Effect of Polyphenols Isolated from *Plantago major L.* and *Plantago lanceolata L.* on Mitochondrial Permeability Transition Pore in Rat Liver

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Abstract

It has been shown that the transport of electrons in the respiratory chain of mitochondria, ion-transport processes, the balance of Ca^{2+} ions and other metabolic processes in the cell are intrinsically connected with lipoperoxidation processes. In particular, in recent years, plant biologically active compounds have been widely used to eliminate oxidative processes developing in the body. Accordingly, we considered it important to study the mechanisms of action of xylopyr-T, glucopyr-T and 2-DVG polyphenols isolated from the plants *Plantago major L.* and *Plantago lanceolata L.*, belonging to the *Plantaginaceae* family, which are widely used in folk medicine. Therefore, *in vitro* experiments, the amount of malondialdehyde and the condition state of mPTP were studied by spectrophotometric method. The obtained results revealed that the studied polyphenols inhibit lipoperoxidation induced by Fe^{2+}/ascorbate the production of malondialdehyde was reduce by xylopyr-T to 81.9 ± 1.2 %, by glucopyr-T to 79.4 ± 1.7 % and by 2-DVG to 75.0 ± 1.8 % at a concentration of 4 µM in rat liver mitochondria. At the same time, mPTP opening in the medium with the substrate succinate (+ rotenone) inhibition 2-DVG polyphenol at a concentration of 100 µM was reduced by 84.5 ± 2.8 %, xylopyr-T polyphenol at a concentration of 200 µM by 67.6 ± 4.8 % and it was shown that glucopyr-T polyphenol at a concentration of 200 µM by 32.5 ± 1.65 % compared to the control. In conclusion, it can be illustrated that the studied polyphenols, along with their high antioxidant properties, show a structure-dependent inhibition of mPTP opening, which is involved in various physiological and pathological processes.

Keywords: Mitochondria, Polyphenols, Malondialdehyde, Mitochondrial permeability transition pore

Introduction

The transport of electrons in the respiratory chain, ion-transport metabolic processes, the balance of Ca^{2+} ions in mitochondria, metabolic processes in cells and other processes are inextricably linked with the processes of lipid peroxidation (LPO) and cause pathological changes in the organs and tissues of the body. Thus, LPO products are involved in the control of transcription of the endogenous antioxidant system [1]. Preadipocytes are periodically exposed to the cytotoxic action of fatty acids. Long-term continuation of this state induces Ca^{2+}-dependent cyclosporin A (CsA)-sensitive mitochondrial permeability transition pore (mPTP) of mitochondrial inner membranes, which in turn reduces mitochondrial membrane potential, oxygen consumption, and the ability to synthesize ATP [2]. Ca^{2+} ions are one of the main 2nd messengers in the cell and affect many biological processes. In regulating the amount of Ca^{2+} ions in cells, mitochondria control their saturation and release. Thus, Ca^{2+} ions perform 2 functions: First, they control ATP production and mitochondrial metabolism in vital physiological processes; second, they have been shown to control cell death, cancer development and metastasis in pathophysiological processes [3,4]. mPTP is a supramolecular structure located at the border of the inner and outer membranes of mitochondria. It has been shown that mPTP is involved in various cellular reactions, from the physiological control of mitophagy to the activation of apoptosis and necrosis, but its molecular structure remains unknown. Oxidative stress, a high concentration of inorganic phosphate ions, a low concentration of adenine

nucleotides causes mPTP. As a result, it was shown that the mitochondrial permeability transition pore opens in the mitochondrial membrane and thus the membrane becomes porous that allows any molecule to pass up to 1,500 Da [5-7]. Classical mPTP is activated under the influence of Ca^{2+} ions in the presence of inorganic phosphate and the substrate of the respiratory chain, as a result, reactive oxygen species (ROS) are formed, the membrane depolarizes, mitochondria swell, Ca^{2+} ions are released from the matrix, and its sensitivity to CsA increases. This process has been shown to be inhibited by the antioxidants [8]. Likewise, recent evidence has highlighted that of mPTP opening can be a pharmacological target in the treatment of various diseases, and it is important to identify new and useful mPTP modulators [9].

