

## EFFECT OF *PERILLA FRUTESCENS* EXTRACTS AND ROSMARINIC ACID ON RAT HEART MITOCHONDRIAL FUNCTIONS

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**Abstract:** *Perilla frutescens* L. due to its aromatic, antibacterial, anti-inflammatory and antioxidant traits has been traditionally used as medicinal plant in Eastern Asia. Alterations of mitochondria are interconnected with many chronic diseases. Bioactives of herbal extracts can modulate mitochondrial effects and be beneficial in prevention of mitochondrial related chronic diseases. Direct effects of the red-leaf form *P. frutescens* extract (PFE) and the green-leaf form *P. frutescens* var. *crispa* f. *viridis* extract (PCE) were evaluated investigating activities on the oxidative phosphorylation and antioxidant activity in the rat heart mitochondria *in vitro*. HPLC-MS analysis was applied for the identification of phenolic compounds. Cell with a Clark-type oxygen electrode was used for mitochondrial respiration measurement. The generation of reactive oxygen species was estimated in isolated rat heart mitochondria and determined fluorimetrically. State 3 respiration rate was not affected by lower concentrations, however, it was inhibited at higher concentrations by 22-70% for PFE and by 45-55% for PCE. PFE containing anthocyanins induced the concentration-dependent stimulation (by 23-76%) of the State 4 respiration rate after addition of cytochrome c due to reducing properties. Significant reduction of H<sub>2</sub>O<sub>2</sub> production was observed with investigated concentrations of rosmarinic acid and both perilla extracts. Our results demonstrate that the effect of PFE and PCE extracts on rat heart mitochondria depend on the qualitative characteristics of complex of biologically active compounds. Selective effects on mitochondrial function could enable the regulation of apoptosis or another mechanisms occurring in cells.

**Keywords:** *Perilla frutescens* L., rosmarinic acid, mitochondria, antioxidant, freeze drying

*Perilla frutescens* L. (perilla) is an annual herbaceous plant belonging to the Lamiaceae Lindl. family. Species is originated from East Asia where it has been traditionally used as aromatic, spice and medicinal plant for treatment of various ailments including inflammation, allergies, food poisoning (1, 2). Perilla generally exists as red/purple-leaf and green-leaf forms in characteristics of leaf color (3). Perilla leaves contain a multitude complex of phytochemicals such as phenolic compounds (flavone derivatives, phenolic acids – rosmarinic acid) (4), triterpenic acids (ursolic, oleanolic acids) (5), bioactive monoterpenes (perillyl alcohol, isoeogonolone) (6). Various studies confirmed antioxidant power of phenolic-rich herbal extracts (7-9).

The biochemical targets of phenolic compounds are distributed in cellular compartments (10). Ones of the most important and pivotal in life and death processes of cell are mitochondria. Main sites of effect are: interactions with reactive oxygen species (ROS) formation processes (11); triggering of intrinsic apoptotic pathway mediated by release of cytochrome c (12); effect on mitochondrial permeability transition (MPT) (13); uncoupling of oxidation and phosphorylation or activation of uncoupling proteins UCPs (14). It has been also shown that activation of caspases may be regulated by the redox state of cytochrome c, and the reduced cytochrome c is less potent to caspase activation as compared to oxidized form (15-18). Moreover,

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mitochondria play an important role as a source of ROS in the cell as well as they are critical targets of the damaging effects of ROS (19). The ROS can cause variations in enzyme activity, inhibit protein synthesis and increase the membranes permeability, disturb the cell signaling and growth processes.

Mitochondrial integrity and oxidative phosphorylation efficiency forms the basis of the bioenergy and vitality of the cell. Depending on the concentration and structural peculiarities, biological effect of the phenolic compounds is associated with their antioxidant or pro-oxidant effect (20, 21) as well as with their effects on oxidative phosphorylation (22, 23). Modulation of these effects can be used in prevention of numerous mitochondria related chronic diseases (24, 25).

It is therefore important to investigate the effect of the phenolic-rich herbal extracts on mitochondria energy metabolism, intracellular signaling pathways and pathological processes. To our knowledge the mechanism of action of perilla extracts on heart mitochondrial functions has not been investigated yet.

Therefore, the aim of this study was to evaluate the effects of the red-leaf form *P. frutescens* extract (PFE) and the green-leaf form *P. frutescens* var. *crispa* f. *viridis* extract (PCE) on the oxidative phosphorylation in the rat heart mitochondria and on the antioxidant activity.

## MATERIALS AND METHODS

### Chemicals and plant material

All the used solvents were of HPLC grade. Methanol, acetonitrile, orthophosphoric acid, acetic acid, chlorogenic acid, rosmarinic acid, caffeic acid, cyanidin chloride and scutellarin hydrate were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland). Ultrapure water (18.2 mΩ/cm) was prepared by Millipore (USA) water purification system. Ethanol was provided by Stumbras (Kaunas, Lithuania). Potassium chloride and magnesium chloride were purchased from Roth (Karlsruhe, Germany). Amplex® Red was purchased from Invitrogen (Carlsbad, USA). All other chemicals used in this study were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

The raw material used for the research was comprised of perilla aboveground part samples collected in the trial area of the Botanical garden of Vytautas Magnus University. Specimens with voucher numbers (No. 10343, 10344) were deposited in the herbarium of Botanical garden of Vytautas Magnus University. Two cultivars of *P. frutescens*

were used: the red-leaf form *P. frutescens* (PF) and the green-leaf form *P. frutescens* var. *crispa* f. *viridis* (PC). The air dry raw material was milled up to the size of the particles passing through the 355 μm mesh. The hydroalcoholic extracts (1 : 10) of PF and PC were prepared by maceration method with 40% ethanol at room temperature initially for 48 h and thereafter until exhaustion. The obtained extracts (*P. frutescens* ethanolic extract – PFE; *P. frutescens* var. *crispa* f. *viridis* ethanolic extract – PCE) were freeze dried for 48 h using a laboratory freeze dryer with shell freezer FD8512S (Ilshin Lab. Co., Ltd., Gyeonggi-do, South Korea).

