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Antiradical and reductant activities of anthocyanidins and anthocyanins, structure–activity relationship and synthesis

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ABSTRACT

Eight anthocyanidins, seven anthocyanins and two synthesized 4′-hydroxy flavyliums were examined as hydrogen donors to DPPH, ABTS and hydroxyl radicals, and as electron donors in the FRAP assay. Most compounds gave better activities than trolox and catechol. A structure–activity relationship (SAR) study showed that, in the absence of the 3-OH group, radicals of the 4, 5 or 7-OH groups can only be stabilized by resonance through pyrylium oxygen, while 3-OH group improved hydrogen atom donation because of the stabilization by anthocyanidin semiquinone-like resonance. Electron donation was also enhanced by the 3-OH group. Both anthocyanidins and their respective anthocyanins showed similar trends and close activities. Different types of sugar unit bonded to the 3-OH group or counter ion had minor effect on activities. The catechol structure improved both hydrogen and electron donation. Compounds lacking the catechol structure had a decreasing order of H-atom and electron donation (Mv > Pn > Pg > Ap > 4′-OH-flavylium) consistent with the decreasing number of their hydroxyl and/or methoxy groups.

1. Introduction

Anthocyanins are glycosides of aglycons called anthocyanidins; both forms are well distributed in the plant kingdom especially in vegetables, fruits and flowers, and are responsible for many of their colors, particularly orange, red, blue and purple. Anthocyanidins are an interesting class of flavonoids characterized by having an oxonium ion, namely 2-phenylbenzopyrylium (flavylium). There are 23 known anthocyanidins and more than 500 anthocyanins that occur naturally. The most common anthocyanidins in plants are cyanidin (Cn), pelargonidin (Pg), peonidin (Pn), delphinidin (Dp), petunidin (Pt) and malvidin (Mv); they vary, as in other flavonoid groups, in their number, and spatial distribution, of hydroxyl and methoxy groups (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The most common anthocyanins are those with the 3-glycoside structure (Kong, Chia, Goh, Chia, & Brouillard, 2003). Functions of anthocyanins in plants include insect attraction for pollination and plant protection from bacteria and oxidative stress. They have shown a wide range of pharmacological applications against various stress conditions and chronic diseases, e.g., inflammation, cognitive decline, neural dysfunction, capillary fragility and permeability, platelet aggregation, cardiovascular complication, liver damage, lipid peroxidation and cancer tumor growth (Kong et al., 2003). Many of these biological activities have been attributed to the potent antiradical and antioxidant activity of anthocyanins. There are two widely accepted mechanisms for radical scavenging activity of phenolic compounds, the hydrogen-atom transfer mechanism (where the antioxidant donates a hydrogen atom to the active radical and gives a stable phenoxyl radical in one step) and the single electron transfer mechanism (where an electron and a proton are transferred in two consecutive steps to give first a radical cation then the phenoxyl radical, respectively). Both mechanisms are thought to work as parallel reactions (Klein & Lukes, 2007; Huang, Boxin, & Prior, 2005).

The antioxidant and antiradical activity of anthocyanidins and anthocyanins are strongly related to their structural features including the kind, number and position of substituents on the flavylium ion. The number and position of hydroxyl and methoxy substituents, as electron donating groups, were found to have a great impact on the antioxidant activity of anthocyanidins (Azevedo et al., 2010; Kähkönen & Heinonen, 2003). Anthocyanidins with catechol (1,2-diphenol) or pyrogallol (1,2,3-triphenol) structures usually showed a much higher antioxidant activity than other analogues as a result of the formation of the very stable semiquinone radicals characterized by extension of the radical delocalization over anthocyanidin rings B and C, and the two

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hydroxyl oxygen atoms (Azevedo et al., 2010; Castañeda-Ovando et al., 2009). The resulting radicals can also be stabilized by hydrogen bonding with neighboring hydroxyl groups (Foti, Barclay, & Ingold, 2002; Pereira, Donate, & Galembeck, 1997). It has been reported that delphinidin (Dp) and Dp-3-glu have a greater DPPH scavenging activity (Kähkönen & Heinonen, 2003) while cyanidin showed more antioxidant activity (Kong et al., 2003) than vitamin E. Glycosylation parameters, i.e., site and number of glycosylation and sugar type, also affect the antioxidant capacity of anthocyanins (Zhao et al., 2014). These effects were contradicted in another study (Wang, Cao, & Prior, 1997). For example, 3-glucosylation can either increase (Pt and Pg), unaffect (Mv and Cn) or decrease (Dp and Pn) the antioxidant activity. Type of sugar showed different effects on anthocyanin activity; while no significant difference in activity was observed between cyanidin-3-glycoside with glucose or galactose, cyanidin-3-arabinoside showed significantly less activity (Kähkönen & Heinonen, 2003). Furthermore, the kind of target free radical can affect the order of anthocyanidin activity; cyanidin exhibited a similar activity to malvidin, but less than that of petunidin, towards the superoxide radical, while cyanidin and petunidin showed similar activities, but higher than that of malvidin, against the peroxynitrite radical (Rahman, Ichiyanagi, Komiyama, Hatano, & Konishi, 2006).

Despite the importance of structure–activity relationship of anthocyanins not only in determining the structural features required to enhance the antioxidant activity but also in finding the source of this enhancement, the SAR of anthocyanins is still poorly understood (Jhin & Hwang, 2014) and have received fewer publications than some other classes of flavonoids (Guzmán, Santiago, & Sánchez, 2009; Kähkönen & Heinonen, 2003). Therefore, this study aims to examine the structural features of anthocyanidins and anthocyanins required to possess high antioxidant activity in addition to studying the effect of these features on the stability of the formed intermediates during the radical scavenging process. The examined structural factors included the number and position of the hydroxyl and methoxy groups, the counter anion and the glycosylation of the 3-hydroxyl group.

