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Comparative Study of the Antioxidative Potential of Common Natural Flavonoids and Isoflavonoids

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The half maximal inhibitory concentration (IC_{50}) values and trolox equivalent antioxidant capacity (TEAC) values were calculated by a 2,2'-diphenylpicrylhydrazyl $(DPPH)^{\cdot}$ assay and a 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid $(ABTS^{+\cdot})$ assay, in order to determine the antioxidative activities of the compounds. On the basis of the DPPH assay, quercetin had the strongest antioxidative potential of the flavonoids, followed in order by fisetin, 7,8-dihydroxyflavone, morin and kaempferol. Quercetin, fisetin and 7,8-dihydroxyflavone had higher antioxidant potentials than butyl hydroxyl anisole. Quercetin had the highest TEAC value amongst the flavonoids and isoflavonoids, followed in order by 3-hydroxyflavone, fisetin, 7,8-dihydroxyflavone and morin. Comparatively, isoflavonoids were found to have significantly weaker antioxidative potential than the flavonoids.

Keywords: $ABTS^{+\cdot}$ assay, antioxidant potential, DPPH assay, isoflavonoids, flavonoids

Oxygen (O_2) gets reduced to oxygen-derived free radicals such as superoxide, hydrogen peroxide, hydroxyl, and nitric oxide radicals during the normal physiological and metabolic processes in the human body [4, 18]. These reactive oxygen species (ROS) generate oxidative stress in the body [9] which affects cell proteins, lipids and carbohydrates, leading to a number of physiological disorders, cancer, cardiovascular disorders, and neurological disorders, and is involved in the process of aging [11]. Many plants often contain substantial amounts of antioxidants such as vitamin C and E, carotenoids, flavonoids and tannins to neutralize excess ROS. These natural compounds can therefore be utilized to scavenge those free radicals from the human body. Antioxidants are widely used as food additives to provide protection against oxidative degradation of foods. Since ancient times, spices added to different types of food to improve flavors have also been recognized for their antioxidant capacities [10]. In order to prolong the storage stability of foods, synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are used for industrial processing. However, these compounds are inappropriate for chronic human consumption.

Flavonoids, plant secondary metabolites, are most commonly known for their antioxidant activities. The antioxidant ability of flavonoids is stronger than other commonly found natural products. Thus, consumers and food manufacturers have become interested in flavonoids for their possible industrial applications, medicinal properties, and especially their putative role in the prevention of cancers, Parkinson's diseases, Alzheimer's diseases, and cardiovascular diseases. Though the physiological evidence has not been well established on flavonoids and isoflavonoids, the beneficial effects of fruits, vegetables, tea, and red wine have been attributed to these groups of compounds. In recent decades, strong interest has focused on screening essential oils and various plant extracts for natural antioxidants because of their good antioxidant properties. The compounds showing high activity to scavenge free radical could be pharmaceutically crucial to develop as a drug against

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many diseases caused due to the free radicals generated within the body during metabolic processes, for example, Age Related Muscular Degeneration (AMD), Parkinson's Diseases, etc. Moreover, the highly active compounds can be further developed as pharmaceutically dynamic molecules by metabolic engineering approaches. In this context, we have carried out a comparative study of the antioxidant potential of commercially available flavonoids and isoflavonoids by three different assays: a preliminary assay on thin layer chromatography-(DPPH assay on TLC), and two commonly used $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ assays.

Fig. 1. Representative DPPH assay on TLC (Q: Quercetin, K: Kaempferol, F: Fisetin).
Yellow spots indicating positive anti-oxidant activity.

The antioxidant effects for flavonoids and isoflavonoids were preliminarily determined by applying $DPPH^{\bullet}$ free radical solvent in flavonoids/isoflavonoids developed TLC following method of Bektas [1]. The purple color of DPPH reagent bleaching by yellow spots was observed on all standards, as shown in Fig. 1, which is the indication of positive antioxidant activity of the compounds. All the tested flavonoids and isoflavonoids along with reference compounds showed the yellow spot on the TLC sprayed by DPPH reagent. Since, the quantification of the anti-oxidant activity by TLC assay could not be carried out, we have analyzed the antioxidant activity of those compounds by DPPH radical scavenging and ABTS radical cation decolorization assays.

