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Inhibitory effects of selected phenolic acids on the oscillations of the Briggs-Rauscher reaction

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Abstract: Phenolic acids are secondary metabolites of aromatic plant that possess prominent antioxidant activity. When an antioxidant is added to an active oscillating Briggs-Rauscher reaction mixture, there is an immediate cessation of the oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added, and a subsequent regeneration of oscillations. In this study, the effects of concentration of the ethanol solutions of selected phenolic acids (gallic, caffeic, chlorogenic, rosmarinic, *p*-coumaric and *m*-coumaric acids) on the oscillatory system Briggs-Rauscher reaction were investigated. The reaction was performed in a constantly stirred reactor, with accurately defined concentrations of reactants, at constant temperature of 25°C. Flow oscillations in the Briggs-Rauscher reaction mixture were monitored as a change in potential between the platinum electrode and silver/silver chloride reference electrode. Relative antioxidant activities of phenolic acids were determined in three ways on the basis of inhibition times. The obtained results showed that the gallic and *p*-coumaric acids have much less antioxidant activity than the caffeic, chlorogenic and rosmarinic acids. The ability to inhibit oscillations of the Briggs-Rauscher reaction mixture is not showed for *m*-coumaric acid.

INTRODUCTION

Phenolic acids are widespread plant secondary metabolites. They belong to the subclass of polyphenols with more than 8000 of natural compoundsthat possess one common structural feature, a phenol (an aromatic ring bearing at least one hydroxyl group). According to the basic structure they are divided into hydroxybenzoic and hydroxycinnamic acids.

Caffeic, vanillic, ferulic and *p*-coumaric acids are found in almost all plants. Other acids are found in selected plants or foods (Robbins, 2003). The hydroxycinnamic acid class, which includes *p*-coumaric, caffeic and ferulic acids, occur most frequently as simple esters with hydroxy carboxylic acids or D-glucose. In contrast, the hydroxybenzoic acid class, such as *p*-hydroxybenzoic, gallic and ellagic acids, is present mainly in the form of glucosides (Ota *et al*., 2011).

Although the role of phenolic acids as secondary metabolites in plants is not fully clarified, it is considered to participate in many processes such as nutrient, protein synthesis, enzyme activity, photosynthesis and others.

Phenolic acids have prominent antioxidant activity. Hydroxycinnamic acids has better antioxidant properties than most hydroxybenzoic acids (Rice-Evans *et al*., 1996; Robards *et al*., 1997).

Gallic acid and its derivatives have potential for combating oxidative damages, cancer manifestations and microbial infestations. Large number of research studies are available to show its ability for the treatment of diabetes, ischemic heart diseases, ulcer and other ailments (Nayeem *et al*., 2016).

Caffeic acid, one of the most prominent naturally occurring cinnamic acids, is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma and allergic reactions (Robbins, 2003).

Chlorogenic acid is an ester formed between caffeic acid and quinic acid, and is one of major polyphenol compounds found in numerous plant species, including coffee beans, apples, and blueberries. Chlorogenic acid protects cells from oxidative stress induced by UVB radiation (Cha *et al*., 2014). Moreover, chlorogenic acid protects mesenchymal stem cells against oxidative stress (Li *et al*., 2012).

Rosmarinic acid has antioxidant and anti-inflammatory effects and is used for the treatment of asthma and reactive airway diseases, allergic disorders such as allergic rhinitis, otitis media, chemical sensitivity and multiple allergen reactivity (Stansbury, 2014).

The *p*-coumaric acid has beneficial effects on human health through their prevention of degenerative pathologies, such as cardiovascular disease and cancer (Ota *et al.*, 2011).

The *m*-coumaric acid is a polyphenol metabolite from caffeic acid, formed by the gut microflora and the amount in human biofluids is diet-dependant (Konish and Kobayashi, 2004; Mennen *et al*., 2016).