Therefore, we studied the effect of polyphenols isolated from plants - *Plantago major L* and *Plantago lanceolata L* - hexahydroxydiphenol-*l*-(*o*-2-*o*-galloyl-*β-D*-glucopyranoside)-*l*-(*o*-*β-D*-xylopyranoside) diester (xylopyr-T), hexahydroxydiphenoyl-*l*-(*o*-*β-D*-glucopyranoside)-2-(*o*-4-*D*-galloyl-*β-D*-glucopyranoside) diester (glucopyr-T) and 2-*o*-bis-digalloyl-4,6-valoneyl-*β-D*-glucose (2-DVG) on Fe^{2+}/ascorbate-induced malondialdehyde formation and Ca^{2+}-dependent CsA-sensitive mPTP in rat liver mitochondria. Structural formulas of the studied hydrolysable tannins are shown in **Figure 1**.

Materials and methods

Materials

The data of the studied polyphenols on isolation and identification from plants *Plantago major L* and *Plantago lanceolata L* are presented in the literature by [10]. These polyphenols are 90 - 95 % pure, and they were kindly represented by Makhmudov *et al.* from the Institute of Bioorganic Chemistry of the Academy of Sciences of the Republic of Uzbekistan. Sucrose, succinate, EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), HEPES (N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)), calcium chloride, rotenone, KH_2PO_4 and potassium chloride were purchased from Sigma-Aldrich (USA). Cyclosporine A and EDTA (ethylenediaminetetraacetic acid tetrasodium salt dihydrate) were purchased from Wako (Japan). Other drugs were purchased from Chembiogen (Uzbekistan).
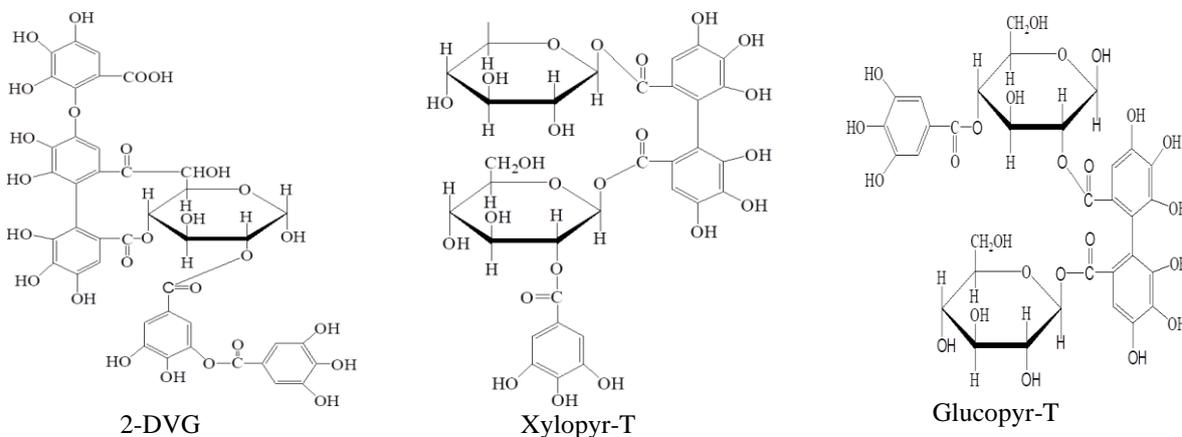

Figure 1 Structural formulas of the studied hydrolyzable tannins.

Liver mitochondria isolation

Preparation of liver mitochondria was performed as previously described from albino Wistar rats in medium containing 250 mmol sucrose, 10 mmol tris-chloride, 1 mmol EDTA, pH 7.4. Nuclei and fragments of cells were precipitated at 600× g for 10 min at 0 ± 2 °C in a RS-6 centrifuge with an angle-type rotor. Mitochondria were obtained from the supernatant by centrifugation at 5,000× g for 20 min [11]. The obtained mitochondria were suspended in the isolation medium without EDTA in a ratio of 10:1. During the experiment, the mitochondrial suspension was kept in an ice bath. The protein concentration of prepared mitochondrial suspension was examined by the Biuret method using bovine serum albumin as a standard [12]. Animal studies were carried out in accordance with the bioethical regulations developed by the Institute of Biophysics and Biochemistry at the National University of Uzbekistan.

