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A-51 as A Natural Calcium Channel Blocker: An Integrative Study Targeting Hypertension

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Abstract

This study investigates the vasorelaxant and antihypertensive potential of compound A-51 through an integrative approach involving *in vitro*, *in vivo*, and molecular docking analyses. A-51 induced concentration-dependent relaxation of rat aortic rings pre-contracted with 50 mM KCl, achieving 76.0% ± 2.7% relaxation at 60 µM, with an IC_{50} of 31.54 µM. In phenylephrine (1 µM)-induced contractions, the effect was even stronger, with 80.0% ± 1.7% inhibition observed at 45 µM. Endothelium removal reduced the relaxant response by 25.0% ± 2.4%, indicating partial dependence on endothelial nitric oxide pathways. *In vivo*, A-51 administered at 25 mg/kg significantly lowered systolic blood pressure from 98.0 ± 8.9 mmHg to 84.3 ± 8.3 mmHg and diastolic pressure from 74.8 ± 7.3 mmHg to 58.0 ± 5.6 mmHg within 2 h. In adrenaline-induced hypertensive rats, A-51 at 50 mg/kg reduced systolic pressure from 160.3 ± 11.6 mmHg to 93.3 ± 9.2 mmHg within 3 h, demonstrating its antihypertensive efficacy under pathophysiological conditions. Molecular docking studies revealed strong binding affinities between A-51 and calcium-regulating proteins, including Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger, with binding energies of −6.6 kcal/mol, supporting its role as a calcium modulator. Together, these findings highlight A-51's multi-target mechanism of action involving vascular smooth muscle relaxation and endothelial support. Its notable efficacy, combined with natural origin, positions A-51 as a promising candidate for further development as a novel antihypertensive agent.

Keywords: A-51, Vasorelaxation, Blood pressure, Calcium channels, Nitric oxide, Molecular docking

Introduction

Hypertension remains a pressing global health issue and is one of the leading contributors to cardiovascular morbidity and mortality worldwide. According to recent WHO estimates, over 1.28 billion adults aged 30 - 79 years are affected by hypertension, with the majority residing in low- and middle-income countries where detection and treatment remain suboptimal. Despite the availability of a wide array of pharmacological antihypertensive agents—including ACE inhibitors, beta-blockers, and calcium channel blockers—these treatments are often limited by adverse side effects, poor patient compliance, and variable therapeutic efficacy due to genetic and environmental factors [1,2]. This unmet clinical need has fueled a

growing interest in the use of natural compounds as complementary or alternative therapeutic options for the management of hypertension. Among the most extensively studied classes of phytochemicals are polyphenols, flavonoids, and tannins, owing to their potent antioxidant, anti-inflammatory, and vasomodulatory properties [3,4]. Epidemiological studies have linked higher dietary intake of these compounds with improved vascular function and reduced blood pressure, further strengthening their potential role in cardiovascular prevention [5]. A number of natural products—such as resveratrol, quercetin, berberine, and curcumin—have been reported to exert antihypertensive effects through a variety of mechanisms including modulation of nitric oxide synthesis, suppression of angiotensin-converting enzyme (ACE), and inhibition of calcium influx via L-type voltage-gated calcium channels [6-8]. For instance, quercetin has been shown to inhibit Ca^{2+} influx in vascular smooth muscle cells by blocking L-type Ca^{2+} channels, while resveratrol enhances endothelial nitric oxide production, leading to vasodilation [9]. Despite these advances, the detailed mechanisms by which many natural compounds interact with calcium signaling pathways or modulate calcium-handling proteins such as SERCA, NCX, or RyR2 remain incompletely characterized. Recent computational and biochemical studies have begun to shed light on these interactions, but much remains to be elucidated [10]. In this context, the present study aims to investigate the antihypertensive properties of A51, a biologically active propyl ester of 4-hydroxy-3,5-dimethoxybenzoic acid. Using a comprehensive multiscale strategy that integrates *in vitro* cellular assays, *in vivo* pharmacodynamic models, and *in silico* molecular docking and dynamics simulations, we evaluated the compound's modulatory effects on calcium homeostasis, its influence on systemic blood pressure, and its binding affinity toward key calcium transport-associated proteins. This combined experimental and computational approach is designed to clarify the underlying mechanisms of action of A51 and to provide scientific justification for its development as a novel plant-derived calcium channel modulator with therapeutic potential in hypertension.

Materials and methods

Chemical profile of 4-Hydroxy-3,5-dimethoxybenzoic acid (A-51)

4-Hydroxy-3,5-dimethoxybenzoic acid, also known as a structural analog of sinapic acid, is a naturally occurring phenolic compound characterized by 2 methoxy groups at the 3- and 5-positions, a hydroxyl group at the 4-position, and a carboxylic acid moiety. It belongs to the class of hydroxybenzoic acids and is commonly found in various plant species as part of their secondary metabolite profile. This compound is known for its antioxidant and anti-inflammatory properties and has shown potential in modulating vascular function. Its structural similarity to gallic and sinapic acid derivatives suggests possible interaction with calcium regulatory pathways, including calcium ion channels and transporters. The presence of both electron-donating methoxy and hydroxyl groups on the aromatic ring enhances its reactivity towards reactive oxygen species (ROS) and contributes to its vasoprotective activity. In this study, the propyl ester derivative of 4-hydroxy-3,5-dimethoxybenzoic acid (referred to as A-51) was synthesized and evaluated for its vasorelaxant and antihypertensive activity using *in vitro* aortic ring assays, *in vivo* blood pressure monitoring, and *in silico* docking analysis against key calcium-handling proteins.

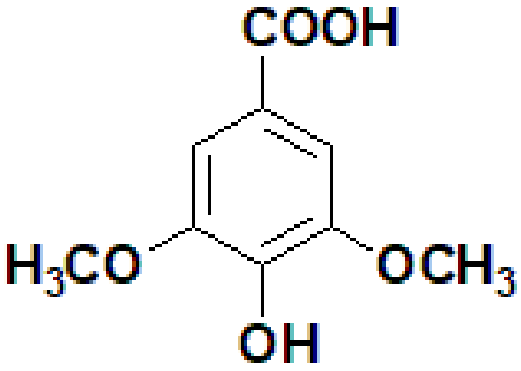


Figure 1 4-Hydroxy-3,5-dimethoxybenzoic acid (A51).

Chemicals

Phenylephrine, phentolamine, and verapamil (≥ 98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal ethics

All preoperative and experimental protocols were read and approved slowly by the Institutional Committee for Animal Use and Care. The animals lived in vivarium rooms under controlled conditions, relative humidity of 55% - 65% and ambient temperature of 22 ± 2 °C, and had free access to water and normal laboratory chow. All animal handling and care procedures rigorously followed the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Ethical approval for this research was provided by the Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, Animal Ethics Committee (Protocol No. 133/1a/h, 4 August 2016).

Tissue preparation

All surgical procedures were carried out under sodium pentobarbital anesthesia to avoid pain in animals. The experiment employed thoracic aortic tissue of healthy adult male Wistar rats weighing between 200 - 250 g. Euthanasia was performed using cervical dislocation. The aorta was dissected following thoracotomy, meticulously freed of the perivascular adipose and connective tissue, and sectioned into 3 - 4 mm vascular rings. These aortic segments were placed in a 5 mL organ bath of Krebs-Henseleit physiological buffer, which consisted of (in mM): NaCl 120.4, KCl 5, $NaHCO_3$ 15.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.5, and HEPES, pH 7.4. In some of the assays, a calcium-free Krebs solution with the addition of 1 mM EGTA was utilized in order to identify responses that were calcium-dependent. Throughout the experiment, the solution was aerated with carbogen gas (5% CO_2, 95% O_2) and kept at 37 °C using a DAIHAN ultrathermostatic water bath system [11].

Aortic-ring contraction studies

Aortic rings were mounted in a Radnoti isometric transducer system (USA) using platinum wire hooks and equilibrated for 60 min under normal physiological conditions. Each tissue sample was preloaded with the initial preload of 1 g (10 mN). The contractile activity was captured from the isometric transducer onto a signal amplifier and digitally onto a Go-link analog-to-digital converter attached to a computer. Analysis and data collection were performed with Origin Pro software, version 8.5 SR1 (EULA, Northampton, MA, USA). Contractile tension (mN) of the aortic tissue *in vitro* was expressed as a percentage for statistical analysis purposes [12]. Vascular smooth muscle activity was confirmed by an experimental apparatus based on Vandier *et al*. (2002). The 5 mL organ bath was connected to a recirculating reservoir system of Krebs-Henseleit solution. Temperature was strictly controlled with a thermostat, and the buffer solution was bubbled constantly with a 95% O_2 and 5% CO_2 gas mixture. Isometric tension of the mounted aortic segments was recorded continuously with a Grass Instruments isometric transducer (USA) and displayed with the GoLink amplifier and data acquisition system [13].

