

Scavenging Activity of Reactive Oxygen Species by Polyphenols Extracted from Different Vegetal Parts of Celandine (*Chelidonium majus*). Chemiluminescence Screening

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Celandine (Chelidonium majus) is a wide-spread plant in Europe, Asia and North America. Its leaves, flowers and roots are used in medicine for stimulating the production of bile and pancreatic digestive enzymes; also, celandine extracts are thought to have anti-cancerous, anti-inflammatory and antimicrobial actions. Ethanolic extracts were used for the dosage of polyphenols and flavonoids and also for the evaluation of the decrease of chemiluminescence intensity produced by the reaction of luminol with reactive oxygen species (hydrogen peroxide, superoxide anion, hydroxyl radical, singlet oxygen, hypochlorite anion and peroxy radical). The obtained results showed that the following relation describes the concentration of total polyphenols and flavonoids in the studied vegetal parts: flowers>leaves>roots>seeds>stems. The polyphenolic extracts exhibited different scavenging activities, depending on their origin and reactive oxygen species that were tested. Hydroxyl radical and hypochlorite anion were strongly scavenged by celandine roots extract. Superoxide anion was scavenged the best by celandine leaves extract. Celandine flowers extract scavenged the best peroxy radical and singlet oxygen. The extract prepared from celandine leaves had the best capacity to scavenge hydrogen peroxide.

Keywords: free radicals, scavenging activity, antioxidant activity, celandine

The term of reactive oxygen species (ROS) include free radicals, chemical species capable of independent existence and possessing one or more unpaired electrons, and non-radical species that can easily lead to free radical reactions in living organisms. These compounds include hydroxyl radical (HO·), superoxide anion (O₂⁻), peroxy radical (RCOO·), nitric oxide (NO·) hydrogen peroxide (H₂O₂), singlet oxygen (¹Δg or ¹O₂), ozone (O₃) and hypochlorous acid (HOCl) [1]. In living systems, ROS are produced by cellular metabolism and by the action of exogenous environmental agents. They are generated by a process known as redox cycling and are catalyzed by transition metals, such as Fe²⁺ and Cu²⁺ [2]. Because ROS production is a naturally occurring process, varieties of enzymatic and nonenzymatic mechanisms have developed to protect cells against ROS [3]. Uncontrolled ROS production can damage essential complex molecules such as lipids, proteins, nucleic acids and carbohydrates, which can further initiate or propagate the development of many diseases, such as cancer, liver injuries and cardiovascular diseases, iron overload, rheumatoid arthritis, Parkinson's disease, motor neuronal diseases, diabetes [4-9].

Polyphenols constitute one of the most common and widespread groups of substances in flowering plants, occurring in all vegetative organs, as well as in flowers and fruits. They are considered secondary metabolites involved in the chemical defense of plants against predators and in plant-plant interferences [10, 11]. Late researches demonstrated that polyphenols have an ideal chemical structure for ROS scavenging activity, and they are more effective than tocopherol and ascorbate [12, 13].

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extracts are thought to have anti-cancerous, anti-inflammatory and antimicrobial actions.

Because the existing studies on celandine extracts are focused especially on the biological effects of alkaloids, chelidone, chelidoxanthine and coptisine, the purpose of this study was to obtain polyphenolic extracts from celandine roots, stems, leaves, flowers and seeds and to analyze their capacity to annihilate reactive oxygen species by luminol chemiluminescence assay.

Experimental part

Plant material collection

Celandine was harvested in the morning, in the period of maximum flowering. The roots, stems, leaves, flowers and green seeds were shade dried and grounded into powder form by a grinder.

Extraction of total phenolics

1000 mg of the ground powder of the plant samples were weighed and kept in a magnetic stirrer for 1 h after adding 10 mL of 60% ethanol; the samples were then kept in a water bath for 3 h. The obtained extracts were centrifuged for 15 min at 10,000 rpm. The supernatants were collected and stored for analyzing total phenols and total flavonoid contents and for determining the capacity to annihilate reactive oxygen species.

Spectrophotometric determination of total phenolic content

The total polyphenols content (TPC) was evaluated by an assay based on the reaction of Folin-Ciocalteu reagent [14]. Briefly, 500 μL of the extract was transferred to a 100 mL Erlenmeyer flask and the final volume was adjusted to 46 mL by addition of distilled water. 1 mL of Folin-Ciocalteu reactive solution was added and incubated at room temperature for 3 min. 3 mL of 2% sodium carbonate solution was added and the mixture was shaken on a

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shaker for 2 h at room temperature. The absorbance was measured at 760 nm using an Jasco UV-VIS spectrophotometer.

