TiS Trends in Sciences
TRENDS IN SCIENCES 2027; 24(2): 13371
SPRINT Scholarly Publishing and Repository Integration Center
https://doi.org/10.48048/tis.2027.13371
RESEARCH ARTICLE

Integrated *In Vitro* and *In Silico* Evaluation of the Vasorelaxant Mechanisms of Pulicarin and Chrysoeriol via Modulation of Ca^{2+} Entry in Rat Aortic Smooth Muscle

Anvarbek Bogbekov[1,*], Izzatullo Abdullaev[2], Sirojiddin Omonturdiev[2,5], Shakhlokhon Ismailova[7], Azamjon Mukhtorov[1], Nurali Ergashev[6], Esokhon Komilov[5,6], Khaydarali Ruziboev[1], Anvar Zaynabiddinov[4], Ulugbek Gayibov[2], Elmira Nazirova[2], Kamila Eshbakova[3] and Takhir Aripov[2]

[1]*Department of Biophysics and Bioinformatics, National University of Uzbekistan, Tashkent, Uzbekistan*
[2]*Laboratory of Plant Cytoprotectors, Institute of Bioorganic Chemistry named after A.S. Sadykov Academy of Sciences of the Republic of Uzbekistan, Tashkent, Uzbekistan*
[3]*The Institute of the Chemistry of Plant Substances named acad. S.Yu. Yunusov of the Academy of Sciences of the Republic of Uzbekistan, Tashkent, Uzbekistan*
[4]*Department of Human Physiology and Safety, Andijan State University, Andijan, Uzbekistan*
[5]*National University of Uzbekistan named after Mirzo Ulugbek, Physiology of Humans and Animals, Faculty of Biology and Ecology, University Street, Tashkent, Uzbekistan*
[6]*Institute of Biophysics and Biochemistry, National University of Uzbekistan, Tashkent, Uzbekistan*
[7]*Department of Human and Animal Physiology, Namangan, Uzbekistan*

([*]Corresponding author's e-mail: anvarbekbogbekov23@gmail.com)

Received: 16 January 2026, Revised: 7 March 2026, Accepted: 14 March 2026, Published: 10 September 2026

Abstract

Flavonoids are increasingly recognized for their vasoprotective properties, yet the precise molecular mechanisms underlying their vascular actions remain insufficiently defined. This study examined the vasorelaxant effects of two naturally occurring flavonoids, pulicarin and chrysoeriol, combining functional in vitro assays with in silico molecular docking. In isolated rat aortic rings, both compounds produced a clear concentration-dependent inhibition of KCl (50 mM)-induced contraction, a model that selectively activates voltage-dependent Ca^{2+} channels (VDCCs). Pulicarin reduced contraction by 7.9 ± 3.2% at 5 µM and 72.8 ± 3.1% at 30 µM (IC_{50} = 17.58 µM), whereas chrysoeriol produced 3.1 ± 3.9% and 74.8 ± 3.2% relaxation at the same concentrations (IC_{50} = 18.18 µM). Co-incubation with verapamil further diminished KCl-evoked contraction, indicating partial overlap with classical VDCC blockade. In phenylephrine-stimulated rings, both flavonoids markedly suppressed receptor-operated Ca^{2+} channel (ROCC)-mediated contraction, reducing phentolamine-resistant tone to 37.5 ± 4.2% and 39.7 ± 3.6%, respectively. In endothelium-intact tissues, pulicarin and chrysoeriol produced robust relaxation (74.6 ± 3.3% and 72.4 ± 3.6%), which was significantly attenuated in endothelium-denuded preparations and in the presence of L-NAME, demonstrating partial dependence on endothelial nitric oxide signaling. Docking analyses revealed high-affinity binding of both flavonoids to VDCC pore-forming regions and regulatory residues, supporting their inhibitory action on Ca^{2+} influx. Collectively, these findings indicate that pulicarin and chrysoeriol exert potent vasorelaxant effects via combined VDCC and ROCC inhibition together with endothelium-dependent NO signaling.

Keywords: Vasorelaxation, Voltage-dependent Ca^{2+} channels, Receptor-operated Ca^{2+} channels, Pulicarin, Chrysoeriol, Aortic smooth muscle

Introduction

Cardiovascular diseases (CVDs) remain the primary cause of mortality worldwide and are strongly associated with endothelial dysfunction, increased vascular smooth muscle contraction, and dysregulation of vascular tone. Hypertension, in particular, is characterized by enhanced vasoconstrictor signaling, reduced nitric oxide (NO) bioavailability, excessive intracellular Ca^{2+} accumulation, and impaired activity of endothelial-derived relaxing factors. These pathophysiological alterations increase peripheral vascular resistance and promote long-term cardiovascular complications [1]. Therefore, the search for novel vasorelaxant molecules capable of modulating vascular reactivity continues to be a major focus of modern pharmacology. Medicinal plants of the Asteraceae family have long been used in traditional medicine across Asia for treating inflammatory, hepatic, and circulatory disorders. *Pulicaria gnaphalodes* and *Inula caspica* are 2 ethnopharmacologically important species known for their rich phytochemical composition, particularly flavonoids, terpenoids, and phenolic acids. Despite numerous reports on their antioxidant, antimicrobial, and anti-inflammatory activities, their direct effects on vascular function and potential cardiovascular benefits remain poorly studied. Among their secondary metabolites, pulicarin (isolated from *Pulicaria gnaphalodes*) and xrezeario-type flavonoids (characteristic for *Inula caspica*) have recently gained scientific interest due to their structural similarity to known vasorelaxant and cardioprotective flavonoids. Flavonoids are well recognized for their ability to modulate vascular tone through multiple mechanisms, including activation of endothelial NO synthase, inhibition of voltage-dependent Ca^{2+} channels, opening of potassium channels, suppression of reactive oxygen species, and interference with G-protein–mediated vasoconstrictor pathways. However, no experimental or computational studies have yet explored the vascular actions of pulicarin and xrezeario [2]. Advances in computational chemistry have enabled in silico molecular docking to become a powerful complementary tool for predicting the interaction of natural compounds with molecular targets that regulate vascular tone. Docking approaches allow the identification of ligand-binding conformations, estimation of binding affinity (ΔG), detection of key residue interactions, and prediction of potential biological pathways. When combined with classical in vitro aortic ring assays, *in silico* models provide mechanistic insight and strengthen the interpretation of pharmacological outcomes. Given the traditional medicinal relevance of *Pulicaria* and *Inula* species, the structural diversity of their flavonoids, and the global need for safer, plant-derived antihypertensive agents, a combined pharmacological and computational investigation is warranted [3].

Materials and methods

Chemicals

Extracts obtained from *Pulicaria gnaphalodes* and *Inula caspica* were utilized as the primary plant materials in the present study. The authenticated plant samples were supplied by “Bioton” LTD, located in Tashkent, Uzbekistan. Additional chemical reagents required for the pharmacological experiments—including phenylephrine, phentolamine, and verapamil—were procured from Sigma-Aldrich Chemie, a division of Sigma-Aldrich (St. Louis, MO, USA). *Pulicaria gnaphalodes* and *Inula caspica* are herbaceous species belonging to the Asteraceae family and are widely distributed across Central Asia, particularly in arid and semi-arid regions of Uzbekistan, Turkmenistan, Iran, and Kazakhstan. These plants typically grow in dry steppes, foothill zones, sandy soils, and disturbed roadside habitats, where they are naturally adapted to hot and drought-prone environments [4]. *Pulicaria gnaphalodes* is characterized by its narrow gray-green leaves and compact clusters of small yellow flower heads, whereas *Inula caspica* displays elongated lanceolate leaves and bright yellow capitulate inflorescences, a common morphological feature of the Asteraceae family. Both species have been traditionally used in Central Asian and Middle Eastern folk medicine for treating inflammatory conditions, digestive disturbances, respiratory disorders, and various types of wounds. Phytochemical investigations show that Pulicaria and Inula species contain diverse bioactive secondary metabolites, including flavonoids, sesquiterpene lactones, and phenolic acids [5]. Of particular relevance are pulicarin, a representative flavonoid of Pulicaria gnaphalodes, and xrezeario-type

flavonoids commonly found in Inula caspica. These compounds have demonstrated antioxidant, antimicrobial, and anti-inflammatory properties in previous studies, and emerging evidence suggests that they may also possess cardiovascular potential by modulating vascular tone and reducing oxidative stress. Owing to their chemical composition and pharmacological promise, both plants represent valuable sources for experimental vascular research [6].

- *Angiosperms*
  - *Asterales* Link
    - *Asteraceae* Giseke
    - *Asteroideae* Lindl.
    - *Inuleae* Cass.
    - *Inula* L.
    - *Inula caspica* F.K.Blum ex Ledeb.



Figure 1 *Inula caspica*: Botanical features and regional distribution.

- *Angiosperms*
  - *Asterales* Link
    - *Asteraceae* Giseke
    - *Asteroideae* Lindl.
    - *Inuleae* Cass.
    - *Pulicaria* Gaertn.
    - *Pulicaria gnaphalodes* (Vent.) Boiss.



Figure 2 *Pulicaria gnaphalodes* (Vent.) Boiss: Botanical features and regional distribution.

Animal ethics

All surgical and experimental protocols were evaluated and approved by the Institutional Animal Care and Use Committee. Animals were kept in a controlled vivarium environment with regulated temperature (22 ± 2 °C), relative humidity of 55% - 65%, and ad libitum access to standard laboratory feed and water. Animal housing, handling, and experimental procedures were carried out in strict accordance with the European Directive 2010/63/EU for the protection of animals used in scientific research. Ethical authorization for this study was provided by the Animal Ethics Committee of the Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan (Protocol No. 133/1a/h, approved on August 4, 2016).

Tissue preparation

All procedures involving animals were carried out in accordance with institutional guidelines and were approved by the local animal care and use committee.

The rats were housed under standard laboratory conditions, with controlled humidity (55% - 65%) and a stable temperature of 22 ± 2 °C. Euthanasia was performed by cervical dislocation, after which the thoracic aorta was rapidly removed and transferred to a 5 mL organ bath filled with Krebs–Henseleit solution (**Figure 2**). The composition of the solution included 120.4 mM NaCl, 5 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.5 mM glucose, and HEPES, with the pH adjusted to 7.4. In certain experiments, a Ca^{2+}-free Krebs solution containing 1 mM EGTA was used instead [8]. The organ bath was maintained at 37 °C using a DAIHAN WATER BATH ultrathermostat and continuously aerated with a carbogen gas mixture (95% O_2 and 5% CO_2). The isolated aorta was carefully freed from surrounding fat and connective tissue and then cut into 3 - 4 mm segments for subsequent vascular reactivity studies (FAO. Directive 2010/63/EU on the protection of animals used for scientific purposes. Off J Eur Union, 2010).

Aortic-ring contraction studies

The aortic rings were mounted on a Radnoti isometric force transducer using platinum wire hooks and allowed to stabilize for 60 min prior to recording. Each preparation was adjusted to a resting tension of 1 g (10 mN) (**Figure 3**). Contractile responses generated by the vascular rings were transmitted to a signal amplifier and continuously monitored on a computer through a Go-Link digital converter. Data acquisition and analysis were performed using OriginLab OriginPro v.9.0 SR1 (EULA, Northampton, MA, USA). The isometric tension values (mN) recorded under in vitro conditions were processed and expressed as percentages of the maximal response, following established methodologies [9].