### Quantitative analysis of phenolic compounds by HPLC

Chromatograph Waters 2695 with 2 detectors Waters 2487 UV/Vis and Waters 996 DAD (Waters, Milford, USA) was used for the analysis. The analytes were distributed by ACE C<sub>18</sub> analytic column (5 μm, 150 × 4.6 mm) with the guard column – ACE 5 μm C<sub>18</sub> (Aberdeen, Scotland). Ten microliters of the test sample was injected. The analysis was performed at room temperature.

### Anthocyanidin analysis

Acid mediated hydrolysis was performed prior to HPLC analysis. The anthocyanin content was converted to anthocyanidin - cyanidin. The detection was performed at the wavelength of 520 nm. The flow speed was 1 mL/min. Eluent A was acetonitrile (ACN), eluent B was orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub>). The following alteration of the gradient was used: 0 min – 7% A (93% B), 37 min – 25% A (75% B), 40 min – 7% A (93% B), 45 min – 7% A (93% B). Cyanidin was identified by comparing the analyte retention time to standard retention time and spectrum achieved by the DAD detector. Total anthocyanidin content is presented in cyanidin equivalents.

### Analysis of phenolic acids and flavones

The detection was performed at the wavelength of 290 nm. The flow speed was 1 mL/min. Eluent A was acetic acid of 0.5%, eluent B – methanol. Gradient alteration: 0 min. – 90% A and 10% B; 40 min. – 20% A and 80% B; 50 min. – 10% A and 90% B; 52 min. – 0% A and 100% B; 53 min. – 90% A and 10% B; 60 min. – 90% and 10% B. Rosmarinic acid, caffeic acid, chlorogenic acid and scutellarin were identified by comparing the analytes retention times with standard retention times and spectrum achieved with the DAD detector. Combined detection system (HPLC-MS-DAD) was

applied for the essence analysis for identification of the flavones (apigenin-7-O-caffeoylglucoside, luteolin-7-O-diglucuronide and apigenin-7-O-diglucuronide). HPLC pump Waters 1525 connected to the DAD detector Waters 996 and through flow separator connected to MS detector Waters ZQ 2000 (Waters, Milford, MA, USA) equipped with quadrupole mass analyzer was used under the negative electrospray ionization conditions. M/z interval from 100 to 950 a.u. was selected for the test. Capillary voltage was 3 kV, the voltage of extractor was 30 V, and temperature of the source was 120°C. M/z ratio, mass fragments, DAD spectra, retention time are used to identify separate components and to relate the data of the both systems. Retention times, molecular mass of the compound, mass fragments,  $\lambda_{\max}$  absorption were compared with literature data. Quantity of the flavone glycosides was calculated as equivalents of luteolin-7-O-glucoside.

#### Ethics statement

All experimental procedures were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (License No. 0006). The experimental procedures used in the present study were performed according to the permission of the Department of Public Food and Veterinary Service of Lithuania (No. 0155 July 12<sup>th</sup>, 2007). Adult male Wistar rats weighing 200–250 g were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment.

#### Preparation of rat heart mitochondria

Hearts of male Wistar rats were excised and rinsed in ice-cold 0.9% KCl solution. Subsequently, the tissue was cut into small pieces and homogenized in a buffer (10 mL/g tissue) containing 180 mM KCl, 10 mM Tris/HCl and 1 mM EGTA (pH 7.7) for 45 s. The homogenate was centrifuged at  $750 \times g$  for 5 min and the supernatant obtained was centrifuged at  $6800 \times g$  for 10 min. The mitochondrial pellet was suspended in buffer containing 180 mM KCl, 20 mM Tris/HCl and 3 mM EGTA (pH 7.35) to approximately 50 mg/mL protein, and stored on ice. Biuret method was applied for protein quantity determination in the mitochondria suspension using bovine serum albumin as a standard (26).

#### Mitochondrial respiration measurement

Thermostatically controlled cell with a Clark-type oxygen electrode was used for mitochondrial

respiration measurement at 37°C in 1.5 mL measuring medium of the following composition: 150 mM KCl, 10 mM Tris-HCl, 5 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , pH 7.2, with substrates: 1) 5 mM pyruvate and 5 mM malate or 2) 15 mM succinate (+ 2 mM amyltal). It is estimated that the medium contains 420 nmol O/mL of dissolved oxygen. Mitochondrial respiration rate in the second metabolic state ( $V_2$ ) was registered by adding 0.5 mg/mL of mitochondria and the respective substrate into the medium, and after adding 1 mM ADP the mitochondrial respiration rate was registered in the third metabolic state (State 3;  $V_3$ ). State 4 respiration rate was measured after addition of atractyloside, an inhibitor of ADP/ATP transport. Finally, cytochrome c was added during State 4, and the State 4 respiration rate in the presence of cytochrome c ( $V_4 + \text{cyt c}$ ) was registered. *P. frutescens* and *P. frutescens* var. *crispa* f. *viridis* ethanolic extracts (14.6 mg/mL) were added to the measuring cell (1.58 mL) during the phase of State 2 ( $V_2$ ) respiration in different quantities (9, 46, 69, 92, 184 and 323  $\mu\text{g/mL}$ ). Standard solution of rosmarinic acid was prepared by dissolving the acid in water (0.07 mg/mL). Mitochondrial respiration rate was expressed in nmol O/min/mg of mitochondrial protein.