Determination of malondialdehyde

In addition, liver mitochondria for purification from sucrose residues were recentrifuged in potassium chloride isolation medium to quantify malondialdehyde (MDA). The composition of the isolation medium

was 175 mmol KCl, 25 mmol tris - HCl, pH 7.4 [13]. Liver mitochondria were diluted in large quantities in this medium, and the isolation was recentrifuged at $5,000\times$ g for 20 min at 0 ± 2 °C. Mitochondria were suspended in isolation medium at a ratio of 10:1 and stored in an ice bath. LPO was induced in mitochondria by adding 10 µmol $FeSO_4$ and 200 µmol ascorbate to the incubation medium. In this case, the formation of MDA reacts with thiobarbituric acid to form a trimetine complex containing 1 MDA molecule and 2 thiobarbituric acid molecules, and this complex gives high optical absorption at a wavelength of 532 nm. Its molar extinction coefficient is $\varepsilon = 1.56\times10^5$ cm^{-1} M^{-1}. The composition of the incubation medium for LPO was as follows: KCl - 125 mmol, tris-HCl - 10 mmol, pH 7.4. Mitochondria (4 mg/mL protein) were incubated with Fe^{2+}/ascorbate for 15 min at 37 °C. At the end of the incubation, 220 µmol 70 % trichloroacetic acid was added to stop the biochemical process and precipitate proteins. After that, the sample was centrifuged at $3,000\times$ g for 15 min to completely precipitate the proteins in the sample. Two milliliter of the supernatant was taken, 1 mL of a 0.8 % solution of thiobarbituric acid was added, and the samples were boiled for 15 min until a trimetine complex was formed. Then, the optical density of the sample was measured on a spectrophotometer at a wavelength of 532 nm.

Mitochondrial permeability transition pore analysis

mPTP was assessed by the rate of Ca^{2+}-dependent mitochondrial swelling by recording the light scattering of the mitochondrial suspension at 540 nm [14]. The incubation medium was as follows: Sucrose - 200 mmol, KH_2PO_4 - 1 mmol, succinate - 5 mmol, EGTA - 20 µmol, HEPES - 20 mmol, tris-HCl - 20 mmol, rotenone - 2 µmol, pH - 7.2, and mitochondrial protein 0.3 - 0.4 mg/mL.

Statistical analysis

All values were expressed as mean ± standard error mean (SEM) calculated using the Origin 6.1 (OriginLab, USA). Differences between groups were assessed using the t-Test (Two Populations) system. Differences were regarded as significant if $p < 0.05$ was adapted.

Results and discussion

It is known that under the influence of the Fe^{2+}/ascorbate system, mitochondria swell and increase in size. In various diseases, the lability of mitochondrial membranes increases, the membrane potential of mitochondria decreases, complex I of the respiratory chain is inhibited, and at the same time, complex II is hyperactivated due to partial compensation. It has been shown that this complex state of imbalance is one of the factors leading to the formation of ROS in the mitochondrial matrix and simultaneously in the cell cytosol, which reduces glutathione stores and enhances the LPO process [15]. The death of BRAFV600E melanoma cells induced by BAY 87-2243 led to the opening of mPTP, depolarization of the mitochondrial membrane potential, an increase in ROS, an increase in the LPO process and a decrease in glutathione due to the inhibition of complex I of the mitochondrial respiratory chain [16].

For the immediate results, in experiments *in vitro* xylopyr-T polyphenol isolated from *Plantago major L.* had an inhibitory effect on the process of MDA formation induced by Fe^{2+}/ascorbate under the influence of different concentrations of xylopyr-T (**Figure 2(A)**). It was found that 1 µmol concentrations of polyphenol inhibit the formation of MDA in rat liver mitochondria induced by Fe^{2+}/ascorbate by 13.9 ± 3.1 % compared with the control. Additionally, 2 and 4 µmol concentrations of polyphenol inhibited the formation of MDA in mitochondria stimulated with Fe^{2+}/ascorbate by 58.8 ± 3.9 and 81.9 ± 1.2 %, respectively, compared with the control. Therefore, this polyphenol exhibits high antioxidant properties at low micromolar concentrations.
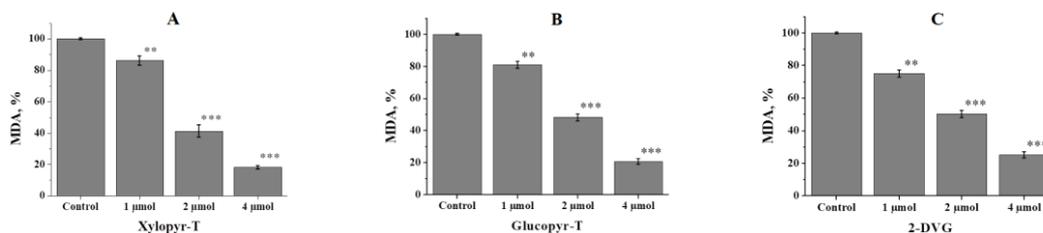

Figure 2 Effect of xylopyr-T (A), glucopyr-T (B), and 2-DVG (C) on LPO in mitochondria induced by Fe^{2+}/ascorbate. The y-axis shows the inhibition of MDA formation in mitochondria under the influence of Fe^{2+}/ascorbate, and the abscissa shows the applied concentrations of polyphenols ($**$ - $p < 0.01$, $***$ - $p < 0.001$; n = 5).

