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Veronica Moorman

Montserrat Rabago-Smith

Evan D. Kindl

Imani R. Williams

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Montserrat Rabago Smith,[a] Evan D. Kindl,[a] Imani R. Williams,[a,b] Veronica R. Moorman[a,c]

[a] Department of Chemistry, Biochemistry, Chemical Engineering, and Applied Biology, Kettering University, Flint, MI 48504, United States

[b] Present address: College of Veterinary Medicine, Tuskegee University, Tuskegee, AL, USA

[c] To whom correspondence should be addressed: vmoorman@kettering.edu

ABSTRACT: Flavonoids are antioxidants known to be abundant in edible plants. Seven 5,7,3',4'-tetrahydroxy substituted flavonoids representing each major flavonoid class were used as cytochrome *c* reductants to systematically investigate the redox role of their C-rings. Additional examples of flavonoids and benzenediols were investigated to better understand the role of the B-ring. Pseudo-first order rate constants (k_{obs}) and apparent bimolecular rate constants (k_{app}) values were calculated from spectroscopic measurements. Of the seven flavonoids tested, five yielded measurable observed reduction rate constants. Butein (a chalcone) had the highest apparent bimolecular rate constant (k_{app}), followed by taxifolin (a flavanonol), catechin (a flavanol), eriodictyol (a flavanone), and luteolin (a flavone). Quercetin (a flavonol) and cyanidin (an anthocyanidin), however, reduced cytochrome *c* but k_{app} rate constants were unable to be calculated. Neither this trend nor trends in observed rate constants correlated with flavonoid pK_a, solvent accessible surface area, polar surface area, reduction potential, antioxidant ability, resonance, or radical scavenging efficiency. Weak correlation, however, was found with degrees of freedom and the number of redox involved electrons. While some cytochrome *c* reduction rates have been reported, this study is the first to systematically investigate the role of the structure of the flavonoid C-ring across a full set of flavonoids with identical B-rings.

Keywords: cytochrome *c*; reduction; flavonoid; apparent bimolecular rate constant; uv/vis spectroscopy

1. Introduction

Flavonoids are ubiquitous polyphenolic pigments known to be present in a variety of plant sources. They are best known for their antioxidant activity,[1] which is based on their ability to transfer electrons with labile hydrogens. However, the specific mechanisms for their ability to confer antibacterial,[2] anti-cancer,[3] anti-cardiovascular distress,[4] antidiabetic,[5] anti-inflammatory,[6] antiviral,[7] and anti-neurodegenerative properties[8] remains largely elusive. As such, they have become of great interest over the past few decades, particularly in regards to their interactions with human proteins[9-12]

Chemically, these flavonoids have structures based on the generic C6-C3-C6 polyphenolic structure 2-phenylchromane. This structure consists of two phenolic rings (the A- and B-rings) and a six-membered heterocyclic ring (the C-ring). The A-ring is condensed to the C-ring, while the B-ring is a substituent on the 2-position of the C-ring. Differences based on the hydroxyl, ketone, and other substituents at the 3 or 4 position, as well as the incorporation of a double bond between carbons 2 and 3 of the C-ring, further subclassify these flavonoids into flavones (4: ketone, 2-3: alkene), flavonols (4: ketone, 2-3: alkene, 3: hydroxyl), flavanones (4: ketone), flavanonols (4: ketone, 3:hydroxyl), flavan-3-ols (3:hydroxyl), anthocyanidins (3-4:alkene, 1-2:alkene, 3:hydroxyl, 1:O^+), and the two-ringed chalcones (no C ring).[11] While there are over 20 million possible flavonoid chemicals based on these classes,[13] only about 4,000 are known to exist in nature,[14] many of which are based on 2-phenyl-5,7-chromanediol where the 5 and 7 carbons of ring A are hydroxylated. 3',4'-Hydroxy substituted 2-phenyl-5,7-chromanediols (also known as 5,7,3',4'-hydroxy substituted flavonoids), in particular, are abundant in edible plant sources[13, 15, 16] and an example from each flavonoid class was investigated as a part of the present study, as noted in Table 1, in regards to their ability to reduce the protein cytochrome *c*.

Cytochrome *c* is a small, highly conserved heme-containing protein located in the inner membrane space of mitochondria in eukaryotes and in the periplasm of prokaryotes. It is a necessary component of the electron transport system and is also involved in apoptotic pathways.[17] *In vivo*, the oxidation state of the iron in its heme prosthetic group (either ferric, Fe^{3+}, or ferrous, Fe^{2+}) is crucial to its specific functions. Since reduced cytochrome *c* has distinct absorption peaks at 550 and 520 nm not present in the oxidized form,[18] its reduction can be tracked *in vitro* spectroscopically. This paper reports mammalian cytochrome *c* reduction through observed pseudo-first order rate constants (k_{obs}) for multiple reductant concentrations, their apparent bimolecular rate constants (k_{app}), and explanations for their observed differences when comparing different flavonoids. Previous studies in this lab and others[19-23] have investigated rates of flavonoid-induced cytochrome *c* reduction, but none to date have investigated a full series of compounds containing identical A- and B-rings. This study conducted on a biologically-relevant series of flavonoids is therefore the first to systematically investigate the role of the C-ring structure on a flavonoid's ability to reduce cytochrome *c*.

2. Materials and methods

2.1. General Experimental Procedures.

Oxidized equine cytochrome *c* stocks were prepared in 0.30 M Tris (pH 7.8) at approximate 8 μM (as determined using an ε_{550} of 8.4 $mM^{-1}cm^{-1}$)[24] and stored at 4 °C for no more than 48 hours. Reductants were dissolved at 20.0 mM in pure dimethyl sulfoxide (DMSO) and later diluted to 2X the working concentration using 0.30 M Tris (pH 7.8) and DMSO for a 90:10 buffer:DMSO ratio. Individual experiments were initiated by adding into wells of a 96-well plate equal volumes of oxidized cytochrome *c* (7.98 ± 0.37 μM in 0.3 M Tris at pH 7.8) and reductant (2.00-2000 μM in 10% DMSO and 90% 0.3 M Tris at pH 7.8) and mixing without introducing air bubbles. Final reductant concentrations therefore varied from 1 μM to 1000 μM (above 50 μM for cases where k_{app} was determined), while the cytochrome *c* remained constant (4.0 μM). Reaction kinetics were followed for each reductant using at least

four different reductant concentrations, see below. Samples without reductant and/or cytochrome *c* were also plated for each run as controls and/or blanks. Each condition was tested in quadruplicate.