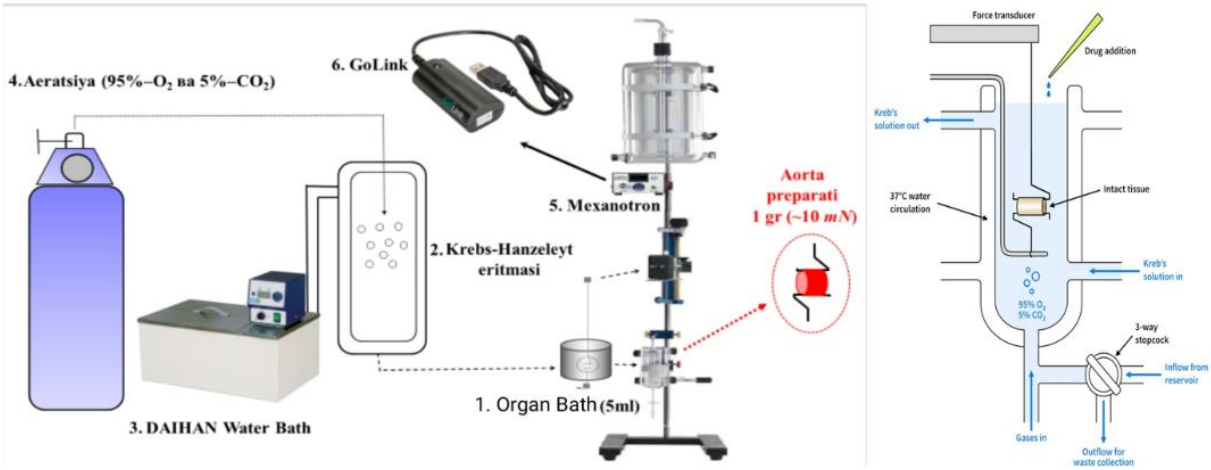


Figure 2 Diagram of the experimental setup used to monitor isometric contractions in isolated rat aortic vascular tissue. (1) The organ bath (5 mL) is connected to a dedicated reservoir for solution circulation. (2) Krebs-Henseleit solution is used to maintain physiological conditions. (3) A thermostat ensures stable temperature regulation. (4) The system is continuously aerated with a gas mixture containing 95% oxygen and 5% carbon dioxide. The aortic tissue is mounted within the experimental chamber for contraction assessment. (5) An isometric transducer (Grass Instrument, USA) captures mechanical responses, while (6) GoLink devices handle signal amplification and system integration.

Blood pressure measurements

Tail-cuff plethysmography was performed with the Sistola device (Neurobotics, Russia) after 3 days of acclimatization in order to reduce variability caused by stress. Blood pressure is measured in triplicate at every session of measurement for obtaining trustworthy data [14]. All the experiments were carried out at the "BFM Pharmacology and Screening Laboratory" and the "Plant Cytoprotectors" Laboratory of the A. Sodikov Institute of Bioorganic Chemistry. Data acquisition and processing were carried out by using AcqKnowledge 4.2 for MP150 software (**Figure 3**).

Figure 3 The "Sistola" device (Neurobotics, Russia) used for non-invasive measurement of arterial blood pressure in rats via the tail artery.

Statistics

Statistical processing and plots were performed using Origin Pro version 9 (USA). Vascular contractile responses were given as percent maximum contraction induced by phenylephrine (10 mM) or potassium chloride (50 mM). Values are presented as the mean of 4 - 6 independent experiments (n = 4 - 6). Paired t-tests were utilized to test within-groups, and unpaired t-tests are utilized for testing differences among different experimental groups. A *p*-value less than 0.05 was adopted as an indicator of statistical significance [15].

Molecular docking "software and databases"

All the software programs used in the current study were accessible free of charge for educational use. Structural data of biological macromolecules involved in calcium signaling and control were retrieved from the Protein Data Bank (PDB), an internationally recognized database of 3-dimensional biomolecular structures [16]. Target proteins were L-type calcium channel Cav1.2 (PDB ID: 6jp5), R-type calcium channel Cav2.3 (PDB ID: 7xlq), sodium-calcium exchanger NCX1 (PDB ID: 8sgi), ryanodine receptor type 2 RyR2 (PDB ID: 5c33), and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA, PDB ID: 6rb2). A PubChem database was also utilized to download reference compounds and target flavonoids of interest for investigation. PubChem combines massive pharmacology data, molecular targets, chemical structures, and biological pathways and combines every DrugCard, which contains over 80 fields of data, including small molecule and protein target fields (see **Table 1**).

Structures of the PDB and docking results were visualized using PyMOL (version 1.2), a Python-based molecular graphics software package (http://www.pymol.org). AutoDock 4.2, a software developed by The Scripps Research Institute (www.scripps.edu), was used for molecular docking studies. Docking inputs and parameter values were prepared in advance with AutoDock Tools (ADT), a straightforward graphical user interface for the setup and running of docking simulations. AutoDock provides a robust computational framework for predicting the binding conformation and interaction of small molecule compounds and macromolecular targets of known 3-dimensional structure [17].

Calculation of Inhibition Constant (Ki) from Binding Energy. In the molecular docking analysis, the strength of an interaction of a target protein and a ligand is usually quantified as the binding free energy (ΔG) in kilocalories per mole (kcal/mol) [18]. The binding energy can be utilized to determine the inhibition constant (Ki), the binding affinity of a ligand, from a straightforward thermodynamic formula.

$$Ki = e^{\wedge} \frac{\Delta G x 1000}{RxT} \quad (1)$$

where:

- 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 1000 converts kcal to cal.

Results and discussion

Investigation of the involvement of L-type and R-type Ca^{2+} channels in the vasorelaxant action of A-51

50 mM KCl is observed to contract aortic smooth muscle via the process of activation of voltage-dependent L-type calcium (Ca^{2+}) channels predominantly [19]. Extracellular rise in K^+ causes depolarization of the membrane, which changes the membrane potential and causes opening of the channels for the entry of calcium into smooth muscle cells and vasoconstriction. In the current research, we examined the vasorelaxant activity of the A51 compound on KCl-induced contraction of isolated rat aortic rings. The findings indicated that A51 provoked relaxation of pre-contracted tissues dose dependently. Particularly, within the range of 5 - 60 μM, A51 notably reduced KCl (50 mM)-induced contractions from 5.0% ± 2.2% to 76.0% ± 2.7% compared with the control group (**Figure 4(A)**). These results indicate that A51 blocks depolarization-stimulated calcium entry, potentially through alteration of L-type Ca^{2+} channel function on smooth muscle cell membrane [20]. Through inhibition of extracellular calcium influx to the cell required for contraction, A51 lowers intracellular calcium and relaxes vascular smooth muscle. To recapitulate further, if this effect is specific to L-type Ca^{2+} channels, a comparative pharmacology experiment was performed using the prototype L-type calcium channel blocker verapamil. When co-administered with A51, following administration of a submaximal dose of verapamil (0.1 μM)—that partially inhibited KCl-induced contraction—an additive 15.0% ± 2.7% relaxation was evoked. An additive effect implies a synergistic or complementary effect between verapamil and A51 (**Figure 4(B)**). While not as potent as synthetic blockers, the observed IC_{50} places A51 within a feasible therapeutic window, particularly for long-term management of vascular dysfunction where high potency is not always preferable due to safety concerns. This value, alongside concentration-dependent

relaxation of precontracted aortic rings, supports the notion that A51 primarily acts by inhibiting voltage-dependent L-type calcium channels. By preventing extracellular calcium influx into vascular smooth muscle cells, A51 reduces cytosolic calcium concentrations, leading to muscle relaxation and vasodilation. The similarity in mechanism with verapamil further highlights A51's potential as a plant-derived alternative or adjunct therapeutic agent in the treatment of hypertension and other vascular disorders characterized by dysregulated calcium signaling [21].