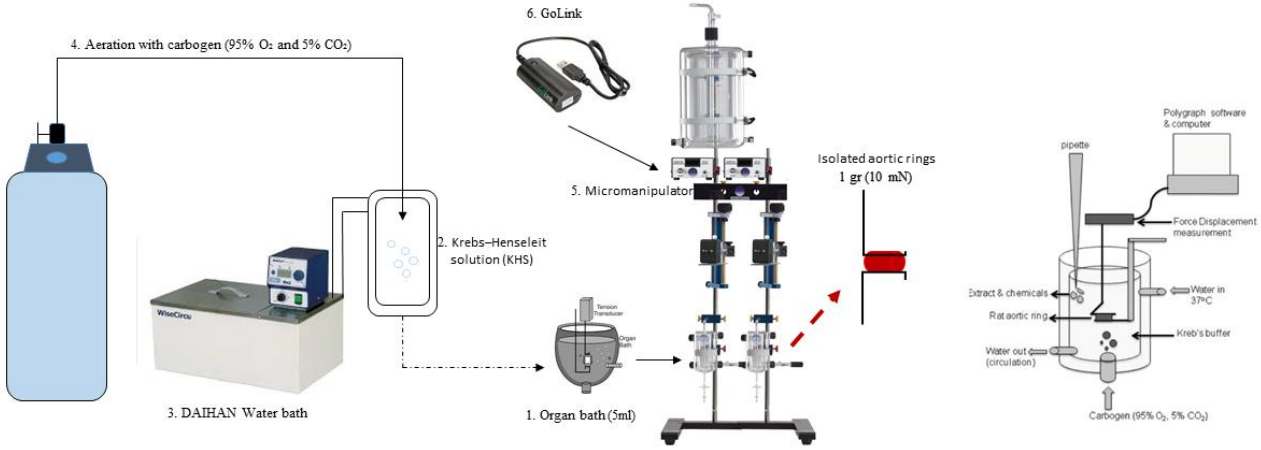


Figure 3 A schematic illustration of the apparatus used to regulate and measure the isometric contraction of isolated rat aortic vascular muscle. 1) The organ bath (5 mL) circulates through a specialized reservoir. 2) The Krebs-Henseleit solution ensures physiological conditions. 3) A thermostat maintains a constant physiological temperature. 4) The system is aerated with a gas mixture of 5 % CO_2 and 95 % O_2. The contractile activity of the aortic preparation is secured in the experimental chamber. 5) An isometric transducer (Grass Instrument, USA) monitors the contraction, and 6) GoLink devices provide signal amplification.

Molecular docking "software and databases"

All computational tools employed in this study are freely accessible for academic research. Three-dimensional structures of the target proteins were retrieved from the Protein Data Bank (PDB), an internationally recognized repository for structural data of biological macromolecules Berman *et al.* 2000. Because calcium homeostasis plays a central role in vascular smooth muscle contraction and relaxation, several Ca^{2+}-regulating proteins were selected for *in silico* analysis. The following crystallographic structures were downloaded from the PDB database: The L-type voltage-gated calcium channel Cav1.2 (PDB ID: 6jp5), the R-type calcium channel Cav2.3 (PDB ID:

7xlq), the sodium–calcium exchanger NCX1 (PDB ID: 8sgi), the cardiac ryanodine receptor RyR2 (PDB ID: 5c33), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase SERCA (PDB ID: 6rb2). Chemical structures of the flavonoid compounds examined in this work, including pulicarin and xrezeario, as well as the reference standard molecules, were obtained from the PubChem database [10].

PubChem serves as an extensive chemical and bioinformatics platform that integrates detailed pharmaceutical, pharmacological, and structural information for small molecules and their molecular targets. Each chemical entry contains multiple fields of curated data, including physicochemical properties, biological activities, and protein-interaction information. All downloaded protein structures (PDB format) were inspected and prepared using PyMOL (version 1.2), a Python-based molecular visualization and editing software (www.pymol.org). PyMOL was also used for visualizing and interpreting the resulting ligand–protein docking poses. Molecular docking simulations were performed using AutoDock 4.2, developed by The Scripps Research Institute (www.scripps.edu) [11].

AutoDock Tools (ADT) served as the graphical interface to prepare the macromolecules and ligands, assign partial charges, define rotatable bonds, set grid box parameters, and manage docking configurations. AutoDock provides a robust platform for predicting the most favorable binding conformations and interactions between small molecules, such as flavonoids, and target proteins with known three-dimensional structures [12].

Preparation of ligand and protein files

Before docking could be performed, the retrieved PDB structures required preprocessing, as files obtained from the Protein Data Bank are typically not directly suitable for docking studies. Structural issues such as missing hydrogen atoms, the presence of crystallographic water molecules, co-crystallized ligands, ions, and additional non-relevant heteroatoms necessitated correction. Each protein structure was imported into AutoDock Tools (ADT). Crystallographic water molecules and non-essential heteroatoms were removed. Co-crystallized ligands were deleted prior to docking calculations; however, they were retained separately for validation purposes when applicable. All crystallographic heteroatoms, including calcium ions and other non-protein components, were removed during receptor preparation to avoid potential artificial electrostatic bias in docking simulations under the rigid receptor model. This approach ensured consistent electrostatic treatment across all targets. The structures were visually inspected in PyMOL to evaluate missing residues, and no critical residues within the functional or channel pore regions were absent in the selected models. Polar hydrogen atoms were added, and Kollman united atom charges were assigned to the protein structures to ensure appropriate electrostatic representation. Protonation states were adjusted to approximate physiological pH (7.4) conditions according to the standard procedures implemented in AutoDock Tools. The prepared receptor file was then saved in the .pdbqt format, which includes atom types and partial charge information required for docking calculations. Ligand structures underwent a comparable preparation process. The molecules were loaded into ADT, hydrogens were added, and Gasteiger charges were assigned. Nonpolar hydrogens were merged according to standard AutoDock protocols. ADT automatically identified the appropriate rotatable bonds and root atom for each ligand. The final ligand files were exported in the .pdbqt format, containing structural coordinates, rotatable bond definitions, and assigned partial charges necessary for docking simulations [12].

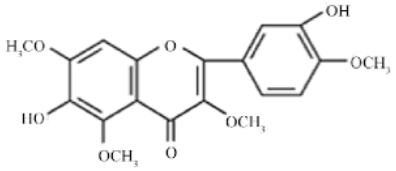
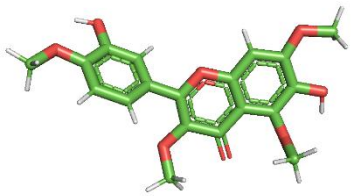
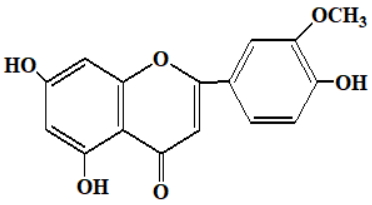
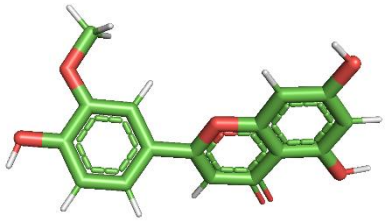
Calculation of inhibition constant (Ki) from binding energy

In molecular docking analyses, the interaction strength between a ligand and its target protein is commonly represented as the binding free energy (ΔG), expressed in kcal/mol. The corresponding inhibition constant (Ki) can be estimated from the calculated binding energy by applying the standard thermodynamic equation:

$$\mathrm{Ki} = \mathrm{e}\text{^}\frac{\Delta G \times 1000}{R \times T}$$

- Ki is the inhibition constant (in mol/L).
- ΔG is the binding free energy (in kcal/mol).
- R is the universal gas constant = 1.987 cal/(mol·K).
- T is the temperature in Kelvin (usually 298.15 K).
- The factor 1,000 converts kcal to cal [13].

Table 1 2D and 3D structures of ligands selected for molecular docking analysis.

Ligands	2D	3D
Pulicarin		
Chrizoeriol		

Statistics

Statistical analyses and graphical outputs were generated using OriginPro 8.5 software (Microsoft, USA). Contractile responses were expressed as percentages relative to the maximal tension produced by phenylephrine (10 mM) or KCl (50 mM). Data are reported as mean values obtained from 5 to 8 independent aortic ring preparations (n = 5 - 8). For comparisons involving paired datasets, a paired t-test was applied, whereas differences between separate experimental groups were evaluated using an unpaired t-test. A probability level of $p < 0.05$ was considered statistically significant

Results and discussion

***In vitro* experiments in rats aortic smooth muscle**

Dose-dependent activity of voltage-gated L-type and R-type Ca^{2+} channels using specific blockers

It is well known that contraction of aortic smooth muscle induced by KCl (50 mM) is mediated by the activation of voltage-dependent Ca^{2+} channels located in the plasma membrane. Elevation of extracellular K^+ reduces the membrane potential, leading to membrane depolarization, the opening of voltage-gated Ca^{2+} channels, and a subsequent increase in intracellular Ca^{2+} concentration, ultimately enhancing contractile force [13]. Based on this mechanism, the present study evaluated the vasorelaxant effect of the flavonoid pulicarin on KCl-induced contraction of isolated rat aortic rings. Pulicarin produced a clear concentration-dependent relaxation within the 5 - 30 µM range. At 5 µM, pulicarin reduced the amplitude of contraction by 7.9 ± 3.2% relative to the control, while at 30 µM the inhibitory effect increased markedly to 72.8 ± 3.1%. The half-maximal inhibitory concentration (IC50) of pulicarin was calculated to be 17.58 µM, indicating a moderate to strong ability to suppress high-K^+-induced vascular contraction. These findings suggest that pulicarin effectively attenuates depolarization-induced Ca^{2+} influx and may act as a potential modulator of voltage-dependent Ca^{2+} entry in vascular smooth muscle (**Figure 3**).

In a separate series of experiments, the vasorelaxant effect of the flavonoid chrysoeriol was evaluated on KCl (50 mM)-induced contraction of isolated rat aortic rings. Chrysoeriol produced a marked concentration-dependent relaxation in the range of 5 - 30 µM. At 5 µM, chrysoeriol decreased the amplitude of KCl-induced contraction by 3.1 ± 3.9% compared with the control, whereas at 30 µM the inhibitory effect increased substantially, reaching 74.8 ± 3.2%. The calculated half-maximal inhibitory concentration (IC50) of chrysoeriol was 18.18 µM, indicating its strong ability to suppress depolarization-induced vascular contraction (**Figure 3**).

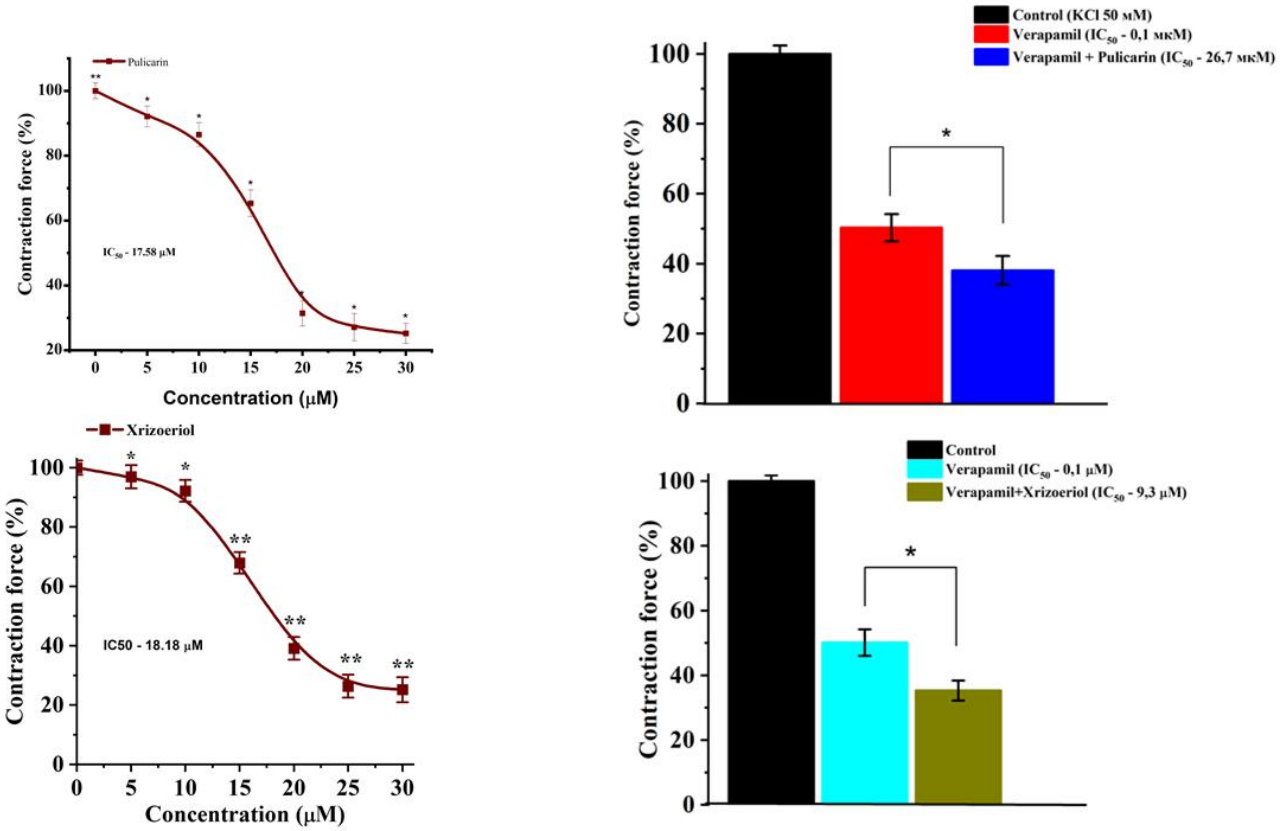


Figure 3 Pulicarin and chrysoeriol suppress KCl-evoked vascular contraction and enhance verapamil-mediated Ca^{2+} channel blockade.

