression equations ($p<0.001$) were found with $R^2$ of 98.39% and 99.67% for antioxidant activity measured using ABTS+ and FRAP assays respectively. The

following equations (Equation 1 and 2) showed the relationship between TBC and TPP with antioxidant activity.

$$TEAC = 178.8 + 8.07\ TBC + 1.051\ TPP \quad (1)$$

$$FRAP = -5.3 + 6.87\ TBC + 4.173\ TPP \quad (2)$$

Both TBC and TPP were significant predictors of antioxidant activity measured using FRAP assay whereas only TBC was a significant predictor of antioxidant activity measured by ABTS+ assay. This analysis provides positive support to our hypothesis that betalains are main contributors to antioxidant activity in betalain-rich extracts.

## 5.4 Betalains and their cellular antioxidant and anti-inflammatory activities

The consumption of betalains has been linked to the prevention of diseases caused by chronic inflammation, such as cancer, arthritis, diabetes, bowel, and heart disease (Rahimi et al., 2019; Moreno-Ley et al., 2021). Therefore, the present study assessed the anti-inflammatory and cell-protective activities of the betalains using an inflammatory cell model (**Chapter 4**). The studies conducted by Tan et al. (2015), Yang et al. (2016), and Ahmadi et al. (2020) claimed that betanin could down-regulate the expression of pro-inflammatory markers iNOS, COX-2, TNF-α, IL-6, IL-1β and NF- κB protein levels while reducing ROS generation. The findings of the present study are in agreement with these results and showed that betanin dose-dependently reduced pro-inflammatory cytokines and other markers as well as ROS production in the concentration range 1 μM - 100 μM. Moreover, betacyanins (betanin and neobetanin) showed better attenuation of pro-inflammatory cytokine expression and scavenging of ROS compared to the betaxanthins (vulgaxanthin I and indicaxanthin). Tesoriere et al. (2014) demonstrated that indicaxanthin (5 – 25 μM) dose-dependently down-regulated the expression of IL-6, IL-8, $PGE_2$, and NO while inhibiting NF-κB activation and reducing protein expression of COX-2, iNOS, and NOX-1 in Caco2 cells stimulated by IL-1β. Similar results were reported in the

present study demonstrating that indicaxanthin (10 µM) down-regulated mRNA expression of IL-6 (28%), iNOS (27%), and COX-2 (30%).

However, Allegra et al. (2014a) reported pro-oxidant behaviour of indicaxanthin in LPS-stimulated RAW 264.7 cells. They demonstrated that whilist indicaxanthin inhibited the activation of NF- κB signaling pathway and therefore down-regulated of mPGES-1, it subsequently, activated ROS production and up-regulated the expression of COX-2 and hematopoietic prostaglandin D synthase (H-PGDS). These observations suggested that the pro-oxidant activity of indicaxanthin at a relatively higher concentration (100 µM) allows the production of anti-inflammatory mediators inducing the prostaglandin metabolism in LPS-stimulated macrophages. The phenomenon of pro-oxidant behaviour of betaxanthins (indicaxanthin and vulgaxanthin I) was observed in the present study with RAW 264.7 cells even at low concentrations (1 µM – 25 µM). In contrast, betacyanins (betanin and neobetanin) showed a significant reduction of oxidative stress by reducing ROS generation.

Betalains have been linked to Nrf2 activation, however, so far there is not much literature available, apart from betanin. Esatbeyoglu et al. (2014) reported that betanin (15 µM) induced Nrf2 activation moderately by 20% in a dual-luciferase reporter assay and increased expression of Nrf2 traget HO-1 by 15% on protein level via Western Blotting in human liver hepatoma cells. Further, Krajka-Kuźniak et al. (2013) showed that treatment with betanin (2, 10, and 20 µM) resulted in the translocation of Nrf2 from the cytosol to the nucleus while increasing the mRNA and protein levels of GSTP, GSTT, GSTM, and NQO1 in both THLE-2 and HepG2 cells. The results of the present study provide evidence that betanin (10 µM) induced mRNA levels of HO-1 and γGCS by 66% and 40% in LPS-stimulated murine macrophages. When compared to betaxanthins, betacyanins showed generally more potent induction of HO-1 and γGCS whereas betaxanthins, especially indicaxanthin did not change these targets at any concentration (10, 50, and 100 µM).

The present study used betalains in a wide range of concentrations (0.4 µM – 100 µM) which can be considered physiologically feasible to assess their anti-inflammatory properties and the effects on ROS production with macrophage cells (RAW 264.7). For instance, the total betalain content of fresh beetroot juice ranged between 0.8 – 1.3 g/L (mean value 1.05 g/L) (Wruss et al., 2015) and assuming that one person drinks 500 mL of beetroot juice per time, ~525 mg of total betalain content was ingested by a person. Therefore, we can roughly assume that intestinal cells are exposed to the ~2.37 mM betalain content. It has been shown that the consumption of red beetroot juice led to betalain concentrations in human plasma reaching the micromolar range (0.2 – 6.9 µmol/L) after 3 hrs (Tesoriere et al., 2003). In contrast, Clifford et al. (2017) reported that betanin was not detectable in the human plasma at any time point (1-8 hrs) after consumption of beetroot juice. In addition, Sawicki et al. (2018) found 87.65 ± 15.71 nmol/L betalain levels in blood plasma after the first and second week of consumption of fermented beetroot juice (200 mL/ day, 41.8 ± 1.9 mg betalains/ 200 mL of juice) by healthy humans. Therefore, we can assume that betalains are poorly absorbed and available in nanomolar to micromolar ranges based on the aforementioned literature. The present study showed that betalains can reduce ROS production even at nanomolar concentrations (400 and 800 nM) and exhibited anti-inflammatory properties at all the tested micromolar concentrations.

Further, betalains may have potential effects on other cell signaling pathways such as Protein kinase C (PKC) pathway (involved in phosphorylation of target proteins to activation and inhibition of many cellular processes), Mitogen-activated protein kinases (MAPKs) signaling cascade (involve in gene expression associated with the regulation of inflammation and cytokine expression), phosphatidylinositol 3-kinase/protein kinase B PI3/Akt pathway (one of the strongest intracellular signal transduction pathway that promotes metabolism, proliferation, and cell survival), which has shown to be modulated by other bioactives such as polyphenols (Mansuri et al., 2014). This means, there is a lack of *in vitro* and *in vivo* studies available to confirm the effect of betalains on cell signaling pathways and cross-talks of pathways related to inflammation. Therefore, further research is warranted to investigate in

more detail the effects of individual betalains on different cell signaling pathways and resulting protein signatures.

Overall, the data of present study show differential effects on antioxidant and anti-inflammatory properties of betalains. Further, it was assumed that betanin is the main active compound in betalain-rich extracts which may basically contributing to antioxidant and anti-inflammatory properties, while different betalains contribute differently to the overall antioxidant and anti-inflammatory activity of the extracts due to structural differences.

## 5.5 Conclusions

Low ethanol concentrations (30% ethanol v/v) are the most suitable solvent to extract both betalains and polyphenols from the red beetroot powder using an ultrasound-assisted extraction method. Rapid loss of total betalain content can be seen at room temperature irrespective of the solvent used to extract the betalains. Further, moderate loss of antioxidant activity parallel to the betalain content over time highlighted the potential contribution of betalains, polyphenols, and their metabolites and/or degradation products to antioxidant activity. Comparative extraction results demonstrated that the beetroot pomace which is the by-product of the beetroot juice industry can provide good pigment yield similar to the whole beetroot samples.

Flash chromatography can effectively purify betanin, neobetanin, vulgaxanthin I, and indicaxanthin from beetroot and prickly pear sources while resulting in purification yields ranging from 1.87 – 4.87 mg/g. Percentage purity of betanin and indicaxanthin were more than 95% whereas percentage purity of vulgaxanthin I and neobetanin were 79% and 52%, respectively, based on HPLC analysis. Betacyanins were more potent as antioxidants than betaxanthins while betanin demonstrated strongest antioxidant capability in comparison to other purified compounds.

Betanin dose-dependently decreased intracellular ROS production while betaxanthins (vulgaxanthin I and indicaxanthin) showed pro-oxidant behaviour increasing ROS production in $H_2O_2$ stimulated macrophages. All purified betalains

significantly down-regulated the secretion of pro-inflammatory cytokines (IL-6, iNOS, IL-1β, and COX-2) and up-regulated the synthesis of the anti-inflammatory cytokine IL-10 while showing moderate induction of cell-protective markers (HO-1 and γGCS). Betacyanins showed more tendency of down-regulating pro-inflammatory cytokines and scavenging ROS compared to betaxanthins.

## 5.6 Future directions

The present study established a comprehensive workflow for isolation, purification, and characterization of betalains from its predominant sources. The four isolated betalains were applied in a macrophage cell model to assess their potential anti-inflammatory properties.

Recommendations for further work directly related to the research are summarized as below;

- Optimization of enzyme assisted extraction (EAE) of betalains from plant materials: Proposed to carry out investigations using pectinase, cellulase, protease and amlylase enzymes to evaluate the best combination of enzymes to extract betalains. Further, studies need to be carried out to find the optimum conditions for enzyme concentrations, treatment temperature and time after finalizing the enzyme or enzyme combination.

- Optimization and tailoring of flash chromatography method to further enhance the purity of vulgaxanthin I and neobetanin: Proposed modifications are to change the solvent gradient along with column volume, change pH of the crude extract, and change the organic solvents such as methanol, ethanol, and isopropanol. These suggested alterations will allow overcoming the problem of sugar contamination in the samples. Moreover, other types of betalain could be isolated, including betalamic acid and different types of vulgaxanthin.

- Scaling up the flash purification method: Betalain purification in the present study was conducted using a small-scale development method using a column size of 60 g. It is suggested to trial the scale-up experiments using a larger column size such as 120 g or 200 g to obtain purified compounds for further laboratory analysis. Further, the following parameters should be held constant when scaling up the optimized method; concentration of the sample, elution solvents, elution gradient, and column volumes, solvent flow velocity, monitoring wavelengths, column brand, and sample load percentage.

- Identification of other bioactive compounds collected during the purification: The primary focus of the present study was to develop an effective method for the purification of betalains from two common betalain sources (beetroot and prickly pear). However, it was observed that there was a capability of separating some other compounds such as polyphenols during the flash chromatography process. Identification of those fractions would provide useful information to discover other compounds which can be purified in the same process from the main betalain sources.

Further recommendations to increase the awareness of this research area can be summarized as below;

- Establish the effects of betalains on a cellular level on a more global scale. The interactions with further cell signaling pathways through transcriptomic and proteomic analysis. Profiling of complex responses to compound treatment is important to explore the mode of actions of betalains and provide useful information for medical and pharmaceutical research.

- Investigating the interactions of betalains with other compounds such as polyphenols to identify potential synergistic or antagonistic effects: In fact, the majority of food supplements contain a combination of individual compounds. Therefore, it is of high relevance to study the interactions of individual compounds to understand the contribution of each of these in a complex mixture.

- Bioavailability of betalains: The biological activity of compounds greatly depends on their bioavailability. So far, betalain absorption, metabolism, and excretion are not fully described. Therefore, understanding the bioavailability of this pigment group as well as in combination with the food matrix is important to understand their effects on human health.

