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Original Article

Comparing the Effects of Kaempferol, Galangin and Apigenin Flavanoids on Basis of Its Structural Differences in Increasing of Paraoxonase 1 Activity and Attenuating Oxidative Stress Markers in Rats

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Background and Aims: Flavonoids as polyphenolic naturally occurring compounds have antioxidant activity. There is increasing evidence suggests that flavonoids may affect the activity of enzymes. Paraoxonase 1 is calcium - dependent enzyme that is present in high density lipoproteins. This enzyme has an important role in the prevention of low density lipoprotein oxidation. We investigated the effects of kaempferol, galangin and apigenin from two different chemical subclasses of flavonoids on serum paraoxonase 1 activity and stress oxidative parameters in male rats.

Materials and Methods: 40 rats (weighting 250±20 g) were randomly divided into four groups. Each group subdivided into two equal subgroups. Subgroups received a dose of 10 mg/kg or 20 mg/kg of flavonoid. Flavonoids were dissolved in ethanol 10%, and given by oral gavage once a day for two months. After that, paraoxonase activity was measured by spectrophotometric method regarding the amount of para Nitro phenol production at a wavelength of 412 nm. The malondialdehyde and total antioxidant capacities were measured respectively by the thiobarbituric acid and the 1,1-Diphenyl-2-picryl-hydrazyl reduction method.

Results: The results obtained from the direct effect of selected flavonoids on augmentation serum paraoxonase activity and prevention of malondialdehyde production in comparison with the control group was as follows: kaempferol> Galangin > apigenin.

Conclusions: Results confirm that structural differences in C3-OH and number of hydroxyl groups could have been an important role in increasing serum paraoxonase 1 activity and reduction of oxidative stress parameters.

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Introduction

Flavonoids are polyphenolic compounds with over 8000 individual known compounds. They have potential antioxidant activity and multiple reduction capacities, which are due to their ability to reduce free radical formation and to scavenge free radicals [1]. The pharmacological effects of flavonoids consist of anti-inflammatory, cardio-protective and anticancer activities are due to their antioxidant activities [2-4]. Flavonoids exert its antioxidant effects by removing reactive oxygen species during oxidative stress [5, 6]. In recent decades, the potential application of flavanoids has driven the scientists towards using this drug in treating many abnormalities. Flavonoids usually subdivided, according to their chemical structure, into several subclasses including flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones [7]. Flavones such as apigenin (5,7,4'-OH) can be found in celery and parsley, while flavonols such as galangin (3,5,7-OH) and kaempferol (3,5,7,4'-OH) are abundant in tea. These compounds are based on the flavan nucleus, the number, positions, and types of substitutions influence radical scavenging and chelating activity [8-10].

The paraoxonase (PON) enzyme family, comprising PON1, PON2, and PON3, has been shown to protect against oxidative stress, principally in the blood circulation and to inhibit macrophage foam cell formation and atherogenesis [11]. The PON1 protein is synthesized mainly by the liver and attached to high density lipoproteins, it has a crucial role in the prevention of low density lipoproteins oxidation [12]. The PON2 is not released into the serum, but is ubiquitously expressed in cells in nearly every human tissue. PON3, Like PON1, is found in liver cells and in plasma at a concentration of about two orders of magnitude lower than that of PON1. Recent studies on the function of paraoxonase have focused on its possible influence on cardiovascular physiology, lipid metabolism, and potential antiatherogenic actions [13]. Various *in vitro* and *in vivo* studies in animals and humans have provided initial evidence that antioxidants can increase PON1 activity, possibly by protecting the enzyme from oxidative stress-induced inactivation [14].

Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [15]. The antioxidant activity of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [8]. Based on the previous studies, flavonoids compounds with different structures, number and positions of the hydroxyl groups have different antioxidant effects [9, 16]. In this research, the direct effect of kaempferol, galagininand apigenin from two different subclasses with different number of hydroxyl group on serum paraoxonase 1 activity as an antioxidant marker in male rats was studied.

Material and Methods

Kaempferol, Galangin, apigenin and paraoxon were purchased from Sigma Chemical Company, (St. Louis, MO, USA) and the

calcium chloride was purchased from Merck. Experiments were carried out according to the guidelines of animal Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Animals

40 adult male Wistar rats (250±20 g) were bought from the Pasteur Institute, Karaj, Iran. They were maintained in the animal house of Yazd university of medical sciences with free access to standard rat chow diet and tap water. The rats were housed five per polycarbonate cage and were kept under controlled conditions of temperature 22±2°C, a relative humidity of 50-60%, and 12-h light/dark cycles. Rats were fed with water and food. After 2 weeks of acclimation, they were randomized into four groups, three flavonoid treatment groups and one vehicle group. Treatment groups subdivided into two equal subgroups that received one dose (10 mg/kg or 20 mg/kg) of flavonoid in 10% ethanol solution by oral gavage once a day for two months [17-20]. The rats in the vehicle group underwent the same experimental protocol, received the same volume/weight of the vehicle. The treatment doses selected based on Hu, et al. [21] and preliminary study.

