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Flavone: The Molecular and Mechanistic Study of How a Simple Flavonoid Protects DNA from
Oxidative Damage

A thesis
presented to
the faculty of the Department of Biochemistry and Molecular Biology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biomedical Science

by
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December 2003

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ABSTRACT

Flavone: The Molecular and Mechanistic Study of How a Simple Flavonoid Protects DNA from
Oxidative Damage

by

Jennifer D. Dean

Dietary flavonoids are ubiquitous and are marketed as supplements. Characterized as antioxidants, they offer protection against a number of degenerative diseases. Flavonoid mechanics involve free radical scavenging, metal chelation, and substrate association. The skeletal structure of flavonoids is a fused ring system modified by hydroxyl, sugar, and carbohydrate additions. Flavone is a structurally simple flavonoid. Quercetin and its glycosidic analog rutin are complex structures. Using a DNA oxidation/cleavage assay, flavone reduces DNA nicking by 91%. Depending on the solvent system used, quercetin can either increase or decrease DNA oxidation. Rutin exhibits neither pro- nor antioxidant activity. The molecular interactions responsible for these results are defined for flavone. 1) Flavone intercalates into DNA and saturates DNA at a 1/3.5 flavone:DNA molar ratio. 3) Flavone reduces iron-dependent DNA oxidation. 4) Flavone interacts with quercetin to enhance DNA protection. These results characterize the primary activities of a simple flavonoid.

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LIST OF ABBREVIATIONS

antioxidant index	AO
distilled water	dH ₂ O
dimethyl sulfoxide.....	DMSO
dimethylformamide	DMF
dithioerythritol.....	DTE
ethidium bromide	EBR
hydrogen peroxide.....	H ₂ O ₂
hydroxy radical.....	HO•
non-detergent sulfo-betaine.....	NDSB
proxyl fluorescamine.....	PF
sodium phosphate buffer	NPB
supercoiled-to-nicked-circular-conversion.....	SNCC
sucrose bromophenol blue.....	SBOB
tris-borate-ethylenediamine-tetraacetic acid	TBE
Tris-hydrochloric acid (10mM) Ethylenediamine-tetraacetic acid (1mM), pH 8.4.....	T ₁₀ E ₁
ultraviolet	UV
visible	Vis

CHAPTER 1

INTRODUCTION

Antioxidants are essential to good health. Diets rich in antioxidants contribute to a lower incidence of several major chronic diseases¹⁻². In particular, cancer development and/or growth is inhibited by antioxidants. Antioxidants delay or prevent the oxidation of a given substrate by free radicals³. Normal metabolic processes produce free radicals^{4,5}. Under normal conditions, these molecules function as physiological signals. However, free radicals are highly reactive and unstable⁶. Characterized by unpaired electrons in their outer orbit, free radicals also can cause oxidative damage to cells and tissues. Cellular antioxidant enzymes neutralize many free radicals. However, the delicate balance between radical generation and neutralization can be disrupted. Additional radicals can be generated by exogenous sources (*i.e.*, ultra-violet (uv) light, drugs and their metabolites, and smoke). Consequently, free radicals accumulate and cause extensive damage to cellular macromolecules⁷. Antioxidants are an ancillary defense against such accumulation. For example, flavonoids prevent hydroxy radical-induced damage⁸. Hydroxy radicals may accumulate because there is no cellular enzyme to neutralize the species. They are generated by Fenton chemistry via the reaction of H₂O₂ and iron (Fig. 1). Flavonoids inhibit hydroxy radical production by chelating the transition metal⁹. They also scavenge hydroxy radicals by donating an electron to neutralize the species^{7,10}. In addition, some flavonoids may associate with the oxidizable substrate to prevent direct hydroxy radical damage. Lipid peroxidation and protein/DNA oxidation studies document these activities^{7,11,12}. However, most activity studies focus on the relationship between flavonoid structure and antioxidant mechanism⁷.



Figure 1. The Production of Hydroxy Radicals by Fenton Chemistry

They suggest that the antioxidant activity of flavonoids is due to hydroxyl group substitution about the flavonoid skeleton. Modification to any chemical structure changes the way it reacts with other compounds. The same is true for flavonoids. The addition or substitution of a hydroxyl group, or sugar moiety, may alter reactions between flavonoids and hydroxy radicals. Metabolism is an example of where flavonoids would undergo such modifications^{13,14}. Take for instance quercetin's mode of digestion; quercetin is reduced to several simpler flavonoids in the digestive tract. It is necessary to evaluate the activity of these simpler flavonoids and determine their contribution to flavonoid antioxidant activity. Flavone is the rudimentary structure of many common flavonoids (Fig. 2B). A type of building block, flavone's chemical structure can undergo certain additions to produce more highly substituted flavonoids. Addition of hydroxyl groups to flavone produces quercetin (Fig. 2C). The glycosidic analog of quercetin, rutin, results from sugar addition (Fig. 2D).

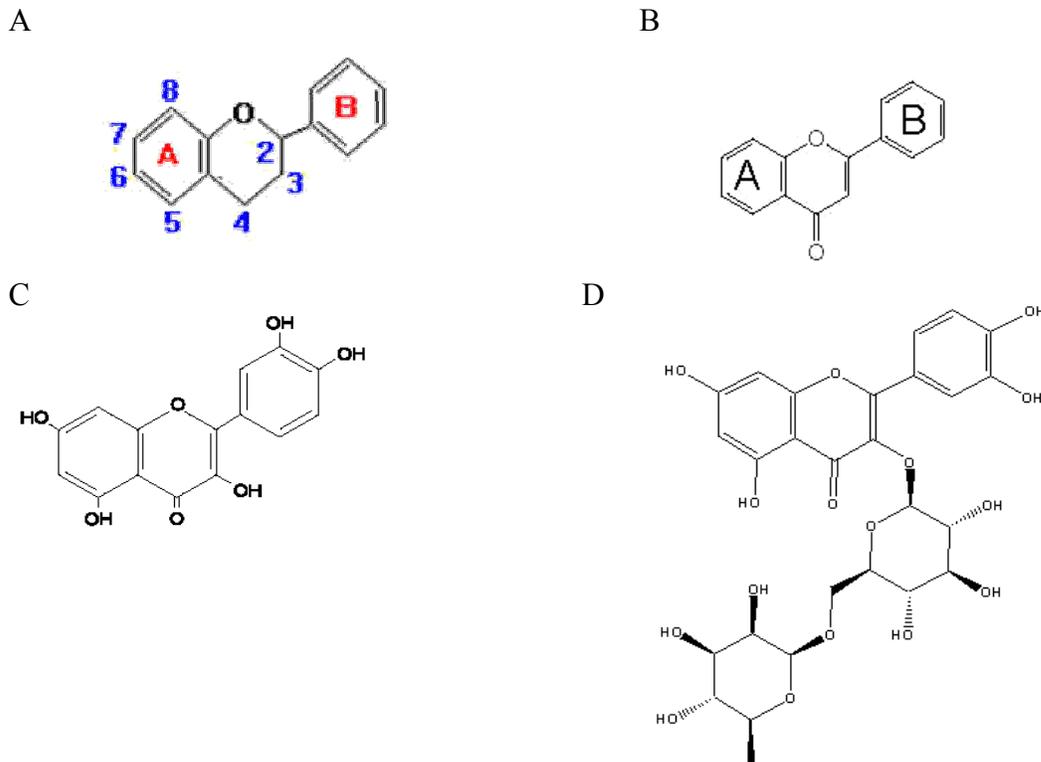


Figure 2. Structure of Common Flavonoids. A. Rudimentary skeletal structure of all flavonoids. B. Carbonyl addition to C-4 with 2,3 conjugated double bond to produce flavone. C. Hydroxyl group addition pattern of quercetin. D. Addition of rutinose to quercetin produces rutin.

Previous *in vivo* and *in vitro* studies suggest that flavone is not an efficient antioxidant because it does not efficiently scavenge free radicals^{7,8}. Flavonoids that neutralize free radicals do so by electron donation. While flavone is an aromatic molecule, the lack of hydroxyl substituents prevents it from donating a large number of electrons. Flavone is planar and hydrophobic, features characteristic of an intercalator. It also must be capable of some type of molecular interaction with other species. Therefore, it is possible that flavone could use intercalation along with other flavonoid mechanisms for antioxidant activity. The data shown here conclude that flavone intercalates DNA and interacts with iron to prevent hydroxy radical damage and generation.

The hypothesis of this thesis is that flavone can protect DNA from oxidative damage despite the lack of hydroxyl substituent attachments. Through molecular interactions with the components of the hydroxy radical generating system and the oxidative target, DNA, flavone will decrease the rate of nicking to the DNA phosphodiester backbone. The biological effects of flavonoids are dependent on the fundamental activities of their rudimentary skeletal structures. The molecular interactions of these simple flavonoids could contribute to a better understanding of flavonoid activity within the body. Further, the evaluation of simple flavonoids gives future research a basis for comparison of the more complex flavonoid structures.

CHAPTER 2

MATERIALS AND METHODS

DNA Isolation

pTZ19U DNA (United States Biochemicals), a 2865 base-pair plasmid, was grown in *E. coli* bacterium, strain DH5 α IQ, overnight in Luria Bertini medium with 25 μ g/ml ampicillin (Sigma Chemical Co.). Plasmid DNA was isolated employing the Promega wizard kit and its μ micromolar concentration (1.516 μ M = (75.85 μ M/50 μ g) and purity were measured spectrophotometrically¹⁵. Calf thymus DNA (Sigma Chemical Co.) was extracted with a chloroform:isopropyl alcohol solution (24:1 chloroform:isopropyl ratio) and dialyzed with 10mM NPB.

Reagents

Flavone, quercetin, and rutin (Sigma Chemical Co.) were prepared as 250 mM stock solutions in dimethylformamide (DMF). Ferrous-EDTA was prepared at a 1:1.5 molar ratio of, 0.1M ferrous ammonium sulfate (Fe²⁺(NH₃)SO₄) (Sigma Chemical Co.) and 0.15 M tetra-sodium EDTA (pH 9.5) (Sigma Chemical Co.), stored as 500 μ L aliquots at -20°C. H₂O₂, 0.46 M, was prepared fresh weekly by dilution from a 9.06 M stock solution (Fisher Chemical Co.) into 10mM sodium phosphate buffer (NPB), pH 7.1. Dithioerythritol (DTE, Sigma Chemical Co.) was prepared as a 0.1 M stock and stored as 10 μ L aliquots at -80°C. Proxyl fluorescamine (PF, Molecular Probes) was prepared as a 53.3 mM stock in 1.4 M dimethylsulfoxide (DMSO, Sigma Chemical Co.). NPB buffer was prepared as a 10 mM stock, pH 7.1 in distilled water (dH₂O).