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# Appendix A

## Supporting information of Chapter 1

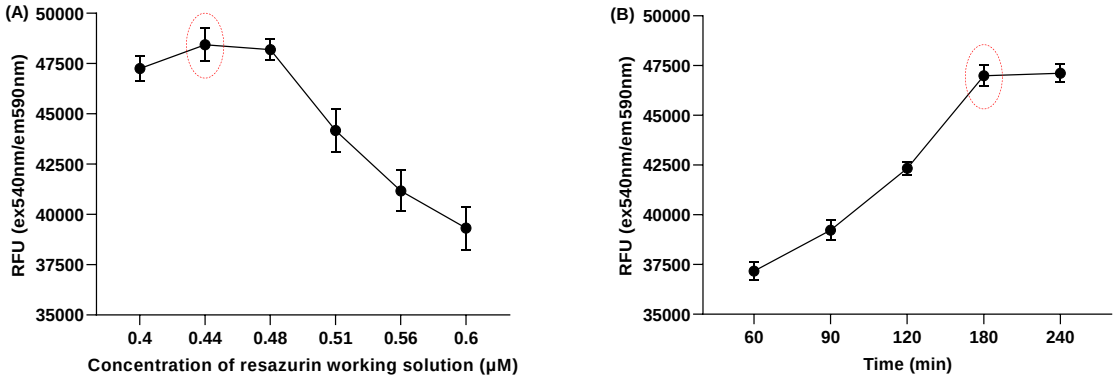


**Figure A.1** Changes of fluorescence signals (RFU) for different concentration of resazurin (A) and incubation time (B). Fluorescence measured at ex/em 540/590 nm. Experiments were conducted using RAW 264.7 cells.

# Appendix B

## Supporting information of Chapter 2

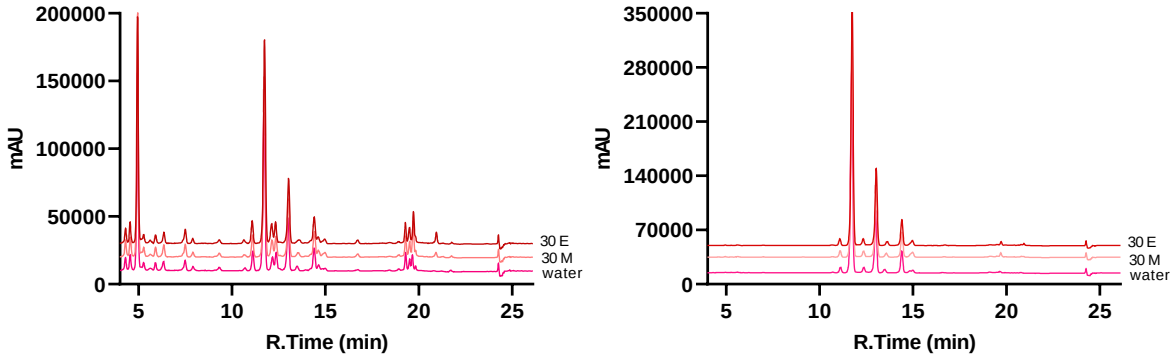


**Figure B.1** Betalain pattern of extracts. Presented are the different betalain compounds extracted with water, 30% methanol (30 M) and 30% ethanol (30 E).

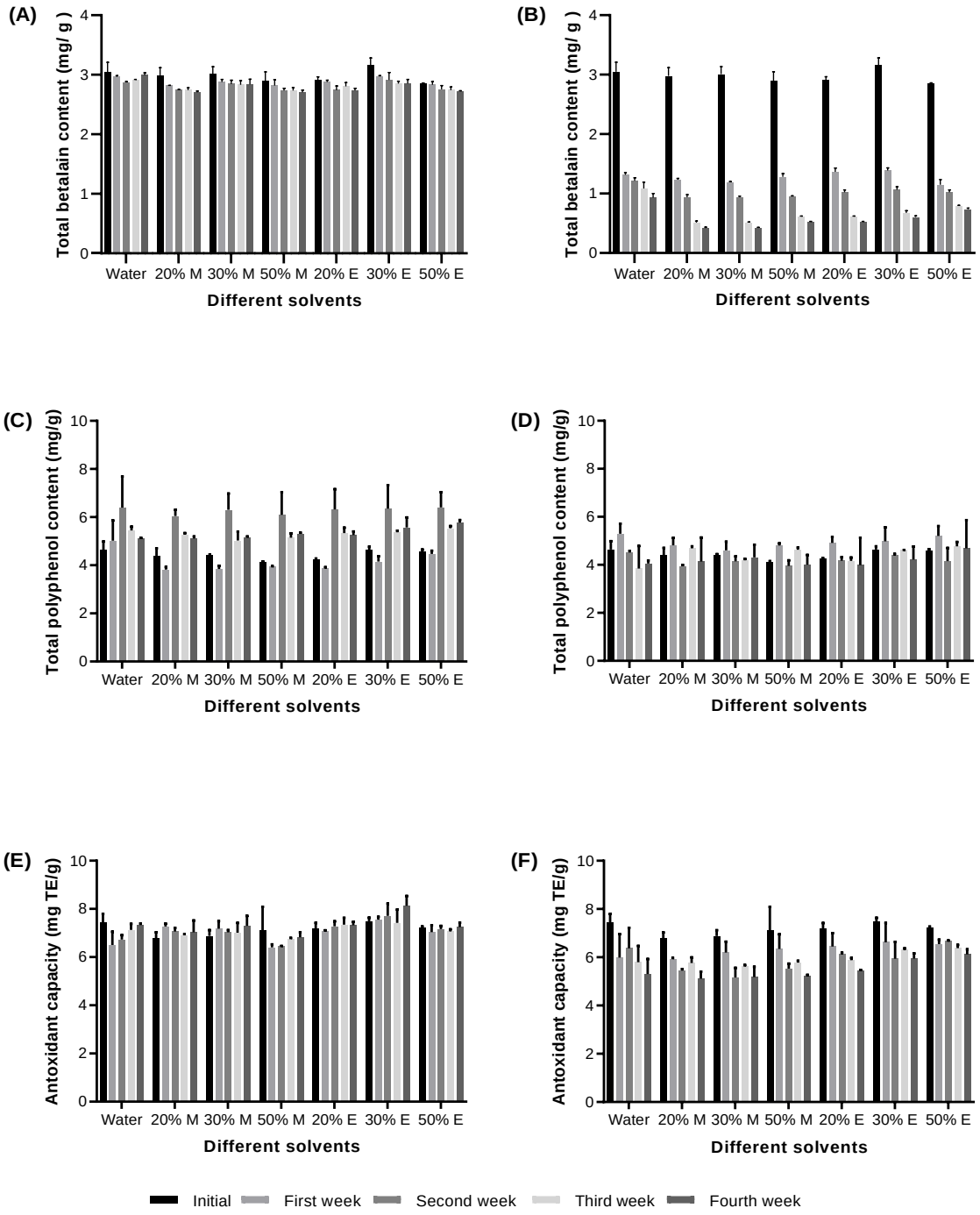


**Figure B.2** Variation of total betalain content, total polyphenol content and antioxidant activity (TEAC –Trolox equivalent antioxidant capacity) of the different extracts during a 4-week storage at -20 °C (A), (C), (E) and room temperature (B), (D), (F). Data are mean with ± SD (n = 2, independent extractions).

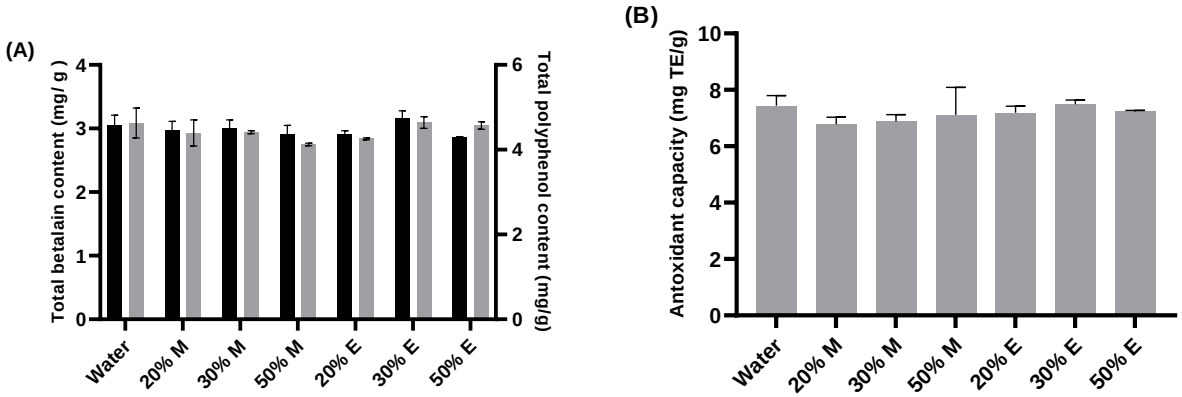


**Figure B.3** Effects of water, methanol and ethanol solvent mixtures on total betalain content, total polyphenol content and antioxidant activity in red beetroot extracts (initial values). Data are mean with ± SD of three independent extractions.

**Table B.1** Pearson correlation coefficients (r) between colour values (L, a, b, c, h) and total betalain content.

| Colour trait | Total betalain content (mg/g) | |
|---|---|---|
| | R | P value |
| L* | -0.9074 | <0.0001 |
| a* | 0.8967 | <0.0001 |
| b* | -0.8807 | <0.0001 |
| c* | 0.5930 | <0.0001 |
| H | -0.9256 | <0.0001 |
| Lab | 0.9820 | <0.0001 |
| a/b | 0.9840 | <0.0001 |

L*= lightness or darkness; a*= redness or greenness; b*= yellowness or blueness; c*= chroma; h= hue angle, a/b – ratio that indicate the quantitative development of colour

