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Effect of Flavonoids on PTP and Dehydrogenase Activity in Liver Mitochondria

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The inner mitochondrial membrane contains various ion channels and protein complexes that participate in cellular bioenergetics by synthesizing ATP. In addition, these structures are involved in cellular signaling through the generation of reactive oxygen species, thereby contributing to the regulation of physiological processes and the development of pathological conditions. Thus, mitochondria play a crucial role in the functioning of cells and tissues. Therefore, in in vitro studies, we investigated the effects of various flavonoids on the mPTP of rat liver mitochondria, as well as on the activities of succinate–ferricyanide reductase (succinate dehydrogenase) and NADH–ferricyanide reductase (NADH dehydrogenase). The results demonstrated that flavonoids exert an inhibitory effect on the mPTP of rat liver mitochondria, forming the following order based on their half-maximal inhibitory concentrations: apigenin > quercetin > isorhamnetin > oroxylin A > pulicarin > chrysoeriol > kaempferol > kaempferol-7-O-rhamnoside > hyperoside. Moreover, pre-incubation of liver mitochondria with 50 μM NADH for 10 minutes enhanced the inhibitory effect of flavonoids on the mPTP. Under these conditions, apigenin, oroxylin A, quercetin, kaempferol, chrysoeriol, hyperoside, and pulicarin exhibited strong inhibitory effects, whereas the remaining flavonoids showed weaker inhibition. In addition, at concentrations of 10 and 50 μM, the flavonoids hyperoside, kaempferol, kaempferol-7-O-rhamnoside, isorhamnetin, quercetin, afzelin, and pulicarin increased succinate–ferricyanide reductase activity, while the other flavonoids exhibited very weak or no effect. At the same time, a strong inhibitory effect of quercetin, isorhamnetin, kaempferol-7-O-rhamnoside, hyperoside, and kaempferol on NADH–ferricyanide reductase activity was observed, whereas the remaining studied flavonoids demonstrated only weak inhibitory effects. Overall, the results indicate that the flavonoids investigated in vitro possess distinct and compound-specific properties.

Keywords: Afzelin; Apigenin; Chrysoeriol; Hyperoside; Isorhamnetin; Kaempferitrin; Kaempferol; Kaempferol-7-O-rhamnoside; Liver mitochondria; Mitochondrial permeability transition pore; NADH dehydrogenase; Oroxylin; Pulicarin; Quercetin; Succinate dehydrogenase.

Various types of ion channels are located in the inner membrane of mitochondria. Among them, the mitochondrial permeability transition pore (mPTP), situated between the outer and inner mitochondrial membranes, plays a role in both physiological and pathological processes.[1] The characteristics of the mPTP were first described by R.A. Haworth and D.R. Hunter in 1979.[2] In mitochondria, excessive accumulation of Ca²z ions leads to a pathological increase in reactive oxygen species (ROS).[3-5] Naturally, excessive accumulation of Ca²z ions in mitochondria induces the opening of the mPTP. This allows the free passage of small molecules with a molecular weight of up to 1,500 Daltons into the mitochondrial matrix.[6,7] As a result, this leads to depolarization of the mitochondrial membrane and a decrease in ATP production, ultimately triggering cell death.[7] Moreover, the mPTP regulates the efflux of Ca²z ions from mitochondria and participates in Ca²z signaling during normal cellular function. The mPTP is a complex structure, and its composition has been reported to include cyclophilin D (CypD), adenine nucleotide translocase (ANT), hexokinase-2 (HK2), voltage-dependent anion channel (VDAC), benzodiazepine receptor (BzR), phosphate carrier (Pi), and glycogen synthase kinase-3â (GSK-3â).[8] However, recent studies suggest that F€ F -ATP synthase may also be a component of the mPTP.[6] However, debates regarding the structure of the mPTP are still ongoing. In mitochondria, CypD, considered a regulatory protein of the mPTP, promotes its opening, whereas cyclosporin A (CsA), a specific inhibitor of the mPTP, interacts with CypD to maintain the pore in a closed state.[9,10]

Ca²z ion homeostasis in mitochondria is considered one of the key factors regulating oxidative metabolism and cell death.[3] MPTP-related cell death occurs as a result of the pore transitioning to an open state.[4] The redox couple NADz (â-nicotinamide adenine dinucleotide)/ NADH (reduced â-nicotinamide adenine dinucleotide) also participates in the regulation of Ca²z ion homeostasis in mitochondria. A decrease in the NADz /NADH ratio is considered a condition associated with mitochondrial dysfunction.[11] In diabetic cardiomyopathy, a decrease in the NADz /NADH ratio and an increase in the acetyl-CoA/ CoA ratio have been reported. NADz serves as a co-substrate for deacetylases and sirtuins, playing a crucial role in redox signaling. Acetyl-CoA acetylates lysine residues in proteins, leading to the disruption of mitochondrial integrity.[12] Pyridine nucleotides NADH and NADPH play a crucial role in mitochondrial energy production. The intracellular NADz /NADH ratio regulates the rate of ATP synthesis by controlling NADH-dependent dehydrogenases and modulates post-translational protein modifications through the activation of NADz -dependent enzymes.[13] Furthermore, the intracellular NADz /NADH ratio has been shown to maintain redox balance and antioxidant activity. It has been demonstrated that the NADz /NADH ratio in the cytosol and mitochondria responds differently to acute metabolic disturbances.[14] It has been shown that inhibition of soluble adenylyl cyclase (sAC) leads to an increase in the extracellular lactate/pyruvate ratio and the cytosolic NADH/NADz ratio. Additionally, sAC inhibition has been found to specifically suppress complex I of the mitochondrial respiratory chain. This effect supports ATP production via glycolysis and oxidative phosphorylation, thereby maintaining cellular energetic homeostasis.[15] In chronic obstructive pulmonary inflammation in rats, administration of NADH has been shown to provide protection against disease development and to stabilize the antioxidant defense system of the lungs.[16]

Mitochondrial complex I (NADH:ubiquinone oxidoreductase, NADH dehydrogenase) is the largest multimeric enzyme complex of the mitochondrial respiratory chain, responsible for electron transport and for establishing a proton gradient across the inner mitochondrial membrane to drive ATP synthesis.[17,18] Mitochondrial complex I exists in a supercomplex with complexes III and IV of the respiratory chain and is considered a major source of deleterious ROS.[18] It is well established that mitochondrial complex I serves as a key entry point for electrons into oxidative metabolism within the respiratory chain. Consequently, various mutations in nuclear and mitochondrial genomes can lead to NADH dehydrogenase dysfunction, which contributes to the development of diverse pathologies.[19,20] Specifically, alterations or the absence of the NUIM subunit (NADH dehydrogenase [ubiquinone] iron-

sulfur protein 8) of mitochondrial complex I lead to a reduction in enzyme activity and an increase in ROS production.[21]