#### Measurement of $\text{H}_2\text{O}_2$ generation

The generation of ROS was estimated as the release of  $\text{H}_2\text{O}_2$  from isolated rat heart mitochondria (0.25 mg/mL) and determined fluorimetrically using Ascent Fluoroskan plate reader (Thermo Fisher Scientific). Mitochondria (0.25 mg/mL) were incubated for 30 min in the same medium as used for mitochondrial respiration (at 37°C) supplemented with pyruvate (6 mM) plus malate (6 mM), antimycin A (0.016  $\mu\text{g/mL}$ ), and without (for control) or with different amounts of PFE and PCE (36.5–200.7  $\mu\text{g/mL}$ ). After incubation, Amplex Red (5  $\mu\text{M}$ ) and horseradish peroxidase (2 U/mL) were added, and fluorescence (excitation at 544 nm, emission at 590 nm) was measured. Amplex Red fluorescence response was calibrated by adding known amounts (nmol) of  $\text{H}_2\text{O}_2$ .

#### Statistical analysis

Statistical data analysis packages SPSS 17.0 and Microsoft Excel were used in analysis of the data. All experiments were carried out in triplicate of three separate experiments and expressed as the mean  $\pm$  standard error. Statistically significant differences were determined using Student's *t*-test. Level of significance  $\alpha = 0.05$  was chosen.

## RESULTS

### Phenolic composition of *P. frutescens* extracts (PFE and PCE)

Anthocyanins, flavones and phenolic acids were determined in *P. frutescens* and *P. frutescens* var. *crispa* f. *viridis* (PCE and PFE) extracts. During HPLC-DAD-ESI/MS analysis 4 flavone glycosides were identified by applying the negative ionization model in the PFE and PCE extracts (27). Rosmarinic acid was found to be predominant phenolic compound in PFE and PCE extracts (Table 1). Main qualitative difference between the extracts is that PCE does not contain anthocyanins. Amounts of luteolin-7-O-diglucuronide and scutellarin are significantly greater in PFE extract  $27.43 \pm 2.3$  and  $10.49 \pm 3.4$  mg/g, respectively ( $p < 0.05$ ). Notable amount of apigenin-7-O-diglucuronide  $15.18 \pm 2.1$

mg/g was detected in PCE extract. Chlorogenic and caffeic acids were minor components among identified compounds in both extracts of *P. frutescens* (Table 1).

### Effects of PFE and PCE on mitochondrial oxidative phosphorylation

In order to compare the direct effects of red leaf form *P. frutescens* (PFE) and green-leaf form *P. frutescens* var. *crispa* f. *viridis* (PCE) extracts on mitochondrial oxidative phosphorylation, the mitochondrial respiration rates in various metabolic states were measured. In a first series of experiments, the effects of PFE and PCE on the pyruvate and malate (mitochondrial Complex I dependent substrates) oxidation (Tables 2, 3) were assessed.

As can be seen in Tables 2 and 3, none of the investigated extracts had any significant effect on

Table 1. Amounts of phenolic compounds in *P. frutescens* extracts (mg/g in dry extract of PFE and PCE and ng/ $\mu$ L in working solution).

Compounds	PFE		PCE	
	mg/g	ng/ $\mu$ L	mg/g	ng/ $\mu$ L
Anthocyanins <sup>a</sup>	$2.79 \pm 0.68$	$40.73 \pm 0.15$	n.d.	n.d.
Apigenin-7-O-caffeoylglucoside <sup>b</sup>	$2.44 \pm 0.43$	$35.58 \pm 0.34$	$2.06 \pm 0.56$	$30.01 \pm 0.65$
Luteolin-7-O-diglucuronide <sup>b</sup>	$27.43 \pm 2.30$	$400.47 \pm 8.80$	$4.17 \pm 0.57$	$60.81 \pm 1.33$
Apigenin-7-O-diglucuronide <sup>b</sup>	$11.65 \pm 1.34$	$170.15 \pm 5.30$	$15.18 \pm 2.13$	$221.56 \pm 6.40$
Scutellarin	$10.49 \pm 3.40$	$153.26 \pm 6.71$	$6.50 \pm 1.12$	$94.84 \pm 5.32$
Rosmarinic acid	$23.50 \pm 3.20$	$343.20 \pm 9.31$	$24.33 \pm 2.74$	$355.25 \pm 10.31$
Chlorogenic acid	$0.28 \pm 0.01$	$4.05 \pm 0.34$	$0.69 \pm 0.10$	$10.02 \pm 2.43$
Caffeic acid	$1.54 \pm 0.70$	$22.48 \pm 0.34$	$0.79 \pm 0.11$	$11.47 \pm 1.73$
Total	$80.13 \pm 4.27$	$1169.93 \pm 34.45$	$53.71 \pm 2.13$	$783.98 \pm 20.84$

<sup>a</sup> – amount of anthocyanidins was expressed as equivalents of cyanidin; <sup>b</sup> – amount of flavone was expressed as equivalents of luteolin-7-O-glucoside.

Table 2. Effect of *P. frutescens* ethanolic extract (PFE) on the rat heart mitochondrial function (pyruvate + malate substrate).

