universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Protective effects of anthocyanins and degradation products on oxidative stress in human colon carcinoma cells“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften UG2002
Betreut von / Supervisor:	Univ. prof. Dr. Doris Marko

Acknowledgements

First, I would like to thank Prof. Doris Marko for the interesting topic, scientific expertise and the chance to work and learn in her workgroup. I would also like to thank my lab colleagues and mainly my co-supervisor Dr. Gudrun Pahlke for all the helpful discussions and advice, which most certainly made working and writing easier.

Further, I am ever so grateful for my family, which has always been far away but still close and supportive, my friends who accept that social time was limited, and last but not least my partner who has always supported me through high and low during the whole time of this thesis project.

Thank you!

Declaration of originality

I hereby declare that this master´s thesis and the work reported was originated and composed entirely by me.

Furthermore I confirm that the information derived from published and unpublished work of others has been acknowledged in the text and that references are given in the index of literature.

This work has not been submitted previously or concurrently – as far as I am aware of – to any examining board.

Vienna, January 2018 Signature: _____

Katarina Ahlberg

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$^1O_2$	Singlet molecular oxygen
AAPH	2,2′-Azobis(2-amidinopropane) dihydrochloride
ABAP	2,2´-Azobis (2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	2,2´-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AC	Antioxidative capacity
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism, Elimination
Al	Aluminium
ANOVA	Analysis of variance
ARE	Antioxidant Response Element
ATCC	American Type Culture Collection
ATP	Adenosin triphosphate
bw	body weight
C2BBe1	Caco-2 brushborder expressing 1
CGE	Cyanidin-3-glc-equivalents
Cu	Copper
Cul3	Cullin 3
Cy	Cyanidin
Cy-3-glc	Cyanidin-3-glucoside
Cy-3-sam	Cyanidin-3-sambubioside
Cy-3-sam-5-glc	Cyanidin-3-sambubioside-5-glucoside
DAD	Diode array detector
DCF	2´,7´-dichlorofluorescein
DCFH	2´,7´-dichlorofluorescin
DCFH-DA	2´,7´-dichlorofluorescin diacetate
DMEM	Dulbeccos Minimal Essential Medium
DMSO	Dimethyl sulfoxide
Dp	Deplhinidin
DPPH	2,2-di(tertoctylphenyl)-1-picrylhydrazyl
E3	Ubiquitin-protein isopeptide ligase
EDTA	Ethylenediaminetetraacetic acid
EPIC	European Prospective Investigation into Cancer and Nutrition
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fe	Iron
Gal	Galactose
GIT	Gastrointestinal tract
Glc	Glucoside
GLUT2	Glucose transporter 2
GSKE	Grape Skin Extract
GSTs	Glutathione S-transferases
H_2O_2	Dihydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HO-1	Heme oxygenase-1
$HO^{\bullet}$	Hydroxyl radical
HPLC	High-performance liquid chromatography
IC50	Half maximal inhibitory concentration
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
KCl	Potassium chloride
Keap1	Kelch-like ECH-associated protein 1
KH2PO4	Monopotassium phosphate
LDH	Lactate dehydrogenase
Megal	Methyl-galactose
Mg	Magnesium
Mo	Molybdenum
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
Mv	Malvidin
$Na_2HPO_4 \cdot 2\ H_2O$	Disodium hydrogen phosphate dehydrate
NHANES	National Health and Nutrition Examination Survey
NQO1	Quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2 [NF-E2]-related factor 2
$O_2^{\bullet -}$	Superoxide anion
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
Ovrflw	Overflow
P/S	Penicillin/Streptomycin
PBS	Phosphate-buffered saline
PCA	Protocatechuic acid
Pg	Pelargonidin
PGA	Phloroglucinol aldehyde
Pn	Peonidin
Pt	Petunidin
ROO•	Peroxyl radical
ROS	Reactive Oxygen Species
SCF	Scientific Committee on Food
SGLT	Sodium glucose linked transporter
SOD	Superoxide dismutase
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TRAP	Total radical-trapping antioxidant parameter
TRIS	Tris (hydroxymethyl-) aminomethane
Trolox	6-hydroxy-2,5,7,8,-tetramethychroman-2-carboxylic acid
U	units
UGTs	UDP-glucuronosyltransferases
WST-1	Sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt

1 Introduction

Anthocyanins are colouring plant substances of great interest concerning protection against several chronic and degenerative diseases such as cancer (Cooke et al., 2005), cardiovascular disease (Cassidy, 2017; Thompson et al., 2017) and diabetes (Gowd et al., 2017). Research on mechanisms of action, effectiveness and possible toxicity is needed in order to be able to utilize the full potential of these promising pigments. This thesis is a part of the European project AnthoPLUS, where pure anthocyanins of different complexity in their side chain decoration are produced and assessed for their quality and properties (AnthoPLUS). This thesis focuses primarily on the toxicity and antioxidative potential of anthocyanins contained in elderberries (*Sambucus nigra*) in C2BBe1 (Caco-2 brushborder expressing 1) human colon carcinoma cells. The respective main metabolites are tested as well regarding production of reactive oxygen species (ROS), in particular the influence on intracellular stress levels and possible protective effects against an induced high ROS-level as measured with the DCF Assay. In this context the question is addressed whether potential degradation products contribute to the antioxidant effects of anthocyanins.

After a brief overview of anthocyanins in general, the theoretical part focuses on cyanidin-based anthocyanins, on their prevalence, bioavailability and effects, while going in to more detail on assays with which antioxidative activity and toxicological effects can be quantified, in order to give a base to the following presentation of the utilized assays, the experimentally obtained data and the discussion thereof.

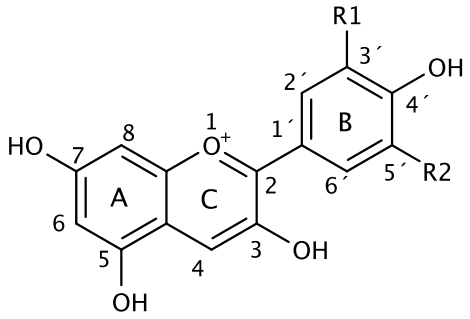
2 Theoretical background

2.1 Anthocyanins

Anthocyanins are a subclass of the large group of secondary plant metabolites flavonoids, consisting of phenolic rings with different side chains. The name derives from the Greek, with “anthos” = flower and “kyanos” = blue. Anthocyanins occur mainly in berries, flowers and fruit, where they are responsible for the blue, purple and red colours, although they are also present in vegetables, roots, legumes and cereals (Fleschhut et al., 2006; Kamiloglu et al., 2015).

2.2 Chemical structure

Anthocyanins are glycosides of derivatives of 2-phenylbenzopyrylium or flavylium salts with different numbers of hydroxy- and/or methoxy groups, different nature, number and position of attached sugar moieties even with aliphatic or aromatic acids (Kong et al., 2003). The sugar moieties are often bound with β-3-*O*-glycosidic or β-3,5-*O*-diglycosidic bonds. Figure 1 shows the aglycons of the most common anthocyanins, termed anthocyanidins, which are rarely found in their free form in plant dietary material (Keppler & Humpf, 2005).



Anthocyanidin	R_1	R_2
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Malvidin (Mv)	OCH_3	OCH_3
Pelargonidin (Pg)	H	H
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OH	OCH_3

Figure 1: Anthocyanidin structures, modified according to Kamiloglu et al. (2015)

According to Kong and colleagues the most common anthocyanins in edible plant parts are cyanidin-based with 50%, followed by pelargonidin, peonidin, delphinidin, petunidin and malvidin, all around 12 – 7 %. The 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides are common, with 3-glycosides and in particular cyanidin 3-*O*-glucoside (Cy-3-glc) being by far the most common. (Kong et al., 2003)

2.3 Exposure

The average consumption of anthocyanins in the US was estimated with data from the National health and nutrition survey study (NHANES) to be 12.5 mg/day (Wu et al., 2006) whereas in Europe the range lay between 18.73 and 64.88 mg/day with a mean of 29.44 mg for men and 33.52 mg for women as estimated through the EPIC-study (European prospective investigation into cancer and nutrition) and nutrition databases (Zamora-Ros et al., 2011).

2.3.1 Food content

Food content can be considerably higher, reaching 2 - 4 g/kg fresh weight of blackcurrants or blackberries, values which increase as the fruit ripens (Manach et al., 2004). Consumption data are derived from food frequency questionnaires and dietary recalls which may imply an underestimation of intake due to poor anthocyanin representation of food composition databases. A single serving of berries and juices may contain 400 - 500 mg anthocyanins (Kay et al., 2009). Table 1 presents some examples of the anthocyanin content of berries.

Table 1: Anthocyanin content of selected berries, a) Fan-Chiang and Wrolstad (2005) , b) Jakobek et al. (2007), c) Prior et al. (2001) d) Stevenson (2012), e) Oertel et al. (not published) , f) Anton et al. (2013)

Source	Anthocyanins (mg/L or mg/kg)
Blackberry, fresh	700 – 2010[a]
Blackcurrant, juice	1544[b]
Blueberry, fresh	570 – 5030[c]
Chokeberry, juice	3042[b]
Cranberry	3600[c]
Elderberry, juice	1190[e] – 4188[b]
fresh	2729[f]

2.3.2 Dietary supplements

Amounts in commercially available dietary supplements can vary greatly, for example containing up to 12 g elderberry extract per serving of syrup, or around 30-40 mg anthocyanins per capsule with a recommended daily intake (according to producer) of 594 mg extract containing 13 % anthocyanins, hence equalling 78 mg anthocyanins/day (Natures Answer; Schweikart; Sunday Natural).

2.3.3 Food additives

Anthocyanins are approved as a food additive (E163) with an ADI (acceptable daily intake) of 2.5 mg/kg bodyweight (bw) set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982. The Scientific Committee on Food (SCF) in 1975 did not, and the European Food Security Authority (EFSA) could not set a numerical ADI in their 2013 re-evaluation due to the currently inadequate toxicological data acquisition. Aqueous grape skin and blackcurrant extracts were however not seen as likely to be of safety concern. As a food colouring substance a maximum permitted level of 200 mg/kg food in fruit-flavoured breakfast cereals is authorized, and in most other foods anthocyanins from fruits and vegetables are permitted at *quantum satis*. The mean exposure to E163 was estimated to 0.7 - 1.9 mg/kg bw/day for adults (high level exposure 1.1 - 3.8 mg/kg bw/day) whereas the mean estimated exposure for toddlers and children was about 1.5 - 4 mg/kg bw/day. The JECFA and European commission have different specifications for anthocyanins, although both are only available for grape skin extract (GSKE) and blackcurrant extract. GSKE contains glucosides of peonidin, malvidin, delphinidin and petunidin, whereas blackcurrant extract contains cyanidin-3-rutinoside, delphinidin-3-rutinoside, cyanidin 3-glucoside and delphinidin 3-glucoside. (EFSA, 2013)

2.4 Anthocyanin degradation and metabolism

Most anthocyanins are highly reactive and can easily degrade or react with constituents of the media, forming colourless or brown compounds and pigments. The presence of oxygen, light, enzymes and pH have a large effect on anthocyanin colouring and stability (Jackman et al., 1987). Since in this thesis only cyanidin-based structures were tested, focus will be on these.

2.4.1 pH dependant structure

The pH of the solution directly influences the chemical form and colour of the anthocyanins. At pH 1, the flavylium cation with its red and purple hues is the predominant form, followed by the quinoidal blue species through rapid loss of a proton at pH 2 - 4 and the colourless carbinol pseudobase and chalcone between pH 5 and 6 (figure 2). McGhie & Walton describe a slower hydration of the flavylium form to the colourless hemiketal form. Above pH 7 the

anthocyanins degrade differently depending on their substituted groups. (Castañeda-Ovando et al., 2009; McGhie & Walton, 2007)

At pH 1-3, while in their reddish flavylium forms, anthocyanin 3-glucosides are stable in aqueous solution, even after 60 days of storage at 10°C, whereas as the pH is shifted towards neutrality (pH 5 - 7) the colours turn bluish and the stability decreases. (Cabrita et al., 2000)

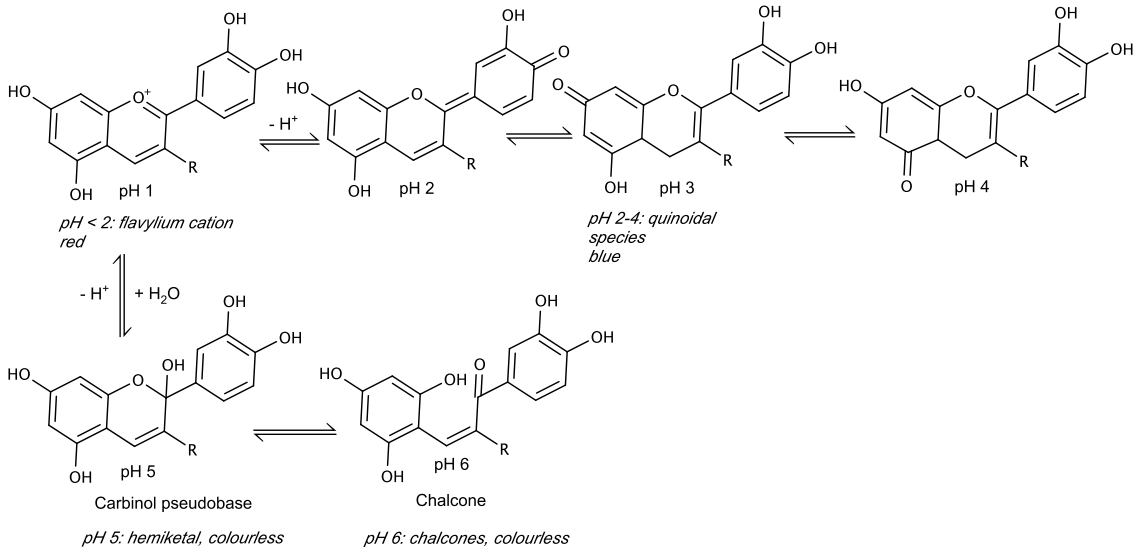


Figure 2: pH-dependency of cyanidin-based anthocyanins, modified after Castañeda-Ovando et al. (2009)

Anthocyanins can also be stabilized and colour be retained through a phenomenon called co-pigmentation. Pigments can form molecular or complex associations with other organic compounds or metallic ions, forming electron rich systems, which protect the electron-poor flavylium ion against nucleophilic attacks at the position 4. Anthocyanins with o-di-hydroxyl groups in the B ring (Cy, Dp, Pt) are known to form complexes with metals such as Al, Fe, Cu, Sn, Mg and Mo, producing blue hues (Castañeda-Ovando et al., 2009).

2.4.2 Degradation

Fleschhut et al. (2006) found that the anthocyanidins Mv, Cy, Pn and Dp had almost completely disappeared after a 1-hour incubation of 160 µM at neutral pH. After 30 minutes the HPLC/DAD/MS showed peaks assumed to be the dimerization products of two Cy due to the reactive quinoid form, a major pH form in neutral media. The corresponding phenolic acid, in this case

protocatechuic acid (PCA), was also present. The mono- and di-glucosides are more stable in neutral media, with the sugar moieties preventing the degradation of the unstable diketone to phenolic acid and aldehyde. Incubations with human faecal flora on the other hand showed a rapid degradation of the glycosides to their phenolic acids and aldehyde, with the colonic microbiota responsible for the deglycosylation (Fleschhut et al., 2006). This degradation process is pictured in figure 3.

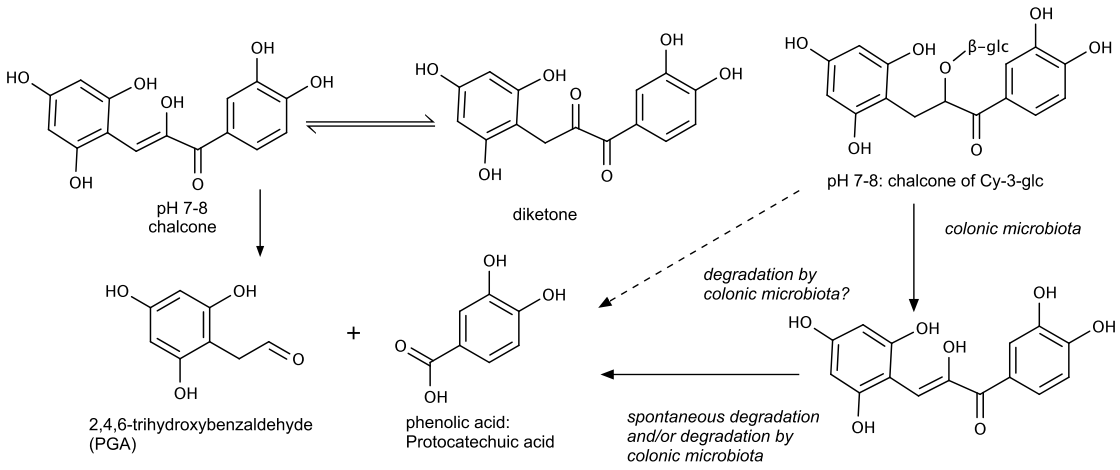


Figure 3: Degradation process of cyanidin and Cy-3-glc, after Fleschhut et al. (2006)

2.4.3 Metabolism and bioavailability

2.4.3.1 Bioavailability

The bioavailability of anthocyanins has been described as very poor compared with other flavonoids. Flavonoids are often detected in plasma and urine in the form of glucuronidated and/or sulphated derivatives, which for anthocyanins with some exceptions has not been the case, as only unchanged glycosides were found. According to Manach et al. (2005) this might be due to the anthocyanin metabolites being very unstable, as proven in a study by Felgines et al. (2003) where many metabolites degraded during the freezing of the acidified samples, whereas in the freshly processed samples >80 % of the total metabolites of strawberry anthocyanins were identified as glucuronides.

The extensive pre-systemic metabolism of anthocyanins could be an explanation for the observed low bioavailability (Fang, 2014b) of around 1 %

only (Kamiloglu et al., 2015). This was shown in the study of Czank et al. (2013) in humans consuming 500 mg $^{13}C_5$-labeled Cy-3-glc with a minimum relative bioavailability of 12.3 ± 1.3 % and metabolites reaching a 42 times higher serum concentration peak, which occurred later than the parent anthocyanin peak. Absorption, distribution, metabolism and elimination (ADME) of anthocyanins and their mechanism of action is still not quite clear, and since there are already notable effects regarding reduced cardiovascular disease and antioxidative effects the question arises whether their bioavailability and/or consumption has been underestimated or if anthocyanins are potent at nanomolar serum concentrations. (Czank et al., 2013)

2.4.3.2 Metabolism and distribution

Mouth

The metabolism of anthocyanins starts to a certain extent in the mouth. During the short residence time anthocyanins can be degraded by microbiota, temperature and binding to salivary proteins as well as the aglycones being detached by oral native or microbiota-related glycosidases. (Lila et al., 2016; Mallery et al., 2011)

Stomach

A quick absorption of anthocyanins into the bloodstream after consumption suggests that the stomach is an important site of absorption. There seems to be a consensus on phenolic acids (PCA in the case of cyanidin-based anthocyanins) and phloroglucinol aldehyde (PGA) being the main degradation products, however whether the phenolic acids are formed in the gastrointestinal lumen before being absorbed or in the intestinal wall after absorption is still discussed (Fang, 2014b; Vitaglione et al., 2007). A study on rats fed a diet with blackberry anthocyanin extract for 15 days found indications that anthocyanins were absorbed from the stomach as intact glycosides, since only native anthocyanins (68.6 nmol Cy-3-glc/g tissue) were detected 3 hours after the last meal (Talavera et al., 2005). This concurs with the results of simulated gastric digestion experiments which showed no significant anthocyanin degradation, only loss of anthocyanin aglycones (Woodward et al., 2011). The organic anion carrier bilitranslocase seems to have a higher affinity to the anthocyanin glycosides than to their aglycones. It rapidly delivers anthocyanins to the portal

circulation followed by transit through the liver and into the systemic circulation (Lila et al., 2016).

Small intestine

Anthocyanins are efficiently and rapidly absorbed from the small intestine. They appear quickly in the circulation and are excreted in bile and urine as both intact anthocyanins and as metabolites such as methylated, glucuronidated and sulphated derivatives. Possible transporters for anthocyanin glycosides are discussed to be glucose transporters such as SGLT (sodium glucose linked transporter) and the GLUT2 (glucose transporter 2) transporter, whereas prior to passive diffusion of the aglycones brush border enzymes like the lactase phloridzin hydrolase may be responsible for the hydrolysation forming the more hydrophobic anthocyanidins (Fernandes et al., 2014; Lila et al., 2016).

The jejunum may exhibit the largest absorption capacity of the gastrointestinal tract (GIT) as found in a study on rats where the jejunum contained the highest amount of Cy-3-glc (605 nmol/g tissue) while also phase II metabolites such as methylated, glucuronidated forms and cyanidin were found (Talavera et al., 2005). Within 120 minutes of simulated duodenal digestion no phenolic acid degradation products were found, while significant losses of Cy-3-glc and anthocyanin aglycones has been observed (Talavera et al., 2005; Woodward et al., 2011). Along the passage through the GIT the amount of phenolic acids increases as it is catabolized over time and enterohepatic recycling of anthocyanins adds to the pool of anthocyanin-derived metabolites (Lila et al., 2016).

Large intestine

Incubations with gut microbiota from human faeces shows that the anthocyanins were deglycosylated by the colon bacteria to smaller phenolic compounds such as PCA (Fleschhut et al., 2006) or conjugates of the aglycone, e.g. Cy-3-glc to Cy and cyanidin-3-rutinosid to Cy-3-glc (Aura et al., 2005). This was taken further by Keppler and Humpf (2005) who incubated with microbiota isolated from the caecum of freshly slaughtered pigs under strict anaerobic conditions, finding that the released aglycons degraded spontaneously into phenolic acids and aldehydes with high stability. Czank et al. (2013) however found that Cy-3-glc degradation products and phase II conjugates appeared

rapidly in the serum and concluded that some degradation occurred in the small intestine, suggesting that colonic microbiota may not be needed for cleaving the anthocyanin ring.

Excretion

The excretion of anthocyanins takes place in urine and bile in the intact form and as metabolites as well as in volatile metabolites in breath (Fang, 2014a) and metabolites in faeces. During the first 6 hours after ingestion urine, followed by breath, is considered the main elimination pathway, whereas after 6 hours the elimination through faeces dominates. Mean recoveries as measured by Czank (2013) after ingestion of 500 mg [^{13}C]-Cy-3-glc administered to humans were 5.37 ± 0.67% (urine), 6.91 ± 1.59% (breath) and 32.13 ± 6.13% (faeces). Earlier studies has shown a recovery of 0.26 - 2.67% of ingested non labelled anthocyanins and their metabolites (Fang, 2014a). Vitaglione et al. (2007) determined PCA to be the main metabolite of Cy-3-glc after human consumption of blood orange juice, since 73 % of the ingested Cy-3-glc was detected in urine as PCA.

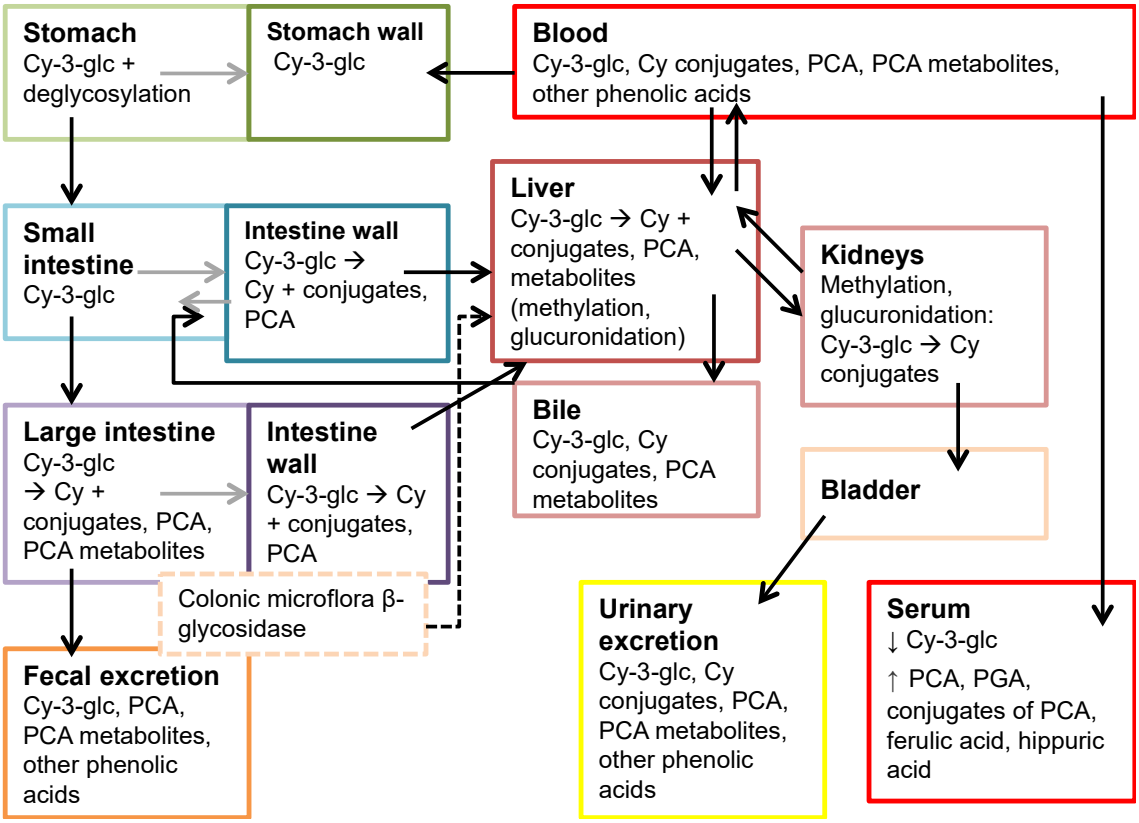


Figure 4: Schematic diagram of absorption and metabolism of anthocyanins, modified from Fang (2014b), Czank et al. (2013), Fernandes et al. (2014), McGhie and Walton (2007)

Serum

The tracer study by Czank (2013) reports that the Cy-3-glc peak serum concentration of 0.14 ± 0.05 µmol/L was reached around 1.8 h after consumption, with the degradation products (PCA and PGA) reaching 5 times higher concentrations. The major metabolites (phase II conjugates of PCA, ferulic acid and hippuric acid peaked 6-24 h post consumption at concentrations between 0.94 ± 0.37 and 2.35 ± 0.15 µmol/L. The serum C_{max} of Cy-3-glc after a more natural consumption of 71 mg cyanidin-glycosides as contained in one litre of blood orange juice was measured at 1.9 nmol/L and that of PCA 492 nmol/L 0.5 and 2 h post consumption (Vitaglione et al., 2007) whereas the maximum plasma concentration of total anthocyanins after the intake of 720 mg anthocyanins contained in 12 mg elderberry extract of 97.7 nmol/L was reached within 71.3 min (Cao et al., 2001).

Caco-2 cells

Since the Caco-2 cell line exhibits morphological and functional characteristics of small intestinal cells it is often used for cellular permeability studies of polyphenols as well as their original purpose of cytotoxic screening of anti-tumour drugs (Kamiloglu et al., 2015). The cells are described in chapter 8.1.1 (Materials and Methods), of colonic origin and are often used as a colon cancer model (Forester & Waterhouse, 2010). Incubations with Cy-3-glc indicate that a metabolism of PCA and PGA in Caco-2 cells takes place with occurrence of glucuronide and sulphate conjugates (Kay et al., 2009).

2.5 Test substances

2.5.1 Cyanidin-3-glucoside

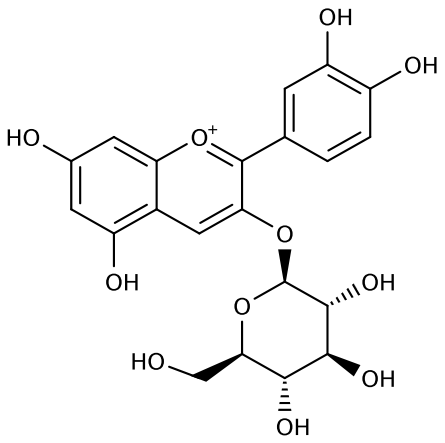


Figure 5: Chemical structure of cyanidin-3-glucoside

Cyanidin-3-glucoside, kuromanin with its common name, is the most widespread anthocyanin (Kong et al., 2003) and the main anthocyanin of blackberries and elderberries, making up 89.65% of anthocyanins detected with mass spectrometry (MS) in blackberry juice (Oertel & Matros, unpublished), and depending on the variety and fruition of the elderberries, between 204 – 586 mg CGE (Cy-3-glc-equivalents)/ 100 g fruit (Sidor & Gramza-Michałowska, 2015). The monoglucoside is composed of the aglycon cyanidin with glucose *O*-glycosidic bound to C3.

2.5.2 Cyanidin-3-sambubioside

Cyanidin-3-sambubioside (Cy-3-sam) is, together with Cy-3-glc one of the two major anthocyanins of elderberries (*Sambucus nigra*) (Sidor & Gramza-Michałowska, 2015). Attached to the cyanidin at position 3 of the C-ring is the disaccharide sambubiose (2-*O*-β-*D*-xylopyranosyl-β-*D*-glucopyranose, figure 6A).

Elderberries (figure 6B) were found to contain a range of Cy-3-sam between 122.2 - 630.8 mg / 100 g fresh weight, varying with study and cultivar, while the percentage of Cy-3-sam of total anthocyanin in the juice thereof also varied, from minor to main component with 85.55% anthocyanins identified as Cy-3-sam as measured with MS-fragmentation and photometric assays (Oertel et al.; Sidor & Gramza-Michałowska, 2015).

