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Mitochondrial and Pharmacokinetic Insights into 3,5,7,2',6'-Pentahydroxyflavanone: Respiratory Modulation, Calcium Handling, and Membrane Stability

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Abstract

This study investigates the mitochondrial effects and pharmacokinetic profile of 3,5,7,2',6'-pentahydroxyflavanone (PHF), a naturally occurring flavonoid. *In vitro* results showed that PHF at 40 and 60 µg/mg protein reduced glutamate-supported state 3 respiration by 6% - 10% and slightly increased state 4 respiration, leading to a decrease in respiratory control ratio (RCR) and ADP/O ratio. For succinate as substrate, PHF at the same doses reduced V_3, V_4, and VDNP respiration rates by 15% - 33% and increased the ADP/O ratio up to 1.44-fold, indicating enhanced coupling efficiency. Enzymatic assays revealed that PHF selectively suppressed succinate dehydrogenase and oxidase activities by up to 25% without affecting NADH-linked enzyme systems. Notably, PHF reduced mitochondrial calcium uptake by 10.7% - 47.8% in a dose-dependent manner (100 - 500 mg/kg, i.p.) and attenuated the activity of membrane-bound oxidases under phospholipase A_2 stress, suggesting membrane-stabilizing effects. ADMETlab predictions indicated favorable drug-like properties (MW 286.05, logP 2.25, TPSA 111.13), high plasma protein binding (PPB 95.2%), and acceptable oral bioavailability ($F_{50\%}$: +++). PHF was predicted to inhibit CYP1A2, CYP2D6, and CYP3A4 enzymes but not act as their substrate. Toxicity alerts were minimal, though genotoxicity (0.956) and eye irritation (0.996) probabilities were elevated. Taken together, PHF demonstrates mitochondria-targeted bioactivity and a moderate ADMET profile, positioning it as a potential lead compound for cytoprotective drug development.

Keywords: 3,5,7,2',6'-pentahydroxyflavanone, Mitochondrial respiration, Oxidative phosphorylation, Calcium accumulation, Membrane stabilization, Dehydrogenase activity, ADMET profiling, Cytochrome P450, Flavonoid, Cytoprotection, Pharmacokinetics, *In vitro*, *In vivo*

Introduction

Mitochondria are critical organelles that regulate cellular energy production through oxidative phosphorylation (OXPHOS), maintain redox balance, and mediate calcium homeostasis and apoptosis [1]. Mitochondrial dysfunction is a central event in many pathological processes, especially under toxic stress and oxidative overload. Disruptions in mitochondrial respiration, membrane potential, and enzyme activities often lead to impaired cellular metabolism and cell death. Flavonoids are widely studied for their antioxidant and cytoprotec-

tive properties [2]. Among them, Flavosan, a synthetic flavonoid compound, and 3,5,7,2',6'-pentahydroxyflavanone (PHF), a polyhydroxylated flavanone, have shown potential in modulating mitochondrial function and counteracting oxidative stress. These compounds can influence mitochondrial respiration, enzyme activity (including oxidases and dehydrogenases), and lipid peroxidation, which are key parameters in assessing mitochondrial health. Earlier studies have demonstrated that certain flavonoids can affect oxygen consumption, improve ATP production efficiency, and protect mitochondrial membranes from damage [3]. However, detailed mechanisms by which these compounds influence mitochondrial function under toxic stress, particularly *in vivo*, remain to be clarified. One of the potent experimental models for mitochondrial toxicity involves envenomation with Central Asian cobra (Naja naja oxiana) venom, which disrupts respiratory and oxidative balance in liver mitochondria [4]. The ability of flavonoids to correct such venom-induced mitochondrial impairments has not been thoroughly studied. This research explores the mitochondrial-modulating and antitoxic potential of Flavosan and PHF under both *in vitro* and *in vivo* conditions. Special attention is given to their effects on oxygen consumption, oxidative phosphorylation efficiency, calcium transport, and lipid peroxidation processes [5]. These investigations contribute to the understanding of flavonoid-based strategies for mitochondrial protection and detoxification in conditions of xenobiotic and venom-induced stress.

Materials and methods

Chemicals

The following reagents were used in the experiments: Tris-HCl, ADP, ATP, and succinic acid were purchased from Reanal (Hungary), while cytochrome c was obtained from Sigma (USA). All other chemicals and solvents were of analytical or chemically pure grade, supplied by manufacturers in the Russian Federation. Phospholipase A_2 enzymes were isolated from the venom of the Central Asian cobra (*Naja naja oxiana*) and used in relevant experiments [6].

The flavonoid compound used in this study was 3,5,7,2',6'-pentahydroxyflavanone, also known by its chemical names: 2-(2,6-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one, 3,5,7,2',6'-pentahydroxyflavone, Viscidulin I, CAS Number: 92519-95-4 This compound belongs to the class of polyhydroxylated flavanones and is characterized by 5 hydroxyl groups located at positions 3, 5, 7, 2', and 6' (**Figure 1**). Its molecular formula is $C_{15}H_{10}O_7$, and the molecular weight is 302.24 Da. 3,5,7,2',6'-Pentahydroxyflavanone was isolated and purified with the assistance of the research team from the Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan. Due to its high hydroxylation, the compound exhibits potent antioxidant and potential mitochondria-targeted bioactivity, making it a suitable candidate for assessing mitochondrial function under both physiological and toxicological conditions [7].

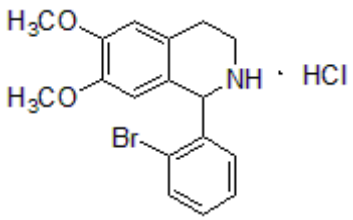

Figure 1 3,5,7,2',6'-pentahydroxyflavanone.

Animal ethics

All preoperative and experimental procedures were carefully reviewed and approved by the Institutional Committee for Animal Use and Care. The animals were housed in a vivarium under standardized conditions, including a relative humidity of 55% - 65%, a controlled ambient temperature of 22 ± 2 °C, and unrestricted access to water and standard laboratory chow. All aspects of animal care and handling were conducted in full compliance with the European Directive 2010/63/EU, which governs the protection of animals used for scientific research. Ethical clearance for this study was granted by the Animal Ethics Committee of the Institute of Bioorganic Chemistry,

Academy of Sciences of the Republic of Uzbekistan (Protocol No. 133/1a/h, dated 4 August 2016).

