Effect of *Perilla frutescens* aqueous extract on free radical production by human neutrophil leukocytes

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Key words: *Perilla frutescens; antioxidant; lucigenin; luminol; chemiluminescence.*

Summary. *Objective. The present study was intended to evaluate the antioxidant properties of aqueous extract of the Perilla frutescens (L.) Britton.*

Material and methods. The antioxidant properties of Perilla frutescens were analyzed employing neutrophil leukocytes stimulated by the nonopsonized Escherichia coli. The neutrophil leukocytes were affected by adding an aqueous extract of Perilla. The generation of the reactive oxygen species by neutrophil leukocytes was investigated using assessment of luminol- and lucigenin-dependent chemiluminescence.

Results. We found out that the treatment of neutrophil leukocytes with the Perilla aqueous extract inhibited the release of reactive oxygen species, measured as luminol- and lucigenin-dependent chemiluminescence, by about 30% and more than 90%, respectively.

Conclusion. The results of this study show that the aqueous extract of the Perilla frutescens inhibits significantly free radical production by neutrophil leukocytes, which was especially obvious when the lucigenin-dependent chemiluminescence assessment method was applied.

Introduction

An increasing body of evidence now available implicates the participation of reactive oxygen species (ROS) in the pathogenesis of a variety of diseases (1–3). It is likely that the role of reactive oxygen species is common to both bacterial and host-mediated pathways of tissue damage (4). Indeed, ROS were implicated in the pathogenesis of more than 100 conditions (5). Neutrophil leukocytes (NL) are recognized as a particularly rich source of ROS (1, 6, 7). The yields of superoxide ion and other ROS released by NL can be measured by assessment of lucigenin- and luminol-dependent chemiluminescence (CL) (8, 9).

In treatment of chronic inflammatory diseases as well as during the experiments, different scavengers of free radicals, such as rutin, aspirin, indomethacin, mannitol (10), vitamin E, probucol (11), or hormone preparations (1) are used to reduce the action of ROS.

In addition, in recent years many researches have been focused on the free radical scavenging activity of plants and their extracts (12–14). They exist widely in the kingdom *Plantae* and are important components in many Chinese traditional medicines. Much attention has been paid to flavonoids due to their diverse pharmacological effects such as antioxidant (15, 16), antiatherosclerotic (17), antibacterial (18), and anticancer actions (19).

There is a growing interest in natural antioxidants found in plants because of the worldwide trend towards the use of natural additives in food and cosmetics. Herbs and spices are one of the most important targets to search for natural antioxidants from the point of view of safety. Among the herbs of the *Labiatae* family and its extracts are the first marketed natural antioxidants (20). It is known that a dry purified extract of the *Perilla frutescens* (L.) Britton (family *Labiatae*) has different chemical compounds in the preparation (21, 22). The preparations of this plant also have strong antioxidant properties; they protect cells and decelerate their aging. In addition to that, active substances of the plant affect alteration processes in the focus of the inflammation (23, 24).

The usage of *P. frutescens* in medicine is rather wide. Asian experts in medicinal herbs administered P. frutescens as an antitussive and a medicine for respiratory diseases, as means of the prevention of flu, in cases of poisonings with seafood, and in cases of improper nutrition (25). The preparations of this plant have antimicrobial, desensitizing, and other effects (26). It has been known for years that *P. frutescens* has antibacterial, antiseptic, antipyretic, spasmolytic, antiseizure, antiasthmatic, antitussive, expectorant,

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restorative, and tonic properties (27). *Perilla* possesses immunomodulatory actions (28).

The aim of the present study was to investigate the effect of aqueous extract of the *Perilla frutescens (L.) Britton*, prepared from the overground part of the plant by simplified technology, upon the generation of ROS by peripheral venous blood leukocytes. The generation of ROS by leukocytes was investigated using luminol- and lucigenin-dependent CL assessment. The stimulation of the above-mentioned cells was performed utilizing nonopsonized *Escherichia coli* ATCC25922 (*E. coli*).