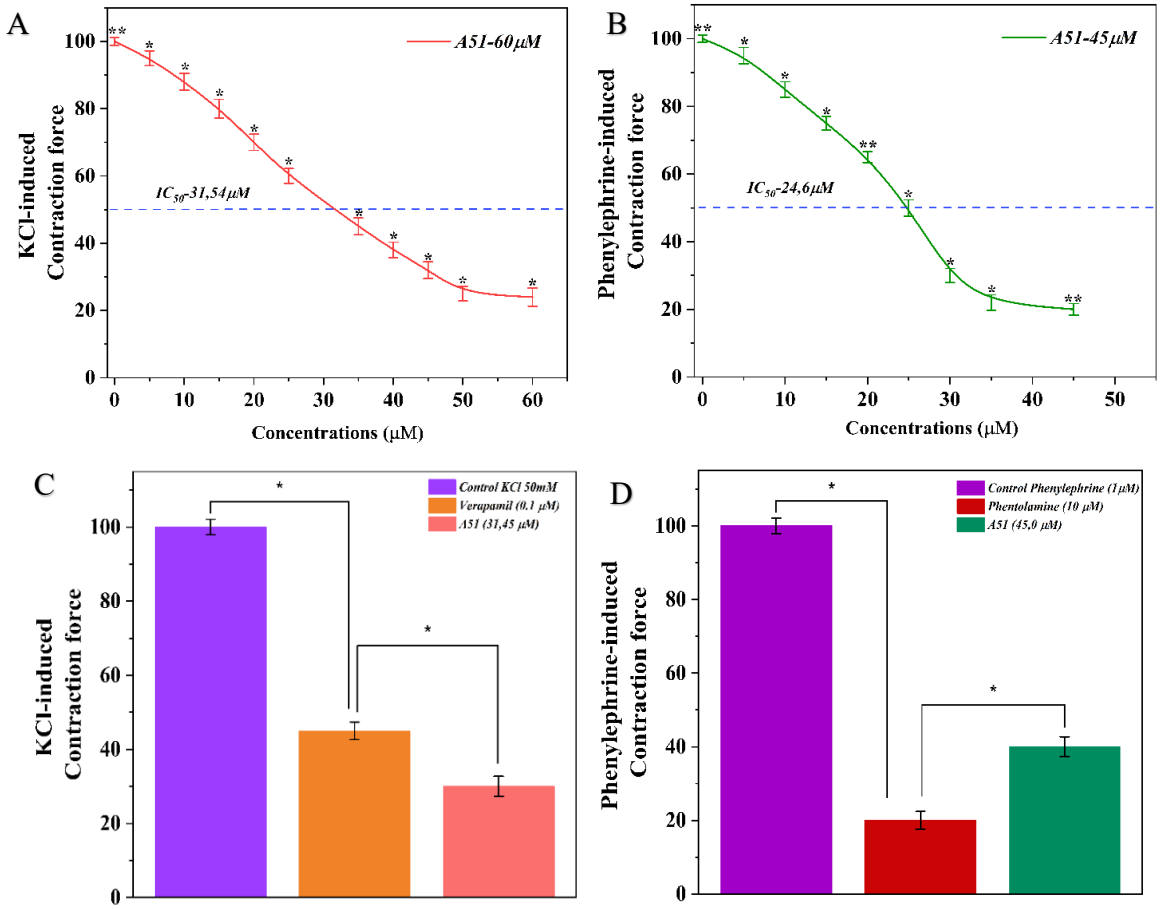


Figure 4 (A) Dose-dependent, linear vasorelaxant effect of compound A-51 on KCl-induced contraction. (B) Comparison of A-51 with the calcium channel blocker verapamil. (C) Effect of A-41 on receptor-operated Ca^{2+} ion channels. (D) Confirmation of A-51's mechanism using the α-adrenergic antagonist phentolamine. *Data represent mean ± SEM, n = 3 - 4, p < 0.05.*

It is well known that vascular smooth muscle contraction is controlled by not just voltage-gated L-type Ca^{2+} channels, but also by intracellular calcium control mechanisms, particularly those of the SR. These internal calcium stores and ROCCs serve to significantly regulate intracellular calcium concentrations and thus smooth muscle tone [22]. To investigate the possible role of A51 in the regulation of receptor-activated pathways, we tested its action on phenylephrine (1 μM)-induced contraction of rat aortic rings. Phenylephrine, being an agonist of α-adrenoceptors, causes vasoconstriction primarily through the release of calcium from the SR and activation of ROCCs on the plasma membrane. A51 in our study showed significant vasorelaxant activity, significantly inhibiting phenylephrine-induced contraction. At its highest dose employed (45 μM), A51 inhibited contractile response by 80.0% ± 1.7% against untreated control (**Figure 4(C)**). The above hints that A51 would initiate its relaxant effect by inhibiting receptor-operated calcium entry and consequently intracellular Ca^{2+} increase and muscle contraction. To expand this mechanism further,

comparative experiments were done with phentolamine, an α-adrenoceptor selective blocker, and flavonoids that were known to interfere with receptor-mediated calcium signaling. Phentolamine (10 μM) itself inhibited phenylephrine-induced contraction by 80.0% ± 2.4%. Surprisingly, when phentolamine was pre-incubated with A51 (45 μM), the contraction was inhibited to an additional 60.0% ± 2.7% (**Figure 4(D)**). This additive inhibition is consistent with the suggestion that A51 prevents α-adrenoceptor-mediated calcium signaling, and most likely does so by preventing ROCCs, and possibly also by preventing downstream intracellular mobilization of calcium. The repeatable reduction in contractile force with both A51 and phentolamine suggests that A51's vasorelaxant effect is at least partly mediated by receptor-operated inhibition of calcium influx, in addition to its previously characterized action on L-type Ca^{2+} channels [23].

A study on how endothelial mechanisms mediate the relaxant response elicited by A-51

The vascular endothelium is essential for maintaining vascular tone through the release of local mediators, most notably nitric oxide (NO), a principal vasodilator derived from endothelial cells. When the endothelium becomes structurally or functionally impaired—a condition known as endothelial dysfunction (ED)—it significantly contributes to the pathogenesis of cardiovascular disorders such as hypertension and atherosclerosis [24,25].

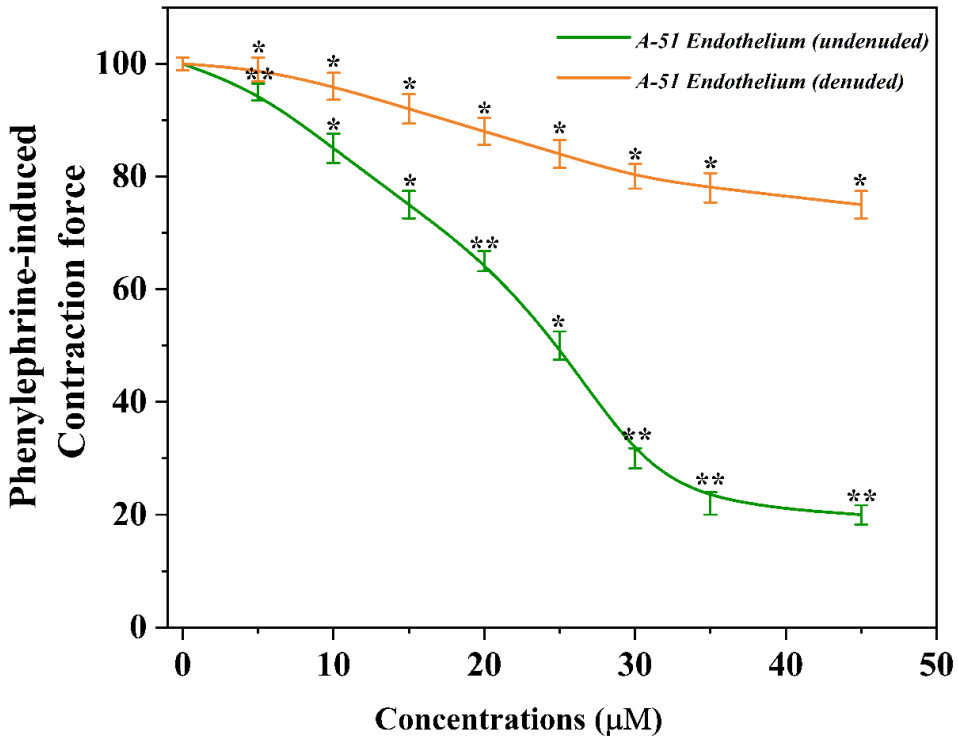


Figure 5 Relaxant effect of A-41 on the contraction induced by Phe 1 μM in the undenuded and denuded rat aortic blood vessel endothelial layer. Contraction force elicited by 1 μM Phe was taken as 100% of control. (In all cases *$p < 0.05$, **$p < 0.01$; n=5).

