CHEMILUMINESCENT DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY IN MEDICINAL PLANT MATERIAL

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Medicinal plant material is one of the sources of antioxidants for the human body. Chemiluminescence analysis is one of the common methods of determining the content of antioxidants in plant materials. In our work, chemiluminescence analysis was used to determine the total antioxidant capacity (TAC) of fruit decoctions of mountain-ash, rose and hawthorn, as well as raspberry fruit infusion. Experiments established the kinetics of the chemiluminescence of a system consisting of horseradish peroxidase, hydrogen peroxide and luminol. Concentrations and volumes of components of the system were chosen such that strong antioxidants (ascorbic acid) and antioxidants of average force (quercetin) were completely oxidized during measurement (10 minutes). A method for TAC calculation based on changes in chemiluminescence light sum in the presence of plant samples was proposed and substantiated. Analysis of chemiluminescence kinetics showed that antioxidants of average force dominate in the objects studied, including flavonoids and weak antioxidants (tocopherol and others). Comparison of the calculated TAC values for the objects under study and their chemical analysis data showed that products containing the same amount of antioxidants with different ratios of antioxidants by types might vary in their ability to protect the body against the harmful effects of free radicals. The technique described is a promising one for the study of plant objects containing a mixture of different types of antioxidants.

Keywords: free radical, antioxidant, antioxidant activity, total antioxidant capacity, chemiluminescence, luminol

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ХЕМИЮЛИМНЕСЦЕНТНАЯ МЕТОДИКА ОПРЕДЕЛЕНИЯ ОБЩЕЙ АНТИОКСИДАНТНОЙ ЕМКОСТИ В ЛЕКАРСТВЕННОМ РАСТИТЕЛЬНОМ СЫРЬЕ

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Лекарственное растительное сырье является одним из источников антиоксидантов для организма человека. Среди методов определения содержания антиоксидантов в растительных объектах распространенный метод хемилюминесцентного анализа. В настоящей работе он был использован для оценки общей антиоксидантной емкости (ОАЕ) отваров плодов рябины, шиповника и настоев плодов малины. В опыте регистрировали кинетику хемилюминесценции в системе, состоящей из пероксидазы хрища, перекиси водорода и люминола. Концентрация и объем компонентов системы в пробе были подобраны так, чтобы сильные антиоксиданты (аскорбиновая кислота) и антиоксиданты средней силы (кверцетин) полностью окислялись за время измерения (10 мин). Предложен и обоснован способ расчета ОАЕ на основе изменения светосуммы хемилюминесценции в присутствии растительных образцов. Анализ кинетики хемилюминесценции показал, что в изученных объектах преобладают антиоксиданты средней силы, в том числе флавоны и слабые антиоксиданты (токоферол и др.). Сопоставление рассчитанных значений ОАЕ для изучаемых объектов и данных их химического анализа показало, что продукты, содержащие одно и то же количество антиоксидантов с разными их соотношениями по типам, могут различаться по способности защищать организм от вредного воздействия свободных радикалов. Описанная методика перспективна для изучения растительных объектов, содержащих смесь антиоксидантов различных типов.

Ключевые слова: свободный радикал, антиоксидант, антиоксидантная активность, общая антиоксидантная емкость, хемилюминесценция, люминол


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Free radicals generated in the body cause damage to the cell membranes, which in turn triggers various diseases [1]. The destructive oxidative effects of radicals are checked by the body's antioxidant defense system, in which low-molecular compounds — radical interceptors (traps) — play an important role. Medicinal plant material and herbal preparations are one of the sources of antioxidants [2]. Understanding the antioxidant capacity of these plant materials and preparations helps to enhance their preventive and therapeutic actions.

The main techniques used to identify antioxidants are considered in [3–8]. However, identifying antioxidants as chemical compounds does not provide a complete picture of the protective properties of the object being studied: they are caused not only by the amount of a particular antioxidant but also by the activity of each of them. Antioxidant activity (AOA) is the constant (k_AOA) of the reaction rate of a free radical and antioxidant. The chemiluminescence technique (CL) is used to determine the total number of radicals that react with antioxidants in a sample (total antioxidant capacity, TAC); when mathematical modeling of CL kinetics is used, the technique enables to also determine the rate of formation and reaction of radicals with antioxidants, i.e. AOA ([9–11]).

