ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATING THE BIOAVAILABILITY AND BIOACTIVITY OF BLACK CARROT POLYPHENOLS IN AN IN-VITRO EPITHELIAL-ENDOTHELIAL CO-CULTURE MODEL

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SİYAH HAVUÇ POLİFENOLLERİNİN IN-VITRO EPİTELYAL-ENDOTELYAL HÜCRE KÜLTÜR MODELİ KULLANILARAK BİYOYARARLILIK VE BİYOAKTİVİTESİNİN İNCELENMESİ

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Ceren OZKAN, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 506131530, successfully defended the thesis entitled "INVESTIGATING THE BIOAVAILABILITY AND BIOACTIVITY OF BLACK CARROT POLYPHENOLS IN AN IN-VITRO EPITHELIAL-ENDOTHELIAL CO-CULTURE MODEL", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my beloved family,

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November 2017

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SUMMARY

It has attracted attention to consume polyphenol rich fruits and vegetables and utilise their byproducts recently. Black carrot is one of those foods that is rich in polyphenols especially in anthocyanins. These compounds have numerous health benefits to living organisms including protection to neurodegenerative diseases, cancer and cardiovascular diseases. In order to have these health benefits polphenols should be bioavailable to the organism. This study was performed to test bioavailability and bioactivity of black carrot anthocyanins. Besides raw black carrot samples, pomace and peel samples were also studied. In order to simulate absorption and bioactivity of polyphenols in black carrot, pomace and peel, in vitro cell models were used. Transport experiments were carried out with Caco-2 cells and co-culture experiments were designed with both Caco-2 and Eahy926 cell lines. Results were monitored with HPLC-DAD. In co-culture experiments nitric oxide assay (NO), intracellular reactive oxygen species assay (ROS) were used, as well as enzyme-linked immunosorbent assays (ELISA) to evaluate the anti-inflammatory effect of black carrot anthocyanins. Both in transport and co-culture experiments, digested samples gave better results than undigested samples. In bioavailability experiments digested samples were found to have a basal recovery between 1.3 – 5.3 % of initial anthocyanin concentration whereas undigested samples were found between 0.8 – 2.7 % of initial values. A similar result was seen in transport of phenolic acids. No cytotoxic effect was observed after 24 hours prior to treatment (TEER values were found between 74-93 % of initial values after 24 hours). The anti-inflammatory effect was determined in cells treated with black carrot, pomace and peel samples by using MCP-1, VEGF, ICAM-1 and IL-8 markers. In these

experiments, black carrot anthocyanins were found to have anti-inflammatory effect with decrease of these markers up to 92 %. This co-culture model was found to be applicable for bioavailability and bioactivity studies.

SİYAH HAVUÇ POLİFENOLLERİNİN IN-VITRO EPİTELYAL-ENDOTELYAL HÜCRE KÜLTÜR MODELİ KULLANILARAK BİYOYARARLILIK VE BİYOAKTİVİTESİNİN İNCELENMESİ

ÖZET

Polifenoller açısından zengin gıdalar ve bu gıdalardan elde edilen ürünler, sağlık açısından pek çok faydası bulunması sebebiyle tüketiciler tarafından ilgi görmektedir. Bu ilgi her geçen gün artmakta, bilimsel çalışmalar ile elde edilen veriler bu artışı desteklemektedir. Günlük diyette polifenollere yer vermenin organizmaya pek çok faydası bulunmaktadır. Nörodejeneratif hastalıklara, kansere ve kardiyovasküler hastalıklara karşı koruyucu rol oynaması, bağışıklık sistemini güçlendirmesi ve sindirim sistemini düzenlemesi gibi özellikler bu faydalardan birkaçıdır. Polifenol içeriği bakımından önemli bir yere sahip olan siyah havuç, özellikle antosiyaninler bakımından zengindir. Siyah havuç başta Türkiye, Orta Doğu ve Uzak Doğu olmak üzere pek çok coğrafyada binlerce yıldır yetiştirilmekte ve tüketilmektedir. Taze meyve olarak tüketilmesinin yanı sıra siyah havuç suyu, konsantresi ve siyah havuçtan üretilen fermente içecekler de sıkça tüketilmektedir. Fakat polifenollerin sağlığa yararlı olabilmesi için, tüketim miktarının yanında organizmadaki biyoyararlığı da önemlidir. Taze siyah havucun yanı sıra, havuç kabuğu ve posasının da karşılaştırıldığı bu çalışmada, siyah havuç antosiyaninlerinin biyoyararlılığı ve biyoaktivitesi araştırılmıştır. Araştırmalarda canlı organizmaya en yakın sonuçlara ulaşabilmek için *in-vitro* hücre modeli kullanılmıştır. Taze siyah havuç, kabuk ve posa örnekleri ön soğutmalı öğütücü ile toz haline getirilmiş ve kimyasal ekstraksiyon işlemi uygulanmıştır. Çalışmalarda hem sindirilmiş hem de sindirilmemiş örneklerine yer verilmiştir. Sindirim iki aşama şeklinde tasarlanmıştır. İlk aşamada örnekler 2 saat boyunca 10 mL mide solusyonuna maruz bırakılmış, ikinci aşamada ise ince bağırsak sindirimi, örneklerin 30 mL duodenal sıvı ve 15

mL safra sıvısı karışımı bir solusyonda 4 saat bekletilmesi ile simule edilmiştir. Organizmadaki emilimin incelendiği transport deneyleri Caco-2 hücreleri ile, çalışmanın ikinci kısmında biyoaktivitenin incelendiği deneyler ise Caco-2 – Eahy926 hücreleri ile birlikte tasarlanan hücre kültür modeli kullanılarak yürütülmüştür. Hücre kültürleri %70-80 konsantrasyona ulaşınca 1:3 – 1:5 oranlarında bölünmüş ve haftada 3 defa besiyerleri yenilenmiştir. Transport deneyleri 6 bölmeli transwell plateler kullanılarak yapılmıştır. Her bir bölmede yaklaşık $6{,}0x10^5$ hücre olacak şekilde ekilen Caco-2 hücreleri kültür besiyerinde 21 gün boyunca çoğaltılmıştır. Siyah havuç, kabuk ve posa örnekleri ön deneyler sırasında transport besiyeri olarak seçilen HBSS (Hank's Balanced Salt Solution) içerisinde çözdürülerek transport düzeneğine eklenmiştir. Caco-2 ve EA.hy926 hücreleriyle tasarlanan kültür modelinde çalışmanın ikinci kısmı olan biyoaktivite incelenmiştir. Bu aşamada 12 bölmelik transwell plateler kullanılmış ve hücre kültür modelinin 4. Gününde Caco-2 hücreleri siyah havuç ve ürünlerinden hazırlanan örneklere maruz bırakılmıştır. Deney öncesinde bazal kısma, kardiyovasküler hastalıklardan kaynaklanan enflamasyonun oluşturulmak üzere 1 ng mL^{-1} TNF-α eklenmiş ve 3 saat boyunca beklenmiştir. 4 saat süreyle 37°C'de inkübe edilen hücrelerin apical ve bazal kısımları toplanmış ve analiz edilmek üzere -80 ° C'de saklanmıştır. Deney sonuçları HPLC-DAD kullanılarak görüntülenmiştir. HPLC analizi, çalışma öncesinde örneklerden çıkarılan fenolik bileşik profili kullanılarak yapılmıştır. Caco-2 – Eahy926 hücre kültür modeli ile yapılan biyoaktivite çalışmalarında nitrik oksit (NO) ve hücre içi reaktif oksijen türleri (ROS) ve sitotoksisite analizleri yapılmıştır. Bunun yanında ELISA (enzyme-linked immunosorbent assays) testi ile siyah havuç antosiyaninlerinin antienflamatuar etkisi incelenmiştir. Yapılan tüm çalışmalar tek yönlü ANOVA ile istatistiksel olarak değerlendirilmiştir. HPLC analizlerine göre antosiyanin profilinde en yüksek oranda bulunan 5 antosiyanin başta cyanidin-3-xylosylferuloyl-glucosylgalactoside olmak üzere; cyanidin-3-xylosylglucosyl-galactoside, cyanidin-3-xylosylgalactoside, cyanidin-3-xylosylsinapoyl-glucosyl-alactoside, cyanidin-3-xylosylcoumaroyl-glucosylgalactoside şeklinde belirlenmiştir. En yüksek oranda bulunan fenolik asitlerin ise başta Klorojenik asit olmka üzere, Neoklorojenik asit ve Kafeik asit olduğu tespit edilmiştir. Edinilen sonuçlara göre, çalışmanın her iki kısmında da, sindirime uğramış

örnekler sindirilmemiş örneklere göre daha iyi sonuçlar vermiştir. Transport çalışmalarında, sindirilmiş örneklerle yapılan deneylerde bazal kısımda bulunan antosiyanin konsantrasyonu başlangıç konsantrasyonunun % 1.3 – 5.3 arasında değişirken, sindirilmemiş örneklerde bu oran % 0.8 – 2.7 aralığında bulunmuştur. Benzer şekilde fenolik asitler de transport deneylerinde, sindirilmiş örneklerle çalışılan düzenekte bazal kısımda başlangıç konsantrasyonunun % 4 - 7 aralığında bulunurken, sindirilmemiş örneklerde bazal kısımda bu oran % 1.6 - 3.3 aralığında bulunmuştur. Uygulamanın 24 saat sonrasında yapılan hücre içi toksisite deneylerinde, TEER değerleri başlangıç değerlerinin % 74 – 93 aralığında bulunmuş, örneklerin uygulandığı hücrelerde herhangi bir toksik etki yaratmadığı gözlenmiştir. Elde edilen bir başka bulgu, transport deneylerinde açillenmemiş antosiyaninlerin transport membranından geçme oranının açillenmiş antosiyaninlere göre daha yüksek olmasıdır. Yapılan hücre içi reaktif oksijen türleri analizinde sonuçların TNF-α varlığı ile değiştiği gözlemlenmiştir. Enflamasyon koşulları altında değilken sindirilmiş siyah havuç posası örneğinin ROS seviyesini önemli derecede artırdığı tespit edilmiştir. Fakat TNF-α uygulanmış deney düzeneğinde, sindirilmiş siyah havuç posası örneğinin inkübasyon sonrası ROS seviyesinin %20 oranında azaldığı görülmüştür. Sonuçlar değerlendirildiğinde sindirilmiş ve sindirilmemiş siyah havuç ve sindirilmiş posa örneklerinin TNF-α uygulanmış endotelyal hücrelerde oksidatif stresi azaltabildiğine ulaşılmıştır. Endotelyal hücreler tarafından sentezlenen nitrik oksit seviyesinin belirlenmesi için yapılan analizde (NO) sindirilmiş ve sindirilmemiş siyah havuç, kabuk ve posasının, nitrik oksit üretimini, enflamasyon varlığında ve yokluğunda artırdığı belirlenmiştir. Siyah havuç, kabuk ve posa örnekleri uygulanmış deney düzeneğinde, MCP-1 (monocyte chemoattractant protein-1), VEGF (vascular endothelial growth factor), IL-8 (interleukin-8) ve ICAM-1 (intercellular adhesion molecule-1) belirteçleri kullanılarak, siyah havuç antosiyaninlerinin antienflamatuar etkisi incelenmiştir. Yapılan deneylerde TNF-α uygulanmış örneklerde belirteçler enflamasyon bulunmayan örneklere göre çok daha yüksek bulunmuştur. Sağlıklı koşullar altında IL-8 konsantrasyonunun, sindirilmiş siyah havuç örneğinin uygulandığı hücrelerde azaldığı görülmüştür. Aynı zamanda sindirilmemiş siyah havuç ve posa örneklerinde de yüksek miktarda azalma tespit edilmiştir. TNF-α

aktivitesinin bulunduğu durumda IL-8, TNF-α bulunmayan hücrelere kıyasla, sindirilmiş ve sindirilmemiş kabuk ve posa örnekleri tarafından azaltılmıştır. Enflamasyon bulunmadığı durumda, VEGF salgısı hem sindirilmiş hem de sindirilmemiş siyah havuç, kabuk ve posa örnekleri tarafından azaltılmıştır. Özellikle sindirilmemiş posa ve kabuk bu belirteçte kayda değer bir fark göstermiştir. TNF-α' ya maruz bırakılmış hücrelerde VEGF salgısındaki azalma sindirilmiş ve sindirilmemiş örneklerin her ikisi için de çok daha yüksektir. MCP-1 salınımının TNF-α varlığında önemli düzeyde arttığı görülmüştür. Fakat tüm örnekler MCP-1 seviyesinin azaltılması yönünde etki etmiştir. ICAM-1 belirteci de hem sindirilmiş hem sindirilmemiş örnekler ile azalma yönünde sonuçlar vermiştir. Deneyler sonucunda siyah havuç antosiyaninlerinin enflamasyonu önleyici rol oynadığı, belirteçlerin %92 oranına varan bir azalma gösterdiği tespit edilmiştir. Çalışmada kullanılmak üzere tasarlanan Caco-2 – Eahy926 hücre kültür modeli, yapılan deneylerde literatürdeki çalışmalarla paralel sonuçlar vermiş, biyoyararlılık ve biyoaktivite çalışmalarında kullanılmaya uygun bulunmuştur.

1. INTRODUCTION

Fruits and vegetables started to be investigated many years ago. They have gained attention due to their bioactive compounds, vitamins and minerals, which can result in health benefits. It is proven that consumption of fruit and vegetables can prevent cardiovascular diseases or cancer (Capanoglu et al, 2012).

Carrot or *Daucus carota L.* is one of the most widely consumed vegetables because of its flavor and health benefits (Mandel and McCauley, 2015). This vegetable originates from Turkey and the Middle and Far East, where it has been known for at least 3000 years (Montilla et al, 2011; Schwarz et al, 2004) and are still traditionally grown and consumed in Turkey, Afghanistan, Egypt, Pakistan, India and the Far East (Kammerer et al, 2004b). Mostly black carrot is processed into juice, concentrate and traditional lactic acid fermented beverages as 'Shalgam' and "Kanji" or consumed as fresh (Turkyilmaz et al, 2012; Turker and Erdogdu, 2006).

Anthocyanins are a major group of water-soluble plant pigments that are responsible for red, purple and blue color of many fruits, vegetables and cereal grains. They naturally occur as glycosides form of anthocyanidins and may be acylated with aliphatic or aromatic acids (Turker and Erdogdu, 2006). The main natural sources of anthocyanin-based colorants which contain acylated anthocyanins are reported as red radishes, red potatoes,red cabbages and black carrots and particularly berries as strawberry, blackberry, blueberry, raspberry and also red grape, red wine and other blue, red or purple fruits (Netzel et al, 2007; Kamiloglu et al, 2015b). Anthocyanins have been reported to be protective against cardiovascular disease, and claimed to have antioxidant, anticancer, antidiabetic, antimutagenic, and antiinflammatory properties (Netzel et al, 2007; Capanoglu et al, 2012).

In order to show health benefits, these compounds should be bioavailable. Transport of anthocyanins through the gut epithelium is one of the most important phases where bioavailability is evaluated. This factor can be investigated using *in vivo* studies and also *in vitro* models (Kamiloglu et al, 2015a). Polyphenols are not so active compounds within the human body,

because they have a low intrinsic activity and also they are barely absorbed from the intestine. Better understanding about the role of anthocyanins in human body can be possible by evaluating their biochemical reactions. For simulating the gastrointestinal system in human, *in vitro* digestion methods are being used. In addition to being rapid and safe, these methods do not have the same ethical restrictions as *in vivo* methods do. Although they are only static models of digestion, *in vitro* methods have been reported as having good correlation with results from human and animal studies (Kamiloğlu et al, 2015b). As an *in vitro* model, transepithelial transport assays are prevalently used to simulate biological barriers in human body by using cell culture lines such as Caco-2 cells which is used as an intestinal cell wall model (Fernandes et al, 2015).

The impact of polyphenols on the communication mechanisms between intestine and endothelium is currently unexplored. In general, there is an increasing interest in the co-cultivation of different cell lines to study cross-talk mechanisms between different tissues as an alternative for animal research. Co-cultivation models with intestinal and endothelial cell lines are rare (Kunzt et al, 2015a, Olejnik et al, 2016) and therefore the development of combined in vitro models may enhance the knowledge about this communication. In this study, human colon cancer cell lines (Caco-2) and human endothelial cell lines (EAhy926) were used as an *in-vitro* co-culture model to investigate bioavailability and bioactivity of anthocyanins in digested and undigested black carrot, pomace and peel samples.

