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Investigation of Antioxidant Activity of Gallic, Protocatechuic and Vanillic Acids using the Briggs-Rauscher Reaction as Tool

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Abstract: Hydroxybenzoic acids are an important class of polyphenols because their strong antioxidative activity. In this study, the antioxidant activity of three hydroxybenzoic acids was investigated using the Briggs-Rauscher reaction. Besides individual gallic, protocatechuic, and vanillic acids at concentrations of 250, 500, and 1000 µM respectively, the various mixtures of two and three hydroxybenzoic acids were also tested. The highest antioxidant activity showed protocatechuic acid at a concentration of 1000 µM while the lowest antioxidant activity was observed for vanillic acid at a concentration of 250 µM. The most of investigated hydroxybenzoic acids mixtures showed some degree of antagonistic effect. The highest antagonistic effect was found for the equimolar mixture of protocatechuic and vanillic acid at concentration of 250 µM. On the contrary, the equimolar mixture of gallic acid and vanillic acid at the same concentration indicated a high synergistic effect.

INTRODUCTION

Phenolic acids are an important group of secondary metabolites with a basic chemical structure of C_6-C_1 (hydroxybenzoic acids) or C_6-C_3 (hydroxycinnamic acids), consisting of a phenolic ring and a carboxyl substituent (Saxena, Saxena and Pradhan, 2012; Kahkeshani, Farzaei, Fotouhi et al., 2019). Gallic acid, protocatechuic acid, and vanillic acid are the most common members of hydroxybenzoic acids.

Gallic acid (GA) exists in different forms in many plants (oak, carob, sumac), vegetables (onions, potatoes), fruits (strawberries, grapes, pineapple, banana, lemon, mango), nuts (walnut, hazelnut), olive oil, coffee, tea, and wine. It has antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, antimelanogenic, antimutagenic, anticancer, cardioprotective, gastroprotective, and neuroprotective activities among others (Brglez Mojzer, Knez Hrnčić, Škerget et al., 2016; Nayeem, Asdaq, Salem et al., 2016; Fernandes and Saldago, 2016; Kahkeshani et al., 2019). Because of its many biological and pharmacological activities, gallic acid and its derivates are commonly used in the food and pharmaceutical industries (Mota, Queimada, Pinho et al., 2008; Kahkeshani et al., 2019). It is soluble in water unlike other phenolic acids (Brglez Mojzer et al., 2016).

Protocatechuic acid (PCA) is widely distributed in many fruits (plums, grapes, gooseberries), medicinal plants (rosemary, hibiscus, melissa), spices (anis), nuts (almond), rice, olive oil, and white wine (Kakkar and Bais, 2014). It has various biological activities like antioxidant, antibacterial, anticancer, antidiabetic, antiviral, anti-inflammatory, analgesic, etc. (Kakkar and Bais, 2014).

Vanillic acid (VA) is an oxidized form of vanillin, and is widely used as flavoring, additive, and preservative in the food industry. It is found in some fruits (mango, strawberry), cereals (wheat, rice), herbs, spices, green tea, juices, beer, and wine (Zuo, Wang and Zhan, 2002; Kumar, Prahalathan and Raja, 2011; Palafox-Carlos, Gil-Chávez, Sotelo-Mundo et al., 2012; Almeida, Cavalcante, Vicentini et al., 2016). It has antibacterial, antimicrobial, antifilarial, antioxidant, anti-inflammatory properties, free-radical scavenging ability, cardioprotective, chemopreventive, and hepatoprotective effects (Itoh, Isoda, Kondoh et al., 2009; Itoh, Isoda, Kondoh et al., 2010; Raja and Mol, 2010; Kim, Kim, Um et al., 2010; Kumar et al., 2011; Almeida et al., 2016).

When more than one antioxidant is present in a sample, synergistic effect of those compounds towards increasing antioxidant activity can be noticed, yet the mechanism behind those interactions is still not thoroughly explained. Synergistic antioxidant activity depends on the type of compounds and their concentration in the mixture. A particular combination may show synergism at one concentration ratio while antagonism at the other (Sonam and Guleria, 2017).
Interactions between phenolic acids can lead to the changes in overall antioxidant activity. Because phenolic acids are usually present in the form of mixtures in nature, it is impossible to explore their interactions on the total antioxidant activity of samples (Skroza, Generalić Mekinić, Svilović et al., 2015).
In this study, the antioxidant activity of individual hydroxybenzoic acids (gallic acid, protocatechuic acid, and vanillic acid) and different mixtures of two or three hydroxybenzoic acids were investigated using the Briggs-Rauscher reaction method. This method is based on the inhibitory effects of antioxidants on the oscillations of the Briggs-Rauscher reaction mixture (Cervellati, Höner, Furrow et al., 2001).

