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# THE EFFECTS OF ISOLATED FRACTIONS OF RED PEPPER *CAPSICUM ANNUUM L.* ON THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AND LIPID PEROXIDATION

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**ABSTRACT**

Fruit proteins, seed proteins and capsaicinoids fractions were isolated from red pepper of *Capsicum annum L.* plants family and their effects on mitochondrial permeability transition pore (MPTP) and lipid peroxidation of rat liver were studied *in vitro*. Seed proteins did not influence to MPTP; however fruit proteins caused MPTP to open and led to mitochondrial membrane permeabilization. Opening of the MPTP causes massive swelling of mitochondria; capsaicinoids fractions inhibited the swelling process of mitochondria and caused the closed state of the MPTP. Fruits and seeds protein fractions from red pepper did not reduce the effect of $Fe^{2+}$/ascorbate-induced mitochondrial swelling and had no effect on the accumulation of malondialdehyde (MDA) in the membranes of mitochondria. Capsaicinoid fraction had a concentration-dependent inhibitory effect on the $Fe^{2+}$/ascorbate-dependent swelling of mitochondria and of lipid peroxidation. Half-maximal inhibitory concentration ($IC_{50}$) on the swelling of mitochondria fraction was 2 µg/ml. Capsaicinoids fraction prevented the effect of $Fe^{2+}$/ascorbate on mitochondria and reduced the accumulation of MDA in membrane. Complete inhibition of lipid peroxidation was shown at a 50 µg/ml capsaicinoids concentration. Capsaicinoids, reducing the membrane destructive effects of $Fe^{2+}$/ascorbate, had antioxidant properties and a protective effect on mitochondria. The obtained results showed the presence of different compounds in red pepper differently affecting MPTP and lipid peroxidation.

**Keywords:** Red pepper, proteins, capsaicinoid, mitochondria, MPTP, lipid peroxidation

## INTRODUCTION

Mitochondria are the organelle where cell respiration, oxidative phosphorylation, synthesis of most cellular ATP and regulation of apoptosis take place; they are also the main intracellular source of reactive oxygen species (ROS). Since these metabolic processes involve dozens of different protein complexes such as ion channels and ion transporting systems, effects of plant compounds on them are very complex and often difficult to interpret and are subject of intensive investigation. Production of free oxygen radicals in cell and mitochondrial respiratory chain, increasing peroxidation reaction depending on them and damages to membranes are considered as the main mechanisms of different pathological condition in organisms. Mitochondria supplies energy to all processes taking a part in a cell; mitosis, meiosis, necrosis, apoptosis, muscle contraction, conservation of ion homeostasis, $Ca^{2+}$ signalization, substitution of vesicles, excretion of hormones and neuromediators (**Armstrong, 2007; Smaili *et al.*, 2009**). Mitochondrial permeability transition pore (MPTP), megapore, $Ca^{2+}$-sensitive cyclosporine A pore are very significant for the mitochondrial function and for the life and death of plant and animals cells (**Halestrap, 2009; Juhaszova *et al.*, 2008**). Hence MPTP are in exposed conformations during different pathological conditions, they increase the permeability and make cells pass to more conductive condition (Lemasters et al., 1998), and thus they cause the necrotic death of cells (**Jacobson *et al.*, 2002**). Compounds isolated from *Capsicum* plants were demonstrated to cause cancer cells death with apoptosis (**Jung *et al.*, 2001; Mori, 2006**), and similar antioxidant activity (**Lee *et al.*, 1995**).