The TPC was expressed as gallic acid equivalents (GAE) in mg/100 mL extract (mg GAE/100 mL).

Spectrophotometric determination of flavonoid content

Quantitative determination of flavonoids was made by colorimetric assay described by [15]. Diluted alcoholic extracts and standard solution of (+)-catechin (10 mg/100 mL) were added to 10 mL volumetric flask containing 4 mL of distilled water. To the above mixture, 0.3 mL of 5% NaNO₂ was added. After 5 min, 0.3 mL of 10% AlCl₃ was added. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. Then, the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. The total flavonoid content was calculated and expressed as mg of (+)-catechin equivalents/100 mL (mg CE/100 mL).

Assay procedure for chemiluminescence emission kinetics and for quenching effects of alcoholic extracts on reactive oxygen species

For the evaluation of chemiluminescence emission kinetics and for the evaluation of quenching effects, all extracts were diluted with dimethyl sulfoxide (DMSO) in ratio 1:100 (v/v).

The annihilation of hydroxyl radical

For hydroxyl radical scavenging effect assays, HO· was generated by a Fenton-type reaction system [16]. Briefly, in a tube containing 50 µL FeSO₄ (0.4 mmol/L) and 50 µL H₂O₂ (1.5%), after incubation at 30°C for 2 min, 50 µL sample or PBS (in control experiment) and 600 µL luminol (LH₂) (0.1 mmol/L) were added into the mixture and the chemiluminescence (CL) intensity was measured.

Superoxide anion scavenging activity

O₂⁻ was generated in a pyrogallol autooxidation system [17]. The reaction mixture contained 50 µL pyrogallol (3.125 · 10⁻⁴ mol/L), 200 µL carbonic acid/buffer saline solution (CBSS) pH 10.2 containing 0,1 mol/L EDTA, 10 µL polyphenolic extract (DMSO was used in control) and 400 µL luminol (1 · 10⁻³ mol/L). The CL intensity was measured immediately for 170s.

Peroxy radical scavenging activity

5 mM linolenic acid in normal butanol (n-BuOH) was aerobically oxidized in a water bath at 37°C for 60 min. To 6 µL of alcoholic extract, 900 µL of the oxidized solution were added and then the mixture was incubated at 37°C for 3 min. After adding 300 µL of 8 µM luminol in n-BuOH, the CL intensity was measured [18].

Extract	Total phenolics (mg GAE/100 ml)	Total flavonoids (mg CE/100 ml)
Roots	219.8 ± 10.3	145.7 ± 7.9
Stems	52.7 ± 4.2	36.75 ± 2.9
Leaves	285.3 ± 11.9	218.3 ± 16.8
Flowers	381.7 ± 23.2	299.5 ± 18.4
Seeds	99.6 ± 7.1	42.3 ± 3.2

The annihilation of hydrogen peroxide

H₂O₂ scavenging activity was assayed according to the method described by [19] with slight modifications. Briefly, the reaction mixture consisted in 100 µL of luminol (2.5 · 10⁻⁵M), 440 µL 30mM Tris pH 8.5 and 10 µL sample or 10 µL Tris (as control). 50 µL of 30 mM H₂O₂ were added to trigger the CL reaction.

The annihilation of singlet oxygen

Singlet oxygen scavenging activity was made as described by [18], with the only difference that instead of lactoperoxidase it was used horseradish peroxidase. To evaluate the quenching effect of chemiluminescence generated by luminol - [¹O₂] system (LH₂ - ¹O₂), to 6 µL sample, 300 µL of 0.4% H₂O₂ in 100 mM acetate buffer (pH 4.5), 300 µL of 80 mM NaBr in acetate buffer and 0.8 mM luminol in acetate buffer were added. The mixture was incubated at 37°C for 10 min. The CL intensity of the mixture was measured after adding 300 µL of a 10 µg/mL solution of horseradish peroxidase in acetate buffer.

The annihilation of hypochlorite anion

Hypochlorite anion (ClO⁻) scavenging activity was evaluated as described by [18]. Briefly, 900 µL of 0.53 mM luminol in 50 mM borate buffer (pH 9.5) were added to 6 µL of sample. After incubation at 37°C for 10 min, 300 µL of 40 µM NaClO in borate buffer were added to the mixture and then the CL intensity was measured.

For evaluation of CL emission kinetics, the CL measurements were performed at room temperature for 170 s (2min 50s), in test tubes (Φ12 x 75 mm), using a Berthold luminometer. CL emission was counted at 5 s. The intensity of CL is given as the relative light units per second (RLU/s). Five measurements were made and an average value was calculated, obtaining a maximum 10% relative scattering of the results from the mean value.