All the methods for measurements of the antioxidant activity are based on the generation of free radicals in a reaction mixture and the effects of added antioxidants on some properties of the radical or of the mixture: absorbance, quenching of chemiluminescence, electric potential, ect. These properties change depending on the amount of antioxidants added with respect to those of a reference mixture (Cervellati *et al*., 2002; Shalaby and Shanab, 2013). The Briggs-Rauscher oscillating reaction method is relatively new and inexpensive method for meausuring antioxidant activity. This method is based on the inhibitory effects by antioxidants on the oscillations of the Briggs-Rauscher reaction mixture. When antioxidants are added to an active oscillating Briggs-Rauscher reaction mixture, some of which cause an immediate cessation of the oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added in a wide range of concentration, and a subsequent regeneration of the oscillations (Cervellati *et al*., 2001; Cervellati *et al*., 2002; Furrow *et al*., 2004). The inhibition time (t_{inhib}) is defined as the time elapsed between the end of the addition of the antioxidant and the first regenerated oscillation. Relative antioxidant activity with respect to a substance chosen as standard can then be determined on the basis of inhibition time (Hönor and Cervellati, 2002; Hönor *et al*., 2002).

In this study, the inhibitory effects of concentration of the ethanol solutions of selected phenolic acids (gallic, caffeic, chlorogenic, rosmarinic, *p*-coumaric and *m*-coumaric acids) on the oscillatory system Briggs-Rauscher reaction, which consisted of hydrogen peroxide, malonic acid, manganese(II) sulfate monohydrate, potassium iodate, sulfuric acid and starch as indicator, were investigated.

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), chlorogenic acid was obtained from Acros Organics, (Geel, Belgium), gallic acid, caffeic acid, rosmarinic acid, *p*-coumaric acid and *m*-coumaric acid were obtained from Sigma (St. Louis, USA).

Preparation of the solutions for the Briggs-Rauscher reaction

For the Briggs-Rauscher oscillating reaction (Marković and Talić, 2013; Dacić and Gojak-Salimović, 2016) the following mixture should be prepared: 0.067 mol/L potassium iodate, 1.5 mol/L hydrogen peroxide, 0.0267 mol/L sulfuric acid, 0.050 mol/L malonic acid, 0.0067 mol/L manganese(II) sulfate monohydrate and 0.01% fresh starch. In our study, for achieving the Briggs-Rauscher oscillating reaction, three stock solutions were prepared daily. **Solution A**: 43 g potassium iodate and 4.5 mL 96% sulfuric acid were dissolved in distilled water and diluted to 1 L; **Solution B**: 15.6 g malonic acid, 3.4 g manganese(II) sulfate monohydrate and 3 g starch were dissolved in distilled water and diluted to 1 L; **Solution C**: 500 mL of 30% hydrogen peroxide was diluted to 1 L.

Preparation of the solutions of selected phenolic acids

In the concentration range used in this work all of selected phenolic acids are ethanol soluble.

Apparatus

Oscillations of the Briggs-Rauscher reaction were monitored potentiometrically by recording the potential of the reaction mixture using a platinum electrode and $Ag/AgCl/KCl_{(sat)}$ reference electrode (+197 mV vs. SHE). The electrodes were connected to a pH multimeter (Phywe, Model 13702.93). The accuracy of the multimeter was ±1 mV. All measurements were conducted at constant temperature (25±0.5°C) using a thermostating system. The reaction mixture was stirred by a magnetic stirrer (600 rpm).

Procedure

The Briggs-Rauscher reaction mixture was prepared by mixing the appropriate amounts of stock solutions of reagents. For each measurement 10 mL of each solution A and B were mixed into the double-wall thermostated beaker equipped with a magnetic stir bar and placed on a stirring plate. The 10 mL of solution C was used to initiate the oscillations. After the third oscillation, 1 mL of ethanol solution of phenolic acid at corresponding concentration was added to an active Briggs-Rauscher reaction mixture. Typical potentiometric recordings for a non-inhibited and an inhibited Briggs-Rauscher reaction mixture are shown in Figure 1 and Figure 2. The inhibition times were then measured from the recordings.