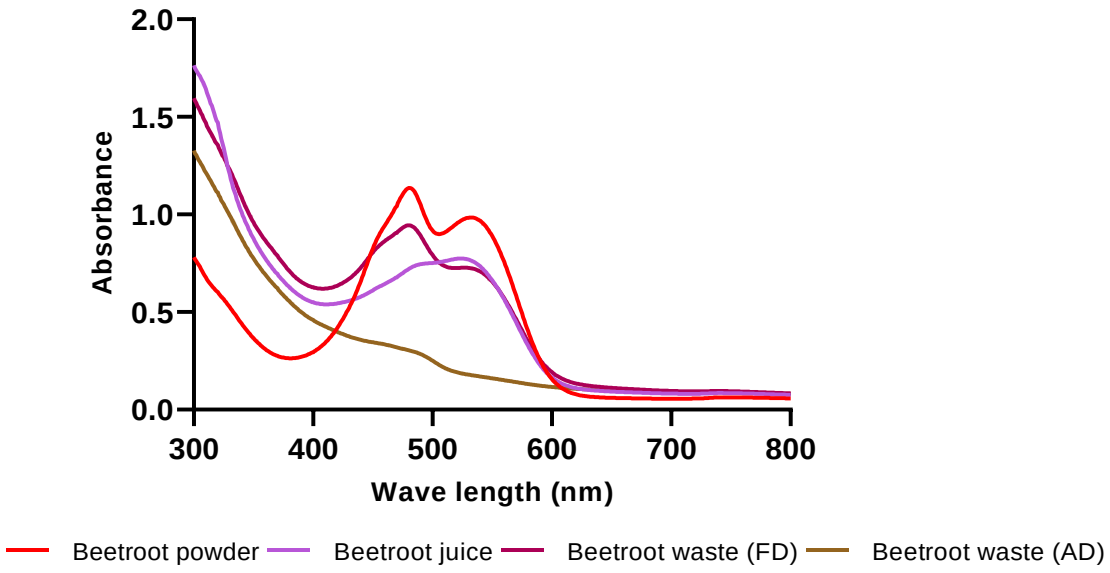


**Figure B.4** UV-vis scan of beetroot samples showing the characteristics at 536 nm and 486 nm wavelength.

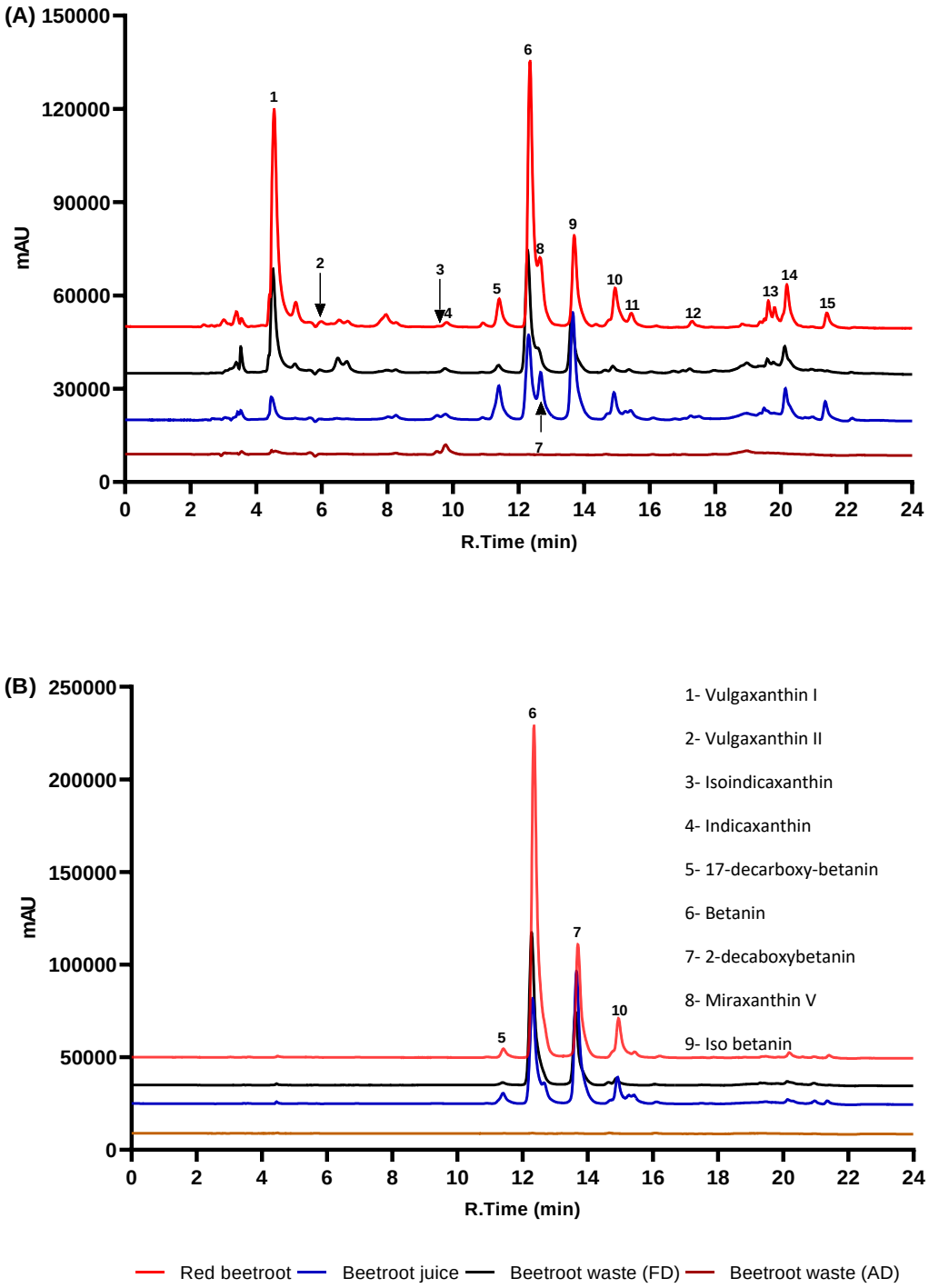


**Figure B.5** HPLC-DAD chromatograms of different red beetroot samples. Elution monitored at 486 nm (betaxanthins, A) and 536 nm (betcayanins, B). ESI-MS positive ion mode was used to identify the peaks.

# Appendix C

## Supporting information of Chapter 3

**Table C.1** HPLC/MS identification of fractions collected from red beetroot extract used for betanin purification.

| Colour of the fraction | Fraction No. | [M+H] m/z | Compound/s |
|---|---|---|---|
| | F1 | 507 | 2-decarboxybetnin/isobetanin |
| | F2 | 340 | Vulgaxanthin I |
| | F3 | 340 | Vulgaxanthin I |
| | F4 | 340 | Vulgaxanthin I |
| | F5 | 340 | Vulgaxanthin I |
| | F6 | 340 | Vulgaxanthin I |
| | F7 | 340, 341, 551 | Vulgaxanthin I, Vulgaxanthin II, Betanin |
| | F8 | 340, 341, 347, 551 | Vulgaxanthin I, vulgaxnthin ii, miraxanthin V, betanin |
| | F9 | 551 | Betanin |
| | F10 | 551 | Betanin |
| | F11 | 551, 551 | Betanin, iso betanin |
| | F12 | 551, 551 | Betanin, iso betanin |
| | F13 | 551, 551, 505 | Betanin, iso betanin |
| | F14 | 551, 551, 505 | Betanin, iso betanin |
| | F15 | 551, 551. 505, 549 | Betanin, iso betanin, , neobetanin |
| | F16 | 551, 505, 549 | Betanin, , 17-decarboxyneobetanin, neobetanin |
| | F17 | 551, 505, 549 | Betanin, , 17-decarboxyneobetanin, neobetanin |
| | F18 | 551, 505, 549 | Betanin, , 17-decarboxyneobetanin, neobetanin |
| | F19 | 551, 505, 549 | Betanin, , 17-decarboxyneobetanin, neobetanin |

**Table C.2** HPLC/MS identification of fractions collected from red beetroot extract used for neobetanin purification.

| Colour of the fraction | Fraction No. | [M+H] m/z | Compound/s |
|---|---|---|---|
| | F1 | 325, 326, 340 | Isoleucine-bx, vulgaxanthin III, vulgaxanthin I |
| | F2 | 325, 326, 340 | Isoleucine-bx, vulgaxanthin III, vulgaxanthin I |
| | F3 | 325, 326, 340 | Isoleucine-bx, vulgaxanthin III, vulgaxanthin I |
| | F4 | 325, 326, 340 | Isoleucine-bx, vulgaxanthin III, vulgaxanthin I |
| | F5 | 340, 551, 551 | Vulgaxanthin I, betanin, isobetanin |
| | F6 | 340, 551, 551 | Vulgaxanthin I, betanin, isobetanin |
| | F7 | 340, 551, 551 | Vulgaxanthin I, betanin, isobetanin |
| | F8 | 309, 551, 551 | Indicaxanthin, betanin, iso betanin |
| | F9 | 551, 551 | Betain, isobetanin |
| | F10 | 551, 551 | Betanin, iso betanin |
| | F11 | 551, 551, 507, 311 | Betanin, isobetanin, Valine-bx |
| | F12 | 551, 551, 549 | Betanin, isobetanin, neobetanin |
| | F13 | 551, 551, 549 | Betanin, isobetanin, neobetanin |
| | F14 | 551, 551, 549 | Betanin, isobetanin, neobetanin |
| | F15 | n/a | |
| | F16 | n/a | |
| | F17 | n/a | |

**Table C.3** HPLC/MS identification of fractions collected from yellow beetroot.

| Colour of the fraction | Fraction No. | [M+H] m/z | Compound/s |
|---|---|---|---|
| | F1 | n/a | |
| | F2 | 325, 326, 340 | Isoleucine-bx, vulgaxanthin III, vulgaxanthin I |
| | F3 | 340 | Vulgaxanthin I |
| | F4 | 340 | Vulgaxanthin I |
| | F5 | 340 | Vulgaxanthin I |
| | F6 | 340 | Vulgaxanthin I |
| | F7 | 340 | Vulgaxanthin I |
| | F8 | 340 | Vulgaxanthin i |
| | F9 | n/a | |
| | F10 | 326, 340 | Vulgaxanhtin III, vulgaxanthin I |
| | F11 | 325, 340, 341, 326 | Isoleucine-bx, vulgaxanthin I, vulgaxanthin II, vulgaxanthin III |
| | F12 | n/a | |
| | F13 | 309, 309 | Iso-indicaxanthin, indicaxanthin |
| | F14 | 309, 309 | Iso-indicaxanthin, indicaxanthin |
| | F15 | 309 | Indicaxanthin |
| | F16 | 309 | Indicaxanthin |
| | F17 | n/a | |
| | F18 | 375, 347 | Miraxanthin V, Portulacaxanthin II (Tyrosine-bx) |
| | F19 | 375, 347 | Miraxanthin V, Portulacaxanthin II (Tyrosine-bx) |
| | F20 | n/a | |
| | F21 | n/a | |
| | F22 | n/a | |

**Table C.4** HPLC/MS identification of fractions collected from yellow prickly pear extract.

| Colour of the fraction | Fraction No. | [M+H] m/z | Compound/s |
|---|---|---|---|
| | F1 | n/a | |
| | F2 | n/a | |
| | F3 | n/a | |
| | F4 | 312, 278 | n/a |
| | F5 | 312, 278 | n/a |
| | F6 | 312, 278 | n/a |
| | F7 | 312, 294 | n/a |
| | F8 | 312, 294 | n/a |
| | F9 | 312, 294 | n/a |
| | F10 | 312, 294, 309 | n/a , indicaxanthin |
| | F11 | 309 | Indicaxanthin |
| | F12 | 309 | Indicaxanthin |
| | F13 | 309 | Indicaxanthin |
| | F14 | 309 | Indicaxanthin |
| | F15 | 309 | Indicaxanthin |
| | F16 | n/a | |
| | F17 | n/a | |
| | F18 | n/a | |
| | F19 | n/a | |
| | F20 | n/a | |
| | F21 | n/a | |
| | F22 | n/a | |
| | F23 | n/a | |
| | F24 | n/a | |
| | F25 | n/a | |

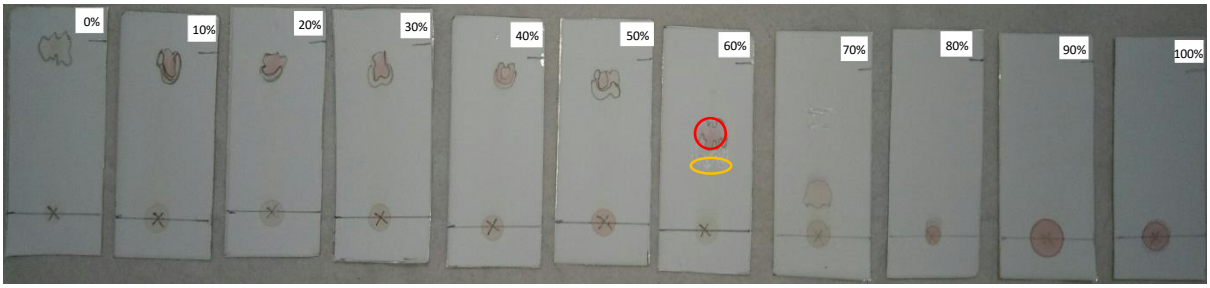


**Figure C.1** Representative TLC separation of red beetroot extract run in different concentrations of acetonitrile (0 – 100% v/v) with 0.1% formic acid using Silica gel 60 $F_{254}$ aluminium plates. Red and yellow circles showed betacyanin and betaxanthin fractions with acetonitrile/formic acid (60:0.1 v/v), respectively.