The most common modification of the chemiluminescent technique of determining the total antioxidant capacity is based on the use of luminol as a chemiluminescence activator [12–15]. The sample is placed in a chemiluminescence cuvette with addition of luminol, hydrogen peroxide and compounds capable of forming radicals by spontaneous decay (thermolysis), for example 2,2'-Azobis(2-aminopropene) dihydrochloride (AAPH): AAPH $\rightarrow$ 2P$^\bullet$.

In the presence of molecular oxygen, alkyl radical P$^\bullet$ forms peroxyl radical ROO$^\bullet$: ROO$^\bullet$ + O$_2$ $\rightarrow$ ROO$^\bullet$. Next, the peroxyl radical oxidizes luminol-based chemiluminescent probe (LH2) to form a radical luminol (LH)$$ ROO^\bullet$ + LH$_2$ $\rightarrow$ ROOH + LH$^\bullet$. Through formation of intermediate compounds (luminol hydroperoxide and luminol endoperoxide), LH$^\bullet$ forms the molecule of the final product of luminol oxidation (aminophthalic acid) in an electronically excited state, which generates photons and as a result, chemiluminescence is observed [9]. CL intensity is proportional to the rate of formation of photons, which in turn is proportional to stationary concentration of LH$^\bullet$ in the system. While interacting with radicals, antioxidants interrupt the above-described transformation chain and prevent photon formation.

Compounds subject to thermolysis are not the only possible source of radicals in analysis of the antioxidant capacity of a sample by chemiluminescence method. Alternatives include horseradish peroxidase/hydrogen peroxide compound [13, 16], hemin-hydrogen peroxide [8], cytochrome c/cardiolipin/hydrogen peroxide [11], and others. The mechanism of peroxidation of luminol is considered in Cormier et al. [17]. CL kinetic curves for these systems reflect two reaction stages: step of increase in CL intensity and the stage of plateau or gradual recession of luminescence when CL intensity is either constant or decreases slowly. Authors in [15] described two approaches to measuring the total antioxidant capacity, taking into account this curve feature. TRAP (Total Reactive Antioxidant Potential) method is based on measurement of CL latency $\tau$ and can be used to determine such antioxidants as Trolox and ascorbic acid — they are characterized by high constant of the rate of radical reaction, and for this reason can be called powerful antioxidants [11]. They are completely oxidized during the latent period. The TAR (Total Antioxidant Reactivity) technique is used to measure the degree of suppression of chemiluminescence $q$ on the plateau or at the maximum chemiluminescence curve:

$$ q = \frac{(I - I_0)}{I_0}, $$

where $I$ is CL intensity without an antioxidant, while $I_0$ is CL intensity in the presence of an antioxidant. This method is used if the system contains predominantly weak antioxidants with low constants of rate of interaction with radicals — much lower than the luminol constant [11].

The action of antioxidants is characterized not only by indicators $r$ and $q$. As can be seen from [8, 11], the action of such antioxidants as uric acid in genni/H$_2$O$_2$/luminol or tocopherol, rutin and quercetin in cytochrome c/cardiolipin/H$_2$O$_2$/luminol system is characterized by a change in the maximum rate of CL rise ($v_{\text{max}}$). As shown by results of mathematical modeling of kinetics, the constants of the constants of the reaction rate of such antioxidants with radicals are close to luminol constant. Therefore, such antioxidants may be regarded as medium-strength antioxidants [11].

If the test material, particularly plant materials, contained only one type of antioxidants, their content would have been characterized by one of the above three indicators ($r$, $q$ or $v_{\text{max}}$). But a plant material contains a mixture of antioxidants of different strength. To address this problem, some authors [8, 18–20] used chemiluminescence light sum change $\Delta S$ over a specific time calculated by the formula:

$$ \Delta S = S_I - S_{I_0}, $$

where $S_I$ and $S_{I_0}$ are CL light sums for a predetermined time $t$ in the control and test samples respectively. The time should be sufficient to oxidize all the antioxidants in the system, i.e. for the CL curve of the test sample to reach the level of the CL curve of the control sample. The latter suggests that researchers should not only establish the luminescence light sum, but also record the CL kinetics curve within quite a long time. However, this is not always done.

Since all measurable indicators depend on the instrument and measurement conditions, the antioxidant effect of a substance in a system is usually compared with the effect of an antioxidant taken as the standard, for example Trolox [8, 21].