In the next series of experiments, the involvement of voltage-dependent Ca^{2+} channels in the vasorelaxant effects of the investigated flavonoids was assessed by examining their interaction with verapamil, a specific blocker of these channels. For this purpose, a concentration of verapamil (0.1 μM) that produced approximately half-maximal inhibition of KCl (50 mM)-induced contraction was used. When aortic rings precontracted with KCl were incubated with verapamil (0.1 μM) together with pulicarin (17.58 μM) or chrysoeriol (18.18 μM, IC50), an additional reduction in contractile amplitude by 11.9 ± 4.1% and 14.7 ± 3.1%, respectively, was observed (**Figure 3**). These results indicate that both flavonoids exert potent vasorelaxant activity and significantly suppress high-K^+-induced contraction. The observed relaxation is likely related to the inhibition of voltage-dependent Ca^{2+} channels located in the sarcolemma of vascular smooth muscle cells, leading to a reduction in Ca^{2+} entry. The findings obtained with verapamil further support the hypothesis that pulicarin and chrysoeriol may act, at least in part, through interference with voltage-dependent Ca^{2+} channel function [14].

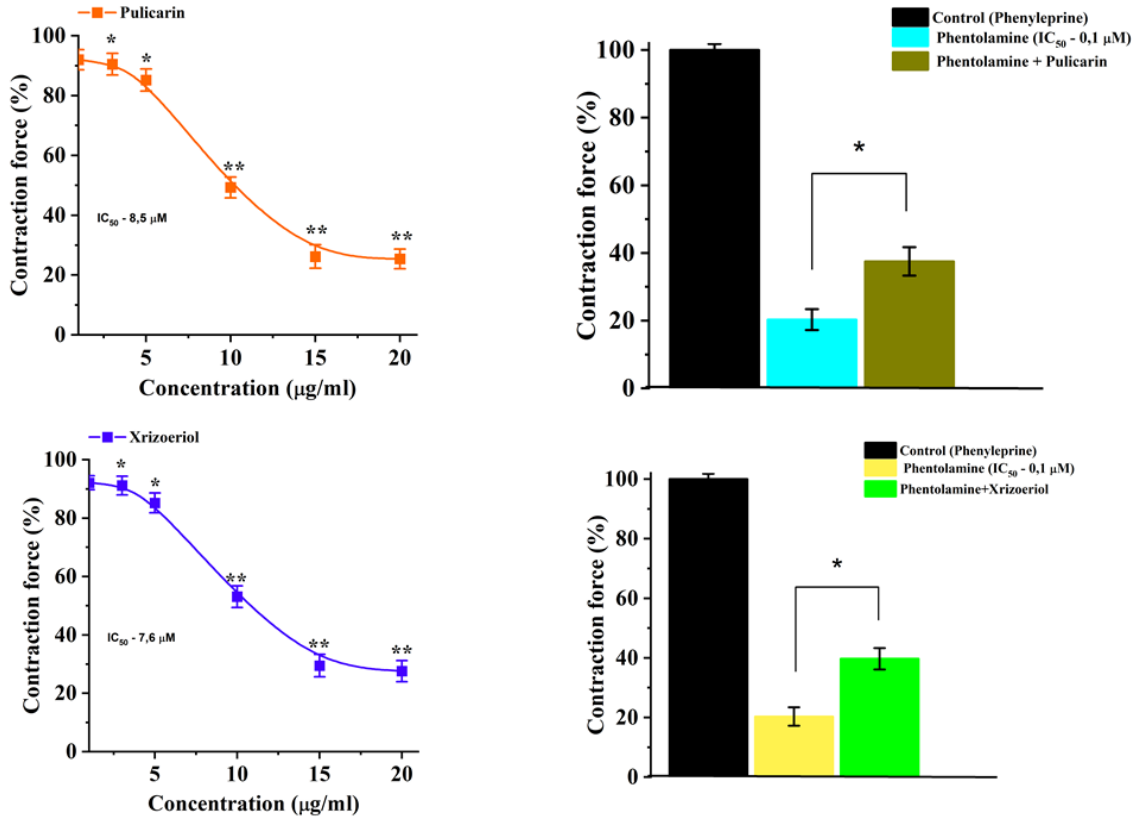


Figure 4 Concentration-dependent inhibition of phenylephrine-induced contraction by pulicarin and chrysoeriol and their interaction with phentolamine in isolated rat aortic rings.

These findings suggest that the relaxant effect of the extract may be mediated, at least in part, through the inhibition of receptor-operated Ca^{2+} channels. To further examine this possibility, the actions of pulicarin and chrysoeriol were compared with those of phentolamine (10 μM), a well-established α-adrenoceptor antagonist. As expected, phentolamine alone markedly reduced phenylephrine-induced contraction, producing an inhibition of 81.7 ± 3.1%. Under these conditions, when pulicarin and chrysoeriol were applied in the presence of phentolamine, the remaining contractile response decreased to 37.5 ± 4.2% and 39.7 ± 3.6%, respectively. These results indicate that both flavonoids significantly diminish receptor-operated contractile activity. Taken together, the data support the conclusion that the vasorelaxant properties of pulicarin and chrysoeriol are associated with the blockade of receptor-operated Ca^{2+} channels, a notion further strengthened by the findings obtained using the α-adrenoceptor antagonist phentolamine [15].

Study the role of endothelium in the relaxant effect of pulicarin and chrizoeriol

The endothelium, a single-cell layer lining the inner surface of blood vessels, plays a central role in maintaining vascular homeostasis and regulating blood flow. One of its most important functions is the production of signaling molecules such as nitric oxide (NO), a potent vasodilator essential for the control of vascular tone and blood pressure. Endothelial dysfunction, resulting from an imbalance in vasoactive mediators, is closely associated with the development of cardiovascular diseases including hypertension and atherosclerosis [16]. Early endothelial injury can initiate a cascade of pathological events that eventually culminate in widespread vascular impairment. Nitric oxide is synthesized in endothelial cells by endothelial nitric oxide synthase (eNOS), which converts L-arginine into NO. The activity of eNOS is regulated by intracellular calcium levels and the calcium-binding protein calmodulin, allowing the controlled production of physiologically relevant amounts of NO [17].

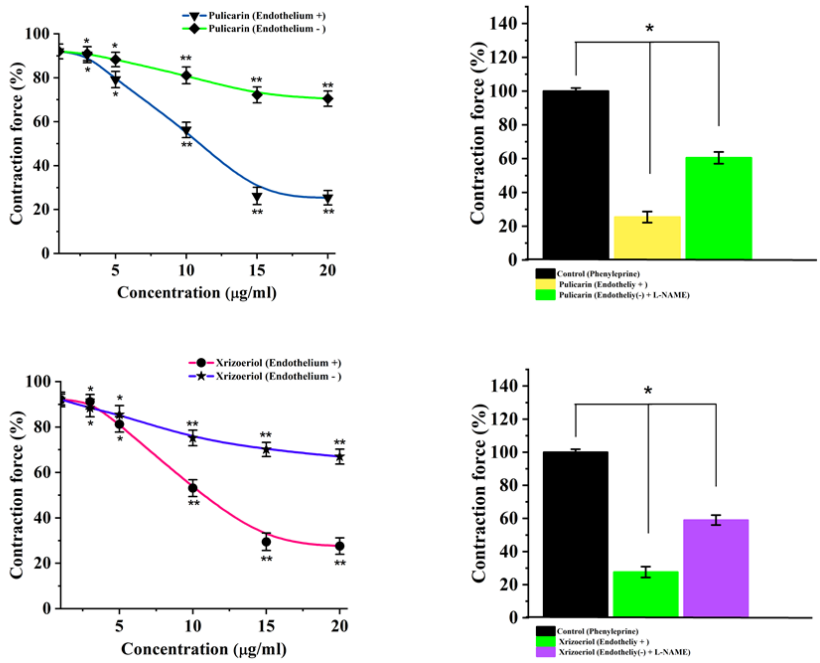


Figure 5 Endothelium-dependent vasorelaxant effects of pulicarin and chrysoeriol and attenuation of relaxation by the eNOS inhibitor L-NAME in phenylephrine-precontracted aortic rings.

Experimental findings from the present study demonstrated that pulicarin and chrysoeriol markedly reduced phenylephrine (1 μM)-induced contraction in endothelium-intact aortic rings by 74.6 ± 3.3% and 72.4 ± 3.6%, respectively. However, when the endothelial layer was mechanically removed, the relaxant effects of both flavonoids were significantly diminished, with inhibition reduced to only 29.5 ± 3.4% for pulicarin and 23 ± 3.3% for chrysoeriol. Further confirmation of endothelium-dependent involvement was obtained using the eNOS inhibitor L-NAME (100 μM). Under these conditions, the vasorelaxant responses were substantially attenuated, with pulicarin and chrysoeriol decreasing phenylephrine-induced contraction by only 39.5 ± 3.5% and 41 ± 3%, respectively. These results strongly suggest that the vasorelaxant actions of pulicarin and chrysoeriol are mediated, at least in part, through the endothelial NO–dependent pathway.

***In silico* molecular docking studies of *pulicarin and xrizoeriol* compounds targeting Ca^{2+} ion channels**

Ca^{2+} L type channel

Voltage-dependent L-type calcium channels (LTCCs), belonging to the Cav1.x family, represent a major class of high-voltage–activated calcium channels widely expressed in cardiac muscle, vascular smooth muscle, neurons. non-excitable tissues. These channels are heteromultimeric complexes in which the pore-forming alpha1 subunit (Cav1.1–Cav1.4) determines ion selectivity, voltage sensitivity. pharmacological properties. Auxiliary beta, alpha2-delta. gamma subunits regulate channel trafficking, membrane stability. gating kinetics. L-type Ca^{2+} channels play key roles in several key physiological processes [18]. In the heart, Cav1.2 channels provide the main source of inward Ca^{2+} current that initiates Ca^{2+}-induced Ca^{2+} release and triggers myocardial contraction. In vascular smooth-muscle cells, activation of LTCCs allows a rapid influx of Ca^{2+}, leading to myosin light-chain phosphorylation and strong vasoconstriction; so, Cav1.2 activity is a major determinant of vascular tone and arterial blood pressure. In neurons, Cav1.2 and Cav1.3 channels modulate neurotransmitter release, synaptic plasticity. activity-dependent gene expression through Ca^{2+}-sensitive signaling pathways. The activity of LTCCs is tightly controlled by membrane voltage, intracellular Ca^{2+}, calmodulin, protein phosphorylation [19]. neurohumoral signals. For example, beta-adrenergic stimulation enhances Cav1.2 function via PKA-mediated phosphorylation, increasing Ca^{2+} entry during excitation. Conversely, Ca^{2+}-dependent inactivation limits excessive Ca^{2+} loading and maintains

homeostasis. Dysregulation of L-type Ca^{2+} channels contributes to pathologies. Increased expression or hyperactivation of Cav1.2 is associated with hypertension, coronary vasospasm, cardiac hypertrophy. arrhythmias. In the central nervous system, excessive Ca^{2+} influx through LTCCs disrupts calcium homeostasis and contributes to neurodegenerative processes. L-type Ca^{2+} channels are also key pharmacological targets Mansoor *et al.* 2023. Dihydropyridines such as nifedipine and amlodipine, phenylalkylamines such as verapamil. benzothiazepines such as diltiazem block these channels by stabilizing their inactive state, thereby reducing Ca2+ influx and producing vasodilation. Recently, natural compounds, especially flavonoids, have been shown to modulate Cav1.2 activity and induce vascular relaxation by suppressing Ca^{2+} entry. so, understanding the structural organization, regulatory mechanisms. ligand interactions of L-type Ca^{2+} channels is key for the development of novel therapeutic agents targeting cardiovascular disorders [20].