Pyruvate + malate	Control	PFE 9 $\mu$ g/mL	PFE 46 $\mu$ g/mL	PFE 92 $\mu$ g/mL	PFE 184 $\mu$ g/mL	PFE 323 $\mu$ g/mL
V <sub>2</sub>	$50.5 \pm 2.1$	$53.4 \pm 1.1$	$52.1 \pm 2.9$	$47.2 \pm 2.2$	$44.5 \pm 2.9$	$46.2 \pm 2.0$
V <sub>2</sub> + PFE		$53.7 \pm 0.4$	$51.5 \pm 2.1$	$48.5 \pm 2.2$	$50.1 \pm 2.9$	$59.4 \pm 2.2$
V <sub>3</sub>	$222.8 \pm 11.1$	$210.7 \pm 12.7$	$194.2 \pm 19.5$	$174.3 \pm 10.9^*$	$87.1 \pm 9.9^*$	$66.1 \pm 8.3^*$
V <sub>4</sub>	$48.2 \pm 1.6$	$53.3 \pm 2.9$	$49.5 \pm 1.8$	$46.5 \pm 2.1$	$40.5 \pm 5.4$	$47.3 \pm 5.3$
V <sub>4</sub> + cyt c	$90.6 \pm 2.9$	$90.3 \pm 3.3$	$98.8 \pm 9.3$	$111.4 \pm 8.3^*$	$124.8 \pm 8.9^*$	$159.4 \pm 7.4^*$
RCI	$4.5 \pm 0.3$	$3.9 \pm 0.3$	$3.6 \pm 0.3$	$3.7 \pm 0.1^*$	$1.9 \pm 0.2^*$	$1.4 \pm 0.1^*$

V<sub>2</sub> – mitochondrial respiration rate in the second metabolic state with 0.5 mg/mL mitochondria and 5 mM pyruvate + 5 mM malate. V<sub>3</sub> – mitochondrial respiration rate in the third metabolic state, with 1 mM ADP. V<sub>4</sub> – mitochondrial respiration rate in the fourth metabolic state, with 0.12 mM atractiloside. V<sub>4</sub> + cyt c – mitochondrial respiration rate in the fourth metabolic state with 32  $\mu$ M cytochrome c. RCI – mitochondrial respiration control index; n = 3; \*p < 0.05.

Table 3. Effect of *P. frutescens* var. *crispa* f. *viridis* ethanolic extract (PCE) on the rat heart mitochondrial function (pyruvate + malate substrate).

Pyruvate + malate	Control	PCE 9 $\mu\text{g/mL}$	PCE 46 $\mu\text{g/mL}$	PFE 69 $\mu\text{g/mL}$	PCE 92 $\mu\text{g/mL}$	PCE 184 $\mu\text{g/mL}$
$V_2$	47.7 $\pm$ 2.5	52.8 $\pm$ 0.7	52.9 $\pm$ 3.9	55.5 $\pm$ 0.3	47.4 $\pm$ 3.2	46.8 $\pm$ 0.2
$V_2$ + PCE		54.6 $\pm$ 1.8	53.6 $\pm$ 2.9	63.9 $\pm$ 0.3	45.5 $\pm$ 2.0	52.3 $\pm$ 0.7
$V_3$	321.7 $\pm$ 8.3	311.5 $\pm$ 6.6	289.5 $\pm$ 15.1	307.5 $\pm$ 0.8	175.5 $\pm$ 2.6*	144.7 $\pm$ 2.9*
$V_4$	39.0 $\pm$ 1.9	46.0 $\pm$ 1.3	43.6 $\pm$ 7.7	57.2 $\pm$ 0.5	28.8 $\pm$ 0.8	46.1 $\pm$ 0.2
$V_{4+\text{cyt c}}$	89.2 $\pm$ 2.7	93.1 $\pm$ 1.6	87.8 $\pm$ 4.1	103.5 $\pm$ 1.4	82.3 $\pm$ 5.9	73.7 $\pm$ 0.3
RCI	6.8 $\pm$ 0.4	5.8 $\pm$ 0.1	5.5 $\pm$ 0.2	5.5 $\pm$ 0.1	3.7 $\pm$ 0.2*	3.1 $\pm$ 0.6*

$V_2$  – mitochondrial respiration rate in the second metabolic state with 0.5 mg/mL mitochondria and 5 mM pyruvate + 5 mM malate.  $V_3$  – mitochondrial respiration rate in the third metabolic state, with 1 mM ADP.  $V_4$  – mitochondrial respiration rate in the fourth metabolic state, with 0.12 mM atractiloid.  $V_{4+\text{cyt c}}$  – mitochondrial respiration rate in the fourth metabolic state with 32  $\mu\text{M}$  cytochrome c. RCI – mitochondrial respiration control index; n = 3; \*p < 0.05.

the mitochondrial State 2 respiration rate. Maximal, i.e., ADP stimulated mitochondrial respiration rate ( $V_3$ ) was not affected by lower concentrations of both extracts (Tables 2, 3), however higher concentrations (92  $\mu\text{g/mL}$ , and higher) induced the inhibition (by 22-70% for PFE and by 45-55% for PCE, respectively (p < 0.05), depending on the concentration used. The highest applied PFE (323  $\mu\text{g/mL}$ ) and PCE (184  $\mu\text{g/mL}$ ) extract concentration inhibited the  $V_3$  rate by 70 and 55%, respectively (Tables 2, 3). For comparison of both extracts effect on the maximal mitochondrial respiration rate by oxidizing the mitochondrial Complex II-dependent substrate - succinate, further experiments were carried out. We found that PFE (at the concentration range of 9–46  $\mu\text{g/mL}$ ) and PCE (9–69  $\mu\text{g/mL}$ ) had no significant effect on the mitochondrial respiration rates in the States 2 and 3. Statistically significant (44–54% for PFE and 19–30%, for PCE, respectively) decrease in the mitochondrial respiration rate in the State 3 ( $V_3$ ) was observed at the higher PFE (92–323  $\mu\text{g/mL}$ ) and PCE (92–184  $\mu\text{g/mL}$ ) concentrations (Figs. 1, 2). In summary, the inhibitory effects of both extracts (PFE and PCE) on ADP-stimulated respiration are more expressed on pyruvate malate (Complex I)-dependent oxidation than on Complex II (succinate) oxidation.