Isolation of liver mitochondria

Liver mitochondria were isolated from white outbred rats (180 - 220 g) using a standard differential centrifugation technique [8,9]. The isolation buffer consisted of 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, adjusted to pH 7.4. Following decapitation, the abdominal cavity was carefully opened, and the liver was rapidly excised and transferred into a beaker containing ice-cold isolation buffer. After weighing, the liver tissue was initially minced and then further homogenized using a Teflon-glass homogenizer at a tissue-to-buffer ratio of 1:6 (w/v). The homogenate underwent a first centrifugation at 1,500 rpm (approximately 400×g) for 7 min at 0 - 2 °C using an RS-6MC centrifuge equipped with an angled rotor, to eliminate coarse debris and unbroken cells. The supernatant was subsequently subjected to a second centrifugation at 6,000 rpm (about 5,000×g) for 15 min at 0 - 2 °C to pellet the mitochondria. The resulting mitochondrial pellet was separated from the supernatant, and any residual liquid or lipid contamination adhering to the tube walls was carefully removed with filter paper. The isolated mitochondria were then resuspended in EDTA-free isolation buffer at a dilution of 10:1 (buffer:tissue) to prepare the working mitochondrial suspension. This suspension was stored in an ice-cooled container until further use in experiments. Protein content in the mitochondrial preparation was quantified using the Biuret assay [10].

Assessment of lipid peroxidation in mitochondria

Lipid peroxidation (LPO) in isolated mitochondria was evaluated using an Fe^{2+}/ascorbate-induced oxidative system. This model promotes oxidative damage to mitochondrial membranes, leading to membrane permeability loss and resulting in mitochondrial swelling, which was monitored spectrophotometrically. The incubation medium consisted of 125 mM KCl and 10 mM Tris-HCl, adjusted to pH 7.4. LPO was initiated by the addition of 10 μM ferrous sulfate ($FeSO_4$) and 600 μM ascorbate. Mitochondrial swelling, as an indirect indicator of peroxidative damage, was measured at a mitochondrial protein concentration of 0.3 - 0.4 mg/mL in the cuvette [11].

Measurement of mitochondrial Ca^{2+} accumulation

Calcium accumulation in liver mitochondria was assessed based on the membrane potential–dependent uptake of Ca^{2+} along the electrochemical gradient ($\Delta\mu H^+$), which is primarily maintained by the activity of the respiratory chain and potassium ion diffusion potential. The energy-dependent uptake occurs via the mitochondrial calcium uniporter, independent of other ion exchanges. Measurements were conducted using a pH-metric method that monitors Ca^{2+}/H^+ exchange across the inner mitochondrial membrane. Sequential additions of $CaCl_2$ to the mitochondrial suspension led to Ca^{2+} uptake accompanied by proton extrusion. Once the accumulation threshold was exceeded, Ca^{2+} release was observed, indicating membrane permeability changes and activation of endogenous phospholipases. The incubation medium contained 120 mM KCl, 10 mM Tris-HCl buffer (pH 7.4), 5 mM succinate, 1 mM KH_2PO_4, and 1 μg/mL rotenone. The system was calibrated using a known concentration of HCl solution [12,13].

Temperature-induced changes in mitochondrial oxidase activity

To evaluate the thermal sensitivity of mitochondrial oxidases, experiments were performed using once-frozen liver mitochondria thawed in a standard incubation medium containing 250 mM sucrose and 10 mM Tris-HCl (pH 7.4). Control and flavonoid-treated mitochondrial suspensions were incubated at 37 °C for 180 min. Thermal exposure in the absence of oxygen and respiratory substrates led to structural destabilization of the mitochondrial membrane, including an increase in non-bilayer domains [14,15]. This promoted the activation of endogenous lipolytic and proteolytic enzymes by Ca^{2+} ions, facilitating their access to membrane-bound enzyme complexes. Following incubation, the activity of membrane-associated oxidases - specifically rotenone-sensitive NADH oxidase and succinate oxidase - was determined. Enzyme activity was expressed in nanograms of oxygen uptake per minute per milligram of mitochondrial protein.

Effect of phospholipase A_2 and trypsin on mitochondrial oxidase activity

The stability and activity of mitochondrial rotenone-sensitive NADH oxidase and succinate oxidase systems were evaluated under the influence of phospholipase A_2 (PLA_2) and trypsin. Experiments were conducted at 20 °C using an incubation buffer containing 250 mM sucrose and 10 mM Tris-HCl (pH 7.4). Phospholipase A_2, a member of a diverse enzyme family, hydrolyzes the ester bond at the Sn-2 position of glycerophospholipids, generating lysophospholipids and free fatty acids. Mitochondrial suspensions were first treated with a selected flavonoid (20 µg per mg of protein) and mixed thoroughly. After 5 min, PLA_2 was added at a concentration of 0.04 µg/mg protein. Following an 80-minute incubation, oxidase enzyme activities were measured. For comparison, control samples were treated with PLA_2 alone (same concentration) without flavonoids. In a separate set of experiments, the effects of trypsin on oxidase activity were assessed under similar conditions. Enzymatic activity was expressed as nanograms of oxygen consumed per minute per milligram of mitochondrial protein [16,17].

Statistical analysis

Statistical analysis and graphical representations were carried out using Origin Pro software, version 9 (USA). Data are presented as the mean ± standard error from 4 to 6 independent experiments (n = 4 - 6). Paired t-tests were applied for comparisons within the same group, while unpaired t-tests were used to evaluate differences between experimental groups. A *p*-value of less than 0.05 was considered statistically significant.