2.2. Spectroscopy.

All spectroscopy was performed using a SpectraMax M2 Multiplate Reader (Molecular Devices) set to 24.0 °C using UV-vis transparent polystyrene 96-well plates (Dot Scientific). For the absorbance methods described here, a tunable monochromator (1 nm increments) with a wavelength bandwidth of ≤ 4.0 nm, full width half maximum, wavelength accuracy ±2.0 nm across wavelength range and wavelength repeatability of ±0.2 nm was utilized. The instrument utilizes a xenon flash lamp light source and a silicon photodiode as a photodetector. Kinetic studies were monitored at 550 nm over 60 minutes (increments of 20 or 34 seconds). Spectra scanning wavelengths inclusive of 500 to 600 nm were also taken.

2.3. Statistical Analysis.

All quadruplicate data were averaged for either individual wavelength (spectra data) or time points (kinetics data). External referencing was undertaken by subtracting blank absorbance averages (of wells containing no cytochrome *c* or reductant) on an individual plate from all other absorbance averages. The total concentration of cytochrome *c* was calculated using an average across all times in cytochrome *c*-only wells, assuming near complete oxidation (as confirmed through spectra data), and an ε_{550} for oxidized cytochrome *c* of 8.4 $mM^{-1}cm^{-1}$ as reported by van Gelder and Slater[24]. This extinction coefficient value was also used in the following equation to determine the concentration of reduced cytochrome *c* for averages at each time point for kinetic data: $[\text{cytochrome } c]_{reduced} = A_{550} - 8.4*[\text{cytochrome } c]_{total}/21.1$, where 21.1 is the reduced-oxidized extinction coefficient also in mM^{-1}. Spectra were also used to confirm a lack of reduction-indicating absorbance peak at 550 nm for each reductant. K_{obs} values (the desired pseudo-first order rate constant) were calculated in Origin 7 (OriginLab, Northampton, MA) by plotting the above determined reduced cytochrome *c* concentrations over time and fitting the resulting plot to the equation f(x)= d*(1-exp(-b*x))+c, where b is the k_{obs}. K_{app} values (the desired bimolecular rate constant) were also calculated in Origin 7 by determining the slope of a best fit line to a plot of k_{obs} versus reductant concentration. Errors reported for these rate constants were calculated during the fitting process. Statistical analyses of the k_{app} fits were done using KaleidaGraph 4.5 (Synergy Software, Reading, PA).

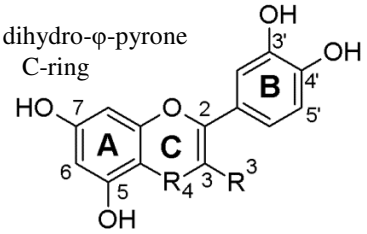
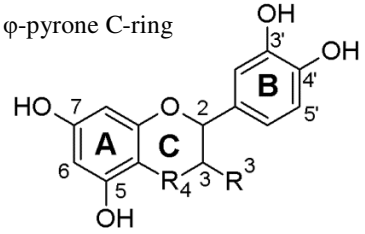
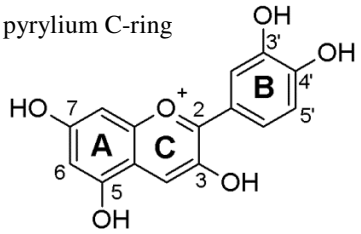
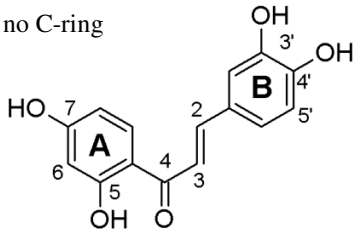
2.4. Materials.

The chemicals (-)-epicatechin (**14**), (+)-catechin (**3**), (-)-catechin gallate (**15**), quercetin (**2**), (±)-eriodictyol (**4**), resorcinol (**9**), dimethyl sulfoxide (DMSO) and equine heart cytochrome *c* were purchased from Sigma-Aldrich. Rutin (**16**), chrysin (**12**), 1,2-cyclohexanediol (**10**) and catechol (**8**) were purchased from Acros Organics. Butein (**7**), cyanidin (**6**), luteolin (**1**) and (±)-taxifolin (**5**) were purchased from Cayman Chemical Company. 6,7-Dihydroxyflavone (**13**) was purchased from ICC Chemical Company, while 5,7-dihydroxychromone **(11**) was purchased from APExBIO. Tris base (tris(hydroxymethyl)aminomethane) was purchased from Dot Scientific and titrated with hydrochloric acid purchased from Fisher to form 0.30 M Tris (pH 7.8) buffer. All compounds were purchased with at least 95 % purity and used without further purification

3. Results and Discussion

5,7,3',4'-Tetrahydroxy substituted flavonoids were chosen as the focus for this study because of their natural abundance, known antioxidant abilities, and promise as medicinally beneficial compounds[9,11]. As summary of their structure, pK_a, and biological availability are shown in Table 1.

Table 1. 5,7, 3',4',-Hydroxy Substituted Flavonoids Compared in Current Study.

2-(3'4'-dihydroxyphenyl-5,7-chromanediols		R^3	R_4	pK_a (C#) [a]	common plant sources
dihydro-φ-pyrone C-ring 	luteolin (**1**) *(flavone)*	H	C=O	6.57 (7)	celery, thyme, green peppers, chamomile[16]
	quercetin (**2**) *(flavonol)*	OH	C=O	6.38 (7)	broccoli, citrus, capers, apples, onions[16]
φ-pyrone C-ring 	(+)-catechin (**3**) *(flavanol)*	OH	CH_2	9.00 (3')	tea, cocoa, wine[16]
	(±)-eriodictyol (**4**) *(flavanone)*	H	C=O	7.85 (7)	citrus[16]
	(±)-taxifolin (**5**) *(flavanonol)*	OH	C=O	7.74 (7)	citrus and onions[25]
pyrylium C-ring 	cyanidin (**6**) *(anthocyanidin)*	H	CH_2	6.01 (3)	berries, apples, plums, red cabbage, red onion[16]
no C-ring 	butein (**7**) *(chalcone)*	H	C=O	7.11 (3')	cashews, legumes, coffee, peppers[26]

[a] Lowest pK_a theoretically calculated using ref. [27]

3.1. Reduction of cytochrome *c* is accomplished through ortho-substituted dihydroxy phenyl ring. 5,7, 3',4' substitution is not, however, the only tetrahydroxy substitution pattern known in tetrahydroxy substituted flavonoids. Flavonoids with diphenols as rings A and B often have either an ortho- (an adjacent 1,2) or a meta- (1,3) substitution pattern. 5,7,3',4'-Tetrahydroxy substituted compounds, for example, are ortho-substituted on the B ring (3',4'-) and meta-substituted on the A ring (5,7-). While other studies have indicated that it is the ortho substitution that is necessary for flavonoid redox ability (see for example references [28-30]), it was important to explicitly demonstrate the role of each diphenol ring in our present system.