2. Materials and methods

2.1. Chemicals and instruments

The anthocyanidins used were apigeninidin (Ap), pelargonidin (Pg), cyanidin (Cn), delphinidin (Dp), peonidin (Pn), petunidin (Pt), malvidin (Mv) and quercetagetinidin (Qu). The anthocyanins used were pelargonidin-3-glucoside (Pg-3-glu), cyanidin-3-glucoside (Cn-3-glu), delphinidin-3-glucoside (Dp-3-glu), peonidin-3-glucoside (Pn-3-glu), petunidin-3-glucoside (Pt-3-glu), malvidin-3-glucoside (Mv-3-glu) and malvidin-3-galactocoside (Mv-3-gal). Other chemicals used were 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-Azinobis-(3-ethylbenzo thiazoline-6-sulfonic acid ammonium salt) (ABTS), 2,4,6-Tripyridyl-s-triazine (TPTZ), hydrogen peroxide, potassium persulfate, ferrous sulfate, ferric chloride, EDTA, and methanol. All chemicals were purchased from Sigma–Aldrich or Fluka Chemical companies. FT-IR was Shimadzu (Affinity-1); scan range was 400–4000 cm^{-1}. ^1H NMR spectra were recorded on a Bruker Ultra Shield Plus instrument at 600 MHz at Aramco Company in Dammam, SA. The chemical shifts are expressed in (ppm) downfield from tetramethylsilane (TMS) as internal standard. Deuteriodimethylsulphoxide (DMSO-d_6) was used as a solvent. The prepared compounds were dried by Labconco freeze drier. UV–Vis spectra were recorded in methanolic solution of the analyzed compounds on a Thermo Fisher Scientific instrument model EVO 300LC Evolution 300. The scan covered the range 200–800 nm.

2.2. Evaluation of the radical scavenging activity

2.2.1. DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of compounds was measured according to Brand-Williams, Cuvelier, and Berset (1995) with some modifications. Methanolic DPPH solution was prepared at 0.1 mM (0.004%) concentration then stored at −20 °C. A compound (25 μl, 3 mM) or 25 μl methanol (as a control) was completed to 2.5 ml by DPPH solution (final concentration 30.0 μM). Absorbance (A) was measured at 517 nm at various intervals until a steady state was reached; methanolic solution of the compound served as blank. All experiments were done in duplicates. The inhibitory percentage of DPPH was calculated according to the following equations:

$$\%\text{DPPH radical scavenging activity} = \frac{A517(\text{control}) - A517(\text{Exp})}{A517(\text{control})} \times 100$$

where A517(Exp) and A517(control) are the absorbance of experiment and control respectively at the steady state condition.

A trolox standard curve of nine concentrations in the linear range of 1.2–6.0 mM was prepared, and the trolox equivalent (TE_{DPPH}), defined as mM trolox has the same activity of 1 mM compound, was calculated.

2.2.2. ABTS radical scavenging activity (TEAC method)

The method of Arnao, Cano, and Acosta (2001) with some modifications was adopted. Stock solutions of 7.4 mM ABTS in water and 2.6 mM potassium persulfate ($K_2S_2O_8$) were prepared. Before use, equal volumes of the two solutions were mixed and allowed to react in the dark for 12–16 h at room temperature to generate the ABTS radical cation. The solution was then diluted with methanol until an absorbance of 1.1 at 734 nm (diluted ~30-fold) was achieved. A compound (20 μL, 1.5 mM in methanol) or methanol (control) was diluted to 3.0 ml with the freshly prepared $ABTS^{+\cdot}$ solution (final concentration 10.0 μM) then the decrease in absorbance was recorded after 6 min in the dark at 734 nm against a blank of methanolic solution of the compound. All experiments were done in duplicates. A standard curve of trolox (1.0–6.5 mM) was prepared. Results are presented as trolox equivalent of antioxidant capacity (TE_{ABTS} or TEAC value). The % of $ABTS^{\cdot+}$ scavenging activity was calculated by the following equation:

$$\%\text{ of ABTS}^{\cdot+}\text{ scavenging activity} = \frac{A734(\text{control}) - A734(\text{Exp})}{A734(\text{control})} \times 100$$

where A734(Exp) and A734(control) are the absorbance of experiment and control respectively at the steady state.

2.2.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the compounds was measured based on the method of Halliwell, Gutteridge, and Arouma (1987), with a slight modification to match the high anthocyanin and anthocyanidin activities as follows: 200 μl deoxyribose solution (5.6 mM), 200 μl H_2O_2 (2.1 mM) and 200 μl of the compound (0.05 mM) or oxygen free water (control), were placed in a test tube. The Fenton reaction was initiated by the addition of 200 μl EDTA (0.2 mM) and 200 μL $FeSO_4$ solution (40 μM in 1 mM HCl); all solutions were oxygen free. The reaction mixture (1 ml) with a final tested compound concentration 10 μM was heated at 100 °C for 15 min then the reaction was stopped by addition of 1 ml 10% trichloroacetic acid (TCA). A one ml solution of 2% thiobarbituric acid (TBA) and 0.04% butylated hydroxyanisole (BHA) dissolved in NaOH (100 mM) was added. The mixture was heated at 100 °C for 15 min then cooled and the absorbance (A) was measured at 532 nm; oxygen free water containing the same

compound concentration served as blank. All experiments were done in triplicates. The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\%\text{OH radical scavenging activity} = \frac{\text{A532(control)} - \text{A517(Exp)}}{\text{A532(control)}} \times 100$$

where A532(Exp) and A532(control) are the absorbance of experiment and control respectively at the steady state condition.

A trolox standard curve in the linear range of 25–800 μM was prepared and the trolox equivalent (TE_{OH}) was calculated.

2.3. Reducing power determination by FRAP assay

The reducing power of the anthocyanidins and anthocyanins were performed by the ferric reducing ability of plasma (FRAP) assay as described by Benzie and Strain (1996) with some modifications. Three stock solutions (300 mM acetate buffer (3.1 g NaOAc $3H_2O$ and 16.0 ml HOAc in liter buffer solution, pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution) were prepared. A FRAP working solution was freshly prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution then warmed at 37 °C. The studied compounds (150 μl, 0.1 mM) were diluted to 3.0 ml with the FRAP solution (final concentration 5.0 μM). The increase in absorbance was measured at 593 nm after 4 min; the blank contained all reagents except using methanol instead of the assayed compound. All experiments were done in duplicates. %FRAP was expressed relative to the highest compound in reducing power according to the following equation:

$$\%\text{FRAP} = \frac{\text{A593(Exp)}}{\text{A593(Strongest reductant)}} \times 100$$

where A593(Exp) is the absorbance of the experiment at a steady state.

