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Electrochemical and spectrometric study of antioxidant activity of pomiferin, isopomiferin, osajin and catalposide

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ABSTRACT

The antioxidant properties of pomiferin, isopomiferin, osajin and catalposide are evaluated. The electrochemical behaviour of these compounds at a carbon paste electrode was studied using square wave voltammetry. Oxidative signals, optimized frequencies and appropriate pH acetate buffer conditions were determined. The detection limits (3 S/N) for pomiferin, isopomiferin, osajin and catalposide were estimated to be 50 pg/ml, 800 pg/ml, 40 pg/ml and 10 ng/ml, respectively. Furthermore, spectrometric test was employed with 2,2-diphenyl-1-picrylhydrazyle (DPPH) to evaluate the antioxidant activities of these compounds. Based on the obtained results, the highest antioxidant activity measured by DPPH tests was found at pomiferin followed by isopomiferin. The activities of osajin and catalposide were undetectable. The protective effects of pomiferin, isopomiferin, osajin and catalposide on DNA exposed to oxygen radicals *in vitro* were also studied. Changes in height of oxidative signals for the four bases (guanine, thymine, adenine and cytosine) were measured for DNA exposed to oxygen radicals, generated by Fenton's reaction, non-oxidized ssDNA (50 μg/ml) displayed well developed signals; however, after oxidative damage the observed oxidative signals decreased. Significant protective effects were observed for pomiferin and osajin. Decreased effect was observed for isopomiferin while a further reduced protective effect was seen for DNA exposed to catalposide. Based on the obtained results, pomiferin had the highest antioxidant activity followed by isopomiferin, osajin and catalposide.

1. Introduction

Extracts from different parts of *Derris malaccensis* (Leguminosae) and osage orange, *Maclura pomnifera* (Moraceae) may be used as potential antidiabetic drugs [1–9]. Flavonoid compounds, such as pomiferin, isopomiferin and osajin (prenylated isoflavones), isolated from these plants possess biologically significant properties; specifically, their antidiabetic and antioxidant capabilities [1,7,9] are of great interest. The iridoid glucoside catalposide, isolated from *Catalpa ovata* also exhibits antioxidant capabilities [10]. The chemical formulae of pomiferin, isopomiferin, osajin and catalposide are shown in Fig. 1. Chromatographic methods coupled with UV/vis and/or mass spectrometry are commonly used analytical methods to study and quantify these compounds [7,11–16]. Recently, electrochemical methods were utilized for the same purpose [13,16,17].

The main aim of this work was to evaluate the antioxidant properties of pomiferin, isopomiferin, osajin and catalposide. Basic electrochemical behaviour of these compounds was studied at carbon paste electrodes by using square wave voltammetry. Spectrometric test with 2,2-diphenyl-1-picrylhydrazyle (DPPH) was employed to evaluate their antioxidant activities. The protective effect of pomiferin, isopomiferin, osajin and catalposide to DNA exposed to oxygen radicals *in vitro* was also examined.

