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Determination of phenolic compounds and their antioxidant activity in *Erigeron acris* L. extracts and pharmaceutical formulation by flow injection analysis with inhibited chemiluminescent detection

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ABSTRACT

It was found that the chemiluminescence (CL) produced from the reaction of luminol with iodine in the alkaline medium was strongly inhibited by plant phenolic compounds. Based on this finding, a new flow injection CL method was developed for the determination of caffeic acid and 6′-caffeoylerigeroside. The latter compound was isolated for the first time from *Erigeron acris* L. herb. The method was simple, rapid and sensitive with a detection limit of 4×10^{-3} ng mL^{-1} (caffeic acid) and 0.18 ng mL^{-1} (6′-caffeoylerigeroside), linear range of 0.1–1.5 ng mL^{-1} (caffeic acid) and 1–200 ng mL^{-1} (6′-caffeoylerigeroside), relative standard deviation of 3.3% for 10 measurements of 0.45 ng mL^{-1} caffeic acid and 2.9% for 40 ng mL^{-1} 6′-caffeoylerigeroside. This method was successfully applied to determine the content of phenolic compounds/antioxidant activity of *E. acris* L. extracts and phenolic acids content in pharmaceutical formulation. A possible mechanism of the inhibition of the proposed CL system was discussed.

1. Introduction

Erigeron acris L., blue fleabane, from the Asteraceae family, is a biennial or perennial plant occurring in dry grasslands and fields [1]. In folk medicine it was applied to relieve toothache and arthritic pains [2]. Phytochemical studies showed the presence of polyphenolic compounds in this plant. Flavonoid aglycones (quercetin, luteolin, apigenin and kaempferol), luteolin 7-*O*-glucoside, scutellarin and erigeroside were isolated from the herb [3–5]. Polyphenols in general play a key role in the antioxidant activity of plant sources. Many publications concern searching for methods to measure that activity, most of which are spectrophotometric methods based on the capacity of the sample to inhibit the production of free radicals generated in the system [6].

Over the past few years, there have been increasing attention to the chemiluminescence (CL) as a sensitive and fast assay for the screening of antioxidant activity. Among different chemiluminogenic species exploited for the evaluation of antioxidant activity, luminol seems to be the most commonly used CL reagent. The assays employing a luminol CL detection are based on the scavenging of free radicals (including reactive oxygen species) involved in the sequence leading to an electronically excited 3-aminophtalate dianion (3-APA*), which emit the light on return to its ground state [7]. The determination of antioxidants is mainly based on the inhibiting of CL derived from few enzymatic reactions: luminol–H_2O_2–horseradish peroxidase [8,9], luminol–xanthine–xanthine oxidase [8,10,11], luminol–hypoxanthine–xanthine oxidase [12], but also luminol–H_2O_2–Co(II)/EDTA [13], luminol–H_2O_2–Fe(II) [14], luminol–ClO^- [15] and luminol-2,2′-azo-bis-(2-amidinopropane) [16] CL system. Majority of those methods are manual, only three employs flow injection methodology [10,13,15]. CL detection combined with flow injection analysis considerably reduces the time of analysis and is very useful for studying the inhibition effect of antioxidants on unstable oxygen species with a short lifetime (e.g. $O_2^{\bullet -}$) [10].

To the best of our knowledge, there are no reports using the CL of luminol–I_2 system for the determination of antioxidant activity. As far in the literature there are only few studies which exploited luminol–I_2 detection system for the CL determination of different analytes [17–20]. Babko et al. [17] as first found that luminol–I_2 detection system could be used to measure iodine concentration. Min et al. [18] have reported use of a sequential injection analysis (SIA) system for the analysis of penicillin, based on the formation of penicilloic acid by penicillinase, which was quantified by its quenching effect on the CL originating from luminol oxidized by I_2. Ratanawimarnwong et al. [19] used gas-diffusion

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flow injection analysis (FIA) system with luminol–I_2 detection for the determination of iodide in the pharmaceutical products. Iodide in a sample was oxidized by $K_2Cr_2O_7$ to iodine which diffused through the PTFE membrane into an acceptor stream of iodide solution and reacted with luminol to produce CL. Nalewajko et al. [20] applied luminol–I_2 detection system for the determination of catecholamines in pharmaceutical formulations (FIA-CL) and human urine samples (HPLC-CL).

In this paper, the inhibiting effect of polyphenols isolated from *E. acris* on the CL of luminol oxidized by iodine in the flow injection mode has been investigated for the first time. Because of the difficulty in measuring each antioxidant component separately and interactions among antioxidants, the FIA-CL method have been developed to assess the total content of phenolic compounds/antioxidant capacity of diethyl ether and ethyl acetate fractions of flowers, leaves and roots of *E. acris*. For comparative studies, the ability to scavenge a stable, free 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) was investigated as well. It was also intended to evaluate the antioxidant activity of 6′-caffeoylerigeroside (isolated from *E. acris* for the first time) and to compare it with a reference antioxidant, the Trolox molecule. Additionally, in order to assess the accuracy of the developed CL method, the total content of phenolic acid expressed as a caffeic acid equivalent was determined using the pharmaceutical formulation Immunofort.

2. Experimental

2.1. General techniques

^{1}H, ^{13}C NMR, DEPT, ^{1}H COSY, HSQC, heteronuclear multiple bond coherence (HMBC) spectra were recorded on a Bruker Avance II 400 apparatus. HPLC analysis was performed using the Waters system (Milford, MA, USA) equipped with a Waters 600E pump, a 600 Controller and a 996 PDA detector scanning between 190 nm and 400 nm. The data were collected and analysed with the Millenium^{32} Chromatography Manager V4.0 Software. Separation was carried out using a Symmetry C_{18} column (5 μm; 3.9 mm × 150 mm, Waters Corp., USA). The compounds were detected at 275 nm.