The addition of 1 mL of ethanol, without phenolic acid does not interrupt the oscillations. All samples were run at least in duplicate and results were expressed as mean values.

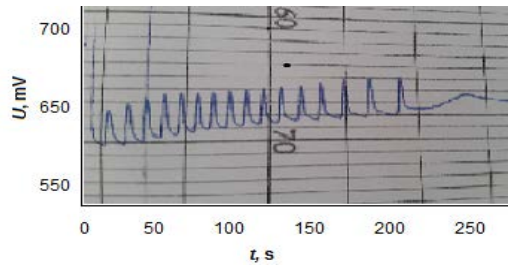

Figure 1: Recording of the potential versus time of non-inhibited Briggs-Rauscher reaction

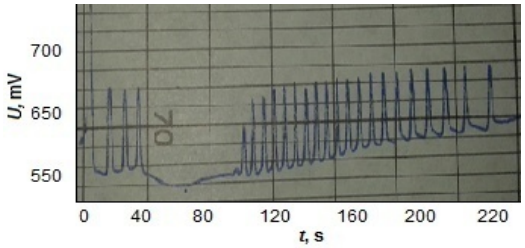

Figure 2: Recording of the potential versus time when 1 mL of a solution of gallic acid (100 mg/L) was added to 30 mL of an active Briggs-Rauscher reaction mixture after the third oscillation

RESULTS AND DISCUSSION

Our previous work showed the ability of chlorogenic acid to inhibited oscillations of the Briggs-Rauscher reaction mixture at room temperature (Dacić and Gojak-Salimović, 2016). In this study, the effects of various concentration of the ethanol solutions of selected phenolic acids (gallic, caffeic, chlorogenic, rosmarinic, *p*-coumaric and *m*-coumaric acids) on the oscillatory system Briggs-Rauscher reaction were evaluated at 25°C.

In all samples except *m*-coumaric acid, the inhibition times increased with increased concentration, and linearity was found in a wide concentration range of phenolic acid added (Figure 3). Below a certain concentration of phenolic acid added (different for each phenolic acid), the behavior deviates from linearity. At low concentrations of phenolic acids added, the inhibition times become too low to be measured as well for some other antioxidants (Cervellati *et al*., 2000). There is a threshold under which inhibition time cannot be detected and we believe that the straight-lines curve toward zero under these lower limits. At high concentrations of phenolic acids the amplitudes of resumed oscillations becomes too low, until up to a given concentration (different for each phenolic acid) oscillations do not restart.

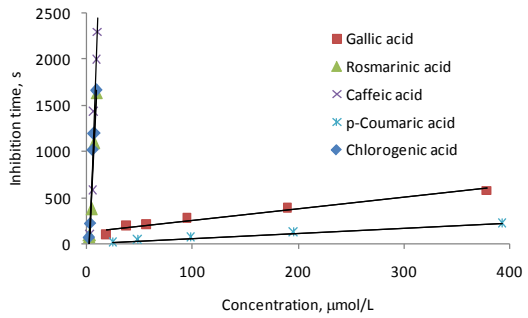

Figure 3: Straight lines of inhibition time versus concentration for the phenolic acids studied

As shown in Figure 3, the slopes of the straight lines are different, so the calculation of the relative antioxidant activity will depend on the substance chosen as standard and the concentration of the sample. The parameters of the straight lines and *R*-squared values are reported in Table 1.