Mitochondrial respiration control index (RCI) showing the mitochondrial coupling between oxidation and phosphorylation was assessed. RCI was not affected at lower concentrations by both extracts, whereas a concentration dependent decline in RCI (from 24 up to 69% and from 46 up to 62%, respectively, substrates pyruvate + malate (Figs. 1, 2), p < 0.05) was observed when 92  $\mu\text{g/mL}$  and higher con-

centrations of PFE and PCE were used. Effect on RCI when oxidizing succinate was lower (30-40% and 24-33%, respectively), at 184-323  $\mu\text{g/mL}$  of PFE (Fig. 1) and 92-184  $\mu\text{g/mL}$  of PCE (Fig. 2).

In order to find out why the inhibition of the mitochondrial respiration rate is observed in the third metabolic state when high concentrations of perilla extracts were used, we investigated the effect of these extracts on the mitochondrial respiration rate uncoupled from the phosphorylation (i.e., from ATP synthesis),  $V_{\text{CCCP}}$ . (Table 4). Our results show (Table 4) that the uncoupled respiration rate decreased similarly as State 3 (by 45% and by 47%, substrate pyruvate + malate; and by 43-27%; substrate succinate) for both extracts (PFE and PCE, respectively) respiration rate showing that it might be due to the inhibition of mitochondrial respiratory chain at Complex I and Complex II.

The most important difference between the anthocyanin-containing red perilla extract PFE and green perilla extract PCE (which contains no anthocyanins) is that PFE induced a powerful concentration dependent stimulation of the State 4 respiration rate when external cytochrome c was added at State 4. Thus, the State 4 respiration rate in the presence of cytochrome c ( $V_{4+\text{cyt c}}$ ) increased by 23, 38 and 76%, respectively, after addition of 92, 184 and 323  $\mu\text{g/mL}$  of PFE (Table 2). Meanwhile, PCE had no effect on this parameter at any used concentration (Table 3).

Finally, we measured the effect of rosmarinic acid, predominant compound in the both extracts of perilla leaves, on the heart mitochondrial oxidative phosphorylation, in order to find out the direct effects on mitochondrial respiration. Rosmarinic

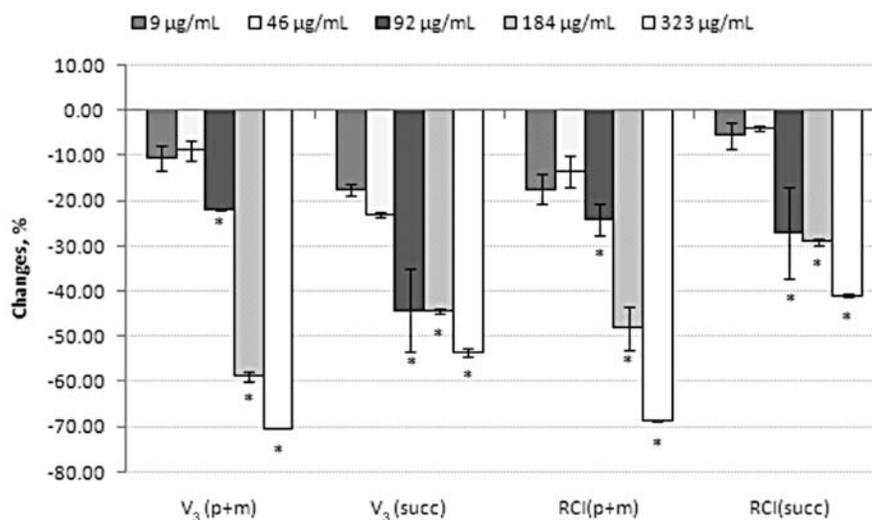


Figure 1. Effects of *P. frutescens* extract (PFE) on mitochondrial State 3 respiration rate ( $V_3$ ), respiratory control index (RCI). Substrates were 5 mM pyruvate + 5 mM malate or 15 mM succinate (+ 2 mM amytal).  $p < 0.05$  vs. respective control;  $n = 3$ .

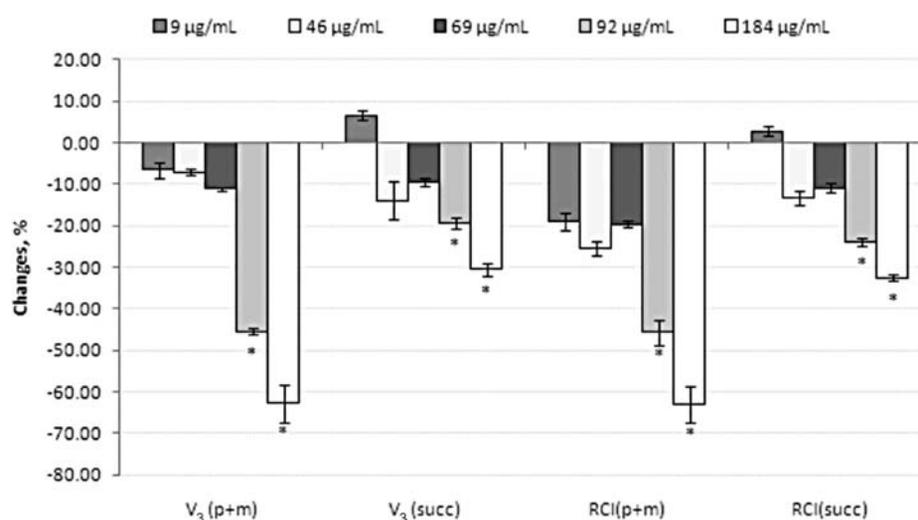


Figure 2. Effects of *P. frutescens* var. *crispa* f. *viridis* extract (PCE) on mitochondrial State 3 respiration rate ( $V_3$ ), respiratory control index (RCI). Substrates were 5 mM pyruvate + 5 mM malate or 15 mM succinate (+ 2 mM amytal).  $p < 0.05$  vs. respective control;  $n = 3$ .