In our *in silico* experiments, we evaluated the interaction of our flavonoids, pulicarin and chrysoeriol, with the L-type calcium channel (PDB ID: 6jp5). Using Discovery Studio, we identified the ligand-binding active site of the channel and determined its coordinates as center_x = 168.279698, center_y = 176.439484, and center_z = 180.397000. Docking analysis revealed that pulicarin binds to the L-type Ca^{2+} channel with a binding energy of –8.2 kcal/mol. According to thermodynamic principles, a more negative binding energy indicates a stronger and more stable interaction between the ligand and the protein. To further characterize the inhibitory potential of the ligand, we calculated the inhibition constant (Ki). Ki represents the concentration of ligand required to occupy 50% of the target protein's active sites under equilibrium conditions, and it is a widely accepted quantitative parameter for evaluating binding affinity and inhibitor strength. Lower Ki values correspond to tighter binding and stronger inhibitory activity. Based on the docking-derived binding energy, the Ki value for pulicarin was estimated to be 0.92 μM, suggesting that it acts as a potent inhibitor of the L-type calcium channel. Our in vitro experiments supported these findings, showing that both pulicarin and chrysoeriol effectively inhibit L-type Ca^{2+} channels in vascular smooth-muscle preparations. The combination of *in vitro* and *in silico* results therefore provides strong evidence that these flavonoids exert their vasorelaxant effects, at least in part, through direct inhibition of Cav1.2 channels. The interaction of pulicarin with the L-type Ca^{2+} channel (PDB ID: 6jp5) is interpreted structurally.

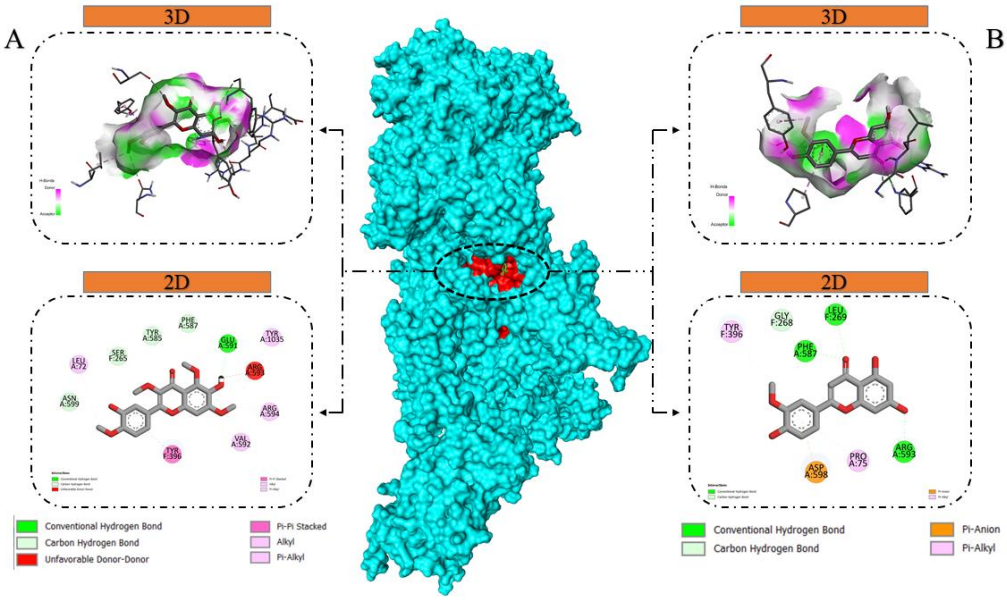


Figure 6 Binding interactions of pulicarin (A) and chrysoeriol (B) with the L-type Ca^{2+} channel: 3D surface visualization and 2D interaction maps.

The interactions are described in terms of the amino acids involved and how they help the ligand preferentially bind to the L-type Ca^{2+} channel. GLU A:591 Contains a standard hydrogen bond. In respect of the channel's structure, Glu591 is one of several Glu's (see below), Typically Glu's provide stabilisation to the transmembrane helices of the channel through their ability to associate hydrophilically with the surrounding electrostatic surroundings of the channel's inner vestibule. When bound to ligand, Glu591 provides an excellent anchor point for the ligand as Glu591 has a free Robyane and free Hydrogen forming an excellent donor. The multiple H-bond donating sites on Pulicarin will give Glu591 a significant advantage with regard to forming H-bonds with ligands providing a greater binding stability. ASN A:599; SER A:265; TYR A:585; PHE A:587: carbon-hydrogen (C-H) bonds. Two polar amino acids, Asparagine and Serine, both with polar side chains, play key roles in positioning ligands between calcium channels. The side chain amides from Asparagine, and hydroxyls from Serine, provide C-H bonds that stabilize a ligand during the binding process with calcium channels; they are geometrically important to the positioning of the ligand onto the active site of the calcium channel. While Tyrosine and Phenylalanine are both aromatic and hydrophobic groups, they do possess partially polar surfaces, which allow them to simultaneously create C-H bonds with the planar flavonoid rings. The contribution from these items enables Pulicarin to be located in a proper orientation, while also promoting π-surface complementarity. ARG A:593: unfavorable donor-donor interaction. Typically, arginine residues (Arg) help stabilize negative charges located within the channel pore domain and/or interact with phospholipid head groups. Due to its positively charged guanidinium group, which usually acts as a strong hydrogen-bond donor, a donor-donor interaction will typically indicate some sort of steric or electrostatic repulsion in that position. Mildly unfavorable interactions, such as that for arginine residue 593 (Arg593), indicate that Arg593 is not optimally aligned with both hydrogen bonding groups of the ligand, but is instead close. However, this repulsion can also work to help "lock" the ligand into a fixed orientation by limiting unnecessary rotations. TYR F:396 – π-π stacking. Tyrosine is one of the aromatic residues found inside the hydrophobic channel cavity where π-π stacking mainly occurs due to the positioning of the aromatic ring of tyrosine in line or slightly offset of the aromatic rings of pulicarin. Additionally, flavonoids have a planar benzopyran backbone which will allow for strong interactions with aromatic residues to occur, creating a significant contribution to the binding affinity through the provision of strong hydrophobic and van der Waals stabilization of the transmembrane domain. VAL A:592, ARG A:594, TYR A:1035 – alkyl and π-alkyl interactions. Valine hydrophobic residue typically found on the lipid-occupied side in transmembrane helices, creates a perfect lipophilic pocket to facilitate van der Waals interactions with the hydrophobic portion of pulicarin. Arginine, although a charged residue, contains aliphatic carbons throughout its side chain that may create π-alkyl interactions with aromatic ligands. Tyrosine creates an aromatic environ. In summary, Pulicarin interacts with the polar, aromatic, and aliphatic regions of the L-type Ca^{2+} channel via hydrogen bonding, π interactions, and van der Waals interactions, respectively. Ultimately, these three types of interactions provide a basis for the binding affinity and inhibition potential of Pulicarin to the L-type Ca^{2+} channel (**Figure 6(A)**).

Table 2 Binding energies (kkal/mol) of pulicarin and chrysoeriol against major Ca^{2+}-handling channels and transporters.

Flavonoids	Ca^{2+} L type channel	Ca^{2+} R type channel	Ca^{2+}-ATPases	NCX1	RyR2	SERCA
Pulicarin	−8.2	−7.8	−9.1	−8.0	−6.8	−7.4
Xrizoeriol	−8.6	−8.6	−9.3	−8.2	−7.5	−7.9

The docking data indicate that the R-type Ca^{2+} channel has a strong interaction with chrysoeriol with an estimated binding energy of −8.6 kcal/mol, which equates to an inhibition constant (Ki) of 0.467 μM and a very strong inhibitory potential. The ligand is located within the binding pocket of the R-type Ca^{2+} channel at

the following coordinates: Center_x = −14.791000, center_y = −8.417000, and center_z = 10.185000. The R-type Ca^{2+} channel contains a number of critical structural and functional residues that interact with and bind chrysoeriol. Located in the channel's hydrophobic core, phenylalanine (PHE A:587) serves to create a hydrophobic pocket used for binding non-polar ligands, including chrysoeriol. Additionally, PHE A:587 provides a π-stacking interaction with hydroxyls and can stabilize chrysoeriol by hydrogen bonding. Another structurally critical residue associated with the channel's hydrophobic core is leucine (LEU F:269), which is a highly hydrophobic transmembrane residue that maintains the integrity of the lipid-facing section of the channel and helps shape the binding pocket to allow for van der Waals interactions between the ligand and channel. Arginine (ARG A:593), which tends to be involved in stabilizing the negative charges on either side of the channel pore, also acts as a hydrogen-bond donor to help anchor chrysoeriol at a defined angle. Glycine's (GLY F:268) variable size and flexibility make it a frequent inhabitant of the narrower curvature regions of transmembrane helices, including the binding site in R-type channels (**Figure 6(B)**). The structural contribution of glycine facilitates an improved fit for the ligand within the channel, due in part to the existence of C-H bonds at the ends of glycine's backbone. Another important aromatic residue in the binding pocket of the R-type channel is tyrosine (TYR F:396). Tyrosine's π-alkyl interactions with the planar aromatic rings of chrysoeriol provide a number of stabilizing forces (hydrophobic and van der Waals) for chrysoeriol. With the negatively charged carboxyl group, ASP A:598 is involved in electrostatic regulation of the pore, as well as forming π-anion interactions with the electron-rich aromatic system of the ligand, which increases binding affinity. PRO A:75 typically is a propene that introduces kinks or bends into transmembrane helices, which assists in defining a local geometry of the binding pocket. PRO also assists in hydrophobic packing through π-alkyl contacts and enhances the overall stability of the ligand–channel complex. All of these residues together provide a polar, aromatic and hydrophobic environment that allows chrysoeriol to bind to R-type Ca^{2+} channels with a high affinity and a strong potential for inhibition.

Ca^{2+} R type channel

R-type calcium channels (Cav2.3) are classified as a unique subtype of high-voltage-activated (HVA) Ca^{2+} channels that are involved in many different neuronal as well as neuroendocrine physiological functions. Although R-type channels also have some electrophysiological features in common with N-type and P/Q-type channels, their pharmacological profile is distinct because R-type channels are not susceptible to the inhibitory effects of classical calcium channel blockers, such as dihydropyridines, ω-conotoxins, and ω-agatoxins [22]. This pharmacological distinction gave rise to the name "R-type," with R signifying "resistance." The structural features of Cav2.3 channels result from the presence of the alpha1E subunit, which forms the pore of the channel and senses the change in voltage. The beta and alpha2-delta auxiliary subunits modulate the expression, localization to membranes, and kinetic behavior of Cav2.3 channels. Cav2.3 channels are found primarily in the brain, specifically in areas such as the hippocampus, cortex, amygdala, and cerebellum, where Ca^{2+} signaling in dendrites is critical for the proper functioning of these regions. R-type calcium channels (Ca^{2+}) are responsible for allowing the entry of Ca^{2+} into cells following depolarization, thus giving the cell excitability, participation in synaptic remodeling, and enhancement of long-term potentiation (LTP) Muller *et al.* 1998. These channels have a major role in regulating Ca^{2+} in the dendritic spines and influence the post-synapse's ability to respond. Additionally, Cav2.3 channels are thought to play roles in the release of neurotransmitters, secretion of hormones, awareness of pain, and pain processing. In some neurons, R-type current also helps form burst patterns of action potentials and back-propagate action potentials. Cav2.3 dysfunction or abnormal expression has been reported for several neurological disorders such as epilepsy, chronic pain, anxiety, and degenerative diseases. In addition, these channels maintain Ca^{2+} homeostasis in the dendrites; therefore, abnormal Cav2.3 causing excitotoxicity and impairing synaptic integration. Historically, there was a general belief that pharmacologically targeting R-type channels was difficult. However, recent studies have revealed that there are now natural compounds, peptides and synthetic chemicals that can modify the actions of R-type channels [24]. Because of the significant number of

structures and the fact that they can be gated in a variety of ways, Cav2.3 seems to be an excellent target for drug discovery by virtue of it having potential use as a neuroprotective drug and also having applications in pain management. The results of the docking analysis indicated that pulicarin binds to the R-type calcium channel by interacting with a specific site within the active site region. The coordinates for this binding pocket are −14.791000, −8.417000, and 10.185000, respectively, with binding energy calculated at −7.8 kcal/mol and the approximate inhibition constant (Ki) of around 1.9 μM.