A standard curve of trolox was prepared in the linear range of 0.1–1.0 mM. The reducing power is presented as the FRAP value expressing the trolox equivalent, TE_{FRAP} (mM trolox/mM compound).

2.4. Synthesis of 4′-hydroxy-flavylium products

2.4.1. Synthesis of 2,4′-dihydroxy chalcone

To a solution of salicylaldehyde (8 mmol), ethanol (4 ml) and aqueous sodium hydroxide (10%, 4.0 ml), *p*-hydroxy acetophenone (8 mmol) was added. The solution was heated and refluxed for approximately 60 min then cooled in an ice-water bath. Concentrated hydrochloric acid was added until the product began to precipitate. The product was cooled for 24 h then filtered and washed with 5 ml of cold water. The crude product was recrystallized from a methanol–water solvent (45% yield). UV–Vis (MeOH): 260 and 320 nm. IR (pellet): $\bar{\nu}$O–H (3400 cm^{-1}), $\bar{\nu}$C=O (1680 cm^{-1}), $\bar{\nu}$C=C (1500, 1590 and 1640 cm^{-1}). ^1H NMR (DMSO-d_6): protons on C3′ and C5′ (6.64 ppm, d, *J* 8.1 Hz), C2 and C6 (7.37 ppm, d, *J* 8.1 Hz), C4 and C5 (6.36 ppm, m), C3 and C6 (7.17 ppm, m), Hα (6.69 ppm, d, *J* 9.0 Hz), Hβ (7.72 ppm, d, *J* 9.0 Hz), OH (10.2 ppm, s).

2.4.2. Synthesis of 4′-hydroxy flavylium ferric chlorides

To a solution of 4.0 mmol 2,4′-dihydroxy chalcones in a minimum amount of acetic acid (~1.0 ml), a concentrated hydrochloric acid (1.0 ml) was added. The resulting solution was heated in a hot water bath until a deep red color was formed (~30 min.) then cooled at room temperature. A solution of ferric chloride in acetic acid (16.2 g $FeCl_3$/100 ml glacial acetic acid) was added until no more precipitate was produced. The precipitate was filtered and washed with diethyl ether to remove the acetic acid. Purification was done by recrystallization from acetic acid to give 57% dark orange product. UV–Vis (MeOH): 295, 360 and 440 nm. IR (pellet): $\bar{\nu}$O–H 3400 cm^{-1}, $\bar{\nu}$=C–H 3010 cm^{-1}, $\bar{\nu}$C=C 1440, 1530 and 1580 cm^{-1}, $\bar{\nu}$C–O 1190 and 1340 cm^{-1}. ^1H NMR (DMSO-d_6): protons on C3′ and C5′ (7.20 ppm, d, *J* 9.0 Hz), C2 and C6 (7.78 ppm, d, *J* 9.0). C3 (8.82 ppm, d, *J* 9.0 Hz), C4 (9.37 ppm, d, *J* 9.0 Hz), ring A protons m (6.64, 6.80, 8.32 and 8.61 ppm).

2.4.3. Synthesis of 4′-hydroxy flavylium chlorides

A solution of equimolar (8 mmol) of salicylaldehyde and *p*-hydroxy acetophenone in distilled EtOH was cooled to 0 °C in conical flask (25 mL). Gaseous HCl (generated by addition of 98% H_2SO_4 on solid NaCl) was gently bubbled through the solution for 90 min. The mixture was kept at 4 °C for 5 days then filtered. More precipitate was collected after addition of diethyl ether. The precipitate was washed with diethyl ether and dried by freeze drier to give an 81% yield of dark orange product. UV–Vis (MeOH): 300 and 455 nm. IR (pellet): $\bar{\nu}$O–H 3350 cm^{-1}, $\bar{\nu}$=C–H 3010 cm^{-1}, $\bar{\nu}$C=C 1440, 1530 and 1580 cm^{-1}, $\bar{\nu}$C–O 1180 and 1340 cm^{-1}. ^1H NMR (DMSO-d_6): protons on C3′ and C5′ (7.20 ppm, d, *J* 9.0 Hz), C2 and C6 (8.20 ppm, d, *J* 9.0). C3 (8.61 ppm, d, *J* 9.0 Hz) C4 (9.27 ppm, d, *J* 9.0 Hz), ring A protons m (6.84, 8.56 ppm).

2.5. Statistical analysis

One-way ANOVA and linear regressions were performed using SPSS package (version 16). The ANOVA test was used to examine the significant difference among compounds' activity. The LSD method was used to discriminate among means at a 95% confidence level where significance level $\leqslant 0.05$ is considered significant. Linear regressions were assessed by the correlation coefficient (R^2), standard error of the estimate (SE), the number of data point (n), the least significant difference (p), and the 5%-confidence intervals (in parentheses).

3. Results and discussion

3.1. General

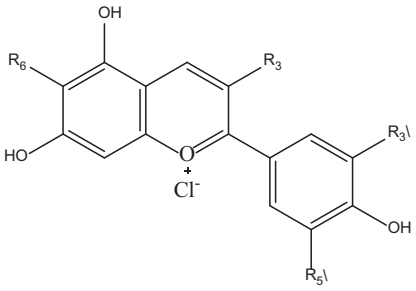
To examine the antioxidant activity of anthocyanins and their structure–activity relationship (SAR), a series of 15 anthocyanidins and anthocyanins (Table 1) along with the synthesized 4′-hydroxy flavylium compounds were examined as radical scavengers against DPPH, ABTS and hydroxyl radicals, besides their activity as reductants in the FRAP assay. Two commonly antioxidants known to have good antioxidant activities namely trolox, (as a monophenol), and catechol, (as *ortho* diphenol), were also inspected for comparison.