Here, catechol (**8**) and resorcinol (**9**) were first studied to confirm the effects of isolated ortho- and meta- hydroxyl substituted phenols. The ortho substituted diphenol catechol was able to reduce cytochrome *c* when using 0.10-1.0 mM concentrations with a calculated apparent bimolecular rate constant (k_{app}) of 20.6 ± 3.51 x 10^{-4} μM^{-1}, and, for example, an observed pseudo-first order rate constant (k_{obs}) 0.11635 ± 0.0044 min^{-1} at 100 μM reductant concentration. In contrast, the meta-substituted diphenol

resorcinol showed no ability to reduce cytochrome *c* as shown by the calculated concentration of reduced cytochrome *c* after an hour to be within error of zero at any of the concentrations tested (0.10-1.0 mM). This difference in reducing ability is illustrated in Figure 1 through the comparison of absorption spectra where the characteristic 520 and 550 nm peaks is present after the addition of catechol (**8**) but not of resorcinol (**9**). (The para-hydroxy substituted phenol hydroquinone/quinol is also known to reduce cytochrome *c*,[31] but does not model a substitution pattern present in flavonoids and thus is not described here.) This finding is consistent with previous studies[30] and with the documented redox properties of these compounds, since compounds with low reduction potentials typically make good reductants[32] and the reduction potential of resorcinol (0.300 V) is known to be much higher compared to that of catechol (0.139 V).[33,34]

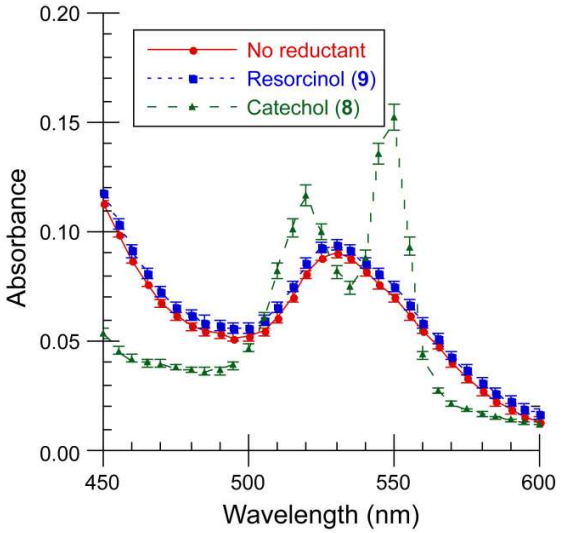


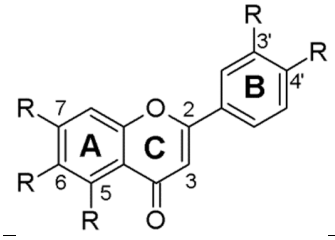
Figure 1. Spectra of 4 μM cytochrome *c* at pH 7.8 without reductant (red circle marker with solid line) and after 1 hour with 1.0 mM of resorcinol (**9**) (blue square with small dashed line) or catechol (**8**) (green triangle with long dashed line). Error bars indicate standard deviation.

To further evaluate the importance of the resonance found in both catechol and resorcinol, 1,2-cyclohexanediol (**10**) (which is a cyclohexane ring with a 1,2-dihydroxy substitution pattern) was also tested. Despite having the vicinal dihydroxyl groups of catechol (**8**), no reduction of cytochrome *c* was observed over a range of 0.1-1 mM 1,2-cyclohexanediol (**10**). Therefore, it appears as though it is the resonance between the hydroxyl groups that is essential for cytochrome *c* reduction in this system, which requires both the vicinal dihydroxyl groups and aromaticity of the base ring.

The importance of 1,2-dihydroxy substituted phenyl rings for this flavonoid system was next tested in a system of molecules all containing dihydroxychromones, with A-ring hydroxyl groups in either an ortho- or a meta- conformation. The compounds tested are all related to the flavone luteolin (**1**), whose C-ring contains a 2,3-double bond and a 4-keto, see Table 1. Results from these studies are tabulated in Table 2 and explained below. Because most flavonoids contain A-rings that are fused with an additional C-ring, it was important to establish that this structural difference does not change the lack of reducing ability of a meta-substituted ring, as demonstrated in the case of resorcinol (**9**). Similar to resorcinol (**9**), 5,7-dihydoxychromone (**11**) (which contains no B-ring nor ortho-substituted hydroxyls) resulted in no reduction of cytochrome *c* over the course of one hour for any concentration tested (up to 500 μM). Chrysin (**12**) is identical to 5,7-dihydoxychromone (**11**) only with the additional of unsubsituted phenyl ring at the 2-position of the C-ring and yet did appear to have a slight ability to reduce cytochrome *c*, although k_{obs} rates were more than an order of magnitude slower than any other reductant at the same