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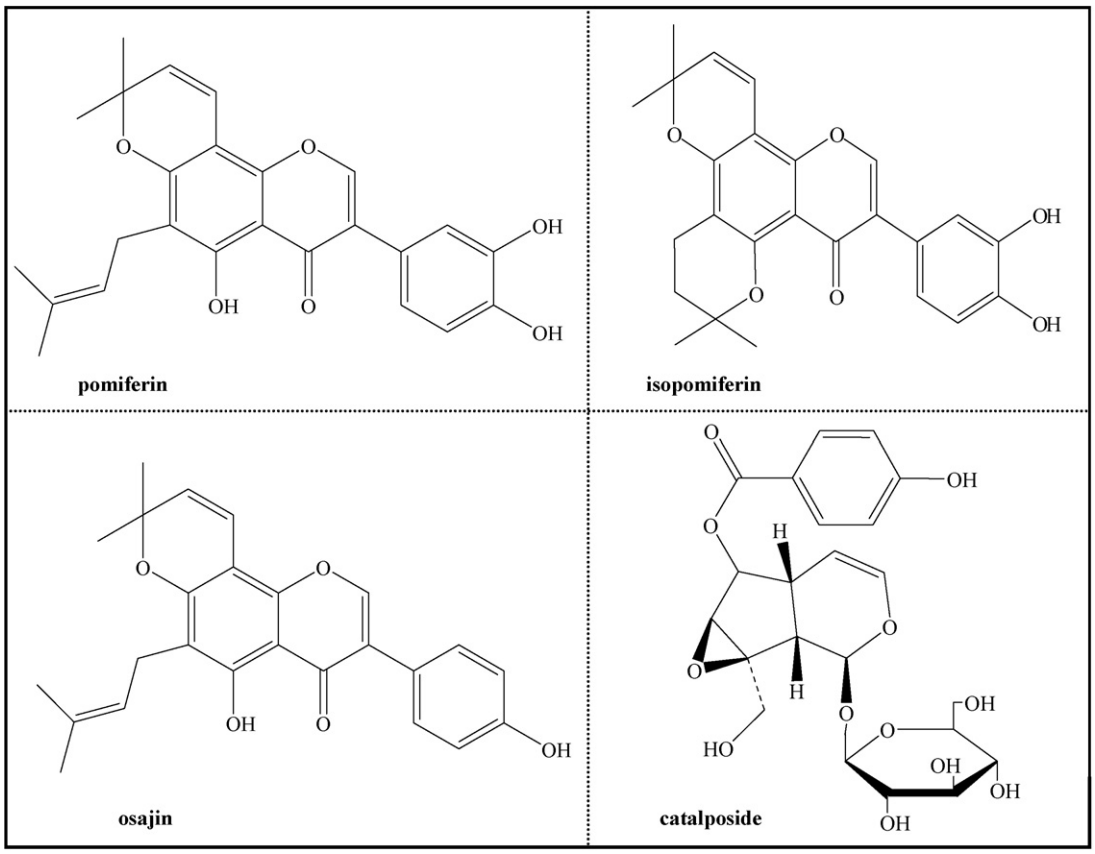

Fig. 1. Formulae of the flavonoids (pomiferin, isopomiferin and osajin) and iridoid glycoside (catalposide) in this study.

2. Experimental

2.1. Chemicals

Pomiferin, isopomiferin, osajin and catalposide were isolated from osage orange, *Maclura pomnifera* (Moraceae) as previously published [2,9,18]. All other chemicals used were purchased from Sigma–Aldrich unless noted otherwise. 10 μg/ml standard stock solutions were prepared with HPLC grade methanol (Sigma–Aldrich, USA) and stored in the dark at −20 °C. Working standard solutions were prepared daily by diluting the stock solutions. Lyophilised polymerized DNA (Reanal, Hungary) was isolated from chicken erythrocytes (MW = 400,000 g/mol).

2.2. Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated by a set of WTW buffers (Weilheim, Germany).

2.3. Electrochemical measurements

Electrochemical measurements were performed with CH Instruments Electrochemical Workstation (USA) using a three electrodes cell system. A carbon paste working electrode, a Ag/AgCl/3M KCl reference electrode and a platinum wire counter electrode were utilized. Phosphate buffer (0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4 in ratio 4:6 (v/v), pH 6.98) and/or acetate buffer (0.2 M CH_3COOH + 0.2 M CH_3COONa) were used as the supporting electrolytes. For optimization of pH of acetate buffer see Section 3. Square wave voltammetric parameters were as follows: initial potential 0.0 V, end potential 1.2 V, pulse amplitude 49.85 mV and step potential 1.05 mV. The frequency was optimized. For other details see Section 3. The instrument was controlled by programme CHI, Version 7.12 (Auxtin USA). For smoothing and baseline correction the GPES 4.9 software supplied by EcoChemie was employed. All experiments were carried out at room temperature.

2.4. Preparation of carbon paste electrode

The carbon paste (about 0.5 g) was made of 70% carbon powder (10–50 μM) and 30% mineral oil (Sigma–Aldrich; free of DNase, RNase, and protease). This paste was housed in a teflon body having a 2.5 mm diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper [19–21].

2.5. Spectrophotometric determination of antioxidant properties of the target molecules

Spectrophotometric measurements were carried out using an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed on a cooled sample holder (4 °C) and automatically pipetted into plastic cuvettes with a 0.5 cm path length. Incubation proceeded at 37 °C. To measure antioxidant activities of pomiferin, isopomiferin, osajin or catalposide the following procedures were used: (i) 2,2-diphenyl-1-picrylhydrazyle (DPPH, 9.5×10^{-5} M, 450 μl) dissolved in ethanol was pipetted; (ii) the

solution was stirred; (iii) 5 μl of flavonoid at three various concentrations (0.0005, 0.002 or 0.01 M) were pipetted into cuvettes with DPPH; (iv) the solution was stirred; (v) absorbance of the mixture was scanned at 510 nm for 20 min in 16-s intervals. The instrument was controlled using programme BS-200 (Mindray, China).