Results and discussion

Effects of 3,5,7,2',6'-pentahydroxyflavanone on mitochondrial respiration and oxidative phosphorylation

The influence of 3,5,7,2',6'-pentahydroxyflavanone (PHF) on the mitochondrial oxidation of glutamate and succinate was evaluated across various metabolic states. The corresponding results are presented in **Table 1**. At lower concentrations, PHF exerted minimal effect on glutamate-supported respiration and oxidative phosphorylation. However, increasing its concentration to 40 and 60 µg/mg mitochondrial protein resulted in a noticeable decline in glutamate oxidation in the phosphorylating state (V_3), while simultaneously enhancing respiration in the resting state (V_4). This imbalance led to a decrease of 6% - 10% in both the respiratory control ratio (RCR) and ADP/O ratio, indicating a mild uncoupling effect [18]. These findings suggest that only at higher concentrations does PHF moderately suppress oxidative phosphorylation efficiency, likely by reducing the coupling between oxidation and ATP synthesis during NAD-linked substrate utilization. In contrast, PHF affected succinate oxidation differently. At low doses, PHF had negligible effects on succinate-supported respiration across all metabolic states. However, at increased concentrations (40 and 60 µg/mg), a dose-dependent inhibition of succinate oxidation was observed. Interestingly, this was accompanied by a paradoxical increase in ADP/O ratio, whereas the RCR remained relatively unchanged. Specifically, at 40 µg/mg, succinate oxidation in States V_3, V_4, and uncoupled (V_DNP) decreased by 1.15-, 1.18-, and 1.14-fold respectively compared to control, while the ADP/O ratio increased by 1.21-fold. At 60 µg/mg, further reductions in respiration rates were recorded: V_3, V_4, and V_DNP decreased by 1.24-, 1.27-, and 1.33-fold, respectively, with a corresponding ADP/O increase of 1.44-fold [19]. These findings indicate that PHF may enhance the efficiency of ATP synthesis per oxygen atom consumed during succinate oxidation, possibly by facilitating proton transfer or reducing proton leak. Physiologically, such a shift may help meet cellular ATP demand more efficiently, particularly under conditions where NAD-linked substrates are limited. This is consistent with the theoretical yield difference between substrates, as oxidation of NAD-dependent substrates yields ~3 ATP molecules, whereas succinate yields only ~2.

Table 1 Effect of flavosan on mitochondrial respiration and oxidative phosphorylation (M ± m; n = 4).

Parameters	Respiratory rate, nanogram oxygen atom/min mg protein			
	Flavosan, µg/mg protein			
	Control	20	40	60
V3 (α-Kg)	53.3 ± 3.1	52,4±3,6	51.8 ± 3.4	50.0 ± 3.3
V4	15.2 ± 2.0	15,9±2,7	16.3 ± 3.1	16.0 ± 2.8
VDNF	59.0 ± 3.4	58,4±3,8	56.6 ± 4.0	54.2 ± 3.6
NKCH	3.50 ± 0.15	3.29 ± 0.12	3.18 ± 0.13	3.12 ± 0.14
ADF/O	2.76 ± 0.12	2.68 ± 0.12	2.53 ± 0.11	2.60 ± 0.11
V3 (St)	117.9 ± 4.9	115.3 ± 5.2	100.0 ± 4.2**	89.6 ± 4.0***
V4	39.8 ± 2.4	38.0 ± 3.6	32.6 ± 3.2*	29.2 ± 3.1**
VDNF	180.0 ± 7.2	186.7 ± 8.8	155.6 ± 6.0***	120.0 ± 5.2***
NKCH	2.96 ± 0.16	3.03 ± 0.12	3.00 ± 0.11	3.06 ± 0.12
ADF/O	1.64 ± 0.08	1.72 ± 0.09	1.99 ± 0.07**	2.36 ± 0.08***

Note: Here and in other tables, the confidence interval is expressed as follows: * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$.

Effects of 3,5,7,2',6'-pentahydroxyflavanone on mitochondrial dehydrogenase and oxidase activities

The impact of 3,5,7,2',6'-pentahydroxyflavanone (PHF) on mitochondrial dehydrogenase and oxidase enzymes localized within the inner mitochondrial membrane was systematically investigated (**Table 2**). At lower concentrations, PHF had negligible influence on NADH dehydrogenase and rotenone-sensitive NADH oxidase activities [20]. Similarly, the activities of succinate dehydrogenase and succinate oxidase were unaffected at minimal PHF levels (**Figure 2**). However, as PHF concentration increased to 40 and 60 µg/mg protein, a dose-dependent suppression of both succinate dehydrogenase and oxidase activities was observed. Specifically, activity decreased by 1.15 - 1.16-fold at 40 µg/mg, and by 1.24 - 1.25-fold at 60 µg/mg protein, suggesting a selective inhibitory effect on FAD-linked respiratory enzymes. Further analysis examined the temporal dynamics of NADH oxidase activity during incubation at 20 °C. In control mitochondria, rotenone-sensitive NADH oxidase activity increased over time, showing 1.26-, 1.48-, and 1.67-fold elevations at 60, 120, and 180 min, respectively. In contrast, mitochondria incubated with PHF exhibited much slower increases (1.10-, 1.18-, and 1.25-fold, respectively), indicating that PHF delays enzyme activation, likely by stabilizing the membrane and reducing NADH diffusion to its binding site [21,22].

Table 2 Effect of PHF on mitochondrial dehydrogenase and oxidase activities (M ± m; n = 4).

PHF, µg/mg protein	NADH-dehydrogenase	%	RS NADH-oxidase	%
Control	8.88 ± 0.41	100	64.0 ± 3.8	100
20	8.74 ± 0.40	98.4	62.3 ± 3.9	97.4
40	8.50 ± 0.35	95.7	60.7 ± 3.4	95.0
60	8.35 ± 0.32	94.0	60.9 ± 3.7	94.9
IC_{50}	Ns	ns	Ns	ns
PHF, µg/mg protein	Succinate dehydrogenase	%	Succinate oxidase	%

PHF, μg/mg protein	NADH-dehydrogenase	%	RS NADH-oxidase	%
Control	24.22 ± 1.43	100	195.8 ± 9.9	100
20	23.49 ± 1.70	97.0	189.3 ± 9.4	96.7
40	20.71 ± 1.38*	85.5	165.0 ± 6.5**	84.3
60	18.38 ± 1.21***	75.9	146.6 ± 5.2***	74.9
IC_{50}	ns	ns	ns	ns

Note: Here the reliability coefficient is: *$p < 0.05$; **$p < 0.02$; ***$p < 0.001$.