Studying mitochondria and investigating in molecular levels are very significant for the estimation of medicinal properties of plants. Because mitochondria and MPTP are considered as specific "targets" not only for pathogens but also for pharmacological agents (**Szewczyk *et al.*, 2002**). In folk medicine plants of *Capsicum annuum L* family are well-known with their medicinal properties since ancient times. These plants are commonly named as leguminous red peppers. Capsaicinoids are the main compounds giving bitter taste to pepper fruits. Yet 20 different capsaicinoids have been identified (**Legin, 1996**). Capsaicine and its analogs efficiently treat bronchial asthma, enuresis, inflammations of intestines, and different chronic deceases such as rheumatoid arthritis, osteoarthritis, neuralgia, and diabetic neuropathy (**Kenji *et al.*, 2010**). However, the effects of capsaicinoids and proteins from red pepper *Capsicum annuum L.* on membranes, mitochondria and in molecular levels have not been studied.

The aim of the work was to investigate the effects of different fractions of red pepper *Capsicum annuum L.* on the MPTP state and lipid peroxidation in rat liver mitochondria *in vitro* experiments.

## MATERIAL AND METHODS

### Isolation of capsaicinoids

Individual capsaicinoids were isolated from the aggregate of capsaicinoids by HPLC Agilent Technologies 1200, equipped with diode - array detector, using reverse phase analytical column 4.6 x 250 mm ZORBAX 300SB-C18, 5мм. Solutions: A - 0.1% $CF_3COOH$, B-$CH_3CN$. The concentration gradient of acetonitrile in minutes: 5% / 0-5 min, 70% / 35-40 min, 5% / 45 min. Flow rate 1ml/min, absorption 269 nm (**Zivavitdinov and Ishimov, 2009**).

### Identification of capsaicinoids

Research on mass spectrometry was performed on LC-MC Waters ZQ - 4000 by direct introduction of individual substances in the mass spectrometer under the following conditions: ionization source: ESI +, flow drying gas: 450 l/h, the temperature of the drying gas: 250 °C, the voltage skimmer cone at 30, 35, 50, 70V, mass range of 100 - 400 m/z, the ionization method: positive, mobile phase: water - methanol (1:9), flow rate: 0.25 mL / min. To obtain mass spectra at different voltages cone skimmer each sample re-introduced into the injector of the mass spectrometer.

### Isolation of proteins sum

Proteins sum was extracted from fruit and seed materials used for the extraction of capsaicinoids. For that purpose, alcohol in filtrated unit was fully evaporated.

Remained dry mass was extracted with 0.01 M Tris-HCl buffer (pH 7.5) for 8 hours at room temperature. Extract filtered and proteins were precipitated with 90% ammonium sulphate. Precipitate was isolated by centrifugation (8000 r/min), dialyzed and lyophilized. The proteins were quantified by Lowry method (**Lowry *et al.*, 1951**).

**Isolation of mitochondria**

Mitochondria were isolated from the livers of white rats, weighed 120-140 g, by conventional differential centrifugation (**Schneider, Hogeboom, 1951**). Composition of isolation medium: 250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, and pH 7.4.

**Measurement of the mitochondrial inner membrane permeability**

The kinetics of swelling of mitochondria was studied by the changes of optical density at 540 nm. Composition of the incubation medium (IM) for energized mitochondria: 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 20 mM Tris-HCl, 1 mM $KH_2PO_4$, 20 mM Hepes, 2 μM rotenone (**He *et al.*, 2003**).

**Lipid peroxidation as measured by MDA contents**

Induction of non-enzymatic $Fe^{2+}$/ascorbate-dependent lipid peroxidation was performed by adding 10 mM $FeSO_4$ and 600 mM ascorbate. IM contained 125 mM KCl, 10 mM Tris-HCl, pH 7.4. Antioxidant activity of the tested compounds was measured by inhibition of $Fe^{2+}$/ascorbate-dependent swelling process of rat liver mitochondria at 540 nm on a photometer.

Formed MDA was quantified by using a molar extinction coefficient (e = $1.56 \cdot 10^5$ $M^{-1}$ $cm^{-1}$) with the following formula: MDA nmol/mg protein = D/1.56·30.