The extent of the decrease in the absorbance of $DPPH^{\bullet}$ in the presence of antioxidants correlates with the free radical scavenging potential of the antioxidant. The free radical scavenging capacities of the compounds measured by $DPPH^{\cdot}$ assay [2] modified by Sanchez-Moreno *et al.* [14] are presented in Table 1. The percentage inhibition of $DPPH^{\bullet}$ (IC_{50} value) was recorded at 30-min incubation at room temperature for varied concentrations of different

Table 1. IC_{50} value and TEAC value of different flavonoids and isoflavonoids along with reference compounds determined by DPPH and ABTS assay respectively.

Compounds		DPPH assay	ABTS assay	
		IC_{50} value (μg/ml)	r^2 value	TEAC value
Flavonoids	Quercetin	55.88	0.9724	2.177
	Fisetin	62.92	0.9933	1.129
	7,8-dihydroxyflavone	84.04	0.9676	1.028
	Morin	168.08	0.9928	1.007
	Kaempferol	179.52	0.9987	0.91
	Hespertin	*	0.9532	0.608
	Chrysin	*	0.9492	0.066
	Apigenin	*	0.9821	0.076
	3-hydroxyflavone	*	0.9819	1.181
	Naringenin	*	0.9759	0.029
Isoflavonoids	BiochaninA	*	0.9846	0.030
	Daidzein	*	0.9336	0.090
	Genistein	*	0.992	0.083
Reference Compounds	Gallic acid	33.33	0.9772	5.454
	Butyl hydroxy anisole	151.03	0.9901	1.388
	Trolox	nd	0.9902	1.00

All the values were determined in triplicate. Notes: nd: not detected, *indicates more than 500 μg/ml.

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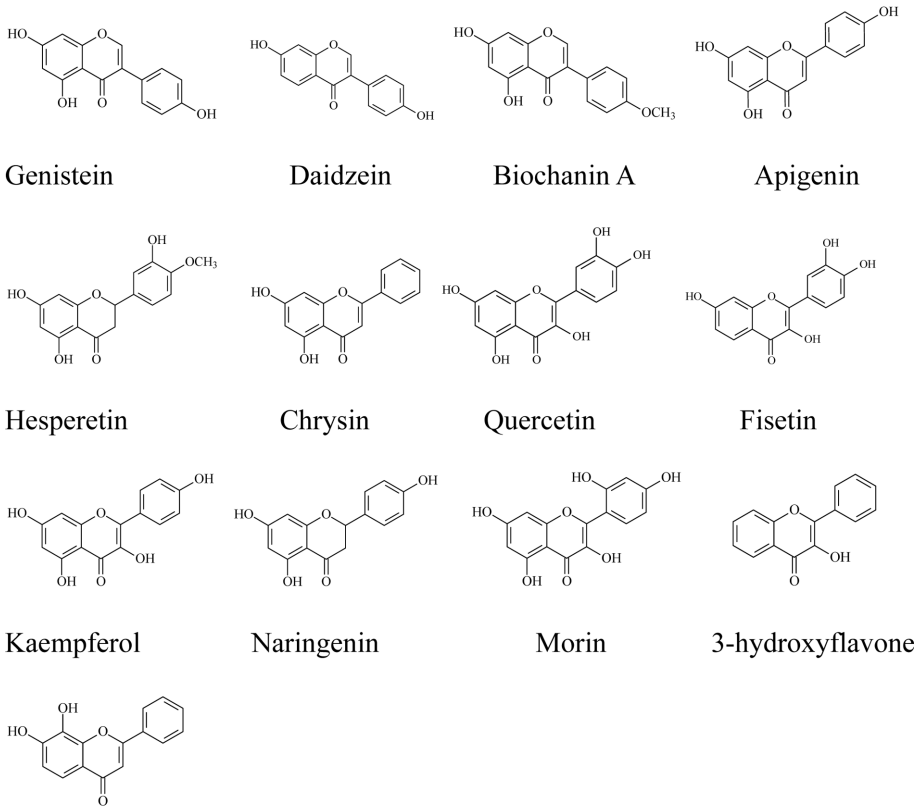