2. LITERATURE REVIEW

2.1. Black Carrot Polyphenols

Carrot or *Daucus carota L.* is part of *Apiaceae* family and one of the most widely consumed vegetables because of its flavor and health benefits (Mandel and McCauley, 2015). This two years old vegetable *c*ultivars are subdivided into the eastern or anthocyanin group (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) and the western or carotene group (*Daucus carota* ssp. *sativus* var. *sativus*) (Kammerer et al, 2004a). Black or purple carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) originate from Turkey and the Middle and Far East, where it has been known for at least 3000 years (Montilla et al, 2011; Schwarz et al, 2004) and are still traditionally grown and consumed in Turkey, Afghanistan, Egypt, Pakistan, India and the Far East (Kammerer et al, 2004b). Ereğli district in Konya, Turkey has the major production of black carrot, followed by Adana. Mostly black carrot is processed into juice, concentrate and traditional lactic acid fermented beverages as 'Shalgam' and "Kanji" or consumed as fresh (Turkyilmaz et al, 2012; Turker and Erdogdu, 2006). Because of the processing, a huge amount of byproducts as pomace and peel are produced. It has been reported that in 2009 and 2010, 2700 and 6900 tons of pomace from black carrot was produced in Turkey, respectively. Most of the fruits and vegetables rich in anthocyanins, have also byproducts which is used as source of anthocyanin (Kamiloglu et al, 2015a).

Black carrot was not cultivated in Europe before the 12th century and is considered to be the origin model of all modern orange carrots, which were produced by Dutch growers around 1750 (Schwarz et al, 2004). Nowadays, although orange carrots are commonly consumed, interest to black carrots is also increasing (Kamiloglu et al, 2015b).

Black carrots have high nutritional content that is shown in Table 2.1. They are rich in sugars minerals fiber, protein and polyphenols. On the contrary, they contain low level of fat . It has been reported that black carrot consists of dry matter in the range of 142.3-159.6 g/kg. (Tatoglu, 2014).

Table 2.1 : Nutritional content of black carrot.

Nutrients	**Amount (100 g^{-1})**
Sugars	
glucose	1.10-5.60 g
fructose	0.14-4.36 g
saccharose	1.20-3.31 g
Minerals	
calcium	33 mg
magnesium	17 mg
potassium	256 mg
phosphorus	29 mg
iron	0.26 mg
zinc	0.15 mg
Fibers	2.48 g
Fat	0.14 g
Protein	0.7-1.38 g

Black carrot has an appealing bluish-purple color which attracts interest as a natural food colorant (Netzel et al, 2007). However, mostly they have low stability to heat, light and increased pH values. Due to increasing demand for natural food, pigments have started to be replaced by natural colorings originating from plants. Today many varieties of black carrot are commonly used in confectionery, jams, jellies, preserves, frozen desserts, soft drinks, juices, candies or other fermented productions as a healthier alternative to synthetic colorants like FD and C Red 40 because of their high pigment content (Schwarz et al, 2004; Kammerer et al, 2004a; Ersus and Yurdagel, 2007). For black carrot to be used as a food colorant no declaration is required with an E-number on labels. Furthermore, coloring foods with black carrot juice may provide health benefits (Khandare et al, 2011).

Anthocyanins (Greek ‘anthos’ = flower and ‘kyaneos’ = blue) are a major group of water-soluble plant pigments that are responsible for red, purple and blue color of many fruits, vegetables and cereal grains. Anthocyanins are part of the flavanoids class and contain derivatives of glycosylated salts (anthocyanidins) of polyhydroxy and/or polymethoxylated 2- phenylbenzopyrolium (flavylium). They naturally occur as a glycoside form of anthocyanidins and may be acylated with aliphatic or aromatic acids (Turker and Erdogdu, 2006). The main natural sources of anthocyanin-based colorants which contain acylated anthocyanins are

reported as red radishes (Giusti and Wrolstad, 1996), red potatoes (Rodriguez-Saona et al, 1999), red cabbages (Dyrby et al, 2001) and black carrots (Stintzing et al, 2002) and berries such as strawberry, blackberry, blueberry, raspberry and also red grape, red wine and other blue, red or purple fruits (Kamiloglu et al, 2015b). Anthocyanin content of some foods are shown in Table 2.2.

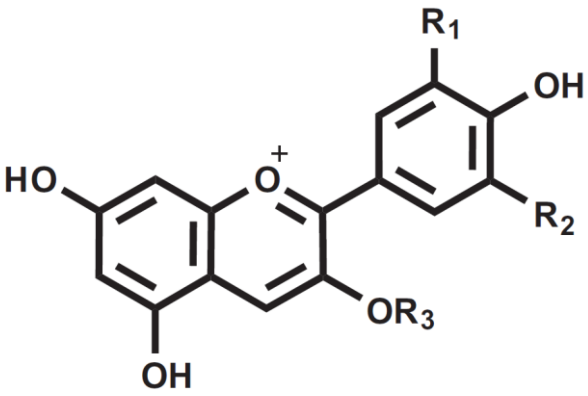
Table 2.2 : Anthocyanin content of some foods (Clifford, 2000).

Commodity	**Content (mg/liter or mg/kg)**
Blackberry	1150-6600
Blueberry	825-4200
Boysenberry	1609
Cherry	20-4500
Chokeberry	5060-10000
Cranberry	600-2000
Cowberry	1000
Currant (black)	1300-4000
Elderberry	2000-10000
Grape (red)	300-7500
Loganberry	774
Orange, Blood (juice)	2000
Plum	20-250
Raspberry (black)	1700-4277
Raspberry (red)	100-600
Raspberry (red) single strength juice	4-1101
Sloe	1600
Strawberry	150-350
Cabbage (red)	250
Eggplant	7500
Onion	250
Rhubarb	2000
Wines (red)	240-350
Wines (Port)	140-1100

In a research which was published 40 years ago, the daily intake of anthocyanins was reported to be 180 mg/day during winter and 215 mg/day during summer in the United States (Kuhnau, 1976). Recently researches have showed that this value is much lower because of variations in dietary sources. In 2006, the intake of anthocyanins has been reported approximately 82 mg/day in Finland and 12.5 mg/day in the United States (Wu et al, 2002). It has been reported that the intake estimates of approximately 10 mg anthocyanidins/day for adults do not meet the recommended intakes of fruits and vegetables, which is 34.4–36.0 mg/day (Wallace and Giusti, 2014).

Anthocyanins have been reported to have a role in a number of biological pathways linked with protection against cardiovascular disease, metabolic syndrome, vision problems, and neurodegenerative diseases. In addition, pharmacological and health promoting properties such as antioxidant, anticancer, antidiabetic, antimutagenic, antiinflammatory and antiaging properties and therapeutic purposes of anthocyanins have been reported (Espin et al, 2000; Kong, 2003; Bagchi et al, 2004; Einbond et al, 2004; Lila, 2004; Netzel, 2007; Zafra-Stone et al, 2007; Patras et al, 2010; Ziberna et al, 2012). Many authors also indicate advantageous biological properties of anthocyanins, such as inhibition of DNA damage in tumor cells, inhibition of digestive enzymes, prevention of lipid oxidation, inhibition of thrombocyte aggregation, etc. (Withrowa-Rajchert et al, 2009). Also anthocyanins, anthocyanidins, and anthocyanin-rich extracts from different vegetables or fruits have been shown to prevent the growth of human cancer cells from leukemia, breast, prostate, uterine, lung, vulva, stomach, melanoma, and colon cancer (Reddivari et al, 2007; Hui et al, 2010). In animal studies it has been shown that anthocyanins prevent tumors of the breast, skin, stomach, esophagus, and colon (Kocic et al, 2011).

The most widespread anthocyanins known in fruits and vegetables are derivatives of six anthocyanidins, which are pelargonidin (Pg3glc), cyanidin (Cy3glc), peonidin (Pn3glc), delphinidin (Dp3glc), petunidin (Pt3glc) and malvidin (Mv3glc) (Figure 2.1) However, it has been reported that 539 anthocyanins have been isolated from plants so far (Fernandes et al, 2014; Kamiloglu et al, 2015b).



Anthocyanins	R1	R2
Pg3glc	H	H
Pn3glc	OCH3	H
Cy3glc	OH	H
Mv3glc	OCH3	OCH3
Pt3glc	OCH3	OH
Dp3glc	OH	OH

Figure 2.1 : Representation of the general structure of anthocyanins (flavylium form). R_3 is a sugar moiety.

The sugar moieties can be different but are usually glucose, rhamnose, galactose or arabinose. It also may be a mono or disaccharide, and acylated with a phenolic or aliphatic acid. The difference of these compounds can be seen in the aromatic B ring (Fernandes et al,2014).

Due to their highly reactive nature, anthocyanins are sensitive to degradation reactions. Oxygen, temperature, light, various enzymes and pH are the factors which may affect their stability during processing or storage. Due to pH difference in human body, they may occur in different molecule forms as flavylium cation, carbinol base, chalcone, quinonoidal base and anionic quinonoidal base. This has been shown with colors in Figure 2.2 (Fernandes et al, 2014).

The vegetables rich in anthocyanins are the families Brassicaceae (red cabbage, radish), Apiaceae (purple-black carrot), Solanaceae (purple potato), and Convolvulaceae (purple sweet potato). Anthocyanins based on cyanidin are

characteristically found in red cabbage and black carrot, while pelargonidin, peonidin-, and petunidin-glycosides are extensively found in radish, purple potato, and sweet potato, respectively (Shamina et al, 2007).

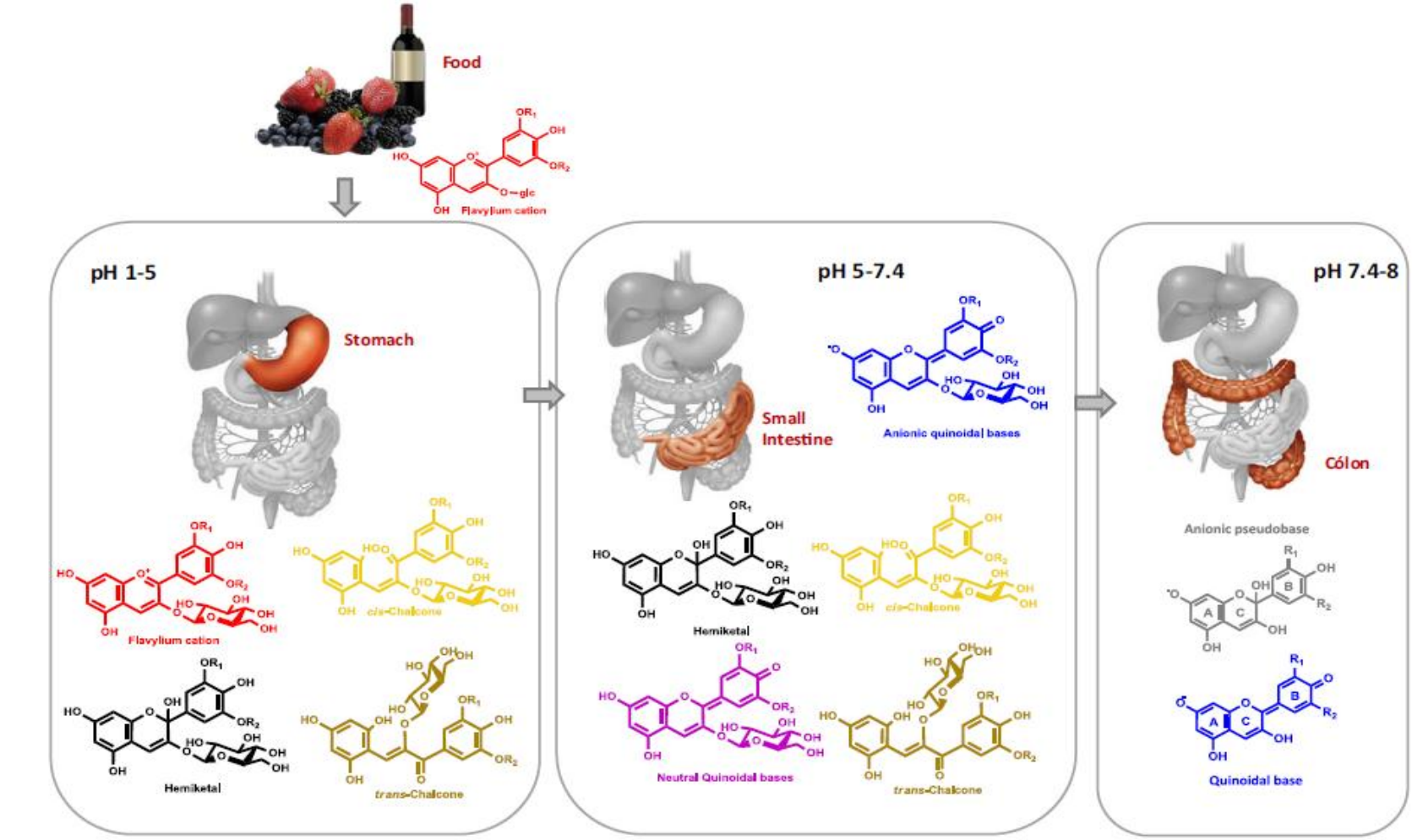


Figure 2.2 : Schematic representation of the form of anthocyanin equilibrium according to the GI tract pH (Fernandes et al, 2015).

In addition to extraordinary physical properties such as attractive color, black carrot has also an outstanding nutritional composition. It has been reported that black carrot has the highest value of the total amount of phenolics, compared to other carrot varieties. The total amount of phenolics in black (purple) carrot was 74.64 mg/100 g, whereas the corresponding values in white, yellow, and orange varieties ranged from 7.72 to 16.21 mg/100 g. Furthermore black (purple) carrot has been found to contain 2.2 and 2.3 times more α- and β-carotenes than orange carrot (Table 2.3) (Alasalvar et al, 2001). It has also attracted attention because of the level of anthocyanins. The amount of anthocyanins in black carrots (*D. carrota*) have been reported to be 4–1799 mg/100 g dry matter, while the amounts in plums (*Prunus domestica*) and apples (*Malus sylvestris*) are 5–449, and 1–117 mg/100 g fresh weight, respectively (Wallace and Giusti, 2014).

Table 2.3 : Content of sugars, vitamin C, and *α*- and *β*-carotenes in different raw carrot varieties[a] (Alasalvar et al, 2001).

	Sugars (g/100 g)				**Relative**	**Vitamin C**	***α*-carotene**	***β*-carotene** (μg/100
Color	fructose	glucose	sucrose	total	Sweetness[b]	(mg/100 g)	(μg/100 g)	g)
orange	1.34 ± 0.04[c]	1.44 ± 0.06[c]	2.69 ± 0.13[c]	5.47 ± 0.10[c]	6.07 ± 0.09[d]	5.33 ± 0.36[c]	3990 ± 912c	6935 ± 208c
purple	0.58 ± 0.03[d]	0.69 ± 0.09[d]	4.11 ± 0.23[d]	5.38 ± 0.29[c]	5.62 ± 0.26[c]	nm[g]	8725 ± 811d	16130 ± 593d
yellow	1.31 ± 0.19[c]	1.77 ± 0.21[c]	1.96 ± 0.11[ef]	5.04 ± 0.49[c]	5.54 ± 0.57[cd]	1.98 ± 0.06[d]	tr[h]	Tr
white	1.47 ± 0.14[c]	1.59 ± 0.19[c]	2.33 ± 0.31[cf]	5.39 ± 0.47[c]	6.05 ± 0.50[cd]	1.25 ± 0.09[e]	nd[i]	Nd

[a]Data are expressed as mean ± SD of three determinations on a fresh weight basis. [b]Relative sweetness was calculated relative to sucrose (fructose, 1.73; glucose, 0.74; and sucrose, 1.00). [c-f] Means (SD followed by the same letter, within a column, are not significantly different ($p > 0.05$). [g]nm, not measured (due to pink color). [h]tr, trace. [i]nd, not detected.

Since black carrot has extraordinary quality parameters, the anthocyanin profile has been studied in detail and it was found that black carrot mainly contains cyanidin-based pigments (Gläßgen et al, 1992; Mazza and Miniati, 1993; Baker et al, 1994; Dougal et al, 1998; Kammerer et al, 2004a; Turker et al, 2004; Cevallos-Casals and Cisneros-Zevallos, 2004; Kirca et al, 2006). In addition to

cyanidin glycosides, trace amounts of pelargonidin and peonidin glycosides have been reported (Elham et al, 2006).

Based on literature reports, as major compounds the structure of the two nonacylated anthocyanins cyanidin 3-xylosyl(glucosyl)galactoside and cyanidin 3-xylosylgalactoside, as well as the three monoacylated anthocyanins cyanidin 3-xylosyl(sinapoylglucosyl)galactoside, cyanidin 3-xylosyl(feruloylglucosyl)galactoside and cyanidin 3-xylosyl(coumaroylglucosyl)galactoside were characterized (Figure 2.3) (Kammerer et al, 2004a; Montilla et al, 2011; Schwarz et al, 2004; Stintzing et al, 2002). Also molecular structures of these major compounds in black carrot juice are shown in Figure 2.4 (Schwarz et al, 2004).