Supercoiled-to-Nicked-Circular-Conversion (SNCC) Assay

The supercoiled-to-nicked-circular-conversion (SNCC) assay is a first-order kinetic analysis of the conversion of supercoiled DNA (Form I) to nicked circular DNA (Form II). The assay uses Fenton chemistry to produce OH[•] for the exposure of DNA to an oxidant. Separation of the bands, as seen by a gel mobility shift between the supercoiled and nicked DNA forms, by gel electrophoresis allows for quantitative measurement. Figure 3 illustrates the logic of the SNCC assay. The reaction parameters are set so the conversion of Form I to Form II DNA with respect to time is linear. Therefore, kinetic rates of the reaction can be determined by linear regression analysis. Additionally, precise calculations of the kinetic rates of the conversion reaction are dependent upon the availability of the substrate. Early time points are those best suited for measuring the initial rate of nicking. During the first minutes of the reaction, the Form I DNA substrate should remain in excess. A single nick of the DNA backbone determines the initial rate of nicking. This type of nicking represents the conversion of Form I, supercoiled DNA to Form II, nicked circular DNA. However, if multiple nicks or double strand breaks occur, Form II DNA can be converted to linear Form III. Form III is linear DNA. The appearance of Form III DNA signifies that all of the Form I substrate has been exhausted and that Form II DNA is being nicked further (Fig. 3). When this occurs, the reaction cannot be measured by first order kinetics since the slope of the line would not represent the conversion of Form I to Form II DNA. Figure 4 illustrates the results of a non-linear reaction *i.e.*, a reaction that has run out of substrate. In this example, the Fenton reactants were too concentrated. The production of hydroxy radicals quickly surpassed the availability of supercoiled substrate. Therefore, at later time points, total conversion of Form I DNA to Form II DNA occurred. If the reaction had proceeded, Form II DNA would have been converted to Form III. The slope of line A represents excessive nicking of Form I and Form II DNAs (Fig. 4). The corrected slope, line B includes only the initial time points. These points more accurately reflect the conversion of Form I DNA to Form II DNA while the Form I substrate is still in excess.

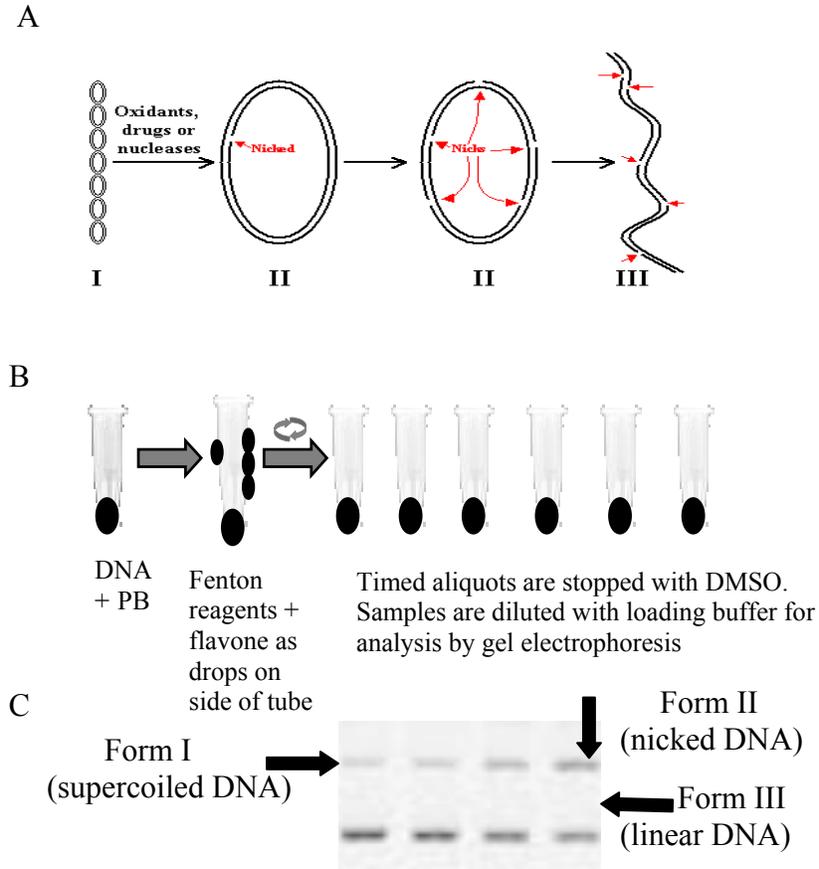


Figure 3. Detailed Diagram of the SNCC Assay. A. An overview of the nicking reaction and that converts Form I to Form II DNA and the result of excess nicking of Form II to yield linear Form III DNA. B. *In vitro* nicking assay design. C. Illustration of the gel electrophoresis pattern of a DNA nicking reaction and indication of where Form III DNA would appear if Form II DNA experienced multiple nicks.

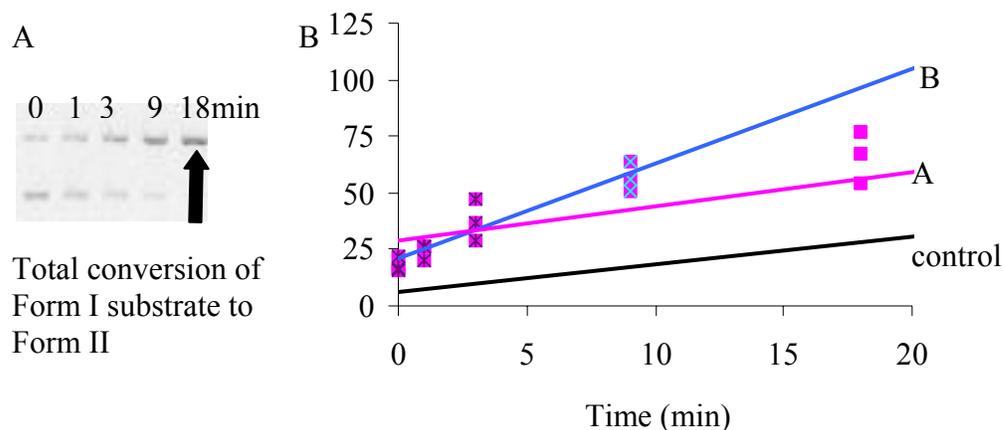


Figure 4. Linear Regression Analysis of Nicking Reaction that has Run Out of Substrate. A. Gel analysis of a non-linear SNCC reaction. Vertical arrow illustrates total conversion of Form I DNA. Trendline A represents a non-linear reaction. Trendline B is a plot of form II band's fluorescent intensity vs. exposure time of the Fenton reaction. Time points used to calculate trendline: A (0, 1, 3, 9, 18 min.), B (0, 1, 3, 9 min.).

The control reaction was initiated by premixing 7 μ L of DNA (421.7 μ M) and 26.3 μ L NPB (10 mM) in a 0.6mL microcentrifuge tube. The Fenton reagents, 0.54 μ L Fe²⁺EDTA (1mM), 0.54 μ L H₂O₂ (2.5 nM), and 0.54 μ L DTE (1mM uM), were added as individual drops on the side of the tube. A quick flick of the tube to mix the reagents with the DNA began the reaction. Timed aliquots of 5 μ L were removed and added to 2 μ L of DMSO (2M) stop solution. Aliquots were prepared for agarose gel electrophoresis by the addition of SBOB-T₁₀E₁ (sucrose bromphenol blue tris-hydrochloric acid ethylenediamine tetraacetic acid, 45 μ L). Samples (10 μ L) were run at 3.41 V hr/cm on a 1% agarose gel in 50 mM TBE (50 mM tris base, 50mM boric acid and 1.12 mM EDTA) + EBR (ethidium bromide 0.25 μ g/mL) running buffer. The gel was rinsed for 2 x 15 minutes in dH₂O and the fluorescent image was captured digitally using the Alpha Innotech 2200 Imaging system. The fluorescent intensity of the ethidium-stained Form II DNA was measured by spot density analysis. The control + flavonoid, control + flavone, control + rutin and control + quercetin reactions differed from the control reaction only in the addition of the respective flavonoid/solvent as an individual drop on the side of the tube. Premix reactions of flavone + DNA, flavone +

Fe²⁺EDTA, and flavone + quercetin, also were identical to the control reaction except for the required pre-incubation (10 min) of the premix components before the start of the reaction.

SNCC Assay Data Analysis

The plot of time (min) vs. Form II band intensity was analyzed by linear regression to obtain the slope of the line. This value corresponds to the relative rate of HO·-dependent nicking of the DNA¹⁶. A significant decrease in slope compared to the control reaction indicates an increase antioxidant activity measured as an increase in DNA protection against oxidative cleavage. The slope also was used in the following equation to obtain an antioxidant index (AO).

$$AO \equiv 1 - [(control + flavonoid\ slope) \div control\ slope] \times 100\%$$

The antioxidant index is a percent measurement of antioxidant efficacy that can range from 0% (no DNA protection over the control) to 100% (total DNA protection). A negative percent value is indicative of pro-oxidation activity of the additive, (*i.e.*, increased DNA nicking over the control). The corresponding R² values of each line also are indicated on the plots. These values represent how well the data points fit the line.

Solvent Selection

Various solvents were evaluated in the SNCC assay to determine their effect on DNA oxidation. The required solvent must be compatible with the SNCC assay and with DNA integrity. Therefore, it cannot have significant antioxidant or DNA denaturing activity. Many organic solvents that readily dissolve flavonoids protect DNA from hydroxy-radical induced damage. Ethanol, methanol, and acetonitrile readily dissolved flavonoids but exhibited antioxidant properties in the SNCC assay. As seen in Figure 5, ethanol dissolved flavone but its antioxidant activity was so high that the antioxidant activity of flavone could not be detected. Therefore, the antioxidant activity of flavone could not be evaluated in this solvent. Other solvents pose similar problems. NDSB-195 also has antioxidant activity comparable to flavone while NaOH dissolves flavonoids but denatures DNA. To address these issues many solvents were screened in the SNCC assay. A solvent that exhibits no significant antioxidant activity is dimethylformamide

(DMF). DMF does not interfere with the Fenton reaction or damage DNA. Further, DMF can be used in spectrophotometric studies since it has no significant absorption or fluorescence.

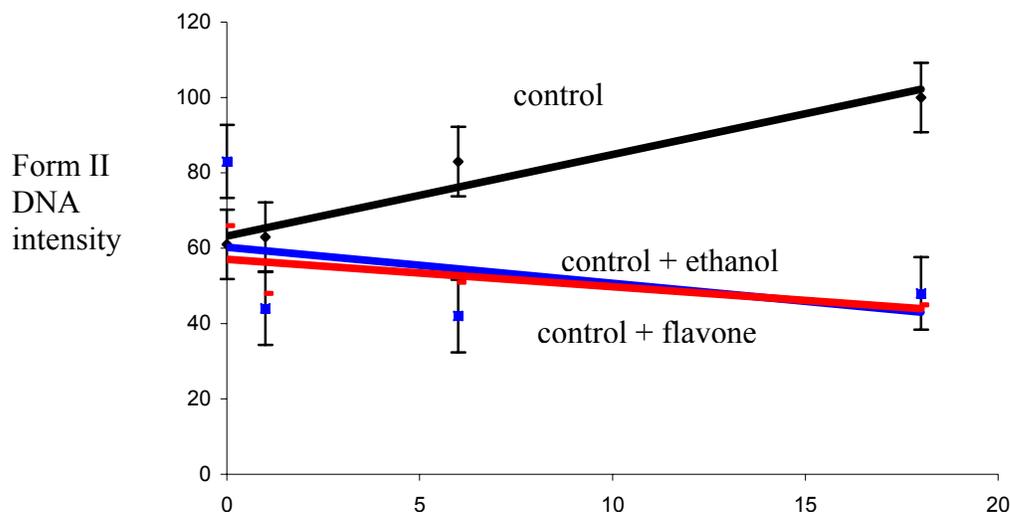


Figure 5. Antioxidant Effects of Flavonoid Solvent System. Linear regression analysis of the SNCC assay illustrating the antioxidant activity of ethanol and its incompatibility as a flavonoid solvent. The slope of the control + ethanol is not statistically different from the control + flavone slope. The antioxidant activity of ethanol far exceeds that of flavone. Flavone's antioxidant activity cannot be evaluated in this solvent. Each trendline represents the average result of triplicate reactions. Error bars = standard error of the mean.