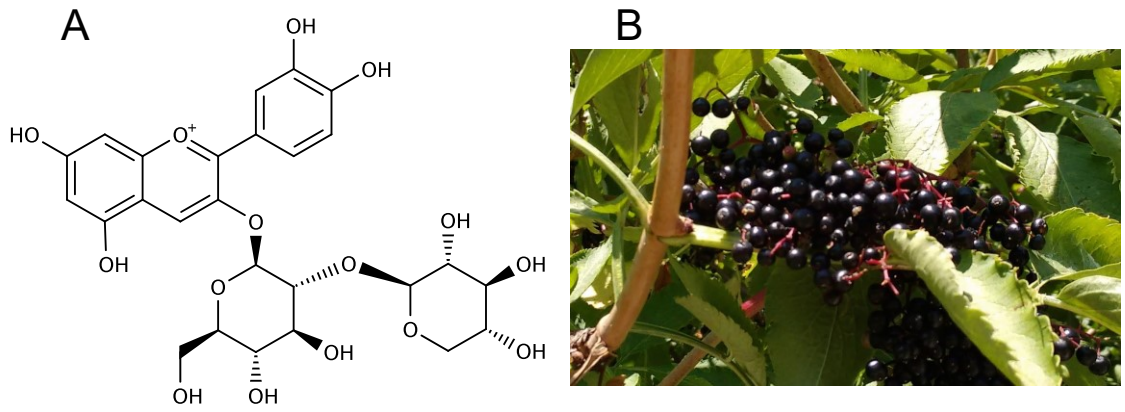


Figure 6: A) Chemical structure of cyanidin-3-sambubioside, B) ripe elderberries

Elderberry products are often used as a remedy against the common cold and influenza, where extract in form of capsules or syrup is consumed to reduce the time of recovery. This may be explained by the presence of anthocyanins counteracting oxidative stress, by the antibacterial and antiviral activities or by regulating the immune system regarding pro- and anti-inflammatory cytokines (Sidor & Gramza-Michałowska, 2015). The use of these products according to manufacturer's instructions could provide for example 78 mg anthocyanins per day in capsules of 5.94 g extract (Sunday Natural) and up to 48 g extract per day in elderberry syrup (Natures Answer).

2.5.3 Cyanidin-3-sambubioside-5-glucoside

Cyanidin-3-sambubioside-5-glucoside (Cy-3-sam-5-glc) is one of the main anthocyanins of elderberries, forming up to 55.5% of the total cyanidin content, the content varying widely within and between studies from 14 to 82 mg Cy-3-glc equivalents (CGE) / 100 g fresh weight (Sidor & Gramza-Michałowska, 2015). Data from an elderberry juice extraction however, using the more precise analytical method MS, yielded only 14.12% of the anthocyanins as Cy-3-sam-5-glc (Oertel et al.), consistent with 15% in juice extracts measured with HPLC (Jakobek et al., 2007). The structure of Cy-3-sam-5-glc is identical with the previously mentioned cyanidin-3-sambubioside except for the addition of a second glucose with an O-β–glycosidic bond to C5 at the A-ring (figure 7).

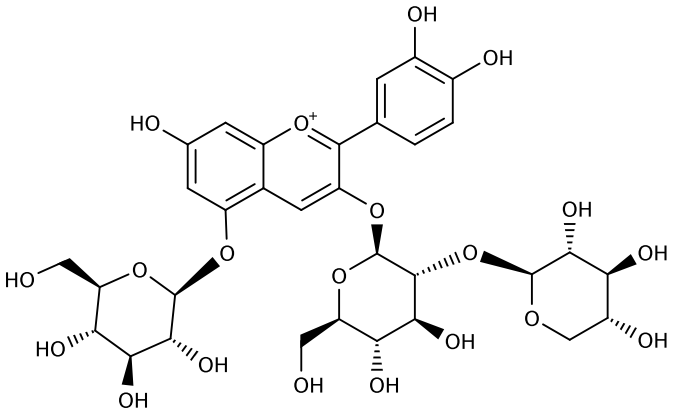


Figure 7: Chemical structure of cyanidin-3-sambubioside-5-glucoside

2.5.4 Cyanidin

The aglycon cyanidin is isolated as the chloride salt of the 3,3´,4´,5,7-pentahydroxyflavylium ion, which form metallic brown-red needles (RÖMPP, 2011). Bound with glucose on carbon in position 3 it forms the most common anthocyanin cyanidin-3-glucoside. The cyanidin glycosides can be hydrolysed by beta-glycosidase reactions in the intestine and by colonic microbiota, releasing the unstable aglycon (Galvano et al., 2004).

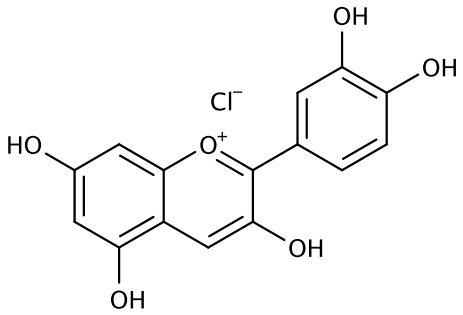


Figure 8: Chemical structure of cyanidin chloride

The mean intake of cyanidin in Europe is calculated as 12.01 mg/day for men and 15.09 mg/day for women, which is 40.1% respectively 45% of the total daily anthocyanin intake. The EPIC study shows a lower intake of cyanidin in northern Europe than in central and southern parts. Fruits, nuts and seeds were the food group with the largest percentage consumption (55.5 - 76.6%), with “apple and pears” and “stone fruits” contributing with 21.3 - 34.9% of the cyanidin intake, while berries with their high amounts of anthocyanins only contributed with 2.6 - 9.6%. Leafy vegetables reached a percentage of 14.3% in

southern Europe, while only 5.5% was reached in northern Europe. Non-alcoholic beverages with fruit and vegetable juices and carbonated drinks were a large source of cyanidin in central and northern Europe with 21.7 - 26.5%, while wine reached a lower percentage of 1.2 - 2.6%. (Zamora-Ros et al., 2011)

2.5.5 Phloroglucinol aldehyde

Phloroglucinol aldehyde, or with its IUPAC name 2,4,6-trihydroxybenzaldehyde, is as previously mentioned recognized as a main degradation product and metabolite of anthocyanins. As can be seen in figure 3, the hydroxylation of the B ring of the parent anthocyanin results in a phenolic acid and phloroglucinol aldehyde.

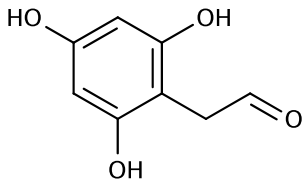


Figure 9: Chemical structure of phloroglucinol aldehyde

2.6 Stability

2.6.1 Glycosides and aglycon

According to Fleschhut et al. (2006) the anthocyanin-monoglucosides and even more the anthocyanin-diglucosides are rather stable under neutral pH conditions, where the sugar moieties can prevent the degradation of the unstable α-diketone intermediates to phenolic acid and the aldehyde. This was confirmed by Kay et al (2009) who found that the aglycon cyanidin was almost completely lost immediately upon incubation in cell free Dulbeccos minimal essential medium (DMEM) media whereas 57% of Cy-3-glc was lost after 4 h of incubation, producing the degradation products PCA and PGA. There was no significant loss of the phenolic components when incubated at the same conditions. Caco-2 cell incubations showed no significant differences in loss of Cy-3-glc or occurrence of PCA and PGA which suggests a spontaneous chemical breakdown of the compound and not an enzymatic deglycosylation induced by the Caco-2 cells (Kay et al., 2009)

2.6.2 Phloroglucinol aldehyde

In contrast to phenolic acids only very small amounts of phloroglucinol aldehyde (PGA) were detected after 24 h incubation at physiological conditions in sterilized inoculum filtrate (Keppler & Humpf, 2005) or cell culture media, where less than 10% was recovered and even 1 h incubation resulted in over 20% loss (Forester & Waterhouse, 2010). Kay et al (2009) on the other hand found no significant loss of either protocatechuic acid (PCA) or PGA after 4 h incubation in phosphate buffered saline (PBS) or cell culture media. Due to aldehydes being highly reactive Forester and Waterhouse assume that condensation with free amino-groups of amino acids or proteins and the formation of imines occurs. In non-sterilized inoculum filtrate PGA in small amounts was detected as an oxidation product (Kay et al., 2009).

2.6.3 Impact of catalase

Polyphenols have been reported to be able to form H_2O_2 when incubated in cell culture media, with Dulbecco's Minimum Essential Media (DMEM) producing the largest quantity (Long et al., 2000). These artefacts may pose a problem due to accumulating H_2O_2 interfering with cell viability and influencing the effectiveness of the tested compound. The ROS generation of the anthocyanin metabolites can be reduced by adding catalase to the cell culture media, which should decompose the excessive H_2O_2 (Forester et al., 2014). The addition of 100 U/ml catalase is enough to diminish the effect as tested with delphinidin, cyanidin and cyanidin-3-glucoside, whereas for PGA there was no enhanced H_2O_2 formation (Esselen et al., 2011; Forester et al., 2014; Kern et al., 2007).

2.7 Toxicological properties

The growth inhibiting properties of anthocyanins and the corresponding anthocyanidins on tumor cells has been studied in several cell lines with the incentive of finding anticarcinogenic or chemopreventive effects. An inhibitory effect in the growth of human cancer cells of several different origin such as breast, uterus, vulva, colon and lung was hereby evident (Cooke et al., 2005). In this case the anticarcinogenic effect was interesting but the main goal was to rule out cytotoxic effects on the cells, which may lead to a false positive ROS-reduction in the DCF assay evaluating the antioxidant effects of the test compounds. Since the research of the present thesis deals with cyanidin-based

anthocyanins in C2BBe1 colon cells, the focus will be put on colon cells and cyanidins.

2.7.1 Cytotoxicity test systems

Several test systems may be used such as the colorimetric assay sulforhodamine B assay where the optical density of the pink dye as measured with a photometer correlates linearly with the cellular protein content (Skehan et al., 1990) as explained in more detail in chapter 8.2.1 (Material and Methods). The tetrazolium salt based assays MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and WST-1 (sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt) are also based on colorimetric measurements of absorbance where the yellow dye MTT is reduced to purple formazan by living, metabolically active cells, using the activity of various intracellular dehydrogenase enzymes (Mosmann, 1983) whereas the soluble formazan of WST-1 is formed by extracellular transport of electrons across the plasma membrane of dividing cells (Berridge, Herst, & Tan, 2005). The LDH Assay is based on the enzyme cytoplasm lactate dehydrogenase, which due to the intact membrane being impermeable for this enzyme, is only released from damaged cell membranes. The activity of LDH is directly proportional to the absorbance of a formazan salt formed during the assay and linearly correlated to the cell concentration (Allen, 1994)

2.7.2 Berry extracts

Two major anthocyanins contained in elderberry (cyanidin-3-sambubioside and cyanidin-3-sambubioside-5-glucoside) have so far not been widely examined as single compounds but only as component of elderberry extracts.

Elderberry extract (with and without a simulated gastrointestinal digestion) reduced the metabolic activity of human non-tumorigenic colon cells NCM460 and caused cytotoxicity with an IC_{50}-value at 10.71 mg/ml (digested: 12.81 mg/ml) as measured with the MTT Assay. Unpublished data of the same group showed that treatment with the purified anthocyanin extract of Cy-3-sam and Cy-3-glc (250 µg/ml) had no effect on viability and proliferation (Olejnik et al., 2016), which, when calculated as 100% Cy-3-sam would equal 430 µM.

Incubation of colon cancer cells HT29 with a blackberry extract has shown an IC_{50} of 120 µg/ml without, and over 200 µg/ml with catalase, using the SRB assay (Esselen et al., 2011). The highest used concentration of 50 µg/ml did not show any significant differences in Caco-2 cell metabolism or viability in the CellTiter-Glo assay and MTT assay (Elisia & Kitts, 2008). Similarly, incubations with bilberry extract show a significant cytotoxicity only at the highest concentration of 500 µg/ml in HT29 cells (Schantz, 2010), whereas in Caco-2 cells and also in HT29 cells in later experiments no significant cytotoxic effect was apparent in the Alamar blue assay (Juadjur et al., 2015). HCT116 colon cells were significantly inhibited only after 48 hours by 1 - 4 mg/ml bilberry extract (Katsube et al., 2003).

2.7.3 Cyanidin and glycosides

As can be seen in table 2, the inhibitory effect of cyanidin and cyanidin-3-glucoside has been tested in several different colon cell lines with different test systems, producing vastly different outcomes such as a massive decrease of viability (82%) in HCT-116 (Katsube et al., 2003), whereas the same concentration (200 µM cyanidin) only decreased the growth of Caco-2 cells by around 20% (Renis et al., 2008). The effect of Cy-3-glc proves to be just as heterogeneous, since a 40% growth inhibition was found in Caco-2 cells after incubation of 200 µM, compared with Elisia and Kitts (2008) who did not find any growth inhibition even when using concentrations ranging up to 1.2 mM, both incubating for 24 hours and using the MTT assay. Olsson et al. (2004) measured a massive growth inhibition of 88% incubating 200 µg/ml (445 µM) in HT29 cells using the WST-1assay whereas Esselen et al. (2011) using 300 µM in the SRB Assay did not find any inhibition. This heterogeneous effect even included a growth promotion of 4.1% by 100 µg/ml (222.5 µM) Cy-3-glc in the study by Olsson et al.

The use of catalase may play a significant role for the heterogeneous reports as the two vastly different outcomes of no growth inhibition up to 300 µM and an IC_{50} of 57 µM measured during the same study with and without the addition of catalase to incubation media shows (Esselen et al., 2011).

Table 2: Inhibitory effect of cyanidin and Cy-3-glc in human colon cancer cells, adapted after Cooke et al. (2005)

Compound	Cell line	Inhibitory effect	Reference
Cyanidin	Caco-2	No inhibition up to 100 µM (MTT, 68h)	Cvorovic et al. (2010)
	Caco-2	Growth inhibition ca. 20%, 200 µM (MTT, 24h)	Renis et al. (2008)
	HT29	IC_{50} 57 µM (SRB), 72h No inhibition 300 µM with catalase	Esselen et al. (2011)
	HT29	IC_{50} 57 µM (SRB), 24h	Marko et al. (2004)
	HT29	IC_{50} 63 µM *(DNA dye Hoechst,* 72h)	Kang et al. (2003)
	HCT-116	IC_{50} 85 µM *(DNA dye Hoechst,* 72h)	Kang et al. (2003)
	HCT-116	Growth inhibition (82%) 200 µM *(viability test, 48h), 100 µM ca. -40%*	Katsube et al. (2003)
Cy-3-glc	Caco-2	Growth inhibition ca. 40%, 200 µM (MTT, 24h)	Renis et al. (2008)
	Caco-2	No inhibition up to 0.5 mg/ml = 1.12 mM (MTT, 24 h)	Elisia and Kitts (2008)
	HT29	No growth inhibition 300 µM (SRB, 72h)	Esselen et al. (2011)
	HT29	Growth inhibition (88%), 200 µg/ml = 445 µM (WST-1, 24h)	Olsson et al. (2004)

In Caco-2 cells both cyanidin and Cy-3-glc (200 µM) show a dose dependent cell proliferation decrease as assessed with a clonogenic test while the MTT assay also indicates a decrease of cell viability (Renis et al., 2008), whereas 100 µM cyanidin has not proved to be inhibitory on cell growth. Incubations with the drug resistant LoVo and LoVo/ADR cell lines on the other hand show cytotoxicity for cyanidin with IC_{50} values of 47 and 26 µM respectively (Cvorovic et al., 2010).

2.7.4 Phloroglucinol aldehyde

Phloroglucinol aldehyde has shown a potent growth inhibiting effect in HT29 cells detected after 72 h incubation with an IC_{50} of 160 ± 17 µM (using catalase and SRB assay) (Esselen et al., 2011) whereas another group previously found the cell growth of Caco-2 inhibited with an IC_{50} of 76.7 µM, attributed to an induction of apoptosis since only a low cytotoxicity (<19.8%, LDH-Assay) was measured (Forester & Waterhouse, 2010). By measuring the intracellular content of ATP (adenosine triphosphate), cell viability was found to be decreased only by the highest tested concentration of 100 µM PGA in Caco-2 cells in a later study by the same group, compared to other metabolites such as methyl-galactose and galactose which caused a dose- and time-dependent decrease of viability. PGA was more effective in HT29 than in other cell lines,

and proliferation was even increased in HCT-15 and SW-480 cells (Forester et al., 2014).

2.8 Beneficial properties

A wide range of beneficial properties of anthocyanins has been described. Studies conducted on cells from various organs, animal trials and human clinical trials show that anthocyanins have antioxidant (Mazza, Kay, Cottrell, & Holub, 2002; Shih, Yeh, & Yen, 2007), anti-inflammatory (Morais et al., 2016; Serra, Paixao et al., 2013) and anti-carcinogenic effects (Cooke et al., 2005; Lin et al., 2016) as well as showing positive effects concerning cardiovascular health (Thompson et al., 2017). Since the focus of this thesis is on the antioxidant effects of cyanidin-based anthocyanins and a potential protection against oxidative stress, mainly these beneficial properties and respective anthocyanins will be further discussed.

2.8.1 Oxidative stress

Oxidative stress is described as an imbalance in the cells normal production of antioxidants and oxidants. The formation of reactive oxygen species (ROS) are as pro-oxidants a part of normal aerobic life. If however the production exceeds the capability of the cellular antioxidant defence systems, oxidative damage and pathophysiological events might occur (Sies, 1991). Six major reactive oxygen species have been shown to cause oxidative damage in the human body, namely the non-radicals hydrogen peroxide (H_2O_2), singlet molecular oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$), superoxide anion ($O_2^{\bullet -}$), the long lived peroxyl radicals ($ROO^\bullet$) and the very reactive and short-lived hydroxyl radical ($HO^\bullet$) (Huang, Ou, & Prior, 2005; Sies, 1991). The biological defence system of living cells includes enzymatic antioxidants that convert ROS to more harmless species, for example the conversion of $O_2^{\bullet -}$ to oxygen and H_2O_2 by superoxide dismutase (SOD), followed by the breakdown of H_2O_2 by catalase to water and oxygen. For $ROO^\bullet$, $HO^\bullet$, $^1O_2$ and $ONOO^-$ no such enzymatic scavengers are known, which is where non-enzymatic antioxidants such as vitamins C and E and phytochemicals such as anthocyanins play their role (Huang et al., 2005).

2.8.2 Anthocyanins and antioxidant activity

Anthocyanin content and their antioxidant activity contribute to fruits and vegetables protective effects concerning degenerative and chronic diseases. Substances where a free electron or hydrogen atoms can be donated to reactive free radicals are easy to oxidize and are thereby often good antioxidants. The anthocyanidins cyanidin, delphinidin and petunidin all have o-dihydroxyl substituents at the B-ring (vicinal hydroxyl groups), making them susceptible to oxidation. The antioxidant capacity of cyanidin is proposed to be based on a free radical mechanism where a very stable semiquinone is formed by stabilizing the phenoxyl radical with oxygen (Castañeda-Ovando et al., 2009) as seen in figure 10.

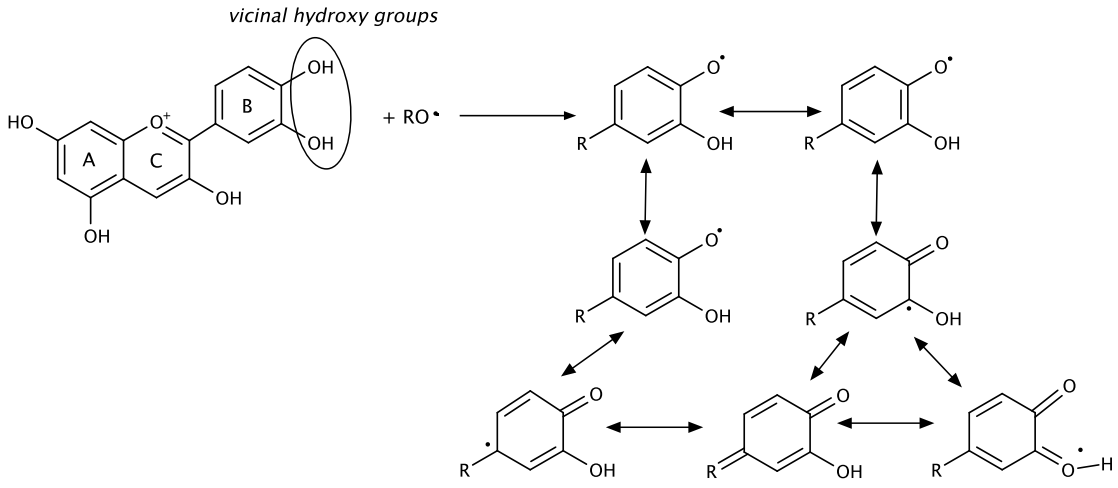


Figure 10: Stabilization of Cy semiquinone radical. R = Benzopyroliumcation. Modified after Castañeda-Ovando et al. (2009)

The antioxidant effect of anthocyanins however may not be only due to free radical scavenging but also through acting on the antioxidant response element (ARE) through the Keap1-Nrf2 pathway, hereby promoting the expression of ARE-regulated phase II enzymes (Lin et al., 2016; Shih et al., 2007).

The Keap1-Nrf2-pathway (figure 11) is one of the most important regulators of the expression of molecules with intracellular antioxidant functions. Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor 2) is constantly ubiquinated by a Keap1 (Kelch-like ECH-associated protein 1) complex (Cul3-Keap1 ubiquitin E3 ligase complex) and rapidly degraded. When, however, electrophilic or oxidative stress is present, reactive cysteine residues of Keap1 are modified, E3 ligase activity declines, Nrf2 is stabilized and translocated to the nucleus, where

it can bind to ARE (antioxidant response elements) of phase II detoxification enzymes such as HO-1 (haem oxygenase), NAD(P)H, NQO1 (quinone oxidoreductase 1), UGTs (UDP-glucuronosyltransferases) and GSTs (glutathione S-transferases), thus regulating their transcription (Dinkova-Kostova et al., 2002; Gorrini et al., 2013; Taguchi et al., 2011).

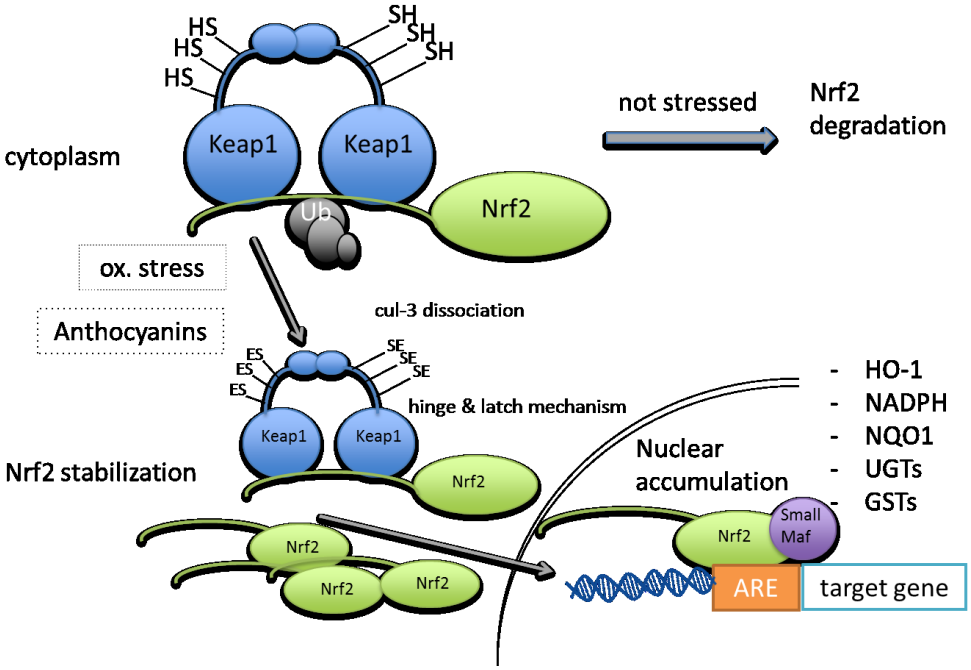


Figure 11: Nrf2-Keap1 Pathway, modified according to Taguchi et al. (2011)

24 hours incubation of rat liver cells with cyanidin, malvidin and delphinidin showed a significant induction of the phase II enzymes as well as a repression of cell death and apoptosis induced by H_2O_2, which lead the authors to the conclusion that anthocyanins indeed can stimulate the antioxidant system to resist oxidant-induced injury as well as modulate the defence systems against oxidative stress through the ARE-regulated phase II enzyme expression (Shih et al., 2007). A Nrf2 luciferase reporter gene assay using CHO cells treated with blueberry extract, its main anthocyanin Cy-3-glc, aglycon Cy and several degradation products however showed a significantly increased ARE-promoter activity only by PGA. Similarly, only PGA was also able to significantly increase transcription levels of HO-1 and Nrf2 in HT29 colon carcinoma cells, while neither berry extract, Cy-3-glc or protocatechuic acid made a significant difference (Kropat et al., 2013).

2.8.3 Antioxidant properties of anthocyanins

2.8.3.1 In vitro research

There are many *in vitro* assays available for measuring the antioxidant capacity. Some of the most common include the Trolox equivalent antioxidant capacity (TEAC) assay, the oxygen radical absorbance capacity (ORAC) assay, the DPPH (2,2-di(tertoctylphenyl)-1-picrylhydrazyl) assay and the total radical-trapping antioxidant parameter (TRAP) assay (Huang et al., 2005).

The TEAC assay is based on the capacity of the tested substance to scavenge $ABTS^{\bullet+}$ (2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) in comparison to the antioxidant standard Trolox (6-hydroxy-2,5,7,8,-tetramethychroman-2-carboxylic acid), a water soluble vitamin E analogue. The TEAC assay was established by Miller and Rice-Evans and later improved. It measures the extent of the decolourization of the blue/green $ABTS^{\bullet+}$ after addition of antioxidants and is calculated relative to Trolox as a function of concentration and time. TEAC values of common antioxidants ascorbic acid (1.05), α-tocopherol (0.97) and glutathion (1.28) (Re et al., 1999) are close to Trolox in antioxidant effect when compared with the powerful antioxidants Cy (3.47) and its glucoside (2.68). Cyanidin seems to be one of the more active anthocyanidins when compared to Pg (2.26), Pn (2.45) and Mv (2.95) although with slightly lower values than Dp (3.91) (Ali et al., 2016)

The capacity of an elderberry extract (from freeze-dried powder) to scavenge $ABTS^{\bullet+}$ was determined to be 15.71 ± 1.09 µM Trolox equivalents/ml (Olejnik et al., 2016), whereas extract from air-dried berries only reached 0.89-1.97 µM TE/ml (Duymus et al., 2014). After the freeze-dried extract underwent a simulated gastrointestinal digestion the TEAC values had decreased to 5.23 ± 0.35 µM Trolox equivalents/ml, which was associated with significant losses (88.4%) of anthocyanins such as cyanidin-3-sambubioside and cyanidin-3-glucoside (Olejnik et al., 2016).

The oxygen radical absorbance capacity assay (ORAC) was developed by Cutler and Cao (1993), and improved by Ou et al. (2001). The fluorescence intensity of the synthetic probe fluorescein (3´,6´-dihydroxyspiro[isobenzofuran-1[3H], 9´[9H]-xanthen]-3-one), is measured with a fluorescence reader at λ

$485_{ex}/525_{em}$ nm when initiated with peroxyl radical generator AAPH. The fluorescence intensity decreases with time as the fluorescein is consumed, but in the presence of an antioxidant the fluorescein decay is inhibited. This inhibition is plotted to curves compared with Trolox standards (Huang et al., 2005). Cy-3-glc has been shown to have an ORAC value of 2.26 (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002) to 3.49 µMol TE/ µMol anthocyanin, while Cy was slightly less potent with 2.24 µMol TE/ µMol. The addition of different sugars proved to have different effects on the antioxidant activity, as a 3-glycosylation with galactose decreased, whereas glucose and rhamno-glucose increased ORAC activity (Wang et al., 1997). 5´glycosylation with a third sugar was shown to reduce antioxidant values, as shown with cyanidin-3-xylosyl-galactoside and cyanidin-3-xylosyl-glucosyl-galactoside (Stintzing et al., 2002).

The free radical scavenging potential of substances is often also tested by how well they can bleach the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH has an odd electron and gives a strong absorption band at λ 517 nm using visible spectroscopy. When a free radical scavenger is present and the electron is paired off the absorption vanishes with a stoichiometric decolourization (from deep violet) with respect to the number of electrons taken up (Acquaviva, 2003). The pure Cy shows a medium TE-Value of 1.296 compared with other anthocyanidins (Dp: 1.691, Mv: 1.334, Pg: 0.78) while its glucoside produced the slightly lower value 1.193 TE (Ali et al., 2016). Acquaviva. (2003) however found the opposite ratio, with Cy-3-glc being equivalent to Trolox and more potent than the aglycon.

2.8.3.2 Cell based research

Antioxidant activity is often determined using the substance DCFH-DA (2,´7´-dichlorofluorescin diacetate) in cell culture experiments where fluorescence emission reflects on the cells intracellular ROS-production, and thereby on the potency of the anti- or prooxidant substance incubated (Wang & Joseph, 1999). A detailed description of the DCF Assay can be found in chapter 8.2.2.

An antioxidant activity has been seen for cyanidin and a weaker effect by cyanidin-3-glucoside where a decrease in ROS production was measured using the DCF assay after 24 hours incubation in Caco-2 cells (Renis et al., 2008) as

well as a dose-dependent inhibition of fluorescence increase after a one hour incubation with cyanidin and delphinidin and subsequent generation of hydroxyl radicals with ABAP (2,2´-azobis (2-amidinopropane) dihydrochloride). In drug-resistant LoVo/ADR cells on the other hand both delphinidin and cyanidin were instead inducing the formation of free radicals in a dose-dependent manner (Cvorovic et al., 2010). In Caco-2 cells incubated for three hours with a purified blackberry extract containing the sole anthocyanin cyanidin-3-glucoside with a subsequent peroxyl radical generation a suppression of fluorescence development in a concentration-dependent manner was also evident (Elisia & Kitts, 2008).

The elderberry anthocyanins have, as mentioned in chapter 2.7.2 concerning their growth inhibitory potential, also not been tested as single compounds regarding their antioxidative effects, but only as extracts. Elderberry extract has shown a dose-dependent inhibition of fluorescence intensity by 13%, 20% and 40% when incubated with non-tumorigenic colonic NCM460 cells at 0.01, 0.1 and 1 mg/ml (17.2 µM, 172 µM, 1720 µM) respectively, when incubated in the presence of 100 µM H_2O_2. After the simulated gastrointestinal digestion of the extract prior to 30 minutes cell incubation the effect was only significant at the highest concentration of 1 mg/ml with an inhibition of ROS generation of 22% (Olejnik et al., 2016).

As a comparison with other dark berries, a bilberry extract was shown to induce a significant decrease of intracellular ROS in a concentration dependent manner in both Caco-2 (at 50 µg/ml) and HT29 cells (250 µg/ml) (Schantz, 2010).