Material and methods

Patient selection. Patients for the study were selected from a large number of individuals treated in the Department of Odontology at Kaunas University of Medicine Hospital. The patients with acute oral inflammatory disorders were not included in the investigation. The age of the examined patients ranged from 18 to 50 years. All the patients gave the informed consent. For performing the assessment of luminol-dependent and lucigenin-dependent CL, leukocytes were obtained via natural erythrocyte sedimentation from peripheral venous blood of 22 patients (12 females and 10 males).

Laboratory studies. Twenty milliliters of venous blood were collected from each subject by venipuncture and anticoagulated with heparin (20 U/mL).

The test tubes with blood were positioned at an angle of 45 degrees and kept for 1 hour at 37°C. Then 2 mL of the supernatant plasma rich in leukocytes was aspirated from each tube and placed in plastic cuvettes for chemiluminescent examination. The examination was performed at a temperature of 37°C.

ROS generation was investigated by luminol- and lucigenin-dependent CL assessment method as proposed by Korkina et al. (10). The luminol- and lucigenin-dependent CL was registered by a scintillation counter Delta-300 (in the Department of Biochemistry at Kaunas University of Medicine) 15, 30, 45, 60, 75 minutes from the beginning of stimulation, i.e. until it reached its maximal value.

It is known that neutrophil leukocytes make a major contribution to the total CL response of whole blood or isolated cell suspensions (29). Therefore, the intensity of nonstimulated and stimulated CL induced by NL ($I_{(NL)}$) can be calculated (10) from the CL intensity of the total leukocyte fraction ($I_{(leuk)}$) using the equation:

$I_{(NL)}=I_{(leuk)}\times 100V/vcn$

Where: v – the cell suspension volume; V – the volume of the cuvette; c – the leukocyte concentration; n – the NL content (percentage).

The CL response was expressed as mean±standard error of the mean (SEM) peak values of relative light units/min (cpm) per 1×10^6 neutrophils.

Reagents. Luminol, lucigenin and Hank's balanced salt solution were obtained from Sigma Chemical Co St. Louis. Mo (USA). Plastic vials and other disposable pieces of plastic were obtained from Care Rot GmbH and CoKG.

E. coli samples were grown in the Laboratory of Microbiology at Kaunas University of Medicine Hospital. Specimens of *E. coli* 24-hour-old culture were used for the investigations.

Preparation of the P. frutescens extract. The dry aqueous extract of *P. frutescens* leaves was manufactured at Kaunas Technology University, using the lyophilization technique. *P. frutescens* extract was obtained from dried, chopped, and prepared raw material. The raw material was extracted in the shaker (Mylab Platform Shaker Universal PSU 20) via double hot extraction at room temperature for 2 hours. After that the mixture was filtered using Buchner's filter funnel, and the resulting filtrate was lyophilized in a lyophilizer (MAXI dry lyo) until the dry substance was obtained. The dry substance residue of the aqueous solution of the aqueous extract of *P. frutescens* leaves was 0.32 g/mL (32%). For experimental investigations, sterile solution was diluted 1:1000 with sterile Hank's solution.

The final dilution of *P. frutescens* aqueous extract in the samples was 1:10 000. The final luminol and lucigenin concentration was 50 μM. The final *E. coli* concentration was 6×10^6 cells/mL. All solutions were prepared using deionized water of high purity.

Statistical analysis. The statistical significances of the differences between the samples that were affected by the *Perilla frutescens (L.) Britton* aqueous extract and the controls were assessed using the Wilcoxon signed-ranks test.

Results

The results of measurements of luminol- and lucigenin-dependent CL of stimulated peripheral venous blood leukocytes and *P. frutescens* extract effect upon them are given in Fig. 1 and Fig. 2.