The percentage of quenching effect against the reactive oxygen species was calculated using equation (1).

$$\text{Quenching effect \% (S\%)} = \frac{I_0 - I}{I_0} \times 100 \quad (1)$$

where I₀ is the CL intensity generated from the reference system (control) (RS) and I represent CL intensity generated for the sample. The concentrations of generated radicals could not be estimated.

Results and discussions

The quantitative determination of the total phenolic content, expressed as mg gallic acid equivalents/100 mL alcoholic extract, and total flavonoid content of the extracts expressed as mg (+)-catechin equivalents/100 mL are presented in table 1.

Table 1
QUANTITATIVE DETERMINATION OF
THE TOTAL PHENOLIC CONTENT
AND TOTAL FLAVONOID CONTENT
OF CELANDINE EXTRACTS

The obtained results showed that the distribution of polyphenols in studied vegetal parts of celandine is different, the highest quantities of polyphenols being quantified in roots, flowers and leaves. The highest concentration of total polyphenols and total flavonoids was determined in celandine's flowers extract (381.7 ± 23.2 mg GAE/100 mL, respectively 299.5 ± 18.4 mg CE/100 mL), while the lowest concentrations were recorded in celandine stems extract (52.7 ± 4.2 mg GAE/100 mL, respectively 36.75 ± 2.9 mg CE/100 mL) (table 1).

Kinetics and quenching effects of alcoholic extracts on reactive oxygen species

Luminol (LH₂) reacts with ROS to yield a compound in an excited electronic state which returns to ground state with production of light (chemiluminescence). The decrease of CL intensity in time, under reference system (RS) signal, corresponds to the annihilation of ROS by an antioxidant, and the increase of CL intensity in time, upper RS signal corresponds to the formation of free radicals by a prooxidant [20, 21].

The annihilation of hydroxyl radical

In figure 1 it is shown the effect of celandine polyphenolic extracts on CL emission kinetics produced by luminol – hydroxyl radical reaction (LH₂ – •OH) in PBS pH 7.4. It was observed that the extracts obtained from celandine roots, stems and flowers decreased the intensity of CL emission during the tested 170 s comparatively to the RS. Thus, these extracts scavenged hydroxyl radical and they were considered to have an antioxidant action. Celandine seeds extract increased CL intensity, which indicates the generation of supplementary quantities of free radicals in the system and therefore a prooxidant effect. The extract obtained from celandine leaves manifested an antioxidant activity only in the zero moment (0.0s); after this moment, the extract generated free radicals, which increased the intensity of CL comparatively to reference system (RS).

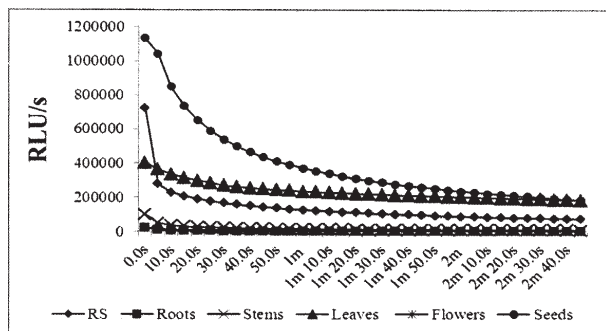


Fig. 1. The effect of celadine polyphenolic extracts on CL emission kinetics produced by LH₂ - •OH reaction, in PBS pH 7.4

Alcoholic extract	S%					
	HO•	O ₂ ⁻	RCOO•	H ₂ O ₂	¹ O ₂	ClO ⁻
Roots	97.04	97.99	20.49	80.51	86.37	5.23
Stems	81.76	98.31	-6.35	60.46	48.97	1.49
Leaves	-31.07	99.16	-26.35	97.56	93.70	-93.03
Flowers	95.44	98.94	29.76	94.60	95.57	0.69
Seeds	-277.38	98.77	19.03	89.90	94.32	0.91

The calculation of the percentage of quenching effect after 5 s of reaction confirmed the above results. Thus, the highest quenching effect was recorded for roots extract (97.04%), while the lowest quenching effect was observed in case of stems extract (81.76%). Polyphenolic extracts obtained from celandine leaves and seeds generated in the used Fenton-like system supplementary quantities of free radicals, which, in reaction with luminol, increased the CL intensity. Thus, in the presence of these two extracts, the quenching effect was -31.07%, respectively -277.38% (table 2).