2.6. Fenton's reaction with DNA

To study the effects of flavonoids and catalposid on DNA, damaged by oxidation Fenton's reaction was carried out [22]. DNA (100 μg per test tube) was briefly exposed to $FeCl_2$ and H_2O_2 mixed in a stoichiometric ratio of 1:1. Applied concentrations of these reagents were 1, 2 or 4 mM. To investigate the influence of flavonoids on this reaction, pomiferin, isopomiferin, osajin and catalposide were added to the test tubes. The reactions were carried out in the presence of 10 mM NaCl and 10 mM phosphate buffer (10 mM NaH_2PO_4 + 10 mM Na_2HPO_4 in ratio 2:8 (v/v), pH 7.4) at 37 °C for 15 or 30 min and stopped by adding 100 μl of 100 mM EDTA solution. Reaction solutions were then measured by square wave voltammetry with CH Instruments.

2.7. Descriptive statistics

MICROSOFT EXCEL® (USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless noted otherwise.

3. Results and discussion

The antioxidant capabilities of the investigated compounds have been examined in several earlier studies [1,7,9]. Although, their use as antioxidant drugs has not been thoroughly investigated, it has been suggested that they can potentially be used for the treatment of diabetes and/or tumour diseases.

3.1. Electrochemical behaviour of the target molecules

3.1.1. Pomiferin

The square wave voltammograms of the compounds of the interest (10 μg/ml) were measured at a carbon paste electrode in the presence of acetate buffer (pH 4.3). The oxidative region of the voltammogram curves were baseline corrected and smoothed using GPES 4.9 software. A signal sample of pomiferin at potential 0.85 V is shown in Fig. 2A. The influence of experimental conditions on the signal of pomiferin was further studied, specifically the change of the pomiferin oxidative signal with increasing frequency (Fig. 2A). A sharp increase was observed in peak height up to a frequency of 250 Hz. At higher frequencies of up to 540 Hz, the peak height enhanced gradually. The signals measured at frequencies higher then 540 Hz showed relative standard deviations exceeding 50%. A frequency of 250 Hz was used for optimal results. pH of the supporting electrolyte (range from 3.4 to 5.2) also contributed to signal intensities. The highest signal was measured in the presence of acetate buffer at pH 4.3 (Fig. 2B). The dependence of pomiferin peak height on its concentration and its measurement in the presence of acetate buffer at pH 4.3 and 250 Hz are shown in Fig. 2C. The signal is proportional to the pomiferin concentration in the tested range (from 0.1 to 100 ng/ml). The linear calibration curve ($y = 241.2x - 42.7$, $R^2 = 0.9942$) for pomiferin within the range from 0.1 to 1 ng/ml is shown in Fig. 2C. The relative standard deviation (R.S.D.) was below 5%. The detection limit (3 S/N) for pomiferin was estimated to be 50 pg/ml. The detection limits were calculated according to Long and Winefordner [23], whereas N was expressed as the standard deviation of noise determined in the signal domain.

3.1.2. Isopomiferin

Square wave voltammetric measurements of isopomiferin are shown in Fig. 3. The dependence of isopomiferin peak height on frequency is complex compared to pomiferin dependence. The highest signal was measured at 550 Hz and is shown in Fig. 3A. Two peaks, the first at potential 0.80 V and a second at potential of about 0.73 V, were observed. To evaluate the dependence between peak height and frequency, the first peak was studied. The electrochemical behaviour of isopomiferin was compared to pomiferin and their dependence on isopomiferin peak height in relation to various pH of acetate buffer (the range from 3.4 to 5.2) was examined as well. The highest signal resembled the observed signal at pH 4.3, but at higher pH values the isopomiferin signal was absent (Fig. 3B). The dependence of the peak height on isopomiferin concentration is shown in Fig. 3C. The linear calibration curve ($y = 78.8x + 158.1$, $R^2 = 0.9983$) for pomiferin within the range 1–12 ng/ml is shown in inset Fig. 3C. The R.S.D. was below 7% and the detection limit was 800 pg/ml.