Serum paraoxonase activity measurement

Serum paraoxanase activity was measured according to Beltowsky protocol [17]. Briefly, 40 µl of serum was added to 460 µl of cocktail (Tris-HCL 100 mM, Cacl2 2 mM and paraoxon 2 mM pH 8). The amount of hydrolysis of paraoxon to p-nitrophenol was measured using a spectrophotometric UV at a wavelength of 412 nm at 25°C. Paraoxonase activity was expressed as U/L serum.

Lipid peroxidation assay

Lipid peroxidation (as malondialdehyde) level was measured by the method of Buege and Aust [18]. Briefly, 100 µl serum was incubated reagent containing 0.375% thiobarbituric acid, 15% trichloroacetic acid, 0.25 M HCl, and 6.8 mM 2, 6-ditert-butyl-4-methylphenol for 60 min. in a boiling water bath. After centrifugation at 3000 rpm for 15 min. the absorbance of the supernatant was recorded at 532 nm by using 1, 1, 3, 3-tetraethoxypropane as an external standard. The lipid peroxidation was expressed as Malondialdehyde (MDA) in micromoles per liter of serum.

Free radical scavenging capacity assay

The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidative potential of various natural products [19]. Free radical scavenging capacity was measured by adding 20 µl of serum to 380 µl phosphate buffer (pH 7.4) and 400 µl DPPH in methanol. Later, it was incubated for 30 min. at room temperature. Samples absorbance versus blank (we used methanol instead of rat serum) at a wavelength of 520 nm was read with ELISA reader and reduction of DPPH was calculated using the following formula:

$$DPPH\% = [(A - Ax) / A] * 100\%$$

A: absorbance of DPPH "solution with methanol

Ax: absorbance of a DPPH "solution with plasma

Statistical analysis

Analysis of two-way ANOVA was used to compare the means of different flavanoids treatment and two doses of each treatment. Each experiment was repeated separately at least three times. Results are presented as mean ± SEM and significance was accepted at $p<0.05$.

Results

Effect of selected flavonoids on serum paraoxonase activity in treated rats

The effects of a two-month treatment of kaempferol, galangin and apigenin in 10 and 20 mg/kg body weight of rats on serum paraoxanase activity were showed (Fig. 1). All Flavonoid treatments significantly increased serum paraoxanase activity in comparison with the vehicle group ($p<0.05$). The difference between the doses in each treatment group was not significant except in kaempferol treatment group ($p>0.5$). Among tested flavonoids, kaempferol showed the most effect on increasing of serum paraoxanase activity in dose-dependent manners.

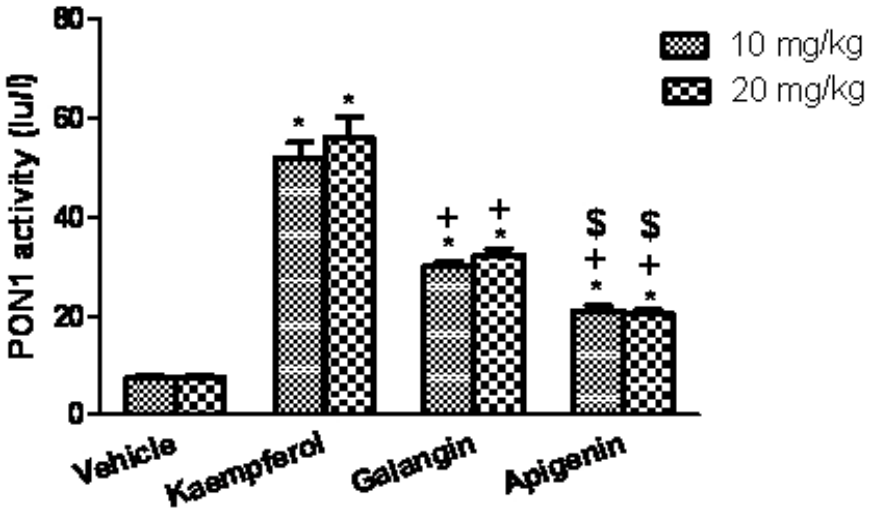


Fig. 1. Serum paraoxonase activity of flavonoid treated groups. All flavonoid treatments augment serum paraoxonase activity in comparison with the vehicle group. Kaempferol showed the most effect ($P<0.05$). * $p<0.05$ compared with vehicle group, + $p<0.05$ compared with kaempfero, $ $p<0.05$ compared with galangin