Fig. 2. Structures of flavonoids and isoflavonoids used in this study.

compounds. The IC_{50} value was also checked after different time intervals of incubation time (10 min, 20 min and 30 min) of compounds with freshly prepared DPPH$^{\bullet}$ radical. The inhibition percentage of the DPPH radical reached to maximum and remained constant after 30 min of incubation (data not shown). The determined IC_{50} values were thus compared with a reference compound as well as between the flavonoids and isoflavonoids after 30 min of mixing of reaction mixture with DPPH radical. The reference compound, gallic acid, was the most potent among all the tested compounds. The result also revealed that among all the flavonoids and isoflavonoids used in this study (Fig. 2), quercetin was a highly potent antioxidant with IC_{50} value of 55.88 μg/ml and TEAC value of 2.177. In DPPH assay, quercetin was followed by fisetin and 7,8-dihydroxyflavone, morin and kaempferol. After quercetin, however, 3-hydroxyflavone was stronger in $ABTS^{+\bullet}$ decolorization assay [12], followed by fisetin, 7,8-dihydroxyflavone, morin, kaempferol, and hespertin. All isoflavonoids (biochanin A, genistein, daidzein) and the other flavonoids (apigenin, chrysin, naringenin) had relatively weaker antioxidant potential (Table 1). Their IC_{50} value is higher than 500 μg/ml. The time dependent inhibition of $ABTS^{+\bullet}$ was also determined. The inhibition of $ABTS^{+\bullet}$ was almost constant after 6 min of initial mixing of radical with antioxidant (Fig. 3). Hence, the TEAC value of the compounds were determined by measuring $ABTS^{+\bullet}$ decolorization at 6 min after the mixing of the radical mixture and test compound. The TEAC values were determined from the calibration curve for each substrate and compared with each other, along with Trolox (Table 1). The influences of both the antioxidant concentration and the reaction duration on the inhibition of the radical cation absorption were taken into account when determining the antioxidant activity. The flavonols like quercetin found to have good antioxidant potential because of the formation of intramolecular hydrogen bond as explained by van Acker *et al.* [16]. Quercetin and its derivatives are found to have the highest antioxidant activity [6].

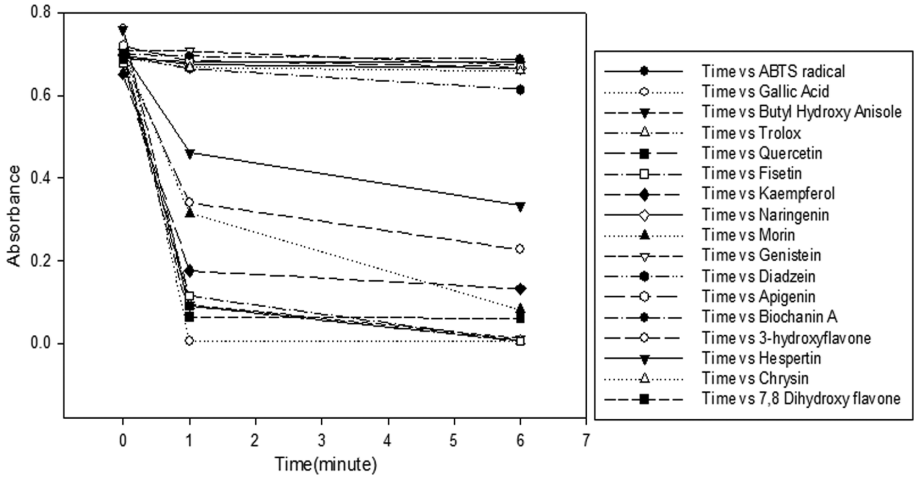

Fig. 3. The effects of the duration of interaction of specific antioxidants on the suppression of the absorbance of the $ABTS^{+}$ at 734 nm.