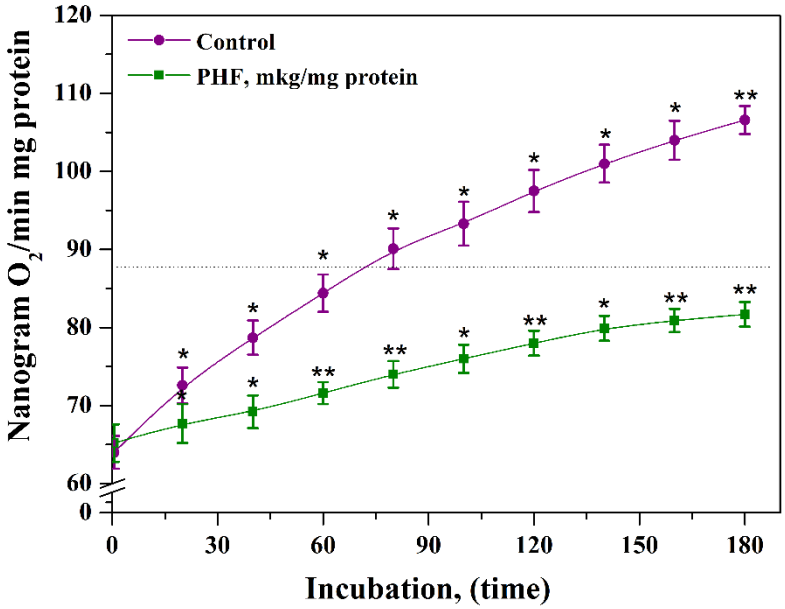

Figure 2 Changes in rotenone-sensitive NADH-oxidase activity upon incubation of mitochondria with PHF at 20 °C (n = 8 - 10).

Experiments conducted at physiological temperature further confirmed this membrane-stabilizing effect (**Figure 3(A)**). After 60 min of incubation at 37 °C, control mitochondria displayed a 1.21-fold increase in NADH oxidase activity, while no change was observed in the PHF group [23]. At longer time points (180 - 300 min), NADH oxidase activity in controls decreased sharply (1.25 - 1.78-fold), whereas the PHF-treated group showed significantly milder reductions (1.09 - 1.22-fold), reinforcing the conclusion that PHF preserves enzymatic integrity under stress. The same pattern was observed for succinate oxidase: While control mitochondria showed a biphasic activity curve (initial activation followed by decline), PHF-treated mitochondria maintained stable activity throughout the incubation (**Figure 3(B)**). At 360 min, succinate oxidase activity in the control dropped by 1.69-fold, while PHF-treated mitochondria exhibited no significant loss, supporting PHF's role in maintaining membrane structural stability [24].

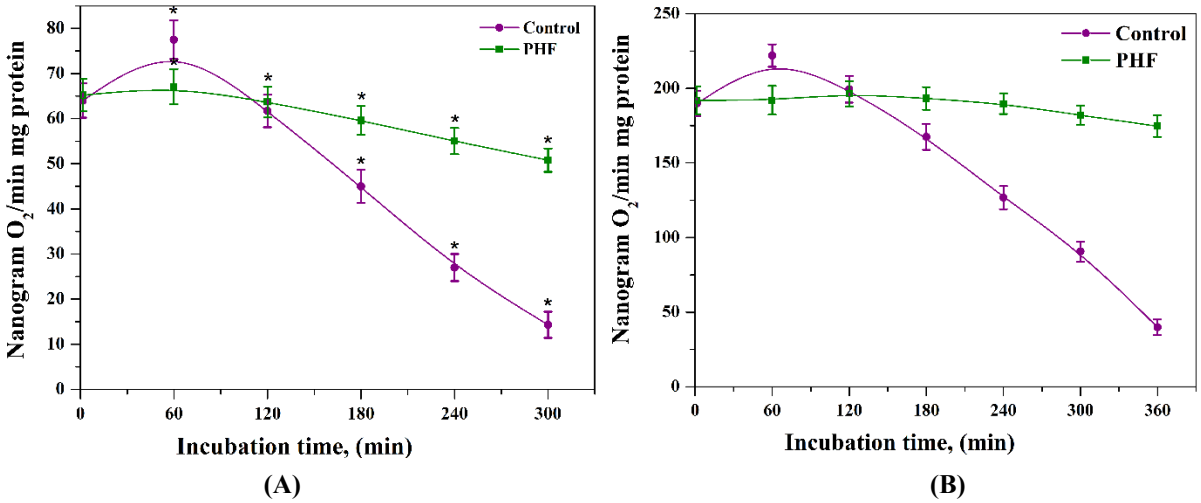

Figure 3 Changes in rotenone-sensitive NADH-oxidase (A) and succinate oxidase (B) activities upon incubation of mitochondria with PHF at 36.7 °C (n = 8 - 10).

Membrane stabilization by PHF was further validated in experiments involving phospholipase A_2 (PLA_2), an exogenous membrane-disrupting enzyme isolated from *Naja naja oxiana* venom. When control mitochondria were incubated with PLA_2 at 20 °C, NADH oxidase activity sharply increased (1.25-fold at 20 min), indicating enhanced membrane permeability. However, in PHF-treated mitochondria, the same activity rose by only 1.07-fold. Over time, PLA_2-induced activity declined to baseline in both groups, but the reduction was significantly less pronounced in PHF-treated mitochondria (60 - 80 min: 1.08 - 1.20-fold vs. 1.50 - 1.78-fold in controls). Similarly, succinate oxidase activity in control mitochondria rapidly declined under PLA_2 exposure (20 - 80 min: 1.28 to 1.82-fold decreases), whereas PHF co-treatment substantially mitigated this drop (only 1.07 to 1.23-fold reductions). This differential effect is attributed to the peripheral localization of succinate dehydrogenase, which makes it more accessible to PLA_2, compared to NADH dehydrogenase, which resides deeper within the membrane [25,26].