Ultraviolet-Visible Absorbance Spectroscopy

Ultraviolet (UV)-Visible (Vis) spectra were measured in a 96-well quartz microtiter plate at 2 nm slits using Molecular Devices Spectra Max PLUS 384 spectrophotometer. Final concentrations of flavone were obtained by diluting the 250 mM stock with 10 mM NPB to a 1mM working stock. The working stock was diluted to 300 μ L to obtain final concentrations. Spectra were corrected by subtracting the absorbance of flavone + additive minus solvent and any additional fluorophores. Flavone was mixed with the Fenton components to determine molecular interactions. Table 1 displays these mixing

reactions. Reaction mixture concentrations were comparable to those used in the SNCC assay.

Table 1. Mixing of flavone with Fenton components for studies of molecular interactions by UV-Vis spectroscopy*.

Reaction mixture	Flavone (F) (uM)	DNA (D) (uM)	Iron (I) (uM)	Quercetin or Rutin (Q or R) (uM)
F only	125			
F + D	125	41		
F + I	100		100	
F + Q	125			125
F + R	100			125

*Concentration values represent the final concentration of the components in the assay mix.

Fluorescence Spectroscopy

Initial excitation values were obtained on a Jobin Yvon Horiba Fluoromax 3 fluorimeter. Using the Fluoro-max software, discovery scans simultaneously measured the excitation and emission spectra of flavone. Flavone produced one broad emission peak, 315-515 nm with $\lambda_{\max} = 384$ when excited at 300 nm. Though it is recommended to choose an excitation wavelength well below the emission spectra start value, exciting below 300nm reduces the sensitivity of the detection. Fluorometry allows for the highly selective and rapid study of changes due to mixing of substances¹². DNA and iron do not fluoresce. However, spectra were corrected by subtracting any significant solvent fluorescence. To follow the changes in flavone's fluorescence in component mixtures, samples were measured in a microtiter plate at 2 nm slits using Molecular Devices Spectra Max Gemini XS fluorimeter. Fluorometric assays also were used to determine saturation ratios. If flavone's emission spectrum is sensitive to DNA or iron associations, stoichiometry will elucidate the concentration dependence of such interactions.

Mixing Reactions

Flavone + DNA or Iron

Flavone's spectral changes were monitored upon the addition of DNA or iron. Final concentrations of flavone (100 μM), Fe^{2+} EDTA (100 μM), and DNA (300 μM) were obtained by diluting stock solutions to 300 μL with NPB. Emission spectra (315-515 nm) were obtained with 300 nm excitation and reagent ratios proportional to those used in the SNCC assay.

Flavone/DNA Ratios

Saturation ratios were obtained by monitoring flavone's spectral changes upon addition of increasing concentrations of DNA. Flavone's peak fluorescence was at $\lambda_{\text{em}} = 384\text{nm}$ at $\lambda_{\text{ex}} = 300\text{ nm}$. Flavone (50 μM) was titrated with increasing amounts of calf thymus DNA (2900.5 μM) until the addition of DNA no longer induced changes in fluorescence. Separate flavone samples also were titrated with identical amounts of NPB to account for volume changes. Saturation ratios were determined by plotting the average of triplicate measurements of flavone's fluorescence units vs. the DNA : flavone ratio.

Flavone + DNA + Ethidium

Flavone's intercalative efficacy also was measured by the ability of flavone to prevent ethidium intercalation. Ethidium + DNA fluorescence was monitored at $\lambda_{\text{ex}} = 518\text{ nm}$ and $\lambda_{\text{em}} = 530\text{-}780\text{ nm}$. Ethidium (10 μM) was titrated with aliquots of a premixed DNA/flavone complex (3 DNA base pairs per 1 molecule of flavone) at the following EBr:DNA ratios: 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5. Endpoint values of ethidium/DNA maximum fluorescence ($\lambda_{\text{em}} = 605\text{nm}$) were plotted against DNA : EBR ratios to determine if pre-intercalated flavone affects subsequent ethidium intercalation.

Flavone and Hydroxy Radical Scavenging

Hydroxy radical scavenging is the primary antioxidant mechanism of many flavonoids⁷. Although flavone's structure does not contain the hydroxyl groups reported to be necessary for high-efficiency electron donation, it may scavenge free radicals via a separate mechanism. The radical scavenging activity of flavone was evaluated using a fluorescent probe. Proxyl fluorescamine is a molecule that reacts with the by product of a $\text{HO}\cdot$ scavenging event. DMSO neutralizes $\text{HO}\cdot$ and as a result forms a stable methyl

radical ⁷. This stable radical reacts with the fluorescent probe proxyl fluorescamine to form a stable complex and with enhanced fluorescence at $\lambda_{\text{ex}} = 390 \text{ nm}$, $\lambda_{\text{em}} = 455\text{-}555 \text{ nm}$. This excitation is well beyond that of flavone (300 nm). The reaction between DMSO and $\text{HO}\cdot$ will decrease if flavone scavenges $\text{HO}\cdot$. Consequently, less methyl radical will interact with proxyl fluorescamine and its fluorescence will decrease. To examine flavone's scavenging activity, proxyl fluorescamine was monitored for changes in fluorescence when exposed to Fenton reagents. In the control reaction, Fenton reagents, (100 μM Fe^{2+} , 100 μM DTE, and 5.1 μM H_2O_2) were added to a microtiter well containing proxyl fluorescamine (0.5 μM) and DMSO (126 μM). The addition reactions were identical to the control but included 125, 250, and 375 μM flavone. Changes in the proxyl fluorescamine/DMSO solution spectra were monitored in the control and control + flavone reactions. DNA was not included in these Fenton reactions.

CHAPTER 3

RESULTS

The previously described methods were used to answer some fundamental questions about flavone's antioxidant activity. These results describe the possible mechanisms used by flavone to prevent DNA damage by hydroxy radicals.

Does Flavone Protect DNA from Oxidative Damage?

Hypothesis: Flavone can protect the DNA phosphodiester backbone from hydroxy radical-induced nicks. The control reaction of the SNCC assay was modified by the addition of 250 μ M flavone. This control + flavone reaction was compared to the control reaction to determine the antioxidant efficacy of flavone. Agarose gel analysis of the samples is illustrated in Figure 6A. Conversion rates of Form I to Form II DNA in the control reactions are greater than in the control + flavone reactions. Statistical analysis was carried out by plotting Form II nicked DNA band intensities of both the control and control + flavone samples as a function of time. Linear regression analysis of each triplicate reaction revealed a statistically significant decrease in the slope of the control + flavone reaction compared to the control (Fig. 6B). Using the AO index equation, flavone was determined to provide 91% DNA protection over the control. Therefore, flavone is protecting DNA by one or more of the earlier described flavonoid mechanisms (*i.e.*, metal-flavonoid interactions, substrate association, and/or free radical scavenging). Further, if flavone is protecting DNA from hydroxy radical-induced damage, how does it compare with the antioxidant activity of other common flavonoids?

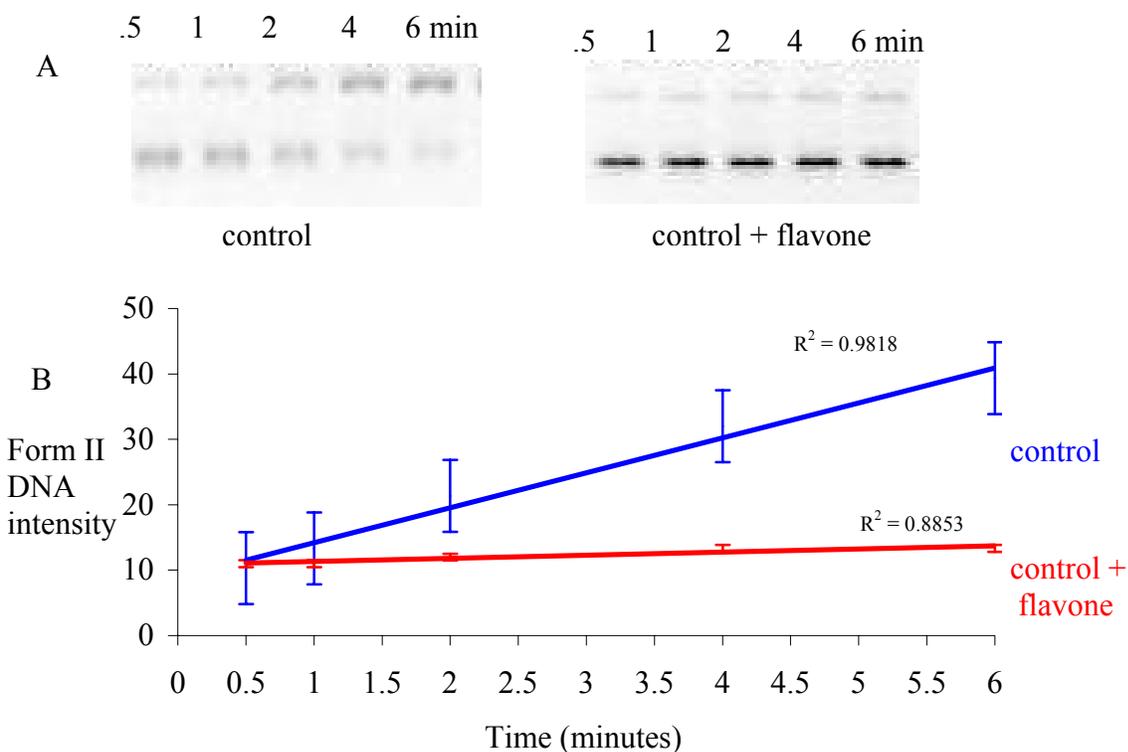


Figure 6. SNCC Assay and Statistical Analysis of Control + Flavone Reactions. A. Gel analysis of control and control + flavone reactions. The Form I substrate in the control reaction was excessively nicked to determine the extent of flavone's antioxidant activity but did not go beyond the linear range of the reaction (*i.e.*, appearance of Form III DNA). B. Linear regression analysis of the control and control + flavone reactions; nicked DNA band intensities are plotted over time. Slope values: control (5.339), flavone (0.4824). Each trendline represents the average result of triplicate reactions. C. Calculation of the AO value. Error bars = standard error of the mean.

Does Quercetin Reduce DNA Damage in the SNCC assay?

Hypothesis: Quercetin increases oxidation of DNA in the Fenton reaction.

Strick et al.¹⁷ reported that quercetin oxidatively damaged DNA *in vivo*. To test if quercetin would damage DNA in a simple system, quercetin (dissolved in NDSB-195) was added to the SNCC assay in the same manner as flavone. The reaction rates of control only and control + quercetin were compared by gel electrophoresis (Fig. 7A) and linear

regression analysis (Fig 7B). Quercetin increased DNA oxidation. The slope of the control + quercetin was greater than that of the control reaction. AO calculations show quercetin increases DNA oxidation 71% over the control. However, as discussed previously, quercetin is a solvent dependent pro-oxidant. In this assay, quercetin was dissolved in NDSB-195. As later data will show, depending on the solvent system used quercetin also exhibits antioxidant activity. Therefore, to directly compare the antioxidant activity of flavone and quercetin, the same solvent system must be used to dissolve both flavonoids.