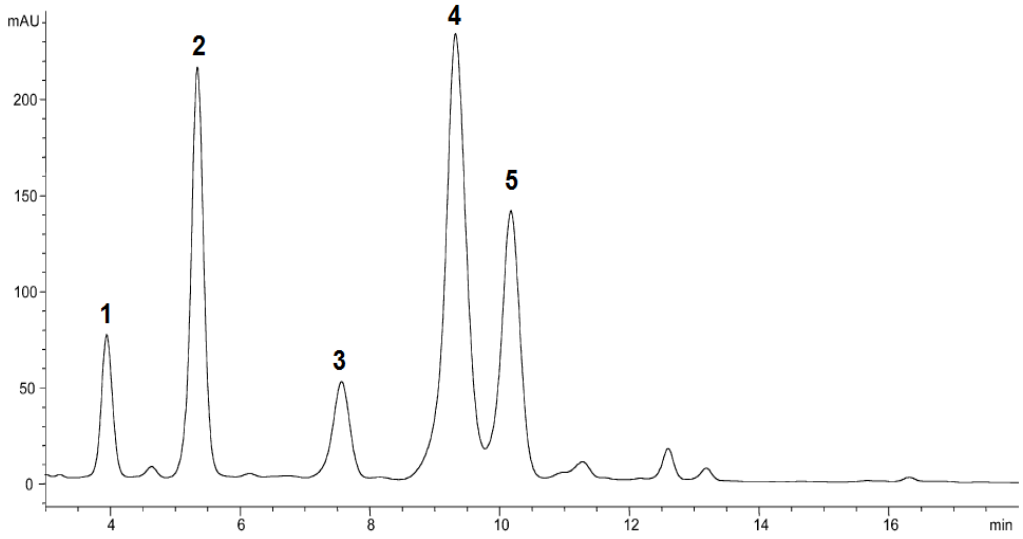


Figure 2.3 : HPLC separation of anthocyanins from a carrot root extract (520 nm). Peak assignment: (1) cyanidin 3-xylosylglucosylgalactoside (cya 3-xylglcgal), (2) cya 3-xylgal, (3) sinapic acid derivative of cya 3-xylglcgal, (4) ferulic acid derivative of cya 3-xylglcgal, (5) p-coumaric acid derivative of cya 3-xylglcgal (Kammerer et al, 2004a).

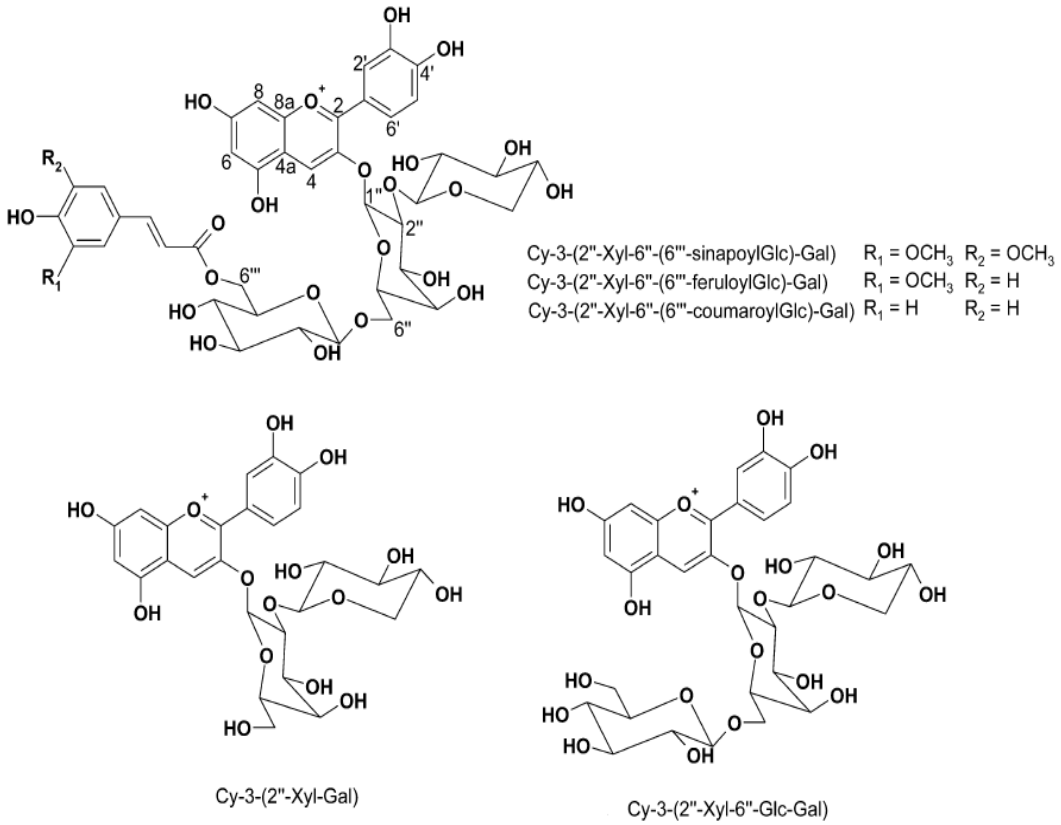


Figure 2.4 : Structures of main anthocyanins in black carrot juice (Cy: cyanidin; Xyl: *β*-D-xylopyranose; Gal: *β*-D-galactopyranose; Glc: *β*-Dglucopyranose) (Schwarz et al, 2004).

Reports in literature indicate that black carrot has a high anthocyanin content, up to 1750 mg/kg fresh weight (Mazza and Miniati, 1993). Kammerer *et al.* (2004a) have found 45.4 mg/kg to 17.4 g/kg dry matter and the feruloyl derivative of cyanidin xylosylglucosylgalactoside as the predominant pigment comprising 43–84% of the total anthocyanin contents in black carrot. In another study, 41% of anthocyanins have been found to be acylated as cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside (27.5%) and cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside (13.5%) (Stintzing et al, 2002). After determination of all phenolic compounds, Schwarz *et al.* (2004) reported that chlorogenic acid (243.3mg/L) was the major phenolic compound. Caffeic acid was found as 30.3 mg/L whereas ferulic acid was 20.5 mg/L and only a small amount of coumaric acid was found (2.3 mg/L) (Figure 2.5).

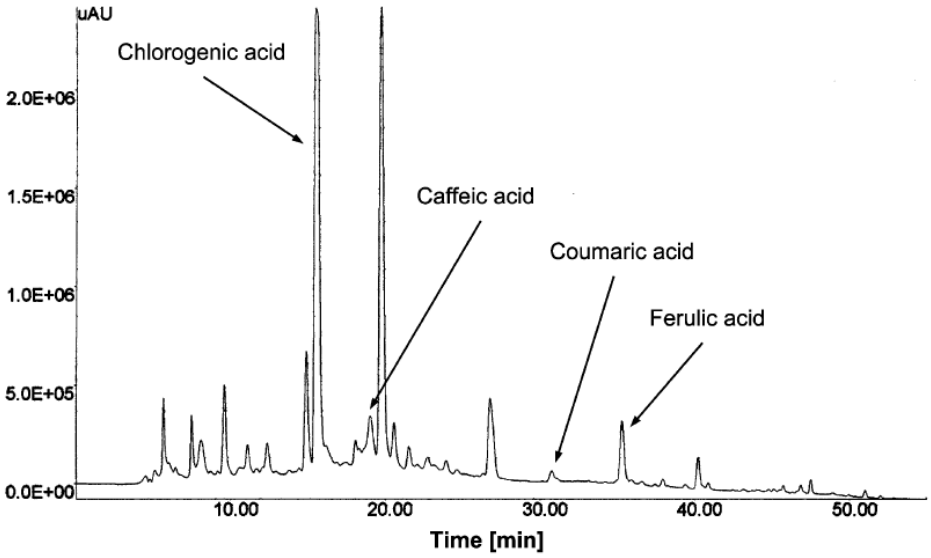


Figure 2.5 : HPLC chromatograms of black carrot cinnamic acid derivatives at 323 nm. (Schwarz et al, 2004).

2.2. Methods Used to Assess the Bioavailability of Polyphenols

Bioavailability is defined by the Food and Drug Administration (FDA) as 'rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.' There are also other definitions of terms related to the absorption of xenobiotics based on literature (Fang, 2014):

Bioavailability: The extent to which the xenobiotic can be used by the body.

Systemic availability: The proportion of the dose of a xenobiotic that reaches the systemic circulation intact after oral administration

Apparent systemic availability: For xenobiotics that undergo extensive first-pass metabolism, apparent bioavailability is the proportion of the dose that reaches the systemic circulation intact after oral administration.

Total systemic availability: The proportion of the dose of a xenobiotic that is absorbed through the gastrointestinal wall and enters the systemic circulation both in its original form and as metabolite(s) produced by first-pass metabolism.

Disposition: The process of getting a xenobiotic or its active metabolite(s) to their site(s) of action(s) in the body in appropriate concentration(s).

With these definitions, bioavailability and disposition are suitable terms to express the processes which involve polyphenols in the human body.

Bioavailability can also be defined as the fraction of a nutrient or non-nutrient that is available for the human body for physiological functions and/or storage. For polyphenols, this principally involves the following digestive processes: 1) release of polyphenols from the food matrix; 2) changes in polyphenols during gastric/ small-intestinal digestion; 3) cellular uptake of aglycons and some conjugated polyphenols by enterocytes; 4) microbiological fermentation of nonabsorbed polyphenols or those re-excreted via bile or the pancreas to yield additional metabolites, 5) phase I/II enzyme modifications that occur upon uptake (in the small intestine/ colon); 6) transport in the bloodstream and subsequent tissue redistribution; and 7) excretion via the kidney or re-excretion into the gut via bile and pancreatic juices (Bohn, 2014).

Since anthocyanins are highly reactive molecules that become different molecule forms in aqueous solution changing with the pH, they are the least bioavailable ones in flavanoid class. This may be critical at the point of determination of anthocyanin stability and occurrence in biological matrices such as plasma, urine, faeces, tissues or organs. Sample preparation is very important to quantify the exact amount of anthocyanins. In view of these challenges, they are very difficult to track during in vivo experiments and also to evaluate their bioavailability (Fernandes et al, 2015).

Transport of anthocyanins through the gut epithelium is one of the most important phases when bioavailability is evaluated. This factor can be investigated using *in vivo* studies and also *in vitro* models (Kamiloglu et al, 2015a). In numerous studies, it has been tried to achieve this goal including *in vivo* (human trials and animal studies) and *in vitro* (cell-based assays) studies (Figure 2.6). Human and animal studies are highly complicated and also have some disadvantages such as chemical instability and inadequate analytical methodology which are easy to control in an *in vitro* study. Contrary to this, an

in vitro method can be simple, more convenient, and less expensive (Yi et al, 2006).

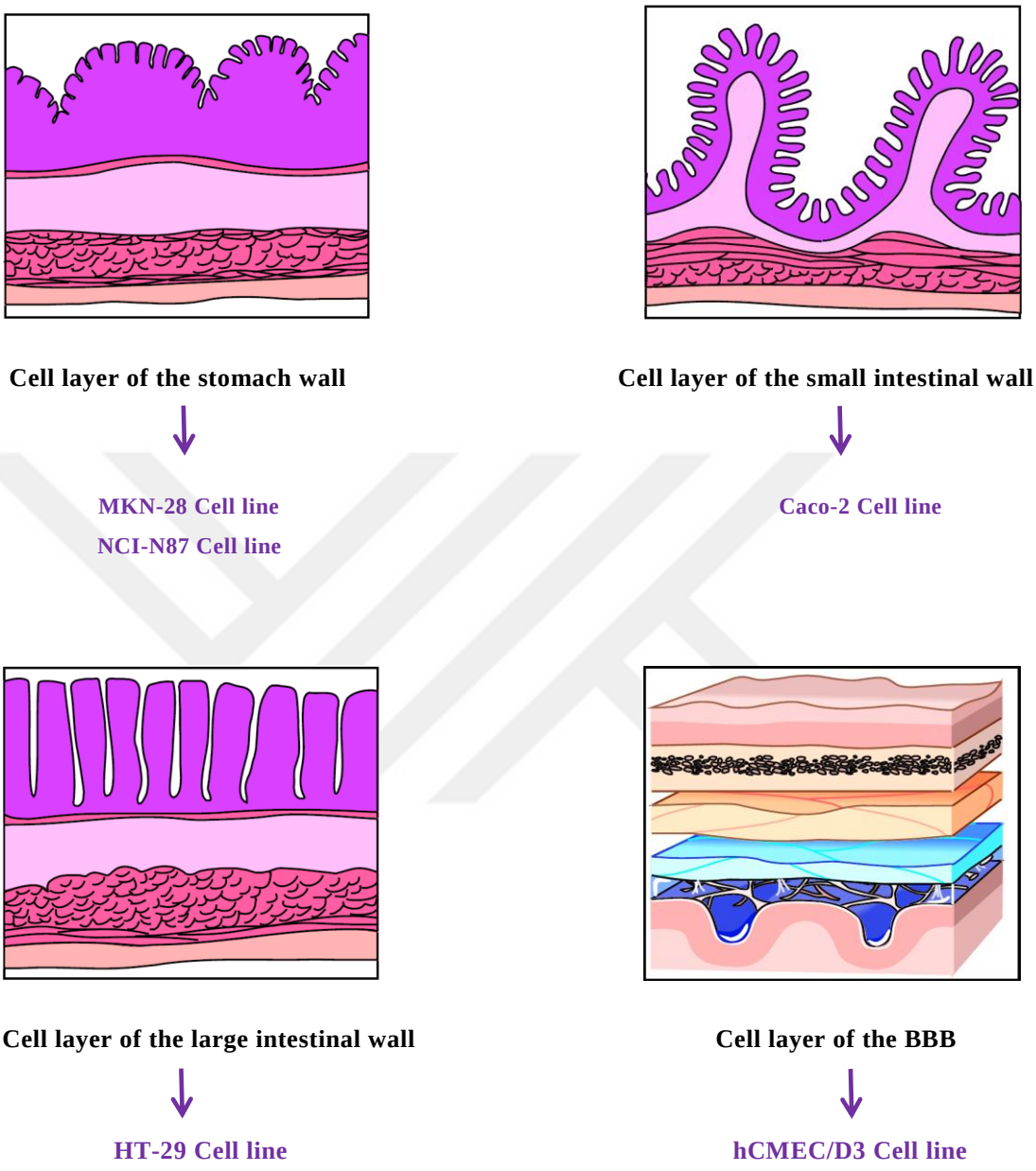


Figure 2.6 : Some of the in vitro human cell-based models available to study anthocyanins absorption (Fernandes et al, 2015).

As an in vitro model, transepithelial transport assays are prevalently used to simulate biological barriers in the human body by using cell culture lines such as Caco-2 cells which is used as an intestinal cell wall model. However, as in vivo studies reported that they appear in plasma quickly, flavonoids including anthocyanins may also be absorbed at the stomach level (Fernandes et al, 2015).

The variety of available human models are limited to study gastric absorption and metabolization of drugs or nutrients. However there are several in vitro models for intestinal absorption and metabolism studies. As animal models, rat and porcine gastric mucosa (Perioli et al. 2013) are the most similar models used to simulate human metabolism. The only available models amongst cell line based ones, are primary cultures of guinea pig gastric mucous epithelial cells (Kavvada, 2005) and one human gastric cancer cell line NCI-N87 cell line (Lemieux et al. 2011). However it has never been applied in nutritional research (Fernandes et al, 2015).

Metabolism of chlorogenic acids in gastric epithelial monolayers has been already defined (Farrell et al. 2011). Since the main results of polyphenol gastric absorption are only reported with animal studies (rat), the gastric absorption in the human body is still unclear. A human adenocarcinoma gastric cell line MKN-28 has recently been used as a model of human gastric barrier for evaluating mechanisms of nutraceutical transport (Fernandes et al. 2012). With this cell model line, anthocyanin transport studies have been carried out and transport efficiency has been reported as increasing by incubation time. However, there were no differences observed with different anthocyanins used (Dp3glc, Cy3glc and Mv3glc). In this gastric cell barrier model, anthocyanin transport efficiency was found to be in a range of 5 to 8 % (Table 2.4).