4′-Hydroxy flavylium chloride and ferric chloride were synthesized as outlined in Fig. 1 to examine separately the effects of the 4′-OH group on ring B, and 5,7-diOH groups on ring A, on activity; the three hydroxyl groups present in most common anthocyanidins.

3.2. Scavenging DPPH, ABTS and hydroxyl radicals

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical is a reactive hydrogen acceptor frequently used in determining the radical scavenging activity of various natural and synthetic compounds since it is a stable colored radical that can be obtained pure and used in known concentrations (Nishizawa, Kohno, Nishimura, Kitagawa, & Niwano, 2005). The ABTS assay depends on the oxidation of the colorless ABTS molecules by oxidizing agents, e.g., potassium persulfate to the bluish-green radical cation $ABTS^{\cdot+}$ which can abstract a hydrogen atom from good hydrogen atom donors to give the colorless $ABTSH^+$ radical cation (Lien, Ren, Bui, & Wang, 1999).

Table 1
Structures, maximum absorbance (λ_{max}), colors and common names of the studied anthocyanidin and anthocyanin chlorides.



Anthocyanidins & anthocyanins	R_3	R_6	$R_{3'}$	$R_{5'}$	λ_{max}	Color
Apigeninidin (Ap)	H	H	H	H	481	Orange
Pelargonidin (Pg)	OH	H	H	H	524	Orange-red
Cyanidin (Cn)	OH	H	OH	H	538	Bluish-red
Delphinidin (Dp)	OH	H	OH	OH	551	Bluish-red
Peonidin (Pn)	OH	H	OMe	H	538	Bluish-red
Petunidin (Pt) or myrtillidin	OH	H	OMe	OH	549	Bluish-red
Malvidin (Mv)	OH	H	OMe	OMe	547	Bluish-red
Quercetagetinidin (Qu)	OH	OH	OH	H	522	Orange-red
Callistephin (Pg-3-glu)[1]	Oglu	H	H	H	514	Orange
Kuromanin1 (Cn-3-glu)	Oglu	H	OH	H	527	Orange-red
Myrtillin (Dp-3-glu)	Oglu	H	OH	OH	541	Bluish-red
Pn-3-glu	Oglu	H	OMe	H	526	Orange-red
Pt-3-glu	Oglu	H	OMe	OH	540	Bluish-red
Oenin chloride (Mv-3-glu)	Oglu	H	OMe	OMe	539	Bluish-red
(Mv-3-gal)[1]	Ogal	H	OMe	OMe	540	Bluish-red

[1] Glu and gal stand for glucoside and galactoside respectively.

A hydroxyl radical differs from the other used free radicals in being one of the most reactive radical formed naturally in biological systems (Cheng, Ren, Li, Chang, & Chen, 2002). The radical scavenging activities and the reducing power of anthocyanidins and anthocyanins are shown in Table 2. Results are expressed in both, the percentage activity and trolox equivalent. The final concentrations of the assayed compounds in each test experiment (30.0, 10.0 and 10 μM in % DPPH, ABTS and OH assays respectively) were chosen so that the anthocyanidin and anthocyanin activities varied over most of the percentage scale (0–100%) for better differentiation among the compounds' activities and accurate correlation with their structures. Trolox equivalent, on the other hand, is independent of antioxidant concentration in the assay solution, and is thus better used to compare activities towards different radicals or oxidants.

Results presented in Table 2 show that most of the studied anthocyanidins and anthocyanins have higher activities than that of trolox, a strong antioxidant that bears the basic phenolic

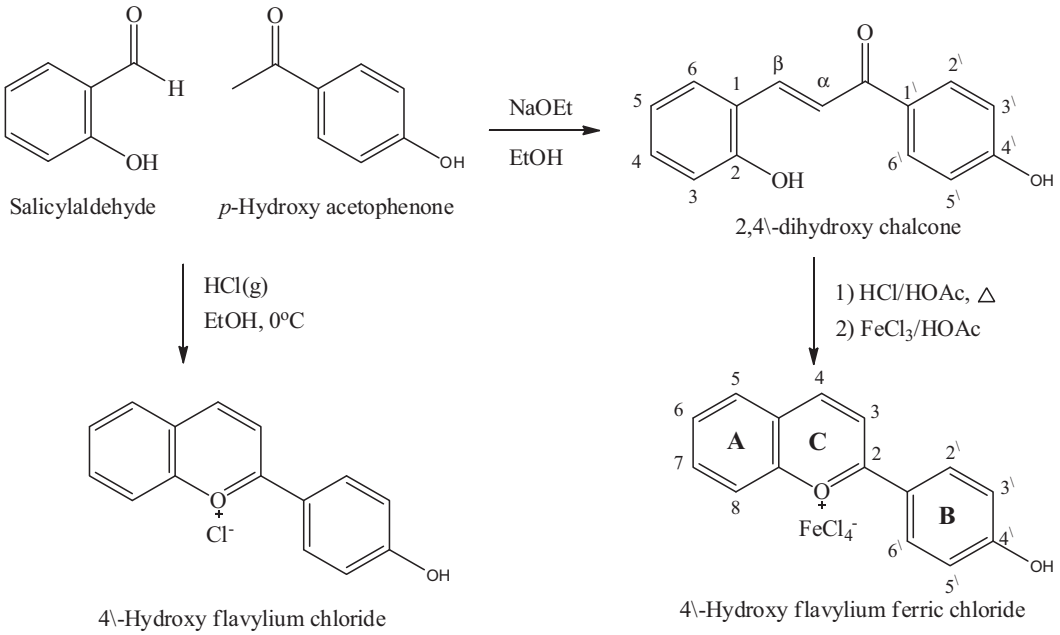


Fig. 1. Synthesis of 4′-hydroxy flavylium ferric chloride and chloride.

structure of vitamin E. The most active compounds towards all radicals were Qu, Dp, Pt and their anthocyanins, with TE_{DPPH}, TE_{ABTS} and TE_{OH} higher than 1.473 (Pt-3-glu), 3.583 (Pt) and 1.698 (Dp-3-glu) respectively, while the least active compounds against all radicals were 4′-hydroxy flavyliums, Ap, Pg and Pg-3-glu with TE_{DPPH}, TE_{ABTS} and TE_{OH} less than 0.835 (Pg-3-glu), 2.258 (Pg) and 0.819 (Pg-3-glu), respectively. Compounds with a trolox equivalent <1.0 in the hydroxyl radical assay were 4′-hydroxy flavyliums, Ap, Pg and Pg-3-glu, in the DPPH assay were the same compounds plus Pn-3-glu, and in the ABTS assay were only 4′-hydroxy flavyliums. Anthocyanidins and anthocyanins showed generally more activity, higher trolox equivalent, towards ABTS than DPPH and hydroxyl radicals.