Mitochondrial protein concentrations were determined by Biuret assay (**Gornall *et al.*, 1949**), using bovine serum albumin as standard. The amount of mitochondrial protein was 0.5 mg per 1 ml of the IM.

**Drugs and chemicals**

These given chemical reagents were used: EGTA, EDTA, cyclosporine A ("Sandoz", Switzerland), rotenone, tris-HCl (Serva, Germany), Sucrose (Russia), $CaCl_2$ ("Sigma", USA).

**Data analysis**

The results were statistically analyzed using the software "Origin 6.1". The data was evaluated using parametric Student's t-test, we expressed as M ± m. Deemed authentic results are expressed at * - P<0.05; ** - P<0.01; ***- P<0.001.

**RESULTS AND DISCUSSION**

Fruits and seeds of red pepper *Capsicum annuum L* were freed from the plants and capsaicinoids were extracted with ethyl alcohol and the sum compiled 0.63%. Proteins sum isolated from fruits were 0.346 mg/g and seeds 0.433 mg/g. Molecular weights of proteins in fractions were studied by SDS electrophoresis. We found 53, 48, and 28 kDa proteins in the fruit and 74, 72, 50, 36, 34, 28, 26, 25, 20 and 18 kDa proteins in the seeds.

First we conducted experiments in calcium-free IM. Under these conditions the effects of fractions on permeability of mitochondria membrane is studied. Fruit and seed proteins sum did not significantly affect the mitochondria swelling (Table 1). Capsaicinoids fraction in 130 μg/ml concentration did not affect membrane *permeability* transition, either and in higher concentrations (200-500 μg/ml) they caused the mitochondria to swell. Thus, in calcium-free IM fruit and seed proteins, and capsaicinoids fractions in small concentrations did not directly influence. Capsaicinoids fraction, in higher concentrations had a damaging effect on the membrane of mitochondria.

**Table 1** The effects red pepper fractions on mitochondrial swelling in calcium-free incubation

| Fraction | *Control* | Swelling of mitochondria, % Concentration (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **10** | **30** | **65** | **100** | **130** | **200** | **350** | **500** |
| **Fruit proteins** | 100.0±1.6 | 100.1±1.4 | 102.2±1.5 | 103.1±1.6 | 101.0±1.8 | 103.0±1.2 | 102.0±1.4 | 105.3±1.7 | 108.2±1.0* |
| **Seed proteins** | 100.1±1.7 | 100.2±1.5 | 101.0±1.5 | 103.0±1.7 | 104.1±1.9 | 101.0±1.3 | 104.2±1.1 | 107.1±1.5 | 109.4±1.4* |
| **Capsaicinoids** | 100.0±1.5 | 101.3±1.7 | 105.3±1.2 | 102.2±1.1 | 104.1±1.8 | 103.1±1.1 | 117.4±1.3* | 162.4±2.3** | 201.1±3.1*** |

(* - P<0.05; ** - P<0.01; ***- P<0.001, compared with control; n=5).

Experimental results showed that proteins sum isolated from the fruits and seeds of red pepper, in lower and higher concentrations, did not meaningly influence to mitochondrial membrane permeability in calcium-free medium, thus, they do not have membrane activity. High concentrations of capsaicinoids fractions in calcium-free medium induced mitochondrial swelling what constitutes a violation of the barrier function of mitochondrial membranes.

The effects of three fractions - fruit proteins, seed proteins and capsaicinoids on MPTP were studied. It is known that high levels of $Ca^{2+}$ can induce MPTP opening and mitochondrial swelling, which prompts mitochondrial massive release of $Ca^{2+}$ and reactive oxygen species into the cytosol which, in turn, compromises mitochondrial function. We studied the effects of $Ca^{2+}$ in IM. 10 μM $Ca^{2+}$, included in IM, caused mitochondria membrane permeabilization process. In this condition MPTP passed from low- to high-conductance state; for 6 minutes mitochondria swelling increased by 23 ±2.5% compared to control (Figure 1).