acid content accounts for 29% in PFE and 45% in PCE of all the identified compounds in the dry extract. Moreover, during the HPLC-ABTS research it was determined that rosmarinic acid determines up to 60% of the raw material antioxidant activity (27). The rosmarinic acid content selected for the experiments meet the determined content in the extracts. The study revealed that rosmarinic acid did not have any significant effect on the mitochondrial respiration rates in any metabolic state when oxidiz-

ing pyruvate and malate (Table 5). Such an effect was neither detected in the succinate oxidation case (data not presented).

#### Effects of PFE and PCE on mitochondrial $H_2O_2$ production

In the last experiments we tested the effect of perilla extracts and rosmarinic acid on  $H_2O_2$  production by isolated rat heart mitochondria. Significant reduction of  $H_2O_2$  generation was determined using

selected concentrations of PFE, PCE and rosmarinic acid (Fig. 3). At a concentration of 36.5  $\mu\text{g/mL}$ , PFE and PCE reduced  $\text{H}_2\text{O}_2$  generation by 20% and 40% as compared with control ( $p < 0.05$ ). At higher concentration of 73  $\mu\text{g/mL}$ , PCE extract also showed more potent activity than PFE reducing peroxide generation up to 64% when compared to control (Fig. 3) whereas PFE induced the reduction by 40%. Higher amounts of PFE caused the decrease of  $\text{H}_2\text{O}_2$  production by 79% (not shown), similarly as PCE at lower (36.5  $\mu\text{g/mL}$ ) concentration used. The powerful antioxidant rosmarinic acid at concentration of 0.35, 2.1 and 4.2  $\mu\text{g/mL}$  reduced peroxide generation by 27, 48 and 69%, respectively, thus similarly, as both extracts alone and was practically responsible for the full antioxidant activity of crude PFE and PCE extract.

## DISCUSSION

Scientific data concerning the effect of phenolic compounds on mitochondrial functions is ambivalent. In many researches, phenolic compounds are ineffective or inhibit the mitochondrial respiratory chain at Complex I (the activity of NADH-oxidase) and at Complex II activity succinate-oxidase (10). Flavonols, isoflavones and flavones are the most potent inhibitors of NADH oxidase (28, 29). Our data revealed that the perilla leaf extracts (both, PFE and PCE) containing flavone glycosides (apigenin-7-O-caffeoylglucoside, luteolin-7-O-diglucuronide, apigenin-7-O-diglucuronide, scutellarein-7-O-glucuronide) at higher concentrations inhibit Complex I dependent pyruvate + malate oxidation more potent as com-

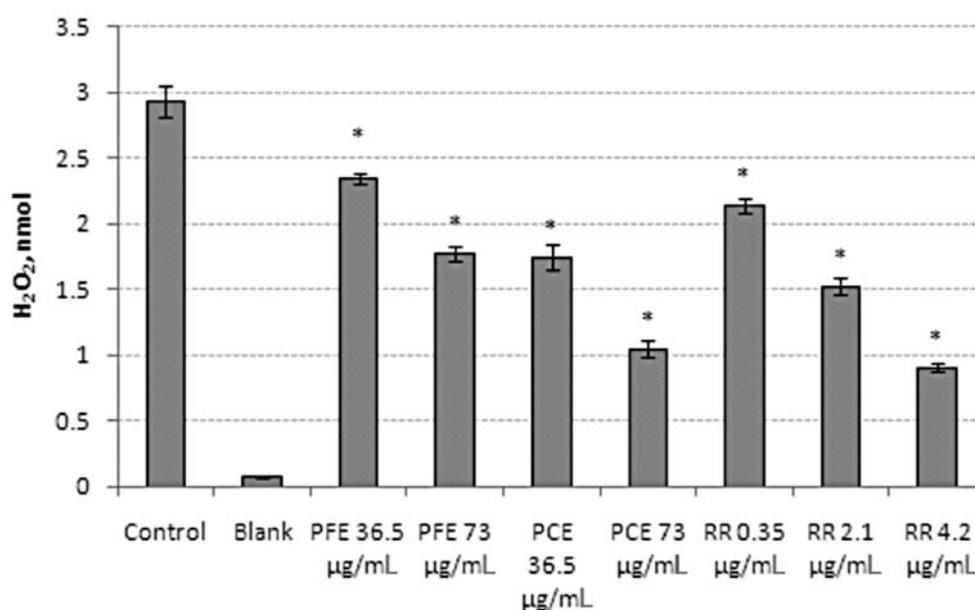


Figure 3. Effect of *P. frutescens* extracts (PCE and PFE) and rosmarinic acid on production of peroxide by isolated rat heart mitochondria. Standard incubation media was used,  $n = 3$  separate measurements, with three reiterations within each.  $p < 0.05$  vs. respective control;  $n = 3$

Table 4. Effects of *P. frutescens* ethanolic extract (PFE) and *P. frutescens* var. *crispa* f. *viridis* ethanolic extract (PCE) on uncoupled from phosphorylation respiration rate ( $V_{\text{CCCP}}$ ) of mitochondria energized with pyruvate + malate and succinate.