The data of the Fig. 1 show that the diluted 1:10 000 *P. frutescens* aqueous extract reduced peripheral blood NL, stimulated by nonopsonized *E. coli,* luminol-dependent CL; however, this reduction was not reliable ($P>0.05$) 15 min after the start of NL stimulation. Whereas, it was statistically significant

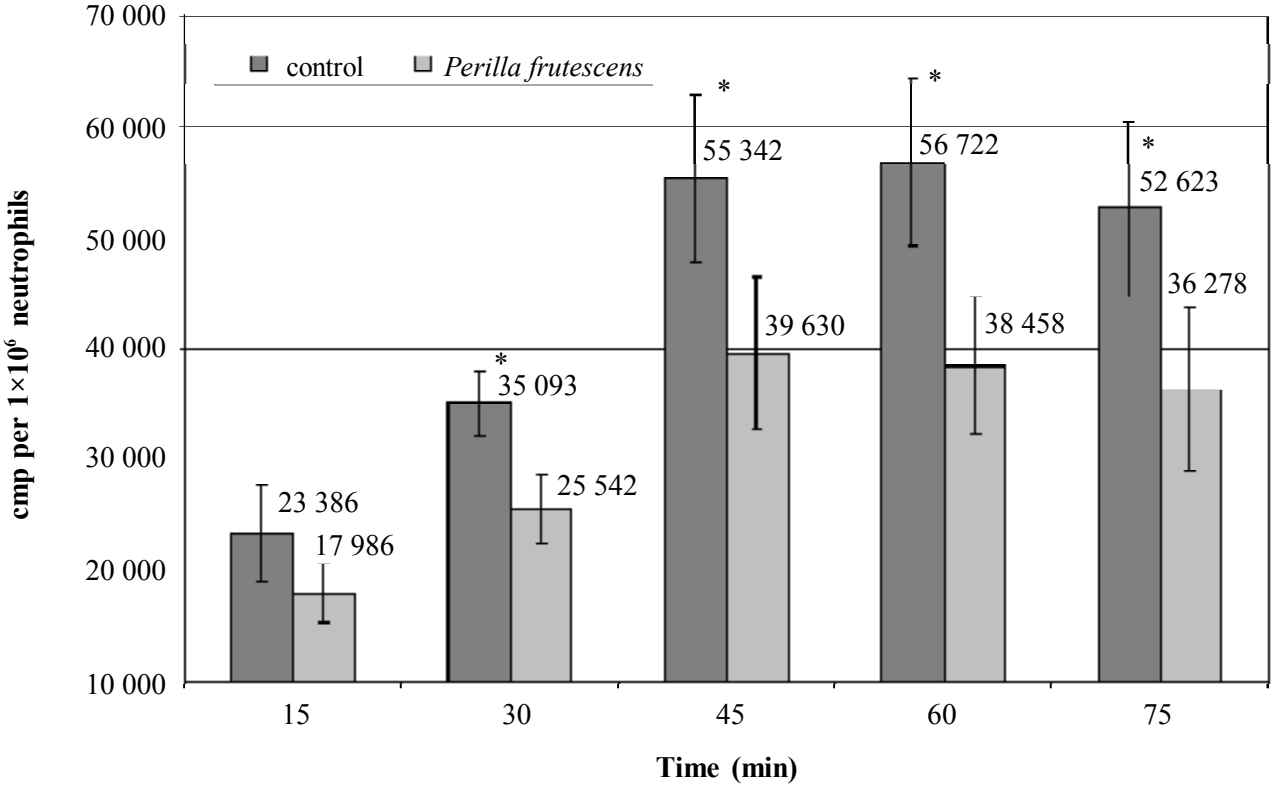

Fig. 1. **Effect of *Perilla frutescens* aqueous extract on luminol-dependent chemiluminescence of blood neutrophils**