2.8.3.3 In vivo research

Several human intervention studies concerning the antioxidant effect of anthocyanins have been conducted, mainly within the frame of the development of cardiovascular diseases, where plasma biomarkers such as the antioxidant capacity (AC) of the plasma as evaluated with the ORAC-, TEAC-, or FRAP-assay were measured. Several studies show an increase in plasma antioxidative capacity after the intervention with an anthocyanin rich juice or supplement (Kropat, 2012), such as the significant increase of plasma TEAC (+17%) and TRAP (+28%) one hour after ingestion of 400 ml elderberry juice

(Netzel et al., 2005) or the significantly increased serum ORAC (+15%) four hours after ingestion of a blueberry supplement (Kay & Holub, 2002). Longer interventions with juices however did not significantly change the antioxidant capacity (Bub et al., 2003; Duthie et al., 2006; Riso et al., 2005). So far no human intervention studies with the pure anthocyanins have been conducted, and the antioxidant effects of fruit juices and extracts may be at least to some extent caused by other compounds (Kropat, 2012).

Table 3: Selection of human intervention studies on antioxidative capacity (AC) of anthocyanin rich products. ↑: increased, ↔: unchanged. a) Netzel et al. (2005), b) Kay and Holub (2002), c) Jensen et al. (2008), d) Bub et al. (2003), e) Riso et al. (2005), f) Duthie et al. (2006). Modified after Kropat (2012)

Product	**Dose** *(juice / fruit)*	**Duration**	**Outcome**
Elderberry juice[a]	710 mg *(400 mL)*	Single dose	↑ AC plasma
Blueberries (freeze-dried)[b]	1200 mg *(100 g)*	Single dose	↑ AC plasma
Multifruit juice[c]	177 mg *(120 mL)*	Single dose	↑ AC plasma
Anthocyanin rich fruit juice [d]	69 mg/day *(330 mL)*	2 weeks	↔ AC plasma
Blood orange juice[e]	28 mg/day *(600 mL)*	3 weeks	↔ AC plasma
Cranberry juice[f]	2.1 mg/day *(750 mL)*	2 weeks	↔ AC plasma

3 Aim of the thesis

The aim of this thesis was to investigate the antioxidant properties of elderberry anthocyanins in human colon carcinoma cells C2BBe1 and compare the effects depending on the different side chains and sugar moieties. Since a low absorption rate of the anthocyanins has been reported even though major effects can be seen with nanomolar serum-concentrations (Czank et al., 2013), the question arose whether the potency of the anthocyanins lays in the anthocyanin itself or in its metabolized forms, out of which for this thesis the aglycon cyanidin and the common metabolite of anthocyanins, phloroglucinol aldehyde was chosen.

To rule out that any toxicity is reducing the amount of cells in the antioxidative assays and thus a falsified reduction of ROS levels is recorded, first a cytotoxicity assay measuring the growth inhibiting properties of all test compounds is conducted. The dichlorofluorescein (DCF) assay is used to investigate the impact of a short time incubation of the substances on the intracellular ROS-formation. A longer incubation time is used to assess the magnitude of the compounds to strengthen the cells capability to withstand extraordinary stress, which in this case was induced by 1 mM hydrogen peroxide (H_2O_2).

4 Results

In this chapter the results of the experimental work conducted within the frame of this thesis are presented. A detailed description concerning assay protocols, practical implementation, statistical analysis and materials can be found in chapter 8 (Material and Methods).

4.1 Antiproliferative effects of elderberry anthocyanins and their degradation products

In order to rule out that any possible cytotoxicity or antiproliferative effect may influence the result of the measurements on oxidative stress, first a sulforhodamine B (SRB) assay was performed. The SRB assay measures the optical density (absorbance) of cellular protein stained with the dye sulforhodamine B, showing a high linear correlation between protein content and optical density (Skehan et al., 1990). 1 mM dihydrogen peroxide, proven in a previous master thesis (Janson, 2015) to have a strong cytotoxic effect in C2BBe1 cells, was used as a positive control.

The results of the cell growth/ cytotoxicity assay show no statistically significant change in cell proliferation for cells incubated 24 hours with any of the test substances compared to solvent control (0.8 % acidified DMSO) as calculated with non-normalized data (absorbance at λ = 570 nm) using Oneway ANOVA and in the case of Cy-3-sam-5-glc with Kruskal-Wallis ANOVA due to the fact, that these data did not pass the test for normal distribution since the experiment was only repeated four times. The data are presented as test over control (T/C) in (%) (figure 12). A slight tendency towards cell growth reduction at the highest concentration can be observed for all substances (test over control (T/C) data for Cy-3-glc: 87.99% ± 12.11%, Cy-3-sam: 81.52 ± 13.93% and Cy-3-sam-5-glc: 77.33 ± 10.07% (figure 12A) and PGA: 86.16 ± 9.25% (figure 12B)) except for the aglycon cyanidin, which shows growth promoting effect (112.75 ± 12.61%) at the highest concentration yet with no statistical significance (figure 12A).

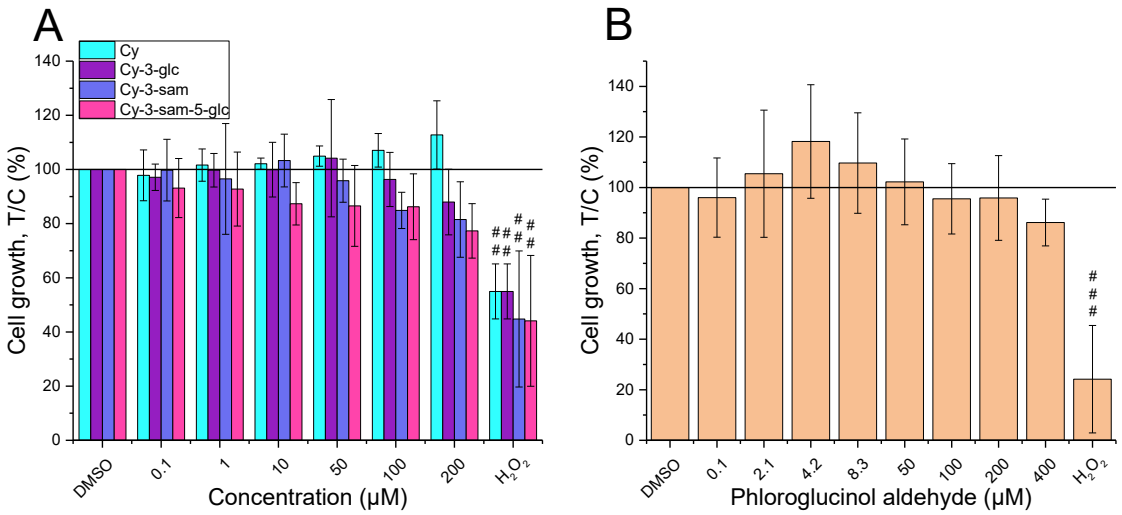


Figure 12: Effect of (A) Cy, Cy-3-glc,Cy-3-sam[x] and Cy-3-sam-5-glc[x] and (B) PGA on cell growth of C2BBe1 human colon tumor cells after incubation for 24 h

Measured with the SRB assay. The absorbance was measured with a Cytation3 microplate reader (λ=570 nm) in at least triplicates from at least 5 (Cy-3-sam-5-glc n=4) independent experiments and normalized to solvent control (T/C in %). Solvent: (A) 0.8% DMSO, 100U/ml catalase, 0.0001% HCl (conc.) (B) 0.8% DMSO, 100 U/ml catalase. H_2O_2 (1mM) served as positive control. Plotted is the mean ± SD of the T/C-values. Non normalized data outliers were eliminated with Nalimov and used for statistics. For Cy, Cy-3-glc and Cy-3-sam and PGA, no significant differences to DMSO solvent were found calculated with ANOVA (Bonferroni) (α = 0.05:*). Student's *t*-test (α = 0.01: ##, 0.001: ###) for positive control vs respective solvent control (medium). Kolmogorov-Smirnov test for normal distribution and Brown-Forsythe test for variance homogeneity was performed. No significant differences to DMSO solvent were found for Cy-3-sam-5-glc using Kruskal-Wallis ANOVA (α = 0.05). Mann-Whitney-U-test (α = 0.01: ##) for positive control against solvent.
[x]: Approx. amount (according to MS): Cy-3-sam-equivalients: 63.57% Cy-3-sam, 36.43% Cy-3-glc. Cy-3-sam-5-glc equivalents: 63.73% Cy-3-sam-5-glc, 24.92% n.i., 11.34% Cy-3,5-diglucoside (Oertel et al.)

The minor non-significant proliferative effect of Cy in C2BBe1 at 200 µM fits with the heterogeneous literature, as Renis et al. (2008) describes a growth inhibition of around 20% for 200 µM Cy (24 h), whereas at 100 µM, with a longer incubation time (68 h), no toxicity could be seen (Cvorovic et al., 2010). For HT29 cells the range spans from 57 µM IC_{50} to no inhibition up to 300 µM which may be attributed to the use of catalase in the latter case (Esselen et al., 2011; Kang et al., 2003; Marko et al., 2004). HCT-116 cells on the other hand displayed a massive growth inhibition of 82% when incubated with 200 µM Cy, a value dropping to about 40% at 100 µM (Katsube et al., 2003), concurring with the IC_{50} of 85 µM of another work group (Kang et al., 2003).

The prevalent heterogeneous literature on Cy-3-glc ranges from a growth inhibition of ca. 40% at 200 µM (Renis et al., 2008) to no inhibition at 0.5 mg/ml (1.12 mM) in Caco-2 cells and for HT29 cells a growth inhibition of 88% at 200 µg/ml (445 µM) using the WST-1 assay (Olsson et al., 2004) yet no inhibition at 300 µM (Esselen et al., 2011) when tested with the SRB assay. Together with the literature and since our results for 200 µM Cy-3-glc amounted to a non-significant growth inhibition of 12.01%(T/C: 87.99 ± 12.11%), we assume Cy-3-glc not to be cytotoxic at the highest concentration tested (200 µM).

The results of elderberry anthocyanin Cy-3-sam so far can only be compared with the purified Cy-3-sam from elderberry extract incubated with non-tumorigenic colon cells NCM40, which in line with our findings did not display any significant effects on viability and proliferation up to a concentration of 250 µg/ml (430 µM) (Olejnik et al., 2016).

The results show a lower inhibitory effect for PGA as has been previously measured in Caco-2 cells, the parental clone of C2BBe1, with an IC_{50} of 76.7 µM and cell viability decreased at 100 µM (72 h incubation time) (Forester et al., 2014; Forester & Waterhouse, 2010). In HT29 cells an IC_{50} of 160 µM was determined (Esselen et al., 2011), although the differences in growth inhibition to our measurements may be attributed to the longer incubation time (72 h) compared to the 24 hour incubation.

To see whether any of the anthocyanins differed in effect from each other, a comparison between the anthocyanins at the same concentrations was performed with Kruskal-Wallis ANOVA, which showed no significant differences in cell growth (shown in figure 13).

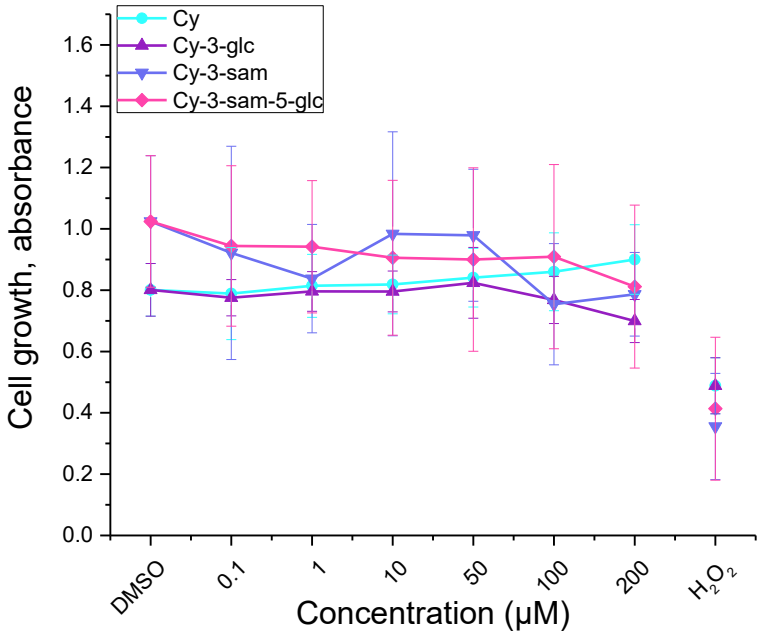


Figure 13: **Comparison of the effect of Cy, Cy-3-glc, Cy-3-sam[x] and Cy-3-sam-5-glc[x] on cell growth of C2BBe1 cells after 24 h incubation**

Measured with the SRB assay. Presented are the means ± SD of the absorbance measured at λ=570 nm with a Cytation3 microplate reader in at least triplicates from at least 5 independent experiments (except Cy-3-sam-5-glc, n=4). Solvent: 0.8% DMSO, 0.0001% HCl (conc.), 100 U/ml catalase. H_2O_2 (1mM) served as positive control. Plotted is the mean ± SD of the absorbance-values. Data outliers were eliminated with Nalimov and used for statistics. No significant differences to eachother were found calculated with ANOVA (Bonferroni) (α = 0.05:*). [x] : Approx. amount (according to MS): Cy-3-sam-equivalients: 63.57% Cy-3-sam, 36.43% Cy-3-glc. Cy-3-sam-5-glc equivalents: 63.73% Cy-3-sam-5-glc, 24.92% n.i., 11.34% Cy-3,5-diglucoside (Oertel et al.)

4.2 Antioxidant effects

To examine the effect of the anthocyanins on the intracellular ROS level of the cells the dichlorofluorescein assay (DCF assay) was performed following the optimized protocol of the master thesis of Theresa Janson (2015).The non-fluorescent dichlorofluorescin-diacetate (DCFH-DA) enters the cell where present ROS can oxidize the DCFH-DA, leading to the fluorescent DCF. The resulting fluorescence, which correlates with ROS levels, can be quantified (LeBel, Ischiropoulos, & Bondy, 1992; H. Wang & Joseph, 1999). C2BBe1 cells were incubated with substances for 1 h to assess the impact on intracellular ROS-levels.

All anthocyanins tested showed significantly lower fluorescence than the solvent control as calculated with “non-normalized” relative fluorescence data from 0.01 µM for Cy-3-glc (P (t_{30}) = 0.041; P (t_{45}) = 0.029) reaching P <0.001 from 0.1 µM onward, indicating anti-oxidative properties. Cy-3-sam results were also significant from 0.01 µM (P <0. 01, reaching P <0.001 from 0.1 µM), 0.1 µM for Cy-3-sam-5-glc (P (t_{15}) = 0.025, reaching P<0.001 from t_{30}) and 1 µM for Cy (P<0.01: $t_{30,}$ P<0.001 from t_{30}). A clear dose dependent decrease in ROS can be seen in figure 14 where data are plotted normalized to solvent control (figure 14A-D) as well as the relative fluorescence shown in figure 15).

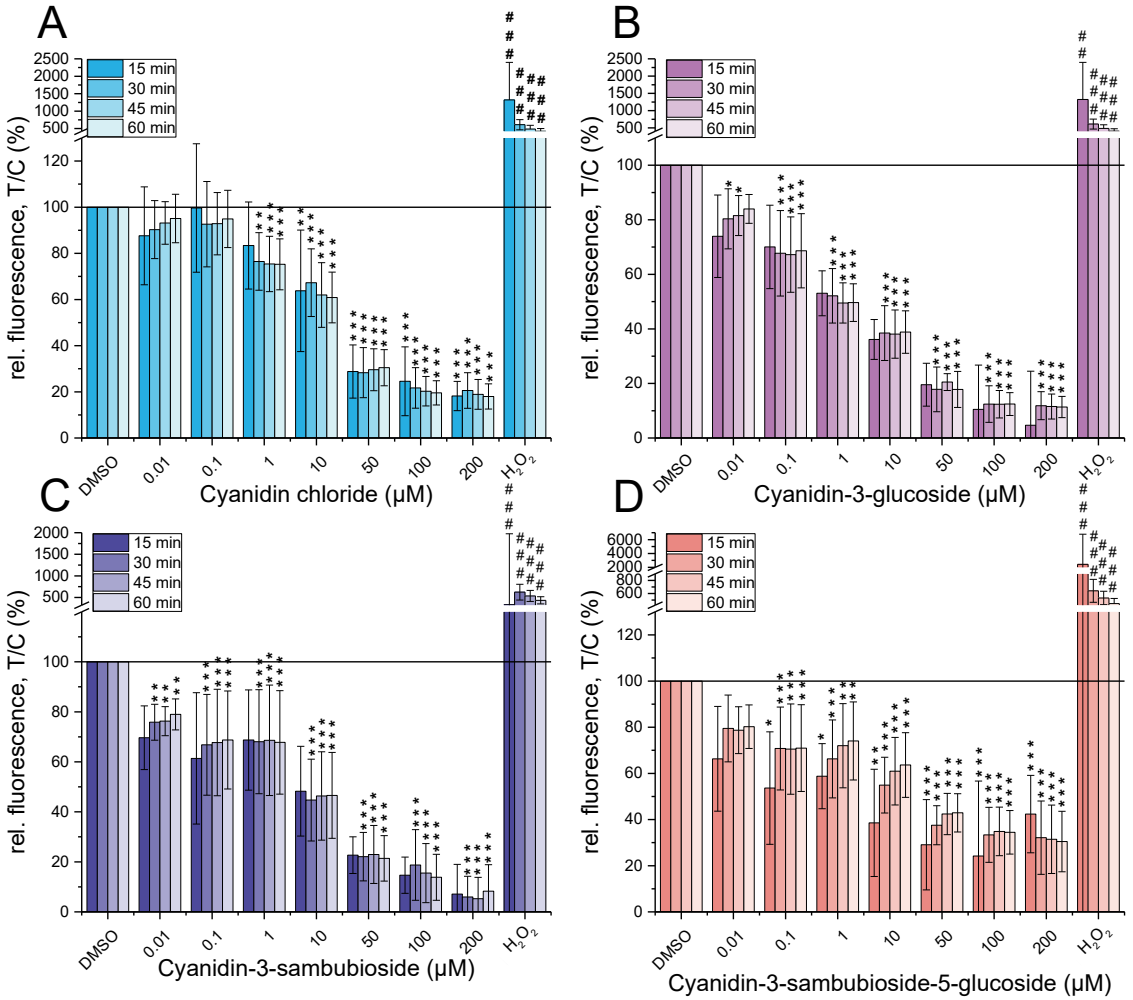


Figure 14: Effect of Cy, Cy-3-glc, Cy-3-sam^x and Cy-3-sam-5-glc^x on cellular ROS levels in C2BBe1 cells during 1h incubation measured with the DCF assay

Cells were pre-incubated with DCFH-DA followed by 1h incubation with Cy, Cy-3-glc, Cy-3-sam^x or Cy-3-sam-5-glc^x while fluorescence intensity is quantified with a Cytation3 photometer (excitation: λ: 485 nm, emission: λ: 528 nm. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and normalized to solvent control (T/C in %). Solvent ctrl: 0.8% DMSO, 0.0001% HCl (conc.), 100 U/ml catalase. 1mM H_2O_2 served as positive control. Outliers of non-normalized data were eliminated with Nalimov Statistical analysis using 1) for A, B t_{30}-t_{60}, C t_{30}-t_{60}, D and 2) for B t_{15}, C t_{15}.

1) Significant differences to DMSO solvent calculated with ANOVA (Bonferroni) (α = 0.05:*; 0.01:**, 0.001:***) and Student's *t*-test (α = 0.001: ###). Kolmogorov-Smirnov test for normal distribution and Brown-Forsythe test for variance homogeneity was performed.
2) Significant differences to solvent control calculated with Kruskal-Wallis ANOVA (α = 0.05:*; 0.01:**, 0.001:***) and Mann-Whitney-U test (α = 0.001: ###). Variance homogeneity was calculated with Brown-Forsythe test and found significant different, hence non-parametric tests for significance were chosen.

[x] : Approx. amount (according to MS): Cyanidin-3-sambubioside-equivalients: 63.57% Cy-3-sam, 36.43% Cy-3-glc. Cy-3-sam-5-glc equivalents: 63.73% Cy-3-sam-5-glc, 24.92% n.i., 11.34% Cy-3,5-di-glc (Oertel & Matros)

The non-parametric test of Kruskal-Wallis ANOVA had to be performed for the datasets of the time point t_{15} for both Cy-3-glc and Cy-3-sam due to inhomogeneous variances. A significant difference in mean rank was calculated for both ($P<0.001$), as shown in table 4. Highlighted in red is the strongest difference.

Table 4: Mean ranks of separate Kruskal-Wallis ANOVA for Cy-3-glc and Cy-3-sam at t_{15}

KWANOVA , mean rank		
	Cy-3-glc	**Cy-3-sam**
Conc. (µM)	15 min	15 min
DMSO	39.82	49.00
0.0001	30.75	45.00
0.01	35.83	45.17
0.1	48.67	27.09
1	30.67	28.67
10	33.44	26.20
50	29.44	15.60
100	25.33	13.80
200	17.00	7.17
Prob >χ^2	**1.02E-06**	**2.98E-06**

The most potent effect is observed with Cy-3-sam at 200 µM with 8.27 ± 10.61% of ROS (table 5, red highlighted) compared to the solvent control, although there was no clear decreasing effect beyond 50 µM anymore because the curves stagnate already. For a more detailed overview of the relative decrease see table 5 with data obtained at time point t_{60}, normalized to solvent control. A one way ANOVA was performed for all concentrations except 0.0001 µM where no normal distribution can be assumed due to only four replicate experiments.

Table 5: Impact of Cy, Cy-3-glc, Cy-3-sam and Cy-3-sam-5-glc on ROS production in C2BBe1 cells after 1 h incubation. The mean values ± standard deviation normalized to solvent control at t_{60} are listed, samples with*: n=4, no statistic evaluation (no normal distribution)

Mean T/C values, t_{60}				
Conc. (µM)	**Cy**	**Cy-3-glc**	**Cy-3-sam**	**Cy-3-sam-5-glc**
0.0001*	93.40 ± 10.03	88.29 ± 10.28	81.63 ± 20.81	110.14 ± 14.08
0.01	95.11 ± 10.52	83.98 ± 5.29	78.99 ± 6.18	80.24 ± 3.85
0.1	77.16 ± 36.32	68.64 ± 16.61	68.74 ± 19.60	70.97± 18.79
1	75.25± 11.03	49.63 ± 6.90	67.79 ± 20.68	74.06± 16.92
10	60.88 ± 10.99	38.83 ± 7.78	46.59 ± 17.18	63.64 ± 14.01
50	30.46 ± 7.81	17.80 ± 6.60	21.40 ± 9.08	42.92 ± 8.30
100	19.15 ± 5.49	12.45 ± 4.18	13.84 ± 9.19	34.48 ± 9.45
200	18.00 ± 5.47	11.37 ±3.91	8.27 ± 10.61	30.48± 13.09

Our results are in line with reports in the literature where both delphinidin and cyanidin (25-100 µM) showed a dose-dependent inhibition of time-dependent fluorescence increase in Caco-2 cells after a ROS-induction by ABAP (Cvorovic et al., 2010). Elderberry extract on the other hand did not have any effect on intracellular ROS level in NCM460 colon cells at concentrations of 0.01 - 1 mg/ml, whereas a high dose of gastrointestinal-digested extract (10 mg/ml) resulted in a significant increase of fluorescence intensity, although without physiological significance due to the high concentration (Olejnik et al., 2016).

Figure 15 show the non-normalized relative fluorescence in order to see the curve of the fluorescence change and choose a timepoint for comparison of the substances.

Since the data of time point t_{60} show the most significant differences from solvent control and the curve shows a clear trend this time point was chosen for comparison between the different anthocyanins (fig. 16).

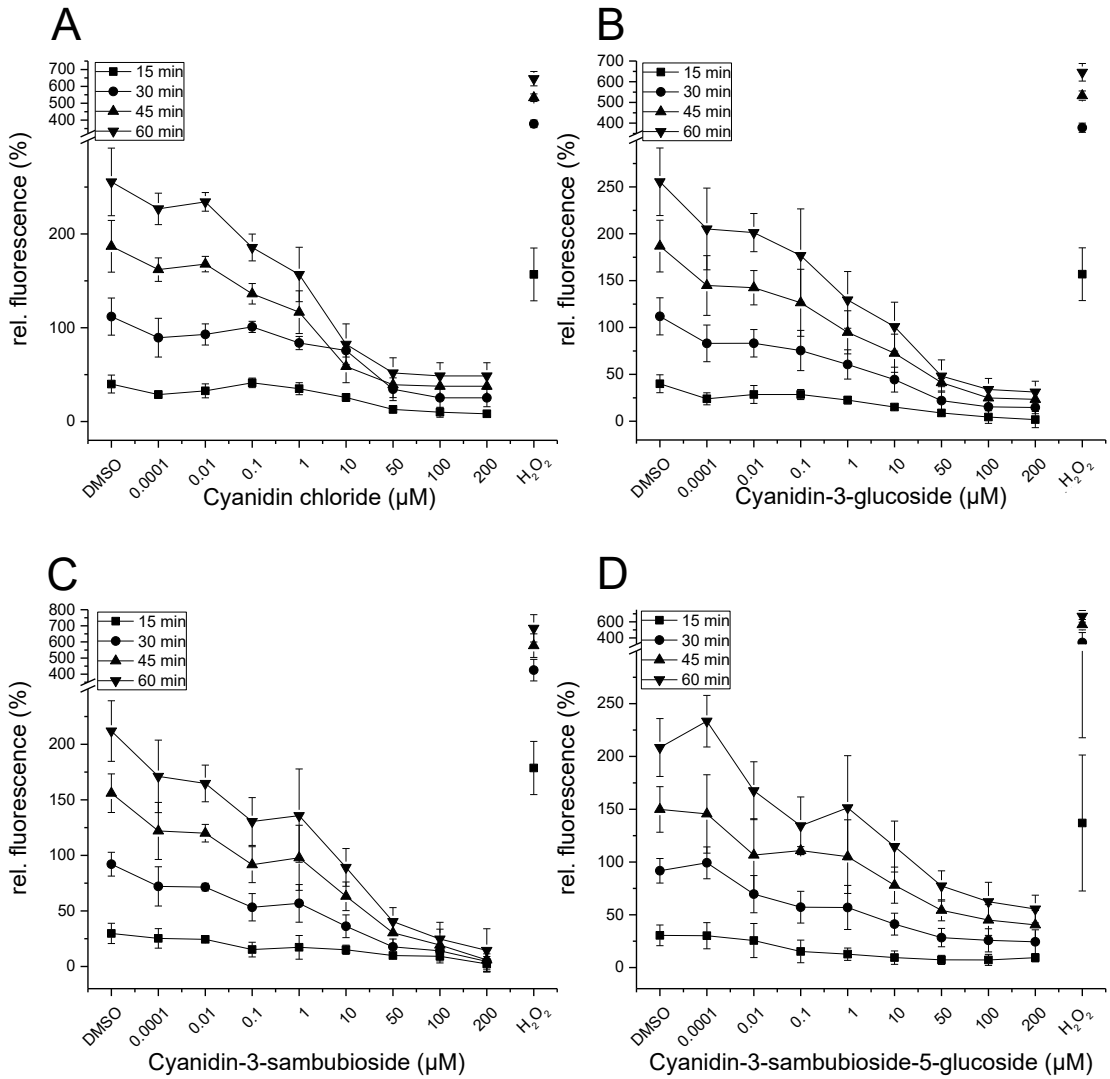


Figure 15: Effect of Cy, Cy-3-glc, Cy-3-sam^x and Cy-3-sam-5-glc^x on intracellular stress in C2BBe1 cells, non-normalized data

Cells were pre-incubated with DCFH-DA followed by 1h incubation with Cy, Cy-3-glc, Cy-3-sam^x or Cy-3-sam-5-glc^x while fluorescence intensity is quantified with a Cytation3 photometer (excitation: λ: 485 nm, emission: λ: 528 nm. Plotted is the mean and SD of fluorescence change from t_0, calculated from at least triplicates from at least 5 independent experiments.Solvent ctrl: 0.8% DMSO, 0.0001% HCl (conc.), 100 U/ml catalase. 1mM H_2O_2 served as positive control. Outliers of non-normalized data were eliminated with Nalimov. Statistics shown in figure 14.

The graphical plot shows that Cy-3-sam and Cy-3-sam-5-glc decrease ROS levels significantly better than Cy at 0.01 µM (P = 0.001, P = 0.026) (figure 17). With increasing concentration Cy-3-sam-5-glc slowly reaches the same and even higher fluorescence than Cy with statistical significance at 100 µM, indicating less potency compared to Cy to reduce cellular ROS. whereas Cy-3-glc with higher concentrations reaches significant differences to Cy and Cy-3-sam-5-glc (shown in figure 16 with A) data normalized to solvent control and B) non normalized relative fluorescence data).

As can be seen in figure 16 B the solvent control show a significantly higher fluorescence in the Cy and Cy-3-glc incubations compared to Cy-3-sam-5-glc, possibly due to different cell densities, hence the comparison with normalized data.

