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A Comparitive Study on Antioxidant Activity of Flavonoids: Structure-Activity Relationships

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ABSTRACT In recent times flavonoids received greater attention as a group of "bioactive" compounds because of their potent antioxidant activities. In the present study, the antioxidant activities of several flavonoids have been examined using DPPH and ABTS-free radical scavenging assays. In addition, the ABTS-radical cation scavenging activities of flavonoids were compared with that of vitamin E (Trolox, TEAC). Flavonoids inhibited DPPH-free radicals with varying potency, from highest to lowest: quercetin, fisetin, baicalein, rutin, hesperetin, naringenin, and chrysin. Against the ABTS-free radicals, the order of potency of the flavonoids is (highest to lowest, in brackets TEAC values) quercetin (3.154), rutin (3.032), naringenin (2.686), fisetin (2.578), baicalein (2.237), hesperetin (2.155), chrysin (2.141). Both antioxidant assays demonstrated the free radical scavenging capabilities of flavonoids, and the TEAC values suggest higher potencies for flavonoids compared to vitamin E. The results also suggest that number of hydroxyl substitutions, presence of sugar substitutions and presence of double bond at C-2 and C-3 positions on the basic ring skeleton of the flavonoids are important determinants for antioxidant activity.

Key Words: flavonoids, free radicals, antioxidant, structure-activity relationships

INTRODUCTION

Flavonoids are polyphenolic compounds found ubiquitously in nature and are categorized according to their chemical structure (C_6-C_3-C_6) into different subclasses [1-2]. The main flavonoid subclasses include flavonols, flavones, and flavanones [1-2]. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). In recent times flavonoids have received considerable interest as "bioactive" compounds because of their potential beneficial effects on human health [3]. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and cardiovascular protective activities [4]. Most of these biological activities of the flavonoids are attributed to their potent antioxidant and free radical scavenging activities [4-5]. Flavonoids may provide health promoting effects by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body. The contribution of the flavonoids to the endogenous antioxidant defense system may be substantial considering that the total daily intake of flavonoids can range from 50 to 800 mg, compared to the average daily intake of other dietary antioxidants, such as vitamin C (70 mg), vitamin E (7-10 mg) or carotenoids (2-3 mg). The antioxidant capacity of flavonoids depends upon their chemical structure [6]. The position of hydroxyl groups and other substitutions in the basic ring skeleton of flavonoids are important determinants for their antioxidant and free radical scavenging activities. Antiradical (antioxidant) activity of flavonoids has been attributed to their electron-donating ability; hence changes in the chemical structure of the flavonoids have dramatic effects on their antioxidant activity [6]. In this study, several flavonoids, representing different subclasses of flavonoids, were analyzed and compared for their antioxidant activities using DPPH- [7-8] and ABTS- [9-10] free radical scavenging assays and possible antioxidant structure-activity relationships among different flavonoids were determined. Further, ABTS-free radical scavenging activity of flavonoids was compared with that of Trolox (vitamin E).

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picryl-hydrazyl, 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid, potassium pyrosulafate, Trolox (vitamin E), and phosphate buffered saline sachets were purchased from Sigma. The flavonoids tested in this study were purchased from Fluka Chemicals and from Sigma Chemicals.