**P*<0.01 vs *Perilla frutescens.*

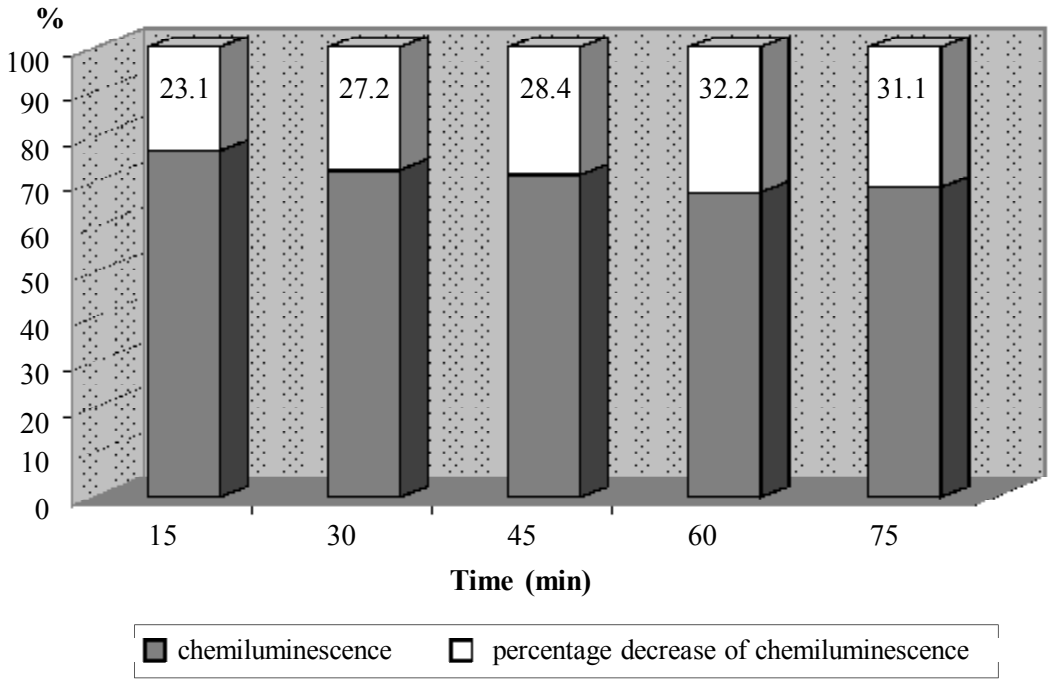

Fig. 2. **Percentage decrease of luminol-dependent chemiluminescence of activated neutrophils pretreated with *Perilla frutescens* aqueous extract**

(P<0.01) 30 min after the stimulation start and remained similarly reliable at 45, 60, and 75 minutes of the stimulation.

Luminol-dependent CL of peripheral blood NL stimulated by nonopsonized *E. coli* reached its maximum at 45–60 minutes of the stimulation and remained almost unchanged even at 75 minute of the stimulation.

The data of our study have shown that the *P. frutescens* aqueous extract inhibited the luminol-dependent CL of NL stimulated by nonopsonized *E. coli* by 27–32% (Fig. 2).

The data of the Fig. 3 have revealed that the diluted 1:10 000 aqueous extract of *Perilla* may reduce significantly (P<0.001) lucigenin-dependent chemiluminescence of the peripheral blood NL stimulated by nonopsonized *E. coli*. The above-mentioned CL reduction under the influence of the *P. frutescens* extract remains similar at 15, 30, 45 minutes of the stimulation. The above-mentioned lucigenin-dependent CL under influence of the diluted 1:10 000 *P. frutescens* extract reached its maximum at 30 minute of the stimulation by the nonopsonized *E. coli*.

It is necessary to notice a very significant inhibitory effect of aqueous extract of the *P. frutescens* on the lucigenin-dependent CL of NL stimulated by nonopsonized *E. coli* (Fig. 4), which exceeds 90%.

Discussion

In the present study, we have investigated the effects of the *Perilla frutescens* aqueous extract on the generation of reactive oxygen species by stimulated NL. For this study, we had chosen the chemiluminescence method, which requires less quantity of test compounds, and is of high sensitivity, simpler, and less expensive (15). The advantages of the method are emphasized by the other authors (30).