Many authors have used the horseradish peroxidase/hydrogen peroxide system to analyze the total antioxidant capacity of plant material. In [22, 23], CL latency (TRAP method) was used to estimate the amount of antioxidants in samples, while in [18–20], the area under the CL curve was used. However, these studies did not provide a clear justification of the choice of a particular indicator for TAC assessment. The aim of the study was to determine how the ratio of different types of antioxidants affects TAC and to modify the chemiluminescence method in such a way as to be able to more accurately estimate TAC in a plant material. To this end, we set a number of tasks. First, to compare the CL kinetics of the test objects with the kinetics of the standard three types of antioxidants (strong, medium and weak) in order to understand what type of antioxidants contributes most to the TAC of the test objects. Second, to calculate the TAC of the test objects by measuring the reduction in CL light sum under the influence of these objects in comparison with the effect of an antioxidant that contributes most to the TAC.

METHODS

The test objects were industrial samples of the fruits of hawthorn, mountain-ash and wild rose produced by herbal
company Krasnogorskslesredstva (Russia), as well as naturally grown raspberry fruits collected by the authors in Moscow Oblast and dried at a temperature of 60–80 °C until they had no juice left in them and were deformed under pressure.

The reagents used for analysis of antioxidant capacity by chemiluminescence method were: KH₂PO₄, 20 mM buffer solution (pH 7.4); horseradish peroxidase (activity 112 U/mg, M = 44173.9), 1 mM aqueous solution; Luminol (5-amino-1,2,3,4-tetrahydro-1,4-phthalazinedione, 3-Aminophthalic acid hydrazide, M = 177.11), 1 mM aqueous solution; hydrogen peroxide (H₂O₂, M = 34.01), 1 mM aqueous solution; antioxidant solutions (ascorbic acid, quercetin, tocopherol). All reagents were produced by Sigma-Aldrich, USA.

Hawthorn, mountain-ash and wild rose fruit decoctions and raspberry fruit infusion were prepared according to the procedure of the State Pharmacopoeia of the USSR set out in the general pharmacopoeial article “Infusions and decoctions” [24].

The total antioxidant capacity was determined through chemiluminescence on chemiluminometer Lum-100 (DiSoft, Russia) using PowerGraph 3.3 software. To determine the TAC in the plant material, 40 µl of luminol at a concentration of 1 mM, 40 µl of horseradish peroxidase at a concentration of 0.1 µm, 10 to 50 µl of decoction or infusion (depending on concentration) and phosphate buffer in the quantity necessary to bring the total sample volume to 1 ml were placed in the cuvette of the device. The cuvette was mounted on the device and CL was recorded, observing the background signal. After 48 s of observing the background signal, 100 µl of H₂O₂ at 1 mM concentration was added into the cuvette and CL observation continued for 10 minutes. Four samples with different concentrations of each of the plant objects were prepared. Later, the TAC of the samples of decoctions and infusions were recalculated to quercetin.

Concentrations of luminol, horseradish peroxidase and hydrogen peroxide were selected such that it was possible to determine the antioxidant capacity of aqueous extracts of medicinal plants in a reasonable time (no more than 10 min). Within this time, the chemiluminescence curves for the antioxidants ascorbic acid and flavonoid quercetin (the main antioxidants of plant materials) reached a plateau, indicating complete destruction of antioxidants in the system. Dilutions of the test samples and concentration of solutions of standard antioxidants (indicated in the captions of the figures) were selected such that all the CL kinetic curves were measured with the same device sensitivity.

The antioxidant capacity was calculated from the change in area \( \Delta S \) under chemiluminescence kinetic curve (light sum) by adding a substance containing an antioxidant. To this end, \( S_\text{r} \) was calculated for the system without an antioxidant and the area \( S_\text{r} \) (characterizing the system in which the antioxidant was added) subtracted therefrom. The value of \( \Delta S \) depends on chemiluminometer sensitivity and measurement conditions. The ratio \( \Delta S/C \cdot V \) (where \( C \) is the concentration of the test biological material in the cuvette, in g/l, and \( V \) — the volume of the cuvette in liters) expresses the antioxidant capacity of 1 g of the test plant material.