Endothelial dysfunction (ED) is caused by vasoconstrictor and vasodilator mediator imbalance, normally triggered by oxidative stress and modifiable risk factors including smoking, diet, and metabolic disease [26]. Nitric oxide (NO) is a predominant endothelial function mediator and, in endothelial cells, is generated from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS). Its enzymatic activity is calcium-calmodulin dependent and is inhibited by complex formation with caveolin, a membrane-associated protein. Physiologic stimuli, i.e., acetylcholine or bradykinin, cause eNOS dissociation from caveolin to enhance NO production. Interestingly, low-dose statins also increase NO production through

inhibition of the eNOS-caveolin complex, one component of their vascular protective effect [27]. However, oxidative stress will likely considerably interfere with this pathway through increasing production of reactive oxygen species (ROS) that catalyze NO breakdown, thus reducing its bioavailability and vasodilatory effect. Under normal physiological conditions, NO induces relaxation of vascular smooth muscle cells through the NO/cGMP/PKG pathway, which decreases intracellular levels of Ca^{2+} with the result of vasodilation [28]. To clarify the involvement of the endothelium in the vasorelaxant activity of A51, additional experiments were performed on endothelium-denuded rat aortic rings. Denudation of the endothelium caused a marked loss (25.0% ± 2.4%) of the vasorelaxant activity of A51 (**Figure 5**). The finding suggests that the endothelium—and, likely, NO signaling—is primarily responsible for mediating the relaxant effect of A51.

Molecular docking study of A-51 with aortic ion channels

To understand the molecular mechanisms of our *in vitro* results better, molecular docking tests involving the A51 compound were conducted. Tests were performed to compare the binding affinity and inhibitory activity (Ki values) of A51 with various major calcium-regulating ion channels taking part in vascular smooth muscle control. Docking simulations demonstrated information regarding A51's interaction at the molecular level, which accounted for the vasorelaxant activity *in vitro*.

A51 was docked against a panel of targets for regulation of calcium in the aorta, which are detailed below:

- L-type Ca^{2+} channel
- R-type Ca^{2+} channel (Cav2.3)
- Ryanodine receptor 2 (RyR2)
- SERCA (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase)
- Ca^{2+}-ATPase
- Na^{+}/Ca^{2+} exchanger (NCX)

Docking results yielded variable binding affinities with −5.5 to −6.6 kcal/mol of binding energies. Highest contacts were against Ca^{2+}-ATPase and NCX, both of which offered the lowest binding energy of −6.6 kcal/mol and were indicative of stronger binding and possible functional modulation. The corresponding Ki values also indicated this trend, where NCX and Ca^{2+}-ATPase have the lowest Ki (highest affinity) and the highest Ki (lowest affinity) is being shown by the R-type Ca^{2+} channel, indicating a weaker inhibitory interaction. The rank order for the decreasing binding affinity (i.e., increasing Ki values) among the proteins tested was R-type Ca^{2+} > RyR2 > SERCA > L-type Ca^{2+} > Ca^{2+}-ATPase ≈ NCX.

These data suggest an interaction potency gradient between the calcium-regulatory proteins and are consistent with the speculation that A51 has at least partial vasodilatory activity through modulation of intracellular calcium transport, specifically through actions on Ca^{2+}-ATPase and NCX, both pivotal in calcium clearance and vascular regulation (**Table 1**).

Voltage-gated L-type Ca^{2+} ion channel

Calcium ions (Ca^{2+}) are essential eukaryotic cell secondary messengers and are essential regulators of many physiological and biochemical processes. Of their many cellular functions, entry of Ca^{2+} is especially important in the regulation of cardiac, vascular smooth muscle, and neuronal tissue responses. One of the major pathways of calcium entry into intracellular compartments is through VDCCs, with L-type calcium channels (Cav 1 family) being among the most important because of their almost universal distribution and basic functions in cell signaling. These channels have imperative roles in cardiac and vascular smooth muscle cells, as calcium entry maintains muscle contraction, secretion of hormones, expression of genes, and signal transduction [29].

Regulation and function of L-type calcium channels are closely associated with numerous pathophysiological conditions such as cardiovascular disease, hypertension, neurological disorder, and metabolic syndrome. Thus, L-type channels have become highly useful targets for physiological research as well as pharmacological treatment. Computational modeling and experimental approaches have been heavily utilized over the last few years with a focus to elucidate structural dynamics, regulation mechanisms, and interaction of L-type channels with bioactive molecules—especially flavonoids—increasing their therapeutic relevance. In this research, the biological significance of L-type calcium channels, modulators of

their activity, and flavonoid-derived ligand inhibitory activity are investigated [30]. When the A51 ligand was docked onto the Ca^{2+} L-type calcium channel, its binding energy was –5.6 kcal/mol, indicating a stable interaction. The inhibitory constant (Ki) was determined from this binding energy as 78.7 mM, which categorizes the ligand as a weak inhibitor for the L-type channel. To induce functional inhibition, the ligand will have to contact essential amino acid residues necessary for channel function. The A51 compound forms normal contacts with significant amino acid residues of the L-type Calcium channel protein. (**Table 1**)

It makes classical hydrogen bonds with Asparagine (ASN A:596), Phenylalanine (PHE A:587), and Arginine (ARG A:593) residues, which are accountable for stabilizing the binding. These residues fall within pore-forming or voltage-sensing domains, and their presence as hydrogen bonding could be responsible for the gating and conductance function of the channel.

Moreover, A51 also forms alkyl and π-alkyl contacts with Tyrosine (TYR A:1035) and Valine (VAL A:592), amino acids most commonly located in hydrophobic core regions of the transmembrane domain, enabling ligand accommodation and immobilizing the molecule in the binding pocket.

In addition, Leucine (LEU F:269) is involved in 2 hydrogen bond donor interactions that can contribute to the stability of the proper channel domain and ligand-binding region structure.

These molecular interactions show that A51 is an excellent binder to the calcium channel, possibly to play a role in blocking calcium entry and thereby contribute to its vasorelaxant and calcium-modulating action, which is vital in antihypertensive treatment.

Receptor mediated Ca^{2+}R-type calcium channels

R-type calcium channels (Cav2.3) are members of the family of voltage-dependent calcium channels (VDCCs) but are a member of the Cav2 subfamily. They are also referred to as “R-type” as they are resistant to conventional blockers of calcium channels and have intermediate electrophysiological characteristics intermediate between T-type (transient, low-voltage activated) and L-type (long-lasting, high-voltage activated) calcium channels [31]. They are important in neuronal excitability, transmitter release, as well as some cardiac and vascular responses.

In molecular docking experiments, A51 had a comparably weak binding affinity towards the R-type calcium channel with an approximate −6.1 kcal/mol binding energy, reflecting unsatisfactory interaction stability. The inhibition constant (Ki) was obtained from this value as 33.85 mM, thus rendering A51 is a weak Cav2.3 channel inhibitor.

Docking analysis determined that A51 binds to a number of precise amino acid residues in the R-type channel with a series of non-covalent interactions:

- LEU A:743 - in conventional hydrogen bonding and π-sigma interactions. Leucine at this position could be implicated in supporting the ligand in the pore mouth or within the voltage-sensing region owing to its hydrophobic character.
- PHE A:699, ILE A:1136, ILE A:1137, ALA A:1140, PHE A:1141 - participate in alkyl and π-alkyl interactions, generally involved in hydrophobic stabilization. The residues can be expected to be located within the transmembrane helices or in the nearby hydrophobic pockets of the binding cavity for permitting ligand orientation and partial occupancy of the binding cavity.

These contacts, structurally important as they are, are not adequate to induce major conformational adjustments in the channel or to block calcium conductance effectively. Therefore, A51’s modulatory action on R-type channels should be minimal, and this would imply that it is probably not an important target for this compound’s vasorelaxant or calcium-modulating activity.