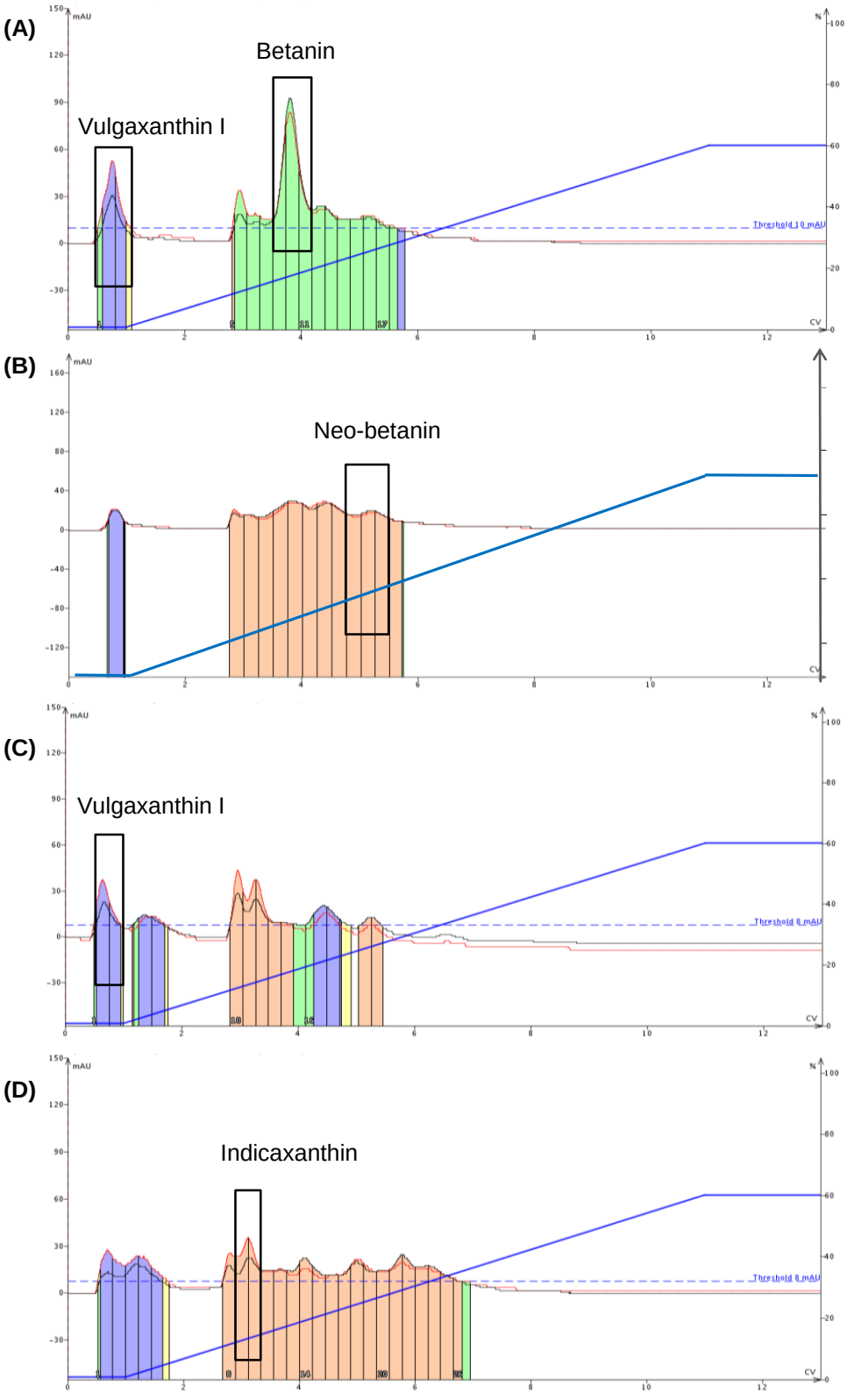


**Figure C.2** Flash purification of betalains (A) betanin and vulgaxanthin I from red beetroot extract (B) neo betanin from red beetroot extract (C) vulgaxanthin I from yellow beetroot extract and (D) indicaxanthin from yellow prickly pear extract.

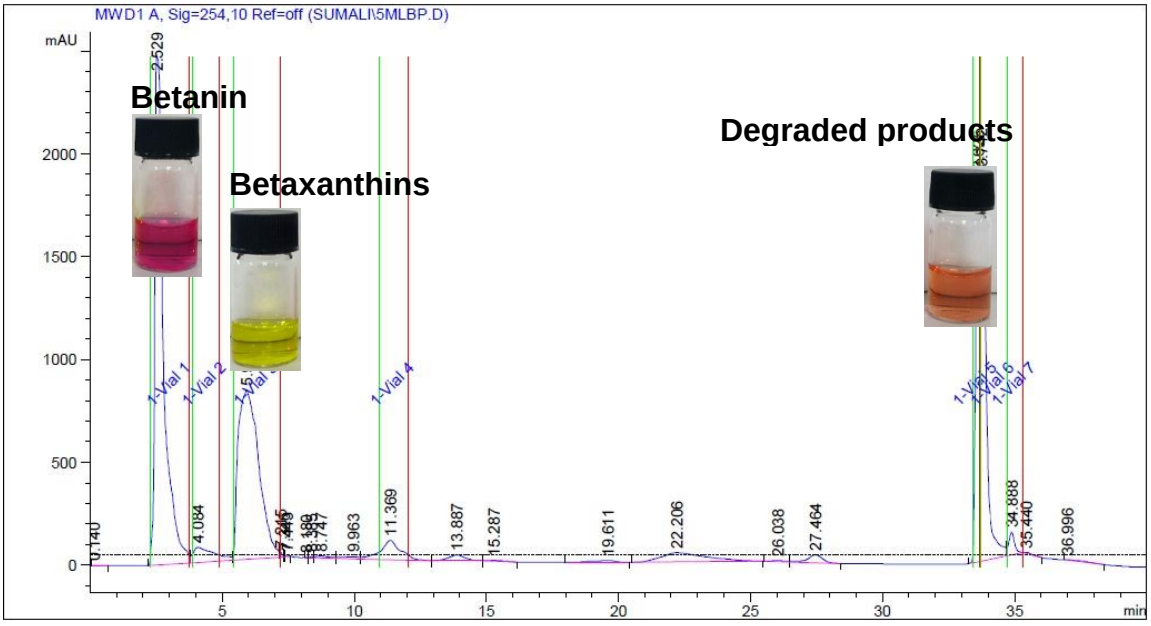


**Figure C.3** Raw data chromatogram of preparative HPLC for purification of betanin. Injection volume was 5 mL of concentrated red beetroot extract. Chromatographic conditions: mobile phase A – water/acetic acid (99.9:0.1 v/v), B - acetonitrile/water/acetic acid (60:39.9:0.1 v/v); flow rate: 5 mL/min.

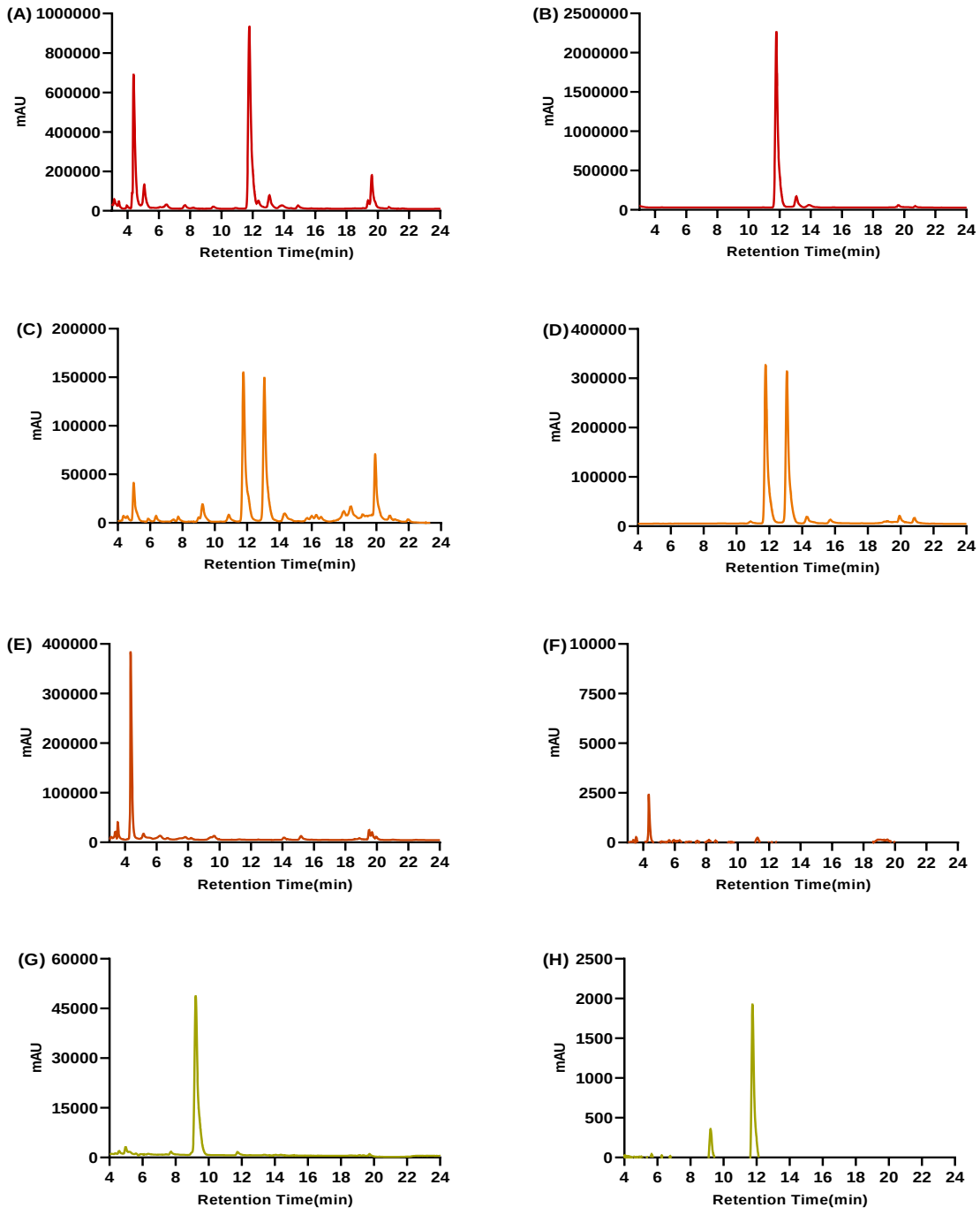


**Figure C.4** HPLC-DAD chromatograms of raw extracts used for the purification. (A), (B) red beetroot extract used for betanin and vulgaxanthin I purification, (C), (D) red beetroot extract used for neobetanin purification, (E), (F) yellow beetroot extract used for the vulgaxanthin I purification (G), (H) yellow prickly pear extract used for indicaxanthin purification. UV-Vis monitoring of A, C, E, and G at 486 nm and B, D, F and H at 536 nm. Chromatographic conditions: Mobile phase A – water/formic acid (98:2 v/v), B – methanol; flow rate: 0.95 mL/min; column oven: 40 °C.