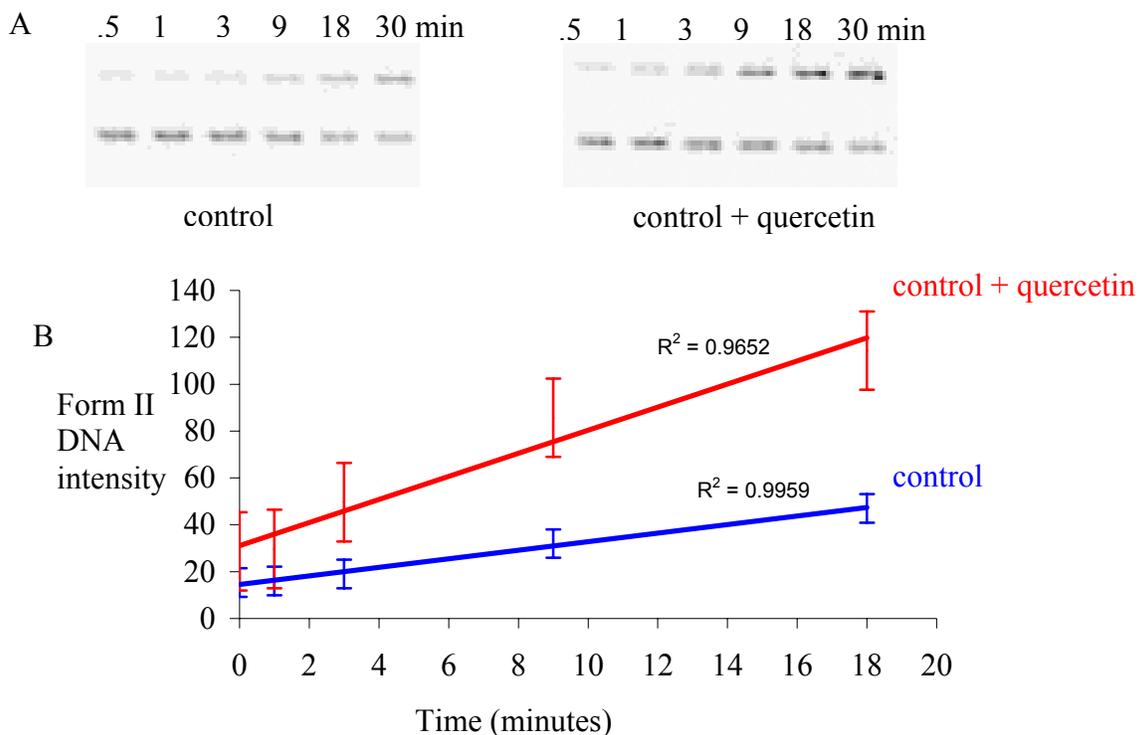


Figure 7. SNCC Assay and Statistical Analysis of Control + Quercetin Reactions. The plot indicates that when dissolved in NDSB-195, quercetin exhibits pro-oxidant activity. A. Gel analysis of control and control + quercetin reactions. B. Linear regression analysis of data points in A; nicked DNA band intensities are plotted over time (30 min. time point not shown). Each trendline represents the average result of triplicate reactions. Error bars = standard error of the mean.

Rutin is quercetin's glycosidic analog. The purpose of the following experiment was to evaluate if the sugar addition to quercetin would alter its antioxidant activity. As previously mentioned, when digested, flavonoid undergo certain modifications. This is an example of how these types of modifications might affect the overall antioxidant activity of a flavonoid.

Antioxidant Activity of Rutin

Hypothesis: Rutin protects DNA from oxidative damage. Previous studies report that as an antioxidant, rutin binds iron to prevent redox cycling and preferentially scavenges the superoxide radical¹⁸. To test if rutin was able to prevent redox cycling in the Fenton reaction and directly prevent hydroxy radical-induced DNA damage, rutin was added to the SNCC assay. The gel analysis is illustrated in Figure 8. The slope of the control + rutin reaction is not statistically different from that of the control (data not shown). Although rutin may bind iron, the production of hydroxy radicals in the Fenton reaction is not affected. Further, these data suggest that rutin does not scavenge the hydroxy radical and that sugar addition does affect the overall activity of the flavonoid.



Figure 8. SNCC Assay Analysis of Rutin. Gel analysis of control and control + rutin reactions showing no statistical difference between the control and rutin reactions revealing rutin is neither an antioxidant nor a pro-oxidant.

Flavone appears to be an antioxidant in the SNCC assay. Though structurally simple, it has protected DNA from hydroxy radical-induced damage. However, the question still remains as to how flavone is able to prevent DNA oxidation. The following results identify a direct interaction between flavone and SNCC assay components.

Premix Reactions in the SNCC Assay

Flavone + DNA premix

Hypothesis: Is there a direct interaction between flavone and DNA that protects against hydroxy radical-induced damage. To investigate specific molecular mechanisms of flavone's antioxidant activity the SNCC assay was modified by premixing flavone and DNA for 10 minutes before the addition of Fenton reagents. The results of the assay are illustrated in Figure 9. The rate of DNA nicking was reduced by flavone when it was premixed with DNA (Fig. 9A). The slope of the premix reaction was statistically different from the control and from the control + flavone reactions (Fig. 9B). The AO calculation indicated that the flavone + DNA premix inhibited DNA nicking by 96% over the control and 5% over the control + flavone reaction. In addition, the y-intercept of the flavone/DNA premix is significantly different from the control. This suggests that in the absence of Fenton reagents, flavone may intercalate DNA and reduce the availability of ethidium intercalation sites. Consequently, ethidium fluorescence is affected. Thus, flavone may protect DNA through a direct interaction with the substrate and intercalate to reduce the interaction between DNA and other molecules.

Does Flavone Interact with Fenton Components?

Flavone interacts directly with the substrate but does it also interact with other components of the Fenton reaction? The Fenton reaction is iron dependent. The redox cycling of iron is necessary for hydroxy radical production. If flavone interacts with iron, it may slow the production of hydroxy radicals and prevent DNA oxidation. Further, iron binds DNA. In an iron dependent system, any reaction that involves iron is brought into close proximity of the DNA. Therefore, if flavone interacts with iron it also may reduce iron-DNA binding and prevent the radical generating system from reacting near the DNA. However, there are consequences to such an interaction. As the results will show, if flavone interacts with iron, it would not be able to as intercalate DNA as efficiently. The following data will attempt to provide evidence for flavone/iron interactions. Further the results will determine whether this mechanism provides more DNA protection than the interaction between flavone and DNA.

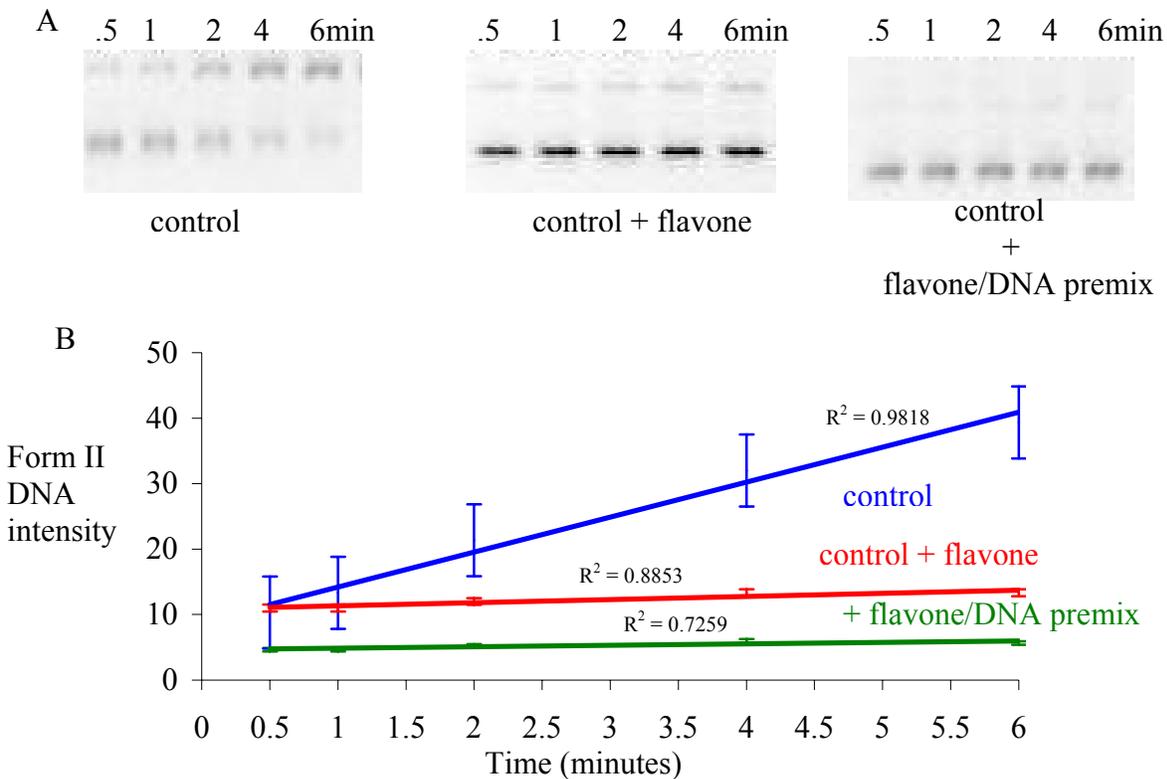


Figure 9. SNCC Assay and Linear Regression Analysis of Flavone/DNA Premix Reactions. The B Plot illustrates that flavone/DNA premixes offer increased antioxidant protection. A. Gel analysis of control, control + flavone and control + flavone/DNA premix reactions. B. Linear regression analysis of the data in A; nicked DNA band intensities are plotted over time. Each line represents the average result of triplicate reactions. Error bars = standard error of the mean

Does Flavone Associate with Iron to Reduce Hydroxy Radical Generation?

Hypothesis: Flavone interacts with iron to slow the production of hydroxy radicals in the Fenton reaction. Flavone + iron premix: To further analyze flavone's molecular mechanism, the SNCC assay was modified by premixing flavone and iron for 10 minutes before the start of the reaction (Fig. 10A). As seen in figure 10B the premix of flavone + iron was less effective in reducing the oxidative damage than was the control + flavone reaction. The AO calculation indicated flavone + iron premix inhibits DNA oxidation by 80%. These data suggest that flavone interacts with iron to prevent hydroxy radical-induced damage. However, this interaction limits the availability of free flavone. With limited availability, flavone's association with DNA is less efficient. Therefore, the data

suggest that like flavone/DNA premixes, flavone/iron premixes reduce DNA damage. However, they do not increase DNA protection more than flavone/DNA premixes or the simultaneous flavone addition reactions.