Similarly, antioxidant capacity \( \Delta S_\text{r} \) of the standard antioxidant solution, e.g. quercetin, placed in the same reaction mixture volume, was calculated. The ratio \( \Delta S_\text{r}/C_\text{r} \cdot V \) (where \( C_\text{r} \) is the weight concentration of the antioxidant in the cuvette, g/l) expresses the antioxidant capacity of 1 g of the antioxidant. For each of the standard antioxidants, a signal from the solutions of several concentrations were observed to make sure that calculations were performed within linear dependence, and results obtained are reproducible. Indeed, a linear dependence \( \frac{\Delta S_\text{r}}{C_\text{r}} = k \cdot C_\text{r} \) of the signal on the concentration at which stoichiometric ratio \( k \) was calculated was obtained. According to Fisher’s exact test, the \( k \) values obtained for standard antioxidants are statistically significant with a 0.975 probability. Then, a signal from four concentrations for each of the four plant samples was observed. A linear dependence of the signal on the concentration \( \Delta S = k \cdot C \) at which stoichiometric coefficient \( k \) was calculated was obtained for all the samples. With a probability of 0.975 (Fisher’s exact test), the \( k \) values obtained for the plant samples are statistically significant. Total antioxidant capacity of the plant material in terms of the weight of a standard antioxidant (mg%) was calculated using the formula

\[
\text{TAC} = \frac{k \cdot 10^6}{C_\text{r}}
\]

The values were presented as the arithmetic mean ± standard deviation (M ± S) at p <0.05.

RESULTS

The study of chemiluminescence kinetics in the presence of sodium ascorbate (fig. 1) showed that this antioxidant is characterized by latency when CL is almost completely suppressed. Its duration is proportional to the amount of the antioxidant in the system. In this case, neither the slope of the CL curves nor the CL intensity at the plateau changes. This is due to the fact that ascorbic acid is a strong antioxidant that intercepts all radicals formed in the system, including luminol radicals and CL does not develop until the entire ascorbate is oxidized.

The action of tocopherol (fig. 2) was manifested in the form of a decrease in the CL plateau intensity, which is characteristic of weak antioxidants, though tocopherol is considered to be one of the most powerful antioxidants. Possibly, this discrepancy is due to the fact that in our experiment, the free radicals were in aqueous solution, whereas the effect of tocopherol is typically studied in non-polar media. Tocopherol had medium-strength antioxidant properties in [11], where the cytochrome c/cardiolipin complex was used as the source of radicals; reaction with luminol proceeded within this complex. After studying the effect of different concentrations of quercetin on our system (fig. 3) and comparing the kinetic curves for it and sodium ascorbate and tocopherol, it was observed that the main effect of quercetin manifested in the form of a change in the slope angle of curves, i.e. the speed of CL development, which is typical for medium-strength antioxidants.

The CL curves for all the decoctions studied (fig. 4) resemble the curves for quercetin with a slight decrease in CL intensity at the end, i.e. at the exit to the plateau. As shown in [11], such behavior is characteristic of medium-strength antioxidants, which, in our case, can include polyphenols – flavonoids and tannins. For raspberry fruit infusion (fig. 4, D), decrease in chemiluminescence at the plateau level is noticeable, which is characteristic of weak antioxidants [11] such as tocopherol in this case. In terms of quercetin and tocopherol, raspberry fruit infusion contains 4.7 ± 0.9 mmol/g quercetin and 11.9 ± 0.8 mmol/g of tocopherol.

Comparing the chemiluminescence curves obtained for different concentrations of the four investigated aqueous water extracts from plant materials showed that the contribution of medium-strength and weak antioxidants in the total antioxidant...
capacity of the sample decreased in series: raspberry fruit infusion, rosehip decoction (fig 4, A), mountain-ash fruit decoction (fig. 4, B), hawthorn fruit decoction (fig. 4, C). The values of $\Delta S$ based on concentrations $C$ of the test substance in the cuvette and the total antioxidant capacity values expressed in terms of quercetin are shown in the table.

DISCUSSION

Data obtained in the course of experiments and TAC values (calculated based on the data) of the test objects were compared with their content of main antioxidants identified by chemical analysis methods [25–29]. Despite the fact that there is undeniable positive correlation between the total amount of antioxidants and TAC in different objects, there are still notable differences between these indicators. For example, the total content of flavonoids, tannins, and ascorbic acid is greater than the TAC calculated for all the test objects, except hawthorn fruit decoction (see table).