In the blood–brain barrier (BBB) absorption, transport of anthocyanins and their metabolites through hCMEC/D3 cells was reported as changing as a function of time (Faria et al, 2014). Anthocyanin metabolites were found with higher transport efficiency than the native anthocyanins (Table 2.4). No other bioreaction of metabolites was determined as a result of cell metabolism and transport in BBB cell model was described as in a lipophilicity-dependent way (Faria et al. 2014).

Another cell model originated from colorectal origin with epithelial morphology is the HT-29 cell line. This cell line has been used to evaluate absorption, secretion and transport by intestinal cells. It has been seen that HT-29 cells remain as a non-polarized monolayer without differentiation under standard conditions. However, by using different inducers or in adjusted conditions the

morphology of the cells is changing. These changes can also result from the development of an apical brush-border membrane and can be characterized by a redistribution of membrane antigens. Other human intestinal cell lines such as the HCT-116 and SW480 cell.

Table 2.4 : Anthocyanins absorption studies using Gastric and BBB models.

Model	**Source**	**[Anthocyanin]**	**Duration (h)**	**Transport efficiency**	**References**
Gastric (MKN-28)	*Commercial standards* Dp3glc Cy3glc Mv3glc	500 µM	3	*Apical pH 3.0* 6.38 7.96 10.44 *Apical pH 5.0* 6.95 7.06 8.25	Fernandes et al. (2012)
	Red wine	50 µg/mL	3	*Apical pH 5.0* 5.56	Oliveira et al. (2015)
BBB (hCMEC/D3)	*Commercial standards* Dp3glc Cy3glc Mv3glc *Synthetized metabolites* 4'Me-Dp3glc 3' and 4' Me-Cy3glc	100 µM	18	12 % 16 % 20 % 18 % 19 %	Faria et al. (2014)

Lines which are mainly used in unraveling cancer-related mechanisms, are less popular for the simulation of the human intestinal epithelium as well as the HuTu-80 cell line, a model for duodenal cells (Kamiloglu et al, 2015a).

Fogh and co-workers have isolated the Caco-2 cell line in 1977 from a human colon adenocarcinoma. Caco-2 cell line model has been extensively used and studied in the field of drug permeability and absorption, drug resistance mechanisms and for the study of cytotoxic effects of anti-tumor drugs (Kamiloglu et al, 2015a; Yi et al, 2006). The Caco-2 cell lines have been used to study transport of phytochemicals such as quercetin, epicatechin, and carotenoids (Walgren et al, 1998; Vaidyanathan and Walle, 2001; Konishi et al, 2003; Boyer et al, 2004) and much valuable information has been obtained using this model system (Yi et al, 2006). For many years, it has also been used to evaluate cellular permeability of polyphenols widely (Manna et al, 2000; Deprez et al, 2001; Konishi and Kabayashi, 2004; Manzano and Williamson, 2010).

In numerous studies, Caco-2 cells have been confirmed to differentiate in culture conditions and display behaviour of mature enterocytes as characteristics. The cells on the surface of the top medium side generate a brush border that resembles the luminal membrane of the intestinal epithelium. The cells on the bottom medium side that are attached to the permeable membrane develop into the basolateral membrane. Altough Caco-2 cells are originated from colon cells, they show characteristics of small intestinal cells morphologically and functionally (Kamiloglu et al, 2015a).

2.3. Bioavailability of Anthocyanins and Phenolic Acids

2.3.1. Bioavailability of Polyphenols

Polyphenols are one of the most common compounds in the human diet but they are not so active compounds within the human body because they have a low interior activity and also they are barely absorbed from the intestine. In addition, they are highly metabolized and rapidly eliminated. Metabolism of anthocyanins in the human body takes place as it is shown in Figure 2.7 (Kay, 2006).

Most polyphenols in food occur as esters, glycosides, or polymers and these substances cannot be absorbed in their native form although the aglycones can be

absorbed from the small intestine. They must be hydrolyzed by enzymes in intestines or by the colonic microflora to be absorbed.

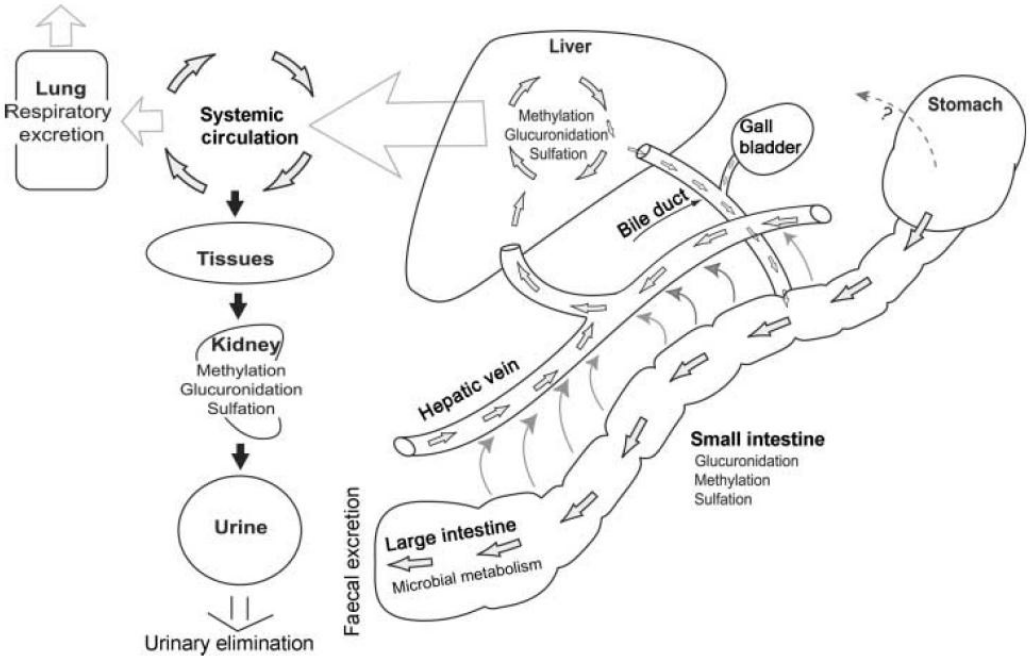


Figure 2.7 : Potential route for anthocyanin absoprtion, metabolism and elimination (Kay, 2006).

The efficiency of absorption is often reduced when the microbiota is involved because the microbiota also degrades the released aglycones and produces various simple phenolic acids . In the pathway of absorption, polyphenols are conjugated in the small intestine and later in the liver. This detoxification process mainly consists of reactions as methylation, sulfation, and glucuronidation. It restricts the potential toxic effects of many xenobiotics and provides opportunity to their elimination in the bile and urine system by increasing their hydrophilicity. The conjugation mechanisms are highly efficient, and aglycones are generally either absent in blood or present in low concentrations after consumption of nutritional doses. Circulating polyphenols are mostly bound to albumin. Although the ability of polyphenols to accumulate within specific target tissues is still unclear, they can penetrate tissues. Especially tissues in which they are metabolized need to be further investigated. Polyphenols and their derivatives mainly undergo urinary and biliary elimination. They are transferred via the biliary route into the duodenum and are

exposed to the action of bacterial enzymes as β-glucuronidase at the end of the intestine. After this action they may be reabsorbed. This cycle between liver and intestine may cause a longer presence of polyphenols within the body (Manach et al, 2004).

2.3.2. Anthocyanin Transport Through Caco-2 Cells

Studies investigating anthocyanin absorption by Caco-2 cells are presented in Table 2.5 (Kamiloglu et al, 2015a). The majority of these studies suggest that unlike other flavonoids, anthocyanins could be transported through Caco-2 monolayers in intact glycone forms, with the exceptions of black currant and some grape anthocyanins. Steinert *et al*. (2008) reported that anthocyanins from black currant, namely Dp-3-Glu, Dp-3-Rut, Cy-3-Glu and Cy-3-Rut, were not detected in any serosal solution. Also it has been demonstrated that Dp-3-Glu from grape extract was not transported (Kuntz et al, 2015a). Similarly, diglucosylated Mv-3,5-DGlu and Pn-3,5-DGlu from grape/blueberry extract were not transported in quantifiable concentrations (Kuntz et al, 2015b; Kamiloglu et al, 2015a).

Nevertheless, studies that observed anthocyanin transport, reported very low transport efficiencies. The transport efficiency of anthocyanins from blueberry extracts averaged *ca*. 3%–4% (<1% in Dp-3-Glu) (Yi et al, 2006). Similarly, only about 1% of the red grape skin anthocyanins passed through a Caco-2 cell monolayer and reached the basolateral side (Faria et al, 2009). The percentage of transported monomeric anthocyanin glycosides from açaí fruit ranged from 0.5% to 4.9% (Pacheco-Palencia et al, 2010), whereas according to Cardona *et al*. (2015) the transport rate of açaí anthocyanins was 1.2%. Transport efficiencies of Mv-3-Glu and Cy-3-Glu standards were found to be 4% and 0.8%–2.4%, respectively (Fernandes et al, 2012; Zou et al, 2014). Moreover, Cy-3-Glu-Rut recovery from sour cherry fruit and nectar was *ca*. 0.5%–4% (Toydemir et al, 2013). Trace amount of Pg-3-Glu, the predominant anthocyanin from strawberry extract, was found on the basolateral side of the epithelium (Kosińska-Cagnazzo et al, 2015). Transport efficiency of the major grape anthocyanin (Mv-3-Glu) was 0.35% (Kuntz et al, 2015a), while the absorption rates of Mv-3-Glu, Pn-3-

Glu, Pt-3-Glu, Dp-3-Glu and Cy-3-Glu from grape/blueberry extract were 0.005%–0.06% (Kuntz et al, 2015b). These results are in line with *in vivo* studies showing a very low bioavailability of anthocyanins, with <1% of the ingested amount reaching the plasma or being excreted in the urine (Netzel et al, 2001; Frank et al, 2003; Kurilich et al, 2005; Charron et al, 2009; Milbury et al, 2010). Few studies compared the transport efficiency of anthocyanins across Caco-2 cells with other polyphenols. The transport of both Mv-3-Glu and catechin through Caco-2 cells was found to be time dependent and reached approximately to the same value (4%) after 120 min of incubation (Fernandes et al, 2012). Similarly, the recovery of epicatechin in the basolateral side (1%–4%) was also about the same with Cy-3-Glu-Rut

Table 2.5 : Studies investigating anthocyanin absorption by Caco-2 cells (Kamiloglu et al, 2015a).

Sample	Anthocyanins	Anthocyanin Concentration	Cell Origin	Cell Differentiation	Incubation Time	Key Findings	Reference
Blueberry	Dp-3-Glu, Cy-3-Gal, Cy-3-Glu, Pt-3-Glu, Pn-3-Gal, Pn-3-Glu, Mv-3-Glu	50 μg/mL	ATCC	20–26 days	0–120 min	Transport efficiency of ACNs averaged ca. 3%–4% (<1% in Dp-3-Glu); Glucose-based ACNs had higher bioavailability than galactose-based ACNs	Yi et al. (2006)
Black currant extract	Dp-3-Glu, Dp-3-Rut, Cy-3-Glu, Cy-3-Rut	180 μM	DSMZ	19–21 days	0–80 min	ACNs were not detected in any serosal solution	Steinert et at. (2008)
Red grape skin	Dp-3-Glu, Cy-3-Glu, Pt-3-Glu, Pn-3-Glu, Mv-3-Glu	200 μg/mL	ATCC	25 days	4 days of pre-treatment + 6 min	Only ca. 1% of ACNs are transported; ACN transport significantly increased in the presence of ethanol; Cells pre-treated with ACNs showed ca. 50% increased transport; GLUT2 may be responsible for ACN transport	Faria et al. (2009)
Açaí pulp	Cy-3-Rut, Cy-3-Glu	50–500 μg/mL	ATCC	21 days	30–120 min	Transport efficiency of ACNs was 0.5%–4.9%; Presence of polymeric ACNs decreased transport of monomeric ACN glycosides (up to 40.3%)	Pacheco-Palencia et al. (2010)
Standard	Cat-Mv-3-Glu, Mv-3-Glu	100 μM	n/a	21 days	30–120 min	Transport efficiency of Mv-3-Glu was 4%; Absorption efficiency of Cat-Mv-3-Glu was lower than Mv-3-Glu (ca. 3%)	Fernandes et al. (2012)
Sour cherry fruit and nectar	Cy-3-Glu-Rut	55 μM	ATCC	23–24 days	360 min	Cy-3-Glu-Rut recovery was ca. 0.5%–4%; Cy-3-Glu-Rut transported 3 times more efficiently from nectar than fruit; Sucrose and citric acid enhanced the transport of Cy-3-Glu-Rut (ca. 5-fold); SPE reduced the transport efficiency of Cy-3-Glu-Rut by 5–10-fold	Toydemir et al. (2013)

Table 2.5 : *(Con*

Sample	Anthocyanins	Anthocyanin Concentration	Cell Origin	Cell Differentiation	Incubation Time	Key Findings	Reference
Standard	Cy-3-Glu	37.5 μM	n/a	20–26 days	60 min	Nano-encapsulated Cy-3-Glu with apoferritin was more efficiently transported compared to free Cy-3-Glu	Zhang et al. (2014)
Standard	Cy-3-Glu	10–40 μM	ATCC	13 days	30–120 min	Phloridzin and phloretin inhibited the absorption of Cy-3-Glu; SGLT1 and GLUT2 are probably involved in the absorption of Cy-3-Glu	Zou et al. (2014)
Açaí concentrate	Cy-3-Glu, Cy-3-Rut	500 μg/mL	ATCC	18–21 days	0–120 min	Transport rate of ACNs was 1.22%; Phospholipids from soy lecithin and terpenes from cold pressed citrus oil increased the transport of ACNs	Cardona et al. (2015)
Strawberry	Pg-3-Glu, Pg-3-Mal-Glu, Cy-3-Glu	16.3 mg/100 g	ATCC	21 days	120 min	Trace amount of Pg-3-Glu was transported	Kosińska-Cagnazzo et al. (2015)
Grape	Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Cy-3-Glu, Dp-3-Glu	1766.1 μg/mL	ATCC	21 days	30–240 min	Mv-3-Glu, Pn-3-Glu, Pt-3-Glu and Cy-3-Glu were transported, whereas Dp-3-Glu was not transported; Transport efficiency of major anthocyanin (Mv-3-Glu) was 0.35%	Kuntz et al. (2015a)
Grape/ blueberry extract	Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Dp-3-Glu, Cy-3-Glu, Mv-3,5-DGlu, Pn-3,5-DGlu	2613 μM	ATCC	21 days	0–90 min	Absorption rates of Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Dp-3-Glu and Cy-3-Glu were 0.005%–0.06%; Mv-3,5-Dglu and Pn-3,5-DGlu were not transported in quantifiable concentrations	Kuntz et al. (2015b)

ACN: anthocyanin; ATCC: American type culture collection; Cy-3-Gal: cyanidin-3-galactoside; Cy-3-Glu: cyanidin-3-glucoside; Cy-3-Glu-Rut: cyanidin-3-glucosylrutinoside; Cy-3-Rut: cyanidin-3-rutinoside; Dp-3-Glu: delphinidin-3-glucoside; Dp-3-Rut: delphinidin-3-rutinoside; DSMZ: German collection of microorganisms and cell cultures; GLUT2: glucose transporter 2; Mv-3-Glu: malvidin-3-glucoside; Mv-3,5-DGlu: malvidin-3,5-diglucoside; n/a: not available; Pg-3-Glu: pelargonidin-3-glucoside; Pg-3-Mal-Glu: pelargonidin-3-malonyl-glucoside; Pn-3-Gal: peonidin-3-galactoside; Pn-3-Glu: peonidin-3-glucoside; Pn-3,5-DGlu: peonidin-3,5-diglucoside; Pt-3-Glu: petunidin-3-glucoside; SGLT1: sodium-dependent glucose transporter 1; SPE: solid phase extraction.

(0.5%–4%) (Toydemir et al, 2013). Reported transport of some other flavonoids through Caco-2 cells was 30% for quercetin, 17% for genistein and 6% for epicatechin (Tian et al, 2009; Kamiloglu et al, 2015a).

Transport of anthocyanins can be majorly effected by their aglycone structure. Dp-3-Glu from blueberry extract showed a lower transport efficiency compared to Mv-3-Glu and Pn-3-Glu. This can be evaluated as a result of the higher number of hydroxyl groups in Dp. Also the greater hydrophobic structure of Mv may be the reason of transferring more to cells and tissues . In addition, structures of anthocyanins have an effect on their stability. It has been shown that in black currant the loss of delphinidins was higher than cyanidins (Steinert et al, 2008). Variety of sugar moiety and polymeric structure of anthocyanins may also effect their absorption by Caco-2 cells. It has been reported that glucose-based anthocyanins had a higher bioavailability than galactose-based anthocyanins in blueberry extracts (Yi et al, 2006). However, no differences are found between the respective glucose and rutinose sugar moieties in black currant anthocyanins. This shows that the sugar variety may have a minor effect on anthocyanin stability (Steinert et al, 2008; Kamiloglu et al, 2015a).