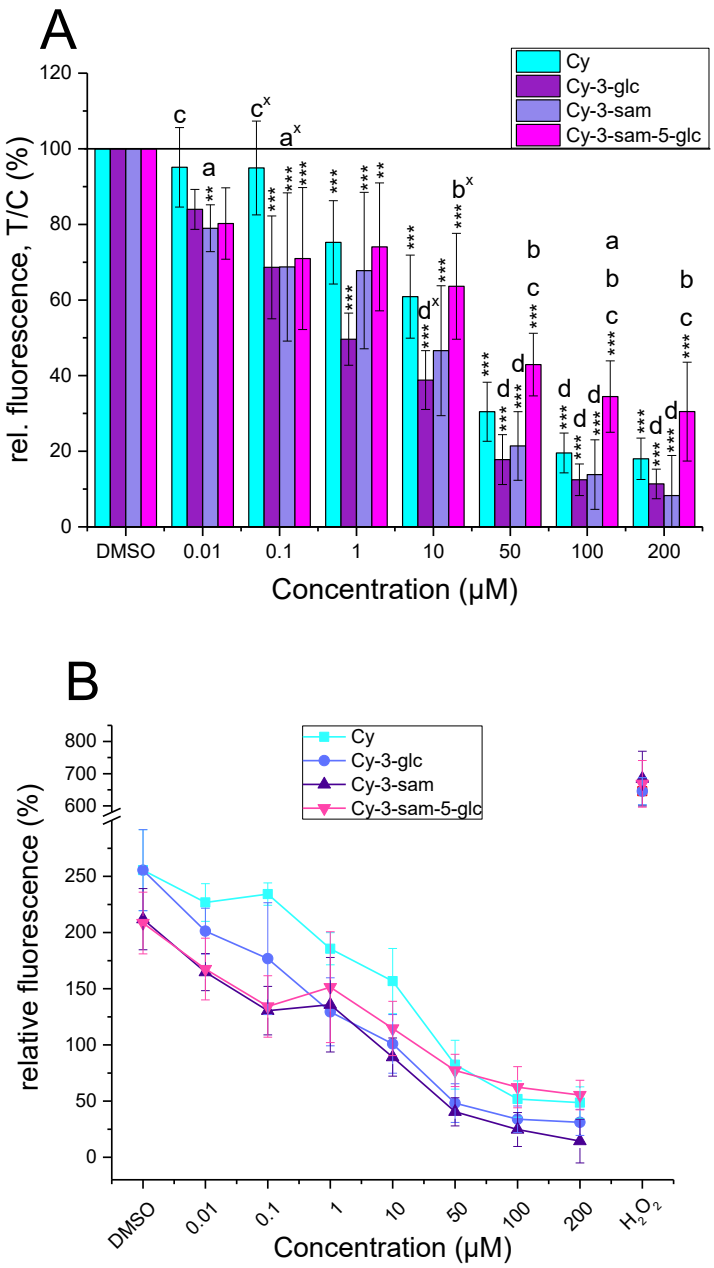
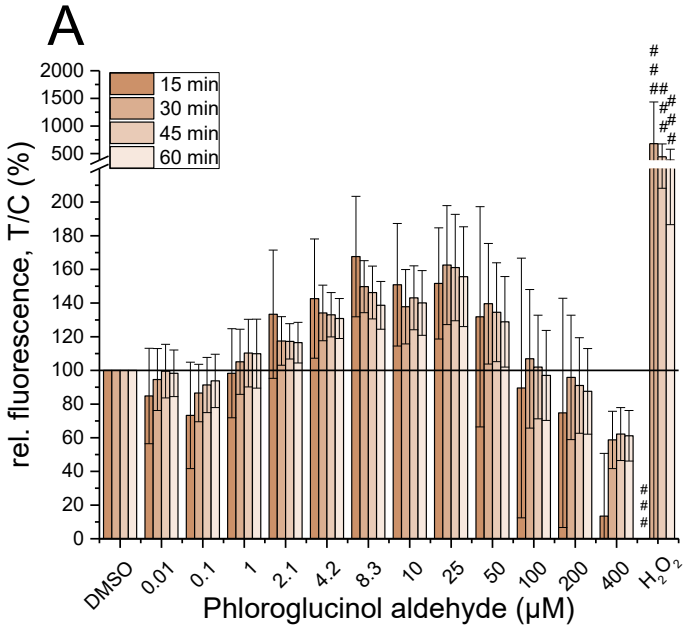


Figure 16: Comparison of the effect of Cy, Cy-3-glc, Cy-3-sam^x and Cy-3-sam-5-glc^x on cellular ROS in C2BBe1 cells at 1h of incubation. A) Data normalized to solvent control and presented as T/C in %, B) non normalized fluorescence change related to t_0 in %.

One way ANOVA and posthoc Bonferroni (or for x^x marked: Kruskal Wallis ANOVA due to inhomogenous variances) was performed for every concentration level with normalized data. Significantly different fluorescence change between the substances (α = 0.05) noted with a) Cy, b) Cy-3-glc, c) Cy-3-sam, d) Cy-3-sam-5-glc. Significant differences to DMSO solvent calculated with ANOVA (Bonferroni) (α = 0.05:*; 0.01:**, 0.001: ***) and Student's *t*-test (α = 0.001: ###) for each substance separate.

The aglycon Cy has at low concentrations (≤ 0.1 µM) a lower antioxidant impact than the glycosides. The ROS level in C2BBe1 cells is by Cy significantly higher than by Cy-3-sam at 0.01 and 0.1 µM, whereas at higher concentrations (≥ 10 µm) Cy-3-sam-5-glc shows the highest fluorescence values, indicating higher cellular ROS production than in presence of Cy-3-glc and Cy-3-sam. At the concentrations of 50 and 200 µM, the potency of Cy-3-glc is joined by Cy-3-sam, whereas at 100 µM the Cy is significantly less potent in reducing ROS than all other substances. For an overview of the normalized data see table 5 on page 33.

Despite being a main degradation product PGA does not appear to lower intracellular ROS levels. Instead, there seem to be an increase in ROS with a peak at 25 µM in all measured time points (t_{60} T/C: 155.7 ± 29.6%), indicative for a potential pro-oxidant effect. Only the highest concentration of PGA (400 µM, t_{60} T/C: 61.2 ± 16.0%) shows a lower ROS-level compared to the solvent control as seen in figure 17. Two different ways of data presentation were chosen, figure 17A shows the results normalized to solvent control, whereas in figure 17B the mean ± SD of fluorescence change from the measurement at t_0 is plotted.



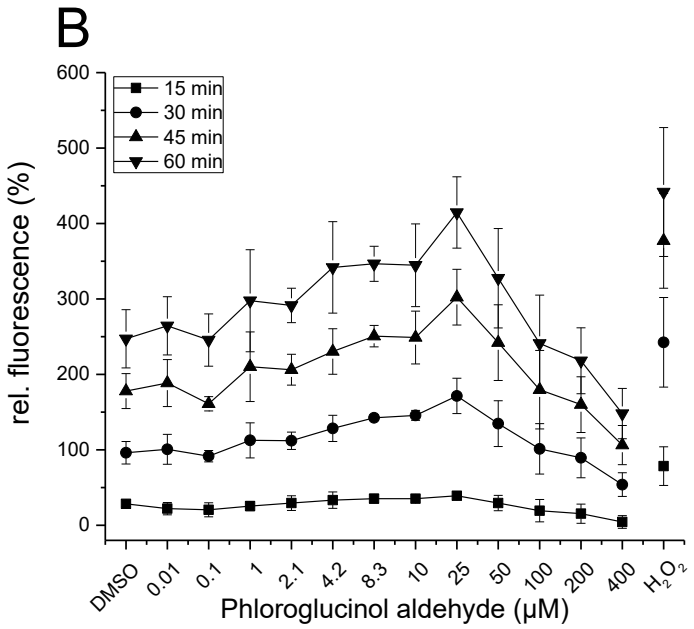


Figure 17: Effect of PGA on cellular ROS level of C2BBe1 cells during 1h incubation measured with the DCF assay.

Cells were pre-incubated with DCFH-DA followed by 1h incubation with PGA while fluorescence intensity is quantified with a Cytation3 photometer (excitation: λ: 485 nm, emission: λ: 528 nm. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and normalized to solvent control (T/C in %) (A). Solvent: 0.8% DMSO, 100 U/ml catalase. 1mM H_2O_2 served as positive control. Outliers of non-normalized data were eliminated with Nalimov. Statistical analysis with non-normalized data (B) showed no significant differences to solvent calculated with Kruskal-Wallis ANOVA (α = 0.05), Mann-Whitney-U-test (α = 0.001: ###) for pos. ctrl vs. medium. Kolmogorov-Smirnov test shows a normal distribution.

Table 6: Ranking by Kruskal-Wallis ANOVA of results from 1 h incubations with PGA of C2BBe1 cells measured with the DCF Assay.

PGA: Kruskal-Wallis ANOVA, Mean rank				
Conc. (µM)	**15 min**	**30 min**	**45 min**	**60 min**
DMSO	63.57	41.56	42.60	43.94
0.01	45.00	45.40	49.20	53.40
0.1	41.56	35.14	30.00	43.13
1	53.40	59.80	63.20	67.60
2.1	66.83	60.83	63.00	68.17
4.2	77.00	76.33	76.67	87.43
8.3	87.00	90.17	89.00	92.67
10	88.29	93.40	86.00	87.71
25	98.40	105.00	105.40	108.40
50	68.59	78.29	79.28	80.06
100	45.46	46.92	45.77	43.31
200	33.27	37.73	32.27	29.64
400	11.56	7.44	7.67	7.78
Prob>χ^2	**6.95E-07**	**8.92E-10**	**3.76E-10**	**1.39E-10**

Due to significantly different variance homogeneity (Brown-Forsythe test) a Kruskal-Wallis ANOVA was performed with the non-normalized relative fluorescence data, showing a significant difference ($P < 0.001$) in median values

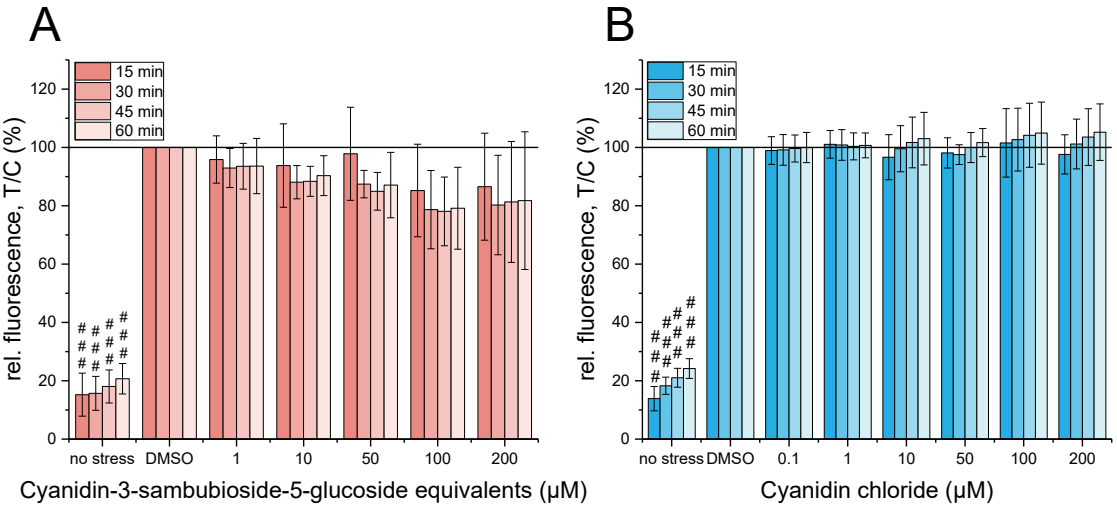
as shown in the ranking of table 6. Highlighted in red is the highest mean rank at 25 µM PGA in comparison to the solvent control DMSO highlighted in green.

Other anthocyanin degradation products have been shown to decrease ROS production, such as the grape phenolic compounds gallic acid (GA, 1 and 10 µM) and syringic acid (SA,10 µM) (S. Wang et al., 2015) as measured in Caco-2 cells with the DCF assay. SA as well as protocatechuic acid (PCA) show a reduced ROS level at ≥ 5 µM (Völkle, 2016), thus the rather pro-oxidant effect of PGA was not expected.

4.3 Protective effect against H_2O_2 induced stress

A modified version of the DCF assay (protective DCF assay) was used to assess whether and to which extent a 24h incubation with anthocyanins and respective metabolites can modify the cells response to oxidative stress induced by 1 mM H_2O_2. It is hypothesised that possibly strengthening mechanisms of the cells such as the Nrf2/Keap1 signalling pathway are involved to protect from H_2O_2-stress. This effect can be measured by the DCF assay as a reduction in fluorescence and thus in ROS.

After 24h incubation with PGA, Cy and Cy-3-sam-5-glc no statistically significant differences to the solvent control are evident as seen in figure 18.This indicates that after this incubation time the substances are not showing any antioxidant effect and do not protect the cells against the induced ROS.



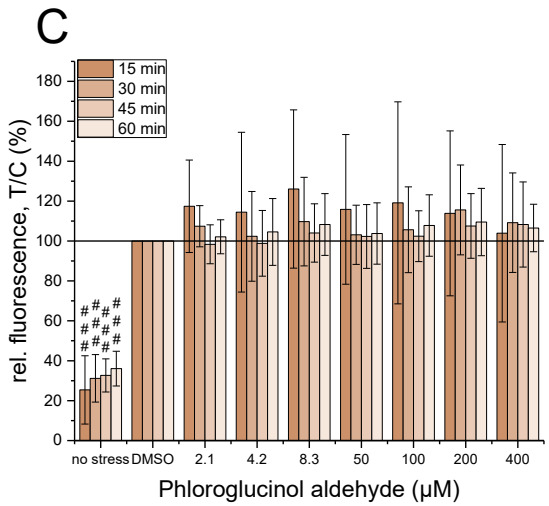


Figure 18: Effect of Cy-3-sam-5-glc[x], Cy and PGA on H_2O_2 induced oxidative stress in C2BBe1 cells after 24h of pre-incubation.

Cells were stressed for 1h with 1 mM H_2O_2 while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and is normalized to solvent control (T/C in %). Solvent: A, B) 0.8% DMSO, 0.0001% HCl (conc.), 100U/ml catalase: C) 0.8% DMSO, 100 U/ml catalase. Stressor: 1mM H_2O_2 in colourless medium. Non-normalized data outliers were eliminated with Nalimov. Statistical analysis with non-normalized data using 1) for A, B, and 2) for C
1) Significant differences to DMSO solvent as calculated with ANOVA (Bonferroni) (α = 0.05) and Students t-Test (α = 0.001: ###). Kolmogorov-Smirnov Test for normal distribution and Brown-Forsythe Tests for variance homogeneity was performed.
2) Significant differences to solvent as calculated with Kruskal-Wallis ANOVA (α = 0.05:*) and Mann-Whitney-U-Test (α = 0.001: ###). Variance homogeneity was calculated with Brown-Forsythe test and found sig. different, hence non-parametric tests for significance were chosen.
[x] : Approx. amount (according to MS): Cy-3-sam-5-glc-equivalents: 63.73% Cy-3-sam-5-glc, 24.92% n.i., 11.34% Cy-3,5-di-glc. (Oertel et al.)

In contrast, for Cy-3-glc a statistically significant difference to the solvent control was evident at the highest concentration of 200 µM Cy-3-glc (t_{30}: $P<0.05$, t_{45}: $P<0.01$, t_{60}: $P<0.001$) as calculated with Oneway ANOVA and a Bonferroni posthoc test (figure 19A). The same properties can be assumed for Cy-3-sam, where due to significantly different variance homogeneity (as calculated with the Brown-Forsythe test) a Kruskal-Wallis ANOVA was performed, which shows significant differences in median values (t_{15}: $P<0.05$, t_{30}: $P<0.01$, t_{45}, t_{60}: $P<0.001$) at 200 µM, with the ranking shown in table 7 (figure 19B). Cy-3-glc, Cy-3-sam and Cy-3-sam-5-glc (figure 18C) all show an apparent trend to a dose dependent reduction in fluorescence and thus in H_2O_2 –induced ROS, as shown in figure 19, yet with a statistical significance only at the highest concentration of 200 µM (T/C at t_{60} of Cy-3-glc: 60.69 ± 11.31%; Cy-3-sam: 61.54 ± 17.13%) and only for Cy-3-glc and Cy-3-sam. In the case of Cy-3-sam-5-glc (T/C at t_{60}: 81.76 ± 23.60%) a trend without statistical significance and

high standard deviations (figure 18A) is observed. Statistics are performed on non-normalized fluorescence change data (see figure 22).

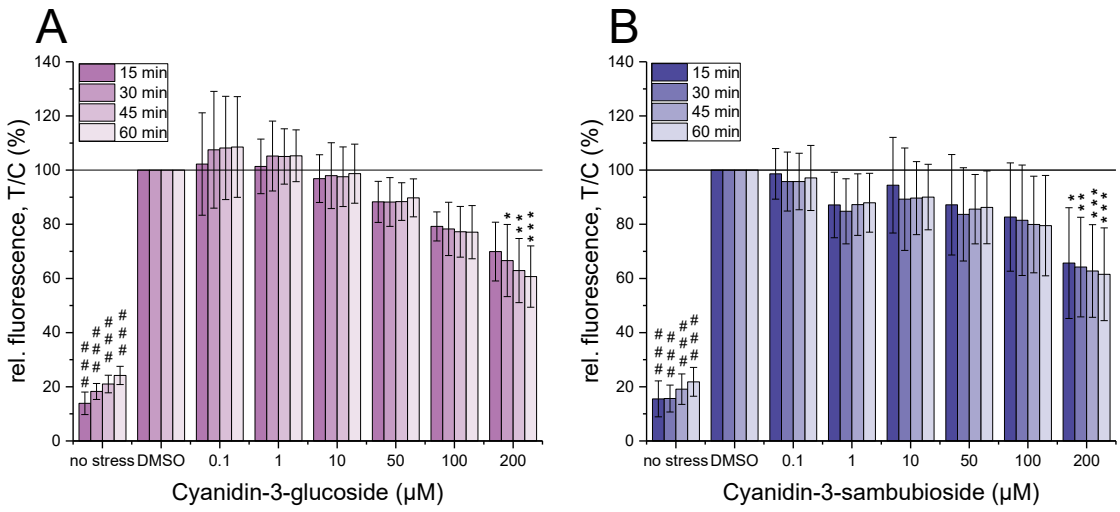


Figure 19: Effect of Cy-3-glc and Cy-3-sam[x] on H_2O_2 induced oxidative stress in C2BBe1 cells, normalized to solvent control

Cells were stressed for 1h with 1 mM H_2O_2 while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and is normalized to solvent control (T/C in %). Solvent: 0.8% DMSO, 0.0001% HCl (conc.), 100U/ml catalase: Stressor: 1mM H_2O_2 in colourless medium. Non-normalized data outliers were eliminated with Nalimov. Statistical analysis with non-normalized data using 1) for A, and 2) for B
1) Significant differences to DMSO solvent as calculated with ANOVA (Bonferroni) (α = 0.05) and Students t-Test (α = 0.001: ###). Kolmogorov-Smirnov Test for normal distribution and Brown-Forsythe Tests for variance homogeneity was performed.
2) Significant differences to solvent as calculated with Kruskal-Wallis ANOVA (α = 0.05:*) and Mann-Whitney-U-Test (α = 0.001: ###). Variance homogeneity was calculated with Brown-Forsythe test and found sig. different, hence non-parametric tests for significance were chosen.
[x]: Approx. amount (according to MS): Cy-3-sam-equivalients: 63.57% Cy-3-sam, 36.43% Cy-3-glc. (Oertel et al.)

Table 7: Mean ranking of Kruskal-Wallis ANOVA with Cy-3-sam

Cy-3-sam Kruskal-Wallis ANOVA, Mean rank				
Conc. (µM)	15 min	30 min	45 min	60 min
DMSO	39.82	38.80	42.22	43.00
0.1	48.67	45.20	44.60	46.40
1	30.67	23.67	23.67	24.56
10	33.44	30.67	32.57	30.38
50	29.44	24.83	27.71	29.71
100	25.33	23.22	22.78	22.78
200	17.00	12.89	10.78	11.11
Prob>χ^2	**0.0212**	**0.0019**	**3.35E-04**	**3.21E-04**

The colour of the incubation media after the 24h pre-incubation of the cells was diverse, with a stronger antioxidant effect the darker the colour appeared. All anthocyanins showed a dark reddish/purple colour while in acidified DMSO-

solvent solution. The colour of Cy disappeared almost immediately upon mixing with medium, while Cy-3-sam-5-glc showed a slowly dispersing, more reddish colour, and Cy-3-glc and Cy-3-sam in particular still showed a dark purple colour at higher concentrations after 24h incubations (figure 20). Since the aglycon is reported to have a low stability in cell culture media while the glycosides remain stable with a degradation of 57% (Cy-3-glc) compared with 96% for Cy after 4 h incubation (Fleschhut et al., 2006; Kay et al., 2009) this may together with the possible uptake via glucose transporters (Fernandes et al., 2014) be an explanation for the missing protective effect of the aglycon and the notable effect of the glycosides.

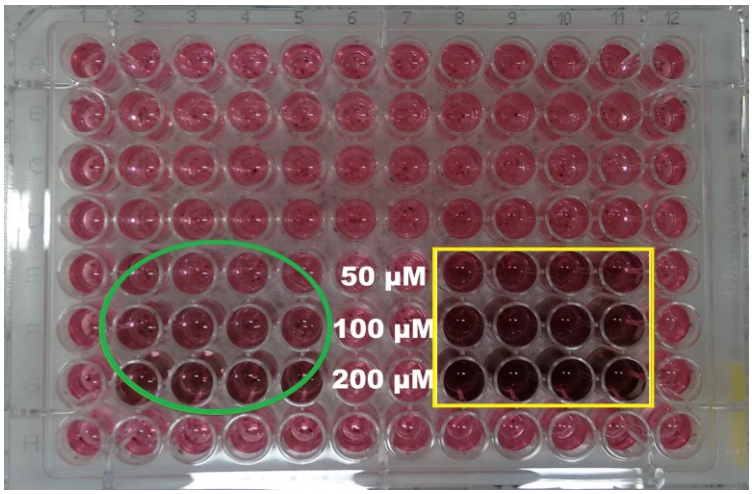


Figure 20: Visible colour-differences in higher concentrations (50, 100, 200 µM) of Cy-3-sam-5-glc (green ellipse) and Cy-3-sam (yellow square) after 24h incubation, pictured in transparent 96 well plate.

Using Oneway ANOVA and Bonferroni posthoc test the comparison between anthocyanins and the aglycon at each separate concentration with normalized data showed that Cy-3-sam lowered the fluorescence intensity induced by hydrogen peroxide significantly better than Cy, first at 1 µM ($P<0.05$) and at 200 µM ($P<0.001$) (figure 21). Cy-3-glc lowered fluorescence significantly better than Cy at 100 µM ($P<0.05$) and 200 µM ($P<0.001$).

The two monoglycosides, Cy-3-glc and Cy-3-sam, seem rather similar in their protective effect against hydrogen peroxide induced ROS as shown in figure 21.

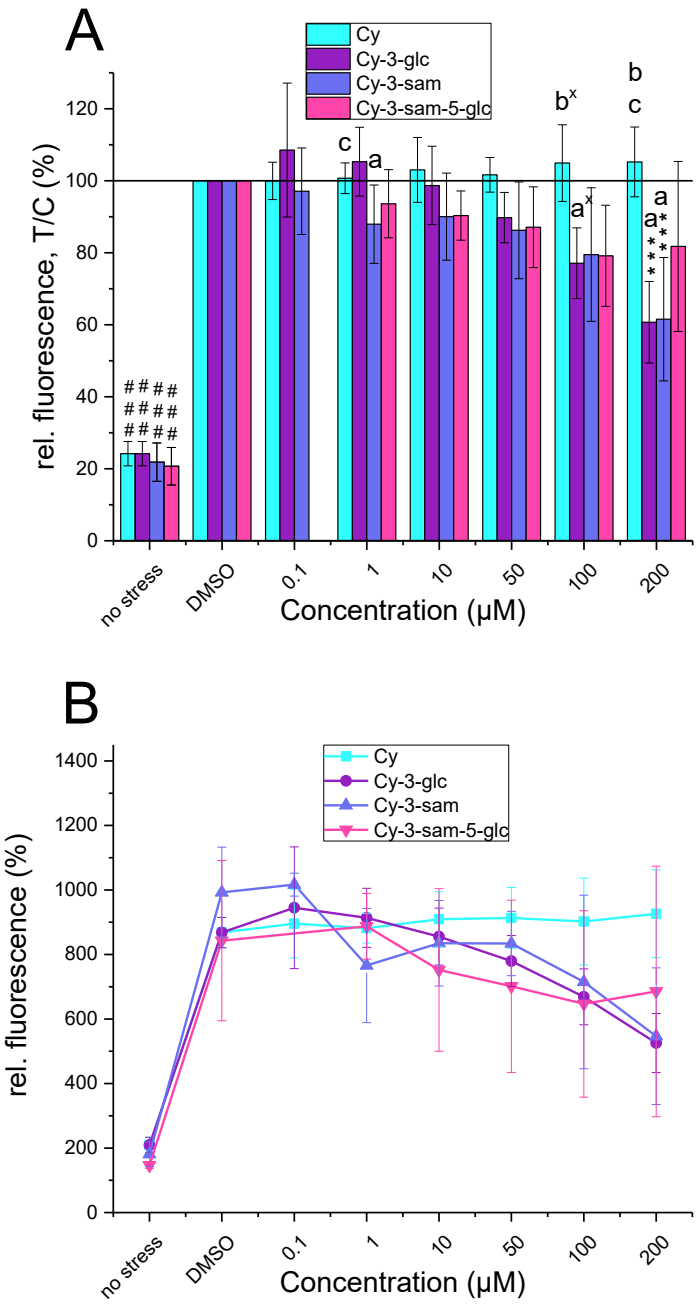


Figure 21: Comparison of the effect of Cy, Cy-3-glc, Cy-3-sam and Cy-3-sam-5-glc on H_2O_2-induced stress in C2BBe1 after 24h pre-incubation. A) Data plotted as T/C (%), B) Data (non-normalized) plotted as fluorescence change

Cells were stressed for 1h with 1 mM H_2O_2 while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and is normalized to solvent control (T/C in %). Solvent:0.8% DMSO, 0.0001% HCl (conc.), 100U/ml catalase: Stressor: 1mM H_2O_2 in colourless medium. Non-normalized data outliers were eliminated with Nalimov. Data is plotted at t_i = 60 min. ANOVA and posthoc Bonferroni (or x^x: Kruskal Wallis ANOVA) was performed for every concentration level with normalized data. Significantly different fluorescence change between the substances (α = 0.05) noted with a) Cy, b) Cy-3-glc, c) Cy-3-sam, d) Cy-3-sam-5-glc. Significant differences to DMSO solvent calculated with ANOVA (Bonferroni) (α = 0.05:*; 0.01:**, 0.001: ***) and Student's *t*-test (α = 0.001: ###) for each substance separate.
^{x}: Approx. amount (according to MS): Cy-3-sam-equivalents: 63.57% Cy-3-sam, 36.43% Cy-3-glc. Cy-3-sam-5-glc-equivalents: 63.73% Cy-3-sam-5-glc, 24.92% n.i., 11.34% Cy-3,5-di-glc. (Oertel et al.)

Table 8: Protective effect of Cy, Cy-3-glc, Cy-3-sam and Cy-3-sam-5-glc against H_2O_2-induced stress. Listed are the means ± standard deviation of T/C values (normalized to solvent control) at the time point t_{60}

Mean T/C values, t_{60}				
Conc. (µM)	**Cy**	**Cy-3-glc**	**Cy-3-sam**	**Cy-3-sam-5-glc**
0.1	99.97 ± 5.17	108.54 ± 18.60	97.10 ± 12.02	-
1	100.69 ± 4.27	105.29 ± 9.56	87.94 ± 10.87	93.61± 9.46
10	103.03 ± 9.01	98.69 ± 10.90	90.04 ± 12.10	90.33 ± 6.85
50	101.66 ± 4.82	89.76 ± 7.01	86.25 ± 13.44	87.11 ± 11.22
100	104.91 ± 10.65	77.09 ± 9.82	79.50 ± 18.54	79.15 ± 14.06
200	105.24 ± 9.70	60.69 ± 11.31	61.54 ± 17.14	81.76 ± 23.60

Our results concerning Cy contradict the results of Cvorovic et al. (2010) who found a dose-dependent inhibition of fluorescence increase while incubating Cy with Caco-2 cells and stressing with the hydroxyl radical-generator ABAP. Although it should be noted that both Dp and Cy were even inducing the formation of free radicals in LoVo/ADR cells, and that the incubation time was only one hour. A slightly longer incubation (3 hours) with Cy-3-glc in Caco-2 cells on the other hand showed a significant protection against 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced ROS-formation (Elisia & Kitts, 2008).

So far no DCF experiments with Caco-2 and elderberry anthocyanins have been conducted, the results from the non-tumorigenic colonic NCM460 cells with elderberry extract however are very much in line with our own results, as an inhibition of fluorescence intensity of 13%, 20% and 40% by 0.01, 0.1 and 1 mg/ml was apparent (Olejnik et al., 2016). Although, one must bear in mind that other compounds with possible antioxidant activity are still present in the fruit extract.

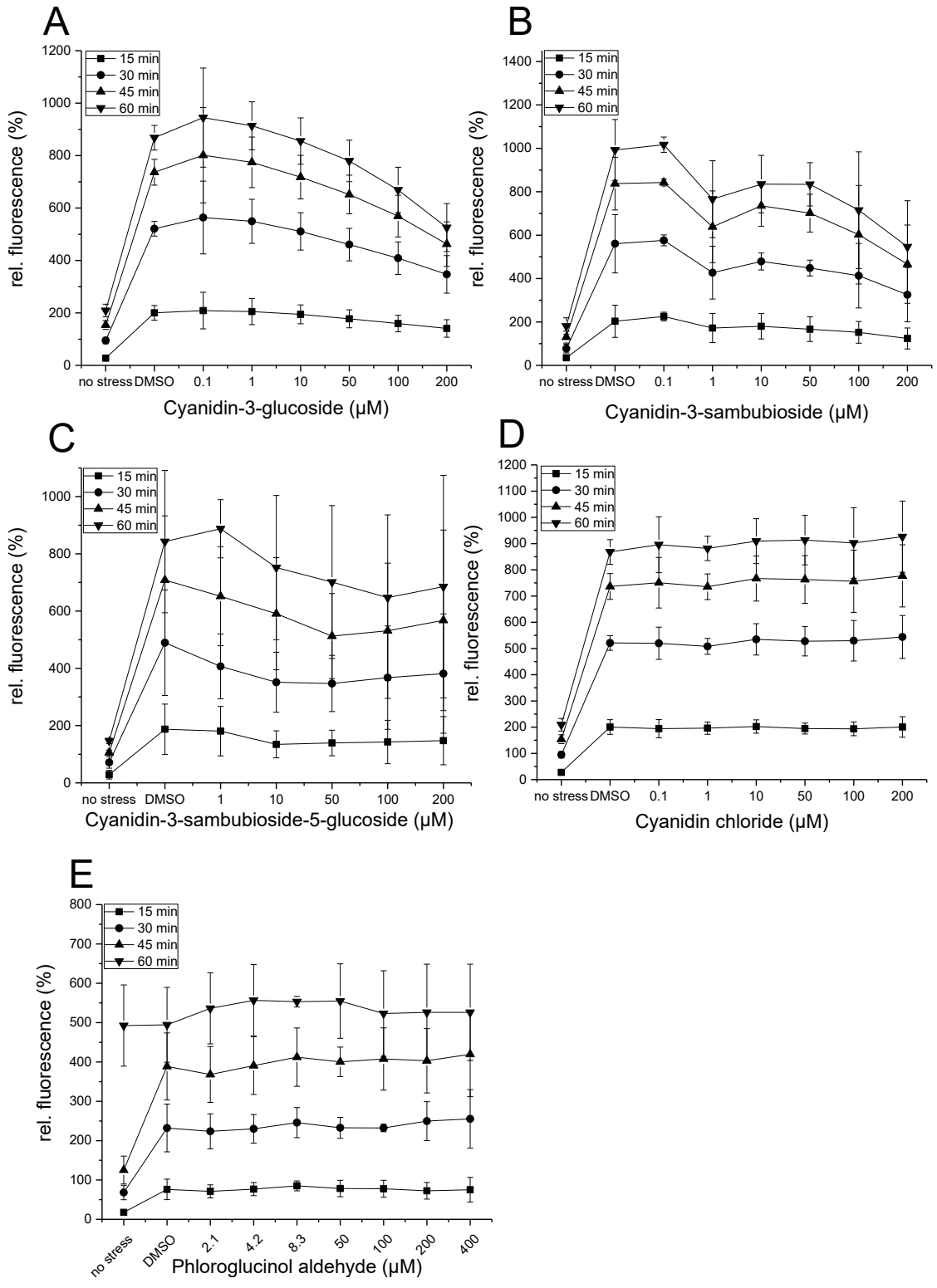


Figure 22: Effect of Cy-3-glc, Cy-3-sam^x, Cy-3-sam-5-glc^x, Cy and PGA on H_2O_2 induced oxidative stress in C2BBe1 cells, non-normalized data plotted as fluorescence change

Cells were stressed for 1h with 1 mM H_2O_2 while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments. Plotted is the fluorescence change from t_0. Solvent: 0.8% DMSO, 0.0001% HCl (conc.), 100U/ml catalase: Stressor: 1mM H_2O_2 in colourless medium. Non-normalized data outliers were eliminated with Nalimov. Statistical analysis shown in fig. 18-19.