SERCA (sarcoplasmic reticulum Ca^{2+}ion channel)

As a P-type ATPase, SERCA operates via a phosphorylation/dephosphorylation mechanism, in which conformational changes pump 2 calcium ions uphill against the concentration gradient per molecule of hydrolyzed ATP. Structurally, SERCA is a single polypeptide chain with a transmembrane domain, which crosses the ER or SR membrane, and a cytoplasmic domain, where ATP binding and hydrolysis occur [32]. Functionally, SERCA serves the important function of relaxing the muscle through calcium pumping out of the cytosol after contraction, reducing intracellular calcium

levels and promoting relaxation of muscle fibers. In non-muscle cells outside muscle tissue, SERCA expression in a broad range of non-muscle cells has also been reported, where the enzyme serves functions in calcium signaling, folding of proteins, and control of calcium-dependent pathways of enzymes.

Previous studies have indicated that augmentation of SERCA activity via pharmacological therapy or gene therapy is therapeutically advantageous in the clinical management of diseases like heart failure, skeletal muscle disease, and neurodegenerative disease via improvement of calcium cycling and cell homeostasis. The Molecular docking experiment showed that A51 ligand binds to the SERCA (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase) protein with the binding energy of −6.1 kcal/mol (**Table 1**). The Inhibition constant (Ki) was derived from the binding energy and was determined to be 33.85 mM, and A51 was determined to be a moderate SERCA activity inhibitor.

The interaction profile reveals a number of non-covalent bonds between A51 and important amino acid residues of the SERCA structure:

- GLY A:227 and LEU A:41 are involved in classical hydrogen bonding, which is important in stabilizing ligand orientation in the active or allosteric binding pocket. LEU A:41, in particular, is close to the transmembrane domain and can play a role in structural flexibility in the ATPase conformation cycle.
- PRO A:160 makes a carbon hydrogen bond with the ligand. Proline residues tend to create structural turns or kinks in the protein backbone, and interaction at this position may affect local folding critical for channel gating or ion translocation.
- THR A:230 has an unfavorable donor-donor interaction, indicating steric or electrostatic hindrance that may prevent optimal binding at this position.
- LEU A:41 and ALA A:142 are engaged in alkyl and π-alkyl interactions, respectively, that contribute hydrophobic stabilization to the binding pocket. They are typically from the transmembrane helices, where ligand accommodation and membrane protein function are enabled by hydrophobic interactions.

Collectively, these interactions suggest that A51 has the ability to modulate SERCA activity by binding or altering functionally significant sites to alter the calcium transport dynamics. While not so coveted for full inhibition, partial modulation of SERCA would nonetheless be capable of modulating intracellular calcium homeostasis necessary for relaxation and signaling of the muscle.

RyR2 (Ryanodine) receptor

Ryanodine receptor 2 (RyR2) is a giant intracellular Ca^{2+} release channel of the SR membrane of cardiac muscle cells. RyR2 is an important mediator of excitation–contraction coupling and is essential for initiating and coordinating cardiac muscle contraction. When there is electrical depolarization of the cardiac cell membrane, L-type calcium channels (Cav1.2) open and permit the entry of a small quantity of Ca^{2+} into the cytoplasm [33]. The incoming calcium serves as a signal that triggers RyR2 to release a much greater quantity of Ca^{2+} stored in the SR — a process termed calcium-induced calcium release (CICR). The resulting increase in intracellular calcium ultimately results in sarcomere contraction and effective cardiac output. Apart from its function in muscle contraction, RyR2 also serves a very important function in circulatory dynamics maintenance, regulation of blood pressure, and oxygen supply of peripheral tissues. Its normal function helps in heart rhythm stabilization and is an important constituent in the prevention of arrhythmias, heart failure, and sudden cardiac death [34]. RyR2 function is regulated by post-translational modifications like phosphorylation and oxidation, by pharmacological agents, and by genetic factors. RyR2 mutations or dysregulation is highly implicated in deadly conditions like catecholaminergic polymorphic ventricular tachycardia (CPVT), congestive heart failure, and fatal arrhythmias.

Due to its pivotal position within cardiac calcium cycling and electrical stability, RyR2 is an extremely attractive pharmacologic target. It is of significant interest to understand its regulation and pathophysiologic change for the development of new therapeutic approaches in the treatment of cardiovascular disease.

According to our molecular docking calculation, the A51 ligand possesses a comparatively weak binding affinity against the RyR2 (Ryanodine Receptor 2) receptor with an estimated binding energy of –5.5 kcal/mol, which equals an estimated Ki value of 93.16 mM (**Table 1**). This indicates A51 as a weak inhibitor

of the RyR2 channel. Although the binding affinity is low, the interaction involves discrete amino acid residues that are perhaps structurally or regulatory important for receptor activity.

Residue-level contacts were uncovered by docking analysis as follows:

- GLU B:711 forms a normal hydrogen bond with A51. Glutamate residues of this region preferentially engage in calcium coordination or in gating mechanisms in intracellular calcium channels.
- GLU B:712, GLU A:701, and GLY A:709 are implicated in carbon hydrogen bonding, indicating weak but possibly stabilizing interactions in the ligand binding site. These are likely to be at the luminal or cytosolic boundary and would likely influence RyR2's conformational adaptation upon release of calcium.
- TYR A:703 is implicated in π-alkyl interaction, which would likely contribute to hydrophobic stabilization in the binding pocket and help anchor the ligand close to the regulatory domains of the channel.

While these interactions are too subtle to completely abolish RyR2 function, they indicate that A51 interacts with structurally significant regions of the protein and can potentially have subtle modulatory effects on calcium release via the channel. Since RyR2 is involved in cardiac excitation–contraction coupling, even subtle modulators such as A51 would be of physiological relevance, particularly under conditions of impaired calcium homeostasis.

Ca^{2+}-ATPase

Ca^{2+}-ATPase is an important membrane-bound enzyme that helps to regulate intracellular calcium balance through virtue of its active transport of the calcium ions (Ca^{2+}) from the cytosol into intracellular organelles—like the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER)—or out of the cell through the plasma membrane. This energy-requiring process is ATP-hydrolysis based and is necessary for the maintenance of calcium homeostasis, particularly during increased cellular activity [35]. By effectively pumping off excess calcium from the cytoplasm, Ca^{2+}-ATPase becomes critical in averting overload of calcium that would otherwise interfere with many cellular processes. In cardiac and muscle cells, the enzyme is crucial for the relaxation of muscles because it pumps away directly cytosolic Ca^{2+} after the contraction of the muscle, and in doing so, the muscle is able to revert back to resting state. In addition to muscle physiology, calcium ions serve as second messengers in numerous essential cellular processes, including transmission of nerve impulses, hormone secretion, intracellular signal transduction cascades, and gene expression. Such events are under precise control by the spatial and temporal distribution of Ca^{2+}, which is under predominantly controlled Ca^{2+}-ATPase activity.

Disfunction or blockage of Ca^{2+}-ATPase was found to lead to the accumulation of calcium within the cytoplasm, and such has been implicated in some pathological states like heart failure, muscular dystrophy, and neurodegenerative diseases. Because of its central role in both physiology and pathology, Ca^{2+}-ATPase represents a very promising therapeutic target, and modulation of the activity of this enzyme may hold new means for treating disorders implicating dysregulation of calcium. Most drugs are affecting heart function by promoting the action of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In our molecular docking experiment, the A51 ligand displayed a weak binding interaction with Ca^{2+}-ATPase, which resulted in a binding energy of −6.1 kcal/mol. This is approximately an estimated inhibition constant (Ki) of 33.85 mM and represents weak inhibitory activity (**Table 1**). Although affinity is weak for binding, the interaction profile indicates that A51 may bind to functionally important residues within the structure of Ca^{2+}-ATPase and likely alter its regulatory function. Docking analysis identified a number of important non-covalent interactions between Ca^{2+}-ATPase amino acid residues and A51:

- LYS A:514, ARG A:559, and ALA A:516 have normal hydrogen bonds with A51, which are needed for the stabilization of the ligand in the ATPase catalytic or regulatory sites. The residues can be involved in nucleotide binding or can be involved in the maintenance of structural conformation for ion transport.
- PHE A:487 is involved in a π-π stacking interaction of aromatic ring positioning. This is common in the facilitation of ligand orientation stability in

hydrophobic or aromatic-containing areas of membrane proteins.

- LYS A:492 and ALA A:516 engage in alkyl and π-alkyl contacts, a common hydrophobic contact that can be used to stabilize ligand anchoring in the lipid-facing or transmembrane segments of the protein.