Table 1: Parameters of straight-lines equations (t_{inhib}= *m*(antioxidant) + *q*) and *R*-squared values

Antioxidant	*m* (μmol/L)$^{-1}$ s	*q* (s)	R^2
Caffeic acid	326.0	–1055	0.978
Chlorogenic acid	228.7	–419.4	0.955
Rosmarinic acid	230.8	–480.1	0.970
Gallic acid	1.156	148.6	0.995
p-Coumaric acid	0.551	4.125	0.997

The relative antioxidant activity were calculated as relative activity with respect to concentrations (*rac*), relative activity with respect to slopes (*ras*) and relative activity with respect to inhibition times (*rat*) (Cervellati *et al*., 2001). Caffeic acid was chosen as standard.

Relative antioxidant activity with respect to concentrations (*rac*) is the ration between concentrations of the chosen standard and samples that give the same inhibition time:

$$rac = [std]/[smp]$$

The concentration of standard that should give the same inhibition time of the sample was calculated from the straight-line equation of the chosen standard.

Relative antioxidant activity with respect to slopes (*ras*) is the ratio between the slope of the straight line of the sample and that of the standard:

$$ras = \text{slope(smp)}/\text{slope(std)}$$

Relative antioxidant activity with respect to inhibition times (*rat*) is the ratio between the inhibition time of the sample and that of the standard at the same concentration:

$$rat = t_{inhib}(\text{smp})/t_{inhib}(\text{std})$$

The chosen concentration must be specified together with the *rat* values and must be in the linear concentration range of the standard and of all the examined substances. The obtained *rac*, *ras* and *rat* values are reported in Table 2.

Table 2: Relative antioxidant activities with respect to concentrations, slopes and inhibition times

Antioxidant	Concentration (μmol/L)	*rac*	*ras*	*rat*
Caffeic acid	3.85	1	1	1
Chlorogenic acid	2.71	1.420	0.700	0.872
Rosmarinic acid	2.95	1.305	0.707	0.850
Gallic acid	44.5	0.086	0.035	0.085
p-Coumaric acid	355	0.011	0.017	0.005

The *rac* values were calculated at an inhibition time of 200 s. On the basis of *rac* values, the order of antioxidant activity of the studied phenolic acids is: chlorogenic acid > rosmarinic acid > caffeic acid > gallic acid > *p*-coumaric acid. When possible, it is convenient to calculate a mean value of *rac* in the linear concentration range of the sample and the standard. The value $(rac)_m$ is more significant than the *rac* value calculated at only one inhibition time (Cervellati*et al*., 2002).

On the basis of *ras* values, the order of antioxidant activity of the studied phenolic acids is: caffeic acid > rosmarinic acid ≈ chlorogenic acid > gallic acid > *p*-coumaric acid. This method of relative activity calculation is useful for comparison of the effect of changes in sample concentration with the effect of changes in the reference concentration, within the linear ranges (Cervellati *et al*., 2001).

The *rat* values were calculated at concentration 9 μmol/L. On the basis of *rat* values, the order of antioxidant activity of the studied phenolic acids is: caffeic acid > chlorogenic acid > rosmarinic acid > gallic acid >*p*-coumaric acid. This method of relative activity calculation has the same limitations asthe *rac*. The advantage is that the activity is reffered to a given specified concentration (Cervellati *et al*., 2001).

The ability to inhibit oscillations of the Briggs-Rauscher reaction mixture is not showed for *m*-coumaric acid. Recording of the potential versus time when 1 mL of a solution of *m*-coumaric acid (2500 mg/L) was added to 30 mL of active Briggs-Rauscher reaction mixture after the third oscillation are reported in Figure 4.

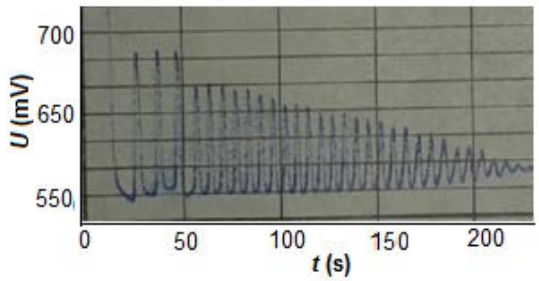

Figure 4: The effect of the *m*-coumaric acid on the active Briggs-Rauscher reaction mixture

The oscillatory time (the duration of the oscillatory regime) and numbers of oscillations depends on the concentration of studied phenolic acids. Variation of oscillations parameters in the Briggs-Rauscher reaction mixture with concentration of *m*-coumaric acid are reported in Table 3.