5 Discussion

Anthocyanins are discussed to have several positive therapeutic effects, many of them based on their antioxidant activity and the suppressing of excessive ROS-production (Olejnik et al., 2016), which may prevent or ease oxidative stress-related diseases and thus act cardioprotective, neuroprotective, anti-inflammatory, anticarcinogenic and retaining of the integrity of DNA (Zafra-Stone et al., 2007), making them interesting for further studies.

There are many *in vitro* studies concerning the chemical antioxidant activity of the most common anthocyanins and of anthocyanin-rich extracts, it is however questionable if these effects are of relevance *in vivo*, since none of the assays consider the uptake, metabolism and bioavailability of the compounds. Cell culture experiments are of great value as model systems to screen for effects and mechanisms in a biological system (Wolfe & Liu, 2007) where it is possible both to test high concentrations in order to screen for toxicity and to use biologically relevant concentrations that may *in vivo* reach the studied cells. The concentrations used in this thesis span both points of interest, as Esselen et al. (2011) calculated that based on an ileostomy-study where up to 85% of ingested blueberry extract could reach the colon (Kahle et al., 2006), 200 g blackberries (50 mg total anthocyanins / 100 g FW) would supply comparable concentrations in colon cells as proven bioactive in their study (max. test concentration: 100 µM Cy and Cy-3-glc). Analogue, considering 134.94 mg Cy-3-sam/ 100 g fresh weight of elderberries (Anton et al., 2013), a concentration of 197.4 µM may be reached in the colon, concentrations which showed a protective effect against ROS-induction in the protective DCF assay with Cy-3-glc and Cy-3-sam. When calculated with the lower percentage of Cy-3-glc (28.3%) arriving in the stoma-bags (Kahle et al., 2006) it would still be 65.7 µM, which was potent in lowering the intracellular ROS-production as measured with the DCF assay.

Seeing to the purely chemical antioxidant activity often the anthocyanins have a higher ORAC value than their respective glycosides, which is attributed to the reactiveness of the aglycon (Stintzing et al., 2002), while for cyanidin and Cy-3-glc this was however the opposite case (H. Wang et al., 1997), agreeing with the cell culture DCF data, where the glucoside showed a higher ROS-reducing

potential than the aglycon. Cy-3-glc and Cy-3-sam showed a very similar pattern in both the DCF assay and the protective version, whereas the dose-response curve of Cy-3-sam-5-glc was less steep in the DCF assay, and although showing a similar curve as Cy-3-glc and Cy-3-sam in the protective DCF assay, at the highest concentration it plateaued and was not significantly different to solvent control. On the level of chemical structure the addition of a third sugar moiety may reduce the antioxidant impact, as has been shown with cyanidin-3-xylosyl-galactoside and Cy-3-xyl-glc-gal (Stintzing et al., 2002).

An uptake study with elderberry anthocyanins in aortic endothelial cells could show that all four anthocyanins (Cy-3-glc, Cy-3,5-di-glc, Cy-3-sam and Cy-3-sam-5-glc) could be found in the cell membrane, however only Cy-3-glc and Cy-3-sam were detected in the cytosol. Significantly more Cy-3-glc than Cy-3-sam were detected in cytosol, which the authors attribute to the bulkier structure of the Cy-3-sam and that it may be degraded to Cy-3-glc while inside the cell (Youdim, Martin, & Joseph, 2000). The non-significant results of the protective DCF concerning Cy-3-sam-5-glc may thus be attributed to the poor cellular uptake of the larger glycoside, although uptake studies in the same cell line would be necessary for confirmation.

The intracellular ROS level was effectively lowered by all incubated anthocyanins from 0.01 - 0.1 µM onwards, although not by the degradation product of Cy (PGA), which acted rather as a pro-oxidant with a peak maximum at 25 µM (155.7 ± 29.6% compared with solvent control). As a comparison, other anthocyanin degradation products such as syringic acid and protocatechuic acid showed similar ROS-decreasing effects as cyanidin when tested in our group (Völkle, 2016). PCA was able to reduce cellular ROS by 25.3% already at a concentration of 0.1 µM (figure 28, appendix). Another interesting effect was seen in the comparison of the single anthocyanins and the combination of the two major elderberry anthocyanins in the proportion as present in the juice, named AnthoKit (Cy-3-sam 88%, Cy-3-sam-5-glc 12%). The AnthoKit showed an even stronger decrease of ROS-levels than the single compounds (figure 29, appendix, AnthoKit data by Schier (2017)). A similar observation was seen in the chemical antioxidant activity, as pigment extracts were shown to have 3- to 6-fold higher ORAC values than those of the isolated

pigments, such as the elderberry extract ORAC-value of 9.22 compared with Cy-3-glc (2.26). A possible synergism within the extract was discussed (Stintzing et al., 2002).

The result of the experimental work mainly agrees with the heterogeneous literature concerning the substances already tested in cell culture experiments, as reported in chapter 4 (Results) with the exception of the protective effect attributed to Cy in the experiments of Cvorovic et al. (2010), although a direct comparison proves difficult due to different test systems, cell lines, modifications of the assays and use of different stressors.

6 Conclusion

Elderberry anthocyanins proved to have considerable potential in decreasing intracellular ROS levels in C2BBe1 cells, with Cy-3-glc and Cy-3-sam showing the most potent impact as an apparent IC_{50} was reached at 1 µM (Cy-3-glc) and 10 µM (Cy-3-sam), while at a concentration of 200 µM ROS was reduced by 88.63 and 91.73% respectively in comparison to the solvent control. The largest glycoside, Cy-3-sam-5-glc, while being similarly effective at lower concentrations, only reached a reduction of fluorescence by about 70% compared to solvent control at the highest test concentration 200 µM. A similar picture was evident in the protective DCF assay, where only Cy-3-glc and Cy-3-sam proved to significantly protect the cells from H_2O_2-induced ROS at the highest concentration tested (200 µM). Concerning the degradation products, cyanidin caused a similar decrease in intracellular ROS as the triglycoside, whereas PGA instead lead to increased ROS levels, thus acting rather pro-oxidant. Concluding, the monoglucoside and the diglycoside proved to be the most potent elderberry anthocyanins with respect to antioxidant properties, while according to different sources both also being the most abundant in elderberry juice (Sidor & Gramza-Michałowska, 2015). Still open for discussion is why the triglycoside was less efficient and not protective against H_2O_2-induced ROS, this is where uptake- and stability studies are indicated. The mechanism of the protective effects of Cy-3-sam and Cy-3-glc in C2BBe1 cells is not known. Experiments concerning the modulation of Nrf2-dependant gene transcription could add for clarity, and have been started already.

7 Summary

Anthocyanins are phenolic compounds responsible for the blue, purple and red colours of berries, fruit and flowers (Kamiloglu et al., 2015). The most common anthocyanidin in edible plant parts is cyanidin, which accounts for ca. 50%, with cyanidin-3-glucoside being by far the most common anthocyanin (Kong et al., 2003). The average consumption in Europe amounts to a range from 18.73 to 64.88 mg anthocyanins /day (Zamora-Ros et al., 2011), whereas food content can be significantly higher, reaching 2 - 4 g anthocyanins/kg fresh weight of blackcurrants or blackberries (Manach et al., 2004) and over 4 g/L in elderberry juice (Jakobek et al. (2007). The goal of this thesis was to characterize the predominant elderberry anthocyanins Cyanidin-3-glucoside (Cy-3-glc), Cyanidin-3-sambubioside (Cy-3-sam) and Cyanidin-3-sambubioside-5-glucoside (Cy-3-sam-5-glc) and their selected metabolites cyanidin (Cy) and phloroglucinol aldehyde (PGA) in the context of their antioxidant and ROS-protecting properties in a colon cell model system using C2BBe1 cells.

Since very little research on elderberry anthocyanins in their pure form has been published, first a toxicity assay in the form of the sulforhodamine B Assay was conducted in order to rule out any antiproliferative effect distorting the result of the following experiments concerning antioxidant activity. None of the compounds significantly reduced the proliferation of the cells as compared to solvent control after 24 hours incubation.

All anthocyanins and the aglycon Cy induced a potent reduction of intracellular ROS-production as assessed with the DCF Assay, with Cy-3-glc and Cy-3-sam showing the strongest effect compared to the solvent control, decreasing as far as 11.37 ± 3.91% and 8.2 ± 10.61% by 200 µM after 60 minutes incubation. The aglycon caused similar effects as Cy-3-sam-5-glc, whereas the metabolite PGA caused a significant induction of ROS-production with a peak at 25 µM (155.7 ± 29.6%). In order to determine whether the compounds are capable of strengthening the cells against extracellular ROS-accumulation, they were stressed with 1 mM H_2O_2 after a 24-hour incubation with the compounds, and fluorescence was again measured with the DCF Assay. After one-hour stress, incubation with 200 µM Cy-3-glc and Cy-3-sam decreased oxidative stress significantly (T/C 60.69 ± 11.31; 61.54 ± 17.14) whereas none of the other

compounds acted protective. The exact mechanism of the protective effect is still open, and it would be crucial to conduct uptake- and stability experiments in order to see to what extent the compounds are taken up by the cells, and to what extent the larger anthocyanins are degraded to structurally less complex anthocyanins and metabolites.

8 Material and Methods

8.1 Cell culture

8.1.1 Caco-2 Brush Border expressing 1 (C2BBe1)

In this thesis a clone (C2BBe1, ATCC® CRL-2102™) of the common adherent cell line Caco-2 was used. The Caco-2 cells are enterocytes from the colorectal adenocarcinoma of a 72 years old Caucasian male isolated by Fogh et al. in the 1970s ((ATCC); Sambuy et al., 2005).

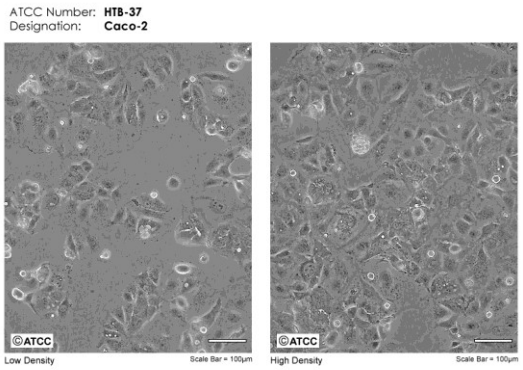


Figure 23: not differentiated Caco-2 cells (ATCC)

In their work on Caco-2 as a model system for intestinal epithelial permeability Hidalgo et al. (Hidalgo, Raub, & Borchardt, 1989) described a moderate growth for 4 days with near confluence at day 3, followed by a rapid growth phase until steady state was reached at day 6 where cells were strongly interdigitated, contained numerous desmosomes and formed a polarized cell monolayer with microvilli. At day 16 the monolayer was 30 µm high with morphology similar to that of the small intestine including columnar shape and well-defined brush border.

In an initial study on the parental Caco-2 cell line Peterson and Mooseker found that it is highly heterogeneous concerning the morphology and the brush border expression, which also was lost by many cells after several passages. In order to achieve a more heterogeneous population they cloned the parental cell line using limiting dilution and chose the cultivated cells for their morphological homogeneity, apical villin localization and stability of the brushborder expression (Peterson & Mooseker, 1992). Two clones, the C2BBe1 and C2BBe2 were chosen as the best concerning these criteria, and of the two C2BBe1 reaches confluence faster and trypsin can dissociate the cells during cell passage easier. When allowed to grow beyond confluence C2BBe1 cells differentiate and form a polarized monolayer with an apical brushborder, which is comparable to the morphology and protein content of the human enterocytes. The study of Peterson & Mooseker (1992) shows that the brush border

cytoskeleton of the confluent, polarized C2BBe-cells is comparable to that of the human enterocyte.

8.1.2 Thawing cells

The frozen stocks were kept at -196 °C in liquid nitrogen. To take cells into culture the vial was rapidly thawed in a 37°C water bath while keeping the cap above the surface. The vial was decontaminated with 70% alcohol and the cells suspended in 10 ml 37°C complete medium. In order to remove the freezing medium (high DMSO content), cells were centrifuged according to working group praxis at 350 *g* (RCF) for 6 minutes. ATCC suggests doing this at 125 *g* for 5-7 minutes ((ATCC)). The media above the cell pellet was carefully removed, and the cell pellet re-suspended in 10 ml previously prepared, sterile filtrated, complete growth medium with 1% P/S (penicillin/streptomycin) and 20% FBS (foetal bovine serum). The cells were initially cultured in 25 cm^2 flasks for several days until cells had attached to the bottom and formed islands of confluent cells. Medium was changed every third day if cells were not yet confluent. The cells were first passaged after approximately one week into larger flasks (T75).

8.1.3 Cultivation

The C2BBe1 cells were cultivated in 75 cm^2 flasks at 37°C, 5% CO_2 and 95% air moisture. Dulbecco´s Modified Eagle Medium (DMEM) GlutaMAX™ with 4.5 g/L glucose and sodium pyruvate was used as cell culture medium. The GlutaMAX™ formulation contains L-alanyl-L-glutamine, which is a stabilized form of L-glutamine that minimizes degradation and ammonia build-up (Thermo-Fischer). To complete the medium it was substituted with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The fetal bovine serum contains important growth factors, cytokines, and hormones, binding- and transport proteins, as well as factors for attachment and spreading of the cells. (Gstraunthaler & Lindl, 2013) In order to reduce the risk of contamination through bacteria the combination of the antibiotics penicillin (5000 units/ml) and streptomycin (5000 µg/ml) was used routinely. Mycoplasms are a common and often unnoticed source of contamination. Their small size (0.2-2 µm) allows them to pass most filters (around 0.2 µm) and they are resistant against β-

lactame-antibiotics (Gstraunthaler & Lindl, 2013). The cells used in this thesis were tested by Eva Attakpah and proven negative for mycoplasms.

8.1.4 Sub-cultivation

In this thesis the C2BBe1 cells were used in a non-differentiated state, which means they have to be sub-cultivated at a regular basis in order to keep them in log-state of growth where the proliferation is the highest. The adherent cells must be loosened from the growth surface before reaching complete confluence, brought to suspension, and a smaller amount of cells is cultivated in a new tissue culture flask with fresh complete growth medium (Gstraunthaler & Lindl, 2013). The C2BBe1 cells were passaged twice a week at 80 - 90% confluence using PBS (phosphate buffered saline solution) to wash away cell debris and medium rests which could hamper the effect of the trypsin used to detach the cells. Trypsin is an alkaline protease from the bovine pancreas, which by cleaving the peptide bonds of lysine or arginine breaks the cell-matrix-connections and thus detaches the cell from the bottom of the culture flask. The trypsin acts for 3 - 10 minutes until the cells are free from the bottom of the flask. In order to avoid selection of cells with poor anchorage this process should be complete. Fresh complete growth media is added to stop the trypsin digestion through the trypsine inactivation by the protease inhibitors contained in FBS. (Gstraunthaler & Lindl, 2013)

Protocol

- Remove used growth medium with sterile Pasteur pipette, not touching the cell layer
- Wash away rests of growth medium and dead cells with 10 ml 37°C PBS (phosphate buffered saline solution), applied with pipette on the side of the bottle and gently rocked so that PBS covers the cell matrix. Remove PBS with sterile Pasteur pipette
- Add 1.5 ml 37°C trypsin solution, covering all cells by gently rocking the flask, incubate for 5 - 8 minutes in incubator at 37°C, 5% CO_2 and 95% air moisture
- Add 8.5 ml 37°C complete growth medium and allow covering all cells in order for the FBS to stop the digestion of the cell membranes. Use a 5 ml

sterile pipette to wash the bottom of the flask several times with cell suspension to loosen rests and disperse cell clusters

- Count cells with Neubauer hemocytometer using trypan blue staining to distinguish viable from dead cells
- Add appropriate amount of cell suspension (around 1.2 - 1.5 million cells for growth time 3 - 4 days corresponding to ca. 1 - 2 ml) to new flask (or old if cells are completely removed from the bottom) to a total of 20 ml 37°C complete growth medium

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Passage number

The cells in this thesis were used between passage 18 and 40. The age of the cells, e.g. number of passages in culture can influence functions and activities of the cells. The expression of the glucose transporter GLUT-5 and sucrose-isomaltase was shown to increase with the number of passage, as well as the trans-epithelial electrical resistance (TEER) and the proliferation rate, which was increased in late passages. The passage number also influenced the expression of hormones and metabolic activities. The review of Sambuy et al. describes early passages of Caco-2 and clones as around 30 - 50 and late passages around 90 - 110 (Sambuy et al., 2005), which places our cells in the younger category.

8.1.5 Counting cells with Neubauer hemocytometer

The initial number of cells (inoculum) is for most adherent cells crucial for their proliferation. In order to achieve optimal growth where the cells don´t grow too slowly or reach confluence and contact inhibition too fast it is important to count the cells before seeding the appropriate amount. In this thesis a Neubauer hemocytometer was used. It was originally used to count red blood cells, hence the name. The hemocytometer is a thick glass microscope slide with etchings in four large squares containing four small squares each (figure 24). These 1 mm^2 large and 0.1 mm deep squares form chambers with a volume of 0.1 µl where cell suspension is drawn through the capillary forces from the edge of the thin glass slide covering the chamber.

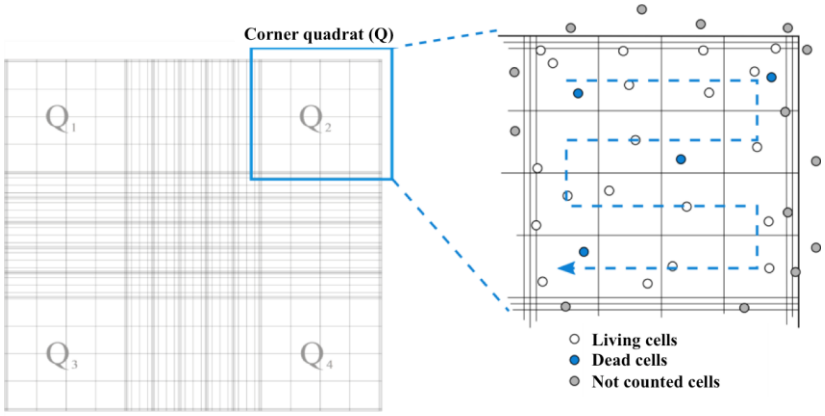

Figure 24: Neubauer hemocytometer, modified after Gehrke (2012), Gstraunthaler and Lindl (2013)

In order to achieve a representative cell count at least four large squares (Q_{1-4}) and the mean value thereof should be counted. All cells inside the lines and only the cells on two on the outer lines of the large square should be counted (Gstraunthaler & Lindl, 2013).

Protocol Counting Cells

- Clean chamber and cover slide with 70% ethanol. Lightly moisturize the cover slide and place in the middle of the chamber. “Newton rings” should appear when correctly placed.
- Stain a small aliquot of the cell suspension with trypan blue.
- Apply 10 µl stained cell suspension to the edge of the hemocytometer, right next to the cover slide. The capillary forces will draw the suspension into the chamber.
- Use a microscope with 10x objective to count the cells in each square
- The mean value of all large squares is multiplied with the trypan blue dilution factor and 10^4, which results in cell concentration per ml.

Trypan blue is a sour stain, which as anion binds to proteins (pH 7.5). Living cells should not be stained whereas dead cells will be blue. Only the vital cell count should be used for the inoculum.

$$\text{Percentage vital cells} = \frac{\text{unstained cells}}{\text{unstained cells+stained cells}} \text{ x } 100.$$

(Gstraunthaler & Lindl, 2013)

8.1.6 Freezing cells

In order to secure the supply of cells, stocks were prepared in two varieties, according to ATCC guidelines (A) and according to lab praxis (B) at a cell passage as low as possible and frozen in liquid nitrogen for later use. Anti-freezing agent containing medium was prepared in order to rapidly freeze the cells and to secure good vitality. Because of its low freezing point DMSO was used as anti-freezing agent to prevent the formation of ice crystals in and outside the cells. DMSO enters the cell and partly binds or replaces the water, thus preventing crystals and dehydration of the cytoplasm. The addition of a higher content of FBS might also have a protecting effect. (Gstraunthaler & Lindl, 2013)

Protocol Freezing Cells

- Passage cells according to protocol (2 ml trypsin, 4 ml medium for a 125 cm^2 flask)
- Count cells, portion around 4 million cells per ml in either A) complete growth medium containing 5% DMSO as end concentration or B) FBS with 10% DMSO as end concentration.
- Prepare 0.5 ml freezing media (A: 15% DMSO, B: 30% DMSO) for each 1.5 ml cryovial
- Add 1 ml cell suspension immediately before freezing.
- Place in freezing container (Mr. Frosty®) containing isopropyl alcohol at -80°C for at least 4 h to assure slow freezing (1°C/minute) and thereby better cell vitality before final storage in liquid nitrogen at -196°C. (Thermo-Fischer)

8.1.7 Incubation conditions

All cells were left to settle and anchor to the 96 well plates at 5 % CO_2, 37°C and 95% relative air humidity for 24 hour before the experiments were started. The incubation time with the anthocyanins for the SRB and the protective DCF assays was set at 24 hours. The DCF assay experiments were first incubated for up to 3 hours (with PGA), before incubation time was set for one hour during microplate-reader measurement. All incubations were under above-mentioned conditions.

To be able to compare the results with previous data of the work group the same solvent (80% DMSO with 20% water) was kept for solving the chemically stable PGA. Anthocyanins however are only stable at a low pH, which is why the 80% DMSO was acidified. Cabrita and colleagues reported that all anthocyanin-3-glucosides in their study were very stable even after 60 days in an aqueous solution at pH 1-3 (Cabrita et al., 2000). 3% acetic acid had been previously used in the work group but proved to be not acidic enough. After pH-measurements 0.1% HCl (conc., 37%) was chosen. At this concentration the solvent pH was low enough (2.87) to stabilize the flavylium-form and at the same time kept the growth medium at a physiological pH (7.88). All test substances were vortexed well and kept for 1 minute in an ultrasonic bath to ensure proper dissolving. The final dilution in incubation medium (containing 100 U/ml catalase) was prepared immediately before use and all dilutions were well vortexed. The final concentration of DMSO amounted to 0.8% in all experiments.

8.2 Test Systems

8.2.1 Sulforhodamine B assay

The Sulforhodamine B (SRB) assay is an *in vitro* colorimetric cytotoxicity assay, which measures the cellular protein content of adherent and suspension cultures growing in 96-well microtiter plates. It was first developed by Skehan et al. (1990). It is a sensitive, inexpensive method with a high linear correlation between protein content and optical density. Cells are first fixed at the bottom of the microtiter plate wells by addition of trichloroacetic acid directly in the growth medium. Fixing medium is removed and washed with tap water after which the fixed cells are air-dried. The dried cellular protein is stained with sulforhodamine B solution, which is a pink aminoxanthene dye with two sulfonic groups. While mildly acidified through the 1% acetic acid used for solving the

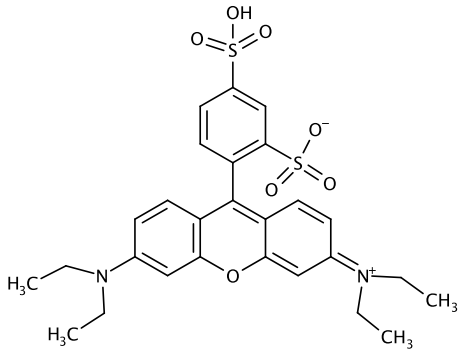

Figure 25: Structure formula SRB

SRB compound and washing off excess dye, the SRB binds stoichiometrically to basic amino acid residues of the proteins in the TCA-fixed cells. The cellular protein is once again air-dried, after which the bound SRB dye is brought into solution with Tris-base and the optical density is measured with a microtiter plate reader at 570 nm. (Skehan et al., 1990)

8.2.1.1 Protocol SRB assay

According to Theresa Janson's optimized and adapted SRB protocol for C2BBe1

Appliances and materials:

- Microtiter plate reader Cytation 3, Gen5 Software
- Ultrasonic bath
- 96-well microtiter plate (sterile, for tissue culture)
- Multistep-pipette
- Brown injection needle (0.45x25 mm)

Reagents:

• Catalase (3356 U/mg)	100 U/ml medium: Solve 2.98 mg in 100 µl cold PBS, dilute 1:100 in anthocyanin incubation medium
• H_2O_2	1 mM → 1M stock solution in H_2O_{bidest}, dilute 1:1000 in colourless medium for pos. ctrl
• DMEM Glutamax	High glucose (4.5 g/L), 10% FBS, 1% P/S
• Trichloroacetic acid (TCA)	50% (w/v): 12.5 g in 25 ml H_2O_{bidest}
• Acetic acid (100%)	1 % (v/v): 10 ml in 1 L H_2O_{bidest}
• SRB	0.4% (w/v): 1 g in 250 ml 1% acetic acid
• TRIS-base	10 mM: 1.2114 g in 1 L H_2O_{bidest}, adjust to pH 10 with HCl- or NaOH solution

Optimized protocol:

- **Seed cells:**
  - 15.000 cells/well in 96 well plate and 200 µl medium (for a total cultivation of 48 h)
  - 24 h growth in incubator at 5% CO_2, 37°C, humidified atmosphere

- **Incubation with substance**
    - Weigh substance and solve it in acidified 80% DMSO for a 20 mM stock solution using ultrasonic bath for 1 minute and vortex (use dark tubes and turn off laminar light, anthocyanins are light sensitive) or thaw solved acidified aliquots.
    - Weigh catalase: 2.98 mg solved in 100 µl cold PBS, vortex well, dilute 1:100 with medium for 100 U catalase/ml medium (catalase is used when incubating ROS-producing substances, not in H_2O_2-pos-ctrl + medium ctrl)
    - Make dilution series: stock dilution 0.01, 0.1, 1, 5, 10, 20 mM, then dilute stock dilution 1:100 in medium for 0.1, 1, 10, 50, 100, 200 µM
    - Make solvent control: dilute acidified 80% DMSO 1:100 with medium (100 U/ml catalase)
    - Make positive control: 1 M H_2O_2 stock in water, dilute 1:1000 with medium (no catalase): 1 mM H_2O_2, prepare ctrl medium (no catalase)
    - Aspire cell culture medium using brown injection needle (0.45x25 mm) and pump on lowest setting
    - Add 200 µl incubation medium with substance, solvent- (0.8% acidified DMSO) and positive control (end conc.1 mM) (use 100 µl/well if substance is limited)
    - Incubate for 24h
- **Protein fixation**
    - Add 20 µl 50% TCA to incubation medium
    - Incubate in refrigerator (4°C) for 1h
    - Flick fixing fluid in the sink with a swift hand movement
    - Wash 4x with tap water (use plastic tube on the tap and low water pressure)
    - Let dry without lid overnight in a drawer (room temperature)

- **Protein dye**
    - Add 50 µl SRB reagent to dried proteins
    - Incubate for 30 minutes in a dark drawer at room temperature
    - Flick dyeing solution in the sink (be cautious of staining splashes)

- Wash 2x with tap water and 2x with 1% acetic acid until washing water is clear
- Let dry without lid overnight in a dark drawer (room temperature)

- **Photometric measurement**
  - Add 100 µl TRIS-base (pH 10)
  - Use the shake-option in the plate reader for 5 minutes (slow, orbital, 300 sec)
  - Absorption measurement at λ = 570 nm

- **Data evaluation**
  - Form mean of at least triplet measurements
  - Norm the test substance to the mean of the control, multiply with 100 → T/C (%)
  - **Statistical analysis**: For n ≥ 5 perform ANOVA (Bonferroni) for substance-concentrations comparing with solvent control and Student´s *t*-test for positive control + respective solvent control if all requirements are met, if not use Kruskal-Wallis ANOVA and Mann-Whitney-U-test.

8.2.2 Dichlorofluorescein assay

Keston and Brandt (1965) first described the use of 2´,7´-dichlorofluorescin diacetate (DCFH-DA) in a fluorometric analysis to detect the formation of hydrogen peroxide. The method to quantify oxidative stress and thereby the potency of pro-oxidants in cells was then further developed by Wang and Joseph (H. Wang & Joseph, 1999) using a fluorescent microplate reader.