3.3. Reducing power of anthocyanidins and anthocyanins

The method differs from the previous determinations in that it does not involve a hydrogen atom transfer reaction but rather determines the reducing power of the tested compounds; in other words, it measures the ability of the anthocyanidins and anthocyanins to donate an electron rather than a hydrogen atom. The method depends on the reduction of the colorless ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) by abstracting an electron from a reductant. The reducing power was expressed as percentage (%FRAP at 5.0 μM compound) relative to the most active compound, (Qu, 100%) and as trolox equivalent, TE_{FRAP} (Table 2). Results showed that all the used anthocyanidins and anthocyanins, except for Ap and monohydroxy flavyliums, have a good electron donating ability with $TE_{FRAP} > 1.0$. TE_{FRAP} values, for example, 5.90, 3.77 and 3.72 for Qu, Pt-3-glu and Dp-3-glu respectively. Compounds with a catechol or pyrogallol moiety on ring A or B gave the highest reducing capacity (%FRAP > 36.41, $TE_{FRAP} > 2.152$). These results are consistent with previously reported results where the TE_{FRAP} of anthocyanins ranged from 0.9 to 5.2, with the highest activities shown for those with *o*-diphenols (Jordheim, Aaby, Fossen, Skrede, & Andersen, 2007).

3.4. Structure activity relationship (SAR)

The scavenging activity against DPPH, ABTS and OH radicals and the reducing power (FRAP assay) presented in Table 2 and Fig. 2 suggest the following SAR features:

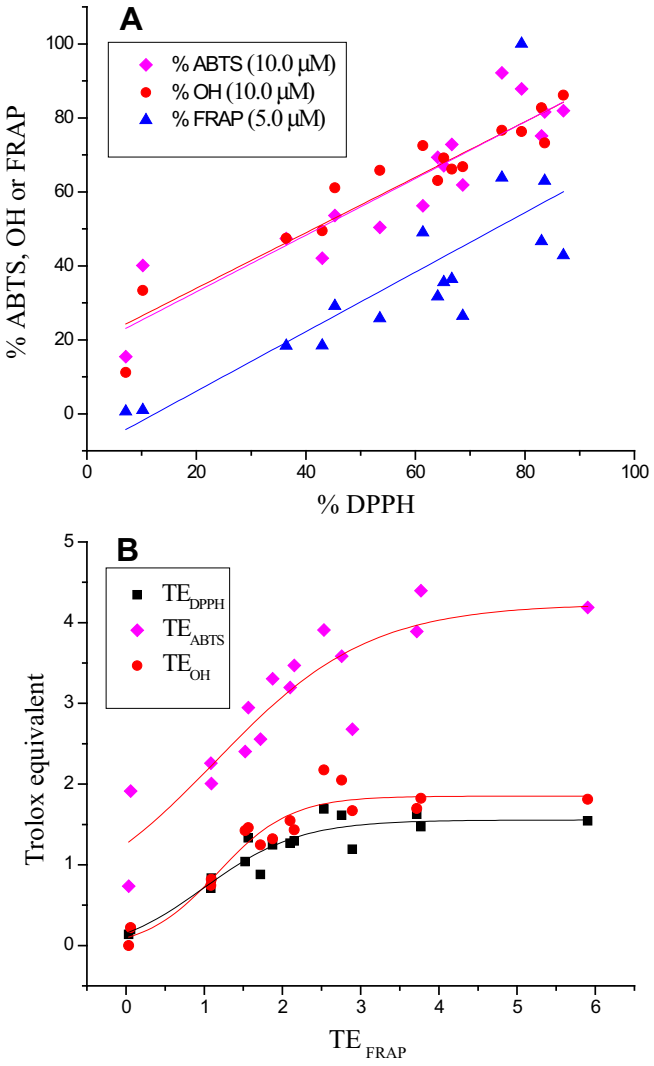


Fig. 2. Linear correlation of %DPPH with %ABTS, %OH and %FRAP (A), and correlation of TE_{FRAP} with TE_{DPPH}, TE_{ABTS} and TE_{OH} (B).

Table 2
Radical scavenging activities and reducing power of anthocyanidins and anthocyanins[3].