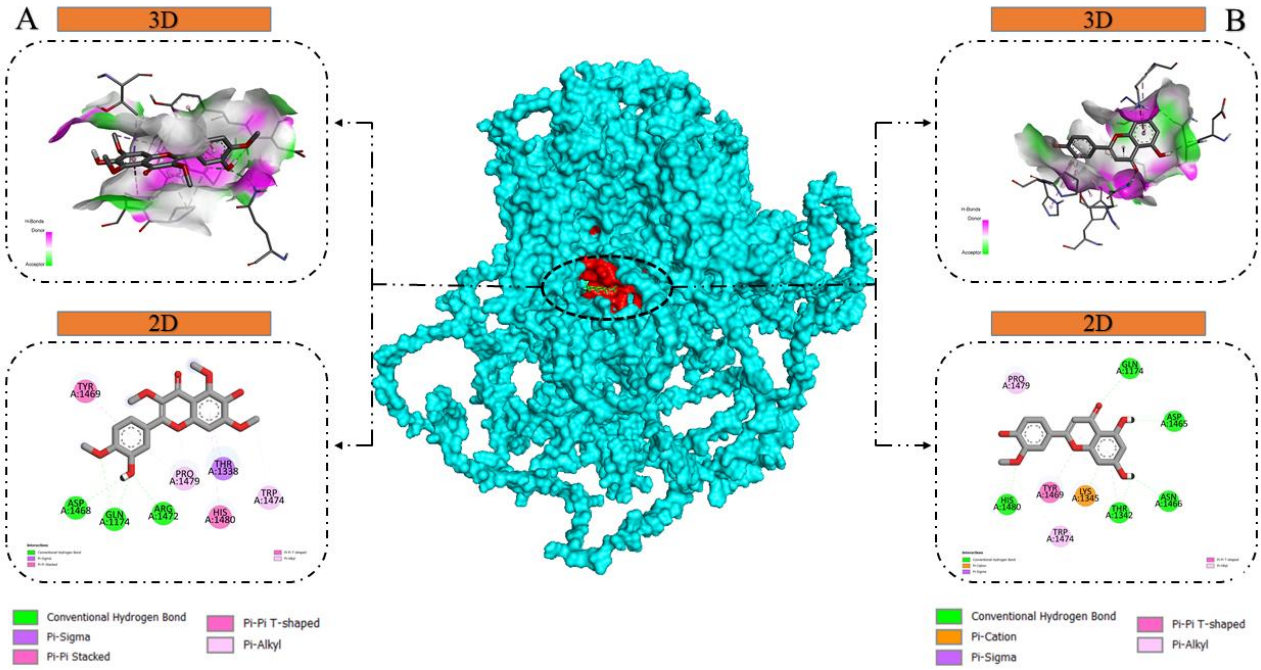


Figure 7 3D binding poses and 2D interaction profiles of pulicarin and chrysoeriol within the active site of the R-type Ca^{2+} channel.

Pulicarin has a moderate to strong inhibitory effect at this site. Pulicarin creates multiple stabilizing interactions within the binding pocket. Three amino acids, aspartate (ASP A:1468), glutamine (GLN A:1174), and arginine (ARG A:1472), contribute to the formation of traditional hydrogen bonds that provide strong polar anchor points to properly orient pulicarin in the small cavity of the active site. Tyrosine (TYR A:1469) makes a T-shaped π–π interaction with the aromatic portion of pulicarin, and histidine (HIS A:1480) contributes to π–π staking interactions, both of which increase the ability of pulicarin to properly fit and bind in this small active site pocket. Moreover, the contribution of THR A:1338 also provides a π–sigma interaction that aids the fine positioning of the ligand [25]. The π–alkyl interactions established by PRO A:1479 and TRP A:1474 serve to improve the hydrophobic packing of the flavonoid scaffold and lock in the ligand in an optimal orientation. The combination of these hydrogen-bonding, π-based, and hydrophobic interactions accounts for the stability of the pulicarin–channel complex, while establishing its ability to inhibit the R-type Ca^{2+} channel (**Figure 7(A)**). Based on the docking study, chrysoeriol was found to have a strong interaction with the R-type Ca^{2+} channel based on a binding energy calculated at −8.6 kcal/mol. This corresponds to an inhibition constant of approximately Ki = 0.49 μM, indicating a high level of inhibitory activity. The analysis reveals that chrysoeriol has several different types of stabilizing interactions made within the cavity of the active site. Multiple residues comprised of GLN A:1174, ASP A:1465, ASN A:1466 and THR A:1342 create conventional hydrogen bonds with chrysoeriol that serve to provide a polar anchoring point for its directionality within the binding site as well as assisting with proper orientation. HIS A:1480 has

created 2 separate contributions; one interacts through a conventional hydrogen bond formed by its imidazole ring and simultaneously creates a π-alkyl interaction within the aromatic system of the ligand. The combination of these two interactions provides greater stability of the active complex due to both a polar and hydrophobic interaction. Also, TYR A:1469 creates a π–π T-shaped interaction with the planar benzopyran rings of chrysoeriol, enhancing aromatic complementarity. TRP A:1474 and PRO A:1479 create π-alkyl interactions to enhance hydrophobic packing around the ligand, thereby further decreasing the binding energy. Additionally, LYS A:1345 engages in a π-cation interaction, strengthening electrostatic complementarity between the positively charged lysine side chain and the electron-rich aromatic core of the flavonoid (**Figure 7(B)**). Together, these hydrogen-bonding, aromatic, and hydrophobic interactions create a highly cooperative binding environment that explains the strong affinity and inhibitory potency of chrysoeriol toward the R-type Ca^{2+} channel.

Ca^{2+}-ATPases (calcium ATPases)

Calcium ions play a central role in the regulation of numerous physiological processes, including muscle contraction, neurotransmitter release, gene expression, and cell survival. Because of their broad signaling functions, intracellular Ca^{2+} levels must be precisely maintained within a narrow range. One of the key systems responsible for this regulation is the family of Ca^{2+}-ATPases, ATP-driven membrane pumps that transport calcium ions against their concentration gradient. These enzymes are localized primarily in the plasma membrane and in the membranes of intracellular organelles such as the sarcoplasmic and endoplasmic reticulum. Ca^{2+}-ATPases use the energy derived from ATP hydrolysis to remove excess Ca^{2+} from the cytosol and store it either outside the cell or within organelles, ensuring rapid restoration of basal calcium levels after stimulation. Two major types are generally described: the plasma membrane Ca^{2+}-ATPase, which extrudes Ca^{2+} to the extracellular space, and the sarco/endoplasmic reticulum Ca^{2+}-ATPase, which pumps Ca^{2+} into the lumen of internal stores. By tightly regulating intracellular Ca^{2+}, these pumps maintain the proper conditions for Ca^{2+}-dependent signaling pathways and prevent cytotoxic calcium overload. In smooth muscle cells, Ca^{2+}-ATPases have a particularly important functional role. Smooth muscle contraction depends on a rise in cytosolic Ca^{2+}, which activates calmodulin and the myosin light-chain kinase pathway. After contraction is initiated, relaxation requires efficient removal of Ca^{2+} from the cytoplasm. Ca^{2+}-ATPases rapidly lower intracellular Ca^{2+} levels by pumping it back into the sarcoplasmic reticulum or out of the cell, thereby terminating the contractile signal. This activity is essential for controlling vascular tone, airway resistance, gastrointestinal motility, and other smooth muscle functions. Impairment of Ca^{2+}-ATPase activity can therefore contribute to abnormal smooth muscle contractility, hypertension, vascular dysfunction, and related pathologies. In our docking analysis, the interaction between Ca^{2+}-ATPase and the flavonoid Pulicarin at the defined grid center (center_x = 27.060000, center_y = 1.672000, center_z = 2.679000) resulted in a binding energy of −9.1 kcal/mol. This corresponds to an estimated Ki value of approximately 0.2 μM, indicating a strong inhibitory potential. Docking with the second flavonoid, chrysoeriol, produced an even lower binding energy of −9.3 kcal/mol, which translates to an estimated Ki value of about 0.14 μM. These results suggest that chrysoeriol exhibits a slightly higher affinity for Ca^{2+}-ATPase compared to pulicarin.

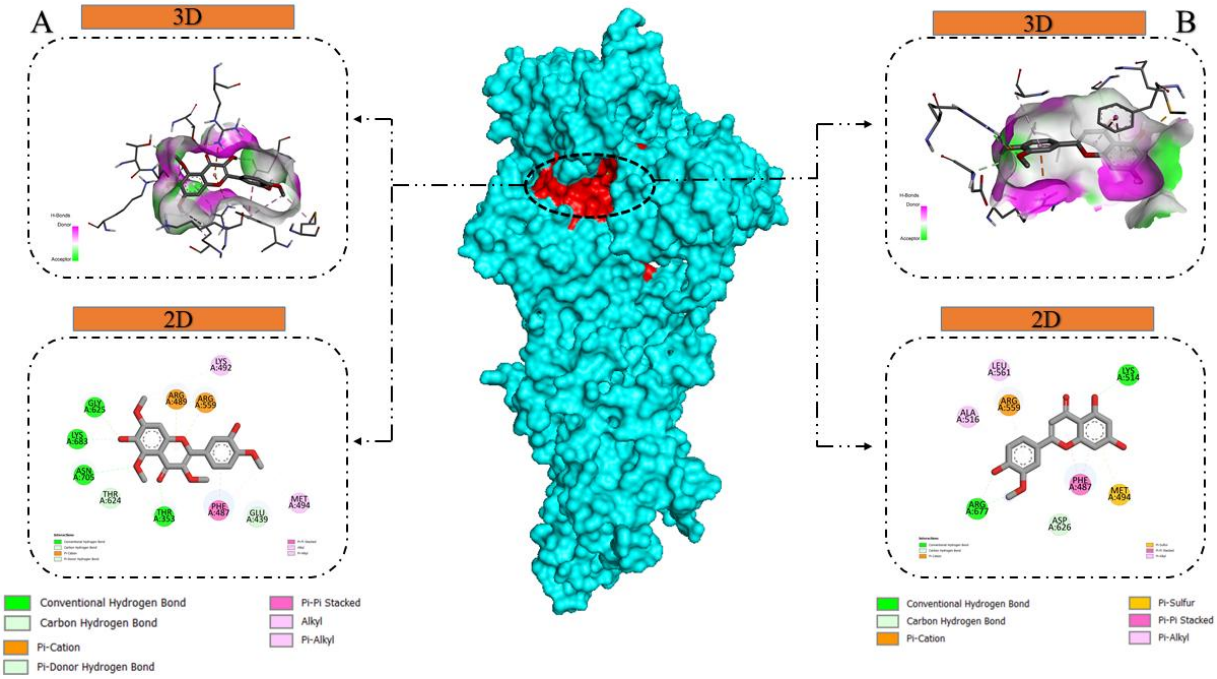


Figure 8 Binding interactions of pulicarin (A) and chrysoeriol (B) with the Ca^{2+}-ATPases (calcium ATPases): 3D surface visualization and 2D interaction maps.