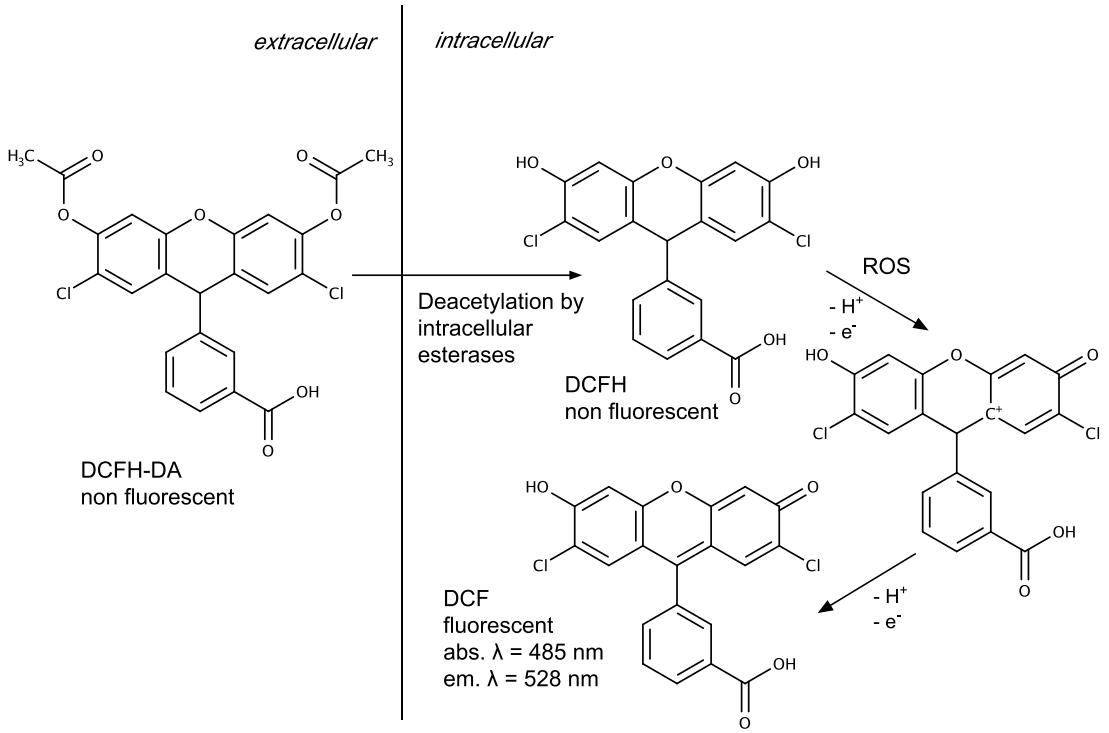

Figure 26: Mechanism of DCFH-DA, adapted after LeBel et al. (1992)

The non-ionic, nonpolar DCFH-DA crosses the intact cell membrane where it is hydrolysed by intracellular esterases to nonfluorescent 2´,7´-dichlorofluorescin (DCFH, figure 26). In the presence of reactive oxygen species (ROS) it is oxidized to highly fluorescent 2´,7´-dichlorofluorescein (DCF) (LeBel et al., 1992). When excited at wavelength λ = 485 nm DCF emits light which is measured at λ = 528 nm (Wang & Joseph: 530 nm) by a microplate reader. The emitted light reflects the ROS in the cells.

Data analysis was performed according to Wang and Joseph (H. Wang & Joseph, 1999) who calculated the percentage of increase in fluorescence per well instead of only fluorescence net change.

The use of the formula $\frac{Fti-Ft0}{Ft0}$ x 100% (with Ft_i = fluorescence at time point i and Ft_0 = first measurement) reflects the changes over time in the same well, while accounting for variability among wells and cancelling out the background fluorescence (H. Wang & Joseph, 1999). This assay was used to evaluate the efficacy of anthocyanins against the formation of intracellular ROS.

The protocol used in this thesis was first optimized by Theresa Janson and contains new minor modifications for example the use of a modern plate reader.

8.2.2.1 Protocol DCF assay

Modified from Theresa Janson's optimized protocol for C2BBe1.

Appliances and materials:

- Microtiter plate reader Cytation 3, Gen5 Software
- 96 well plate (black, sterile)
- Infrared light lamp
- Dark 15 ml falcon tubes
- Multichannel pipette
- Washing trays for PBS and DCF reagent
- Brown injection needle (0.45x25 mm)

Reagents:

• DCFH-DA	10 µM: Solve 0.48729 mg in DMSO to C = 1 mM, aliqute and store at -20°C; dilute 1:100 in 37°C PBS
• Catalase	100 U/ml medium: Solve 2.98 mg in cold PBS, dilute 1:100 in anthocyanin incubation medium
• H_2O_2	1 mM → 1M stock solution in H_2O_{bidest}, dilute 1:1000 in colourless medium
• DMEM Glutamax	High glucose (4.5 g/L), 10% FBS, 1% P/S
• Colourless DMEM	High glucose (4.5 g/L), 0% FBS, 0% P/S
• 37°C PBS	

Optimized protocol:

1. **Seed cells:**

- 15.000 cells/well (for a total cultivation of 48h) in 96 well plate and 200 µl medium / well
- 24h growth in incubator at 5% CO_2, 37°C, humidified atmosphere

2. **Prepare substances**

- Weigh substance and solve it in acidified 80% DMSO for a 20 mM stock solution → ultrasonic bath for 1 minute and vortex. (Anthocyanins are

light sensitive; use dark tubes and turn off laminar light) or thaw frozen aliquot

- Weigh catalase: 2.98 mg solved in 100 µl cold PBS, vortex well, dilute 1:100 with medium for 100 U catalase/ml medium (catalase is used when incubating ROS-producing substances)
- Make dilution series with stock solution: 0.01, 0.1, 1, 5, 10, 20 mM

3. **Incubate with DCFH-DA** (under infrared light)

- Beforehand: weigh DCFH-DA in dark tube and solve in DMSO (1 min ultra-sonic bath, vortex). Aliquot 70 µl (for 1 plate, or 140 µl for 2 plates) in dark 15 ml falcon tubes, store at -20°C.
- Add 7 ml PBS to 70 µl DCFH-DA stock solution to make working solution. Vortex.
- Aspire cell culture medium using brown injection needle (0.45x25 mm) and pump on lowest setting
- Wash with 100 µl PBS (multichannel pipette) and aspire
- Add 100 µl DCFH-DA working solution with multichannel pipette
- Incubate 20 minutes in incubator, keep dark

4. **Incubate with substance**

- <u>During DCFH-DA incubation</u>:

- Make dilution series with **colourless** medium: dilute stock dilution and solvent (acidified 80% DMSO) 1:100 in medium (100 U/ml catalase) for 0.1, 1, 10, 50, 100, 200 µM
- Make positive control 1 M H_2O_2, dilute 1:100 for 1 mM H_2O_2 and control (colourless medium, no catalase)

- Aspire DCFH-DA solution
- Wash 2x with PBS, aspire
- Add 100 µl incubation medium with substance, solvent- and positive control with its respective solvent control

5. **Measure fluorescence immediately**, then every 15 minutes for 1h (excitation at λ 485 nm, emission at λ 528 nm). Use plate reader protocol: incubate at 37°C, 5% CO_2, Settings: read from bottom, use lid, gain settings: 100, if positive control tends to overflow the reader add an extra read at gain = 90 (less sensitive, but yields data)

6. **Data evaluation (H. Wang & Joseph, 1999)**

- Form percentage of mean fluorescence change: $\frac{\mathrm{Fti-Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\%$
  - F_{ti}: fluorescence after i minutes
  - F_{t0}: fluorescence at first measurement
- Form means of the replicates
- Norm means of the test substances on the mean of the control, multiply with 100: T/C $(\%) = \frac{mean\left(\frac{\mathrm{Fti-Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\right)\mathrm{treated}}{mean\left(\frac{\mathrm{Fti-Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\right)\mathrm{control}}\ x\ 100\%$
- **Statistical analysis**: For n ≥ 5 perform ANOVA (Bonferroni) for substance-concentrations comparing with solvent control and Student´s t-Test for positive control + control if all requirements are met, if not use Kruskal-Wallis ANOVA and Mann-Whitney-U-Test.

8.2.3 Protective dichlorofluorescein assay

A modified version of the DCF assay was developed by Theresa Janson (2015) and used to assess the anthocyanins capability to strengthen the cells ability to withstand ROS induced by H_2O_2. The cells were incubated with the anthocyanins for 24 h prior to oxidative stress induction by an up to 1 h incubation with 1 mM H_2O_2 during the previously described DCF assay. During this time intracellular antioxidative protective mechanisms may be induced and thereby less DCFH-DA oxidizes to fluorescent DCF, hence showing a lower signal than solvent control containing stressor (Schaefer et al., 2006; Wolfe & Liu, 2007).

8.2.3.1 Protocol protective DCF assay

Modified from Theresa Janson's optimized protocol for C2BBe1

Appliances and materials:

- Microtiter plate reader Cytation 3, Gen5 Software
- 96 well plate (black, sterile)
- Infrared light lamp
- Dark 15 ml falcon tubes
- Multichannel pipette
- Washing trays for PBS and DCF reagent
- Brown injection needle (0.45x25 mm)

Reagents:

- DCFH-DA — 10 µM: Solve 0.48729 mg in DMSO to C = 1 mM, dilute 1:100 in 37°C PBS
- Catalase — 100 U/ml medium: Solve 2.98 mg in 100 µl cold PBS, dilute 1:100 in anthocyanin incubation medium
- H_2O_2 — 1 mM → 1M stock solution in H_2O_{bidest}, dilute 1:1000 in colourless medium
- DMEM Glutamax — High glucose (4.5 g/L), 10% FBS, 1% P/S
- Colourless DMEM — High glucose (4.5 g/L), 0% FBS, 0% P/S
- 37°C PBS

Optimized protocol:

1. **Seed cells:**

- 15.000 cells/well (for a total cultivation of 48h) in 96 well plate and 200 µl medium/well
- 24 h growth in incubator at 5% CO_2, 37°C, humidified atmosphere

2. **Incubate with substances**

- Weigh substance and solve it in acidified 80% DMSO for a 20 mM stock solution→ ultrasonic bath for 1 minute and vortex, (use dark tubes and turn off laminar light when working with light sensitive substances) or thaw frozen aliquot
- Make dilution series with stock solution: 0.01, 0.1, 1, 5, 10, 20 mM

- Dilute substance and solvent (acidified 80% DMSO) 1:100 in DMEM Glutamax (100 U/ml catalase) for 0.1, 1, 10, 50, 100, 200 µM solutions
- Aspire cell culture medium using brown injection needle (0.45x25 mm) and pump on lowest setting
- Add 200 µl incubation medium with substance and solvent control (0.8% acidified DMSO) (100 µl if substance is scarce)
- Incubate for 24 h

3. **Incubate with DCFH-DA** (under infrared light)

- Beforehand: weigh DCFH-DA in dark tube and solve (1 min ultra-sonic bath, vortex). Aliquot 70 µl (for 1 plate, or 140 µl for 2 plates) in dark 15 ml falcon tubes, store at -20°C.
- Vortex 7 ml PBS with 70 µl DCFH-DA stock solution for working solution.
- Aspire cell culture medium (brown injection needle (0.45x25 mm), pump on lowest setting)
- Wash with 100 µl PBS (multichannel pipette) and aspire
- Add 100 µl DCFH-DA working solution with multichannel pipette
- Incubate 20 minutes in incubator, keep dark
- Prepare 1 M H_2O_2 →dilute 1:1000 in ***colourless medium*** (no catalase)→ 1 mM
- Aspire DCFH-DA solution
- Wash 2x with PBS, aspire
- Add 100 µl 1 mM H_2O_2 or colourless medium as negative control

4. **Measure fluorescence immediately**, then every 15 minutes for 1h (excitation at λ 485nm, emission at λ 528 nm). Use plate reader protocol: incubate at 37°C, 5% CO_2, Settings: read from bottom, use lid, gain settings: 100, if positive control tends to overflow the reader add an extra read at gain = 90 (less sensitive, but yields data)

5. **Data evaluation** (H. Wang & Joseph, 1999)

Form percentage of mean fluorescence change: $\frac{\mathrm{Fti}-\mathrm{Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\%$

- F_{ti}: fluorescence after i minutes
- F_{t0}: fluorescence at first measurement

- Form means of the replicates
- Norm means of the test substances on the mean of the control, multiply with 100: T/C $(\%) = \frac{mean\left(\frac{\mathrm{Fti}-\mathrm{Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\right)\mathrm{treated}}{mean\left(\frac{\mathrm{Fti}-\mathrm{Ft0}}{\mathrm{Ft0}}\ \mathrm{x}\ 100\right)\mathrm{control}}\ x\ 100\%$
- **Statistical analysis:** For n ≥ 5 perform ANOVA (Bonferroni) for substance-concentrations comparing with solvent control and Student´s t-Test for positive control + control if all requirements are met, if not use Kruskal-Wallis ANOVA and Mann-Whitney-U-Test.

8.3 Statistical analysis

In order to make statistically sound analysis several tests were performed in dependence of their outcome. Many statistical tests such as the often-used analysis of variance (ANOVA) and the Student's *t*-test are based on the assumption of a normal distribution of data (OriginLab). The Kolmogorov-Smirnov test was chosen as a test of normal distribution. When normality was proven a One-way ANOVA was performed with the Brown-Forsythe test to control for variance of homogeneity. The ANOVA is used to detect significant differences and was applied to the solvent control and the test-groups with the addition of the Bonferroni posthoc test for multiple comparison of the means of the groups, which controls the overall type I error (OriginLab). The ANOVA results were only considered when the variances were homogenously distributed. If this was not the case the non-parametrical test Kruskal-Wallis ANOVA was performed, which is a method that tests for differences of the samples medians instead of means (OriginLab). A simple checklist of the different tests can be seen in figure 27.

Each experiment had either a positive (1 mM H_2O_2) or a negative control (no stressor) in order to determine the success of the assay. When normal

distribution and variance-homogeneity was assured Student's two-sample *t*-test for independent groups was performed between positive/negative control and its own control to assure that a significant difference between the mean values was prevalent. The non-parametrical Whitney-Mann-U test was performed to recognize a significant difference between the two distributions on the basis of ranked data when samples were either not normally distributed or variances of homogeneity were significantly different.

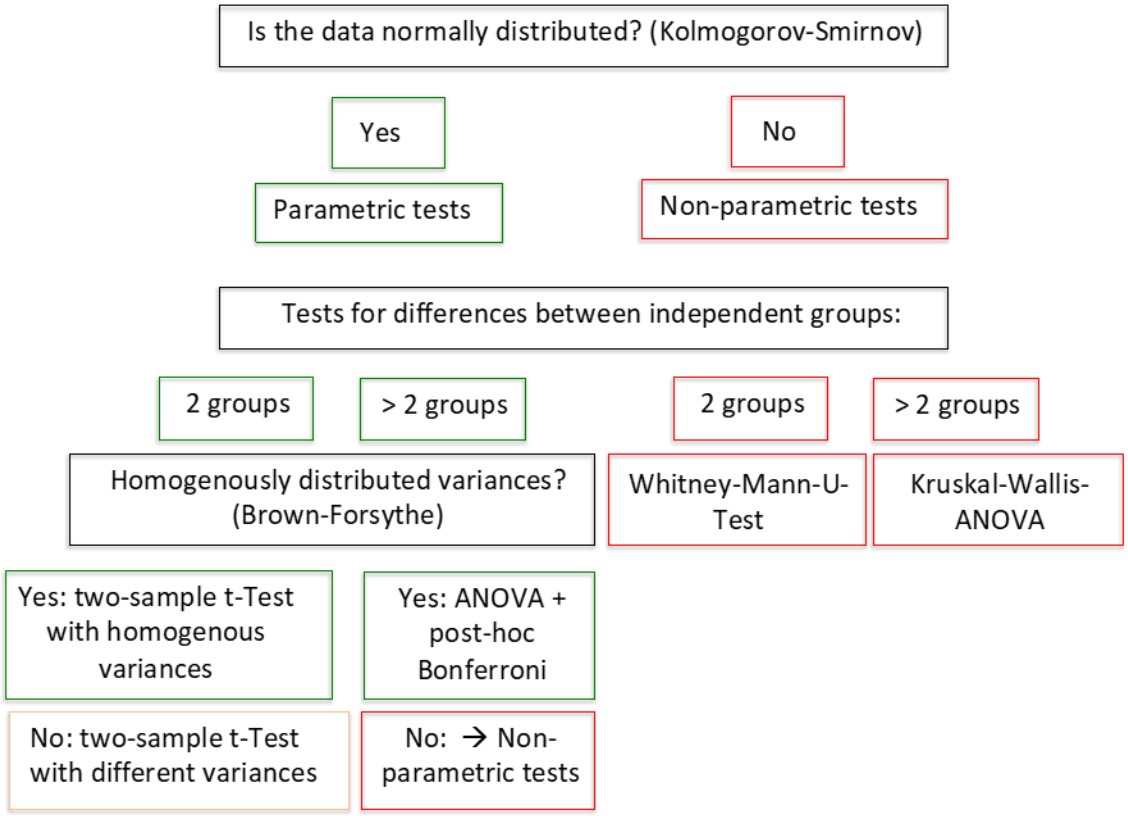

Figure 27: Statistics procedure checklist modified after Hager

An elimination of outliers in non-normalized data was performed using the Nalimov test in Microsoft Excel in each separate experiment with more than five replicates. All statistic tests were performed on non-normalized data with OriginPro 9.1., except for comparisons between substances where normalized data was tested.

8.4 Index of materials

Cell line:	**Source:**
• Human colon carcinoma cell line Caco-2 brushborder-expressing-1 (C2BBe1), CRL-2102™	ATCC

Reagents for cell culture:	**Manufacturer:**
• Dulbeccos Modified Eagle Medium (DMEM), GlutaMAX™ + Glucose (4.5 g/L) + Sodium pyruvate + GlutaMAX + Phenol red - HEPES	Gibco™, Thermo Fischer Scientific Ref: 31966-021
• Dulbeccos Modified Eagle Medium (DMEM) + Glucose (4.5 g/L) + HEPES (25 mM) - Sodium pyruvate - Phenol red	Gibco™, Thermo Fischer Scientific Ref: 21063-029
• Fetal Bovine Serum Ref: 10270-106	Gibco™, Thermo Fischer Scientific Prepared: heat inactivate in water bath at 56°C for 30 minutes. Store aliquots at -20°C
• PBS *(Phosphate Buffered Saline)*	Prepared: 1710 mM NaCl (100 g/l) 100 mM Na_2HPO_4 (14.2 g/l) 34 mM KCl (2.5 g/l) 18 mM KH_2PO_4 (2.4 g/l) 10x PBS: Solve in H_2O_{bidest}; set pH to 7.4; Fill up to 1 l with H_2O_{bidest}; dilute to 1x PBS with H_2O_{bidest}; autoclave, store at 4°C

• Penicillin/Streptomycin (P/S): 10.000 U/ml	Invitrogen, Carlsbad, USA
• Trypan blue solution (0.4%)	Sigma-Aldrich Chemie GmbH, München, D
• Trypsin (Bovine pancreas 3.6 U/mg)	Carl Roth GmbH & Co KG, Karlsruhe
• Trypsin solution	Prepared: 500 mg Trypsin 250 mg EDTA 100 ml 10x PBS Solve in H_2O_{bidest}; stir over night on ice; set pH to 7-7.4; Fill up with H_2O_{bidest} to 1 l; sterile filtration; store aliquots at -20°C.

Materials cell culture:	**Manufacturer:**
• 96 well plates, yellow, sterile	Sarstedt AG & Co, Nürnbrecht, D
• 96 well plates, black, sterile	Costar®, Corning, NY, USA
• Combitips for multistep pipette, sterile (0.1 ml, 1 ml, 5 ml, 10 ml)	Eppendorf AG, Hamburg, D
• Cryotubes, Cryopure 1.8 ml	Sarstedt AG & Co, Nürnbrecht, D
• Falcon tubes (15 ml, 50 ml), dark and clear	Sarstedt AG & Co, Nürnbrecht, D
• Filter for syringe, sterile	Sarstedt AG & Co, Nürnbrecht, D
• Flasks, tissue culture, T175, T75, T25	Sarstedt AG & Co, Nürnbrecht, D
• Needles, brown 0.45x25mm	Henke Sass Wolf, Tuttlingen, D
• Neubauer hemocytometer	Marienfeld Superior, Lauda Königshofen, D
• Pasteur pipettes, glass	Carl Roth GmbH & Co KG, Karlsruhe, D
• Pipette tips, not sterile (10 µl, 200 µl, 1000 µl), autoclaved	Sarstedt AG & Co, Nürnbrecht, D
• Serological pipettes, sterile	Sarstedt AG & Co, Nürnbrecht, D

(5, 10, 25 ml)	
• Syringe for filtration (10 ml)	Omnifix®, Braun Melsungen AG, Melsungen, D
• Washing trays, 25 ml	Carl Roth GmbH & Co KG, Karlsruhe, D

Chemicals:	**Manufacturer:**
• 2´,7´-Dichlorofluorescindiacetate (DCFH-DA) >98%	Sigma-Aldrich Chemie GmbH, München, D, LOT: 064M400SV, 095M4006V
• Acetic acid (100%)	Carl Roth GmbH & Co KG, Karlsruhe, D
• Catalase From bovine liver (3356 U/mg)	Sigma-Aldrich Chemie GmbH, München, D CAS: 9001-05-2, LOT: SLBB1799V
• Dimethyl sulfoxide (DMSO) >99.5%	Carl Roth GmbH & Co KG, Karlsruhe, D LOT: 035220262
• Disodium phosphate (Na_2HPO_4)	Carl Roth GmbH & Co KG, Karlsruhe, D
• Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co KG, Karlsruhe, D
• Hydrochloric acid, 37%	Carl Roth GmbH & Co KG, Karlsruhe, D LOT: 105222328
• Hydrogen peroxide, 30%	Carl Roth GmbH & Co KG, Karlsruhe, D Rotipuran ®, LOT: 451176116
• Monopotassium phosphate (KH_2PO_4)	Carl Roth GmbH & Co KG, Karlsruhe, D
• Sodium chloride (NaCl)	Carl Roth GmbH & Co KG, Karlsruhe, D
• Sodium hydroxide (NaOH) (pH-meter)	Carl Roth GmbH & Co KG, Karlsruhe, D

• Sulforhodamin B sodium salt (SRB)	Sigma-Aldrich Chemie GmbH, München, D
• Tris-(hydroxymethyl)-aminomethan TRIS Pufferan® 99,9%,	Carl Roth GmbH & Co KG, Karlsruhe, D LOT: 085225265

Test substances:	**Manufacturer:**
• Phloroglucinol aldehyde (2,4,6-trihydroxybenzaaldehyde)	Sigma-Aldrich Chemie GmbH, München, D CAS: 487-70-7, LOT: 0001451801
• Cyanidin chloride, HPLC>98%	TransMIT for PlantMetaChem, Gießen, D Ref. C018, LOT: 07-14
• Cyanidin-3-glucoside, purified, blackberry kit	AnthoPLUS, Applied Biochemistry Group, IPK-Gatersleben, D
• Cyanidin-3-sambubioside equivalents, purified Anthocyanin fraction 2, elderberry kit	AnthoPLUS, Applied Biochemistry Group, IPK-Gatersleben, D
• Cyanidin-3-sambubioside-5-glucoside equivalents, purified Anthocyanin fraction 1, elderberry kit	AnthoPLUS, Applied Biochemistry Group, IPK-Gatersleben, D

8.5 Index of devices

Device	**Model / Manufacturer**
• Autoclave	Systec DX-150, Systec GmbH, Weitenberg, D
• Centrifuges	Rotina 420R, Hettich Zentrifugen, Tuttingen, D Micro centrifuge SD 229VAC, 6000RPM, Carl Roth GmbH & Co KG, Karlsruhe, D
• Freezing Container	Mr. Frosty™ , Thermo Fischer Scientific, Waltham, MA, USA
• Incubators	Heracell 240i CO_2 Incubator, Thermo Fischer

	Scientific, Waltham, MA, USA
• Laminar flow	Herasafe KS, Thermo Fischer Scientific, Waltham, MA, USA
• Magnetic stirrer	IKA® RCT basic safety control, IKA-Werke GmbH & Co KG, Staufen, D
• Microscope	Axiovert 40C, Zeiss, Oberkochen, D Objective: A-Plan, 5x/0,12 Ph 0, A-Plan, 10x/0,25 Ph 1
• Microplate Reader	Cytation3 imaging reader, Gen5 Software BioTek® Instruments, Winooski, Vermont, USA
• Micro Scale	DeltaRange Microbalance XP26DR, Mettler Toledo, Greifensee, CH
• pH-meter	Mettler Toledo Seven Easy pH, Electrode: Mettler Toledo, InLab Expert Pro, Mettler Toledo, Greifensee, CH
• Pipettor	Pipetus ®, Hirschmann Laborgeräte GmbH & Co KG, Eberstadt, D
• Pipettes	Eppendorf Research pipettes 10-1000 µl, Eppendorf Research plus, multichannel, 100 µl, Eppendorf AG, Hamburg, D Multistep pipette, AutoRep M, Rainin pipettes®, Mettler Toledo
• Ultrasonic waterbath	Ultrasonic cleaner, USC300T, VWR Österreich, Wien, A
• Vacuum pumps	Vakusafe comfort, IBS tecnomara GmbH, Fernwald, D
• Vortex	Vortex Genie 2, Scientific Industries, Bohemia, NY, USA Biovortex V1, Peqlab, Biotechnologies GmbH, Erlangen, D
• Water bath	GD100, Grant Instruments, Cambridge, UK

9 Index of Literature

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10 Appendix

Table of content

10.1 Zusammenfassung

Anthocyane sind phenolische Verbindungen die für die blaue, lila und rote Farbe von Beeren, Obst und Blumen verantwortlich sind (Kamiloglu et al. 2015). Das am häufigsten vorkommende Anthocyanidin in essbaren Pflanzenteilen ist Cyanidin (Cy) mit etwa 50% und Cyanidin-3-glucosid (Cy-3-glc) als häufigstes Anthocyan (Kong et al. 2003). Der durchschnittliche europäische Verzehr liegt zwischen 18,73 und 64,88 mg Anthocyane/d (Zamora-Ros et al., 2011) Der Gehalt an Anthocyanen in Lebensmitteln kann jedoch deutlich höher sein, mit z.B. über 4 g/L in Holunderbeerensaft (Jakobek et al., 2007). Das Ziel dieser Arbeit war, die vorherrschenden Holunderbeeren-Anthocyane Cy-3-glc, Cy-3-sam (Cyanidin-3-sambubiosid) und Cy-3-sam-5-glc (Cyanidin-3-sambubiosid-5-glucosid) samt ihren ausgewählten Metaboliten Cy und Phloroglucinolaldehyd (PGA) bezüglich ihrer antioxidativen und ROS-schützenden Wirkung in C2BBe1-Kolonzellen zu untersuchen .

Da nur wenige Daten über Holunderbeeren-Anthocyane in ihrer Reinform vorliegen, wurden die Verbindungen zuerst mit einem Toxizitätstest (Sulforhodamin B-Test) hinsichtlich wachstumsstörender Effekte überprüft. Damit sollen potenzielle Artefakte aufgrund zytotoxischer Wirkung bei den antioxidativen Tests ausgeschlossen werden. Keine der Substanzen hatte eine signifikante Wirkung auf das Zellwachstum nach 24 Stunden Inkubation.

Alle Anthocyane und das Aglykon Cy bewirkten eine potente Abnahme der intrazellulären ROS-Produktion gemessen mit dem DCF-Assay. Cy-3-glc und Cy-3-sam zeigten dabei die stärksten Effekte im Vergleich zur Lösungsmittelkontrolle. Nach 60 Minuten Inkubation bewirkte die höchste Konzentration von 200 µM eine ROS-Bildung von jeweils nur 11.37 ± 3.91% und 8.2 ± 10.61% im Vergleich zur Lösungsmittelkontrolle von 100%. Das Aglykon hat ähnliche ROS-reduzierende Effekte wie Cy-3-sam-5-glc verursacht, Im Gegensatz dazu führte der Metabolit PGA zu einer signifikanten Zunahme der ROS-Produktion mit dem Peakmaximum bei der Konzentration 25 µM (155.7 ± 29.6%). Um abschätzen zu können ob die Substanzen die Zellen gegen ROS-Akkumulation schützen können, wurden die Zellen nach einer 24-stündigen Substanz-Inkubation für 1 h mit 1 mM H2O2 gestresst und ROS anschließend mit

dem DCF-Assay gemessen. Nach Vorinkubation mit 200 µM Cy-3-glc und Cy-3-sam zeigten die Zellen signifikant reduzierte ROS-Spiegel und damit eine protektive Wirkung gegen H2O2 (T/C 60.69 ± 11.31; 61.54 ± 17.14). Keine der anderen Substanzen wirkte protektiv.

Der genaue Wirkmechanismus für den protektiven Effekt ist noch nicht völlig geklärt. Aufnahme und Stabilitätsexperimente wären wichtig, um zu sehen in welchem Ausmaß die Substanzen von den Zellen aufgenommen werden und ob die größeren mehrfach-substituierten Anthocyane zu strukturell weniger komplexe Anthocyane und Metaboliten abgebaut werden.

10.2 Abstract

Introduction: Cyanidin is the most common anthocyanidin in edible plants, accounting for ca. 50% of anthocyanins, with Cy-3-glc being the most common glycoside. The average European consumes 18.73 - 64.88 mg anthocyanins /day, although food or supplement content can be considerably higher. The goal of this work was to characterize the main anthocyanins of elderberries, Cy-3-sam, Cy-3-sam-5-glc and Cy-3-glc, together with selected metabolites Cy and PGA concerning their antioxidative potential in a colon carcinoma cell model.

Material and Methods: Colon carcinoma cell line C2BBe1, a clone of Caco-2, was chosen due to its comparability to normal human enterocytes. The compounds were solved in 80% DMSO, which for the anthocyanins and Cy was acidified with HCl in order to keep the flavylium structure stabile. First, a proliferation assay in the form of the SRB Assay was performed after a 24-h incubation with all compounds, after which the effect on intracellular ROS was measured with a Cytation3 platereader using the DCF Assay and up to one hour incubation of the substances. The protective effect of the compounds was further investigated with a 24-h incubation, after which a ROS-induction with 1 mM H_2O_2 was set and fluorescence was measured within the protective DCF Assay.

Results: None of the compounds reduced proliferation significantly, although a slight tendency at highest concentration could be seen. All of the anthocyanins and Cy significantly reduced ROS-production in a dose-dependent manner in the DCF Assay, with Cy-3-glc and Cy-3-sam being most potent, with 200 µM resulting in 11.37 ± 3.91% and 8.2 ± 10.61% of solvent control fluorescence after 60 min. PGA showed a significant ROS-induction, with a peak at 25 µM (155.7 ± 29.6%). Only Cy-3-glc and Cy-3-sam showed a protection against induced ROS, lowering the fluorescence, in a dose dependant manner, significantly after 60 min stressor to 60.69 ± 11.31% and 61.54 ± 17.14% compared with solvent control.

Conclusion: Elderberry anthocyanins effectively reduce intracellular ROS in C2BBe1 cells, and Cy-3-glc and Cy-3-sam act as a protective agent at 200 µM against H_2O_2-induced ROS. Further experiments on uptake and stability are needed to investigate the protective mechanism of these promising pigments.