DPPH-assay

The DPPH-assay measures the electron or hydrogen donating activity of antioxidant substances and hence provides a measure of free radical scavenging antioxidant activity of respective substance. This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical 1, 1-diphenyl-2-picryl-hydrazyl ($DPPH^*$). $DPPH^*$ is a purple-colored stable free radical and in the presence of antioxidant substance, which can donate an electron, $DPPH^*$ was reduced to the yellow-colored diphenylpicrylhydrazine. $DPPH^*$-solution (0.2 mM) was prepared by dissolving 7.886 mg of 1, 1-diphenyl-2-picrylhydrazyl molecules in 100 ml of methanol. The flavonoid solutions were prepared in stock (10 mM) in methanol and on the day of experiment the stock solution was serially diluted with methanol to the range of 0.1 μM – 10 mM. The UV absorbance of DPPH-free radicals (2.9 mL of DPPH solution in methanol) at 517 nm in the presence of different concentrations of the flavonoids (total volume 0.1 mL) was measured spectrophotometrically. The absorbance was read against blank prepared in identical way but without the addition of flavonoid solution- 2.9 mL of DPPH solution + 0.1 mL of methanol. Substrate blank readings (2.9 mL of methanol + 0.1 mL of flavonoid solution) were also recorded for each concentration. These readings were deducted from the absorbance obtained in the presence of DPPH.

ABTS-assay

The ABTS assay is based on the inhibition by antioxidants of the absorbance of the ABTS-free radical cation ($ABTS^{*+}$), which has a characteristic long-wavelength absorption spectrum showing maximum absorption maxima at 415 nm. $ABTS^{*+}$ was produced by incubating 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS, 7 mM) with 2.45 mM potassium pyrosulafate in purified water and allowing the mixture to stand in the dark at room temperature for 24 hrs before use. Following the 24 hrs reaction period the $ABTS^{*+}$ working solution (35 μM) was prepared by diluting the stock solution of $ABTS^{*+}$ with phosphate buffered saline (PBS, pH 7.4). The flavonoid solutions were prepared in stock in methanol and on the day of experiment the stock solution was serially diluted with methanol to the range of 1 μM – 1 mM. The flavonoid solutions, at different concentrations (total volume of 20 μL) were added to 180 μL of $ABTS^{*+}$ solution and complete mixing of reactants was achieved by bubbling three to four times using plastic pipettes. After 4 min of the incubation at room temperature the UV absorbance of $ABTS^{*+}$ at 415 nm was measured spectrophotometrically. The absorbance was read against blank prepared in identical way but without the addition of flavonoid solution (180 μL $ABTS^{*+}$ + 20 μL methanol). Substrate blank readings (180 μL PBS+ 20 μL flavonoid solutions) were also recorded for each concentration. These readings were deducted from the absorbance obtained in the presence of $ABTS^{*+}$.

Data Presentation and Statistical Analysis

The DPPH-and ABTS-free radical inhibitory activities of various flavonoids were given as mean ± SEM of 4 or 5 determinations. And the observed differences among inhibitory activities of various flavonoids were tested for statistical significance using student's t test, and 'p' value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The antioxidant activities of several flavonoids (Table 1), representing different subclasses of falvonoids, were measured by means of $DPPH^{\cdot}$ and ABTS-free radical scavenging assays. Our results showed that all the flavonoids tested concentration-dependently inhibited both DPPH-, and ABTS free radicals (Fig.1), but with a higher sensitivity towards later. At the maximum concentration tested flavonoids inhibited DPPH-free radicals with a varying potency (in brackets % maximum inhibition): baicalein (81.29 ± 2.27), chrysin (4.83 ± 0.07), fisetin (80.33 ± 0.04), hesperetin (37.86 ± 0.33), naringenin (11.61 ± 0.75), rutin (79.39 ± 0.29), and quercetin (88.01 ± 0.41). However, in ABTS-assay at the maximum concentration tested, the flavonoids inhibited $ABTS^{*+}$ - free radicals with a potency order (from

higher to lower, in brackets TEAC values) quercetin (3.154), rutin (3.032), naringenin (2.686), fisetin (2.578), baicalein (2.237), hesperetin (2.155), and chrysin (2.141).