Superoxide anion scavenging activity

The effect of celandine polyphenolic extracts on CL emission kinetics produced by LH₂ – O₂⁻ reaction, in carbonic acid/buffer saline solution (CBSS) pH 10.2 is showed in figure 2. Comparatively to reference system, all polyphenolic extracts scavenged superoxide anion, manifesting an antioxidant activity. The highest capacity to scavenge superoxide anion was recorded for leaves extract, while the lowest capacity was observed in case of roots extract.

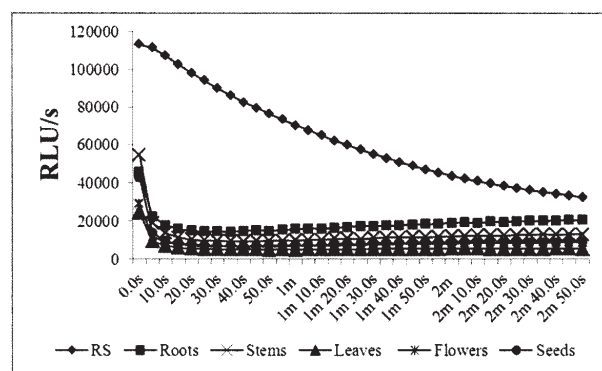


Fig. 2. The effect of celadine polyphenolic extracts on CL emission kinetics produced by LH₂ – O₂⁻ reaction, in carbonic acid/buffer saline solution (CBSS) pH 10.2

The results obtained after calculation of percentage of quenching effect after 5 s of reaction demonstrated that all extracts strongly scavenged superoxide anion generated by pyrogallol autooxidation, the highest activity being recorded for celandine leaves extract (99.16%) and the lowest for celandine roots extract (97.99%) (table 2).

Peroxy radical scavenging activity

In figure 3 is shown the effect of celandine polyphenolic extracts on CL emission kinetics produced by LH₂ - RCOO• reaction. In case of peroxy radical, resulted from aerobic oxidation of linoleic acid, the extracts manifested either an antioxidant or a slight prooxidant action; an antioxidant

Table 2
THE PERCENTAGE OF QUENCHING EFFECT (S%) AGAINST ROS, 5 S AFTER THE BEGINNING OF THE REACTION

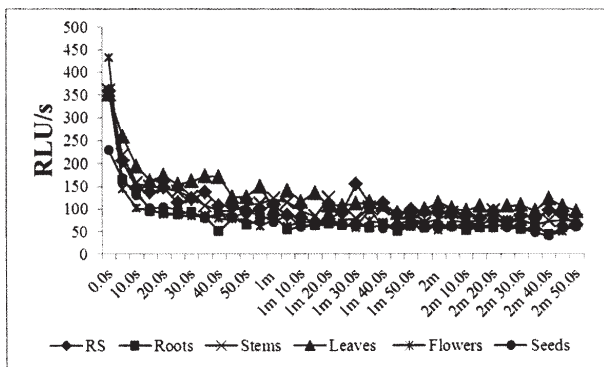


Fig. 3. The effect of celandine alcoholic extracts on CL emission kinetics produced by LH_2 - RCOO reaction in n-BuOH

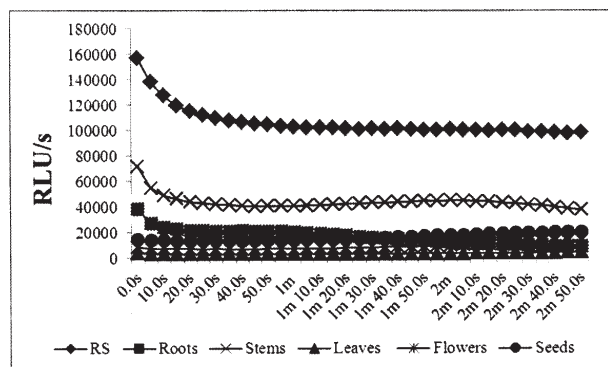


Fig. 4. The effect of celandine alcoholic extracts on CL emission kinetics produced by LH_2 - H_2O_2 reaction, in Tris-HCl buffer pH 8.5

activity during the analyzed 170 s was recorded for the extracts obtained from celandine roots, flowers and seeds, while stems and leaves extracts had an prooxidant activity during the whole interval.

In the first 5 s of experiment, the strongest effect of CL quenching was manifested by celandine flowers polyphenolic extract (29.76%) (table 2).

The annihilation of hydrogen peroxide

Graphical representation of CL intensity depending on time demonstrates that, comparatively to reference system, all tested extracts have the capacity to scavenge hydrogen peroxide, the best activity being recorded for the extracts prepared from celandine leaves, flowers and seeds (fig. 4). In the first 5 s of reaction, all extracts decreased the CL emission (over 60%), the highest percent of quenching effect being calculated for celandine leaves extract (97.56%) and the lowest for celandine stems extract (60.46%) (table 2).