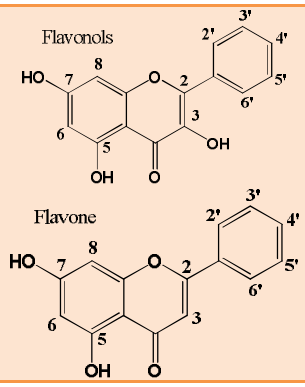
The differences between two doses (10 and 20 mg/kg) of galangin, apigenin and kaempferol were not significant. Results of experiments repeated three times and expressed as mean±Standard deviation(SD).

Effect of selected flavonoids on MDA production in treated rats

To assess the effect of selected flavonoids (Table 1) on lipid peroxidation, the levels of malondialdehyde were measured after two months treatment in rats by oral gavage once a day. All flavonoids (kaempferol, galangin and apigenin) showed inhibitory effects on MDA production, but not in dose-dependent manners (Fig. 2). Among tested flavonoids, kaempferol is more effective in inhibition of MDA

production than others are. There was not any significant difference between the two doses of all flavonoid treatment groups (Results of experiments repeated three times and expressed as mean ± SD).

Table 1. Structure of selected flavonoids: kaempferol (3,5,7,4 '-OH), Galangin (3,5,7-OH) and Apigenin (5,7,4'-OH).

Structure	Name	Substituents 3'	4'	5'
	Kaempferol	H	OH	H
	Galangin	H	H	H
	Apigenin	H	OH	H

Free radical scavenging capacity of flavonoids

To test the effect of flavonoid treatment on the free radical scavenging capacity of serum was used DPPH method. In this test, a solution of 2, 2-diphenyl-1-picrylhydrazyl stable free radical was decolorized after reduction with an antioxidant. Three selected flavonoid increased serum free radical scavenging capacity of treated rats, but the effect of Kaempferol than others flavonoids was greatest. Although 20 mg/kg dose of all flavonoid showed the more effect on serum Free radical scavenging capacity than 10 mg/kg dose, but these differences were not significant in any of flavonoid treatment groups. (Fig. 3)

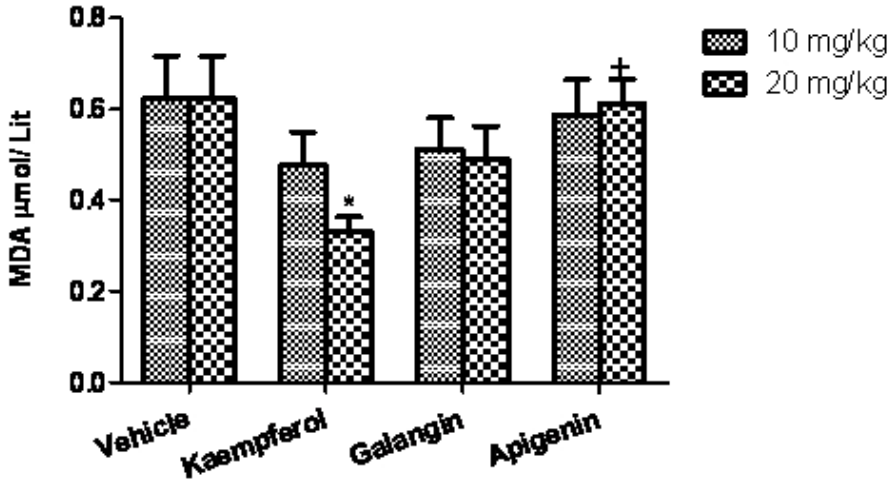


Fig. 2. MDA production in flavonoid treated groups. All flavonoid treatments reduced MDA production in comparison with the vehicle group. The most reduction was seen in kaempferol treated groups. * Flavonoid treated groups compared with vehicle ($p<0.05$), + galangin and apigenin groups compared with kaempfero ($p<0.05$)