concentration, reduction did not go to completion over the time allotted, and the p-value for this k_{app} fit was 0.13 indicating that this reduction is not statistically significant. It is also of note that 6,7-dihydroxyflavone (**13**), which contains an ortho-substituted diphenol on ring A but no hydroxyls on its B ring, was also able to reduce cytochrome *c* (with a bimolecular apparent rate constant of 9.45 x $10^{-5} \pm 4.55 \times 10^{-5}$ min^{-1} μM^{-1} and an observed rate constant at 100 μM of 0.02671 ± 0.00107 min^{-1}). The observed rate constant (at 100 μM reductant concentration) for the ortho substituted 6,7-dihydroxyflavone (**13**) is therefore approximately 100 times faster compared to the meta substituted chrysin (**12**) indicating the preferred mechanism for reduction of cytochrome *c* occurs when diphenols are adjacent to each other[35]. Their k_{app} values (as shown in Table 2), however, are of the same order of magnitude, showing that their k_{obs} rate constants change as a function of reductant concentration at roughly equal rates. These values can also be compared with the reducing ability of luteolin (**1**) (a compound containing the base of chrysin (**12**) with ortho-substituted dihydroxyls on the B-ring), which easily reduced cytochrome *c* with an observed rate constant at 100 μM of 0.0328 ± 0.00205 min^{-1} and with an apparent bimolecular rate constant of $2.86 \times 10^{-4} \pm 2.52 \times 10^{-5}$ min^{-1} μM^{-1}. Both types of rate constants were higher than 6,7-dihydroxyflavone (**13**). In comparing the rate constants of these four compounds, it can be concluded that chromones and flavones with ortho-diphenols are more likely to reduce cytochrome *c* compared to the flavones with meta-diphenol substitutions, with the strongest reductions occurring as a result of B-ring substitutions.

Table 2. Related Chromones and Flavones' Reactions with Cytochrome *c* within 60 Minutes.



	substitution on ring A	B	k_{obs} (min^{-1}) at 100 μM [reductant]	k_{app} (min^{-1} μM^{-1} [reductant])
6,7-dihydroxyflavone (**13**)	6,7-dihydroxy (ortho)	no substitutions	0.02671 ± 0.00107	$9.45 \times 10^{-5} \pm 4.55 \times 10^{-5}$
5,7,3',4'-tetrahydroxy flavone (**1**) (Luteolin)	5,7-dihydroxy (meta)	3',4'-dihydroxy (ortho)	0.03283 ± 0.00205	$2.86 \times 10^{-4} \pm 2.52 \times 10^{-5}$
5,7-dihydroxy flavone (**12**) (Chrysin)	5,7-dihydroxy (meta)	no substitutions	None	None
5,7-dihydroxychromone (**11**)	5,7-dihydroxy (meta)	no ring present	None	None

3.2. 5,7,3',4'-Tetrahydroxyphenol examples of all flavonoids classes reduce cytochrome c with a range of rate constants.

The 5,7,3',4'-tetrahydroxy flavone luteolin (**1**) was also compared against other 5,7,3',4'-tetrahydroxy flavonoids, representing each of the other different flavonoid classes: quercetin (**2**) (a flavonol), (+)-catechin (**3**) (a flavan-3-ol), (±)-eriodictyol (**4**) (a flavanone), (±)-taxifolin (**5**) (a flavanonol), cyanidin (**6**) (an anthocyanidin) and butein (**7**) (a chalcone). For each compound, the observed pseudo-first order rate constants (k_{obs}) at various concentrations were calculated and were used to calculate the apparent bimolecular rate constant (k_{app}). These values were then compared against known physical values. The observed pseudo-first order rate constants (k_{obs}) at the same concentration (100 μM) were used to compare relative rates for cytochrome *c* reduction by different flavonoids, while the apparent bimolecular rate constants (k_{app}), which are independent of the reductant concentration, relate the effect that

flavonoid concentration has on changing the rate of the reaction. Both sets of values were tabulated in Table 3 and shown graphically in Figure 2.

Table 3. K_{app} and k_{obs} (at 100 μM Reductant Concentration) Rate Constants for the Reduction of Cytochrome *c* by Various 5,7,3',4'-Hydroxyphenols Reductants.

reductant	k_{obs} (min^{-1}) at 100 μM [reductant]	k_{app} (x 10^{-4} min^{-1} μM^{-1})
luteolin (**1**)	0.0328 ± 0.00205	2.86 ± 0.252
quercetin (**2**)	--- [a]	---
(+)-catechin (**3**)	0.127 ± 0.00340	7.07 ± 0.428
(±)-eriodictyol (**4**)	0.0687 ± 0.00200	4.75 ± 0.183
(±)-taxifolin (**5**)	0.0673 ± 0.00299	9.90 ± 0.536
cyanidin (**6**)	--- [b]	---
butein (**7**)	1.27 ± 0.155	112. ± 8.77

[a] At 100 μM, k_{obs} was unable to be fit. Reduction occurred prior to measurements. This was true for all concentrations attempted with an excess of reductant.
[b] At 100 μM, k_{obs} was able to be fit, but the intense color at this (and all concentrations) contributed to high error and therefore unusable data.

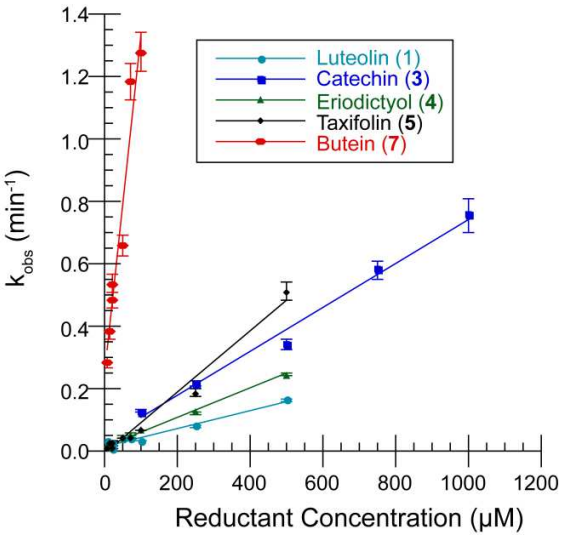


Figure 2. K_{obs} values for the reduction of cytochrome *c* by 5,7,3',4'-tetrahydroxyphenol flavonoids as a function of flavonoid concentration. Slopes indicate k_{app} values (min^{-1} μM^{-1}). Error bars indicate standard deviation.