In the following experiments, we studied the effect of various concentrations of the polyphenol glucopyr-T on the LPO process induced by Fe^{2+}/ascorbate (**Figure 2(B)**). From the results obtained, it was found that glucopyr-T at a concentration of 1 µmol reduced the formation of MDA in rat liver mitochondria under the influence of Fe^{2+}/ascorbate by 19.0 ± 2.2 % compared with the control. It was found that the formation of MDA in liver mitochondria was inhibited by 51.8 ± 2.1 % compared with the control under the influence of this polyphenol at a concentration of 2 µmol. Additionally, under the influence of 4 µmol polyphenol, it was found that the formation of MDA in the mitochondria of the liver of rats was inhibited by 79.4 ± 1.7 % compared with the control. It can be seen that this polyphenol, like xylopyr-T polyphenol, has high antioxidant properties.

In experiments, we studied the effect of polyphenol 2-DVG on the LPO process induced by inducers of the Fe^{2+}/ascorbate system in rat liver mitochondria (**Figure 2(C)**). The results obtained showed that 2-DVG at 1 µmol inhibited LPO induced by Fe^{2+}/ascorbate by 25.0 ± 2.8 % compared with the control and at a concentration of 2 µmol polyphenol by 50.0 ± 2.1 % compared to control. This polyphenol at 4 µmol inhibited Fe^{2+}/ascorbate-induced MDA formation by 75.0 ± 1.8 % in mitochondria and exhibited high antioxidant properties. The results obtained show that all 3 polyphenolic compounds exhibit high antioxidant properties and prevent LPO in rat liver mitochondria *in vitro*.

According to the literature data, polyphenols, especially condensable and hydrolysable tannins, exhibit higher antioxidant properties than flavonoids [17-19].

Succinate-induced membrane potential-dependent electron transport sensitizes mitochondria to mPTP opening. Succinate inhibits electron transport as a result of mPTP-induced membrane potential depolarization. Respiration controlled by complex I slows down with the opening of mPTP, and its activity is maintained in the presence of substrates related to complex II, which is consistent with the inhibition of respiration provided by complex I due to leakage of NADH (nicotinamide adenine dinucleotide reduced) from the mitochondrial matrix. ROS formed in complex III of the respiratory chain do not increase the sensitivity of mitochondria to mPTP opening. Thus, it has been shown that the metabolic flow and the metabolic environment of the cell provide a functional response of mitochondria to excessive accumulation of Ca^{2+} ions in mitochondria [20]. It can be seen that substrates of the mitochondrial respiratory chain play an important role in the functional state of cells. We have previously examined various respiratory chain substrates for mPTP, in particular pyruvate-malate [21] and glutamate-malate [22], and in this study, we examined the succinate-dependent state of mPTP. $CaCl_2$ (10 - 50 µmol) was used as a mitochondrial mPTP inducer.

The chemical modification of biologically active compounds isolated from plants affects their membrane-active properties. Therefore, we investigated the effect of the polyphenols 2-DVG, glucopyr T and xylopyr T on mPTP. First, we studied the effect of the polyphenol 2-DVG at 25, 50, 75 and 100 µmol concentrations on mPTP in the presence of the FAD (flavin adenine dinucleotide) -dependent respiratory chain substrate succinate (5 mmol) and rotenone (2 µmol) (**Figures 3(A)** and **3(A')**). As seen from the results obtained, when rat liver mitochondria were added to the incubation medium without the presence of Ca^{2+} ions and without exposure to biologically active compounds, there was no reaction. In further studies, the addition of 10 - 50 µmol Ca^{2+} ions to the incubation medium caused strong mitochondrial swelling. This situation causes rupture of the outer mitochondrial membrane and release of proapoptotic proteins and cytochrome c from the intermembrane space into the environment [23,24]. This, in turn, leads to cell death. We then continued our studies by adding 5 µmol of CsA, a specific inhibitor of mPTP, to the incubation medium to confirm that our study subject was indeed an mPTP, and this showed that our study subject was indeed an mPTP. Continuing the study of mPTP, it was found that under the influence of 25 µmol polyphenol, 2-DVG inhibited Ca^{2+}-dependent mPTP by 10.3 ± 2.6 % compared with the control. Additionally, under the influence of 50 µmol polyphenol, calcium ion-induced swelling of mitochondria was inhibited by 34.3 ± 4.1 % compared with the control. Under the influence of 75 µmol 2-DVG, mPTP opening was inhibited by 72.4 ± 4.1 % compared with the control. The influence of 100 µmol polyphenol on mPTP, was inhibited by 84.5 ± 2.8 % compared with the control. As seen from the above results, it was found that the polyphenol 2-DVG inhibits mPTP, showing high antioxidant properties. At the same time, the half-maximal inhibitory concentration of polyphenol 2-DVG on rat liver mPTP was $IC_{50} = 60.0 \pm 3.5$ µmol.