The annihilation of singlet oxygen

The intensity of CL produced by the reaction of luminol with singlet oxygen was much lower in the presence of polyphenols extracted from celandine leaves, flowers and roots, comparatively to reference system (fig. 5). The polyphenols extracted from celandine seeds strongly scavenged singlet oxygen in the first 30 s of reaction; after that it was recorded the increase of CL intensity, probably due to the generation of new free radicals. The lowest singlet oxygen scavenging activity was observed in case of celandine stems extract. Calculations of percentage of quenching effect, after 5 s of reaction, show that the highest capacity to scavenge singlet oxygen was calculated for celandine flowers extract (95.57%) and the lowest for celandine stems extract (48.97%) (table 2).

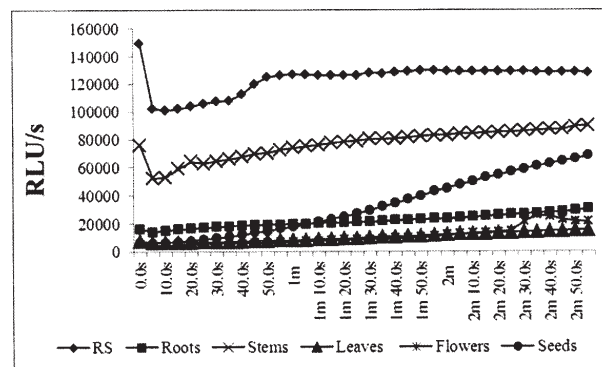


Fig. 5. The effect of celandine polyphenolic extracts on CL emission kinetics produced by LH_2 - 1O_2 reaction, in acetate buffer pH 4.5

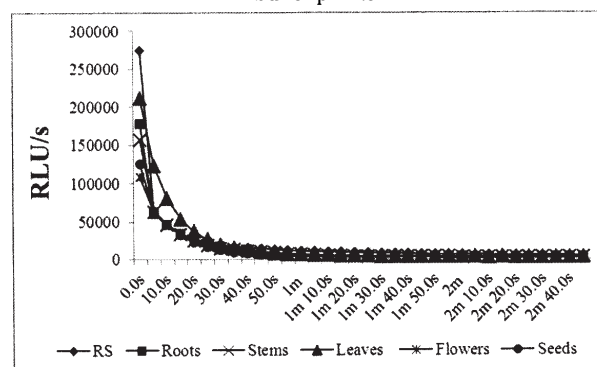


Fig. 6. The effect of celandine polyphenolic extracts on CL emission kinetics produced by LH_2 - ClO^- reaction, in borate buffer pH 9.5

The annihilation of hypochlorite anion

The extracts obtained from celandine roots, stems, flowers and roots presented antioxidant activity against hypochlorite anion; thus, CL intensity produced by the reaction of luminol with hypochlorite anion was lower in the first 20 s of reaction, comparatively to the reference system (fig. 6). The highest hypochlorite anion scavenging activity was recorded in case of celandine seeds extract. In the first 30 s of reaction, celandine leaves extract had a prooxidant activity, the intensity of CL being higher than the one of reference system.

The results obtained after the first 5 s of reaction showed that celandine roots extract scavenged the best hypochlorite anion (5.23%). The value calculated for celandine leaves was -93.03%, which indicates a prooxidant activity in the first 5 s of reaction, probably due to the generation of a supplemental quantity of free radicals capable to produce chemiluminescence in reaction with luminol (table 2).

Conclusions

In this study it was investigated the content in polyphenols and total flavonoids of five alcoholic extracts obtained from celandine roots, stems, leaves, flowers and seeds.

Polyphenolic extracts were used to determine their capacity to scavenge some reactive oxygen species (HO ; O_2^- ; $RCOO^-$; H_2O_2 ; 1O_2 and ClO^-).

The following relation describes the concentration of total polyphenols and flavonoids in the studied vegetal parts: flowers > leaves > roots > seeds > stems.

The polyphenolic extracts exhibited different scavenging activities, depending on their origin and reactive oxygen species that were tested.

Hydroxyl radical and hypochlorite anion were strongly scavenged by celandine roots extract. Superoxide anion

was scavenged the best by celandine leaves extract. Celandine flowers extract scavenged the best peroxy radical and singlet oxygen. The extract prepared from celandine leaves had the best capacity to scavenge hydrogen peroxide.

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