No	Compound	%DPPH (30.0 μM)[2]	TE_{DPPH}	%ABTS (10.0 μM)[2]	TE_{ABTS}	%OH (10.0 μM)[2]	TE_{OH}	%FRAP (5.0 μM)[2]	TE_{FRAP}
1	Ap	10.20(±0.29)^{a}	0.198	40.12(±1.36)^{a}	1.913	33.33(±1.20)	0.220	1.00(±0.04)^{a}	0.059
2	Pg	36.43(±1.55)	0.708	47.36(±1.61)^{b,c}	2.258	47.46(±1.28)^{a}	0.743	18.37(±0.57)^{b,h}	1.085
3	Cn	66.67(±2.45)^{b}	1.296	72.80(±2.99)^{d,e}	3.471	66.1(±2.38)^{b}	1.434	36.41(±1.39)^{c}	2.152
4	Dp	87.04(±3.69)^{c}	1.691	81.96(±3.94)^{f}	3.908	86.1(±1.89)	2.175	42.87(±1.88)	2.531
5	Pn	53.49(±2.27)^{d}	1.039	50.37(±1.71)^{b,j}	2.402	65.82(±1.45)^{b,c}	1.424	25.80(±0.80)^{d}	1.524
6	Pt	83.03(±3.05)^{c,e}	1.614	75.14(±3.08)^{d}	3.583	82.71(±2.23)	2.049	46.69(±2.05)^{e}	2.757
7	Mv	68.64(±2.91)^{b}	1.334	61.84(±2.54)^{g}	2.949	66.78(±2.40)^{b,d}	1.459	26.49(±1.01)^{d}	1.565
8	Qu	79.41(±2.25)^{e,f,g}	1.543	87.86(±4.22)^{h}	4.189	76.27(±2.06)^{e}	1.811	100.00(±4.38)	5.904
9	Pg-3-glu	42.99(±1.82)^{h}	0.835	42.06(±1.43)^{a,c}	2.006	49.49(1.78)^{a}	0.819	18.47(±0.57)^{b,h}	1.091
10	Cn-3-glu	61.38(±2.26)^{i}	1.193	56.23(±1.91)^{i}	2.681	72.5(±1.60)^{f}	1.671	49.00(±2.15)^{e}	2.893
11	Dp-3-glu	83.63(±3.55)^{c,f}	1.625	81.59(±3.92)^{f}	3.891	73.22(±1.98)^{f}	1.698	62.95(±2.76)^{f}	3.717
12	Pn-3-glu	45.31(±1.67)^{h}	0.881	53.6(±1.82)^{i,j}	2.556	61.06(±2.20)^{g}	1.347	29.12(±1.11)^{d,g}	1.719
13	Pt-3-glu	75.8(±3.22)^{g,j}	1.473	92.17(±4.43)^{h}	4.395	76.61(±2.07)^{e}	1.823	63.86(±2.80)^{f}	3.770
14	Mv-3-glu	64.07(±2.36)^{b,i}	1.245	69.34(±2.84)^{e,k}	3.306	63.05(±2.27)^{c,g}	1.321	31.73(±1.21)^{g}	1.873
15	Mv-3-gal	65.17(±2.40)^{b,i}	1.266	67.06(±2.75)^{g,k}	3.198	69.15(±1.52)^{d}	1.547	35.54(±1.56)^{c}	2.099
16	4-OHFvFeCl$_4$[1]	7.39(±0.21)^{a}	0.182	14.98(±0.51)^{l}	0.714	13.56(±0.49)^{h}	<0.001	0.40(±0.02)^{a}	0.024
17	4-OHFvCl	7.12(±0.20)^{a}	0.138	15.44(±0.52)^{l}	0.736	11.19(±0.40)^{h}	<0.001	0.60(±0.02)^{a}	0.036
18	Catechol	78.46(±2.88)^{e,j}	1.525	38.68(±1.31)^{a}	1.844	72.20(±1.95)^{f}	1.660	20.28(±0.63)^{b}	1.197
19	Trolox	53.63(±1.52)^{d}	1.042	21.83(±0.74)	1.041	57.63(±2.07)	1.120	16.06(±0.70)^{h}	0.949

[1] Fv stands for flavylium.
[2] Final concentration of the examined compound.
[3] Means (±SD) sharing the same letter in one column are significantly not different at significance level 0.05.

The 4′-hydroxy flavylium chloride (Table 2, entry 17) showed low scavenging activity towards all radicals (%DPPH 7.12, %ABTS 15.44, %OH 11.19) since the resulted phenoxyl radical can only be stabilized by a 4′-OH radical resonance through the pyrylium oxygen as presented in Fig. 3. Different counter ions (Cl^- and $FeCl_4^-$) showed insignificant effects on the flavylium ion activity in all tests.

In apigeninidin (Ap), the presence of the 5,7-dihydroxyl groups caused an insignificant difference in activity towards DPPH (%DPPH 10.20) but a significant increase in %ABTS (40.12) and %OH (33.33). Despite the more hydroxyl groups in Ap, its radical, upon dehydrogenation, can only give 5- or 7-OH radical resonance through the pyrylium oxygen similar to that of the 4′-hydroxyl group as depicted in Fig. 3. Apigeninidin showed different behavior towards different radicals with no activity towards hydroxyl and nitric oxide radicals, but a good activity towards lipids and ascorbyl radicals (Boveris et al., 2001). Chrysin has a similar structure to that of apigeninidin but with no 4′-OH group. Both anthocyanidins gave similar scavenging activities towards the ABTS radical cation (Rice-Evans, Miller, & Paganga, 1996) indicating similar stabilization effects of 4′-OH radical resonance and 5- or 7-OH radical resonance.

Pelargonidin (Pg) has a similar structure to Ap but with an extra hydroxyl group in position 3; it gave significantly higher activities against all radicals. The higher activity can be attributed to the 3-OH radical resonance between 3-OH and 5-, 7- or 4′-OH groups, and the formation of a stable diketonic product as illustrated in Fig. 3, which pushes the hydrogen transfer reaction forward. Radicals formed on 5-, 7- or 4′-hydroxyl groups can also be stabilized by the same resonance in the opposite direction. This resonance effect is similar to that reported for the catechol structure where stable semiquinone radicals are formed leading to stable diketones (Ali & Ali, 2015; Castañeda-Ovando et al., 2009). The presence of the 3-OH group conjugated with a 2–3 double bond was previously reported to enhance the antioxidant activity in other classes of flavonoids; this structural feature permits coplanarity of ring B with rings A and C, allowing extension of conjugation and electron delocalization leading to extra stability of the resulting radicals (Balasundram, Sundram, & Samman, 2006; Heim, Tagliaferro, & Bobilya, 2002).

Cyanidin (Cn), which has more hydroxyl groups in 3′ position than Pg, showed much higher scavenging activity (%DPPH 66.7%). The higher activity of Cn is due to its catechol structure that allows the well-known stabilization of a semiquinone radical and the formation of a stable quinone product (Ali et al., 2013; Bendary, Francis, Ali, Sarwat, & El Hady, 2013; Castañeda-Ovando et al., 2009) as illustrated in Fig. 3. Petunidin (Pt) showed more scavenging activity against all radicals (e.g., %DPPH 83.03) than that of Cn since it bears a catechol structure but one more OMe group. It can also be observed that the strongest reductants in the FRAP assay were Dp, Pt and Cn, and their anthocyanins plus Qu, indicating that a catechol moiety enhances not only radical scavenging activity but also the electron donating ability of anthocyanidins and anthocyanins as mentioned above.