In the following experiments, we investigated the effect of xylopyr-T polyphenol on rat liver mPTP at 50, 100, 150 and 200 µmol concentrations as a substrate of succinate (5 mmol) with rotenone (2 µmol) (**Figures 3(B)** and **3(B')**). Initially, the state of liver mPTP was studied with a Ca^{2+} inducer (10 - 50 µmol) and CsA blocker (5 µmol), as in experiments with 2-DVG. Although the antioxidant properties of xylopyr-T polyphenol were close to those of 2-DVG polyphenol, it showed an inhibitory effect on rat liver mPTP compared to 2-DVG polyphenol at higher concentrations. It was found that under the influence of 50 µmol

xylopyr-T polyphenol on mPTP under the influence of Ca^{2+} ions, high-amplitude opening was inhibited by 18.4 ± 2.5 % compared with the control. Under the influence of 100 µmol polyphenol, mPTP was inhibited by 41.3 ± 6.0 % compared with the control. It was also found that under the influence of a 150 µmol polyphenol on mPTP, it was inhibited by 46.9 ± 7.1 % compared with the control. Finally, when exposed to a concentration of 200 µmol polyphenol, mPTP was inhibited by 67.6 ± 4.8 % compared to the control. The results obtained show that xylopyr-T polyphenol has a milder inhibitory effect on rat liver mPTP compared to higher concentrations of 2-DVG polyphenol, leading to stabilization of the mitochondrial membrane in a stress state caused by Ca^{2+} ions. The half-maximal inhibitory concentration of this polyphenol on rat liver mPTP was $IC_{50} = 167.6 \pm 5.2$ µmol.

We also investigated the effect of 50, 100, 150 and 200 µmol concentrations of the polyphenol glucopyr-T in the incubation medium with succinate (+ rotenone), which is considered a respiratory chain substrate, on the CsA-sensitive Ca^{2+}-dependent rat liver mPTP (**Figure 3(C)** and **3(C')**). As seen from the results obtained, the polyphenol glucopyr-T at a concentration of 50 µmol inhibited the swelling of rat liver mitochondria induced by 10 - 50 µmol Ca^{2+} by 6.1 ± 1.68 % compared with the control. A higher 100 µmol concentration of this polyphenolic compound inhibited mPTP by 13.2 ± 1.28 % compared to the control. It was found that 150 and 200 µmol concentrations of the polyphenol glucopyr-T inhibited the swelling of rat liver mitochondria by 22.6 ± 2.5 and 32.5 ± 1.65 %, respectively, compared with the control. The polyphenol glucopyr-T has a weak inhibitory effect on rat liver mPTP, despite its high antioxidant effect compared to the 2 polyphenols studied above. With a weak inhibitory effect of this polyphenol on mPTP, it was not possible to determine the half-maximal inhibitory concentration.
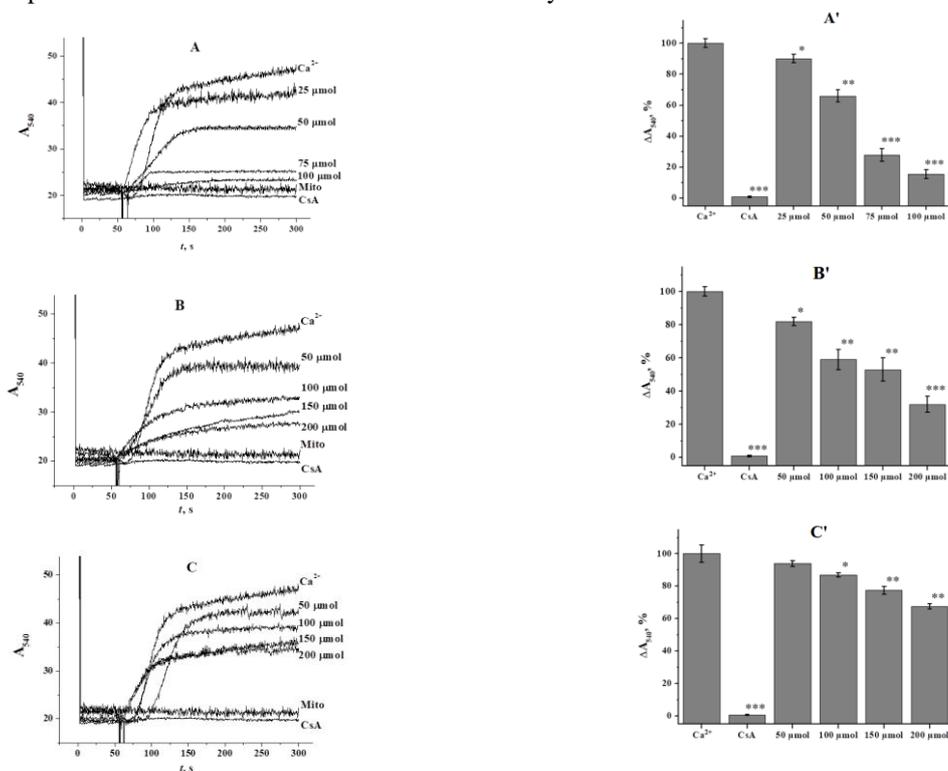