3.1.3. Osajin

Osajin measurements by SWV showed well developed oxidative signals at a potential of 0.75 V. The peak height of osajin enhanced with increasing frequency within the tested range (from 20 to 550 Hz). At frequencies higher then 550 Hz the signals were not repeatable with R.S.D. above 40%. Similar behaviour was also observed for pomiferin. The pH dependence was similar to those measured for isopomiferin with a maximum pH of 4.8. The difference is the sharp decrease in peak height (up to 80% in comparison with the signal height at pH 4.8) observed at pH 5.0 compared to pH 4.6 for isopomiferin (Fig. 4B). Although all target molecules have similar chemical structures, the slight difference might be due to changes in their physico-chemical properties. Free hydroxyl groups in pomiferin are likely to be responsible for its behaviour at various pH values. The dependence of the peak height on osajin concentration is shown in Fig. 4C. Its shape slightly differs from the pomiferin and isopomiferin dependences. The linear calibration curve ($y = 265.3x - 16.5$, $R^2 = 0.9908$) for pomiferin within the range from 0.1 to 1 ng/ml is shown in Fig. 4C. The R.S.D. was below 4% and the detection limit 40 pg/ml.

3.1.4. Catalposide

Catalposide due to its sugar embodied structure showed different electrochemical behaviour. The electrochemical signal of catalposide (10 μg/ml) is shown in Fig. 5A. The signal was observed at the potential of 0.80 V. The dependence of the catalposide peak height on frequency increased from 20 to 200 Hz and the signal decreased slightly up to 350 Hz. At frequencies higher than 350 Hz, R.S.D. of the signals exceeded 40%. We also observed different pH dependence of catalposide signals compared to pomiferin, isopomiferin and osajin (Fig. 5B). The highest signal was determined at pH 4.8. In contrast, the lowest signals were measured at the most acidic pH values. Catalposide's electrochemical behaviour may be due to the presence of its sugar moiety. The dependence of the peak height on osajin concentration is shown in Fig. 5C. Its shape slightly differed from the other studied molecules. The linear calibration curve ($y = 633.3x - 55.2$, $R^2 = 0.9990$) for catalposide within the range from 0.1 to 1 μg/ml is shown in Fig. 5C. The R.S.D. was below 9% and the detection limit 10 ng/ml.

3.2. Antioxidant activity—spectrophotometric analysis

Attempts have been made to find correlation between antioxidant capacity and oxidation potential. However some results seem to be contradictory and unclear, nevertheless, the correlation

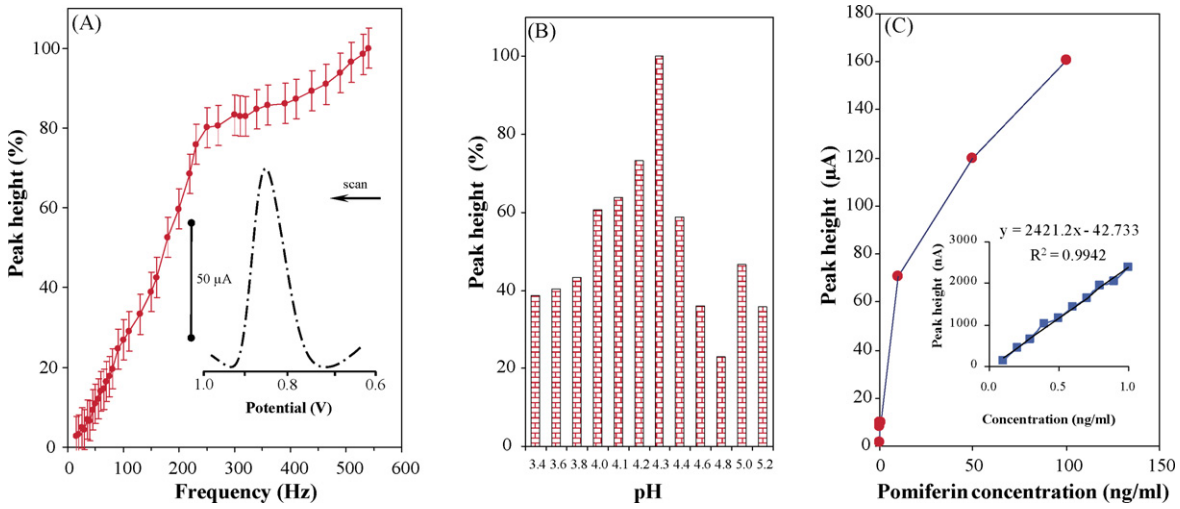

Fig. 2. Square wave voltammetric behaviour of pomiferin at carbon paste electrode. (A) The dependence of the peak height on frequency, (B) pH of acetate buffer and (C) concentration of pomiferin. In inset in (A): typical SW voltammogram of pomiferin (10 μg/ml). In inset in (C): the calibration curve for pomiferin within the range of 0.1–1 ng/ml.