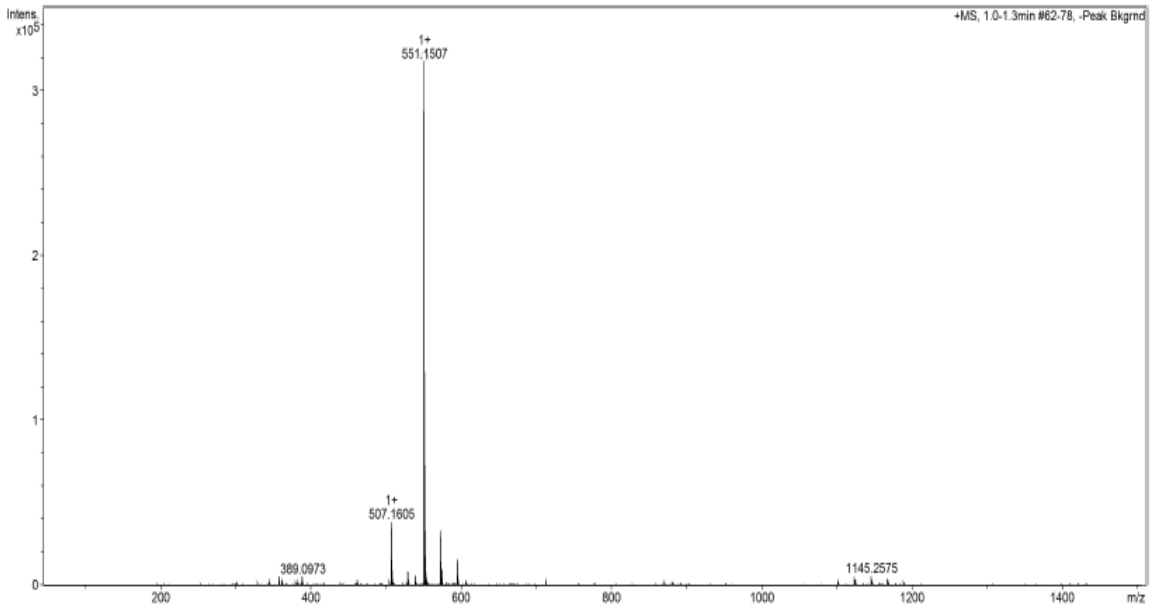


**Figure C.5** Positive ionization MS chromatogram for purified betanin, *m/z* 551 $[M + H]^+$ using Maxis Impact HD Mass Spectrometer (Bruker MaXis impact, Bremen, Germany).

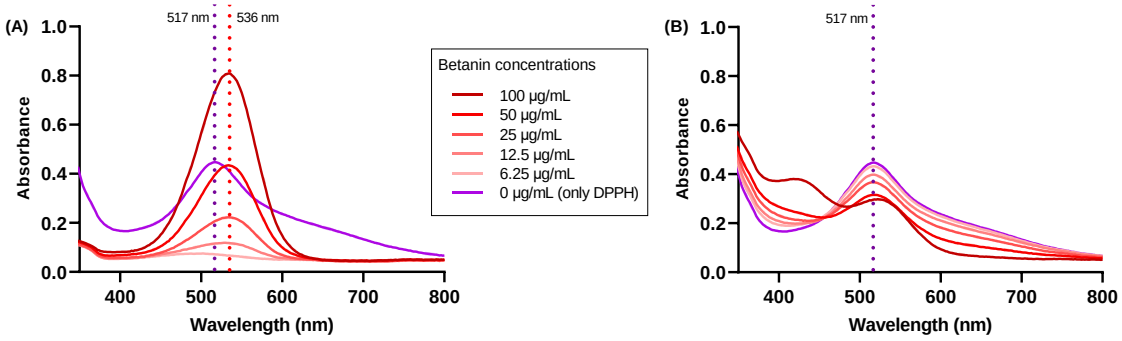


**Figure C.6** Absorbance scan of (A) different concentrations of betanin without DPPH radical and (B) with DPPH radical (n = 3).

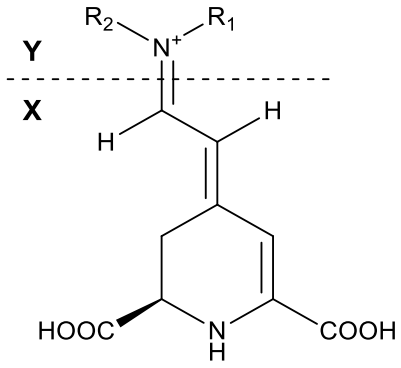


**Figure C.7** Common structure for all betalains, (X) – Betalamic acid moiety and (Y) R1 – cyclo DOPA for betacayanins, R2 – amino acid or amino acid derivatives for betaxanthins.

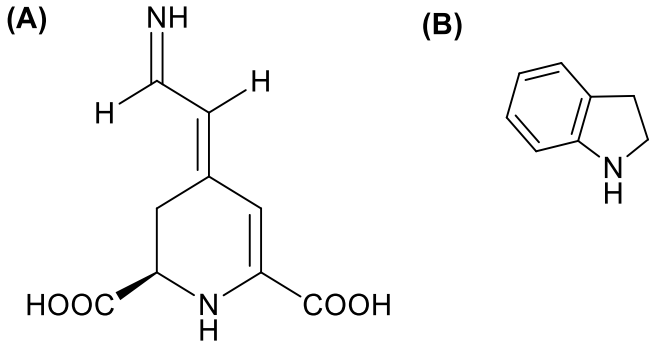


**Figure C.8** Structure of imino and tetrahydropyridine groups (A) and common structure of indole group (B).

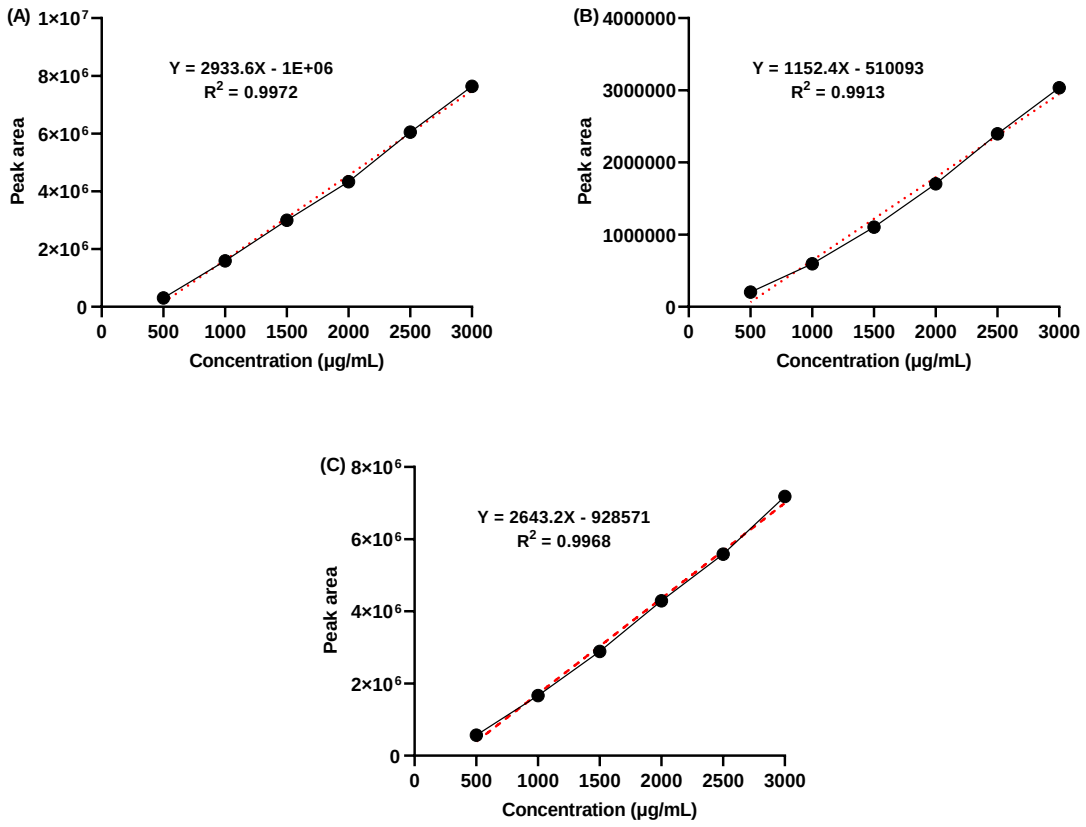


**Figure C.9** Calibration curves for (A) sucrose, (B) glucose and (C) fructose using UFLC$_{XR}$ system with evaporative light scattering detector (ELSD).