The presence of polymeric anthocyanins in açaí fruit decreased the transport of monomeric anthocyanins glycosides in a dose-dependent manner by up to 40.3% (Pacheco-Palencia et al, 2010). Similarly, the absorption efficiency of flavanol-anthocyanin dimer Catechin-Mv-3-Glu, an anthocyanin derivative reported in grape skins and red wine, was lower than Mv-3-Glu (*ca.* 3%) (Fernandes et al, 2012). The presence of other food components has been shown to have a major impact on anthocyanin transport (Kamiloglu et al, 2015a).

Solid phase extraction (SPE) of sour cherry extracts reduced the transport efficiency of Cy-3-Glu-Rut by 5–10-fold (Toydemir et al, 2013). Although some *in vivo* reports (Bub et al, 2001; Andlauer et al, 2003) claim that ethanol has no influence on anthocyanin absorption, it was shown as improving anthocyanin transport through Caco-2 cells (Faria et al, 2009). The ethanol concentration used in the cell culture study (1%), which was non-toxic to Caco-2 cells (Faria et al, 2009), is much lower than the actual ethanol concentration in red wine. Therefore, this can be explained with the difference in the doses used (Faria et

al, 2009). Citric acid was reported to increase anthocyanin transfer through Caco-2 cells (Toydemir et al, 2013). This can be associated with anthocyanins being more stable at low pH values (Fang, 2014; Oliveira et al, 2015). In addition, phospholipids from soy lecithin and terpenes from citrus oil increased the transport of anthocyanins in açaí fruit, and it has been reported that a combination of phospholipids and terpenes are the most effective for the transport through Caco-2 cells (Cardona et al, 2015). These results represent that transport of anthocyanins in the Caco-2 cell line model can be effected by several factors including the loss of anthocyanins because of spontaneous metabolic conversions (Kamiloglu et al, 2015a).

It has been suggested that anthocyanins could interfere with the transporters responsible for their own transport. Anthocyanins have sugar moieties especially glucose residues, therefore these anthocyanin transporters were suggested to be the glucose transporters. SGLT1 and GLUT2 are the main hexose transporters in Caco-2 cells. SGLT1 is an energy-dependent and sodium-dependent cotransporter and GLUT2 is a facilitated transporter. SGLT1 occurs only on the apical membrane and GLUT2 was known to be only in the basolateral membrane and rarely on the apical membrane. However, in the past few years, it has been found that GLUT2 occurs on the apical side and can be gathered to the membrane in the presence of a large amount of glucose and become the main transporter responsible for glucose uptake (Faria et al, 2009; Zou et al, 2014; Kamiloglu et al, 2015a).

In a study evaluating red grape skin anthocyanins, an inhibitory effect (about 60% decrease) was seen because of interfering with glucose (Faria et al, 2009). Pn-3-Glu from strawberry extract also effected glucose uptake and transport to the basolateral side by inhibiting activities of the glucose transporters (Manzano and Williamson, 2010). In addition, it is reported that exposure to anthocyanin rich berry extract derived from blueberry, bilberry, cranberry, elderberry, raspberry seeds and strawberry significantly reduce SGLT1 and GLUT2 expressions (Alzaid et al, 2013). Inhibition studies conducted using the pharmacological agents, phloridzin, an inhibitor of SGLT1, or phloretin, an inhibitor of GLUT2, revealed that the absorption of Cy-3-Glu was significantly inhibited in the presence of these agents (Zou et al, 2014). These data suggest that anthocyanins may prevent hyperglycemia by

decreasing glucose transporter expressions (Kamiloglu et al, 2015). However, the exact mechanism of anthocyanin absorption is still unclear.

The low stability of anthocyanins has a direct effect on their health benefits, therefore, an encapsulation process has been studied to improve anthocyanin bioavailability (Betz et al, 2012; Oidtmann et al, 2012; Robert and Fredes, 2015). Compared to free Cy-3-Glu, the nano-encapsulated Cy-3-Glu with apoferritin was found to have a higher transport efficiency through a Caco-2 cell monolayer (Zhang et al, 2014). As a result of another process, sour cherry nectar has shown three times more efficient transport of Cy-3-Glu-Rut than sour cherry fruit through a Caco-2 cell monolayer (Toydemir et al, 2013).

2.3.3. Anthocyanin Metabolism by Caco-2 Cells

Polyphenols are exposed to some transformations in the human body. Phase I transformation consists of oxidation, reduction and hydrolysis. Phase II biotransformations that occur more intensively happen in the liver and the intestine and consist of conjugation reactions as methylation, glucuronidation and acetylation (Cardona et al, 2013). It has been demonstarted that anthocyanins can be transformed to phenolic acids and aldehydes under biological conditions spontaneously (Galvano et al, 2008). In addition, under cell culture conditions, protocatechuic acid (PCA) and phloroglucinaldehyde (PGA), which are derived from the A and B rings of the main compound are found as the main metabolites of Cy-3-Glu and Cy (Figure 2.8). These metabolites can be further conjugated to glucuronide and sulfate conjugates enzymatically (Kay et al, 2009). Another Phase II reaction of anthocyanins, methylation, alters the number of hydroxyl and methoxyl groups in ring B in comparison with the native compound. In a study, methylated metabolites of Cy-3-Glu, Dp-3-Glu and Pt-3-Glu were found to display some antiproliferative activity for the Caco-2 cell line (Fernandes et al, 2013). In addition, some other anthocyanin metabolites as gallic acid, 3-*O*-methylgallic acid, and PGA were found to reduce cell proliferation in Caco-2 cells more than native anthocyanins (Forester and Waterhouse, 2010). Consequently when health benefits of anthocyanins are evaluated, the potential of metabolites should be considered as well (Kamiloglu et al, 2015a)

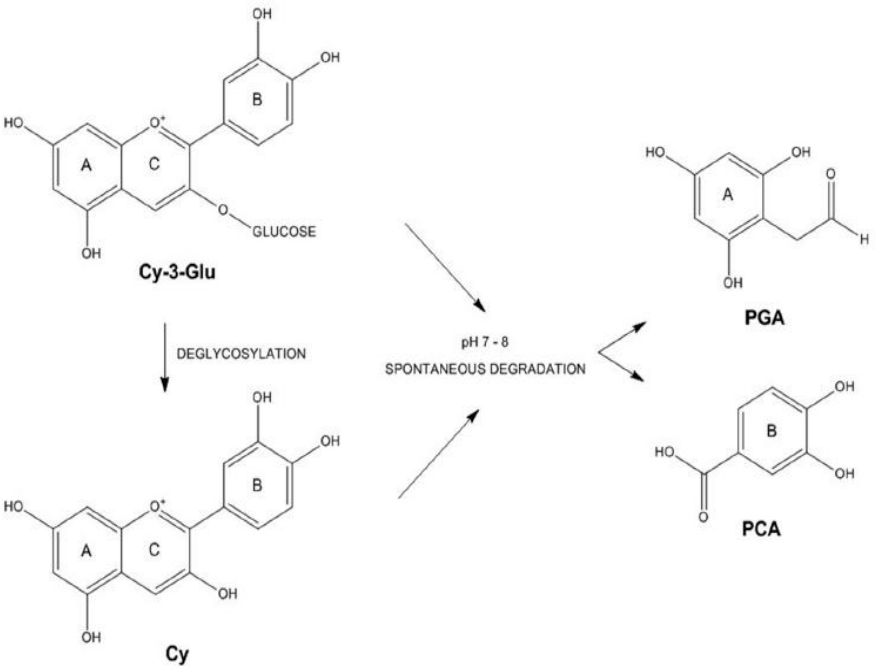


Figure 2.8 : Metabolites (PGA: phloroglucinaldehyde; PCA: protocatechuic acid) of cyanidin-3-glucoside (Cy-3-Glu) and cyanidin (Cy) (Kamiloglu et al, 2015a).

3. MATERIALS AND METHODS

3.1. Plant Material

Black carrot and its industrial by-product pomace were collected in triplicate from an established processing plant (Erkonsatre Co.) in Konya, Turkey. Peel was obtained manually from whole black carrots. All samples were ground to a fine powder in liquid nitrogen using a precooled grinder (IKA A11 basic, IKA-Werke GmbH and Co., Staufen, Germany) and subsequently lyophilized (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 h and stored at −20 °C until further analysis.

3.2. Chemical Extraction

For each sample, three independent extractions were carried out. Lyophilized powder (0.1 g) was treated with 5 mL of 75% aqueous methanol containing 0.1% (v/v) formic acid. The treated samples were sonicated in an ultrasonic bath (Elma S60H elmasonic, Singen, Germany) for 15 min and subsequently centrifuged at 3000 *g* and 4 °C for 10 min (Sigma Laboratory Centrifuge 4K15, Osterode am Harz, Germany), and the supernatants were collected. This extraction protocol was repeated once more for the pellet, and the two supernatants were pooled to a final volume of 10 mL. Prepared extracts later referred as undigested samples were dried and stored at −20 °C.

3.3. In Vitro Digestion

The simulated digestion system consist of two stages i.e. a gastric and small intestine stage. Stages of *in vitro* digestion are shown in Figure 3.1.

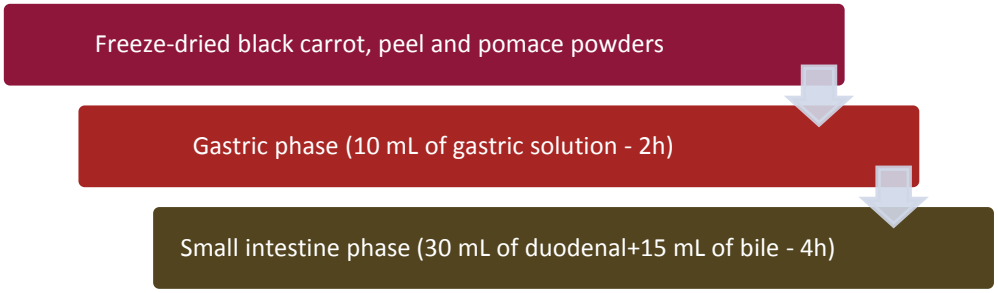


Figure 3.1 : Stages of *in vitro* digestion.

Sixty-two mL of distilled water was added to 3 grams of freeze dried black carrot, peel and pomace powders in penicillin bottles (65 mL distilled water for the blank). After mixing, 15 mL of sample was immediately taken as time point zero (t=0). After addition of 10 mL gastric juice, samples were incubated in a shaker for 2 hours at 37˚C (pH 3). Gastric solution contains; 16.5 g/L sodium chloride (NaCl) (Sigma-Aldrich, Steinheim, Germany), 2.1 g/L sodium dihydrogen phosphate (NaH_2PO_4) (Sigma-Aldrich), 4.92 g/L potassium chloride (KCl) (Sigma-Aldrich), 2.4 g/L calcium chloride dehydrate ($CaCl_2.2H_2O$) (Sigma-Aldrich), 0.54 g/L urea (Chem-Lab NV, Zedelgem, Belgium), 2.1 gr/L mucin (Sigma-Aldrich), 6 g/L bovine serum albumin (Sigma-Aldrich) and 6 g/L pepsin (Sigma-Aldrich). After 2 hours, 15 mL of sample was collected. For the small intestine stage, 15 mL sample was collected from gastric phase, 30 mL of duodenal and 15 mL of bile medium were added in penicillin bottles. Samples were incubated at 37˚C for 4 hours (pH 7). Duodenal medium contained 7.01 g/L NaCl, 0.56 g/L KCl, 0.2 g/L $CaCl_2.2H_2O$, 5.61 g/L sodium bicarbonate ($NaHCO_3$) (Chem-Lab NV), 0.08 g/L potassium dihydrogen phosphate (KH_2PO_4) (Chem-Lab NV), 0.5 g/L magnesium chloride 6 aqueous ($MgCl_2.6H_2O$) (Chem-Lab NV), 0.1 g/L urea, 1 g/L bovine serum albumin, 3 g/L pancreatin (Sigma-Aldrich) and 0.5 g/L lipase (Sigma-Aldrich), whereas; bile medium contained 5.26 g/L NaCl, 0.38 g/L KCl, 0.22 g/L $CaCl_2.2H_2O$, 5.79 g/L $NaHCO_3$, 0.25 g/L urea, 1.8 g/L bovine serum albumin and 6 g/L of bile (Sigma-Aldrich). After 4 hours, 15 mL of samples, later referred to as digested samples, were collected, centrifuged at 3000 *g* and 4 °C for 10 min and stored at -20˚C until further analysis (Kamiloglu et al, 2015b).

3.4. Maintenance of Cell Cultures

The Caco-2 human colon cancer cell lines and human endothelial cell lines EAhy926 were obtained from American-Type Culture Collection (ATCC). The cells were grown in 25 cm^2 canted neck tissue culture flasks (Sarstedt Co., Essen, Belgium) using DMEM supplemented with Glutamax (4.5 g/L glucose), 10% heat-inactivated FBS and 1% non-essential amino acids (NEAA). Cells were maintained in a humidified atmosphere of 10% CO_2 in air at 37 ºC (Memmert CO_2 incubator, Memmert GmbH and Co., Nurnberg, Germany). Medium was changed three times per week and cells were sub-cultured at 70-80% confluence. For sub-culturing, the cells, washed with 3 mL phosphate buffered saline (PBS), were detached from the bottom of the flask with 2 mL trypsin-EDTA wash and subsequent incubation at 37 ºC and 10% CO_2 in air for 3-5 min. A volume of suspension (depending on the cell concentration and passage number) was seeded to new flasks, and the final volume was made up to 4 mL with the DMEM medium with Glutamax, 10% FBS and 1% NEAA. This sub-culturing procedure enabled to split the cells in culture flask to the new flasks with concentrations of 1:3 to 1:5 of original concentrations those in culture flask, depending on the confluency of the cells.

3.5. Transport Experiments

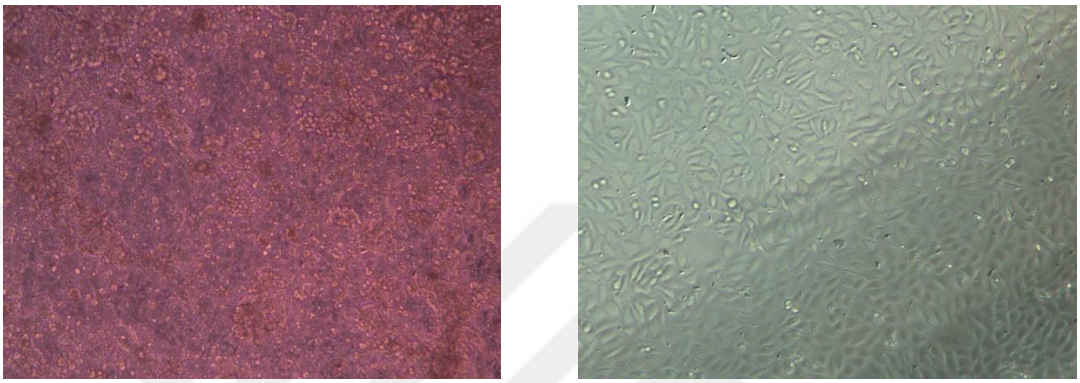
For transport experiments, Caco-2 cells in flasks, having a passage number less than 50, were harvested using the trypsinization protocol described above and resuspended in DMEM medium with Glutamax, 10% FBS, 1% penicillin-streptomycin (Pen Strep, Gibco, Life Technologies) and 1% NEAA. One hundred microliter of this cell suspension was mixed with 100 µL trypan blue stain (0.4%) and the number of viable cells were determined using a 0.1 mm depth Burker counting chamber under a AE30/31 inverted microscope (MOTIC, VWR, Leuven, Belgium). They were seeded in 6-well Transwell plates (0.4 µm pore diameter, 24 mm insert, Corning Costar, VWR) at a concentration of approximately $6.0x10^5$ cells per well. Culture medium with cells was added to the apical chamber and without cells to the basal chamber in volumes of 2 mL and 2.5 mL, respectively. Cells were allowed to grow and differentiate to

confluent monolayers for 21 days post seeding. Culture medium was replaced two/three times a week. Experiments were carried out using black carrot, peel and pomace samples dissolved in Hank's Balanced Salt Solution (HBSS), which was selected as transport medium based on preliminary studies. In order to use sample concentrations that do not negatively influence the monolayer integrity of Caco-2 cells, studies were performed in which different concentrations were analyzed during several hours of exposures to cells. Based on these results, 5 mg dry-weight/mL concentration was chosen for black carrot and pomace. For peel, a higher concentration (7 mg dryweight/ mL) was selected to compensate the anthocyanin content. In each sample, these concentrations correspond to equal amounts (30 ± 2 μM) of cyanidin-3- xylosyl-feruloyl-glucosyl-galactoside, which is the major anthocyanin compound in black carrots. On the day of the transport experiment (day 21), to ensure that the monolayers exhibit the properties of a tight biological barrier, transepithelial electrical resistance (TEER) values were monitored using an automated tissue resistance measurement system (REMS, World Precision Instruments, Hertfordshire, UK). Then, the transport medium was changed with HBSS, and preincubated for 1 h. The pH of the samples in HBSS were adjusted to 6.5, which is the pH of the upper gastrointestinal tract and loaded into the apical compartment of the culture wells (2 mL). HBSS alone (2.5 mL) was adjusted to pH 7.5 and loaded into the basolateral compartment. The Caco-2 cells were incubated with black carrot, peel and pomace samples for 4 h at 37 °C and 10% CO_2. Apical and basolateral sides were sampled every 2 h, stabilized by adjusting the pH to 2 with formic acid, and stored at −20 °C until HPLC analysis.