Although A51 does not exhibit high-affinity binding, the interactions are clear in showing that it would be a weak modulator of Ca^{2+}-ATPase activity. Given the enzyme's such prominence in muscle function and calcium homeostasis, partial modulation could have physiological implications in situations of pathological regulation of calcium.

NCX (sodium and calcium exchanger)

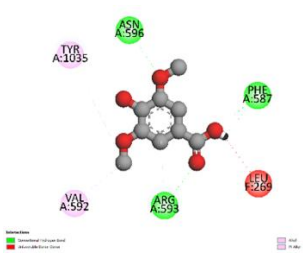
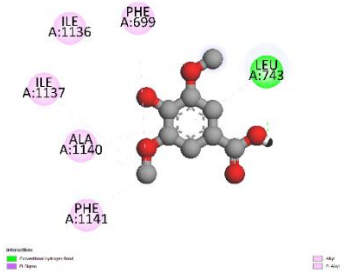
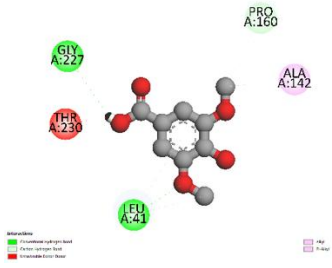
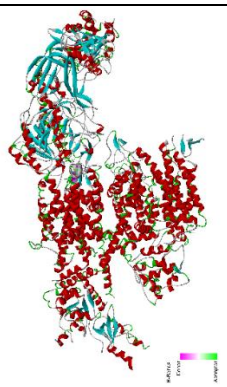
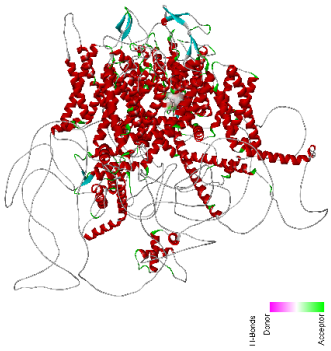
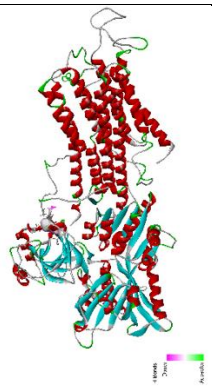
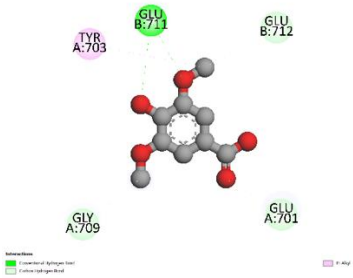
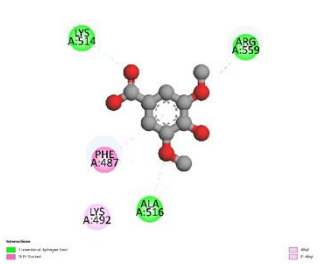
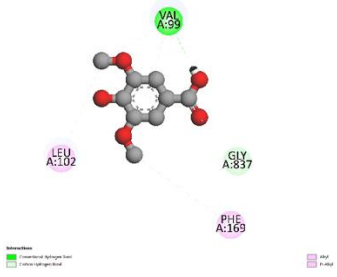
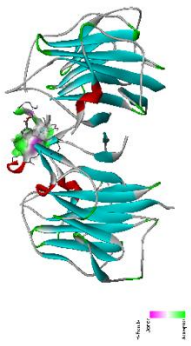
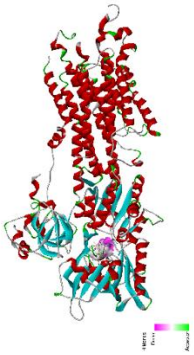
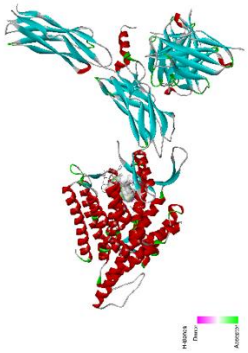
The Na^{+}/Ca^{2+} exchanger (NCX) is an essential plasma membrane-bound ion transport protein that ensures the bidirectional exchange of calcium (Ca^{2+}) and sodium (Na^{+}) ions across the plasma membrane, hence its significant contribution to intracellular calcium homeostasis. Works largely in its forward mode, NCX extrudes a single Ca^{2+} ion from the cytoplasm upon the influx of 3 Na^{+} ions using the electrochemical gradient of sodium as its driving force. This is an ATP-independent process and electrogenic, and therefore NCX is very energy efficient in states of energy deficiency. NCX activity is especially crucial in excitable cells like cardiomyocytes and neurons because it serves to maintain low cytosolic concentrations of Ca^{2+} within the cell rapidly after cell activation [36]. With the intervention of extrusion of Ca^{2+}, NCX collaborates with other mechanisms of clearing calcium such as SERCA and Ca^{2+}-ATPase, thereby preventing calcium overload, which otherwise leads to cytotoxicity, mitochondrial injury, or cell death.

In addition to its homeostatic role, NCX also has a fundamental role in physiological processes such as cardiac relaxation, synaptic transmission, and neuroprotection in states of stress such as ischemia, oxidative stress, and excitotoxicity. Dysregulation of the activity of NCX is involved in the pathology of numerous diseases such as cardiac arrhythmias, heart failure, epilepsy, and neurodegenerative disease. Being a master regulator of calcium homeostasis and disease pathophysiology, NCX is now an active drug target. Ongoing research is focused on the characterization of selective NCX modulators, inhibitors, or promoters of disease-specific action, with the hope of normalization of calcium balance and improved clinical outcome in cardiac and neurological disease. In our molecular docking studies, the A51 ligand demonstrated moderate binding affinity to Na^{+}/Ca^{2+} exchanger (NCX) protein with an estimated –6.0 kcal/mol for its predicted binding energy and 40.070 mM for its predicted inhibition constant (Ki). These estimates indicate that A51 has moderate inhibitory action and forms stable, specific interactions with functionally relevant residues of NCX protein. The docking outputs yielded a series of non-covalent A51-amino acid contacts that are relevant to the NCX structure:

- A51:99 VAL is in a conventional hydrogen bond with A51, suggesting a stabilizing polar interaction near or within a transmembrane segment that is contributing towards the conformational stability of the ion transport pathway.
- GLY A:837 is engaged in a carbon hydrogen bond, a less rigid but stabilizing interaction shared by flexible loop or hinge regions, which may influence local structural mobility upon ion exchange.
- VAL A:99, LEU A:102, and PHE A:169 also engage in alkyl and π-alkyl interactions that reach hydrophobic stabilization within the cleft of binding. Residues would be largely located within the membrane-spanning segment, where hydrophobic interactions are chiefly important for ligand placement and conformational transition during ion translocation.

These interactions collectively indicate that A51 can be a weak modulator of NCX function, perhaps the rate or efficiency of Na^{+}/Ca^{2+} exchange. Considering the critical role the exchanger plays in cardiac relaxation and neuronal signaling and calcium removal during stress, this modulation could be of physiological and therapeutic importance during conditions of calcium disequilibrium or excitotoxicity.

Table 1 Molecular docking results of A-51 with various calcium ion channels.

Channels	L-type Ca^{2+} (A)	Ca^{2+} R-type (B)	SERCA (C)
2D			
3D			
Ki value	78.7mM	33.85 mM	40.070 mM
Affinity (Kcal/mol)	−5.6	−6.1	−6.0
2D			
3D			
Ki value	93.16 mM	33.85 mM	40.070 mM
Affinity (Kcal/mol)	−5.5	−6.1	−6.0

Effect of A51 compound on blood *Pressure in vivo* studies

The effect of the A51 compound on blood pressure was evaluated *in vivo* using the tail cuff method. Rats were divided into 4 groups, each receiving different doses of the A51 compound: 25 and 50 mg/kg. Prior to the experiment, the baseline blood pressure (0 h) of all animals was measured. Afterward, the A51 compound was administered intravenously, and its effect on the cardiovascular system was monitored for 3 h, with measurements taken every hour.

Effect of A51 compound at 25 mg/kg dose:

In the control group, baseline values were 98.0 ± 8.9 mmHg for systolic blood pressure and 74.8 ± 7.3 mmHg for diastolic pressure. Blood pressure in the experimental group treated with 25 mg/kg of A51 responded dynamically during the 3 h observation period:

- At 1 h, systolic pressure temporarily increased to 101.3 ± 10.0 mmHg, and diastolic pressure increased to 79.3 ± 7.8 mmHg, suggesting a transient increase at an early point.