A number of nonvolatile compounds, such as flavonoids, terpenoids, phenolics, glycosides, and others, also have some biological activities. Further research has isolated such constituents as apigenin, ascorbic-acid, beta-carotene, caffeic acid, citral, dillapiol, elemicin, limonene, luteolin, myristicin, perillaldehyde, protocatechuic acid, quercetin, rosmarinic acid, and more, too numerous to mention. An infusion of the plant is useful in the treatment of asthma, colds, cough and lung afflictions, influenza prevention, nausea, vomiting, abdominal pain, constipation, food poisoning and allergic reactions (especially from seafood), and to restore health and balance (21, 22,

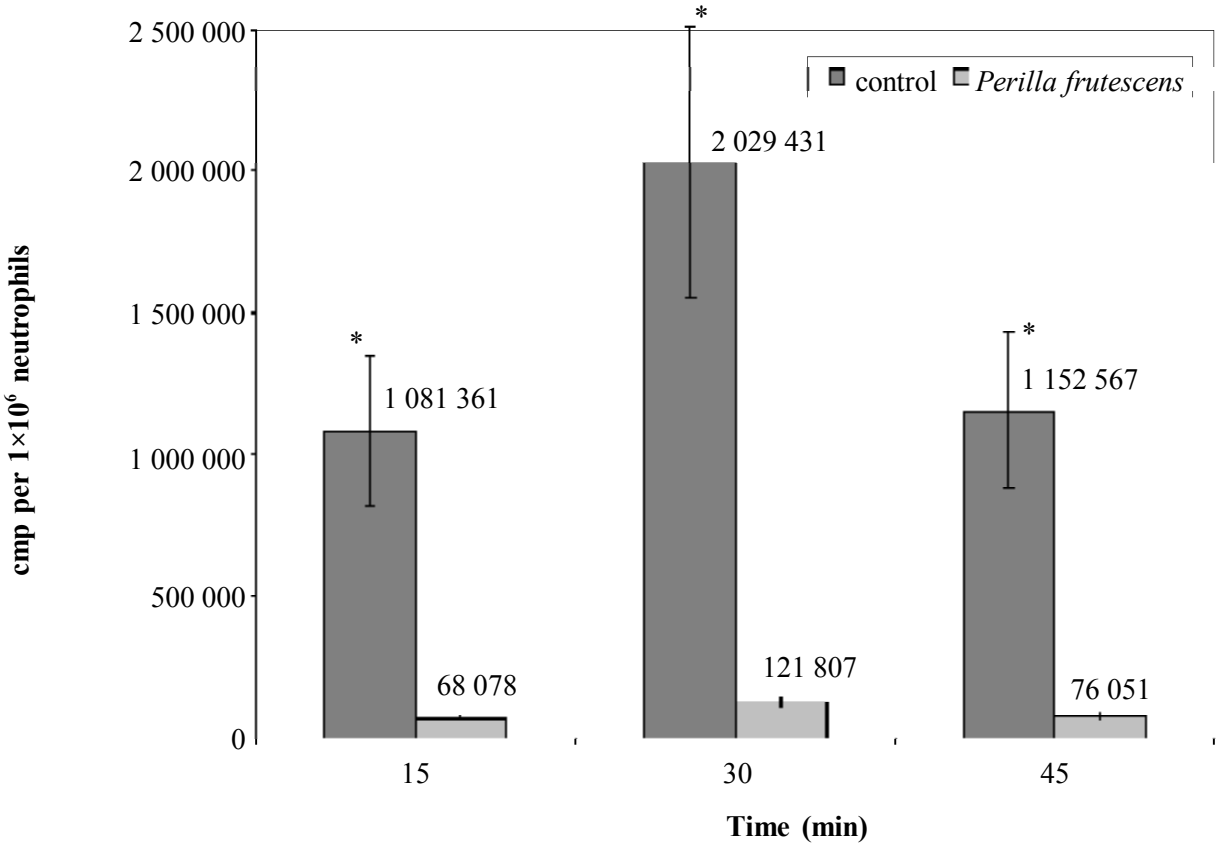

Fig. 3. **Effect of *Perilla frutescens* aqueous extract on lucigenin-dependent chemiluminescence of blood neutrophils**

*P<0.001 vs *Perilla frutescens*.