10.3 Datapoints for each measurement

10.3.1 Sulforhodamine B assay

10.3.1.1 Phloroglucinol aldehyde

24 h incubation with PGA (µM), 0.8% DMSO, pos. ctrl. 1 mM H_2O_2

Non normalized data

24h incubation with PGA (µM), (*Nalimov outliers, removed for statistics*)

N	DMSO	0.1	2.075	4.15	8.3	50	100	200	400	Medium	H_2O_2
1	1.25					1.01	0.93	1.01	0.90	0.96	0.11
2	1.26			1.40	1.60	1.46	1.36	1.39	1.22	1.18	0.29
3	1.14			1.16	1.26	1.18	1.06	0.97	0.90	0.96	0.10
4	1.16		1.32	1.45	1.30	1.24	1.24	1.16	1.02	1.19	0.14
5	0.85		0.64	0.82	0.77	0.68	0.62	0.67	0.72	0.77	0.11
6	0.75		1.15	1.20	1.16	0.95	0.87	0.90	0.71	0.95	0.10
7	*1.68*		*1.90*	1.91	1.89	1.70	*1.97*	*1.91*	*1.53*	*2.37*	*0.80*
8	0.55	0.47	0.46		0.49		0.50			0.56	0.38
9	0.48	0.51	0.46		0.45		0.48			0.66	0.39
10	0.58	0.65	0.65		0.54		0.54			0.56	0.10
11	0.64	0.68	0.65		0.71		0.62			0.78	0.10

Normalized to solvent control

24 h incubation with PGA (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean T/C (%) ± SD

Conc.	Mean	*SD*
DMSO	100.00	*0.00*
0.1	96.01	*15.68*
2.075	105.47	*25.16*
4.15	118.22	*22.46*
8.3	109.68	*19.86*
50	102.24	*16.98*
100	95.53	*13.91*
200	95.87	*16.74*
400	86.16	*9.25*
Medium	100.00	*0.00*
H_2O_2	24.18	*21.24*

10.3.1.2 Cyanidin

Non-normalized data

24h incubation with Cy (µM)

N	DMSO	0.1	1	10	50	100	200	Medium	H_2O_2
1	0.83	0.85	0.81	0.86	0.90	0.93	1.08	0.79	0.54
2	0.82	0.76	0.80	0.81	0.82	0.82	0.84	0.64	0.34
3	0.92	1.00	0.98	0.96	0.96	1.04	0.91	0.95	0.58
4	0.69	0.59	0.75	0.70	0.71	0.70	0.77	1.21	0.49
5	0.75	0.76	0.72	0.77	0.81	0.82	0.91	0.94	0.50

Normalized to solvent control

24 h incubation with Cy (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean T/C (%) ± SD

Conc.	Mean	*SD*
DMSO	100.00	*0.00*
0.1	97.85	*9.38*
1	101.63	*5.96*
10	102.12	*2.07*
50	104.94	*3.74*
100	107.07	*6.19*
200	112.75	*12.61*
Medium	100.00	*0.00*
H_2O_2	54.97	*10.15*

10.3.1.3 Cyanidin-3-glucoside

Non-normalized data

N	DMSO	0.1	1	10	50	100	200	Medium	H_2O_2
1	0.83	0.80	0.85	0.85	0.91	0.81	0.73	0.79	0.54
2	0.82	0.75	0.77	0.71	0.69	0.74	0.60	0.64	0.34
3	0.92	0.86	0.88	0.85	0.82	0.85	0.77	0.95	0.58
4	0.69	0.71	0.76	0.74	0.96	0.78	0.74	1.21	0.49
5	0.75	0.76	0.73	0.83	0.73	0.65	0.66	0.94	0.50

Normalized to solvent control

24 h incubation with Cy-3-glc (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean T/C (%) ± SD

Conc.	Mean	*SD*
DMSO	100.00	*0.00*
0.1	97.12	*4.85*
1	99.72	*6.21*
10	99.93	*10.10*
50	104.17	*21.68*
100	96.32	*10.01*
200	87.99	*12.11*
Medium	100.00	*0.00*
H_2O_2	54.97	*10.15*

10.3.1.4 Cyanidin-3-sambubioside

Non-normalized data

24h incubation with Cy-3-sam, (*Nalimov outliers, removed for statistics*)

N	DMSO	0.1	1	10	50	100	200	Medium	H_2O_2
1	1.09		0.95	1.10	1.04	0.85	0.75	1.04	0.13
2	0.67	0.71	0.88	0.65	0.69	0.57	0.53	0.58	0.31
5	1.22	1.23	1.01	1.25	1.15	0.97	0.90	1.24	0.32
6	0.98	0.84	0.79	0.93	0.82	0.86	0.82	0.98	0.70
7	1.15	1.33	1.47	1.41	1.20	1.19	0.90	1.06	0.61
8	0.55	0.51	0.56	0.57	0.47	0.52	0.57	0.55	0.41

Normalized to solvent control

24 h incubation with Cy-3-sam (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean T/C (%) ± SD

Conc.	Mean	*SD*
DMSO	100.00	*0.00*
0.1	99.75	*11.37*
1	96.51	*20.44*
10	103.28	*9.74*
50	95.84	*7.95*
100	84.90	*6.69*
200	81.52	*13.93*
Medium	100.00	*0.00*
H_2O_2	44.80	*25.13*

10.3.1.5 Cyanidin-3-sambubioside-5-glucoside

Non-normalized data

N	DMSO	0.1	1	10	50	100	200	Medium	H_2O_2
1	1.09		0.89	1.00	0.84	1.05	0.80	1.04	0.13
2	0.67	0.55	0.68	0.53	0.59	0.49	0.48	0.58	0.31
5	1.22	1.11	1.26	1.19	1.31	1.19	1.13	1.24	0.32
6	0.98	1.06	1.01	0.80				0.98	0.70
7	1.15	1.06	0.87	1.01	0.86	0.91	0.84	1.06	0.61

Normalized to solvent control

Conc.	Mean	*SD*
DMSO	100.00	*0.00*
0.1	93.14	*10.91*
1	92.76	*13.65*
10	87.32	*7.81*
50	86.54	*14.92*
100	86.24	*12.16*
200	77.33	*10.07*
Medium	100.00	*0.00*
H_2O_2	44.11	*24.14*

24 h incubation with Cy-3-sam-5-glc (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean T/C (%) ± SD

10.3.2 DCF assay

10.3.2.1 Phloroglucinol aldehyde

Incubation with Cy (µM), 0.8% DMSO, 1 mM H_2O_2

15 min (t_{15}) PGA (µM), (*Nalimov outliers, removed for statistics*)

N	DMSO	0.01	0.1	1	2.075	4.15	5	8.3	10	25	50	100	200	400	Medium	H_2O_2
1	*13.39*						25.11		28.64		36.61	31.75	27.58	4.76	-3.49	32.77
2	25.98						30.13		33.93		27.79	23.04	14.14	3.56	*-22.55*	*-9.67*
3	29.40					51.13		*58.46*			*57.75*	41.61	33.91	19.62	17.46	71.12
4	32.72				39.99	40.55		41.81			37.41	32.34	20.01	10.88	10.50	98.05
5	24.21				19.25	26.75		29.39			20.43	21.40	15.08	9.74	-5.91	52.01
6	*15.09*				24.89	29.83		33.44			37.34	29.47	20.73	-7.84	7.06	76.78
7	24.75				28.29	31.66		39.96			38.05	11.80	-3.60	-0.74	*-16.44*	81.87
8	*16.24*				21.50	16.74		27.92			12.34	11.06	11.89	-5.20	18.25	74.12
9	22.84				42.69	36.90		38.91			39.67	37.49	29.75	4.29	23.76	101.02
10	27.73		6.85								23.83	-6.64	-2.62		7.81	37.19
11	32.14		8.96								30.44	2.03	2.02		20.63	60.29
12	30.95		25.40								20.59	7.44	*-35.95*		9.96	58.11
13	31.98		24.60								11.32	9.04	*-35.87*		21.07	82.62
14	31.04	33.74	33.63	36.30					38.65	34.62	20.94				-0.18	116.24
15	*19.12*	18.66	17.05	24.54					31.60	36.60	32.34				3.76	77.06
16	30.21	25.16	31.52	23.09					41.11	47.25	45.33				9.07	124.06
17	21.31	20.84	20.38	22.12					37.74	36.93	26.35				2.00	105.94
18	32.25	11.85	16.22	21.29					35.07	40.55	41.52				-3.03	83.20

30 min (t_{30}) PGA (µM), (*Nalimov Outliers, removed for statistics*)

N	DMSO	0.01	0.1	1	2.075	4.15	5	8.3	10	25	50	100	200	400	Medium	H_2O_2
1	71.38						116.57		*120.73*		132.27	136.18	118.33	65.50	*4.68*	158.23
2	68.48						91.95		*104.90*		100.67	90.45	75.24	40.98	*-5.66*	*37.41*
3	121.89					*188.69*		*212.97*			*208.12*	161.70	127.06	79.14	65.92	225.96
4	109.65				124.15	142.23		143.82			139.16	128.28	100.13	67.73	74.47	302.21
5	107.14				103.56	137.04		139.71			110.41	105.45	76.05	45.92	42.68	179.65
6	88.68				124.25	146.44		148.16			170.37	141.67	111.18	46.78	71.71	261.59
7	85.60				95.16	104.10		137.85			153.78	64.75	102.51	56.36	23.76	246.77
8	90.89				110.35	110.33		140.78			97.58	90.15	68.05	27.03	85.44	244.33
9	93.80				114.73	130.63		144.43			143.99	118.97	101.72	55.41	79.73	244.36
10	105.84		80.87								112.02	56.99	45.94		39.62	*94.38*
11	*141.14*		101.10								145.98	81.17	57.11		88.47	145.55
12	92.36		86.08								106.34	71.81	*-27.22*		28.26	146.71
13	100.96		91.20								87.56	68.85	*-23.57*		65.49	242.41
14	118.50	132.05	*137.23*	147.34					155.56	154.23	130.70				43.98	340.08
15	99.53	107.73	*116.71*	116.36					146.09	207.45	180.70				52.27	249.38
16	98.46	88.18	100.95	85.64					142.98	173.84	178.39				49.52	313.61
17	84.73	83.15	92.78	98.04					137.59	147.48	121.91				49.88	287.24
18		92.40	88.14	115.39					146.24	174.43	179.03				34.58	291.64

45 min (t_{45}) PGA (µM), (*Nalimov Outliers, removed for statistics*)

N	DMSO	0.01	0.1	1	2.075	4.15	5	8.3	10	25	50	100	200	400	Medium	H_2O_2
1	154.58						236,94		215.27		238.50	240.37	209.79	134.72	*24.95*	281.99
2	*126.50*						167,73		192.57		185.79	169.24	147.43	86.28	*8.91*	*89.70*
3	220.43					*325.96*		*363.59*			320.18	267.95	215.32	138.08	122.33	371.69
4	203.39				230.47	254.31		247.25			245.96	226.05	182.18	132.29	122.47	485.61
5	202.99				201.89	253.00		260.56			204.98	193.04	147.46	94.15	87.22	285.43
6	167.50				215.88	261.53		263.35			287.03	235.96	190.41	94.08	125.46	419.40
7	146.66				172.77	186.85		225.87			260.92	108.51	170.70	115.62	50.62	345.32
8	173.83				219.83	215.66		260.95			186.42	169.95	129.02	60.43	144.45	393.07
9	166.89				196.40	210.85		246.30			237.40	194.90	145.60	101.00	120.93	359.02
10	199.61		160.56								202.10	119.21	99.21		91.37	*175.17*
11	*254.41*		*109.79*								262.48	160.37	121.94		156.46	267.36
12	151.96		149.00								187.52	125.06	*-18.92*		47.65	*236.39*
13	167.68		154.01								155.74	125.20	*-14.62*		105.21	349.96
14	205.55	238.96	*251.01*	278.84					293.71	281.88	235.15				86.45	398.91
15	185.95	194.93	*213.81*	220.83					274.63	362.52	321.54				104.66	431.46
16	164.66	163.04	175.71	155.80					265.66	303.88	305.56				95.00	411.57
17	157.02	163.88	167.12	183.35					243.49	264.90	220.46				102.60	421.03
18	*248.07*	182.02	160.11	212.26					256.72	298.37	299.75				65.01	435.06

60 min (t_{60}) PGA (µM), (*Nalimov Outliers, removed for statistics*), ovrflw: overflow → fluorescence too high for platereader

N	DMSO	0.01	0.1	1	2.075	4.15	5	8.3	10	25	50	100	200	400	Medium	H_2O_2
1	207.82						272.95		272.95		298.90	301.65	269.01	183.09	*39.90*	358.14
2	182.98						239.64		272.58		255.99	233.92	205.52	119.75	*29.00*	*126.89*
3	312.63					445.05		*486.70*			416.95	355.28	288.86	192.07	172.98	473.77
4	289.37				304.39	350.61		340.51			343.71	306.46	247.72	181.50	172.78	*638.94*
5	294.33				290.32	369.84		363.47			283.62	270.21	207.28	138.69	139.27	375.79
6	237.97				294.59	363.14		353.46			366.16	296.77	254.24	124.62	187.37	522.58
7	209.69				258.53	259.20		308.19			350.93	147.83	227.64	160.07	79.42	406.60
8	249.95				324.50	310.18		374.86			262.65	232.35	181.08	94.10	202.34	476.34
9	233.31				275.89	294.78		339.02			315.07	256.31	195.74	138.24	165.00	433.59
10	284.31		235.34								274.93	170.07	145.79		135.96	*236.04*
11	*352.71*		288.12								349.83	220.51	175.93		218.53	316.58
12	209.33		208.10								255.70	168.65	*-10.59*		67.28	310.66
13	226.69		212.51								210.59	172.99	*-5.37*		132.10	432.18
14	291.47	323.91	*373.91*	400.26					411.78	389.64	324.89				125.68	ovrflw
15	273.17	276.47	306.25	316.56					394.01	492.17	433.71				149.69	570.78
16	228.16	227.92	245.97	220.38					369.00	419.92	428.15				137.38	ovrflw
17	224.10	234.01	238.52	260.11					338.39	367.99	309.51				151.97	525.42
18	*347.08*	259.49	229.59	291.11					353.93	403.53	413.53				90.27	539.85

Normalized to solvent control

Incubation with PGA (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± *SD*

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.01	84.82	*28.35*	94.57	*18.41*	99.57	*15.93*	98.28	*13.85*
0.1	73.26	*31.59*	86.58	*17.06*	91.34	*16.41*	93.77	*15.84*
1	98.30	*26.47*	105.14	*19.33*	110.27	*20.09*	109.95	*20.50*
2.075	133.38	*38.09*	117.48	*14.44*	117.27	*10.51*	116.50	*12.06*
4.15	142.65	*35.44*	134.17	*16.52*	133.07	*13.20*	130.83	*11.86*
8.3	167.63	*35.78*	149.75	*15.44*	146.26	*15.68*	138.66	*14.21*
10	150.87	*36.42*	137.86	*22.08*	143.14	*18.99*	140.07	*19.21*
25	151.67	*33.00*	162.56	*35.35*	161.12	*31.60*	155.67	*29.64*
50	131.82	*65.42*	139.62	*35.81*	134.55	*29.37*	128.87	*26.92*
100	89.57	*77.12*	106.90	*41.17*	102.02	*30.77*	97.04	*26.73*
200	74.75	*68.09*	95.86	*36.95*	91.03	*28.37*	87.55	*25.45*
400	13.48	*37.22*	58.73	*17.02*	62.19	*15.76*	61.16	*15.03*
Medium	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
H_2O_2	-3169.96	*15834.26*	681.26	*754.34*	442.21	*233.98*	383.78	*197.23*

10.3.2.2 Cyanidin

Normalized to solvent control

Incubation with PGA (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± *SD*

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.01	84.82	*28.35*	94.57	*18.41*	99.57	*15.93*	98.28	*13.85*
0.1	73.26	*31.59*	86.58	*17.06*	91.34	*16.41*	93.77	*15.84*
1	98.30	*26.47*	105.14	*19.33*	110.27	*20.09*	109.95	*20.50*
2.075	133.38	*38.09*	117.48	*14.44*	117.27	*10.51*	116.50	*12.06*
4.15	142.65	*35.44*	134.17	*16.52*	133.07	*13.20*	130.83	*11.86*
8.3	167.63	*35.78*	149.75	*15.44*	146.26	*15.68*	138.66	*14.21*
10	150.87	*36.42*	137.86	*22.08*	143.14	*18.99*	140.07	*19.21*
25	151.67	*33.00*	162.56	*35.35*	161.12	*31.60*	155.67	*29.64*
50	131.82	*65.42*	139.62	*35.81*	134.55	*29.37*	128.87	*26.92*
100	89.57	*77.12*	106.90	*41.17*	102.02	*30.77*	97.04	*26.73*
200	74.75	*68.09*	95.86	*36.95*	91.03	*28.37*	87.55	*25.45*
400	13.48	*37.22*	58.73	*17.02*	62.19	*15.76*	61.16	*15.03*
Medium	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
H_2O_2	-3169.96	*15834.26*	681.26	*754.34*	442.21	*233.98*	383.78	*197.23*

Non-normalized fluorescence change data

Incubation with Cy (µM), 0.8% DMSO, 1 mM H_2O_2

15 min (t_{15}) Cy (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	45.31			42.75	40.08	22.25	11.29	9.09	9.62	14.58	121.99
2	43.39			33.85	25.73	26.53	8.75	7.86	3.66	*3.59*	134.57
3	39.03		29.20	37.73	30.90	29.31	19.11	17.89	9.60	15.35	138.82
4	53.66		35.57	41.23	38.29	22.87	12.54	11.98	8.32	19.11	185.48
5	49.10	26.37	42.38	48.61	42.80	21.35	13.07	5.13	10.40	21.57	196.85
6	28.47	26.43	33.91	43.55	32.72	31.67				21.33	172.04
7	28.06	27.46	20.17	*2.92*						13.72	148.61
8	32.86	34.77	35.26	*13.76*						18.77	

30 min (t_{30}) Cy (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	138.91			*134.32*	*129.94*	77.19	38.52	26.59	28.77	*80.35*	351.19
2	132.01			106.47	82.09	86.13	29.82	30.65	28.74	65.58	364.29
3	117.97		95.52	104.87	82.64	67.41	48.22	35.15	34.52	68.50	375.21
4	117.17		86.25	91.62	83.33	*44.66*	16.17	10.23	9.54	*46.90*	390.55
5	108.09	80.40	102.24	98.58	95.09	71.82	39.54	20.50	24.83	65.02	*453.75*
6	83.00	76.11	91.14	103.15	75.65	76.40				67.13	372.60
7	85.75	81.10	75.54	*14.24*						*56.21*	414.78
8	112.73	120.19	106.69	*39.94*						61.65	*532.40*

45 min (t_{45}) Cy (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	227.94			*227.15*	*214.04*	138.02	72.53	44.22	41.45	*143.48*	526.21
2	216.30			179.94	136.28	140.99	50.89	47.16	41.52	121.79	512.99
3	191.93		161.77	168.05	127.69	105.96	72.79	48.74	50.65	116.03	555.69
4	187.94		160.30	157.28	133.99	80.63	32.20	17.18	16.65	*80.86*	526.57
5	174.63	135.47	170.04	164.50	154.73	108.74	65.43	37.79	38.36	111.77	*618.89*
6	146.49	134.11	159.96	169.46	128.47	125.48				119.35	511.02
7	156.55	149.28	141.24	*24.89*						*99.10*	565.15
8	193.05	200.58	178.88	*55.32*						112.11	*733.82*

60 min (t_{60}) Cy (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	308.21			312.94	*289.14*	191.70	106.30	62.35	50.17	*200.17*	641.15
2	294.33			245.59	185.70	188.84	70.67	59.53	53.47	171.71	609.96
3	261.29		214.40	243.10	173.27	141.42	96.53	63.91	63.34	162.44	678.78
4	255.48		233.42	223.57	188.44	118.30	51.55	25.12	25.25	*121.59*	627.17
5	237.38	187.84	232.93	225.70	207.84	141.09	87.05	49.84	50.89	162.19	718.76
6	202.04	191.67	229.83	233.49	172.81	159.75				167.29	593.60
7	217.63	211.46	201.11	34.78						*144.08*	649.72
8	268.14	274.66	249.18	67.48						169.74	*851.78*

Normalized to solvent control

Incubation with Cy (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.0001	87.55	*23.18*	91.82	*13.30*	92.10	*10.97*	93.40	*10.03*
0.01	87.61	*21.20*	90.28	*12.56*	93.17	*9.21*	95.11	*10.52*
0.1	99.64	*27.83*	92.64	*18.51*	92.86	*13.49*	94.93	*12.41*
1	83.40	*18.85*	76.49	*12.47*	75.43	*11.99*	75.25	*11.03*
10	63.78	*26.31*	67.29	*14.65*	61.96	*14.02*	60.88	*10.99*
50	28.81	*11.52*	28.32	*10.84*	29.57	*9.06*	30.46	*7.81*
100	24.59	*14.91*	21.70	*8.80*	20.28	*6.39*	19.56	*5.26*
200	18.19	*6.36*	20.57	*7.70*	18.92	*6.46*	18.00	*5.47*
Medium	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
H_2O_2	1323.36	*1073.76*	611.00	*145.40*	486.08	*106.03*	423.51	*76.57*

10.3.2.3 DCF Cyanidin-3-glucoside

Non-normalized fluorescence change data

Incubation with Cy-3-glc (µM), 0.8% DMSO, 1 mM H_2O_2

15 min (t_{15}) Cy-3-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	45.31			42.75	40.08	22.25	11.29	9.09	9.62	14.58	121.99
2	43.39			33.85	25.73	26.53	8.75	7.86	3.66	*3.59*	134.57
3	39.03		29.20	37.73	30.90	29.31	19.11	17.89	9.60	15.35	138.82
4	53.66		35.57	41.23	38.29	22.87	12.54	11.98	8.32	19.11	185.48
5	49.10	26.37	42.38	48.61	42.80	21.35	13.07	5.13	10.40	21.57	196.85
6	28.47	26.43	33.91	43.55	32.72	31.67				21.33	172.04
7	28.06	27.46	20.17							13.72	148.61
8	32.86	34.77	35.26							18.77	*232.32*

30 min (t_{30}) Cy-3-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	138.91					77.19	38.52	26.59	28.77	*80.35*	351.19
2	132.01			106.47	82.09	86.13	29.82	30.65	28.74	65.58	364.29
3	117.97		95.52	104.87	82.64	67.41	48.22	35.15	34.52	68.50	375.21
4	117.17		86.25	91.62	83.33		16.17	10.23	9.54	*46.90*	390.55
5	108.09	80.40	102.24	98.58	95.09	71.82	39.54	20.50	24.83	65.02	*453.75*
6	83.00	76.11	91.14	103.15	75.65	76.40				67.13	372.60
7	85.75	81.10	75.54							*56.21*	414.78
8	112.73	120.19	106.69							61.65	*532.40*

45 min (t_{45}) inc. with Cy-3-glc (µM) (*Nalimov Outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	227.94			*227.15*	*214.04*	138.02	72.53	44.22	41.45	*143.48*	526.21
2	216.30			179.94	136.28	140.99	50.89	47.16	41.52	121.79	512.99
3	191.93		161.77	168.05	127.69	105.96	72.79	48.74	50.65	116.03	555.69
4	187.94		160.30	157.28	133.99	80.63	32.20	17.18	16.65	*80.86*	526.57
5	174.63	135.47	170.04	164.50	154.73	108.74	65.43	37.79	38.36	111.77	*617.89*
6	146.49	134.11	159.96	169.46	128.47	125.48				119.35	511.02
7	156.55	149.28	141.24							*99.10*	565.15
8	193.05	200.58	178.88							112.11	*733.82*

60 min (t_{60}) inc. with Cy-3-glc (µM) (*Nalimov Outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	308.21			312.94	*289.14*	191.70	106.30	62.35	50.17	*200.17*	641.15
2	294.33			245.59	185.70	188.84	70.67	59.53	53.47	171.71	609.96
3	261.29		214.40	243.10	173.27	141.42	96.53	63.91	63.34	162.44	678.78
4	255.48		233.42	223.57	188.44	118.30	51.55	25.12	25.25	*121.59*	627.17
5	237.38	187.84	232.93	225.70	207.84	141.09	87.05	49.84	50.89	162.19	718.76
6	202.04	191.67	229.83	233.49	172.81	159.75				167.29	593.60
7	217.63	211.46	201.11	34.78						*144.08*	649.72
8	268.14	274.66	249.18	67.48						160.74	*851.78*

Normalized to solvent control

Incubation with Cy-3-glc (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) Mean values T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.0001	70.64	*17.92*	85.18	*12.04*	85.94	*10.86*	88.29	*10.28*
0.01	73.96	*15.10*	80.35	*11.02*	81.55	*7.32*	83.98	*5.29*
0.1	70.06	*15.28*	67.72	*15.65*	67.24	*13.82*	68.64	*13.61*
1	53.06	*8.23*	52.13	*9.97*	49.50	*7.38*	49.63	*6.90*
10	36.13	*7.31*	38.48	*10.04*	38.09	*8.84*	38.83	*7.78*
50	19.54	*7.86*	17.84	*8.20*	20.51	*3.09*	17.80	*6.60*
100	10.52	*16.21*	12.43	*6.70*	12.40	*5.03*	12.45	*4.18*
200	4.67	*19.80*	11.84	*5.13*	11.54	*4.60*	11.37	*3.91*
Medium	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
H_2O_2	1323.36	*1073.76*	611.00	*145.40*	486.08	*106.03*	408.31	*68.44*

10.3.2.4 DCF Cyanidin-3-sambubioside

Non-normalized fluorescence change data

Incubation with Cy-3-sam (µM), 0.8% DMSO, 1 mM H_2O_2

15 min (t_{15}) Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	23.43				20.24	12.35	7.80	7.02	4.89	-1.34	*38.25*
2	22.05			17.07	17.05	9.35	9.36	10.38	13.32	0.55	*56.98*
3	*6.36*			4.53	3.16	*-1.35*	*-2.05*	*-2.31*	-4.71	1.18	*26.53*
4	24.42			20.15	19.41	19.77	10.34	11.06	0.02	1.18	137.83
5	32.77			22.81	9.23	17.69	9.96	8.81	3.87	19.59	199.91
6	*57.30*			*32.03*	34.29	16.13	11.54	8.58	-3.53	10.18	*114.97*
7	28.95		23.11	17.34						36.73	197.65
8	22.83		*17.81*	11.99						42.03	182.77
9	36.73		24.15	11.62						45.28	198.24
10	30.46		26.83	14.10						44.44	212.53
11	45.57	26.83	26.64	19.81						20.58	153.36
12	44.42	25.23	24.27	*39.31*						12.14	178.19
13	15.06	13.83	*8.37*	4.47						-4.00	161.82
14	30.27	35.28	21.52	23.64						7.40	164.78

30 min (t_{30}) Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	91.66				76.39	45.11	26.22	20.35	15.86	21.04	*124.23*
2	79.33			66.25	54.04	36.09	26.79	27.69		35.91	*175.24*
3	*42.81*			49.58	42.65	22.70	11.45	9.62	5.13	19.98	*156.24*
4	75.55			53.83	47.30	50.43	16.11	22.47	0.64	49.24	391.62
5	96.79			53.67	41.23	33.96	14.05	0.79	-2.16	58.93	464.89
6	*152.57*		*104.23*	*89.82*	79.37	28.72	11.56	3.61	3.02	60.58	349.06
7	99.88		*84.59*	57.26						*115.48*	482.69
8	87.78		75.31	51.70						*129.79*	542.10
9	88.94		68.32	36.31						*111.43*	450.66
10	84.75		66.73	45.87						86.70	499.10
11	107.44	65.92	72.01	68.47						70.56	344.19
12	109.91	70.13	74.67	*111.23*						58.79	397.37
13	*57.98*	55.55	*47.57*	32.48						38.22	353.84
14	91.00	97.01	71.78	70.91						50.94	400.93

45 min (t_{45}) Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	167.09				138.77	80.88	43.91	28.26	20.83	39.88	*196.18*
2	134.24			113.26	90.51	57.32	39.88	35.76	*44.30*	64.93	*268.86*
3	*75.22*			93.27	78.34	52.79	30.10	19.43	12.22	39.30	*295.46*
4	128.08			87.39	79.22	78.55	26.18	27.95	0.60	94.79	581.22
5	162.06			94.63	69.99	54.19	21.86	0.79	-5.61	102.56	641.98
6	*250.40*		*182.71*	*158.27*	130.29	55.41	19.63	3.45	1.50	109.42	518.92
7	175.87		*149.57*	96.75						*187.43*	690.89
8	153.24		130.95	89.39						*213.13*	*759.58*
9	153.60		116.58	67.84						*168.13*	590.36
10	134.83		107.39	73.99						129.35	669.85
11	172.27	105.67	122.70	113.76						117.62	495.52
12	178.13	114.21	123.65	*173.11*						103.80	536.83
13	*111.75*	107.98	*94.03*	70.20						75.21	473.91
14	156.35	160.24	118.84	107.62						90.04	569.13

60 min (t_{60}) Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	234.20				194.19	114.26	61.23	36.37	24.57	55.94	*251.11*
2	185.77			153.36	119.66	78.16	49.27	41.82	47.97	89.96	*337.85*
3	*107.02*			129.44	107.76	77.09	35.29	23.66	14.76	53.98	*360.55*
4	182.76			130.85	111.39	107.10	38.04	32.83	1.56	136.16	699.04
5	223.98			131.31	97.79	75.48	29.90	6.17	-4.65	147.52	749.18
6	*340.46*		*259.06*	*222.55*	184.18	83.02	29.19	7.37	2.49	154.49	646.61
7	247.60		*212.09*	146.27						*255.89*	828.42
8	211.25		187.22	129.94						*289.62*	*896.06*
9	212.29		165.29	101.86						*225.14*	686.27
10	186.27		150.42	103.18						180.19	787.84
11	235.02	149.64	173.73	164.44						165.50	593.69
12	245.85	156.63	171.83	*235.52*						149.53	633.57
13	163.28	158.67	136.80	99.84						107.60	555.14
14	215.56	219.81	168.27	145.27						126.10	665.67

Normalized to solvent control

Incubation with Cy-3-sam (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100	*0*	100	*0*	100	*0*	100	*0*
0.0001	81.03	*28.63*	81.90	*22.76*	81.14	*21.43*	81.63	*20.81*
0.01	69.64	*12.74*	75.86	*7.18*	76.28	*5.80*	78.99	*6.18*
0.1	61.39	*26.29*	66.81	*20.16*	67.73	*21.28*	68.74	*19.60*
1	68.74	*20.05*	68.05	*20.81*	68.62	*22.08*	67.79	*20.68*
10	48.25	*17.95*	44.73	*16.36*	46.36	*17.68*	46.60	*17.18*
50	22.68	*7.33*	22.09	*9.69*	22.96	*11.59*	21.40	*9.08*
100	14.66	*7.23*	18.75	*14.12*	15.51	*11.81*	13.84	*9.19*
200	7.12	*11.88*	5.98	*8.27*	5.26	*8.56*	8.27	*10.61*
Medium	100	*0*	100	*0*	100	*0*	100	*0*
H_2O_2	330.58	*1644.09*	623.00	*182.59*	535.59	*127.59*	433.18	*83.09*