# Appendix D

# Supporting information of Chapter 4

**Table D.1** Total betalain and polyphenol content of red and yellow beetroot samples.

| Property | Red beetroot (mg/g) | Yellow beetroot (mg/g) |
|---|---|---|
| Total betalain content | 2.71 ± 0.02 | 1.05 ± 0.02 |
| Betacyanin content | 1.52 ± 0.01 | 0 |
| Betaxanthin content | 1.19 ± 0.01 | 1.05 ± 0.02 |
| Total polyphenol content | 9.51 ± 0.90 | 3.79 ± 0.12 |

Data present in mean ± SD

**Table D.2** HPLC-MS data (negative ionization mode) for identification of polyphenols present in different beetroot sources.

| No | Compound | Retention time (min) | $\lambda_{max}$ | $[M-H]^-$ | Red Beetroot | Yellow Beetroot |
|---|---|---|---|---|---|---|
| 1 | Catechin | 4.29 | 282 | 289 | + | - |
| 2 | Cochliophilin A | 4.98 | 283 | 281 | + | - |
| 3 | *p*-coumaric acid | 5.33 | 282 | 163 | + | + |
| 4 | Unknown | 5.35 | 264 | 147 | - | + |
| 5 | Caffeic acid | 5.67 | 265 | 179 | + | + |
| 6 | *N-trans*-feruloylmethoxytyramine | 6.13 | 278 | 342 | + | + |
| 7 | Ferulic acid | 6.39 | 274 | 193 | + | + |
| 8 | Unknown | 7.10 | 261 | 151 | - | + |
| 9 | Chlorogenic acid | 7.77 | 281 | 353 | + | + |
| 10 | Gallic acid | 9.25 | 282 | 169 | + | + |
| 11 | Unknown | 14.76 | 278 | 197 | + | + |
| 12 | Rosmarinic acid | 16.65 | 265 | 359 | + | - |
| 13 | *N-trans*-feruloyltyramine | 21.75 | 274 | 312 | + | - |
| 14 | Quercetin | 52.40 | 361 | 301 | + | - |
| 15 | Betavulgarin | 56.42 | 279 | 311 | + | - |

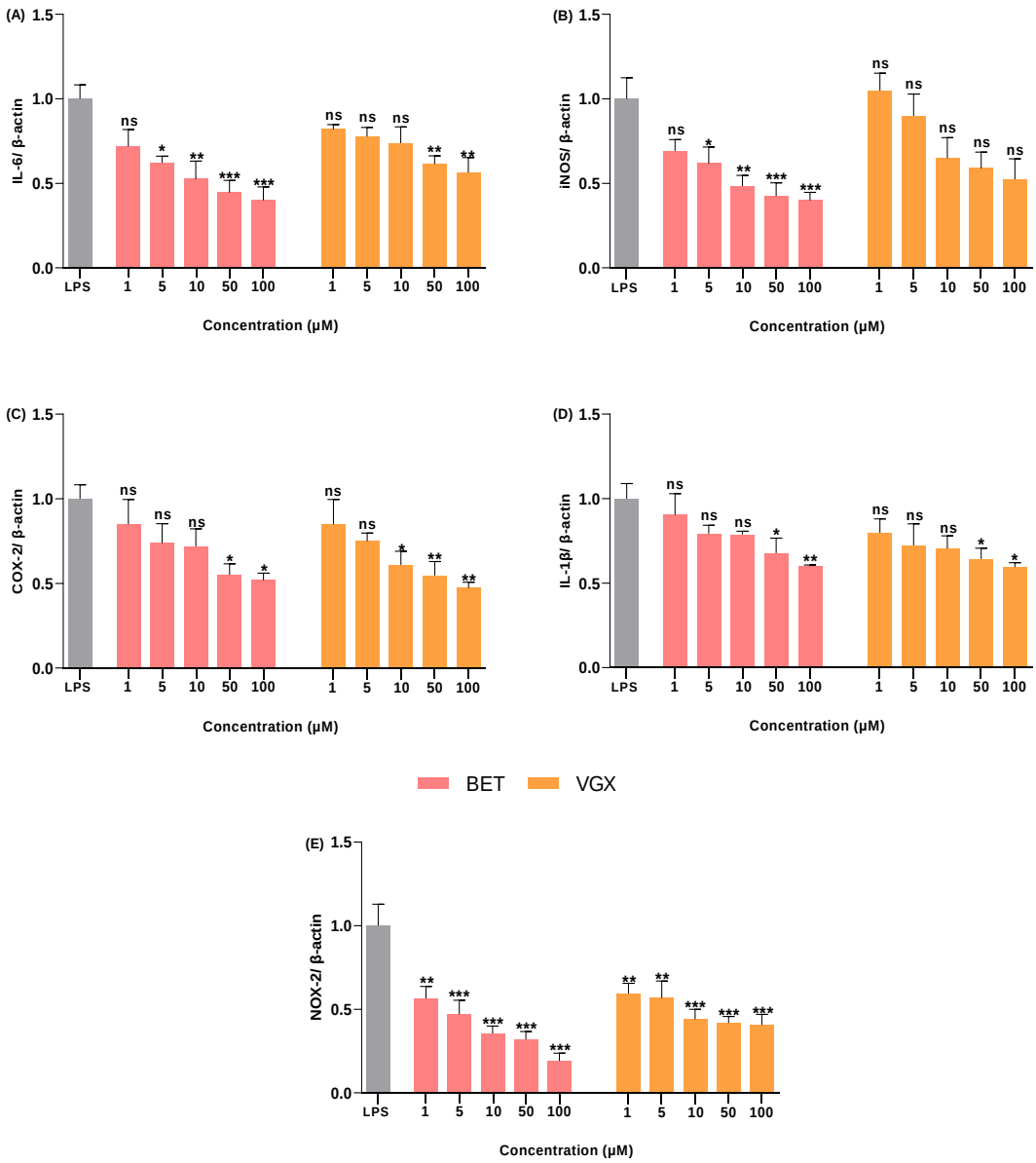


**Figure D.1** Effects of purified betanin and vulgaxanthin I (1, 5, 10, 50 and 100 μM) on gene expression related to pro-inflammatory cytokines (A) IL-6 (B) iNOS (C) COX-2 (D) IL-1β and (E) NOX-2 in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *p < 0.05, ** p<0.01, and *** p<0.001.

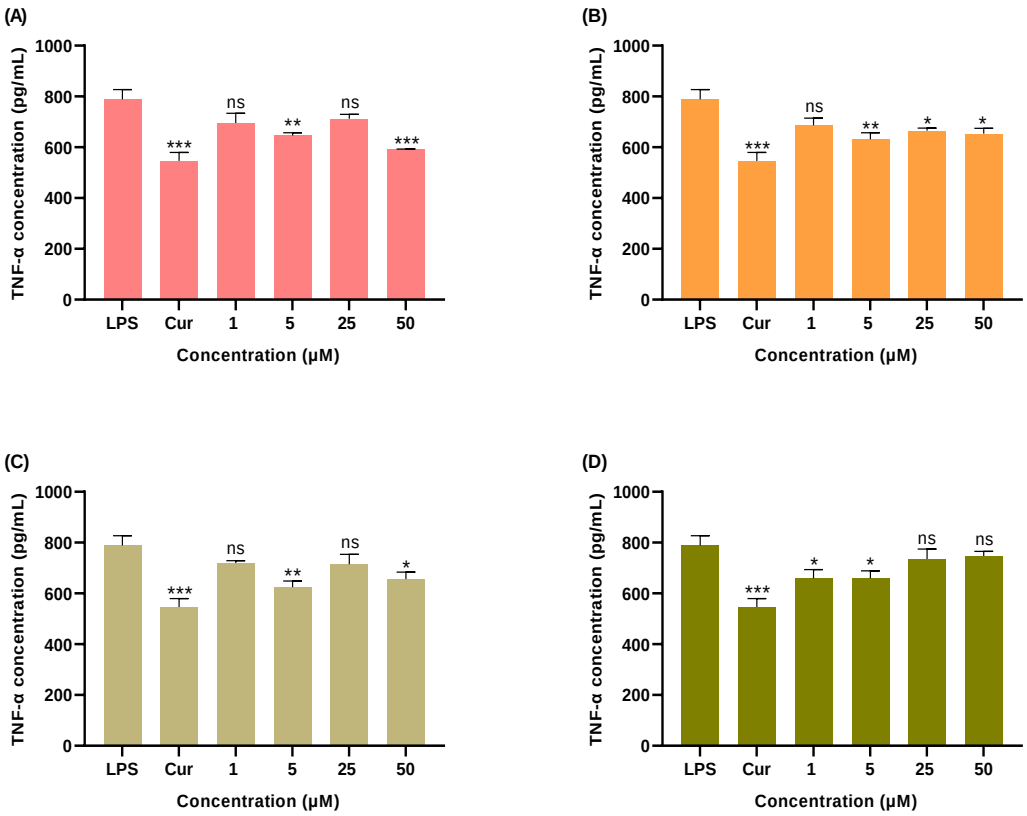


**Figure D.2** Effects of (A) betanin, (B) vulgaxanthin I, (C) neobetanin and (D) indicaxanthin on TNF-α protein secretion in LPS activated RAW 264.7 cells. Data are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *p < 0.05, ** p<0.01, *** p<0.001.

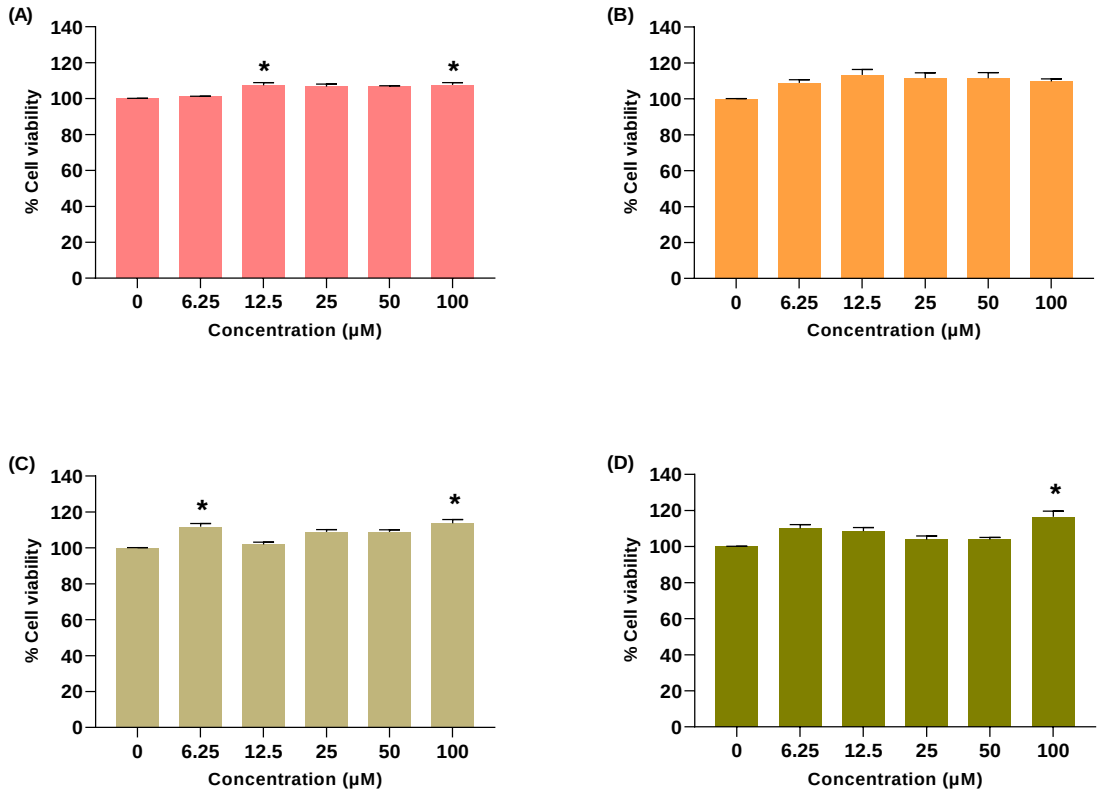


**Figure D.3** Effects of purified betalains on RAW 264.7 macrophage viability. (A) betanin, (B) vulgaxanthin I, (C) neobetanin and (D) indicaxanthin. The values are presented as mean ± SEM of three independent experiments.