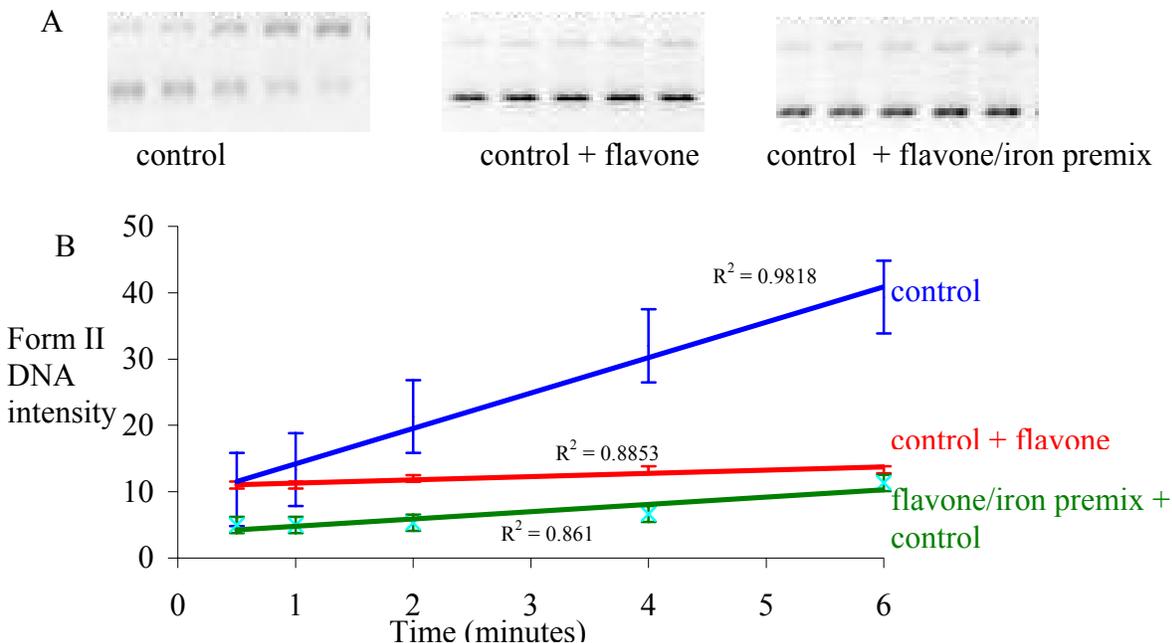


Figure 10. SNCC Assay and Statistical Analysis of Flavone/Iron Premix Reactions. The plot indicates that flavone/iron premixes offer DNA protection but are not as effective as flavone/DNA premixes or the simultaneous addition of flavone with the Fenton reagents. A. Gel analysis of control, control + flavone, and control + flavone/iron premix reactions. B. Linear regression analysis of the data in A; nicked DNA band intensities are plotted over time. Each trendline represents the average result of triplicate reactions. Error bars = standard error of the mean.

Flavonoids exist ubiquitously in fruits and vegetables. Their antioxidant activity in the body may result from interactions between flavonoids. Therefore, combination studies that involve flavone and other common flavonoids may result in enhanced DNA protection. The following data questions whether flavone and quercetin interact to produce the observed antioxidant effect.

Does Flavone Interact with other Flavonoids?

Hypothesis: Flavone interacts with other flavonoids to enhance their antioxidant activity. Flavone + quercetin premix: To assess the antioxidant efficacy of flavone combined with other flavonoids, the SNCC assay was modified by premixing flavone + quercetin before addition of the Fenton reagents. In this assay, quercetin was dissolved in DMF. Quercetin exhibited 43% DNA protection over the control. When dissolved in DMF, quercetin exhibits antioxidant activity. In contrast to data presented earlier, this suggests that quercetin is a solvent dependent pro-oxidant. Therefore, depending on the solvent used to dissolve quercetin it can exhibit either pro-oxidant or antioxidant activity. By premixing flavone with quercetin (both dissolved in DMF), the antioxidant activity of quercetin was increased (Fig 11 A). As illustrated in Figure 11B the slope of the control + quercetin reaction is greater than that of the control + flavone/quercetin premix reaction. The premix reaction increased quercetin antioxidant activity by approximately 1.6 fold. Table 2 compares the slopes of each reaction. The slope of the flavone only reaction is 0.4824. The slope of the quercetin-only reaction is 2.405. The numeric average of these 2 slopes is 1.444. This number is significantly (p -value = 0.023) different from the slope value of the flavone/quercetin premix reaction (1.138). Therefore, when quercetin is dissolved in DMF, it does exhibit antioxidant properties. Further, a premix of flavone and quercetin enhances DNA protection. The difference in slope between the different reactions reveals that the protective effect of a flavone/quercetin premix is greater than an additive effect: it is not a result of the antioxidant activity of flavone plus the antioxidant activity of quercetin. The data suggest that flavone and quercetin are complementary antioxidants. Because the antioxidant effect of the flavone and quercetin premix is greater than the additive effect, the increased protection seems to result from the interaction between flavone and quercetin. Therefore, these data suggest that certain flavonoids work collectively to prevent hydroxy radical-induced oxidation.

The SNCC assay results reveal details of the possible antioxidant mechanisms of flavone. Flavone affords DNA protection by interacting with DNA, with iron, and with other flavonoids. However, the SNCC assay is not designed to characterize the specific

interactions that might occur between flavone and the other assay components (*i.e.*, DNA, iron, and other flavonoids).

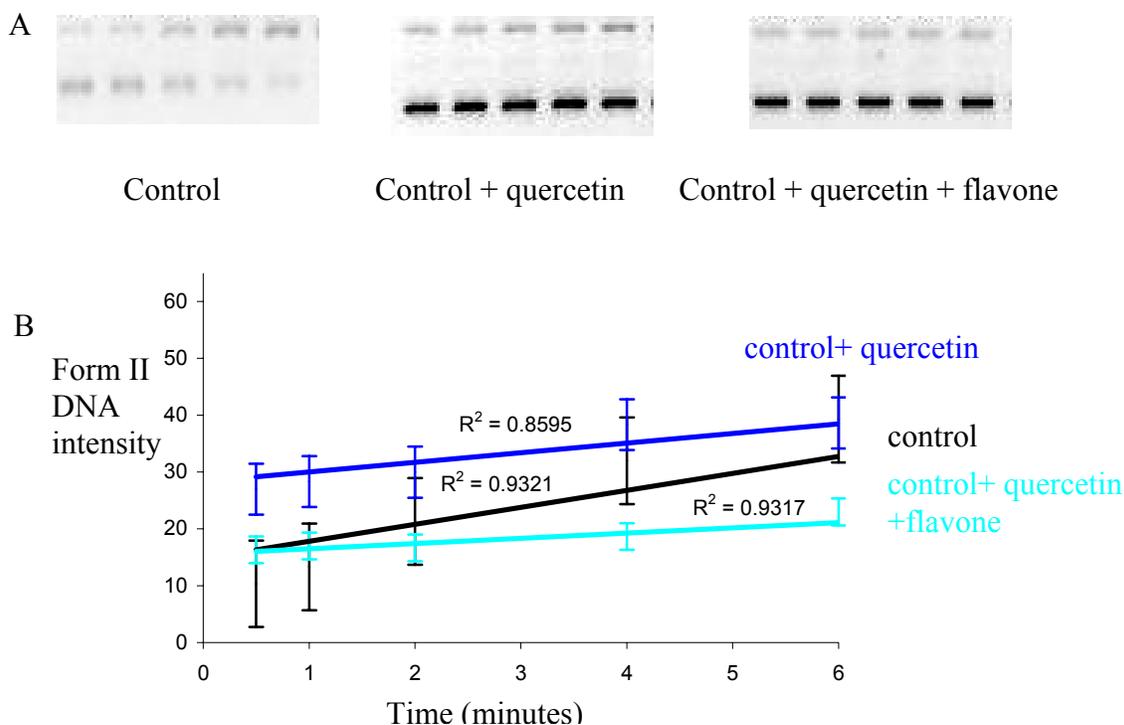


Figure 11. SNCC Assay and Statistical Analysis of Flavone/Quercetin Premix Reactions. Flavone and quercetin were equimolar (284 μ M). The plot indicates that flavone and quercetin premix reactions are more effective antioxidants than quercetin only. The plot also demonstrates that when dissolved in DMF, quercetin is an antioxidant. A. Gel analysis of control, control + quercetin and control + quercetin/flavone reactions. B. Linear regression analysis of the control, control + quercetin, and control + quercetin/flavone reactions; nicked DNA band intensities are plotted over time. Each trendline represents the average result of triplicate reactions. Error bars = standard error of the mean.

Table 2. Comparing the slope values of quercetin, flavone, and quercetin/flavone premix

Reaction	Slope
control only	5.339
control + quercetin	2.405
control + flavone	0.4824
control + (quercetin/flavone)	1.138
[(control + quercetin) + (control + flavone)] / 2	1.444

UV-Visible and fluorescence spectroscopy are tools that characterize molecular interactions by changes in the spectral pattern of component chromophores and fluorophores, respectively. They are independent measures of molecule : molecule interactions. The following experimental analyses use the spectral properties of flavone and other fluorophores to describe the molecular interactions responsible for flavone's antioxidant activity.

Analysis of Molecular Interactions between Flavone, DNA, and Iron by UV-Visible Spectrophotometry

Flavone, DNA, and Iron

Figure 12 illustrates the change in flavone spectra upon addition of DNA and iron. Flavone's absorbance decreases (a hypochromic shift) with the addition of DNA. This implies that flavone's electronic structure is changing in the presence of DNA. Flavone absorbs energy from the incident light; energy that is dispersed by molecular movements in the solvent. If intercalated, flavone's molecular movement is restricted by the helical structure of the DNA. This loss of rotational movement limits the amount of energy that can be dissipated causing a decrease in absorption capability. In contrast, in the presence of ferrous or ferric iron flavone's absorbance spectra increases (a hyperchromic shift). The increase in absorbance indicates an associative energy exchange between flavone and iron. Therefore, flavone's interaction with iron is different than it's interaction with DNA.

Flavone, Quercetin, and Rutin

Flavone and quercetin collectively reduced DNA oxidation. Therefore, the molecules are interacting within the reaction solution. To characterize this interaction, equimolar flavone and quercetin and flavone and rutin were analyzed by UV-Vis spectroscopy. The absorbance of flavone was monitored after the addition of either quercetin or rutin (Fig.13). The absorbance spectra of flavone was corrected by subtracting the absorbance of quercetin and rutin from flavonoid mixture samples. Flavone's absorbance decreases in the presence of equimolar quercetin. In contrast, the flavone's spectrum is significantly increased in the presence of rutin. This increase is a hyperchromic shift and illustrates strong electronic coupling between flavone and rutin. Fluorescence spectroscopy complements UV-Vis spectral data. However, fluorescence is a more sensitive analytical tool. The changes observed in the fluorescence spectra of flavone will provide additional evidence for the characterization of flavone's molecular interactions.

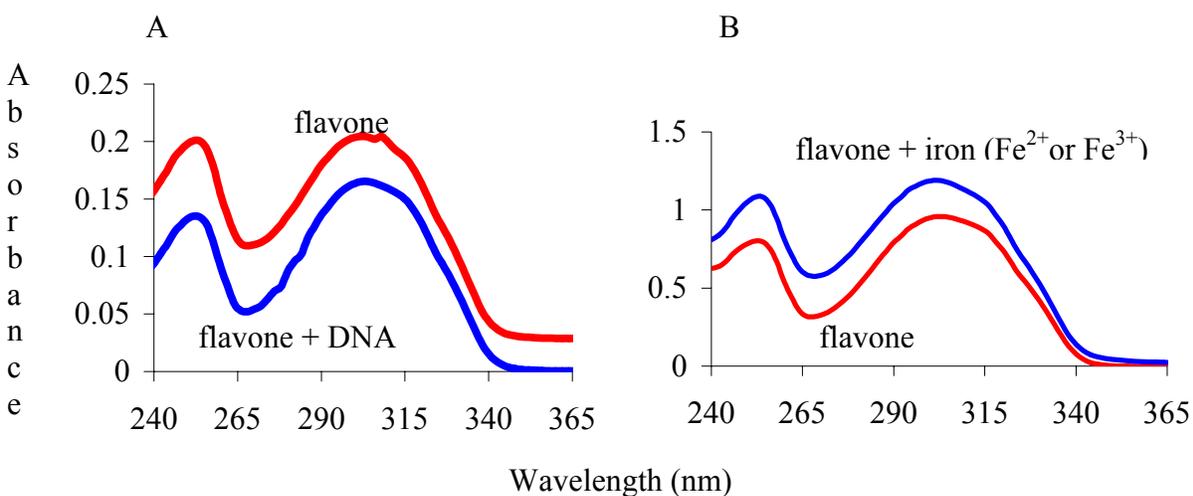


Figure 12. Changes in Absorbance Spectra of Flavone with Additions of DNA or Iron.