Other researchers have also shown that chemical analysis results and TAC value determined by chemiluminescent do not coincide often. In [19], the total antioxidant capacity determined in a peroxidase/luminol/hydrogen peroxide system correlated with the content of triterpene compounds. However, in another study [18] conducted by the same authors where another plant was used as the test object, no correlation was found between TAC and the content of a group of substances, including flavonoids.

Such differences are related to at least three factors. First, antioxidant activity matters, i.e. the rate of reaction of antioxidants with radicals, which is different for different antioxidants included in the plant sample. According to Izmailov [11], the rate constant for the corresponding reactions for mexidol, tocopherol and quercetin correspond as $0.04 : 2 : 60$. Secondly, each antioxidant molecule entering into a chemical reaction can intercept different numbers of radicals. According to [8], quercetin, uric and ascorbic acid intercepted $3.6 \pm 0.1$, $1.4 \pm 0.1$ and $0.5 \pm 0.2$ radicals per reacting antioxidant molecule respectively (hemin/H$_2$O$_2$/luminol system was used). Thirdly, the results of the study could have been affected by the presence of peroxidase activity in the plant samples, as in [23], as well as by the presence of calcium in the samples, which, as shown in [30], can, under certain conditions, enhance the activity of peroxidase horseradish. This usually results in higher CL intensity on the plateau than on the control curves, which, however, we didn’t observe.

The first factor severely limits the use of such indicator as a change in light sum because chemiluminescence measurement time should be more than the time of expenditure of all antioxidants in the sample. You know when this moment arrives only by measuring the chemiluminescence kinetics. Furthermore, the contribution of weak antioxidants in TAC is sharply undervalued since the time of their complete oxidation arrives only by measuring the chemiluminescence kinetics. Of all antioxidants in the sample. You know when this moment arrives only by measuring the chemiluminescence kinetics.

Stoichiometric coefficient of the antioxidant is even greater in value. The number of radicals $n$ intercepted by them is equal to

$$n = p \cdot \Delta m,$$

where $p$ is the stoichiometric coefficient, and $\Delta m$ is the change in antioxidant concentration during measurement: in this case, $\Delta m = 100 \text{µM}$.
The difference in the light sum of the luminescence in the absence and presence of an antioxidant is proportional to

\[ S = k \cdot n \]

where \( k \) is the coefficient, constant under the same measurement conditions.

The total number of intercepted radicals is equal to

\[ n = \sum p \cdot m \]

where \( p \) is the stoichiometric coefficient of a particular antioxidant, and \( m \) is its concentration during measurement. The total number of intercepted radicals is obviously not equal to the total amount of antioxidants since \( p \) coefficients are not equal to one and also differ significantly for different antioxidants.

The method considered in this paper allows to determine the total antioxidant capacity, whereas chemical analysis allows to determine the total content of antioxidants in a product. Therefore, chemiluminescence method is more informative than chemical analyses.

**CONCLUSIONS**

The conditions selected by us for evaluation of the total antioxidant capacity of plant materials by observing chemiluminescence kinetics in a system consisting of horseradish peroxidase, hydrogen peroxide and luminol (concentration of the components are 4 nM, 100 µM and 40 µM, respectively; 20 mM phosphate buffer, pH 7.4) ensured oxidation of strong antioxidants (ascorbic acid) and medium-
strength antioxidants (quercetin) within 10 minutes. Such duration of measurement is convenient and provides the required measurement quality.

Analysis of chemiluminescence kinetics showed that in the test objects (fruit decoctions of mountain-ash, rosehips, hawthorn and raspberry fruit infusion), the main antioxidants were medium-strength antioxidants, including flavonoids, as well as weak antioxidants (tocopherol and others). Based on reduction in the chemiluminescence light sum, the total antioxidant capacity of the test objects was calculated. Comparison of TAC values obtained with chemical analysis results showed that products containing the same amount of antioxidants in different ratios may differ by their ability to effectively protect the body against the harmful effects of free radicals. The technique described is a promising one for the study of plant materials that contain a mixture of different antioxidants. In addition, the technique is simple and not expensive. A combination of measurement of chemiluminescence kinetics with mathematical modeling of reactions will allow not only to automate the process of determining the total antioxidant capacity but also to determine the contribution of individual groups of antioxidants in the indicator.

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