These findings suggest that PHF protects mitochondrial enzymes by preserving membrane integrity, thereby restricting both endogenous and exogenous lipolytic and proteolytic enzymes from accessing their catalytic targets. Thus, 3,5,7,2',6'-pentahydroxyflavanone acts as a functional membrane stabilizer, supporting mitochondrial resilience under enzymatic and thermal stress.

Protective role of PHF against phospholipase A_2-induced oxidase instability

Our results demonstrate that 3,5,7,2',6'-pentahydroxyflavanone (PHF) selectively reduces the activity of succinate dehydrogenase and succinate oxidase, while having negligible effect on NADH dehydrogenase and rotenone-sensitive NADH oxidase under normal conditions. These findings suggest substrate-specific modulation of mitochondrial oxidative enzymes. Moreover, PHF significantly stabilizes the inner mitochondrial membrane, thereby limiting access of both endogenous and exogenous lipolytic and proteolytic enzymes to membrane-bound dehydrogenases and oxidases. This was further supported by experiments involving phospholipase A_2 (PLA_2), a membrane-disrupting enzyme isolated from *Naja naja oxiana* venom [27,28].

When control mitochondria were incubated with PLA_2, the activity of rotenone-sensitive NADH oxidase increased sharply within the first 20 min (1.25-fold), indicating increased membrane permeability (**Figure 4(A)**). In contrast, mitochondria pretreated with PHF showed only a 1.07-fold increase during the same interval. At 40 min, enzyme activity in both groups returned to baseline, suggesting temporary activation [29,30]. However, with prolonged incubation (60 and 80 min), enzyme activity in control mitochondria decreased significantly (1.50- and 1.78-fold), while in the PHF-treated group, the reductions were considerably smaller (1.08- and 1.20-fold), with absolute differences of 0.42 and 0.58, respectively.

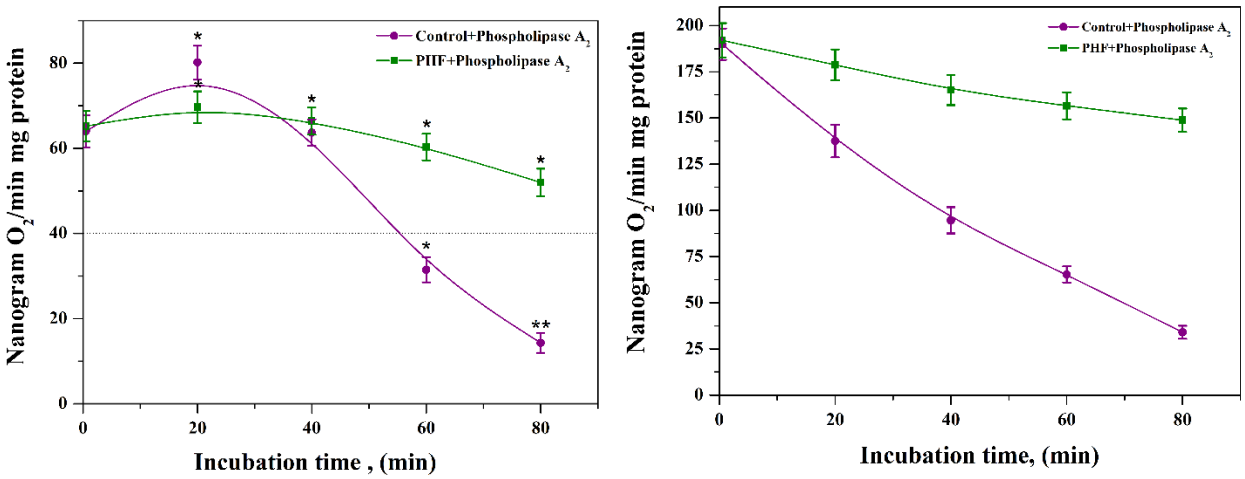

Figure 4 Changes in rotenone-sensitive NADH-oxidase (A) and succinate oxidase (B) activities upon incubation of mitochondria with PHF and phospholipase A2 (n = 4).

Succinate oxidase was even more sensitive to PLA_2-induced damage (**Figure 4(B)**). In control mitochondria, its activity decreased rapidly at all time points (20, 40, 60 and 80 min: 1.28, 1.50, 1.64, and 1.82-fold reductions). Meanwhile, in PHF-treated mitochondria, the decline was much milder (1.07, 1.14, 1.16, and 1.23-fold reductions). This difference may be explained by the spatial localization of the enzyme complexes. NADH dehydrogenase is embedded deep within the central region of the inner membrane, making it less accessible to PLA_2, whereas succinate dehydrogenase is peripherally located, allowing easier access by the enzyme. PHF appears to form a stabilizing barrier that inhibits PLA_2 from reaching the active sites of these complexes. Taken together, these findings suggest that PHF enhances mitochondrial membrane stability, thereby preventing phospholipase A_2 from disrupting critical enzymatic functions. This protective effect is especially important under conditions of toxic or oxidative stress, positioning PHF as a potential mitochondria-targeted membrane stabilizer.

Effect of 3,5,7,2',6'-pentahydroxyflavanone on mitochondrial Ca^{2+} accumulation

Mitochondria are known not only for producing ATP - the universal energy currency of the cell - but also for their capacity to accumulate and buffer intracellular Ca^{2+}. Compared to the endoplasmic or sarcoplasmic reticulum, mitochondria possess a significantly higher storage capacity for Ca^{2+} and actively participate in maintaining cytosolic Ca^{2+} homeostasis. Under physiological conditions, cytosolic free Ca^{2+} levels remain low (10^{-7} - 10^{-8} M), while mitochondrial Ca^{2+} uptake occurs efficiently only when local Ca^{2+} concentrations transiently rise, particularly at the interfaces with the endoplasmic reticulum (up to 10^{-3} - 10^{-2} M). This transient mitochondrial Ca^{2+} influx plays a dual role: at moderate levels, it stimulates ATP synthesis via activation of key dehydrogenases of the TCA cycle. However, excessive Ca^{2+} accumulation may lead to overproduction of reactive oxygen species (ROS), opening of mitochondrial permeability transition pores, mitochondrial swelling, and ultimately, rupture of the outer membrane and organelle dysfunction [31].