Furthermore, regarding complex II of the respiratory chain – succinate dehydrogenase (SDH), this enzyme is unique in that it participates both in electron transport within the respiratory chain and in the tricarboxylic acid (TCA) cycle.[22,23] Recent findings indicate that SDH functions as a tumor suppressor in carcinogenesis, whereas succinate is regarded as an oncometabolite.[23] Dysfunction of the SDH enzyme has been shown to contribute to the development of both cancer and neurodegenerative diseases.[24] During ischemia, the accumulation of succinate occurs as a result of the reverse activity of the SDH enzyme. Additionally, this process is influenced by the breakdown of purine nucleotides and the partial reverse operation of the malate/aspartate cycle, which correlates with the amount of fumarate. Upon reperfusion, the accumulated succinate is rapidly oxidized by SDH, leading to the overproduction of ROS through reverse electron transport at mitochondrial complex I.[25] In *in vivo* studies, 30 minutes of renal ischemia-reperfusion has been shown to induce mitochondrial dysfunction, and prolonging the ischemic period exacerbates this process. Following ischemia-reperfusion, the accumulation of succinate in the cytosol leads to an increase in H, O, production, which is dependent on mitochondrial complex II. Changes in LDH activity during ischemia-reperfusion indicate the development of cell necrosis.[26] During cardiac ischemia/reperfusion, reverse electron transport (RET)-dependent ROS production at mitochondrial complex I, driven by succinate, has been proposed to induce the opening of the mPTP, thereby contributing to cardiac injury. It has been demonstrated that in rabbit heart mitochondria, the opening of the mPTP, induced by Ca²z or alamethicin, leads to static ROS generation at complex II. Simultaneously, ischemia inhibits mitochondrial complexes II and III, resulting in an accumulation of succinate and fumarate within the mitochondrial matrix and promoting their efflux. Upon reperfusion, the activation of static ROS production facilitates the transition of the mPTP to the open state. Notably, this process has been reported to occur following ROS generation via reverse electron transport in the mitochondrial respiratory chain.[27] ROS generated in mitochondria play a critical role in cellular signaling. However, pathological conditions such as ischemia-reperfusion injury disrupt ROS homeostasis, leading to cell death.[28]

Thus, it has been emphasized that, in the study of various pathological processes developing in the organism, mitochondrial respiratory chain complexes I, II, and IV, ATP-sensitive potassium channel activity (mitoK_{ATP}), adenine nucleotide translocase (ANT), and the mPTP may serve as pharmacological targets for various drugs and biologically active compounds.[29] Therefore, in the present study, the effects of flavonoids on Ca^{2+}–induced mitochondrial swelling (mPTP opening), as well as on the activities of succinate–ferricyanide reductase (succinate dehydrogenase) and NADH–ferricyanide reductase (NADH dehydrogenase), were investigated.

MATERIALS AND METHODS

Chemicals

Kaempferol, kaempferol-7-O-rhamnoside, kaempferitrin and afzelin, belonging to the group of flavonoids, were isolated from *Geranium rotundifolium.*[30] The purity of these compounds ranged between 86–90%. Other flavonoids and all reagents which are used in the experiments purchased by Sigma Aldrich company.

Isolation of liver mitochondria. Liver mitochondria were isolated by differential centrifugation.[31,32] The isolation medium consisted of 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4). After decapitation, the rat's abdominal cavity was opened, and the liver was excised and placed in a beaker containing ice-cold isolation medium. The liver mass was measured, then mechanically homogenized. The tissue was further homogenized in a Teflon homogenizer at a 1:6 g/mL ratio with the isolation medium. The homogenate was subjected to the first centrifugation at 1500 rotation/min (400 × g) for 7 min at 0-2°C using an RS-6MC centrifuge with an angled rotor. This step removed large tissue fragments and cell debris. The resulting supernatant was centrifuged again at 6,000 rpm (5000 × g) for 15 min at 0-2°C. The mitochondria, which had settled at the bottom of the tube, were separated from the supernatant. Any remaining liquid and lipid droplets on the tube walls were removed using filter paper. The purified

mitochondria were resuspended in isolation medium without EDTA at a 10:1 g/mL dilution to prepare a mitochondrial suspension, which was stored in a special ice-cooled container for experiments. Protein concentration in mitochondria was determined using the Biuret method.[33]

Determination of mPTP. The state of the Ca^{2+}-dependent CsA-sensitive mPTP was assessed spectrophotometrically at 540 nm by monitoring changes in mitochondrial swelling kinetics.[34] The incubation medium contained 200 mM sucrose, 20 ìM EGTA, 20 mM Tris, 20 mM HEPES, 1 mM KH_2PO_4, 5 mM succinate, and 2 ìM rotenone (pH 7.4). Mitochondrial protein concentration was 0.3-0.4 mg/mL, and the temperature was maintained at 26°C.

Measurement of succinate-ferricyanide-reductase (succinate dehydrogenase) activity. Succinate ferricyanide-reductase activity in rat liver mitochondria was studied using a spectrophotometric method based on the change in optical density at 420 nm.[35] The enzyme activity was determined by measuring the reduction of $K_3[Fe(CN)_6]$ over a 5 min period at 37°C. The composition of the incubation medium used for determining enzyme activity included: 100 mM phosphate buffer, 1 mM sodium azide, 100 ìM $K_3[Fe(CN)_6]$, and 5 mM succinate (pH 7.8). The total volume of the incubation medium was 3 ml.

Measurement of NADH-ferricyanide-reductase (NADH dehydrogenase) activity. The activity of the mitochondrial NADH-ferricyanide-reductase enzyme was determined spectrophotometrically by recording the change in optical density at 420 nm.[36] This change occurred during the reduction of $K_3[Fe(CN)_6]$ at 25/ °C over 5 min. The incubation medium composition (mM) was as follows: tris-HCl – 100, NaN_3 – 1, $K_3[Fe(CN)_6]$ – 0.1–0.2, NADH – 0.27, with a total volume of 3 ml

Animal ethics. The Animal Use Committee of our institution approved all experimental procedures and preoperative care guidelines. Animals were housed in standard vivarium conditions (humidity: 55-65%, temperature: 22 ± 2°C) with free access to drinking water and laboratory food. All manipulations with animals were carried out in accordance with the European Convention for the Protection of Animals Used for Scientific Purposes (1998) and the International Bioethical Guidelines of the Institute of Biophysics and Biochemistry of the National University of Uzbekistan (BEC/IBB-N44/2024/14-1). All operations were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Albino Wistar rats weighing 180-220g were used in the experiments.

Statistics. Statistical analysis and graphical illustrations were performed using OriginPro 8.5 (USA). Data were obtained from 5 to 8 independent experiments (n = 5-8). A paired t-test was used for analyzing combined data, while an unpaired t-test was applied for comparisons between individual

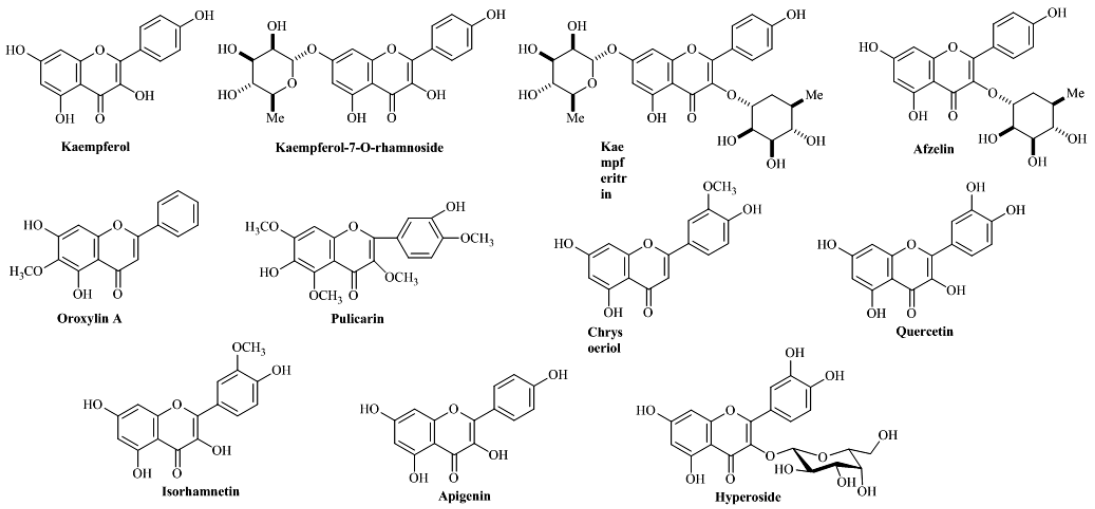

Fig. 1. Structural formulas of the studied flavonoids

groups. Statistical significance was set at levels: $p< 0.05$.