A. Corrected UV-Vis spectra of flavone and flavone + DNA. Hypochromic shift indicates dissipation of flavone's absorption energy in presence of DNA. B. Spectra of flavone and flavone + iron. Increase in spectra indicates an associative energy exchange between flavone and iron. Spectra represent triplicate trials. Error bars = standard error of mean.

Analysis of Flavone's Antioxidant Mechanisms by Fluorescence Spectroscopy

Flavone and DNA

Flavone's molecular interactions with DNA and with iron also were monitored by fluorescence (Fig. 14). Flavone's fluorescence decreased upon the additions of DNA. The spectral blue shift is similar to that seen in the insertion of a chromophore into the hydrophobic region of a protein, and is a strong indicator of intercalation because the helical core of DNA is hydrophobic. If flavone is an intercalator, what is the DNA : flavone molecular saturation ratio? The following experiment measures this value in order to determine the ratio at which all flavone molecules are intercalated into the DNA helix.

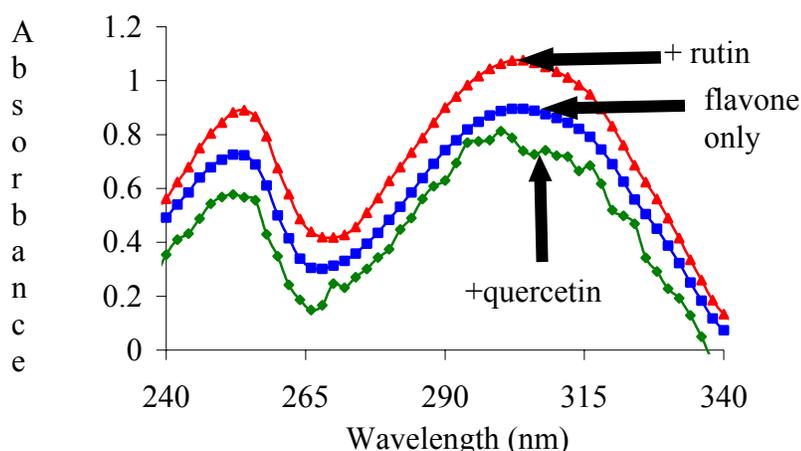


Figure 13. Corrected UV-Visible Absorption Spectra of Flavone with Quercetin or Rutin. Flavone's spectra increases with rutin addition indicative of electron coupling between the molecules. Decrease with the addition of quercetin is characteristic of intramolecular stacking of the planar molecules in the solution. Absorption scan of flavone, flavone + quercetin, and flavone + rutin. This hypochromic shift represents the intramolecular aromatic stacking of flavone and quercetin, limiting the random motion of each

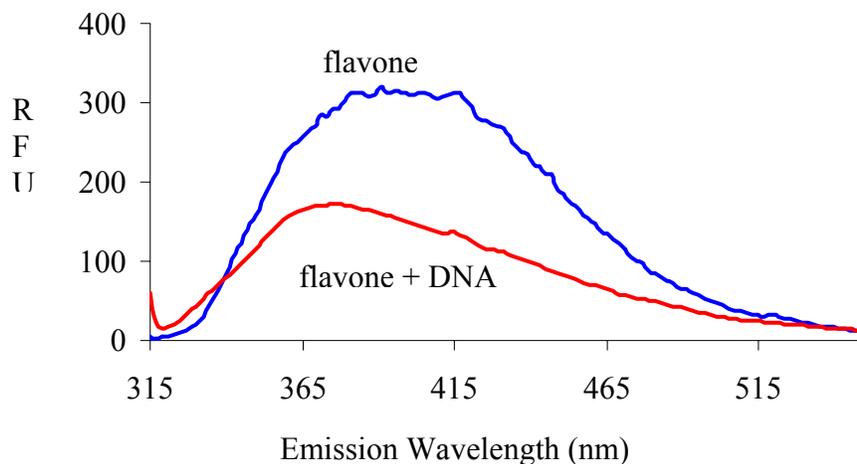


Figure 14. Fluorescence Spectra of Flavone and Flavone + DNA. Flavone's spectra decreases and shifts towards the blue spectrum with DNA; characteristic shifts of an intercalator. DNA and flavone concentrations are: 300uM DNA in bp and 100uM flavone. DNA:flavone ratio is 3:1. Excitation = 320 nm Emission = 315-515 nm.

DNA Titration of Flavone

To determine the saturation ratio of flavone molecules to base pairs of DNA, 50 uM flavone was titrated with increasing concentrations of DNA. Endpoint measurements of flavone's maximum emission peak (384 nm) were recorded. Flavone's decrease in fluorescence over each DNA addition was plotted against the ratio of DNA : flavone (Fig. 15). A saturation ratio of ~3.5 base pairs of DNA per one flavone molecule was determined by linear regression analysis, with a p-value of 0.038.

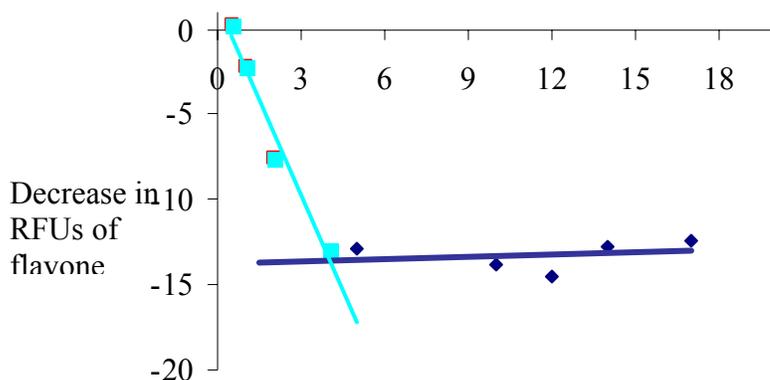


Figure 15. Reduction of Flavone's Fluorescence Intensity during DNA Titration. Flavone (125 μM) titrated with DNA (2900.5 μM). Saturation ratio is 3.5 base pairs of DNA per flavone molecule. Data points represent average of triplicate samples. (p-value of intersection is 0.038)

Flavone, Ethidium, DNA

The approximate ratio of DNA : flavone was 3.5 base pairs of DNA per one molecule of flavone. If flavone intercalates, its presence within the DNA should create 2 conditions for competing intercalators. Primarily, flavone should decrease the number of intercalation sites for a competing intercalator. Secondly, flavone intercalation should unwind the DNA helix. These phenomenon are best evaluated using an intercalator whose DNA binding ratio is similar to that of flavone. Ethidium intercalates into DNA at a 4:1 saturation ratio. When intercalated, ethidium's fluorescence increases 25 times¹⁹. To determine if flavone can reduce ethidium intercalation, DNA and a DNA/flavone premix were titrated into an ethidium solution. Endpoint measurements of ethidium's maximum emission at 605 nm were monitored for changes (Fig. 16). In comparison, the ethidium that was titrated with DNA only produced significantly higher fluorescence than ethidium titration with the flavone/DNA premix. Initially, flavone decreased the intercalation of ethidium. However, the fluorescence of the ethidium increased after reaching the 4 : 1 DNA : ethidium saturation ratio. This increase suggests additional ethidium intercalation. Therefore, the combined intercalation of flavone and ethidium may lengthen the DNA helix and reduce repulsive forces that normally inhibit ethidium intercalation above saturation values.

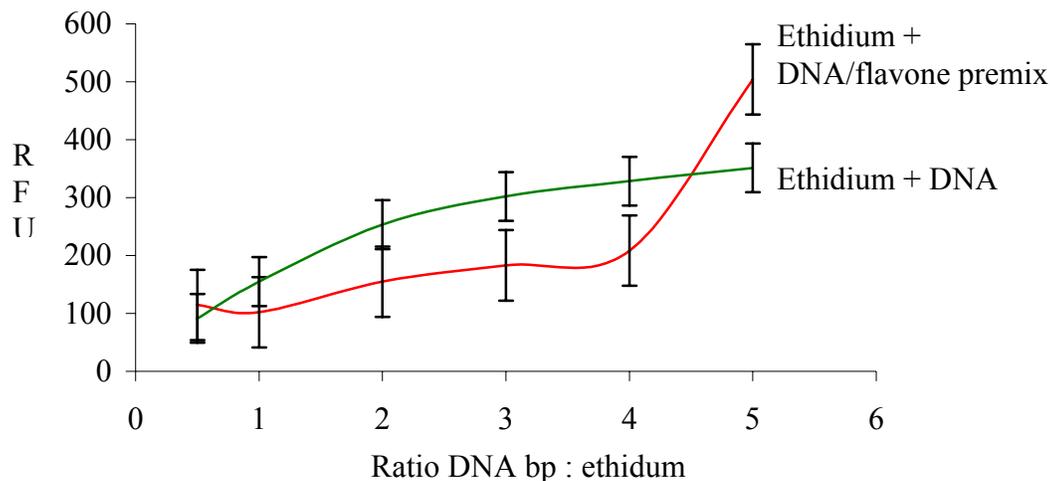


Figure 16. Fluorescence Spectra of Ethidium Titrated with DNA or a DNA/Flavone Premix. Measurements of ethidium fluorescence ($\lambda_{\text{ex}} = 518 \text{ nm}$ and $\lambda_{\text{em}} = 530\text{-}780 \text{ nm}$) were taken with each addition of DNA or a DNA + flavone premix. Ethidium's fluorescence values were plotted against the ratio of DNA:ethidium. While the DNA concentration increased during the titration, the ratio of DNA : flavone was maintained at 3.5:1. Flavone decreases ethidium's intercalation into DNA and lengthens the DNA helix. Measurements are averages of triplicate trials. Error bars = standard error of mean.

Flavone and Iron

To analyze the interaction of flavone with iron, flavone spectra were compared to those obtained from the flavone and iron mixtures. Figure 17 illustrates the molecular interaction of flavone and iron. Flavone's fluorescent intensity decreases and shifts to higher wavelength (red shift), upon addition of either the free or chelated forms of iron. Collectively, the decrease and the red shift represent an energy exchange between flavone and iron, consistent with the hyperchromic shift observed in the absorbance spectra.

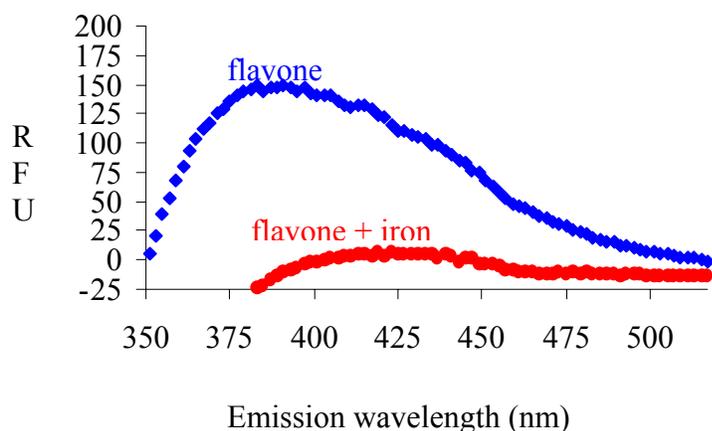


Figure 17. Decrease in Flavone's Fluorescence Spectrum with Iron Addition. Molar ratio is 1:1 (100 μ M flavone and 100 μ M iron). Flavone's spectra $\lambda_{ex} = 300$ nm is quenched and shifts towards the red spectrum. These changes illustrate exchanges of energy.