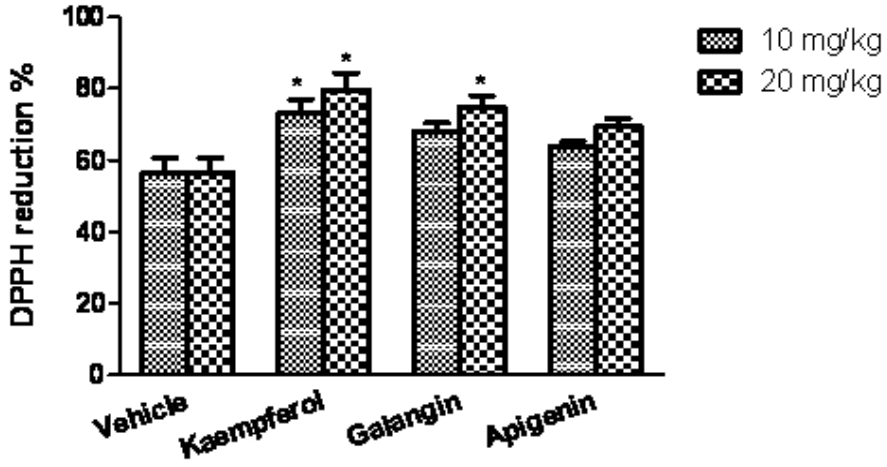


Fig. 3. DPPH reduction in flavonoid treated groups. All selected flavonoid increased serum free radical scavenging capacity of treated rats in comparison with the vehicle group ($p<0.05$), but the significantly increase was seen by kaempferol in two doses and for galangin only in 20 mg/kg. * flavonoid treated groups compared with vehicle ($p<0.05$)

There was not any significant difference between the two doses of all flavonoid in the treatment groups. (Results of experiments repeated three times and expressed as mean±SD)

Discussion

Flavonoids have beneficial effects in a multitude of disease states, including cardiovascular disease, cancer, and neurodegenerative disorders. Many of the biological actions of flavonoids have been attributed to their antioxidant properties, but the precise mechanisms by which flavonoids exert their beneficial or toxic actions remain unclear. Recent studies have considered that their classical hydrogen-donating antioxidant activity is improbable to be the sole explanation for cellular effects of flavonoids because they are widely metabolized in vivo, resulting in a significant alteration in their redox potentials [22-24].

Our result confirms that all three selected flavonoid in two doses increased serum PON 1 activity in the treated male rat group compared to non-flavonoid treated rats. Atrahimovich , et al demonstrated the same effects of flavonoid on recombinant paraoxonase 1 activity in vitro. On the other hand, for the first time our study showed that kaempferol had more influence on paraoxonase activity than galangin and apigenin in vivo in the same dose. This observation could be explaind based on the more binding affinity of kaempferol for PON 1 enzyme that has been proven by Atrahimovich et al. [14]. Unlike in rats, in human quercetin another flavonoid had not have any effect on PON 1 activity after two weeks, but increased PON1 expression in mice

liver by dietary quercetin after six week was established [25]. Serum paraoxonase activity was changed in rats in our study, but in humans, it may not be related to the differences in the flavonoid, flavonoid concentrations administered, differences in the duration of the experimental trials (8 vs. 2 weeks) and the differences between rats and metabolism of flavonoids in humans.

In terms of chemical structure, flavonols showed a stronger amplifying effect on paraoxonase activity than flavanone (apigenin), suggesting that C3-OH is crucial to augment paraoxonase activity. We also observed that the kaempferol flavonol with 4′-hydroxyl groups was more effective at the augmentation of paraoxonase activity than galangin flavonol without 4′-hydroxyl group, supporting an important role for the 4′-hydroxyl groups in flavonoids to influence paraoxonase activity [14, 16]. Moreover, this effect of kaempferol is a dose-dependent manner.

In addition, kaempferol showed more capacity in free radical scavenging than galangin and apigenin. Futhermore galangin had more capacity in free radical scavenging than apigenin too. These results accumulatively supported finding of Seyoum A et al that ranked flavonoids capacity in free radical scavenging according to their structures [16]. Their results demonstrated more capacity of kaempferol and galangin with 3-OH than apigenin without 3-OH in free radical scavenging in vitro [16].

In our experiment, kaempferol had greater antioxidant activity in prevention of lipid peroxidation than galangin and epigenin, but in Yang et al. study galangin had more antioxidant activity than kaempferol. This inconsistency may be related to circumstance of the experiment *(In vivo vs. In vitro)* and the metabolism of kaempferol and galangin in rats [26]. Surprisingly, the antioxidant activity of kaempferol in high dose (20 mg/kg) had no significant difference with galangin avtivity.

Conclusion

Taken together kaempferol, a flavonol with 4′-hydroxyl groups, had the most effect on augmentation serum paraoxonase activity, prevention of lipid peroxidation and free radical scavenging in vivo. The results can be taken into account for the development of flavonoids with a high therapeutic index

Conflict of Interest

The author declared no conflict of interest.

Acknowledgment

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