RESULTS

Initially, the study focused on the state of mPTP located in the inner membranes of rat liver mitochondria. In this setup, the response of mitochondria to Ca^{2+} ions (10 ìM) in the absence of any biologically active substances was assessed, and no reaction was observed (data not shown). Continuing the experiments, when the mPTP inducer, Ca^{2+} ions (10 ìM),[27] was added to the incubation medium together with

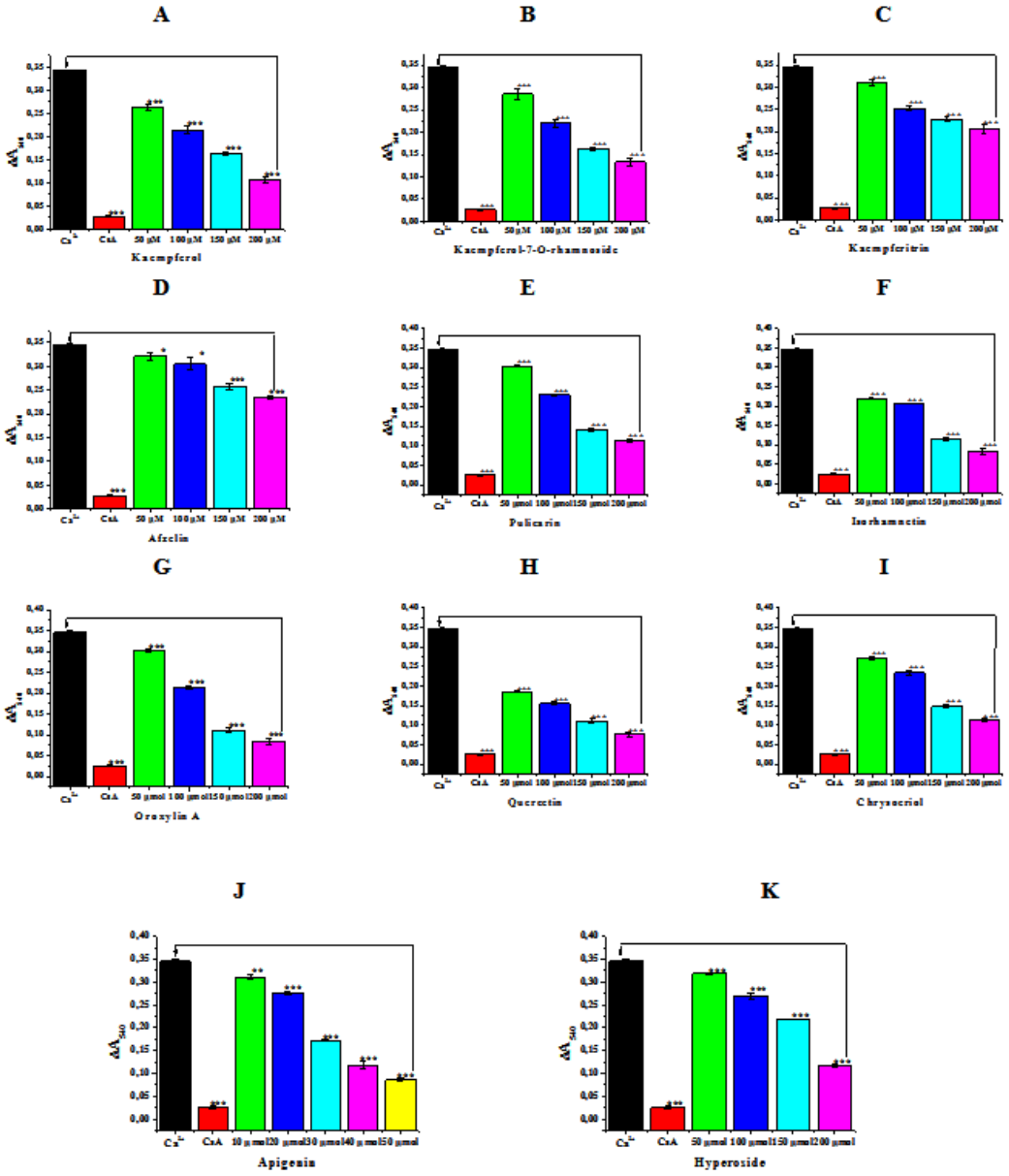

Fig. 2. Effect of flavonoids on mPTP in rat liver. Ca^{2+}-dependent high-amplitude mitochondrial swelling, and complete inhibition of mPTP with CsA (5 ìM). And also, a study of the inhibitory effect of flvonoids and polyphenols with various concentrations - kaempferol (A), kaempferol-7-Î-rhamnoside (B), kaempferitrin (C) and afzelin (D), Pulicarin (E), Isorhamnetin (F), Oroxylin À (G), Quercetin (H), Chrysoeriol (I), Apigenin (J), Hyperoside (K) (* -p < 0,05, ** -p < 0.01, *** -p < 0.001; n = 8).

liver mitochondria, mitochondria exhibited high-amplitude swelling (Fig. 2).

To confirm that the object under investigation was indeed mPTP, CsA, 5 ìM, a specific inhibitor of the mPTP modulator CypD,[37] was applied, which prevented 95–100% of the high-amplitude Ca^{2+}-induced mitochondrial swelling (Fig. 2). These results clearly indicate that the object studied in rat liver mitochondria is mPTP.

Subsequently, the effects of flavonoids isolated from plants on mPTP were investigated. The influence of different concentrations of the tested flavonoids on mPTP in rat liver mitochondria was studied in the presence of the mPTP inducer, Ca^{2+} ions (10 ìM). Initially, the effects of kaempferol at concentrations of 50, 100, 150, and 200 ìM on mPTP were examined (Fig. 2, A). At a concentration of 50 ìM, kaempferol inhibited mPTP by 23.8±0.52% relative to the control. At 100 ìM, it suppressed Ca^{2+}-induced mitochondrial swelling by 37.6±1.49% compared to the control. At 150 ìM, kaempferol inhibited the transition of mitochondria to a high-amplitude open conformational state by 52.5±1.32%, and at 200 ìM, it inhibited the opening of mPTP by 69.8±3.5% relative to the control. The half-maximal inhibitory concentration (IC_{50}) of kaempferol was determined to be 138.6±4.91 ìM (Fig. 3, A).