Flavone associates with iron and DNA in experiments designed to look at individual interactions. However, is flavone able to interact with both DNA and iron within the same solution? The answer to this question would provide further evidence that flavone uses multiple mechanisms to decrease hydroxy radical-induced oxidative damage. The following experiment examines the changes in flavone's spectral pattern with the addition of DNA and subsequent addition of iron.

Flavone and DNA/Iron Interactions

To determine whether flavone interacts with both iron and DNA, the spectra of 100 μ M flavone was monitored for changes (Fig. 18). With the addition of 300 μ M DNA, flavone's spectra decreases as in previous data. However, with the addition of iron to the flavone/DNA mix, flavone's spectra shifts toward the red spectrum and increases. This decrease indicates that flavone continues to interact with iron at DNA : flavone saturation ratios. The interaction between flavone/DNA and flavone/iron may not be independent antioxidant mechanisms. Flavone may be intercalating DNA to prevent radical-induced nicks and at the same time interacting with iron to slow the production of free radicals and prevent the iron/DNA binding that brings the radical generating system close to the DNA.

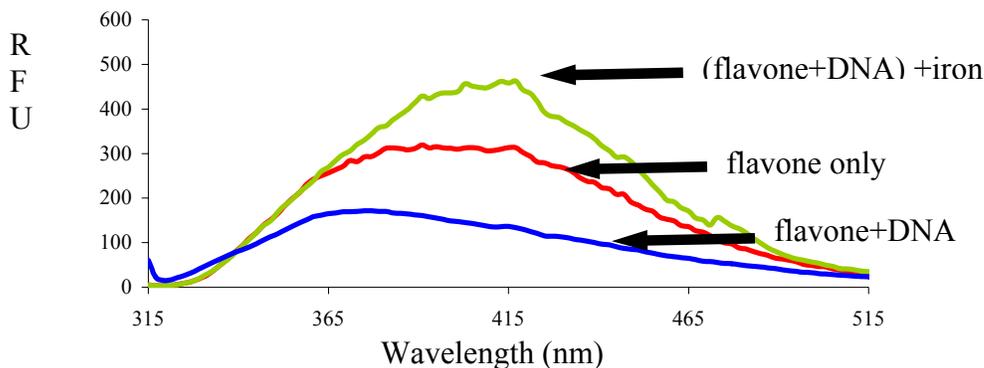


Figure 18. Fluorescence Spectra of Flavone with the Addition of DNA and Iron. Plot illustrates that intercalated flavone may still react with iron. Measurements of flavone's fluorescence (100 μM) were taken after the addition of DNA (300 μM) and iron (100 μM). Flavone's fluorescence decreased with the addition of DNA but subsequently increased with the addition of iron. Excitation = 300nm Emission = 315-515nm.

Collectively, the previous results indicate that flavone's antioxidant mechanism includes interactions with DNA and iron. However, does flavone interact directly with the hydroxy radical. As previously stated, many flavonoids are able to neutralize free radicals. These flavonoids are structurally similar to flavone but have hydroxyl group substituents about their ringed structures. Flavone has no such substituents and should not efficiently scavenge free radicals. To verify that flavone is not an efficient free radical scavenger, the following competition assay was performed. The study compares flavone's hydroxy radical scavenging ability against the strong radical scavenger DMSO. Flavone and Proxyl Fluorescamine

The radical scavenging activity of flavone was determined by monitoring changes in the fluorescence spectra of proxyl fluorescamine. The stable methyl radical intermediate formed when DMSO neutralizes free radicals interacts with proxyl fluorescamine to increase the latter's fluorescence. The Fenton reaction mixture of 100 μM Fe^{2+} , 100 μM DTE, and 5.15 μM H_2O_2 produces hydroxy radicals. After 10 minutes, the reaction was the titrated with DMSO and proxyl fluorescamine. Simultaneously, 125 μM flavone was added to a duplicate set of reactions to determine if flavone could scavenge radicals more

efficiently than DMSO. Figure 19 illustrates that upon addition of flavone, at a 1:1 flavone:DMSO ratio, there is no change in the spectra of PF. This indicates that flavone does not compete with DMSO for free radical scavenging at concentrations proportional to those in the SNCC assay. A decrease in fluorescence did occur when flavone was in excess of DMSO. DMSO is not usually present in the Fenton reaction or in cells. Therefore, flavone may scavenge free radicals but higher concentrations in this assay are required to observe its effects.

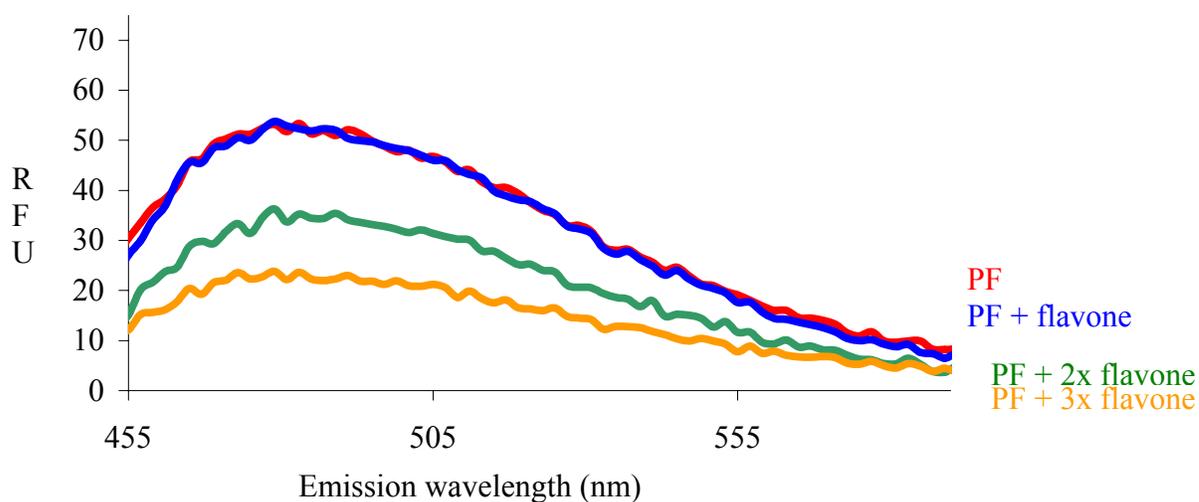


Figure 19. Flavone vs. DMSO: A Radical Scavenging Competition Study. Fluorescence spectra of 0.5 μM proxyl fluorescamine upon addition of flavone (1x = 125 μM , 2x = 250 μM , 3x = 375 μM) at 1:1, 2:1, and 3:1 flavone : DMSO ratios. The plot shows no decrease in PF fluorescence with the addition of flavone at a 1:1 flavone : DMSO (125 μM) ratio. Data indicates that flavone is not an efficient free radical scavenger at these concentrations.

CHAPTER 4

DISCUSSION

Numerous *in vivo* and *in vitro* studies document the antioxidant activities of flavonoids². These compounds are natural antioxidants that reduce the damaging effects of free radicals. In biological systems, the antioxidant activities of flavonoids are well defined. Flavonoids scavenge free radicals, chelate transition metals, and bind the oxidizable substrate to prevent oxidative damage. The molecular interactions of flavonoids are not well defined. A wide range of theories surround the specific molecular relationships that exist between flavonoids and their prevention of free radical oxidation. Previous studies illustrate the interaction of flavonoids and transition metals²⁰. Quercetin and rutin reduce ferric iron to ferrous. This activity was illustrated by monitoring the spectra of an absorbant dye that binds ferrous iron. When quercetin or rutin were combined with ferric iron, the absorbance intensity due to ferrous dye increased. Therefore, quercetin and rutin were found to effectively reduce ferric iron to the ferrous state. This study and others have established a relationship between certain flavonoids and transition metals²⁰⁻²².

Past studies also described the free radical scavenging activity of flavonoids⁸. These studies rank flavonoids based on their ability to scavenge free radicals. They suggest that the anti-radical activity of flavonoids increases with the number of hydroxyl group substituent attachments at specific positions on the rings⁷ (Fig. 2). Rutin and quercetin ranked 6th and 7th, respectively, while flavone was 25th out of a total 29 flavonoids. Flavonoids also have been evaluated in DNA oxidation studies. Quercetin promotes iron-dependent DNA oxidation by reducing complexed iron and also intercalates into DNA to independently cause DNA cleavage^{5,17,23}. *In vivo*, 25 uM quercetin induced cleavage of the myelogenous lymphoblastic leukemia gene, a concentration 4-8 times lower than the amount of flavone or rutin needed to produce similar *in vivo* damage. Additional studies showed that quercetin is mutagenic and damages DNA⁵.

In contrast, many studies cite the DNA-protective effects of flavonoids. Jagetia et al,²⁴ found that naringin, a highly substituted flavonoid, was able to prevent chromosomal aberrations in mouse bone marrow. Studies such as the ones cited here give an overview of flavonoid activity. However, they do not give a detailed account of flavonoid mechanisms, possible molecular interactions, and overall antioxidant effects within a specific system. Further, these studies fail to describe the activity of a simple flavonoid, data that could be used to determine the effect of substituent attachments. This thesis attempts to resolve this issue. These flavonoids, flavone, quercetin, and rutin, were characterized by their ability to protect DNA from hydroxyl-radical induced damage. The molecular interactions and corresponding mechanisms of antioxidant activity were extensively characterized for flavone.

Does Flavone Scavenge Free Radicals?

Free radical scavenging is one of the most commonly studied antioxidant mechanisms. Many studies characterize antioxidant activity solely on the ability of a compound to neutralize free radicals. The radical scavenging activity of flavonoids has been reported to be dependent upon the number and position of hydroxyl groups on their ringed structures. Quercetin is a highly effective radical scavenger. Husain et al.⁸ report that, relative to the control quercetin scavenges 48% of the hydroxyl radicals present in solution. This study also reported that flavone was not an efficient scavenger. Flavone only scavenged 4% of the hydroxy radicals. Figure 19 confirms this finding. Flavone did not decrease the fluorescence of PF when added to the reaction series. Hydroxylation of flavonoids is necessary for effective radical scavenging. Therefore, flavone must use other molecular interactions to prevent DNA oxidation.

How Does Flavone Protect DNA from Oxidative Damage?

Flavone has minimal modifications from the rudimentary flavonoid structure (Fig. 2B). Flavone bears only the addition of a carbonyl group at C₄ and a C₂-C₃ double bond and is the primary backbone of nearly all flavonoids. Past studies suggest that flavone's lack of substituent attachments prevents high antioxidant efficacy. Many of these studies were based on the radical scavenging ability of flavonoids. As discussed above, flavone

does not efficiently scavenge free radicals. However, in the SNCC assay, flavone reduces hydroxyl radical-induced DNA damage by 91%. In contrast, quercetin exhibits pro-oxidant activity (AO = -71%) when dissolved in NDSB-195 and only 48% antioxidant activity when dissolved in DMF. Quercetin's glycosidic analog rutin neither enhances nor prevents DNA oxidation. Therefore, adding hydroxyl groups at specific positions on flavone to yield quercetin has a negative effect on antioxidant activity as measured in the SNCC assay. Quercetin binds iron, but also intercalates into DNA²⁵. Iron-bound quercetin that intercalates DNA may bring the hydroxy radical generating system closer to the phosphodiester backbone. Iron has 6 electrochemical coordination sites. Consequently, unless quercetin is able to bind all the sites, iron can still react with H₂O₂ to promote radical-induced DNA damage. Additionally, intercalated and iron-bound quercetin cannot scavenge free radicals as effectively as it would free in solution. If structural additions to flavonoids are of such consequence, the specific molecular interactions of flavone are integral to understanding the differences in flavonoid activity. The simplicity of the compound and its activity provides the primary foundation for more complex studies. In the SNCC assay flavone's molecular interactions appear to be limited to iron and DNA associations. Flavone is planar, hydrophobic, and electron rich. These structural properties hint that flavone may intercalate into DNA and interact with iron to prevent hydroxy radical-induced damage.