2.2. Reagents and solutions

6′-Caffeoylerigeroside, luteolin, apigenin, scutellarin were isolated from *E. acris*, caffeic, *p*-coumaric, ferulic, gallic, chlorogenic, *p*-hydoxybenzoic acids, kaempferol, isoquercitrin were purchased from Fluka (Steinheim, Germany), syringic, vanillic, protocatechic acids were supplied by Roth (Karlsruhe, Germany). The stock solutions (120 mg L^{-1}) of investigated phenolic compounds were prepared in methanol and diluted with water to obtain an appropriate concentration of working solutions. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was provided by Randox Laboratories Ltd. (United Kingdom). DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was supplied by Sigma–Aldrich Chemie (Steinheim, Germany). The methanolic solution of DPPH• (6×10^{-5} mol L^{-1}) was prepared daily, before measurements. The water used to prepare the solutions was purified in a Milli-Q Plus water purification system (Millipore S.A., Molsheim, France). Chloroform ($CHCl_3$), diethyl ether (Et_2O), ethyl acetate (EtOAc) were purchased from POCH, Gliwice, Poland. Methanol (MeOH) was from Merck (Darmstadt, Germany) in HPLC grade purity. Luminol was supplied by Fluka (Steinheim, Germany), I_2, KI, NaOH by POCH (Gliwice, Poland) all of analytical grade. A 2.5×10^{-2} mol L^{-1} stock luminol solution was prepared in 0.06 mol L^{-1} or 0.15 mol L^{-1} NaOH and was kept in the dark at +4 °C. The 5×10^{-2} mol L^{-1} stock I_2 solution was prepared in 100 mL of water containing 4 g of KI.

2.3. Plant material and extract preparation

Leaves, inflorescences and roots of *E. acris* were collected in June 2006 near Białystok, situated in the northeast of Poland. A voucher specimen is deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Białystok (Poland).

Five grams of each source were extracted twice, with 250 mL and with 100 mL of MeOH under reflux. Than, after evaporation of the solvent, the residue was dissolved in hot water and exhaustively extracted with $CHCl_3$, Et_2O, EtOAc. Et_2O and EtOAc had the higher content of polyphenol compounds so they were chosen for further studies. After evaporation of solvent, residues from Et_2O and EtOAc fractions were dissolved in methanol and diluted with water to fit the concentration of analytes to the linear calibration range.

Additionally the total phenolic acid content was analysed using a herbal drug. The herbal preparation Immunofort (Leki Natury, Poland) was purchased from a local pharmacy. It contains in 100 g: *Echinaceae herbae succus* (40.0 g), *Aroniae fructi succus* (30.0 g), *Urticae herbae succus* (10.0 g) and *Bardanae ridicis succus* (10.0 g) and is standardized on phenolic acids (declared amount 1.0 mg of phenolic acids expressed as a caffeic acid equivalent in a single dose). The pharmaceutical formulation was diluted with 1.25×10^{-3}% methanolic carrier stream to obtain the proper concentration of analytes.

2.4. Isolation and identification of new compounds from E. acris herb

Extract preparation was as previously described [3]. From methanolic extract of the *E. acris* herb, after separation on a Sephadex LH-20 column, 14 fractions were obtained, fractions 1–10 eluted with 50% MeOH and 11–14 eluted with MeOH. In fraction 5 erigeroside and in fraction 6 scutellarin were recognized. From fraction 7, 395 mg of compound **1** was obtained. Fraction 8, containing a mixture of compounds, on polyamide column was separated using EtOAc–MeOH step gradient as the eluent. From sub-fractions eluted with EtOAc–MeOH (5:5–3:7, v/v), 512 mg of compound **2** was obtained.

Isolated compounds were identified by spectral analysis.

Compound **1**, yellowish needles from MeOH; ^{1}H NMR (DMSO-d_6) δ (ppm): 8.16 (1H, s, H-2), 8.05 (1H, d, J = 5.6 Hz, H-6), 7.46 (1H, d, J = 15.88 Hz, H-7″), 7.04 (1H, d, J = 1.92 Hz, H-2″), 6.99 (1H, dd, J = 8.16 and 1.92 Hz, H-6″), 6.77 (1H, d, J = 8.12 Hz, H-5″), 6.39 (1H, d, J = 5.64 Hz, H-5), 6.25 (1H, d, J = 15.88 Hz, H-8″), 4.88 (1H, d, J = 7.12 Hz, H-1′), 4.42 (1H, d, J = 1.64, H-6′a), 4.18–4.13 (1H, m, H-6′b), 3.26–3.21 (3H, m, H-2′, 3′, 4′). ^{13}C NMR (DMSO-d_6) δ (ppm): 144.12 (C-2), 145.76 (C-3), 172.4 (C-4), 116.21 (C-5), 155.76 (C-6), 99.99 (C-1′), 73.09 (C-2′), 76.35 (C-3′), 69.75 (C-4′), 74.0 (C-5′) and 63.2 (C-6′).

The ^{1}H NMR spectrum of compound **1** exhibited aromatic protons at δ_H 7.04, 6.99, 6.77 and two trans olefinic protons at δ_H 6.25 and 7.46 (d, J = 15.88 Hz), indicating the presence of (*E*)-caffeic acid moiety. On the other hand, a singlet was observed at δ_H 8.16 and doublets at δ_H 8.05 and 6.39 (J = 5.6 Hz), together with a γ-pyrone carbon signal at δ_C 172.4 originating in pyromeconic acid. Assignment of the remaining ^{1}H and ^{13}C resonances of the sugar and the signal of the anomeric proton appearing at δ_H 4.88 (d, J = 7.12 Hz) indicate that the sugar residue was a β-D-glucopyranosyl. The connectivity between the glucose and caffeic acid moiety was confirmed by HMBC experiment, which showed a correlation peak between H-6′a, H-6′b (δ_H 4.42 and 4.18, respectively) of glucose