The binding between the flavonoids and Ca^{2+}-ATPase occurs because the ligands interact with specific amino acids located in an important region of the protein. Pulicarin forms hydrogen bonds with ASN A:705, LYS A:683, GLY A:625, and THR A:353, while THR A:624 and GLU A:439 participate in carbon–hydrogen and donor–acceptor interactions. The ligand is also stabilized by strong π–cation interactions with ARG A:489 and ARG A:559. In addition, PHE A:487 forms a π–π stacking interaction with the aromatic ring of the flavonoid, and MET A:494 together with LYS A:492 contributes through alkyl and π–alkyl contacts (**Figure 8(A)**). All of these interactions help the ligand fit tightly into the binding pocket and increase its inhibitory effect on the protein. These amino acids play structural and functional roles in Ca^{2+}-ATPase. Many of them are located near regions that help the protein bind calcium or change its shape during the pumping cycle. Polar residues such as asparagine, threonine, glycine, and lysine easily form hydrogen bonds, which explains why the ligand attaches to them. Charged residues like arginine and glutamate help stabilize the ligand through electrostatic forces. Aromatic residues such as phenylalanine create a hydrophobic pocket that allows the flavonoid's ring structure to stack and stay in place. Hydrophobic residues like methionine and the aliphatic chain of lysine further strengthen the binding. The ligand binds to these amino acids because together they form a pocket that matches the chemical structure of the flavonoid. This makes the interaction stable and allows the compound to interfere with the normal activity of Ca^{2+}-ATPase. The interaction between chrysoeriol and Ca^{2+}-ATPase is formed through several specific contacts with key amino acids in the protein. Chrysoeriol forms conventional hydrogen bonds with ARG A:677 and LYS A:514, while the aromatic residue PHE A:487 participates in a π–π stacking interaction that helps stabilize the ligand. Additional stabilization is provided by π–alkyl interactions with ALA A:677 and LEU A:561. The ligand also forms a carbon–hydrogen bond with ASP A:626, and a strong π–cation interaction with ARG A:559. MET A:494 contributes through a π–sulfur interaction, which further anchors the ligand within the binding pocket. These combined interactions allow chrysoeriol to fit tightly into the Ca^{2+}-ATPase active region and support its inhibitory effect on the protein (**Figure 8(B)**).

NCX1

The sodium–calcium exchanger 1 (NCX1) is a membrane transport protein that plays a central role in maintaining intracellular calcium balance in excitable

and non-excitable cells. NCX1 operates by exchanging intracellular Ca^{2+} for extracellular Na^{+} across the plasma membrane. Under normal physiological conditions, the exchanger works in its forward mode, extruding one Ca^{2+} ion in exchange for the entry of three Na^{+} ions. This mechanism is essential for rapidly lowering cytosolic Ca^{2+} concentration after cell activation and for preventing calcium overload. NCX1 is highly expressed in the heart, vascular smooth muscle, neurons, and other tissues where calcium signaling is tightly regulated. In cardiac and vascular cells, NCX1 is a key component of the calcium removal system that restores relaxation after contraction. Its activity directly influences excitation–contraction coupling, vascular tone, neurotransmission, and many Ca^{2+}-dependent signaling pathways. The exchanger can also operate in reverse mode when the intracellular sodium concentration rises or when the membrane potential becomes depolarized. In this state, NCX1 allows Ca^{2+} to enter the cell in exchange for Na^{+} efflux. Reverse-mode activity is important in certain physiological processes but may contribute to pathological calcium overload during ischemia, oxidative stress, or excessive sympathetic stimulation. Dysregulation of NCX1 has been linked to cardiovascular diseases, arrhythmias, hypertension, neurodegeneration, and cell injury caused by disturbed ion homeostasis. Because of its dual transport capacity and strong influence on intracellular Ca^{2+} dynamics, NCX1 continues to be an important target in studies of cellular physiology, pharmacology, and disease mechanisms [30]. The interaction of our flavonoids with the NCX1 channel was evaluated using a docking grid centered at coordinates center_x = 142.026713, center_y = 158.751000, and center_z = 153.700000. The docking analysis showed that pulicarin produced a binding energy of −8.0 kcal/mol, corresponding to an estimated Ki value of approximately 1.34 µM. Chrysoeriol exhibited an even stronger interaction with a binding energy of −8.2 kcal/mol, which translates to an estimated Ki of about 0.96 µM. These Ki values indicate that both flavonoids possess a strong inhibitory potential toward the NCX1 channel, with chrysoeriol showing slightly higher affinity.

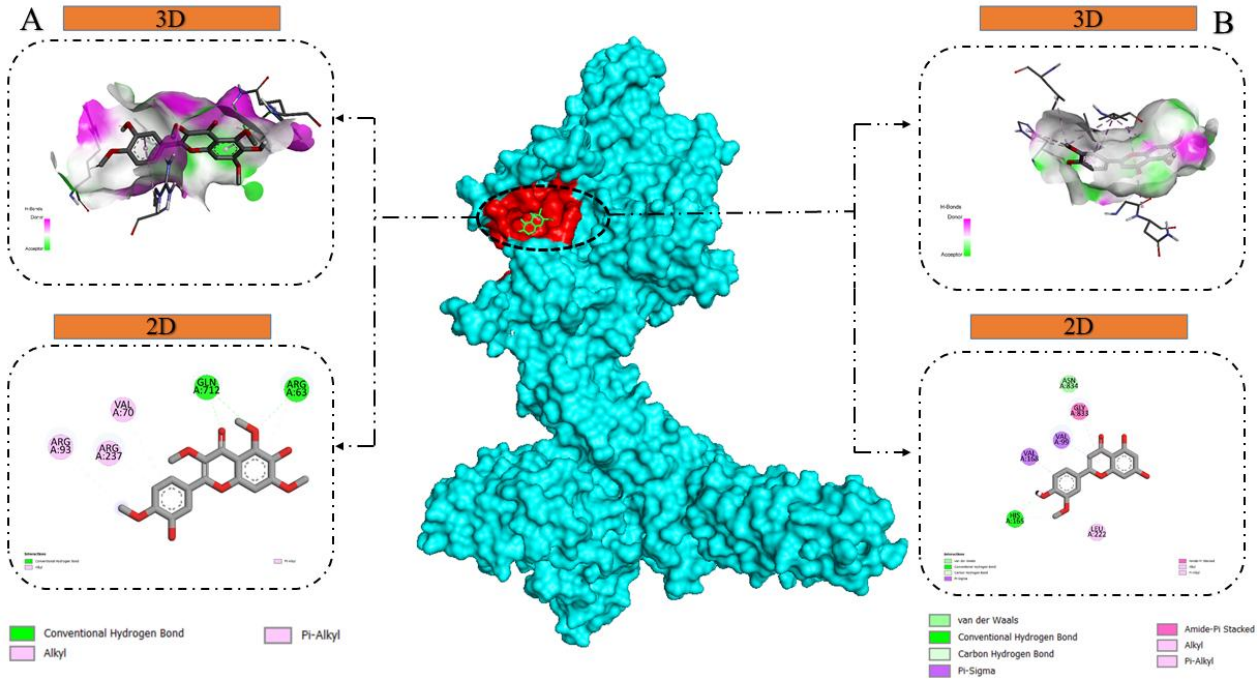


Figure 9 Binding interactions of pulicarin (A) and chrysoeriol (B) with the NCX1:3D surface visualization and 2D interaction maps.

In the NCX1 protein, the amino acids involved in ligand binding are located in regions that contribute to the stability of the exchanger and support its ability to move Na^{+} and Ca^{2+} across the membrane. Each residue has chemical properties that match the functional groups of the flavonoids, which is why these compounds bind

to them. For pulicarin, the interacting residues are ARG A:93, ARG A:237, VAL A:70, GLN A:712, and ARG A:63. Arginine residues provide strong positive charge and can form hydrogen bonds as well as π–alkyl contacts with aromatic rings, which explains why pulicarin attaches to ARG A:93, ARG A:237, and ARG A:63. Valine at position 70 is hydrophobic and easily interacts with the non-polar parts of the flavonoid. GLN A:712 has a polar side chain that forms stable hydrogen bonds, helping to hold the ligand in place (**Figure 9(A)**). For chrysoeriol, the interacting residues include ASN A:834, HIS A:165, VAL A:99, VAL A:168, GLY A:833, LEU A:222, and again VAL A:99 in different interaction types. ASN A:834 and GLY A:833 create small flexible pockets that fit the ligand through van der Waals or amide–π interactions. HIS A:165 can form hydrogen bonds because of its imidazole ring. Valine residues at positions 99 and 168 form hydrophobic surfaces that support π–sigma or carbon–hydrogen contacts with the aromatic ring of the ligand. LEU A:222 is also hydrophobic and stabilizes the ligand through alkyl interactions (**Figure 9(B)**). These amino acids naturally form part of a hydrophobic and partially polar cavity inside NCX1, and the chemical features of both flavonoids match this environment. For this reason, the ligands bind strongly to these specific residues and can interfere with the normal functioning of the exchanger.

RyR2

The ryanodine receptor type 2 (RyR2) is a large intracellular calcium release channel located in the membrane of the sarcoplasmic reticulum, predominantly expressed in cardiac muscle cells and also present in vascular smooth muscle. RyR2 plays a central role in the regulation of intracellular calcium dynamics by mediating the rapid release of Ca^{2+} from internal stores into the cytosol. This release is essential for excitation–contraction coupling, where a small influx of calcium through voltage-dependent channels triggers a much larger calcium release through RyR2, generating a coordinated contractile response. RyR2 is a highly regulated channel whose activity is influenced by cytosolic Ca^{2+}, luminal Ca^{2+}, Mg^{2+}, ATP, phosphorylation state, and interactions with associated proteins such as calmodulin, FKBP12.6, and calsequestrin. Proper control of RyR2 opening and closing is necessary to maintain rhythmic calcium cycling and prevent uncontrolled Ca^{2+} leakage. Dysregulation of RyR2 function can lead to abnormal calcium release, impaired relaxation, arrhythmias, and cellular injury. Mutations or altered regulation of this receptor have been linked to conditions such as catecholaminergic polymorphic ventricular tachycardia, heart failure, and stress-induced cardiac dysfunction. Because RyR2 directly shapes intracellular calcium signals, it is considered a key therapeutic target in studies of cardiac physiology and calcium-dependent pathologies. Understanding how small molecules, natural compounds, and regulatory proteins interact with RyR2 provides important insights into mechanisms that control calcium homeostasis under both physiological and pathological conditions.

In our *in silico* experiments, we evaluated the interaction of our flavonoids with the RyR1 receptor. Docking studies using a grid centered at coordinates center_x = 9.181367, center_y = 17.019000, and center_z = 27.203000 revealed binding energies of −6.8 kcal/mol and −7.5 kcal/mol for pulicarin and chrysoeriol, respectively. From these binding energies, the estimated inhibition constants (Ki) were calculated to be approximately 10 μM for pulicarin and 3 μM for chrysoeriol. These low micromolar Ki values indicate that both flavonoids exhibit a relevant inhibitory potential toward RyR1, with chrysoeriol showing a higher affinity and stronger inhibition than pulicarin.