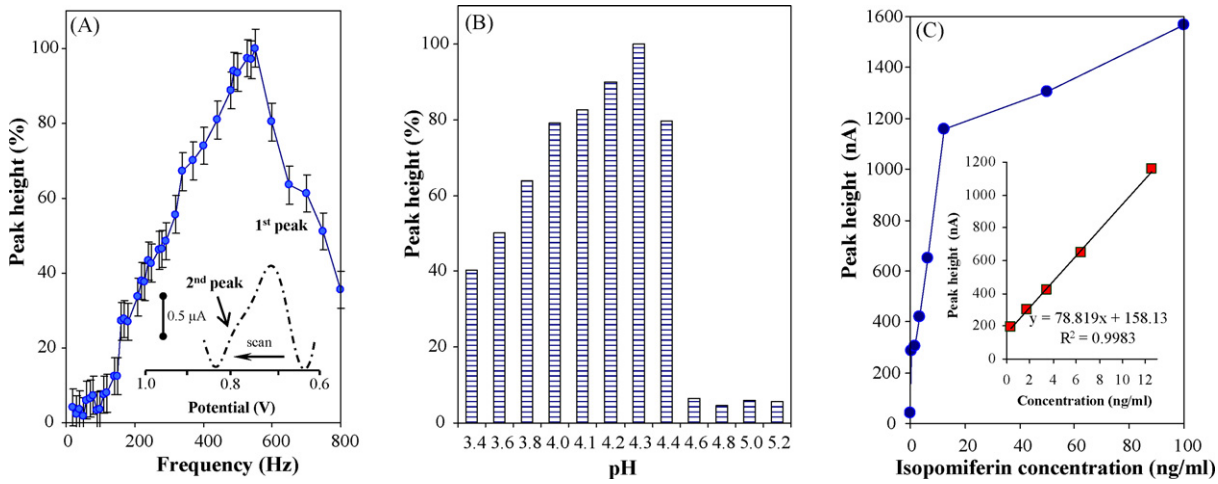

Fig. 3. Square wave voltammetric electrochemical behaviour of isopomiferin at carbon paste electrode. (A) The dependence of the peak height on frequency, (B) pH of acetate buffer and (C) concentration of isopomiferin. In inset in (A): typical SW voltammogram of isopomiferin (10 μg/ml). In inset in (C): the calibration curve for isopomiferin within the range of 1–12 ng/ml.

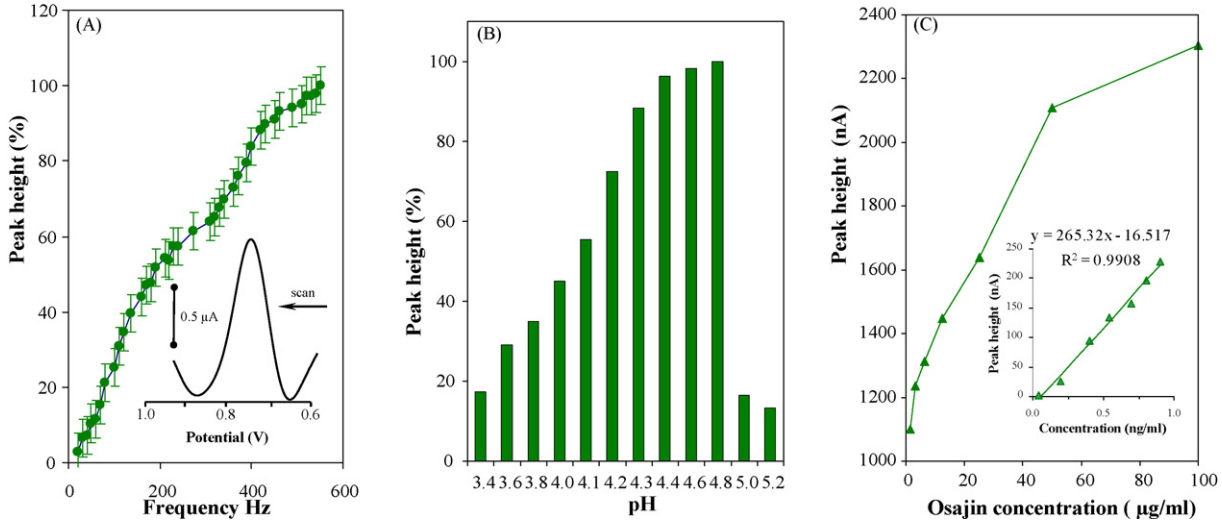

Fig. 4. Square wave voltammetric electrochemical behaviour of osajin at carbon paste electrode. (A) The dependence of the peak height on frequency, (B) pH of acetate buffer and (C) concentration of osajin. In inset in (A): typical SW voltammogram of osajin (10 μg/ml). In inset in (C): the calibration curve for osajin within the range of 0.1–1 ng/ml.