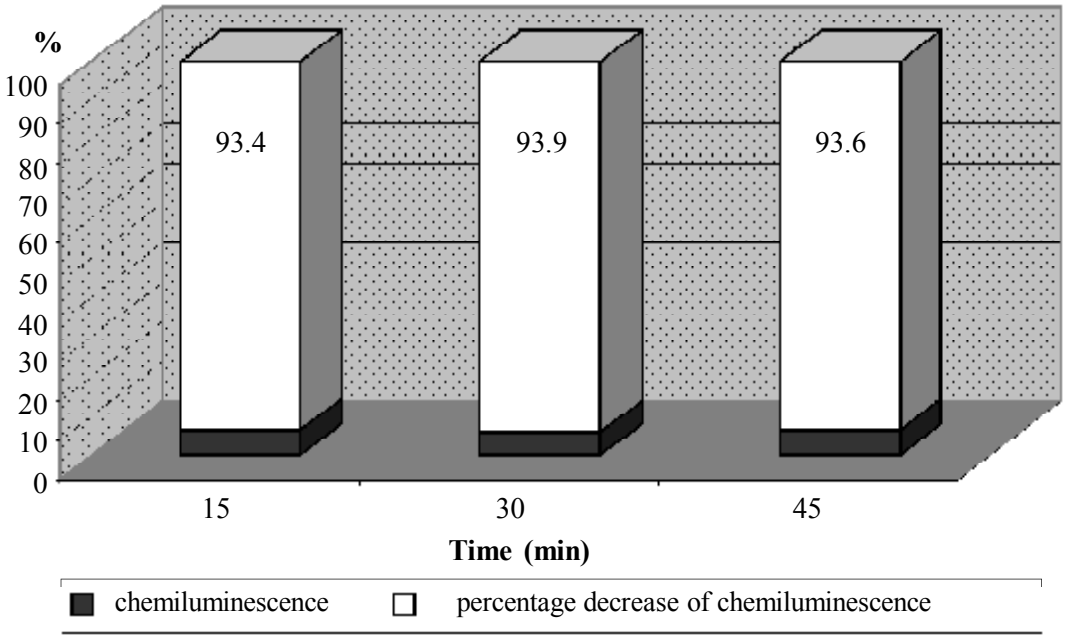

Fig. 4. **Percentage changes of lucigenin-dependent chemiluminescence of activated neutrophils pretreated with *Perilla frutescens* aqueous extract**

27). It is considered that increasing usage of *Perilla* products must induce more detailed investigations of biologic activity and immunomodulative qualities as well (28, 31, 32). *Perilla* seeds contain a drying oil with high content of multiply unsaturated fatty acids (60% α-linolenic acid, 15% both linoleic and oleic acid); their medicinal value is sometimes matter of great exaggeration. *Perilla* contains the pseudotannins and antioxidants typical of the mint family (33, 34). The extract of *Perilla* contains flavones (luteolin, apigenin, scutellarin) and rosmarinic acid, which have antioxidant activity (34–36).

The results of the study have shown that the *Perilla frutescens* aqueous extract may reduce the luminol-dependent CL of the peripheral venous blood NL stimulated by nonopsonized *E. coli* (Fig. 1) up to 30%. It is known (37–39) that luminol-dependent CL is conditioned by the myeloperoxidase system, which plays an important role in the host response, especially in the oxygen-mediated defense mechanisms (40). It was determined that the flavonoids inhibit 30–70% of the luminol-dependent CL of the leukocytes in concentration-dependent manner (10). The other authors have stated that flavonoids decrease the production of hydrogen peroxide by neutrophils in concentration-dependent manner (30) and significantly inhibit luminol-dependent leukocyte CL in vitro (41, 42).

It is difficult to compare the values of the changes of the luminol-dependent CL of neutrophil leukocytes induced by flavonoids obtained in vivo and in vitro, as flavonoids are metabolized in vivo to monocyclic phenolic acids. Phenolic acids have virtually no effect on the chemiluminescence related to superoxide formation that is measured by lucigenin but inhibit luminol-dependent CL (43). Consequently, that part of the in vivo pharmacological activity of flavonoids may readily be accounted for by phenolic acids.