- At 2 h, there was a significant hypotensive effect with widespread reduction of systolic pressure to 84.3 ± 8.3 mmHg and diastolic pressure dropping to 58.0 ± 5.6 mmHg.

There was some recovery at the 3 h with increasing systolic pressure to 113.3 ± 11.2 mmHg and diastolic pressure to 86.0 ± 8.5 mmHg, which was above baseline levels.

Most notably, the group to which 25 mg/kg A51 was administered exhibited the greatest and most consistent decrease in blood pressure with increasing return towards baseline, hence reflecting an excellent balancing and effective antihypertensive effect. With these results, 25 mg/kg was thus established as the best and most optimal dose in the induction of controlled reduction in blood pressure.

Effect of A51 compound at 50 mg/kg dose:

In the control group, systolic blood pressure was 125.0 ± 12.4 mmHg, and diastolic blood pressure was 93.5 ± 9.2 mmHg. In the group receiving 20 mg/kg of A51, the blood pressure changes were as follows:

- 1 h: Systolic blood pressure decreased to 89.0 ± 8.8 mmHg, and diastolic blood pressure decreased to 64.0 ± 6.2 mmHg.
- 2 h: Systolic blood pressure increased again to 124.5 ± 12.3 mmHg, and diastolic blood pressure decreased to 92.0 ± 9.0mmHg.
- 3 h: Blood pressure partially deareased, with systolic blood pressure reaching 108.3 ± 10.7 mmHg, and diastolic blood pressure returning to 81.3 ± 8.1mmHg

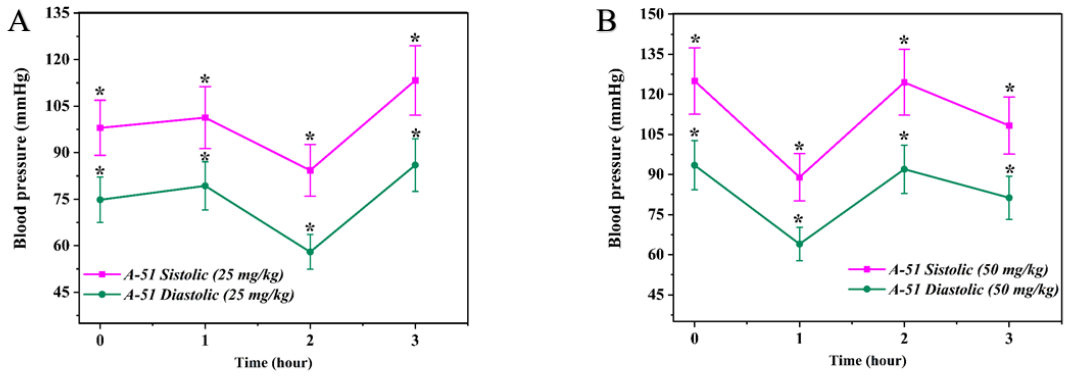


Figure 6 Doses decrease in systolic and diastolic blood pressure was observed following the administration of A51 compound at doses of 25 and 50 mg/kg. These results indicate that the antihypertensive effect of the A51 compound is dose-dependent (n = 4, $p < 0.05$).

The dramatic drop in blood pressure observed at the dose of 50 mg/kg and subsequent failure of stabilization point to possible dangers of the drug at this dose, but compared to the 25 mg/kg dose, the duration

of the reduction was shorter. This dose also demonstrated a significant effect. Further studies are required to explore the mechanism of action of the compound in greater detail (**Figure 7**).

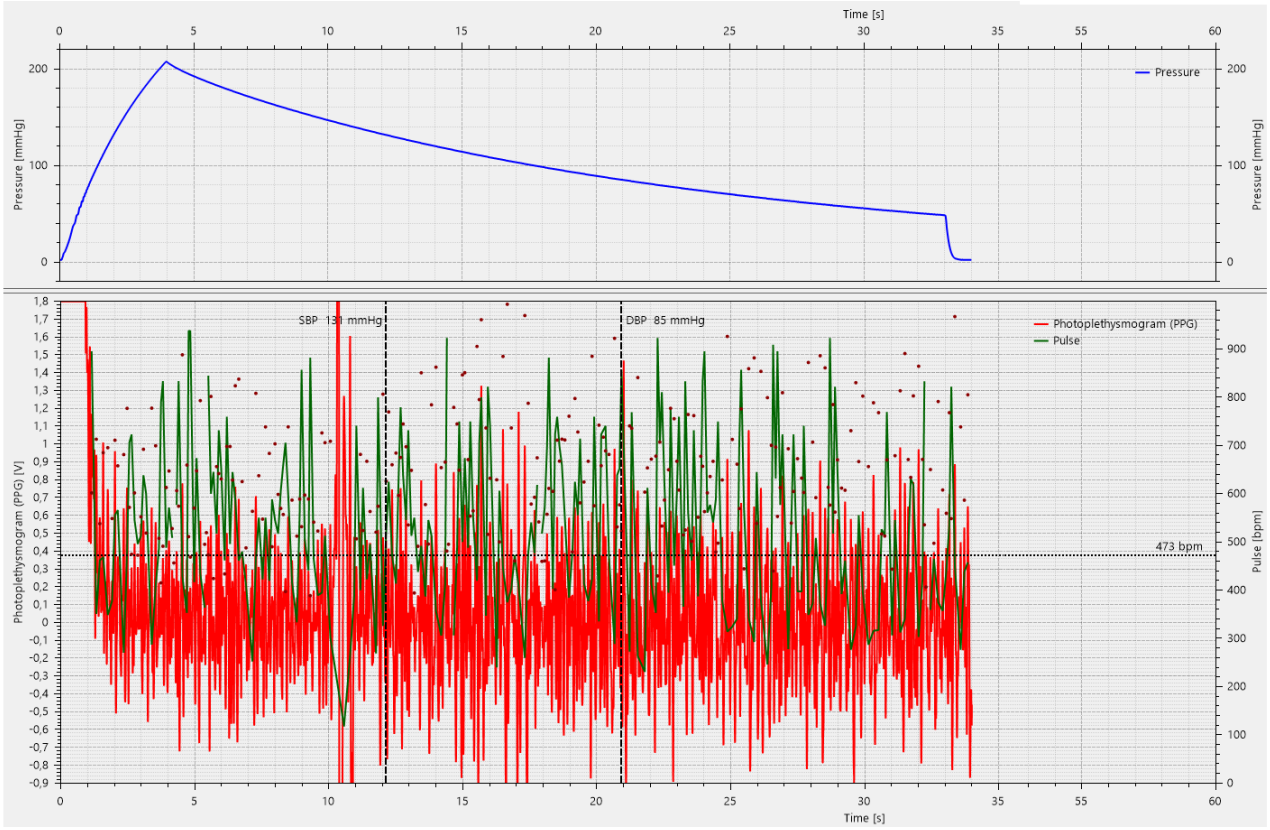


Figure 7 Original systolic recordings obtained using the Neurobotics software. (These results were used as sample data for statistical analysis.) (Control Group).

Evaluation of the effect of A51 compound in the adrenaline-induced hypertension model

Before the main experimental procedures were carried out, the optimum dosage of the A51 compound was found, and all the subsequent experiments were performed with the chosen dosage.

The rats were randomly assigned to 3 groups: A health control group, an adrenaline-induced model group of hypertension, and an experimental group to which the A51 compound was administered after adrenaline treatment. All 3 groups consisted of healthy male rats (n = 4), each with a weight of 300 - 350 g. Baseline diastolic blood pressure (DBP) and systolic blood pressure (SBP) were recorded in all animals at the commencement of the study using the non-invasive Tail-Cuff method. The following parameters were taken into account:

- In a control group, SBP was 106.3 ± 10.5 mmHg and DBP was 76.8 ± 7.4 mmHg.
- In an A51-treated group (50 mg/kg), SBP was 94.0 ± 9.3 mmHg and DBP was 68.8 ± 5.8 mmHg.

These initial values were control points with reference to which the cardiovascular response of both groups could be measured quantitatively throughout the experiment.