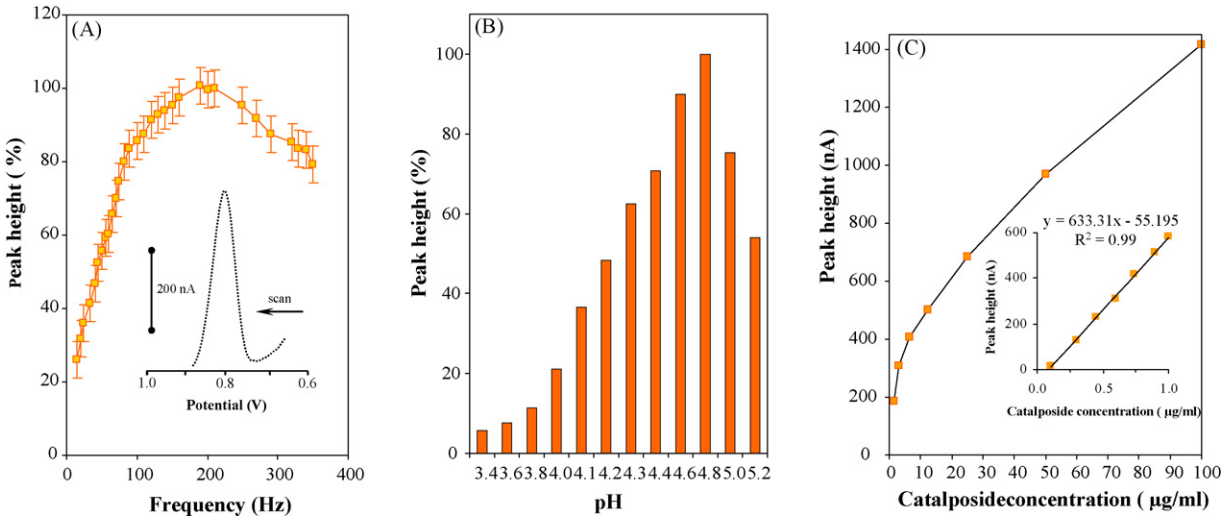

Fig. 5. Square wave voltammetric electrochemical behaviour of catalposide at carbon paste electrode. (A) The dependence of the peak height on frequency, (B) pH of acetate buffer and (C) concentration of catalposide. In inset in (A): typical SW voltammogram of catalposide. In inset in (C): the calibration curve for catalposide within the range of 0.1–1 μg/ml.

between antioxidant capacity and oxidation potential is present. Oxidation potentials for well known antioxidants such as vitamin C or rutin are about 0.4 V. As described in Section 3.1, target molecules' oxidation signals occur at about 0.8 V. Based on these facts it can be concluded that oxidation potential cannot be considered as one and the only hallmark for evaluation of antioxidant capacity [24–31]. Antioxidant activity of pomiferin, isopomiferin, osajin and catalposide were determined using a spectrophotometric method in which the second of the two absorption maxima of DPPH at 310 and 530 nm disappears (colour change from purple to yellow) if DPPH radical is reduced in the presence of other instable radicals or hydrogen donors [32,33]. This method is reported to be highly specific and suitable for the determination of antioxidant activity [34]. Trolox was used as the antioxidant standard. As it is shown in Fig. 6A spectral changes of DPPH after pomiferin, isopomiferin and trolox addition are apparent. On the contrary, no colour change was observed with osajin and catalposide which is in agreement with previously published data [18].

The time course of the reactions was further studied using an automatic analyser commonly used in clinical laboratories. The sample and reagents were pipetted into cuvettes, stirred and incubated as described in Section 2. The changes, measured at 510 nm in 16-s intervals for 20 min are shown in Fig. 6B. Pomiferin, isopomiferin and trolox (2 mM) showed marked changes, while osajin and catalposide did not. Isopomiferin had twice as much antioxidant activity as pomiferin and trolox. Additional experiments on antioxidant activity of pomiferin, isopomiferin, osajin, catalposide and trolox were carried out using four times lower concentrations of the tested compounds and the same concentration of DPPH. Under these conditions pomiferin had similar antioxidant activity as trolox and osajin while catalposide did not show antioxidant activity. Isopomiferin displayed approximately twice as high antioxidant activity (not shown) versus the other studied compounds.