3.6. Co-culture Experiments

For the co-culture setup, Caco-2 cells (Figure 3.2A) were seeded on 12-well Transwell plates (0.4 μm pore diameter, Elscolab, Kruibeke, Belgium) at a concentration of 250,000 cells per well. Fifteen days after confluency of the Caco-2 cells, EA.hy926 (Figure 3.2B) cells were seeded on the basolateral compartment of the Transwell plate, at a cell density of 300,000 cells per well. The EA.hy926 cells were grown in the co-culture system until they reached confluency on the third day. On the fourth day of co-culture, Caco-2 cells were

treated apically with undigested and digested samples of black carrot, peel and pomace as explained in 3.5. Transport experiments section. Prior to this treatment, the basolateral compartment in the co-culture was incubated for 3 h with 1 ng mL^{-1} TNF-α in HBSS in order to induce inflammation associated with cardiovascular diseases. The co-culture was incubated at 37°C at 10% CO_2 and samples of culture medium of apical and basal compartments were collected after 4 h and immediately stored at -80 °C prior to analysis.



(A) **(B)**

Figure 3.2 : Caco-2 (A) and EA.hy926 (B) cells exposed to black carrot polyphenols in a co-culture set-up.

3.7. HPLC-DAD Analysis

Polyphenol profiles of samples were determined based on a previous study performed by our group (Kamiloglu et al, 2015c) where the identification of black carrot polyphenols was carried out using an UPLC-ESI-MS^E system. Samples were passed through 0.45 μm membrane filters and injected into a Thermo Dionex Ultimate 3000 HPLC (Thermo Fischer Scientific, Landsmeer, The Netherlands) coupled with a diode array detector (DAD). A Grace Smart RP C18 column (250 x 4.6 mm, 5 μm) was used as the stationary phase. The following solvents with a flow rate of 1 mL/min and injection volume of 10 μL were used for spectral measurement at 312 and 520 nm: TFA/MQ water (1:1000, v/v; eluent A) and TFA/acetonitrile (1:1000, v/v; eluent B). The linear gradient was used as follows: 0 min, 5% B; 0−45 min, 35% B, linear; 45−47 min, 75% B, linear; 47−54 min, 5% B. The content of anthocyanin glycosides was quantified

using cyanidin-3- O-glucoside, whereas phenolic acids were quantified using their authentic standards.

3.8. Cytotoxicity assay

After the transport, to analyze the cytotoxic effects of black carrot polyphenols on cells, the tetrazolium bromide (MTT) assay is applied. MTT assay is based on the conversion of the yellow MTT to purple formazan in the mitochondria of living cells. Briefly, 200 µL of MTT dissolved in PBS (5 mg/mL) was added to 1 mL cell culture medium and incubated in the dark at 37°C for 2 h. After 2 h, the medium was removed, the formazan crystals were dissolved in 2 mL DMSO and the absorbance was measured at 570 nm with a Bio-Rad multiplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The results were expressed as percentage compared to untreated cells.

3.9. Determination of Intracellular Reactive Oxygen Species (ROS)

The inhibition of intracellular ROS was monitored in endothelial cells through the reaction with the oxidant sensitive fluorogenic probe H_2-DCFDA (2,7-dichlorodihydrofluorescein diacetate) (Sigma-Aldrich, St. Louis, MO, USA). The non-fluorogenic compound is converted by intracellular deacetylases to DCFH, which upon oxidation by ROS is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). After the transport experiments, endothelial cells were incubated with 20 µM H2-DCFDA in HBSS for 30 min. The cells were then washed with phosphate buffered saline (PBS) and lysed with 1% Triton for 30 min. The samples were then centrifuged at 10000 x g for 10 min, and fluorescence of supernatants was immediately measured on a Spectramax Fluorescent Plate Reader ($\lambda_{ex/em}$=485/535 nm) (Molecular Devices, CA, USA).

3.10. Enzyme-Linked Immunosorbent Assays (ELISA)

The secretion of IL-8, VEGF and ICAM-1 in the co-culture was determined in cell culture media collected at time points 4 h from the basolateral compartment and analysed using the human IL-8 TMB, human VEGF TMB and human ICAM-1 ABTS ELISA kits (Peprotech, London, UK), respectively, following the manufacturer's instructions.

3.11. Determination of Nitric Oxide (NO) Production in Endothelial Cells

The production of NO in endothelial cells was monitored using the Griess colorimetric assay (Sigma-Aldrich) (Simão et al, 2012). Concentrations of nitrite (NO_2) were quantified through a six-point matrix-matched standard curve of sodium nitrite ($NaNO_2$) (0-20 μmol L^{-1}). In the procedure, samples of culture medium were mixed with an equal volume of the Griess reagent. After 15 min at room temperature (18 °C), absorbance was read at 540 nm.

3.12. Statistical analysis

All analyses were performed with four biological and three technical replicates (at least three measurements from four different wells) and reported as mean ± standard deviation. Data were subjected to statistical analysis using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test.

4. RESULTS AND DISCUSSION

4.1. Phenolic Profile

Anthocyanin content of black carrot, pomace and peel was determined for both apical and basolateral compartments after 4 hours incubation using HPLC-DAD.

Five major anthocyanins could be identified at 520 nm. A chromatogram of the anthocyanin composition of the black carrot, pomace and peel under investigation is shown in Figure 4.1. The anthocyanin profile corresponded to those reported previously (Kammerer et al, 2004a; Montilla et al, 2011; Suzme et al, 2014; Olejnik et al, 2016). The major anthocyanins were cyanidin-based with different sugar moieties. Two of them were non-acylated (cyanidin-3-xylosylglucosyl-galactoside and cyanidin-3-xylosylgalactoside), and three were acylated with sinapic acid (cyanidin-3-xylosylsinapoyl-glucosyl-alactoside), ferulic acid (cyanidin-3-xylosylferuloyl-glucosylgalactoside) and coumaric acid (cyanidin-3-xylosylcoumaroyl-glucosylgalactoside) whereas the predominant anthocyanin is cyanidin-3-xylosylferuloyl-glucosylgalactoside. Major phenolic acids are found as neochlorogenic acid, chlorogenic acid and caffeic acid whereas the predominant phenolic acid is chlorogenic acid (Table 4.1.).

In black carrot and peel samples, the anthocyanin profile of both apical and basal compartments was found similar whereas pomace samples were found to contain a much higher amount of cyanidin-3-xylosylgalactoside, i.e. almost the same as the predominant anthocyanin cyanidin-3-xylosylferuloyl-glucosylgalactoside.

Table 4.1 : Polyphenols in undigested and digested black carrot, pomac peel.

	Undigested (µmol L^{-1})			Digested (µmol L^{-1})		
	Black carrot	Pomace	Peel	Black carrot	Pomace	Peel
Cyanidin-3-xylosylglucosyl-galactoside	2.6±0.2	6.8±0.1	0.9±0.1	0.1±0.1	2.0±0.1	0.2±0.0
Cyanidin-3-xylosylgalactoside	2.2±0.4	38.7	4.2±0.3	0.1±0.0	1.9±0.3	0.3±0.1
Cyanidin-3-xylosylsinapoyl-glucosylgalactoside	6.1±0.3	12.1±0.2	4.1±0.0	0.1±0.0	2.7±0.3	0.1±0.0
Cyanidin-3-xylosylferuloyl-glucosylgalactoside	33.4±0.4	29.9±0.3	27.7±0.5	1.1±0.1	14.6±1.7	2.0±0.0
Cyanidin-3-xylosylcoumaroyl-glucosylgalactoside	4.4±0.2	5.5±0.2	3.0±0.2	0.1±0.0	1.8±0.4	0.2±0.0
Neochlorogenic acid	0.6±0.0	2.0±0.2	0.0±0.0	0.4±0.0	4.7±1.0	0.1±0.0
Chlorogenic acid	25.8±0.2	62.3±0.3	4.8±0.0	1.0±0.4	18.1±0.4	1.4±0.1
Caffeic acid	2.9±0.2	3.4±0.3	0.5±0.0	1.7±0.0	1.5±0.0	1.8±0.0

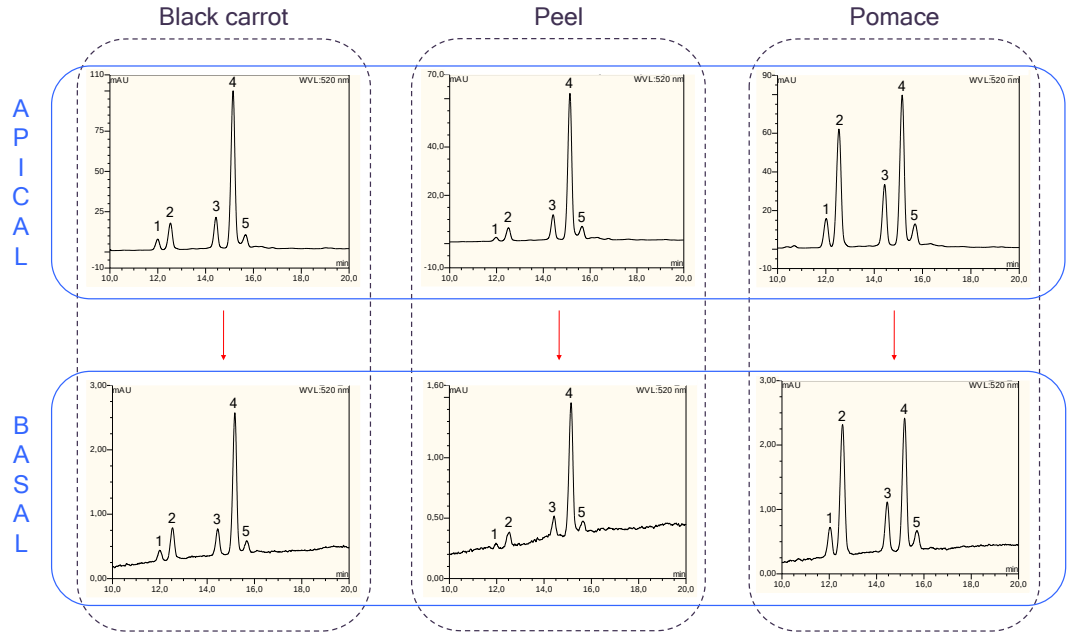


Figure 4.1 : Representative HPLC-DAD chromatograms (recorded at 520 nm) of black carrot, peel and pomace anthocyanins in the apical side after 4 h incubation (upper panel); and in the basolateral side after 4 h incubation (lower panel). Numbers refer to the anthocyanin peaks: (1) cyanidin-3-xylosylglucosyl-galactoside; (2) cyanidin-3-xylosylgalactoside; (3) cyanidin-3-xylosylsinapoyl-glucosylgalactoside; (4) cyanidin-3-xylosylferuloyl-glucosylgalactoside; (5) cyanidin-3-xylosylcoumaroyl-glucosylgalactoside.

4.2. Transport Experiments

The Caco-2 cell culture model was used in order to determine the permeability of the anthocyanins of black carrot, pomace and peel across the intestinal barrier. Transport experiments of anthocyanins and phenolic acids were carried out as it has explained in materials and methods section.

TEER values were found to be between 93% - 106% of the initial values after 1 hour of incubation with HBSS. After 4 hours incubation with samples, a decrease in a range of 25% - 33% was monitored, whereas TEER values measured after 24 hours incubation in growth medium were found between 74% - 93% of the initial values (Table 4.2). It has been monitored that TEER values of digested samples are slightly lower than undigested samples. This may be explained by the content of the digestive solution including salts or minerals.

The TEER measurement indicated a high integrity of the Caco-2 monolayer in both cultures, before and after the transport experiment. Although a significant decrease in TEER values was noticed after 4 hours incubation with black carrot, pomace and peel samples ($p<0.001$) , values were measured close to normal TEER values compatible with the literature after 24 hours (Olejnik et al, 2016). In addition, the blanks behaved the same, so it is the HBSS, or other manipulations that have caused the effect and not the black carrot compounds. These indicates that no permanent damage was observed in Caco-2 cells treated with samples.

Table 4.2 : TEER ($\Omega.cm^2$) measurements performed in growth medium and during incubation in HBSS.

Samples	t = 0 h (Growth medium)	t = 1 h (HBSS)	t = 1+4 h (HBSS + Samples)	t = 24 h (Growth medium)
Undigested				
Blank	1408 ± 113	1390 ± 180 (99%)[a]	1198 ± 77 (85%)	1285 ± 124 (91%)
Black carrot	1272 ± 62	1349 ± 50 (106%)	1065 ± 40 (84%)	1178 ± 56 (93%)
Peel	1547 ± 50	1631 ± 69 (105%)	1111 ± 14 (72%)	1232 ± 47 (80%)
Pomace	1577 ± 99	1466 ± 75 (93%)	1091 ± 38 (69%)	1286 ± 119 (82%)
Digested				
Blank	1382 ± 60	1401 ± 37 (101%)	965 ± 66 (70%)	1138 ± 12 (82%)
Black carrot	1464 ± 61	1453 ± 41 (99%)	1080 ± 53 (74%)	1143 ± 13 (78%)
Peel	1592 ± 49	1536 ± 31 (96%)	1106 ± 14 (69%)	1196 ± 36 (75%)
Pomace	1656 ± 42	1578 ± 31 (95%)	1165 ± 43 (70%)	1222 ± 12 (74%)

[a] Numbers in brackets represent the TEER values relative to the initial TEER value, expressed as percentages.

The transport of the anthocyanins of black carrot, pomace and peel was evaluated at the chosen concentration explained in 3.5. Transport experiments are shown in Table 4.3. The results indicated that black carrot anthocyanins were able to cross the Caco-2 cell monolayer and their transport to the basolateral side increased with time. Following the 2-hour transport experiment, between 0.1 – 1.2 % of initial values of anthocyanins in digested samples was transported through the Caco-2 monolayer whereas the basal recovery was between 1.3 – 5.3 % after 4 hours incubation. In addition, transport of anthocyanins in undigested samples was found to be in a range of 0.2 – 0.6 % and 0.8 – 2.7 % of the initial anthocyanin concentration after 2 hours incubation and 4 hours incubation, respectively.

Cyanidin-3-xylosylglucosyl-galactoside, cyanidin-3-xylosylgalactoside, cyanidin-3-xylosylsinapoyl-glucosylgalactoside, and cyanidin-3-xylosylcoumaroyl-glucosylgalactoside could not be detected in the basolateral side of digested black carrot and peel samples after 2 hours incubation whereas basal recovery of anthocyanin acylated with ferulic acid was found to be 0.9%

and 1.2% in black carrot and peel samples, respectively. This can be explained because of the low concentration of these compounds below the detection limit of the HPLC. The transport of phenolic acids in black carrot, peel and pomace is shown in Table 4.4. Basal recovery of phenolic acids in digested samples of black carrot, peel and pomace was detected to be between 4 – 7 % of the initial concentration after 4 hours incubation, whereas the transport of phenolic acids in undigested samples was found to be between 1.6 – 3.3 % at the same time point. Neochlorogenic acid could not be found in basal compartment of black carrot and peel samples after 2 hours of incubation and in basal compartment of undigested peel samples after 4 hours of incubation. This may be explained by the low concentration of neochlorogenic acid below the dedection limit of the HPLC. These findings correspond with the transport results of phenolics in the literature (Cardona et al, 2015; Kosinska-Cagnazzo et al, 2015; Kuntz et al, 2015a). Toydemir et al. (2013) observed cyanidin-3-(2G-glucosylrutinoside) transport from morello cherries across Caco-2 cells with transport efficiencies of 3–4%. Similar transport efficiencies (1%) were reported by Faria et al. (2009) who tested the uptake of ACNs from grape skin extracts as well as of M3G as single compound. Notably, Steinert et al. (2008) could not observe any transport of ACNs when applied to Caco-2 cells.