Does Flavone Interact with Iron to Reduce Hydroxy Radical Generation in the Fenton Reaction?

The SNCC assay is based on the generation of hydroxy radicals by the reaction of Fe²⁺ with H₂O₂ (Fenton chemistry). Redox cycling of the iron (Fe²⁺ to Fe³⁺) is needed for the reaction to proceed²⁶ (Fig. 1). Flavonoids that interact with either form of iron reduce redox cycling and, as a result, the production of hydroxy radicals. By inhibiting the reaction machinery, flavonoid/iron associations should correlate with a reduction in DNA oxidation. Though this reduction is not apparent in quercetin studies, flavone/iron interactions contribute to flavone's antioxidant effect. As illustrated in Figure 12, flavone interacts with either Fe²⁺ or Fe³⁺. Flavone's absorbance intensity increases (hyperchromic effect) with the addition of iron, indicating that flavone is undergoing an

electronic transition. Specifically, it signifies the promotion of an electron to a higher energy state²⁶. A flavone/iron interaction of this type may reduce iron availability for the Fenton reaction. If flavone associates with Fe^{2+} or Fe^{3+} , redox cycling in the Fenton reaction would occur at a slower rate. Additionally, if flavone reacts with both forms simultaneously, the radical generating system would suffer. In the SNCC assay the concentration of flavone is 5 times greater than iron. As seen in Figure 17, at a 1:1 flavone : iron ratio, flavone's fluorescence dissipates and shifts towards the red spectrum. The decrease and red shift results from an energy transfer between flavone and iron¹². The exchange reduces the energy available for flavone to fluoresce. Therefore, at SNCC assay concentrations, flavone would sufficiently react with iron to reduce the production of hydroxyl radicals. In either case, flavone's association with iron contributes to its overall DNA protective effect.

Does Flavone Intercalate DNA?

The structural conformation of flavone is characteristic of a classical DNA intercalator²⁷. Flavone is a planar, electron-rich, hydrophobic compound. As a planar ring system, it could stack with the DNA base pairs and protect the phosphodiester backbone from oxidative damage. Additionally, flavone intercalation may cause extension of the DNA duplex. In the presence of DNA, the absorbance spectrum of flavone decreases. This hypochromic shift indicates flavone helical ordering of flavone in the DNA helix¹². The limitation on flavone's molecular movements causes a decrease in its ability to absorb light energy. A classic example of a similar hypochromic shift is the decreased absorbance of DNA bases upon double helix formation from random coils. The hypochromic effect seen in absorption studies is repeated in fluorescence. Flavone's fluorescence intensity also decreases with the addition of DNA. However, the fluorescence spectrum is also characterized by a blue shift. Intercalators are characterized by shifts towards the blue spectrum due to their movement. Ethidium, a common intercalator, experiences the same type of shift in the presence of DNA. Flavone intercalation also may cause expansion of the DNA helix. Figure 15 illustrates a DNA (bp) : flavone saturation ratio of 3.5:1. This indicates 1 flavone molecule intercalates every 3rd to 4th base pair on average. In comparison, one molecule of

ethidium intercalates every fourth DNA base pair (data not shown). Charge (+) repulsion prevents the ethidium molecules from proximal intercalation. However, when flavone intercalates DNA, it expands the DNA helix that could reduce ethidium's charge repulsion. As a result more sites become available for ethidium intercalation. Ethidium does not fluoresce significantly at 605 nm unless it is intercalated¹⁹. Upon intercalation, ethidium's fluorescence increases by ~25 fold. Ethidium's fluorescence does not significantly change in the presence of a 1:3 DNA : flavone premix. However, as ethidium approaches the 4:1 DNA/ethidium saturation ratio, its fluorescence intensity is reduced. This indicates that the intercalated flavone is affecting ethidium's intercalation. Beyond the 4:1 DNA : ethidium saturation ratio, there is an increase in ethidium fluorescence. This increase suggests that flavone's intercalation may stretch the DNA helix and allow ethidium to bind DNA at additional sites. Therefore, in the presence of a flavone/DNA premix, the intercalation of ethidium and flavone at their DNA saturation ratios, may lengthen the DNA helix and allow for increased ethidium intercalation.

Are Flavone/DNA or Flavone/Iron Premixes More Effective?

The SNCC assay can be modified to determine if flavone/DNA or flavone/iron premixes increase DNA protection. As illustrated by fluorescence and UV-Visible spectroscopy, flavone interacts with both DNA and iron to prevent hydroxyl radical-induced DNA damage. Under normal assay protocol, DNA is exposed to the Fenton reagents and flavone simultaneously¹⁶. However, premixes of flavone/iron and flavone/DNA illustrate that flavone intercalation of DNA is a more effective mechanism than the interaction between flavone and iron. The simultaneous addition of flavone and Fenton reagents to DNA offers a 90% DNA protection. This protection increases to 96% if flavone and DNA are premixed before Fenton exposure. This indicates that flavone's intercalation into DNA yields better DNA protection if the 2 molecules are pre-incubated. In contrast, a premix of flavone and iron does not increase DNA protection. The flavone/iron premix reaction is less effective than the simultaneous addition of flavone and Fenton reagents to DNA. The premix afforded 80% DNA protection, 11% and 16% less than simultaneous flavone addition or the flavone/DNA premix reactions, respectively. However, as indicated in the flavone/iron fluorescence studies, flavone may

undergo an energy exchange when reacting with iron. This interaction may reduce the availability of flavone for DNA intercalation. If the structural conformation of flavone no longer favors DNA intercalation, DNA protection would be reduced. These premises illustrate how flavone's direct molecular interaction with DNA and with iron contribute to the prevention of hydroxyl radical-induced damage.

Is Flavone's Antioxidant Mechanism the Collective Effect of DNA and Iron Interactions?

Fluorescence spectroscopy indicated that intercalated flavone is continuing to interact with iron. The initial decrease and blue shift in flavone's fluorescence upon DNA addition indicates intercalation. The subsequent addition of iron to the DNA /flavone mix causes flavone's spectra to significantly increase. The interaction occurring between flavone/DNA and flavone/iron may be a primary mechanism of flavone's antioxidant activity. In this experiment, flavone intercalated into DNA and then interacted with iron. If this occurs flavone then has a triplicate mechanism to defend against hydroxy radical-induced DNA damage. Firstly, flavone could intercalate DNA to prevent the free radicals from extracting an electron from the DNA backbone causing nicks. Secondly, because intercalated flavone may still react with iron, it could slow the production of free radicals. Thirdly, flavone could prevent the iron from binding DNA and delivering the free radical generating system to the DNA. This type of defense would effectively protect the DNA from an iron-dependent radical reaction series.

With the proposed mechanism in place, it is important to characterize flavone's interaction with other flavonoids. The data suggest that flavone interacts directly with both quercetin and rutin. These interactions must be characterized to elucidate other mechanisms flavone may use to prevent free radical damage when other flavonoids are present in solution.

Does Flavone Interact with Other Flavonoids?

As previously stated, flavonoids exist ubiquitously in fruits and vegetables. Their combined antioxidant activity is directly related to molecular interactions between flavonoids. The metabolism of flavonoids is not well understood. Through digestion,

flavonoids can undergo glycosylation or deglycosylation, methylation, and reductions. If a highly substituted flavonoid is broken down into its less-substituted counterparts, their interactions may contribute to an overall antioxidant effect. Flavone's absorbance intensity increases in the presence of rutin. This hyperchromic shift relates to an electronic transition between the 2 flavonoids. Further, the increase illustrates the transition of the vibrational energy level associated with flavone's electronic state. Flavone's absorbance intensity decreases with the addition of quercetin. The hypochromic shift represents stacking of the 2 flavonoids within the solution. As a result, flavone is losing (*i.e.*, sharing) energy and therefore moving towards a lower energy orbital. The structural differences between quercetin and rutin may cause differences in their molecular interactions with other flavonoids. However different, these moieties are interacting with flavone and this interaction may contribute to the *in vivo* antioxidant activity of flavonoids²⁸.

Summary

Cellular DNA oxidative damage is a consequence of hydroxy radical generation in the cell. The type and degree of damage result in a variety of degenerative diseases including cancer. Traditional medicines are combatants against these diseases. However, due to their potentially harmful side effects, use of these types of therapies is limited in western cultures. Alternative and complementary medicinal therapies could potentially extend prevention and treatment of disease well beyond the limits of traditional western medicine. Flavonoid antioxidant activity is an example of such progress. The molecular interactions of flavonoids are responsible for their antioxidant activity. The basic flavonoid flavone is a model compound that could undergo modification reactions to produce more highly substituted flavonoids, and some of these reactions may be reversed through metabolic processes after ingestion. When highly substituted flavonoids such as quercetin are digested, they may be reduced to simpler flavonoids. Therefore, it is important to understand the molecular interactions of the simplest flavonoid to compare the effects of addition and reduction of substituents in relation to antioxidant activity.

Flavone prevents oxidative DNA damage by 91%. Further protection is achieved (96%) by premixing flavone and DNA. This protection is the cumulative effect of flavone's interaction with DNA and with iron. Flavone is an intercalator and this property appears to prevent hydroxy radicals from nicking the DNA backbone.

Flavone also interacts with other common flavonoids. Flavone and quercetin provide collective antioxidant protection. The distribution of flavone in nature makes this finding significant. As previously stated, flavonoids are ubiquitous in fruits and vegetables. Additionally, they also are sold as individual extracts in dietary supplements. Therefore, how these flavonoids react within the same system is of equal importance.

In conclusion, this thesis has shown that flavone is an antioxidant in an iron-dependent hydroxy radical generating system. The suggested mechanistic features and molecular interactions of flavone are DNA intercalation, iron association, and collective antioxidant activity with other flavonoids to prevent hydroxy radical-induced DNA damage.

Future Studies

The data presented in this thesis are a precursor to further intercalation and cellular studies. These results indicate that flavone intercalates DNA at a 3.5:1 DNA : flavone saturation ratio. Additional studies are needed to clearly characterize flavone's interaction with DNA. Some intercalators are base sequence specific²⁹. Other DNA binding molecules may intercalate within the major or minor groove¹⁶. To localize flavone's intercalation of DNA, these base sequence or major/minor groove specific intercalators could be incorporated into a competition study similar to the ethidium/flavone fluorescence assay outlined previously. Future studies should also address mutagenesis. Can flavone prevent mutations of the DNA bases? Preliminary studies indicate that exposing DNA to the Fenton reagents causes base mutations. If flavone is added to the DNA before Fenton exposure, the number of mutations significantly decreases. These studies have begun to define the activity of flavone beyond its molecular interactions. Future studies can evaluate the benefits of flavone and

Fenton component premixes with respect to decreased mutagenesis. These and other studies can use the basic molecular information presented in this thesis to evaluate flavone in more complex biochemical systems.

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