These 5,7,3',4'-tetrahydroxy substituted examples from each flavonoid class were all capable of reducing cytochrome *c* and most of them had similar apparent bimolecular rate constants (k_{app}): between 2.86 and 9.90 x 10^{-4} min^{-1} μM^{-1}, with the exceptions of butein (**7**) and quercetin (**2**) (Table 3). Quercetin (**2**) and cyanidin (**6**) are flavonoid reductants which resulted in no quantitative data due to the fast rate of reduction (in the case of quercetin) and the intense pigmentation of the solution (in the case of cyanidin) even as the reductant concentration was reduced and approached the cytochrome *c* concentration. Butein (**7**), therefore, had the highest calculated apparent bimolecular rate constant (k_{app}), followed by taxifolin(**5**), catechin (**3**), eriodictyol (**4**), and finally luteolin (**1**). On the other hand, the calculated ob-

served rate constants (k_{obs}) at 100 μM of reductant followed a different order: luteolin (**1**) < taxifolin (**5**) < eriodictyol (**4**) < catechin (**3**) < butein (**7**), where cyanidin (**6**) and quercetin (**2**) would presumably have had the largest k_{obs} values had they been able to be measured[19] (Table 3).

While butein (**7**) (the chalcone) had the largest observed rate constant at 100 μM (i.e., it reduced cytochrome *c* the fastest under those conditions) as well as the largest apparent bimolecular rate constant, there appeared to be no other correlations between the two values. In fact, eriodictyol (**4**) and taxifolin (**5**) have similar observed rate constants values (k_{obs}) but almost a two-fold difference in apparent bimolecular rate constants (k_{app}), while butein (**7**) and luteolin (**1**) have similar k_{app} values but k_{obs} values (at 100 μM) with an approximately 10-fold difference. This lack of correlation is not entirely unexpected, as k_{app} values are calculated as the slope of the best-fit line to k_{obs} values over reductant concentration, see Figure 2. It is, however, important to note this since k_{app} values are often equated with reductant ability, yet their ability to reduce at a particular concentration can differ, particularly at lower reductant concentrations. Observed rate constants at higher reductant concentrations may have a better correlation with k_{app} rate constants, but rate constants were unable to be determined at high concentrations for all flavonoids, due to fast reductions and opacity when using certain flavonoids.

In addition to showing similar apparent bimolecular rate constants (ranging from 2.86 to 9.90 x 10^{-4} min^{-1} μM^{-1}), the flavonoids luteolin (**1**), eriodictyol (**4**), taxifolin (**5**) each showed k_{app} fitting intercepts of approximately zero. While the favanol catechin (**3**) visually appears to indicate a non-zero y-intercept, this variable's fitting p-vale was 0.25, well above the 0.05 necessary to reject the null hypothesis of this intercept being equivalent to zero. These results are consistent with a simple bimolecular reaction, between the flavonoid and cytochrome *c*. On the contrary, the chalcone butein (**7**) has a distinct and statistically significant intercept above zero on the y-axis, which suggests a more complicated mechanism when butein is used as a reductant. Interestingly, butein (**7**) is the only reductant tested that has free rotation between ring A and B, and thus it is possible that the large freedom of rotation contributes to this alternate mechanism.

3.3 Cytochrome *c* reduction rate constants do not correlate well with calculated flavonoid properties. As described in Table 3, butein (**7**) (a chalcone with no C-ring), reduced cytochrome *c* with a much higher apparent rate constant (112 x 10^{-4} ± 8.77 x 10^{-4} min^{-1} μM^{-1}) than the other tested compounds. It is hypothesized that this is due to an increase in degrees of freedom at the molecule when compared to other flavonoids. Butein (**7**) is the only flavonoid that has free rotation between rings A and C and its apparent bimolecular rate constant is approximately 11 times higher than the next flavonoid, taxifolin (**5**), which in turn is only four times higher than the lowest flavonoid tested. These non-chalcones contain an ether bridge between ring A and ring B (to form ring C) which restricts movements between ring A and ring B, thus locking the relative positions of ring A and ring B. This restriction appears to decrease both the apparent bimolecular rate and the observed rate constant. Increasing the degrees of freedom in a molecule like butein (**7**), however, could allow for more optimal binding and positioning between cytochrome *c* and the reductant therefore affecting both the apparent bimolecular rate as well as the observed rate constant. Additionally, butein's (**7**) α,β-unsaturated carbonyl chain that joins ring A and B shows full conjugation between the A and B rings (as illustrated in Figure 3), despite the lack of a C-ring. Dziedzic and Hudson have previously noted that butein's antioxidant ability in oil depends on the formation of a resonance stabilized free radical structure,[36] and this structure is likely also formed in the largely aqueous environment studied here.

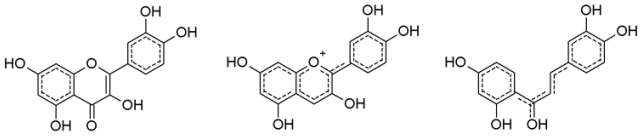


Figure 3. Chemical structures of quercetin (**2**), cyanidin (**6**), and butein (**7**). The resonance of these structures is emphasized through the visualization of the dotted lines.

Chalcones, however, are not the only flavonoid class with a particularly unique structure. The flavonoid cyanidin (**6**) and the anthocyanidin class it represents, are also substantially different from the other flavonoids. Specifically, cyanidin (**6**) is a flavylium salt with an oxycation in its center C-ring with full conjugation between rings A and B (see Figure 3). While not able to be quantified here, its rate of cytochrome *c* reduction is known to be fast.[19] Quercetin (**2**), however, was shown here to be faster than all of the other tested reductants, despite it having resonance of the A and C-rings individually but not across them like the butein (**7**) and cyanidin (**6**).