Figure 3 Effect of polyphenols on Ca^{2+}-dependent mPTP in rat liver. Ca^{2+}-dependent high-amplitude mitochondrial swelling, and complete inhibition of mPTP with CsA (5 µM). And also, a study of the inhibitory effect of polyphenols with various concentrations - 2-DVG (A), xylopyr-T (B) and glucopyr-T (C), and after statistical treatment - 2-DVG (A'), xylopyr-T (B') and glucopyr-T (C') (* - p < 0,05, ** - p < 0.01, *** - p < 0.001; n = 5).

According to the results of the studied polyphenols, the polyphenols 2-DVG, xylopyr-T and glucopyr-T have a specific effect on the CsA-sensitive Ca^{2+}-dependent mPTP of the rat liver in the FAD-dependent respiratory substrate of succinate (with rotenone). At the same time, polyphenol 2-DVG containing a valoneyl group inhibited CsA-sensitive Ca^{2+}-dependent mPTP in rat liver at a concentration of up to 100 µmol, and xylopyr-T polyphenol inhibited CsA-sensitive Ca^{2+}-dependent mPTP in rat liver at a concentration of up to 200 µmol, but the polyphenol glucopyr-T at concentrations up to 200 µmol showed

very weak inhibition of CsA-sensitive Ca^{2+}-dependent mPTP in the rat liver compared to the above polyphenols.

At the same time, in experiments with pyruvate + malate respiratory chain substrates, xylopyr-T and glucopyr-T polyphenols showed a stronger inhibitory effect on rat liver mPTP, and for glucopyr-T it was $IC_{50} = 77.6 \pm 4.8$ µmol [21], in experiments conducted with glutamate + malate, the half-maximal inhibitory concentration of xylopyr-T was $IC_{50} = 101.7 \pm 3.1$ µmol, and the half-maximal inhibitory concentration of glucopyr-T was $IC_{50} = 42.2 \pm 3.8$ µmol [22]. In experiments conducted with the respiratory chain substrates glutamate + malate, the polyphenol glucopyr-T did not show an inhibitory effect on mPTP at concentrations above 50 µmol, and in the succinate substrate (with rotenone), the polyphenol glucopyr-T showed a weak inhibitory effect up to 200 µmol concentration. The studied polyphenols have a substrate-specific effect on mPTP. It was also found that tannins containing hexahydroxydiphenol and valoneyl groups associated with glucose in their molecule have a high inhibitory effect on the activity of poly(ADP-ribose)glycohydrolase. Additionally, the number of monomeric residues in the polyphenol determines how active the hydrolysable tannin is [25].

Conclusions

The results obtained indicate that the studied polyphenolic compounds inhibit the formation of MDA in rat liver mitochondria and exhibit high antioxidant properties, as well as show a structure-dependent inhibition rat liver mPTP opening, exhibiting membrane-active properties and made up the following series, 2-DVG > xylopyr-T > glucopyr-T with a FAD-dependent substrate (succinate + rotenone). Their activity may be associated with ROS inactivation under oxidative stress.

Acknowledgements

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