These findings indicate that, despite the high antioxidant potential of kaempferol,[38] complete inhibition of mPTP was not achieved

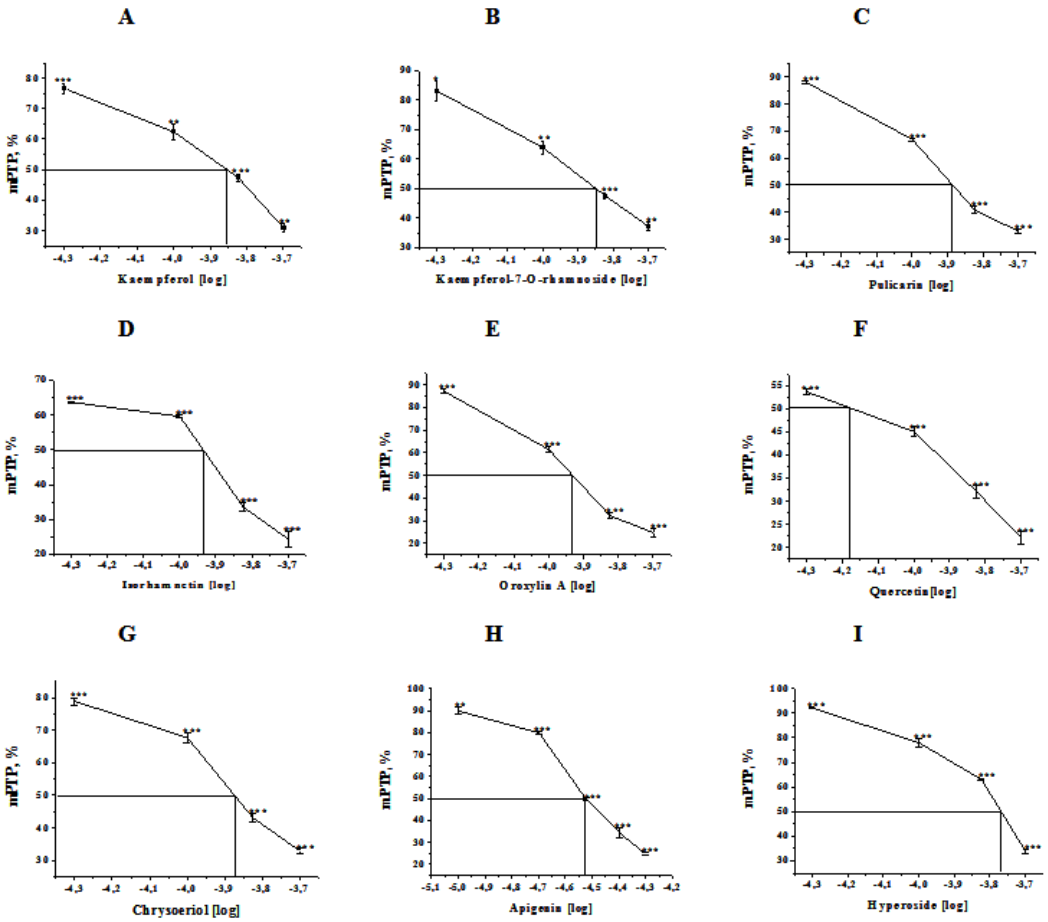

Fig. 3. The half-maximal inhibitory concentration of flavonoids kaempferol (A), kaempferol-7-Î-rhamnoside (B), Pulicarin (C), Isorhamnetin (D), Luteolin (E), Oroxylin À (F), Quercetin (G), Cynaroside (H), Chrysoeriol (I), Apigenin (J), Hyperoside (K) on mPTP in rat liver mitochondria (* -p < 0,05, ** -p < 0.01, *** -p < 0.001; n = 8)

at the concentrations tested. The effects of kaempferol glycosides on mPTP opening induced by Ca^{2+} ions were also investigated. Kaempferol-7-O-rhamnoside, a glycoside of kaempferol, exhibited concentration-dependent inhibition of Ca^{2+}-induced mPTP opening (Fig. 2, B). At a concentration of 50 ìM, this flavonoid inhibited high-amplitude mitochondrial swelling induced by Ca^{2+} by 16.8±0.68% relative to the control. At concentrations of 100, 150, and 200 ìM, kaempferol-7-O-rhamnoside suppressed mPTP transition to the open conformational state by 36.0±1.25%, 51.8±1.07%, and 59.2±3.66%, respectively. The IC_{50} of this kaempferol glycoside