It has been suggested that cytochrome *c* binds to other compounds via ionic interactions of lysine and histidine residues,[37-39] and thus pK_as of the investigated flavonoids were expected to have a strong correlation with cytochrome *c* reduction ability. To evaluate the effect caused by a reductant's charge, compounds were grouped based upon the charges of each ring at the pH studied (7.8): phenols (neutral reductants) and phenolates (negatively charged reductants). As noted above, ortho-diphenols on the B-ring are most important for reduction. The only compound with a deprotonated ortho- phenolate is butein (**7**) (pK_a 7.11). The rest, cyanidin (**6**) (pK_a 8.39), quercetin (**2**) (pK_a 8.63), luteolin (**1**) (pK_a 8.82), catechin (**3**) (pK_a 9.00), taxifolin (**5**) (pK_a 9.61) and eriodictyol (**4**) (pK_a 9.68), are all protonated on their B-ring at pH 7.8. Butein (**7**) (a phenolate) and quercetin (**2**) (a phenol) both have much larger apparent bimolecular rate constants and observed rate constant compared to the other 5,7,3',4'-tetrahydroxyphenols studied (see Table 4). This suggests that protonation of the diphenols in ring B does not correlate to the apparent bimolecular rate constants or observed rate constant values observed. Additionally, the charge on the meta-substituted ring A has no correlation with apparent bimolecular rate constant or observed rate constant trend. Specifically, the A-ring phenolates show k_{app} values between 2.86 and 9.9 x 10^{-4} min^{-1} μM^{-1} while the A-ring phenols show k_{app} values between 4.75 and 112 x 10^{-4} min^{-1} μM^{-1}. Therefore, pK_a values and consequently ionization of 5,7,3',4'-tetrahydroxyphenols in either ring A or ring B does not appear to correlate to the measured apparent bimolecular rate constant or observed rate constant. Neither total solvent accessible surface area nor topological polar surface area correlates well with k_{app} rate constants either.

Table 4. Quantitative Values Calculated for the Flavonoid Reductants used in this Study.

reductant	pK_as (C#)[27] [a]			solvent accessible surface area ($Å^2$)[40]	topological polar surface area ($Å^2$)[27]	resonance across molecule
	A-ring	B-ring	C-ring			
luteolin (**1**)	6.57 (7) 8.07 (5)	8.82 (4') 12.16(3')	---	463.40	107.22	no
quercetin (**2**)	6.38 (7) 7.85 (5)	8.63 (4') 12.82(3')	10.29 (3)	474.90	127.45	no
(+)-catechin (**3**)	9.62 (5) 14.09 (7)	9.00 (3') 10.80(4')	12.85 (3)	476.78	110.38	no
(±)-eriodictyol (**4**)	7.85 (7) 9.05 (5)	9.68 (4') 12.68(3')	---	471.39	107.22	no

(±)-taxifolin (**5**)	7.74 (7) 9.00 (5)	9.61 (4') 12.16(3')	---	479.74	127.45	no
cyanidin (**6**)	6.81 (5) 7.72 (7)	8.39 (4') 11.75(3')	6.01 (3)	474.85	107.22	yes
butein (**7**)	9.19 (7) 12.46 (5)	7.11 (3') 8.56 (4')	N/A	477.10	97.99	yes

[a] Underlined values indicate at least 10% charged species at the pH tested, as determined by pK_a.

3.4. Cytochrome c reduction rate constants do not correlate well with experimentally determined redox values.

It is known that 5,7,3',4'-tetrahydroxyphenols can donate up to two electrons when reducing two equivalents of the iron in ferrylmyoglobin (MnFe),[34] yet when 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to measure radical scavenging efficiency, quercetin (**2**) was found to be a four electron donor, while luteolin (**1**), catechin (**3**), eriodictyol (**4**), and taxifolin (**5**) acted as two electron donors[15] (Table 5). This discrepancy can help to explain quercetin's (**2**) extreme ability to reduce cytochrome *c* much faster than the other tested 5,7,3',4'-tetrahydroxyphenic reductants.

Trolox equivalence antioxidant capacity (TEAC) is often used to report radical scavenging efficiency for flavonoids and other compounds.[41] As noted in Table 5, quercetin (**2**) and cyanidin (**6**) are known to have the highest TEAC values (4.7 mM and 4.4 mM), which are twice as high as catechin (**3**), butein (**7**), luteolin (**1**), eriodictyol (**4**) and taxifolin (**5**) (2.4 mM, 2.4 mM, 2.1 mM, 1.8 mM and 1.9 mM, respectively).[28] On the other hand, while quercetin (**2**) reduced cytochrome *c* so fast in this study that k_{obs} and therefore k_{app} rate constants were unable to be fit and cyanidin (**6**) is known to reduce cytochrome c rather quickly, butein (**7**) had a similar TEAC value (2.4 mM) to the other flavonoids despite its much greater k_{app}. Vitamin C equivalent antioxidant capacity (VCEAC) is another methodology used to report radical scavenging efficiency. Vitamin C equivalents do not follow the same trend as trolox equivalents, nor do they follow the trend of k_{app} cytochrome c reduction rate constants determined here. Based on these results, neither DPPH reduction ability nor TEAC or VCEAC values were predictive for k_{app} bimolecular rate constants for cytochrome c reduction, as visualized in Figure 4.

Table 5. Quantitative Values for Redox Ability of Reductants Used in the Present Study as Reported in the Literature.

reductant	E/V	e⁻ transfer *n* MnFe[34]	DPPH[15]	DPPH reduction k (x 10^3 $M^{-1}s^{-1}$)[15]	TEAC (mM)	VCEAC (mg/L)[42]
luteolin (**1**)	0.41[34 a]	2	2	7.74	2.1[29]	178.3
quercetin (**2**)	0.29[34 a]	2	4	6.43	4.7[29]	229.4
(+)-catechin (**3**)	0.36[34 a]	2	2	1.54	2.4[29]	215.7
(±)-eriodictyol (**4**)	0.36[34 a]	2	2	1.30	1.8[29]	[d]
(±)-taxifolin (**5**)	0.37[34 a]	2	2	1.24	1.9[29]	213.5
cyanidin (**6**)	0.36[43 b]	[d]	[d]	[d]	4.4[29]	240.0
butein (**7**)	0.37[44 c]	[d]	[d]	[d]	2.4[45]	119.5

[a] Versus normal hydrogen electrode (NHE) at pH 7.4

[b] Determined using Ag/AgCl reference in methanol and converted to be comparable to versus NHE[43].

[c] Determined using Saturated Calomel Electrode (SCE) reference at pH 7.5 and converted to be comparable to versus NHE.[46]

[d] Value not known.