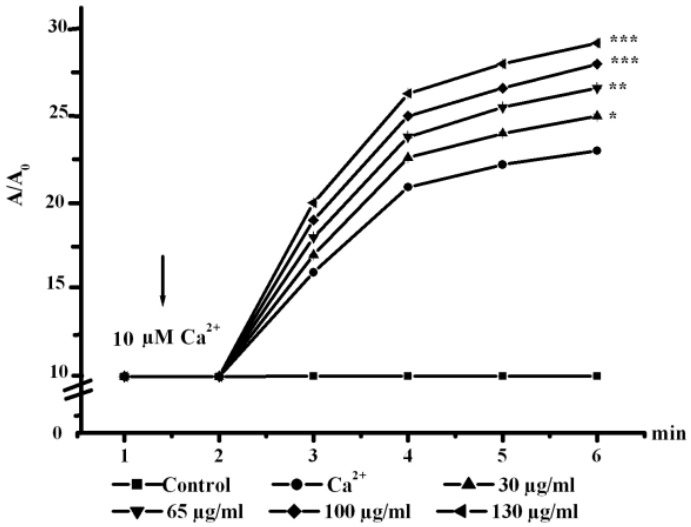


**Figure 1** Effects of red pepper fruit proteins in different concentrations on MPTP condition. In ordinate line the percentage (compared to control calculated as 100%) of mitochondria swelling, in medium containing $Ca^{2+}$, is expressed. In abscissa line duration is expressed in minutes (* - P<0.05; ** - P<0.01; ***- P<0.001, compared with $Ca^{2+}$ swelling; n=5)

The effects of fruit and seed proteins, and capsaicinoids on mitochondrial membrane permeabilization were further studied. The effects of fruit proteins in 30, 65, 100, 130 μg/ml concentrations on MPTP were determined. Doses in 30, 65, 100 μg/ml increased the swelling process of mitochondria respectively to 10.0±2.2%, 18.0 ±2.6%, 25.0±2.3% percentage compared to control. Fruit proteins in 130 μg/ml increased MPTP detection till 31.0 ±3.5% over the control and strengthened mitochondria membrane permeabilization (Figure 1). Thus, red pepper fruit proteins strengthen the influences of $Ca^{2+}$ to MPTP condition and might serve as MPTP inductors.

It is known that activation of the mitochondrial permeability transition in matrix cause volume increase, outer membrane rupture and release of proapoptotic intermembrane space signalling molecules such as cytochrome c (Smaili et al., 2000). The mitochondrial permeability transition is $Ca^{2+}$ dependent, CsA-sensitive and cause mitochondrial dysfunction. MPTP activation is associated with both apoptosis by the mitochondrial pathway and necrosis due to a damage of mitochondria. In this regard that fruit proteins are inductors of MPTP, we can assume that these components will act on the cancer cell death.

In these conditions red pepper seed proteins in 10, 15, 30, 65 µg/ml concentrations did not significantly influence to MPTP and higher concentrations; 100, 130, 200 µg/ml inhibited mitochondria swelling only to 3-6% (results are not shown). Protein fractions were isolated from the fruits and seeds of red pepper had opposite effects on MPTP.

Lower concentration range of capsaicinoids 5-25 µg/ml did not meaningly affect the speed of mitochondria swelling and MPTP condition (Figure 2). Addition of 30 µg/ml of capsaicinoids sum into IM showed that they own membrane activity. In this concentration mitochondria swelling lowered till 24.4±2.0% compared to control. Capsaicinoids concentrations increase in IM inhibited mitochondria swelling more. Capsaicinoids in 130 µg/ml inhibited mitochondrial swelling till 84.4±3.0%, comparing to control (Figure 2). These obtained results demonstrate that capsaicinoids inhibit MPTP and own membrane activity.