Delphinidin (Dp) has more hydroxyl groups in the 5′ position than Cn (pyrogallol structure) on ring B, which increased scavenging activity towards all radicals (e.g., %DPPH 87.04). Quercetagetinidin (Qu) has a pyrogallol moiety on ring A and a catechol moiety on ring B. It gave also higher activity against the three radicals (e.g., %DPPH 79.41) than that of Cn.

According to the previous SAR analysis, peonidin (Pn) should give less activity (%DPPH 53.49) than Cn (%DPPH 66.67) since it has the same structure with methylation of the 3′-hydroxyl group, and thus lacks the catechol structure. On the other hand, it has a higher activity than that of pelargonidin because of the extra 3′-OH group; similar results were obtained by Kähkönen & Heinonen, 2003. As peonidin, malvidin (Mv) lacks a catechol structure but has more 5′-OMe groups; it gave a higher activity (%DPPH 68.64). The electronic effect of substituents has a large influence on both the activation energy and O–H bond dissociation energy of phenols (Foti, Daquino, Mackie, DiLabio, & Ingold, 2008). It was reported previously that methoxy and hydroxyl groups stabilize

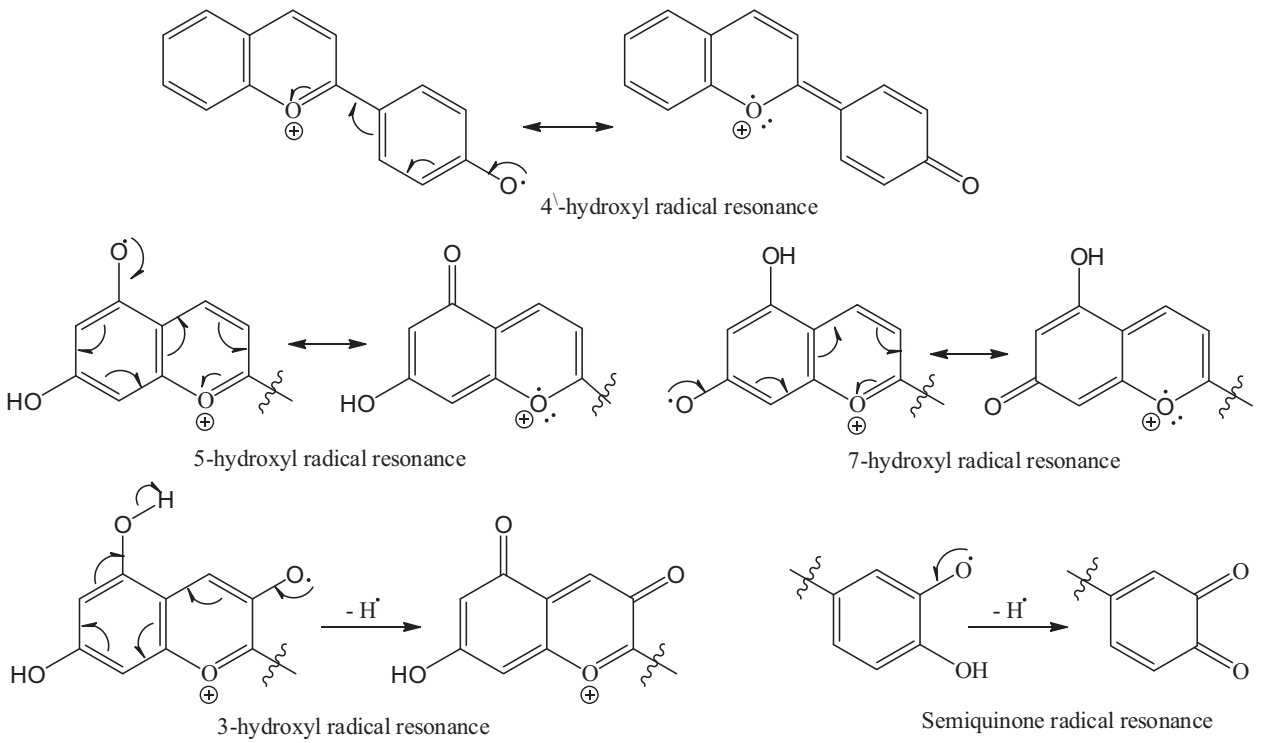


Fig. 3. Stabilization resonance of various anthocyanidin radicals.

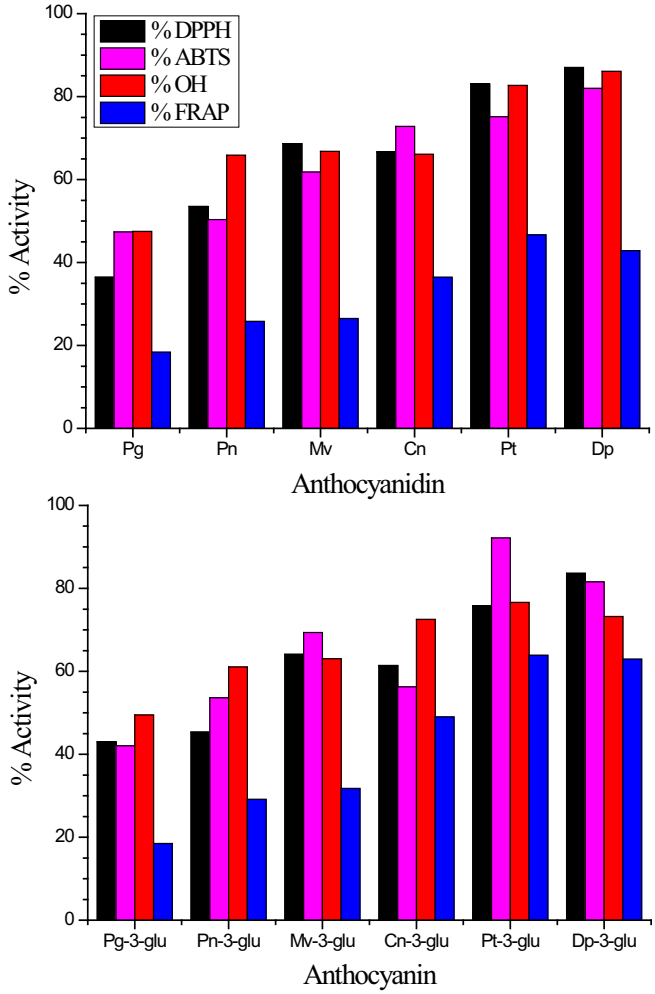


Fig. 4. Radical scavenging and reducing activities of some anthocyanidins and their respective anthocyanins.