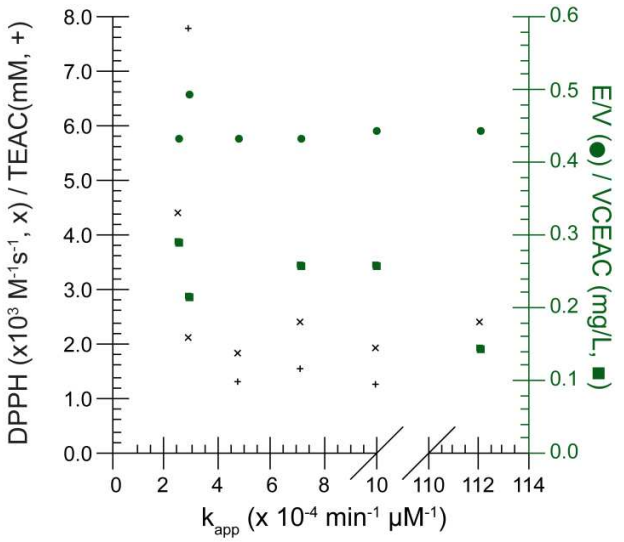


Figure 4. Relationship between k_{app} values as calculated here and reported redox values. DPPH (x) and TEAC (+) are both shown on the left y-axis in black while E/V (●) and VCEAC (■) are shown on the right axis in green.

Studies by other research teams[21, 23] have found some correlation between cytochrome *c*'s apparent bimolecular reduction rate constants (k_{app}) and reduction potentials of flavonoids and other antioxidants. These previous studies, however, they noted a loose overall logarithmic trend with outliers or multiple groupings. Much of these variations were attributed to differing methods for redox potential determination. The present study, therefore, used only three sources for redox potential values and converted them to be comparable. The 5,7,3',4'-hydroxyphenols tested have known redox potentials in the range of 0.29-0.41 V[34, 43-44] as indicated in Table 5. Luteolin (**1**) has the highest reduction potentials of the group (0.41 V versus NHE) and does indeed show the lowest k_{app} value, while quercetin (**2**), with the lowest reduction potential (0.29 V), was too fast to measure using the methodology of the present study. All of the other compounds have similar reduction potentials (0.36-0.37 V versus NHE), despite their differing k_{app} values. Redox potentials therefore correlate only somewhat with k_{app} values for cytochrome *c* reduction calculated here. Additionally, these redox potentials also do not correlate well to the observed rate constants at any concentration tested. Interestingly, cytochrome *c* reduction potential (0.25 V) is lower than any of the 5,7,3',4'-hydroxyphenols tested, which would predict no reaction with any of them. However, reduction occurred even with cyanidin (**6**), a compound that has a reduction potential approximately two times higher compared to the cytochrome *c*. This discrepancy could be attributed to changes in reduction potential of either the cytochrome *c* or the 5,7,3',4'-hydroxyphenol flavonoids after binding[47].

3.5. Stereochemistry of flavonoids does not seem to play a role in cytochrome c reduction ability. Three of the 5,7,3',4'-hydroxyphenols used in the present study (eriodictyol (**4**), taxifolin (**5**), and catechin (**3**)) contain no double bond at the 2-3-carbon position of the C-ring, indicating the possibility of multiple stereochemical isomers. Two of these, eriodictyol (**4**) and taxifolin (**5**), were used as racemic mixtures (±), while the (+)-stereoisomer of catechin (**3**) was used. In order to investigate whether stereochemistry affects reduction rates and rate constants, (-)-epicatechin (**14**) was also to compared with (+)-catechin (**3**). (+)-Catechin (**3**) and (-)-epicatechin (**14**) are in fact epimers (Figure 5). (-)-Epicatechin's 3C hydroxyl and 2C diphenol are located on the same side of the 2-3 C bond (*cis*), compared to (+)-catechin's (**3**) substituents which are located opposite each other (*trans*). The apparent

bimolecular rate constants values for (+)-catechin (**3**) and (-)-epicatechin (**14**) are 7.07 ± 0.428 and 7.71 ± 0.466 x 10^{-4} min^{-1} μM^{-1}, respectively, while the observed rate constant for catechin (**3**) and epicatechin (**14**) at 100 μM are 0.1269 ± 0.0034 and 0.14652 ± 0.00588 min^{-1}, respectively. Thus it appears that the relative location for substituents at positions 3C and 2C do not significantly affects the apparent bimolecular rate constant or the observed rate constant values. Therefore, it is possible that the binding of flavonoids to cytochrome *c* is flexible and could accommodate various chiralities at positions 2C and 3C. These data also indicates that it was appropriate to have used racemic mixtures instead of pure compounds when pure compounds were not readily available. Antioxidant activity assays (including DPPH assays) have been reported for both catechin (**3**) and epicatechin (**14**), which indicates that their ability to act as antioxidants is similar as well.[48] Interestingly, the electron reduction potentials for epicatechin (**14**) and catechin (**3**) are slightly different, 0.33 V and 0.36 V respectively,[34] despite their similar k_{app} and k_{obs} values. This reduction potential difference is generally attributed to the proximity of the hydroxyl to the diphenol,[49] slightly increasing its ability to self-oxidize possibly by long range stabilization of the radical formed,[34] but it does not seem to affect cytochrome *c* reduction.

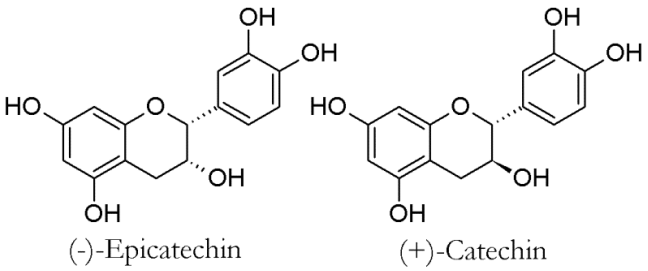


Figure 5. Structures of the epimers (-)-epicatechin (**14**) and (+)-catechin (**3**).

3.6. Common biological modifications of flavonoids at the C3 position lessen their ability to reduce cytochrome c.

Common flavonoid modifications can occur either within their original plant sources or after ingestion in humans and include glycosylation[50], methylation, esterification[51], sulfation[52], and polymerization[53]. These modifications can have a substantial effect of bioavailability in humans, yet studies show conflicting details. For example, *in vivo* studies in human suggest that some cells favor the uptake of glycosidic flavonoids,[54] yet *in vitro* studies using human cells reported that uptake only occurs for non-glycosylated flavonoids[55]. Because of these conflicting reports, direct protein interactions *in vitro* were considered instead of bioavailability. Specifically, two modified flavonoids were tested to compare rate constants with their unmodified versions: (+)-catechin gallate (**15**) (3C-gallated (+)-catechin) amd rutin (**16**) (3C-glycolsylated luteolin) (see Table 6).