EXPERIMENTAL

Reagents

All used reagents were of analytical grade. Potassium iodate, sulfuric acid, hydrogen peroxide, and ethanol were obtained from Semikem (Sarajevo, BiH), malonic acid, manganese(II) sulfate monohydrate, and starch were obtained from Merck (Darmastadt, Germany), gallic acid, protocatechuic acid, and vanillic acid were obtained from Sigma (St. Louis, USA).

Preparation of the solutions of hydroxybenzoic acids

A stock solution of gallic, protocatechuic, and vanillic acids, 2000 μM, were prepared by dissolving pure hydroxybenzoic acid in 2 mL of ethanol and diluting with distilled water to 100 mL. Other solutions of hydroxybenzoic acids in the concentrations of 250, 500, and 1000 μM were obtained by diluting the stock solution with distilled water. Thus solutions were used for the preparation of the mixtures of two and three hydroxybenzoic acids.

Procedure for the determination of antioxidant activity

The antioxidant activity of individual hydroxybenzoic acids and their mixtures was determined by the Briggs-Rauscher reaction. The original procedure (Cervellati et al., 2001) was slightly modified. The Briggs-Rauscher reaction mixture was prepared according to the procedure described in our previous study (Gojak-Salimović and Ramić, 2020). The oscillations of the Briggs-Rauscher reaction mixture were followed potentiometrically at temperature 25±0.5°C. A Pt electrode was used as a working electrode, while Ag/AgCl was used as the reference electrode. After the third oscillation, 1 mL solution of tested hydroxybenzoic acid, at corresponding concentration (or their mixtures) was added to 30 mL of an active, well stirred Briggs-Rauscher reaction mixture.
The total antioxidant activity of individual hydroxybenzoic acids and their mixture was expressed as the inhibition time (t_{inhib}). The inhibition time is defined as the time elapsed between the end of the addition of tested hydroxybenzoic acids (or their mixtures) and the first regenerated oscillation (Cervellati et al., 2001).

RESULTS AND DISCUSSION

Antioxidant activity of selected hydroxybenzoic acids

In this study, the antioxidant activities of gallic, protocatechuic, and vanillic acids were evaluated at different concentrations (250, 500, and 1000 μM) using the Briggs-Rauscher reaction. The obtained results are reported in Table 1.

Table 1: Inhibition times of individual hydroxybenzoic acids

Hydroxybenzoic acid	t_{inhib} (s)		
	250 (μM)	500 (μM)	1000 (μM)
GA	48	117	165
PCA	547	1173	1874
VA	17	394	1351

The inhibition time increased with increased concentration, and linearity was found in the tested concentration range of hydroxybenzoic acid added. The parameters of the straight-lines are reported in Table 2.

Table 2: Parameters of straight-lines equations

Hydroxybenzoic acid	m (μM^{-1} s)	q (s)	R^2
GA	0.147	24.0	0.916
PCA	1.717	196	0.975
VA	1.798	-461	0.996

The obtained results confirmed that the antioxidant activity of the investigated hydroxybenzoic acids varies depending on their structure and concentration. The lowest inhibition time was detected when using vanillic acid at a concentration of 250 μM (17 s), and the longest with protocatechuic acid at a concentration of 1000 μM (1874 s). The inhibition time for certain acids changed significantly with increasing concentration from 250 to 1000 μM. At a concentration of 250 μM antioxidant activity increased in the folowing order: vanillic acid, gallic acid, protocatechuic acid, while at concentrations of 500 and 1000 μM the order was different: galllic acid, vanillic acid, protocatechuic acid.
In the literature, the ranking order of the antioxidant activity of the investigated phenolic acids differed from method to method. Rice-Evans, Miller and Paganga (1996) reported the total antioxidant activity expressed as TEAC values in the following order: gallic acid > *p*-coumaric acid > ferulic acid > vanillic acid > syringic acid > caffeic acid > *m*-coumaric acid > protocatechuic acid > gentisic acid > *o*-coumaric acid > salicylic acid > *p*-hydroxybenzoic acid. Schlesier, Harwat, Böhm et al. (2002) determined the antioxidant activity of four antioxidants and some beverages using six different *in vitro* methods (TEAC, TRAP, DPPH, DMPD, PCL and FRAP). They found that gallic acid was the strongest antioxidant in all methods except the DMPD method.