It is known that MPTP change into the open state when exposed to low concentrations of $Ca^{2+}$. Thus the mitochondria swell rapidly, and compounds with a molecular weight 1.5 kD pass through the pore. Antioxidants and chelators of divalent cations inhibit swelling of mitochondria, i.e. MPTP turn into the closed condition. Capsaicinoid molecules probably affect one of components of MPTP and cause it to close. For example, in various pathologies MPTP is in an open state, resulting in increased ROS formation and lipid peroxidation. The inhibitory effect on the MPTP capsaicinoids might be possibility to use the studied alkaloids up as corrective agents.

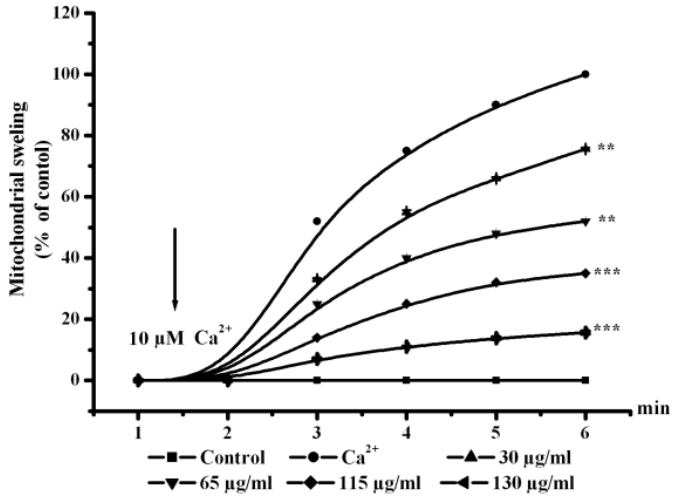


**Figure 2** Effects of capsaicinoids in different concentrations on rat liver mitochondrial swelling. In ordinate line the percentage (compared to control calculated as 100 %) of mitochondria swelling, in medium containing $Ca^{2+}$, is expressed. In abscissa line duration in minutes expressed. (** - P<0.01; ***- P<0.001, compared with $Ca^{2+}$ swelling; n=5).

Further we investigated the effects of red pepper protein fractions and capsaicinoids on $Fe^{2+}$/ascorbate-induced rat liver mitochondria swelling *in vitro.* Obtained results showed that the addition of $Fe^{2+}$/ascorbate in the IM increased the rate of mitochondria swelling (Figure 3). In this case $Fe^{2+}$/ascorbate initiates lipid peroxidation, the products of which violate the barrier function of the mitochondrial membrane, and as a result induced mitochondria swelling. Under these conditions, the addition of capsaicinoids fraction (at a concentration of 1 µg/ml) to the IM, prevented effect of $Fe^{2+}$/ascorbate on mitochondria swelling by 22.0% compared to controls (Figure 3); this indicates the inhibition of lipid peroxidation in the membranes.

When studying the effect of different concentration ranges of capsaicinoids fractions on $Fe^{2+}$/ascorbate-dependent swelling, we defined that the tested fraction has a concentration-dependent inhibitory effect on the swelling of mitochondria. Increasing the concentration of fractions in the IM we observed inhibition of mitochondrial swelling upon the activation of lipid peroxidation. Maximum inhibition of mitochondrial swelling was noted at a concentration of 6 µg/ml of capsaicinoids fraction. Half-maximal inhibitory concentration ($IC_{50}$) of mitochondria fraction on the swelling was 2 µg/ml.

Two isolated protein fractions, from the fruits and seeds of red pepper, did not prevent the effect of $Fe^{2+}$/ascorbate-induced mitochondrial swelling.

The experimental results obtained by our method of mitochondria swelling are indirect. The method is distinguished by the relative simplicity and informativeness; sufficient to establish the antioxidant properties of the test fraction, and a short time of analysis.