Furthermore, the dependence of absorbance on DPPH concentration measured at 510 nm was studied. The dependence had the equation $y = 1 \times 10^8 x - 1163$. This equation was used for the determination of the total amount of reacted radicals. The antioxidant activity was expressed as the number of radical molecules reduced by one molecule of the compound of interest (flavonoids or catalposide) (Table 1). Similar results were obtained by expressing the antioxidant activity as a "total percentage" according to the following equation: $\% = 100 - [(A_{\text{of steady state}}/A_{t0}) \times 100]$. According to this equation, pomiferin (79.8%), has stronger antioxidant properties than its isomer isopomiferin (40.4%) (Table 1). Osajin and catalposide did not exhibit detectable antioxidant activity.

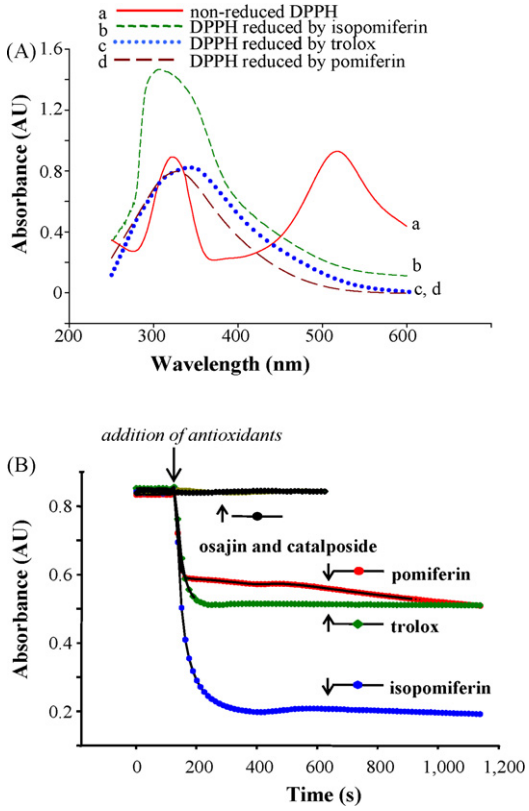

Fig. 6. Evaluation of antioxidant activity of the target molecules by using the DPPH test. DPPH (A) spectra of DPPH, DPPH + isopomiferin, DPPH + pomiferin and DPPH + trolox. To 1500 μl of DPPH (9.5×10^{-5} M), 40 μl of 10 mM of antioxidant was added and spectrum was measured 5 min later. (B) Changes in absorbance of DPPH, DPPH + isopomiferin, DPPH + pomiferin, DPPH + osajin, DPPH + catalposide or DPPH + trolox solutions measured at 510 nm (pomiferin, isopomiferin, osajin, catalposide and trolox—2 mM).

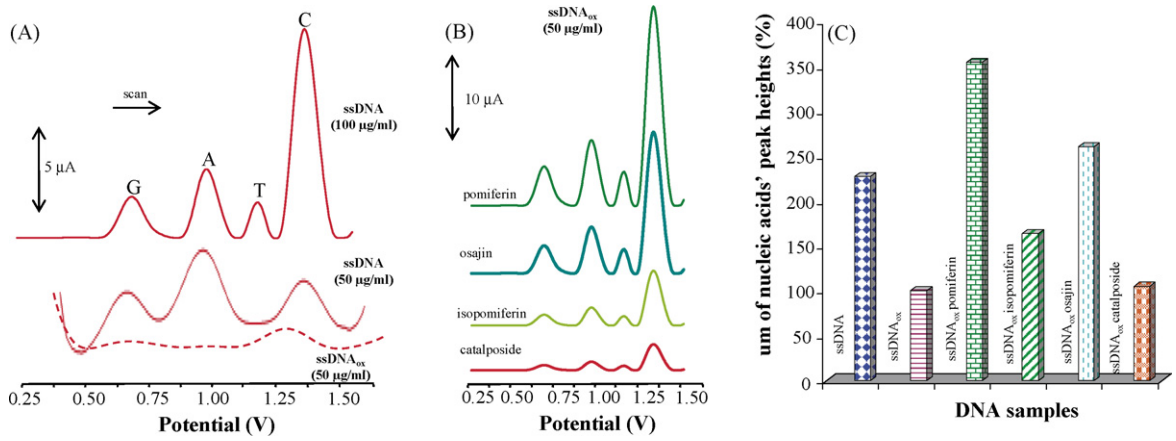

Fig. 7. AdTS SW voltammograms of DNA measured at the carbon paste electrode. (A) Upper voltammogram: 100 µg/ml ssDNA; middle voltammogram: 50 µg/ml ssDNA; lower curve 50 µg/ml ssDNA exposed to Fenton reaction for 15 min. (B) Typical AdTS SW voltammograms of ssDNAox protected by pomiferin, isopomiferin, osajin and catalposide (200 mM) during Fenton reaction. (C) Peak height expressed as the total sum of all oxidative signals of DNA. The highest signal for single nucleic acid represents 100%.