In transport experiments, permeability of non-acylated anthocyanins through the intestinal barrier was determined to be higher than acylated anthocyanins in black carrot, pomace and peel. These findings, which indicate the lower intestinal permeability of the acylated anthocyanins compared to the non-acylated anthocyanins, are consistent with the data reported in the literature (Netzel et al, 2007; Charron et al, 2009; Olejnik et al, 2016) The results obtained in this study indicate that both the acylated and non-acylated cyanidin-based anthocyanins in black carrot have the ability to penetrate the intestinal barrier and may potentially contribute to health-beneficial effects in specific tissues or organs of the human body.

Another remarkable result of transport experiments was observed as the difference in transport efficiencies between undigested and digested samples. Regarding all cyanidin-based anthocyanins, after both 2 hours and 4 hours incubation, digested samples were found to have a higher basolateral recovery

than undigested samples. This result may be explained by the digested samples transporting faster than undigested samples. In addition to this, various enzymes, salts or minerals in digested samples might have interfered with transport agents in the Caco-2 cell barrier. Another interesting observation is that this improved permeability for digested samples is still possible despite the lower apical stability compared to the undigested samples.

Table 4.3 : Apical and basal side recoveries and transport efficiencies of anthocyanins from black carrot, peel and pomace samples.

Sample	t = 2			t = 4		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]
			cyanidin-3-xylosylglucosyl-galactoside			
Undigested						
Black carrot	102.9 ± 3.3	0.5 ± 0.2	0.005 ± 0.002	100.8 ± 2.0	2.1 ± 0.4	0.021 ± 0.004
Peel	94.6 ± 5.8	0.3 ± 0.1	0.003 ± 0.001	96.2 ± 7.9	1.3 ± 0.8	0.013 ± 0.008
Pomace	95.9 ± 0.9	0.6 ± 0.1	0.006 ± 0.001	94.6 ± 0.7	2.5 ± 0.3	0.027 ± 0.003
Digested						
Black carrot	99.7 ± 16.8	nd[c]	-	84.4 ± 14.7	4.6 ± 0.2	0.056 ± 0.010
Peel	77.7 ± 16.2	nd	-	71.8 ± 5.8	2.7 ± 0.5	0.037 ± 0.006
Pomace	89.6 ± 11.1	0.8 ± 0.3	0.009 ± 0.004	68.6 ± 6.8	3.7 ± 0.9	0.053 ± 0.009
			cyanidin-3-xylosylgalactoside			
Undigested						
Black carrot	100.2 ± 2.9	0.5 ± 0.2	0.005 ± 0.002	96.4 ± 2.0	2.4 ± 0.4	0.024 ± 0.003
Peel	94.4 ± 4.0	0.2 ± 0.1	0.002 ± 0.001	94.8 ± 3.6	1.1 ± 0.4	0.011 ± 0.004
Pomace	95.9 ± 0.9	0.6 ± 0.1	0.006 ± 0.001	93.1 ± 0.6	2.7 ± 0.3	0.029 ± 0.004
Digested						
Black carrot	99.9 ± 7.6	nd	-	92.6 ± 9.9	5.3 ± 0.1	0.058 ± 0.007
Peel	91.1 ± 24.5	nd	-	83.1 ± 20.8	3.9 ± 0.1	0.050 ± 0.011
Pomace	88.3 ± 24.5	1.1 ± 0.2	0.013 ± 0.004	64.1 ± 16.9	4.3 ± 1.5	0.068 ± 0.018
			cyanidin-3-xylosylsinapoyl-glucosylgalactoside			
Undigested						
Black carrot	94.5 ± 0.5	0.4 ± 0.2	0.005 ± 0.002	94.2 ± 2.1	1.8 ± 0.3	0.019 ± 0.003
Peel	100.8 ± 1.7	0.5 ± 0.1	0.004 ± 0.001	98.4 ± 0.9	1.1 ± 0.2	0.011 ± 0.002
Pomace	93.8 ± 2.0	0.3 ± 0.1	0.004 ± 0.001	91.7 ± 2.7	1.8 ± 0.3	0.019 ± 0.003

Table 4.3 : (*Cont.)* Apical and basal side recoveries and transport efficiencies of anthocyanins from black carrot, peel and pomace samples.

Sample	t = 2			t = 4		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]
			cyanidin-3-xylosylsinapoyl-glucosylgalactoside			
Digested						
Black carrot	86.9 ± 18.9	nd	-	78.8 ± 11.0	2.7 ± 0.1	0.034 ± 0.005
Peel	82.3 ± 7.5	nd	-	63.3 ± 8.2	3.1 ± 0.1	0.049 ± 0.007
Pomace	81.6 ± 4.7	0.4 ± 0.1	0.004 ± 0.001	70.7 ± 4.9	3.4 ± 0.5	0.048 ± 0.004
			cyanidin-3-xylosylferuloyl-glucosylgalactoside			
Undigested						
Black carrot	93.9 ± 1.7	0.4 ± 0.1	0.004 ± 0.001	94.7 ± 2.0	1.7 ± 0.3	0.018 ± 0.003
Peel	97.1 ± 1.7	0.4 ± 0.1	0.004 ± 0.001	94.1 ± 1.8	1.0 ± 0.2	0.011 ± 0.002
Pomace	93.6 ± 4.4	0.5 ± 0.1	0.005 ± 0.001	92.5 ± 1.9	2.1 ± 0.4	0.022 ± 0.004
Digested						
Black carrot	75.4 ± 14.4	0.9 ± 0.5	0.012 ± 0.006	64.1 ± 4.0	4.7 ± 1.3	0.074 ± 0.020
Peel	76.1 ± 4.1	1.2 ± 0.1	0.015 ± 0.001	60.9 ± 2.2	3.3 ± 0.8	0.054 ± 0.014
Pomace	84.7 ± 8.4	0.4 ± 0.1	0.005 ± 0.002	70.7 ± 6.6	3.4 ± 0.5	0.049 ± 0.008
			cyanidin-3-xylosylcoumaroyl-glucosylgalactoside			
Undigested						
Black carrot	91.9 ± 3.5	0.2 ± 0.1	0.002 ± 0.001	86.1 ± 4.1	1.3 ± 0.4	0.015 ± 0.005
Peel	105.8 ± 6.5	0.3 ± 0.2	0.003 ± 0.002	98.3 ± 11.1	0.8 ± 0.2	0.008 ± 0.003
Pomace	94.0 ± 10.3	0.3 ± 0.1	0.004 ± 0.001	97.7 ± 4.2	1.8 ± 0.5	0.018 ± 0.005
Digested						
Black carrot	83.2 ± 7.6	nd	-	69.0 ± 15.2	1.6 ± 0.3	0.023 ± 0.003
Peel	85.1 ± 12.5	nd	-	65.1 ± 3.6	1.3 ± 0.6	0.019 ± 0.009
Pomace	72.2 ± 15.0	0.1 ± 0.0	0.001 ± 0.000	52.4 ± 6.8	2.1 ± 0.4	0.040 ± 0.005

Table 4.4 : Apical and basal side recoveries and transport efficiencies of phenolic acids from black carrot, peel and pomace samples.

Sample	t = 2			t = 4		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]
			Neochlorogenic acid			
Undigested						
Black carrot	101.7 ± 3.1	nd[c]	-	97.8 ± 6.9	2.1 ± 0.5	0.022 ± 0.004
Peel	98.4 ± 14.9	nd	-	93.7 ± 14.2	Nd	-
Pomace	101.7 ± 0.8	0.4 ± 0.2	0.004 ± 0.002	100.7 ± 1.1	3.3 ± 0.5	0.033 ± 0.005
Digested						
Black carrot	95.8 ± 11.9	nd	-	89.0 ± 13.8	4.8 ± 1.4	0.057 ± 0.027
Peel	93.3 ± 6.4	nd	-	85.5 ± 5.5	4.0 ± 1.6	0.046 ± 0.016
Pomace	94.2 ± 2.3	0.8 ± 0.2	0.008 ± 0.003	81.3 ± 12.6	4.0 ± 0.2	0.050 ± 0.008
			Chlorogenic acid			
Undigested						
Black carrot	95.7 ± 1.5	0.7 ± 0.2	0.007 ± 0.002	94.9 ± 2.8	2.6 ± 0.3	0.027 ± 0.003
Peel	100.4 ± 3.9	0.7 ± 0.5	0.007 ± 0.005	94.9 ± 3.1	2.1 ± 0.5	0.022 ± 0.005
Pomace	96.5 ± 0.4	0.8 ± 0.1	0.008 ± 0.001	93.0 ± 0.8	3.2 ± 0.5	0.034 ± 0.005
Digested						
Black carrot	89.4 ± 15.2	0.2 ± 0.0	0.002 ± 0.001	80.0 ± 14.2	6.1 ± 0.3	0.077 ± 0.011
Peel	84.9 ± 7.0	0.2 ± 0.0	0.003 ± 0.000	82.9 ± 8.4	5.2 ± 0.2	0.063 ± 0.007
Pomace	83.2 ± 16.8	0.7 ± 0.3	0.008 ± 0.002	71.6 ± 9.6	4.5 ± 1.0	0.062 ± 0.007
			Caffeic acid			
Undigested						
Black carrot	89.6 ± 5.2	0.5 ± 0.1	0.006 ± 0.001	85.4 ± 4.5	1.6 ± 0.4	0.018 ± 0.004
Peel	84.8 ± 13.7	0.2 ± 0.1	0.003 ± 0.002	72.8 ± 6.0	2.3 ± 0.2	0.032 ± 0.003
Pomace	99.5 ± 3.0	0.3 ± 0.1	0.003 ± 0.001	91.0 ± 6.6	2.6 ± 1.0	0.028 ± 0.009

[a]Apical and basal recovery (%) = (concentration after transport)/(initial concentration)*100; [b]Transport efficiency = (basal recovery,%)/(apical recovery,%), [c]nd: not detected.

Table 4.4 : (*Cont.)* Apical and basal side recoveries and transport efficiencies of phenolic acids from black carrot, peel and pomace samples.

Sample	t = 2			t = 4		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]
			Caffeic acid			
Digested						
Black carrot	96.9 ± 12.0	2.3 ± 0.6	0.023 ± 0.005	88.7 ± 8.9	7.0 ± 1.0	0.079 ± 0.015
Peel	86.1 ± 2.5	2.2 ± 0.9	0.023 ± 0.010	70.5 ± 7.2	6.4 ± 1.4	0.092 ± 0.025
Pomace	96.4 ± 14.5	2.2 ± 0.1	0.023 ± 0.005	78.4 ± 20.4	5.3 ± 1.1	0.072 ± 0.026

[a]Apical and basal recovery (%) = (concentration after transport)/(initial concentration)*100; [b]Transport efficiency = (basal recovery,%)/(apical recovery,%), [c]nd: not detected.

4.3. Cytotoxicity Assay

After the transport, to analyze the cytotoxic effects of black carrot polyphenols on cells, the MTT assay was applied. The results were expressed as percentage compared to untreated cells (Figure 4.2). No cytotoxicity was observed on Caco-2 cells after treatment of digested and undigested samples with black carrot, pomace and peel. Only a significant difference among the results was found for the undigested pomace sample which increased the mitochondrial activity of the cells.

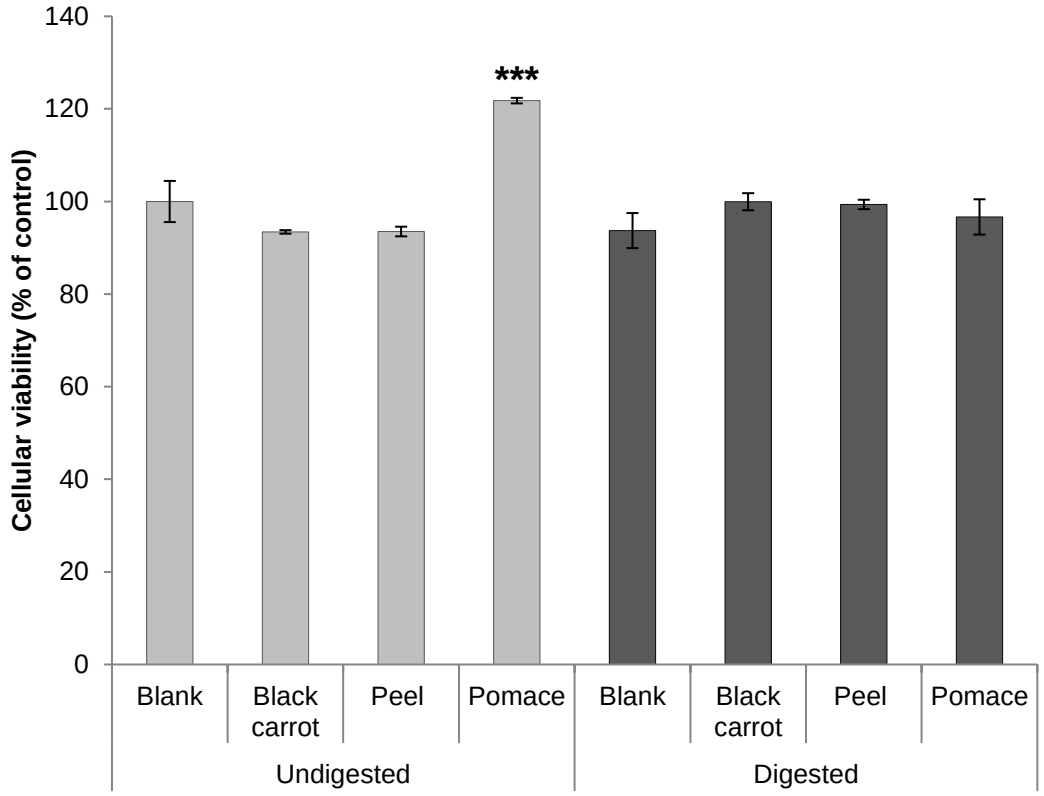


Figure 4.2 : Mitochondrial activity (measured using MTT assay) of differentiated Caco-2 cells exposed to undigested and digested forms of black carrot, peel, pomace and no treatment (blank) for 4 h. Statistical differences compared to undigested blank are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4.4. Intracellular Reactive Oxygen Species (ROS)

A kinetic analysis of the endothelial reactive oxidant species production, following peroxynitrite challenge, revealed an increase in intracellular oxidative

stress after 4 h of incubation. The effect of anthocyanins from black carrot peel and pomace on ROS was found to be dependent on TNF-α treatment. Under non-inflammatory conditions, the effect of the digested pomace sample increased the ROS level significantly ($p<0.01$). On the contrary, in TNF-α treated samples, a decrease of approximately 20% was seen after incubation with digested pomace sample. In addition, under inflammatory conditions both undigested and digested black carrot samples show an upregulatory effect on ROS formation and intracellular levels of ROS were reduced after 4 h incubation. These results indicate that addition of undigested and digested black carrot and digested pomace sample in the co-culture setup may reduce the oxidative stress induced in the endothelial cells by TNF-α. These findings are consistent with literature (Paixao et al, 2011).

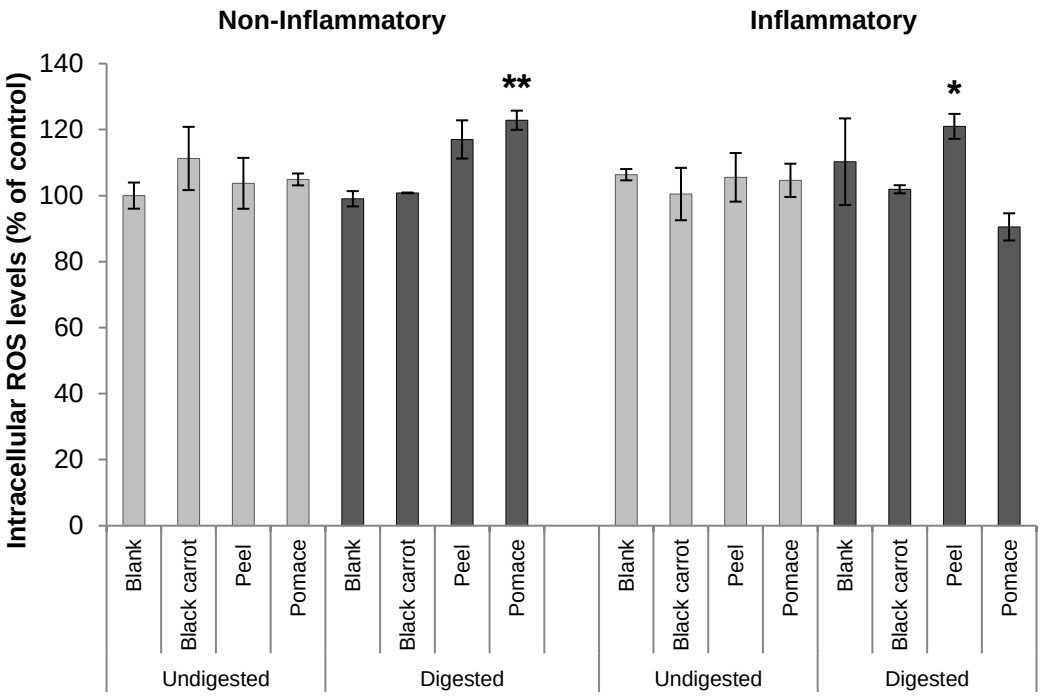


Figure 4.3 : Changes in intracellular ROS levels of endothelial cells in response to 4 h treatment of undigested and digested forms of black carrot, peel and pomace, under TNF-α-induced inflammatory and non-inflammatory conditions. Statistical differences compared to non-inflammatory undigested blank are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, whereas the differences to inflammatory undigested blank are indicated as $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$.