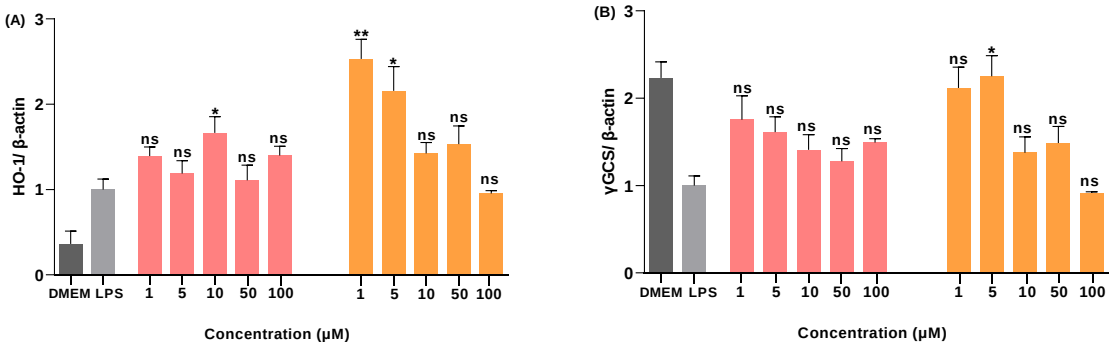


**Figure D.4** Effects of purified betanin and vulgaxanthin I (1, 5, 10, 50 and 100 µM) on gene expression of (A) HO-1 and (B) γGCS in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *p < 0.05.

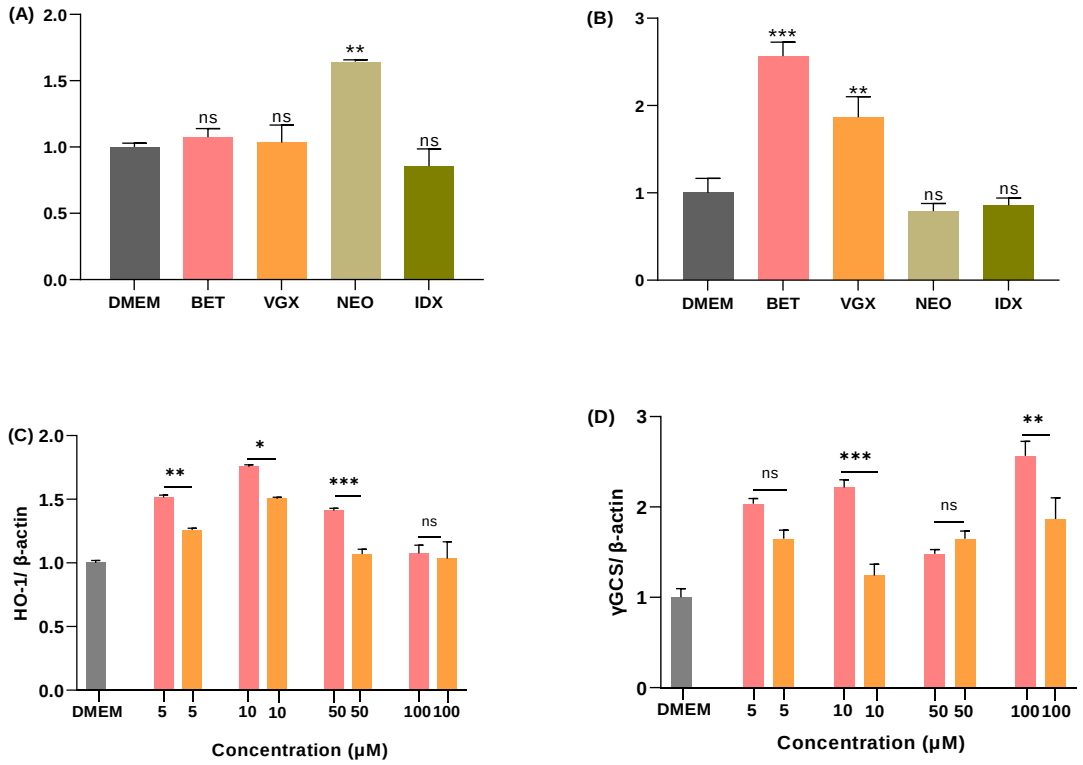


**Figure D.5** Effects of purified betalains on the mRNA expression of (A) HO-1 and (B) γGCS at 100 μM and (C) HO-1 and (D) γGCS with different concentration of betanin and vulgaxanthin I under non-stimulated condition. *p < 0.05, ** p<0.01, *** p<0.001.

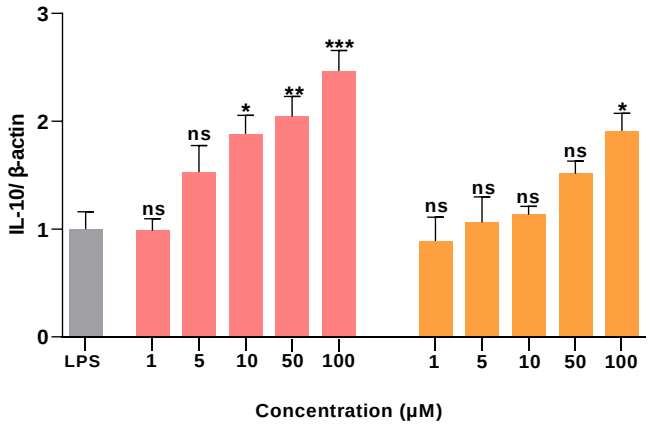


**Figure D.6** Effects of purified betanin and vulgaxanthin I (1, 5, 10, 50 and 100 μM) on IL-10 gene expression in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences to LPS-treated cells: *p < 0.05, ** p<0.01, and *** p<0.001.

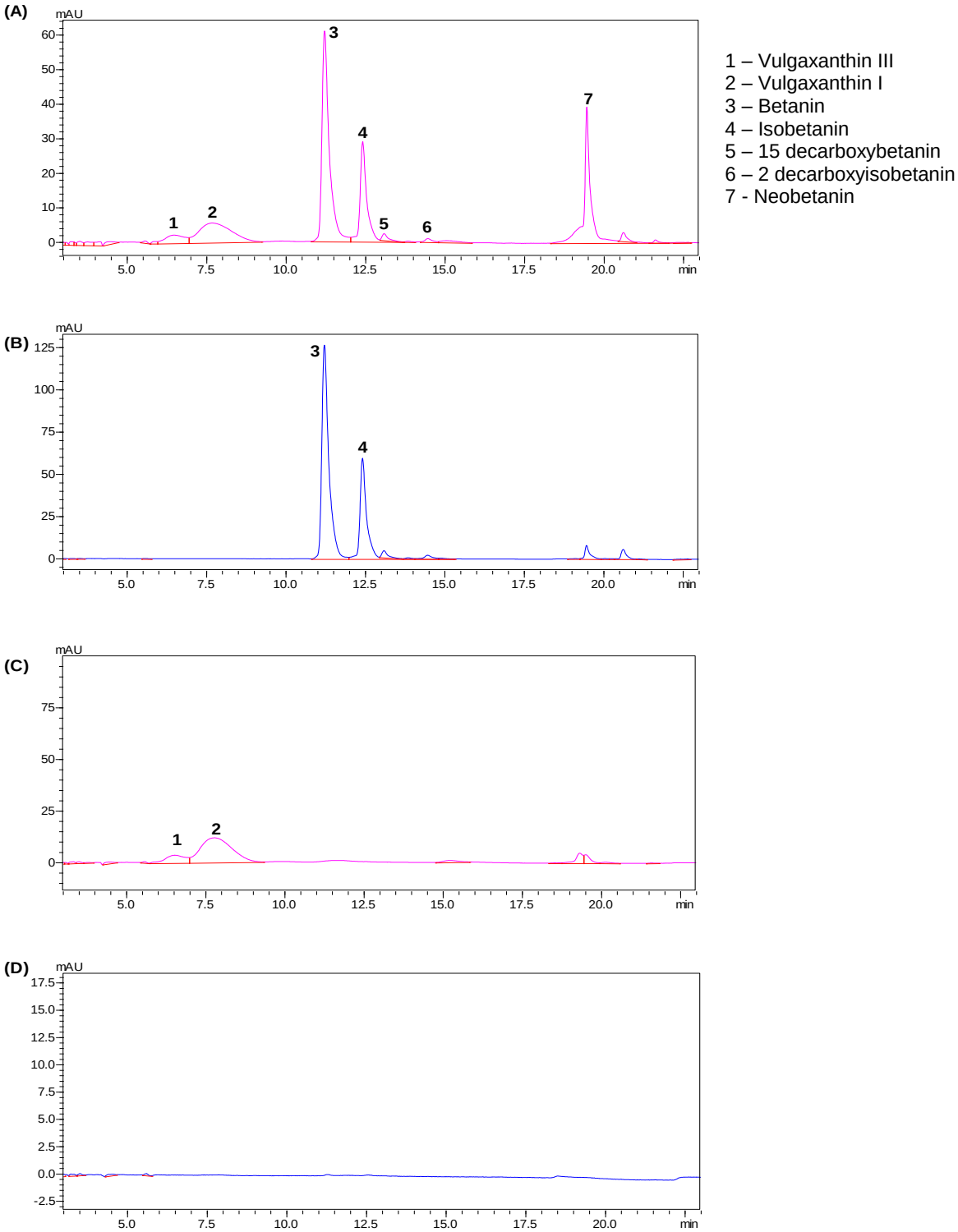


**Figure D.7** HPLC chromatograms of red beetroot (A, B) and yellow beet root (C, D) samples. Elution monitored at 486 nm (betaxanthins, A, C) and 536 nm (betcayanins, B, D). ESI-MS positive ion mode was used to identify the peaks.