Karamać, Kosińska and Pegg (2005) evaluated the radical-scavenging activity of selected phenolic acids using DPPH method. They found that tested phenolic acids exhibited radical-scavengingin the folowing order: gallic acid > gentisic acid > syringic acid > caffeic acid > protocatechuic acid > sinapic acid > ferulic acid > isoferulic acid > vanillic acid > *p*-coumaric acid > *o*-coumaric acid > *m*-coumaric acid > salicylic acid » *p*-hydroxybenzoic acid. Skroza e*t al.* (2015) found the lowest antioxidant activity was observed for gallic acid by the Briggs-Raucher reaction method. Skroza, Šimat, Vrdoljak et al. (2022) examined the antioxidant activity of gallic, gentisic, protocatechuic, syringic, and vanillic acids at concentrations of 2.5 µM and 5 µM using the ORAC method. The ORAC value for certain acids did not change significantly with increasing concentration from 2.5 to 5 µM. At a concentration of 2.5 µM antioxidant activity increased in the folowing order: gallic acid, vanillic acid, syringic acid, gentisic acid, protocatechuic acid, while at a concentration of 5 µM the order was different: galllic acid, syringic acid, gentisic acid, vanillic acid, protocatechuic acid.

Antioxidant activity of hydroxybenzoic acids mixtures

The obtained results for the inhibition time of the mixture of two and three combination hydroxybenzoic acids are reported in Table 3. The results for the inhibition time ranged from 16 s for the mixture of protocatechuic acid and vanillic acid at a concentration of 250 µM (1:1) to 1602 s for the same mixture at a concentration of 1000 µM (1:1). In order to evaluate the impact of interactions between constituents on their antioxidant activity, the inhibition time obtained experimentally for the different mixtures of the investigated hydroxybenzoic acids were compared with theoretical values calculated by adding up the effects of two or three individual hydroxybenzoic acids analyzed separately.

Table 3: Inhibition times for mixtures of hydroxybenzoic acids

Mixtures	**t_{inhib} (s)**		
	250 (µM)	**500 (µM)**	**1000 (µM)**
GA:VA (1:1)	**46 (32)**	81 (255)	323(758)
GA:PCA (1:1)	73 (297)	277 (645)	865(1019)
PCA:VA (1:1)	16 (282)	458 (783)	1602 (1612)
GA:VA:PCA (1:1:1)	53 (204)	256 (561)	**1256 (1129)**
GA:VA (3:1)		122 (186)	
GA:PCA (3:1)		234 (322)	
PCA:VA (3:1)		786 (978)	
GA:VA (1:3)		303 (325)	
GA:PCA (1:3)		839 (909)	
PCA:VA (1:3)		504 (589)	
GA:VA:PCA (2:2:1)		84 (439)	
GA:VA:PCA (2:1:2)		104 (595)	
GA:VA:PCA (1:2:2)		257 (650)	

*The values in parentheses are the sum of antioxidant activities of individual hydroxybenzoic acids at corresponding concentrations.

Our results indicate that most of the investigated mixtures to some extent showed a difference in antioxidant activity when compared to their theoretical values. A slight synergistic effect was observed between gallic acid and vanillic acid at a concentration of 250 µM (1:1). The other investigated of two-component mixtures showed a high antagonistic effect. The mixtures of three hydroxybenzoic acids showed an antagonistic effect except their equimolar combination at concentration of 1000 µM which showed a slight synergistic effect.

Palafox-Carlos et al. (2012) investigated individual antioxidant activity and the interactions of four major phenolic compounds (chlorogenic acid, gallic acid, protocatechuic acid, and vanillic acid) found in 'Ataulfo' mango pulp using the DPPH method. They found that the antioxidant activity decreased in the following order: gallic acid > protocatechuic acid > chlorogenic acid > vanillic acid. The majority of all combinations showed significant synergism while the combination between protocatechuic acid-chlorogenic acid-vanillic acid and gallic acid-vanillic acid had a small antagonism. López-Martínez, Santacruz-Ortega, Navarro et al. (2015) analyzed the interactions and mechanisms of major phenolic acids found in mango (gallic, protocatechuic, chlorogenic, and vanillic acids) and papaya (caffeic, ferulic, and *p*-coumaric acids) using ^{1}H NMR and evaluated the effect of the antioxidant mixtures using the DPPH method. They found that the ability of the phenolic acid to neutralize the DPPH radical decreased in the following order in mango: gallic acid > chlorogenic acid > protocatechuic acid > vanillic acid. The majority of all combinations showed significant synergism while the combination between gallic acid and vanillic acid had a small antagonism.