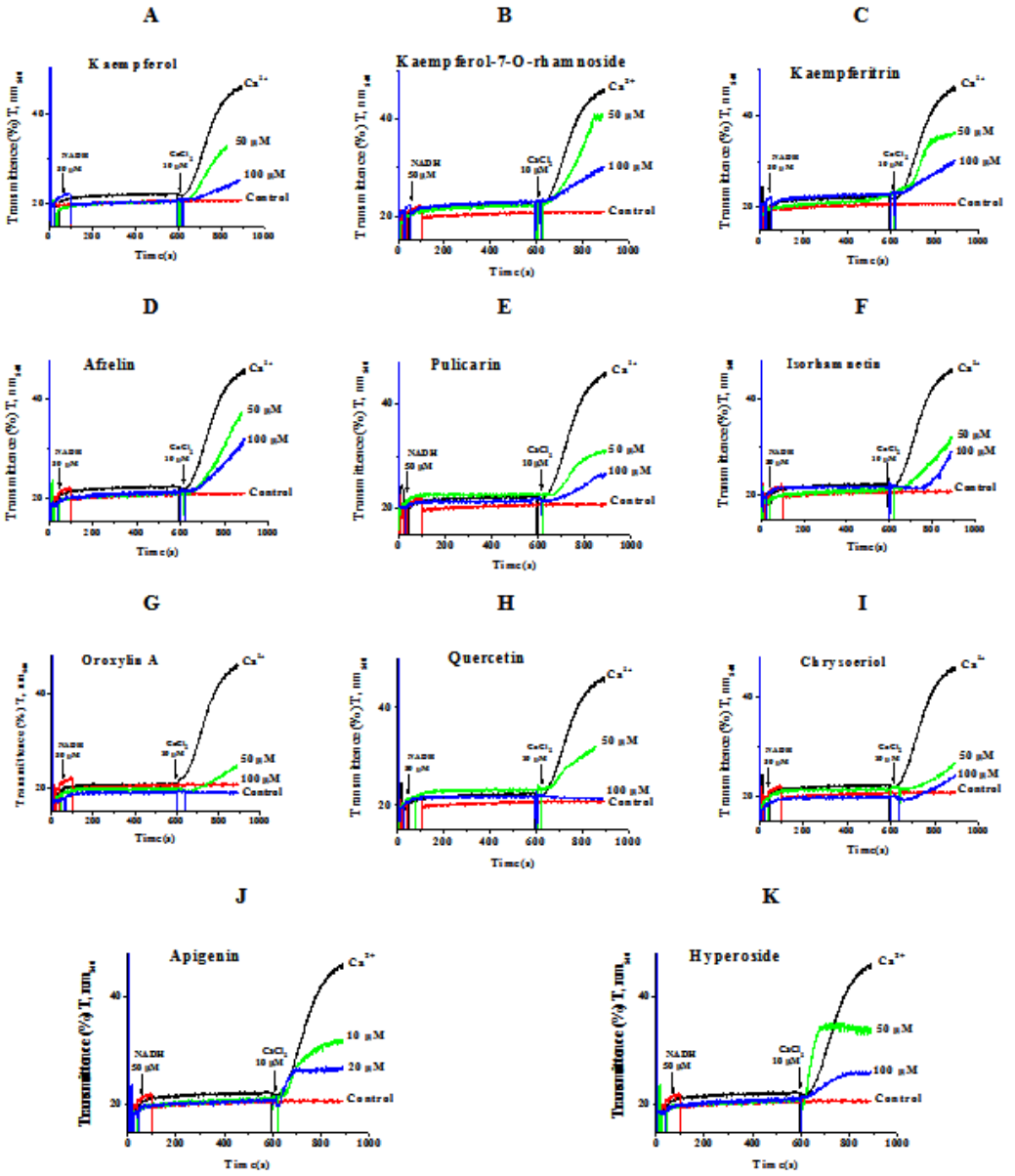

Fig. 4. Effect of various concentrations of flavonoids on Ca^{2+}-dependent mPTP in rat liver, associated with â – Nicotinamide adenine dinucleotide. A – Kaempferol; B – Kaempferol-7-O-rhamnoside; C – Kaempferitrin; D – Afzelin; E - Pulicarin; F – Isorhamnetin; G – Oroxylin À; H – Quercetin; I – Chrysoeriol; J – Apigenin; K – Hyperoside.(* - Ð<0,05, ** - Ð<0,01, *** - Ð<0,001; n=6).

was determined to be 143.0±2.83 ìM (Fig. 3, B). The effects of another kaempferol glycoside, kaempferitrin, on rat liver mitochondria mPTP were also studied at concentrations of 50, 100, 150, and 200 ìM (Fig. 2, C). Initially, mitochondria were incubated with the mPTP inducer Ca^{2+} (10 ìM) and the blocker CsA (5 ìM). At 50 ìM, kaempferitrin inhibited the transition of mPTP to the open conformational state by 9.45±0.17% in the presence of the inducer. At 100 ìM, it suppressed Ca^{2+}-induced high-amplitude mitochondrial swelling by 26.4±0.62% relative to the control. At 150 and 200 ìM, kaempferitrin inhibited the transition to the open conformational state by 33.7±0.8% and 40.3±1.94%, respectively, in the presence of Ca^{2+} ions (10 ìM). The next kaempferol

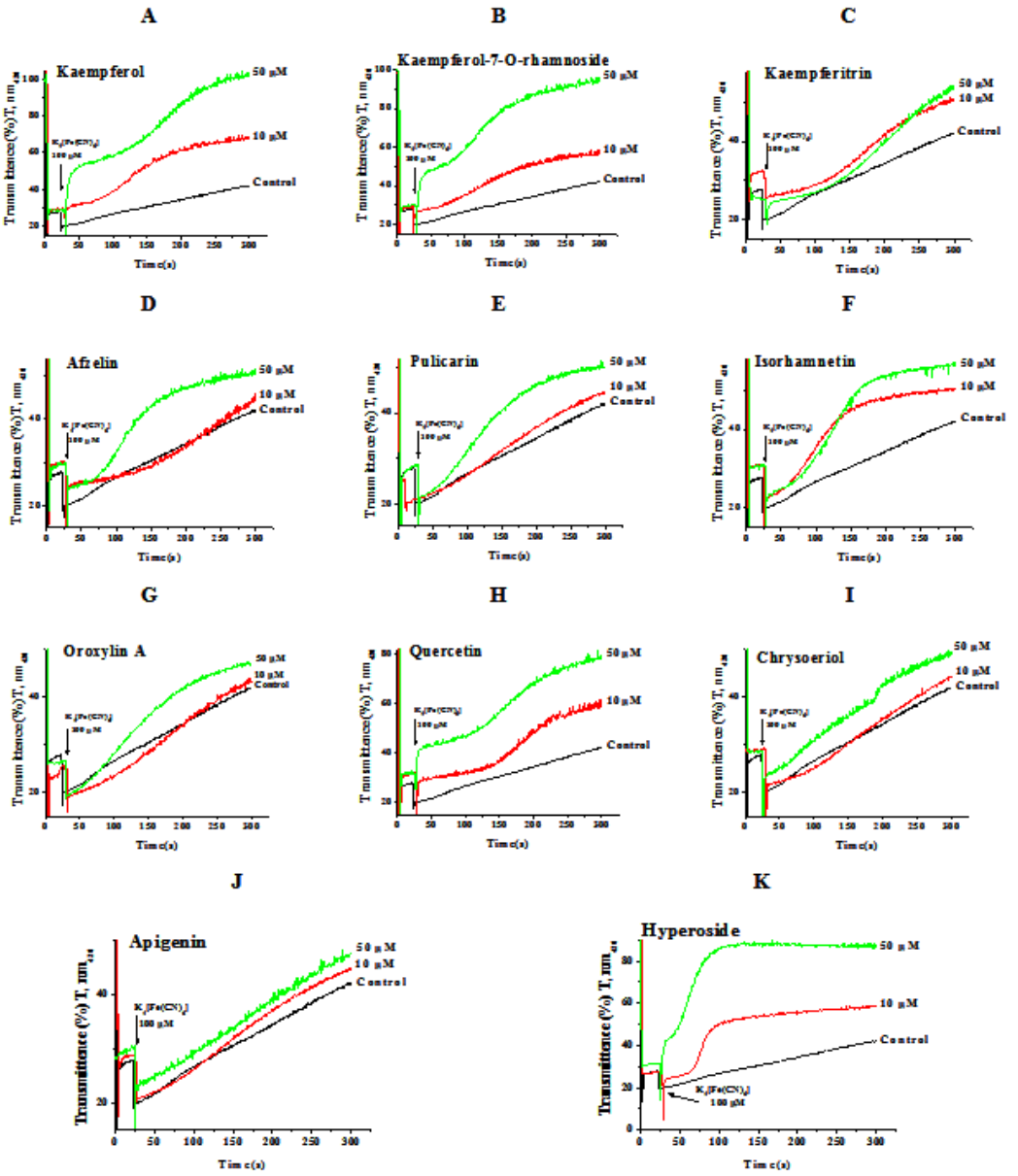

Fig. 5. Effects of selected flavonoids on succinate–ferricyanide reductase activity. A – Kaempferol; B – Kaempferol-7-O-rhamnoside; C – Kaempferitrin; D – Afzelin; E – Pulicarin; F – Isorhamnetin; G – Oroxylin A; H – Quercetin; I – Chrysoeriol; J – Apigenin; K – Hyperoside (n = 5)

glycoside studied was afzelin (kaempferol-3-rhamnoside, 50–200 ìM), which exhibited a distinct effect on mPTP in liver mitochondria induced by Ca^{2+} ions (10 ìM) (Fig. 2, D). Afzelin inhibited high-amplitude mitochondrial swelling induced by Ca^{2+} ions by only 31.6 ± 0.61% at the highest tested concentration of 200 ìM. During the study, the effects of pulicarin on Ca^{2+}-induced high-amplitude swelling of rat liver mitochondria were examined. Pulicarin inhibited mitochondrial swelling by 12.0±0.57% at 50 ìM, 33.2±0.57% at 100 ìM, 59.1±1.2% at 150 ìM, and 67.1±0.8% at 200 ìM (Fig. 2, E). The IC_{50} of pulicarin on mPTP was determined to be 131.8±1.2 ìM (Fig. 3, C). The effects of isorhamnetin on mPTP in rat liver mitochondria were also investigated. At

