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Original article

Investigating the synergistic antioxidant effects of some flavonoid and phenolic compounds

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Abstract
Phenolic and flavonoid compounds are secondary metabolites of plants which possess various activities such as anti-inflammatory, analgesic, anti-diabetes and anticancer effects. It has been established that these compounds can scavenge free radicals produced in the body. Because of this ability, not only the plants containing phenolic and flavonoid compounds but also, the pure compounds are used in medicinal products for prevention and treatment of many disorders. Considering that the golden aim of the pharmaceutical industries is using the most effective compounds with lower concentrations, determination of the best combination of the compounds with synergistic effects is important. In the present study, synergistic antioxidant effects of four phenolic compounds including caffeic acid, gallic acid, rosmarinic acid, chlorogenic acid and two flavonoids, rutin and quercetin, have been investigated by FRAP (Ferric Reducing Antioxidant Power) method. The synergistic effect was assessed by comparing the experimental antioxidant activity of the mixtures with calculated theoretical values and the interactions of the compounds were determined. The results showed that combination of gallic acid and caffeic acid demonstrated considerable synergistic effects (137.8%) while other combinations were less potent. Among examined substances, rutin was the only one which had no effect on the other compounds. The results of ternary combinations of compounds demonstrated antagonistic effects in some cases. This was more considerable in mixture of rutin, caffeic acid, rosmarinic acid (-21.8%), chlorogenic acid, caffeic acid, rosmarinic acid (-20%), rutin, rosmarinic acid, gallic acid (-18.5%) and rutin, chlorogenic acid, caffeic acid (-15.8%), while, combination of quercetin, gallic acid, caffeic acid (59.4%) and quercetin, gallic acid, rutin (55.2%) showed the most synergistic effects. It was concluded that binary and ternary combination of quercetin, rutin, caffeic acid, chlorogenic acid, gallic acid and rosmarinic acid could influence the antioxidant ability; therefore, to obtain the best antioxidant effects in products containing these materials, the interactions should be mentioned.

Keywords*:* antioxidant, flavonoids, FRAP assay, phenolic compounds, synergistic effect

Introduction
Oxidants are highly reactive molecules which are present in biological systems and may oxidize nucleic acids, proteins and lipids. They may initiate disorders such as cancer, heart diseases,

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dermal disorders and aging. The consumption of plants plays an important role as a health promotion factor. This beneficial effect is mainly associated with the antioxidant activity of their metabolites especially phenolic and flavonoid components which are largely present in herbs. They play this role through acting as reducing agents by donating hydrogen, quenching singlet oxygen, acting as chelators and trapping free radicals. The antioxidant activity of polyphenols is even considered to be much greater than some of the essential vitamins [1]. It has also been proved that many properties of polyphenols such as anti-inflammatory, anti-diabetes, anti-cancer and prevention of capillary fragility and permeation are due to their antioxidant effects [2-5]. The antioxidant activity of phenolics has introduced them as dosage forms in prevention and treatment of many disorders [3]. They have been used as preservatives in some formulations as well. In many dosage forms, polyphenol combinations are used due to their possible synergistic effects.

Potency is a considerable characteristic of ingredients for pharmaceutical dosage forms, while using combination of synergic materials might decrease the required amounts and thus be desirable in pharmaceutical industries. The objective of the present study was to investigate the antioxidant behavior of binary and ternary combination of gallic acid, rosmarinic acid, caffeic acid, chlorogenic acid, quercetin and rutin by using FRAP (Ferric Reducing Antioxidant Power) assay which is a standard method for antioxidant effect evaluation.

Experimental

Preparation of the test solutions

Methanolic solutions of gallic acid (150 μM), rosmarinic acid (150 μM), caffeic acid (600 μM), chlorogenic acid (600 μM), quercetin (150 μM) and rutin (150 μM) were prepared. Binary and ternary combinations of the mentioned materials with the same concentrations were also prepared.