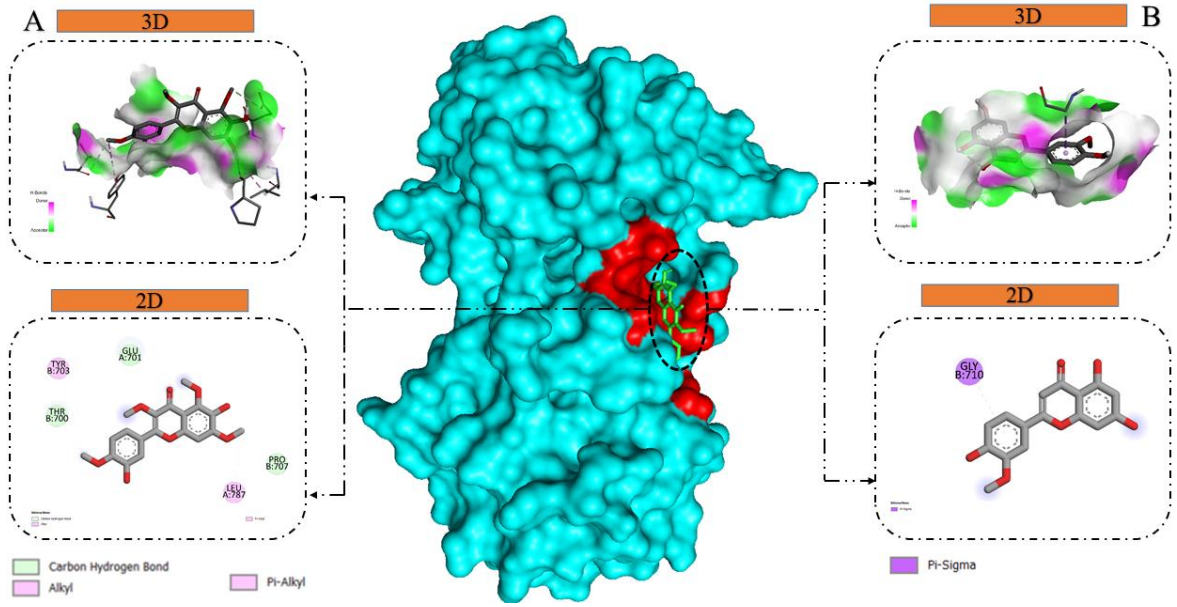


Figure 10 Binding interactions of pulicarin (A) and chrysoeriol (B) with the RyR2: 3D surface visualization and 2D interaction maps.

The amino acids that interact with the flavonoids in the RyR1 receptor are located in regions that help stabilize the channel's structure and support the opening and closing movements that control calcium release from the sarcoplasmic reticulum. These residues create a mixed environment of polar, charged, and hydrophobic surfaces, which together form a natural pocket where small molecules can bind. THR B:700, GLU A:701, and PRO B:707 are positioned near a flexible loop region that contributes to the regulation of the channel's gating. Threonine and glutamate have polar side chains capable of forming carbon–hydrogen contacts, while proline helps define the local shape of the protein backbone. These properties allow pulicarin to form stable carbon–hydrogen bonds with them, giving the ligand an anchoring point in the RyR1 cavity. TYR B:703 and LEU A:787 are hydrophobic residues located in a region that supports the structural packing of the channel (**Figure 10(A)**). Their non-polar side chains create a hydrophobic surface that matches the aromatic and hydrophobic parts of pulicarin. For this reason, pulicarin forms alkyl and π-alkyl interactions with these residues, which stabilize the ligand inside the pocket. GLY B:710 interacts with chrysoeriol through a π–sigma contact (**Figure 10(B)**). Glycine is a small and flexible residue often found in narrow or mobile regions of the protein. Its presence allows the ligand to fit closely and form interactions that require tight spatial alignment. The π–sigma interaction suggests that chrysoeriol's aromatic ring aligns well within this flexible part of the receptor. Overall, the flavonoids bind to these amino acids because their chemical structures match the polar, hydrophobic, and flexible characteristics of the residues in this region of RyR1. This complementary fit allows both compounds to settle into the binding pocket and influence the receptor's function.

SERCA

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is an essential membrane pump responsible for transporting calcium ions from the cytosol into the sarcoplasmic and endoplasmic reticulum. By using the energy derived from ATP hydrolysis, SERCA lowers the intracellular Ca^{2+} concentration and replenishes the internal calcium stores required for signaling. This rapid removal of Ca^{2+} from the cytosol is a key step for muscle relaxation, cellular homeostasis, and the proper functioning of calcium-dependent pathways. SERCA plays a central role in cardiac, skeletal, and smooth muscle physiology. During muscle activation, Ca^{2+} is released into the cytosol to trigger contraction, and SERCA is responsible for removing this calcium to allow relaxation. The activity of SERCA is tightly regulated by proteins such as phospholamban and sarcolipin, as well as by ATP availability and cytosolic

Ca^{2+} levels. Efficient SERCA function ensures stable intracellular calcium cycling and prevents calcium overload, which can otherwise lead to cell injury or dysregulated signaling. Alterations in SERCA expression or activity are associated with various pathological conditions, including heart failure, hypertension, metabolic disorders, and impaired vascular function. Because of its fundamental role in regulating cellular calcium balance, SERCA is widely studied as a therapeutic and pharmacological target. Understanding how natural compounds or synthetic molecules interact with SERCA provides valuable insights into mechanisms of calcium regulation and the development of potential modulators of calcium homeostasis.

When we examined the interaction of our flavonoids with SERCA in silico, docking was performed using a grid centered at coordinates center x = 37.179317, center y = 34.354870, and center z = 58.830812. The resulting binding energies were −7.4 kcal/mol and −7.9 kcal/mol for pulicarin and chrysoeriol, respectively. From these values, the estimated inhibition constants (Ki) were calculated to be approximately 3.7 µM for pulicarin and 1.6 µM for chrysoeriol. These low micromolar Ki values indicate that both compounds exert a strong inhibitory effect on SERCA, with chrysoeriol showing somewhat higher affinity and stronger inhibition than pulicarin.

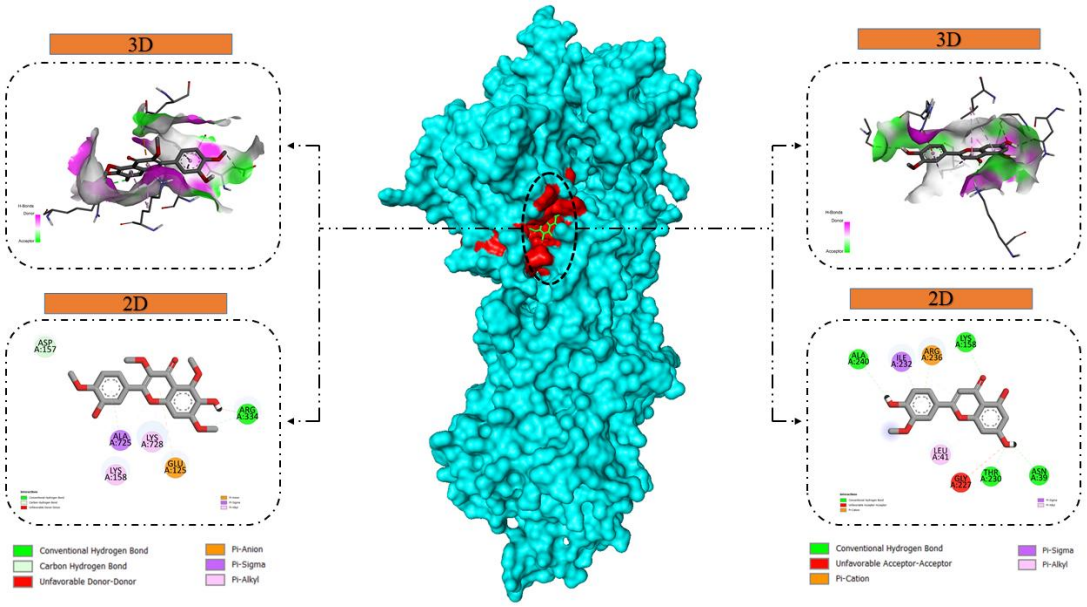


Figure 11 Binding interactions of pulicarin (A) and chrysoeriol (B) with the SERCA: 3D surface visualization and 2D interaction maps.

The amino acid residues that interact with pulicarin and chrysoeriol in SERCA are located in regions that contribute to the structural stability and functional movements of the calcium pump. These residues are part of the transmembrane helices and cytosolic loops that participate in Ca^{2+} binding, ATP-driven conformational transitions, and the overall gating mechanism of SERCA. Because of their chemical properties, these residues create a pocket that matches the polarity, charge, and hydrophobic parts of the flavonoids, which explains why the ligands preferentially bind to them. ARG A:334, LYS A:158, LYS A:728, and ARG A:236 are positively charged residues often found near regions important for stabilizing negative charges during the transport cycle. Their side chains can form hydrogen bonds, π–cation interactions, or electrostatic contacts, making them suitable partners for the polar groups in the flavonoids. ASP A:157 and GLU A:125 are acidic residues that help control the local ionic environment around the Ca^{2+} binding and release pathway (Fig 11A). Their negative charge and ability to participate in carbon–hydrogen or π–anion interactions allow the ligands to anchor themselves in these positions. Residues such as ALA

A:725, ALA A:240, LEU A:41, and ILE A:232 contribute to the hydrophobic core of the protein. These amino acids form non-polar surfaces that interact well with the aromatic rings and hydrophobic regions of pulicarin and chrysoeriol. For this reason, the ligands frequently form π–alkyl, π–sigma, or alkyl interactions with them. GLY A:227 and ASN A:39 occur in flexible regions involved in shaping the binding cavity. Their small or polar side chains help accommodate the ligand and stabilize it through hydrogen bonding or van der Waals interactions (**Figure 11(B)**). Overall, the flavonoids bind to these particular amino acids because their chemical structures complement the electrostatic, hydrophobic, and spatial characteristics of the SERCA binding pocket. This compatibility allows the molecules to fit tightly into the cavity, stabilize their position through several types of interactions, and exert an inhibitory effect on the calcium pump.

Discussion

The present study demonstrates that the naturally occurring flavonoids pulicarin and chrysoeriol produce significant vasorelaxation in rat aortic smooth muscle through mechanisms primarily involving modulation of calcium-dependent signaling pathways. Both compounds produced concentration-dependent relaxation of KCl-induced contraction, a depolarization model that selectively activates voltage-dependent Ca^{2+} channels (VDCCs). The observed IC_{50} values (17.58 μM for pulicarin and 18.18 μM for chrysoeriol) together with the strong inhibition at higher concentrations indicate effective suppression of depolarization-induced Ca^{2+} entry. The enhanced relaxation observed when the flavonoids were co-applied with verapamil suggests that pulicarin and chrysoeriol partially share the pharmacological profile of classical L-type Ca^{2+} channel blockers while potentially affecting additional calcium-handling pathways. Further mechanistic insight was obtained from experiments using phenylephrine, which activates receptor-operated Ca^{2+} channels (ROCCs) through α_1-adrenergic signaling. Both flavonoids markedly reduced phenylephrine-induced contraction, indicating that their inhibitory activity is not restricted to VDCCs but also involves interference with receptor-mediated Ca^{2+} influx. The reduction in vascular tone following phentolamine pretreatment, combined with the additional relaxation produced by pulicarin and chrysoeriol, supports the hypothesis that these compounds attenuate ROCC-dependent calcium mobilization. Taken together, the dual suppression of depolarization-dependent and receptor-mediated Ca^{2+} entry suggests a broad inhibitory effect on smooth-muscle calcium homeostasis. Endothelial signaling also contributes to the observed vasorelaxation. Removal of the endothelium and inhibition of nitric oxide synthesis with L-NAME significantly reduced the relaxant responses, demonstrating partial involvement of the NO pathway. However, relaxation was not completely abolished, indicating that direct smooth muscle mechanisms remain the dominant component. Such dual activity—endothelial stimulation together with direct calcium channel modulation—has been described for several bioactive flavonoids and may explain the robust vascular responses observed in the present experiments. The computational analysis provides structural support for these pharmacological observations. Molecular docking revealed favorable binding interactions between both flavonoids and key residues within calcium channel domains, including ASP1468, GLN1174, ARG1472, TYR1469, and HIS1480. Hydrogen bonding and hydrophobic π-alkyl contacts suggest that pulicarin and chrysoeriol may stabilize channel conformations that restrict Ca^{2+} permeation. The agreement between predicted binding affinity and experimentally observed inhibitory potency strengthens the plausibility of L-type calcium channels as relevant molecular targets. Docking validation was performed where possible using re-docking of co-crystallized ligands under identical grid parameters and AutoDock settings, with pose reproduction evaluated by RMSD analysis. These procedures confirmed the internal consistency of the docking protocol. Nevertheless, docking results should be interpreted cautiously because they represent predicted binding interactions rather than direct functional inhibition. Several methodological limitations should also be considered. Docking simulations were conducted using a rigid receptor model, whereas voltage-gated calcium channels undergo dynamic conformational changes during activation and ion transport. Consequently, static structural models may not fully capture ligand-induced channel modulation. Although the present docking analysis provides useful structural hypotheses, incorporation of receptor flexibility through ensemble

docking or molecular dynamics simulations could further refine the predicted interactions. Molecular dynamics analysis, however, was beyond the scope of the present work. Such simulations could provide additional insight into the stability and dynamic behavior of ligand–channel complexes under physiological conditions. Future computational studies integrating dynamic simulations and comparative analysis with known calcium channel modulators may therefore further clarify the mechanistic basis of the observed vasorelaxant effects.