To evaluate the influence of 3,5,7,2',6'-pentahydroxyflavanone (PHF) on Ca^{2+} uptake, rats were intraperitoneally administered PHF at doses of 100, 200, 300, and 500 mg/kg. Twenty min post-injection, liver mitochondria were isolated and Ca^{2+} transport was measured. The results demonstrated a dose-dependent suppression of Ca^{2+} accumulation: reductions of 10.7%, 21.2%, 29.6%, and 47.8% were observed relative to control. This suggests that PHF significantly inhibits mitochondrial Ca^{2+} uptake in a concentration-dependent manner, thereby potentially protecting against Ca^{2+} overload-induced dysfunction. Since tight regulation of mitochondrial Ca^{2+} levels is essential for maintaining optimal ATP production and preventing pathological ROS generation, the observed inhibitory effect of PHF may confer cytoprotective benefits, particularly under

conditions of elevated cytosolic Ca^{2+} triggered by hormones, neurotransmitters, growth factors, or other signaling molecules (**Figure 5**).

Mechanistically, Ca^{2+} influences energy metabolism both directly - via allosteric activation of target enzymes - and indirectly, through Ca^{2+}-dependent kinases and phosphatases. While moderate mitochondrial Ca^{2+} uptake enhances oxidative phosphorylation, both deficiency and excess of Ca^{2+} can impair ATP synthesis and hydrolysis. It is therefore postulated that PHF may help maintain intracellular Ca^{2+} at physiological levels, supporting mitochondrial efficiency while preventing Ca^{2+}-induced oxidative stress and apoptosis.

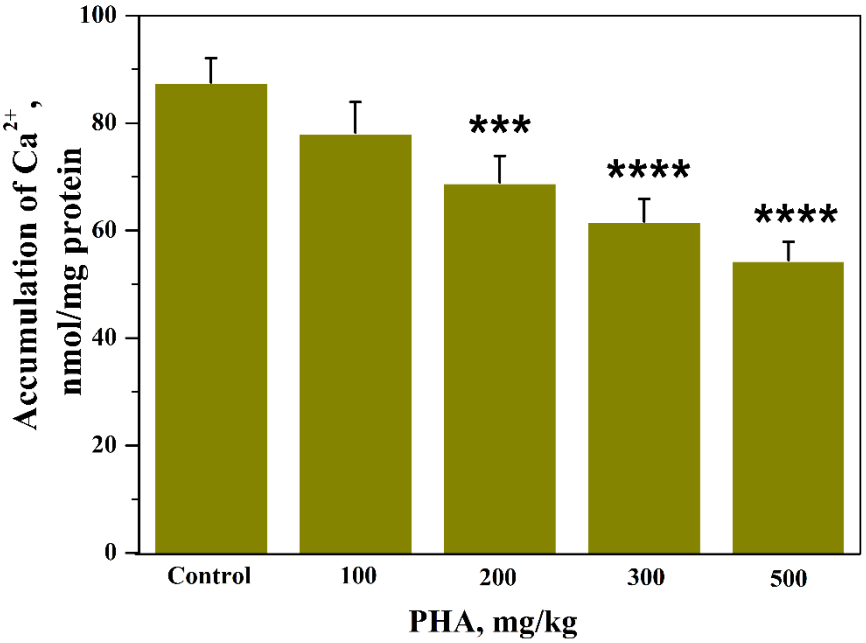

Figure 5 Changes in Ca^{2+} transport in rat liver mitochondria under the influence of PHA (n = 4).

In conclusion, the administration of 3,5,7,2',6'-pentahydroxyflavanone reduces mitochondrial Ca^{2+} accumulation *in vivo*, in proportion to the applied dose. This property may underlie its broader bioenergetic and cytoprotective effects.

Pharmacokinetics and ADMET profile of 3,5,7,2',6'-pentahydroxyflavanone

A comprehensive ADMET analysis was performed to assess the drug-likeness and pharmacokinetic behavior of 3,5,7,2',6'-pentahydroxyflavanone (PHF) (**Figure 6**). The compound displayed favorable physicochemical properties, with a molecular weight of 286.05 Da and a moderate lipophilicity (logP = 2.25; $logD_{7.4}$ = 2.32), falling within Lipinski's Rule of 5 criteria. Its topological polar surface area (TPSA) of 111.13 Å² and high hydrogen bond donor/acceptor count (nHD = 4, nHA = 6) suggest good solubility but moderate membrane permeability [33].

Absorption

The predicted Caco-2 and MDCK permeability values (–5.086 and –4.771, respectively) indicate low passive intestinal absorption. Despite this, the compound demonstrated good oral bioavailability potential with predicted F30% (+++) and F50% (+++), indicating that systemic exposure may still be achieved. PHF is not a known substrate or inhibitor of P-glycoprotein (P-gp), which may reduce the risk of efflux-mediated absorption limitation.

Distribution

PHF exhibited high plasma protein binding (PPB = 95.2%) and moderate volume of distribution (VDss = –0.316 L/kg), suggesting a preference for vascular compartment distribution. The unbound fraction (Fu = 6.9%) reflects sufficient free drug availability for pharmacological action. It also demonstrated inhibitory potential against several hepatic transporters, including OATP1B1, OATP1B3, BCRP, and MRP1, but not

BSEP, implying potential for drug–drug interactions at the hepatic level [34].

Metabolism

PHF is predicted to be both a substrate and inhibitor of several cytochrome P450 isoforms, particularly CYP1A2, CYP2D6, CYP3A4, CYP2C9, and CYP2B6, indicating involvement in complex metabolic pathways. It is not a substrate of CYP3A4 nor CYP2C19, which may limit first-pass metabolism by these enzymes. However, the predicted low HLM (human liver microsomal) stability suggests a short metabolic half-life.