4.5. Nitric Oxide (NO) Production in Endothelial Cells

Endothelium-derived nitric oxide (NO) is normally produced by endothelial cell nitric oxide synthase (ecNOS) (Hou et al, 1999). In mammals including humans, NO is an important cellular signalling molecule involved in many physiological and pathological processes. Certain levels of nitric oxide production are important in protecting organs such as the liver from ischemic damage. The endothelium of blood vessels uses nitric oxide to signal the surrounding smooth muscle to relax, thus resulting in vasodilation and increasing blood flow (Stryer, 1995; Hou et al, 1999).Undigested and digested samples of black carrot, pomace and peel samples increased NO production during both inflammatory and non-inflammatory conditions after 4 hours treatment. It was also observed that the effect of black carrot anthocyanins on TNF-α treated cells is higher than cells without inflammation. In addition to this, digested black carrot sample significantly increased NO production after 4 hours treatment in both TNF-α treated conditions ($p<0.01$). These data are consistent with the stimulating effect of anthocyanins on NO production in literature (Pojer et al, 2013) . It has to be considered that the measured NO could also be produced by the Caco-2 cells in co-culture.

Changes in ROS and NO levels in the co-culture demonstrated a clear effect of the communication between cell lines. Upon inflammation, the consistent decays on cellular viability and intracellular ROS indicated damage of the endothelial cells due to oxidative mechanisms and the combined metabolism in the co-culture. This damage may be delayed by black carrot anthocyanins, but not restored.

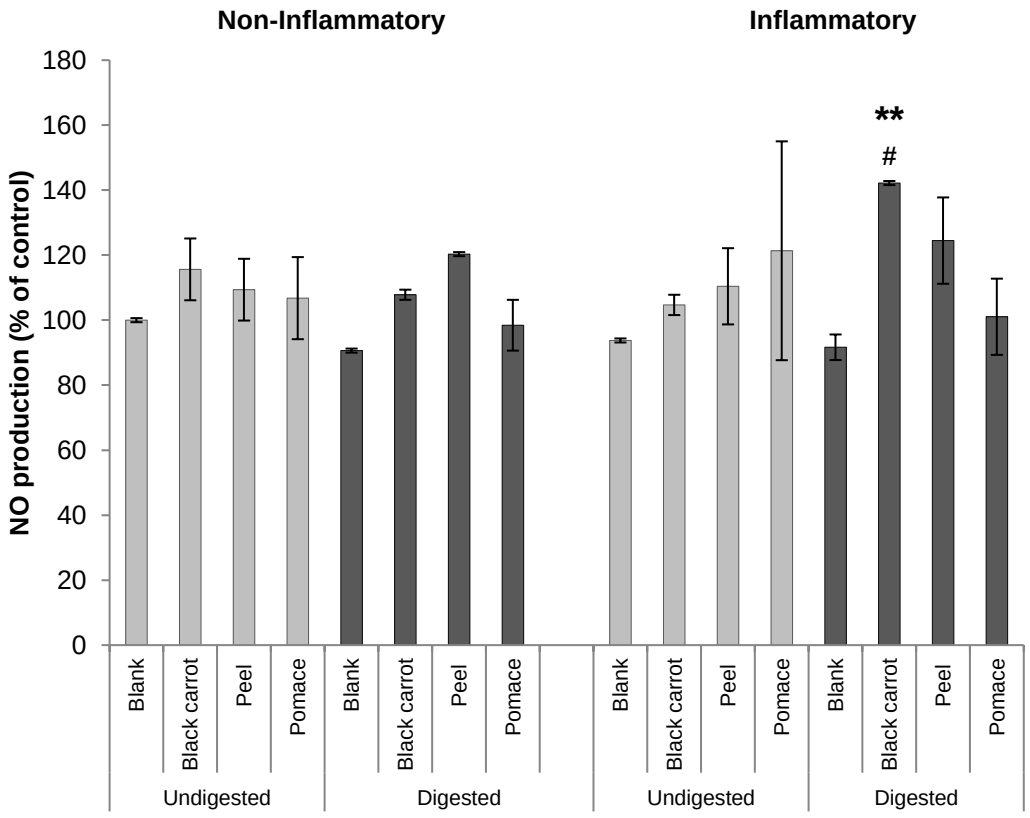


Figure 4.4 : Changes in NO production of endothelial cells in response to 4 h treatment of undigested and digested forms of black carrot, peel and pomace, under TNF-α-induced inflammatory and non-inflammatory conditions. Statistical differences compared to non-inflammatory undigested blank are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, whereas the differences to inflammatory undigested blank are indicated as $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$.

4.6. Enzyme-Linked Immunosorbent Assays (ELISA)

In order to investigate the anti-inflamatory effect of anthocyanins in black carrot, pomace and peel, 4 markers, monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), interleukin-8 and intercellular adhesion molecule-1 (ICAM-1), were used. Results expressed as percentages are shown in Figure 4.5.

In TNF-α-treated cells, the secretion of markers was significantly higher in comparison with basal values of the non-inflammatory condition ($p<0.001$). As expected, secretion of the endothelial growth factor, IL-8 cytokine, MCP-1 and the adhesion molecule were largely increased by induction of inflammation with

TNF-α. In fact, TNF-α modulates the expression of up to 4,000 genes in endothelial cells, mostly related to cell adhesion, inflammation and chemotaxis proteins (Claude et al, 2014).

Under healthy conditions, IL-8 concentrations significantly decreased in co-cultured cells treated with digested black carrot samples ($p<0.001$), also a large decrease was seen in cells treated with undigested black carrot and pomace samples ($p<0.05$, $p<0.01$ respectively). Upon TNF-α activation, the secretion of IL-8 consistently reduced in cells exposed to both undigested and digested peel and pomace samples compared to the untreated cells. Under non-inflammatory conditions, anthocyanins from both digested and undigested black carrot, pomace and peel samples decreased VEGF secretion after 4 hours of treatment. Especially undigested pomace and peel samples showed a significant effect on this marker ($p<0.001$). Under TNF-α induced conditions, decrease in secretion of VEGF was observed much more significantly for both digested and undigested samples ($p<0.001$). MCP-1 expression significantly increased upon TNF-α stimulation. However cells treated with all samples showed a significant decrease on MCP-1 levels. For the ICAM-1 marker, it was also found that both undigested and digested samples showed a significant downregulatory effect after 4 hours treatment compared to untreated cells. These findings confirm the data in literature (Hidalgo et al, 2012; Kuntz et al, 2015a; Olejnik et al, 2016). The secretion of these signal molecules may also be influenced by Caco-2 cells in co-culture.

It was observed that upon exposure to TNF-α, levels of VEGF, NO and ROS were increased in the co-culture. This effect may be explained by activation of mitochondrial Romo1 receptor, boosting ROS formation; and by upregulation of VEGF synthesis in endothelial cells, causing stimulation of the tyrosine kinase receptor VEGFR/Flt (KDR) involved in eNOS phosphorylation and NO activation (Kim et al, 2010). Despite the significant effect of black carrot anthocyanins on reducing secretion of proinflammatory molecules in the co-culture, decreases on ICAM-1, VEGF, MPC-1 and IL-8 concentrations in relation to each control were more pronounced in TNF-α-activated cells. Thus, suggesting that anthocyanins in black carrot, pomace and peel operate through an inhibitory regulation of the inflammatory cascade in endothelial cells, which can

maintain a more sustained effect under pre-existing inflammation. In conclusion, anthocyanins both from digested and undigested black carrot, pomace and peel samples crossed the intestinal cell layer and ameliorated the inflammatory response in endothelial cells by downregulating the expression of pro-inflammatory cytokines, particularly IL-8, and by reducing the levels of adhesion molecules.

These results indicate that this co-culture model is suitable for polyphenol bioavailability studies. Before transport and co-culture experiments, several preliminary experiments were carried out to reach the optimum conditions for both cells and phenolic compounds. Different kinds of media for transport experiments were examined. pH levels in apical and basal compartments were adjusted. No adjustment, adjustment both apical and basal side to pH 7, adjustment of both apical and basal side to pH 7.5 and adjustment apically at pH 6.5 and basally at pH 7.5. Since pH levels in blood and intestinal system are known to be approximately pH 6.5 and pH 7.5 respectively, it was decided to use the apical and basal part as this can simulate a better in-vivo bioavailability setup. It has been observed that medium composition in the transport experiments has a significant importance and that pH adjustment is required for anthocyanin transport experiments and bioavailability studies.

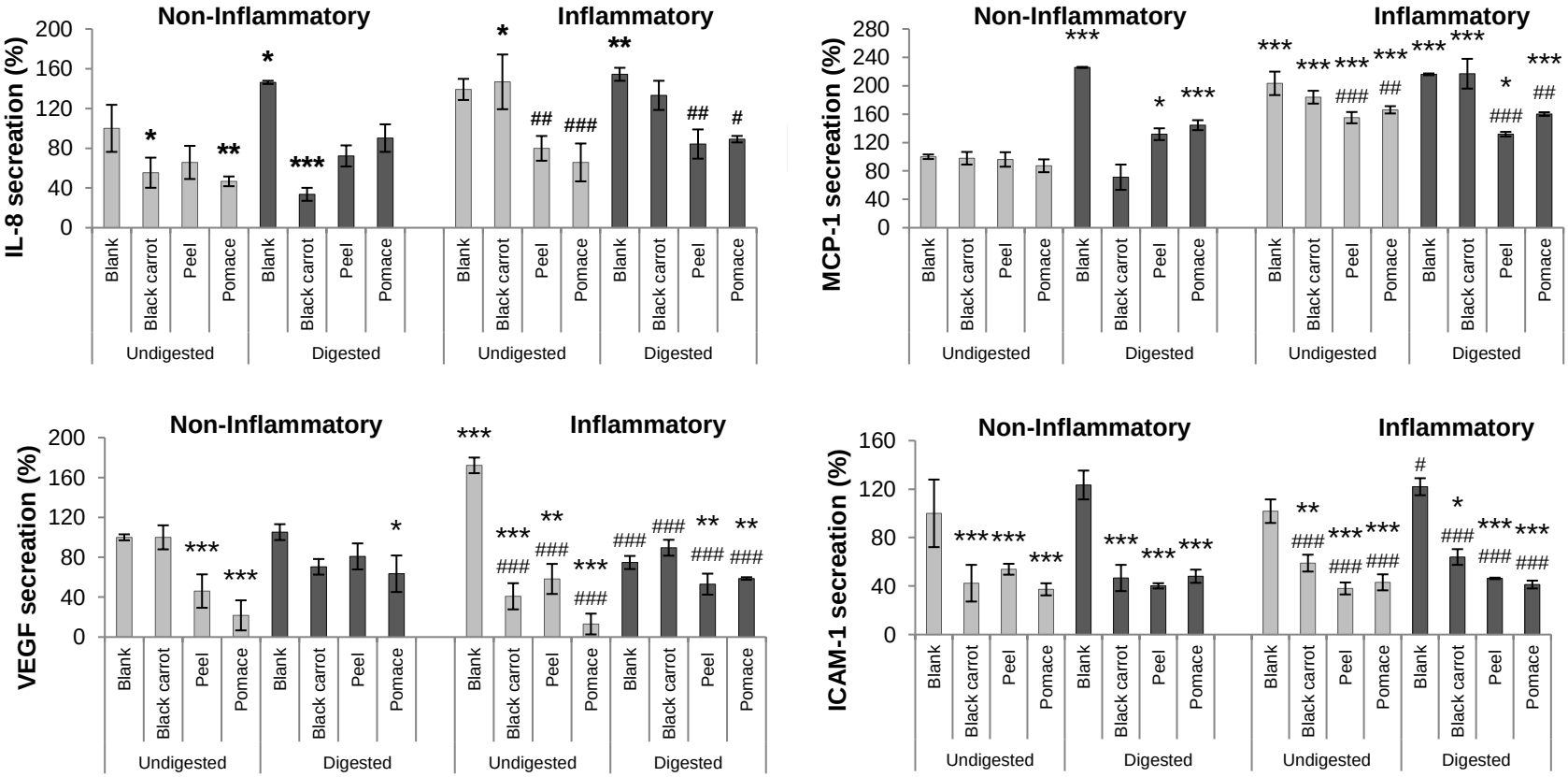


Figure 4.5 : Secretion of IL-8, MCP-1, VEGF and ICAM-1 in endothelial cells in response to 4 h treatment of undigested and digested forms of black carrot, peel and pomace, under TNF-α-induced inflammatory and non-inflammatory conditions. Statistical differences compared to non-inflammatory undigested blank are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, whereas the differences to inflammatory undigested blank are indicated as $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$.

5. CONCLUSION

Studies regarding *in-vitro* bioavailability and bioactivity have been investigated with cell models. In this study we used this co-culture model for the first time with black carrot samples. Transport experiments with Caco-2 cell lines were carried out to determine the biavailability and the co-culture model with Caco-2 and Eahy926 cells was used to evaluate bioactivity of polyphenols in black carrot.

In order to have an optimal simulation of absorption and bioactivity in the human body, several preliminary experiments were carried out. For experimental design, pH was set to 6.5 in apical compartment and to 7.5 in basal compartment to simulate the environment of the intestinal system and blood vessel. In addition, transport medium has been evaluated several times and decided to be HBSS. These preliminary experiments were determined to have optimum conditions to perform the cell culture studies.

Results of transport experiments were monitored with HPLC-DAD. After 4 hours incubation, 5 major anthocyanins of black carrot named cyanidin 3-xylosyl(glucosyl)galactoside, cyanidin 3-xylosylgalactoside, cyanidin 3-xylosyl(sinapoylglucosyl)galactoside, cyanidin 3-xylosyl(feruloylglucosyl)galactoside, cyanidin 3-xylosyl(coumaroylglucosyl)galactoside and 3 major phenolic acids named neochlorogenic acid, chlorogenic acid and caffeic acid were found in the basal compartment in a range of 0.8-5.3 % and 1.6-7 %, respectively. TEER values were found to be close (74-93 %) to initial values after 24 hours, which indicates that black carrot polyphenols have no cytotoxic effect on living cells.

In co-culture experiments, under inflammatory conditions, samples were found to decrease ROS levels. Especially digested black carrot and pomace samples decreased ROS levels 9 % and 19 %, respectively. In the NO assay, digested black carrot sample increased the NO level significantly ($p<0.01$). In particular, with TNF-α treated cells results were more significant than with non-inflammatory ones. No cytotoxicicity was seen in Eahy926 cells in MTT

assay after treatment with samples and cell viability was found to be not different from the blanks. In order to determine the anti-inflammatory effect of black carrot polyphenols, ELISA markers, MCP-1, VEGF, ICAM-1 and IL-8 were used. It has been observed that under inflammatory conditions secretion levels of all markers increased. In addition, it has been found that cells with treated black carrot, pomace and peel samples have lower marker secretion. IL-8, MCP-1, VEGF and ICAM secretion decreased up to 77 %, 68 %, 92 % and 67 % respectively.

In both transport and co-culture experiments, digested samples gave better results than undigested samples. This finding was also important to have better simulation of the polyphenol metabolism in human body by using an *in-vitro* model. Overall black carrot, pomace and peel was found to have a good bioavailability and anti-inflammatory effect on cardiovascular diseases. Although these findings can not be predicted in *in-vivo* studies directly, still, this co-culture model was found to be applicable for bioavailability and bioactivity studies of polyphenols.

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