The results of this study demonstrated that among the flavonoids tested quercetin (3, 5, 7, 3', 4'-pentahydroxy flavone) with five hydroxyl (-OH) substitutions on its basic ring skeleton showed the highest antioxidant activity suggesting that presence of –OH substitutions on the basic ring skeleton of flavonoids was an important determinant of the antioxidant activity of falvonoids [6, 11-12]. Compared with its parent molecule, quercetin, rutin with four –OH substitutions (5, 7, 3', 4') and one sugar substitution at C-3 position on its basic ring skeleton showed significantly lesser antioxidant activity, suggesting that presence of sugar substitution on the flavonoid basic ring skeleton attenuates the antioxidant activity [6, 11-12]. Moreover, fisetin with a similar structure to quercetin except for the absence of –OH substitution at C-5 position showed significantly lesser activity than quercetin, thus supporting our previous observation that antioxidant activity of flavonoids depends on the number of –OH substitutions present on the basic ring skeleton.

The results of this study showed that baicalein, 5, 6, 7-trihydroxy flavone, showed much higher activity than its structural analogue, chrysin (5, 7-dihydroxy flavone). Thus, suggesting that presence of –OH substitution at C-5 position or presence of three continuous –OH substitutions on ring A of flavonoid basic ring skeleton improve their hydrogen or electron donating (antioxidant) abilities. Further, the flavanones (naringenin and hesperetin) tested in this study showed considerably lower antioxidant activity compared to that of flavonols (fisetin, rutin, quercetin), suggesting that presence of double bond between C-2 and C-3 positions of ring C in the basic ring skeleton of flavonoids was an important determinant in their antioxidant activities [6, 11-12].

CONCLUSION

In conclusion, the results of this study showed that flavonoids concentration-dependently inhibited free radicals produced in DPPH, and ABTS-assays, with a higher specificity for later. Number of hydroxyl substitutions, presence of sugar moiety, and unsaturation at C-2 and C-3 positions on the basic ring skelton of flavonoids are the major detriments of antioxidant capacity offlavonoids.

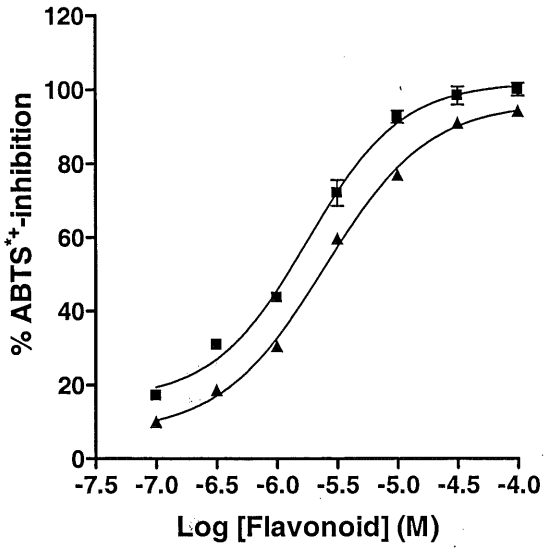
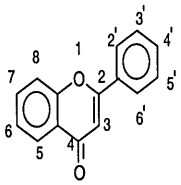
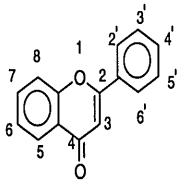
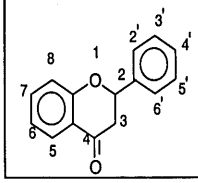


Figure 1: Representative diagram showing concentration-dependent inhibition of ABTS*+ -free radical cation by flavonoids quercetin (■) and naringenin (▲) in ABTS-assay. Symbols represent mean +/- SEM of 6 measurements.

Table 1: Chemical structures of the flavonoids

Generic Structure	Flavonoid	Hydroxylation pattern							
		3	**4**	**5**	**6**	**7**	**3'**	**4'**	**5'**
Flavonol									
	Fisetin	*				*	*	*	
	Quercetin	*		*		*	*	*	
	Rutin	OR_1		*		*	*	*	
Flavone									
	Baicalein			*	*	*			
	Chrysin			*		*			
Flavanone									
	Naringenin			*		*		*	
	Hesperetin		-CH3	*		*	*		

R_1= rutinoside

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