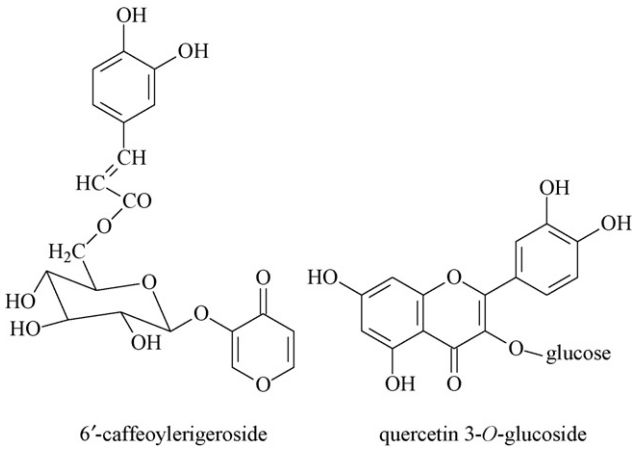

Fig. 1. Structure of compounds isolated from *E. acris* herb.

and the carbonyl carbon resonance (δ_C 166.4 C-9″) of caffeic acid. Therefore, the structure of **1** was identified as pyromeconic acid 6′-caffeoylglucoside, named also 6′-caffeoylerigeroside (Fig. 1). For the first time this compound was isolated from *E. multiradiatus* [21]. This is the second mention of the presence of **1** in the plant kingdom.

Compound **2**, yellow needles from MeOH, after acid hydrolysis gave quercetin as an aglycone and glucose. ^{1}H NMR (DMSO-d_6) δ (ppm): 12.62 (1H, s, H-5-OH), 7.6 (1H,d, J = 2.1 Hz, H-2′), 7.57 (1H, dd, J = 7.0 and 2.0 Hz, H-6′), 6.85 (1H, d, J = 9.0 Hz, H-5′), 6.4 (1H, d, J = 2.0 Hz, H-8), 6.2 (1H, d, J = 1.95 Hz, H-6), 5.45 (1H, d, J = 7.38 Hz, H-1″ anomeric proton of glucose).

The ^{1}H NMR spectrum of compound **2** was corresponding to data in the literature of quercetin 3-*O*-glucoside (isoquercitrin) (Fig. 1) [22].

2.5. HPLC analysis of extracts

Qualitative analysis of Et_2O- and EtOAc-fractions of methanolic extracts of leaves, inflorescences and roots was performed by the HPLC method according to Wen et al. [23]. Mobile phase A was water containing 0.02% trifluoroacetic acid (TFA) and phase B was methanol with 0.02% TFA. The flow rate of the mobile phase was 0.5 mL min^{-1}. The gradient conditions were as follows: 0–5 min, 25% B; 5–10 min, 25–30% B; 10–16 min, 30–45% B; 16–18 min, 45% B; 18–25 min, 45–80% B; 25–30 min, 80% B; 30–40 min, 80–25% B. Gallic, protocatechuic, chlorogenic, vanillic, caffeic, ferulic acid, *p*-hydroxybenzoic, *p*-coumaric acid, previously isolated flavonoid aglycones (quercetin, luteolin, kaempferol, apigenin) and 6′-caffeoylerigeroside were used as standards. Standards and samples were dissolved in 70% MeOH at a concentration of 1 mg mL^{-1}. Compound identification was made by comparison to the retention times and UV–vis spectra. Chromatographic data and the presence of phenolic compounds in Et_2O- and EtOAc-fractions of particular sources were presented in Table 1.

2.6. Chemiluminescence instrumentation and procedure

The configuration of the FI system used for this work is shown in Fig. 2. The solutions of luminol, I_2 and the carrier stream were continuously propelled by the peristaltic pump Minipuls 3 (Gilson, Australia) at an appropriate flow rate to the CL detector (KSP, Poland). The sample solutions were injected with a sample loop of 900 μL into a carrier stream using a four-way rotary injection valve (Model 5041, Rheodyne, USA) and merged with the combined stream of luminol (prepared in an appropriate concentration of NaOH) and I_2 (prepared in 4.82×10^{-4} mol L^{-1} KI) solution in a Perspex T-piece. Two mixing coils of 0.8 mm i.d. (RC_1 and RC_2) of an appropriate length were used to improve the efficiency of CL reactions. The detection system consisted of the flow cell (flat spiral-coiled PTFE tubing 1.0 mm i.d., length of 25 cm in six windings) placed in front of the photomultiplier tube in a light-tight box. The photomultiplier was operated at 1100 V, the control of the system and the data acquisition were performed through special software provided by the manufacturer of the luminometer.

2.7. 1,1-Diphenyl-2-picryl-hydrazyl free radical scavenging method

Radical scavenging activity of plant extracts against a DPPH radical was determined spectrophotometrically by the modified method of Brand-Williams et al. [24]. Diethyl ether and ethyl acetate extract solutions were prepared by dissolving 0.003 g of dry extract in 25 mL of methanol. Three millilitres of methanolic solution of DPPH$^\bullet$ (6×10^{-5} mol L^{-1}) were mixed with 77 μL extract solution. The samples were incubated in the dark for 15 min at room temperature, with the decrease in absorbance of the resulting solution measured at 515 nm. Radical scavenging activity was calculated by using the following formula:

$$\text{Inhibition}\,(\%) = \left[\frac{A_B - A_A}{A_B}\right] \times 100$$

where A_B: absorption of blank sample (t = 0); A_A: absorption of tested extraction solution (t = 15 min).

Table 1
Phenolic compounds recognized by HPLC in Et_2O and EtOAc-fractions from leaves, inflorescences and roots of *E. acris*

Compound	R_t (min)	UV (λ_{max})	Leaves		Inflorescence		Roots	
			Et_2O-fraction	EtOAc-fraction	Et_2O-fraction	EtOAc-fraction	Et_2O-fraction	EtOAc-fraction
Gallic acid	3.31	272	tr	–	–	–	tr	–
Protocatechuic	5.44	260, 295	+	–	++	–	tr	–
Chlorogenic acid	7.51	240, 326	–	–	–	–	–	–
p-Hydroxybenzoic	9.42	256	+	–	+	–	tr	–
Vanillic acid	11.07	260, 292	–	–	+	–	tr	–
Caffeic acid	11.9	323	+	–	+	–	tr	–
p-Coumaric acid	20.42	226, 310	+	–	–	–	tr	–
Ferulic acid	21.74	234, 323	–	–	–	–	tr	–
6′-Caffeoylerigeroside	23.08	251, 328	+	+++	–	++	–	+
Quercetin	30.37	256, 370	–	–	++	–	–	–
Luteolin	30.91	254, 351	–	–	+++	–	–	–
Kaempferol	31.84	265, 365	–	–	+	–	–	–
Apigenin	32.06	267, 340	+	–	++	–	–	–

+++, ++, +, tr: trace, decreasing content in extracts; R_t: the retention time.