10.3.2.5 DCF Cyanidin-3-sambubioside-5-glucoside

Non-normalized fluorescence change data

Incubation with Cy-3-sam (µM), 0.8% DMSO, 1 mM H_2O_2

15 min (t_{15}) Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	23.43				19.31	11.98	10.18	9.78	9.93	2.76	38.25
2	22.05			15.37	13.15	5.91	9.05	9.48	13.76	0.55	56.98
3	*6.36*			4.39	3.38	0.59	-0.18	-2.07	3.00	1.18	26.53
4	24.42			18.38	14.78	17.14	9.89	10.35	10.73	1.18	137.83
5	32.77			18.08	12.71	11.58	7.59	8.68		19.59	199.91
6	*57.30*		53.45	*41.34*	*33.36*				9.16	10.18	114.97
7	36.73		8.27	3.86						*45.28*	198.24
8	30.46		19.09	4.46						*44.44*	212.53
9	45.57	47.87	28.97	33.21						20.58	153.36
10	44.42	41.80	38.33	29.84						12.14	178.19
11	15.06	17.13	9.70	5.48						-4.00	161.82
12	30.27	31.71	21.63	19.86						7.40	164.78

30 min (t_{30}) Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	91.66				77.24	48.95	37.58	30.13	27.29	31.07	124.23
2	79.33			64.95	55.24	37.97	37.10	36.18	40.77	35.91	175.24
3	*42.81*			42.62	36.07	26.92	17.76	10.54	15.82	19.98	156.23
4	75.55			66.55	46.79	53.27	25.36	33.89	26.44	49.24	391.62
5	96.79			60.26	39.17	38.62	24.18	18.29		58.93	464.89
6	*152.57*		*132.99*	*103.84*	87.20				11.60	60.58	349.06
7	88.94		47.90	36.40						*111.43*	450.66
8	84.75		64.15	40.95						*86.70*	499.10
9	107.44	109.86	73.15	71.44						70.56	344.19
10	109.91	105.75	96.06	71.93						58.79	397.37
11	*57.98*	76.90	55.06	42.01						38.22	353.84
12	91.00	104.42	81.14	75.04						50.94	400.93

45 min (t_{45}) Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	167.09				141.70	91.31	67.50	49.56	41.87	55.70	*196.18*
2	134.24			114.31	95.15	68.39	58.93	54.59	60.67	64.93	*268.86*
3	*75.22*			82.72	75.59	57.02	41.38	26.93	33.35	*39.30*	*295.46*
4	128.08			112.72	82.95	99.15	54.76	61.32	42.57	94.79	581.22
5	162.06			107.62	78.93	74.43	48.52	32.90		102.56	641.98
6	*250.40*		*224.57*	*188.08*	156.11				23.91	109.42	518.92
7	153.60		93.23	63.18						*168.13*	590.36
8	134.83		111.71	71.94						129.35	669.85
9	172.27	176.88	125.95	105.31						117.62	495.52
10	178.13	169.53	146.38	110.91						103.80	536.83
11	111.75	138.70	97.67	72.58						75.21	473.91
12	156.35	183.25	134.63	114.29						90.04	569.13

60 min (t_{60}) Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	DMSO	0.0001	0.01	0.1	1	10	50	100	200	Medium	H_2O_2
1	234.20				200.01	133.46	97.13	69.27	56.79	77.79	*251.11*
2	185.77			156.14	130.22	97.61	77.25	69.02	75.34	89.96	*337.85*
3	*107.02*			116.09	108.99	84.97	58.46	39.76	47.62	*53.98*	*360.55*
4	182.76			162.74	121.07	142.91	82.87	85.66	57.16	136.16	699.04
5	223.98			156.74	121.10	114.44	70.74	48.46		147.52	749.18
6	*340.46*		*321.54*	*279.32*	227.08				40.31	154.49	646.61
7	212.29		134.18	90.76						*225.14*	686.27
8	186.27		158.37	101.80						180.19	787.84
9	235.02	242.86	177.76	146.90						165.50	593.69
10	245.85	230.22	203.06	149.70						149.53	633.57
11	163.28	201.38	141.44	106.09						107.60	555.14
12	215.56	259.24	190.39	155.96						126.10	665.67

Normalized to solvent control

Incubation with Cy-3-sam-5-glc (µM), 0.8% DMSO, 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.0001	104.43	*8.06*	111.46	*16.09*	109.79	*13.23*	110.14	*14.08*
0.01	66.31	*22.71*	79.48	*14.47*	78.72	*10.15*	80.24	*9.44*
0.1	53.65	*24.36*	70.79	*17.97*	70.55	*19.56*	70.97	*18.79*
1	58.80	*14.09*	66.28	*16.89*	72.00	*18.25*	74.06	*16.92*
10	38.55	*23.23*	54.91	*12.08*	60.95	*14.64*	63.64	*14.01*
50	29.08	*19.57*	37.56	*8.46*	42.40	*8.95*	42.92	*8.30*
100	24.21	*32.46*	33.37	*11.92*	34.86	*10.52*	34.48	*9.45*
200	42.38	*16.76*	32.14	*15.89*	31.47	*14.82*	30.48	*13.09*
Medium	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
H_2O_2	2435.17	*4349.75*	640.58	*175.48*	531.45	*102.33*	445.34	*78.13*

10.3.3 Protective DCF Assay

10.3.3.1 Protective DCF phloroglucinol aldehyde

24 h incubation with PGA (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2

Non-normalized fluorescence change data

15 min (t_{15}), PGA (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	2.075	4.15	8.3	50	100	200	400
1	12.82	94.23			82.75	90.64	76.51	81.78	82.64	88.26
2	19.45	113.19			103.28	88.96	100.96	80.13	88.64	69.20
3	*32.54*	49.89		73.88	86.24	83.35	99.64	106.82	104.00	101.53
4	19.77	67.06		71.05	62.14	70.13	65.96	60.10	65.30	67.49
5	*3.92*	75.50		93.37	93.19	99.56	98.38	95.00	98.89	109.17
6	25.95	88.56		83.37	64.55	96.23	80.12	94.46	*126.71*	117.55
7	25.43	35.28		49.02	62.18	66.74	43.21	59.77	50.38	41.96
8	12.93	59.14		55.48	60.17	*48.91*	61.56	43.47	55.84	23.92
9	12.50	56.22	61.63						54.73	44.56
10	9.78	60.10	76.93						52.38	42.46
11	16.63	93.60	114.22							91.83
12	20.71	119.92	123.36							105.00

30 min (t_{30}), PGA (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	2.075	4.15	8.3	50	100	200	400
1	76.68	277.52			284.52	295.67	260.28	*271.77*	287.25	265.23
2	60.60	314.44			255.27	246.81	253.73	244.13	253.89	269.56
3	*118.45*	223.95		242.54	223.63	236.12	240.78	230.58	271.96	296.72
4	96.08	234.49		278.39	227.49	247.18	235.86	224.74	237.06	256.47
5	84.41	275.06		265.72	251.97	294.69	263.04	*296.32*	314.73	347.71
6	65.76	180.70		195.50	170.22	218.39	207.23	239.75	320.42	357.02
7	93.76	156.61		188.99	236.93	248.66	199.51	221.88	225.91	206.53
8	38.05	168.28		170.96	190.82	178.90	201.00	*169.03*	221.37	123.81
9	49.91	164.37	175.11						175.61	170.69
10	54.56	193.10	215.97						188.93	167.03
11	58.18	264.34	304.14							278.69
12	70.82	332.06	344.93							324.26

45 min (t_{45}), PGA (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	2.075	4.15	8.3	50	100	200	400
1	147.51	455.78			520.04	506.32	445.58	489.50	492.84	448.73
2	105.72	495.40			401.32	405.59	421.91	421.68	434.48	450.39
3	154.66	400.75		367.18	359.55	366.32	346.94	372.63	417.86	472.29
4	182.66	403.02		459.56	396.17	425.72	410.73	386.14	381.84	444.25
5	140.51	484.16		443.34	413.89	497.65	421.89	525.19	498.27	539.48
6	127.69	337.38		300.73	282.12	325.15	413.03	320.72	*740.95*	565.30
7	171.35	355.76		348.91	436.28	460.13	403.15	447.75	465.12	387.81
8	70.82	273.83		289.19	317.13	311.38	339.44	297.29	375.23	225.26
9	80.44	254.96	263.53						267.31	276.70
10	91.95	302.45	324.48						294.92	278.43
11	108.88	401.00	453.06							438.31
12	121.27	498.99	540.47							507.18

60 min (t_{60}), PGA (µM) (*Nalimov outliers, removed for statistics*), *ovrflw: overflow, too high fluorescence for platereader, removed*)

N	No H_2O_2	DMSO	0.1	2.075	4.15	8.3	50	100	200	400
1	212.06	585.73			686.99	654.71	576.54	636.61	637.64	586.56
2	174.33	636.38			514.78	525.03	546.61	547.46	561.14	578.02
3	212.71	476.90		482.66	458.35	501.41	*485.58*	485.72	534.05	596.87
4	261.52	510.29		595.09	517.07	537.72	548.26	504.71	497.26	575.26
5	197.43	609.85		574.30	560.52	644.30	542.49	667.22	641.98	675.22
6	187.55	ovrflw	ovrflw	ovrflw	ovrflw	ovrflw	ovrflw	ovrflw	ovrflw	ovrflw
7	235.97	465.03		461.17	600.66	627.87	552.00	632.40	617.74	536.79
8	102.93	360.72		358.65	415.27	405.72		409.62	504.20	311.98
9	111.76	327.16	333.18						339.77	358.58
10	126.17	387.81	403.64						374.15	366.84
11	155.86	486.89	OVRF							560.03
12	168.97	572.04	636.14							641.16

Normalized to solvent control

24 h incubation with PGA (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2 (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
No H_2O_2	25.40	*17.11*	31.18	*11.89*	32.66	*8.30*	36.07	*8.70*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.1	115.63	*11.45*	109.33	*5.05*	107.99	*3.96*	106.22	*4.34*
2.075	117.41	*23.16*	107.42	*10.32*	98.34	*9.75*	102.12	*8.52*
4.15	114.45	*40.01*	102.34	*22.47*	98.84	*16.47*	104.55	*16.74*
8.3	126.04	*39.65*	109.72	*22.19*	104.03	*14.62*	108.28	*15.46*
50	115.84	*37.51*	103.09	*14.83*	102.28	*15.98*	103.77	*15.38*
100	119.12	*50.63*	105.65	*21.48*	102.41	*12.73*	107.78	*15.38*
200	113.84	*41.32*	115.58	*22.51*	107.54	*16.22*	109.52	*16.87*
400	103.90	*44.47*	109.20	*24.93*	108.27	*21.33*	106.52	*11.92*

10.3.3.2 Protective DCF cyanidin

24 h incubation with Cy (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2

Non-normalized fluorescence change data

15 min (t_{15}), Cy (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	35.55	172.80	153.32	165.12	163.61	168.46	163.43	169.08
2	31.13	206.28	184.34	194.65	193.23	187.03	216.67	252.35
3	29.37	245.95	248.18	230.75	225.11	225.36	224.95	231.15
4	18.80	189.79	184.93	191.72	224.21	202.39	175.96	165.00
5	22.38	187.41	200.21	198.43	205.77	190.33	187.02	185.98

30 min (t_{30}), Cy (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	113.73	489.97	422.49	456.41	441.45	442.10	428.24	463.43
2	95.23	509.30	513.56	512.51	526.25	506.77	631.94	673.57
3	92.51	555.78	558.18	532.22	538.40	577.41	574.70	558.61
4	79.50	505.05	520.67	510.88	601.53	573.67	511.11	487.23
5	93.11	545.17	584.26	528.67	567.04	537.94	502.30	536.39

45 min (t_{45}), Cy (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	179.22	688.13	600.55	650.15	627.06	629.81	597.41	645.54
2	157.16	706.28	730.63	742.77	756.25	721.34	917.68	956.83
3	141.69	795.12	803.91	768.62	791.11	853.87	813.80	790.72
4	135.92	708.73	760.90	749.35	856.54	833.40	726.89	698.92
5	155.52	783.99	857.16	766.18	801.95	777.28	725.87	792.98

60 min (t_{60}), Cy (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	247.72	841.88	741.74	804.68	774.33	780.64	734.50	785.79
2	211.71	827.24	859.50	889.02	896.98	859.98	1098.88	1135.89
3	191.22	912.85	941.04	917.35	927.06	1012.30	951.87	919.16
4	186.44	833.49	905.61	879.66	1006.93	984.01	862.83	830.76
5	209.19	924.96	1030.26	917.60	941.39	930.01	863.20	959.86

Normalized to solvent control

24 h incubation with Cy (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2, T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
No H_2O_2	13.89	*4.17*	18.27	*2.96*	21.03	*3.23*	24.19	*3.38*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.1	98.93	*4.73*	99.15	*5.26*	99.63	*4.64*	99.97	*5.17*
1	101.07	*4.73*	100.85	*5.26*	100.37	*4.64*	100.69	*4.27*
10	96.65	*7.72*	99.55	*7.91*	101.70	*8.69*	103.03	*9.01*
50	98.13	*5.19*	97.53	*3.37*	99.95	*5.15*	101.66	*4.82*
100	101.56	*11.76*	102.68	*10.77*	104.17	*10.98*	104.91	*10.65*
200	97.60	*6.73*	101.18	*8.52*	103.56	*9.72*	105.24	*9.70*

10.3.3.3 Protective DCF cyanidin-3-glucoside

24 h incubation with Cy-3-glc (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2

Non-normalized fluorescence change data

15 min (t_{15}), Cy-3-glc (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	35.55	172.80	140.97	171.23	174.27	163.99	137.01	126.67
2	31.13	206.28	206.47	216.91	213.28	176.87	173.67	175.50
3	29.37	245.95	324.96	287.50	246.40	235.59	208.88	178.32
4	18.80	189.79	199.33	174.90	154.52	146.52	139.56	111.57
5	22.38	187.41	173.15	175.39	184.42	164.45	138.96	112.29

30 min (t_{30}), Cy-3-glc (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	113.73	489.97	412.92	487.42	467.40	423.74	349.37	325.34
2	95.23	509.30	579.94	600.08	588.19	489.90	466.48	432.15
3	92.51	555.78	784.59	667.28	560.13	530.10	471.59	409.91
4	79.50	505.05	492.77	462.67	412.02	373.00	341.79	262.76
5	93.11	545.17	548.84	528.82	526.39	486.39	414.28	305.46

45 min (t_{45}), Cy-3-glc (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	179.22	688.13	585.14	689.60	651.50	591.92	471.91	429.98
2	157.16	706.28	818.02	832.14	821.15	686.09	650.75	572.31
3	141.69	795.12	1082.59	908.33	764.19	736.51	635.91	526.73
4	135.92	708.73	724.30	682.28	617.59	558.71	502.93	364.69
5	155.52	783.99	797.44	757.99	734.02	684.65	584.14	418.41

60 min (t_{60}), Cy-3-glc (µM)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	247.72	841.88	717.92	842.86	797.28	721.68	562.25	493.65
2	211.71	827.24	965.41	974.16	971.10	812.87	764.55	646.05
3	191.22	912.85	1233.92	1034.78	890.94	869.12	730.28	590.65
4	186.44	833.49	860.32	813.75	742.52	675.12	596.66	418.51
5	209.19	924.96	946.54	902.42	875.48	819.49	690.50	479.19

Normalized to solvent control

24 h inc. with Cy-3-glc (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2, T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
No H_2O_2	13.89	*4.17*	18.27	*2.96*	21.03	*3.23*	24.19	*3.38*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.1	102.24	*18.90*	107.51	*21.56*	108.18	*19.07*	108.54	*18.60*
1	101.37	*10.07*	105.19	*12.89*	105.04	*10.22*	105.29	*9.56*
10	96.85	*8.81*	97.96	*12.17*	97.56	*11.00*	98.69	*10.90*
50	88.28	*7.58*	88.23	*9.02*	88.39	*6.94*	89.76	*7.01*
100	79.22	*5.37*	78.28	*9.83*	77.23	*9.37*	77.09	*9.82*
200	69.92	*10.86*	66.61	*13.31*	62.92	*11.85*	60.69	*11.31*

10.3.3.4 Protective DCF cyanidin-3-sambubioside

24 h incubation with Cy-3-sam (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2

Non-normalized fluorescence change data

15 min (t_{15}), Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	24.73	93.57		89.55	90.52	72.61	66.87	58.48
2	14.00	118.81		102.88	139.97	123.47	107.03	83.68
3	*2.86*	103.79		85.71	106.60	110.66	113.16	99.18
4	41.59	334.75		225.10	193.66	170.56	149.99	137.56
5	36.41	206.85		170.01	214.20	199.96	163.02	111.80
6	40.55	208.99	214.45	185.75	178.82	173.14	160.00	141.91
7	39.72	244.57	211.45	184.73	197.07	172.86	174.61	89.82
8	40.07	214.33	242.05	228.60	230.58	223.32		202.54
9	40.28	278.08	256.05	274.72	270.82	253.98		189.93
10	16.60	199.76	204.44				211.28	
11	53.10	233.31	222.45				223.88	

30 min (t_{30}), Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	68.26	*241.65*		239.45	*225.13*	*180.50*	167.41	145.37
2	50.99	343.21		289.65	*314.11*	*266.57*	227.75	178.41
3	46.46	424.05		407.24	487.29	442.16	478.71	365.42
4	90.47	786.19		520.82	450.91	415.00	378.20	388.83
5	75.55	495.59		358.42	481.86	451.84	384.77	308.64
6	65.14	478.42	*484.11*	419.61	426.33	410.07	392.84	360.79
7	102.33	658.94	538.05	471.86	485.17	461.17	494.02	212.79
8	102.33	532.25	587.37	507.74	541.86	510.68		477.83
9	94.41	698.66	594.70	631.11				491.20
10	77.01	553.46	564.07				580.32	
11	*168.90*	631.99	596.75				614.34	

45 min (t_{45}), Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	109.97	*316.13*		375.00	*328.36*	*268.72*	234.39	205.01
2	94.13	*516.58*		435.03	*429.12*	*371.44*	304.20	243.22
3	114.44	747.99		746.37	781.81	732.71	733.70	576.56
4	138.54	963.04		713.52	636.73	602.77	553.24	584.24
5	111.49	702.87		525.13	688.30	676.59	557.41	435.40
6	99.49	681.61	*696.26*	617.08	616.24	593.84	553.31	510.88
7	172.84	986.07	828.56	723.23	766.30	736.45	742.07	296.73
8	161.23	769.57	841.58	720.60	768.17	721.25		669.71
9	153.50	976.28	855.87	890.05	889.98	846.77		674.90
10	146.01	805.29	826.10				857.86	
11	*263.57*	904.11	861.72				883.11	

60 min (t_{60}), Cy-3-sam (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	0.1	1	10	50	100	200
1	145.34	*454.17*		477.55			284.35	241.69
2	137.63	*650.12*		557.31	605.81		365.28	288.71
3	159.73	881.88		897.02	876.67	854.02	841.52	672.91
4	197.35	1138.54		834.70	754.41	712.40	654.11	691.70
5	152.00	814.84		653.03	841.80	835.38	683.13	523.33
6	138.18	814.62	*832.30*	746.01	742.64	711.38	652.92	606.80
7	236.52	1145.71	975.47	862.60	903.23	867.06	848.30	329.93
8	223.54	933.19	1069.10	821.93	911.38	858.51		777.50
9	212.41	1152.37	1007.27	1042.98	1042.83	1000.04		787.69
10	206.89	972.03	1000.03				1042.41	
11	*340.74*	1079.37	1030.97				1063.53	

Normalized to solvent control

24 h inc. with Cy-3-sam (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2, (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
No H_2O_2	15.54	*6.64*	15.66	*4.98*	19.12	*5.62*	21.83	*5.33*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
0.1	98.63	*9.34*	95.78	*10.90*	95.79	*10.43*	97.10	*12.02*
1	87.13	*12.09*	84.79	*12.02*	87.26	*11.36*	87.94	*10.87*
10	94.42	*17.66*	89.26	*18.90*	89.67	*13.49*	90.04	*12.10*
50	87.20	*18.54*	83.65	*17.23*	85.58	*12.81*	86.25	*13.44*
100	82.65	*19.99*	81.49	*20.40*	79.92	*17.85*	79.50	*18.54*
200	65.67	*20.45*	64.20	*18.39*	62.75	*17.09*	61.54	*17.14*

10.3.3.5 Protective DCF cyanidin-3-sambubioside-5-glucoside

24 h incubation with Cy-3-sam-5-glc (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2

Non-normalized fluorescence change data

15 min (t_{15}), Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	1	10	50	100	200
1	24.73	93.57	84.67	78.60	82.13	67.93	66.99
2	14.00	118.81	122.43	122.85	128.41	91.04	84.44
3	2.86	103.79	88.95	113.23	122.75	114.40	114.32
4	41.59	334.75	320.98	*297.57*	*287.63*	250.85	260.02
5	36.41	206.85	225.48	202.97	200.65	189.79	211.50
6	40.55	208.99	202.75	155.45	162.98		
7	39.72	244.57	219.80				

30 min (t_{30}), Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	1	10	50	100	200
1	68.26	241.65	235.89	197.16	211.46	165.20	163.41
2	50.99	343.21	333.16	293.54	284.02	217.61	202.71
3	46.46	424.05	374.11	403.31	386.20	407.50	374.66
4	90.47	786.19	*767.64*	*730.65*	*731.67*	614.36	662.27
5	75.55	495.59	501.20	455.83	458.06	433.16	505.65
6	65.14	478.42	459.60	407.80	395.99		
7	102.33	658.94	536.26				

45 min (t_{45}), Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*)

N	No H_2O_2	DMSO	1	10	50	100	200
1	109.97	361.13	359.19	283.10	308.85	234.27	226.60
2	94.13	516.58	492.60	461.49	405.90	340.02	313.05
3	114.44	747.99	667.70	685.52	589.97	651.63	580.54
4	*138.54*	963.04	864.73	850.11	*1004.48*	804.77	1011.31
5	111.49	702.87	717.35	651.21	660.71	625.63	707.37
6	99.49	681.61	676.21	613.09	598.46		
7	*172.84*	986.07	784.99				

60 min (t_{60}), Cy-3-sam-5-glc (µM) (*Nalimov outliers, removed for statistics*), *OVRF: Overflow, too high fluorescence for platereader, removed*)

N	No H_2O_2	DMSO	1	10	50	100	200
1	145.34	454.17	*451.47*	355.47	385.67	286.05	268.21
2	137.63	650.12	OVRF	OVRF	469.56	420.46	387.17
3	159.73	881.88	798.37	819.31	677.47	785.79	689.11
4	*197.35*	1138.54	1051.96	1054.48	1138.92	999.23	1249.89
5	152.00	814.84	843.98	777.16	797.91	743.48	833.23
6	138.18	814.62	827.17	753.45	739.05		
7	*236.52*	1145.71	916.68				

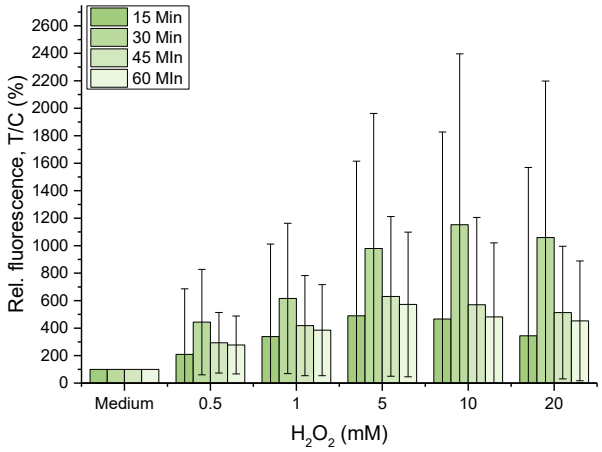
Normalized to solvent control

24 h inc. with Cy-3-sam-5-glc (µM), 0.8% DMSO, stressor t_0: 1 mM H_2O_2, (*Nalimov outliers from non-normalized data removed*) T/C (%) ± SD

Conc.	t_{15}	*t_{15} SD*	t_{30}	*t_{30} SD*	t_{45}	*t_{45} SD*	t_{60}	*t_{60} SD*
No H_2O_2	15.23	*7.36*	15.71	*5.81*	18.05	*5.65*	20.70	*5.23*
DMSO	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*	100.00	*0.00*
1	95.86	*8.10*	92.94	*6.68*	93.54	*7.85*	93.61	*9.46*
10	93.80	*14.30*	88.08	*5.69*	88.37	*5.14*	90.33	*6.85*
50	97.82	*15.95*	87.43	*4.71*	84.96	*6.48*	87.11	*11.22*
100	85.23	*15.86*	78.68	*13.41*	78.08	*11.79*	79.15	*14.06*
200	86.55	*18.34*	80.26	*17.06*	81.32	*20.75*	81.76	*23.60*

10.4 Supporting data

10.4.1 Pretest DCF assay with H_2O_2



C2BBe1 cells are cultured 24h in black 96 well plates (15 000 cells/well) followed by a DCF assay where cells are preincubated with DCFH-DA followed by 1 h incubation with H_2O_2 while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from 3 - 4 independent experiments and normalized to medium (T/C in %)

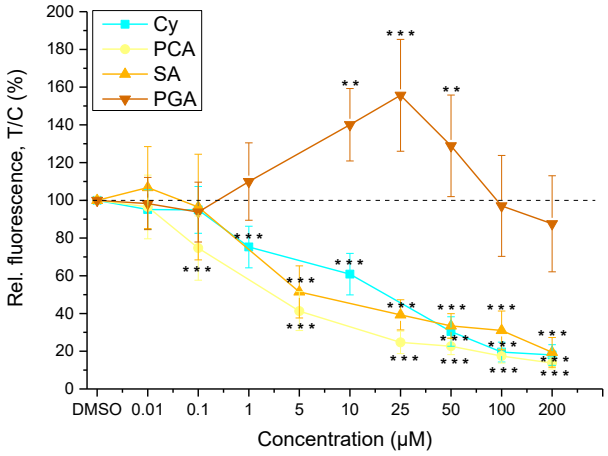
Figure 28: Effect of H_2O_2 on intracellular ROS in C2BBe1 cells

Non-normalized fluorescence change data

t_0: incubation with H_2O_2 (mM), *ovrflw: overflow, too high fluorescence for platereader*

N	Medium	0.5	1	5	10	20
t_{15}						
1	4.07	19.08	41.35	77.17	84.32	57.51
2	8.73	69.71	84.02	103.37	114.83	112.72
3	34.75	83.63	103.47	144.11	154.94	209.38
4	-13.38	61.85	78.06	140.28	202.18	214.58
t_{30}						
1	11.14	66.45	109.85	182.99	191.74	145.51
2	44.20	182.31	220.40	259.46	279.50	267.51
3	96.96	200.42	240.21	302.52	331.02	432.94
4	13.53	135.69	182.10	318.03	413.72	395.18
t_{45}						
1	17.77	104.54	172.59	258.40	261.32	207.91
2	78.17	258.43	323.71	373.32	409.86	393.34
3	158.25	278.06	325.96	396.60	438.10	568.18
4	39.17	189.50	252.35	448.96	*ovrflw*	*ovrflw*
t_{60}						
1	24.14	133.63	210.39	304.95	300.44	254.63
2	103.70	315.74	386.15	427.36	467.77	440.51
3	208.21	325.79	379.02	447.05	459.90	649.31
4	62.28	232.97	311.05	602.06	*ovrflw*	*ovrflw*

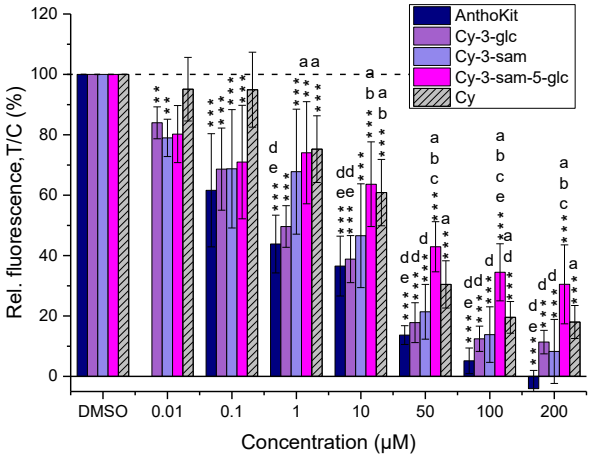
10.4.2 DCF assay protocatechuic acid and syringic acid



C2BBe1 cells are cultured 24h in black 96 well plates (15 000 cells/well) followed by a DCF assay where cells are pre-incubated with DCFH-DA followed by 60 min incubation with Cy, PCA, SA or PGA while fluorescence intensity is quantified with a Cytation3 photometer. Mean and SD of fluorescence change from t_0 was calculated in at least triplicates from at least 5 independent experiments and normalized to solvent control (T/C in %). Solvent: 0.8% 0.0001% HCl (conc.), PGA solvent not acidified, 100 U/ml catalase. Pos.Ctrl: 1mM H_2O_2. Raw data outliers were eliminated with Nalimov. Significant differences to DMSO solvent calculated with ANOVA (Bonferroni) (α = 0.05:*; 0.01:**, 0.001:***) and Students t-Test (α = 0.001: ###). Kolmogorov-Smirnov Test for normal distribution and Brown-Forsythe Tests for variance homogeneity was performed. Data for PCA, SA obtained from Völkle (2016)

Figure 29: Comparison of the effect of Cy, PCA, SA and PGA on intracellular oxidative stress in C2BBe1 cells, t_{60}

10.4.3 DCF Assay: Comparison AnthoKit with cyanidin glycosides and aglycon



Experiment as in Fig. 28. at t_i = 60 min. ANOVA and posthoc Bonferroni was performed for every concentration level with normalized data. Significantly different fluorescence change between the substances (α = 0.05) noted with a) AnthoKit, b) Cy-3-glc, c) Cy-3-sam, d) Cy-3-sam-5-glc, e) Cy Significant differences to DMSO solvent calculated with ANOVA (Bonferroni) (α = 0.05:*; 0.01:**, 0.001: ***) and Students t-Test (α = 0.001: ###) for each substance separate. Data for AnthoKit obtained from Schier (2017)

Figure 30: Comparison of the effect of AnthoKit, Cy-3-glc, Cy-3-sam^x, Cy-3-sam-5-glc^x and Cy on intracellular oxidative stress in C2BBe1 cells, t_{60}