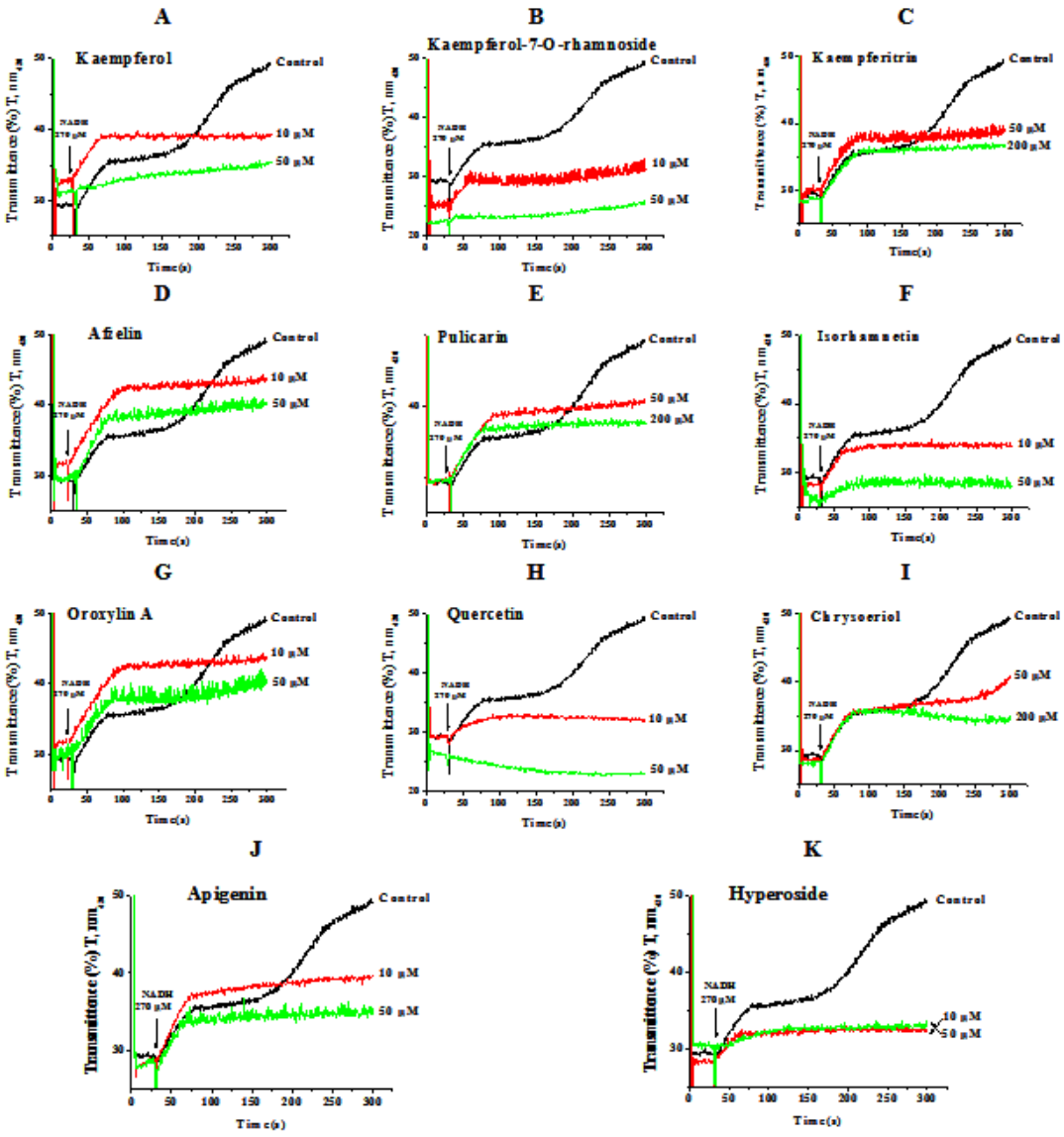

Fig. 6. Effects of selected flavonoids on NADH–ferricyanide reductase activity. A – Kaempferol; B – Kaempferol-7-O-rhamnoside; C – Kaempferitrin; D – Afzelin; E – Pulicarin; F – Isorhamnetin; G – Oroxylin A; H – Quercetin; I – Chrysoeriol; J – Apigenin; K – Hyperoside (n = 5)

50 ìM, isorhamnetin inhibited Ca^{2+}-induced high-amplitude mitochondrial swelling by 36.2±0.3%. At 100, 150, and 200 ìM, inhibition increased to 40.3±0.45%, 66.4±1.1%, and 75.7±2.2%, respectively (Fig. 2, F). The IC_{50} value for isorhamnetin was 116.2 ± 1.0 ìM (Fig. 3, D). Oroxylin A was also evaluated for its effect on mPTP in Ca^{2+}-stimulated rat liver mitochondria. At 50 ìM, Oroxylin A inhibited mPTP opening by 12.8±1.12% relative to the control. At 100, 150, and 200 ìM, inhibition of high-amplitude mitochondrial swelling reached 38.5±1.4%, 67.8±1.3%, and 75.3±2.1%, respectively (Fig. 2, G). The IC_{50} of Oroxylin A was determined to be 117.1±0.63 ìM (Fig. 3, H). Quercetin inhibited Ca^{2+}-induced high-amplitude swelling of rat liver mitochondria more effectively than the previously tested flavonoids. At 50 ìM, quercetin reduced mitochondrial swelling by 46.4±0.8%, while at 100, 150, and 200 ìM, inhibition increased to 55.0±1.1%, 68.0±1.5%, and 77.9±1.5%, respectively (Fig. 2, H). The IC_{50} of quercetin was 67.5±2.97 ìM (Fig. 3, F). The effects of chrysoeriol on mPTP in Ca^{2+}-stimulated rat liver mitochondria were also examined. At 50 ìM, chrysoeriol inhibited the open transition of mPTP by 21.1±1.1% relative to the control; at 100 ìM, inhibition reached 32.3±1.7%; at 150 ìM, 56.9±1.3%; and at 200 ìM, 67.1±0.8% (Fig. 2, I). The IC_{50} of chrysoeriol was 133.8±2.2 ìM (Fig. 3, G). Apigenin was also tested for its effect on Ca^{2+}-induced high-amplitude mitochondrial swelling. At 10 ìM, apigenin inhibited swelling by 10.1±1.74%; at 20 ìM by 20.1±0.93%; at 30 ìM by 50.2±1.4%; and at 40 and 50 ìM, inhibition reached 65.7±2.3% and 75.2±0.8%, respectively (Fig. 2, J). The IC_{50} of apigenin was 29.9±0.16 ìM (Fig. 3, H). Finally, the quercetin glycoside hyperoside was evaluated. At 50 ìM, hyperoside inhibited Ca^{2+}-induced mitochondrial swelling by only 7.8±0.4%; at 100 ìM, inhibition was 22.0±1.6%; and at 150 and 200 ìM, inhibition reached 36.9±1.4% and 66.2±1.68%, respectively (Fig. 2, K). The IC_{50} of hyperoside was 170.6±0.3 ìM (Fig. 3, I).