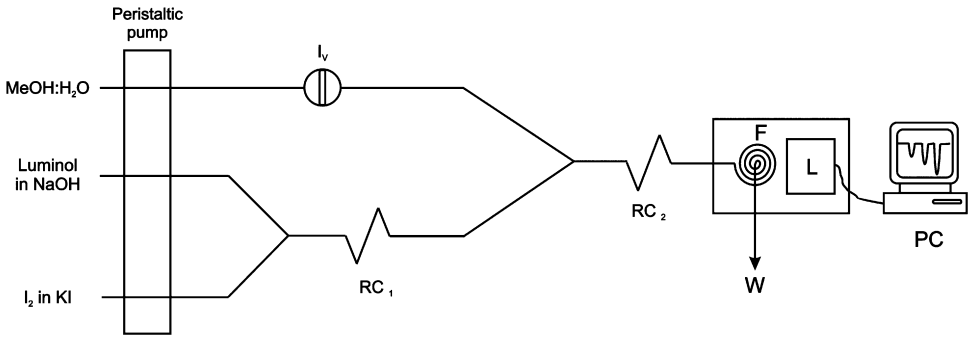

Fig. 2. Schematic diagram of the flow-injection system with CL detection for determination of caffeic acid and 6′-caffeoylerigeroside. RC_1, RC_2: mixing coils; I_V: injection valve; L: luminometer; F: flow cell; PC: computer; W: waste.

2.8. UV–vis absorption and chemiluminescence spectra

Absorption spectra were performed on a model 8452A diode array spectrophotometer (Hewlett-Packard, Germany). CL spectra were monitored using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Ltd., Japan) with the light source switched off.

3. Results and discussion

3.1. Quenching effects of phenolic compounds on CL of luminol

The antioxidant activity of *E. acris* extracts may be related to polyphenol and flavonoid content since it has been reported that these phenolic compounds can act, breaking the chain reaction of lipid, inhibiting CL reactions, scavenging several reactive oxygen species [8], chelate transition metal ions involved in free radical generation [25].

Among several analytical methods for the determination of antioxidant activity, CL is advantageous due to its high sensitivity and rapidity. It is well known that oxidation of luminol in the alkaline medium generates CL, the superoxide anion plays a key role in the formation of the electronically excited product which is induced by a superoxide anion radical ($O_2^{\bullet -}$), as shown in Fig. 3. The luminol radicals generated react with O_2 to yield $O_2^{\bullet -}$. Superoxide anion radical subsequently reacts with a luminol radical to yield an endoperoxide, which decomposes to yield an electronically excited 3-aminophtalate dianion, the emitter of the CL system [7]. When phenolic compounds are added to the solution, the light emission should be reduced, indicating antioxidant activity by scavenging any radical generated in this system. As the proposed procedure involved CL inhibition, it was decided to maximise a background emission by using iodine in order to improve sensitivity. When luminol and iodine are mixed on-line in the FIA-CL detection system, a constant luminescence is produced and registered as a background emission from the CL reaction (I_0). This base line is significantly decreased even when very low concentrations of phenolic compounds are injected into the CL system (I_S). The quantitative detection was based on the net CL intensity calculated by the following formula: $\Delta I = I_0 - I_S$.

In order to elucidate the possible CL mechanism, the emission spectra of luminol–I_2 reaction mixture in the presence and in the absence of phenolic compounds (caffeic acid was chosen as an example) were recorded (Fig. 4). Apparently, luminol is oxidized by iodine to form the excited 3-aminophtalate, because both CL spectra are identical with the characteristic maximum emission at

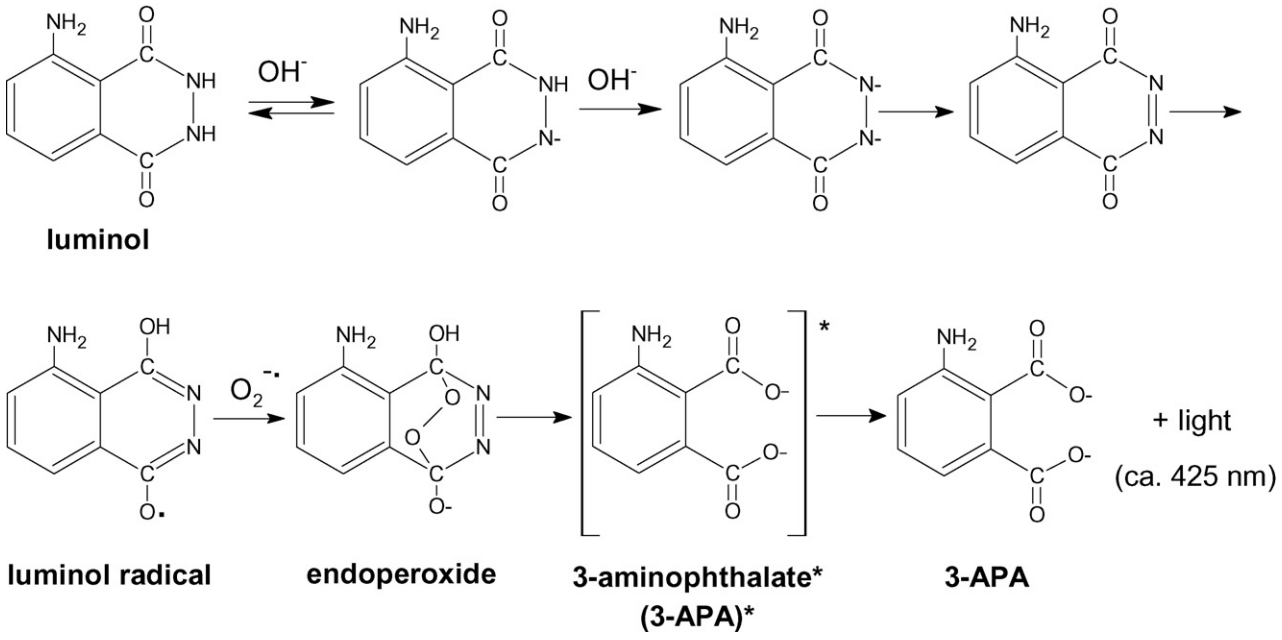

Fig. 3. Chemiluminescent generation with luminol and superoxide anion radical.