Substrate pyruvate + malate				Substrate succinate				
Control	PFE 184 $\mu\text{g/mL}$	Control	PCE 92 $\mu\text{g/mL}$	Control	PFE 184 $\mu\text{g/mL}$	Control	PCE 92 $\mu\text{g/mL}$	PCE 184 $\mu\text{g/mL}$
248 $\pm$ 2.5	135.4 $\pm$ 11.5*	447 $\pm$ 8.9	237 $\pm$ 17.5*	206 $\pm$ 3.2	117.4 $\pm$ 12.2*	309.2 $\pm$ 11.7	276.9 $\pm$ 9.3	225.5 $\pm$ 1.4*

Substrates: 5 mM pyruvate and 5 mM malate; 15 mM succinate (+ 2 mM amytal).  $V_{\text{CCCP}}$  – respiration rate in the presence of 0.19  $\mu\text{M}$  carbonyl cyanidin m-chlorophenylhydrazone (CCCP);  $n = 3$ ; \* $p < 0.05$ .

Table 5. Effect of the rosmarinic acid (RR) on rat heart mitochondrial functions at the oxidation on pyruvate and malate.

	Control	Rosmarinic acid			
		1.6 µg/mL	6.7 µg/mL	22.2 µg/mL	66.5 µg/mL
V <sub>2</sub>	58.4 ± 1.34	64.5 ± 2.3	59.5 ± 3.2	66.2 ± 1.8	65.2 ± 1.4
V <sub>2</sub> + RR		69.8 ± 4.3	57.1 ± 1.7	64.1 ± 2.7	61.5 ± 2.0
V <sub>3</sub>	330.0 ± 34.4	342.0 ± 24.5	355.0 ± 18.6	346.0 ± 23.4	341.0 ± 20.5
V <sub>4</sub>	68.3 ± 6.5	63.1 ± 7.3	70.0 ± 10.5	85.0 ± 5.3	75.0 ± 6.2
V <sub>4+cyt c</sub>	180.0 ± 9.7	164.0 ± 3.2	190.0 ± 3.8	155.0 ± 5.4	143.0 ± 4.3
RCI	5.6 ± 1.2	5.3 ± 0.5	5.9 ± 0.1	5.2 ± 0.5	5.2 ± 0.5

V<sub>2</sub> – mitochondrial respiration rate in the second metabolic state with 0.5 mg/mL mitochondria and 5 mM pyruvate + 5 mM malate. V<sub>3</sub> – mitochondrial respiration rate in the third metabolic state, with 1 mM ADP. V<sub>4</sub> – mitochondrial respiration rate in the fourth metabolic state, with 0.12 mM atractiloside. V<sub>4+cyt c</sub> – mitochondrial respiration rate in the fourth metabolic state with 32 µM cytochrome c. RCI – mitochondrial respiration control index; n = 3; \*p < 0.05.

pared to succinate (Complex II) dependent oxidation.

Hubac et al. (30) also determined that luteolin inhibits NADH oxidase in plant cells. The effects of flavones are more potent on NADH oxidase than on succinate-oxidase (29). It has been shown that Complex II of mitochondrial respiratory chain is more susceptible to the effect of anthocyanins (10), as has been confirmed in our study. As 4 flavone compounds with different B ring hydroxylation and 6 anthocyanin formations are identified in *Perilla* L. species and varieties, the effect on the components of the mitochondrial respiration chain may vary, depending on the complex of the biologically active compounds in the extract and their chemical-biological interaction.

Since the PFE and the PCE extracts inhibited the uncoupled pyruvate and malate oxidation rate (V<sub>CCCP</sub>) by a similar rate (45% and 47%), like they inhibited the mitochondrial ADP-dependent respiration rate (State 3) (by 61% and 55%) and the succinate oxidation (43% and 27% vs. 44% and 22%), it can be proposed that the inhibition of the I and II Complexes activity of the mitochondrial respiration chain is one of the reasons for the inhibition of mitochondrial oxidation. However, this does not deny the assumption that perilla extracts can also affect the phosphorylation subsystem, i.e., ADP/ATP carrier and/or ATP-synthase.

The most important finding is that when exogenous cytochrome c was added to mitochondria during State 4 respiration, a powerful stimulation of State 4 was found with anthocyanin containing (cyanidin derivatives) *P. frutescens* (red perilla) extract (PFE). Thus, PFE (92–323 µg/mL) resulted in the increase in the mitochondrial respiration rate

by 23–78% (Table 2) as compared to the control group. In contrast, PCE obtained from the green forms (*P. frutescens* var. *crispa* f. *viridis*) did not show such an effect. Noteworthy, no anthocyanins were detected in this extract. This once again confirms our and other Lithuanian scientific findings that the anthocyanins are significant for this effect (18, 31). For example, the investigation of the black current berry extract rich in anthocyanins determined that the extract also stimulates the mitochondrial State 4 respiration rate in the presence of exogenous cytochrome c and higher concentrations inhibits the mitochondrial State 3 respiration rate as well (32).

We hypothesize, that the increase of the respiration rate in the State 4 in the presence of added cytochrome c may be associated with the ability of the anthocyanins, ones of the biologically active compounds of the perilla extract, to reduce cytochrome c, as it is revealed by our preliminary experiments and our previous studies investigating the standard anthocyanin solutions or anthocyanins rich herbal extracts (18, 31). Our previous studies revealed that anthocyanins such as cyanidin-3-glucoside, delphinidin-3-glucoside were able to reduce directly cytochrome c and very rapidly, whereas other anthocyanins such as malvinidin-3-glucoside and others had low cytochrome c reducing properties (18). HPLC analysis revealed that PFE extract contained anthocyanins of cyanidin type. Cytochrome c reduction induced by pure anthocyanins or anthocyanin containing plant extracts is a very important fact, because apoptosis, the cellular death process, can be regulated by changing the state of cytochrome c redox. Moreover, the study of Skemiene et al. (18), revealed, that pre-perfusion of

hearts with 20  $\mu\text{M}$  of anthocyanin cyanidin-3-glucoside prevented from ischemia-induced caspase activation. They assumed that the property of anthocyanins to inhibit caspase activation might be due to their ability to reduce cytosolic cytochrome c (18).