the resulted radicals of phenolic compounds, leading to an increase in their antioxidant activity with a greater effect shown by the methoxy group than hydroxyl substituent (Ali & Ali, 2015; Balasundram et al., 2006; Heim et al., 2002). Therefore, comparing the activity of compounds lacking the catechol structure, we find the activity against the three radicals (DPPH, ABTS and OH) decreases in the order of Mv > Pn > Pg > Ap > 4-OH-flavylium respective to the presence of 6, 5, 4, 3 and 1 hydroxyl and/or methoxy groups; the difference is significant in most cases. The same order of activity (Mv > Pn > Pg) was previously found experimentally in the ORAC test (Wang et al., 1997) and predicted theoretically based on the calculation of O–H bond dissociation energies (Guzmán et al., 2009). In addition, the same order was found in the reducing power measured by the FRAP assay (Table 2) suggesting similar factors affecting both the reducing power and the radical scavenging activities of anthocyanidins and anthocyanins. It was observed previously that the reducing power increases with the number of hydroxyl and methoxy groups on ring B (Azevedo et al., 2010; Balasundram et al., 2006). It can also be observed that a compound which lacks a catechol structure, e.g., Mv with four hydroxyl and methoxy groups may have scavenging activities similar or even better (e.g., DPPH and OH assays) than that of a compound possessing a catechol structure, e.g., Cn with less stabilizing substituents.

The discussed SAR features can be applied on the results of scavenging the three studied radicals as well as the electron donating ability of anthocyanidins and anthocyanins, since linear correlations can be drawn between %DPPH and other assays as presented in Fig. 2A and regressions 1–3. Correlations show that the slope of the three regressions are very close (R^2 = 0.75–0.81) indicating not only that factors affecting scavenging the three radicals and reducing power are similar, but also their contributions have similar weights. High correlations (R^2 > 0.9) were recently reported between any two of DPPH, ABTS and FRAP results (Jiméneza et al., 2015). Besides, Fig. 2B shows that anthocyanidins and anthocyanins were more active towards an ABTS radical cation than OH followed by DPPH radicals.

$$\%\text{ABTS} = 17.65(\pm 5.57) + 0.77(\pm 0.09)\%\text{DPPH} \quad \text{(Reg.1)}$$

R^2 0.842, SE 8.393, n 16 (entries 1–16), F 74.40, p 0.000.

$$\%\text{OH} = 18.94(\pm 4.01) + 0.75(\pm 0.06)\%\text{DPPH} \quad \text{(Reg.2)}$$

R^2 0.908, SE 6.041, n 16 (entries 1–16), F 137.70, p 0.000

$$\%\text{FRAP} = -9.98(\pm 10.48) + 0.81(\pm 0.17)\%\text{DPPH} \quad \text{(Reg.3)}$$

R^2 0.623, SE 15.787, n 16 (entries 1–16), F 23.21, p 0.000.

Reg. (3) was improved when Qu, of exceptionally high reducing power, was eliminated to give Reg. (4).

$$\%\text{FRAP} = -6.04(\pm 6.14) + 0.68(\pm 0.10)\%\text{DPPH} \quad \text{(Reg.4)}$$

R^2 0.782, SE 9.176, n 15, F 46.55, p 0.000.

The anthocyanins (Table 2, entries 9–15) gave a similar trend to those of their respective anthocyanidins as presented in Fig. 4. Glycosylation of anthocyanidins may cause an increase or decrease in their activity. In the DPPH test for example, Cn, Pn, and Pt gave significantly higher activities while Pg gave a significantly lower activity than their respective anthocyanins; others, e.g., Dp and Mv showed insignificant difference. The diverse results could be attributed to the contradicted effects of the sugar unit. Jing et al. (2014) showed that the higher activity of 3-glucoside anthocyanins than their aglycons could be due to the electron donating effect of the 3-bulky sugar group. On the other hand, Kähkönen and Heinonen (2003) reported that diglucosidation of anthocyanidins at the 3 and 5 positions lowered the activity which is consistent with prohibition of stabilization by both 3 and 5-radical resonances outlined in Fig. 3. Zhao et al. (2014) have also demonstrated in their recent review that the glycosylation of anthocyanidins usually lessen their antioxidant activity but may also enhance activity depending on the anthocyanidin type and the experimental method.

4. Conclusion

It can be concluded from the present study that the widely distributed anthocyanidins and anthocyanins proved to be strong radical scavengers against a variety of free radicals, i.e., DPPH, ABTS and OH radicals where most of the anthocyanidins and anthocyanins showed more scavenging activity than that of the well-known strong antioxidants trolox and catechol. Anthocyanidins and anthocyanins scavenging activities were sensitive towards the number and position of the hydroxyl and methoxy groups. Compounds with a catechol structure in either ring A or B were generally better hydrogen atom donors because of the stabilization of the resulted radicals and the formation of stable quinone-like products; besides, they were much stronger electron donors. The presence of hydroxyl groups in positions 5, 7 and 4′ only has little effect on both hydrogen atom and electron donation activities, while the presence of a 3-OH group improved both of these activities. The importance of 3-OH group in hydrogen transfer reactions suggests a 3-radical resonance through 5 or 7-OH group forming an anthocyanin semiquinone radicals ending with a stable diketone, similar to that observed for the catechol structure. On the other

hand, anthocyanidin and anthocyanin activities showed a similar trend and were found not sensitive towards the type of counter anion or type of sugar bonded to 3-OH group.

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