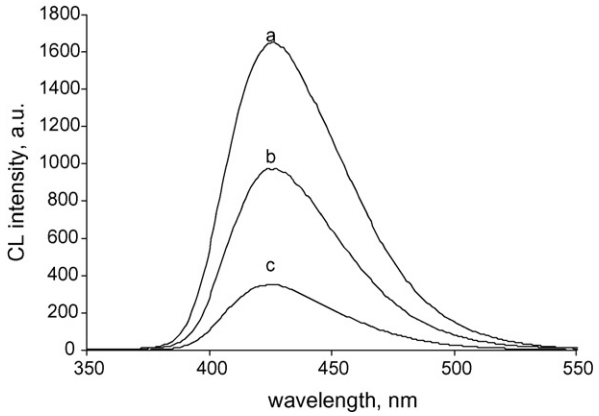

Fig. 4. Chemiluminescence spectra: (a) luminol–I_2; (b) luminol–I_2–caffeic acid (15 ng mL^{-1}); (c) luminol–I_2–caffeic acid (50 ng mL^{-1}). Luminol: 1.4×10^{-3} mol L^{-1}; I_2: 2×10^{-5} mol L^{-1}; NaOH: 6×10^{-2} mol L^{-1}.

about 425 nm. However, the relative CL intensity was lower in the presence of caffeic acid. Thus, the luminophore could be ascribed to 3-aminophtalate.

The CL inhibition of the luminol–I_2 system by phenolic compounds has been also investigated based on the studies of UV–vis absorption spectra (Fig. 5) of phenolic compound (in this case caffeic acid) (a), I_2 (b) and caffeic acid–I_2 system (c). It could be seen that light absorption of the mixed system (c) was different from the algebraic sum of the light absorption of the individual compounds (d), which indicates that there is a chemical reaction between iodine and the phenolic compound. Furthermore, a new absorption band emerges in the range of 390–500 nm, which probably corresponds to an oxidation product of caffeic acid. Hence, the decrement of the CL intensity of luminol is probably partly caused by the consumption of iodine, the oxidant of the luminol–I_2 system.

Additionally, it was observed that deoxidizing of the luminol, iodine and standard solutions by the flow of nitrogen caused about a 20% decrease in the CL intensity of the system investigated. These results as well as results presented in the literature [7] indicated that dissolved oxygen and reactive oxygen species play a significant role in the CL reaction.

Antioxidative activity of the investigated substances could be the result of prevention of the oxidation of luminol. The mechanism of inhibition seems to be very complex. Supposedly, few different pathways may simultaneously take place. Our experiments show that the antioxidants scavenge reactive oxygen species generated in the system as well as compete with luminol for iodine. But other processes, such as quenching excited aminophtalate, as well as scavenging any radicals generated during the oxidation of luminol may occur at the same time [12]. However, the degree of contribution of each process is not obvious. Furthermore, the phenolic compounds content/degree of antioxidant activity seems to be proportional to the negative peak height (ΔI).

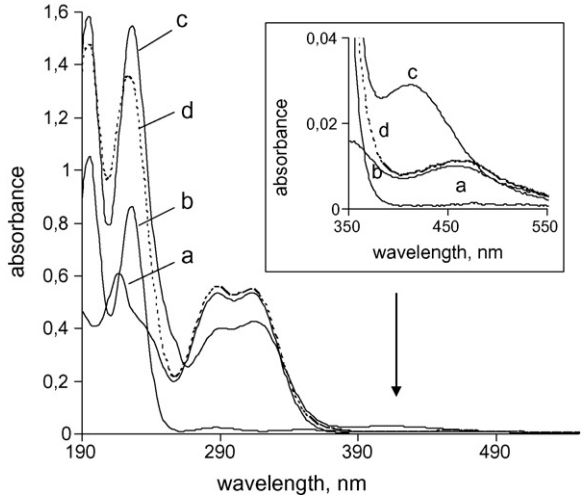

Fig. 5. UV–vis absorption spectra: (a) caffeic acid; (b) I_2; (c) caffeic acid + I_2; (d) algebraic sum of individual absorption spectra a and b. Caffeic acid: 5 μg mL^{-1}, I_2 (in 4.82×10^{-4} mol L^{-1} KI): 9×10^{-6} mol L^{-1}, blank: water.

3.2. Flow injection chemiluminescent determination

3.2.1. Effect of chemical and instrumental variables

Luminol oxidation in the alkaline medium in the presence of iodine is accompanied by strong CL, originating from the excited 3-aminophtalate [7]. Phenolic compounds (e.g. phenolic acids, flavonoids) which are known as potent antioxidants have been found to inhibit CL of the luminol–I_2 system strongly. Caffeic acid and 6′-caffeoylerigeroside exhibit a strong CL quenching effect and were present in all parts of *E. acris* (roots, leaves and flowers). For that reason they were chosen as the standards respectively for the determination of total phenols in Et_2O- and EtOAc-fractions of each part of the plant. In order to determine the parameters that gave the optimum signal for chosen phenolic compounds, a series of univariate searches were performed with respect to the sensitivity on the basis of the negative peak height (ΔI) and the ratio of the signal height to the noise (S/N). To obtain a reliable assay of caffeic acid and 6′-caffeoylerigeroside, the effects of different chemical and instrumental variables were investigated. All these parameters were optimised for 0.45 μg L^{-1} caffeic acid and 40 μg L^{-1} 6′-caffeoylerigeroside.