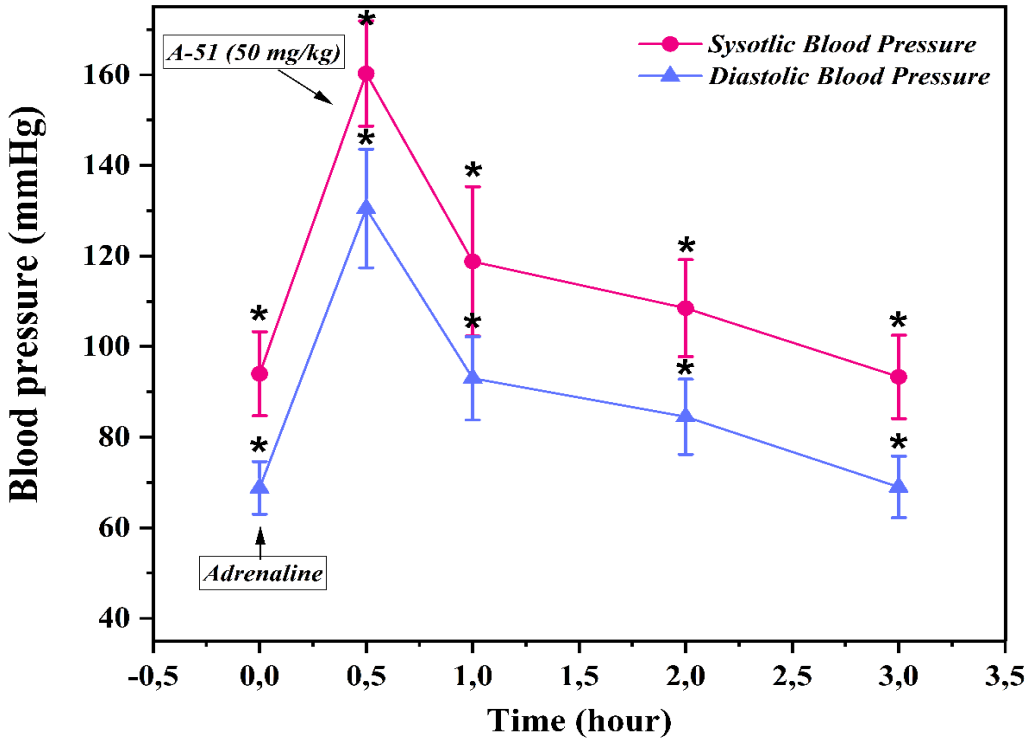


Figure 8 (A) Administration of the A51 compound at a dose of 50 mg/kg resulted in a dose-dependent reduction in systolic and diastolic blood pressure (n = 4, $p < 0.05$).

Following baseline measurement, intravenous injection of adrenaline hydrochloride was conducted in all experimental groups apart from the healthy control group to induce acute hypertension. In accordance with expectation, acute hypertension was detected in the hypertensive model group, and systolic blood pressure (SBP) and diastolic blood pressure (DBP) increased to 160.5 ± 16.3 mmHg and 119.5 ± 11.8 mmHg, respectively.

In an A51 (50 mg/kg)-treated test group with adrenaline administration at the same time, the increase in blood pressure during onset was also identical, with SBP being 160.3 ± 11.6 mmHg and DBP being 119.5 ± 11.8 mmHg, indicating that the compound had not stopped the onset effect of hypertension but could possibly affect its development afterward. In the later half of the research, A51 was given intravenous, and hourly blood pressure was recorded to check its antihypertensive effect. The control group of hypertensive, which was given adrenaline but not A51, was performed to show the natural fall of adrenaline-induced hypertension. In this control group, the blood pressure levels fell in the course of 3 h as follows:

- 1 h: SBP 147.8 ± 11.6 mmHg, DBP 118.8 ± 9.8 mmHg
- 2 h: SBP 140.5 ± 12.5 mmHg, DBP 115.0 ± 10.1 mmHg
- 3 h: SBP 129.0 ± 11.8 mmHg, DBP 98.0 ± 9.2 mmHg (**Figure 8**)

On the other hand, the A51 (50 mg/kg) group registered a much higher blood pressure drop, validating the compound's antihypertensive effect:

- 1 h: SBP 118.8 ± 16.5 mmHg, DBP 93.0 ± 9.2 mmHg
- 2 h: SBP 108.5 ± 10.7 mmHg, DBP 84.5 ± 8.3 mmHg
- 3 h: SBP 93.3 ± 9.2 mmHg, DBP 69.0 ± 6.8 mmHg

All of these results clearly demonstrated that A51 not only decreased blood pressure in an adrenaline-induced hypertensive model but also especially at a dose of 50 mg/kg, which had the maximum and sustained effect throughout time. Not elaborated here, but worthy of mention is the fact that even the dose of 50 mg/kg previously showed a remarkable antihypertensive effect in the previous phases of this study. This again brings the therapeutic potential of A51 in the management of

acute hypertensive attacks closer to reality and deserves continued exploration of its dose-response effectiveness and safety profile.

Discussions

The current study demonstrates that compound A-51 exhibits potent vasorelaxant and antihypertensive effects through a combination of mechanisms involving calcium channel modulation and endothelial function. A-51 induced a concentration-dependent relaxation of aortic rings precontracted with KCl, achieving 76.0% ± 2.7% relaxation at 60 μM, with an IC_{50} of 31.54 μM, suggesting strong inhibition of voltage-dependent L-type Ca^{2+} channels. This effect was confirmed by additive responses when co-administered with verapamil, an established L-type calcium channel blocker, indicating either synergistic interaction or modulation at complementary binding sites. A-51 also significantly inhibited phenylephrine-induced contractions, with up to 80.0% ± 1.7% relaxation at 45 μM, pointing to effective blockade of receptor-operated calcium channels. This effect, enhanced in the presence of phentolamine, supports the hypothesis that A-51 interferes with α_1-adrenoceptor-mediated calcium influx and possibly intracellular calcium release. Removal of the endothelium reduced A-51's relaxant response by 25.0% ± 2.4%, suggesting partial involvement of nitric oxide (NO)-mediated pathways. Since NO is a major vasodilatory mediator synthesized by endothelial nitric oxide synthase (eNOS), A-51 may enhance NO production or reduce its degradation, contributing to improved vascular tone and endothelial function. *In vivo*, A-51 significantly lowered blood pressure in both normotensive and adrenaline-induced hypertensive rats. The 25 mg/kg dose provided optimal antihypertensive response, with systolic blood pressure falling from 98.0 ± 8.9 mmHg to 84.3 ± 8.3 mmHg and diastolic pressure from 74.8 ± 7.3 mmHg to 58.0 ± 5.6 mmHg at 2 h. In hypertensive models, A-51 (50 mg/kg) reversed adrenaline-induced elevations in systolic pressure from 160.3 ± 11.6 mmHg to 93.3 ± 9.2 mmHg, indicating robust and sustained blood pressure-lowering effects. Molecular docking studies further substantiated A-51's mechanism by revealing strong binding affinities to Ca^{2+}-ATPase and NCX (both −6.6 kcal/mol), proteins essential for calcium clearance from the cytoplasm. These interactions suggest A-51 supports intracellular calcium homeostasis not only by limiting calcium influx but also by enhancing its extrusion or sequestration. Moderate interactions with SERCA and RyR2, and weak affinity for R-type calcium channels, indicate target selectivity that favors both functional efficacy and reduced off-target effects.

Taken together, these results confirm that A-51 mediates vasorelaxation and blood pressure reduction via a multi-target mechanism. It modulates L-type and receptor-operated calcium channels, promotes endothelial NO signaling, and regulates intracellular calcium transport. This multifaceted activity makes A-51 a promising candidate for further development as a therapeutic agent in the treatment of hypertension and vascular dysfunction.

Conclusions

In conclusion, the present study provides compelling evidence that compound A-51 exerts significant vasorelaxant and antihypertensive effects through a combination of mechanisms, including blockade of L-type and receptor-operated calcium channels, activation of endothelial nitric oxide signaling, and modulation of calcium-handling proteins such as Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger. The observed IC_{50} of 31.54 μM and the concentration-dependent relaxation of aortic rings confirm its functional potency, while *in vivo* experiments demonstrate its efficacy in reducing arterial blood pressure in both normotensive and hypertensive conditions. These findings not only elucidate the multi-target mode of action of A-51 but also highlight its potential as a promising lead compound for the development of next-generation antihypertensive therapies, particularly those aimed at restoring vascular tone and improving endothelial function. As a nature-derived candidate, A-51 may offer a safer and more holistic alternative or adjunct to existing treatments. Future studies focused on pharmacokinetics, chronic administration, and safety profiling are essential to fully validate its clinical applicability.

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

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