All flavonoids and isoflavonoids are capable of transferring an electron and a hydrogen atom to the $DPPH^{\cdot}$ radical and the $ABTS^{+\bullet}$ radical, respectively. Although these compounds have a similar structure, the radical scavenging potential of either $DPPH^{\bullet}$ or $ABTS^{+\bullet}$ varies according to the compound. The study of structure-activity relationship has shown the importance of position of the hydroxyl group in radical scavenging activities [5]. The substitution at C-3 position and B rings is crucial. However, the activity is influenced by the substituents at position 5 and position 7 [5]. The modification of flavonol at the 3-hydroxyl position by glycosylation weakened the $ABTS^{+\bullet}$ radical scavenging activities because of the lack of a hydroxyl group and steric hindrance [7], and because the C-3 position glycosylation induces torsion of the molecule [16]. Thus, naringenin lacks a 3-hydroxyl group, which might explain its lesser anti-oxidant potential. Interestingly, 7, 8-dihydroxyflavone, although lacking a hydroxyl group in the C-3 position, showed comparatively better antioxidant properties than naringenin. This could be attributed as described by Heijnen [5] and Rice-Evans *et al.* [13]. Surprisingly, isoflavonoids have weaker activity towards both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals. A study of the structure activity relationship of the compounds is essential for determining the exact reason for the different antioxidant activities of similar compounds. In the $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ tests, the flavonoids showed a similar pattern of antioxidant potential. This result is similar with the result shown by Re *et al*. [12], who also reported a similar order of activity, with quercetin having the highest antioxidant potential among the different flavon-3-ols, flavones, and flavanones. However, in the case of naringenin, our result contradicted that of Re *et al*. [12] who reported a higher activity for naringenin compared to that of kaempferol. But, many flavonoids are found to be strong antioxidants effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups [3]. The compounds which were found to have comparatively higher antioxidant activities (quercetin, kaempferol, rutin, morin, hesperetin, fisetin) could play an important role in improving of oxidative stress [8, 15].

The modification of compounds by different functional groups affected the biological activities of the parent molecules. Flavonoids/isoflavonoids exhibiting high activity to scavenge free radicals might be pharmaceutically efficient for development as a drug against many diseases caused by free radicals and can also be developed as an antioxidant supplement in food ingredients. Moreover, such highly active compounds can be further developed as pharmaceutically dynamic molecules by metabolic engineering approaches such as glycosylation, prenylation, methylation and other diverse means to enhance the pharmacodynamics and pharmacokinetics properties of small molecule-based therapeutics and natural products [17]. The screening of antioxidant compounds that are pharmacologically potent with minimal side effects will be helpful for developing potential therapeutics by enhancing their functional

activities and diminishing their harmful effects.

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국문초록

천연물인 플라보노이드와 이소플라보노이드의 항산화 효과 비교연구. 판데이 라메스 프리사드, 코이라라 니런전, 이주호, 이희찬, 송재경*. *선문대학교 제약공학과*

2,2'-diphenylpicrylhydrazyl (DPPH$^{\bullet}$) assay와 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{+}) decolorization assay는 자연상태의 플라보노이드와 이소플라보노이드의 항산화 활성을 확인하는데 사용된다. 억제중간값(half maximal inhibitory concentration (IC_{50}) values)과 트롤록스당량 항산화능값(trolox equivalent antioxidant capacity (TEAC) values)은 DPPH$^{\bullet}$ assay와 ABTS^{+} assay로 계산되었다. DPPH assay 결과, 쿼세틴(quercetin)은 가장 강한 항산화 능력을 가졌고 뒤이어 피세틴(fisetin), 7,8-디하이드록시플라본(7,8-dihydroxyflavone), 모린(morin), 캠퍼롤(kaempferol) 순이었다. 쿼세틴, 피세틴, 7,8-디하이드록시플라본은 부틸하이드록시 아니솔(butyl hydroxyl anisole)보다 더 높은 항산화 능력을 가졌다. 쿼세틴은 플라보노이드와 이소플로보노이드 중에서 TEAC 값이 가장 높았고 뒤이어 3-하이드록시플로본(3-hydroxyflavone), 피세틴, 7,8-디하이드록시플라본과 모린 순이었다. 다른 나머지 플라보노이드와 이소플라보노이드는 트롤록스 보다 더 약한 ABTS^{+} 분해능력(scavenging potential)을 가졌다. 테스트된 13개 플라보노이드/이소플라보노이드에서 이소플라보노이드는 플라보노이드보다 매우 약한 항산화 능력을 보였다.