Excretion

The compound demonstrated a low plasma clearance rate (CL = 1.522 mL/min/kg) and a short elimination half-life ($T_{1/2}$ = 1.547 h), consistent with its limited metabolic stability. These properties may necessitate frequent dosing or formulation modification for sustained plasma levels.

Toxicity profile

Toxicological evaluation revealed low predicted cardiotoxicity (hERG blockers = 0.044), moderate hepatotoxicity (0.452), and genotoxicity potential (0.956). The compound showed no AMES mutagenicity (0.657), moderate oral acute toxicity (rat LD_{50} = 0.507), and relatively low neuro-, nephro-, and hematotoxicity risks. PHF triggered skin sensitization (0.655) but was non-carcinogenic in genotoxic models [35].

In Tox21 assays, PHF was predicted to be positive for aryl hydrocarbon receptor (NR-AhR) activation and oxidative stress response (SR-ARE), as well as MMP pathway activation (SR-MMP +++), which may reflect redox-related biological activity.

Medicinal chemistry alerts

The compound passed major drug-likeness filters including Lipinski, Pfizer, GSK, and Golden Triangle rules, with a QED score of 0.546, indicating moderate drug-like quality. No PAINS alerts or significant reactive functionalities were detected, supporting its suitability for further development. The compound showed no signs of aggregation, chelation, or fluorescence interference, minimizing the risk of assay artifacts [36].

Metabolism

PHF is predicted to be both a substrate and inhibitor of several cytochrome P450 isoforms, particularly CYP1A2, CYP2D6, CYP3A4, CYP2C9, and CYP2B6, indicating involvement in complex metabolic pathways. It is not a substrate of CYP3A4 nor CYP2C19, which may limit first-pass metabolism by these enzymes. However, the predicted low HLM (human liver microsomal) stability suggests a short metabolic half-life [37,38].

Excretion

The compound demonstrated a low plasma clearance rate (CL = 1.522 mL/min/kg) and a short elimination half-life ($T_{1/2}$ = 1.547 h), consistent with its limited metabolic stability. These properties may necessitate frequent dosing or formulation modification for sustained plasma levels [39].

Toxicity profile

Toxicological evaluation revealed low predicted cardiotoxicity (hERG blockers = 0.044), moderate hepatotoxicity (0.452), and genotoxicity potential (0.956). The compound showed no AMES mutagenicity (0.657), moderate oral acute toxicity (rat LD_{50} = 0.507), and relatively low neuro-, nephro-, and hematotoxicity risks. PHF triggered skin sensitization (0.655) but was non-carcinogenic in genotoxic models.

In Tox21 assays, PHF was predicted to be positive for aryl hydrocarbon receptor (NR-AhR) activation and oxidative stress response (SR-ARE), as well as MMP pathway activation (SR-MMP +++), which may reflect redox-related biological activity.

Medicinal chemistry alerts

The compound passed major drug-likeness filters including Lipinski, Pfizer, GSK, and Golden Triangle rules, with a QED score of 0.546, indicating moderate drug-like quality. No PAINS alerts or significant reactive functionalities were detected, supporting its suitability for further development. The compound showed no signs of aggregation, chelation, or fluorescence interference, minimizing the risk of assay.

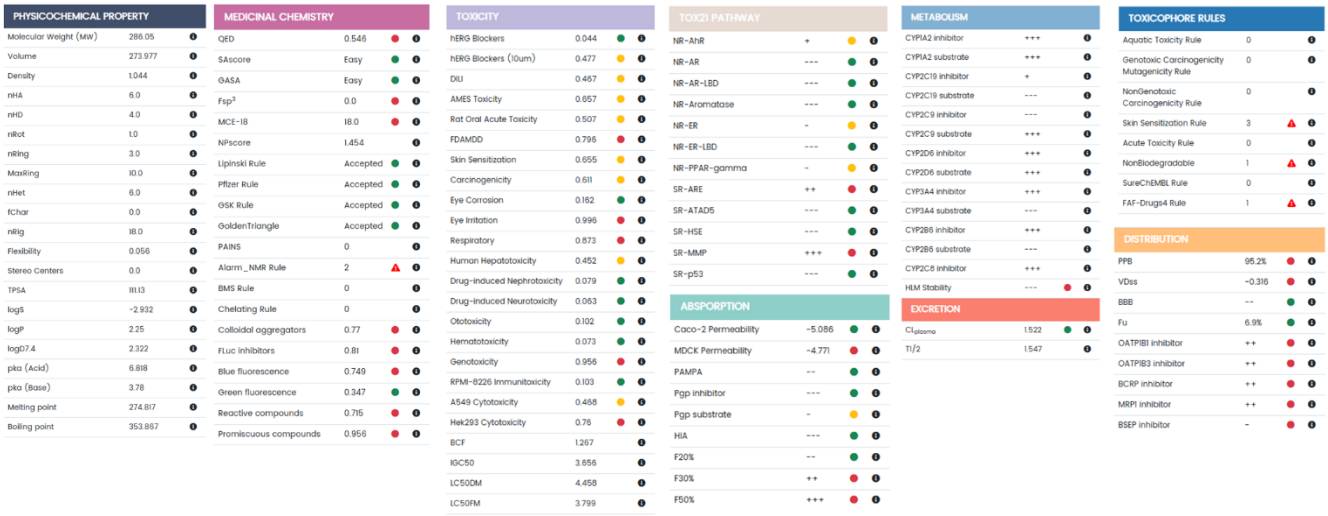

Figure 6 *In silico* prediction of pharmacokinetic and ADMET properties of 3,5,7,2',6'-pentahydroxyflavanone (PHF) based on ADMETlab analysis. The compound demonstrates acceptable physicochemical characteristics, complies with major medicinal chemistry filters (Lipinski, Pfizer, GSK), and shows moderate lipophilicity (logP = 2.25). Absorption parameters suggest low permeability, while distribution is characterized by high plasma protein binding (PPB = 95.2%) and moderate unbound fraction (Fu = 6.9%). The compound acts as both a substrate and inhibitor of multiple CYP450 isoforms, particularly CYP1A2, CYP2D6, and CYP3A4. Toxicological indicators reveal low predicted cardiotoxicity (hERG), moderate hepatotoxicity, and potential for genotoxicity. Colored dots indicate risk levels: Green (favorable), yellow (moderate), red (unfavorable), and red triangles (alerts).