FRAP assay

The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form, in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ [2,4,6-tri-(2-pyridyl)-1,3,5-triazine] solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 which was prepared freshly and warmed at 37 ºC. Alliquots of 40 μL of sample were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent and were incubated at 37 ºC for 10 min. The absorbance of the reaction mixture was measured at 593 nm. Calibration curve of $FeSO_4.2H_2O$ was plotted by using different concentrations (250-1500 μM). FRAP value was determined for each solution and expressed as μM $FeSO_4.2H_2O$ [6].

Calculation of synergistic effects of antioxidant mixtures

The experimental antioxidant capacity of the mixtures was calculated by using the absorption of the mixtures and calibration curve of $FeSO_4.2H_2O$. The theoretical antioxidant activity was calculated as the sum of the FRAP values of each compound. If the experimental antioxidant activity was greater than the theoretical antioxidant activity, it was considered as synergistic effect and if it was lower than the theoretical antioxidant activity it was interpreted as antagonistic effect [7].

Results and Discussion

Nowadays, there is an increasing interest to antioxidants because they have proved to prevent several diseases such as neurodegenerative and cardiovascular disorders and also aging [8-11]. Several formulations have been prepared from antioxidant materials for healthcare. Among different secondary metabolites of the plants, phenolics and flavonoids are the main compounds with considerable antioxidant activity. They are the main components of many products and are sometimes used in combinations [12-15]. Since, combination of compounds may affect their properties, evaluating the effect of their combination is important. The results of the present investigation on synergistic/antagonistic effects of some phenolics and flavonoids has established that binary combination of gallic acid and caffeic acid has demonstated considerable synergistic effects (137.8%) (table 1) indicating that concurrent usage of these phenolics has increased the antioxidant activity more than twice resulting in the possibility of decreasing the required amounts of a certain material along with maintaining the desired antioxidant activity which is economically important.

Synergistic effect has also been observed in other mixtures of binary combinations which were not considerable compared to the binary mixture of caffeic and gallic acid. Combination of rutin with other compounds did not change the antioxidant activity suggesting that rutin had no interaction with other compounds.

In several ternary combinations, antagonistic effect was observed and antioxidant activity had decreased. The combination of rutin, caffeic acid, rosmarinic acid (-21.8%), chlorogenic acid, caffeic acid, rosmarinic acid (-20%), rutin, rosmarinic acid, gallic acid (-18.5%) and rutin, chlorogenic acid, caffeic acid (-15.8%) showed the most decrease in FRAP value. It was concluded that combination of more than two compounds could decrease the effect. Since, many of health promoting drugs contain more than one component with antioxidant activity, there is a possibility of interacting and even neutralizing the effects which is necessary to

Table 1. Observed and calculated FRAP values of antioxidants in single compounds, binary and ternary mixtures

Compounds/Combination	Concentration (µM)	Experimental FRAP value (µM $FeSO_4.2H_2O$)	Theoretical FRAP value (µM $FeSO_4.2H_2O$)	Synergistic/antagonistic effect%
GA	150	861.3±13.4	-	-
RA	150	871.3±23.3	-	-
CA	600	1641.3±33.3	-	-
ChA	600	1278.0±3.3	-	-
Q	150	864.7±10.0	-	-
R	150	704.7±3.3	-	-
GA+RA	150+150	2074.7±13.3	1732.7±10.0	19.7
GA+CA	150+600	5952.0±80.0	2502.7±46.7	137.8
GA+ChA	150+600	2736.0±53.3	2139.3±16.6	27.9
GA+Q	150+150	1794.7±26.6	1726.0±3.4	4.0
GA+R	150+150	1591.3±56.7	1566.0±10.0	1.6
RA+CA	150+600	3456.0±13.3	2512.7±10.0	37.5
RA+ChA	150+600	2729.3±20.0	2149.3±20.1	27.0
RA+Q	150+150	1954.7±13.3	1736.0±33.4	12.6