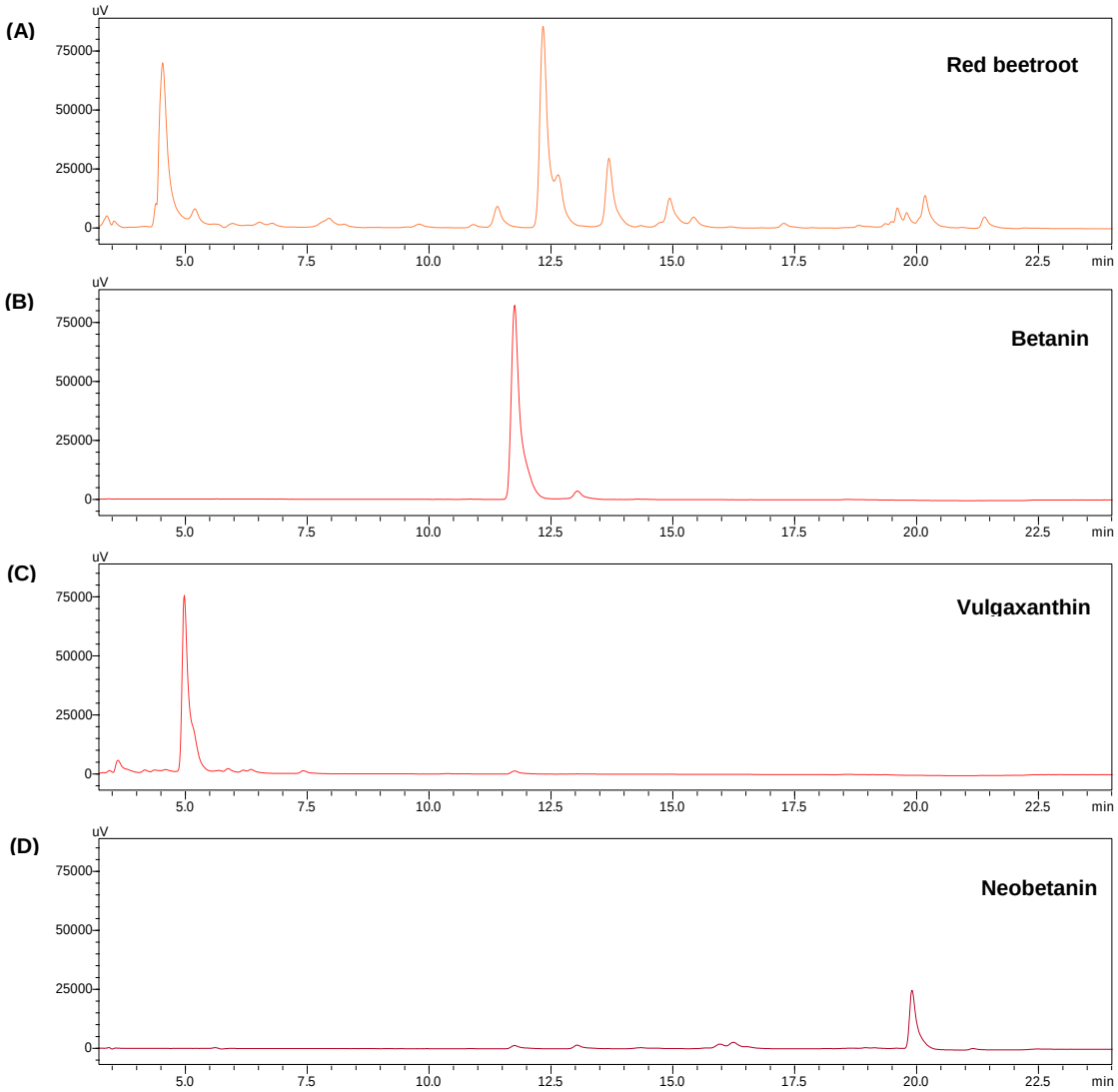


**Figure D.8** HPLC chromatograms of red beetroot extract (A), betanin (B), vulgaxanthin I (C) and neobetanin (D). Elution monitored at 486 nm (A, C and D) and 536 nm (B). ESI-MS positive ion mode was used to identify the peaks.

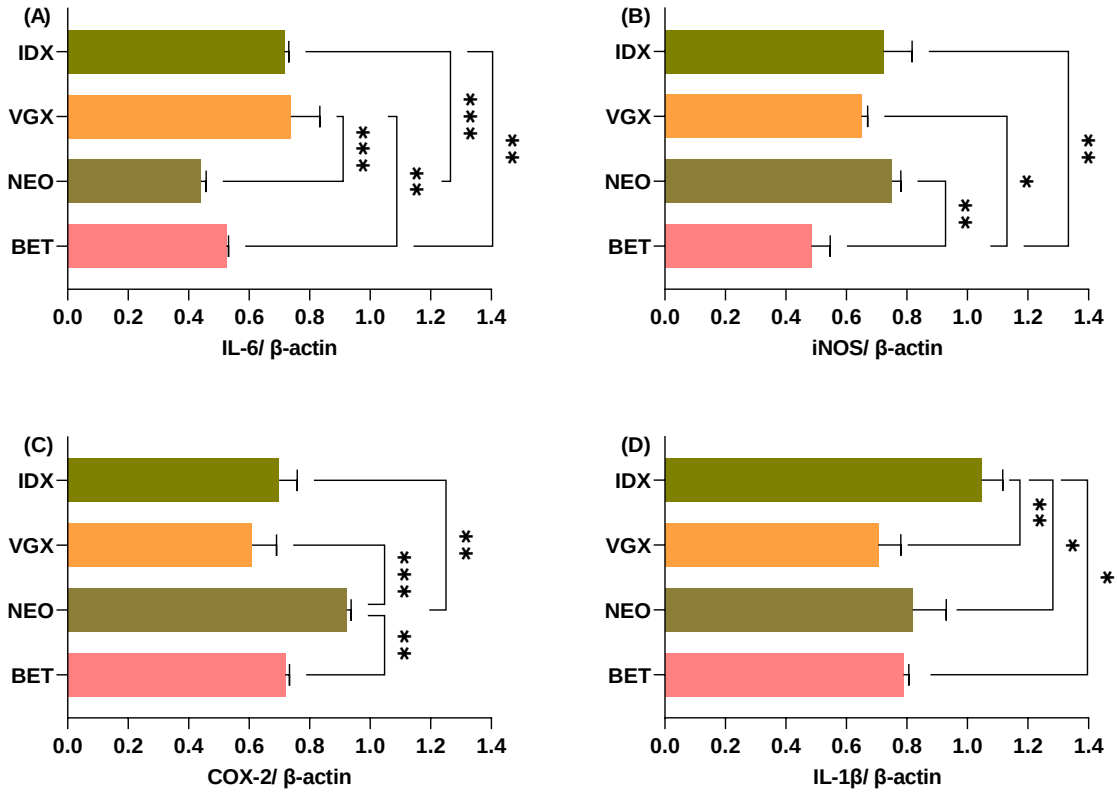


**Figure D.9** Effects of purified betalains on pro-inflammatory cytokine expression (A) IL-6, (B) iNOS, (C) COX-2, and (D) IL-1β at 10 μM concentration. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences: *p < 0.05, ** p<0.01, *** p<0.001**,** Tukey's multiple comparison test.

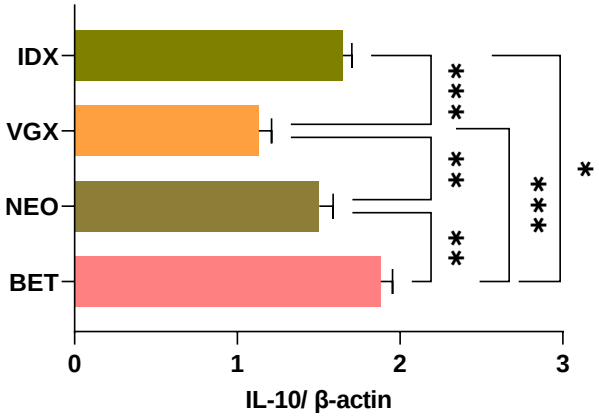


**Figure D.10** Effects of purified betalains (10 μM) on IL-10 gene expression in LPS-stimulated RAW 264.7 macrophages. The values are presented as mean ± SEM of three independent experiments. * indicate significant differences: *p < 0.05, ** p<0.01, *** p<0.001, Tukey's multiple comparison test.

# Appendix E

## Supporting information of Chapter 5

**Table E.1** HPLC-MS data (positive ionization mode) for identification of betalains present in different betalain sources.

| Compound | HPLC – DAD data | | LC-MS | RB | YB | YPP |
|---|---|---|---|---|---|---|
| | Retention time (min) | $\lambda_{max}$ | [M + H] | | | |
| Vulgaxanthin IV | 3.319 | 472 | 325 | - | - | + |
| Isoportulacaxanthin III | 3.502 | 463 | 269 | - | - | + |
| Vulgaxanthin I | 4.525 | 470 | 340 | + | + | + |
| Vulgaxanthin II | 6.452 | 484 | 341 | + | + | - |
| Isoindicaxanthin | 9.444 | 484 | 309 | + | - | - |
| Indicaxanthin | 9.712 | 484 | 309 | + | + | + |
| 17-Decarboxy-betanin | 11.379 | 500 | 507 | + | - | - |
| Betanin | 12.193 | 537 | 551 | + | + | + |
| 2-Decarboxy-betanin | 12.612 | 501 | 507 | + | - | - |
| Miraxanthin V | 12.615 | 484 | 347 | + | - | - |
| Isobetanin | 13.517 | 537 | 551 | + | - | - |
| 15-Decarboxy-betanin | 14.804 | 528 | 507 | + | - | + |
| 2-Decarboxy-isobetanin | 15.274 | 484 | 507 | + | - | - |
| 17-Decarboxy-neobetanin | 17.124 | 484 | 505 | + | - | - |
| 2,17-Bidecarboxy-neobetanin | 18.721 | 484 | 461 | + | - | - |
| Neobetanin | 19.969 | 484 | 549 | + | + | - |
| 2-Decarboxy-neobetanin | 21.164 | 484 | 505 | + | - | - |

RB – red beetroot, YB – yellow beetroot, YPP – yellow prickly pear

**Table E.2** HPLC-MS data (negative ionization mode) for identification of polyphenols present in different betalain sources.

| Compound | HPLC-DAD data | | LCMS [M - H]- | RB | YB | YPP |
|---|---|---|---|---|---|---|
| | Retention time (min) | $\lambda_{max}$ | | | | |
| *trans* cinnamic acid | 4.678 | | 147 | - | - | + |
| Cochliophilin A | 4.76 | 284 | 281 | + | + | - |
| p-coumaric acid | 5.165 | 280 | 163 | + | + | + |
| Unidentified | 5.499 | 275 | 147 | + | + | - |
| N-trans-feruloylmethoxytyramine | 5.643 | 270 | 342 | + | + | - |
| Unidentified | 5.779 | 267 | 342 | + | - | - |
| Epicatechin | 6.553 | 270 | 289 | + | + | - |
| Catechin | 7.252 | 270 | 289 | + | + | + |
| 4-O-caffeoylquinic acid | 7.488 | 272 | 353 | - | - | + |
| Ferulic acid | 7.893 | 292 | 193 | + | + | + |
| Caffeic acid | 8.564 | 267 | 179 | + | + | + |
| Quinic acid | 9.294 | 191 | 270 | - | - | + |
| Gallic acid | 9.807 | 282 | 169 | + | - | - |
| Unidentified | 9.867 | 278 | 304 | + | + | - |
| Unidentified | 12.461 | 273 | 272 | + | - | - |
| Unidentified | 13.874 | 278 | 327 | + | - | - |
| Unidentified | 15.161 | 279 | 349 | + | - | - |
| Syringetic acid | 16.768 | 278 | 197 | + | + | - |
| Quecertin | 19.564 | 361 | 301 | + | + | - |
| Unidentified | 20.308 | 273 | 325 | + | - | - |
| Betavulgarin | 34.160 | 279 | 311 | + | + | - |
| Rosmarinic acid | 34.900 | 265 | 359 | + | + | + |
| Chlorogenic acid | 35.501 | 281 | 353 | + | + | + |

RB – red beetroot, YB – yellow beetroot, YPP – yellow prickly pear