The influence of the concentration of iodine (prepared in 4.8×10^{-4} mol L^{-1} KI) was examined in the range of 6–60 μmol L^{-1}. The background CL (I_0) but also the noise level increased significantly with the increase in the concentration of oxidant. The maximum value of ΔI and S/N ratio was obtained when 20 μmol L^{-1} of iodine was used for the determination of caffeic acid and 15 μmol L^{-1} for 6′-caffeoylerigeroside (Fig. 6A).

Furthermore, the effect of luminol concentration was tested within the range of 0.8–1.8 mmol L^{-1}. With the increase of the concentration of luminol, both ΔI and background CL intensity increased and reached maximum when 1.4 mmol L^{-1} luminol was used for the determination of caffeic acid and 1.2 mmol L^{-1} for the determination of 6′-caffeoylerigeroside, therewith decreased slightly (Fig. 6B). Thus, these values were selected for further studies.

CL of luminol occurs only in the alkaline medium and strongly depends on the concentration of sodium hydroxide. Therefore, the influence of NaOH concentration on the chemiluminescent reaction was examined over the range of 3×10^{-2} to 0.8 mol L^{-1} which is shown in Fig. 6C. The maximum ΔI was observed for 6×10^{-2} mol L^{-1} concentration of sodium hydroxide (caffeic acid) and 0.1 mol L^{-1} (6′-caffeoylerigeroside).

The methanolic stock solutions of the studied phenolic compounds were diluted in water and injected into the methanol–water carrier stream. It was noted that the CL intensity of the investigated system strongly depends on the concentration of methanol in the carrier stream and started to decrease with increasing organic solvent content. It was reported in the literature that this effect is probably connected to the decrease of O_2 solubility in methanol and lower solvent polarity [26]. Therefore, the concentration of methanol in the carrier stream should be as low as possible and was equal to the content of methanol in the most concentrated

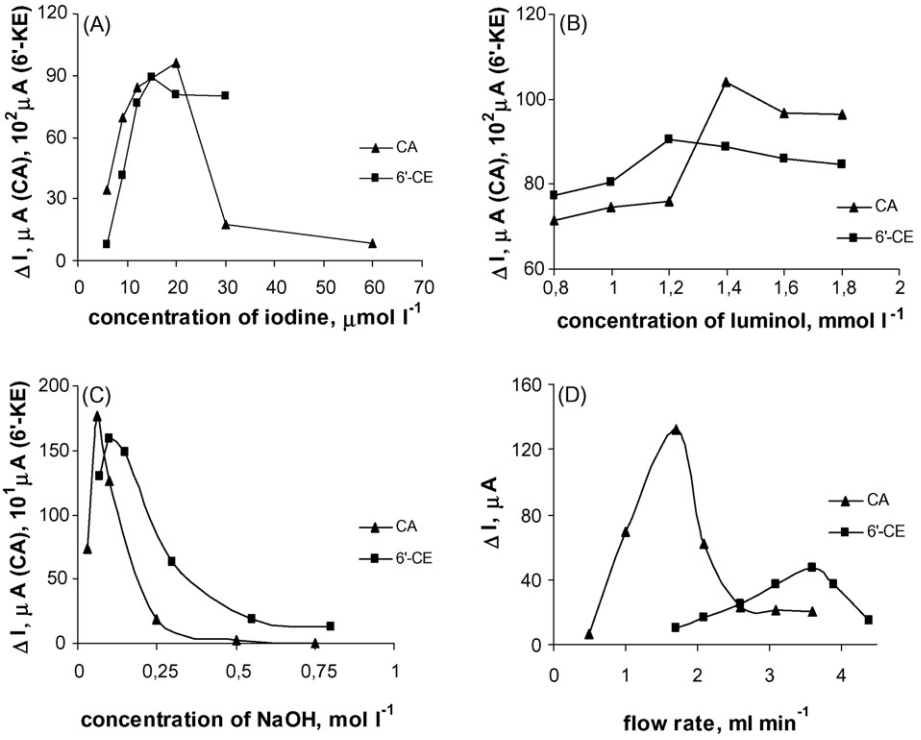

Fig. 6. (A–D) Optimisation of the flow injection system for caffeic acid and 6′-caffeoylerigeroside determination: (A) concentration of iodine (in KI); (B) concentration of luminol; (C) concentration of sodium hydroxide; (D) flow rate. CA: caffeic acid, 6′-CE: 6′-caffeoylerigeroside. Concentration of CA was 0.45 ng mL^{-1} and 6′-CE was 40 ng mL^{-1}.

solution of each analyte in the calibration curve (1.25×10^{-3}% for the determination of caffeic acid and 0.17% for the determination of 6′-caffeoylerigeroside).

The total flow rate of the carrier stream and reagent streams was studied over the range from 0.5 mL min^{-1} to 4.4 mL min^{-1}. The results are shown in Fig. 6D. The negative peak height increased sharply with the increasing flow rate until 1.7 mL min^{-1} (caffeic acid) and 3.6 mL min^{-1} (6′-caffeoylerigeroside) was reached, where the peaks had the highest value. When higher flow rates were used, ΔI decreased significantly. Thus, these values were chosen as the optimal.

Furthermore, the volume of sample injected was varied from 100 μL to 1000 μL by changing the length of the sample loop in the injection valve. It was found that, as the volume of sample injected increased, ΔI become significantly higher until the volume of sample loop of 400 μL (caffeic acid) and 800 μL (6′-caffeoylerigeroside). Then, above these values only small increase of ΔI was observed until the volume of 900 μL. In both cases, 900 μL was selected as an optimum because ΔI reached the maximum value.