Rosmarinic acid is predominant compound in the extracts of *Perilla* L. plants, possessing antioxidant, anti-inflammatory, antibacterial and antiviral activities (33). The mode of action of rosmarinic acid is interrelated with antioxidant mechanisms (34). Even though the rosmarinic acid accounts for 29–45% of all the compounds identified in the PFE and PCE extracts, it is possible to suggest that the effect on oxidative phosphorylation is caused by the other biologically active compounds found in perilla extracts. Phenolic acids such as chlorogenic acid, as we have shown in our previous studies (35), did not affect neither the mitochondrial State 2 nor State 3 respiration rate at the concentration range from 30 ng/mL (36) and up to 300 ng/mL. Higher concentrations inhibit State 3 respiration rate by 10–40% (our unpublished data). Regarding caffeic acid and its effects on the State 3 respiration rate, our unpublished data show the inhibition of mitochondrial State 3 respiration rate by 18–42% at concentration range of caffeic acid at 900–1620 ng/mL. Thus, the possibility exists that reduction of mitochondrial State 3 respiration rate at higher concentrations of PFE and PCE could be partially caused by synergetic effects of phenolic acids such as caffeic and chlorogenic acids and flavones apigenin or luteolin glycosides in different extract samples tested.

This is in agreement with the experiments of other authors demonstrating that flavone derivatives reduce consumption of oxygen in State 3 (29). It has been shown, that flavone hispidulin inhibited mitochondrial respiration at  $V_3$  (29). The amount of hydroxyl groups and their configuration in flavonoid B ring markedly alter the effects to mitochondrial respiration chain (10, 29). Scutellarein, apigenin and hispidulin contains OH group at 4', 7 and 5 positions. At C6 position scutellarein possesses OH group and hispidulin – methoxy group. Melo et al. (37), in experiments with *Hyptis pectinata* L. leaf extract (containing flavone derivatives) effects on mitochondrial respiration, determined that the extract inhibited mitochondrial State 3 respiration rate and had no effect on respiration rate at State 4. Major flavone derivatives identified in *Hyptis pectinata* L. contained OH groups in C3' and/or C4', the same functional groups possess flavones in perilla (C3' and C4' – luteolin, C4' – apigenin derivatives).

Scientific studies confirm that initial target of the effects of flavones on mitochondrial respiration chain is Complex I (29). Flavones inhibit Complex I and II or act on respiratory chain in between coferment Q and cytochrome oxidase (29). The inhibitory effect on NADH dehydrogenase is attributed to hydroxy and methoxy groups in flavonoid B ring. Double bond at C2-C3 and C3 OH group conjugated with 4-oxy group determined the interaction of flavones and mitochondrial inner membrane inhibits mitochondrial respiration chain or induces the uncoupling of oxidation and phosphorylation (10, 11, 38).

In addition, we measured fluorimetrically in the biological system the antioxidant capacity of PFE and PCE. Both extracts reduced the generation of  $\text{H}_2\text{O}_2$  in rat heart mitochondria. The concentration of extracts for antioxidant measurements was chosen that had no suppressive effects on mitochondria. Rosmarinic acid has been found to have major significant effect for antioxidant activity of both extracts. This is in agreement with our results of a previous study evaluating antioxidant activity of PCE using on-line HPLC-ABTS assay, where rosmarinic acid determined about 61% of total antiradical activity (27). Various studies demonstrate significant protective effects of rosmarinic acid at a concentration range of 0.036–20  $\mu\text{g}/\text{mL}$  (39, 40). It is very important to note that in our experiments significant protective effects were already achieved using 0.35  $\mu\text{g}/\text{mL}$  of rosmarinic acid as this concentration corresponds to the rosmarinic acid concentration in plasma determined by Baba et al. (41), in pharmacokinetic study with humans.

## CONCLUSION

Our results demonstrate that the effect of PFE and PCE extracts on rat heart mitochondria depends on the qualitative characteristics of complex of biologically active compounds. One of the main findings of our study is that the red-leaf form *P. frutescens* extract (PFE) containing anthocyanins induced a powerful stimulation of State 4 respiration rate after addition of external cytochrome c, most probably due to their ability to reduce cytochrome c. In contrast, the green-leaf form *P. frutescens* var. *crispa* f. *viridis* extract containing no anthocyanins but greater amounts of apigenin-7-O-diglucuronide as compared to PFE, had no such effect. Both, PFE and PCE at higher concentrations inhibit maximal ADP-dependent mitochondrial respiration rate possibly due to the inhibition of complexes of mitochondrial respiration chain. Pyruvate and malate

(Complex I) dependent oxidation is more sensitive to the effects of the extracts than oxidation of succinate (Complex II).

Our results *in vitro* show, that by using different complexes and concentrations of bioactive compounds, the mitochondrial function could be regulated directly. Consequently, selective effects on mitochondrial function could enable the regulation of apoptosis or another mechanisms occurring in cells. Since certain bioactive compounds can influence mitochondrial oxidative phosphorylation, mitochondriotropic agents i.e., mitochondria targeted phytochemicals might be promising candidates in order to maintain cell viability or regulate apoptotic pathways.

#### Conflict of interest

The authors declare that there is no conflict of interest.

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