The results of our study have shown that *Perilla frutescens* extract markedly inhibits lucigenin-dependent CL. The reduction of peripheral venous blood NL lucigenin-dependent CL that reached more than 90% under the influence of the *Perilla frutescens* aqueous extract is to be considered as especially marked.

The most important agents implicated in the inflammatory injuries to tissue are hydroxyl radicals and superoxide anions. Superoxide anions are regarded as weak restive radicals relative to the hydroxyl radicals, but they can also be converted to the more potent hydroxyl radical (44).

Great oxygen free radical scavenging abilities of bioflavonoids were also stated by the other authors (45). These authors have assessed the concentration-dependent oxygen free radical scavenging abilities of a grape seed proanthocyanidin extract, a group of polyphenolic bioflavonoids, ascorbic acid, and vitamin E succinate as well as superoxide dismutase, catalase and mannitol against biochemically generated superoxide anion and hydroxyl radical using a chemiluminescence assay. At a 100-mg/L concentration, grape seed proanthocyanidin extract exhibited a 78–81% inhibition of superoxide anion and hydroxyl radical

production. Under similar conditions, ascorbic acid inhibited these two oxygen free radicals by approximately 12–19%, while vitamin E succinate inhibited the two radicals by 36–44%. The combination of superoxide dismutase and catalase inhibited superoxide anion by approximately 83%. The above presented results of our investigations and those of the other authors clearly show an especially marked antioxidative activity of flavonoids, especially of those obtained from the *Perilla frutescens*.

Antioxidative activity of flavonoids depends on the number of hydroxyl groups (15, 30). Consequently, the structural types of flavonoids themselves can influence their hydroxyl radical scavenging activities. Perhaps, the construction of flavonoids and their structural types predetermine the antioxidative properties of the flavonoids.

Taking into consideration the especially marked antioxidative properties of *Perilla frutescens*, it is purposeful to further study the construction of the flavones and rosmarinic acid of this plant and their structural types.

Conclusions

The aqueous extract of the *Perilla frutescens (L.) Britton* statistically significantly inhibits production of the reactive oxygen species, especially the superoxide anion, by neutrophil leukocytes.

Krūminės perilės vandeninio ekstrakto įtaka žmogaus neutrofilinių leukocitų laisvųjų radikalų gamybai

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Raktažodžiai: *Perilla frutescens*, antioksidantai, lucigeninas, luminolis, cheminė liuminescencija.

Santrauka. *Tyrimo tikslas*. Nustatyti *Perilla frutescens* (L.) Britton. ekstrakto antioksidacinės savybės.

Tyrimo medžiaga ir metodas. Krūminės perilės ekstrakto antioksidacinės savybės tirtos naudojant neutrofilinių leukocitų stimuliaciją neopsonizuotomis *Escherichia coli* (štamas ATCC 25922). Neutrofiliniai leukocitai buvo paveikti vandeniniu *Perilla frutescens* ekstraktu. Neutrofilinių leukocitų aktyvių deguonies radikalų išskyrimas buvo vertintas naudojant nuo luminolio ir lucigenino priklausomos cheminės liuminescencijos tyrimą.

Rezultatai. Nustatyta, kad, veikiant kraujo neutrofilinius leukocitus vandeniniu krūminės perilės ekstraktu, nuo luminolio ir lucigenino priklausoma cheminė liuminescencija sumažėjo apie 30 ir 90 proc., atitinkamai. Tai rodo aktyvių deguonies radikalų išsiskyrimo slopinamą.

Išvada. Šio tyrimo rezultatai rodo, kad vandeninis *Perilla frutescens* ekstraktas reikšmingai slopina neutrofilinių leukocitų laisvųjų radikalų gamybą – įrodyta taikant nuo lucigenino priklausomos cheminės luminescencijos vertinimo metodą.

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