To improve the efficiency of the chemiluminescent reaction between luminol and iodine and to maximise the inhibiting effect of caffeic acid and 6′-caffeoylerigeroside, two mixing coils (RC_1 and

Table 2
Analytical figures of merit for phenolic antioxidants

Fraction	Investigated compound	Calibration equation	r	Relative response (%)
Diethyl ether	Caffeic acid	$y = (125.2 \pm 0.4)x + (13.8 \pm 0.8)$	0.9999	100[a]
	Protocatechic acid	$y = (7.87 \pm 1.67)x + (3.73 \pm 1.52)$	0.9783	6.29[a]
	Syringic acid	$y = (6.22 \pm 1.11)x + (3.19 \pm 1.01)$	0.9846	4.97[a]
	Apigenin	$y = (4.70 \pm 0.24)x + (1.17 \pm 1.69)$	0.9962	3.75[a]
	Vanillic acid	$y = (2.66 \pm 0.13)x + (4.67 \pm 0.12)$	0.9988	2.12[a]
	p-Hydroxybenzoic acid	$y = (2.20 \pm 0.06)x + (3.03 \pm 0.06)$	0.9996	1.76[a]
	Luteolin	$y = (1.54 \pm 0.14)x + (2.88 \pm 0.43)$	0.9958	1.23[a]
	Gallic acid	$y = (1.48 \pm 0.12)x + (1.05 \pm 0.11)$	0.9998	1.18[a]
	Erigeroside	$y = (1.20 \pm 0.13)x + (3.05 \pm 0.12)$	0.9944	0.96[a]
	p-Coumaric acid	$y = (0.977 \pm 0.066)x + (5.63 \pm 0.17)$	0.9955	0.78[a]
	Chlorogenic acid	$y = (0.870 \pm 0.150)x + (6.10 \pm 0.14)$	0.9854	0.69[a]
	Kaempferol	$y = (0.503 \pm 0.026)x + (1.03 \pm 0.02)$	0.9986	0.40[a]
	Quercetin	$y = (0.153 \pm 0.006)x + (1.06 \pm 0.02)$	0.9992	0.12[a]
	Ferulic acid	$y = (0.200 \pm 0.0001)x + (5.64 \pm 0.001)$	0.9999	0.16[a]
	Trolox	$y = (0.177 \pm 0.013)x + (2.71 \pm 0.001)$	0.9973	0.12[a]
Ethyl acetate	6′-Caffeoylerigeroside	$y = (0.0456 \pm 0.0004)x + (0.448 \pm 0.021)$	0.9982	100[b]
	Isoquercitrin	$y = (0.0365 \pm 0.0027)x - (0.021 \pm 0.283)$	0.9955	80.0[b]
	Scutellarin	$y = (0.0344 \pm 0.0009)x + (0.278 \pm 0.010)$	0.9996	75.4[b]
	Erigeroside	$y = (0.0040 \pm 0.0001)x + (0.006 \pm 0.06)$	0.9998	8.77[b]
	Trolox	$y = (0.0765 \pm 0.0011)x + (1.85 \pm 0.06)$	0.9999	165[b]

[a] Relative response to the slope of caffeic acid.
[b] Relative response to the slope of 6′-caffeoylerigeroside.

Table 3
The content of phenolic compounds expressed as caffeic acid (diethyl ether fraction) and 6′-caffeoylerigeroside (ethyl acetate fraction) equivalents in flowers, leaves and roots of *E. acris* per dry weight

Part of plant	Diethyl ether fraction		Ethyl acetate fraction	
	CL method[a] (mg caffeic acid/g dw)	DPPH inhibition[b] (%)	CL method[a] (mg 6′-caffeoylerigeroside/g dw)	DPPH inhibition[b] (%)
Flowers	0.280 ± 0.005	30.8 ± 0.2	10.4 ± 0.13	30.5 ± 0.2
Leaves	0.538 ± 0.002	46.4 ± 0.1	28.8 ± 0.85	57.9 ± 0.3
Roots	0.069 ± 0.001	8.99 ± 0.06	5.32 ± 0.06	19.4 ± 0.1

[a] Mean of five determinations ± S.D.
[b] Mean of three determinations ± S.D.

RC_2) of 0.8 mm i.d. were used. Generally, with the increase in the length of mixing coils I_0 decreased significantly. The influence of the length of RC_1 coil on CL inhibition was studied from 8 cm to 122 cm. Because of the highest value of ΔI and S/N, 28 cm and 52 cm was selected as the optimal length of the RC_1 coil for the determination of caffeic acid and 6′-caffeoylerigeroside, respectively. The length of the RC_2 coil was tested from 9 cm to 209 cm. In the determination of caffeic acid the maximum negative peak height was obtained when the RC_2 coil was as short as possible (9 cm). In contrast, in the determination of 6′-caffeoylerigeroside, the maximum ΔI was reached when 99 cm-mixing coil was used.

3.2.2. Analytical performance characteristics

With the optimised experimental conditions as mentioned above, working curves were obtained. The negative peak height (ΔI, μA) was linearly proportional to the caffeic acid and 6′-caffeoylerigeroside concentrations (C, ng mL^{-1}) ($n = 6$) in the range of 0.1–1.5 ng mL^{-1} and 1–200 ng mL^{-1}, respectively. The regression equation was obtained as $\Delta I = (125.2 \pm 0.4)C - (13.8 \pm 0.8)$ ($r = 0.9999$) for caffeic acid and $\Delta I = (0.0456 \pm 0.0004)C + (0.448 \pm 0.021)$ ($r = 0.9982$) for 6′-caffeoylerigeroside. The relative standard deviation (R.S.D.) for 10 determinations was 3.3% for 0.45 ng mL^{-1} of caffeic acid and 2.9% for 40 ng mL^{-1} of 6′-caffeoylerigeroside. The detection limit as signal to noise ratio of 3 was 4×10^{-3} ng mL^{-1} (caffeic acid) and 0.18 ng mL^{-1} (6′-caffeoylerigeroside). To the best of the authors' knowledge, 4 ng L^{-1} is the lowest limit of detection among those already existing CL methods for the determination of caffeic acid [27–30,14]. The proposed FIA method is fast and enables to determine 80 samples/h.