The results indicate that the flavonoids studied exhibited a characteristic inhibitory effect on Ca^{2+}-induced opening of mPTP in rat liver mitochondria. Apigenin inhibited high-amplitude mitochondrial swelling at low concentrations up to 50 ìM, whereas quercetin exerted a dose-dependent inhibitory effect on mPTP at 50, 100, 150, and 200 ìM, showing a lower IC_{50} compared to all flavonoids except apigenin. Under the same concentration range (50–200 ìM), kaempferitrin and afzelin failed to reach 50% inhibition of mPTP. In the case of the remaining flavonoids, the inhibition of mPTP in rat liver mitochondria ranged on average from 60% to 75% at maximal concentrations.

It is well established that pyridine nucleotides exert specific effects on the state of mPTP located in the inner mitochondrial membrane. It has been shown that pyridine nucleotides, particularly NADH, can increase the Ca^{2+}-retention capacity (CRC) in mitochondria from various tissues by 1.5- to 2.5-fold. This effect did not significantly alter mitochondrial Ca^{2+} uptake or the concentration of free Ca^{2+} in the medium. Moreover, while reducing Ca^{2+}-induced mitochondrial swelling, NADH did not trigger mitochondrial contraction or repolarization. These findings indicate the existence of an external NADH-dependent regulatory site for mPTP.[39] Based on this, the effects of flavonoids on mPTP in the presence of the pyridine nucleotide NADH were investigated. Experiments were conducted using flavonoid concentrations of 50 and 100 ìM, while for apigenin, 10–20 ìM was used. In these studies, mitochondria were pre-incubated with 50 ìM NADH for 10 minutes, after which the effects of Ca^{2+} (10 ìM) and the flavonoids were assessed. Initially, kaempferol at a concentration of 50 ìM inhibited high-amplitude Ca^{2+}-induced swelling of rat liver mitochondria by 45.5±1.3%, whereas at 100 ìM, inhibition reached 77.6±2.4% (Fig. 4, A). Kaempferol glycoside kaempferitrin at a concentration of 50 ìM inhibited Ca^{2+}-induced swelling of mitochondria by 33.6±1.79%, and at 100 ìM, inhibition reached 66.8±1.47% (Fig. 4, B). Another kaempferol glycoside, kaempferol-7-O-rhamnoside, inhibited Ca^{2+}-induced mPTP opening by 22.9±1.29% and 55.8±1.49% at 50 and 100 ìM, respectively (Fig. 4, C). Afzelin at 50 ìM reduced Ca^{2+}-induced mitochondrial swelling by 22.6±1.27%, and at 100 ìM, by 41.8±1.2% (Fig. 4, D). Pulicarin at 50 and 100 ìM inhibited Ca^{2+}-induced mPTP opening by 63.7±0.27% and 74.9±0.64%, respectively (Fig. 4, E). Isorhamnetin at 50 and 100 ìM inhibited Ca^{2+}-induced mPTP opening by 62.1±0.92% and 46.2±0.9%, respectively (Fig. 4, F). Similarly,

Oroxylin A at concentrations of 50 and 100 ìM inhibited Ca^{2+}-induced mPTP opening by 63.7±0.86% and 86.9±1.15%, respectively (Fig. 4, G). Quercetin at 50 and 100 ìM inhibited Ca^{2+}-induced mPTP opening by 54.0±0.86% and 94.0±1.28%, respectively (Fig. 4, H). Chrysoeriol at 50 and 100 ìM inhibited Ca^{2+}-induced mitochondrial swelling by 59.7±1.03% and 70.7±1.3%, respectively (Fig. 4, I). Apigenin at 10 and 20 ìM inhibited Ca^{2+}-induced mPTP opening by 37.6±0.9% and 61.0±1.4%, respectively (Fig. 4, J). Hyperoside at 50 and 100 ìM inhibited Ca^{2+}-induced mPTP opening by 31.5±0.3% and 58.15±0.4%, respectively (Fig. 4, K). The obtained results indicate that in the presence of NADH, flavonoids more effectively inhibit Ca^{2+}-induced mPTP opening compared to their individual effects. This suggests that the pyridine nucleotide modulates the influence of flavonoids on mPTP status.

In the study, the effects of flavonoids on succinate–ferricyanide reductase activity were investigated. It was found that flavonoids at concentrations of 10 and 50 ìM differentially modulated the enzyme activity. Under the influence of 10 ìM kaempferol, the enzyme activity increased by 25.0±0.67%, while at 50 ìM, it increased by 47.1±1.12% (Fig. 5, A). Kaempferol-7-O-rhamnoside enhanced succinate–ferricyanide reductase activity by 11.6±0.98% at 10 ìM and 25.7±1.1% at 50 ìM (Fig. 5, B). Afzelin also exerted a modest effect, increasing enzyme activity by 7.24±1.5% at 10 ìM and 25.8±1.5% at 50 ìM (Fig. 5, C). Kaempferitrin displayed a weaker activation, increasing enzyme activity by 3.35±0.52% at 10 ìM and 15.3±1.15% at 50 ìM (Fig. 5, D). Pulicarin increased enzyme activity by 17.5±0.3% at 10 ìM and 30.4±1.04% at 50 ìM (Fig. 5, E). Isorhamnetin enhanced the enzyme activity by 22.9±1.2% at 10 ìM and 37.7±1.6% at 50 ìM (Fig. 5, F). Oroxylin A increased the activity by 22.5±1.1% at 10 ìM and 30.3±0.85% at 50 ìM (Fig. 5, G). Quercetin showed a modest effect, enhancing enzyme activity by 9.43±0.51% at 10 ìM and 11.9±0.74% at 50 ìM (Fig. 5, H). Chrysoeriol (Fig. 5, I), apigenin (Fig. 5, J), and hyperoside (Fig. 5, K) increased succinate–ferricyanide reductase activity by 11.0±1.26%, 11.2±0.2%, and 28.1±0.86% at 10 ìM, respectively, and by 16.7±1.4%, 14.6±0.88%, and 32.3±0.7% at 50 ìM, respectively.

The obtained results indicate that the studied flavonoids possess the ability to moderately activate succinate–ferricyanide reductase, an enzyme localized in the mitochondrial respiratory chain. Literature data suggest that the NAD+-dependent deacetylase sirtuin-3 (SIRT3), a protein deacetylase complex, deacetylates the succinate dehydrogenase subunit SdhA, thereby enhancing the activity of mitochondrial respiratory chain complex II.

Moreover, treatment of K562 cell lines with nicotinamide and kaempferol was shown to stimulate SIRT3 expression, reduce acetylation of the SdhA subunit, and increase the activity of complex II of the mitochondrial respiratory chain in these cells. Additionally, acetylation of the hydrophilic surface of SdhA has been proposed to regulate substrate entry into the enzyme's active center.[40] These findings suggest that kaempferol participates in this process, promoting the activation of succinate dehydrogenase. In the conducted experiments, flavonoids generally exhibited a trend toward activating succinate–ferricyanide reductase; however, apigenin, chrysoeriol, kaempferitrin, and oroxylin A displayed very weak activating effects at the concentrations tested.