Table 6. Rate Constants of Modified Flavonoids and their Unmodified Counterparts.

reductant	3C group	k_{app} (min^{-1} μM^{-1})
rutin (**16**)	O-rutinose	4.29 ± 0.64 x 10^{-4}
quercetin (**2**)	OH	---
luteolin (**1**)	H	2.86 ± 0.25 x 10^{-4}
(+)-catechin gallate (**15**)	O-gallate	4.33 ± 0.50 x 10^{-3}
(+)-catechin (**3**)	OH	7.07 ± 0.43 x 10^{-4}

Rutin (**16**) is formed from glycosylation of quercetin (**2**) with rutinose at the C-ring's 3C hydroxyl. This glycsosilation increases the flavonoid's solubility in water, introduces steric hindrance, and removes the hydrogen from the highly oxidizable hydroxyl at 3C, which in turns decreases its capacity for self-oxidation[29, 56]. While the reduction of cytochrome *c* using quercetin (**2**) was too fast to fit for k_{obs} at all reductant concentrations where quercetin (**2**) was in excess, values were able to be determined using rutin (**16**) as the reductant, to give a k_{app} value of 4.29 x 10^{-4} min^{-1} μM^{-1} (see Table 6). Therefore, compared to quercetin (**2**), rutin (**16**) has a much slower reduction of cytochrome *c* (as it was able to be quantified). This is consistent with other studies that have shown negative effects on cytochrome *c* reduction rates using glycosylated 3C-hydroxyl flavonoids and the reduction potentials for quercetin (**2**) and rutin (**16**), 0.29 V and 0.40 V respectively[34]. Additionally, increasing the steric hindrance could interfere with proper binding with cytochrome *c*. Furthermore, the absence of the hydroxyl at C3 eliminates the ability of rutin (**16**) to stabilize a radical intermediate formed during the electron transfer,[57, 58] much like luteolin (**1**) which contains the same base structure with a hydrogen at the C3 position. Despite similar reduction potentials for luteolin (**1**) and rutin (**16**) (0.41 V and 0.40 V respectively[34]) and the steric hindrance of rutin (**16**), rutin (**16**) shows an increased bimolecular apparent rate constant that is 1.5 times when compared with the comparable flavonoid with no 3C oxygen (luteolin (**1**)). This may be attributed to the presence of the oxygen linkage at 3C in rutin (**16**), where luteolin (**1**) has no oxygen at 3C at all. Interestingly the rate constant for the reduction of a superoxide radical by rutin (**16**) and quercetin (**2**) at pH 10 was not affected by glycosylation at C3 when studied by another research group.[59]

(+)-Catechin gallate (**15**) was also used as a cytochrome *c* reductant in this study and is chemically derived from the esterification of catechin (**3**) with a gallate moiety which in characterized by three adjacent hydroxyl groups on a phenyl ring. This esterification of 3C on the C-ring (sp^3) of catechin (**3**) increased the observed rate constant at 100 μM of reductant by approximately two-fold. Furthermore, as tabulated in Table 6, the bimolecular apparent rate constant increased by approximately six times (catechin gallate (**15**) versus catechin (**3**)). This trend has been reported previously[20] and is the result of the ability of the gallate moiety to reduce cytochrome c.

4. Conclusions

In this study, 3',4'-hydroxy substituted 2-phenyl-5,7-chromanediols representing each of the major flavonoid classes (flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanidins, and chalcones) were tested as reducing agents for cytochrome *c*. All were able to elicit reduction, but at varying rates. Quercetin (**2**) (a flavonol) reduced cytochrome *c* too quickly to be quantified in the present study and the pigmentaion of cyanidin (**6**) (an anthocyanidin) obscured results, but apparent bimolecular rate constants (k_{app}) values were calculated for each of the other examples: butein (**7**) (a chalcone) > taxifolin (**5**) (a flavanonol) > catechin (**3**) (a flavanol) > eriodictyol (**4**) (a flavanone) > luteolin (**1**) (a flavone). There was no strong correlation between these k_{app} values and flavonoid pK_a, solvent accessible surface area, polar surface area, reduction potential, antioxidant ability, resonance, or radical scavenging efficiency, yet degrees of freedom and the number of likely electrons transferred seemed to play a role. Additional compounds will need to be tested to more fully understand the exact role of flavonoid structure in cytochrome *c* reduction ability, but this systematic investigation has made it clear that multiple factors contribute. This study was able, however, to confirm the importance of the ortho-dihydroxy substituted phenyl B-ring.

AUTHOR INFORMATION

Corresponding Author

* E-mail: vmoorman@kettering.edu (V. R. Moorman)

Present Addresses

† Present address: College of Veterinary Medicine, Tuskegee University, Tuskegee, AL, USA

ORCID:

Veronica R. Moorman: 0000-0001-7127-1631

Author Notes

The authors declare no competing financial interest.

Contributions

MRS and VRM designed the study. EDK and IRW conducted the experiments with supervision from MRS. MRS, EDK, and VRM analyzed the data. MRS and VRM wrote the manuscript.

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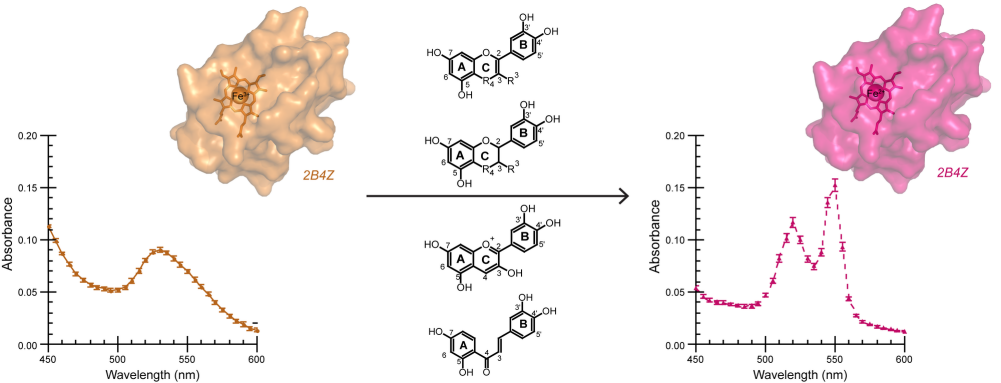
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