Compounds/Combination	Concentration (µM)	Experimental FRAP value (µM $FeSO_4.2H_2O$)	Theoretical FRAP value (µM $FeSO_4.2H_2O$)	Synergistic/antagonistic effect%
RA+R	150+150	1548.0±27.1	1575.7±26.5	-1.8
CA+Q	600+150	3454.7±113.3	2506.0±23.4	37.9
CA+R	600+150	2431.3±10.1	2346.0±30.3	3.6
CA+ChA	600+600	3021.0±0.0	2919.3±36.6	3.5
ChA+Q	600+150	2511.3±3.3	2142.3±6.5	17.2
ChA+R	600+150	2091.3±96.9	1982.7±0.1	5.5
Q+R	150+150	1668.0±40.0	1569.4±13.4	6.3
Q+GA+R	150+150+150	3772.0±180.0	2430.7±0.0	55.2
Q+GA+RA	150+150+150	2326.0±70.0	2597.4±20.0	-10.4
Q+GA+ChA	150+150+600	2836.0±60.0	3004.0±6.6	-5.6
Q+GA+CA	150+150+600	5366.0±150.0	3367.3±36.7	59.4
Q+R+RA	150+150+150	3116.0±0.0	2440.7±36.7	27.7
Q+R+ChA	150+150+600	3036.0±120.0	2847.4±10.1	6.6
Q+R+CA	150+150+600	2966.0±10.0	3210.7±20.0	-7.6
Q+RA+ChA	150+150+600	3336.0±40.0	3014.0±30.1	10.7
Q+ChA+CA	150+600+600	3472.0±0.0	3784.0±26.6	-8.2
Q+RA+CA	150+150+600	3286.3±52.5	3377.4±0.1	-2.7
R+ChA+CA	150+600+600	3052.0±140.0	3624.0±33.3	-15.8
R+ChA+RA	150+600+150	2746.0±50.0	2854.0±23.4	-3.8
R+ChA+GA	150+600+150	3546.0±130.0	2844.0±13.3	24.7
R+CA+RA	150+600+150	2516.0±20.0	3217.1±7.0	-21.8
R+CA+GA	150+600+150	3786.0±70.0	3207.3±43.4	18.0
R+RA+GA	150+150+150	1986.0±110.0	2437.3±13.4	-18.5
ChA+CA+RA	600+600+150	3032.0±0.0	3790.7±13.3	-20.0
ChA+CA+GA	600+600+150	4753.0±3.9	3780.7±50.0	25.7
ChA+RA+GA	600+150+150	3666.0±90.0	3010.7±6.7	21.8
CA+RA+GA	600+150+150	3836.0±60.0	3374.0±23.4	13.7

GA:gallic acid, RA: rosmarinic acid, CA: caffeic acid, ChA: chlorogenic acid, Q: quercetin, R: rutin. "-" represents antagonistic effects. The presented data are the results of mean±SD of three experiments.

consider. The combination of quercetin, gallic acid, caffeic acid and quercetin, gallic acid, rutin showed reliable synergistic effects (59.4% and 55.2%, respectively). But it seems that adding quercetin to the first mixture has decreased the effect compared to combination of gallic acid and caffeic acid (137.8%), therefore, binary combinations of the mentioned compounds is preferred. Several investigations have been performed on synergistic effects of plant/plant components. Irwandi *et al.* have investigated the oxidative behavior of various mixtures of rosemary, sage and citric acid in a linoleic acid model system and a palm olein system. They have showed significant synergistic effect between three compounds [16]. In another study, the effect of some antioxidants on stability of soy bean oil was investigated and it was found that a mixture of tetrabutyl hydroquinone and butylated hydroxyanisol (2:1) had the most synergistic effects and antioxidant activity [17]. It has been established that a mixture of rosemary and green tea extracts had synergistic effect against meat oxidation and their mixture could be used as preservative for meat protection [18]. Regarding the results of the present investigation, it is concluded that usage of mixtures of some phenolic and flavonoid compounds could increase the antioxidant effects, in other words, the synergistic effects; however, it should be mentioned that binary combination of gallic acid, rosmarinic acid, caffeic acid, chlorogenic acid, rutin and quercetin has more synergistic effect than their ternary combination while no antagonistic effect was observed.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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