In the conducted experiments, the effects of flavonoids on NADH–ferricyanide reductase activity were investigated. It was found that flavonoids inhibited enzyme activity in a concentration-dependent manner. Specifically, kaempferol inhibited NADH–ferricyanide reductase activity by 59.7±2.8% at a concentration of 10 μM and by 84.8±1.85% at 50 μM (Fig. 6, A). Kaempferol-7-O-rhamnoside inhibited the enzyme activity by 54.8±2.1% at 10 μM and by 72.3±3.1% at 50 μM (Fig. 6, B). The kaempferol glycoside, kaempferitrin, exhibited weaker inhibitory effects, reducing enzyme activity by 40.5±2.03% at 50 μM and by 51.1±1.13% at 200 μM (Fig. 6, C). Afzelin inhibited NADH–ferricyanide reductase activity by 33.8±1.9% at 10 μM and by 41.9±1.9% at 50 μM (Fig. 6, D). Furthermore, pulicarin inhibited enzyme activity by 34.8±2.05% at 50 μM and by 49.3±2.5% at 200 μM (Fig. 6, E).

Isoramnetin inhibited NADH–ferricyanide reductase activity by 63.5±1.5% at a concentration of 10 μM and by 83.5±2.6% at 50 μM (Fig. 6, F). Oroxylin A inhibited enzyme activity by 33.6±1.0% at 10 μM and by 43.6±0.95% at 50 μM (Fig. 6,

G). Quercetin exhibited a strong inhibitory effect, reducing enzyme activity by 78.8±1.0% at 10 μM and almost completely inhibiting it at 50 μM (Fig. 6, H). Chrysoeriol inhibited enzyme activity by 27.8±1.2% at 50 μM and by 60.2±0.8% at 200 μM (Fig. 6, I). Apigenin inhibited enzyme activity by 33.1±2.4% and 59.4±2.3% at 10 and 50 μM, respectively (Fig. 6, J). Finally, hyperoside inhibited NADH–ferricyanide reductase activity by 68.6±1.4% at 10 μM and by 88.2±1.7% at 50 μM (Fig. 6, K).

The obtained results indicate that flavonoids exhibited a concentration-dependent inhibitory effect on NADH–ferricyanide reductase activity.

DISCUSSION

The obtained results demonstrate that flavonoids at various concentrations exert inhibitory effects on NADH–ferricyanide reductase activity. Among the tested compounds, quercetin, isorhamnetin, kaempferol-7-O-rhamnoside, hyperoside, apigenin, and kaempferol exhibited stronger inhibitory effects on enzyme activity, whereas the remaining flavonoids showed relatively weaker inhibitory effects. Overall, the studied flavonoids differentially modulated mPTP, succinate–ferricyanide reductase, and NADH–ferricyanide reductase activities. In particular, they led to inhibition of mPTP and NADH–ferricyanide reductase activities, while causing an increase in succinate–ferricyanide reductase activity. Furthermore, during the assessment of mPTP status, the use of exogenous NADH was shown to enhance the mPTP-inhibitory properties of the flavonoids.

It is well known that flavonoids are polyphenolic biologically active compounds synthesized in plants, and represent substances possessing antioxidant, anti-inflammatory, antidiabetic, antibacterial, antiviral, immunomodulatory, anticancer, cytoprotective, cytotoxic, lipid-lowering, and other biological activities. [41-44] It was revealed that quercetin, apigenin, and kaempferol, by inhibiting mitochondrial complex I, suppress H_2O_2 formation at low concentrations, while at the same time exhibiting no significant inhibitory effects on mitochondrial complexes II and III.[45] The flavonoid (+)-catechin, in the presence of glutamate–malate substrates, inhibited mitochondrial complex I at nanomolar concentrations, leading to a decrease in membrane potential as well as a reduction in the rates of H_2O_2 and NO formation. The observed decrease in NO production was suggested to be associated with a direct effect of the flavonoid on mitochondrial nitric oxide synthase (mtNOS) activity.[46] Indeed, the studied flavonoids were shown to strongly inhibit mitochondrial complex I, namely NADH–ferricyanide reductase activity (Fig. 6). However, a slight discrepancy was observed between the effects of the studied flavonoids on mitochondrial complex II, i.e., succinate–ferricyanide reductase activity, and the data reported in the literature (Fig. 5). This may be attributed to the use of ferricyanide as an electron acceptor in the determination of SDH activity.[47,48] Furthermore, the increase in succinate–ferricyanide reductase activity in rat muscle mitochondria under the influence of flavonoids suggested that the presence of a hydroxyl group on the C-ring of flavonoids may be one of the main factors contributing to enzyme activation.[49]

It has been shown that peroxidized cardiolipin in mitochondria can act as an inducer for mPTP, similar to Ca^{2+} ions, by lowering the threshold of Ca^{2+}-induced mPTP opening, leading to cytochrome c release from mitochondria and initiating the early phase of apoptosis.[50] At the same time, flavonoids were shown to exert a restorative effect on cytochrome c activity by inhibiting cardiolipin-induced pro-apoptotic peroxidase activity of cytochrome c,[51] and the results obtained in these studies (Fig. 2) indicate that flavonoids may play an important role in correcting mPTP-related mitochondrial dysfunction. In addition, polyphenols and other biologically active compounds have been shown to exert inhibitory effects on mPTP status.[52-56] Flavonoids have been shown to modulate mitochondrial potassium channels, thereby exhibiting cytoprotective properties.[43]

CONCLUSION

Flavonoids were found to exert inhibitory effects on mPTP in rat liver mitochondria, with the following rank order based on their half-maximal inhibitory concentrations: apigenin > quercetin > isorhamnetin > oroxylin A > pulicarin > chrysoeriol

> kaempferol > kaempferol-7-O-rhamnoside > hyperoside. In the presence of exogenous NADH, the inhibitory effects of flavonoids on mPTP were pronounced for apigenin, oroxylin A, quercetin, kaempferol, chrysoeriol, hyperoside, and pulicarin, whereas the remaining flavonoids exhibited relatively weaker effects. Moreover, the flavonoids hyperoside, kaempferol, kaempferol-7-O-rhamnoside, isorhamnetin, quercetin, afzelin, and pulicarin increased succinate–ferricyanide reductase activity, whereas the remaining flavonoids exhibited very weak or no effect. At the same time, quercetin, isorhamnetin, kaempferol-7-O-rhamnoside, hyperoside, and kaempferol showed strong inhibitory effects on NADH–ferricyanide reductase activity, while the other flavonoids exhibited relatively weaker inhibitory effects.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Statement

All manipulations with animals were carried out in accordance with the European Convention for the Protection of Animals Used for Scientific Purposes (1998) and the International Bioethical Guidelines of the Institute of Biophysics and Biochemistry of the National University of Uzbekistan (BEC/IBB-N44/2024/14-1).

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Permission to reproduce material from other sources

Not Applicable.

Author Contributions

Boburbek Yuldoshev: Conceptualization and methodology, investigation, writing-original draft, writing-review, english editing, supervision; Nurali Ergashev: Conceptualization and methodology, writing-original draft, writing-review, english editing, supervision and formal analysis; Esokhon Komilov: Conceptualization and methodology, investigation, funding acquisition, supervision; Mavluda Kenjayeva: Investigation, funding acquisition; Mukhlisa Ikromova: Investigation, funding acquisition; Yulduz Rakhmatillayeva: Investigation, funding acquisition; Hazratova Hulkar: Funding acquisition; Komila Eshbakova: Investigation; Donyor Siddikov: Investigation; Bakhrom Komilov: Investigation; Rustam Makhmudov: Investigation; Asrarov Muzaffar Islamovich: Conceptualization and methodology.

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