Conclusions

The present investigation demonstrates that the naturally occurring flavonoids pulicarin and chrysoeriol exert strong and multifaceted vasorelaxant effects in rat aortic smooth muscle. Both compounds effectively suppressed depolarization-induced contraction by inhibiting voltage-dependent Ca^{2+} channels, as evidenced by their IC_{50} values and the marked reduction of KCl-evoked tension. Their additional ability to attenuate phenylephrine-induced contraction, together with enhanced relaxation in the presence of phentolamine, highlights a complementary blockade of receptor-operated Ca^{2+} channels, indicating a broad interference with calcium influx pathways. The partial reversal of vasorelaxation following endothelial removal and L-NAME treatment confirms the involvement of NO-mediated signaling; however, the persistence of significant relaxation even under these conditions emphasizes that direct smooth muscle mechanisms form the primary basis of their activity. Computational docking analyses further validate these functional results by revealing high-affinity interactions of pulicarin and chrysoeriol with critical amino acid residues in Ca^{2+} channel domains, supporting their proposed role as direct modulators of calcium entry. Collectively, these findings identify pulicarin and chrysoeriol as promising multi-target vasorelaxant agents that act through a combination of endothelial stimulation and inhibition of both VDCC- and ROCC-mediated Ca^{2+} signaling. Their dual functionality suggests significant therapeutic potential for conditions characterized by vascular hypercontractility and impaired calcium homeostasis.

Acknowledgments

We have no grant and we also would like to thanks for Science Academy of Uzbekistan for their great contributions. The research was carried out using institutional resources of the Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan. We would like to thank Academy of Sciences of the Republic of Uzbekistan.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of generative AI tools for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

CRediT Author Statement

Ulugbek Gayibov, **Takhir Aripov**: Conceptualization, Supervision, Project Administration, Writing - Review & Editing. **Izzatullo Abdullaev**: Investigation, Methodology, Data Curation, Formal Analysis, Writing - Original Draft, Methodology, Laboratory Experiments, Data Collection. **Khaydarali Ruziboev**: Validation, Resources. **Anvarbek Bogbekov**: Experimental Design, Investigation, Data Interpretation, Visualization. **Sirojiddin Omonturdiev**: Software, Data Analysis, Figures Preparation. **Kamila Eshbakova**: Literature Review, Reference Management, Writing - Review & Editing. **Anvar Zaynabiddinov**: Supervision, Funding Acquisition, Final Approval of the Manuscript.

Reference

[1] I Abdullaev, U Gayibov, S Omonturdiev, S Fotima, S Gayibova and T Aripov. Molecular pathways in cardiovascular disease under hypoxia: Mechanisms, biomarkers, and therapeutic targets. *The Journal of Biomedical Research* 2025; **39(3)**, 254-269.

[2] I Abdurazakova, A Zaynabiddinov, I Abdullaev, U Gayibov, Z Ziyoyiddinov, L Maxmudov and S

Zhurakulov. Hemodynamic and molecular effects of F-43 in experimental hypertension. *Trends in Sciences* 2026; **23(7)**, 12698.

[3] AV Mahmudov, OS Abduraimov, SB Erdonov, UG Gayibov and LY Izotova. Bioecological features of *Nigella sativa* L. in different conditions of Uzbekistan. *Plant Science Today* 2022; **9(2)**, 421-426.

[4] AA Azamatov, FM Tursunkhodzhaeva, SN Zhurakulov, ZD Boboev, KF Kuchimov, UK Aytmuratova, IS Ortikov, RS Abdurazakova, VI Vinogradova, IZ Abdullaev and UG Gayibov. Anti-Inflammatory Potential of 1-Aryl-6,7-Dimethoxy-1,2,3,4-Tetrahydroisoquinolines: Structure-Activity Relationship and COX-2 Binding. *Molecules* 2026; **31(11)**, 1956.

[5] L Makhmudov, N Tagayalieva, I Abdullaev, U Gayibov, K Baratov, S Usmonov, G Rakhmonova, R Yaqubova, N Vypova and S Karimova. *In silico* Pharmacokinetics and *In vivo* Anti-inflammatory Activity of C-4 Composition. *Biomedical and Pharmacology Journal* 2026; **19(1)**, 410-427.

[6] A Abdullaev, I Abdullaev, A Bogbekov, U Gayibov, S Omonturdiev, S Gayibova, M Turahodjayev, K Ruziboev and T Aripov. Antioxidant potential of *Rhodiola heterodonta* extract: Activation of Nrf2 pathway via integrative *in vivo* and *in silico* studies. *Trends in Sciences* 2025; **22(5)**, 9521.

[7] G Mirzaalimova, A Zaynabiddinov, S Omonturdiev, D Inomjonov, I Abdullaev, U Gayibov and M Kholmirzayeva. *In silico* and *In vitro* Activity of Leonurus turkestanicus on the Aortic Smooth Muscle Ion Channels. *Trends in Sciences* 2026; **23(5)**, 12146.

[8] D Inomjonov, I Abdullaev, S Omonturdiev, A Abdullaev, L Maxmudov, M Zaripova, M Abdullayeva, D Abduazimova, S Menglieva, S Gayibova, M Sadbarxon, U Gayibov and T Aripov. *In vitro* and *in vivo* studies of *Crataegus* and *Inula helenium* extracts: Their effects on rat blood pressure. *Trends in Sciences* 2025; **22(3)**, 9158.

[9] S Sodiqova, S Kadirova, A Zaynabiddinov, I Abdullaev, L Makhmudov, U Gayibov, M Yuldasheva, M Xolmirzayeva, R Rakhimov, A Mutalibov and H Karimjonov. Channelopathy activity of A-41 (propyl ester of gallic acid): Experimental and computational study of antihypertensive activity. *Trends in Sciences* 2025; **22(9)**, 10496.

[10] R Sayidaliyeva, S Kadirova, A Zaynabiddinov, I Abdullaev, L Makhmudov, U Gayibov, M Yuldasheva, M Kholmirzayeva, R Rakhimov, A Mutalibov and H Karimjonov. A-51 as a natural calcium channel blocker: An integrative study targeting hypertension. *Trends in Sciences* 2025; **22(11)**, 10760.

[11] A Khasanov, I Abdullaev, S Kadirova, M Mamajanov, A Zaynabiddinov, S Omonturdiev, L Makhmudov, D Inomjonov, U Gayibov, R Esanov and A Matchanov. N-2 polyphenol targets vascular calcium channels to exert antihypertensive effects: *In vitro* and *in vivo* evaluation. *Trends in Sciences* 2025; **22(12)**, 10782.

[12] Y Mirzayeva, I Abdullaev, U Gayibov, S Omonturdiev, Z Allaniyazova, S Umrkulova and P Usmanov. *In Vitro* and Computational Evaluation of 1-O-Benzoylkarakoline on Vascular Calcium Transport. *Trends in Sciences* 2025; **23(3)**, 11850.

[13] M Zaripova, I Abdullaev, A Bogbekov, U Gayibov, S Omonturdiev, R Makhmudov, N Ergashev, G Jabbarova, S Gayibova and T Aripov. *In vitro* and *in silico* studies of *Gnaphalium uliginosum* extract: Inhibition of α-amylase and α-glucosidase as a potential strategy for metabolic syndrome regulation. *Trends in Sciences* 2025; **22(8)**, 10098.

[14] S Omonturdiev, I Abdullaev, A Khasanov, A Abdullaev, D Inomjanov, U Gayibov, S Gayibova, K Baratov, Y Mirzaeva, M Allamuratov, P Usmanov and T Aripov. Molecular Mechanisms and Experimental Protocols in the Study of Vasorelaxant Activity in Aortic Smooth Muscle Cells. *Trends in Sciences*, 2025; **23(2)**, 11549.

[15] I Abdurazakova, A Zaynabiddinov, I Abdullaev, L Makhmudov, U Gayibov, S Omonturdiev, G Abdullayev, M Xolmirzayeva and S Zhurakulov. Pharmacological Evaluation of F45 on the Cardiovascular System Using In Vitro, In Vivo Models and Molecular Dockings. *Trends in Sciences*, 2025; **22(12)**, 10924.

[16] MR Zaripova, SN Gayibova, RR Makhmudov, AA Mamadrahimov, NL Vypova, UG Gayibov, SM Miralimova and TF Aripov. Characterization of *Rhodiola heterodonta* (Crassulaceae): Phytocomposition, antioxidant and antihyperglycemic activities. *Preventive Nutrition and Food Science* 2024; **29(2)**, 135-145.

[17] S Sodiqova, A Zaynabiddinov, I Abdullaev, U Gayibov and Z Ziyoyiddinov. Antihypertensive Effects of Compound A-42 via Regulation of Calcium-Dependent Ion Transport Systems. *Trends in Sciences*, 2026; **23(9)**, 12797.

[18] G Yao, X Zheng, Y Hu, Y Zhao, B Kong, Y Ti and PL Bu. FBLN7 mediates vascular smooth muscle cell phenotype switching and vascular remodeling in hypertension. *Theranostics* 2024; **14(19)**, 7569-7588.

[19] TF Aripov, UG Gayibov, SN Gaibova, YI Oshchepkova and SI Salikhov. *In vitro* antioxidant and antiradical activity of the total polyphenols of *Rhus coriaria* L. leaves (substances of the antiviral drug "Rutan"). *Khimiya Rastitel'nogo Syr'ya* 2024; **4**, 138-148.

[20] UG Gayibov, EJ Komilov, RN Rakhimov, NA Ergashev, NG Abdullajanova, MI Asrorov and TF Aripov. Influence of a new polyphenol compound from Euphorbia plant on mitochondrial function. *Journal of Microbiology, Biotechnology and Food Sciences* 2019; **8(4)**, 1021-1025.

[21] O Sirojiddin, A Izzatullo, G Ulugbek, I Dolimjon, G Sabina, M Rustamjon, A Takhir, Eİ Torunoğlu and EC Aytar. Cardiovascular effects of *Matricaria chamomilla* extract: Calcium channel modulation and vasorelaxant activity. *The Science of Nature* 2026; **113**, 14.

[22] M Mamajanov, I Abdullaev, G Sotimov, S Mavlanova, Q Niyozov, M Mirzaolimov, A Najimov, E Mirzaolimov, M Raximberganov and U Abdullayev. Mitochondrial and Pharmacokinetic Insights into 3,5,7,2',6'-Pentahydroxyflavanone: Respiratory Modulation, Calcium Handling, and Membrane Stability. *Trends in Sciences* 2025; **22(12)**, 10984.

[23] AG Vakhobjonovna, KE Jurayevich, AIZ Ogli, EN Azamovich, MR Rasuljonovich and AM Islomovich. Tannins as modulators in the prevention of mitochondrial dysfunction. *Trends in Sciences* 2025; **22(8)**, 10436.

[24] I Abdurazakova, A Zaynabiddinov, I Abdullaev, L Makhmudov, U Gayibov, S Omonturdiev, G Abdullayev, M Xolmirzayeva and S Zhurakulov. Pharmacological Evaluation of F45 on the Cardiovascular System Using *In Vitro*, *In Vivo* Models and Molecular Dockings. *Trends in Sciences* 2025; **22(12)**, 10924.

[25] U Gayibov, SN Gayibova, KP Ma'Murjon, FS Tuxtaeva, UR Yusupova, GMK Djabbarova, ZA Mamatova, NA Ergashev and TF Aripov. Influence of quercetin and dihydroquercetin on some functional parameters of rat liver mitochondria. *Journal of Microbiology, Biotechnology and Food Sciences* 2021; **11(1)**, e2924.