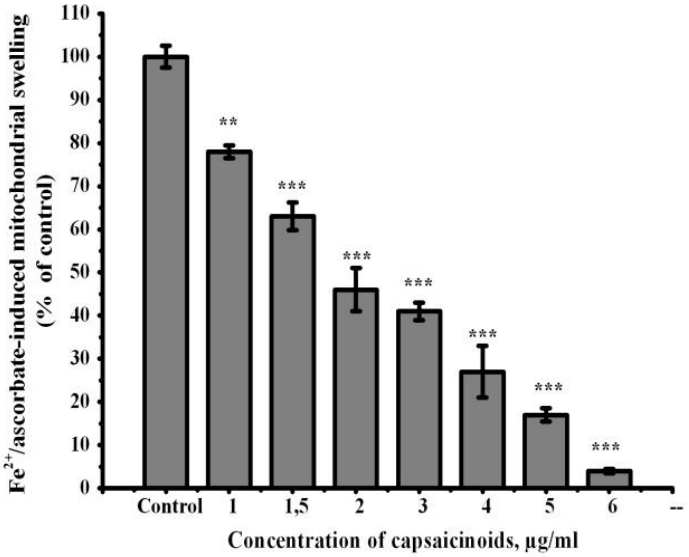


**Figure 3** Effects of capsaicinoids fractions on $Fe^{2+}$/ascorbate-dependent mitochondrial swelling (** - P<0.01; ***- P<0.001, compared with control; n=5).

Experiments have shown that addition of $Fe^{2+}$/ascorbate into the IM increased the MDA accumulation in the membranes of mitochondria (3.22 nmol MDA/mg protein against control 0.21 nmol MDA/mg protein) (Figure 4). Addition of capsaicinoids fractions at a concentration of 1.25 µg/mg protein prevented the effect of $Fe^{2+}$/ascorbate (2.78 nmol MDA/mg of protein) on lipid peroxidation of membranes. Inhibition of lipid peroxidation was also observed under the influence of other concentrations of capsaicinoids. Thus, capsaicinoids fraction at 5.00 µg/mg protein reduced the accumulation until 2.38 MDA nmol/mg protein; at 7.50 µg/mg protein to about 1.70 nmol of MDA/mg of protein, and at 8.75 µg/mg protein reduced the accumulation until 1.21 MDA nmol/mg protein. Complete inhibition of lipid peroxidation observed at a 10.0 µg/mg concentration of capsaicinoids (Figure 4).

Fruit and seeds protein fractions did not influence to $Fe^{2+}$/ascorbate-induced accumulation of MDA, which indicates no antioxidant properties possessed.

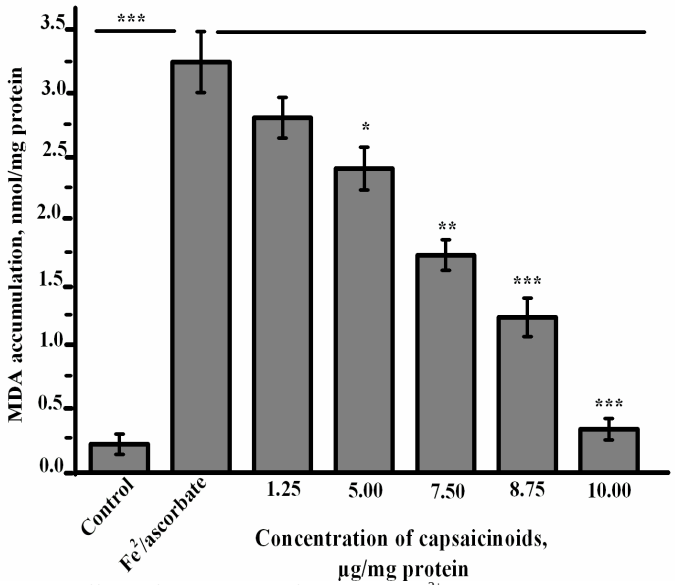


**Figure 4** Effects of capsaicinoids fractions on $Fe^{2+}$/ascorbate-dependent MDA accumulation mitochondrial membranes (* - P<0.05; ** - P<0.01; ***- P<0.001; n=5).