Discussion

The present study comprehensively examined the mitochondrial, enzymatic, and pharmacokinetic effects of 3,5,7,2',6'-pentahydroxyflavanone (PHF), a polyhydroxylated flavonoid with potential bioenergetic and cytoprotective properties. Our findings demonstrate that PHF exerts a substrate-specific influence on mitochondrial respiration, selectively modulates key oxidative enzymes, stabilizes inner membrane integrity under biochemical stress, and exhibits a favorable ADMET profile.

PHF affected mitochondrial oxidative phosphorylation in a concentration- and substrate-dependent manner. When glutamate - a NAD-linked substrate - was used, PHF at higher doses (40 - 60 μg/mg protein) induced a modest decline in V_3-state respiration and an elevation in V_4-state respiration, leading to a mild uncoupling effect reflected by decreased RCR and ADP/O ratios. This suggests a partial dissociation between electron transport and ATP synthesis, consistent with limited interference in NADH-mediated energy metabolism.

In contrast, succinate-supported respiration (FAD-linked) showed a more pronounced dose-dependent inhibition by PHF, with reductions in V_3, V_4, and uncoupled states. Interestingly, this inhibition was accompanied by a paradoxical increase in the ADP/O ratio, implying an enhanced ATP yield per oxygen atom consumed. Such an effect may reflect increased coupling efficiency or altered proton flux across the mitochondrial membrane. The selective suppression of succinate-driven respiration suggests a preferential targeting of FAD-linked complexes, possibly due to their peripheral localization within the inner membrane.

Biochemical assays confirmed this selective inhibition. While NADH dehydrogenase and rotenone-sensitive NADH oxidase remained largely unaffected, both succinate dehydrogenase and succinate oxidase activities were significantly inhibited at higher PHF concentrations. This pattern supports the hypothesis that PHF may exert its effects by stabilizing specific membrane regions or binding to regulatory sites of FAD-dependent complexes. However, due to the steep nature of inhibition within the tested dose range, reliable IC_{50} values could not be determined. As such, "n.s." (not specified) was indicated in the tables. Future studies involving a wider range of PHF concentrations will be

required to determine kinetic constants and dose-response parameters.

Further experiments revealed that PHF delays the time-dependent activation of NADH oxidase during incubation, and protects against activity loss under thermal stress. More strikingly, PHF significantly mitigated enzymatic degradation induced by phospholipase A_2 (PLA_2), a membrane-damaging enzyme. The preservation of NADH and succinate oxidase activities in PHF-treated mitochondria underscores the compound's membrane-stabilizing effect. Given that succinate dehydrogenase is more peripherally located than NADH dehydrogenase, its higher sensitivity to PLA_2 was expected. PHF's ability to shield these complexes may involve interactions with membrane lipids or modulation of lipid bilayer fluidity.

PHF also influenced mitochondrial calcium handling. *In vivo* studies demonstrated a dose-dependent inhibition of Ca^{2+} accumulation by isolated liver mitochondria, suggesting that PHF may prevent mitochondrial calcium overload - a key trigger of oxidative stress, swelling, and apoptosis. This effect likely contributes to the compound's cytoprotective potential, especially under conditions of hormonal or excitotoxic stimulation.

Lastly, the compound exhibited an overall favorable pharmacokinetic and toxicological profile. Although passive permeability was low, PHF showed acceptable oral bioavailability indices and no P-glycoprotein efflux liability. It demonstrated high plasma protein binding, moderate distribution, and low clearance. The compound also passed several medicinal chemistry filters, with no alerts for PAINS, aggregation, or reactivity. Predicted toxicity endpoints were within acceptable limits, although mild hepatotoxicity and genotoxicity potential warrant further investigation.

In conclusion, PHF emerges as a selective modulator of mitochondrial bioenergetics, particularly effective in stabilizing FAD-linked enzymes and preventing membrane disruption under stress conditions. Its dual actions - on respiration efficiency and membrane protection - position PHF as a promising candidate for further development as a mitochondria-targeted cytoprotective agent.

Conclusions

In this study, 3,5,7,2',6'-pentahydroxyflavanone (PHF) demonstrated multifaceted bioenergetic and protective effects on mitochondrial function. PHF selectively inhibited succinate dehydrogenase and oxidase activity while preserving NAD-linked respiratory pathways, indicating a targeted modulation of FAD-dependent enzymes. It also improved oxidative phosphorylation efficiency and reduced mitochondrial calcium overload, suggesting a role in maintaining mitochondrial homeostasis under stress.

Importantly, PHF protected mitochondrial membranes from enzymatic and thermal disruption, likely through stabilization of the inner membrane structure. These findings position PHF as a promising mitochondria-targeted cytoprotective agent.

Given the central role of mitochondrial dysfunction in oxidative stress-related pathologies - such as neurodegenerative diseases, cardiovascular disorders, and metabolic syndromes - PHF holds strong potential as a lead compound for the development of therapeutics aimed at restoring mitochondrial integrity and function in such conditions. Future studies should focus on elucidating its molecular targets, long-term safety, and efficacy in disease models.

Declaration of generative AI in scientific writing

"Only minimal assistance was used from QuillBot for paraphrasing selected sentences. All scientific content, interpretation, and conclusions were developed independently by the authors."

CRediT author statement

Muxtorjon Mamajanov conceived and supervised the study. **Izzatullo Abdullaev** prepared the initial draft. **Gayrat Sotimov** contributed to methodology and revision. **Sadbarxon Mavlanova, Qahramon Niyozov, Mirzohid Mirzaolimov, Akmal Najimov, and Elmurod Mirzaolimov** performed experiments and data analysis. **Mansur Raximberganov** supported administration and funding. **Ulugbek Abdullayev** contributed to conceptualization and supervision. All authors reviewed and approved the final manuscript.

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