Table 1
Antioxidant activity of the studied compounds

	Compound				
	Pomiferin	Isopomiferin	Osajin	Catalposide	Trolox
Number of molecules[a]	1.5	3	0	0	1.5
Antioxidant capacity (%)[b]	40.4 ± 3.6	79.8 ± 5	0	0	41.7 ± 3
Protective effect to DNA[c]	2.5	0.6	1.6	0.04	n.d.

n.d.: not detected.

[a] Number of molecules DPPH reduced by one molecule of the tested compound.

[b] $\% = 100 - [(A_{t\,\text{steady state}}/A_{t0}) \times 100]$.

[c] $(\text{signal}_{DNA}/\text{signal}_{DNAox}) - 1$.

3.3. *Investigation of interaction of pomiferin, isopomiferin, osajin and catalposide with DNA*

Oxidative signals of nucleic acids can be measured at the surface of a carbon paste electrode. For DNA measurements, adsorptive transfer stripping technique (AdTS) coupled with square wave voltammetry was employed to prevent interferences from occurring. The technique involves transferring an electrode from a solution after accumulating a target molecule on its surface followed by rinsing the electrode and immersing it into a supporting electrolyte, where no interferences are present [35–43]. AdTS SW voltammograms of 100 µg/ml ssDNA measured in the presence of phosphate buffer pH 6.98 is shown in Fig. 7A. Oxidative signals of guanine (G), thymine (T), adenine (A) and cytosine (C) can easily be detected in voltammograms [12,44,45]. A recent paper examined the interaction of quercetin and rutin with DNA molecules on carbon paste electrode [12]. Their method of analysing DNA interactions with other molecules appears to be suitable for the rapid examination of flavonoids–DNA interactions. The influence of free oxygen radicals on DNA molecule signals protected and not protected by pomiferin, isopomiferin, osajin or catalposide were investigated using SWV at CPE. The protective effect of the compounds of interest were measured in the presence of phosphate buffer pH 6.98 since flavonoids and catalposide emit low responses at pH values higher than 6. The oxygen radicals were generated by Fenton reaction (1 mM H_2O_2 and 0.5 mM $FeCl_2$) in the presence of 50 µg/ml DNA for 15 min at 37 °C. DNA was analysed using SWV. The non-oxidized ssDNA (50 µg/ml) gave well developed signals; however, after oxidative damaging, observed oxidative signals decreased. The signals of guanine, adenine and thymine were most affected by oxidation (Fig. 7A). The protective effect of the compounds of interest was determined by observing the change in DNA signal, which represents the sum of the peak heights for G, T, A and C. Typical voltammograms of ssDNAox protected by four studied compounds are shown in Fig. 7B. Significant protective effects were observed for pomiferin and osajin. Smaller effect was observed for isopomiferin while a very low protective effect was determined in the case of catalposide (Fig. 7C). Nucleic acids' peak heights of DNA protected by pomiferin or osajin compared to non-oxidized ssDNA might be associated with an ability of pomiferin or osajin to reduce parts of nucleic acids, which were oxidized by oxygen. Protective effects can also be evaluated using the following formula: $(\text{signal}_{DNA}/\text{signal}_{DNAox}) - 1$. According to this equation the highest protective effect was observed for pomiferin and osajin (Table 1). As it is shown in Fig. 6, osajin demonstrated low antioxidant properties. Its protective effect is the most likely due to its ability to interact with DNA molecules. Catalposide is probably not able to interact with DNA molecule; because of this no protective effect to DNA molecule was observed.

4. Conclusion

The correlation between antioxidant activities of certain molecules and their beneficial effects on an organism is a well-known topic studied extensively. Two methods (UV–vis spectrometry – DPPH test and square wave voltammetry – oxidation damaging of DNA) were examined and used to study antioxidant activity. The methods were employed to study pomiferin, isopomiferin, osajin and catalposide activity. Based on the results, pomiferin has the highest antioxidant activity followed by isopomiferin, osajin and catalposide.

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