Hajimehdipoor, Shahrestani and Shekarchi (2014) investigated effects of mixtures between caffeic acid, gallic acid, rosmarinic acid, chlorogenic acid, rutin, and quercetin using FRAP method. They found that combination of rutin, rosmarinic acid, and gallic acid showed an antagonistic effect (-18.5%), while combination of quercetin, gallic acid, caffeic acid (59.4%), quercetin, gallic acid, rutin (55.2%) showed the most synergistic effects. Skroza et al. (2015) investigated the interaction between resveratrol and gallic acid, caffeic acid, catechin, quercetin in equimolar binary mixtures using FRAP, DPPH and the Briggs-Rauscher reaction methods. The high synergism (45.4%) in the mixture of gallic acid and resveratrol were detected using the Briggs-Rauscher reaction method.

Skroza et al. (2022) investigated antioxidant activity of gallic, vanillic, protocatechuic, syringic and gentisic acids using the FRAP method. Besides individual phenolic acids, the equimolar mixtures of two, three, four, and all five acids were also tested at different concentrations (100, 500, 1000 µM). The antioxidant activity decreased in the following order: gallic acid > gentisic acid > syringic acid > protocatechuic acid > vanillic acid. Paper reports the highest synergism for the mixture of protocatechuic, syringic, and gentisic acids at a concentration of 100 µM while the highest antagonism for the mixture of vanillic and protocatechuic acids at same concentration. Also, Skroza et al. (2022) investigated the antioxidant activity of equimolar mixtures of gallic, gentisic, protocatechuic, syringic, and vanillic acids at a concentration of 5 µM using the ORAC method. Only two mixtures of phenolic acids showed an antagonistic effect (gallic acid+syringic acid and gallic acid+vanillic acid), while all the others showed a synergistic effect.

Our previous study (Aljović and Gojak-Salimović, 2017) investigated the antioxidant synergistic and antagonistic effects between ferulic acid, homovanillic acid, and vanillic acid using the Briggs-Rauscher reaction method. The most of the investigated phenolic acids mixtures showed some degree of synergistic effect. Also, the results obtained for the most of investigated two-component and three-component mixtures of ferulic acid, caffeic acid, and rosmarinic acid showed some degree of synergistic effect (Gojak-Salimović and Ramić, 2020).

CONCLUSIONS

Our results suggest differences in the antioxidant activity of gallic acid, protocatechuic acid, and vanillic acid individually as well as in their mixtures. Most of the hydroxybenzoic acid mixtures showed some degree of antagonistic effect. The antioxidant interaction of the investigated hydroxybenzoic acids varies depending on their structure and concentration. It can be concluded that the antioxidant activity of the hydroxybenzoic acids mixtures cannot be predicted from the individual values of their constituents. One method is not enough to fully explain antioxidant activity of the mixtures. For better understanding the antioxidant interactions between the selected hydroxybenzoic acids when present together more studies are needed. Those studies should include various acid combinations, and most preferably additional experimental methods included.

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Summary/Sažetak

Antioksidacijska aktivnost galne, protokatehinske i vanilinske kiseline ispitivana je primjenom Briggs-Rauscher oscilirajuće reakcije. Osim pojedinačnih hidroksibenzojevih kiselina pri koncentracijama 250, 500 i 1000 μM, testirane su i različite kombinacije smjesa dviju i sve tri kiseline. Najbolju sposobnost inhibicije oscilacija Briggs-Rauscher reakcione smjese pokazala je protokatehinska kiselina pri koncentraciji od 1000 μM, dok je najmanja antioksidacijska aktivnost opažena za vanilinsku kiselinu pri koncentraciji od 250 μM. Većina ispitivanih smjesa hidroksibenzojevih kiselina pokazala je određeni stepen antagonističkog efekta. Najveći antagonistički efekat nađen je za ekvimolarnu smjesu protokatehinske i vanilinske kiseline pri koncentraciji od 250 μM. Nasuprot tome, ekvimolarna smjesa galne kiseline i vanilinske kiseline pri istoj koncentraciji pokazala je značajan sinergistički efekat.