Capsaicinoids fraction efficiently inhibited system-induced MDA $Fe^{2+}$/ascorbate accumulation in mitochondria $IC_{50}$, equal 17.80 µg/mg protein. Thus, we found, that capsaicinoids fraction had antioxidant property and had protective effect on mitochondrial membrane, which reduces the damaging effects of $Fe^{2+}$/ascorbate.

The results indicate the antioxidative properties of capsaicinoids. By analyzing the experimental results, we assume that the fraction of capsaicinoids, as they have antioxidative properties, will prevent the formation of lipid peroxides, close

MPTP and finally stabilize the mitochondrial membrane and thus improve energy conversion function of mitochondria. Earlier studies have provided a direct evidence of how capsaicin utilizes mitochondria to cause oxidative stress leading to apoptosis in pancreatic cancer cells (**Pramanik *et al*., 2011**). But, on the other hand, normal pancreatic epithelial cells were resistant to the effects of capsaicin. Mitochondria turn out to be the target of capsaicinoids and a series of mechanisms have been reported to be in relation with the induction of apoptosis through MPTP opening. Our results demonstrate that capsaicin does not open MPTP, on the contrary it closes.

Thus we found among the fractions of red pepper *Capsicum annuum* L., only capsaicinoids fractions have antioxidant properties and have an inhibitory effect on the swelling of mitochondria. In contrast, protein fractions of fruits and seeds of red pepper do not affect the lipid peroxidation in mitochondria.

One of the known mechanisms of mitochondrial dysfunction in various pathologies is the intensification of lipid peroxidation (**Negre-Salvayre *et al.*, 2010**). Induction of lipid peroxidation in the membranes of mitochondria and oxidative stress leads to the change of their permeability, reduce membrane potential, uncouple oxidative phosphorylation (**Batandier *et al.*, 2004**).

Certain plant compounds, biological and pharmacological activities of which are due to their antioxidant properties, lower malondialdehyde (MDA) in the membranes of mitochondria (**Tarakhovsky *et al.*, 2013**). Plants of the genus *Capsicum annuum* are a rich source of capsaicinoids, having high biological activity and similar chemical structures. The effects of capsaicinoids fractions, isolated from red pepper**,** on lipid peroxidation processes of mitochondrial membranes was also measured by estimation of malondialdehyde accumulation (MDA), using $Fe^{2+}$/ascorbate system.

Since MPTP is very significant in the pathogenesis of various pathologies (cancer, ischemic hepatitis, diabetes and others (**Szewczyk, Wojtczak, 2002**)), studied effects of red pepper fractions on mitochondria are promising. Therefore biologically active compounds, isolated from red pepper, are considered as highly significant for the development of new medicinal means expected to influence on MPTP. Also the obtained results are quite important to understand the mechanisms of action of drugs on the basis of red pepper.

## CONCLUSION

In this work we showed that fractions, isolated from the seeds and fruits of red pepper *Capsicum annuum L.* plants family, had effects on the MPTP and $Fe^{2+}$/ascorbate-induced lipid peroxidation in rat liver mitochondria. Obtained results show the presence of compounds in red pepper which affect MPTP differently. Identified in our experiments red pepper seed proteins did not significantly affect MPTP. Unlike seed proteins, fruit proteins interactions with MPTP components caused MPTP to open and increased mitochondrial permeability which led to matrix swelling.

Experiments on mitochondria showed that capsaicinoids fraction has antioxidant properties preventing $Fe^{2+}$/ascorbate-induced mitochondrial swelling and inhibits the accumulation of MDA in the membranes. In contrast to the isolated peptides the capsaicinoids sum inhibits mitochondrial swelling and MPTP passes to closed conformation, causing mitochondrial membrane to be more stable.

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