For the estimation of the total phenols in the *E. acris* extracts, CL inhibition of the luminol–I_2 system by other phenolic compounds identified in the diethyl ether and ethyl acetate fractions was investigated in the optimal experimental conditions for caffeic acid (over the range of 0.1–1.5 ng mL^{-1}; $n = 6$) and 6′-caffeoylerigeroside (over the range of 1–200 ng mL^{-1}; $n = 6$). Moreover, for the purposes of comparison, the calibration graph of Trolox (a vitamin E analogue) often used as a standard for the evaluation of antioxidant activities [28], was performed in the optimum detection conditions for both chosen phenolics. The analytical figures of merit are summarized in Table 2. The slope values of all investigated phenolics present in the diethyl ether and ethyl acetate fraction were much lower than slope values of caffeic acid and 6′-caffeoylerigeroside. The results demonstrate that the FIA-CL method is very sensitive especially for the detection of caffeic acid, which is known as a potent antioxidant, and could be used for the determination of that compound in a different matrix, e.g. food, plant extracts, biological samples (dilution of the sample reduces the effect of the other interfering constituents). It may be the result of strong antioxidant activity of caffeic acid which is known as a superior antioxidant comparing to other hydroxycinnamates in inhibiting low-density lipoprotein oxidation, quenching radicals and singlet oxygen [8]. Additionally, to the best of our knowledge it is the first attempt to estimate the antioxidant activity of 6′-caffeoylerigeroside. Comparing the slope values of a reference antioxidant Trolox and 6′-caffeoylerigeroside indicated, that the latter had nearly half the antioxidant activity of Trolox.

In order to assess the accuracy of the developed CL method, the total content of phenolic acids expressed as a caffeic acid equivalent was determined in the pharmaceutical formulation. To evaluate the selectivity of the CL method for the determination of caffeic acid, the effect of various excipients usually present in pharmaceutical preparations was investigated by using a 0.45-μg L^{-1} caffeic acid solution, which was spiked with increasing amounts of the interfering constituents. An excipient was considered as non-interfering if its presence caused an analytical signal variation lower than ±5% regardless of the CL variation noted in its absence. The tolerable excipient/caffeic acid molar ratios were: higher than 1000 for NaCl, $CaCl_2 \cdot 6H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, EDTA, lactose, and sodium citrate; 45 for glucose; 12.5 for $NaHSO_3$; 2.5 for ascorbic acid and $Na_2S_2O_3 \cdot 5H_2O$.

3.2.3. Method application

3.2.3.1. Analysis of plant extracts. In order to evaluate the utility of the proposed method in real samples, the total phenolic content expressed as caffeic acid (diethyl ether fraction) or 6′-caffeoylerigeroside (ethyl acetate fraction) equivalent was determined in flowers, leaves and roots of *E. acris* according to the previously described procedure (Section 2.3). Table 3 shows the average values of the determinations, calculated and expressed as milligram caffeic acid/6′-caffeoylerigeroside equivalent per gram of dry weight. The antioxidant activity was determined spectrophotometrically by the DPPH$^{\bullet}$ radical scavenging assay described in Section 2.7. It was found that there was a positive correlation between the total phenolic content in each part of plant and its antioxidant activity (Table 3). These results also show that the level of antioxidant activity in a particular part of *E. acris* varies to a great extent. The highest content of phenolic compounds/antioxidant

Table 4
The contents of phenolic compounds expressed as caffeic acid equivalents in the pharmaceutical preparation and recovery data of caffeic acid from pharmaceutical preparation

Pharmaceutical preparation	Labeled amount (mg/dose)	Results by proposed method (mg/dose)[a]			Recovery (%) ($n = 3$)
		Found ± S.D.	Added	Total ± S.D.	
Immunofort	1	0.980 ± 0.027	0.33	1.306 ± 0.028	98.4
			0.67	1.662 ± 0.013	101.9

[a] Mean of three determinations ± S.D.

activity was assayed in leaves, the lowest in roots of the plant tested. *E. acris* can be considered as a good source of natural antioxidants for medicinal use.

3.2.3.2. Analysis of pharmaceutical preparation. The proposed method was also applied to the determination of the total phenolic acid content expressed as a caffeic acid equivalent in the herbal preparation Immunofort according to the procedure described in Section 2.3. The results obtained (Table 4) were in excellent agreement with the nominal contents. To the best of the authors' knowledge, there is no reference method for the estimation of phenolic acid content in the pharmaceutical formulation Immunofort; therefore the accuracy of the results obtained with the proposed method was evaluated through the recovery test. A known amount of standard solution of caffeic acid was added to Immunofort and the results are shown in Table 4. The recovery data confirmed the accuracy of the developed CL method and the absence of interfering matrix effects.

4. Conclusion

In this paper a novel FIA-CL detection method has been established for the determination of phenolic compounds (caffeic acid and 6′-caffeoylerigeroside) based on the phenolic compound-inhibited luminol–I_2 CL in a sodium hydroxide medium. The method has been optimised for the application of different matrix (plant extracts and pharmaceutical preparation). The estimated antioxidant activities of studied extracts from each part of *E. acris* obtained by our CL method are well correlated with those measured and calculated from the DPPH$^{\bullet}$ spectrophotometric method. Among the widely used assays for antioxidant activity [6], the proposed FIA-CL method is advantageous for its high sensitivity which is a few orders of magnitude lower in comparison with the spectrophotometric assays. Majority of the CL methods used for the evaluation of antioxidant activity are manual. Appling of flow injection analysis technique improve precision, sample and reagent consumption, considerably reduce the time of analysis and enable its automation. However, to the best of our knowledge there are only few examples of applying flow injection technique with CL detection to measure antioxidant activity quoted in the literature, based on an acidic potassium permanganate [27] and luminol [10,13,15] CL. Despite two of the above-mentioned FIA-CL methods offer higher sampling rate [15,27], our newly developed method is far more sensitive. Moreover, luminol–I_2 detection system is simple and based on stable and easy-to-handle reagents, there is no necessity to prepare reagents every day as it is in the methods based on the CL of luminol generated in the enzymatic reactions [8,11]. Therefore, it could be applied as an alternative method to screen compounds, plant extracts or beverages for their relative antioxidant potential. According to the high efficiency of the CL reaction between luminol and I_2, it could be used as a post-column detection system in HPLC for simultaneous detection of individual antioxidants present at an even low level in the complex matrix such as plant extracts, food, biological samples.

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