Table 3: Variation of oscillations parameters in the Briggs-Rauscher reaction mixture with concentration of *m*-coumaric acid

Concentration (μmol/L)	Oscillations	
	Duration (s)	Number
0	220	16
24.6	255	22
49.1	420	31
73.7	460	40
122.8	490	53
245.6	420	52
358.6	285	36
491.2	210	28

The largest number of oscillations (60) caused addition 1 mL of *p*-coumaric acid concentration of 500 mg/L to the 30 mL active Briggs-Rauscher reaction mixture. The lowest number of oscillations (23) caused addition 1 mL of gallic acid concentration of 300 mg/L to the 30 mL active Briggs-Rauscher reaction mixture.

The ranking order of the antioxidant activity of antioxidants components of secondary plant products differed from assay to assay. The Briggs-Rauscher reaction method can give useful *in vitro* information on the antioxidant activity at low pH values and has many advantages. Milos and Makota (2012) demonstrated some new possibilities of this method for determine the synergistic or antagonistic effects in mixture of compounds, which often poses a problem when using traditional methods.

CONCLUSIONS

The Briggs-Rauscher oscillating reaction is suitable as an analytical method to determine relative activity of antioxidants. In this study, the effects of concentration of the ethanol solutions of selected phenolic acids (gallic, caffeic, chlorogenic, rosmarinic, *p*-coumaric and *m*-coumaric acids) on the oscillatory system Briggs-Rauscher reaction were investigated. In all samples except *m*-coumaric acid, the inhibition time increased with increased concentration, and linearity was found in a wide concentration range of phenolic acid added. Relative antioxidant activities with respect to concentrations, slopes and inhibition times were calculated. The obtained results showed that the gallic acid and *p*-coumaric acid have much less antioxidant activity than the caffeic acid, chlorogenic acid and rosmarinic acid. The ability to inhibit oscillations of the Briggs-Rauscher reaction mixture is not showed for *m*-coumaric acid. Our future investigation will be focused on the antioxidant synergisms and antagonisms among selected phenolic acids.

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Summary/Sažetak

Fenolske kiseline su aromatski sekundarni biljni metaboliti koji posjeduju značajnu antioksidacijsku aktivnost. Kada se antioksidans doda u aktivnu Briggs-Rauscher reakcijsku smjesu dolazi do neposrednog gašenja oscilacija. Vrijeme inhibicije oscilacija je u proporcionalnom odnosu s količinom i svojstvima dodanog antioksidansa. U ovom radu ispitivan je uticaj koncentracije etanolnih rastvora odabranih fenolskih kiselina (galna, kafena, hlorogenska, ruzmarinska, *p*-kumarinska i *m*-kumarinska kiselina) na oscilirajući sistem Briggs-Rauscher reakcije. Reakcija je izvođena u reakcionom sudu, uz stalno miješanje tačno definisanih koncentracija reaktanata, pri konstantnoj temperaturi od 25°C. Tok oscilacija Briggs-Rauscher reakcijske smjese praćen je potenciometrijskom metodom uz platinsku elektrodu i srebro/srebro hloridnu referentnu elektrodu. Relativne antioksidacijske aktivnosti bazirane na vremenima inhibicije izračunate su na tri načina. Rezultati ispitivanja su pokazali da galna i *p*-kumarinska kiselina imaju mnogo manju antioksidacijsku aktivnost od kafene, hlorogenske i ruzmarinske kiseline. Sposobnost inhibicije oscilacija Briggs-Rauscher reakcijske smjese nije pokazala *m*-kumarinska kiselina.